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Naturally Occurring *Agrobacterium Radiobacter*: A Promising Tool for Biological Management of Crown Gall Disease of Roses

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Abstract

Crown gall disease caused by Agrobacterium tumefaciens is one of its most limiting factors in cut-flower production. In Kenya cut-flower production accounts for about 38 % in floriculture value chain. However, its production faces significant challenges due to pests and diseases. Crown gall disease causes substantial yield loss which warrants an urgent need to explore sustainable management options. The management of crown gall in roses currently includes cultural practices and chemical control methods. The study aimed at evaluating the antagonistic activity of naturally occurring Agrobacterium radiobacter isolates from different flower farms in Nanyuki, Naivasha, Murang'a and Timau against Agrobacterium tumefaciens.

Isolates of Agrobacterim tumefaciens were obtained from young and fresh galls of infected rose plants while isolates of Agrobacterium radiobacter were isolated from soil sample obtained from the greenhouses. Yeast Extract Mannitol Agar supplemented with Congo red dye and Yeast Extract Peptone media were used for study. Complete randomized design was used in isolation of Agrobcterial isolates in the laboratory. Colony morphology and biochemical tests performed included: Gram staining, catalase test, urease test, salt

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tolerance test, 3-ketolactose test, motility test and H_2S production for identification and confirmation of the agrobacterial isolates. Kirby-Bauer disc diffusion technique was used to determine antimicrobial sensitivity patterns of bacteria to antibiotics. Carrot disc assay test was conducted on Agrobacterium tumefaciens for pathogenicity test while in vitro antagonistic test was done on Agrobacterium radiobacter. In the greenhouse experiments, pathogenicity test was carried out using Agrobacterium radiobacter and Agrobacterium tumefaciens isolates on four varieties of rose plants.

Minimum incidence (6 %) of crown gall was observed on Topsun, Fuschiana and H3O Rose varieties upon inoculation with Agrobacterium radiobacter isolates compared to 54 % in the control. In Furiosa variety, there was minimal incidence of crown gall disease on treated plants and in control. There were no significant differences (P>0.05) in the interaction between rose flower variety and treatments on the number of plants with galls. The study confirmed that Agrobacterium radiobacter had the potential in the management of crown gall disease on rose flower plants. Therefore, the study recommends on formulation Agrobacterium radiobacter as a commercial biopesticide and applies it on a larger scale.

Keywords: Rose plants, antagonism, *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*

1. INTRODUCTION

The Kenyan flower industry is the third largest flower exporter by value and volume behind the Netherlands and Colombia on a global level [1]. It contributes about half of fresh horticultural exports and it is estimated that by 2010, the flower industry provided direct employment to over 90,000 and over 500,000 in related industries [2]. However, rose flower production in Kenya is constrained by diseases such as powdery mildew, downy mildew, botrytis and crown gall disease. Its production in Kenya faces significant challenges particularly with regard to pests and diseases with crown gall disease as one of the

major limiting factors. The disease is caused by *Agrobacterium tumefaciens* and is wide spread in rose flower farms and nurseries in Kenya.

Agrobacterium tumefaciens is a soil borne bacterium, a member of family- *Rhizobiaceae* and has worldwide distribution [3]. *Agrobacterium tumefaciens* is a rod shaped, gram negative bacteria found in the soil that causes tumorous growth termed as crown gall disease in dicot plants (Figure 1). They are motile bacteria that grow aerobically without forming endospores [4]. Crown gall is a common disease of dicot plants, including many woody shrubs and various herbaceous plants, stone and pome fruit-trees, grapevines, roses and some ornamental plants [5]. The disease also affects some gymnosperms [6]. It produces crown galls in over 600 species of trees [7] and is considered as the main bacterial disease of stone fruit trees in the nurseries of Mediterranean countries [8]. The galls provide a nutrient rich environment for the growth of *A. tumefaciens* which returns to the soil as the galls decompose [9]. Crown gall disease development occurs when the ideal environment, the virulent pathogen and the susceptible plant host all interact at the same time to cause the disease [10].

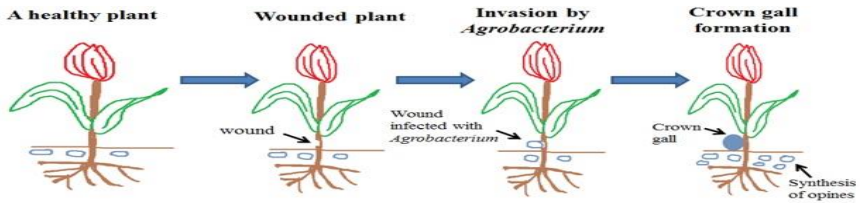

Figure 1: Tumor induced by *Agrobacterium tumefaciens* in plans

The pathogenesis of crown gall is unique and includes the transfer of the part of tumour-inducing (T*i*)-plasmid from *A. tumefaciens* into the chromosome of the plant [11]. Ti plasmid is a piece of circular chromosomal DNA that is generally 190-240 kb in size and usually present in low copy number (1-3 copies) per cell [12]. As a result, plant cells start to produce an

increased amount of hormones leading to uncontrolled tissue proliferation (Figure 2) and synthesis of unusual compounds such as opines derivatives of sugars and special amino acids used by bacteria as nutritional sources [13].

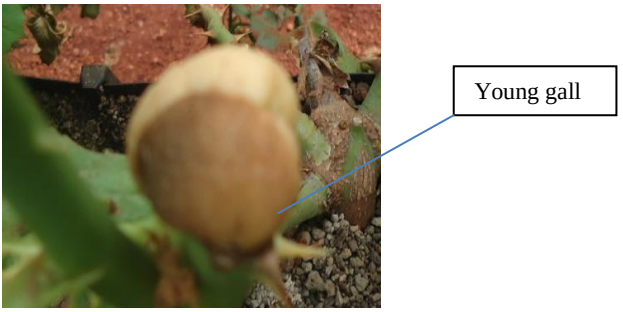

Figure 2: Rose flower plant showing uncontrolled tissue proliferation (gall)

Management of crown gall disease attracted many management strategies including chemicals, pre-plant application of soil sterilizers, soil solarization, herbicides and soil amendments [14[[15]. In biocontrol systems, the pathogen and its antagonistic control agent have to compete for nutrients and space. Many biocontrol agents have been shown to act by antibiosis [16]. Bacteriocins are the most abundant of antimicrobial compounds produced by bacteria and are found in all major phylogenetic bacterial lineages [17].

A biological control approach for crown gall caused by *Agrobacterium tumefaciens* depends on the use of non-pathogenic biological strain *Agrobacterium radiobacter* K84, which produces the bacteriocin-Agrocin 84 which is toxic to certain strains of *A. tumefaciens* [18]. The mode of action Agrocin-84 is based on agrocinopine (analog of agrocin 84) biosynthesis by the plant and its catabolism by the pathogenic strain [18]. The plasmid coded for agrocinopine permease, which is inserted in the bacterial membrane. The inhibitor

agrocin 84, is recognized by this permease, enters the pathogen cells and there it blocks DNA synthesis.

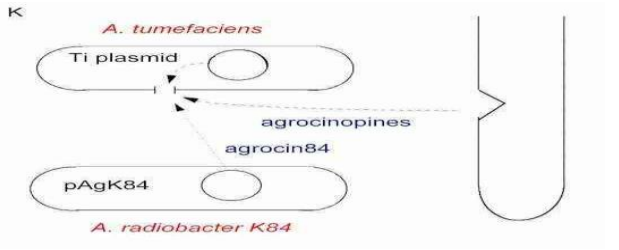

Figure 4: Mode of action of Agrocin 84

Agrobacterium tumefaciens strain C58 contains a PBP called Acc A that mediates the import of the antibiotic agrocin 84, as well as the opine agrocinopine A that acts as both a nutrient and a signaling molecule for the dissemination of virulence genes through quorum-sensing. Currently, there is insufficient information on the occurrence of effective *Agrobacterium radiobacter* in Kenyan soils for management of crown gall disease. Thus, the aim of this work was to test *in vivo* and *in vitro* antagonistic activity of *Agrobacterium radiobacter* isolates naturally occurring in Kenyan soils which has antagonistic effect and potential to control crown gall disease in greenhouse conditions.

2. MATERIALS AND METHODS

2.1 The study sites

A baseline survey was conducted in rose flower farms located in various ecological regions in Kenya namely; Naivasha, Nanyuki, Timau and Murang'a. Naivasha is located in the Great Rift Valley in Kenya within Nakuru County [19]. It is 1890m above sea level, longitude 36° 22' E and latitude of 0°46' S. It experiences bi-modal rainfall distribution [20]. The soils are fertile, thus attractive to floriculturalists and

agriculturalists [19]. The soils here have sodium and pumice content, the latter making the soils permeable with low water-holding capacity [21]. The temperature ranges from 15.9-18.0^{o} C [22], a maximum of 37^{o}C and a minimum of 5^{o}C [20]. The coordinates for the study sites at Naivasha are 0^{o} 01 N 37^{o} 04' E. Nanyuki is located in the leeward side of Mt. Kenya. Its longitudinal extent is between 37^{o} 04'25" and latitudinal extent ranges between 015'S and 1^{o}00'N. It is 1947m above sea level, climatic conditions range from humid in the alpine zone to arid conditions in the lowlands.

2.2 Gall and soil sample collection

Crown gall tissues were collected from infected rose plants from four flower farms in four different ecological regions notably Nanyuki, Timau, Muranga and Naivasha. Gall tissues were collected from five greenhouses in each farm selected on basis of roses infected with the disease and packed in sterilized polythene bags. For soil samples collection was done in the greenhouses of the same farms and at each sampling point, two vertically crossing lines and two concentric circles of radius three metre were drawn. An auger of seven centimeter diameter was used to take four cores of soil in the outer circle. The five subsamples from five greenhouses in each farm were homogenously mixed to constitute a composite sample from which 500g soil was taken and placed in a sterilized polythene bags. All the samples were immediately transferred to Kenyatta microbiology laboratory. Special care was taken to the samples to avoid contamination.

2.3 Gall extraction

Galls were washed using tap water. With the help of a sharp sterilized blade, the galls were diced into small cubes (approximately 2 mm). The cubes were surface sterilized by immersion in 3% sodium hypochlorite (NaOCl) solution for 10-

20 minutes according to Schaad *et al.* [23] and then rinsed in sterilized distilled water three times to remove traces of sodium hypochlorite. Five cubes were crushed in one (1) milliliter of sterilized distilled water with the help of sterilized glass rod in a sterilized Petri plates to form a suspension which was kept undisturbed for ten minutes.

2.4 Isolation of *Agrobacterium tumefaciens*

A loopful suspension of gall extracts was then streaked on two different media that is, Yeast Extract Mannitol Agar (YEMA) supplemented with 25 ppm Congo red and Yeast Extract Peptone Agar (YEP) media. Plates were incubated at 27 ^{0}C for 18-24h and examined for growth and color development. Bacterial colonies were selected based on colonies form, elevation, surface and color. A single colony was picked up after incubation of plates at 27 ± 1°C in a Biochemical Oxygen Demand (BOD) incubator for five days and further re-streaked in fresh media and incubated for another twenty-four hours [23]. This exercise was repeated three times to get a pure single cell bacterial colony, which was transferred to Yeast Extract Mannitol Agar slants. The bacteria growth in each slant was checked after incubation of slants at 27±1^{o}C for five days in a BOD incubator. Isolates were purified on MGY agar media [23]. Purified isolates were cultured on Luria-Bertani (LB) medium described by Miller [25] and preserved in glycerol (25%) stock for further experimentation.

2.5 Isolation of *Agrobacteium radiobacter* from the soil

One gram of the soil particles from each of the sites was suspended in 9 milliliter of sterile distilled water. The suspension was then diluted to 10^{3} by serial dilution method. A loopful of suspension was streaked on Yeast Extract Mannitol Agar (YEMA) supplemented with 25 ppm Congo red dye and Yeast Extract Peptone Agar (YEP) media. A single bacterial

colony was picked up after incubation of plates at 27±1° C in a Biochemical Oxygen Demand (BOD) incubator for three to five days and further re-streaked on the same media in a fresh sterilized plate. The re-streaking after picking a single bacterial colony was repeated three times to get a pure colony [23]. An individual colony of each isolate was further transferred to slants of YEMA [26] which was then incubated at 27±1°C for five days.

2.6 Characterization of Agrobacterial isolates

Morphological characterization

The morphological characterization like color shape, gram staining was carried out for both isolates using 72h old pure cultures. For Gram staining, bacterial smears from 2-3 days colonies were prepared on clean microscope slides. The smears were air-dried and heat fixed by passing the slides over a Bunsen flame and then Gram stained as described by Beck *et al.* [27]. The slides were observed under oil immersion in a compound light microscope at magnification of × 400.

2.7 Biochemical characterization

The biochemical characteristics namely; salt tolerance test, urease test, catalase test, oxidase test, H_2 S production and motility test were carried out for both isolates.

2.8 Salt tolerance

For testing the salt tolerance of isolated cultures, Erlenmeyer flasks with 100ml of Yeast Extract Manitol (YEM) broth having three percent (3%) concentration of sodium chloride was used to isolate the fast growing and slow growing rhizobia from Agrobacterial isolates.

2.9 3-ketolactose test

Isolates of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* were streaked on lactose agar and incubated for 2 days at 28^0C. Visible growths were fully covered with Benedict's reagent. Formation of yellow precipitation around the growth of isolates was observed after 2 h.

2.10 Catalase Production Test

Fresh isolate was transferred to a clean slide using sterilized toothpicks, and thoroughly mixed with a small drop of sterilized distilled water. Next, a drop of 3% hydrogen peroxide (H_2O_2) was added on the smear. The smear was immediately covered with cover slip and bubbles formation was observed.

2.11 Urease Test

A volume of 5ml of Stuart's Urea broth was transferred to 30 ml universal bottle. Heavy inoculants were inoculated into the broth using sterilized toothpicks. All the universal bottles were fixed on orbital shaker for 24 h at 37^0C. Colorimetric change of broth, from yellow to fuschia color was observed.

2.12 Oxidase test

Isolates were streaked on Yeast Mannitol medium and incubated for 24 h at 28^0 C, Isolates were allowed to grow into visible mass and subsequently flooded with few drops of oxidase reagents (0.5 g Tetramethyl-para-phenylenediamine in 50 ml distilled water). The reagent was permitted to flow over growth and color formation was immediately observed after 3 minutes.

2.13 *In vitro* antagonistic activity of *Agrobacterium radiobacter*

The ability of the *Agrobacterium radiobacter* to produce diffusible metabolites was tested according to the agar well diffusion assay (AWDA) as reported by Rhouma *et al*., (2008).

Agrobacterium tumefaciens isolate (10^8 cfu/ml) was transferred individually to 50 milliliters of Luria-Bertani broth medium (LB broth) in a 250 ml Erlenmeyer flask and incubated by shaking at 100 rpm for 2 days at room temperature. Twenty milliliters (20 ml) LBA medium were poured into each sterile Petri dish. One (1) ml of bacterial suspension (10^8 cfu/ml) of *A. tumefaciens* was mixed with 3 milliliters of LBA (0.6 % agar) at 45°C and quickly overlaid on plates containing LB medium, in which wells of 6 millimeter diameter were punched aseptically with a sterile cork borer and a volume (100 µl) of *Agrobacterium radiobacter* was introduced into the wells.

The antagonist (*A. radiobacter*) culture from three different flower farms (Ol Jorowa farm, Likii River farm and Branan farm) coded as A, B and C respectively were shaken vigorously at room temperature (25 ± 2°C), using an orbital shaker at 15,000 rpm for 30 minutes One hundred (100) micro liter of each sample was then filtered through 0.45 micrometer filters under sterile conditions and filled into the wells. Simultaneously addition of saline solution instead of antagonist isolates was served as control. The experimental design was a completely randomized design replicated three times. Plates were incubated at 25°C and subsequently examined for haloes of inhibition around the wells, the size of which was recorded [29].

2.14 Pathogenicity test for *Agrobacterium tumefaciens* using carrot disc assay

The carrots used for the study were obtained from the local market, washed and sterilized with 10 % commercial bleach (NaOCl) followed by washing thrice with sterile distilled water. The carrots were then sliced into thin disc and each disc was overlaid with 100 microliters of inocula (10^8 cfu/ ml). Carrot disc treated with sterile saline solution was used as control. The Petri dishes were sealed by parafilm and incubated for three (3)

weeks at 28^0 C [30]. The discs were examined for development of young galls around meristematic tissue around the central vascular system after three weeks of incubation [31].

2.15 Biocontrol activity on rose plants

Greenhouse experiments were set up to test for *A. radiobacter* isolates antagonism on *A. tumefaciens*. The experiments were carried out in two trials where a randomized split plot design (4 treatments and 4 replicates using 4 rose varieties) was used for the study. Screening of isolates of *A. radiobacter* against *A. tumefaciens* was done by inoculating rose nursery stalk with both agrobacterial isolates. YEMA slants of three days old *A. tumefaciens* and *A. radiobacter* suspended separately in 10 ml sterile distilled water and shaken vigorously to give suspensions of 10^8 cfu/ml were used to conduct the experiment. The varieties of rose plants used for the study were Topsun, Fuschiana, Furiosa and H_3O. Rose flower stalks were wounded with a blunt cylindrical sterilized steel rod of two millimeter diameter at three different portions of the stem to a depth of three millimeter.

In the first treatment, each wounded rose plants were inoculated with 0.004 milliliter suspension measured using a micropipette of each *A. tumefaciens*; the second treatment, rose plant were inoculated with 0.004 milliliter *A. radiobacter* suspension and immediately wrapped with sterilized non-absorbent cotton. The third treatment, rose plants were inoculated with 0.004 milliliter *A. tumefaciens* and after 24 hours of inoculation, 0.004 milliliter suspension of non-pathogenic *(A. radiobacter)* containing 10^8cfu/ml was inoculated on the same wounds after removing the cotton. The wounds were wrapped again immediately with fresh sterilized non-absorbent cotton. The fourth treatment was a control (rose stalks treated with sterile water). This was done in all the four

variety of rose flowers. Wounds were examined for the presence or absence of galls and gall size after four weeks.

2.16 Data analysis

Quantitative data of inhibition zone and number of wounds showing galls and size of galls per replication were subjected to analysis of variance (ANOVA) and where significant, means were separated using Tukey's HSD test at 5 % probability level.

3. RESULTS

3.1 Isolation of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter*

Colonies appeared on the media within 2 days and attained full size in 4-5 days. On solidified agar such as Yeast Extract Mannitol Agar, the colonies of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* were white to cream colored, smooth, convex, glistening circular with entire edges and mucoid. All isolates stained pink in Yeast Extract Mannitol Agar supplemented with Congo red, others red in color. In Yeast Extract Peptone agar (YEP), *A. tumefaciens* isolates colonies were circular, slightly raised, and white to cream colored, translucent slime mucoid and had a smooth margin while *Agrobacterium radiobacter* isolates colonies on YEP were cream to yellow colored, white shiny mucoid and had a smooth, partially raised margin (Tables1and 2). After the Gram stain test, micrographs were observed at magnification of ×400 using an inverted microscope. For both isolates, bacteria were rod-shaped in appearance but for *Agrobacterium radiobacter*, the rods were slightly larger compared to *Agrobacterium tumefaciens*. The colony sizes for both isolates were ranging from 1.0 millimeter to 1.6 millimeter and generally there was a well pronounced growth within 24 hours.

Table 1: Morphological characteristics of *A. radiobacter*

Character	Yeast peptone agar	Yeast extract mannitol agar
Shape	Fluorescent convex	Circular
Color	Cream yellow, white shiny mucous	Red
Surface margin	Smooth, partially raised	Regular
Gram status	Negative	Negative

Table 2: Morphological characteristics of *A. tumefaciens*

Character	Yeast peptone agar	Yeast extract mannitol agar
Shape	Circular, slightly raised	Circular
Color	Cream white, translucent slime	Red/pink
Surface margin	Smooth	Smooth
Gram status	Negative	Negative

3.2 Characterization of pathogenic and antagonistic bacterial isolates

3.2.1 Biochemical test for the isolates

Isolates showed well pronounced growth in higher concentration of 3% sodium chloride, positive oxidase reaction, positive urease reaction, positive motility test and positive catalase test. 3-ketolactose test was carried out as differential procedure between the two strains of *Agrobacterium spp*. *Agrobacterium tumefaciens* isolates showed negative results for 3-ketolactose test while *Agrobacterium radiobacter* isolates showed positive results. For urease test, soil sample showed yellow coloration on the butt and pink coloration on the slant while for gall sample both slant and butt showed pink coloration. For Triple iron sugar test (TSI) on the isolates from soil samples showed pink coloration on the slant and yellow coloration on the butt while isolates from the gall sample showed pink coloration on the slant, gas bubbles at the base of slant and pink coloration on the butt.

Table 3: Biochemical characteristics of *A. tumefaciens* and *A. radiobacter* Isolates

Biochemical tests	*Agrobacterium tumefaciens* isolates				*Agrobacterium radiobacter* isolates			
	A	B	C	D	1	2	3	4
Motility test	+	+	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+	+	+
Urease test	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+
3-ketolactose test	-	-	-	-	+	+	+	-
Salt tolerance test	T	T	T	T	T	T	T	T

[Note +: positive, -: negative, T: tolerant

Agrobacterium tumefaciens isolates A-Ol Jorowa (Naivasha), B-Likii River farm (Nanyuki), C-Branan farm (Muranga) and D-Kisima (Timau).

Agrobacterium radiobacter isolates 1-Ol Jorowa, 2-Likii River farm, 3-Branan farm and 4-Kisima farm.

3.3 Pathogenicity test for *A. tumefaciens* isolates

Agrobacterium tumefaciens isolates from one flower that is, Ol Jorowa flower farm showed pronounced tumors when inoculated in carrot discs. Other isolates from other three flower farms showed no gall or few tumors on the carrot disc. Young galls (tumors) developed at meristematic tissue at the central part of the carrot disc after four weeks of inoculation. No symptoms were noted on un-inoculated control indicating that these strains isolated from *Rosa* spp. were pathogenic.

3.4 *In vitro* antagonistic activity of *A. radiobacter* against *A. tumefaciens*

The inhibition zones from three isolates from three different flower farms coded as A, B and C showed 0.6 mm, 0.53 mm and 0.37 mm in diameter and 0.00 mm in their controls respectively in Agar Well Diffusion Assay (Table 5). There was a significant difference (P= 0.05) in the zones of inhibition detected in antagonistic activity of *A. radiobacter* against A. *tumefaciens*. Isolates in farm A had larger inhibition zones compared to isolates in farm B and C.

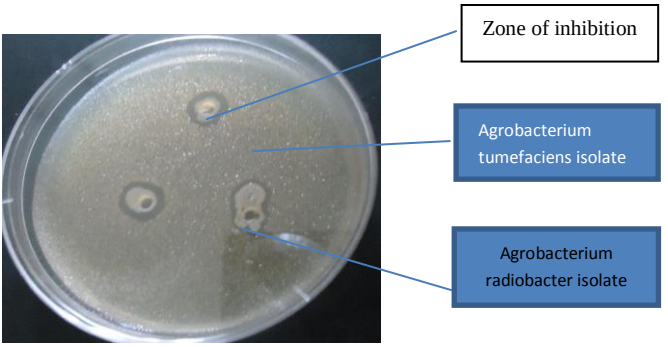

Figure 5: Antagonistic activity of *Agrobacterium radiobcter* isolates against *Agrobacterium tumefaciens*

Table 5: *In vitro* antagonistic effect of *Agrobacterium radiobacter* against *Agrobacterium tumefaciens*

Isolates (*A. radiobacter*)	Zone of inhibition (diameter in mm)
A	$0.600 \pm 0.12^{a*}$
B	0.530 ± 0.12^{a}
C	0.367 ± 0.09^{ab}
D (Control)	0.0 ± 0.0^{b}
p-value	0.0082

Means ± standard error (SE) separated using Tukey's Honest significant difference (HSD) test, *Means within the column followed by the same letters are not significantly different at $p< 0.05$.
Agrobacterium radiobacter isolates A- Ol Jorowa farm, B- Likii River farm and C- Branan farm.

3.5 Biocontrol activity on rose plants

3.5.1 Evaluation of *A. tumefaciens* and *A. radiobacter* isolates for their pathogenicity and antagonism on rose plants

Agrobacterium radiobacter isolates and *A. tumefaciens* isolates from Ol Jorowa farm (Naivasha) were used for antagonism and pathogenicity test. The *A. radiobacter* isolates from the Ol Jorowa farm were used for the study because of greater inhibition *in-vitro* compared to isolates in other farms. *Agrobacterium tumefaciens* isolate screened for their pathogenicity on rose plants showed tumor forming ability.

Agrobacterium radiobacter isolates resulted gall development control. Rose flower stocks treated with *A. tumefaciens* alone had gall sizes ranging from 1.0 cm, 0.57 cm and 1.0 cm in Top sun, Fuschiana and H_3O respectively. The gall sizes in their controls were 1.0 cm, 1.0 cm, 1.0 cm and 1.7 cm in Furiosa, Top sun, Fuschiana and H_3O respectively. There was a significant difference (P= 0.05) and (P= 0.05) on the treatment applied on rose flower plants in trial one and trial two respectively. Similarly, there was a significant difference (P= 0.05) (Table 6a) and (P= 0.05) (Table 6b) in terms of gall size with regard to the treatment applied on rose plants in trial one and two respectively.

Table 6a: Effectiveness of the antagonist (A. *radiobacter*) on tumor formation
TRIAL ONE

	Number of wounds with galls	Gall size (diameter in cm)
Treatment		
Agrobacterium radiobacter	0.1875 ± 0.1001^{ab}	0.06875 ± 0.0435^{b}
A. tumefaciens + A. radiobacter	0.0625 ± 0.0625^{b}	0.00625 ± 0.0063^{b}
Agrobacterium tumefaciens	0.4375 ± 0.1281^{ab}	0.36875 ± 0.1306^{ab}
Control	0.5625 ± 0.1281^{a}	0.79375 ± 0.2242^{a}
Variety		
Furiosa	0.2500 ± 0.1118^{a}	0.1563 ± 0.0584^{a}
Fushiana	0.3125 ± 0.1197^{a}	0.0723 ± 0.1500^{a}
H3O	0.3125 ± 0.1197^{a}	0.5063 ± 0.2020^{a}
Topsun	0.3750 ± 0.1250^{a}	0.4250 ± 0.1974^{a}
P- values		
Treatment	0.0127	0.0001
Variety	0.8959	0.0697
Variety* Treatment	0.7906	0.0224

Means ± standard error (SE) separated using Tukey's Honest significant difference (HSD) test, Mean values followed by the same lowercase within the same column are not significantly different (two-way ANOVA, α= 0.05).

Table 6b: Effectiveness of the antagonist (A. *radiobacter)* on tumor formation
TRIAL TWO

	Number of wounds with galls	Gall size (diameter in cm)
Treatment		
Agrobacterium radiobacter	0.1250 ± 0.0853^{b}	0.1125 ± 0.0774^{b}
A. tumefaciens + *A. radiobacter*	0.1118 ± 0.1188^{ab}	0.11875 ± 0.0564^{b}
Agrobacterium tumefaciens	0.6250 ± 0.1250^{a}	0.3563 ± 0.3563^{ab}
Control	0.5625 ± 0.1281^{a}	0.7625 ± 0.7625^{a}
Variety		
Furiosa	0.2500 ± 0.1118^{a}	0.0563 ± 0.02700^{b}
Fushiana	0.3750 ± 0.1250^{a}	0.3976 ± 0.1518^{ab}
H3O	0.5000 ± 0.1291^{a}	0.7500 ± 0.2405^{a}
Topsun	0.4375 ± 0.1281^{a}	0.1500 ± 0.0619^{b}
P- values		
Treatment	0.0131	0.011
Variety	0.5122	0.0010
Variety* Treatment	0.9700	0.0451

Means ± standard error (SE) separated using Tukey's Honest significant difference (HSD) test, Mean values followed by the same lowercase within the same column are not significantly different (two way ANOVA, $\alpha = 0.05$).

There was no significant difference (P= 0.05) (Table 6a) and (P= 0.05) (Table 6b) on the plants with galls after inoculation in trial one and trial two respectively. In determination of gall sizes on varieties of rose plants used in the study there was no significant difference (P= 0.05) (Table 6a) in trial one but there was a significant difference (P=0.05) in trial two. There was no significant difference (P= 0.05) (Table 6a) and (P= 0.05) (Table 6b) in the interaction between the four varieties rose plants used in the study and the treatment applied in trial one and two respectively.

4. DISCUSSION

The present study reveals the *Agrobacterium radiobacter* isolates from naturally occuring soils in Kenya had antagonistic effect against *Agrobacterium tumefaciens* causing crown gall disease on roses. *In vitro* antagonistic test showed that, there was a significant difference in antagonistic test for *A. radiobacter* with *A. tumefaciens* Isolates (P=0.05) from the

three flower farms. Creation of inhibition zones by *A. radiobacter* against *A. tumefaciens* is an indication that it can be used in the management of galls through antibiosis process. The non-pathogenic strain competes for food and space in mixed inoculations preventing the pathogenic bacterium from becoming established as reported by Farrand [32]. Various microorganisms with antagonistic activity against phytopathogens have been isolated from suppressive soils. In these soils pathogens are either unable to persist or cause low damage to plants and antagonistic microorganism account for a large part in elimination of plant disease. A similar study has been conducted by Mazzol [33].

The non-pathogenic isolates used on the study were isolated from the soils and substrates where rose varieties were planted on greenhouses. *Agrobacterium radiobacter* constitutes important agents for bio-control of soil-borne disease and for plant growth promotion as reported by Rajkumar *et al*. [34].

The greenhouse experiments for the pathogenicity and antagonism test using *A. tumefaciens* and *A. radiobacter* respectively showed there was positive interaction between the treatment and plant varieties on the number plants with galls. In terms of gall sizes there was a negative interaction between the treatment and varieties of rose plants used in the study.

Kawaguchi *et al*. [35] reported the efficacy of non-pathogenic strains *Agrobacterium vitis* VAR03-1 on biological control of crown gall of rose (*Rosa multiflora*) that effectively controlled the crown gall caused by tumorigenic *Agrobacterium tumefaciens*. Benjama *et al*. [36] tested 206 Moroccan isolates of pathogenic *A. tumefaciens* under *in vitro* conditions for their sensitivity against *A. radiobacter* strain K-84 and K-1026 and obtained that strain K-1026 of *A. radiobacter* was more effective than strain K-84.

Results of this study can be an important step in formulating *A. radiobacter* as a commercial biopesticide and

apply it on a larger scale and also establish its ability for root colonization and survival in the rhizosphere. This biological control is solely preventative for the control of crown gall disease of roses. We conclude that *Agrobacterium radiobacter* isolates from naturally occurring soils can offer it as a sustainable yet indigenous biocontrol agent. Thus, an appreciable economic loss and budget incurred on import of synthetic pesticides and their far reaching health hazards could be safeguarded.

5. CONCLUSION

The study showed that crown gall is still one of the important diseases often limiting nursery and greenhouse production of rose flowers in Kenya. Agrobacteria causing this disease are soil-borne pathogens commonly occurring in the soils and other natural environments. In carrot disc assay test, tumor forming ability of isolates from the gall sample was an indication that the isolates were virulence.

The pathogenicity and antagonism test using *A. tumefaciens* and *A. radiobacter* respectively showed there was positive interaction between the treatment and plant varieties on the number plants with galls. In terms of gall sizes there was a negative interaction between the treatment and varieties of rose plants used in the study.

Agrobacterium radiobacter constitutes important agents for bio-control of soil-borne disease. The study therefore confirmed that use of naturally occurring *A. radiobacter* isolates had the potential in the management of crown gall diseases of rose flower stocks in Kenya.

Conflict of Interests

The authors state that there is no conflict of interest.

Authors' Contributions

1. Judith Gitari

Concept development and designs, data collection, data analysis

2. John Maingi

Correction and final approval of publication

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