Full Length Research Paper

Response of arum lily calli to culture filtrate of *Pectobacterium carotovorum* subsp. *carotovorum*

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This report demonstrated that culture filtrate of *Pectobacterium carotovorum* ssp. *carotovorum* isolate ZT0505, the pathogen of bacterial soft rot disease of arum lily (*Zantedeschia* sp.), contained extracellular enzymes and caused arum lily leaf tissue and callus maceration. Arum lily leaf tissue and callus sensitivity to culture filtrate coincided with the host susceptibility to the pathogen. The rates of survival of callus pieces were determined after exposure for various times to culture filtrate. Survival of callus pieces (%) increased with reduction of exposure time from 20 to 14 h and from 14 to 8 h. One out of 30 callus pieces was still viable after 3 cycles of 8 h exposure. Subsequently, the surviving cells in this callus pieces proliferated and differentiated into shoots. Based on this initial work, the callus screening using culture filtrate as selection agent may be useful for *in vitro* selection of soft rot resistant germplasm in arum lily.

Key words: Arum lily, callus, culture filtrate, *in vitro* selection, *Pectobacterium carotovorum* sp. *carotovorum*, soft rot.

INTRODUCTION

Arum lily (*Zantedeschia* sp.), a genus of *Araceae* family, is native to southern Africa and consists of many ornamentally valuable species in two sections of *Zantedeschia* and *Aestivae*. Arum lily has now become an important pot and cut flower worldwide (Snijder, 2004; Wright et al., 2005). Bacterial soft rot caused by *Pectobacterium carotovorum* sp. *carotovorum* (*PCC*, former name *Erwinia carotovora* sp. *carotovora*) is a serious disease of arum lily and a major limiting factor for flower production (Wright, 1998; Snijder, 2004). *PCC* is also pathogenic to other ornamental plants, vegetables and field crops (Barras et al., 1994; Pérombelon and Salmond, 1995). Previous studies on soft rot by *PCC* on potato demonstrate that during the infection process, the

bacteria produce and secrete extracellular enzymes such as protease (Prt), polygalacturonase (Peh), pectate lyase (Pel). These enzymes serve as pathogenicity factors as they degrade the plant cell wall and membrane components, enabling bacteria penetration and colonization of the plant tissue, which results in tissue maceration and plant death (Barras et al., 1994; Pérombelon and Salmond, 1995). The disease is difficult to control. Chemical bactericides are not effective once plant or tuber infection has occurred. Preventive cultural measures, including well-balanced irrigation, mulching and soil ventilation, have been reported to reduce the disease severity; however, because the bacteria are endemic in soil, those methods may not give full control of the disease (Wright et al., 2000, 2005). Therefore combining use of resistant cultivars will be a promising approach in overcoming the disease. However, previous studies indicate that the pathogen can cause tissue maceration of cultivars in both sections of *Zantedeschia* and *Aestivae,* although there is variation for resistance to *PCC* within *Zantedeschia* sp. For instance, *Z. aethiopica* is more tolerant to soft rot than *Aestivae* genotypes (Snijder, 2004). Cultivars that are resistant to the disease are not available.

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In vitro selection is a valuable tool for production of disease-resistant variants that occur during the cultivation of tissue cultures (reviewed by Daub, 1986; Evans, 1989; Karp, 1995) and has been used successfully in many species (Carlson, 1973; Chawla and Wenzel, 1987; Hammerschlag, 1988; Kuksova et al., 1997; Remotti et al., 1997; Kumar et al., 2008; Nasir and Riazuddin, 2008; Sengar et al., 2009). Important steps of this technology are choosing the right selection agent and selection unit and demonstrating that (1) the selection agent plays a role in the disease development and (2) the sensitivity of the selection unit to selection agent correlates with the susceptibility of the plant to the pathogen, indicating that the agent is selective to susceptible cells (Gray et al., 1986; Hartman et al., 1986; Slavov, 2005). In this paper, we studied the response of arum lily calli to culture filtrate of *PCC*, towards establishment of an *in vitro* selection method for obtaining disease resistant germplasm.

MATERIALS AND METHODS

Preparation of *PCC* culture filtrate

A soft rot pathogen, *P. carotovorum* sp. *carotovorum* isolate ZT0 505, was isolated from infected tubers of arum lily (cultivar *Z.* 'Black Magic') grown in the subtropical region around Kunming, China and is deposited in the Microbiological Culture Collection Center, Lab. for Conservation and Utilization of Bio-resources, Yunnan University, China. About 1µl ZT0505 glycerol stock was inoculated in 50 ml LB medium (pH 7.2) in a 250 ml flask. The culture was grown on a shaker (120 rpm) at 28°C, overnight to logarithmic stage, followed by centrifugation at 10,000 rpm for 20 min. The supernatant was filter-sterilized through a 0.22 µm filter.

Enzyme assays

Previously described methods for the plate assay of extracellular enzymes, Prt, Peh and Pel (Chatterjee et al., 1995) were used in this study, except that 30 µl of culture filtrate was applied into each 5 mm well made in the media. The culture filtrate was boiled at 100°C for 10 min and used as control.

For measuring quantitative Pel activity, 1 ml catalysis reaction mixture contained 10 µl of culture filtrate, 1 mM $CaCl_2$, 0.25% sodium polygalacturonate, 0.05 M Tris-HCl, pH 8.0. The catalysis was run for 30 min at 30°C. The reaction was stopped by heating at 100°C for 5 min. The increase in the absorbance of the unsaturated products at 235 nm (OD_{235}) was measured using Nucleic Acid and Protein Analyzer, DU® 640 (BECKMAN COULTER™, USA) and one unit of Pel activity was defined as an increase of 1 OD_{235} unit in 30 min according to Lei et al. (1985).

Pathogenicity test of the culture filtrate

A pathogenicity test was carried out to evaluate the ability of the culture filtrate to produce common soft rot symptoms of arum lily. The bacterial culture of *PCC* ZT0505 was prepared as described above. The bacterial cells were harvested by centrifugation at 5,000 rpm for 30 min, washed once by resuspending the cells in 0.05 M PBS buffer (pH 7.2), followed by centrifugation and resuspending the cells again in the same buffer and cell suspension with about 10^7 cfu ml^{-1} was prepared. Young leaves of cultivar *Z.* 'Black Magic',

belonging to *Aestivae* section and of cultivar *Z. aethiopica* 'Hong Gan', belonging to *Zantedeschia* section, were collected from newly sprouting plants grown in a greenhouse and surface-sterilized by wiping with 75% ethanol for 10 s and then washed with sterile water. 10 µl of the suspensions were inoculated by injection into the cut sites of leaf petioles. The inoculated leaves were incubated at 25°C for 24 h and then amounts of macerated leaf area were visually estimated. Percentages of macerated leaf area in two cultivars were compared as a measure of the bacterium virulence difference in the two cultivars. The experiment was conducted 3 times using 5 leaves per cultivar each time. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.01 was considered as significant.

Similar to the pathogenicity experiment with bacterium suspension, pathogenicity tests of the culture filtuate were conducted using the culture filtrate instead. Young leaves (half parts) of both cultivars were immersed in 30 ml culture filtrate contained in 50 ml tubes, one leaf per tube, incubated at 25°C for 24 h and then amounts of macerated leaf area were visually estimated. Also these experiments were conducted 3 times using 5 leaves per cultivar each time.

Callus initiation

For callus initiation from cultivar *Z.* 'Black Magic', about 0.5 cm long buds were excised from tubers, which were first surface-sterilized with 0.1% $HgCl_2$ for 5 min and then with 1% sodium hypochlorite solution for 20 min, followed by three rinses with sterile distilled water. The excised buds were incubated on MS medium (Duchfa Biochemie, The Netherlands), pH 5.8, solidified with 0.7% agar and supplemented with 3% sucrose, 1.0 mg/l 6-benzylaminopurine (BA) and 0.2 mg/l naphthaleneacetic acid (NAA) in Petri dishes (8.5 x 1.5 cm, diameter x height). Cultures were kept in a growth chamber at 25°C, 60% humidity and with a 16-h-light/8-h-dark cycle (1600 lux) for 2 months to induce callus. For cultivar *Z. aethiopica* 'Hong Gan', microspore-derived callus was used, obtained from anther culture (will be published elsewhere).

Callus response to culture filtrate

Calli were divided into small pieces (about 70 mg fresh weight) and were then immersed in culture filtrate for various periods (8, 14 and 20 h, arbitrarily chosen). Culture filtrate was used full strength or diluted to 67% using MS medium. After immersion treatment, the calli were transferred to test medium (MS medium, pH5.8, supplemented with 3% sucrose, 0.7% agar, 1.0 mg/l BA and 0.1 mg/l NAA) in 300 ml jars and cultured for 15 days under similar conditions as for callus initiation. After this period, calli were judged on the amount of decayed tissue. Fully decayed calli were discarded from the experiment, while from the remaining calli the soft tissue was removed and the vital calli parts were used for another round of exposure to the culture filtrate. Thereafter, with vital calli left a third round of exposure and subsequent cultivation were conducted. In this experiment each treatment started with 30 callus pieces.

RESULTS AND DISCUSSION

Although much is known about extracellular enzymes as pathogenicity factors produced by *PCC* in potato soft rot (Barras et al., 1994; Pérombelon and Salmond, 1995), it was not clear whether *PCC* isolate ZT0505 from arum lily also produces and secretes extracellular enzymes in liquid culture as properties of isolates from different hosts
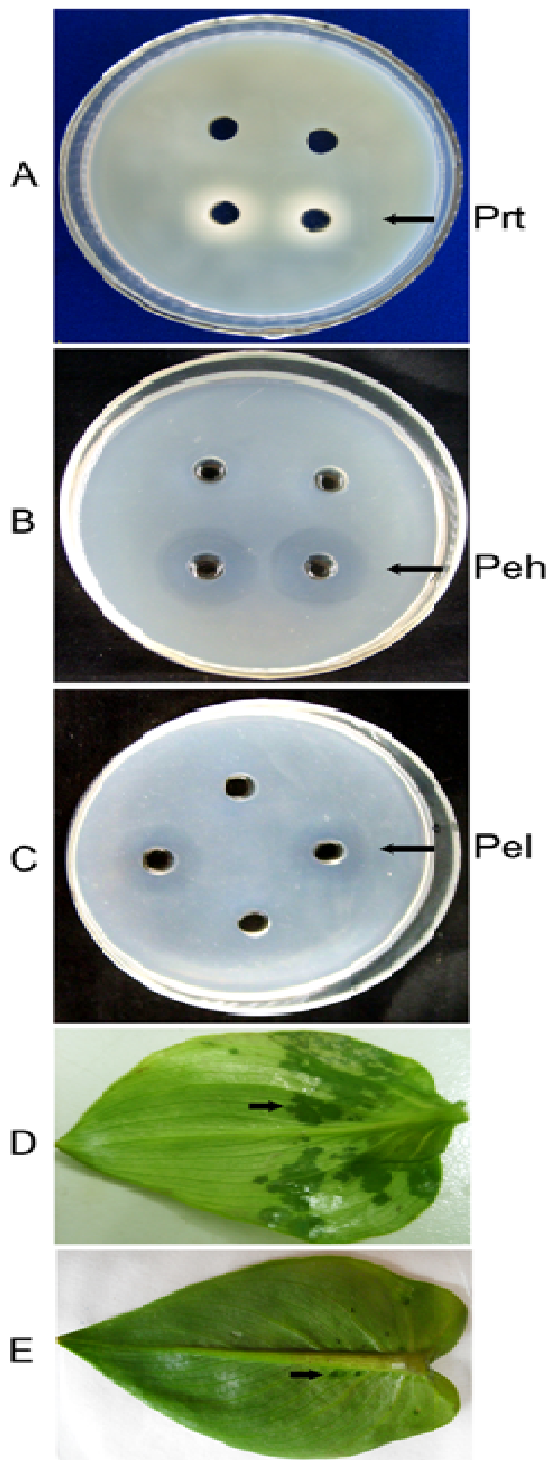
Figure 1. Culture filtrate contained pathogenicity factors. (A, B, C) Plate assays of protease (A), polygalacturonase (B) and pectate lyase (C). Culture filtrate of *PCC* isolate ZT0505 was applied to the wells indicated by arrows. Halos around the wells showed enzymatic activities. The boiled culture filtrate was added into the wells as controls and did not produce halos. (D, E) Leaf maceration caused by culture filtrate in *Z.* 'Black Magic' (D) and *Z. aethiopica* 'Hong Gan' (E). Arrows indicate tissue maceration.

and regions may vary. We initially examined the presence of extracellular enzyme activities in culture filtrate from isolate ZT0505. Culture filtrate produced clear halos around the wells in respective plate assays for Prt, Peh and Pel (Figures 1A, B and C), indicating that these extracellular enzymes were present in culture filtrate. Quantitative assay showed that Pel level was about 20 units of Pel activity on a basis of per ml culture filtrate. Pathogenicity test of the culture filtrate also indicated that the culture filtrate could cause leaf tissue maceration of arum lily. Figures 1D and E showed the results of representative experiment. For *Z.* 'Black Magic', $70.4 \pm 9.2\%$ of culture filtrate-immersed leaf area was macerated, significantly higher than the value of $2.4 \pm 0.1\%$ for *Z. aethiopica* 'Hong Gan'. The maceration was more extensive in *Z.* 'Black Magic' than that in *Z. aethiopica* 'Hong Gan', further demonstrating that culture filtrate contained compounds which play role in the disease development and cultivar *Z.* 'Black Magic' was more sensitive than *Z. aethiopica* 'Hong Gan' in response to the culture filtrate.

To study the response of arum lily calli to culture filtrate, the exposure of calli to the selection agent was performed by immersion of callus pieces into culture filtrate, as large molecule extracellular enzymes serving as important virulence factors are difficult to diffuse from medium to culture tissues. For 20 h exposure, most of callus pieces were completely macerated and turned brown after first cycle of exposure and subsequent cultivation for 15 days. However, there were the partial tissues which turned green in some callus pieces, whereas, the rest part of the callus pieces turned brown. These callus pieces were considered as viable. After the exposure to 67 and 100% culture filtrate, 37 and 30% of callus pieces from the cultivar of *Z.* 'Black Magic' were viable, respectively; in contrast, 47 and 40% of callus pieces from the cultivar of *Z. aethiopica* 'Hong Gan' were viable, respectively (Table 1). The results indicated that the callus response of *Z.* 'Black Magic' was more sensitive to culture filtrate than the callus from *Z. aethiopica* 'Hong Gan', similar to the difference in the leaf tissue response of two cultivars to culture filtrate. On the other hand, the pathogenicity test of *PCC* isolate ZT0505 by inoculation of leaf petioles demonstrated the variation of host susceptibility in the two cultivars. Figure 2 showed the results of representative experiment. For *Z.* 'Black Magic', $40.8 \pm 7.5\%$ of leaf area was macerated, significantly higher than the value of $7.3 \pm 2.6\%$ for *Z. aethiopica* 'Hong Gan'. The pathogen produced more severe soft rot symptom in *Z.* 'Black Magic' than that in *Z. aethiopica* 'Hong Gan'. This is consistent with previous studies by Snijder and van Tuyl (2002). They also found that *Z.* 'Black Magic' are more susceptible to soft rot than cultivars of *Z. aethiopica* species in all tests using patioles, tubers and leaf disks. Arum lily callus sensitivity to culture filtrate coincided with the host susceptibility to the pathogen, indicating that there was selectivity of culture filtrate to callus of susceptible genotype.

After further cycle of 20 h exposure, callus pieces from

Table 1. Percentages of viable callus pieces (n = 30) after exposure to culture filtrate of *PCC* isolate ZT0505 at various intensities.

Exposure time and filtrate strength	Percentage of viable callus pieces (%)					
	Z. 'Black Magic'		*Z. aethiopica* 'Hong Gan'			
	20 h		20 h		14 h	8 h
	67% CF	100% CF	67% CF	100% CF	100% CF	100% CF
After 1 cycle	37	30	47	40	50	70
After 2 cycles	0	0	0	0	3	20
After 3 cycles	0	0	0	0	0	3

Calli were immersed for 8, 14 and 20 h in diluted or full strength culture filtrate (67 and 100% CF). After a culture period for 15 days, viable callus pieces were subjected to a second exposure to culture filtrate and so on for a third time. More treatment combinations were tested with *Z. aethiopica* 'Hong Gan' than with *Z.* 'Black Magic'. Control cultures without exposure to culture filtrate gave 100% viable calli.
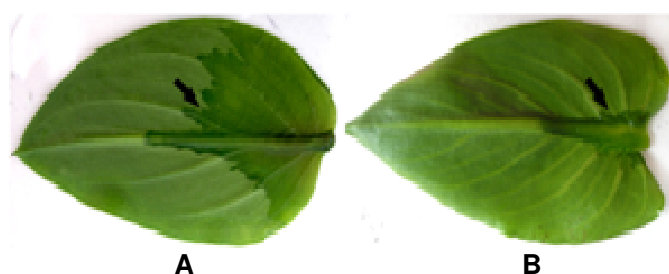

A   **B**

Figure 2. Pathogenicity of *PCC* ZT0505. Bacterial cells of *PCC* ZT0505 were inoculated into the cut sites of leaf petioles, followed by incubation at 25 °C for 24 h and symptoms were photographed. Arrows indicate tissue maceration. (A) Cultivar *Z.* 'Black Magic'. (B) Cultivar *Z. aethiopica* 'Hong Gan'.

A   B

Figure 3. Callus response to culture filtrate. (A) After callus pieces were exposed for 8 h to culture filtrate for 3 cycles, 1 out of 30 callus pieces generated shoots, whereas the rest callus pieces were macerated and turned browning. (B) Callus pieces without the exposure were used as control.

both cultivars were all macerated. We tested callus pieces from *Z. aethiopica* cultivar with reduced exposure time. The rates of viable callus pieces increased with shorter time of exposure after each cycle of the exposure and subsequent cultivation (Table 1). All callus pieces were macerated after 3 cycles of 14 h exposure. However, 1 out of 30 callus pieces was still viable after 3 cycles of 8 h exposure. The surviving cells in this callus pieces proliferated and differentiated into shoots (Figure 3).

The last step of *in vitro* selection will be to test the plants that were regenerated in laboratory for disease resistance under field conditions by artificial inoculation with the pathogen (Hammerschlag, 1988; Remotti et al., 1997; Kumar et al., 2008). Currently, we have not yet finalized *in vitro* selection started at laboratory level as *in vivo* disease resistance test. Nevertheless, the present initial work indicated that (1) culture filtrate contained compounds which play role in the disease development, (2) culture filtrate was selective towards arum lily callus of susceptible genotype and regenerant could be obtained after multi cycles of proper exposure period. The callus screening using culture filtrate as selection agent may thus be useful for selection of arum lily germplasm for soft rot resistance.
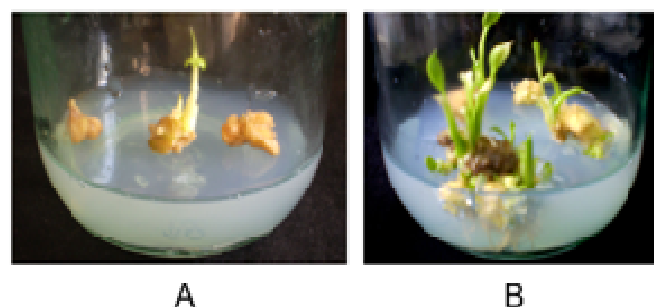
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