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IN-VITRO ANTIBACTERIAL ACTIVITY OF COPTIS CHINENSIS EXTRACT AGAINST *PECTOBACTERIUM CAROTOVORUM* SUB SP. *CAROTOVORUM*

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A B S T R A C T

Soft rot of Zantedeschia, caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), has caused a significant worldwide threat to calla lily production. Hence, in order to effectively manage the disease, an intensive management programme aimed at adequte suppression of the pathogen is paramount. In order to investigate the antibacterial effect of a *Coptis chinensis* extract product against the soft rot- causing bacterium, an *in vitro* study was set up. Bacterial isolate were obtained from rotting calla lily tubers and maintained in Nutrient Broth under refrigeration. Sterile petri plates containing 15 ml of Nutrient agar were prepared and aseptically inoculated with 0.1 ml an overnight grown culture of a standardized *Pectobacterium carotovorum* subsp. *carotovorum* inoculum containing about 1x 10^8 cfu/ml. The inoculum was spread evenly over the whole surface of the plates. After solidification, 1 ml of the different concentrations of *C. chinensis* (1, 10, 25, 50 and 100%) were placed individually at the centre of the inoculated petri plates. Positive check was maintained using Streptomycin sulphate (100ppm) and a negative check using sterile distilled water. Eight replicates were maintained for each treatment and the experiment was repeated twice. Results indicated that the lower concentrations of *C. chinensis* did not cause any inhibition against Pcc. On the other hand, 100% *C. chinensis* made an inhibition zone comparable to that streptomycin sulphate. Our results demonstrated that *C. chinensis* has antibacterial activity and therefore feasible for use in crop protection against soft rots caused by *Pectobacterium carotovorum* subsp. *carotovorum*.

Keywords: Nutrient Agar, Inhibition zone, soft rot, Inoculum.

INTRODUCTION

Remarkable success has been achieved with the use of synthetic pesticides in crop protection. However, with the indiscriminate use of such pesticides, serious issues surrounding development of resistant pest strains have emerged. In response to this, there are concerted efforts aimed at developing alternative pesticides from natural products. Plants generally produce many secondary metabolites which constitute an important source of microbiocides, pesticides and pharmaceutical drug (Ciocan and Băra 2007, Shahid *et al.,* 2013). Use of botanicals in plant disease management assumes special significance by being an eco-friendly and cost-effective strategy which can be used in integration with other strategies for a greater level of protection with sustained crop yields (Inagaki *et al.,* 2008, Ravikumar and Garampalli 2013). The main advantage of naturally derived agents is that they do not enhance antibiotic resistance, a phenomenon commonly encountered with the long-term use of synthetic antibiotics. Several studies have reported the effectiveness of plant extracts in controlling the development of many plant pathogens *in vitro* (An *et al.,* 1998, Balestra *et al.,* 2008, Quattrucci *et al.,* 2013). (Prakash and Karmegam 2012) reported *in vitro* efficacy of four plant extracts vis a viz *Aegle marmelos*, *Aristolochia indica*, *Ocimum canum* and *Plumbago zeylanica* in inhibiting five strains of pathogenic *Xanthomonas campestris* pv. *citri.*

Pectobacterium carotovorum subsp. *carotovotum* is the major cause of soft rot in plants including vegetables and ornamentals such as Zantedeschia. The soft rot disorder is characterized by loss of host tissue structural integrity which is mainly due to production of bacterial pectolytic

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and other macerating enzymes (Perombelon and Kelman 1980, Buonaurio *et al.,* 2002). Plants turn yellow when the disease has initiated, produce a foul smell and can become completely macerated within a few days (Snijder *et al.,* 2004).

Various plant based products have been shown to inhibit the growth of *Pectobacterium carotovorum* subsp. *carotovorum* either *in vitro* or *in vivo*. (Ortega *et al.,* 2003) reported *in vitro* inhibition of *Erwinia carotovor*a subsp. *carotovora* by Chili (*Capsicum annum*) extracts. The ability to inhibit the bacterial growth was attributed to compounds such as *meta*-coumaric and *trans*-cinnamic acids that were present in the extract. *In vitro* potency of essential oils and crude extracts from various plants including *Psidium guajava*, *Thymus vulgaris*, *Rosmarinus officinalis*, *Coriandrum sativum*, *Cuminum syminum* and *Eucalyptus camuldulensis* to inhibit *Pectobacterium carotovorum* subsp. *carotovorum* has been reported by various studies (Alamshahi *et al.,* 2010, Nezhad *et al.,* 2012, Sarah *et al.,* 2012).

Coptis chinensis is a traditional Chinese medicinal herb that has strong antibactrial activity with extensive use in treating dysentry, cholera, leukemia, diabetes, allergies and inflammations (Yan *et al.,* 2008). The antibacterial activity of *C. chinensis* has been attributed to one of its major consttuents, berberine, which also has been shown to possess antimicrobial activities in its pure form to both human and phytopathogenic agents (Hou *et al.,* 2010, Leach 2011, Gan 2012). Berberine has been found to be effective against powdery mildew in cucumber grown under greenhouse conditions (Kuixian *et al.,* 2009), tomato bacterial speckle caused by *Pseudomonas syringae* p.v tomato (Shen *et al.,* 2010) and *Monilinia fruticola*, the causal agent of peach brown rot (Hou *et al.,* 2010). The antibacterial mechanism of berberine has been shown to include inhibition of DNA duplication, RNA transcription and protein synthesis, damage to the bacterial cell surface structure which leads to leakage of Ca^{2+} and K^{+} from the cell (Jian-ling *et al.,* 2011). It has been suggested that there are limited chances of the target organisms developing resistance against the compound since most of the target areas are essential for the functioning of a normal cell.

The aim of this study was to evaluate the antibacterial activity of a *C. chinensis* formulated product against *Pectobacterium carotovorum* subsp*. carotovorum*, the causal organism of calla lily soft rot.

MATERIALS AND METHODS

Collection, Isolation and Identification of the Pathogen: Soft rot-infected calla lily tubers were obtained from Agriflora (K) Ltd farm. The tubers were cleaned, surface-sterilized with 0.5 % sodium hypochlorite solution for 30 seconds, washed with sterile distilled water, and ground in 1 ml of sterile water using sterile mortar under aseptic conditions. The resulting bacterial suspension was left undisturbed for a few minutes. A loopful of the suspension was streaked onto plates containing autoclaved nutrient agar (NA) (Bacto Agar 10g, NaCl 5.0 g, K_2HPO_4 5g, KH_2PO_4 2g, Bacto-peptone 1.0g), and incubated at 28º C for 24 h. Individual colonies (transparent, circular, raised, shiny and creamy white) growing on NA were selected, re-suspended in 1 ml of sterile water, streaked on NA plates, and then incubated at 28°C for another 24 h. The procedure was repeated several times in order to obtain pure cultures. The isolate was subjected successfully to pathogenicity test by leaf disks immersion method. Pure cultures so obtained were maintained at 4°C in the dark for subsequent use (Ni *et al.,* 2010).

***In-vitro* Antibacterial Assay:** The *in-vitro* tests were carried out by direct inoculation method after the failure of disc diffusion method. Sterile 9 cm petri plates were prepared by pouring 15ml of Nutrient Agar (NA) medium and allowed to cool. After solidification, the plates were inoculated with a standardized inoculum containing 1×10^8 cfu/ml bacterial suspension. The suspension was uniformly spread with a sterile cotton swab on the media surface and allowed to dry for 4 minutes. In each of the test test plate, 40 µl of each of the following concentration of *Coptis chinensis* extract 1%, 10%, 25%, 50%, and 100% solutions was applied. A standard reference antibiotic, streptomycin sulphate (100 ppm) and a negative control, sterile distilled water were also included. After incubation at 30° C for 24 h, the resulting inhibition zone was measured in mm using a vernier calliper. The experiment was repeated twice with eight replications in each.

RESULTS

Exposure of *Pectobacterium carotovora* subsp. *carotovora* to *C. chinensis* caused inhibition of the bacterial growth (Fig. 1). There was no distinct inhibition zone (0 mm) for the dilutions (1, 10, 25 and 50%) of *C. chinensis* (Table 1). Undiluted concentration of *C. chinensis* resulted in an inhibition zone (27.62 mm) that was not significantly different in diameter from that caused by Streptomycin sulphate (23.87 mm).

Table 1. Diameter of inhibition zones (mm) caused by *C. chinensis.*

Treatment	Inhibition zone(mm)
100 % *C. chinensis*	27.6200±2.46
1% *C. chinensis*	0.00
10% *C. chinensis*	0.00
100 ppm streptomycin	23.8750±1.639
25% *C. chinensis*	0.00
50% *C. chinensis*	0.00
water	0.00

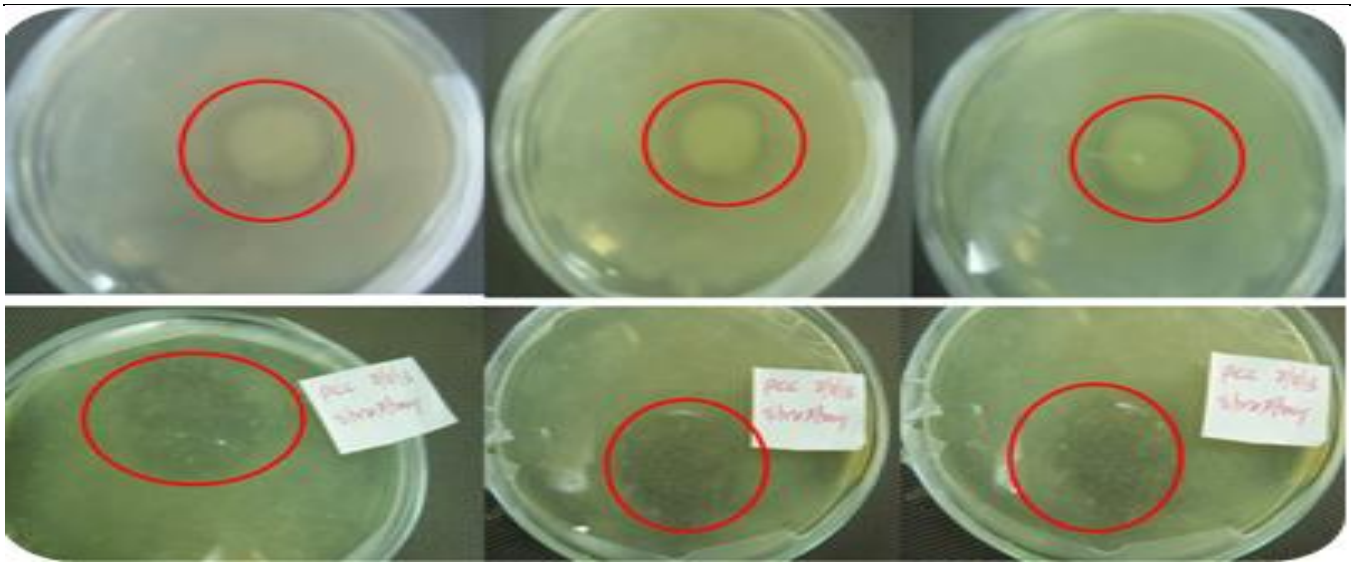

Figure 1. Inhibition zones caused by undiluted C. chinensis extract (1st row) and streptomycin sulphate (2nd row).

DISCUSSION

In our preliminary *in vitro* assays (unpublished), the disc diffusion method was employed but was found to be ineffective, instead, direct inoculation method was utilised. It was suspected that the active compounds in the *C. chinensis* extract were bound irreversibly to the paper discs and therefore a direct inoculation method was employed. Such standard anti-microbial susceptibility testing methods like the agar diffusion and Kirby-Bauer may result to misinterpretation of results especially for extracts with low anti-microbial activity or in cases where the active ingredient(s) may irreversibly bind to the paper discs (Das *et al.,* 2010). Therefore direct inoculation was shown to overcome the challenges associated with the disc diffusion method.

Apart from the undiluted concentration, the other lower concentrations of *Coptis chinensis* extract did not show any inhibition zone against *Pectobacterium carotovora* subsp. *carotovora*. On the other hand, the undiluted concentration of the *C. chinensis* extract product produced an inhibition zone that was not significantly different from that produced by streptomycin sulphate.

These results are in line with those reported by Chung and others (1998) who observed that low density polyethylene films (48 to 55 µm thick) impregnated with either 1.0% w/w *Rheum palmatum* and *Coptis chinensis* extracts or silver-substituted inorganic zirconium retarded the growth of total aerobic bacteria, lactic acid bacteria and yeast on fresh strawberries.

Similarly, Shen *et al*. (2010) reported the effectiveness of a *C. chinensis* extract, 0.5% berberine aqueous solution in the control of tomato bacterial speckle disease caused by *Pseudomonas syringae* pv. tomato. Plant extracts have been reported to possess antibacterial activities against several phytopathogenic bacteria including *Pseudomonas syringae* pv. *syringae* (Balestra *et al.,* 2008, Quattrucci *et al.,* 2013), *Xanthomonas axonopodis* pv. *vesicatoria* (Kotan *et al.,* 2007) and *Clavibacter michigenesis (Pattnaik et al., 2012).* There is no literature indicating the use of *Coptis chinensis* extract against the soft rot bacterium, *Pectobacterium carotovorum* subsp. *carotovorum*. However, *Coptis chinensis* has been shown to have antifungal activities by *in vitro* inhibition of spore-germination and mycelial growth of

Colletotrichum gloeosporioides, *Phytohpthora capsici*, *Pyricularia grisea*, *Rhizoctonia solani*, *Botryosphaeri dothidea* and *Glomerella cingulata (Ahn et al., 2009).* Many authors have correlated the antimicrobial activity of *C. chinensis* to its major constituent, berberine. In their work, (Hou *et al.,* 2010) reported the antimicrobial properties of berberine against phytopathogenic agents both *in vitro* and *in vivo.* The authors observed that berberine excerted its inhibitory effect against *Monilinia fructicola*, *Botrytis cinerea* and *Alternaria solani* in a dose-dependent manner. Other studies have found berberine to be ineffective against gram-negative bacteria such as *Serratia liquefaciens*, *Citrobacter* MFBF and *Providentia stuardii* (Nechepurenko *et al.,* 2010), *Escherichia coli, Staphylococcus aureus* (An *et al.,* 1998). Gram negative bacteria have been shown to be more resistant to antimicrobial agents than Gram-positive bacteria due to their additional outer membrane layer (Leach 2011). This could partly explain why lower concentrations of *C. chinensis* extract used in this study showed no inhibition against the test bacterium.

A berberine-containing extract, *C. chinensis* rhizome, has recently been reported to have inhibitory activity against sortase A and sortase B enzymes. Sortase is a bacterial surface protein anchoring transpeptidase. In *Staphylococcus aureus*, a Gram-positive bacteria, the inhibition of these enzymes has been shown to result in a marked reduction in its virulence and infection potential (Kim *et al.,* 2004, Imanshahidi and Hosseinzadeh 2008). Berberine has been shown to block the adherence of *Streptococcus pyogenes* and *E. coli* to erythrocytes and epithelial cells, and in effect, it is thought to exert an antibiotic effect even against organisms that do not exhibit *in vitro* sensitivity to the alkaloid (Birdsall and Kelly 1997, Imanshahidi and Hosseinzadeh 2008). Berberine and berberine-containing plant extracts also have bacteriostatic effects on streptococci, with an MIC of 30 mg/ml for *S. pyogenes* . Sub-MICs of berberine prevented the adherence of streptococci to host cells, immobilized fibronectin, and hexadecane. Concentrations of berberine below its MIC caused an eightfold increase in release of lipoteichoic acid from the streptococci. Higher concentrations of berberine directly interfered with the adherence of streptococci to host cells either by preventing the complexing of lipoteichoic acid with fibronectin or by dissolution of such complexes once they were formed. Thus, berberine interferes with the adherence of group A streptococci by two distinct mechanisms: one by releasing the adhesin lipoteichoic acid from the streptococcal cell surface and another by directly preventing or dissolving lipoteichoic acid-fibronectin complexes (Sun *et al.,* 1988). The same test concentrations of *C. chinensis* extract used for the *in vitro* assay in the current study were found to reduce disease incidence and severity in our field studies although they showed no *in vitro* inhibition. It has been suggested that some antimicrobial plant extracts when applied to the plants may trigger the accumulation of other defence compounds within the plant or become more potent in the presence of other components involved in the immune response. Similarly, other plant extracts that do not exhibit *in vitro* inhibition may undergo enzymatic processing to make them potent *in vivo (González-Lamothe et al., 2009)*. It is therefore evident that extracts from *C. chinensis* has the capability for *in vitro* inhibition of the soft rot causing bacterium. This is an important step in the management of this bacterium. However, systematic studies need to be conducted in order to establish the actual mode of action of the *C. chinensis* extract both *in vitro* and *in vivo.*

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