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Influence of phenolic acids on indole acetic acid production and on the type III secretion system gene transcription in food-associated *Pseudomonas fluorescens* KM05

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The purpose of these investigations was to evaluate the reduction capability of phenolic acids (ferulic, chlorogenic, gallic, and *p*-coumaric acids) on indole acetic acid synthesis by food-associated *Pseudomonas fluorescens* KM05. Specific genetic primer for the type III secretion system (TTSS) in *P. fluorescens* KM05 was designed and the influence of phenolic acids on its expression was investigated. In the work the ferulic and chlorogenic acids at the concentration of 0.02 and 0.04 μg/ml affected on bacterial growth pattern and the signal molecules production. The phenolic acids, that were appreciable effective against *P. fluorescens* KM05 indole acetic acid production, significantly suppressed TTSS gene.

[**Key words:** Indole acetic acid; Ferulic acid; Chlorogenic acid; Gallic acid; *p*-Coumaric acid; *Pseudomonas fluorescens*; Type III secretion system]

Pseudomonas fluorescens is a common spoilage bacterium of fresh vegetables and minimally processed products of plant origin. This microorganism is responsible for loss of consumer acceptability in the above mentioned foods because it causes vascular plugging in plants, thus leading to their wilting. Therefore, it generates economical losses both in manufactures and in raw material producers (1).

Ongeng et al. (2) and Radziejewska-Kubzdela et al. (3) isolated such microorganisms from chilled vegetables and found out that majority of them belongs to *Pseudomonas* genera (78% of all isolates), including *P. fluorescens*. *P. fluorescens* are phytopathogenic bacteria, existing in intracellular spaces (apoplast) of plants. They elicit maceration of plant tissue and decrease their turgor (4). The type III secretion system (TTSS) is responsible for the process of microbial softening of plant tissue. This system works as a molecular syringe that introduces toxic effectors proteins T3 into cytoplasm of target cells (4). The TTSS process in phytopathogenic bacteria (including *P. fluorescens*) is regulated by cell–cell communication in which indole acetic acid molecules can mediate (5,6). The molecules of indole acetic acid are synthesized in logarithmic and stationary growth phases which is typical for autoinductors of intracellular signaling (*N*-acylhomoserinelactones or AI-2) (7). The indole acetic acid that is synthesized by microorganisms can diffuse freely by bacteria cell wall and accumulate in environment (7,8). The high concentration of indole acetic acid in environment is a controlling factor of virulence of phytopathogenic bacteria. These dependence were presented by Yang et al. (9). Scientists observed that in phytopathogenic bacteria *Erwinia chrysanthemi* 3937 suppression in TTSS gene expression is caused by disorder in indole acetic acid synthesis. The *E. chrysanthemi* 3937 cells with correctly functioning pathway of indole acetic acid were virulent (9). In this case indole acetic acid indirectly influenced on protein synthesis which induced changes in cytoskeleton in plant cells causing creases their cell wall (6,9). The protein cells with creased cell wall are vulnerable to attack of biotic intruders (6). The discovery that TTSS system is signaling molecules-related inspired us to investigate the mechanism of disturbed TTSS gene expression by the limitation of indole acetic acid synthesis.

The results obtained by Brackman et al. (10) and Truchado et al. (11) confirmed that phenolic acids (among others ferulic, chlorogenic, gallic, and *p*-coumaric acids) in herbs, spices and fruits are signaling molecules inhibitors. However, only the influence of these phytochemicals on the decreasing level of autoinducers and the bacterial growth kinetics were investigated up to date (10,11). In the available literature there is no data about the true effect of phenolic acids on gene which is responsible for microorganisms' virulence expression. The researches mentioned above were oriented on the recognition of how the signaling molecules makes it possible to develop effective process minimalizing microbial spoilage of food without the risk of elimination of resistance bacterial forms.

The aim of this work was to compare reduction capability of phenolic acids (ferulic, chlorogenic, gallic, and *p*-coumaric acids) on indole acetic acid synthesis by *P. fluorescens* KM05. First, a specific genetic primer for TTSS system in *P. fluorescens* KM05 was designed and next the influence of phenolic acids on its expression was investigated.

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MATERIALS AND METHODS

Strains *P. fluorescens* KM05 strain used in this study was isolated from storage cabbage that came from a farm in western Poland. Restriction length polymorphism of 16S rRNA gene amplicons and sequencing were carried out for the purpose of bacterial identification. *P. fluorescens* KM05 strain is deposited at the Department of Biotechnology and Food Microbiology strain collection, in Poznan University of Life Sciences, Poland and is freely available upon request.

Vibrio harveyi BB170 and *V. harveyi* BB152 were obtained from the American Type Culture Collection (Rockville, MD, USA). *V. harveyi* BB152 with a *luxS* gene deletion was used as the negative control strain. *V. harveyi* BB170, that specifically responds to signaling molecules with the induction of bioluminescence, was used as the reporter strain.

Indole acetic acid inhibitors Gallic, chlorogenic, *p*-coumaric, and ferulic acids were obtained from Sigma Aldrich (USA). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Germany). All chemicals were of analytical grade and Milli-Q water was used throughout. Phenolic compounds were initially dissolved in DMSO and filtered through a sterile Millex-GP 0.22 μm filter (Millipore, Billerica, MA, USA).

Culture conditions *P. fluorescens* KM05 strain was grown in modified tryptic soy broth (TSB) medium. The modified TSB medium consisted of (g/L): enzymatic digest of casein 17; enzymatic digest of soybean meal 3; sodium chloride 5; dipotassium phosphate 2.5; L-tryptophan 0.5. A comparison of capacity of tested phenolic compounds to inhibit indole acetic acid synthesis was carried out in the modified TSB medium supplemented with phytochemicals to the final concentration of 0.002, 0.02, 0.04 and 0.2 μg/ml. The maximum concentration of DMSO in the assays was 0.1%, which had no effect on bacterial growth. The cultures were incubated at 30°C for 120 h. The pH of the culture media was 7.0.

All strains of *V. harveyi* were grown in Autoinducer Bioassay medium (BD, USA) with shaking (100 rpm/min). The cultures were incubated at 30°C for 24 h. The pH of the culture media was 7.0.

Evaluation of bacterial growth kinetics Biomass buildup was monitored by the pour plate method after every 2 h of cultivation. The Bacterial Growth Kinetics software (Raleigh, USA) was applied for estimation of individual parameters of *P. fluorescens* MK05 growth cycle. It was performed after fitting the observed data to the sigmoidal curves.

Bioluminescence assay The influence of indole acetic acid production by *P. fluorescence* KM05 on light emission by luminescent reporter *V. harveyi* strains (BB152, BB170) was determined using the method of Bassler et al. (12). Briefly, the cell-free supernatants of *P. fluorescens* KM05 were collected by centrifugation (3000 *g*/10 min) after 1, 3, 5, 7, 12, 24, 48, 72, 96 and 120 h of cultivation. Then the cell-free supernatants were passed through a sterile Millex-GP 0.22 μm filter (Millipore). The luminescent reporter *V. harveyi* strains (BB152, BB170) were grown for 24 h at 30°C with shaking (100 rpm/min) in AB medium and diluted 1:5000 in fresh AB medium. The volume of 20 μl of cell-free supernatants was finally added to the diluted *V. harveyi* BB170 cells (180 μl) in wells of a 96-well fluorescent microplate (BD). The fluorescent microplate was then shaken at 30°C for 4 h. To measure levels of background luminescence, samples containing 180 μl of *V. harveyi* BB152 (dilution, 1:5000) and 20 μl of the cell-free supernatants were also included. After incubation, the resulting light production of each sample was measured by a Multimode Microplate Reader Infinite200 ProSeries (Tecan, Switzerland). Results were expressed as relative luminescence units (RLU) of the *V. harveyi* reporter strains. The results were calculated by the following formula:

$$100 - \left[\frac{A}{B} * 100\right] \quad (1)$$

where *A* indicates light emission induced by indole acetic acid produced by *P. fluorescens* KM05 grown in modified TSB medium supplemented with phenolics, and *B* means light emission induced by indole acetic acid produced of *P. fluorescens* KM05 grown in modified TSB medium.

Quantification of indole acetic acid Indole acetic acid content in *P fluorescens* KM05 supernatants was assayed as reported by Patten and Glick (13) after 1, 3, 5, 7, 12, 24, 48, 72, 96 and 120 h of cultivation. The bacterial cells were removed by centrifugation at 4000 *g* for 20 min at 4°C. One ml of cell-free supernatant was mixed with 4 ml of Salkowski reagent, which consisted of 12 g $FeCl_3$ per liter in 7.9 M H_2SO_4. The mixture was left in the dark for 30 min at room temperature. Development of a pink color indicated indole acetic acid. Indole acetic acid content in the samples was evaluated by measuring $O.D._{535}$. Calibration curves were prepared against standard indole acetic acid solutions (Sigma, USA). The percentage inhibition of indole acetic acid production was calculated by the following formula:

$$100 - \left[\frac{C}{D} * 100\right] \quad (2)$$

where *C* means $O.D._{535}$ of supernatant of *P. fluorescens* KM05 grown in modified TSB medium supplemented with phenolics, and *D* indicates $O. D._{535}$ of supernatant of *P. fluorescens* KM05 grown in modified TSB medium.

RNA isolation and cDNA synthesis *P. fluorescens* KM05 cells were cultivated on modified TSB medium supplemented by ferulic and chlorogenic acids and with or without indole acetic acid (Sigma) (1.5 μg/ml) during 72 h. The 72 h fermentation carried out on modified TSB medium was used as a reference probe. Prior to RNA isolation the *P. fluorescens* KM05 were treated with RNAprotect Bacteria Reagent (Qiagen, USA) as recommended by the reagent manufacturer. Total RNA was extracted from cell pellet with TRI Reagent (Sigma) according to the manufacturer's recommendations. The extracted total RNA was treated with the TURBO DNA-free Kit (Life Technologies, USA) to remove residual DNA. Before creating complementary DNA (cDNA), the RNA concentrations in the samples were quantified with a NanoDrop ND-1000 Spectrophotometer (Implen, Germany). First strand of+ cDNA was synthesized from 2 μg total RNA with High Capacity RNA-to-cDNA Kit (Life Technologies) according to the manufacturer's protocol.

Quantitative real-time PCR Quantitative real-time PCR (qRT-PCR) was performed using intercalating dye-based GoTaq qPCR Master Mix (Promega, Germany) with 2 μl of cDNA. To detect transcripts of the TTSS a primer pair was designed based on alignment of several pairs available from GenBank *P. fluorescens* TTSS sequences using PriFi program to target the most conserved sequence region (14). The forward primer of TTSS was 5′ TGATYGTGTCCAACCTGCTGCTGGC 3′ and the reverse was 5′ GAAVAGGCTGTCGAGCAA 3′ which amplify a product of 128 bp. The F0F1 ATP synthase subunit epsilon (atpC) was used as the non-differentially expressed control gene (i.e., endogenous control), the forward primer of atpC was 5′ ACATCGTCAGCGCGGAAGGAGAA 3′ and the reverse was 5′ TCGAGGAAACCA CCGGAGATGTAG 3′ (15). The primers used for real-time PCR were first tested using conventional PCR with DNA isolated from *P. fluorescens* KM05. Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA) under the following conditions: initial cycle 95°C for 10 min and 40 cycles of: 95°C for 15°s; 58°C for 15 s; and 72°C for 30 s followed by melting curve analysis. The comparative threshold cycle (CT) method was used for the analysis of transcriptional levels (16). The results are presented as a $\log_2$ of relative expression of mRNA of TTSS gene (RQ). For reference probe $\log_2$ (RQ) equaled 0.

Statistical analysis The results presented here comprise the average of three independent experiments. Students' *t*-test was used to determine the significant difference ($P < 0.05$) between the inhibition of indole acetic acid production by examined cells. Students' *t*-test was also used to determine the significant difference ($P < 0.05$) between the inhibition of light emission by luminescent reporter *V. harveyi* strains.

RESULTS

Growth curves To characterize the effect of different concentrations of bioactive phytochemicals on *P. fluorescens* KM05 physiology, the growth kinetics studies were carried out (Fig. 1). In the study we particularly focused on the length of lag-phase. The parameter determines the required time for *P. fluorescens* KM05 adaptation for growth-limiting agents in the medium. Beginning of the exponential growth phase by examined cells indicates that bacteria have already started the production of the indole acetic acid molecules (7,8).

In the work, only the content of 0.2 μg/ml of examined phenolics in the culture medium exhibited an inhibitory effect on *P. fluorescens* KM05 growth (data not shown). Compared to the control probe, prolonged the lag-phase and exponential growth phase was obtained when the content of chlorogenic and ferulic acids in the medium were at the level of 0.02 and 0.04 μg/ml, respectively. In the above experimental variants, the stationary phase of bacteria growth started in 18 h of incubation. Delaying the introduction of proliferation indicated to the changes in *P. fluorescens* KM05 physiology in response to the presence of growth-limiting agents. The significant changes in the length of the various stages of development *P. fluorescens* KM05 strain was not observed after exposure of cells to the selected concentrations of gallic and *p*-coumaric acids. In the above experiments, as in the control probe, the average lag-phase lasted 3 h, exponential phase of growth, a further 5 h. The stationary phase of cells growth began in approximately 8 h of cultivation. In all experiments the typical declined phases were observed after 96 h of incubation.

Inhibition of synthesis of indole acetic acid Bioluminescence assay and monitoring of indole acetic acid content in the supernatants of *P. fluorescens* KM05 enabled characterization of the effect of tested phytochemicals on secretion of the signals by examined