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Identification of metabolites for biomarker of nitrogen and potassium use efficiency in oil palm

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Abstract

Nutrient-use efficiency in oil palm is important for economic and environmental reasons. This research aimed to identify biomarkers to discriminate between tolerant and susceptible oil palms to potassium (K) and nitrogen (N) deficiency. A screening of oil palm materials for N or K use efficiency was conducted using an omission trial experiment, where only targeted nutrient was applied as treatment, while all other nutrients were applied as recommended. The treatment was performed in the main nursery for ten months to identify progenies with contrasting traits. Metabolite analysis was performed to identify specific metabolites as biomarkers for N-efficient and K-efficient palms. Samples taken from the roots of the contrasting progenies were treated with liquid nitrogen prior to grinding into a powder for liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis. The LC-HRMS analysis showed 277 metabolites from K and N treatments after data trimming, which were then analysed in MetaboAnalyst 6.0 for biomarker identification. The results showed that some metabolites were statistically significant. Metabolites identified in more than one analysis have a higher likelihood of being considered as biomarkers. In this experiment, we compared PLS-DA, sPLS-DA, and *Random Forest.* However, some identified metabolites were not to occur naturally in the treatment palms. Some amino acids and antioxidants were promising biomarkers to differentiate the N-deficiency-tolerant and K-deficiency-tolerant palms. Thus, the biomarkers facilitate the breeding scheme to create a nutrient-efficient palm planting material.

[Keywords: Antioxidants, biomarkers, LC-HRMS, liquid nitrogen, nutrient-use efficiency]

Introduction

Fertilizer application is crucial for oil palm productivity. Fertilizer application costs up to 60% of the total oil palm maintenance costs. If the fertilizer is not applied correctly, plant production can significantly fall. However, not all fertilizers applied will be absorbed by the plants because most of them will be lost due to leaching, runoff, and evaporation. Nitrogen (N) and potassium (K) are two main nutrients that are significant issues in oil palm fertilizer application. In Indonesia, approximately 90% of the fields showed K deficiency, while N and phosphorus (P) deficiencies were observed in about 50% and 66% of the fields, respectively (Lim et al., 2023). A nutrient-efficient oil palm would help to avoid economic inefficiency and pollution caused by fertilizer losses. To do so, robust biomarkers are necessary to identify palms with tolerance to nutrient stress from susceptible palms. Therefore, a metabolomic approach is a promising tool for finding biomarkers. Liquid chromatography high-resolution mass spectrometry (LC-HRMS) is ideal for compound discovery and metabolite identification with high throughput and high-quality data. It can identify unknown low molecular compounds (less than 200 Da) even if they are not in the reference library (Wallace & McCord, 2020).

Plants that are deficient in nutrients tend to accumulate secondary metabolites as a form of a defence mechanism. The metabolites produced by plants vary in type and concentration depending on the species and condition of the plant. These metabolites can be indicators of nutrient status and stress in plants. The use of GC-MS to create metabolite profiles in plants stressed by N, P, and K nutrients has been carried out by Sung et al. (2015) on tomatoes. Shen et al. (2019) reported that under

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the N stress, rice tended to accumulate metabolites involved in four primary metabolic pathways: sucrose, phenylalanine, amino acid, and tricarboxylic acid cycle metabolism. Meanwhile, Ganie et al. (2020) found that under the N stress, the accumulation of lipid content in the maize root increases due to lipid utilization as an alternate energy source. The primary and secondary metabolites of stress plants have been profiled by Sung et al. (2015), while Kim et al. (2018) profiled primary metabolites, glucose, and sucrose in paprika under N, P, and K scarcity. Putrescine is a commonly used marker for K-deficient plants (Cui et al., 2019). In addition to indicating K deficiency, putrescine is also found in plants stressed by drought and plants stressed by waterlogging (Cui et al., 2020). This suggests a potential relationship between the concentrations of putrescine, spermine, and spermidine and the intensity and type of stress that the plant faces.

Combining various omics approaches has recently proven beneficial in cutting the oil palm breeding cycle and enabling precision breeding. The trend is shifting towards multiomics-assisted breeding. Combining multiple omics approaches in studying oil palm resistance to diseases caused by abiotic stress. Metabolomic and metagenomic factors may interact in a specific way to establish a defence mechanism against stress. Wening et al. (2024) reported that the microbiome in the planting media of K-efficient oil palm seedlings was similar to that in seedlings with sufficient K. Microbial interactions and environmental conditions, such as temperature, humidity, and certain nutrient status, can influence plant resistance to disease or stress. In stressed conditions, microbes such as plant growth-promoting rhizobacteria can produce specific compounds such as phytohormones and antioxidants and even degrade unnecessary compounds (Inbaraj, 2021). On the other hand, the release of certain compounds by microbes can be interpreted as signals that are perceived by plant receptors to activate and build a defence system (Čapek et al., 2018). Based on this concept, the experiment was designed to find specific metabolites to identity N and K use efficiency in oil palms.

Materials and Methods

Palm materials used in this research were DxP saplings at the age of fourteen months after planting. The saplings were derived from a cross between Dura Deli and African Pisiferas. The nursery experiments employed omission trials where only N or K was applied as a treated dose. Deficiency was created by applying the N dose, 50% of the recommended dose, while other fertilizers (K, P, and dolomite) were applied as recommended. The sametreatment was applied for K deficiency, where only K was applied at 50% of the recommended dose, while N, P, and dolomite were applied at the recommended dose. The contrasting progenies were assessed based on a previous experiment (Pangaribuan et al., 2024). The tolerance and susceptibility to the treatment were determined based on morphological and nutrient efficiency parameters. The samples used in this analysis were oil palm seedling roots that were subjected to N and K deficiency for ten months post-transplanting. The transplanting was carried out for three-month-old seedlings in the pre-nursery. Root samples were kept fresh by wrapping them in aluminium foil and soaking them in liquid N until they were sent for further analysis. Samples were shipped via expedited service in dry ice to maintain the condition of the samples.

Metabolite profiling was performed by Corpora Science® using LC-HRMS. As much as 50 mg of each root sample was dissolved in 1 mL of HPLC-grade methanol and vortexed for 1 minute. Sonication was carried out for 30 minutes and continued with centrifugation at 1400 g for 5 minutes. The supernatant formed from this series of processes was filtered using a 0.2 µM nylon filter to be injected into the LC-HRMS instrument. Quality control (QC) was performed by taking 5 mg of each sample and combining them. From the collection of each sample, 60 mg of raw sample was taken as a QC pool. For injection purposes, 5 µL of the sample was used. LC analysis was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC with Binary Pump (Germering, Germany) and a Thermo Scientific™ Accucore™ Phenyl Hexyl column, 100 mm length x 2.1 mm ID x 2.6 µm particle size (Lithuania). Liquid chromatography was performed using MS-grade water with 0.1% formic acid and MS-grade acetonitrile with 0.1% formic acid as the mobile phase with a flow rate of 0.3 mL/min for a total of 25 minutes at a column temperature of 40 ºC. HRMS analysis used a Thermo Scientific™ Orbitrap™ Exploris 240 HRMS (Bremen, Germany) with acquisition mode: full MS/dd-MS2. In this analysis, positive and negative polarities were used alternately. Mass spectral separation was performed with full MS resolution at 60,000 FWHM with scan range 70-1000 m/z and nN as collision gas. Compound identification was performed using Thermo Scientific Compound Discoverer 3.3 (San Jose, USA) with mzCloud library. The databases used were Arita 6549 Flavonoid Database (https://jglobal.jst.go.jp), Lipid Maps (https://lipidmaps.org), NP Atlas (Poynton et al., 2024), Chemspider Database (http://chemspider.com),

BioCyc (https://www.biocyc.org), CheBI (http://github.com/ebi-chebi/ChEBI), CheMBL (https://www.ebi.ac.uk/chembl/), ChemMine (http://chemmine. ucr.edu), Food and Agriculture of the United Nations (www.fao.org), FoodDB (https://foodb.ca), KEGG (https://www.genome.jp> kegg), Nature Chemical Biology (https://nature.com/nchembio), Nature Chemistry (https://nature.com/nchem) and Nature Communications (https://www.nature.com/ncomms). Biomarker identification was performed using Metaboanalyst 6.0 (https://www.metaboanalyst.ca/). Sample normalization was assessed by sum, while data scaling used mean centring. The normalised data was then analysed using PLS-DA and sPLSDA. Potential biomarker metabolites were identified using significant analysis of metabolomics (SAM).

Result and Discussion

Metabolite data obtained from LC-HRMS Orbitrap (Thermofisher™) analysis were filtered by removing metabolites that only appeared in one sample. This was done to avoid false positives that might arise from unbalanced samples. Sometimes, data normalisation that is too strict can eliminate potential data; conversely, normalisation that is too loose can produce false positive information. The normalised data were then analysed using PLS-DA and sPLSDA. Potential biomarker metabolites were identified using significant analysis of metabolomics (SAM). PLS-DA analysis showed that the samples were distributed widely and tended to be clustered based on treatment and response to treatment. 13% of the variation in metabolite concentration produced by oil palm roots subjected to N and K stress can be explained by PC1 and 11% by PC2. Score plot analysis using sPLS-DA showed a distribution of samples that were separated according to treatment and did not overlap. This shows that the metabolites produced by plant roots differ in concentration according to the treatment given and the plant's tolerance to treatment (Figure 1).

LC-HRMS analysis produced 350-490 metabolites from 12 samples. These metabolites were trimmed based on spectral data checked in CFM-ID, the chemical compounds verified in PubChem, the presence in the samples, and their natural existence in the plants. After trimming, 277 metabolites were suitable to be processed in samples tolerant and susceptible to K and N stress. In all of the samples processed, there were variations in the level of regulation against N and K stress. These metabolites showed significant upregulation or downregulation under N and K stress conditions based on fold-change metabolome analysis (Table 1). Metabolites that were significantly increased or decreased only under certain conditions have the potential to be biomarkers. Further analysis using SAM showed that 13 metabolites met the SAM criteria (Table 2). Although the number of metabolites indicated in SAM was less than in FC analysis, it does not mean that all metabolites in SAM were included in FC. Several metabolites stated to be very significant in SAM were not found in the Table 1. This situation could happen because the two methods use different algorithms. Several general analysis models were combined to determine which metabolites could be used as markers (Xia et al., 2009).

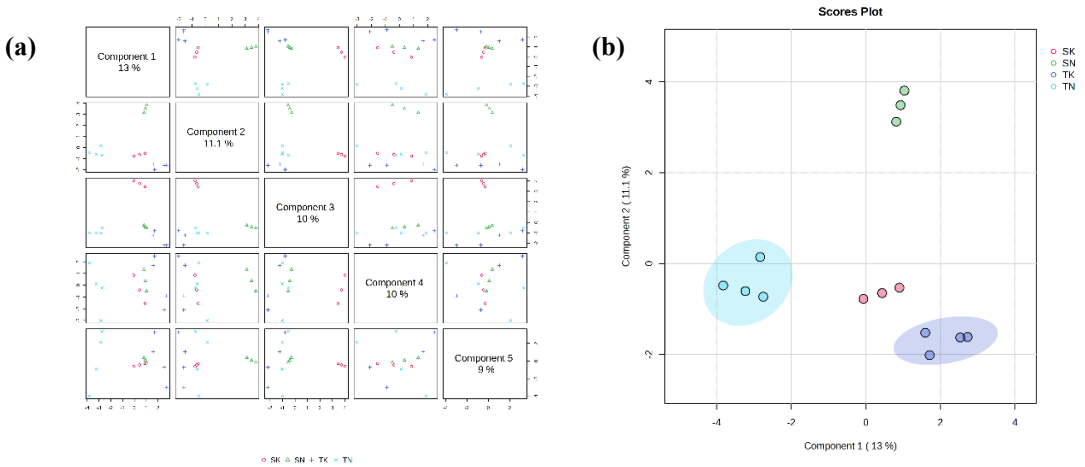


Figure 1. a) Partial least squares discriminant analysis (PLS-DA); b) 2D scores loading plot.
Legends: TK: Tolerant to K deficiency at 50% recommended dose; TN: Tolerant to N deficiency at 50% recommended dose; SK: Susceptible to K deficiency at 50% recommended dose; SN: Susceptible to N deficiency at 50% recommended dose.

Table 1. Oil palm root metabolites fold-change under K and N stress

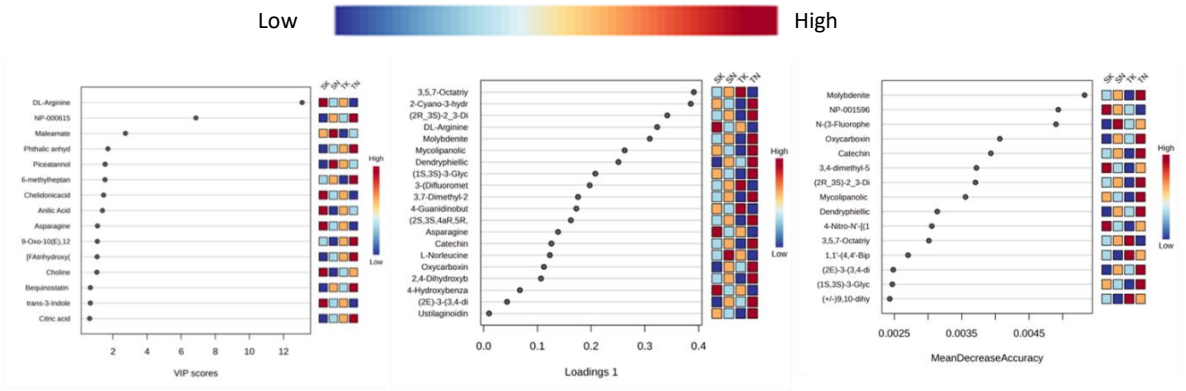
Metabolites	K stress			N stress		
	FC	log2(FC)	Regulated	FC	log2(FC)	Regulated
(+/-)9,10-dihydroxy-12Z-octadecenoic acid	0.37	-1.44	Down	0.36	-1.49	Down
(2S)-2-Isopropyl-3-oxosuccinate	0.40	-1.31	Down	0.32	-1.66	Down
(9Z)-2-Chloro-2-hydroxy-9-octadecenoic acid	0.02	-5.89	Down	na	na	
(R)-3-Amino-2-methylpropanoate	9.63	3.27	UP	na	na	
(R)-Lactaldehyde	na	na		2.31	1.21	Up
1,26-Hexacosanediol	0.01	-6.90	Down	na	na	
1,2-Dinonylnaphthalene	na	na		0.15	-2.69	Down
10_16-Dihydroxyhexadecanoicacid	2.47	1.30	UP	na	na	
10-oxo-nonadecanoic acid	na	na		3.17	1.67	Up
13(S)-HOTrE	0.28	-1.84	Down	na	na	
15-Anilinoretinal	2.16	1.11	UP	na	na	
1-Aminocyclopropane-1-carboxylate	na	na		2.94	1.56	Up
1-Tridecanamine	0.02	-5.34	Down	0.44	-1.20	Down
2-(Acetamidomethylene)succinate	0.04	-4.75	Down	na	na	
2,5-Bis(tert-butylperoxy)-2,5-dimethylhexane	2.05	1.04	UP	na	na	
22alpha-Hydroxy-campest-4-en-3-one	0.41	-1.29	Down	na	na	
2-Cyano-3-hydroxy-3-thioxopropanoic acid	32.17	5.01	UP	0.01	-6.18	Down
2-Furoylglycine;Pyromucuricacid	3.07	1.62	UP	na	na	
3,5,7-Octatriyn-1-ol	na	na		49.17	5.62	Up
3-Dehydroxycarnitine	0.49	-1.03	Down	2.55	1.35	Up
3'-hydroxyacetophenone;3-ACETYLPHENOL	4.22	2.08	UP	na	na	
4-Guanidinobutanal	na	na		3.32	1.73	Up
4-Hydroxybenzaldehyde	na	na		53.93	5.75	Up
4-Oxoproline	na	na		6.54	2.71	Up
5-O-Caffeoylshikimicacid	0.26	-1.95	Down	3.51	1.81	Up
6,8-Pentacosanediol	0.47	-1.10	Down	na	na	
6-Methylquinoline	2.38	1.25	UP	0.48	-1.07	Down
7-Hydroxycoumarine	0.02	-5.87	Down	na	na	
Acetophenone	0.30	-1.72	Down	na	na	
Anadanthoside	na	na		2.76	1.46	Up
Asparagine	na	na		2.51	1.33	Up
Bacillamidin G	0.03	-5.06	Down	na	na	
Bolekolic acid	0.39	-1.36	Down	na	na	
Carboselenoatoiron(1+)	0.14	-2.83	Down	na	na	
Certonardosterol I	na	na		0.40	-1.34	Down
Cissoic acid	0.48	-1.07	Down	na	na	
Citric acid	0.50	-1.00	Down	na	na	
cyclic phosphatidic acid	na	na		0.23	-2.14	Down
Cyclooctene	2.77	1.47	UP	na	na	
D-Glutamate	0.30	-1.74	Down	0.29	-1.77	Down
Diffusoside C	2.93	1.55	UP	0.15	-2.70	Down
Dihomo-Linolenoyl Ethanolamide	na	na		0.02	-6.02	Down

Metabolites	K stress			N stress		
	FC	log2(FC)	Regulated	FC	log2(FC)	Regulated
Dihydrocapsaicin	0.03	-5.29	Down	0.29	-1.77	Down
Diosmetin	3.15	1.65	UP	3.56	1.83	Up
Disulochrin	na	na		2.74	1.45	Up
DL-Arginine	na	na		2.64	1.40	Up
D-Pipecolicacid	0.40	-1.31	Down	na	na	
Ergosta-4,6,8(14),22E-tetraen-3-one	na	na		2.55	1.35	Up
Eriodictyol	na	na		0.45	-1.15	Down
Ethyl 3,3-diethoxypropanoate	39.57	5.31	UP	na	na	
FADH2	5.55	2.47	UP	na	na	
Furfural;Furan-2-carbaldehyde	6.29	2.65	UP	na	na	
Ggamma-L-Glutamyl-L-glutamicacid	na	na		0.01	-6.39	Down
Heptylic acid	0.01	-6.66	Down	0.41	-1.27	Down
Isocordoin	61.72	5.95	UP	na	na	
Isostearamidopropyl dimethylamine	3.67	1.88	UP	na	na	
Itaconic acid	na	na		0.49	-1.03	Down
Kasarin	na	na		13.82	3.79	Up
Lauroyl diethanolamide	4.01	2.00	UP	na	na	
leucyl-4-hydroxyproline	2.49	1.32	UP	na	na	
L-Norleucine	na	na		2.94	1.56	Up
Luteolin	na	na		2.26	1.18	Up
Maleamate	3087.4	11.59	UP	3.13	1.65	Up
Malyngic acid	na	na		0.37	-1.43	Down
Massarilactone D	0.07	-3.89	Down	0.06	-3.94	Down
Mebutamate	0.07	-3.89	Down	na	na	
Medelamine A	na	na		0.019	-5.68	
METHOXYMETHYLMELAMINE	na	na		2.26	1.17	Up
Mycinonic acid III	na	na		0.01	-6.33	Down
N-(2-Phenylethyl)-isobutyramide	na	na		0.35	-1.50	Down
N, N-dimethyl-Safingol	0.005	-7.4621	Down	2.64	1.40	Up
Nitromethanetriol	na	na		0.001	-9.89	Down
NP-016129	na	na		3.9405	1.98	Up
Octadecanamine	na	na		2.0729	1.05	Up
Pantheric Acid C	3.24	1.69	UP	0.49	-1.04	Down
Penispirozine G	na	na		2.24	1.16	Up
Phloretin	0.26	-1.92	Down	na	na	
PHODiA-PG	na	na		0.41	-1.28	Down
Phthalic anhydride	na	na		0.49	-1.03	Down
Pinillidine	na	na		2.40	1.26	Up
Resorcinomycin A	3.03	1.60	UP	na	na	
Scymnol	na	na		3.11	1.66	Up
Sebacicacid	na	na		0.31	-1.69	Down
Sulcatone	0.43	-1.19	Down	na	na	
Suspensolide	0.49	-1.03	Down	na	na	
Tephcalostan C	na	na		0.39	-1.37	Down

Legends: FC = fold change; na = data not available.

Table 2. Significant analysis of metabolomics (SAM)

Metabolites	d.value	stdev	rawp	q.value
Acetophenone	15.83	0.31494	0.0003615	0.036027
(1S,3S)-3-Glycoloyl-3,5,12-trihydroxy-6,11-dioxo-1	12.288	1.4308	0.0005623	0.036027
Diosmetin	11.689	0.27918	0.0006827	0.036027
DL-Arginine	9.9618	1128	0.001245	0.039418
Molybdenite	9.9329	0.46128	0.001245	0.039418
Octadecanamine	8.9961	0.17057	0.0018876	0.041614
2-Cyano-3-hydroxy-3-thioxopropanoic acid	8.8828	0.017731	0.0019679	0.041614
2-Furoylglycine;Pyromucuricacid	8.5495	0.053484	0.0021687	0.041614
22alpha-Hydroxy-campest-4-en-3-one	8.2727	2.061	0.0024498	0.041614
(S)-4-Amino-5-oxopentanoate	8.1564	0.061995	0.0026506	0.041614
Catechin	7.9408	3.3548	0.0028916	0.041614
3-Methoxy-4-hydroxyphenylethyleneglycol	7.1617	0.15718	0.0037349	0.049273
4-Oxoproline	6.7209	0.15978	0.0041767	0.050862



(a) **(b)** **(c)**

Figure 2. Discriminant analysis for biomarker digging using (a) PLS-DA, (b) sPLS-DA, and (c) Random Forest

Differentiating metabolites for tolerant plants from those susceptible to N and K stress was identified using discriminant analysis. PCA analysis using VIP values greater than 1 can differentiate the parameters tested (Xu et al., 2024). However, large omics data sets need a more stringent discriminant analysis to confirm the PCA results and suppress false positives. VIP PLS-DA analysis is a very efficient method for identifying compounds that play an important role in separating samples in PCA. In addition to PCA, Random Forest is a robust and powerful model (Han et al., 2016).

Discriminant analysis using PCA, sPLS-DA, and Random Forest gave varying results (Figure 2). PLS-DA analysis at VIP values above 1 showed several potential compounds such as DL-arginine, piceatannol, maleamate, anilic acid, asparagine, choline, and 3,5,7-octatriyn-1-ol. In the sPLS-DA analysis, potential compounds were obtained, such as DL-arginine, molybdenite, mycolipanolic, asparagine, catechin, and oxycarboxin. The Random Forest analysis obtained potential compounds, such as 3,5,7-octatriyn-1-ol, 4-oxoproline, citric acid, and DL-arginine. Therefore, we decided on the metabolites that appeared in more than one model as biomarker candidates. The more frequently it showed, the stronger its potency as a biomarker (Xia et al., 2009).

The three models (PLS-DA, sPLS-DA, and RF) produce different combinations of potential compounds. However, some compounds appear significantly as differentiators in all three models (DL-Arginine) and compounds that appear in more than one model (3,5,7-Octatriyn-1-ol, Citric acid, Asparagine, Catechin). In addition, some metabolites appear in SAM and FC analysis. The consideration of deciding which metabolites will be used as differentiators could be that those metabolites are statistically significant in various analysis methods, and that the differentiators are naturally present in the plants.

The analyses revealed several promising metabolites; however, some appear challenging to find naturally in plants, such as anilic acid, maleamate, molybdenite, mycolipanolic, and oxycarboxin. For instance, anilic acid is not typically produced by plants but is synthesised industrially as a colourant (Schmidt et al., 2007). While maleamate can be found in some plants, information regarding its metabolism is limited. Wan et al. (2021) noted that maleamate is an inhibitor or suppressor of crabgrass. Molybdenite, a mineral, is occasionally utilised to enrich fertilizers (Li et al., 2024). Its presence in the samples may be attributed to fertilizers applied shortly before sampling. Mycolipanolic is not commonly found in plants, as it is mainly a component of bacterial cell walls (https://www.biocyc.org/). Oxycarboxin, widely used as a systemic fungicide (Sang & Lee, 2020), is also present in plants, although it is unlikely to be present internally due to the fungicide application. Additionally, the metabolite 3,5,7-Octatriyn-1-ol has been documented in studies related to bird's nest salivary (Tong et al., 2020) and has implications for pancreatic health and insulin (Mierzejewski et al., 2024). Anadanthoside is a flavonoid and glycoside with antibacterial activity (https://pubchem.ncbi.nlm.nih.gov/). It is a natural product of a plant well known and belongs to the genus Anadenanthera (Delices et al., 2023).

Other metabolites, such as DL-arginine, asparagine, catechin, acetophenone, and piceatannol, were naturally produced in plants. Acetophenone was only downregulated in K deficiency treatment. Meanwhile, arginine and asparagine were unavailable in K deficiency but upregulated in N deficiency. Catechin and piceatannol were phenol antioxidants (Baranwal et al., 2022; Ahmadpourmir et al., 2024; Al-Jaber et al., 2024). Antioxidants play an important role in defence mechanisms when plants are under stressful conditions. Arginine and Asparagine are amino acids and part of enzymes and proteins. Upregulated amino acids may occur from the breakdown of proteins that produce energy. During stress, amino acids are alternative substrates for energy production when carbohydrates are depleted (Heinemann & Hildebrant, 2021; Trovato et al., 2021). According to this consideration, Isocordoin and Acetophenone appeared to be potential biomarkers for K-efficient stress. At the same time, amino acids, oxoproline, and diosmetine meet the qualification for N-efficient biomarkers in oil palm seedlings. Some metabolites were not naturally found in the oil palm seedling roots. They might present as contamination due to the treatments.

Conclusion

The metabolites produced in the roots of oil palm seedlings under N and K deficiency stress were differentially regulated. Amino acids tend to be upregulated in N-deficiency-tolerant oil palm saplings, whereas acetophenone is downregulated in K-deficiency-tolerant oil palm saplings. Acetophenone and isocordoin are potential biomarkers for K-efficient oil palm. At the same time, oxo-proline and diosmetin are potential biomarkers for N-efficient oil palm based on their metabolite profile in 14-month saplings with a ten-month K/N deprivation treatment.

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