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Evaluation of biological control of *Erwinia amylovora*, causal agent of fire blight disease of pear by antagonistic bacteria

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HIGHLIGHTS

- *Pseudomonas* and *Pantoea* strains showed potential biocontrol activity against fire blight on detached plant organs.
- We investigate the biocontrol potential of *Pantoea agglomerans* strains against *Erwinia amylovora*.
- Antibiotic production of *Pseudomonas* strains were studied under laboratory conditions.

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ABSTRACT

Fire blight caused by *Erwinia amylovora* is one of the most important diseases of pear and apple trees in Kurdistan province, Iran. To develop an effective biocontrol method against the pathogen, a total of 22 bacteria were isolated from above parts of pear trees and screened for *in vitro* antagonistic activity. Ten isolates inhibited the growth of the pathogen were identified by biochemical tests and partial sequencing of 16s rRNA or *recA* genes as *Pantoea agglomerans* (Pa9, Pa10, Pa21), fluorescent *Pseudomonas* sp. (Ps117, Ps170, Ps49, Ps89), *Enterobacter* sp. (En23, En113) and *Serratia* sp. (Se111). These strains were selected for biological control efficacy on immature fruits, detached flowers and leaves under laboratory conditions. All antagonists were able to reduce the disease severity on fruit and flowers. On immature fruits assay, isolates Pa21 and En23 with 83% and 25%, respectively had the highest and lowest effects on disease incidence compared to the control. On flowers, isolates Ps170 with 92% and En23, Ps89 and Se111 with 25% reduction of infection, respectively, had the highest and lowest effects under condition tested. Among strains tested, Ps170 produced amplified fragments corresponding to biosynthesis genes to produce antibiotics PCA, DAPG, Pyrrolnitrin and Pyoluteorin. Based on results obtained in this study, Ps170, Ps117, En113 and Pa21 strains have potential to be used for fire blight control, although their efficacy needs to be evaluating under natural field conditions.

1. Introduction

Pyrus with twelve species is one of the most important fruits growing in temperate regions of Iran. Among species, *Pyrus communis* known as common pear had cultured at about 18,000 hectares (Ministry of Agriculture-Jahad of Iran, 2014). Fire blight disease caused by *Erwinia amylovora* is a destructive disease of pear, apple and other rosaceous plant species and has been worldwide distribution. In Iran, fire blight was first reported in Karaj region (Zakeri and Sharifnabi, 1991). Since then, it has been widespread and is one of the most important diseases of pear and apple trees in several parts of Iran including Kurdistan province (Mollaei and Harighi, 2013; Rahnama and Mazarei, 2002). Nearly all commercial pear cultivars are susceptible to *E. amylovora* (Van der Zwet and Beer, 1995). Effective control of fire blight can be achieved through using of the streptomycin and oxytetracycline but this approach has a risk of pathogenic strains resistant development (McManus et al., 2002). Chemical control by application of copper-based bactericides is effective but it has possible risk of phytotoxicity and pathogen resistance development (Sholberg et al., 2001). The use of chemical inducer of systemic acquired resistance such as Acibenzolar-S-methyl has moderate protection effect from the pathogen (Brisset et al., 2000; Sparla et al., 2004). Due to the high risk of phytotoxicity and resistance of the causal agent to chemical and antibiotics, biological control method is of particular alternative (Vanneste et al., 2004). Various bacteria such as *Bacillus subtilis*, *Lactobacillus plantarum*, *Pseudomonas fluorescens*, *Pantoea agglomerans*, *Rahnella aquatilis*, yeasts and plant extracts have used as biocontrol agents against fire blight

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(Zeller, 2006; Rosello et al., 2013). Among them most numerous studies have been made using *P. agglomerans* and *P. fluorescens* strains (Kenneth and Stochwell, 2000). Some of them became commercially available for fire blight management. *P. fluorescens* A506, *P. agglomerans* E325, *P. agglomerans* P10c, *P. vagans* C9-1 and *B. subtilis* QST713 formulated as BlightBan A506 (Johnson and Stockwell, 1998), Bloomtime FD (Pusey et al., 2008), Blossom Bless (Vanneste et al., 2002), BlightBan C9-1 (Johnson and Stockwell, 2000) and Serenade (Aldwinckle et al., 2002), respectively have been registered as biological products for fire blight control. Some experiments showed that reducing of disease incidence by using antagonist is similar to treatment with antibiotic (Johnson and Temple, 2013). Several mechanisms such as antibiosis, nutrient competition and colonization have been proposed for inhibition of *E. amylovora* and reduction of fire blight incidence. Production of various antibiotics including phenazine, pantocin A and pantocin B is a common feature of *P. agglomerans* strains with potential biocontrol activity for fire blight (Giddens et al., 2002; Wright et al., 2001; Stockwell et al., 2002). The most common mode of actions of other bacterial antagonists such as *Pseudomonas* species are competition for space and nutrients (Vanneste et al., 2004; Cabrefiga et al., 2007; Lindow and Suslow, 2003). In a tritrophic system involving a plant host, biocontrol agent, and a pathogen all three components are subject to environmental conditions. Therefore, develop new biological control agents adopted to specific region and using it instead of commercially available agents might be has a better biological control efficacy. In the case of fire blight, the efficacy of biological control products available is generally low, variable and needs to be used in combination with antibiotics (Ngugi et al., 2011). Therefore, it is still a need to finding new strains with possible novel mechanisms of biological control action. The first aim of this study was to characterize epiphytic bacteria isolated from above parts of pear trees. The second objective was to evaluate the potential of bacterial isolates as antagonist of *E. amylovora* by testing both *in vitro* and *in sito* on detached plant organs.

2. Materials and methods

2.1. Isolation and identification of Bacterial antagonist

The potential bacterial antagonists were isolated from the leaf and blossom surface of healthy pear trees from different locations of Kurdistan province during May to September 2011 and 2012. Samples were collected, placed in plastic bags and used immediately or kept in refrigerator for short period of time. Small tissue pieces were macerated in 2–3 ml of sterile-distilled water for 30 min and the suspension was streaked onto nutrient agar (NA) (Merck) or King's Medium B Agar (KMB) plates (King et al., 1954). The plates were incubated at 26–28 °C for 48–72 h. Identification of bacteria was done by phenotypic properties and partial nucleotide sequencing of *16S rRNA* using PCR with rP1/fD2 primers (Weisburg et al., 1991) or *recA* genes (Waleron et al., 2002). The PCR products were sequenced using an ABI3730XL DNA sequencer (Applied Biosystems) and the sequences were compared with NCBI nucleotide sequence database using blast program. Nucleotide sequences were aligned using the ClustalW program (Ramu et al., 2003) available in BioEdit Sequence Alignment Editor 7.0.9.0 software (Hall, 2011). Alignments were manually adjustment where necessary. Neighbor-joining phylogenetic analysis was performed on datasets by using MEGA version 6.06 (Tamura et al., 2013). A phylogenetic tree was constructed using the neighbor-joining methods (bootstrap analysis with 1000 replicates was conducted) with the same program. *Erwinia amylovora* originally isolated from pear trees with fire blight symptoms in Kurdistan province was used in all experiments (Mollaei and Harighi, 2013).

2.2. Screening of potential antagonists against bacterial pathogen in dual culture assay

Three hundred µL of bacterial pathogen suspension (about 2×10^8 CFU mL^{-1}) was poured into the plates containing nutrient agar medium and maintained at room temperature for 5 min. After which, paper disc (about 10 mm) was immersed in each suspension of antagonistic bacteria spectrophotometrically adjusted (OD_{600nm}) to concentration of about 10^8 CFU mL^{-1} and was spotted at the pathogen-inoculated plates. The plates were incubated at 27 °C for 48–72 h and the width of the inhibition zones was measured. Sterile water spotted in the plates with the pathogen was used as control.

2.3. Antibiotic, protease, hydrogen cyanide and siderophore production by antagonistic bacteria

Screening of antibiotics by antagonistic bacteria was done as previously described (Vanneste et al., 1992). Suspension of bacteria were adjusted to concentration of about 10^8 CFU mL^{-1} and spotted into plates containing NA medium. Plates were incubated at 28 °C for 48 h, then bacterial colonies were discarded from the plates using sterile cotton before exposure to chloroform vapor for 1 h. Subsequently, 300 µl of pathogen suspension (about 2×10^8 - CFU mL^{-1}) was poured into the plates. The plates were incubated at 28 °C for 48–72 h and the width of the inhibition zones was measured. Sterile water spotted in the plates with the pathogen was used as control.

Bacterial isolates were tested for production of protease on skim milk (SKM) agar medium according to method previously described (Smibert and Krieg, 1994). The isolates were spot inoculated into SKM medium and kept at 28 °C for 48 h. Clear halo zone production around the bacterial colony was taken as evidence of extracellular protease production. Production of Hydrogen cyanide (HCN) by antagonistic bacteria was detected according to the method described by Alstrom and Burns (1989). Briefly, 100 µL of bacterial suspension was spread on nutrient agar medium, sealed with parafilm and incubated in an inverted position at 26–28 °C with picric acid indicator papers (5% picric acid/2% Na_2CO_3 solution) placed inside the lids. A color change of indicator from yellow to brown or reddish brown was recorded as HCN production. Siderophore production test was done using CAS agar assay according to a method previously described (Schwyn and Neilands, 1987). The isolates were spot inoculated into CAS agar medium. Plates were incubated at 27–28 °C, and positive results were recorded as a halo zone formation around the colonies.

2.4. Detection of biosynthetic gene targets for known antimicrobials

The presence of gene targets for antibiotics phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (Phl) was screened by PCR using specific primers PCA2a/PCA3b and Phl2a/Phl2b, respectively, according to method described by Raaijmakers et al. (1997). Screening for pyoluteorin and pyoluteorin biosynthetic loci were done by PCR using oligonucleotide primers PrnCf/PrnCr and PltBf/PltBr, respectively. Cycling program was as described by Mavrodi et al. (2001). The amplification products were electrophoresed on 1% agarose gels in 1× TAE buffer at 70 V, stained with 0.5 µg/mL ethidium bromide and photographed.