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RESEARCH

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Co-Cultivation with Azolla Affects the Metabolome of Whole Rice Plant Beyond Canonical Inorganic Nitrogen Fertilization

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Abstract

Azolla spp. are floating ferns used for centuries as biofertilizers to enrich the soil with inorganic nitrogen and improve rice yields. However, the molecular interactions between Azolla and co-cultivated rice plants only recently started to be thoroughly investigated. In this study, we exploited an experiment in which rice plants were grown together with Azolla by maintaining a low and constant concentration of inorganic nitrogen. We employed a combination of non-targeted metabolomics, chemometrics, and molecular networking to dissect the impact of Azolla co-cultivation on the metabolome of rice roots- and leaves, as well as to annotate the metabolites released by Azolla into the growing medium. Our analyses showed that Azolla can synthesize and release a broad range of metabolites in the culture medium, mainly comprising small peptides (i.e., di- and tri-peptides) and flavonoids, that may have stimulated the rice plant growth. We also observed a systemic response in the upregulation of rice metabolites, first in the roots and then in the leaves. Metabolomics analysis indicated that during the first stages of co-cultivation, the impact of Azolla on rice mainly resulted in the accumulation of small peptides, lipids and carbohydrates in roots, as well as flavonoid glycosides and carbohydrates in leaves. Consistent with these results, transcriptomics analysis of rice roots indicated significant changes in the expressions of genes coding for small peptide and lipid transporters and genes involved in the pathways of amino acid salvage and biosynthesis. Overall, our study provides new insights into Azolla's beneficial and growth-promoting effects on rice. Understanding the molecular mechanisms by which Azolla functions as a biostimulant in rice co-culture will facilitate the development of more sustainable and environmentally friendly techniques to increase yields.

Keywords Azolla, Rice, *Oryza sativa*, Metabolomics, Biostimulant, Co-cultivation, Small peptides

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Introduction

The major challenge of the twenty-first century is to sustainably feed a world population expected to reach ~9.7 billion by 2050 (United Nations 2019). Climate change (Das Gupta 2014) and concerns about using chemical fertilizers and pesticides call for innovative strategies to achieve increased yields while decreasing the environmental impact of global crop cultivation (Matson et al. 1997). A strategy for the transition to a more sustainable agricultural production relies on the co-cultivation of crops with companion plants and associated microbes that fix atmospheric nitrogen, thereby acting as soil biofertilizers. An excellent example is the use of the *Anabaena* (*Trichormus*)-*azollae* symbiosis system as a sustainable source of nitrogen in rice cultivation (Watanabe and Liu 1992). Azolla spp. is a small floating fern (Lumpkin & Plucknett 1980) whose leaflets have cavities that provide a microenvironment for the nitrogen-fixing filamentous cyanobacterium *Anabaena* (*Trichormus*) *azollae* (Kumar et al. 2019). *Azolla-Trichormus*, hereinafter referred to as "Azolla", is a unique symbiotic system that persists throughout the fern's life cycle and allows to double its mass in 3–5 days. The Azolla nitrogen-fixing capacity, accounting for 30–40 kg N ha^{-1} in two weeks when growing in nitrogen-free solution (Watanabe et al. 1977), is higher than that achieved by the symbiosis between legumes and Rhizobia and enables the fern to grow in waterlogged habitats poor in nitrogen content (Bhuvaneshwari et al. 2015). Given the high growth rate and great N-fixing potentials, Azolla can cover large water basins in a very short time and further enrich the soil with nitrogen, which is slowly released after plant death and decomposition (Mahanty et al. 2017). The inorganic nitrogen released by Azolla and available to companion crops, such as rice, is about 70% of that of ammonium sulfate (Watanabe et al. 1977). In Indian paddy soils, Azolla decomposed in 8–10 days to benefit to the co-cultivated rice after 20–30 days (Singh 1977). For this reason, Azolla has been used for centuries as biofertilizer in rice paddies in China and Vietnam (Singh 1989; Watanabe 1982; Watanabe et al. 1989, Bhuvaneshwari et al. 2012, 2013; van Hove and Lejeune 2002), and it is still currently used either as green manure or intercropped with rice (Okonji et al. 2012). Thus, the role of Azolla in supplying inorganic nitrogen to rice fields is well documented (Peters and Meeks 1989). In addition, it has been demonstrated that Azolla, following decomposition, increases soil mineral content (N, P, K, Ca, Mg, and Na) and organic matter (Bhuvaneswari et al. 2013).

Studies on free-living extracts of Azolla have indicated that this fern has the potential to produce hormones, vitamins, and other growth-promoting substances that enhance crop growth (Misra & Kaushik 1989a, 1989b; Wang et al. 1991; Mofiz et al. 2024). Moreover, the growth-promoting effect of Azolla starts early, before the end of its life cycle, suggesting that molecules stimulating plant growth are released by the Azolla into the surrounding environment while it is still alive. Evidence has shown an increase in plant height and number of tillers in rice following the addition of Azolla to the soil (Bhuvaneshwari et al. 2015). It has also been shown that co-cultivation of rice with Azolla and associated cyanobacteria boosts rice growth at an early stage by increasing root and shoot growth and, ultimately, enhancing the rice grain weight and protein content (Venkataraman and Neelakantan 1967; Singh and Trehan 1973; Bhuvaneshwari 2012). Thus it has been postulated that Azolla may be a source of growth-promoting compounds released into the water (Wagner 1997), although no report has been published to confirm it. However, the molecular mechanisms by which Azolla exerts its growth-promoting effects on co-cultivated crops remain unclear, and farmers still prefer to rely on chemical fertilizers to control rice yield (Marzouk et al. 2023).

Moving from the companion study by Cannavò et al. (2025), which demonstrated the morphological and transcriptional changes in rice induced by co-cultivation with Azolla, here we employed non-targeted metabolomics to investigate the alterations in the rice plant metabolome triggered by the fern. Specifically, our objectives were to: i) study the Azolla-induced changes of metabolomes in leaves and roots of rice plants at two time points following the onset of Azolla-rice co-cultivation; ii) detect the metabolites released by Azolla into the growth medium and evaluate their potential role in influencing rice phenotype and growth.

Materials and Methods

Plant Material and Experimental Setup

Azolla filiculoides Lam. used in this study was collected, characterized, and grown under controlled conditions in Watanabe solution (Table S1; Watanabe et al. 1992) as described in Costarelli et al., (2021). To set the co-cultivation experiments rice (*Oryza sativa* cv. Kitaake) seeds were sterilized and germinated in Petri dish as reported in Cannavò et al. (2025). Rice plants were grown hydroponically in Yoshida solution (Table S2; Yoshida et al. 1976) by employing expanded clay balls (Atami, Netherlands) as plant support, and grown under controlled environmental conditions in 50/33/11 cm (length/width/depth) boxes filled with 6 L solution and placed in a climatic chamber with a temperature of 25/20 °C (day/night), photosynthetic photon flux density (PPFD) of 220 µmoles m^{-2} s^{-1} provided by fluorescent tubes (Philips, Netherlands) and a 10-h photoperiod.

A set of 6 boxes were prepared to grow 4 rice plants each in Yoshida solution: 3 boxes containing a total of 12 rice plants were co-cultivated with Azolla (+ AZ), and 3 boxes containing other 12 rice plants that were cultivated without Azolla (-AZ) (Fig. 1a). All the boxes were wrapped and darkened with aluminum foil to prevent the development of algae. The pH of Yoshida solution was adjusted with NaOH 1 M to pH = 5.0 and completely replaced every 2 weeks. Leaves and roots of (+ AZ) and (− AZ) rice plants were sampled 40 and 60 days from the onset of hydroponic co-cultivation (doc). The roots and leaves were sampled from three different rice plants

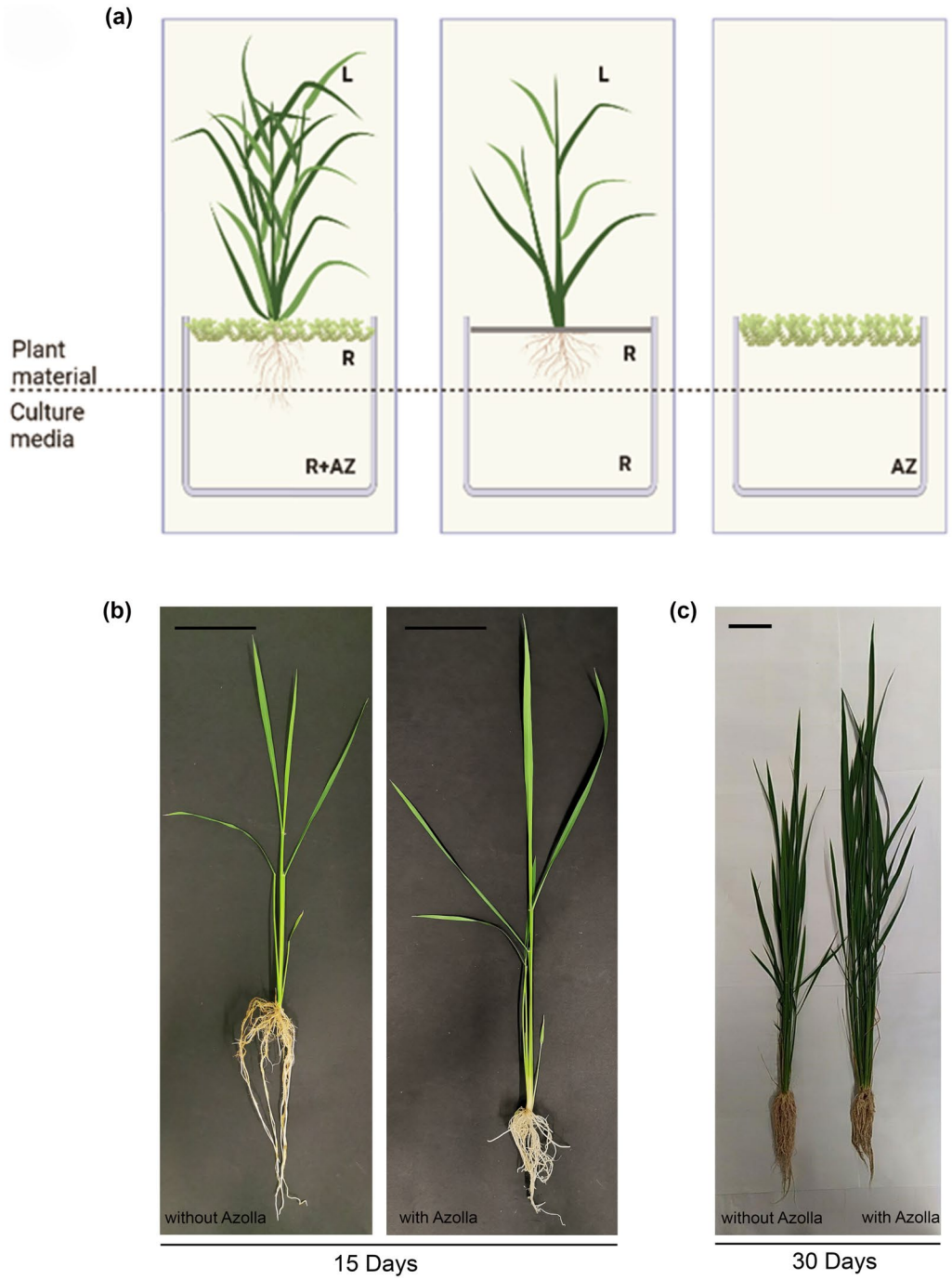


Fig. 1 Schematic diagram of the experimental design (**a**); pictures showing the phenotypic differences of rice plants after 15 days (**b**) and 30 days (**c**) of co-cultivation with- and without-Azolla (the reference bar indicates a length of 5 cm)

from each of the 6 boxes. All samples were flash-frozen in liquid nitrogen, freeze-dried, and stored (at 4 °C) for non-targeted metabolomics analysis. To investigate the metabolites exchanged between rice and Azolla since the early phase of their interaction but when rice plants were already well acclimated to the hydroponic condition, the liquid culture medium was sampled 15 days from the onset of hydroponic cultivation, by collecting 10 ml from each (+ AZ) and (− AZ) boxes. This 15-day time point coincides with the morphological and molecular analyses performed on the same rice plants in the companion study (Cannavò et al. 2025). In addition, 10 mL of liquid culture medium was collected from the 3 boxes where Azolla was grown alone on Watanabe solution, under the environmental conditions described above, and from fresh Watanabe solution as control, and dried by using a Speed Vac (Thermo-Fisher scientific, USA).

Non-Targeted Metabolomics Analysis by UPLC-UHR-QqToF-MS

The extraction of metabolites followed the protocol described in Bertić et al. (2021). Homogenized and powdered (+ AZ) and (− AZ) rice leaf (L), root (R) samples, and lyophilized culture media samples were extracted with cold methanol:2-propanol:H_2O (1:1:1, v/v/v) solution containing 50 µL L^{-1} of internal standard mixture (Table S3). The chemicals (LC–MS hyper grade) methanol/H_2O were purchased from Merck (Darmstadt, Germany) and 2-propanol/acetonitrile from Honeywell (Puchheim, Germany). Due to plant material limitations, we extracted 25 mg of rice leaf with 1000 µL of solvent and 12.5 mg of rice root with 500 µL of solvent, i.e., using the same material-to-solvent ratio. Samples were mixed for 1 min inside a 2 mL polypropylene tube and sonicated in an ultrasonic bath for 10 min at 5 °C. The solution was then centrifuged for 10 min at 10,000 rpm at 5 °C. Four-fifths of the initial extraction volume was recovered and dried by SpeedVac (Univapo 150H, Uniequip, Planegg, Germany). The residue was dissolved in 350 µl of 50% (v/v) acetonitrile in water, mixed for 1 min, centrifuged for 10 min at 10,000 rpm at 5 °C and the supernatant was ready for metabolic analysis. Culture media samples were directly dissolved in acetonitrile/water.

We strictly followed our established non-targeted metabolomics analysis (Ghirardo et al. 2020; Bertić et al. 2021) based on measurements with Ultra Performance Liquid Chromatography (UPLC) Ultra High resolution (UHR) tandem quadrupole/Time-of-Flight (QqToF) Mass Spectrometry (MS). The LC–MS instrument is composed of an Ultimate 3000RS UPLC (Thermo Fisher, Bremen, Germany), a Bruker Impact II (QqToF) and an Apollo II ESI source (Bruker Daltonic, Bremen, Germany). Each sample was measured twice, both on a reversed-phase liquid chromatography (RPLC) column and on a hydrophilic interaction liquid chromatography (HILIC) column (Bertić et al. 2021) to obtain an optimal separation of nonpolar and polar metabolites, respectively (Saba et al. 2001). We analyzed each sample with both RPLC and HILIC columns with MS operated both in positive and negative electrospray ionization modes (for details on chromatography and MS parameters, see Bertić et al. 2021). Data analysis followed Bertić et al. (2021). In short, raw data obtained from LC–MS were manually checked using the software Compass® Data Analysis 4.2 (Bruker Daltonik) for quality control, and corrupted chromatograms were discarded from the analysis. Data were further processed using Metaboscape 4.0 (Bruker) to perform isotope filtering, mass calibration, peak picking, alignments, and peak-groupings based on peak-area correlation. Sample groups (i.e., + AZ and −AZ treatment, 40 and 60 duration of the treatment, L and R plant organ) were created in Metaboscape and only mass-features with > 60% presence at least in one group were retained for analysis. Intensity threshold and recursive counts were defined in Compass® and details on processing parameters can be found in Table S4.

Metabolite annotation was achieved by library comparison (Bertić et al. 2021), and we reported the non-annotated and non-classified metabolites as mass-features (MFs), giving the measured mass-to-charge ratio (*m/z*). For those MFs that were not found in databases, we used the recently developed multi-dimensional stoichiometric compound classification (MSCC) method, which classifies compounds based on their elemental composition in the chemical categories of proteins-related, amino sugars, lipids, carbohydrates, secondary metabolites (Rivas et al. 2018). The elemental composition (sum formula) of MFs was calculated on the basis of the measured accurate mass and isotopic pattern, computed by the 'SmartFormula' algorithm of Metaboscape. The selection of the computed formula is automatically performed by Metaboscape based on the lowest mass deviation (Δ m/z; threshold = 1 ppm) and mSigma to the computed formulas. The mSigma value (range 0–1000; threshold = 50) quantitatively measures the goodness of fit between the measured and theoretical isotopic patterns, by considering mass and intensity deviations (i.e., lower mSigma values mean a better fit). Molecular formulas were further used to calculate H:C, O:C, C: N, C:P, S:C, N:P ratios to depict Van Krevelen diagrams. It should be noted that multiple MF may relate to a single metabolite. Moreover, based on the chemical formula, metabolites were tentatively annotated by using the PubMed open database (https://pubchem.ncbi.nlm.nih.gov/) and National Institute of Standards and Technology (NIST) Chemistry WebBook, SRD 69 (https://doi.org/https://doi.org/

10.18434/T4D303). Systematic classification of tentatively annotated compounds and unknown metabolites were achieved by SIRIUS4 (Dührkop et al. 2019; 2021) using the tools CANOPUS (Djoumbou et al. 2016), CSI:FingerID and COSMIC (Dührkop et al. 2015; Kim et al. 2021; Hoffmann et al. 2022) on molecules that possessed fragmentation spectra (MS/MS) and were found to be statistically significant (adj. p-value < 0.05) between the comparison groups.

Molecular networks were created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu) (Wang et al. 2016). The data were filtered by removing all MS^2 fragment ions within ± 10 Da of the *m/z* of the precursor. MS^2 spectra were window-filtered by choosing only the first 6 fragment ions in the ± 50 Da window across the spectrum. The precursor ion mass tolerance was set to 0.05 Da and the MS^2 fragment ion tolerance to 0.05 Da. Networks were then created in which edges were filtered to have a cosine score greater than 0.70 and more than 6 corresponding peaks. Furthermore, edges between two nodes were retained in the network when each of the nodes appeared in the respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100 and the lowest scoring edges were removed from the molecular families until the molecular family size was below this threshold. The network spectra were then searched in the GNPS spectral libraries. The library spectra were filtered in the same way as the input data. All matches maintained between the network and library spectra had to have a score above 0.7 and at least 6 matching peaks.

Transcriptome Analysis of Rice Roots

RNA-seq on rice roots was performed as described in Cannavò et al. (2025). Briefly, RNA from the roots of three biological replicates per treatment (+ AZ and − AZ) at 15 days after the onset of hydroponic cultivation was isolated with Qiagen RNeasy Plant Mini Kit and treated with Qiagen Rnase-Free Dnase Set. Stranded mRNA libraries were prepared and sequenced in paired-end 150 bp mode on Illumina platform. Raw reads were filtered and quality-trimmed with Fastq-mcf (Aronesty 2011) with options −l 50 −q 30. Reads were mapped on *Oryza sativa* cv. Nipponbare Os-Nipponbare-Reference-IRGSP-1.0 with annotation version 2022–03–11 (downloaded from the RAP-DB database, available at rapdb.dna.affrc.go.jp/download/irgsp1.html) (Kawahara et al. 2013; Sakai et al. 2013). Read alignment and transcript quantification were performed with Rsem v1.3.3 (Li & Dewey 2011). Count tables were imported in R v4.0.2 with package TxImport v1.16.1 (Soneson et al. 2015) and differential gene expression analysis between the + AZ and − AZ samples was performed with DeSeq2 v1.28.1 (Love et al. 2014). The list of differentially expressed (DE) genes with criteria |log2 FC| > 0 and padj ≤ 0.05 was selected to run functional enrichment analysis on ShinyGO v0.76 (Ge et al. 2020), using the set of terms from Gene Ontology (Biological project, Molecular Function and Cellular Component) and KEGG, and using the list of all genes with detectable expression as background.

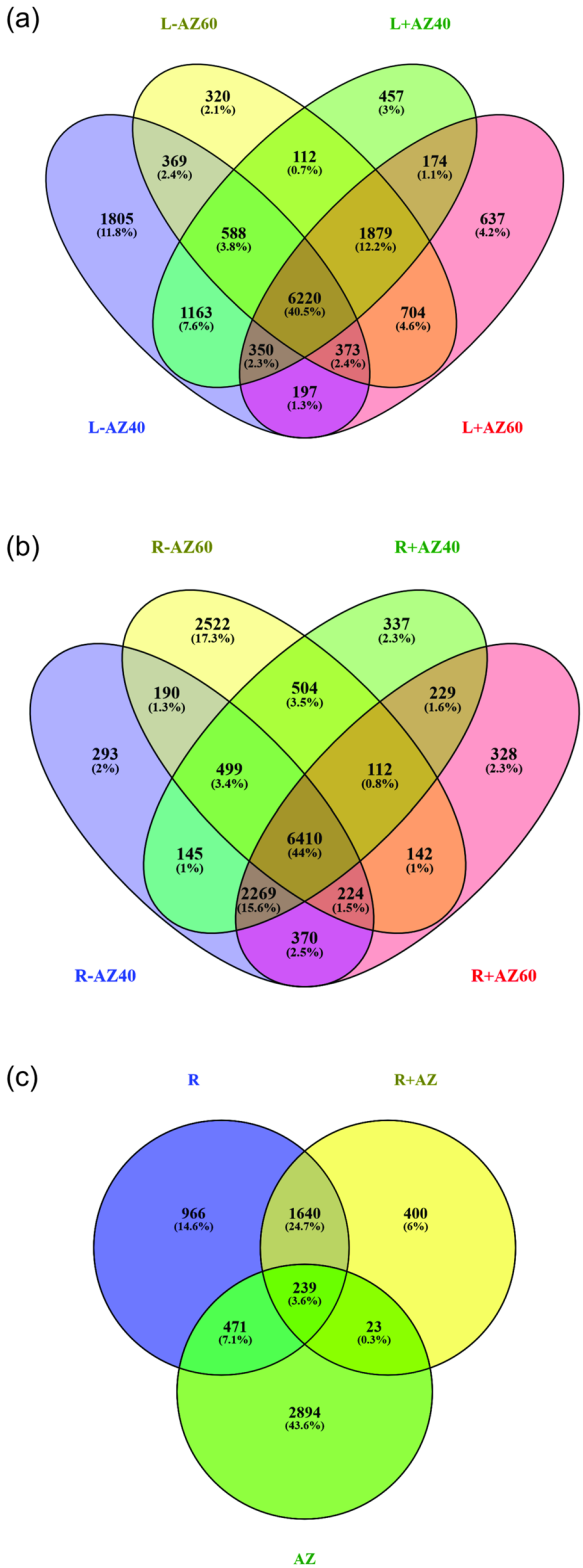
Statistics

All analyses of metabolomic data were performed on 3–6 independent replicates. Multivariate Data Analysis (MDA) was performed according to Bertić et al. (2021) and by using the software SIMCA-P v13.0.3.0 (Umetrics, Umeå, Sweden). Prior to analysis, data were always centered, transformed logarithmically (log10) and Pareto scaled (Eriksson 1999; van den Berg et al. 2006). Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) models were calculated using as Y-variables the rice plant treatment (+ AZ and − AZ) and (40 and 60 doc) duration of the treatment (excluding plant material as Y) and assigning a binary discriminating variable codex to their class (plant organ, treatment and days of treatment). Once the model was created, it was auto-fitted by SIMCA® to the maximum number of significant components, the MFs having a Variance Importance of Prediction (VIP) value > 2 were selected and further analyzed. All MF that had an abundance not statistically different from blanks were removed from the analysis. Significance was tested by t-test after correction for multiple tests with the Benjamini–Hochberg false discovery rate procedure (Benjamini-Hochberg 1995; Glen 2015). Only mass-features with adj. p-values < 0.05 were considered in the result section. Significant perturbations in the metabolome were evaluated with hypergeometric tests, using the function 'phyper' in R v.4.3.1 (R Core Team 2019).

Results

The Impact of Azolla Co-Cultivation on Rice Metabolome

The phenotype of rice roots and aerial organs was significantly modified by co-cultivation with Azolla (Fig. 1b, c) which determined, over time, a higher height, number of leaves and tillers in rice plants co-cultivated with Azolla (Table S5, and as shown in more detail in the companion paper by Cannavò et al. 2025). In our non-targeted metabolomic analysis, we compared the metabolome of leaves and roots of rice co-cultivated with Azolla to those of plants grown without Azolla at two time points, 40 and 60 days after the onset of co-cultivation (doc). Overall, we detected 15,348 and 14,574 metabolite-related mass-features (MFs) (after de-isotoping and peak-grouping of clusters and adducts) in rice leaves and roots, respectively (Fig. 2a, b). Among these, 6220 (40.5%) in leaves (Fig. 2a)



◀ **Fig. 2** Venn diagrams showing metabolites related mass features up- and -downregulated in the metabolome of (**a**) leaf (L), and (**b**) root (R) of rice plants when co-cultivated alone (– AZ) or with Azolla (+ AZ); samples were collected after 40 and 60 days of co-cultivation (doc). **c** Venn diagram showing metabolites related mass features found in the culture media where Azolla (AZ), rice plants (R) and rice plants together with Azolla (R + AZ) were grown, after background correction of the respective culture solution (Watanabe for Azolla, Yoshida for rice cultivated alone or with Azolla)

and 6410 (44%) in roots were found (Fig. 2b), regardless of the presence of Azolla or the rice growth stage. This represents the metabolome of rice (roots and leaves) that is insensitive to either the growth with Azolla or to the plant developmental stage. Although Azolla did not supply inorganic nitrogen to the media (Cannavò et al. 2025), it significantly induced changes in the metabolome of whole rice plants. Specifically, we detected 1268 (8.3%) and 894 (6.2%) MFs in leaves and roots, respectively, that occurred only in plants co-cultivated with Azolla (Fig. 2a, b). In particular, the co-cultivation with Azolla enhanced, over time, the number of metabolites (457 and 637 were detected after 40 and 60 doc, respectively) in rice leaves, whereas it slightly decreased those in roots (337 and 328 at 40 and 60 doc, respectively) (Fig. 2a, b). Besides, in rice plants grown without Azolla, 2494 (16.3%) MFs were found to be regulated in leaves (Fig. 2a) and 3005 (20.6%) in roots (Fig. 2b). In the opposite way to what happened in rice co-cultivated with Azolla, the regulation of metabolites decreased (1805 at 40 doc; 320 at 60 doc) and increased (293 at 40 doc; 2522 at 60 doc) with aging in the leaves and roots of rice grown without Azolla, respectively (Fig. 2a, b). The rice metabolome underwent, as a whole, a lower degree of regulation in plants grown with- than without Azolla, both at leaf- and root-level.

We separated the effects of Azolla co-cultivation on the rice metabolome from those dependent on plant aging by using the multivariate statistical approach OPLS-DA (Fig. 3a, b). This analysis clearly showed significant differences in the metabolomes of both leaves and roots of rice due to Azolla co-cultivation at both 40 and 60 doc (Fig. 3a, b). In leaves, the number of upregulated metabolites was higher than those downregulated at both plant growth stages (Table 1). The impact of Azolla co-cultivation on rice leaf metabolome increased over time, resulting in a higher number of both upregulation and downregulation of metabolites at 60 doc compared to 40 doc (Table 2; Fig. 4—upregulated; Fig. S1—downregulated). Among these metabolites, most (~ 70%) showed

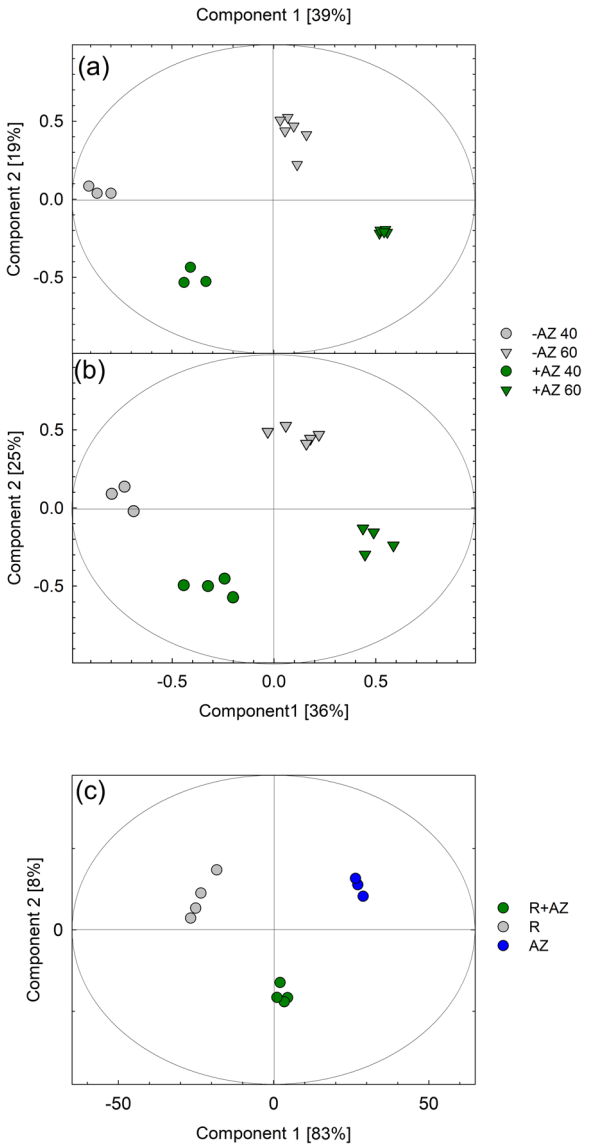


Fig. 3 Score plots of orthogonal partial least square regression discriminant analyses (OPLS-DA) showing the variance of metabolites related mass features in **a** rice leaves and **b** rice roots of plants co-cultivated with Azolla (green colour; + AZ), and without Azolla (grey colour; − AZ). Samples collected at 40 days of co-cultivation (doc) are depicted with circles, and at 60 doc with triangles. **c** Score plot of OPLS-DA of culture media where rice plants were cultivated with Azolla (R + AZ), without Azolla (R), and Azolla without rice plants (AZ). The explained degree of variance of each component is given in parentheses. All OPLS-DA models were statistically significant: (a) p-value = 0.0025; (b) p-value = 0.0011; (c) p-value = 0.0004. Model fitness: (a) R^2X(cum) = 0.255, R^2Y(cum) = 1, Q^2Y(cum) = 0.76; (b) R^2X(cum) = 0.249, R^2Y(cum) = 1, Q^2Y(cum) = 0.77; (c) R^2X(cum) = 0.613, R^2Y(cum) = 0.994, Q^2Y(cum) = 0.809

a low degree of regulation Log_2 fold change (Log_2FC) between 0 and 1 at 40 and 60 doc. In addition, while the number of highly upregulated metabolites (Log_2FC > 3) decreased over time, the number of those highly downregulated (Log_2FC < 3) increased from 40 to 60 doc (Table 1).

A stronger effect on metabolic regulation was found in roots than in leaves. In roots, more than 50% of metabolites increased their level up to double (Log_2FC between 0 and 1) in the presence of Azolla (Table 1). Specifically, in roots of rice co-cultivated with Azolla, a higher upregulation occurred after 40 doc for metabolites with Log_2FC values between 0 and 3 (Table 1; Fig. 4), while a higher downregulation after 60 doc involved those metabolites showing a Log_2FC between −3 and 0) (Table 1; Fig. 4; Fig. S1; Table S6).

We classified the metabolites according to their elemental composition using the multidimensional stoichiometric compound classification (MSCC) method. This grouped compounds into broad classes such as carbohydrates, lipids, protein-related compounds (e.g., amino acids and small peptides), secondary metabolites, amino sugars, and nucleotides. We focused on significant changes (FDR < 0.05) of metabolites strongly associated with Azolla-rice co-cultivation (VIP > 2, OPLS; p < 0.01, CV-ANOVA). Following this approach, we observed a higher number of metabolites changed in rice roots than in leaves (140 vs 7, at 40 doc; 100 vs 20, at 60 doc), with a plant organ-dependent shift between 40 and 60 doc. In fact, in rice roots the metabolome was upregulated at 40 doc, while in leaves it was downregulated at 40 doc compared to 60 doc (Table 2). Specifically, after 40 doc with Azolla, several protein-related metabolites (45; e.g., aminobutyric acid, leucine, dimethylarginine, alanyl-glutamine, alanyl-proline, valine-asparagine, methionine sulfoxide, 1-aminocyclopropane-1-carboxylic acid) and lipid-related metabolites (36; e.g., crotonic acid) increased in the rice root metabolome, whereas after 60 doc, the abundances of protein-related metabolites (124) and lipid-related metabolites (42) decreased (Table 1; Table S6). Interestingly, among the 36 lipid-related metabolites upregulated at 40 doc in rice roots, 7 were also upregulated at 60 doc (FDR < 0.05) including linoleic acid (Table S7). Consistent with these results, transcriptomic analysis of rice roots at 15 doc with Azolla indicated strong changes in the regulation of genes involved in amino acid salvage, i.e. processes leading to the production of amino acids from derivatives (i.e., small peptides) without de novo synthesis (Table 3) and transport of small peptide. In particular, the expression of the six proton-dependent oligopeptide transporter family protein, namely proton-dependent peptide (PTR) transporters, and four ATP synthase (ATP)-binding cassette (ABC) transporters, were strongly affected (Table 3). The expressions of two aminotransferases, involved in amino acids biosynthesis and degradation as well as in

Table 1 Mass features recorded in rice plants co-cultivated with Azolla compared to those cultivated without Azolla and having significance $p < 0.05$ (see Fig. 4 and Fig. S1)

	Up-regulated			**Tot**	**Down-regulated**			**Tot**
	Log_2fold change				**Log_2fold change**			
	0 < 1	**1 < 3**	**> 3**		**-1 < 0**	**-1 <-3**	**< – 3**	
Leaves 40 doc								
Protein-related	136	43	2	*181*	71	25	1	*97*
Lipids	89	26	2	*117*	58	20	…	*78*
Secondary metabolites	28	13	4	*45*	33	18	3	*54*
Amino sugars	21	9	…	*30*	9	4	…	*13*
Carbohydrates	13	2	2	*17*	10	3	…	*13*
Nucleotides	2	2	1	*5*	2	1	…	*3*
Unknown	132	77	3	*212*	123	71	1	*195*
Total	*421*	*172*	*14*	**607**	*306*	*142*	*5*	**453**
Leaves 60 doc								
Protein-r -elated	121	36	3	*160*	122	42	3	*167*
Lipids	84	23	2	*109*	65	20	7	*92*
Secondary metabolites	63	14	…	*77*	63	7	1	*71*
Amino sugars	24	9	…	*33*	30	8	…	*38*
Carbohydrates	26	8	1	*35*	20	6	…	*26*
Nucleotides	1	…	…	*1*	3	1	…	*4*
Unknown	270	61	1	*332*	231	80	3	*314*
Total	*589*	*151*	*7*	**747**	*534*	*164*	*14*	**712**
Roots 40 doc								
Protein-related	132	155	19	*306*	74	89	12	*175*
Lipids	152	148	4	*304*	72	45	6	*123*
Secondary metabolites	42	30	1	*73*	55	32	6	*93*
Amino sugars	23	21	2	*46*	22	16	1	*39*
Carbohydrates	29	13	2	*44*	9	6	…	*15*
Nucleotides	1	1	…	*2*	3	2	…	*5*
Unknown	199	223	9	*431*	177	111	12	*300*
Total	*578*	*591*	*37*	**1206**	*412*	*301*	*37*	**750**
Roots 60 doc								
Protein-related	51	57	4	*112*	239	250	7	*496*
Lipids	55	45	1	*101*	90	87	4	*181*
Secondary metabolites	40	39	2	*81*	46	31	5	*82*
Amino sugars	14	12	…	*26*	35	27	1	*63*
Carbohydrates	13	7	1	*21*	20	21	…	*41*
Nucleotides	2	…	…	*2*	2	1	…	*3*
Unknown	120	96	1	*217*	251	283	7	*541*
Total	*295*	*256*	*9*	**560**	*683*	*700*	*24*	**1407**

phytosiderophore biosynthesis, were also strongly downregulated (Table 3). With respect to lipids, several (14) DEGs related to the biosynthesis/metabolism of fatty acids and their transports were also differentially regulated in the roots of Azolla-cultivated rice plants at 15 doc, of which 11 were upregulated and 3 downregulated (Table S8).

In parallel with the marked reduction in the levels of metabolites in rice root following 60 doc with Azolla, we observed an increase of 20 metabolites in leaves, mainly related to lipids (5), secondary metabolites (3; i.e., flavonoids and phenolics, piperonyl aldehyde) and carbohydrates (2; i.e., xylulose-5-phosphate), as well as to some unknown metabolites (6) (Table 2; Table S3). The increasing number (from 7 to 20), over time, of the

Table 2 Number of strongly affected metabolites in rice plants when co-cultivated with Azolla compared to those grown without Azolla. The accounted metabolites resulted in highly discriminated (VIP > 2; OPLS-DA) and significantly changed (adjusted *p*-values < 0.05; Benjamin Hochberg correction) (see Table S6)

	Up-regulated	Down-regulated
Leaves 40 doc		
Protein-related	…	2
Lipids	…	…
Secondary metabolites	3	2
Amino sugars	…	…
Carbohydrates	1	…
Unknown	3	5
Total	*7*	*9*
Leaves 60 doc		
Protein-related	2	6
Lipids	5	9
Secondary metabolites	3	2
Amino sugars	2	…
Carbohydrates	2	1
Unknown	6	12
Total	*20*	*21*
Roots 40 doc		
Protein-related	45	17
Lipids	36	6
Secondary metabolites	6	8
Amino sugars	1	3
Carbohydrates	4	2
Unknown	48	16
Total	*140*	*52*
Roots 60 doc		
Protein-related	17	124
Lipids	21	42
Secondary metabolites	13	19
Amino sugars	4	11
Carbohydrates	3	14
Unknown	42	151
Total	*100*	*361*

strongly upregulated metabolites in the leaves of rice co-cultivated with Azolla, may suggest that changes in rice metabolome at leaf level occurred later than those at root level following Azolla co-cultivation. Overrepresentation analysis pointed to significant up-regulation of the protein-related metabolism in leaves at 40 doc ($p < 0.01$, hypergeometric test) and carbohydrates at 60 doc ($p < 0.01$), whereas in roots of lipids at 40 doc ($p < 0.001$) and secondary metabolites at 60 doc ($p < 0.001$).

It is worth noting that many significantly regulated metabolites could not be assigned to any of the considered chemical classes by MSCC, and therefore these were referred to as 'unknown' (Table 2; Table S6). However, we employed molecular networking (MN), a technique that can organize and visualize the chemical space in tandem mass spectrometry (MS^2) data, to associate the fragmentation patterns of molecules, i.e., their chemical characteristics, with those that could be annotated through metabolomics databases. Thus, we used MN to link the 'unknown' metabolome to annotated metabolites present in databases. The results of this computational approach highlighted that some of the unknown metabolites whose levels increased at 40 doc were strongly associated to dipeptides (Fig. 5), supporting the observation that Azolla induces the upregulation of nitrogen metabolism in rice roots. Among the annotated metabolites whose levels strongly increase in rice leaves after 60 doc with Azolla, we found a few secondary metabolites (3) and one carbohydrate (1), as well as several metabolites related to flavonoid glycoside metabolism (Fig. 6).

Azolla Released Protein-Related and Flavonoid Compounds in the Culture Medium

We analyzed the chemical compositions of the aqueous solution in which the rice plants, Azolla, and rice co-cultivated with Azolla were grown, after subtracting the compounds present in the original culture media (Watanabe and Yoshida) without plants. We detected a large number of unique metabolites (2894 MFs, 43.6%) into the culture medium when Azolla was cultivated alone (Fig. 2c). In comparison, 966 (14.6%) and 400 (6%) were the MFs only present in the medium hosting rice and rice plants co-cultivated with Azolla, respectively. Overall, the chemical compositions strongly differed, as shown by OPLS-DA analysis ($p < 0.001$; CV-ANOVA) (Fig. 3c; Fig. S1) possibly reflecting the effect of plant growth on two different culture media.

We, therefore, focused our analysis on the molecules released by Azolla in the culture media by comparing the samples growing in the same solution. When comparing co-cultivated rice with rice grown alone in the Yoshida solution, we detected 18 metabolites upregulated, of which 2 masses could be annotated to tri-peptides. The putative release of small peptides from Azolla to the culture media was further investigated by comparing the solutions in which Azolla was grown (in Watanabe) to fresh Watanabe solution. We detected 176 MFs strongly associated with Azolla ($Log_2FC > 2$; VIP > 1.5, $p < 0.001$, CV-ANOVA; Table S6). However, the library search and the MSCC approach were able to annotate and classify only a few of these MF metabolites, specifically in protein-related compounds (8), amino sugar (1), lipid (8), secondary metabolite (4), and carbohydrate (2)

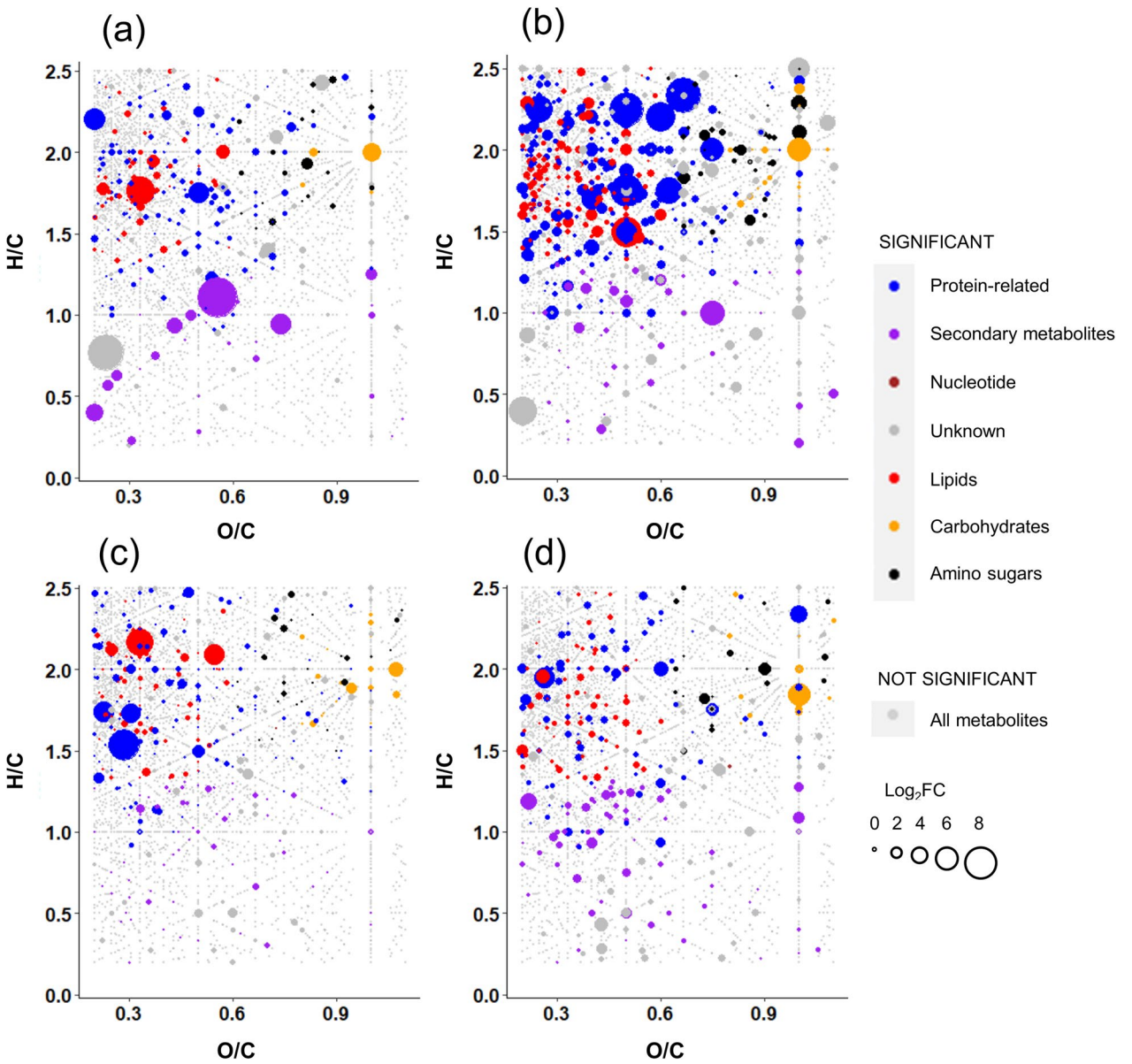


Fig. 4 Van Krevelen diagrams of **a**, **c** leaf material (L) at **a** 40 and **c** 60 days; **b**, **d** root material (R) at **b** 40 and **d** 60 days showing significant (in colour) upregulated metabolites in presence of Azolla. According to assigned chemical formulas, the Van Krevelen diagram combined with MSCC classifies the formula-annotated mass features and assigns them to matched groups. OPLS-DA, (VIP > 1.0). In grey, not significant mass features ($p < 0.05$, 2-way ANOVA, Benjamini-Hockberg corrected). The size of the dots reflects the log fold-change ratios between treatment (+ AZ) and control (– AZ)

(Table S6). Some examples are the putatively annotated (using MS/MS matches on library search) amino acids leucine ($Log_2FC > 10$; VIP = 17), methionine sulfoxide ($Log_2FC = 2.96$; VIP = 1.8), phenylalanine (FC = 2.59; VIP = 1.8); 4-aminobutanoic acid ($Log_2FC = 4.4$; VIP = 1.79); the glycerophospholipid lysophosphatidylcholine ($Log_2FC = 2.16$; VIP = 1.77) and the fatty amide erucamide ($Log_2FC = 3.66$; VIP = 2.02). However, by using molecular networking analysis, we observed a strong association between the remaining significant MFs and small peptides (glutamyl-cysteine, Arg-Ile, Asp-Lys, Lys-Gly-Thr) or flavonoids (e.g., quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, naringenin-7-*O*-glucoside, quercetin-3-*O*-manonylglucoside; Fig. 7).

Table 3 Transporter and aminotransferase-related DEGs in roots at 15 doc. The high levels of oligopeptides in the aqueous solution containing Azolla and in the roots of rice plants co-cultivated with Azolla were associated with the upregulation of several genes encoding proton-dependent peptide transporters (PTR), and ATP synthase (ATP)-binding cassette (ABC) transporters, as well as downregulation of genes encoding for aminotransferase

ID	Gene symbol synonym(s)	Log_2FC	Adj. p-val	Oryzabase gene name synonym(s)
PTR transporters				
Os01 g0871600	OsPOT, POT, OsIROPT1, IROPT1	− 7.76	1.6E−37	Proton-dependent oligopeptide transporter family protein
Os01 g0871500	PTR	− 5.41	1.18E−18	Peptide transporter
Os05 g0411100	PTR	4.68	2.01E−07	Peptide transporter
Os04 g0660900	PTR	5.68	1.05E−05	Peptide transporter
Os10 g0554200	*NRT1.1B, OsNRT1.1B, OsNPF6.5, NPF6.5*	3.05	3.87E−4	Nitrate transporter 1.1B
Os05 g0410900	PTR	2.59	7.00E−3	Peptide transporter
ABC transporters				
Os01 g0533900	OsABCB2, ABCB2, OsPGP2, OsMDR6, OsABCB2_1, OsABCB2_2	1.75	8.20E−05	(ABC) transporter
Os05 g0119000	STAR2	2.13	0.002	(ABC) transporter
Os01 g0836600	*ATP-binding cassette protein subfamily G member 3*	2.61	0.015	ATP-binding cassette (ABC) transporter
Os08 g0398300	ABCA4	− 1.60	0.041	ABC transporter-like domain containing protein
Aminotransferase				
Os09 g0453800	OsIDI4	− 4.01	5.61E−17	Aminotransferase
Os02 g0306401	NAAT1	− 7.29	4.22E−14	Nicotianamine aminotransferase

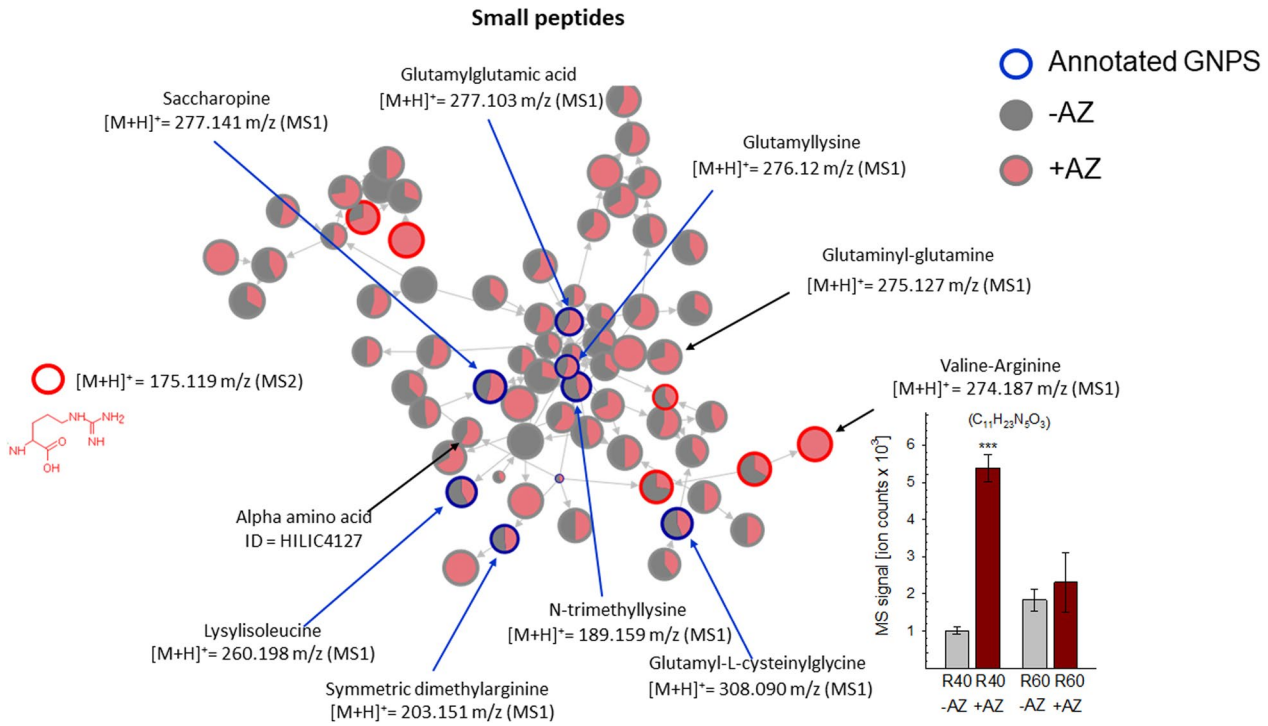


Fig. 5 Molecular networking (MN) showing the upregulation of small peptides in roots of rice co-cultivated for 40 days with Azolla. In the network, nodes (circles) are metabolites connected via edges (nodes) based on the similarity of their mass fragmentation. The pies depict the proportion of the metabolite abundances found in rice plants co-cultivated with- (+ AZ, in red) or without- (− AZ, in grey) Azolla. The blue nodes and their respective protonated ionized masses ($[M+H]^+$) indicate mass features annotated as dipeptides; grey notes are unannotated mass features related to small peptides. The node sizes are the precursor intensities. MN was computed with the LC–MS data measured in HILIC(+)

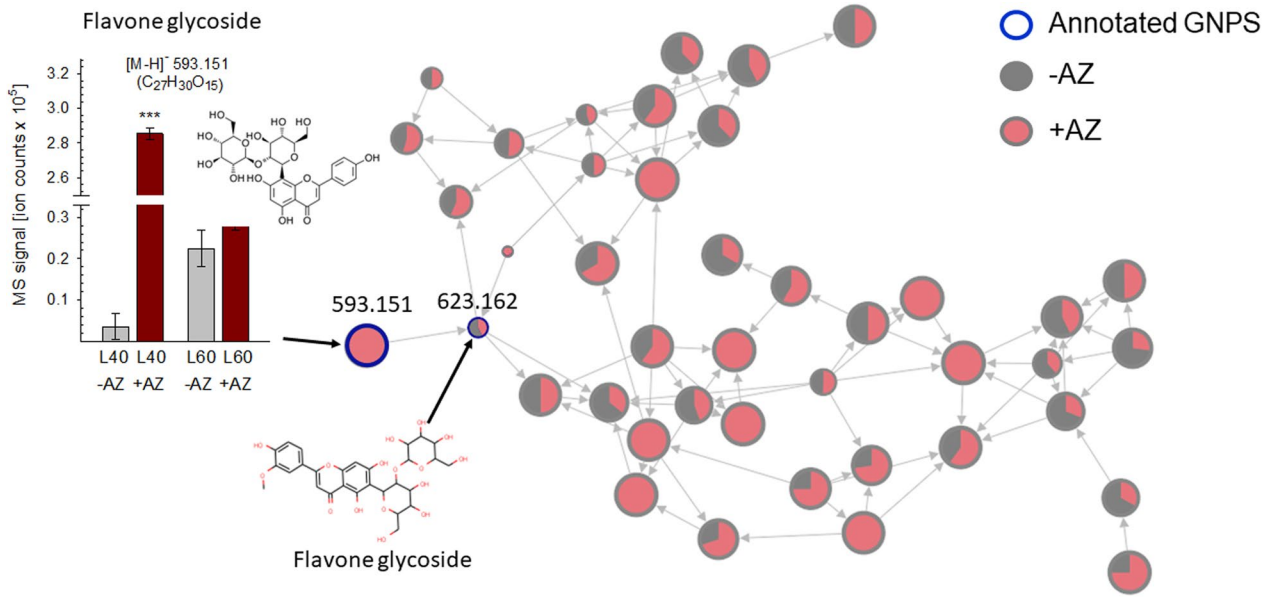


Fig. 6 Molecular networking (MN) showing the upregulation of the flavonoid metabolism in leaves of rice co-cultivated for 40 days with Azolla. In the network, nodes (circles) are metabolites connected via edges (nodes) based on the similarity of their mass fragmentation. The pies depict the proportion of the metabolite abundances found in rice plants cultivated with- (+ AZ, in red) or without- (– AZ, in grey) Azolla. The blue nodes and their respective deprotonated ionized masses ($[M-H]^-$) indicate mass features annotated as flavone glycosides; grey notes are unannotated mass features related to flavonoid metabolisms. The node sizes are the precursor intensities. MN was computed with the LC–MS data measured in RP(–)

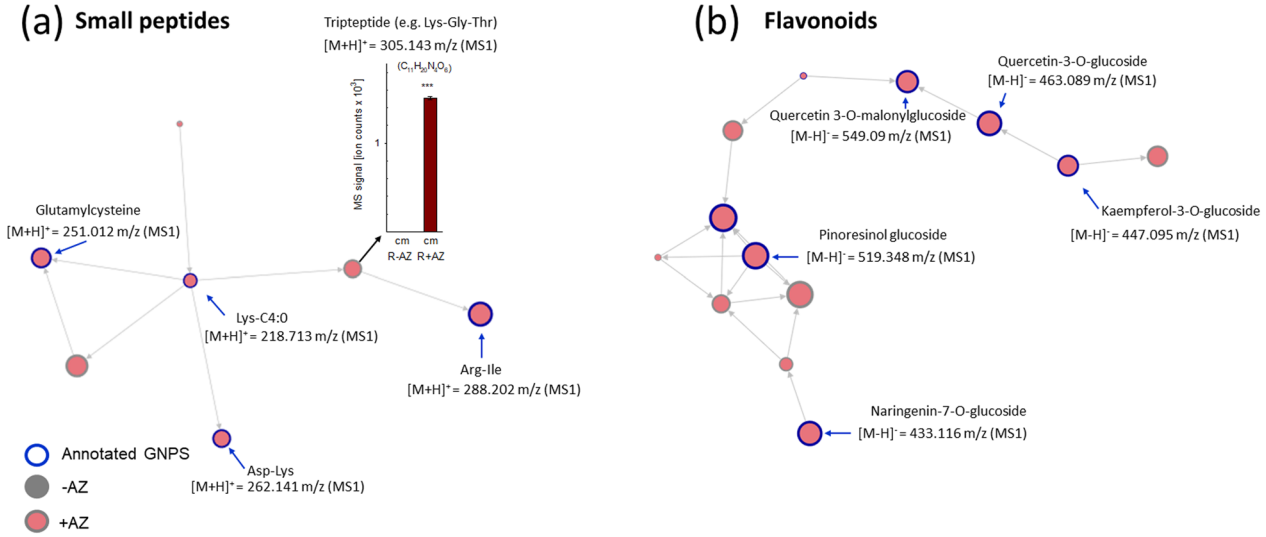


Fig. 7 Molecular networking (MN) showing the presence of **a** small peptides (di- and tripeptides) and **b** flavonoids in the culture media of rice co-cultivated for 15 days with Azolla and compared to the medium of plants growth without Azolla. In the network, nodes (circles) are metabolites connected via edges (nodes) based on the similarity of their mass fragmentation. The pies depict the proportion of the metabolite abundances found in the medium in which rice was cultivated with- (R + AZ, in red) or without (R-AZ, in grey) Azolla. The blue nodes and their respective protonated ionized masses ($[M+H]^+$ in (a) and ($[M-H]^-$) in (b)) indicate mass features annotated as small peptides; grey nodes are unannotated mass features related to small peptides. The node sizes are the precursor intensities. MN was computed with the LC–MS data measured in HILIC(+) (a), and RP(–) (b)

Discussion

In this study, we employed a non-targeted metabolomics analysis on growth media, rice roots, and leaves to deepen our understanding of the factors that induce the morphogenetic changes in rice plants resulting from the co-cultivation with Azolla (as described in the companion study by Cannavò et al. 2025). The collected metabolic dataset was analyzed by combining the multidimensional stoichiometric compound classification (MSCC) with molecular networking (MN) to classify a large number of metabolites, including those not yet present in databases but structurally related to known compounds. Our results show that Azolla can release a wide range of phytochemicals into the aquatic culture medium that have a positive effect on rice growth and development during the early stages of their co-cultivation.

Azolla Releases Metabolites into the Liquid Culture Medium that Impact the Rice Phenotype

The aquatic fern Azolla is known to constitutively produce volatile organic compounds (e.g., isoprene) (Brilli et al. 2022) and non-volatile metabolites (e.g., phenylpropanoids) (Costarelli et al. 2021), which can be enhanced under stressful conditions (Cannavò et al. 2023). Here, we show that Azolla can release a broad range of soluble metabolites, such as small (di-, tri-) peptides, lipids, and flavonoids, into the aquatic culture medium. The ability of Azolla to produce metabolites may be specific to the culture medium and can also be affected by interaction with other plants. This may explain why the metabolites released by Azolla grown alone differ from those detected in the solution where Azolla was co-cultivated with rice. Nevertheless, in both solutions, we mainly detected several metabolites structurally related to protein (e.g., amino acids, di- and tripeptides), flavonoids, and lipids, indicating that Azolla can release these metabolites into the growing solution, regardless its composition (Table S6). Plant roots are known to produce exudates rich in lipids, amino acids, proteins, and other secondary metabolites (Canarini et al. 2019). These exudates have multiple ecological and physiological functions, such as improving plant performance (Baetz and Martinoia 2014) and recruiting growth-promoting rhizobacteria (PGPR) (Narasimhan et al. 2003; Upadhyay et al. 2022) by acting as signaling molecules in plant–microbe interactions (Dennis et al. 2010; Jacoby et al. 2020), also creating a favorable environment for the proliferation of nitrogen-fixing symbiotic cyanobacteria, which positively impact on ecosystem nutrient cycling (Lu et al. 2014).

The metabolites we found released by Azolla in the culture solution have a growth-promoting potential. In particular, the non-protein amino acids, including small peptides or free amino acids (i.e., leucine, phenylalanine, the tripeptide Lys-Gly-Thr) we found in the Azolla growth medium, represent a valuable source of organic nitrogen for rice that may favor both plant growth and stress resistance (Ma et al. 2018), besides having a positive impact on root-associated bacterial communities (Wang et al. 2022a). Among the metabolites we have annotated through MSCC and MN, the putative aminobutyric acid (GABA) may have directly stimulated rice growth, thus it deserves further targeted analysis. Moreover, small peptides can act as hormone-like molecules in plant growth and development (Roy et al. 2018; Feng et al. 2023), function as stress signaling molecules (Chen et al. 2020) able to trigger plant defense responses (Valmas et al. 2023), besides exerting beneficial effects on the microbiome of rice roots (Tejada et al. 2011; Colla et al. 2017). In our study, the small peptides (we have annotated) may have been either directly produced by Azolla, or derived from larger peptides following hydrolysis-mediated by proteolytic enzymes, such as proteases, released from Azolla or rice roots to facilitate uptake (Adamczyk et al. 2010). It is, therefore, reasonable to postulate that the growth promotion effect on rice plants is due to the uptake of small peptides through transporters located on the plasma membrane (Näsholm et al. 2009; Tegeder and Masclaux-Daubresse 2018). Consistent with this hypothesis, the gene expressions of six transporters putatively annotated as *proton-dependent peptide transporters* (*PTR*s) and four *ABC* transporters, were significantly affected in the roots of rice co-cultivated with Azolla. Indeed, the *PTR* gene family comprises a group of membrane transport proteins that are known to facilitate the uptake of di- and tripeptides across cellular membranes (Stacey et al. 2002; Komarova et al. 2008). Among those transporters, eight out of ten were upregulated, and two were markedly downregulated in rice roots (Table 3), suggesting a specific compensatory response to the high levels of di- and tripeptides present in the culture medium, e.g., by feedback inhibition of the transcription factors binding to the promoter regions of *PTR* genes. Moreover, the higher level of di- and tripeptide in the culture medium may lower the demand for amino acid biosynthesis in rice. Accordingly, we note the expressions of genes involved in the de novo aspartate and glutamate biosynthesis (i.e., *aspartate aminotransferase* and *nicotianamine aminotransferase*, respectively; Table 3) to be downregulated in the roots of Azolla co-cultivated rice.

In addition, our analysis detected lipids released by Azolla in the medium (Table S6) that might have been absorbed by rice roots, as highlighted by the differential expression of lipid transporters (Table S8). These lipids found released by Azolla could be involved either in the

synthesis or reinforcement of the external layers of rice root cells (Pereira et al. 2009; Ozturk and Aslim 2010).

We also annotated flavonoids in the Azolla's culture medium (Fig. 7b). Azolla is known to be rich in phenolic compounds (Brouwer et al. 2018; Costarelli et al. 2021), and here we show that Azolla can releases flavonoids into the surrounding medium (Fig. 7b). Flavonoids play a crucial role in plant stress responses by mitigating excess of reactive oxygen species (ROS) and by modulating stress signaling pathways (Daryanavard et al. 2023). Additionally, flavonoids influence phytohormone signaling, thereby contributing to the regulation of plant growth and development (Brunetti et al. 2018).

Overall, the higher number of metabolites we detected in the medium where Azolla was grown alone with respect to those detected in the medium from the co-cultivation with rice (Fig. 2) further suggests that the metabolites released by Azolla are taken up by the rice roots and/or undergo biochemical modifications (e.g., hydrolysis of peptides into free amino acids) due to plant-plant interaction. Since the accumulation of inorganic nitrogen was prevented in our experiments by frequent nutrient solution replacement, it is reasonable to conclude that the metabolites and phytoregulators released by, and exchanged with, Azolla in the culture solution, some of which were taken up by the rice roots, contributed to the morphogenetic changes exhibited by rice plants following co-cultivation with Azolla (Fig. 1: Table S5; Cannavò et al. 2025).

Co-Cultivation with Azolla Impacts both Root and Leaf Metabolomes of Rice Plants

Co-cultivation with Azolla strongly affected the metabolome of rice roots and leaves. To the best of our knowledge, this is the first non-targeted metabolomics investigation of rice plants during the co-cultivation with Azolla. Previous metabolomic studies have been performed on rice plants to track the geographical origins (Hu et al. 2014; Li et al. 2022a), profile therapeutically important metabolites (Kusano et al. 2015; Rajagopalan et al. 2022), and identify biomarkers of seeds yellowing (Liu et al. 2020) and quality deterioration (Wang et al. 2022b).

In this study, we demonstrated that co-cultivation with Azolla initially affected the root- rather than the leaf metabolome, and the upregulated root metabolites were more related to the primary metabolism and, to a lesser extent, to the secondary metabolism (Fig. 4; Table 2–3). Furthermore, we observed an overall increasing number of metabolites being regulated in leaves as rice development progressed, while a decreasing number were regulated in roots, thus indicating a temporal allocation of metabolites from root to shoots. This is in good agreement with the gene expression analysis by Cannavò et al. (2025) of rice roots investigated at 15 days following co-cultivation with Azolla, which revealed already a wide reregulation of genes involved in several plant physiological mechanisms including: amino acid metabolism, phytohormone signaling (such as those responsive to ethylene, cytokinins, auxin, ABA), iron homeostasis, some WRKY transcription factors, and gene related to the antioxidant system. Changes in the root metabolome, particularly in phytohormone signaling, might have impacted the root architecture (Fig. 1b, c), which resulted rather compact in rice plants cultivated with Azolla. However, the increased levels of metabolites in the roots of rice occurring at the first stages of co-cultivation with Azolla, appear to result in part from the direct uptake of metabolites released by Azolla into the culture medium (as discussed above), as well as from the activation of rice metabolic pathways triggered by signaling molecules promoted by the interaction with Azolla-released compounds (as highlighted by Cannavò et al. 2025).

Among the significant changes found in the rice metabolome following co-cultivation with Azolla, we observed a much higher up-regulation than down-regulation of several mass features assigned to protein-related molecules, followed by lipids, secondary metabolites, and carbohydrates (Table 2). This indicates that the interaction between Azolla and rice plants is complex, involving changes in both primary and secondary metabolism similar to those occurring between plants and growth-promoting bacteria (Mashabela et al. 2022). The systemic response in the upregulation of metabolites in rice co-cultivated with Azolla, which initiated in roots and shifted, over time to leaves (Fig. 4), resembles the biostimulant effects shown when seaweed extracts were applied to Arabidopsis, initially affecting the roots and later producing metabolic changes in the leaves (Tran et al. 2023). We also observed a pronounced adjustment of the primary metabolism rather than the secondary metabolism in rice roots when interacting with Azolla. This effect resulted in the accumulation of protein- and lipid-related metabolites and carbohydrates (Table 2). Notably, the elevated levels of small peptides in the roots of rice co-cultivated with Azolla mirrored the enriched levels of small peptides found in the liquid medium where only Azolla was grown alone (Figs. 5, 7). This finding further indicates that rice roots might have absorbed the small peptides produced and released by Azolla into the medium.

One of the most upregulated mass features in rice roots during co-cultivation with Azolla belonging to protein-related metabolites has been assigned to GABA. GABA

is a signaling molecule that has multiple roles in response to abiotic (Nayyar et al. 2014) and biotic (Ramputh and Bown 1996) stresses and in modulating the plant developmental processes (Bouché and Fromm 2004), since it is involved in hormone biosynthesis and nitrogen metabolism (Khan et al. 2021; Pei et al. 2022; Bouché and Fromm 2004). The application of exogenous GABA to Arabidopsis has been shown to promote the direct absorption of these metabolites by the roots through the modulation of several enzymes involved in nitrogen metabolism and nitrogen uptake (Barbosa et al. 2010). In addition, exogenous GABA has been already shown to impact on rice growth by affecting the roots absorption of mineral nutrients, particularly in response to the excess of iron (Zhu et al. 2020). Thus, GABA released from Azolla into the culture medium may have contributed to the changes observed in the phenotype and transcriptional profiles of iron-related genes in rice the roots (as shown in the study of Cannavò et al. 2025).

Among the most abundant lipid-related metabolites stimulated in rice roots following co-cultivation with Azolla, we found a mass feature tentatively assigned to linoleic acid whose levels increased both after 40 and 60 days of co-cultivation. Linoleic acid is a key constituent of cellular membranes, and it is involved in the response to oxidative stress signaling (He and Ding 2020; Saffaryazdi et al. 2020; Liang et al. 2023), as well as in the activation of defense genes (Sumayo et al. 2014). Therefore, roots of rice plants co-cultivated with Azolla richer in linoleic acid may better support the growth of rice plants.

We also showed that co-cultivation with Azolla affects the secondary metabolites in rice leaves, mainly with changes in secondary metabolites (Table 1; Fig. 6). Consistent with our study case, a metabolic reconfiguration was reported in maize leaves treated with biostimulants resulting in differential quantitative profiles of flavonoids and phenolics (Lephatsi et al. 2022). Indeed, our analysis found an increase of a mass feature which we annotated as flavone glycoside in the leaves of rice plants co-cultivated with Azolla (Fig. 6). Flavonoid glycosides produced in leaves help plants to cope with abiotic stress conditions, and their biosynthesis is stimulated during plant growth (Groenbaek et al. 2019). Since the flavonoids detected both in the roots and in the culture medium are structurally different from those we found increased in the leaves, it is likely that the accumulation of flavonoids in the leaves represents an inducible response of rice plants to the interaction with Azolla (Table S3). However, we cannot exclude that, following uptake by the roots from the culture medium, some flavonoids are translocated and undergo further biochemical modifications in the leaves (Buer et al. 2007).

Conclusions

To date, studies have primarily focused on the role of the Azolla as a nitrogen fertilizer. This study reveals that rice benefits from the co-cultivation with Azolla beyond its well-known growth promotion role as an inorganic nitrogen supplier. Our findings demonstrate that the presence of Azolla impacts the metabolome of both rice roots and leaves independently of the inorganic nitrogen levels in the medium. The modification of the rice metabolome induced by Azolla promotes growth and development within a few weeks from the onset of the co-cultivation, occurring well before the agricultural soil is enriched with inorganic nitrogen derived from Azolla decomposition.

Further investigations are required to verify whether the exposure to exogenous small peptides and flavonoids, produced and released by Azolla, can promote the growth (and defense) of neighboring co-cultivated crops. Likewise, it remains to be addressed whether exposure to exogenous metabolites, present in the root exudates of neighboring crops, may affect the metabolism of Azolla. Nevertheless, the current study provides valuable new insights into the beneficial effects of Azolla as a biostimulant to improve rice cultivation in a sustainable and environmentally friendly way.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-025-00788-2.

Supplementary material 1: Figure S1.Van Krevelen diagrams of (a, c) leaf material (L) at (a) 40 and (c) 60 days; (b, d) root material (R) at (b)40 and (d) 60 days showing significant (in colour) downregulated metabolites in presence of Azolla. According toassigned chemical formulas, the Van Krevelen diagram combined with MSCC displays and classifies the formula-annotated mass features and assigns them to matched chemical groups. OPLS-DA, PLS-DA (VIP > 1.0). In grey, notsignificant mass features ($p<0.05$, 2-way ANOVA, Benjamini-Hockberg corrected). The size of the dots reflects thelog2 fold-change ratios between treatment (+AZ) and control (-AZ).

Supplementary material 2: Table S1. List of the macro- and micro-nutrients in the Yoshida solution. Table S2.List of the macro- and micro-nutrients in the Watanabe solution. Table S3.List of the internal standard mixture used for data normalization. Table S4. Metaboscape 4.0 parameters used for processing LC-MS/MS data.

Supplementary material 3: Table S5. Biometric data indicating the number of leaves, height and number of tillers of rice plants cultivated for42 and 63 days with- and without-Azolla (Data are already published in Cannavò et al. 2025, https://doi.org/10.1016/j.rsci.2025.03.004). Data are means standard error ($n = 8$-12). Levene's test has been appliedto verify the homogeneity of variance among the different groups. Asterisks indicate significant differencesbetween the mean values assessed by t-test (**$p <= 0.01$; ***$p <= 0.001$).

Supplementary material 4: Table S6. Dataset of non-targeted metabolomics analysis.

Supplementary material 5: Table S7. Mass features related to lipids shared in roots of rice grown with- and without Azolla.

Supplementary material 6: Table S8. Lipid-related differentially expressed genes (DEGs) in rice roots sampled 15 days since the beginning ofco-cultivation with Azolla. The roots of rice plants co-cultivated with Azolla affected the expression of genesinvolved in fatty acid metabolisms, glycerol-3-phosphate acyltransferases (GPATs), flavin adenine dinucleotide(FAD) coenzymes, and lipid transfer proteins (LTPs).

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Author Contributions
FP, AG, FB: conceptualization; AG, VG, MK: methodology; EC, AG, AS, CP: formal analysis; EC, AC, SC, MC, MCV, LR, AS, CP investigation, FP, AG resources; AG, CP: data curation; FB, AG: writing—original draft; FB, AG, FP, CP, VG, MK review & editing; FB, MK, LR: funding acquisition.

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Data Availability
The datasets generated and analyzed during the current study are openly available at the following link: https://osf.io/b39da/ The RNAseq data are available in the NCBI Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo), accession number GSE278.

Declarations

Competing interests
The authors declare no competing interests.

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