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Comparative Antibacterial Effects of Raw Extracts and Essential Oils of *Ocimum gratissimum* L. against *Ralstonia solanacearum* (Smith)

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ABSTRACT

Raw and processed products of *Ocimum gratissimum* reportedly contain some antibacterial effects. In this study, raw plant extracts and essential oils derived from leaves of *Ocimum gratissimum* were screened for their antibacterial properties on *Ralstonia solanacearum,* the causal agent of bacterial wilt in Irish potato at varying concentrations. The raw extracts were obtained through sequential cold extraction using methanol solvent while the essential oils were extracted through steam distillation. The test products were used at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg mL^{-1}. Antibacterial tests were done using paper disc diffusion inhibition method and antibacterial activity was determined by measuring the size of inhibition zones. The results showed that the five different concentrations of essential oils and plant extracts exhibited highly significant ($p<0.0001$) differences on their effects against growth of *R. solanacearum*. The study confirmed the antibacterial effects of *O. gratissimum* and further demonstrated that the active compound (s) against *Ralstonia solanacearum* is (are) concentrated in the essential oils.

Key words: *Ocimum gratissimum*, *Ralstonia solanacearum*, essential oils, raw extracts, antibacterial effect

INTRODUCTION

Irish potato or cultivated potato (*Solanum tuberosum* L.) is an important crop worldwide providing food, income and employment to over 800 million people globally (Hoffler and Ochieng, 2008). Kenya is the fifth biggest potato producer in Sub-Saharan Africa, with an output of 790,000 tonnes in 2006 (Muthoni and Nyamongo, 2009). The crop ranks as the second most important food crop after maize in Kenya (Felix *et al.*, 2010). It grows in cool and high altitude parts of the country where rainfall is well distributed including slopes of Mt. Kenya and both sides of the Aberdare ranges. The crop is also grown on the highlands on Mau Escarpment, Tinderet, Nandi Escarpment and Cherangani hills (Muthoni and Nyamongo, 2009). The cultivated potato provides high energy content and is easy to produce making it an important part of agriculture (Hoffler and Ochieng, 2008). Potato production is much higher in developed than in developing countries and diseases have been cited as one of the major limiting factors (Muthoni and Nyamongo, 2009; Lemaga *et al.*, 2001; Mureithi, 2000; Otipa *et al.*, 2003).

One of the most destructive diseases known to attack potato plants is bacterial wilt caused by *Ralstonia solanacearum* race 3, biovar 2 (Momol *et al*., 2000). The disease, also known as brown rot, southern wilt, sore eye or Jammy eye, is a major limiting factor to potato crop production in most countries. Other hosts of *R. solanacearum* include economically important crops such as tobacco, pepper, banana, beans, tomato and eggplant (Hayward, 1991; Norman *et al*., 2009). The pathogen invades the roots of the host plant and aggressively colonizes the xylem vessels causing a lethal wilting (Tahat *et al*., 2008). In Kenya, bacterial wilt was first reported in 1940 s and since then it has spread to most potato growing regions. It has been reported that the diseases causes losses ranging from 30 to 70% at altitudes ranging from 1800 to 2800 m (Otipa *et al*., 2003). The disease is considered more serious than even late blight as there are no chemical control procedures and many farmers do not know how to control it (Muthoni and Nyamongo, 2009). In addition, some of the recommended control measures such as crop rotation, use of clean seeds, planting in non-infested soils and growing tolerant varieties (Tahat and Sijam, 2010) have individual practical, technological and economic limitations (Lemaga *et al*., 2001; Muthoni and Nyamongo, 2009; Jinnah *et al*., 2002; Khalequzzaman *et al*., 2002).

The increasing antibiotic resistance of some pathogens that are associated with diseases has increased the interest in the development of new types effective and nontoxic antimicrobial compounds (Sobhy and El-Feky, 2007). Subsequently, the use of agrochemicals is becoming less favourable (Bonjar *et al*., 2006) while the use of plant extracts and phyto-products is gaining attention due to their proven nature specificity, biodegradability, low toxicity and minimum residual toxicity in the ecosystem (Ogbo and Oyibo, 2008; Sobhy and El-Feky, 2007). Much curiosity has been devoted to the Lamiaceae family, especially *Ocimum gratissimum* L. considered as one of the main sources of potential active metabolites (Louis *et al*., 2011). Previous studies have shown that *O. gratissimum*) contains antimicrobial principles (Wagura *et al*., 2011; Lemos *et al*., 2005; Iwalokun *et al*., 2003; Adamu *et al*., 2009). Ocimum oil has been reported to be active against plant pathogens such as *Phytophthora palmivora, Alternaria brassicicola, Aspergillus flavus, Bipolaris oryzae, Fusarium moniliforme, Fusarium proliferatum, Pyricularia grisea* and *Rhizoctonia solani* (Piyo *et al*., 2009). Ocimum oil has also been shown to contain active principles against several species of animal and human bacteria (Sunita and Mahendra, 2008; Akinyemi *et al*., 2004; Lemos *et al*., 2005; Lopez *et al*., 2005).

Guided by the information on potato yield losses caused by bacterial wilt disease, difficulties in the control of the pathogen and the reported antimicrobial activities of *O. gratissimum*, this study was set up to compare the antibacterial effects of raw extracts and essential oils of *O. gratissimum*.

MATERIALS AND METHODS

The study was conducted at Maseno University between September, 2006 and November, 2007. Approximately 4 kg fresh leaves of *O. gratissimum* were collected from Maseno region in Western Kenya and divided into two heaps; one for extraction of plant extracts using methanol solvent and the other for extraction of essential oils. The plucked leaves were washed thoroughly 2 to 3 times with running tap water and once with sterile distilled water.

Extraction of plant extracts: One heap of leaves was shredded and dried as described by Okigbo and Ogbonnaya (2006). They were then ground into fine powder at Kenya Sugar Research Foundation in Kisumu, Kenya in readiness for solvent extraction. Cold extraction of the powdered plant materials using methanol solvent was done sequentially following the method of Eaton

(1989). Known quantity of dry ground leaf material was soaked in the solvent in Erlenmeyer flask and left for four days with occasional shaking. The liquid portion was then filtered using Whatman No.1 filter paper. The filtrate was then concentrated *in vacuo* in a round-bottomed flask using rotary evaporator at 60°C (Junaid *et al.*, 2006). The extracts obtained were kept in vials in readiness for bioassay tests (Eaton, 1989; Llorach *et al.*, 2003).

Extraction of essential oils: The second heap of leaves was air dried under shade, chopped into small pieces and subjected to steam distillation for three h. The distillate was then extracted with petroleum ether which was removed carefully and 34 g of essential oil obtained. The essential oils were diluted with dimethyl sulphoxide to make test concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg mL^{-1} and then stored at-20°C until required (Mbata and Saikia, 2005).

Isolation of wilt bacteria from infected potato tubers: Infected tubers were obtained from a test plot at the National Agricultural Research Laboratories (NARL) fields, Nairobi. These were cleaned under running water to remove adhering soil, air-dried, then cleaned using 97% ethanol to remove any microorganism on its surface. The skin at the end of the stolon was removed using a disinfected scalpel to make vascular tissues visible. A bacterial suspension was prepared using the method described by Priou *et al.* (1999). Approximately 0.5 mL of the bacterial suspension was spread on nutrient agar in Petri dishes. The plates were incubated for 48 h at 28°C and bacterial colonies that were fluidal, flat, pearly white and irregular identified.

Pathogenicity test: The method of Koch's postulates was performed with *Solanum tuberosum* var. Tigoni 381381 as the host. After a 24 h period without water, one side of some potato roots were injured one centimetre from the stem and approximately 20 mL of an aqueous suspension of *R. solanacearum* of 1×10^{7} cfu mL^{-1} was poured around the base of the stem. Five days after inoculation (after the wilting symptoms were exhibited), vascular flow test was run by cutting a piece of potato stem (5 cm long) and suspending it in clear water in a glass container. The cut stem was held with a clip to keep it in a vertical position until smoke like threads streamed downwards from the cut stem (Priou *et al.*, 1999).

Antibacterial assay: The antibacterial effects of the raw extracts and essential oils against *R. solanacearum* were evaluated using the method described by Barry *et al.* (1979) and Souza *et al.* (2005). Inoculation was done by rubbing a sterile cotton swab containing the pathogen on the surface of solidified agar as described by Linnette *et al.* (1974).

Experimental design, data recording and analysis: The experiments were laid down in Randomized Complete Block Design (RCBD) with four replications. The antibacterial activity was recorded as the width (in mm) of clear zones of inhibition surrounding the diffusion discs after 48 h (Reiner, 1982; Baker *et al.*, 1983; Deans and Ritchie, 1987). The data were subjected to ANOVA using SAS version 9.1 and effects declared significant at 5% level. Separation of means was done only for those parameters where the ANOVA was significant, using Least Significant Difference at 5% level of significance [$LSD_{5\%}$] (Steel and Torrie, 1980).

RESULTS

Results of the inhibitory effects of essential oils and plant extracts of *O. gratissimum* against *R. solanacearum* are shown in Fig. 1. The presence of inhibition zones (Fig. 2a-c) depicted

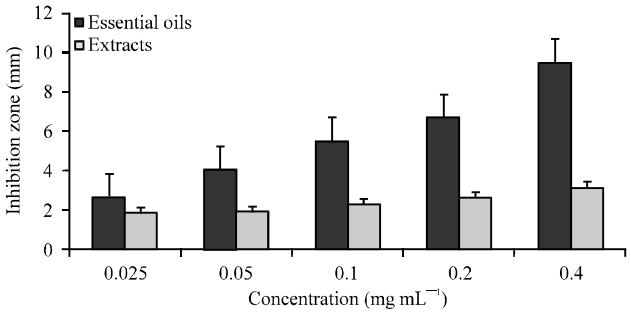

Fig. 1: Antibacterial effects of *O. gratissimum* extracts and essential oils

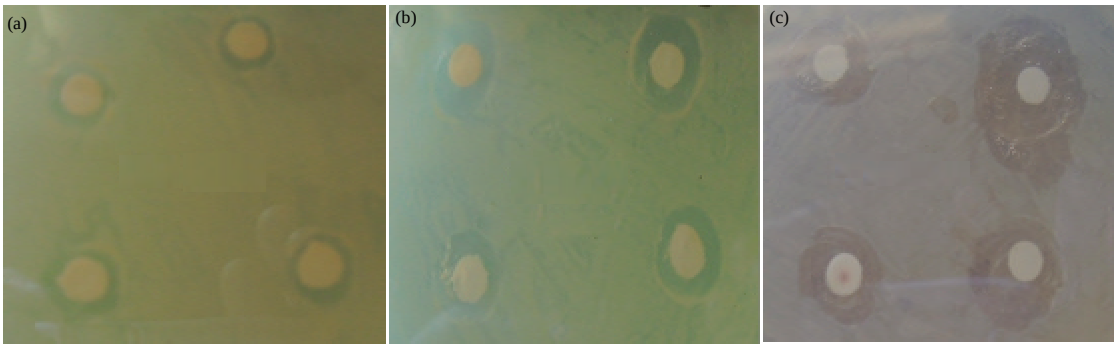

Fig. 2 (a-c): Inhibitory effects of *O. gratissimum* essential oils; (a) 0.05 mg mL^{-1}, (b) 0.2 mg mL^{-1} and (c) 0.4 mg mL^{-1}

antibacterial activity of *O. gratissimum* extracts and essential oil on *Ralstonia solanacearum*. Analysis of variance showed that the five different concentrations (0.4, 0.2, 0.1, 0.05 and 0.025 mg mL^{-1}) of essential oils and plant extracts exhibited highly significant ($p<0.0001$) differences on their effects against growth of *R. solanacearum* (Fig. 1). In both cases, antibacterial activity of the test materials increased as their concentration increased but essential oils were more effective than the plant extracts (Fig. 1).

The inhibitory effects of essential oils increased almost exponentially with every double increase in concentration with each concentration producing significantly different ($p<0.05$) effect from all the rest. The inhibition zones obtained were also distinctively clear and easy to measure (Fig. 2). The highest concentration of 0.4 mg mL^{-1} demonstrated the highest antibacterial activity with inhibition zone of 10.12 mm while the lowest concentration of 0.025 mg mL^{-1} resulted in the smallest inhibition zone of 2.75 mm (Fig. 1). There was no inhibition zone around the discs treated with pure dimethyl sulphoxide (not shown).

The plant extracts were not as effective as the essential oils though they also demonstrated some inhibitory/antibacterial effects against *R. solanacearum*. In fact, the highest concentration of the plant extracts performed statistically the same as the lowest concentration of essential oils. However, like essential oils, the effects of plant extracts were also found to increase with increase in concentration though the increase was not as pronounced as in the case of essential oils. The two lowest concentrations of 0.025 and 0.05 mg mL^{-1} exhibited statistically similar activity where inhibition zone measured 1.84 mm and 1.88 mm respectively. Their efficiency increased marginally to 2.3 mm and then to 2.6 mm when the concentration was raised to 0.1 and 0.2 mg mL^{-1}, respectively. The best results of 3.13 mm were obtained at 0.4 mg mL^{-1} (Fig. 1).

DISCUSSION

The study confirmed the antibacterial effects of *O. gratissimum* as previously reported by other researchers. The presence of antibacterial substances in the plant extracts which caused the inhibition of radial growth *in vitro* agree with reports of other studies (Olayinka, 2009; Mbata and Saikia, 2005). Antibacterial effects of *O. gratissimum* extracts have also been reported by Ntezurubanza *et al.* (1984), Nakamura *et al.* (1999), Iwalokun *et al.* (2003) and Lemos *et al.* (2005). In a study to determine the effects of plant extracts of *O. gratissimum* and other plants on post harvest pathogen of *Persea americana,* Ogbo and Oyibo (2008) observed 100% inhibition with ethanolic extract and over 60% inhibition when unsterilized water was used for extraction. Orafidiya *et al.* (2000) demonstrated that the oil extract of *O. gratissimum* was active against enteroaggregative *E. coli*. The study demonstrated that antibacterial activity of *O. gratissimum* increased with increase in concentration of the active compound. Similar trend have also been reported by Mbata and Saikia (2005).

The inhibitory effects of essential oils were more pronounced than those of plant extracts. Similar observation was made by Adebolu and Salau (2005). The significantly higher activity of essential oils compared to raw extracts was an indication that the oil components of *O. gratissimum* could be the active compounds with antibacterial principles. Akinyemi *et al.* (2004), Lemos *et al.* (2005) and Lopez *et al.* (2005) reported that Ocimum oil is active against several species of bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, Shigella, Salmonella and Proteus) and fungi (*Trichophyton rubrum T. mentagrophytes*, *Cryptococcus neoformans*, *Penicillium islandicum* and *Candida albicans*). The difference observed in antibacterial activity of the essential oils and raw plant extracts can therefore be attributed to the high volatibility of the oil, leading to the escape or evaporation of the oil during leaf drying as well as insufficient release of the oil during extraction. It has been reported that the active principles present in plants are influenced by many factors which include the age of plant, extracting solvent, method of extraction and time of harvesting plant materials (Ajalie and Okigbo, 2005; Okigbo *et al* ., 2005; Okigbo and Ogbonna, 2006).

Previous studies have shown that various species of the genus Ocimum produce oils of diverse characteristics and most with antimicrobial properties (Louis *et al*., 2011; Ntezurubanza *et al*., 1984; Nakamura *et al*., 1999; Lemos *et al*., 2005; Reuveni *et al*., 1984). Matasyoh *et al.* (2008) reported that essential oils derived from leaves of *O. gratissimum* collected from Meru region contained eugenol, methyl eugenol, cis-ocimene and trans-ocimene. A study carried out to analyze essential oils of *O. gratissimum* by Gas Chromatography (Masada, 1976) showed that they contain compounds such as thymol, eugenol and d-limoneme among others. The oils have been reported to be active against species of bacteria and fungi (Matasyoh *et al*., 2008; Mbata and Saikia, 2005; Iwalokun *et al*., 2003; Malik and Singh, 2010). Though the study did not ascertain the chemical compound present in the oil that has the antibacterial properties, eugenol was suspected as the most likely candidate. This component has been demonstrated to have both antibacterial (Nakamura *et al*., 1999) and antihelmintic activities (Pessoa *et al*., 2002).

CONCLUSION

The study confirmed the antibacterial effects of *O. gratissimum* and further demonstrated that the active compound (s) against *Ralstonia solanacearum* is contained in the essential oils. Biologically active essential oils represent a rich potential source of an alternative and perhaps

environmentally more acceptable disease management compounds. Further research is therefore recommended to identify the specific chemical compound(s) that is active against the pathogen.

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