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 adequate 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 isolates 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 1 x 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. carotovorum 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
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 carotovora subsp. carotovora by Chili (Capsicum annuum) extracts. The ability to inhibit the bacterial growth was attributed to compounds such as metabolites 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 cyminum and Eucalyptus camaldulensis 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 antibacterial 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 constituents, 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 fructicola, 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$_2$HPO$_4$ 5g, KH$_2$PO$_4$ 2g, Bacto-peptone 1.0g), and incubated at 28°C for 24 h. Individual colonies (transparent, circular, raised, shiny and creamy white) growing on NA were selected, resuspended 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 $1\times10^8$ 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 carotovorum 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*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibition zone(mm)</th>
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<tbody>
<tr>
<td>100 % <em>C. chinensis</em></td>
<td>27.6200±2.46</td>
</tr>
<tr>
<td>1% <em>C. chinensis</em></td>
<td>0.00</td>
</tr>
<tr>
<td>10% <em>C. chinensis</em></td>
<td>0.00</td>
</tr>
<tr>
<td>100 ppm streptomycin</td>
<td>23.8750±1.639</td>
</tr>
<tr>
<td>25% <em>C. chinensis</em></td>
<td>0.00</td>
</tr>
<tr>
<td>50% <em>C. chinensis</em></td>
<td>0.00</td>
</tr>
<tr>
<td>water</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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 michigenes* (Puttnaik *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, Phytophthora capsici, Pyricularia grisea, Rhizoctonia solani, Botryosphaeria 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 exerted 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.

REFERENCES


Balestra, G. M., A. Rossetti and A. Quattrucci. 2008. Biological control of kiwifruit and tomato bacterial pathogens. In 16th IFOAM Organic World Congress, Modena,Italy (pp. 3-6).


