

# *Arabidopsis thaliana* Metabolites Secreted by Roots during Plant Growth in Phosphorus-Limiting Conditions

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**How to cite this paper:** Abdulkina, L.R., Agabekian, I.A., Chastukhina, I.B., Valeeva, L.R., Romanova, Y.D., Khilyas, I.V., Sharipova, M.R. and Shakirov, E.V. (2019) *Arabidopsis thaliana* Metabolites Secreted by Roots during Plant Growth in Phosphorus-Limiting Conditions. *American Journal of Plant Sciences*, 10, 987-1001.

<https://doi.org/10.4236/ajps.2019.106071>

**Received:** May 17, 2019

**Accepted:** June 18, 2019

**Published:** June 21, 2019

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## Abstract

Phosphorus is one of the most important nutrients required for plant growth and development. While substantial amounts of total phosphorus are present in many soil types, plants are unable to utilize some organic phosphorus sources. The main goal of this study was to characterize the spectrum of secreted plant proteins, organic acids and other metabolites that can potentially contribute to utilization of various phosphorus compounds. Our data indicate that the composition of extracellular proteins secreted by plant roots varies depending on the specific source of P in the growth medium. Furthermore, some root-secreted metabolites, such as citrate, appear to be specific to a subset of ecotypes, while tartrate, succinate and oxalate are secreted by a number of *A. thaliana* ecotypes. We observed secretion of phenolic compounds, such as tannins, and deoxycytidine derivatives. Taken together, while no single secreted polypeptide, organic acid or secondary metabolite can be pinpointed as specific to plant growth in particular phosphorus conditions, our data indicate that *A. thaliana* ecotypes differ in their physiological responses to the source of phosphorus in the growth medium. Overall, these results suggest that physiological changes in plant responses to nutrient limitation are modulated by interactions between soil phosphorus source and the specific genotype of *Arabidopsis* plants.

## Keywords

Natural Variation, Ecotype, Root Exudates, P Deficiency, Secretion

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## 1. Introduction

Phosphorus is one of the most important inorganic nutrients necessary for plant growth, development and physiology. While many soil types harbor substantial amounts of phosphorus-containing molecules, many of these compounds are characterized by low solubility or undergo rapid conversion to insoluble forms [1]. While common agricultural practices routinely rely on the addition of large amounts of phosphate fertilizers, this approach is not sustainable long-term, as it leads to irreversible depletion of non-renewable rock phosphate deposits and has a negative environmental impact due to phosphorus-associated pollution and eutrophication. Thus, an important aspect of modern plant science is to improve agricultural productivity in an environmentally friendly and cost-effective fashion, especially when plants are grown on phosphorus-deficient soils. Such agricultural approaches may include strategies to increase bioavailability of insoluble forms of soil phosphorus. Towards this goal, understanding mechanisms of how plants acquire and respond to various forms of phosphorus from soils may prove to be particularly important.

Plants growing in conditions of phosphorus deficiency can adjust their physiology to utilize alternative sources of phosphorus, including those that are hard to acquire [2]. Such physiological response includes changes in root morphology (inhibition of apical meristem growth and development of additional lateral roots and root hairs), an increase in the expression levels of phosphate transport genes, and induction and secretion of phosphatases into the rhizosphere. Secreted phosphatases have been found in many plants, including *Lupinus albus* [3], *Solanum lycopersicum* [4], *Phaseolus vulgaris* [5], *Nicotiana tabacum* [6] and *Arabidopsis thaliana* [7]. Indeed, secretion of acid phosphatases appears to improve hydrolysis of phosphorus-containing compounds in the rhizosphere [7].

In many agricultural soil types, organic phosphorus-containing compounds make up to 30% - 80% of total soil phosphorus [8] [9]. The effect of various types of secreted phosphatases on plant growth has been studied in experiments with transgenic *A. thaliana* and *Glycine max* plants. Specifically, improved growth was observed for plants expressing secreted phytases or phosphatases when they were grown on synthetic medium containing phytate as the sole source of phosphorus [10] [11] [12] [13]. Furthermore, some transgenic plants expressing extracellular phytases also acquired the ability to grow on natural soils with a high phytate content [14] [15]. Despite these technological advances, alternative routes to improving plant phosphorus metabolism are also being developed, including strategies to engineer better utilization of internal phosphorus reserves of plants.

*A. thaliana* is a common model organism often used for natural variation studies, with many locally adapted populations (ecotypes or accessions) that are broadly distributed throughout the northern hemisphere with a surprisingly wide range of diversification in morphological and physiological traits. In addi-

tion, as a model organism, *A. thaliana* can be grown on Petri dishes with synthetic medium, allowing a detailed molecular analysis of its response to growth on various sources of phosphorus.

Here we investigated the physiological responses of several *Arabidopsis* ecotypes (Col-0, Hi-0, Mt-0, Sf-2) grown under conditions of phosphorus deficiency or on various sources of phosphorus, such as  $\text{Na}_2\text{HPO}_4$  (inorganic phosphate), ATP (adenosine-3-phosphate) and phytate (*myo*-inositol hexakisphosphate sodium salt, IHP). Using a number of different biochemical approaches, we assessed the composition of secondary metabolites secreted by *Arabidopsis* roots in response to environments with different sources of phosphorus. Our results indicate that each *A. thaliana* ecotype differs in its responses to changes in phosphorus sources. Furthermore, root-secreted metabolites also appear to vary depending on the specific ecotype and the source of phosphorus in the medium.

## 2. Materials and Methods

### 2.1. Plant Growth Conditions

Seeds of *Arabidopsis thaliana* ecotypes Col-0, Hi-0, Mt-0 and Sf-2 (ABRC stock numbers CS6673, CS6736, CS1380 and CS6857, respectively) were germinated under sterile conditions on MS medium in Petri dishes [16] under a long-day regime (16 hours of light/8 hours of dark) and 60% humidity at 22°C. After 5 days of growth on MS medium, seedlings were transferred into hydroponic system with standard mineral growth medium [17] supplied with  $\text{Na}_2\text{HPO}_4$  (800  $\mu\text{M}$   $\text{Na}_2\text{HPO}_4$ ), ATP (89  $\mu\text{M}$ , AppliChem) or IHP (133  $\mu\text{M}$  Na-IHP, Sigma-Aldrich) as a sole source of phosphorus or grown with no added phosphorus source (No-P control). All experiments were conducted with three biological replicates (three hydroponic containers for each ecotype and growth condition).

### 2.2. Extracellular Protein and Peptide Analysis

After 21 day of growth on hydroponics, plants were carefully transferred into 50 ml of fresh standard mineral medium supplied with different P sources for 3 hours. Plant growth media were then concentrated at 4°C using ultrafiltration membranes Amicon Ultra-15 3K (3 kDa exclusion limit) [18]. Peptides and polypeptides excreted by *A. thaliana* roots were passed through Supelco Discovery DSC-18 solid phase extraction C18 cartridges (Supelco, PA, USA). After extraction, C18 cartridges were rinsed twice with deionized water, and polypeptide elution was performed with 100% methanol. Residual peptides were then eluted with 80% acetonitrile. To analyze metabolites, growth medium was concentrated and evaporated on a rotary vacuum evaporator Concentrator plus/Vacufuge plus (Eppendorf, Hamburg, Germany) at 200 rpm at room temperature.

UHPLC-ESI-Q-TOF-MS/MS analyses were performed using a Dionex Ultimate 3000 UHPLC connected to a Zorbax Eclipse Plus C-18 column (2.1 × 100 mm, 1.8  $\mu\text{m}$ ) coupled to a Bruker MaXis IMPACT mass spectrometer. Mobile phase consisted of water/acetonitrile/formic acid (phase A was 94.9% water/5%

acetonitrile/0.1% formic acid; phase B was 94.9% acetonitrile /5% water/0.1% formic acid) at a flow rate of 0.4 mL/min [19]. The mobile phase gradient was increased from 0% to 2% (vol/vol) for 1 min, then from 2% to 95% (vol/vol) during 5 min, maintained at 95% for 1 min, then decreased to 2% (vol/vol) of acetonitrile for 1 min until the next injection. Volume of injected sample was 10 µL.

### 2.3. Organic Acids Analysis

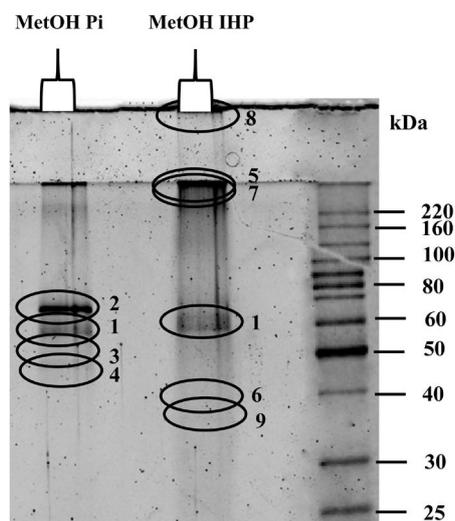
To study organic acids secreted by *A. thaliana*, roots were washed in Milli-Q water and placed in a new volume of MQ water for 8 hours. Identification of organic acids was performed on an Acclaim® Organic Acid OA C18 reverse-phase column (3 µm, 3.0 × 150 mm) using a UltiMate 3000 UHPLC system (Thermo Scientific, Dionex, USA) as described previously [20]. Separation of organic acids was carried out after their elution with 0.1 M phosphate buffer (pH 2.7) at flow rate 0.5 mL/min. Elution of organic acids was monitored at 220 nm. Organic acid standards (succinic, propionic, fumaric, butyric, DL-tartaric, oxalic and citric acids) were purchased from Applichem (HPLC quality).

## 3. Results

### 3.1. Analysis of Proteins Secreted by *A. thaliana* Roots Grown on Media with Different Sources of Phosphorus

*A. thaliana* secretes a number of extracellular proteins under normal and phosphorus-deficient growth conditions [21]. To study protein secretion by the roots of plants grown in conditions of phosphorus deficiency, we initially chose the Mt-0 ecotype of *A. thaliana*. This ecotype is specific to the nutrient-poor and sandy soils on the southern coast of the Mediterranean Sea in the dry subtropical climate of Libya, and we hypothesized that it may have certain physiological adaptations that can make it a good model system for our studies. Plants were grown in two types of conditions: a control environment with inorganic phosphorus (Pi, Na<sub>2</sub>HPO<sub>4</sub>) and phytate (IHP, an organic phosphorus compound that most plants are unable to utilize).

We performed methanol extraction of secreted proteins from plants grown in both conditions (Figure 1). We found only one common protein secreted by plant roots grown in both phosphorus-rich (Pi) and phosphorus-deficient (IHP) media. The identified At5g13610 gene product harbors the DUF155 domain involved in abscisic acid-activated and mitochondria-nucleus signaling pathways (Table 1). In the Pi medium, Mt-0 ecotype also secretes an oxidase (At1g03400 gene product) involved in ethylene biosynthesis, an auxin-responsive protein (At5g66260 gene product) and a Type IV inositol polyphosphate phosphatidylinositol 5-phosphatase 9 (At5PTase9) thought to be involved in cellular responses to abiotic stress (At2g01900 gene product). On the medium with phytate, several unique secreted proteins were also identified: kinase-interacting NET1A and NET1D proteins (At3g22790 and At1g03080 gene products), an allene oxide synthase involved in jasmonic acid signal pathway (At5g42650 gene product), a



**Figure 1.** SDS/PAGE of proteins extracted with methanol. Identified and analyzed protein bands are indicated by black ovals. 1—At5g13610/Q9FNB2, 2—At1g03400.1/Q94A78, 3—At5g66260/Q9FH62, 4—At2g01900/Q9SIS4, 5—At3g22790/Q9LUI2, 6—AT1g03080/F4HZB5, 7—At5g42650/Q96242, 8—At3g28600/F4J0C0, 9—At1g49180/F4I1N8.

**Table 1.** Gene products secreted by Mt-0 ecotype in P-rich (Pi) and P-limiting (IHP) growth conditions.

No.	Gene/Protein Accession	Annotation	P source	Additional References
1	At5g13610/Q9FNB2	Retarded root growth – like protein, DUF155 domain-containing protein. It has mitochondrion organization, located in cytoplasm and is involved in abscisic acid-activated and mitochondria-nucleus signaling pathways. Required for cell division in the root meristem.	Pi, IHP	[22]
2	At1g03400.1/Q94A78	1-aminocyclopropane-1-carboxylate oxydase (ACO), involved in the last step of ethylene biosynthesis as a catalyzer. Regulates glucosinolate biosynthetic processes. Located in cytosol and cell membrane.	Pi	[23]
3	At5g66260/Q9FH62	SAUR-like auxin-responsive protein family. Involved in response to auxin and localized in the mitochondria.	Pi	[24]
4	At2g01900/Q9SIS4	Type IV inositol polyphosphate 5-phosphatase 9 (At5PTase9). Response to cellular salt stress, regulate the clathrin-dependent endocytosis and reactive oxygen species metabolic processes. Has hydrolase activity and is involved in phosphatidylinositol dephosphorylation. Regulates plant adaptations to abiotic stress. Expressed especially in roots.	Pi	[25]
5	At3g22790/Q9LUI2	Protein NETWORKED 1A (NET1A). Involved in filamentous actin binding.	IHP	[25]
6	AT1g03080/F4HZB5	Protein NETWORKED 1D (NET1D). Kinase interacting family protein, localized in plasma membrane. Functions as actin filament binding protein.	IHP	[26]
7	At5g42650/Q96242	Allene oxide synthase, chloroplastic (Cytochrome P450 74A, Hydroperoxiddehydrodrase). Involved in oxylipin biosynthesis as part of lipid metabolism. Regulates senescence, pathogen defence, mechanotransduction and binding of iron ion, heme and oxygen. Located in chloroplast and mitochondrial membranes.	IHP	[27]
8	At3g28600/F4J0C0	AAA-type ATPase family protein, also known as nucleoside triphosphate hydrolase superfamily protein. Located in plasma membrane and has ATP-binding activity. Involved in response to salt stress.	IHP	[28]
9	At1g49180/F4I1N8	Serine/threonine-protein kinase also known as autophagy-related protein 1t (ATG1t). Involved in autophagy cascade mechanism, ATP and nucleic acid binding. Subcellular location in autophagosome.	IHP	[29]

salt-stress responsive nucleoside triphosphate hydrolase involved in ATP binding (At3g28600 gene product) and a serine/threonine protein kinase (At1g49180 gene product) (**Table 1**).

Taken together, our data indicate that the presence or absence of easily extractable phosphorus has a significant effect on the composition of extracellular protein pools secreted by plant roots. Furthermore, our results suggest that the profiles of secreted proteins in Pi and phytate-containing growth conditions overlap only minimally, and most secreted proteins appear to be specific for growth on individual phosphorus source. While gene annotations indicate that at least some of the identified proteins may indeed have a role in phosphorus metabolism (*i.e.* Type IV inositol polyphosphate 5-phosphatase, nucleoside triphosphate hydrolase), the exact functions of each polypeptide in phosphorus acquisition needs to be analyzed further.

### **3.2. Diversity of Organic Acids Secreted by Roots of *A. thaliana* Ecotypes Grown on Media with Different P Sources**

Plants are known to secrete a number of organic acids when grown in conditions of phosphorus deficiency. For example, canola roots secrete citric acid, while barley secretes malic acid, and potato roots secrete a number of organic acids [30]. To study the composition of organic acids secreted by *A. thaliana* roots, we extended our analysis to include three more ecotypes from different climatic zones: Col-0, Hi-0 and Sf-2. The Col-0 ecotype, originally from the plains of Central Europe, is the most commonly used ecotype for many ecological and molecular studies and represents a reference genome for *A. thaliana*. The Hi-0 ecotype (Hilversum, Netherlands) is characteristic of sandy and hilly regions of the Netherlands and grows among moorlands. Sf-2 ecotype (San Feliu, Spain) grows on the slopes of the Pyrenees Mountains down to the Mediterranean coast and is presumably adapted to both the highland conditions and wet climate of western Mediterranean.

Ecotype Hi-0 secreted tartrate under all conditions (Pi, ATP, IHP, noP) (**Table 2**). In addition, Hi-0 also produced succinate and oxalate, but only in the absence of any source of phosphorus in the medium (noP condition), suggesting that these organic acids are secreted in response to severe P limitation. Plants of Col-0 ecotype grown on the Pi medium secreted tartrate, oxalate and citrate (**Table 2**). When this ecotype was grown on ATP as the only source of phosphorus, plants secreted succinate and oxalate. Interestingly, succinate appears to be secreted by both Hi-0 and Col-0 ecotypes, but only in stress conditions (noP for Hi-0 and ATP for Col-0).

To further test the hypothesis that succinate and, possibly, oxalate, are stress related, we analyzed organic acid secretion by two other ecotypes, Mt-0 and Sf-2. Secretion of succinate and oxalate in Mt-0 and Sf-2 ecotypes was observed during growth on several tested media, including Pi medium (**Table 2**). However, citrate was secreted only by the Mt-0 ecotype growing on the medium with ATP. Furthermore, tartrate was also secreted on Pi medium, suggesting that this

**Table 2.** The diversity of organic acids secreted by *A. thaliana* roots (Nd—Not Determined).

Ecotype	Source of phosphorus	Tartrate	Fumarate	Succinate	Oxalate	Malate	Citrate
Hi-0	Pi	+	-	-	-	-	-
	noP	+	-	+	+	-	-
	ATP	+	-	-	-	-	-
	IHP	+	-	-	-	-	-
Col-0	Pi	+	-	-	+	-	+
	noP	nd	nd	nd	nd	nd	nd
	ATP	-	-	+	+	-	-
	IHP	nd	nd	nd	nd	nd	nd
Mt-0	Pi	+	-	+	+	-	-
	noP	nd	nd	nd	nd	nd	nd
	ATP	-	-	+	-	-	+
	IHP	-	-	-	+	-	-
Sf-2	Pi	+	-	+	+	-	-
	noP	nd	nd	nd	nd	nd	nd
	ATP	nd	nd	nd	nd	nd	nd
	IHP	-	-	+	+	-	-

organic acid is typically secreted under growth conditions with abundant P sources. Taken together, while no single organic acid can be pinpointed as a specific metabolite unique to stress conditions, our data indicate that secretion of some organic acids appears to be largely ecotype-specific (*i.e.* citrate for Col-0 and Mt-0), while others are secreted uniformly by several natural populations (*i.e.* tartrate, succinate and oxalate). Furthermore, several tested organic acids (*i.e.* fumarate, malate) were not identified for any of the ecotypes used in this study. These data are in contrast to several previously published studies showing that P deficiency in radish promotes malate and succinate synthesis [31] while in *A. thaliana* rapidly inhibits root growth by activating the malate channel ALUMINUM-ACTIVATED MALATE TRANSPORTER1 [32], indicating that secretion of organic acids can change depending on specific growth conditions and on plant genotypes. Overall, whether secretion of a particular organic acid represents an adaptation factor remains to be further investigated.

### 3.3. Plant Metabolites Excreted by *A. thaliana* Roots Growing on Various Sources of Phosphorus

In addition to proteins and organic acids, plant roots secrete many other metabolites to the rhizosphere. We analyzed metabolites secreted by two *Arabidopsis* ecotypes, Col-0 and Hi-0, which were grown on medium containing inorganic

phosphorus (Pi), medium without any sources of phosphorus (noP) and two types of organic phosphorus (ATP and IHP) (Figure 2). Analysis of excreted metabolites was performed by UHPLC-ESI-Q-TOF-MS/MS, and metabolite identification was performed using the RIKEN MSn spectral database for phytochemicals (ReSpect) <http://spectra.psc.riken.jp/menta.cgi/respect/index> [33].

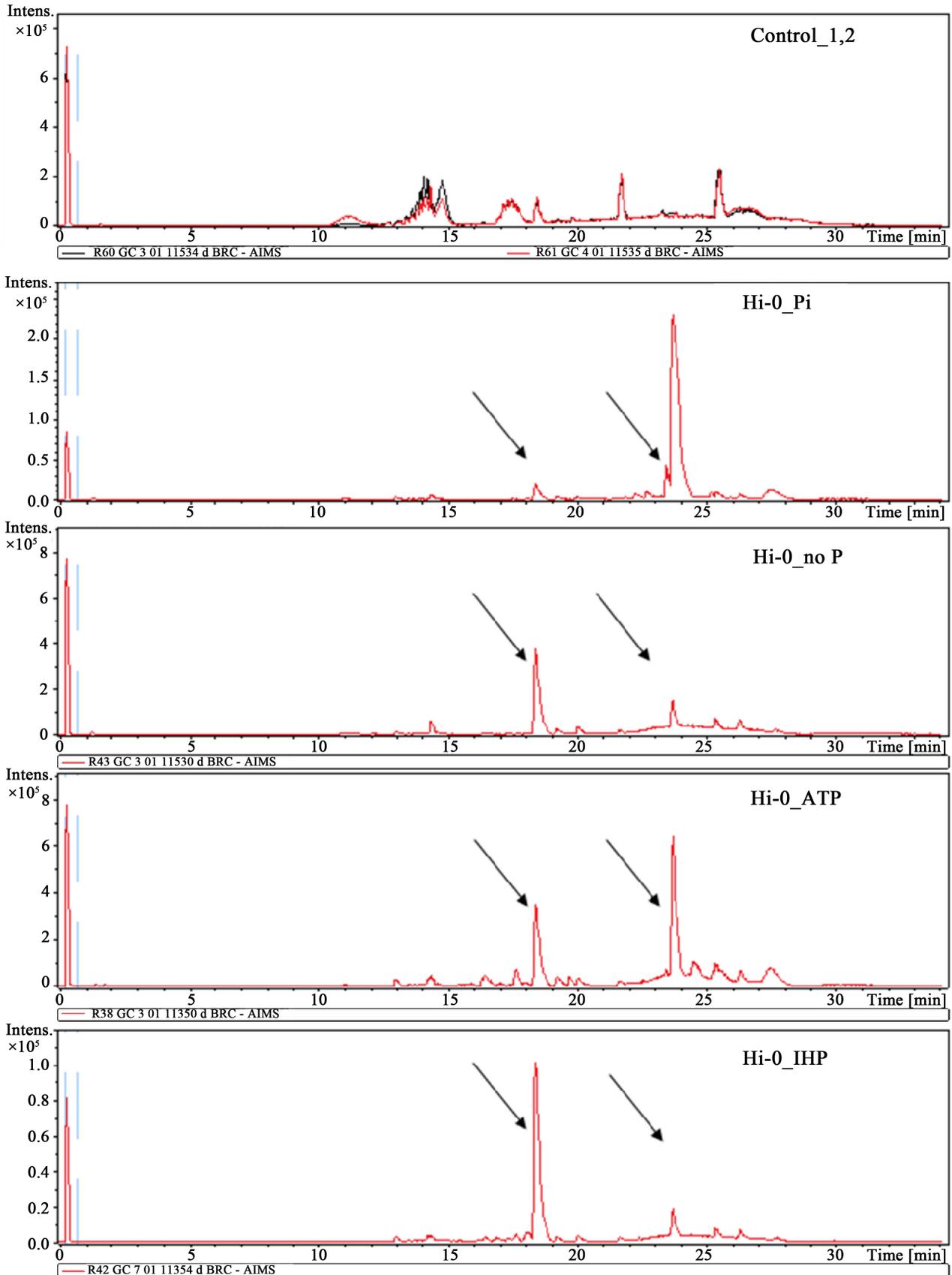
Both Col-0 and Hi-0 ecotypes secreted a compound with retention time (RT) 23.7 min ( $m/z$  281.2792) (Figure 2(a) and Figure 2(b)). This compound corresponds to Oleic acid (molecular formula  $C_{18}H_{34}O_2$ , Figure 3(a)). Interestingly, intracellular concentration of oleic acid in soybean seeds was previously shown to positively correlate with the amount of applied phosphorus fertilizers [34]. Oleic acid is also thought to modulate defense gene expression in *Arabidopsis* [35].

Additionally, Hi-0 ecotype excreted metabolites with RT of 18.5 min ( $m/z$  386.1803 and 408.1623) (Figure 2(a)). The  $m/z$  386.1803 peak was identified as 2'-Deoxycytidine-5'-diphosphate sodium salt (molecular formula  $C_9H_{15}N_3O_{10}P_2$ ) (Figure 3(b)), while the  $m/z$  408.1623 peak corresponds to tannin (molecular formula  $C_{23}H_{22}N_4NaO_2$ ) (Figure 3(c)). Deoxycytidine participates in flowering induction and adaptation to environmental stress [36]. Various modifications of the cytidine base are also involved in plant epigenetic regulations. Current evidence indicates that deoxycytidine likely forms by the action of reactive oxygen species [36] and is located inside plant cells. However, our data indicate it can also be present in exudates.

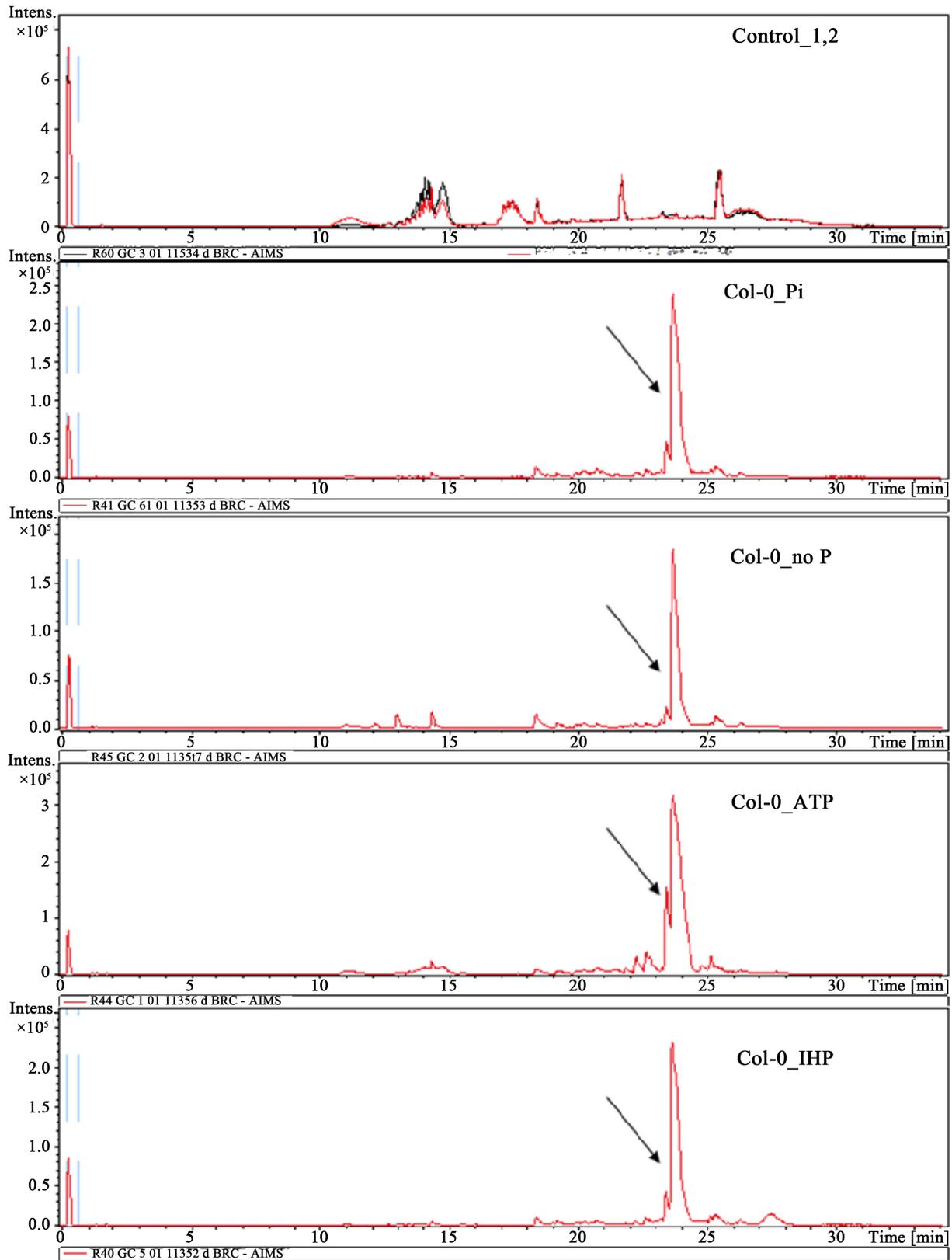
Interestingly, we observed a higher peak for tannin in samples grown in conditions of phosphorus deficiency (noP, IHP). Tannins represent one of the most common and important groups of secondary metabolites in plants, and has previously been associated with plant defense mechanisms in case of biotic stress [37]. Enhanced synthesis of phenolic compounds, including tannins, has also been observed in *Pinus silvestris* under phosphorus deficiencies [38]. Overall, our data indicate that *A. thaliana* ecotypes differ in their physiological response to the source of phosphorus in the growth medium and respond by secreting different polypeptides, organic acids and secondary metabolites.

#### 4. Discussion and Conclusion

Our data provide further confirmation to the notion that *Arabidopsis* ecotypes respond differently to variations in the phosphorus compounds present in the growth medium. Specifically, in response to phosphorus limitation plants secrete a number of proteins, organic acids and secondary metabolites. The spectrum of proteins and metabolites in the root secretome is further modulated by both specific phosphorus compounds in the medium (organic ATP and phytate versus inorganic Pi versus no phosphorus at all) and the nature of *Arabidopsis* ecotypes. In the future, it would also be interesting to analyze the spectrum of secreted proteins and metabolites in response to different concentrations of each P source.

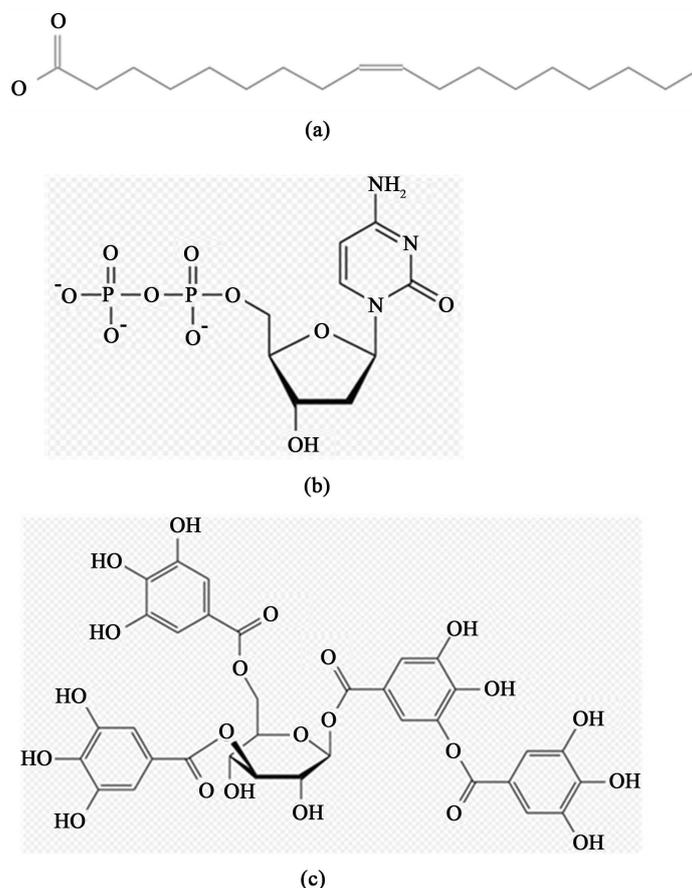


(a)



(b)

**Figure 2.** LC-MS chromatograms of metabolites excreted by roots of the *A. thaliana* Hi-0 (a) and Col-0 (b) ecotypes grown on different sources of phosphorus. Arrows indicate identified metabolites.



**Figure 3.** Molecular formulas of several selected metabolites excreted by *A. thaliana* roots. (a) Oleic acid; (b) 2'-Deoxycytidine-5'-diphosphate sodium salt; (c) Tannin.

### Acknowledgements

This work was supported by the RFBR grants 18-34-00629, 16-34-60200, 15-04-01645A, 18-016-00146 and the Russian Federal Program of Competitive Growth of Kazan Federal University. The authors are grateful to the Interdisciplinary Center for Collective Use at Kazan Federal University (ID RFMEFI59414X0003) sponsored by the Ministry of Education and Science of the Russian Federation. Dr. I.V. Khilyas was supported by the scholarship of the President of the Russian Federation for young scientists and graduate students. This work was supported by DAAD program “Yevgeny Zavoisky”. The authors thank Prof. Dr. Gunter Lochnit, Laboratory of Protein Analytics, Department of Biochemistry, Justus-Liebig University, Giessen, Germany, for help with proteomic analysis.

### Conflicts of Interest

The authors declare no competing interests.

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