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**EFFECT OF POTASSIUM AVAILABILITY ON SOYBEAN METABOLISM BY
INTEGRATED METABOLOMICS AND IONOMICS ANALYSIS**

Ilha Solteira
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GRADUATE PROGRAM IN AGRONOMY

GUSTAVO DOS SANTOS COTRIM

**EFFECT OF POTASSIUM AVAILABILITY ON SOYBEAN METABOLISM BY
INTEGRATED METABOLOMICS AND IONOMICS ANALYSIS**

Dissertation submitted to Engineering College of
Ilha Solteira (FEIS - UNESP) in partial fulfillment
of the requirements for the degree of Master of
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Systems.

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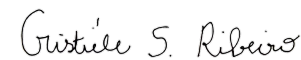
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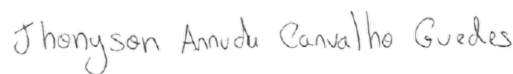
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DEDICATION

*This work is completely dedicated to respectful memory of my grandparents
Jacira Brizola dos Santos and Ranili Custodio Cotrim*

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RESUMO

O potássio (K^+) tem funções fisiológicas vitais nas plantas e sua disponibilidade pode impactar a tolerância de plantas a condições de estresse biótico e abiótico. Anteriormente, estudos limitados investigaram o efeito da adubação potássica no metabolismo da soja. Neste trabalho, a utilização de análises ômicas integradas, metabolômica e ionômica, permitiram investigar a resposta de plantas de soja (*Glycine max*) em campo sob quatro níveis de disponibilidade de K^+ : muito baixa (KVL), baixa (KL), média (KM) e muito alta (KVH). Folhas (V7) e tecidos da vagem (R5.5) coletados foram submetidos à extração e analisados por cromatografia líquida de ultra eficiência acoplada à espectrometria de massa de alta resolução (UPLC-HRMS) e espectroscopia de emissão óptica por plasma acoplado indutivamente (ICP-OES). O emprego de modelos multivariados (PCA-X&Y e O2PLS-DA) permitiram identificar 51 compostos pertencentes a 19 vias metabólicas regulados diferencialmente nos tecidos de plantas que se desenvolveram sob condições contrastantes de disponibilidade de K^+ (KVL vs. KVH). Os níveis de potássio também influenciaram o rendimento de grãos com menores níveis em KVL (2211 kg ha⁻¹) e KL (3737 kg ha⁻¹), diferindo dos tratamentos KM e KVH (4093 e 4096 kg ha⁻¹, respectivamente). Sob baixíssima concentração de K^+ , os teores de Ca^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} e B aumentaram nas folhas jovens e maduras. Não somente, o conteúdo de isoflavonas, coumestanos, pterocarpanos e sojasaponinas mostraram-se positivamente regulados nas folhas severamente deficientes em K^+ , estando estes eventos associados a um possível estado de estresse oxidativo e fotodinâmico decorrentes da deficiência nutricional. A abordagem ômica integrada revelou que a adubação potássica é responsável por promover o aumento da biossíntese de carboidratos, galactolipídeos e flavonóis glicosídicos em folhas e vagens. No entanto, os tecidos da vagem deficientes em K^+ apresentaram aumento no conteúdo de aminoácidos, oligossacarídeos, derivados do ácido benzoico e isoflavonas. O aminoácido asparagina é majoritariamente acumulado nos tecidos deficientes em potássio, sinalizando como possível biomarcador para a deficiência deste macronutriente na soja. Além disso, os resultados indicam que a regulação positiva de metabólitos especializados constitutivos detectados por UPLC-HRMS não estão diretamente associados com o aumento nos níveis de K^+ no solo. Em geral, a presente contribuição melhora nossa compreensão sobre como o metabolismo especializado da soja é dependente e/ou influenciado pela nutrição potássica.

Palavras-chave: *Glycine max*; Fabaceae; Deficiência de Potássio; Fitoalexinas; Metabolismo Especializado; Estresse Abiótico; Metabolômica; Ionômica.

ABSTRACT

Potassium (K^+) has vital physiological functions in plants and its availability can impact the tolerance of species to biotic and abiotic stress conditions. Limited studies have investigated the effect of K^+ fertilization on soybean metabolism. Using integrated omics, ionomics and metabolomics, we investigated the response of field-grown soybean (*Glycine max*) to four rates of soil K^+ availability: very low (KVL), low (KL), medium (KM), and very high (KVH). Soybean trifoliolate leaf (V7) and pod tissue (R5.5) extracts were analysed by ultra-performance liquid chromatography coupled to high-resolution mass spectrometry (UPLC-HRMS) and inductively coupled plasma optical emission spectroscopy (ICP-OES). Multivariate analyses showed that 51 compounds of 19 metabolic pathway maps were regulated in response to K^+ availability. The soybean yield parameters also were influenced in plants under very low (KVL; 2211 kg ha⁻¹) and low (KL; 3737 kg ha⁻¹) differing from KM and KVH (4093 and 4096 kg ha⁻¹, respectively) treatments. Under very low potassium availability, soybean plants promoted the accumulation of Ca^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} , and B in young and old leaves. Not only, isoflavones, coumestans, pterocarpanes, and soyasaponins also were elicited in severely K^+ deficient trifoliolate leaves, which can be associated with oxidative and photodynamic stress status. Potassium fertilization upregulated carbohydrate, galactolipid, and flavonol glycoside biosynthesis in leaves and pod valves, while K^+ deficient pod tissues showed increasing contents of amino acids, oligosaccharides, benzoic acid derivatives, and isoflavones. Additionally, results demonstrate that asparagine content is higher in potassium deficient tissues, which suggests being a biomarker of K^+ deficiency in soybean plants. These results demonstrate that potassium soil fertilization did not linearly contribute to changes in specialised constitutive metabolites of soybean. Altogether, this work provides a reference for improving the understanding of soybean metabolism as dependent on K^+ availability.

Keywords: *Glycine max*; Fabaceae; Potassium deficiency; Phytoalexins; Specialised metabolism; Abiotic stress; Metabolomics; Ionomics.

TABLE OF CONTENTS

1	INTRODUCTION.....	11
2	MATERIAL AND METHODS.....	14
2.1	GENERAL EXPERIMENTAL PROCEDURES.....	14
2.2	EXPERIMENTAL DESIGN AND ASSAY.....	14
2.3	PLANT MATERIALS.....	15
2.4	SOIL CHEMICAL ANALYSIS.....	15
2.5	PLANT EXTRACTION.....	15
2.5.1	Extraction Process for Plant Metabolomics.....	16
2.5.2	Extraction Process for Plant Ionomics.....	16
2.6	INSTRUMENTAL ANALYSES.....	16
2.6.1	Chromatographic Analysis (UPLC).....	16
2.6.2	Mass Spectrometry (ESI-QToF-MS^E).....	17
2.6.3	Inductively Coupled Plasma (ICP-OES).....	17
2.7	YIELD PARAMETERS.....	18
2.8	DATA INTERPRETATION.....	18
2.8.1	Univariate Analysis.....	18
2.8.2	Pre-processing and Chemometrics analysis.....	18
2.8.3	Metabolite Annotation and Molecular Networking.....	19
3	RESULTS AND DISCUSSION.....	21
3.1	SOYBEAN LEAF IONOMICS ANALYSIS.....	21
3.2	SOYBEAN POD IONOMICS ANALYSIS.....	24
3.3	YIELD PARAMETERS.....	26
3.4	CHEMOMETRICS ANALYSIS.....	27
3.5	MOLECULAR NETWORKING AND METABOLIC PATHWAY.....	30
3.6	SOYBEAN LEAVES UNDER K ⁺ HOMEOSTASIS.....	33
3.7	SOYBEAN POD TISSUES UNDER K ⁺ HOMEOSTASIS.....	34
3.8	SOYBEAN LEAVES UNDER K ⁺ DEFICIENCY.....	37
3.9	SOYBEAN POD TISSUES UNDER K ⁺ DEFICIENCY.....	42

4	CONCLUDING REMARKS.....	44
	REFERENCES.....	45
	APPENDIX A – Soil Chemical Attributes.....	57
	APPENDIX B – Leaves Tukey’s t-value.....	58
	APPENDIX C – Leaves Growth Region Tukey’s t-value.....	59
	APPENDIX D – Pod tissues Tukey’s t-value.....	60
	APPENDIX E – Pod tissues Studen’t t-value.....	61
	APPENDIX F – Multivariate Analysis Values.....	62
	APPENDIX G – Putative Metabolite Identification.....	63

1 INTRODUCTION

Potassium (K^+) is an important macronutrient that plays a vital role in metabolic processes, growth, and adaptation to environmental stresses (HASANUZZAMAN *et al.*, 2018; PANDEY; MAHIWAL, 2020). Many physiological and biochemical processes are dependent on K^+ availability, such as cell turgidity regulation, long-distance solute transport, enzymatic regulation (AMTMANN *et al.*, 2008; HUBER; ARNY, 1985), photosynthesis, protein synthesis, and membrane transport (HAFSI *et al.*, 2014; MARSCHNER, 2012). Balanced K^+ nutrition can promote plant tolerance to stress, as flooding (MUGNAI *et al.*, 2011), low temperatures (DEVI *et al.*, 2012), drought (WANG *et al.*, 2013), and salinity (ALMEIDA *et al.*, 2017). In contrast, plants under K^+ deficiency showed oxidative stress status (HAFSI *et al.*, 2014; HASANUZZAMAN *et al.*, 2018; HERNANDEZ *et al.*, 2012) modifying heavily metabolite biosynthesis (ARMENGAUD *et al.*, 2009; CUI *et al.*, 2019; GAALICHE *et al.*, 2019).

Potassium is responsible for activation of nearly 60 enzymes taking part in carbon and nitrogen metabolism (AMTMANN *et al.*, 2008). Plants under adequate K^+ levels synthesise large biomolecules, as cellulose, starch, and proteins (HAZANUZZAMAN *et al.*, 2018). However, under K^+ deficiency the content of small molecules, as free sugars, amino acids, organic acids, and amides increases (PRASAD *et al.*, 2010). Also, we must not disregard that the impact of K^+ supply in plant metabolism is multifaceted, tissue and species-specific, as observed in the responses to stresses in leaves and roots of tomatoes (WEINERT *et al.*, 2021) and oil palm sapling (CUI *et al.*, 2019).

The prevalent view is that a high K^+ availability decreases the severity of diseases, as caused by *Cercospora kikuchii* (ITO *et al.*, 1993; MEYER; KLEPKER, 2007) and *Phakopsora pachyrhizi* (BALARDIN *et al.*, 2006; PINHEIRO *et al.*, 2011) or insect incidence in plants (PERRENOUD, 1990; SEVERTSON *et al.*, 2016). Therefore, farmers are generally instructed to increase K^+ rates to improve crop health (GAO *et al.*, 2018; POTASH AND PHOSPHATE INSTITUTE, 1998; SEIXAS *et al.*, 2020). In spite of the assumption that in most related cases K^+ fertilization decreases the incidence of the diseases, the opposite effect is also reported (DAVIS *et al.*, 2018; PERRENOUD, 1990), as a 63% decrease and 28% increase in insect and mite incidence in plants associated to potassium nutrition (AMTMANN *et al.*, 2008; PERRENOUD, 1990).

Potassium deficiency has also a considerable effect on plant metabolism, including alterations in the profile, concentration, and distribution of many metabolites in different tissues

(AMTANN *et al.*, 2008; HAZANUZZAMAN *et al.*, 2018). Likewise, there is a consensus that specialised metabolism is crucial for plant susceptibility and defence against insects or pathogens (AMTMANN *et al.*, 2008; DAVIS *et al.*, 2018; GAO *et al.*, 2018). Nevertheless, how plants under K^+ contrasting availability can cope with biotic stresses remains unclear, and according to Marschner (2012) the understanding of the impacts of K^+ availability on specialised metabolism remains incipient when compared to the K^+ effect on physiological and primary metabolism processes.

Increase content of reducing sugars (primary metabolism) and glucosinolates (specialised metabolism) are potentially relevant to insects and/or pathogens, as detected in *Arabidopsis thaliana* under K^+ deficiency (ARMENGAUD *et al.*, 2009; TROUFFLARD *et al.*, 2010). Similarly, plants of *Hordeum vulgare* under K^+ deficiency increased jasmonic acid and other oxylipins content sensitive to the pathogen *Blumeria graminis* (DAVIS *et al.*, 2018). In *Glycine max* (L.) Merr. (Fabaceae) field experiments with K^+ fertilization revealed multiple inducing mechanisms improving plant resistance to cyst nematode *Heteroda glycines* via root exudation of phenolic acids and plant pathogen-related genes (GAO *et al.*, 2018). Furthermore, the metabolomic analysis revealed differences in gentisic acid and shikimic acid content from exudates of soybean roots, when plants are grown under low K^+ conditions (TANTRIANI *et al.*, 2020).

Previous studies of soybean metabolite content influenced by K^+ fertilization used only methodologies that allowed the detection of few target compounds (HU *et al.*, 2015; SEGUIN; ZHENG, 2006; VYN *et al.*, 2002) or analytical methods covering primary metabolism (TANTRIANI *et al.*, 2020). Certainly, these results improved our knowledge, but not enough to understand how soybean under K^+ deficiency or adequate nutrition has constitutive specialised metabolites that can respond differently in plant interactions. Recently, studies comprising integrated omics greatly contributed to elucidating the influence of nutrients on plant metabolism (CUI *et al.*, 2019; TANTRIANI *et al.*, 2020; ZHAO *et al.*, 2020). Ionomics and metabolomics analyses showed that K^+ deficiency and waterlogging in sunflower are not simply additive stresses, but also influence respiration as well nitrogen metabolism when associated with biomarkers (CUI *et al.*, 2019). However, it remains unknown whether K^+ availability can influence the constitutive content of soybean specialised metabolites with a recognized role in biotic and abiotic interactions; thus, research methodologies based on untargeted metabolomics allow investigating of metabolome changes on a larger scale (FENG *et al.*, 2020; YANG *et al.*, 2018).

We hypothesize that K^+ rates in soil fertilization can promote an increase in specialised metabolite profiles of soybeans. This work aimed to advance a step towards understanding the effect of K^+ nutrition on plant interactions, and it is expected that these results can provide new insights into the importance of potassium in soybean metabolism. Therefore, we carried out untargeted metabolomics and ionomics analysis to compare the metabolic profile of trifoliolate leaves and pod tissues (valves and immature seeds) of soybean influenced by K^+ availability. The contributions of this work allowed us to suggest the correlation of the soybean metabolite phenotype with K^+ status and list its importance previously reported in biotic and abiotic interactions.

2 MATERIAL AND METHODS

2.1 GENERAL EXPERIMENTAL PROCEDURES

Leucine-enkephalin (Waters Technologies, USA) and quercetin ($\geq 95\%$ HPLC, Sigma-Aldrich, USA) were used as external control standards. Formic acid LiChropur™ (98-100% HPLC, Merck, Germany), acetonitrile and methanol Chromasolv™ ($\geq 99.9\%$ LC-MS, Honeywell, Germany) were the solvents for running the samples in chromatographic analysis. Reference standards were applied to compare retention time, MS¹ and MS² data including, daidzin, genistin, glycitin, malonyl-daidzin, malonyl-genistin, malonyl-glycitin, acetyl-daidzin, acetyl-genistin, acetyl-glycitin, daidzein, genistein, glycitein, and coumestrol ($\geq 95\%$ HPLC; Sigma-Aldrich, USA). All aqueous solutions were prepared using a Milli-Q™ system-producing ultrapure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$; Millipore, USA).

2.2 EXPERIMENTAL DESIGN AND ASSAY

The experiments were carried out at Embrapa Soybean Experimental Station ($23^{\circ}18'37''\text{S}$, $51^{\circ}17'68''\text{W}$; 590 m asl) in Londrina, Paraná State, Brazil, set on a Rhodic Hapludox (Staff, 2014). This area has flat and smooth wavy topography, with 760 g kg^{-1} of clay in the no-tillage system (0 to 20 cm layer) of cultivation. The region has a humid subtropical climate (Cfa) according to the Köppen climate classification (ALVARES *et al.*, 2013). Potassium (K^+) availability conditions were tested after application of 0, 40, 80, and $160 \text{ kg K}_2\text{O ha}^{-1}$ (KCl; 60% K_2O). Subsequently, the soils at 0 to 0.2 m depth sites were randomly collected (15 sub-samples) and homogenized to form a composite sample at each experimental unit in October 2019 using a soil auger (0.05 m diameter) (Appendix A). Soil samples were analysed and allowed us to consider four levels (treatments): very low (KVL), low (KL), medium (KM), and very high (KVH) K^+ availability. The nutrient soil content interpretation was based on Pauletti and Motta (2019) for regional conditions (Paraná State, Brazil).

Seeds of *Glycine max* (L.) Merr. (Fabaceae) cv. BRS 1010 IPRO (VI maturity group) were sown on November 2nd, 2019, with a row spacing of 0.5 m and 15 seeds per linear metre, expecting a density of $300,000 \text{ plants ha}^{-1}$. The experimental design was a randomized block design, with plots of 4 m x 8 m, composed of four lines of 6 m, and six replicates. Before sowing, seeds were treated with cobalt (2 g ha^{-1}), molybdenum (20 g ha^{-1}), and inoculated with 2 mL kg^{-1} of *Bradyrhizobium japonicum* SEMIA 5079 (OLIVEIRA JUNIOR *et al.*, 2020). Soil fertilization was carried out with 111 kg ha^{-1} of triple superphosphate – TSP (45% P_2O_5) as recommended by Pauletti and Motta (2019).

2.3 PLANT MATERIALS

Potassium is a very mobile nutrient in plants (MARSCHNER, 2012); therefore, potential differences in leaf metabolomic profiles can vary according to tissue maturity. Thus, we sampled trifoliolate leaves of soybean plants at the V7 stage (FEHR; CAVINESS, 1977) when in vegetative stage the highest K^+ uptake occurs (BENDER *et al.*, 2015), and collected old and young leaves. Leaf samples were classified according to the growth region, lower third (LT), medium third (MT), and upper third (UT). These tissues were collected 49 days after sowing (DAS) and immediately packed in liquid nitrogen (six replicates). The fresh mass was measured on an AY220 Marte™ analytical balance (Shimadzu, Brazil) and stored at -80 °C in an ultrafreezer (Indrel Scientific, Brazil) at the Embrapa Chemical Ecology Laboratory. Subsequently, leaves were lyophilized at -55 °C for 48 h in an L101 freeze-dryer (Liotop, Brazil). Previous studies have associated potassium-deficient soils with the incidence of diseases caused by the pathogen *C. kikuchii* in soybean pods (MEYER; KLEPKER, 2007; SEIXAS *et al.*, 2020). Therefore, we also collected soybean pods (R5.5 stage) at 98 DAS, and immature seeds (IS) were detached from the valves (PV) and separately maintained using the same storage process previously described for the analysis of trifoliolate leaves.

2.4 SOIL CHEMICAL ANALYSIS

The collected soil samples were oven-dried at 45 °C for 48 h and sieved through a 2 mm. Extractable Ca^{2+} , Mg^{2+} , and Al^{3+} in 1 mol L⁻¹ KCl (MEHLICH, 1953) and plant-available P and K^+ were evaluated using Mehlich-1 solution (MEHLICH, 1984). Soil pH was determined in 0.01 mol L⁻¹ CaCl₂ and organic carbon (OC) following the procedures described by Walkley and Black (1934). Chemical attributes from the experimental area are available in Appendix A.

2.5 PLANT EXTRACTION

The plant material (soybean leaves, immature seeds, and pod valves) was ground in a mortar with liquid nitrogen for metabolomics and ionomics analyses. Ultra-performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-QToF-MS^E) was used for metabolomics analysis at the Embrapa Chemical Ecology Laboratory. Plant ionomics was carried out in inductively coupled plasma optical emission spectroscopy (ICP-OES) at the Embrapa Plant and Soil Analysis Laboratory.

2.5.1 Extraction Process for Plant Metabolomics

Metabolomic analysis was performed using a 90 mg aliquot of plant tissues in 15 mL Falcon tubes. For metabolite extraction, 3 mL of 80% methanol (MeOH: H₂O; 80:20; v/v) was added to the plant tissue. Subsequently, samples were placed in an LSUC2-500-22.5 (60 Hz frequency, 1300 W power; Logen Scientific, Brazil) ultrasonic bath for 20 min, vortexed for 10 s and centrifuged at 5,500 rpm, at 4 °C for 20 min in a Sorvall™ Legend™ X1R centrifuge (Rotor 75003623; Thermo Fisher Scientific, USA). The supernatant (3 mL) was filtered with Millex-HV Durapore™ 0.45 µm Acrodisc Filter Syringes (SLHVX13NL, PVDF; Merck Millipore, Germany) into glass tubes for drying in N₂ flux in MA 4006 dry-block (400 W power; Marconi Equipamentos, Brazil). Samples were placed in a freezer at -20 °C until performing the analyses. For chromatographic analysis, samples were solubilized in 80% MeOH and filtered on Millex-HV Durapore™ 0.22 µm Acrodisc Filter Syringes (SLHVX13TL, PVDF; Merck Millipore, Germany), aiming at a final concentration of 2.5 mg mL⁻¹.

2.5.2 Extraction Process for Plant Ionomics

Plant elemental content analysis was performed using a 150 mg aliquot of plant sample freeze-dried and ground in a mortar. Subsequently, the samples of plant tissue were digested in 6 mL nitric acid (HNO₃: H₂O; 50:50; v/v) and 2 mL hydrogen peroxide (H₂O₂) in a MARSXpress microwave oven (CEM Corporation, Canada). The conditions followed 10 min of the heating ramp, maintaining a constant temperature at 170 °C for 15 min under 2 MPa pressure and a power of 1600 W (USPEA, 1996).

2.6 INSTRUMENTAL ANALYSES

2.6.1 Chromatographic Analysis (UPLC)

Chromatographic analyses were performed using ACQUITY UPLC™ ultra-performance liquid chromatography (Waters Corporation, USA) equipment set in a binary solvent system and sample manager. Aliquots (5 µL) of plant extracts were injected through an autosampler at 20 °C into an ACQUITY UPLC HSS C18 SB stationary reversed-phase column (100 mm x 2.1 mm; 1.8 µm; Waters Corporation, USA). The column temperature was maintained at 40 °C, with a flow rate of 0.4 mL min⁻¹. The mobile phase gradient was as follows: (A) binary elution system composed of Milli-Q™ ultrapure water and 0.1% formic acid (H₂O:

CH₂O₂; v/v) and (B) acetonitrile with 0.1% formic acid (ACN: CH₂O₂; v/v). The gradient system was set 2-95% B (0-15 min), 100% B (15.1-17 min) and reconditioned with 2% B (17.1-19.1 min). Samples were injected in triplicate, randomly, and included blanks.

2.6.2 Mass Spectrometry (ESI-QToF-MS^E)

A XevoTM QToF-MS mass spectrometer (Waters Corporation, USA) equipped with a ZSprayTM source operating in negative electrospray ionization (ESI⁻) was used for analyses. Data acquisition mass range was set at 100 to 1100 Da and scan time at 0.25 s. MS Tune was configured for MS^E Centroid mode data acquisition, 6 V low collision energy on MS¹, and MS² function with ramp ranging from 20 to 40 V. The following MS parameters were applied in the analyses: 2.6 kV capillary voltage, 35 V sampling cone voltage, 150 °C supply temperature, and 1.5 V extraction cone voltage.

Nitrogen was produced by an NM30LA-MS high-purity N₂ generator (Peak Scientific InstrumentTM, Scotland), which was used as the cone gas, desolvation temperature, and flow at 350 °C and 500 L h⁻¹, respectively. Argon ≥ 99.9% purity (White Martins, Brazil) was used as a collision gas at a pressure of 1.3×10⁻² mbar. Mass accuracy and reproducibility were ensured by leucine-enkephalin infusion into the lock mass system (400 ng mL⁻¹), [M-H]⁻ ion *m/z* 554.2615, and every 20 s on the LockSpray flow probe at 20 µL min⁻¹. The instrument was calibrated using a sodium formate standard solution and MassLynxTM 4.1 software (Waters Corporation, USA) used for data acquisition. Quercetin-3-*O*-rhamnoside was used as an external control standard (*t_R* = 5.64 min; *m/z* = [M-H-Rha]⁻ 301.0348) analysed every 10 injections, these data of stability were registered and reported in Appendix G.

2.6.3 Inductively Coupled Plasma (ICP-OES)

Plant tissue ionomics analyses were performed in inductively coupled plasma optical emission spectroscopy (ICP-OES) OptimaTM 8300 (Perkin Elmer, USA). The macro and micronutrient contents were measured using the calibration curve processed for each batch of samples (Table 1 and Table 2).

2.7 YIELD PARAMETERS

At maturity soybean (R8 stage, 131 DAS), three rows of soybean 6 m in length were mechanically harvested to estimate the 100-seed dry weight (g) and soybean grain yield (kg ha⁻¹). The weight and moisture of the harvested seed were recorded, and the final seed yield was calculated based on a standard method of seed moisture content of 130 g kg⁻¹ (13%) water content (Table 3), as also described by Firmano *et al.* (2020).

2.8 DATA INTERPRETATION

2.8.1 Univariate Analysis

Yield parameters and ionomics data were submitted to analysis of variance (ANOVA), and means were compared using Tukey's test (p -value ≤ 0.05). Statistical comparisons between leaves from different growing regions (LT, MT, and UT) were performed using ANOVA + Tukey's test, and pod tissues (PV and IS) by two-sample t-test. ANOVA and Student's t-test were analysed in MinitabTM 18.1 software (Minitab Inc., USA)

2.8.2 Pre-processing and Chemometrics analysis

Soybean leaf and pod extracts from K⁺ treatments (KVL, KL, KM, and KVH) were analysed in a UPLC-QToF-MS^E system, and raw mass data were processed using MarkerLynx XSTM 4.1 software (Waters Corporation, USA). The processing parameters for chromatographic data were adjusted by retention time interval of 0 to 14 min. Mass acquisition amplitude was maintained from 100 to 1100 Da, mass tolerance was set at 0.04 Da, and the minimum peak intensity limit was considered at 50 counts. The isotopic data removal function was employed with smoothing (Savitzky-Golay); the noise elimination level was 9, and the window and mass retention times were set at 0.02 and 0.20, respectively. The preprocessing dataset was imported into SIMCATM EZinfo software (UmetricsTM, Sweden) for multivariate statistical analysis and the Pareto method was used for data scaling (SALDANHA *et al.*, 2020).

Metabolomics (X) and ionomics (Y) analysis data arrays were submitted to multivariate analysis. Firstly, the dataset was employed the unsupervised model, based on principal component analysis (PCA-X&Y), followed by the supervised model, two-way orthogonal partial least squares discriminant analysis (O2PLS-DA). These chemometric tools help to reduce the dimension of the dataset and enable the visualization of variability sources. These models can join two multivariate matrices in a single model, allowing to visualize the

interaction and variance of both datasets (X-variable: untargeted metabolomics analysis; Y-variable: targeted ionomics analysis). Models were evaluated from the R^2 values representing the model's explanatory capacity, while Q^2 suggested its predictive capacity (Appendix F). Supervised multivariate models were generated for comparative treatments KVL vs. KVH, and S-Plot was performed for variable selection.

Potential markers were annotated based on four conditions: (I) selection of metabolites observed within the quadrant $p(\text{corr}) [1] \geq 0.6$ and $p[1]$ (loadings) ≥ 0.06 on the S-Plot graph; (II) variable importance in projection (VIP) value ≥ 1 was used; (III) variables were selected when p -value ≤ 0.05 in the CV-ANOVA test; and (IV) false discovery rate (FDR), proposed by Benjamini and Hochberg (1995) was performed for calculating p -value transformation into q -value (TUGIZIMANA *et al.*, 2016; VANDERPLANCK; GLAUSER, 2018; ZHAO *et al.*, 2017; ZHU *et al.*, 2018). Fold change was calculated by dividing the mean intensity values of metabolites in KVL vs. KVH treatments (Table 4, Table 5, Table 6, and Table 7).

2.8.3 Metabolite Annotation and Molecular Networking

Metabolomics data were imported into MS-DIAL 4.70 software (TSUGAWA *et al.*, 2015, 2019) for deconvolution and MS^E spectra alignment; posteriorly, putative identification was performed using MS-FINDER 3.52 (TSUGAWA *et al.*, 2016). Heuristic rules were considered (SUMNER *et al.*, 2007), and the mass tolerance was adjusted to 0.02 Da. Metabolites were putatively identified according to metabolic standards initiative (MSI) of level 2.1 (SCHRIMPE-RUTLEDGE *et al.*, 2016; SUMNER *et al.*, 2007; TSUGAWA *et al.*, 2019). These metabolites were annotated based occur previously reported in *G. max* or Fabaceae plants using the Human Metabolome Database (HMDB; <https://hmdb.ca/>), PubChem Database (<https://pubchem.ncbi.nlm.nih.gov/>), LipidMaps (LMSD; <https://www.lipidmaps.org/>), MassBank Database (<https://mona.fiehnlab.ucdavis.edu/>), ChemSpider (<http://www.chemspider.com/>), Kyoto Encyclopedia of Genes and Genomes Database (KEGG; <https://www.genome.jp/kegg/compound/>), Chemical Entities of Biological Interest (ChEBI; <https://www.ebi.ac.uk/chebi/>), FoodB (<https://foodb.ca/>), KnapSack (<http://www.knapsackfamily.com/>), and PlantCyc database (<https://plantcyc.org/>). A feature-based molecular networking (FBMN) workflow (NOTHIAS *et al.*, 2020) on global natural products social molecular networking (GNPS) (WANG *et al.*, 2016) was created, and Cytoscape 3.8 (SHANNON *et al.*, 2003) using yFiled Layout Algorithms 1.1 and chemViz2 1.1 were used for molecular network manipulation, visualization, and analysis (Figure 3).

Chemical ontology descriptions, MS² fragments, average intensity, mass theoretical, and measured data are available in Appendix G.

3. RESULTS AND DISCUSSION

3.1 SOYBEAN LEAF IONOMICS ANALYSIS

We evaluated the profile of nutrients and metabolites in soybean tissues under four soil nutrient conditions, very low (KVL; $0.05 \text{ cmol}_c \text{ dm}^{-3}$), low (KL; $0.09 \text{ cmol}_c \text{ dm}^{-3}$), medium (KM; $0.13 \text{ cmol}_c \text{ dm}^{-3}$), and very high (KVH; $0.50 \text{ cmol}_c \text{ dm}^{-3}$) K^+ availability by Mehlich-1 extraction (Appendix A). Potassium leaf content increased in response to K^+ rates applied to the soil, as expected (Table 1). Potassium accumulation evaluated in very high K^+ soil availability (KVH) was 3.4-fold (16.22 g kg^{-1}) at UT, averaging 4.3-fold (12.08 g kg^{-1}) in MT and LT leaves in comparison to very low K^+ availability (KVL) treatment (Table 1 and Appendix B). Plant K^+ content was associated with soybean tissue maturity (Table 1 and Appendix C). We observed that the upper third (UT) leaves had the highest K^+ levels compared to the medium (MT) and lower third (LT) leaves, respectively. Previously, the high mobility of potassium was reported by Marschner (2012) and Mengel *et al.* (2001) who also related that due to higher demand for K^+ in metabolic activities, potassium is often transported, redistributed, and accumulated primarily in the young tissues.

Leaf calcium (Ca^{2+}) and magnesium (Mg^{2+}) contents decreased significantly with increasing K^+ rates; phosphorus (H_2PO_4^-) and sulphur (S) levels were not altered by treatments (Table 1). However, the mean content of S tended to be higher in MT and UT ($> 2.0 \text{ g kg}^{-1}$), compared with the LT (Table 1 and Appendix C). Other authors reported a reduction in Ca^{2+} and Mg^{2+} content by increases in K^+ soil fertilization (DIEM; GODBOLD, 1993; FIRMANO *et al.*, 2020; HAFSI *et al.*, 2014). The high K^+ level in the soil solution could competitively inhibit the uptake of Ca^{2+} and Mg^{2+} by the plant root absorption site (CASTRO *et al.*, 2021; FIRMANO *et al.*, 2020). In fact, the increased content of these macronutrients ($\text{Ca}^{2+}/\text{Mg}^{2+}$) was also detected by Hafsi *et al.* (2014) in leaves under K^+ deficiency, which they suggested being associated with alleviating the effects of nutritional deficiency. Management programs to obtain high K^+ rates or getting soils with high natural contents of K^+ can lead to a lower uptake of Mg^{2+} and even Ca^{2+} , that generally triggers yield reductions (CASTRO *et al.*, 2021).

The contents of micronutrients in soybean old leaves (LT), such as manganese (Mn^{2+}), iron (Fe^{2+}), copper (Cu^{2+}), and boron (B) significantly increased by 1.04-, 1.11-, 1.23-, and 1.88-fold under KVL compared to KVH (Table 1). Regardless of the treatment, Fe^{2+} and Mn^{2+} concentrations were lower in MT and UT leaves in comparison to LT (Table 1 and Appendix C). However, zinc (Zn^{2+}) leaf content in the three growing regions (LT, MT, and UT) was reduced when soybean plants grown under very low potassium availability (KVL).

These experimental results suggest that soybean leaves can resist low potassium availability by accumulating Ca^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Cu^{2+} , and B. Integrated omics of wild soybean (*Glycine soja*) under low phosphate availability showed an accumulation of Ca^{2+} , Mg^{2+} , Fe^{3+} , NO_3^- and S in young leaves, and reduced Zn^{2+} levels as related by Shen *et al.* (2021). These results suggest that this interaction (P and K^+ deficiency) should be investigated in the future for a better understanding of the mechanisms of soybean tolerance to nutrient deficiency.

Iron usually acts as a mineral regulatory element and defends plants against stresses by activating plant enzymatic antioxidants (TRIPATHI *et al.*, 2018). The reduction in boron levels in the soybean leaves in conditions of increased K^+ rates (Table 1) was probably associated with its low phloem mobility (MARSCHNER, 2012). Moreover, a reduction in B availability may also occur due to low Ca^{2+} uptake, revealed in plants that grown under high K^+ soil content, which reduced the B requirement (FIRMANO, 2017; FIRMANO *et al.*, 2020). In general, our results demonstrate that the content of Mn^{2+} , Zn^{2+} , and Cu^{2+} in the leaves are poorly or non-responsive to increasing K^+ levels in the soil. In fact, the concentration of these nutrients in soybean leaves (cv. BRS 1010 IPRO) were also not affected in the agronomic assays reported by Firmano (2017), being within or above the class of contents considered appropriate in soybean plants (HARGER, 2008).

Table 1 - Ionomics analysis of soybean trifoliolate leaves (cv. BRS 1010 IPRO) in response to K⁺ soil fertilization

Treatments		Leaf DW (g)	P	K ⁺	Ca ²⁺	Mg ²⁺	S	Zn ²⁺	Mn ²⁺	Fe ²⁺	Cu ²⁺	B
			(g kg ⁻¹)					(mg kg ⁻¹)				
KVL	LT	0.22	2.47 ^{aB}	2.73 ^{cB}	22.9 ^{aA}	11.37 ^{aA}	1.67 ^{aB}	43.17 ^{bA}	279.72 ^{aA}	398.17 ^{aA}	12.30 ^{aA}	64.75 ^{aA}
KL	LT	0.24	2.44 ^{aB}	7.25 ^{bB}	19.9 ^{aA}	5.78 ^{bA}	1.75 ^{aB}	50.83 ^{aA}	282.28 ^{aA}	389.08 ^{bA}	9.95 ^{bA}	38.59 ^{bB}
KM	LT	0.25	2.39 ^{aB}	8.84 ^{bB}	14.5 ^{bA}	4.17 ^{cA}	1.65 ^{aC}	51.39 ^{aA}	270.50 ^{bA}	353.50 ^{cA}	8.95 ^{bA}	37.67 ^{bB}
KVH	LT	0.25	2.41 ^{aB}	12.02 ^{aB}	12.0 ^{bA}	3.27 ^{cA}	1.62 ^{aB}	50.39 ^{aA}	268.44 ^{bA}	358.17 ^{cA}	9.95 ^{bA}	34.32 ^{bB}
KVL	MT	0.31	2.60 ^{aA}	2.87 ^{cB}	20.1 ^{aB}	9.38 ^{aB}	2.27 ^{aA}	35.94 ^{bB}	248.22 ^{aB}	269.33 ^{aB}	10.60 ^{aA}	58.38 ^{aA}
KL	MT	0.37	2.62 ^{aA}	7.97 ^{bB}	16.2 ^{bB}	5.07 ^{bB}	2.23 ^{aA}	44.44 ^{aB}	251.61 ^{aB}	226.50 ^{bB}	9.60 ^{aA}	37.33 ^{bB}
KM	MT	0.35	2.59 ^{aA}	9.40 ^{bB}	12.2 ^{cA}	3.67 ^{cB}	2.14 ^{aB}	46.50 ^{aB}	246.61 ^{aB}	200.67 ^{cB}	8.22 ^{aA}	29.90 ^{cC}
KVH	MT	0.36	2.56 ^{aA}	12.14 ^{aB}	11.5 ^{cA}	3.02 ^{cAB}	2.16 ^{aA}	47.06 ^{aB}	254.06 ^{aB}	196.17 ^{cB}	8.57 ^{aB}	30.85 ^{cB}
KVL	UT	0.38	2.60 ^{aA}	4.70 ^{dA}	17.2 ^{aC}	7.75 ^{aC}	2.39 ^{aA}	25.06 ^{bC}	137.28 ^{aC}	263.16 ^{aB}	11.07 ^{aA}	61.95 ^{aA}
KL	UT	0.44	2.61 ^{aA}	9.88 ^{cA}	11.0 ^{bC}	3.53 ^{bC}	2.37 ^{aA}	28.00 ^{aC}	133.44 ^{aC}	221.83 ^{bB}	9.32 ^{bA}	44.48 ^{bA}
KM	UT	0.44	2.57 ^{aA}	13.64 ^{bA}	9.60 ^{cB}	3.07 ^{bC}	2.31 ^{aA}	28.56 ^{aC}	128.50 ^{aC}	182.67 ^{cB}	8.18 ^{bA}	41.35 ^{bA}
KVH	UT	0.43	2.53 ^{aA}	16.22 ^{aA}	9.50 ^{cB}	2.78 ^{bB}	2.37 ^{aA}	27.89 ^{aC}	130.06 ^{aC}	183.19 ^{cB}	7.48 ^{bC}	39.75 ^{bA}

Note: Leaf DW – leaf dry weight; KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; LT – lower third; MT – medium third; UT – upper third; The data presented are representations of the mean ($n = 6$) and results of ANOVA + Tukey's test ($p \leq 0.05$); Different lower case letters indicate differences amongst K⁺ availability conditions (KVL, KL, KM, and KVH) in the same soybean tissue; Different capital letters indicate differences amongst soybean trifoliolate leaves (LT, MT, and UT) in the same K⁺ treatment.

Source: The Autor (2022).

3.2 SOYBEAN POD IONOMICS ANALYSIS

Ionomics analysis showed that the K^+ content increased 1.6-fold in immature seeds (IS), and 5.0-fold in pod valves (PV) when KVH treatment was compared to KVL (Table 2 and Appendix D). The increased content of K^+ in immature seeds was similar for KVH and KM, but higher than that assimilated by IS of plants under KL and KVH treatment. In general, our results obtained for K^+ accumulation in soybean seeds were similar to those reported by Parvej *et al.* (2015) and Vyn *et al.* (2002). The seeds, as propagation organs of the species, tend to receive a greater nutrient amount, even in plants with a nutritional deficiency status (Marschner, 2012).

The pod valves were more affected by low potassium availability (KVL and KL treatments); they presented mean levels of K^+ accumulation $> 2.50 \text{ g kg}^{-1}$, compared with immature seeds ($> 7.07 \text{ g kg}^{-1}$). Other macronutrients (P, Ca^{2+} , Mg^{2+} , and S) were not significantly altered by treatments in soybean pod tissues. In general, the P content observed in IS ($< 3.23 \text{ g kg}^{-1}$) is higher than detected in PV ($< 2.63 \text{ g kg}^{-1}$) by the two-sample t-test, except in the KM-PV vs. KM-IS (Table 2 and Appendix E). However, the accumulation of Ca^{2+} and Mg^{2+} was greater in PV (> 7.03 and 3.73 g kg^{-1} , respectively).

The concentration of Zn^{2+} and Mn^{2+} were higher in the PV of soybean plants in soils with medium (KM) and very high K^+ availability (KVH) (Table 2). The same tendency was observed for Mn^{2+} in IS. These results indicated that low K^+ soil availability increased Mn^{2+} accumulation in soybean pod tissues (R5.5 stage). Copper content (Cu^{2+}) responded only in pod valves when soybean plants were exposed to very low and low potassium treatments (KVL and KL). Immature seeds (IS) showed increased Fe^{2+} concentration in KVL compared to other K^+ treatments ($< 53.6 \text{ mg kg}^{-1}$). Under our experimental conditions, no significant changes in the content of Zn^{2+} , Cu^{2+} , and B were observed in IS, and Fe^{2+} and B in PV considering the four K^+ availability conditions (Table 2).

The effect of potassium availability on the elemental content of vegetative and reproductive tissues investigated reveals that Ca^{2+} and Mg^{2+} responded to K^+ rates only in leaves, but this interaction did not occur in soybean pod tissues (R5.5 stage). Furthermore, plants grown under very low and low K^+ content (KVL and KL) did not accumulate Ca^{2+} , Mg^{2+} , Mn^{2+} , and B in pod tissues, as observed in the results of trifoliate leaves (especially LT leaves). However, Cu^{2+} concentration increase occurred only in pod valves and leaves under low K^+ availability (Table 1 and Table 2) and Fe^{2+} content only responded to immature seeds (IS) in comparison to pod valves.

Table 2 - Ionic analysis of soybean pods (cv. BRS 1010 IPRO) sampled at R5.5 growth stage in response to K⁺ soil fertilization

Treatments		P	K ⁺	Ca ²⁺ (g kg ⁻¹)	Mg ²⁺	S	Zn ²⁺	Mn ²⁺	Fe ²⁺ (mg kg ⁻¹)	Cu ²⁺	B
KVL	PV	2.63 ^{aB}	2.50 ^{dB}	7.03 ^{aA}	4.47 ^{aA}	1.06 ^{aB}	6.83 ^{cB}	33.00 ^{cA}	69.33 ^{aA}	10.27 ^{aB}	27.13 ^{aA}
KL	PV	2.55 ^{aB}	5.13 ^{cB}	7.37 ^{aA}	4.47 ^{aA}	1.13 ^{aB}	9.97 ^{bA}	40.67 ^{bA}	71.67 ^{aA}	10.57 ^{aB}	29.30 ^{aA}
KM	PV	2.57 ^{aA}	10.53 ^{bA}	9.50 ^{aA}	3.93 ^{aA}	1.19 ^{aB}	11.20 ^{aA}	68.67 ^{aA}	68.67 ^{aA}	8.70 ^{bB}	34.30 ^{aA}
KVH	PV	2.53 ^{aB}	12.57 ^{aA}	8.37 ^{aA}	3.73 ^{aA}	1.05 ^{aB}	13.60 ^{aA}	65.67 ^{aA}	71.33 ^{aA}	7.53 ^{bB}	31.77 ^{aA}
KVL	IS	3.23 ^{aA}	7.07 ^{bA}	2.37 ^{aB}	1.53 ^{aB}	1.76 ^{aA}	11.43 ^{aA}	24.00 ^{bB}	58.67 ^{aA}	21.73 ^{aA}	15.97 ^{aB}
KL	IS	3.14 ^{aA}	7.87 ^{bA}	2.37 ^{aB}	1.43 ^{aB}	1.73 ^{aA}	10.50 ^{aA}	23.67 ^{bB}	51.67 ^{bB}	18.97 ^{aA}	15.73 ^{aB}
KM	IS	3.15 ^{aA}	11.47 ^{aA}	2.47 ^{aB}	1.50 ^{aB}	1.91 ^{aA}	12.83 ^{aA}	36.00 ^{aB}	52.33 ^{bB}	20.23 ^{aA}	17.52 ^{aB}
KVH	IS	3.12 ^{aA}	11.73 ^{aB}	2.43 ^{aB}	1.50 ^{aB}	1.87 ^{aA}	11.70 ^{aA}	35.00 ^{aB}	53.67 ^{bA}	19.50 ^{aA}	17.87 ^{aB}

Note: KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; IS - immature seeds; PV – pod valves; The data presented are the mean values ($n = 6$); Different lower case letters indicate differences amongst K⁺ availability conditions (KVL, KL, KM, and KVH) in the same soybean tissue by Tukey's test ($p \leq 0.05$); Different capital letters indicate differences amongst soybean pod tissues (PV and IS) in the same K⁺ treatment by two-sample t-test ($p \leq 0.05$).

Source: The Autor (2022).

An indirect effect of the increase of K^+ fertilization reported by Firmano *et al.* (2020) was greater Mn^{2+} accumulation in soybean seeds, with stabilization of this concentration after soil rate fertilization ($> 120 \text{ kg K}_2\text{O ha}^{-1}$). The Mn^{2+} increase content in IS can be associated with its activity in energetic metabolism (RANADE-MALVI, 2011). The high K^+ availability can also promote the major metabolic activity of plants, which leads to greater Mn^{2+} content in seeds (FIRMANO, 2017). Overall, our results are consistent in the relationship of nutritional balance of macro and micronutrients when soybean plants were under the contrasting conditions of K^+ availability (MARSCHNER, 2012).

3.3 YIELD PARAMETERS

The dry weight of 100 seeds and the yield parameters increased with the K^+ rates (Table 3). The 100-seed dry weight was higher in the KVH (15.5 g) and KM (15.6 g) than in the KL (14.1 g) and KVL (12.4 g) treatments, as expected. The yield followed the same trend; the highest means were achieved by KVH (4096 kg ha^{-1}), and KM (4093 kg ha^{-1}) treatment plots, in contrast to KL (3737 kg ha^{-1}) and KVL (2211 kg ha^{-1}). Parvej *et al.* (2015) related yield increase of 40 and 60% when soybean grown under medium and high K^+ soil fertility (81 and 91 $\text{mg K}^+ \text{kg}^{-1}$, respectively), compared with the low K^+ treatment (61 $\text{mg K}^+ \text{kg}^{-1}$). A previous study in the same edaphoclimatic conditions of ours conducted by Firmano *et al.* (2020) showed that soybean yield stabilised when the K^+ soil content extracted by Mehlich-1 reaches $0.15 \text{ cmol}_c \text{ dm}^{-3}$. In our analysis, the potassium soil concentration in KM treatment was $0.13 \text{ cmol}_c \text{ dm}^{-3}$ and $0.50 \text{ cmol}_c \text{ dm}^{-3}$ in KVH (Appendix A), justifying the soybean yield similarity between both treatments.

Table 3 - Soybean (cv. BRS 1010 IPRO) grains yield parameters as function of K^+ soil availability

Treatments	100-Seeds DW (g)	Yield (kg ha^{-1})
KVL	12.4 ^c	2211 ^c
KL	14.1 ^b	3737 ^b
KM	15.6 ^a	4093 ^a
KVH	15.5 ^a	4096 ^a

Note: KVL – very low K^+ availability; KL – low K^+ availability; KM – medium K^+ availability; KVH – very high K^+ availability. The data presented are the mean values ($n = 6$) obtained from ANOVA. Means followed by the same letter in the column do not differ from each other by Tukey's test ($p \leq 0.05$).

Source: The Autor (2022).

3.4 CHEMOMETRICS ANALYSIS

The PCA-X&Y models indicate the response of the metabolite profile to K^+ availability. In our study, we established two-blocks (X = intensities of metabolites; Y = ion concentration) as a bidirectional multivariate method that aims to separate the covariance between two data sets. Graphs of PCA-X&Y indicate the response of metabolite profile to K^+ availability. PCA-X&Y_A was created for trifoliolate leaf samples and shows 404 variables (Figure 1a). The Pareto scale scored 21 components, explained by 83% $R^2X_{(cum)}$ variance. The first two main components accounted for 45.3% of the total variance ($R^2X [1] = 0.317$ and $R^2X [2] = 0.136$). PCA-X&Y_A also showed distant points representative of the leaf growth regions (LT, MT, and UT) in PC1. The O2PLS-DA_B model exhibited similar clustering, with better separation of the treatments in leaf samples (Figure 1b). Cluster I have 9.1 to -14.5 variance in PC2, and in general, the O2PLS-DA_B score plot showed two main components, cluster I (KL, KM, and KVH) and cluster II (KVL), observable by PC2.

Loading plots of trifoliolate leaves reveal the influence of K^+ content measured by ionomic analysis (Figure 1c), as previously reported (2.1 section). Potassium fertilization had a greater contribution to cluster I (KL, KM, and KVH), predominantly on the upper third (UT) leaves in both treatments (Figure 1b, c); Zn^{2+} and Mn^{2+} content also contribute to PC2, but mostly for the lower third (LT) and medium third (MT) leaves. The concentration of Ca^{2+} , Mg^{2+} , Cu^{2+} , Fe^{2+} , and B were associated with the leaves of plants that grow under very low potassium availability (KVL).

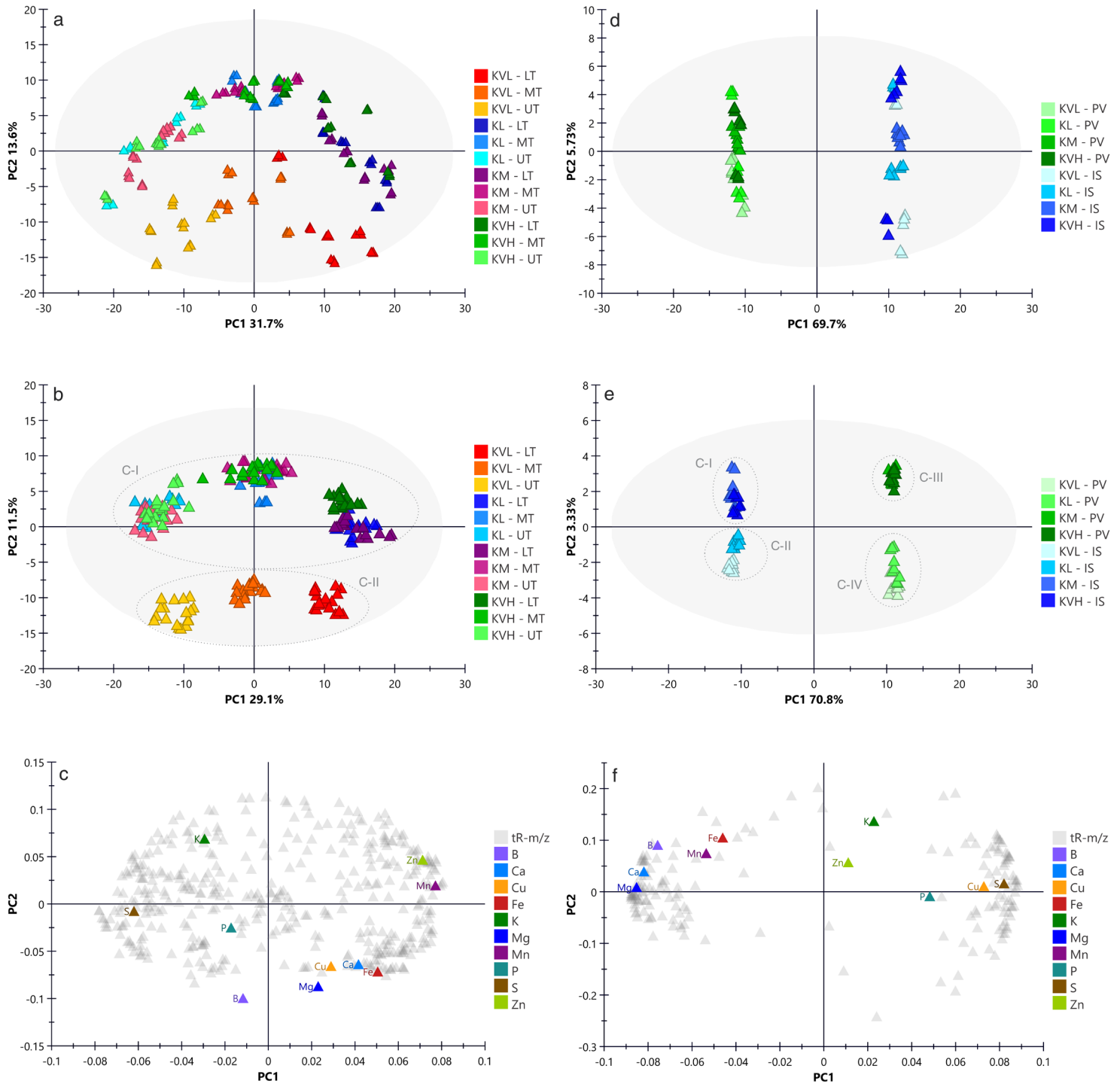
In the PCA-X&Y_D model, the pod tissue samples dataset used was composed of 221 variables, with seven components explaining 89% of the variance in $R^2X_{(cum)}$ (Figure 1d). Two main components explained 75.4% of the variance ($R^2X [1] = 0.697$ and $R^2X [2] = 0.057$), revealing low treatment separation characteristics for PC2. Pod valves (PV) and immature seeds (IS) samples were distant from the projection (PC1). The use of O2PLS-DA_E (supervised model) was necessary for treatment distinctions (Figure 1e) since only PCA-X&Y_D did not clearly explain the relationship between the K^+ rates and metabolite content in pod tissues. The clusters in the supervised multivariate model (O2PLS-DA_E) from the pod tissues and the results of ANOVA (Tukey's test) were similar in regard to K^+ availability (Table 2). Four groups were observed in the O2PLS-DA_E score plot, cluster I (KM-IS and KVH-IS), cluster II (KL-IS and KVL-IS), cluster III (KM-PV and KVH-PV), and cluster IV (KL-PV and KVL-PV), represented by Figure 1e.

Chemometric models showed that K^+ rates applied in soil fertilization were represented by PC2, while different sampling leaf regions (LT, MT, and UT) and pod tissues (PV and IS) were explained by PC1 (Figure 1). The positive quadrant of PC2 in the plot of IS and PV metabolic profiles reveals that medium and very high potassium availability (KM and KVH) treatments showed a correlation of K^+ content measured in ionomic analysis (Figure 1f). However, the variance between the K^+ availability (KVL, KL, KM, and KVH) was significantly lower in pod tissues (3.33% by PC2; Figure 1e) compared to trifoliolate leaves (11.5% by PC2; Figure 1b). These results suggest that potassium rates promote more variance in leaf metabolite content than in pod valves and immature seeds, respectively.

The O2PLS-DA models (Appendix F), established from the metabolomics/ionomics datasets for soybean leaves and pods, explained 99% $R^2Y_{(cum)}$ and predicted 98% $Q^2Y_{(cum)}$ of the total variance, indicating the adequacy of the model, and allowing the establishment of S-Plot graphs, using only KVL vs. KVH treatments. We observed that soybean under very high potassium availability (KVH) accumulated differentially 17 and 7 compounds in leaves and pods, respectively, compared with the lowest K^+ availability (Table 4 and 5). Under very low potassium availability, O2PLS-DA models (KVL vs. KVH) revealed that 21 and 18 metabolites were upregulated in trifoliolate leaves and pod tissues, respectively (Table 6 and 7). These experimental results indicated that K^+ rates did not contribute to linearly increasing constitutive specialised metabolites in soybean development stages sampled, rejecting our initial hypothesis. However, our data showed that under very low potassium availability soybean metabolism had the highest metabolic shift, as also observed previously by Shen *et al.* (2021) with *G. soja* deficient in phosphorus.

Figure 1 - Unsupervised and supervised chemometric models of soybean plants under four soil K^+ availability. These models allowed us to correlate the metabolomics data (404 and 221 molecular features to soybean leaves and pod tissues, respectively as X input) with ionomics (10 factors as Y input) coherently. (a) PCA-X&Y_A scatter plot of trifoliolate leaves. (b) O2PLS-DA_B score plot highlighting the identified two clusters (C-I and C-II) of trifoliolate leaves. (c) O2PLS-DA_B loading plot of trifoliolate leaves, the loadings (factor) in the graph represent macro and micronutrients quantified by ICP-OES that contribute to the O2PLS-DA model. (d) PCA-X&Y_D scatter plot of soybean pod tissues. (e) O2PLS-DA_E score plot highlighting the identified four clusters (C-I, C-II, C-III, and C-IV) of soybean pod tissues. (f) O2PLS-DA_E loading plot of soybean pod tissues, the loadings (factor) in the graph represent nutrients quantified by ICP-OES that contribute to the O2PLS-DA model. List of abbreviations: LT –

lower third leaves; MT – medium third leaves; UT– upper third leaves; IS - immature seeds; PV – pod valves; KVL – very low K^+ availability; KL – low K^+ availability; KM – medium K^+ availability; KVH – very high K^+ availability.



Source: The Autor (2022).

The multivariate analysis grouped the leaf samples according to the growth region (LT, MT, and UT). The progressive metabolite accumulation may be a response to observed differences in tissue maturity, also reported by Song *et al.* (2014) and Yuk *et al.* (2011). These authors associated the expressive accumulation of coumestrol and glyceollin VII in soybean leaves occur at R7/R8 stage. Likewise, wild soybean under low phosphate (Pi) conditions promoted the transportation and reuse of sugars and amino acid metabolites and mobilize Pi from hexose-phosphate from old to young leaves (SHEN *et al.*, 2021). Unlike K⁺, phosphorus is a structural component of many molecules in plants, which justifies major metabolome reprogramming. However, potassium in plants is more associated with catalysis or activation of enzymes (CUI; TCHERKEZ, 2021; MARSCHNER, 2012). Soybean under potassium homeostasis should have a sufficient level of this nutrient for enzymatic activity, not resulting in an increase in the contents of constitutive specialised metabolite, as shown by our experiment. Otherwise, K⁺ deficiency leads to relevant metabolic changes (LU *et al.*, 2019; TROUFFLARD *et al.*, 2010), as also observed to low nitrogen (LIU *et al.*, 2019) or phosphorus (SHEN *et al.*, 2021) conditions.

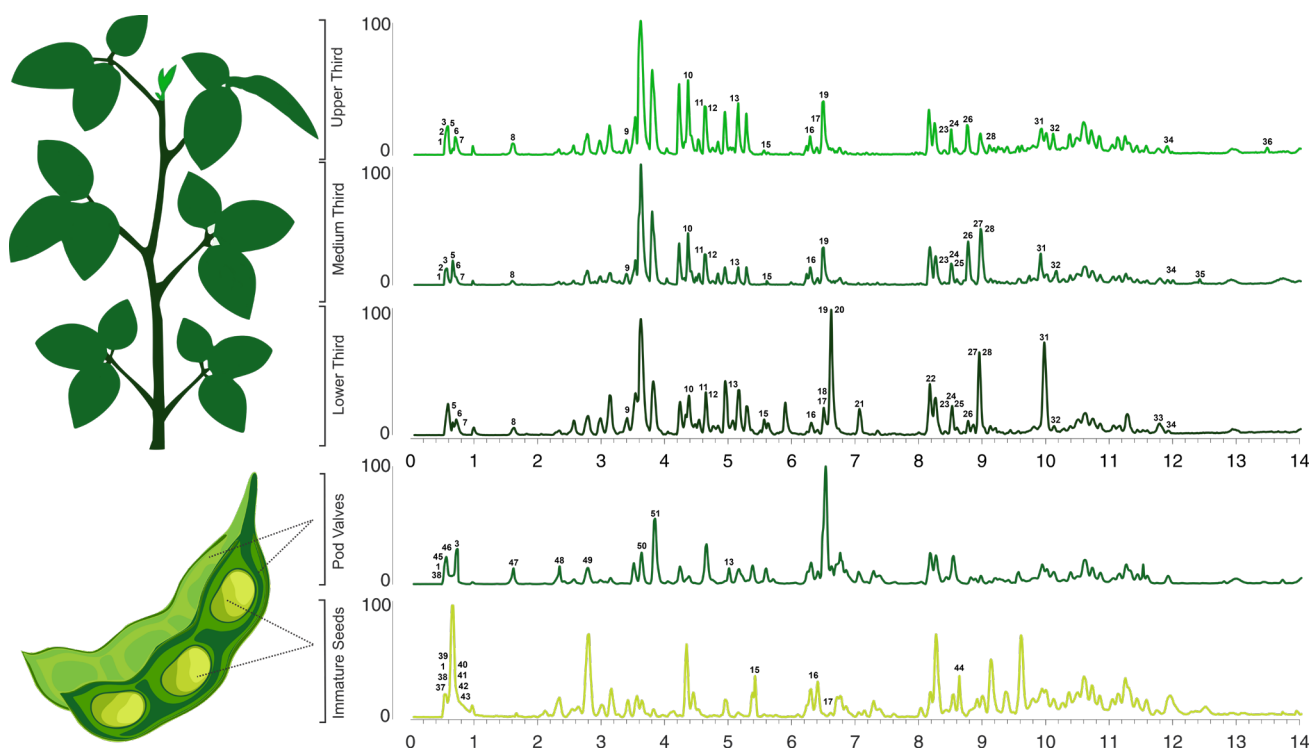
3.5 MOLECULAR NETWORKING AND METABOLIC PATHWAY

Feature-based molecular networking analysis was carried out to gain a comprehensive overview of the differentially regulated metabolites in response to K⁺ availability, complementing the data indicated by the base peak intensity (BPI) in chromatograms and the differential chemical profile of soybean tissues (Figure 2). Molecular networking showed the connection of 902 nodes, 1430 edges, and 311 components that resulted in 89 spectral molecular families putative annotated through an automated library spectral matching (Figure 3). The features highlighted by the chemometric analysis were identified and color-coded in the molecular network. This strategy allowed us to highlight the compounds impacted by K⁺ availability and relate them to chemical structures. Spectra not clustered into molecular families were represented as self-loop nodes at the bottom of the network; colored nodes represent ontology groups of soybean metabolites regulated by K⁺ fertilization. The lack of comprehensive spectral libraries associated with characteristics of our MS¹ and MS² data (MS^E/DIA – data-independent acquisition) can explain the considerable number of spectra not clustered.

Chemometrics analysis showed 51 metabolites differently regulated in response to K⁺ availability (Figure 2). The chemical ontology analysis of metabolites influenced by K⁺

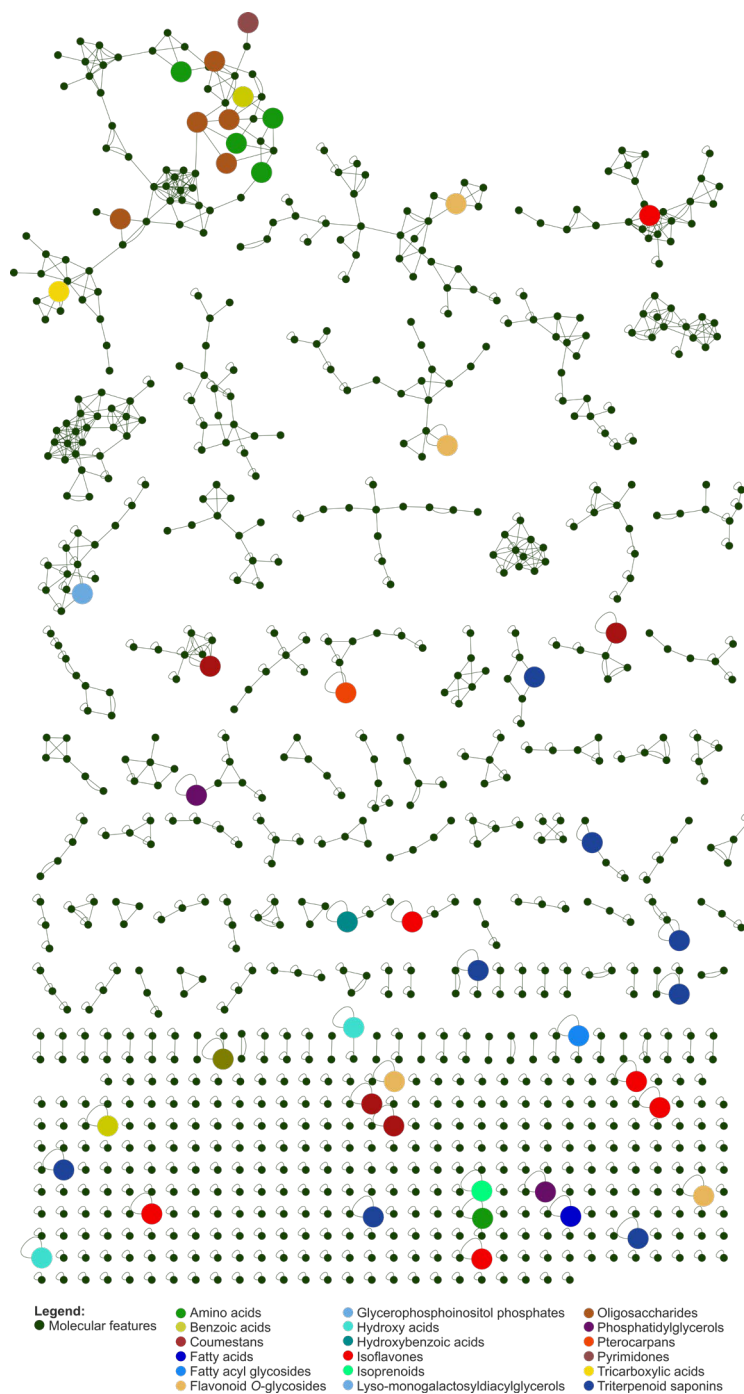
fertilization showed regulation of sixteen phenolic compounds, members of the groups of flavonols, isoflavones, coumestans, and pterocarpan; eight lipids classified as jasmonic acid conjugates, fatty acids, and membrane lipids; Additionally, nine terpenoids were putatively identified as sesquiterpenoid and triterpenoid saponins, and seven were carbohydrates; besides of eleven compounds classified as organic acids, amino acids, and nucleic acids (Appendix G).

Figure 2 - Soybean tissues representation and base peak intensity (BPI) mass chromatograms (UPLC-QToF-MS^E) in negative ion mode (ESI⁻) displaying comparative metabolomic profile differences. The corresponding list of metabolites annotated in the chromatograms is available in Appendix G



Fifty-one annotated metabolites of soybean plants influenced by contrasting soil potassium availability were correlated using the KEGG database. These compounds were linked to nineteen metabolic pathways, such as alanine, aspartate, glutamate, histidine, β -alanine, citrate cycle, glyoxylate and glucuronate interconversion, nitrogen, galactolipid, glycerophospholipid, α -linolenic acid, purine and pyrimidine metabolism, arginine, aminoacyl-tRNA, galactosylcyclitol, phenylpropanoid, flavonol, isoflavonoid, sesquiterpenoid, and triterpenoid biosynthesis. Overall, these KEGG pathways belong to the pathway maps that include carbohydrates, amino acids, nucleotides, lipids, specialised metabolites, and energetic metabolism (Appendix G).

Figure 3 - Complete feature-based molecular network (FBMN) in global natural product social molecular networking (GNPS) of soybean trifoliolate leaves and pod tissues influenced by K⁺ availability. Nodes represent MS² spectra and are connected based on spectral similarity defined (cosine score ≥ 0.8), matched fragment ion (5), and network TopK (10), encompassing 902 nodes and 1430 edges organized in 89 spectral molecular families. Large coloured nodes represent metabolites influenced by K⁺ nutrition identified by chemometrics models and representative chemical ontology



Source: The Autor (2022).

3.6 SOYBEAN LEAVES UNDER K⁺ HOMEOSTASIS

Flavonol glycosides, as kaempferol 3-glucosyl-(1→2)-gentiobioside, kaempferol-3-*O*- α -L-rhamnopyranosyl-(1→6)- β -D-galactopyranoside, and kaempferol-3-*O*-rutinoside were upregulated in soybean leaves of fertilized plants (KVH) when compared to very low potassium availability (KVL) treatment (Table 4). Flavanols are usually associated with pathogen, insect resistance (DILLON *et al.*, 2017), and plant protection from UV-B effects (HARBORNE; WILLIAMS, 2000). Previous studies also associated that K⁺ nutrition provoked an increase in phenolic compounds, such as ferulic, cinnamic, salicylic, and rosmarinic acids (GAO *et al.*, 2018; NGUYEN *et al.*, 2010; PRASAD *et al.*, 2010). Indeed, K⁺ favours metabolic reactions and the turgidity of cells, maintaining the leaf orientation for major light interception (MALAVOLTA, 1980); these events suggest that differential regulation of phenolic compounds can be associated with UV-B.

Monogalactosyldiacylglycerol (18:3) and lyso-monogalactosyldiacylglycerol (16:0/0:0) content also were upregulated (6.1- and 3.3-fold, respectively) in leaves of upper third, when soybean plants grown under KVH compared to KVL treatment (Table 4). In rice, galactolipid biosynthesis was downregulated in seedlings deprived of K⁺ nutrition (SHANKAR *et al.*, 2013). Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are major galactolipids constituents of chloroplast photosynthetic membranes (MURAKAWA *et al.*, 2014), deficiency of these metabolites is associated with plant light sensitivity (JARVIS *et al.*, 2000). Nitrogen deficiency in *A. thaliana* led to a decrease in MGDG and a concomitant increase in DGDG, resulting in a coordinated breakdown of galactolipids and chlorophyll (GAUDE *et al.*, 2007).

Soyasaponins can occur constitutively in any soybean tissue (TSUNO *et al.*, 2018; YATES *et al.*, 2021). In our analyses, soybean plants under potassium homeostasis (KVH) showed differential regulation (upregulation) of three triterpenoid saponins, as soyasaponin Ba (3.4-fold), soyasaponin γ g (3.9-fold), and soyasaponin Bc' (3.4-fold) in the UT leaves (Table 4). Nevertheless, soybean leaves under very low potassium availability (KVL) also revealed upregulation of four additional soyasaponins. Soyasaponin Bc' and soyasaponin γ g have the same structure, except for the conjugate 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moiety. Upregulation of soyasaponin γ g and soyasaponin Bc' biosynthesis (> 3.3-fold) were also detected after abiotic stress, as occurred in common bean seeds of plants grown under severe drought conditions (HERRERA *et al.*, 2019). Shankar *et al.* (2013) revealed that when K⁺ privation is imposed on rice seedlings, gene expression of terpenoid biosynthesis is

reduced. Moreover, other macronutrients, such as nitrogen and phosphate availability can alter saponin content (SZAKIEL *et al.*, 2011).

3.7 SOYBEAN POD TISSUES UNDER K^+ HOMEOSTASIS

Metabolomics analysis of pod tissues showed increased concentration of kaempferol-3-*O*-digalactopyranoside (1.5-fold), malonyl-genistin (1.5-fold), β -D-glucopyranosyl-11-hydroxyjasmonic acid (1.7-fold), trihydroxy-octadecadienoic acid (2.7-fold), and soyasaponin Bb' (1.8-fold) in plants grown under KVH in contrast with KVL treatment (Table 5). Isoflavone content increases in soybean seeds when plants are grown under K^+ rates were reported by Bellaloui *et al.* (2013) and Vyn *et al.* (2002). Especially, Seguin and Zheng (2006) described the difference in isoflavonoid content was not significant when K^+ was applied in medium-to high-fertility soils. In fact, we expected that the upregulation of flavonoids and isoflavones in soybean plants caused by K^+ fertilization would be higher in immature seed extracts. Nevertheless, it is necessary to consider that this regulation is also dependent on yearly environmental factors (drought and temperature), edaphic conditions, and potassium soil content.

Table 4 - Putative metabolite identification from soybean leaves upregulated under very high potassium (KVH) availability by UPLC-QToF-MS^E

tr (min)	Formula	Identification	Chemical Ontology	Lower Third (LT)			Medium Third (MT)			Upper Third (UT)		
				KVL-LT vs. KVH-LT			KVL-MT vs. KVH-MT			KVL-UT vs. KVH-UT		
				FC	FDR	VIP	FC	FDR	VIP	FC	FDR	VIP
0.66	C ₁₂ H ₂₂ O ₁₁	Sucrose	Carbohydrates	—	—	—	2.1	4.17E ⁻⁰²	2.2	3.3	4.00E ⁻⁰²	1.8
0.73	C ₅ H ₁₀ O ₅	D-xylose	Carbohydrates	16.1	3.57E ⁻⁰³	1.6	15.1	4.17E ⁻⁰³	1.7	11.7	3.33E ⁻⁰³	1.2
0.73	C ₄ H ₆ O ₅	Malic acid	Beta hydroxy acids	7.8	7.14E ⁻⁰³	1.2	9.9	1.25E ⁻⁰²	1.3	2.1	3.33E ⁻⁰²	0.7
3.40	C ₃₃ H ₄₀ O ₂₁	Kaempferol-3-glucosyl-(1→2)-gentiobioside	Flavonols	1.4	1.79E ⁻⁰²	2.2	1.4	3.33E ⁻⁰²	2.2	1.4	5.00E ⁻⁰²	1.1
4.35	C ₂₇ H ₃₀ O ₁₅	Kaempferol-3- <i>O</i> - α -L rhamnopyranosyl-(1→6)- β -D-galactopyranoside	Flavonols	4.9	1.07E ⁻⁰²	1.7	4.8	8.33E ⁻⁰³	2.1	5.0	4.67E ⁻⁰²	0.6
4.51	C ₂₇ H ₃₀ O ₁₅	Kaempferol-3- <i>O</i> -rutinoside	Flavonols	4.6	1.43E ⁻⁰²	1.8	3.5	2.50E ⁻⁰²	2.1	2.9	1.67E ⁻⁰²	1.8
5.03	C ₂₄ H ₂₂ O ₁₃	Malonyl-genistin*	Isoflavones	1.4	3.93E ⁻⁰²	2.4	—	—	—	—	—	—
6.30	C ₁₈ H ₃₂ O ₅	Trihydroxy-octadecadienoic acid	Fatty acids	1.3	4.64E ⁻⁰²	1.3	1.3	4.58E ⁻⁰²	1.9	1.3	4.33E ⁻⁰²	1.4
6.49	C ₁₅ H ₁₀ O ₅	Genistein*	Isoflavones	1.6	2.86E ⁻⁰²	3.0	—	—	—	—	—	—
6.61	C ₄₈ H ₇₈ O ₁₉	Soyasaponin Ba (V)	Triterpenoid saponins	1.4	2.14E ⁻⁰²	1.2	—	—	—	3.4	3.67E ⁻⁰²	1.1
8.70	C ₄₈ H ₈₆ O ₁₈ P ₂	Phosphoinositide phosphate	Glycerophosphoinositol phosphates	—	—	—	2.4	2.92E ⁻⁰²	1.4	2.5	2.33E ⁻⁰²	1.3
9.57	C ₄₈ H ₇₄ O ₁₇	Soyasaponin γ g	Triterpenoid saponins	—	—	—	—	—	—	3.9	3.00E ⁻⁰²	1.5
9.90	C ₄₅ H ₇₄ O ₁₁	Monogalactosyldiacylglycerol (18:3)	Monogalactosyldiacylglycerols	2.8	2.50E ⁻⁰²	2.1	8.7	1.67E ⁻⁰²	4.1	6.1	6.67E ⁻⁰³	3.7
10.15	C ₃₁ H ₅₈ O ₁₄	Lyso-monogalactosyldiacylglycerols (16:0/0:0)	Lyso-monogalactosyldiacylglycerols	—	—	—	2.2	2.08E ⁻⁰²	1.9	3.3	1.00E ⁻⁰²	2.7
11.74	C ₂₂ H ₄₃ O ₉ P	Phosphatidylglycerol (16:1(9Z)/0:0)	Phosphatidylglycerols	1.9	3.21E ⁻⁰²	1.8	—	—	—	2.2	2.00E ⁻⁰²	2.4
11.90	C ₄₁ H ₆₆ O ₁₃	Soyasaponin Bc' (IV)	Triterpenoid saponins	2.1	3.57E ⁻⁰²	1.2	2.0	3.75E ⁻⁰²	1.5	3.4	1.33E ⁻⁰²	1.8
13.64	C ₄₆ H ₇₅ O ₁₀ P	Phosphatidylglycerol (18:2/22:6)	Phosphatidylglycerols	—	—	—	—	—	—	2.9	2.67E ⁻⁰²	2.0

Note: Metabolites were annotated by level 2.1 of the metabolomics standards initiative; t_R – retention time; KVL – very low K⁺ availability; KVH – very high K⁺ availability; LT – lower third; MT – medium third; UT– upper third; “ * ” - identified using authentic standards; “ – ” - not significant; O2PLS-DA and S-Plot models were performed using p -value ≤ 0.05 and false discovery rate (FDR) with q -value ≤ 0.05 (Appendix G); Importance variable in score projection (VIP ≥ 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVH vs. KVL treatments (metabolite content is higher in KVH than KVL treatment); MS¹ and MS² data, chemical ontology and KEGG Pathway are available in Appendix G.

Source: The Autor (2022).

Table 5. Putative metabolite identification from soybean pod tissues upregulated under very high potassium (KVH) availability by UPLC-QToF-MS^E

t_R (min)	Formula	Identification	Chemical Ontology	FC	FDR	VIP
Immature Seeds						
6.33	C ₁₈ H ₃₂ O ₅	Trihydroxy-octadecadienoic acid	Fatty acids	2.7	4.58E ⁻⁰²	1.7
8.61	C ₄₂ H ₆₈ O ₁₄	Soyasaponin Bb' (III)	Triterpenoid saponins	1.8	8.33E ⁻⁰³	1.9
Pod Valves						
0.70	C ₆ H ₈ O ₇	Citric acid	Tricarboxylic acids	1.6	2.00E ⁻⁰²	1.1
0.70	C ₄ H ₄ N ₂ O ₂	Uracil	Pyrimidones	1.5	4.00E ⁻⁰²	2.2
2.79	C ₁₈ H ₂₈ O ₉	<i>β</i> -D-glucopyranosyl-11-hydroxyjasmonic acid	Fatty acyl glycosides	1.7	3.33E ⁻⁰³	1.3
3.86	C ₂₇ H ₃₀ O ₁₆	Kampferol-3- <i>O</i> -digalactopyranoside	Flavonols	1.5	4.33E ⁻⁰²	1.8
5.00	C ₂₄ H ₂₂ O ₁₃	Malonyl-genistin*	Isoflavone	1.5	4.67E ⁻⁰²	2.3

Note: Metabolites were annotated by level 2.1 of the metabolomics standards initiative; t_R – retention time; KVL – very low K⁺ availability; KVH – very high K⁺ availability; “*” – identified using authentic standards; O2PLS-DA and S-Plot models were performed using *p*-value ≤ 0.05 and false discovery rate (FDR) with *q*-value ≤ 0.05 (Appendix G); Importance variable in score projection (VIP ≥ 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVH vs. KVL treatments (metabolite content is higher in KVH than KVL treatment); MS¹ and MS² data, chemical ontology and KEGG Pathway are available in Appendix G.

Source: The Autor (2022).

3.8 SOYBEAN LEAVES UNDER K⁺ DEFICIENCY

The metabolomic profile of leaf samples from plants grown in KVL showed differential regulation at 21 metabolites, compared to KVH (Table 6). Under K⁺ deficiency, soybean leaves revealed upregulation of amino acids, benzoic acid derivatives, pyrimidines, tricarboxylic acids, isoflavones, coumestans, pterocarpans, phosphatidylglycerols, and triterpenoid saponins content. The phytoalexins (isoflavonoids and soyasaponins) were the major class of specialised metabolites elicited in K⁺ deficient soybean leaves (Table 6 and Figure 4).

The amino acids mostly accumulated in UT trifoliolate leaves of plants in KVL were allantoic acid (3.3-fold) and L-asparagine (16.6-fold) compared to KVH treatment (Table 6). L-asparagine was also a differentially regulated metabolite in PV (2.4-fold) and IS (1.6-fold) in KVL treatment (Table 6 and 7). Both metabolites are participants in soybean nitrogen metabolism (alanine, aspartate, glutamate, and purine metabolism) by KEGG Metabolic Pathway (Appendix G). Previously, L-asparagine was differentially regulated in Fabaceae plants under potassium deficiency (STEWART; LARHER, 1980). L-asparaginases (EC 3.5.1.1) have been associated with K⁺ dependence for catalytic activity, promoting L-asparagine hydrolysis in L-aspartate and ammonium (BRUNEAU *et al.*, 2006; LEA *et al.*, 2007). However, changes in the content of L-asparagine can favour pest infestation, as *Aphis glycines* in soybeans (SEVERTSON *et al.*, 2016; WALTER; DIFONZO, 2007) and *Myzus persicae* in *Brassica napus* (SEVERTSON *et al.*, 2016). Therefore, based on previous studies (AMTMANN *et al.*, 2008; WALTER; DIFONZO, 2007) and our results, we consider that L-asparagine and other amino acids changes can affect soybean pest infestation and such susceptibility are important issues for future research.

Four soyasaponins were upregulated in leaves from plants under very low potassium availability (KVL), as malonyl-dHex-Hex-HexA-soyasapogenol B (≤ 2.6 -fold), soyasaponin Be (≤ 2.6 -fold), soyasaponin β a (≤ 4.9 -fold), and soyasaponin β g (≤ 4.7 -fold). However, the regulation of soyasaponin Be and malonyl-dHex-Hex-HexA-soyasapogenol was restricted to MT and UT leaves, being undetectable in the LT (Table 6). Interestingly, the soyasaponins elicited in plants under deficiency were heterosides containing soyasapogenol B (aglycone) plus three moiety units conjugated to position C-3. Soyasaponin β a and soyasaponin β g have a DDMP moiety and pisumsaponin I, a malonyl group, both in the C-22 hydroxyl position. DDMP-conjugated saponins showed pro-oxidative and superoxide radical (O₂⁻) scavenging activity, and this property was attributed to the DDMP moiety (OKUBO; YOSHIKI, 2000; YOSHIKI; OKUBO, 1995). DDMP saponins accumulation in K⁺ deficient plants can be

associated with oxygen radical controllers in soybean leaves under oxidative stress (YOSHIKI *et al.*, 1998).

Isoflavonoids (isoflavones, coumestans, and pterocarpanes) were predominantly upregulated compounds in soybean plants under KVL treatment (Table 6). The isoflavonoids have been frequently detected in soybean leaves (HOFFMANN-CAMPO *et al.*, 2001; PIUBELLI *et al.*, 2005) and pods (DA GRAÇA *et al.*, 2016; PIUBELLI *et al.*, 2005) under biotic interactions. This class of flavonoid can be observed, as constitutive (phytoanticipins) or induced (phytoalexins) metabolites (GRAHAM *et al.*, 1990; SIMONS *et al.*, 2011; TIKU, 2020). Our results indicated upregulation of isoflavones genistein (1.3-fold), malonyl-genistin (≤ 2.9 -fold), acetyl-genistein (≤ 2.5 -fold), and daidzein (≤ 16.1) in leaves of plants under K⁺ soil deficiency (Table 6).

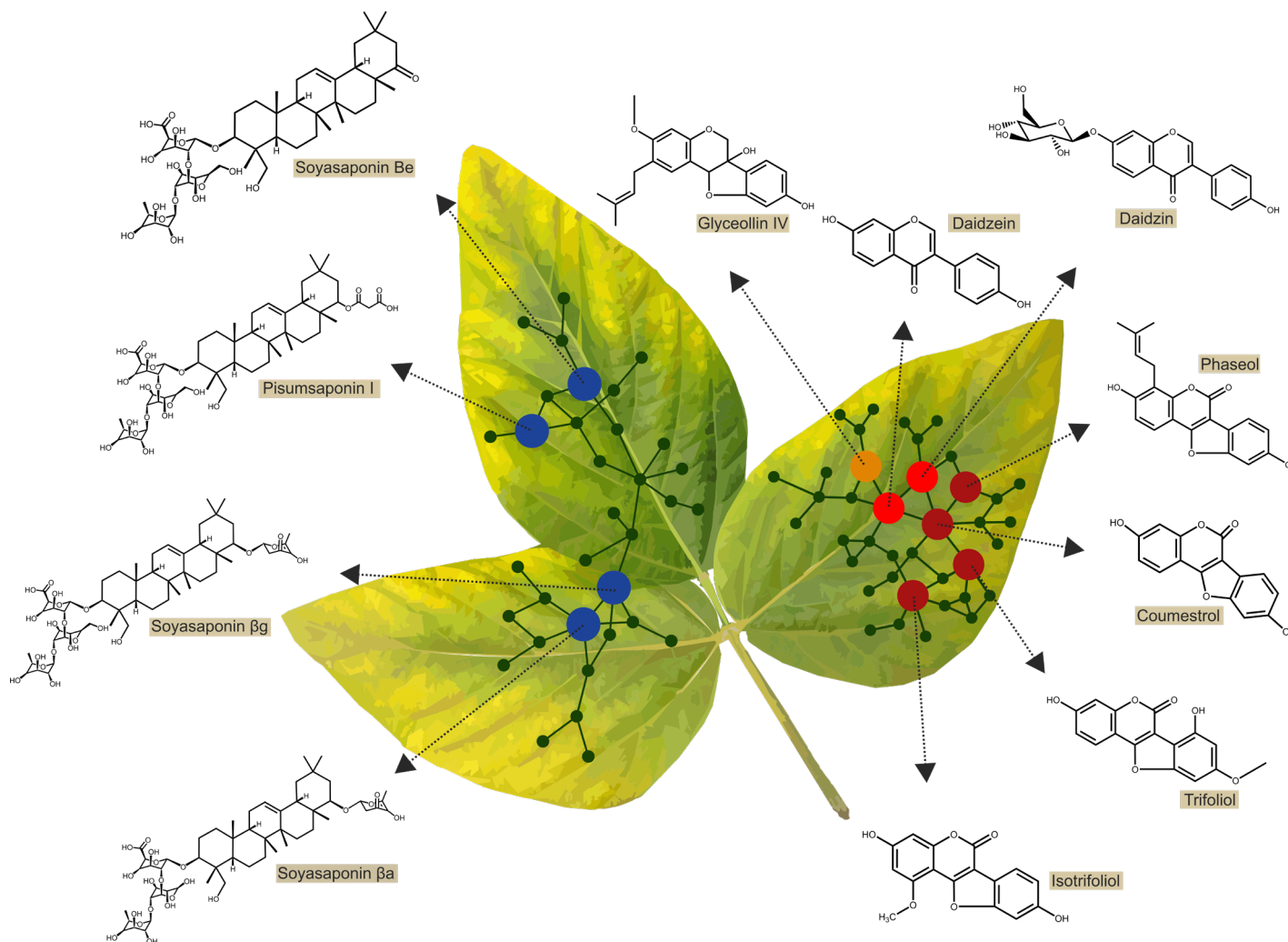
Chemometrics models showed that glycosylated isoflavones were predominantly accumulated in leaf extracts from medium (MT) and upper third (UT) of soybean plants. The biosynthesis of aglycone daidzein in LT leaves was 2.7-fold upregulated in KVL compared with KVH. Interestingly, the differential regulation of this compound was 16.1-fold greater in UT, and no changes were detected in MT leaves. These experimental results suggest that the LT leaves primarily had to cope with potassium deficiency at V7 stage, when soybean samples were collected. Potassium is a highly mobile nutrient in the plant, its redistribution was therefore expected and revealed by the ionic analysis. In addition, although different tissues had different metabolites depending on the tissue maturity, they are part of the same organism that has complex and induced responses. The high daidzein upregulation in upper third (UT) leaves can be associated with induced mechanisms. This condition suggests that UT leaves were preparing to deal with the stress caused by K⁺ lacking, a situation that already occurred in the older leaves. Our results demonstrate that K⁺ deficiency accumulated not only the aglycones but also glycosidic isoflavones, corroborating previous investigations after stress by herbicide acifluorfen (COSIO *et al.*, 1985), ethylene application (BAN *et al.*, 2020), *Nezara viridula* (PIUBELLI *et al.*, 2003), and *Spodoptera litura* herbivory (MURAKAMI *et al.*, 2014).

Table 6 - Putative metabolite identification from soybean leaves upregulated under very low potassium (KVL) availability by UPLC-QToF-MS^E

tr (min)	Formula	Identification	Chemical Ontology	Lower Third (LT)			Medium Third (MT)			Upper Third (UT)		
				KVH-LT vs. KVL-LT			KVH-MT vs. KVL-MT			KVH-UT vs. KVL-UT		
				FC	FDR	VIP	FC	FDR	VIP	FC	FDR	VIP
0.64	C ₄ H ₈ N ₂ O ₃	L-asparagine	Amino acids	—	—	—	8.0	2.31E ⁻⁰²	1.0	16.6	3.57E ⁻⁰³	1.8
0.68	C ₄ H ₈ N ₄ O ₄	Allantoic acid	Amino acids	—	—	—	—	—	—	3.3	2.86E ⁻⁰²	1.3
0.71	C ₄ H ₄ N ₂ O ₂	Uracil	Pyrimidones	3.1	3.33E ⁻⁰³	1.7	2.1	3.85E ⁻⁰³	1.7	2.4	1.07E ⁻⁰²	1.6
0.72	C ₆ H ₈ O ₇	Citric acid	Tricarboxylic acids	2.4	6.67E ⁻⁰³	2.6	1.7	7.69E ⁻⁰³	2.6	2.0	7.14E ⁻⁰³	2.7
1.60	C ₁₃ H ₁₆ O ₉	Gentisic acid 2-β-D-glucoside	Benzoic acids	1.3	4.67E ⁻⁰²	1.0	2.6	1.15E ⁻⁰²	2.6	3.0	1.79E ⁻⁰²	2.3
4.60	C ₂₁ H ₂₀ O ₁₀	Genistin*	Isoflavones	—	—	—	1.2	5.00E ⁻⁰²	1.9	1.5	1.43E ⁻⁰²	3.7
5.03	C ₂₄ H ₂₂ O ₁₃	Malonyl-genistin*	Isoflavones	—	—	—	1.6	3.85E ⁻⁰²	2.6	2.9	3.21E ⁻⁰²	3.1
5.14	C ₂₃ H ₂₂ O ₁₁	Acetyl-genistin*	Isoflavones	—	—	—	1.6	3.08E ⁻⁰²	1.3	2.5	3.57E ⁻⁰²	1.2
5.47	C ₁₅ H ₁₀ O ₄	Daidzein*	Isoflavones	2.7	5.00E ⁻⁰²	1.0	—	—	—	16.1	4.64E ⁻⁰²	1.1
6.49	C ₁₅ H ₁₀ O ₅	Genistein*	Isoflavones	—	—	—	—	—	—	1.3	5.00E ⁻⁰²	2.4
6.51	C ₁₆ H ₁₀ O ₆	Isotrifoliol	Coumestans	2.1	4.00E ⁻⁰²	1.4	—	—	—	ND	ND	ND
6.61	C ₁₅ H ₈ O ₅	Coumestrol	Coumestans	2.4	3.67E ⁻⁰²	4.5	—	—	—	—	—	—
7.04	C ₁₆ H ₁₀ O ₆	Trifoliol	Coumestans	3.5	3.33E ⁻⁰²	2.3	—	—	—	ND	—	ND
8.15	C ₂₀ H ₁₆ O ₆	Unknown	Unknown	9.2	2.00E ⁻⁰²	1.5	—	—	—	ND	—	ND
8.40	C ₅₁ H ₈₀ O ₂₁	Malonyl-dHex-Hex-HexA-soyasapogenol B (Pisumsaponin I)	Triterpenoid saponins	—	—	—	1.8	2.69E ⁻⁰²	1.1	2.6	2.50E ⁻⁰²	1.5
8.50	C ₄₈ H ₇₆ O ₁₈	Soyasaponin Be (Dehydrosoyasaponin I)	Triterpenoid saponins	—	—	—	2.1	1.54E ⁻⁰²	1.0	2.6	2.14E ⁻⁰²	1.3
8.52	C ₂₁ H ₂₂ O ₅	Glyceollin IV	Pterocarpan	7.0	2.67E ⁻⁰²	1.3	—	—	—	—	—	—
8.95	C ₂₀ H ₁₆ O ₅	Phaseol (4-prenyl-coumestrol)	Coumestans	3.4	2.33E ⁻⁰²	5.2	—	—	—	—	—	—
8.98	C ₅₃ H ₈₂ O ₂₀	Soyasaponin βa	Triterpenoid saponins	4.9	1.33E ⁻⁰²	2.2	2.9	3.46E ⁻⁰²	2.4	2.3	3.93E ⁻⁰²	2.1
9.11	C ₅₄ H ₈₄ O ₂₁	Soyasaponin βg	Triterpenoid saponins	4.7	1.00E ⁻⁰²	2.1	3.0	1.92E ⁻⁰²	2.3	2.0	4.29E ⁻⁰²	1.8
12.47	C ₂₂ H ₄₅ O ₉ P	Phosphatidylglycerol (16:0/0:0)	Phosphatidylglycerols	4.8	1.67E ⁻⁰²	1.2	—	—	—	—	—	—

Note: Metabolites were annotated by level 2.1 of the metabolomics standards initiative; tr – retention time; KVL – very low K⁺ availability; KVH – very high K⁺ availability; LT – lower third; MT – medium third; UT – upper third; “*” – identified using authentic standards; “–” – not significant; “ND” – not detected; O2PLS-DA and S-Plot models were performed using p -value ≤ 0.05 and false discovery rate (FDR) with q -value ≤ 0.05 (Appendix G); Importance variable in score projection (VIP ≥ 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVL vs. KVH treatments (metabolite content is higher in KVL than KVH treatment); MS¹ and MS² data, chemical ontology and KEGG Pathway are available in Appendix G. **Source:** The Autor (2022).

Figure 4 - Isoflavonoids (isoflavones, coumestans, and pterocarpan) and triterpenoid saponins (soyasaponins) as phytoalexins upregulated in soybean leaves under very low potassium availability



Source: The Autor (2022).

Leaves under very low K^+ availability (KVL) showed a higher daidzein content that was exclusively differently regulated under stress (KVL vs. KVH). This aglycone is a direct precursor of soybean phytoalexin biosynthesis, as coumestans and pterocarpan (GRAHAM *et al.*, 1990; KUC, 1995). Chemometrics models revealed that leaves under nutritional stress upregulated biosynthesis of coumestans, as coumestrol (2.4-fold), isotrifoliol (2.1-fold), trifoliol (3.5-fold), phaseol (3.4-fold), as well as, gliceollin IV (7.0-fold) a pterocarpan (Table 6). The upregulation of these phytoalexins is likely associated with metabolism disorders and plant oxidative stress status (AHMAD *et al.*, 2014; HERNANDEZ *et al.*, 2012). Our results suggest that this also occurs in soybean leaves under severe K^+ deficiency. Phytoalexins can be autotoxic to cells, therefore upregulation of biosynthesis is generally associated with defence mechanisms. Previously, coumestans elicitation occur when *Phaseolus vulgaris* leaves were injured by sulphur dioxide, ozone, and herbicides (RUBIN *et al.*, 1983). Ozone fumigation, a highly reactive and cytotoxic agent, increased daidzein and coumestan contents in soybean (KEEN; TAYLOR, 1975). Phaseol and other prenylated isoflavones were also detected in soybean cotyledons after lactofen (diphenyl ether) treatment (CHENG *et al.*, 2011).

Previously published investigations were consistent in associating that potassium deficiency leads to status for the uncontrolled generation of reactive oxygen species (ROS) in plants (AHMAD *et al.*, 2014; CAKMAK, 2005; HERNANDEZ *et al.*, 2012; MIAO *et al.*, 2010). ROS are mainly produced in chloroplasts and peroxisomes (ASADA, 2000; POSPÍŠIL, 2016) when plants are exposed to nutrient deficiency, salinity, and water deficit (CAKMAK, 1994; FOYER *et al.*, 1994). Interestingly, ROS generation is enhanced when plants are under abiotic stress and also exposed to light, causing photo-oxidative damage to chloroplasts (CAKMAK; MARSCHNER, 1992; ISSAWI *et al.*, 2018; MARSCHNER; CAKMAK, 1989), and photodynamic stress status (ISSAWI *et al.*, 2018). Under low K^+ availability and sufficient contents of other nutrients, plants usually showed chlorotic and necrotic tissues when exposed to light (CAKMAK, 2005; MARSCHNER *et al.*, 1996), however, these symptoms were absent when plants were maintained under shading (MARSCHNER; CAKMAK, 1989). Phosphorus and iron deficiency typically cause chlorotic symptoms (MARSCHNER, 2012). Nevertheless, in our experiment, independently of K^+ availability, phosphorus content remained unaltered, and iron content increased in leaves severely K^+ deficient, supporting our findings that leaf symptoms were only associated with K^+ deficiency.

The metabolomic profile of soybean plants under potassium deficiency showed differential regulation of DDMP saponins that are oxygen radical controllers in *G. max* (YOSHIKI *et al.*, 1998). Isoflavones and pterocarpan (e.g., daidzein, isotrifoliol, and phaseol)

are excellent free radical inhibitors and lipid oxidation reducers (BOUÉ *et al.*, 2008; LEE *et al.*, 2005; LI *et al.*, 2017; TODA; SHIRATAKI, 1999). Based on previous works and our results, we suggest that soybean metabolism was reprogrammed for the biosynthesis of non-constitutive specialised metabolites (phytoalexins) for handling oxidative damage caused by potassium starvation.

3.9 SOYBEAN POD TISSUES UNDER K⁺ DEFICIENCY

Eighteen metabolites were differentially regulated in K⁺ deficient soybean pod tissues (Table 7). Unlike in leaves, amino acids were the main class of metabolites differentially regulated in the pod tissues. L-glutamine and L-asparagine were upregulated (≤ 2.4 -fold) in immature seeds (IS) and pod valves (PV), and the amino acids belonging to the nitrogen metabolism pathway, as ureidoglycine (1.9-fold in PV) and allantoic acid (1.6-fold in PV) were not differentially regulated in IS.

Metabolomic profile analyses indicated positive regulation of the isoflavones daidzein (1.5-fold) and genistein (17.8-fold) in IS, and daidzin (2.3-fold) in PV tissues when plants grew under K⁺ deficiency (Table 7). In our analysis, genistein is highly regulated in IS; the conjugated metabolite genistein (genistin) was also induced by the stink bug, *Nezara viridula*, whose damage (seed punctures) are directly imposed in seeds (PIUBELLI *et al.*, 2003), but the concentration increases 3.1-fold. Additionally, higher regulation of genistein was detected when soybean plants were under abiotic stress, such as salt stress, flooding, and shading (LIU *et al.*, 2017; VANTOAI *et al.*, 2012; WU *et al.*, 2008).

Low K⁺ availability also showed upregulation of protocatechuic acid-4-*O*- β -glucoside (1.4-fold) and gentisic acid-5-*O*- β -glucoside (2.1-fold) in PV, and L-histidine (1.8-fold) only in IS (Table 7). Protocatechuic and gentisic acids (benzoic acid derivatives) are effective antioxidants and inhibitors of lipid peroxidation (MORAN *et al.*, 1997; RICE-EVANS *et al.*, 1997), whereas L-histidine is associated with energetic storage compounds, control of hydroxyl radicals (ZS-NAGY; FLOYD, 1984) and singlet oxygen (¹O₂) (WADE; TUCKER, 1998). Our results demonstrate that K⁺ status promoted upregulation of many compounds in soybean pods and leaves (reproductive and vegetative structures), many of them are considered antioxidants; therefore, the biosynthesis of these compounds might have similar purposes, as a mechanism to control oxidative stress status in soybean plants under very low K⁺ availability.

Table 7. Putative metabolite identification from soybean pod tissues upregulated under very low potassium (KVH) availability by UPLC-QToF-MS^E

t_R (min)	Formula	Identification	Chemical Ontology	FC	FDR	VIP
Immature Seeds						
0.62	C ₆ H ₉ N ₃ O ₂	L-histidine	Amino acids	1.8	2.50E ⁻⁰²	2.4
0.64	C ₅ H ₁₀ N ₂ O ₃	L-glutamine	Amino acids	2.4	3.33E ⁻⁰²	1.4
0.65	C ₄ H ₈ N ₂ O ₃	L-asparagine	Amino acids	1.6	4.17E ⁻⁰²	1.8
0.66	C ₁₈ H ₃₂ O ₁₅	Oligosaccharide unknown	Oligosaccharides	2.8	2.92E ⁻⁰²	2.3
0.66	C ₁₇ H ₃₀ O ₁₅	Oligosaccharide unknown	Oligosaccharides	2.1	3.75E ⁻⁰²	1.8
0.67	C ₆ H ₁₂ O ₇	Galactonic acid	Hydroxy acids	3.9	1.25E ⁻⁰²	1.8
0.67	C ₁₉ H ₃₄ O ₁₆	Ciceritol	Oligosaccharides	1.7	5.00E ⁻⁰²	1.3
0.69	C ₁₉ H ₂₀ O ₁₂	3,5-dihydroxyphenyl 1- <i>O</i> -(6- <i>O</i> -galloyl- β - <i>D</i> -glucopyranoside)	Oligosaccharides	1.6	1.67E ⁻⁰²	4.0
5.41	C ₁₅ H ₁₀ O ₄	Daidzein*	Isoflavones	1.5	2.08E ⁻⁰²	2.4
6.50	C ₁₅ H ₁₀ O ₅	Genistein*	Isoflavones	17.8	4.17E ⁻⁰³	2.8
Pod Valves						
0.64	C ₅ H ₁₀ N ₂ O ₃	L-glutamine	Amino acids	2.1	2.67E ⁻⁰²	1.1
0.65	C ₄ H ₈ N ₂ O ₃	L-asparagine	Amino acids	2.4	1.33E ⁻⁰²	2.1
0.66	C ₁₁ H ₂₀ O ₁₀	3- <i>O</i> - β - <i>D</i> -galactopyranosyl-L-arabinose	Monosaccharides	2.9	1.67E ⁻⁰²	1.3
0.68	C ₃ H ₇ N ₃ O ₃	Ureidoglycine	Amino acids	1.9	3.67E ⁻⁰²	2.0
0.69	C ₄ H ₈ N ₄ O ₄	Allantoic acid	Amino acids	1.6	2.33E ⁻⁰²	2.0
0.73	C ₄ H ₆ O ₅	Malic acid	Beta hydroxy acids	2.2	5.00E ⁻⁰²	1.2
0.74	C ₁₃ H ₁₆ O ₉	Gentisic acid 2- β - <i>D</i> -glucoside	Benzoic acids	2.1	6.67E ⁻⁰³	1.4
1.62	C ₁₃ H ₁₆ O ₉	Protocatechuic acid 4- <i>O</i> - β -glucoside	Benzoic acids	1.4	3.00E ⁻⁰²	1.5
2.34	C ₂₁ H ₃₂ O ₁₀	Dihydrophaseic acid 4- <i>O</i> - β - <i>D</i> -glucoside	Sesquiterpenoids	1.6	1.00E ⁻⁰²	2.3
3.64	C ₂₁ H ₂₀ O ₉	Daidzin*	Isoflavones	2.3	3.33E ⁻⁰²	1.3

Note: Metabolites were annotated by level 2.1 of the metabolomics standards initiative; t_R – retention time; KVL – very low K⁺ availability; KVH – very high K⁺ availability; “*” – identified using authentic standards; O2PLS-DA and S-Plot models were performed using p -value ≤ 0.05 and false discovery rate (FDR) with q -value ≤ 0.05 (Appendix G); Importance variable in score projection (VIP ≥ 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVL vs. KVH treatments (metabolite content is higher in KVL than KVH treatment); MS¹ and MS² data, chemical ontology and KEGG Pathway are available in Appendix G. **Source:** The Autor (2022).

4 CONCLUDING REMARKS

Untargeted metabolomics reveals 51 primary and specialised metabolites that act in 19 biochemical pathways that were influenced by K^+ availability. Potassium is not a structural component of plant metabolites in comparison with phosphorus and nitrogen, but under very high K^+ availability, the upregulation of flavonoids, galactolipids, and carbohydrates may be resulting from enzymatic activity increasing. In contrast, the biochemical mechanisms of soybeans in response to very low K^+ availability are associated to: (I) accumulation of Ca^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} , and B in young and old leaves; (II) increase of Fe^{2+} and Cu^{2+} content in immature seeds and pod valves, respectively; (III) leaf metabolism reprogramming for the biosynthesis of phytoalexins, such as isoflavones, coumestans, pterocarpanes, and soyasaponins; (IV) enhancement of the amino acids, mono and oligosaccharides, benzoic acid derivatives, and isoflavones content in soybean pods (IS and PV); (V) L-asparagine accumulation in vegetative (leaves) and reproductive (pod tissues) structures.

Overall, the results suggest that soybean metabolism reprogramming is associated with oxidative stress caused by low K^+ availability, and shows an increased content of phytoalexins (antioxidant agents), as isoflavonoids and triterpenoid saponins. Additionally, results suggest that L-asparagine is a promising soybean biomarker under K^+ deficiency.

Although the literature shows that plants fertilized with K^+ are more tolerant/resistant to biotic and abiotic stresses, our data reveals that increasing fertilization rates did not promote a substantial increase in the content of constitutive specialised metabolites in soybean, refuting our initial hypothesis. We understood that this interaction is more complex and thus further work using integrated omics is certainly required, to understand whether the profile of metabolites induced by biotic elicitors (e.g., insects or pathogens) may be associated to K^+ content in plants.

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APPENDIX A – Soil Chemical Attributes

Soil chemical attributes after potassium application at 0 – 0.2 m soil layer in October 2019

Treatments	pH (0.01 M CaCl ₂)	H ⁺	Al ³⁺	Ca ²⁺	Mg ²⁺	K ⁺	CEC _{pH7.0}	V	P	OC	K ⁺ Sat
		(cmol _c dm ⁻³)						%	(mg dm ⁻³)	(g dm ³)	%
KVL	4.42	4.55	0.16	2.21	1.60	0.05	8.57	45	22.1	14.8	0.58
KL	4.35	4.86	0.22	2.08	1.51	0.09	8.76	42	24.4	14.2	1.02
KM	4.39	4.77	0.13	2.10	1.47	0.13	8.60	43	21.8	15.4	1.51
KVH	4.55	4.27	0.12	2.06	1.34	0.50	8.29	47	20.7	16.0	6.03

Note: CEC – cation exchange capacity at pH 7.0; V% – base saturation; OC – Organic carbon; K⁺ Sat – saturation (%) of K⁺ in CEC_{pH7.0} [(K⁺/CEC_{pH7.0})*100]; KVL – without K⁺ fertilization; KL – 40 kg K₂O ha⁻¹; KM – 80 kg K₂O ha⁻¹; KVH – 160 kg K₂O ha⁻¹; Ca²⁺, Mg²⁺, and Al³⁺ (KCl 1 mol L⁻¹); P and K⁺ (Mehlich-1).

Source: The Autor (2022).

APPENDIX B – Leaves Tukey's t-value

Tukey simultaneous tests for mean differences of ionic analysis of soybean leaf growth region in response to K⁺ soil fertilization. The means of samples are available in Table 1

Treatments			P	K ⁺	Ca ²⁺	Mg ²⁺	S	Zn ²⁺	Mn ²⁺	Fe ²⁺	Cu ²⁺	B
			<i>p</i> -value									
LT	KM	x KL	-1.64 ^{NS}	1.96 ^{NS}	-4.71 ^{***}	-5.11 ^{***}	-1.79 ^{NS}	0.46 ^{NS}	-6.80 ^{***}	-8.98 ^{***}	-1.55 ^{NS}	-0.31 ^{NS}
	KVH	x KL	-1.22 ^{NS}	11.91 ^{***}	-6.42 ^{**}	-7.95 ^{***}	-2.15 ^{NS}	-0.27 ^{NS}	-8.21 ^{***}	-11.71 ^{***}	0.05 ^{NS}	-2.06 ^{NS}
	KVL	x KL	0.79 ^{NS}	-11.28 ^{***}	2.58 ^{NS}	17.64 ^{***}	-1.49 ^{NS}	-4.58 ^{***}	-1.48 ^{NS}	3.78 [*]	3.65 ^{***}	12.63 ^{***}
	KVH	x KM	0.41 ^{NS}	7.94 ^{***}	-1.71 ^{NS}	-2.84 ^{NS}	-0.37 ^{NS}	-0.73 ^{NS}	-1.41 ^{NS}	0.27 ^{NS}	1.60 ^{NS}	-1.75 ^{NS}
	KVL	x KM	2.43 ^{NS}	-15.25 [*]	7.30 ^{***}	22.74 ^{***}	0.29 ^{NS}	-5.04 ^{***}	5.33 ^{***}	6.46 ^{***}	5.20 ^{***}	12.94 ^{***}
	KVL	x KVH	2.01 ^{NS}	-23.19 ^{***}	9.00 ^{***}	25.58 ^{***}	0.66 ^{NS}	-4.31 ^{***}	6.74 ^{***}	6.98 ^{**}	3.59 ^{***}	14.69 ^{***}
MT	KM	x KL	-0.83 ^{NS}	1.08 ^{NS}	-4.40 ^{***}	-3.65 [*]	-0.80 ^{NS}	1.66 ^{NS}	-1.43 ^{NS}	-3.15 [*]	-0.41 ^{NS}	-3.24 [*]
	KVH	x KL	-1.67 ^{NS}	11.85 ^{***}	-5.19 ^{***}	-5.34 ^{***}	-0.67 ^{NS}	2.11 ^{NS}	0.74 ^{NS}	-2.90 [*]	-0.29 ^{NS}	-2.83 [*]
	KVL	x KL	-0.19 ^{NS}	-14.49 ^{***}	4.26 ^{***}	11.25 ^{***}	0.41 ^{NS}	-7.21 ^{***}	-0.91 ^{NS}	3.52 [*]	1.06 ^{NS}	9.18 ^{***}
	KVH	x KM	-0.84 ^{NS}	1.77 ^{NS}	-0.79 ^{NS}	-1.69 ^{NS}	0.13 ^{NS}	0.45 ^{NS}	2.17 ^{NS}	-0.46 ^{NS}	0.71 ^{NS}	0.41 ^{NS}
	KVL	x KM	0.65 ^{NS}	-18.57 ^{***}	8.66 ^{***}	14.90 ^{***}	1.21 ^{NS}	-8.87 ^{***}	0.52 ^{NS}	5.66 ^{***}	1.83 ^{NS}	12.42 ^{***}
	KVL	x KVH	1.48 ^{NS}	-26.34 ^{***}	9.45 ^{***}	16.59 ^{***}	1.09 ^{NS}	-9.32 ^{***}	-1.65 ^{NS}	6.12 ^{***}	1.71 ^{NS}	12.01 ^{***}
UT	KM	x KL	-1.29 ^{NS}	9.57 ^{***}	-4.14 [*]	-1.76 ^{NS}	-0.66 ^{NS}	0.38 ^{NS}	-0.82 ^{NS}	-3.78 [*]	-1.62 ^{NS}	-2.03 ^{NS}
	KVH	x KL	-0.53 ^{NS}	16.14 ^{***}	-3.56 [*]	-1.83 ^{NS}	-0.05 ^{NS}	-0.08 ^{NS}	-1.96 ^{NS}	-3.68 [*]	-1.24 ^{NS}	-1.20 ^{NS}
	KVL	x KL	-0.34 ^{NS}	-13.16 ^{***}	10.59 ^{***}	15.92 ^{**}	-0.99 ^{NS}	-7.00 ^{***}	1.90 ^{NS}	4.05 ^{**}	4.04 [*]	11.32 ^{***}
	KVH	x KM	0.76 ^{NS}	6.57 ^{***}	-0.11 ^{NS}	-1.07 ^{NS}	0.61 ^{NS}	-0.45 ^{NS}	0.86 ^{NS}	0.10 ^{NS}	-1.33 ^{NS}	-1.16 ^{NS}
	KVL	x KM	0.94 ^{NS}	-22.73 ^{***}	13.03 ^{***}	17.68 ^{**}	-0.33 ^{NS}	-4.98 [*]	1.73 ^{NS}	7.83 ^{***}	6.66 ^{***}	13.36 ^{***}
	KVL	x KVH	0.19 ^{NS}	-29.30 ^{***}	13.14 ^{***}	18.75 ^{**}	-0.94 ^{NS}	-6.93 ^{***}	1.87 ^{NS}	7.73 ^{***}	8.28 ^{***}	14.52 ^{***}

Note: KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; LT – lower third; MT – medium third; UT– upper third; The data presented indicate the *t*-value, and superscript characters represents significance levels ($p > 0.05$ ^{NS}; $p < 0.05$ ^{*}; $p < 0.010$ ^{**}; $p < 0.001$ ^{***}) by Tukey's test ($n = 6$). **Source:** The Autor (2022).

APPENDIX C – Leaves Growth Region Tukey's t-value

Tukey simultaneous tests for mean differences of ionic analysis. The means of samples are available in Table 1 for a comparison between three soybean leaf growth regions (LT, MT, and UT) in response to K⁺ soil fertilization with data generated by ionic analysis

Treatments				P	K ⁺	Ca ²⁺	Mg ²⁺	S	Zn ²⁺	Mn ²⁺	Fe ²⁺	Cu ²⁺	B
				<i>p</i> -value									
KVL	MT	x	LT	8.00***	0.51 ^{NS}	-2.83*	-3.54**	10.40***	-6.28***	-12.5***	-11.16***	-2.27 ^{NS}	-2.21 ^{NS}
	UT	x	LT	7.24***	7.51***	-5.73***	-6.45***	12.39***	-14.91***	-57.23***	-11.74***	-1.65 ^{NS}	-0.97 ^{NS}
	UT	x	MT	-0.77 ^{NS}	7.00***	-2.90*	-2.91*	2.00 ^{NS}	-8.62***	-44.73***	-0.57 ^{NS}	0.62 ^{NS}	1.24 ^{NS}
KL	MT	x	LT	12.64***	1.68 ^{NS}	-4.00**	-3.60**	5.44***	-3.90**	-16.42***	-23.45***	-1.15 ^{NS}	-0.82 ^{NS}
	UT	x	LT	11.99***	6.16***	-9.74***	-11.31***	7.16***	-13.95***	-79.53***	-24.24***	-2.09 ^{NS}	3.85**
	UT	x	MT	-0.64 ^{NS}	4.48***	-5.69***	-7.71***	1.72 ^{NS}	-10.05***	-63.11***	-0.79 ^{NS}	-0.93 ^{NS}	4.67***
KM	MT	x	LT	5.14***	1.70 ^{NS}	-2.27 ^{NS}	-2.71*	9.53***	-3.04**	-8.01***	-17.32***	-2.08 ^{NS}	-7.62***
	UT	x	LT	4.53***	14.37***	-4.85***	-5.97***	12.56***	-13.72***	-48.19***	-19.41***	-2.18 ^{NS}	3.22**
	UT	x	MT	-0.61 ^{NS}	12.67***	-2.58*	-3.25**	3.03**	-10.68***	-40.11***	-2.09 ^{NS}	-0.09 ^{NS}	10.84***
KVH	MT	x	LT	3.47**	0.26 ^{NS}	-1.61 ^{NS}	-1.32 ^{NS}	4.46***	-2.57*	-6.37***	-7.08**	-3.27**	-1.70 ^{NS}
	UT	x	LT	4.10***	8.92***	-4.55***	-2.56*	6.14***	-17.34***	-62.77***	-7.59***	-5.77***	2.58*
	UT	x	MT	0.63 ^{NS}	8.66***	-2.94*	-1.24 ^{NS}	1.69 ^{NS}	-14.77***	-56.40***	-0.51 ^{NS}	-2.50*	4.28***

Note: KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; LT – lower third; MT – medium third; UT – upper third. The data presented indicate the *t*-value, and superscript characters represents significance levels ($p > 0.05$ ^{NS}; $p < 0.05$ *; $p < 0.010$ **; $p < 0.001$ ***) by Tukey's test ($n = 6$).

Source: The Autor (2022).

APPENDIX D – Pod tissues Tukey's t-value

Tukey simultaneous tests for mean differences of ionomic analysis. The means of samples are available in Table 2 and represent the data of soybean pod tissues in response to K⁺ soil fertilization

Treatments				P	K ⁺	Ca ²⁺	Mg ²⁺	S	Zn ²⁺	Mn ²⁺	Fe ²⁺	Cu ²⁺	B
				<i>p</i> -value									
PV	KM	x	KL	0.52 ^{NS}	11.03 ^{***}	0.58 ^{NS}	-0.93 ^{NS}	0.83 ^{NS}	5.45 ^{***}	4.80 ^{***}	-0.43 ^{NS}	6.41 ^{**}	2.02 ^{NS}
	KVH	x	KL	-0.15 ^{NS}	15.19 ^{***}	1.44 ^{NS}	-1.09 ^{NS}	-0.96 ^{NS}	7.33 ^{**}	4.29 ^{**}	-0.10 ^{NS}	-3.17 [*]	1.00 ^{NS}
	KVL	x	KL	0.98 ^{NS}	-5.38 ^{***}	-0.48 ^{NS}	0.00 ^{NS}	-0.92 ^{NS}	-6.24 ^{***}	-3.61 [*]	-0.35 ^{NS}	-0.28 ^{NS}	-0.87 ^{NS}
	KVH	x	KM	-0.67 ^{NS}	4.15 ^{**}	-1.63 ^{NS}	-0.98 ^{NS}	-1.79 ^{NS}	0.88 ^{NS}	-0.51 ^{NS}	0.33 ^{NS}	-1.41 ^{NS}	-1.02 ^{NS}
	KVL	x	KM	0.46 ^{NS}	-16.41 ^{***}	-1.56 ^{NS}	0.58 ^{NS}	-1.75 ^{NS}	-7.67 ^{**}	-6.12 ^{***}	0.08 ^{NS}	8.48 ^{**}	-0.78 ^{NS}
	KVL	x	KVH	1.13 ^{NS}	-20.57 ^{***}	-1.92 ^{NS}	0.61 ^{NS}	0.04 ^{NS}	-10.13 ^{***}	-5.60 ^{***}	-0.25 ^{NS}	10.69 ^{***}	-1.87 ^{NS}
IS	KM	x	KL	0.65 ^{NS}	5.93 ^{***}	0.94 ^{NS}	1.51 ^{NS}	0.87 ^{NS}	1.68 ^{NS}	4.40 ^{**}	0.12 ^{NS}	0.71 ^{NS}	1.82 ^{NS}
	KVH	x	KL	0.27 ^{NS}	6.37 ^{***}	0.62 ^{NS}	1.52 ^{NS}	0.54 ^{NS}	0.86 ^{NS}	4.05 ^{**}	0.47 ^{NS}	0.15 ^{NS}	2.17 ^{NS}
	KVL	x	KL	1.75 ^{NS}	-1.32 ^{NS}	0.00 ^{NS}	2.27 ^{NS}	0.82 ^{NS}	0.67 ^{NS}	0.12 ^{NS}	-9.97 ^{**}	1.43 ^{NS}	0.24 ^{NS}
	KVH	x	KM	-0.38 ^{NS}	0.44 ^{NS}	-0.31 ^{NS}	0.00 ^{NS}	-1.09 ^{NS}	-0.82 ^{NS}	-0.36 ^{NS}	0.35 ^{NS}	-0.56 ^{NS}	0.35 ^{NS}
	KVL	x	KM	1.10 ^{NS}	-7.25 ^{***}	-0.94 ^{NS}	0.76 ^{NS}	0.93 ^{NS}	-1.01 ^{NS}	-4.28 ^{**}	-7.61 ^{**}	0.72 ^{NS}	-1.58 ^{NS}
	KVL	x	KVH	1.48 ^{NS}	-7.69 ^{***}	-0.62 ^{NS}	0.76 ^{NS}	-1.24 ^{NS}	-0.19 ^{NS}	-3.93 ^{**}	8.86 [*]	1.28 ^{NS}	-1.93 ^{NS}

Note: KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; IS - immature seeds; PV – pod valves. The data presented indicate the *t*-value, and superscript characters represents significance levels ($p > 0.05$ ^{NS}; $p < 0.05$ ^{*}; $p < 0.010$ ^{**}; $p < 0.001$ ^{***}) by Tukey's test ($n = 6$).

Source: The Autor (2022).

APPENDIX E – Pod tissues Studen't t-value

Two-sample t-test comparison of nutrients content between soybean immature seeds and pod valves in response to K⁺ soil fertilization. Data were obtained by ionomic analysis and the means of samples are available in Table 2

Treatments			P	K ⁺	Ca ²⁺	Mg ²⁺	S	Zn ²⁺	Mn ²⁺	Fe ²⁺	Cu ²⁺	B
			<i>p</i> -value									
KVL	IS	x PV	6.90 ^{***}	10.02 ^{***}	-8.81 ^{***}	-14.87 ^{***}	13.92 ^{***}	2.95 [*]	-4.45 ^{**}	-1.58 ^{NS}	7.93 ^{***}	-5.51 ^{***}
KL	IS	x PV	15.88 ^{***}	4.56 ^{***}	-18.19 ^{***}	-22.07 ^{***}	11.52 ^{***}	0.25 ^{NS}	-5.00 ^{***}	-23.69 ^{***}	4.17 ^{**}	-20.35 ^{***}
KM	IS	x PV	0.82 ^{NS}	1.28 ^{NS}	-10.41 ^{***}	-26.22 ^{***}	8.20 ^{***}	0.51 ^{NS}	0.001 ^{**}	-2.30 [*]	5.57 ^{**}	-7.90 ^{***}
KVH	IS	x PV	7.39 ^{***}	-2.48 [*]	-14.80 ^{***}	-15.98 ^{***}	22.83 ^{***}	-1.25 ^{NS}	-6.41 ^{***}	-2.41 ^{NS}	13.63 ^{***}	-6.63 ^{***}

Note: KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; IS - immature seeds; PV – pod valves. The data presented indicate the *t*-value, and superscript characters represents significance levels (*p* > 0.05 ^{NS}; *p* < 0.05 ^{*}; *p* < 0.010 ^{**}; *p* < 0.001 ^{***}) by two-sample t-test (*n* = 6).

Source: The Autor (2022).

APPENDIX F – Multivariate Analysis Values

Multivariate statistical parameter values obtained and used to assure the quality of unsupervised and supervised models generated from leaf and pods tissue sample analysis

Model Type	Treatments	Samples	Components	$R^2X_{(cum)}$	$R^2Y_{(cum)}$	$Q^2Y_{(cum)}$	<i>p</i> -value
PCA-X&Y	Leaves	ALL	22	0.836	--	0.714	--
O2PLS-DA	Leaves	ALL	23	0.831	0.806	0.695	1.010E ⁻³¹
O2PLS-DA (I)	KVL (-1) vs. KVH (1)	LT	3	0.695	0.990	0.983	2.210E ⁻²⁴
O2PLS-DA (II)	KVL (-1) vs. KVH (1)	MT	2	0.354	0.994	0.986	4.392E ⁻²⁸
O2PLS-DA (III)	KVL (-1) vs. KVH (1)	UT	3	0.610	0.993	0.987	8.162E ⁻²⁶
PCA-X&Y	Pod Tissues	ALL	7	0.893	--	0.883	--
O2PLS-DA	Pod Tissues	ALL	10	0.912	0.532	0.438	1.606E ⁻¹¹
O2PLS-DA	KVL (-1) vs. KVH (1)	IS	4	0.874	0.995	0.972	5.537E ⁻⁰⁷
O2PLS-DA	KVL (-1) vs. KVH (1)	PV	2	0.560	0.993	0.983	2.162E ⁻¹¹

Note: $R^2X_{(cum)}$ and $R^2Y_{(cum)}$ represent the cumulative model variation in matrix X and Y, respectively; $Q^2Y_{(cum)}$ – predicted cumulative variation in matrix Y. *p*-value is obtained from cross-variance analysis (CV-ANOVA) on supervised models; KVL – very low K⁺ availability; KL – low K⁺ availability; KM – medium K⁺ availability; KVH – very high K⁺ availability; LT – lower third; MT – medium third; UT – upper third; ALL – total number of samples; IS – immature seeds; PV – pod valves.

Source: The Autor (2022).

APPENDIX G – Putative Metabolite Identification

External control standard parameters in UPLC-ESI-QToF-MS^E analysis

External Control Standard	t _R (min)	m/z [M-H-Rha] ⁻	Mass Error (ppm)	Average Intensity
1	5.64	301.0356	-2,657500063	602
2	5.64	301.0327	6,975937666	608
3	5.64	301.0368	-6,643750158	610
4	5.63	301.0331	5,647187634	613
5	5.63	301.0329	6,31156265	641
6	5.63	301.0333	4,982812618	581
7	5.63	301.0328	6,643750158	615
8	5.63	301.0343	1,66093754	633
9	5.65	301.0351	-0,996562524	572
10	5.64	301.0347	0,332187508	621
11	5.63	301.0334	4,650625111	606
12	5.62	301.0331	5,647187634	695
13	5.63	301.0351	-0,996562524	670
14	5.63	301.0352	-1,328750031	636
15	5.63	301.0341	2,325312555	672
16	5.62	301.0333	4,982812618	652
17	5.62	301.0327	6,975937666	699
18	5.64	301.0351	-0,996562524	633
19	5.63	301.0335	4,318437603	688
20	5.63	301.0332	5,315000126	578
21	5.64	301.0333	4,982812618	613
22	5.64	301.0342	1,993125047	688
23	5.64	301.0329	6,31156265	679
24	5.64	301.0334	4,650625111	663
25	5.64	301.0338	3,321875079	681
26	5.64	301.0336	3,986250095	645
27	5.64	301.0327	6,975937666	673
28	5.64	301.0331	5,647187634	599
29	5.64	301.0328	6,643750158	612
30	5.64	301.0329	6,31156265	581
31	5.63	301.0341	2,325312555	589
32	5.64	301.0335	4,318437603	663
33	5.64	301.0332	5,315000126	620

Source: The Autor (2022).

Putative metabolite identification from soybean leaves upregulated under very high potassium (KVH) availability by UPLC-QTof-MSE

[illegible]

Note: Metabolites were annotated by level 2 of the metabolomics standards initiative; ID - identification corresponding to the chromatogram in Figure 2B; RT - retention time; KVL - very low K⁺ availability; KVLH - very high K⁺ availability; LT - lower titer; MT - medium titer; UT - upper titer; **** - identified using authentic standards; * - -, not significant; O2PLS-DA and S-Plot models were performed using * -value < 0.05 and fold discovery rate (FDR) with * -value < 0.05 (Data S1); Importance variable in score projection (VIP > 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVLH vs. KVL treatments (metabolic content is higher in KVLH than KVL treatment). Source: The Author (2022).

Putative metabolite identification from soybean leaves upregulated under very low potassium (KVL) availability by UPLC-QToF-MS^a

[illegible]

Putative metabolite identification from soybean pod tissues upregulated under very high potassium (KVH) availability by UPLC-QToF-MS^E.

ID	t _R (min)	Theoretical Mass [M-H] ⁻	Measured Mass [M-H] ⁻	Mass error (ppm)	Formula	Identification	Metabolic Pathway	KEGG Pathway Maps	MS ² Fragments	Chemical Ontology	FC	FDR	p-value	VIP	Average Intensity (Normalized)				
Immature Seeds																			
16	6.33	327.22	327.2165	3.6	C ₁₈ H ₃₂ O ₅	Trihydroxy-octadecadienoic acid	α-Linolenic acid metabolism	Lipid Metabolism	229.14; 211.13	Fatty acids	Lipids	2.7	4.58E-02	7.71E-03	1.7	22.076	21.069	29.845	54.049
44	8.61	795.45	795.4537	-0.1	C ₄₂ H ₈₄ O ₁₄	Soyasaponin Bb (III)	Sesquiterpenoid and triterpenoid biosynthesis	Metabolism of specialized metabolites	457.36	Triterpenoid Saponins	Terpenoids	1.8	8.33E-03	6.17E-07	1.9	36.165	46.776	71.010	64.387
Pod Valves																			
5	0.70	191.02	191.0191	3.2	C ₈ H ₁₆ O ₅	Citric acid	Citrate cycle (TCA cycle)	Carbohydrate metabolism	133.01; 111.01	Tricarboxylic acids	Organic Acids	1.6	2.00E-02	1.31E-03	1.1	77.660	85.646	109.146	121.518
4	0.70	111.02	111.0197	2.6	C ₄ H ₈ N ₂ O ₂	Unclel	Pyrimidine metabolism; β-Alanine metabolism	Amino acid metabolism		Pyrimidones	Nucleic acids	1.5	4.00E-02	3.82E-03	2.2	24.847	28.545	34.085	37.506
49	2.79	387.17	387.1648	3.2	C ₁₉ H ₃₄ O ₅	β-D-glucopyranosyl-11-hydroxyjanmonic acid	α-Linolenic acid metabolism	Lipid Metabolism	207.10	Fatty acid glycerides	Lipids	1.7	3.33E-03	1.03E-10	1.3	192.814	243.316	319.373	336.843
51	3.86	609.15	609.1433	4.6	C ₂₇ H ₅₀ O ₁₅	Kampferol-3-O-β-glactopyranoside	Flavone and flavonoid biosynthesis	Metabolism of specialized metabolites	285.04	Flavonoids	Phenolic Compounds	1.5	4.33E-02	6.55E-03	1.8	70.633	69.390	109.179	104.644
13	5.00	517.10	517.1006	-3.6	C ₂₇ H ₅₀ O ₁₃	Malonyl-genistin*	Isoflavonoid biosynthesis	Metabolism of specialized metabolites	431.09; 269.04	Isoflavone	Phenolic Compounds	1.5	4.67E-02	1.75E-02	2.3	126.593	165.430	149.170	183.297

Note: Metabolites were annotated by level 2.1 of the metabolomics standards initiative; ID - identification corresponding to the chromatograms in Figure 2; IR - retention time; KVL - very low K⁺ availability; KVH - very high K⁺ availability; * * - identified using authentic standards; O2PLS-DA and S-Plot models were performed using p-value ≤ 0.05 and false discovery rate (FDR) with q-value ≤ 0.05 (Data S1); Importance variable in score projection (VIP ≥ 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVH vs. KVL treatments (metabolite content is higher in KVH than KVL treatment). Source: The Author (2022).

Putative metabolite identification from soybean pod tissues upregulated under very low potassium (KVH) availability by UPLC-QToF-MS^E.

	t _R (min)	Theoretical Mass [M-H] ⁻	Measured Mass [M-H] ⁻	Mass error (ppm)	Formula	Identification	Metabolic Pathway	KEGG Pathway Maps	MS ² Fragments	Chemical Ontology	FC	FDR	p-value	VIP	Average Intensity (Normalized)				
						Immature Seeds									KVL - IS	KL - IS	KM - IS	KVH - IS	
37	0.62	154.06	154.0629	-4.6	C ₈ H ₁₆ N ₂ O ₂	L-histidine	β-Alanine metabolism; Histidine metabolism	Amino acid metabolism	128.90	Amino acids	Amino acids	1.8	2.50E-02	2.17E-03	2.4	140.095	96.775	86.250	79.474
38	0.64	145.06	145.0623	-3.1	C ₇ H ₁₄ N ₂ O ₃	L-glutamine	Arginine biosynthesis; Purine metabolism; Pyrimidine metabolism	Amino acid metabolism	127.05; 109.10	Amino acids	Amino acids	2.4	3.33E-02	3.08E-03	1.4	33.926	20.379	13.040	14.540
1	0.65	131.05	131.0467	-3.7	C ₈ H ₁₆ N ₂ O ₃	L-asparagine	Alanine, aspartate and glutamate metabolism	Amino acid metabolism	114.01; 113.03	Amino acids	Amino acids	1.6	4.17E-02	5.29E-03	1.8	94.881	69.908	73.774	59.041
39	0.66	487.17	487.1678	-2.0	C ₂₄ H ₄₀ O ₁₅	Oligosaccharide unknown	Galactosylcyclitol biosynthesis	Carbohydrate metabolism	341.10	Oligosaccharides	Carbohydrates	2.8	2.92E-02	2.41E-03	2.3	50.510	30.024	15.769	18.578
40	0.66	473.15	473.1527	-3.2	C ₂₃ H ₄₀ O ₁₄	Oligosaccharide unknown	Galactosylcyclitol biosynthesis	Carbohydrate metabolism	341.10	Oligosaccharides	Carbohydrates	2.1	3.75E-02	3.17E-03	1.8	110.974	72.463	72.246	54.766
41	0.67	195.05	195.0518	-4.1	C ₈ H ₁₆ O ₅	Galactonic acid	Galactose metabolism	Carbohydrate metabolism	154.05; 131.04	Hydroxy acids	Organic Acids	3.9	1.25E-02	7.45E-05	1.8	42.119	42.967	31.232	10.712
42	0.67	517.18	517.1756	3.4	C ₂₉ H ₅₀ O ₁₆	Ciceritol	Galactosylcyclitol biosynthesis	Carbohydrate metabolism	439.08; 341.10	Oligosaccharides	Carbohydrates	1.7	5.00E-02	2.99E-02	1.3	46.416	38.999	27.092	27.866
43	0.69	439.09	439.0857	5.6	C ₂₈ H ₅₀ O ₁₂	3,5-dihydroxyphenyl 1-O-(6-O-galloyl-β-D-glucopyranoside)	Galactosylcyclitol biosynthesis	Carbohydrate metabolism	341.10	Oligosaccharides	Carbohydrates	1.6	1.67E-02	2.20E-04	4.0	183.332	151.581	149.694	118.212
15	5.41	253.05	253.0494	4.8	C ₁₃ H ₂₄ O ₄	Daidzein [*]	Isoflavonoid biosynthesis	Metabolism of specialized metabolites	253.04	Isoflavones	Phenolic Compounds	1.5	2.08E-02	8.72E-04	2.4	176.069	132.954	134.566	119.826
17	6.50	269.05	269.0445	3.8	C ₁₅ H ₂₄ O ₅	Genistein [*]	Isoflavonoid biosynthesis	Metabolism of specialized metabolites	269.04	Isoflavones	Phenolic Compounds	17.8	4.17E-03	4.29E-07	2.8	68.216	30.418	4.661	3.846
						Pod Valves									KVL - PV	KL - PV	KM - PV	KVH - PV	
38	0.64	145.06	145.0622	-2.4	C ₈ H ₁₆ N ₂ O ₃	L-glutamine	Arginine biosynthesis; Purine metabolism; Pyrimidine metabolism	Amino acid metabolism	127.05; 109.1	Amino acids	Amino acids	2.1	2.67E-02	1.57E-03	1.1	30.838	17.528	12.816	14.735
1	0.65	131.05	131.0465	-2.2	C ₈ H ₁₆ N ₂ O ₃	L-asparagine	Alanine, aspartate and glutamate metabolism	Amino acid metabolism	114.01; 113.03	Amino acids	Amino acids	2.4	1.33E-02	2.60E-04	2.1	77.823	34.115	32.499	32.306
45	0.66	311.10	311.1001	-5.6	C ₁₉ H ₃₄ O ₁₀	3-O-β-D-galactopyranosyl-L-arabinose	Galactosylcyclitol biosynthesis	Carbohydrate metabolism	—	Monosaccharides	Carbohydrates	2.9	1.67E-02	1.24E-03	1.3	20.051	13.254	10.229	6.875
46	0.68	132.04	132.0418	-2.7	C ₈ H ₁₆ N ₂ O ₃	Ureidoglycine	Purine metabolism	Amino acid metabolism	—	Amino acids	Amino acids	1.9	3.67E-02	3.07E-03	2.0	56.085	47.729	48.290	28.270
3	0.69	175.05	175.0477	-2.5	C ₈ H ₁₆ N ₂ O ₄	Allantoic acid	Purine metabolism	Amino acid metabolism	132.04; 89.10	Amino acids	Amino acids	1.6	2.33E-02	1.39E-03	2.0	91.941	75.770	73.205	55.118
6	0.73	133.01	133.0149	-5.0	C ₈ H ₁₆ O ₅	Malic acid	Citrate cycle (TCA cycle)	Carbohydrate metabolism	133.01; 115.0	Beta hydroxy acids	Organic Acids	2.2	5.00E-02	3.83E-02	1.2	38.899	28.329	29.955	18.071
8	0.74	315.07	315.0713	2.7	C ₁₇ H ₃₀ O ₆	Genistic acid 2-β-D-glucoside	Phenylpropanoid biosynthesis	Metabolism of specialized metabolites	152.01; 108.02	Benzoic acids	Organic Compounds	2.1	6.67E-03	3.01E-06	1.4	28.123	27.270	13.078	13.008
47	1.62	315.07	315.0727	-1.8	C ₁₇ H ₃₀ O ₆	Protocatechuic acid 4-O-β-glucoside	Phenylpropanoid biosynthesis	Metabolism of specialized metabolites	152.01; 108.02	Benzoic acids	Organic Compounds	1.4	3.00E-02	1.78E-03	1.5	75.417	78.325	49.313	49.908
48	2.34	443.19	443.1928	-1.2	C ₂₃ H ₄₀ O ₁₀	Dihydrochastic acid 4-O-β-D-glucoside	Sesquiterpenoid and Triterpenoid biosynthesis	Metabolism of specialized metabolites	303.11; 201.02; 135.04	Sesquiterpenoid	Terpenoids	1.6	1.00E-02	1.54E-05	2.3	108.841	77.233	60.854	70.391
50	3.64	415.10	415.1046	-2.8	C ₂₃ H ₄₀ O ₆	Daidzin [*]	Isoflavonoid biosynthesis	Metabolism of specialized metabolites	253.04	Isoflavone	Phenolic Compounds	2.3	3.33E-02	2.30E-03	1.3	28.765	25.604	6.797	12.517

Note: Metabolites were annotated by level 2.1 of the metabolomics standards initiative; ID - identification corresponding to the chromatograms in Figure 2; IR - retention time; KVL - very low K⁺ availability; KVH - very high K⁺ availability; * * - identified using authentic standards; O2PLS-DA and S-Plot models were performed using p-value ≤ 0.05 and false discovery rate (FDR) with q-value ≤ 0.05 (Data S1); Importance variable in score projection (VIP ≥ 1.0) were considered; Fold change (FC) refers to metabolite relative intensity values between KVL vs. KVH treatments (metabolite content is higher in KVL than KVH treatment). Source: The Author (2022).