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Modulation in phenolic root exudate profile of *Abelmoschus esculentus* expressing activation of defense pathway

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ABSTRACT

Phenolics play a key role in communication between plants and microbes in the rhizosphere. In this study, shikimic, gallic, fumaric, ferulic, vanillic acid and quercetin in root exudates of *Abelmoschus esculentus* act as chemoattractants of endophytic *Alcaligenes faecalis* strains, BHU 12, BHU 16 and BHU M7. *In vitro* chemotaxis assay showed that BHU 12 expressed highest chemotactic movement (CFU $\sim 50 \times 10^{12}$) towards *A.esculentus* root exudates followed by BHU 16 and BHU M7 (CFU$\sim 9 \times 10^{12}$), thereby confirming their ability to colonize the host rhizoplane region. However, BHU 16 expressed highest biofilm formation ability followed by BHU 12 and BHU M7. Assessment of chemotactic and biofilm formation potential towards individual phenolic acids revealed BHU 12 to be maximally attracted towards 1 µM shikimic acid (2×10^{15}) while BHU 16 towards 1 mM vanillic acid (6.5×10^{12}) and BHU M7 towards 1 mM ferulic acid (3.5×10^{12}), thereby confirming the phenolic acid components responsible for particularly attracting the endophytic isolates. Upon colonization, the endophytic isolates modified the phenolic profiles of root exudates *in planta* in a manner so as to plausibly attract more of the beneficial rhizospheric microbiota as well as self-fortification against pathogenic microbes. This hypothesis was verified by monitoring the changes in phenolic components of *A. esculentus* root exudate owing to *S. rolfsii* infection, a disastrous soil-borne pathogen. Thus, on the whole, the work provides intricate details of plant-endophyte interactions for biotic stress management through careful manipulation of root exudates, thereby aiding in sustainable agriculture.

1. Introduction

The rhizosphere region represents a dynamic ecosystem with the diverse flora, fauna and microbes continuously interacting with each other in a variety of complex reactions (Whipps, 2001; Singh et al., 2016a). Governed by root exudates, these interactions primarily mediate promotion of plant performance in terms of growth and defense (Dakora and Phillips, 2002; Bais et al., 2004, 2006; Badri and Vivanco, 2009; Bertin et al., 2003; Sun et al., 2012; Haichar et al., 2014). Plant root exudates contain not only ions, free oxygen, water, enzymes, mucilage, carbon-containing primary and secondary metabolites, but also phenolics, which selectively stimulate growth of rhizospheric soil microbiota by generating redox reactions in soils thereby altering the composition of microbial communities in different root parts (Northup et al., 1998; Hättenschwiler and Vitousek, 2000; Verbon and Liberman, 2016). Moreover, phenolics subjected to abiotic and biotic reactions contribute to mineralization of soil phosphorus and nitrogen as well as humification (Kefeli et al., 2003; Halvorson et al., 2009). Phenolics are also reported to chelate metals and improve soil porosity leading to an increase in the mobility and bioavailability of essential elements, such as magnesium, potassium, calcium, zinc, copper, manganese, iron, boron and molybdenum, for plant roots (Cesco et al., 2012; Pii et al., 2015).

The phenomenon of chemotaxis and biofilm formation serve as the basic prerequisites for effective bacterial colonization (Sood, 2003; Timmusk et al., 2005; Li et al., 2013; Kimani et al., 2016).Most of the phenolics of root exudates serve as chemotactic signals for a number of soil microorganisms that recognize them and move towards plant roots in the carbon-rich environment of rhizosphere (Perret et al., 2000; Taylor and Grotewold, 2005). In line with the above context, Singh et al. (2016b) reported enhanced chemoattraction of *Bacillus subtilis* towards rice plant due to rutin, a bioflavanoid, which further stimulated antioxidant pathway of the host. The signal system of plants plays an effective role as it screens the approaching microbes by differentially

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stimulating the growth of beneficial ones through the process of root exudation and simultaneously inducing defense mechanisms against pathogens to prevent parasitic interactions. Thus it may be hypothesized that plant species acquire resistance against potential soil-borne pathogens by releasing allelochemicals as root exudates (Dixon, 2001; D'Auria and Gershenzon, 2005; Baetz and Martinoia, 2014). Several reports indicate defensive attributes of root exudates, for instance, *Ocimum basilicum* challenged with *Pythium ultimum* released rosmarinic acid, a caffeic acid derivative with antimicrobial property (Bais et al., 2002). Similarly, Bais et al. (2005) clarified root exudate mediated resistance offered by *Arabidopsis thaliana* to *P. syringae* pathovars. Baetz and Martinoia (2014) suggested that alterations in root exudate components drastically modify the rhizospheric microbiota. In line, Verbon and Liberman (2016) advocated that root exudates influence quorum sensing in soil bacteria while the rhizospheric microbes, in turn modify root exudate composition and induce systemic resistance within host plants.

Abelmoschus esculentus (okra) is a multipurpose crop owing to the various uses of its tissues (Mihretu et al., 2014). While, the fruit serves as a culinary delicacy (Ndunguru and Rajabu, 2004), the mucilage can be utilized as a renewable source of biodegradable material, due to its plasticity, high water solubility, viscosity and elasticity (BeMiller et al., 1993). For these reasons *A. esculentus* is one of the most sought after vegetable crops with India ranking first in its production and export (NCPAH, 2016). However, the yield can be negatively affected by several forms of biotic stresses, such as the attack by fungal phytopathogens, for instance, *Sclerotium rolfsii*, which requires warm and humid temperature conditions for its activity.

Plant inoculation with endophytic microbes protects the host plant from several forms of environmental stresses (Ray et al., 2015, 2016; Mishra et al., 2015). *Alcaligenes faecalis* is well known for its various plant growth promotional attributes, such as, IAA production, phosphate solubilization and ammonia production which eminently aids in root growth as well as antagonistic activity against common soil borne phytopathogens owing to production of hydrogen cyanide and proteolytic activity. These attributes support the candidature of *A. faecalis* as a suitable plant growth promoting and biocontrol agent (Ray et al., 2016; Ndeddy Aka and Babalola, 2016).

This study aims to identify the root exudate phenolics responsible for chemotactically attracting endophytic *A. faecalis* when *A. esculentus* plants were grown under hydroponic conditions. The changes in the composition of root exudate phenolics due to the presence of endophytic microbes were analyzed by high performance liquid chromatography (HPLC). Changes were also observed in root exudate composition in case of okra infected with *S. rolfsii* which enabled the evaluation of basic defense acquired by the host. Thus, this study provides a novel description of sequential changes occurring in phenolic acid compositions of root exudates due to priming by beneficial microbes followed by challenge with harmful phytopathogens.

2. Materials and methods

2.1. Okra root exudates preparation

Seeds of *A. esculentus* (cv. Ujjwal) were surface sterilized using 5% sodium hypochlorite for 10 min followed by three successive rinses with sterile distilled water. The surface sterilized seeds were transferred to 0.5% water agar plates and incubated at 28 ± 2 °C for 2–3 days until emergence of the radicle. For each extraction, 30 seedlings with approximately 2 cm radicle length were transferred to a 50 ml conical flask containing 5 ml of sterile chemotaxis buffer (10 mM potassium phosphate, 0.1 mM ethylene diamine tetra acetic acid(EDTA), 1 mM magnesium sulphate; pH 7.0) and incubated at 28 ± 2 ° C for 24 h. The root exudates released in the buffer were collected and sterilized by passing through a 0.22 µm filter and stored at −80 °C (Yao and Allen, 2006). Total protein concentration of root exudates was determined according to Bradford assay (Bradford, 1976).

2.2. HPLC analyses of A. esculentus root exudates

The collected root exudates were fractionated with ethyl acetate (1:1). The organic fraction was collected and refractionated three times with ethyl acetate to completely collect the organic components of root exudates. The ethyl acetate solution was evaporated and the resulting phenolic components were dissolved in HPLC grade methanol and filtered through 0.22 µm millipore filter.

Analysis of phenolic acids in the exudate was performed according to Singh et al. (2014). The pure phenolic acid standards used for HPLC were purchased from Sigma-Aldrich (St. Louis, MO, USA). Injection volume of the sample was 20 µl. The HPLC, Shimadzu LC-10A (Japan) was equipped with dual pump LC-10A binary system, UV detector SPD-10A, Phenomenex (Torrance, USA) and C18 column (RP-Hydro, 4 µm, 250 mm × 4.6 mm). The mobile phase included 1% acetic acid (A) and acetonitrile (B) with the following gradient elutions: 0 min 82% A plus 18% B at the flow rate of 1 ml min^{-1} for 15 min; 68% A plus 32% B at the flow rate of 1 ml min^{-1} for 25 min; 50% A plus 50% B at the flow rate of 1 ml min^{-1} for 30 min. After 30 min, the run was stopped and optical density of the eluents was detected at 254 nm wherein the peaks of samples were compared with their standard peaks.

2.3. Chemotaxis and biofilm formation by the endophytic bacterial isolates in presence of A. esculentus root exudates

2.3.1. Chemotaxis assay

Qualitative analysis of chemotactic response of the endophytic isolates, BHU 12, BHU 16 (isolated from *A. esculentus* leaf) and BHU M7 (isolated from *Andrographis paniculata* leaf) towards each of the HPLC identified phenolic components of okra root exudates was performed according to Singh et al. (2016b). 24 h old bacterial cultures (10 µl) were spot inoculated on swarm agar plates comprising of 0.8% nutrient broth, 0.5% glucose and 0.4% agar. Each of the HPLC identified phenolic acids at three different concentrations, i.e. 1 mM, 1 µM and 1 nM were incorporated into the plates in form wetted filter paper discs. The plates were further incubated at 28 ± 2 °C for 10 h for assessing chemotactic motility towards the different phenolic acids.

Quantitative assessment of chemotactic ability of BHU 12, BHU 16 and BHU M7 towards each of the HPLC identified phenolic acids was evaluated according to Tan et al. (2013) with slight modifications. A 4-cm 25-gauge needle attached to a 2 ml syringe was used to contain 100 µl of crude root exudate and their individual phenolic acids at concentrations of 1 mM, 1 µM and 1 nM, respectively in different experimental setups. The needle was pricked and inserted into an eppendorf tube (200 µl) containing 100 µl of bacterial suspension of each of the strains and the entire setup was incubated in dark under sterilized conditions at 28 ± 2 °C. Bacterial chemotaxis towards crude root exudates was assessed after 2, 4 6, 8, 10 and 12 h, whereas that towards each phenolic acid was evaluated after 1 h.

After the required time interval, the syringe content was transferred to an eppendorf tube containing 1 ml of sterile normal saline and serially diluted. The individual dilutions were plated on nutrient agar plates, incubated at 28 ± 2 °C and observed after 24 h for appearance of bacterial colonies.

2.3.2. Biofilm formation assay

In vitro biofilm formation by BHU 12, BHU 16 and BHU M7 on solid surface in presence of okra root exudates and its phenolic acids was assessed according to Ray et al. (2015). The strains were grown upto mid-log phase in nutrient broth (NB) medium (HiMedia). Aliquots from the respective cultures were inoculated in NB diluted with 1/10 vols of okra root exudates and the individual HPLC identified phenolic acids at concentrations of 1 mM, 1 µM and 1 nM, respectively. 100 µl of the mixture were added to each well of a 96 well PVC microtitre plate. The