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Proceeding Paper

Phenolic Compounds as Biomarkers of Interactions between the Endophyte *Klebsiella oxytoca* and the Common Duckweed, *Lemna minor* L. †

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Abstract: The common duckweed (*Lemna minor* L.) as a model organism is experiencing a form of a renaissance. In this study, our focus was on the interactions between duckweeds and a rhizosphere-associated bacterial strain, *Klebsiella oxytoca* (Access. No. MK212915). Five distinct phenolic compounds were identified by liquid chromatography–mass spectrometry: luteolin 6,8-di-C-hexoside, p-hydroxybenzoic acid, caffeic acid, apigenin 6-C-(2″-pentosyl)hexoside and p-coumaric acid. All of the identified compounds reflect the colonization of the plant by *K. oxytoca*. This paper is another call for all plant physiologists to focus their research on *L. minor* and to analyze different aspects of complex plant/bacterium interactions.

Keywords: biomarkers; phenolic compounds; *Klebsiella oxytoca*; duckweed; LC–MS

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1. Introduction

The common duckweed (*Lemna minor* L.) is a cosmopolitan, miniature, fast-reproducing higher aquatic plant of simplified morphology. In the last decade, the common duckweed has experienced a form of renaissance, after decades of being largely substituted by *Arabidopsis thaliana* [1]. Its simple and small genome, as well its high vegetative reproduction rates, low requirements for in vitro growth and ability to thrive even under unfavorable conditions, makes the common duckweed, as well as many other related species of the same family (Lemnaceae), an almost ideal model organism for a wide variety of studies. One of the important traits of the common duckweed is its ability to co-exist with a large number of various microorganisms in its natural habitat, which makes it particularly well-suited for research on plant–microorganism interactions [1,2]. In this work, we analyzed phenolic compounds associated with the co-cultivation of an endophytic bacterium, *K. oxytoca*, and duckweed (*L. minor*). These compounds correspond to various stages of colonization of the plant by bacteria and antioxidative responses of the plant to bacterial presence. Therefore, we propose that they can be used as biomarkers of interactions between these two species.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

L. minor was collected from a pond in the garden of the Institute for Biological Research "Siniša Stanković" in Belgrade (44°48′14.44″ N, 20°27′54.47″ E). Sterile duckweed cultures (two–four fronds) were maintained according to our previous protocol: in Murashige and Skoog medium at 24 ± 2 °C (under fluorescent light of 40 μmol m^{-2} s^{-1} with 16 h light/8 h dark photoperiod) [3]. Nutrition medium was replaced every 7 days.

2.2. Bacterial Strain Klebsiella oxytoca 14bg Access. No. MK212915 and Growth Conditions

K. oxytoca was isolated and identified in our previous studies and selected for the ensuing experiments due to its high resistance to phenol, ability to eliminate phenol and its positive effect on the multiplication rates of the duckweeds *in vitro*. This strain was also identified as an endophyte [4]. Bacterial monoculture containing *K. oxytoca* was grown and maintained on a Luria–Bertani (LB) medium and prepared according to the previously applied protocol [4].

2.3. Co-Cultivation of K. oxytoca and Duckweed

In our previous work, we observed a change in color of nutrition MS medium (from colorless to green-brownish) during experiments with multiplication rates of duckweeds. This change in color appeared only in duckweed specimens with *K. oxytoca*, and not with other bacteria, and was more pronounced in phenol-free MS media than in phenol-supplemented MS media (with 500 mg L^{-1} of phenol) [4]. This prompted further investigation. Briefly, an overnight culture of *K. oxytoca* in 5 mL of LB medium was used to inoculate sterile MS medium containing duckweeds. Overnight culture was briefly centrifuged, the supernatant discarded and the remaining debris resuspended in sterile MS medium (1 mL) which was then transferred to a flask containing sterile MS medium (100 mL) with 100–150 surface-sterilized duckweeds. Duckweeds and bacteria were then co-cultivated for 3 days. After 3 days, samples of this MS medium were taken for LC–MS analysis.

2.4. Liquid Chromatography–Mass Spectrometry (LC–MS)

MS nutrition medium with duckweeds and *K. oxytoca* was analyzed using LC–MS. System used for LC–MS was: UHPLC Accela 6000-ESI-LTQ Orbitrap XL with column C18 with 1.7 microns diameter, from the manufacturer Thermo Fisher Sci, Waltham, MA, USA. Gradient elution with two elution buffers was performed. Elution buffers A and B were (A) 0.1% aqueous solution of formic acid (HCOOH) and (B) acetonitrile.

2.5. Bibliographical Analysis

Bibliographical analysis was conducted using the Publish or Perish software [5] and the data was retrieved from the Google Scholar database.

3. Results

3.1. Change of Color of MS Medium

A change in color of MS medium used for co-cultivation of *K. oxytoca* and duckweeds (from colorless to green-brownish) was apparent after 48 h of co-cultivation (Figure 1). The color became darker as the co-cultivation progressed.

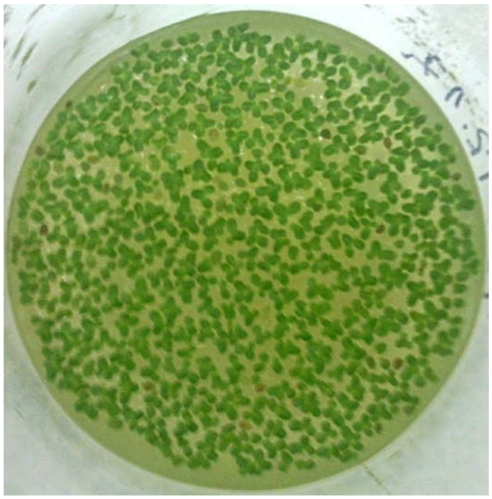

Figure 1. Appearance of color in MS medium used for co-cultivation of *L. minor* and K. oxytoca.

3.2. Bibliographical Analysis

Bibliographical analysis of publications containing key words "duckweed phenolic compounds liquid chromatography mass spectrometry bacteria rhizosphere" showed that there are 489 research papers published between 1960 and 2021 corresponding to these search criteria. The majority of these papers (457) were published after 2001.

When the keywords "duckweed phenolic compounds liquid chromatography mass spectrometry Klebsiella oxytoca" were used as the search criteria, only 17 papers published between 2001 and 2021 were retrieved. Out of these, 10 articles were presented with an h index.

3.3. LC–MS

LC–MS detected five phenolic compounds: luteolin 6,8-di-C-hexoside, p-hydroxybenzoic acid, caffeic acid, apigenin 6-C-(2″-pentosyl) hexoside and p-coumaric acid (Table 1).

Table 1. Phenolic compounds detected by LC–MS in MS medium used for co-cultivation of *K. oxytoca* and *L. minor*.

No.	t_R, min	Compound Name	Molecular Formula, $[M–H]^-$	Calculated Mass, $[M–H]^-$	Exact Mass, $[M–H]^-$	Δ ppm	MS^2 Fragments, (% Base Peak)	MS^3 Fragments, (% Base Peak)	MS^4 Fragments, (% Base Peak)
1	5.16	Luteolin 6,8-di-C-hexoside	$C_{30}H_{25}O_{14}^-$	609.14611	609.14380	3.79	591(10), 519(30), **489**(100), 429(10), 399(20), 369(15)	471(10), 399(30), **369**(100)	**341**(100), 313(40), 298(30)
2	5.66	*p*-Hydroxybenzoic acid	$C_7H_5O_3^-$	137.02442	137.02415	1.97	109(10), **93**(100)	–	–
3	5.91	Caffeic acid	$C_9H_7O_4^-$	179.03498	179.03513	−0.84	**135**(100)	135(60), 117(15), **107**(100), 91(55), 79(15)	–
4	6.24	Apigenin 6-C-(2″-pentosyl)hexoside	$C_{26}H_{27}O_{14}^-$	563.14063	563.13922	2.50	443(10), 431(10), **413**(100), 341(10), 311(20), 293(40)	**293**(100)	275(20), 265(60), 249(90), 221(40), **175**(100), 173(70)
5	6.60	*p*-Coumaric acid	$C_9H_7O_3^-$	163.04007	163.03984	1.41	**119**(100)	119(60), 101(20), 93(25), **91**(100), 72(10)	–

4. Discussion

The observed change in the color of the MS medium used for the co-cultivation of *K. oxytoca* and *L. minor* was probably due to release of p-hydroxybenzoic, caffeic and p-coumaric acids, known for their brownish color. Luteolin 6,8-di-C-hexoside is abundant in many plants, where it has immunomodulatory effects [6]. p-Hydroxybenzoic acid is accumulated in plants that are under bacterial attack; it is also an intermediary compound in bacterial metabolism of some monoaromatic compounds [7]. Caffeic acid is an essential biomolecule of all plants, as it is a lignin precursor. However, it is an uncommon metabolyte in bacteria [8]. Apigenin 6-C-(2″-pentosyl)hexoside is also a flavonoid and is found in root exudates. Its synthesis is stimulated during the endophytic colonization of roots [9]. p-Coumaric acid is also associated with the endophytic colonization of roots and with plants' defense responses [10]. Furthermore, it has antimicrobial properties [11]. Therefore, all the detected biomolecules can be associated with the endophytic colonization of the duckweed root, with the plant–bacteria signalization, and with bacterial modulation of plants' antioxidative responses. In our previous work [3], we also demonstrated the

importance of bacterial modulation of the antioxidative response of L. minor. Namely, the presence of bacteria H. paralvei modulated the expression of plants' peroxidases and alleviated phenol-induced stress on the plants. Similar processes might be happening in K. oxytoca–*L. minor* interactions as well, and the aforementioned phenolic compounds might in turn function to regulate bacterial activity.

Bibliographical analysis revealed that this specific interaction between *L. minor* and *K. oxytoca* is insufficiently investigated. Since the *K. oxytoca* strain 14bg used in this study [4] was identified as an endophyte with a potential positive effect on the multiplication rates of the duckweeds and an ability to remove phenol from MS medium, more analyses would contribute to their better application in bioremediation and/or agriculture. In the decade of re-discovery of *L. minor* as a model organism, the future of this research area looks more promising than ever [1].

5. Conclusions

Five phenolic compounds that reflect the interactions between endophytic *K. oxytoca* strain 14bg and L. minor, the common duckweed, were identified by LC–MS. According to the findings presented in this paper, interactions between the common duckweed and bacterium *K. oxytoca* are under-investigated and, therefore, more research is needed.

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