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Aqueous Plant Extracts as Possible Quorum Sensing Inhibitory (QSI) Agents against Soft Rot caused by *Pectobacterium carotovorum* in Tobacco

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The study involved estimation of the Quorum Sensing Inhibition (QSI) potential of aqueous extracts of three of plant leaves *viz.* pine needle, green tea and mugwort against the soft rot pathogen, *Pectobacterium carotovorum* in Tobacco system. In an agar well diffusion assay, the extracts showed QSI activity against the bio-indicator bacteria, *P. aureofaciens* strain 30-84. However the extracts at the tested concentration did not show bactericidal/antibiotic effect. In the initial screening performed *in vitro*, using tobacco seedlings, all the extracts under study showed QSI activity upon challenge inoculation with *P. carotovorum*. Green tea leaf extracts rendered the highest level of disease suppression (93%) followed by leaf extracts of Mugwort and pine needle. Experiments were repeated under greenhouse conditions and found that the crude extracts significantly reduced disease symptoms upon challenge with the pathogen. The results suggested that commercial pine needle, mugwort and green tea extracts could significantly destabilize the QS mediated pathogenicity in *P. carotovorum* - Tobacco system and be effectively applied for the control of soft rot. The extracts are also expected to work well with QS mediated pathogenicity in other plants, which requires further detail study. Also it offers an environmentally friendly means of plant protection.

Key words: Quorum Sensing Inhibition, *Pectobacterium carotovorum,* soft rot, Tobacco, Plant extracts.

Pectobacterium carotovorum subsp. carotovora is a Gram-negative enterobacterium that causes soft-rot in tobacco, potato and other crops. The virulence regulation systems in *P. carotovorum* has been reported to be mediated by a process termed quorum sensing (QS), which is a population density -dependent cell-to-cell communication mechanism used by many Gram-negative bacteria. In *Erwinia*, an arsenal of plant cell wall-degrading enzymes is produced in a cell density-dependent manner, which causes maceration of plant tissue. However, QS is central not only to controlling the production of such destructive enzymes, but also to the control of a number of other virulence determinants and secondary metabolites.

Bacterial QS has been well studied over the past 20 years and has been reported to be mediated through diffusible signaling molecules, enabling communication between bacterial cells (Whitehead *et al*. 2001). Most Gram-negative bacteria, including *Pectobacterium carotovorum*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*, use N-acyl-L-homoserine lactones (AHLs) as signal molecules. The AHLs are structurally diverse and differ in their acyl chain

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length. Many *Erwinia* species, including *P. carotovorum*, produce N-(3-oxohexanoyl) - L-homoserine lactone (3-oxo-C6-HSL) and N-hexanoyl-L-homoserine lactone (C6-HSL), which are signal molecules of a QS system consisting of an AHL synthase (ExpI) and an autoinducer receptor protein (ExpR) that control several virulence factors (Cui *et al*. 2005).

An interference with this cell density-dependent communication mechanism has been suggested to be a promising strategy to control the bacterial coordinated behavior that also leads to several of the virulence factors. Several chemicals and enzymes have been identified as quorum sensing inhibitors (QSI) in recent years that target the key components of bacterial QS systems. The halogenated furanone compounds produced by seaweed, *Delisea pulchra* were noticed because of their QSI activities (de Nys *et al*. 1993). Several plants have been suggested to have evolved strategies to interfere with the bacteria's AHL signaling system to prevent them from initiating a pathogenic attack. Such interference could include the production of signal mimics, signal blockers or signal-degrading enzymes or the production of compounds that block the activity of the AHL producing enzymes. Teplitski *et al*. (2000) reported AHL inhibitory activities in exudates from pea seedlings. Several other plant based compounds like salicylic acid (Rosenberg *et al*. 2008), urosolic acid (Ren *et al*. 2005), cinnamaldehyde (Brackman *et al*. 2008), extract from garlic (Bjarnsholt *et al*. 2005) and cranberries (Yamanaka *et al*. 2007) have all shown various degrees of QSI properties against a number of bacteria in various studies. The current study evaluated three different plant extracts for their efficiency as QSI agent against soft rot in tobacco caused by *P. carotovorum* SCC1.

MATERIALS AND METHODS

Pseudomonas aureofaciens strain 30-84 produce phenazine antibiotics, and the synthesis of it has been demonstrated to be regulated by a quorum sensing system that utilizes the AHL molecule N-hexanoyl-L-homoserine lactone (C6-HSL) (wood *et al*. 1997). The strain can be used as an indicator for determining QSI compounds (McLean *et al*. 2004) as the pigment production in 30-84 is readily inhibited by AHL analogues and other antagonists. *P. aureofaciens* 30-84 were cultured on R2A agar plates. *Pectobacterium carotovorum* SCC1 was used as the pathogenic bacterium for inducing soft-rot in tobacco and was grown in Luria-Bertani (LB) agar/broth. All strains were maintained in LB broth as glycerol (30%) stocks at –80°C and small samples were subcultured into fresh medium (when necessary) for the assays.

Aqueous extract of leaves of green tea (*Camellia sinensis*), pine (*Pinus Sylvestris*) and mugwort (*Artemisia vulgaris*) were prepared after washing 3 times with double distilled (DD) water. 50g each of the plant parts were ground with 150ml DD water, allowed to settle for 48h and supernatant collected after filtering through Whatman No.1 paper. The supernatant was sterile filtered using 0.2um syringe filter and stored at 4 °C. Sterile extractions in dilutions of 1/5, 1/10, 1/20 and 1/50 were screened for QSI activity in a well-diffusion assay by adding sterile crude extract preparation into the well (6 mm) punched in the solidified R2A agar plate in which the indicator strain *P. aureofaciens* 30-84 was inoculated. The plates were incubated at 25°C for 48 h and the diameter of the QSI zones surrounding the wells was measured. QSI is indicated by the lack of pigment production of the indicator culture in the vicinity of the test sample. Growth inhibition of the indicator culture indicates possible antibiotic production (McLean *et al*. 2004).

Tobacco cv. *Xanthi* seed were stored at 5ºC and were surface sterilized *via* 2 min of washing in sterile water, followed by 10 min of soaking in 75% ethanol and 10 min of immersion in 3% (vol/vol) sodium hypochlorite. The seeds were rinsed four times in an excess of sterile distilled water. The seeds were placed on MS agar (0.8%) with addition of 3% sucrose in multiwell plates (12-well, Falcon), 1 ml in each well. Tobacco seeds were grown in a growth chamber at 25 °C with a 14 h light and 10 h dark regime. Six weeks after seeding, the plants were sprayed with the crude extracts (1/5 dilution). Negative control was maintained by spraying with sterile water. The phytopathogenic bacteria, *P. carotovorum* SCC1 were grown for 48 h in LB broth and harvested with centrifugation, then washed in sterile water and resuspended in sterile distilled water at OD 600nm = 1.0 (1×10^8 colony-forming units/ml). Five days after the

treatment with the crude extracts, the leaves were challenged with SCC1 (suspensions of 1×10^8 CFU/ml). Leaves treated with sterile distilled water served as negative controls. Two days after pathogen challenge, soft-rot disease was rated by counting the number of leaves showing disease symptoms.

Tobacco cv. *Xanthi* seed were surface sterilized as above, and then held in sterile tubes for 2 days of vernalization at 4ºC in darkness. For determination of QSI activity of the crude extracts against SCC1, the sterilized seeds were planted into sterile soil-less medium (peat moss/vermiculite/perlite, 7:3:3, vol/vol/vol) using 500 cm^3 of this matrix in a 10.5-by-10.5-by-9-cm pot, with 15 to 30 seeds per pot. The seedlings were grown with a cycle of 16 h of light and 8 h of dark under 40-W fluorescent lights (80 µmol photons m–2s–1). The temperature was maintained at 26 ± 1ºC with a relative humidity of 60%. Seedlings were watered with 20 ml of sterile water per pot at 2-day intervals. After 6 weeks of growth, the plants were sprayed with the crude extracts (1/5 dilution) and challenge inoculated with SCC1 suspensions as described above. Two days after pathogen challenge, soft-rot disease was rated and the data analyzed.

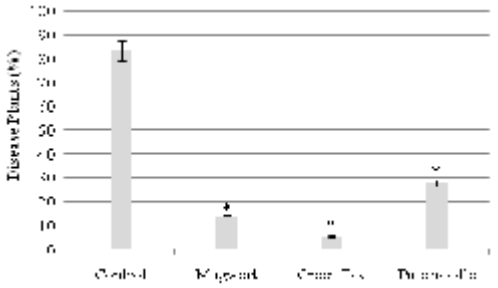
RESULTS AND DISCUSSION

Plants living in an environment with a very high bacterial cell density were long suspected to have protective mechanisms against bacterial infections. Not surprisingly, several plant extracts have been shown to be able to control bacterial pathogenesis by interfering with quorum sensing. Use of plant derived natural compounds for plant disease control without the use of growth-inhibitory agents that unavoidably select for resistant organisms (Richards and Melander 2009) is highly favored and is an environmental friendly strategy. The plants used in the study are known for their medicinal use in traditional medicines. The mugwort plant contains essential oils, flavonoids, triterpenes, and coumarin derivatives. It was also used as an anthelminthic. Pine tree needle extracts have been used as natural disinfectants. Various components including catechin polyphenols of green tea and their health benefits have been described.

In the study, we screened leaf extracts of the three medicinal plants for their ability to inhibit QS mediated virulence in the plant pathogen, *P. carotovorum*. As in Table 1, significant inhibition of pigment production by the bio indicator strain, 30-84 was detected with all of the three crude extracts screened; indicating interference with the AHL mediated QS activity. From measurements of the diameter of zone of inhibition of pigment production, it was clear that the QSI activity is concentration dependent. At the tested minimum dilution of 1/5, the extracts showed the highest activity of inhibition of pigment production. At the highest dilution tested (1/50), the green tea leaf extract showed QSI activity, but for extracts of pine needle and mugwort, there were no activity. The reduced QSI activity at increasing dilutions could presumably due to the dilution effect of the active components in the extract. Also the fact that the aqueous extract itself (not other expensive solvents & complicated procedures of extraction) contains active compounds that inhibits QS, adds

Table 1. QSI activity of the aqueous extracts of leaves of green tea, pine needle and mugwort against the indicator bacterium, *P. aureofaciens* 30-84. The zone of inhibition of pigment production by 30-84 indicated QSI activity

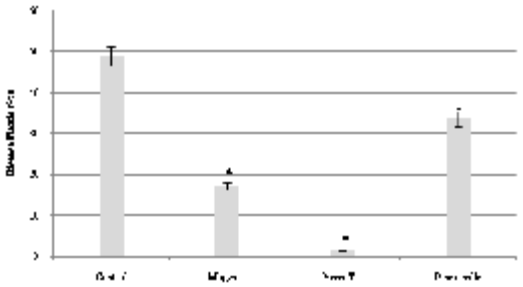
Treatment	Diameter of zone of inhibition of pigment production (mm)		
	Pine leaf	Green tea	Mugwort
1/5 dilution	1.43	1.41	1.17
1/10 dilution	0.68	0.81	0.59
1/20 dilution	0.39	0.64	0.37
1/50 dilution	0.00	0.34	0.00



* statistically different ($P<0.05$) based on Fisher's protected least significant difference (LSD)

Fig. 1. Tobacco plant (Xanthi nc) protection against *Pectobacterium carotovorum* SCC1 by various plant extracts: *in vitro* study

Fig. 2. Tobacco plant (*Xanthi* nc) protection against *Pectobacterium carotovorum* SCC1 by various plant extracts: Greenhouse study



* statistically different (P<0.05) based on Fisher's protected least significant difference (LSD)

Fig. 3. Tobacco plant (Xanthi nc) protection against *Pectobacterium carotovorum* SCC1 by various plant extracts: Greenhouse study

to the economic feasibility of application of the technology. The extracts at the tested concentration did not show growth-inhibitory action against the indicator strain, which confirmed that the plant extracts do not exert antibiotic effect. Similar studies conducted elsewhere with Plant extracts such as vanilla, *Tremella fuciformis*, *Conocarpus erectus*, *Quercus virgiana*, pea seedlings and other various higher plants have been reported to possess anti-QS activity against QS indicator strains (Ponnusamy *et al*. 2009; Adonizio *et al*. 2008).

In the initial screening performed *in vitro*, using tobacco seedlings, all the extracts under study showed QSI activity after challenge inoculation with SCC1. There were significant disease protection in plants treated with extracts of Green tea (93%), Pine leaves (67%) and Mugwort (83.3) (Figure 1). Results of *in vitro* experiments encouraged us to explore further and check the activity under greenhouse conditions. All extracts were tested under greenhouse conditions and found that the green tea extract rendered the highest level of disease protection (97%), followed by mugwort (64.5%) and controlled soft rot effectively (Figure 2, 3). In broth studies, our results indicated that all plant extracts tested, mugwort, pine needle and green tea extracts showed significantly high disease protection (Fig.3). Even though pine needle extracts showed significant QSI activity in the well-diffusion assay and also showed considerable disease suppression activity *in vitro* (67%), the percent disease suppression in the greenhouse trial was only 31.4%, the reasons for which could not be explained. Green tea catechins have been previously reported to significantly decrease the production of virulence factors in *Porphyromonas gingivalis* (Sakanaka *et al*. 2004). A different group indicated that phytochemicals from tea significantly diminished the transfer of the conjugative R plasmid in *E. coli* a process also related to QS (Zhao *et al*. 2001). Compounds from other natural sources having an anti-QS activity include also L-canavanine from *Medicago sativa;* garlic extract (used at 2%) and isolated components and some of their derivatives (Bjarnsholt *et al*. 2005; Persson *et al*. 2005; Rasmussen *et al*. 2005a) and the polyphenol curcumin (Rudrappa, and Bais, 2008). QS inhibitory activities have also been found in numerous plant extracts including *Pisum sativum, M. sativa, M. truncatula, Allium sativum, Callistemon viminalis*, bean sprout, chamomile, *Daucus carota, Capsicum chinense, B. buceras*, and *C. erectus* (Adonizio *et al*. 2006; 2008; Bjarnsholt *et al*. 2005; Gao, 2003; Keshavan *et al*. 2005; Rasmussen *et al*. 2005b). A recent study

identified QS inhibitors in a variety of medicinal plants commonly found in Southern Florida (Adonizio *et al*. 2006).

There are many reports that have indicated the presence of anti-QS activity in natural products including plants (Vattem *et al*. 2007; Allison *et al*. 2008), although the mechanism of action is not very well determined. QS Mechanisms can be inhibited in various ways: through signal mimicry (such as with furanones or synthetic signal analogs) resulting in a decrease in QS gene expression, through enzymatic degradation of homoserine lactones, through ant activator proteins or negative transcriptional regulator homologs homologs (Gonzalez *et al*. 2006; Whitehead *et al.* 2001). The well-studied QSI agent, Furanones (from marine algae) act by mimicking the AHL signal, presumably by occupying the binding site on the putative regulatory protein, rendering it highly unstable and accelerating its turnover rate, and thus resulting in the rapid disruption of the QS-mediated gene regulation (Eberl *et al*. 1999). Vattem *et al*. (2007) suggested that natural compound mediated QSI activity could be the result of a combination of two different mechanisms mainly, the ability of phytochemicals to interfere with the activity of AHL and to modulate the synthesis of AHL's by the bacteria. However, the ability of natural extracts to modulate QS in bacteria especially by inhibiting the synthesis of the signal molecule has never been indicated. At the same, the chances could not be ruled out that the plant-extracts may directly influence virulence factor production in bacteria in a QS independent manner, as it has been shown by Rasmussen *et al* (2005a, 2005b) that cyclic disulphides and trisulphides from garlic inhibited LuxR and LuxR based QSI in *P. aeruginosa*, even without any antibiotic properties. As the plant crude extracts contain a mixture of various active compounds, it has been estimated that majority of it have one or few major constituents and a variety of other minor compounds. Thus, it is difficult to comment on the exact mode of action on QS system and the exact molecule that renders it.

CONCLUSION

In conclusion, the present study evidenced the QSI potential of three of plant extracts, pine leaf, green tea and mugwort against the soft rot pathogen, *P. carotovorum*. The results suggest that commercial mugwort and green tea could be effectively applied to the control of QS mediated pathogenicity. As the type of extractant has role in the bioactive fraction showing the activity, the crude extracts need to be extracted in other solvents as well in order to have a detailed understanding on various molecules that are involved in QSI activity. Further studies needed to determine the active ingredients in the crude extracts to explore the full potential of the technology. Inhibition of QS offers new hope in combating plant pathogens as it doesn't impose harsh selective pressure for the development resistant strains. Furthermore, QS-inhibitory compounds that are plant based, are not expected to eliminate beneficial bacteria existent in the host.

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