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Antibacterial effect of Artemisia and ginger extracts in controlling *Agrobacterium tumefaciens* in roses

Alfred Njagi¹, Methuseloh Mang'erere Nyamwange²,
Ezekiel Mugendi Njeru², Jonah Kiprono Birgen¹

¹Department of Plant Sciences, Kenyatta University, P. O. Box 43844-00100, Nairobi, Kenya, ²Department of Biochemistry, Microbiology and Biotechnology, Kenyatta University, P. O. Box 43844-00100, Nairobi, Kenya

ABSTRACT

Rose is the world's most traded cut flowers with 74 % being produced in Kenya. Pests like spider mites, caterpillar, aphids, thrips, nematodes and diseases such as crown gall, downy mildew, powdery mildew and botrytis highly compromise rose production. Crown gall disease caused by *Agrobacterium tumefaciens* is the most problematic disease of roses in Kenya, causing production loss of up to 60 % depending on the age and variety of rose. An experiment to determine the antibacterial effect of Artemisia and ginger extracts was carried out *in vitro*, where the inhibitory zones around filter discs soaked with extracts on Muller Hinton agar was established. The extracts were emulsified with dimethylsulfoxide (50 %) and minimum inhibitory concentrations of Artemisia (125 mg/ml), ginger (62.5 mg/ml) and mixture of Artemisia and ginger (31.25 mg/ml) were used to soak the filter discs whereas the commercial recommended rate of copper hydroxide of 6.25 mg/ml was used. Artemisia and copper hydroxide (commercial antibiotic) had highest inhibition zone of 12.80 mm compared to ginger 10.60 mm. Mixture of Artemisia and Ginger had slightly lower inhibition zone (10.20 mm) though not significantly different from ginger ($P > 0.001$). An eight-month greenhouse experiment was also done to determine the efficacy of the extracts on inoculated rose plants. The results showed that crown gall incidence and gall weight were low but not significantly different from copper hydroxide and Artemisia. Crop vigor, which was indicated by stem length was highest for Artemisia treatment with an average of 73.54 cm followed by copper hydroxide (67.25 cm) while ginger and mixture of ginger and Artemisia had 53.44 cm and 64.70 cm respectively. From the results of this research, Artemisia and Ginger extracts are promising alternative to control crown gall and possibly other diseases in field crops. Artemisia performance compares well with copper hydroxide and therefore the best alternative to replace copper hydroxide.

Key words: Roses, Crown gall, Artemisia, ginger, *Agrobacterium tumefaciens*

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***Corresponding Author:**

Methuseloh Mang'erere

Nyamwange,

E-mail: nmethu1992@gmail.

com

INTRODUCTION

Rose production in Kenya is hampered by diseases such as crown gall disease, caused by *Agrobacterium tumefaciens* (Furuya *et al.*, 2004). The bacteria are found worldwide and affect species belonging to over ninety-three plant families (Kado, 2002; Rhouma *et al.*, 2006). In Kenya, the first crown gall incidence was noted in 1998 but has currently spread across all rose production regions (Arim, 2011). Crown gall infected flowers show galls, slow growth, stunting, leaves chlorosis and general decreased production (Agrios, 2005). Depending on age and variety of flowers, about 60% production loss is possible following infestation by *Agrobacterium tumefaciens* (Arim, 2011).

Currently, there is no effective conventional pesticide for crown gall control. However, dipping the roots and the crown of rose

seedlings in a solution containing nonpathogenic *Agrobacterium radiobacter* bacteria strain 84 before planting offers protection (Kado, 2002; Arim, 2011), though many farmers have reported re-infection, implying that *Agrobacterium radiobacter* offers short-lived protection. Copper compounds are widely used by farmers as conventional chemicals against crown gall although its usage in disease management is seldom satisfactory owing to pathogen developing resistance, chemical residues and phytotoxicity reported in many plant species (Agrios, 2005).

Breeding for pest and disease resistance in roses has not been explored because varieties grown depend on dynamic customers' tastes and preferences. Moreover, some rose varieties remain in the market for a very short period, making breeding for resistant uneconomical (Arim, 2011). Careful cultural activities, control of chewing insects and nematodes prevent

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natural wounds that form the route of infection for the roses (Burr and Otten, 1999). Other methods which have been used for controlling plant pathogenic microorganisms include using synthetic fungicides and bactericides. However, extensive reliance on these chemicals has brought about disease and pests' resistance (Brent and Holloman, 1998). Further, most synthetic chemicals have been forbidden in many countries owing to their toxicity and negative impact on yields and environment, therefore the need to turn to organic chemicals as alternative (Arim, 2011).

In this study, we hypothesized that the antimicrobial effects of Artemisia and ginger extracts control crown gall disease in roses by *Agrobacterium tumefaciens*. The specific objectives of the study were to (1) to determine whether Artemisia and Ginger extracts inhibit growth of *Agrobacterium tumefaciens in vitro*; (2) to determine the efficacy of Artemisia and Ginger extracts in management of crown gall disease in the greenhouse.

MATERIALS AND METHODS

Study Site

Isolation and characterization of *Agrobacterium tumefaciens*, extraction of Artemisia and ginger rhizome crude extracts as well as *in vitro* testing of antibacterial effects on *Agrobacterium tumefaciens* was carried out at Kenyatta University Microbiology Laboratory, Kiambu County in Kenya. Greenhouse experiments were set up in Gatoka farm (1631 meters above sea level) in Muranga County. The region has two rainfall seasons with annual rainfall of 835 mm and temperature range of 21 to 35 °C. The soils are predominantly deep loamy, well weathered and very fertile. The main agricultural products from the county include flowers, coffee, fruits, maize, beef and dairy (Ntale and Litondo, 2013).

Isolation and Characterization of *Agrobacterium Tumefaciens*

Young actively growing galls from a rose plant were surface sterilized with 3 % sodium hypochlorite and rinsed several times with sterile water. The galls were grinded using sterile mortar and pestle, centrifuged at 70 rpm for 30 minutes. The supernatant was inoculated on Congo red yeast extract mannitol agar which was then incubated at 28 °C for ten days (Aysan and Sahin, 2003). Red stained bacteria colonies from Congo red yeast extract mannitol agar was inoculated on selective Yeast peptone glucose agar, incubated in dark at 28 °C for two days and growth analyzed. Gram stain and biochemical tests such as motility, urease, citrate, catalase and Ketolactose enzyme production were performed on isolated colonies.

Extract Preparation and Phytochemical Analyses of Plant Extracts

Fresh Artemisia leaves were thoroughly washed under running water, air dried for seven days at room temperature and ground into fine powder using a sterile mortar and pestle. Ginger

rhizomes, acquired from supermarket were dried in an oven at 40 °C for 72 hours. The powdered materials were separately soaked in methanol at the ratio of 1:4, poured in an air tight plastic and kept in refrigerator at 4 °C for 48 hours (Agbo et al., 2000). The mixtures were filtered with cheese cloth, followed by Whatman filter paper, concentrated separately to remove methanol using Rotary Evaporator in a vacuum at 40 °C until 10 % of their original was achieved (Atata et al., 2003; Mariita et al., 2010). The extracts were further concentrated to complete dryness in a water bath and transferred to vials which were kept at 4 °C for laboratory uses. Chemical tests to identify the bioactive constituents in the Artemisia and Ginger extracts were carried out using the standard procedures (Trease and Evans, 2012).

Determination of Minimum Inhibitory Concentrations (MICs)

Minimum Inhibitory Concentration was achieved through broth micro dilution, using a 96- well microtiter plate in accordance with CLSI guidelines (Wayne, 2011). Extracts were initially emulsified in 50 % Dimethylsulfoxide (DMSO) solution. Through serial two-fold dilutions; Ginger, Artemisia and mixture of ginger and Artemisia extracts of concentration 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.6 mg/ml, 7.81 mg/ml and 3.9 mg/ml were put in different wells. Copper hydroxide was used as a positive control at manufacturers recommended rate of 62.5mg/ml whereas DMSO was used as a negative control. The set up was done in triplicate. About 0.5ml of *A. tumefaciens* inoculum was dispensed in the wells of micro titer plate containing the extracts and incubated at 37 °C for 24 hours. Growth of bacteria was examined as a function of turbidity using Varioscan Flash (Kelava and Cavar, 2011).

Assay of Antimicrobial Activity using Disc Diffusion Method

Two days old *A. tumefaciens* suspension was swabbed on Petri dishes containing sterilized Muller Hinton Agar. Sterile filter discs were soaked in MIC solutions of Ginger, Artemisia and mixture of Ginger and Artemisia. The extracts impregnated discs were air dried and placed on the agar plates using sterile forceps. The set up was done in triplicate. The plates were maintained for 2 hours at 4 °C and then incubated for 24 hours at 37 °C. After incubation, the average mean diameters of inhibitory zones formed around each disc were computed in millimeters.

Greenhouse Experimental Design

The experiment was laid out in a completely randomized design with six treatments and five replicates for each treatment. Seedlings were transplanted into individual buckets of diameter 20 cm and 4.5litres volume. The buckets contained soil that was sterilized by autoclaving for 15 minutes at 121 °C at 15 psi to remove soil borne pests and diseases. Treatments with Artemisia extract, Ginger extracts, Mixture of ginger and Artemisia and

copper hydroxide was done once per week for nine months from January to September, 2016. Two controls, one with plants inoculated with *A. tumefaciens* and was not treated and the other with non-inoculated plants that as well received no treatments was set up. Irrigation and sprays for other diseases and pests were done as programmed. Records of disease incidence, weights of the galls and length of harvested stems were evaluated at three months interval for ten months.

Inoculation of the Seedlings

Less than three days old YEMA slant of *A. tumefaciens* was suspended in 10ml of sterile distilled water. The suspension was then shaken vigorously to give a suspension of 10^8 cfu/ml. A sterile steel wire was used to make 3 mm deep wounds at three locations on the seedling stems where 0.004 ml of *A. tumefaciens* suspension was inoculated. The wounds were then wrapped with water-soaked cotton wool for one week when successful inoculation was expected to have taken place.

Data Analyses

The results were subjected to Analysis of Variance (ANOVA) using Minitab software to test the significant difference of plants extracts in bacterial growth inhibition (*in vitro*). The efficacy of the plant extracts in the greenhouse was tested through analysis of the disease incidence and severity based on weight of the galls and the stem length (Moore, 1997; Masood *et al.*, 2010). Readings of the above parameters was recorded and analyzed for the month of March, May, July and September of the year 2016. Significant means were separated using Tukey's test.

RESULTS

Isolation and Morphological Characterization of *Agrobacterium Tumefaciens*

The inoculums from the crown galls absorbed Congo Red dye from yeast extract mannitol agar (YEMA). Mannitol in the YEMA was the carbon source while the yeast extract was the nitrogen source and growth factors for *Agrobacteria tumefaciens*. Magnesium in the medium provided cations required for bacterial growth. The red stained isolates from YEMA, when streaked on yeast peptone glucose agar established as whitish-cream, raised-convex with smooth margins. The contaminant colonies from the galls neither absorbed Congo Red dye nor established on peptone glucose agar.

Inhibition Growth of *Agrobacterium Tumefaciens In vitro* by Artemisia and Ginger Extracts

Mean inhibition zones of *A. tumefaciens* around the disc soaked in Artemisia extract and that soaked in copper hydroxide were the same (12.80 mm). These zones were significantly higher ($P < 0.001$) than mean zones of inhibition by ginger (10.60 mm) and the mixture of ginger and Artemisia (10.20 mm) as shown in Table 1.

Table 1: Zones of inhibition of *Agrobacterium tumefaciens in vitro*

Treatment	Mean zone of inhibition
Ginger	10.60 (0.67) b
Copper hydroxide	12.80 (0.37) a
Control (DMSO)	6.00 (0.00) c
Ginger+Artemisia	10.20 (0.37) b
Artemisia	12.80 (0.37) a

The mean standard errors are presented in parentheses. Values followed by the same letter in the same column are not significantly different at $P < 0.05$ (Tukey's HSD test)

Efficacy of Artemisia and Ginger Extracts in Management of Crown Gall Disease in the Greenhouse

Stems harvested in March from all treatments did not vary in length (Table 2). In May, lengths of stems harvested from artemisia (79.22 cm) and from uninoculated (78.41 cm) treatments were significantly ($P < 0.001$) taller than those from other treatments. In July, the length of stems from artemisia (77.84 cm) and uninoculated (78.23 cm) were significantly ($P < 0.001$) taller than the other treatments. In September, the length of stems from artemisia treatment (79.44cm) and uninoculated plant (77.42 cm) were significantly taller ($P < 0.001$) than the stems from other treatments as shown in Table 2.

Comparison of gall weights from various treatments showed a significant difference ($P < 0.001$) in the gall weights as shown in Table 3. The negative control (uninoculated plants) had significantly ($P < 0.001$) lower gall weights than other treatments. Inoculated plants that were not treated had the highest gall weight of 52.94 g while the non-inoculated plants had the least gall weight of 0.06 g. The gall weights obtained from Copper hydroxide, artemisia, ginger and mixture of ginger and artemisia were not significantly different (Table 3).

DISCUSSION

Isolation and Characterization of *Agrobacterium Tumefaciens*

Agrobacterium tumefaciens isolates from crushed fresh galls absorbed Congo red dye and showed a well pronounced growth in glucose peptone agar. The isolates were gram negative rods, motile, catalase positive, urease positive, citrate positive and exhibited negative reaction to both indole and hydrogen sulfide tests. These properties were in agreement with those reported by Islam (2010), for physical and biochemical characteristics of *A. tumefaciens*. Unlike most bacteria, *Agrobacterium tumefaciens* is known to produce ketolactose enzyme which causes precipitation of cuprous oxide in Benedict's reagent found in lactose broth, differentiating the isolates from *Rhizobium* species (Aysan and Sahin, 2003).

In vitro and *In vivo* antibacterial Activity of Ginger and Artemisia Extracts

All plant extracts produced zones of inhibition greater than 9 mm against *Agrobacterium tumefaciens*, hence the extracts

Table 2: Length of stems harvested (cm) in the greenhouse in different treatments

Treatment	March	May	July	September
Ginger	47.82 (0.52) a	62.08 (0.62) d	55.02 (0.43) c	48.84 (0.38) d
Artemisia	57.63 (0.47) a	79.22 (0.88) a	77.84 (0.62) a	79.45 (0.67) a
Copper hydroxide	48.42 (0.55) a	74.09 (0.83) b	70.63 (0.52) b	75.87 (0.49) b
Ginger + Artemisia	50.67 (0.45) a	69.45 (0.65) c	74.28 (0.58) b	64.41 (0.42) c
Inoculated	47.22 (0.48) a	60.84 (0.54) d	54.24 (0.38) c	47.48 (0.35) d
Uninoculated	59.81 (0.49) a	78.41 (0.85) a	78.23 (0.64) a	77.42 (0.58) a
P-value	0.056	0.001	0.001	0.001

The mean standard errors are presented in parentheses. Values followed by the same letter in the same column are not significantly different at $P < 0.05$ (Tukey's HSD test)

Table 3: Gall weights harvested in the greenhouse in different treatments

Treatment	Mean gall weight (g)
Copper hydroxide	30.57 (1.55) b
Ginger + Artemisia	32.29 (1.36) b
Ginger	33.19 (1.82) b
Artemisia	32.47 (1.39) b
Inoculated	52.94 (1.06) a
Non-inoculated	0.06 (0.06) a

The mean standard errors are presented in parentheses. Values followed by the same letter in the same column are not significantly different at $P < 0.05$ (Tukey's HSD test)

are considered active (Celikel and Kavas, 2008). Both Artemisia and Copper hydroxide had similar antibacterial effect with an inhibition zone of 12.8 mm. The high contents of phenols and flavonoids in artemisia extracts could be responsible for inhibition of growth of *A. tumefaciens*. Ginger and mixture of ginger and Artemisia also had remarkable activity against *A. tumefaciens*. This further demonstrates that a mixture of extracts from both ginger and artemisia could be used to control *A. tumefaciens* infection. Dimethylsulfoxide (negative control) did not have any antibacterial activity. In another work done on *A. Absintium* in USA, the extracts exhibited zones of 13 mm which is comparable with the results of this study (Mikicinski, 2012).

The disease severity, which was showed by weight of the galls and harvested stem length was indirectly related to the size of zone of inhibition achieved by the extracts *in vitro*. As such, the treatment with the biggest zone of inhibition had the least incidence and the treatment with the smallest zone had the highest incidence. The higher the galls weight, the higher the severity of the crown gall. These results were in agreement with work done by Masood (2010). The performance of the mixture of ginger and artemisia was significantly lower than its individual plant extracts, a possibility of antagonism of secondary metabolites during their activity. Crop growth vigor, analyzed through getting the average length of harvested stem also followed the same trend as the gall weights. As such, the higher the gall weight the shorter the stem length and vice versa.

The antibacterial effects noted *in vitro* and *in vivo* were attributable to the secondary metabolites in *Artemisia annua* and *Zingiber officinale* extracts. Flavonoids, one of the phenolic compounds found in both *Artemisia annua* and *Zingiber*

officinale extracts has been found to have antimicrobial activity against plant pathogens *in vitro* (Cowan, 1999). They work by forming antibacterial complexes with extracellular soluble proteins and bacterial cell wall in response to infection (Trease and Evans, 2012; Orhan *et al.*, 2010). If a combination of phenolic compounds is present, like the case in *Artemisia annua* (flavonoids and tannins), they work synergistically to achieve better antimicrobial activity (Yadav and Agarawala, 2011). Due to this fact, the antibacterial effect of *Artemisia annua* is better than *Zingiber officinale* since the latter did not contain tannins.

Tannins act by binding the poline rich protein that interferes with protein synthesis (Sanchez *et al.*, 2015). They also affect microbes through enzyme inhibition and substrate deprivation. Alkaloids act by interfering with the impulse transmission, alters the cell membrane while others affect synthesis of functional protein (Creelman and Mullet, 1997). Saponins bind with the cell membrane cholesterol and form complexes that make pores on the cell surface of the pathogen (Gauthier *et al.*, 2009; Melzig *et al.*, 2011).

CONCLUSIONS

Ginger and Artemisia extracts demonstrated antibacterial effects both *in vitro* and *in vivo*, pointing a possibility of replacing synthetic chemicals with effective plant compounds in managing crop diseases and pests. In the greenhouse experiments, the performance of artemisia extract was significantly better than that of ginger and the mixture of ginger and artemisia. The performance of the mixture of ginger and artemisia was lower than its individual plant extracts, demonstrating a possibility of antagonism of the secondary metabolites. The performance of copper hydroxide and that of artemisia extracts were not significantly different signifying the possibility of replacing copper hydroxide with artemisia extracts.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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