*Supplementary Data File*

# Data-driven characterisation of metabolome reprogramming during early development of sorghum seedlings

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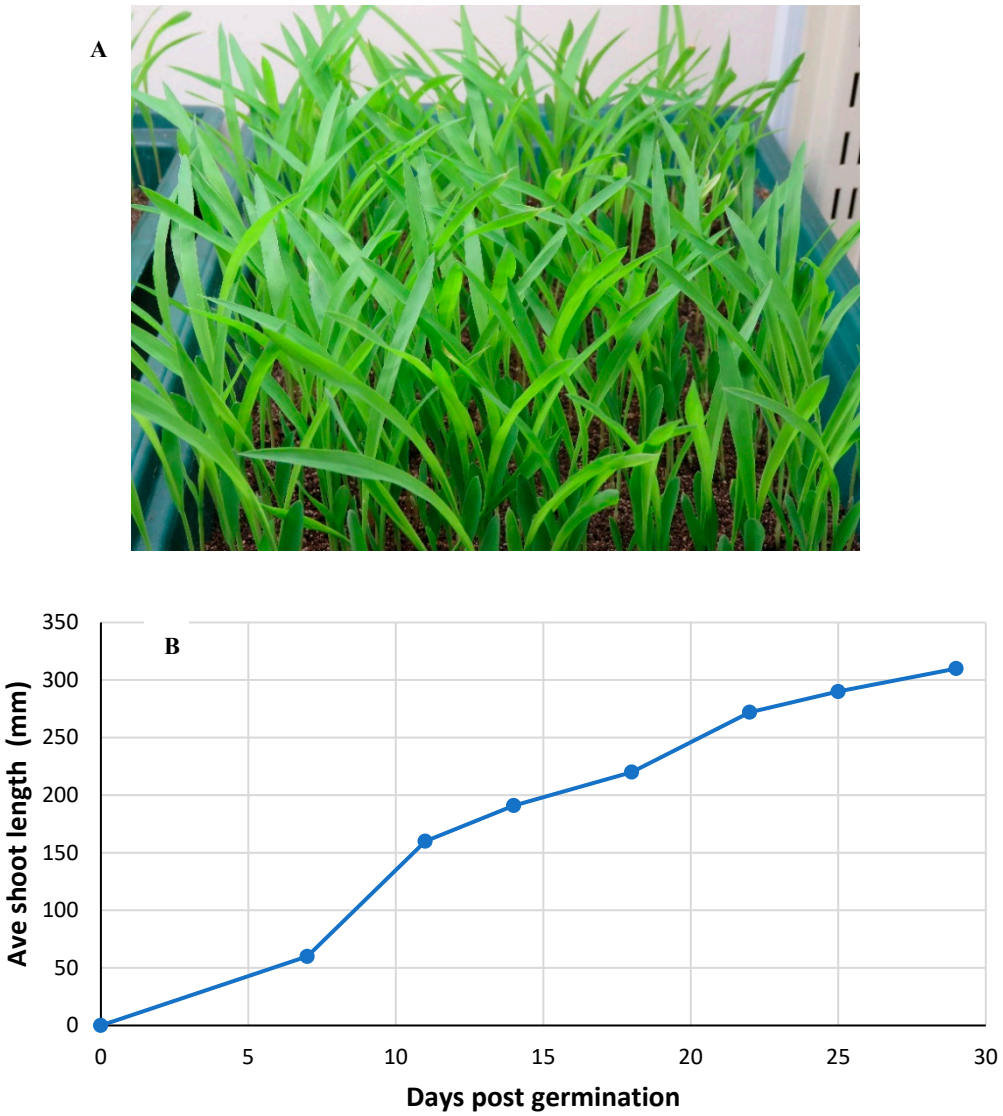



**Figure S1.** Growth of *Sorghum bicolor* seedlings cv. NS 5511. (**A**) Sorghum seedling growth at day 7 post germination. (**B**) Graph depicting average shoot length at different days.

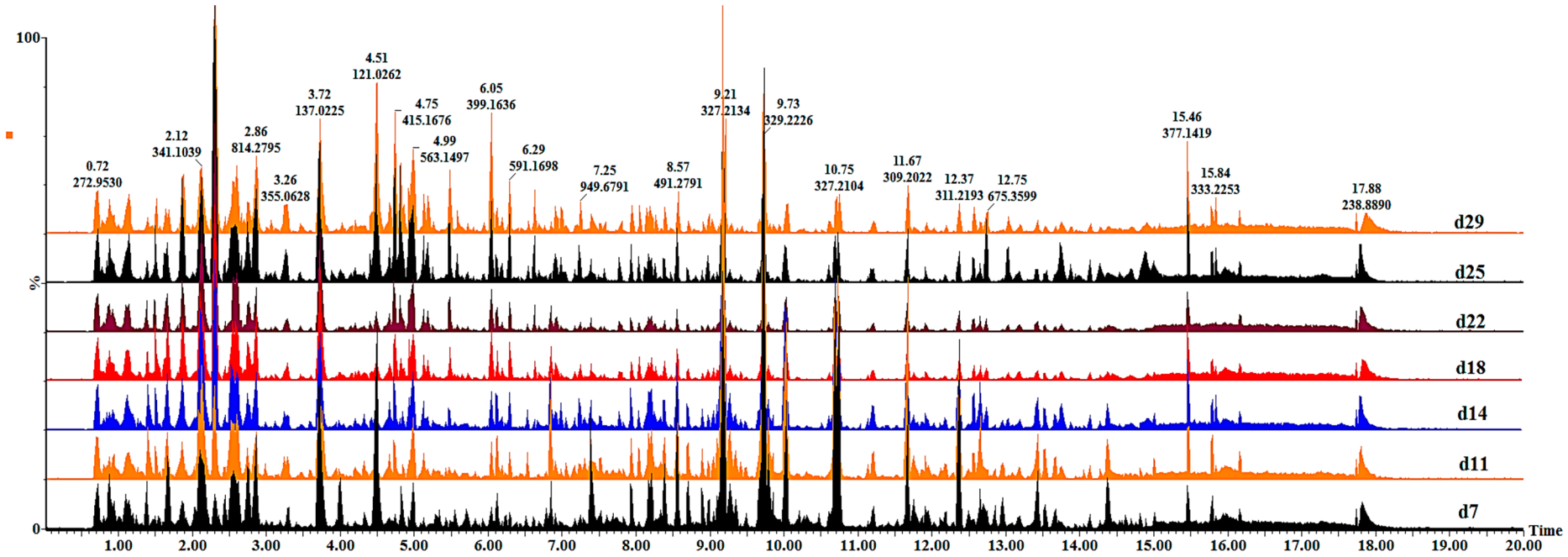



**Figure S2A.** UHPLC-MS base peak intensity (BPI) chromatograms from methanolic extracts derived from sorghum seedlings in ESI negative ionisation mode. Sample extracts were prepared from plant material harvested at the indicated time intervals. Variation in the displayed chromatograms, linked to changes in the metabolite composition at different developmental stages, can be visually observed from d 7 to d 29.

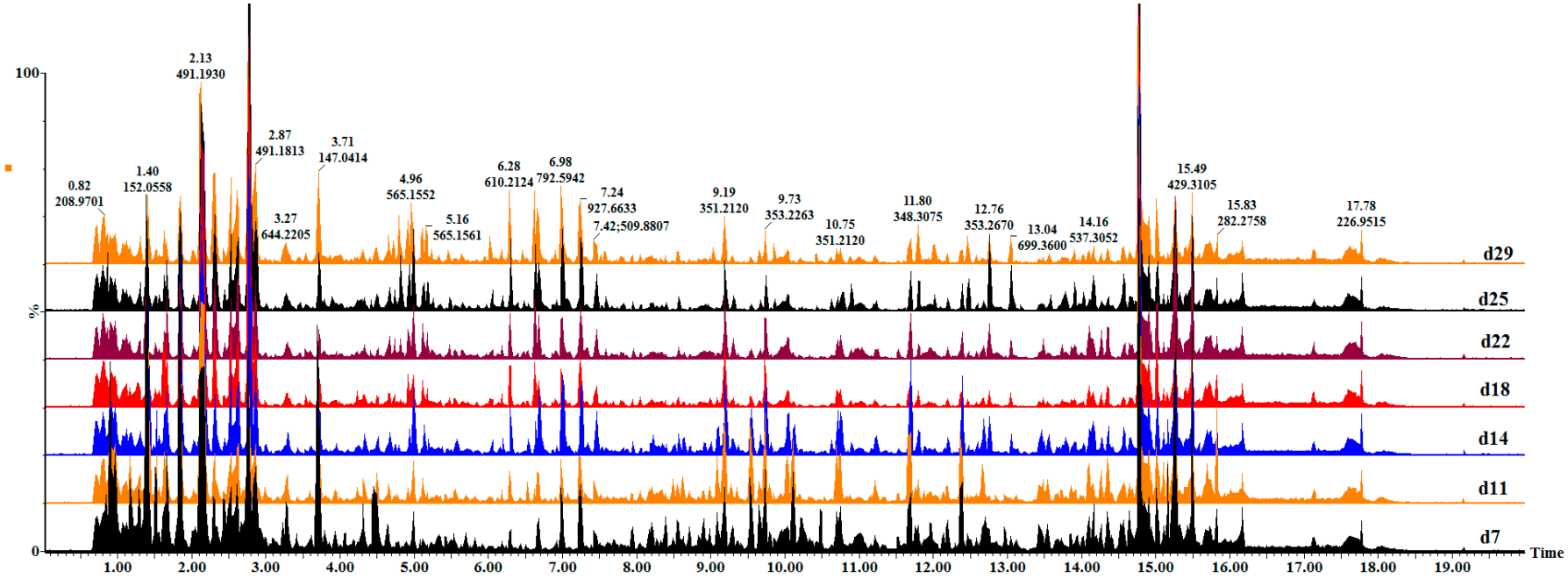



**Figure S2B.** UHPLC-MS BPI chromatograms of methanolic extracts derived from sorghum seedlings in ESI positive ionisation mode. Sample extracts were prepared from plant material harvested at the indicated time intervals. Variation in the displayed chromatograms, linked to changes in the metabolite composition at different developmental stages, can be visually observed from d 7 to d 29.

**Table S1**. Classification and annotation of metabolites from leaf extracts of *Sorghum bicolor* seedlings from different development stages (days 7, 14 and 29 post-germination).

| Metabolite | *m/z* | Rt (min) | Adduct | Molecular formula | *p*-value ** | Fold change *** | Stage (Early, Mid, Late) |
|---|---|---|---|---|---|---|---|
| **Amino acids and derivatives** | | | | | | | |
| Phenylalanine | 180.092 | 2.59 | $[M-H\_NH_3]^-$ | $C_9H_{11}NO_2$ | $6.71 \times 10^{-6}$ | 0.6 | E,M,L |
| Tyrosine | 182.081 | 1.13 | $[M+H]^+$ | $C_9H_{11}NO_3$ | $1.77 \times 10^{-31}$ | 2.1 | E,M,L |
| Dhurrin | 334.090 | 2.60 | $[M+H\_Na]^+$ | $C_{14}H_{17}NO_7$ | $2.49 \times 10^{-29}$ | 1.4 | E,M,L |
| Tryptophan | 205.097 | 2.69 | $[M+H]^+$ | $C_{11}H_{12}N_2O_2$ | $4.47 \times 10^{-11}$ | 3.6 | E,M,L |
| **Organic acids** | | | | | | | |
| Citric acid / Isocitric acid | 191.018 | 1.10 | $[M-H]^-$ | $C_6H_8O_7$ | $2.60 \times 10^{-6}$ | 0.7 | E,M,L |
| Benzoic acid | 121.028 | 4.46 | $[M-H]^-$ | $C_7H_6O_2$ | $1.76 \times 10^{-20}$ | 0.6 | E,L |
| **Flavonoids** | | | | | | | |
| Apigenin 8-*C*-glucoside (vitexin) | 431.099 | 5.55 | $[M-H]^-$ | $C_{21}H_{20}O_{10}$ | $7.57 \times 10^{-5}$ | 1.4 | E,M,L |
| Apigenin 7-*O*-glucoside (apigetrin) | 431.098 | 6.33 | $[M-H]^-$ | $C_{21}H_{20}O_{10}$ | $2.38 \times 10^{-3}$ | 1.0 | E,M,L |
| Apigenin 6-*C*-xyloside-8-*C*-glucoside (vicenin-1) | 563.142 | 4.87 | $[M-H]^-$ | $C_{26}H_{28}O_{14}$ | $2.01 \times 10^{-4}$ | 2.7 | E,M,L |
| Apigenin 6,8-di-*C*-glucoside (vicenin-2) | 593.151 | 4.45 | $M-H]^-$ | $C_{27}H_{30}O_{15}$ | $2.00 \times 10^{-5}$ | 2.7 | E,M,L |
| Apigenin 6-*C*-glucosyl-8-*C*-xyloside (vicenin-3) | 563.139 | 5.09 | $[M-H]^-$ | $C_{26}H_{28}O_{14}$ | $1.03 \times 10^{-8}$ | 2.7 | E,M,L |
| Apigenin 7-*O*-neohesperidoside (rhoifolin) | 577.156 | 6.06 | $[M-H]^-$ | $C_{27}H_{30}O_{14}$ | $3.58 \times 10^{-8}$ | 3.8 | E,M,L |
| Luteolin 7-*O*-glucoside (luteoloside) | 447.091 | 5.71 | $[M-H]^-$ | $C_{21}H_{20}O_{11}$ | $4.44 \times 10^{-13}$ | 1.6 | E,M,L |
| Luteolin 7-*O*-neohesperidoside (lonicerin) | 593.179 | 5.51 | $[M-H]^-$ | $C_{27}H_{30}O_{15}$ | $2.20 \times 10^{-15}$ | 1.3 | E,M,L |
| Naringenin 7-*O*-β-D-glucoside (prunin) | 433.114 | 5.91 | $[M-H]^-$ | $C_{21}H_{22}O_{10}$ | $1.44 \times 10^{-16}$ | 2.3 | M,L |
| Naringin | 625.175 | 3.33 | $[M-H\_FA]^-$ | $C_{27}H_{32}O_{14}$ | $8.31 \times 10^{-9}$ | 30.6 | E,M,L |
| Sophoraflavanone G | 423.182 | 5.46 | $[M-H]^-$ | $C_{25}H_{28}O_6$ | $1.21 \times 10^{-10}$ | 30.6 | M,L |
| Quercetin 3-rhamnoside-7-rhamnoside | 595.165 | 4.51 | $[M-H]^-$ | $C_{27}H_{32}O_{15}$ | $2.87 \times 10^{-9}$ | 1.4 | E,M,L |
| Quercetin 3-*O*-rhamnoside (quercitrin) | 447.092 | 4.61 | $[M-H]^-$ | $C_{21}H_{20}O_{11}$ | $9.02 \times 10^{-17}$ | 1.8 | E,M,L |
| Hesperidin | 609.181 | 4.80 | $[M-H]^-$ | $C_{28}H_{34}O_{15}$ | $1.18 \times 10^{-10}$ | 6.5 | E,M,L |
| Unknown flavonoid | 581.149 | 4.33 | $[M-H]^-$ | $C_{26}H_{30}O_{15}$ | $6.18 \times 10^{-15}$ | 55.1 | E,M,L |
| Unknown flavonoid | 611.158 | 3.10 | $[M-H]^-$ | $C_{27}H_{32}O_{16}$ | $6.18 \times 10^{-15}$ | 55.1 | E,M,L |
| **Hydroxycinnamic acids and derivatives** | | | | | | | |
| 7-Hydroxycoumarin | 161.024 | 1.87 | $[M-H]^-$ | $C_9H_6O_3$ | $3.91 \times 10^{-12}$ | 1.3 | E,M,L |
| *p*-Coumaric acid | 163.039 | 3.65 | $[M-H]^-$ | $C_9H_8O_3$ | $6.40 \times 10^{-2}$ | 0.9 | E,M,L |
| Coumaroyl glucose | 327.107 | 7.22 | $[M+H]^+$ | $C_{15}H_{18}O_8$ | $1.00 \times 10^{-4}$ | 2.1 | E,M,L |
| Caffeic acid derivative | 475.143 | 1.92 | $[M-H]^-$ | $C_{20}H_{28}O_{13}$ | $1.81 \times 10^{-15}$ | 1.5 | E,M,L |
| 2-*O*-Caffeoylglyceric acid | 267.048 | 4.38 | $[M-H]^-$ | $C_{12}H_{12}O_7$ | $1.48 \times 10^{-3}$ | 0.5 | E,M,L |
| 3-Feruloylquinic acid | 367.099 | 3.75 | $[M-H]^-$ | $C_{17}H_{20}O_9$ | $5.98 \times 10^{-9}$ | 1.3 | E,M,L |
| Coniferyl acetate | 221.081 | 7.42 | $[M-H]^-$ | $C_{12}H_{14}O_4$ | $4.67 \times 10^{-6}$ | 2.7 | E,M,L |
| Sinapoyl (sinapyl/sinapic) alcohol | 209.074 | 6.72 | $[M-H]^-$ | $C_{11}H_{14}O_4$ | $1.11 \times 10^{-7}$ | 0.5 | E,M,L |
| Sinapaldehyde glucoside I | 371.130 | 6.53 | $[M+H]^+$ | $C_{17}H_{22}O_9$ | $4.70 \times 10^{-29}$ | 4.3 | E,M,L |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1,3-*O*-Coumaroyl-feruloylglycerol | 413.121 | 9.03 | $[M-H]^-$ | $C_{22}H_{22}O_8$ | $9.84 \times 10^{-12}$ | 0.4 | E,M,L |
| Sinapaldehyde glucoside II | 415.125 | 5.68 | $[M+FA-H]^-$ | $C_{17}H_{22}O_9$ | $6.87 \times 10^{-2}$ | 3.8 | E,M,L |
| **Fatty acids and derivatives** | | | | | | | |
| Octadecatetraenoic acid (OTA) | 275.199 | 13.44 | $[M-H]^-$ | $C_{18}H_{28}O_2$ | $1.64 \times 10^{-17}$ | 0.5 | E,M,L |
| 15-Hydroxylinoleic acid/15-Hydroxy-9,12-octadecadienoic acid | 295.226 | 14.30 | $[M-H]^-$ | $C_{18}H_{32}O_3$ | $5.10 \times 10^{-3}$ | 1.2 | E,M,L |
| 9,14-Dihydroxy-10,12-octadecadienoic acid (ODA-2OH)(II) | 311.219 | 11.81 | $[M-H]^-$ | $C_{18}H_{32}O_4$ | $5.87 \times 10^{-17}$ | 0.5 | E,M,L |
| 9,12,13-Trihydroxy-10-octadecenoic acid (ODA-3OH)(IV) | 329.229 | 9.60 | $[M-H]^-$ | $C_{18}H_{34}O_5$ | $1.45 \times 10^{-5}$ | 0.6 | E,M,L |
| Trihydroxyoctadecadienoic acid II (ODA-3OH)(II) | 327.213 | 11.05 | $[M-H]^-$ | $C_{18}H_{32}O_5$ | $1.54 \times 10^{-5}$ | 0.5 | E,M,L |
| **Phytohormones and derivatives** | | | | | | | |
| Indole-3-acrylic acid/ N-Ac-indole-3-carboxaldehyde | 188.076 | 2.71 | $[M+H]^+$ | $C_{11}H_9NO_2$ | $2.27 \times 10^{-10}$ | 3.7 | E,M,L |
| Indole-3-acetyl-leucine | 333.120 | 3.25 | $[M+H\_NaNa]^+$ | $C_{16}H_{20}N_2O_3$ | $9.16 \times 10^{-11}$ | 7.8 | E,M,L |
| Salicylic acid | 137.031 | 3.69 | $[M-H]^-$ | $C_7H_6O_3$ | $1.50 \times 10^{-1}$ | 0.9 | E,M,L |
| Salicylic acid 2-*O*-beta-D-glucoside | 299.074 | 1.62 | $[M-H]^-$ | $C_{13}H_{16}O_8$ | $7.21 \times 10^{-17}$ | 1.7 | E,M,L |
| Zeatin riboside | 352.183 | 3.16 | $[M+H]^+$ | $C_{15}H_{21}N_5O_5$ | $1.35 \times 10^{-28}$ | 1.2 | E,M,L |
| Abscisic acid | 265.155 | 3.35 | $[M+H]^+$ | $C_{15}H_{20}O_4$ | $2{,}34 \times 10^{-9}$ | 1.8 | E,M,L |
| Traumatic acid | 297.129 | 3.90 | $[M+H\_FA-Na]^+$ | $C_{12}H_{20}O_4$ | $1.3 \times 10^{-10}$ | 1.8 | E,M,L |
| Riboflavin | 443.118 | 5.58 | $[M-FA-NaNa]^-$ | $C_{17}H_{20}N_4O_6$ | $8.10 \times 10^{-3}$ | 0.3 | E,M,L |

* Annotation was according to level 2 as stipulated by the Metabolomics Standards Initiative [12].
** The *p*-value indicates the probability of a null hypothesis, that there is no difference between two groups that are being analysed, (7 vs. 14 d, 7 vs. 29 d, and 14 vs. 29 d), with *p*-values < 0.05 indicating the null hypothesis can be rejected.
*** Fold change indicates the relative changes in metabolite concentrations between two groups, based on averaged signal intensities. Fold change values differ depending on the stages compared (e.g. L/E) and only the highest values are reported for increases (>1) and lowest values for decreases (<1).

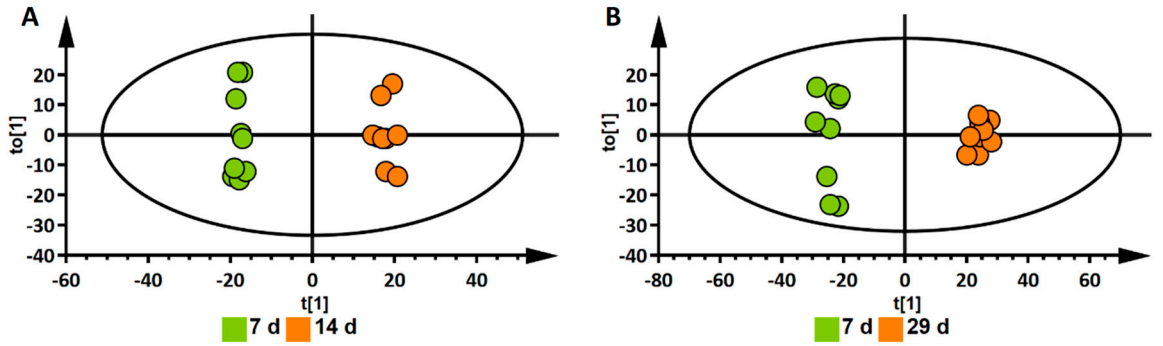



**Figure S3.** OPLS-DA of differentially occurring metabolites in extracts from *Sorghum bicolor* seedlings following ESI (+/-) UHPLC-MS analysis. OPLS-DA scores plots of the predictive component t[1] and the first orthogonal component t0 [1]. (**A**) 7-d *vs*. 14-d and (**B**) 7-d *vs*. 29-d group samples. The 14-d *vs*. 29-d version of the same plot is presented in the main text.

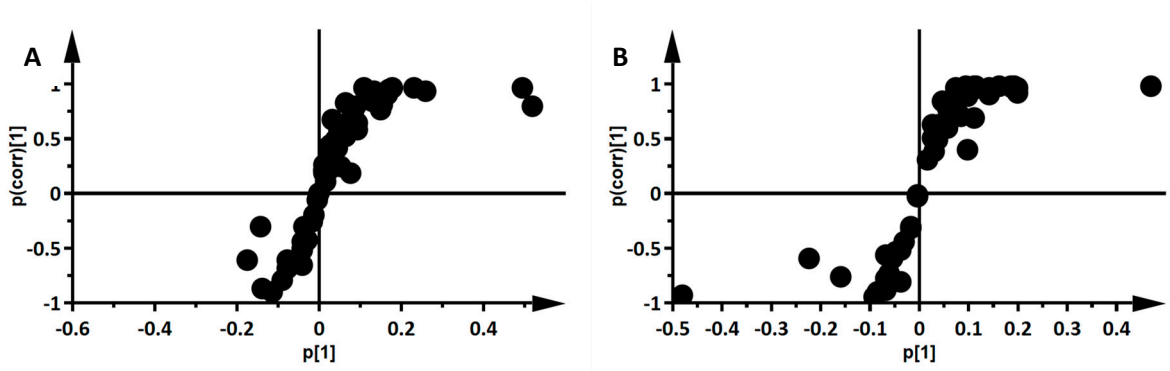



**Figure S4.** OPLS-DA S plots of discriminant biomarkers occurring in extracts from *Sorghum bicolor* seedlings following ESI (+/-) UHPLC-MS analysis. Discriminant biomarkers are found at each end of the S-plot at **(A)** 7-d *vs*. 14-d and **(B)** 7-d *vs*. 29-d growth stages. The covariance (variable magnitude) and correlation (reliability) of the variables in the model (indicated with black dots), are represented on the axes as p[1] and p(corr)[1] respectively. The *m/z* features located at the extreme ends of the plot show a positive association (high magnitude and high reliability) to the respective conditions being compared, while those in the middle can be regarded as shared features.

**Table S2.** Pathway enrichment analysis based on the presence of metabolites present in hydromethanolic extracts from developing *Sorghum bicolor* seedlings.

| Pathway names | Hits | Raw *p** | FDR** | Impact | Matched metabolites |
|---|---|---|---|---|---|
| **Arranged by pathway impact > 0.1** | | | | | |
| Biosynthesis of secondary metabolites | 1 | 0.09 | 1.0 | 1.00 | 4-Coumarate |
| Isoquinoline alkaloid biosynthesis | 1 | 0.10 | 1.0 | 0.50 | Tyrosine |
| Phenylalanine metabolism | 1 | 0.18 | 1.0 | 0.47 | Phenylalanine |
| Flavone and flavonol biosynthesis | 2 | 0.01 | 0.42 | 0.15 | Quercitrin, quercetin rhamnoside, glucoside |
| Tryptophan metabolism | 1 | 0.40 | 1.0 | 0.12 | Tryptophan |
| Riboflavin metabolism | 1 | 0.18 | 1.0 | 0.12 | Riboflavin |
| Tyrosine metabolism | 1 | 0.25 | 1.0 | 0.11 | Tyrosine |
| Glyoxylate and dicarboxylate metabolism | 1 | 0.41 | 1.0 | 0.10 | Isocitrate |
| **Arranged by FDR <0.5, raw *p*-value<0.1** | | | | | |
| Phenylpropanoid biosynthesis | 4 | 0.008 | 0.40 | 0.06 | Phenylalanine, Coumarate, Sinapoyl-OH, Sinapald-Glu |
| Aromatic amino acids biosynthesis | 3 | 0.006 | 0.40 | 0.02 | Tyrosine, Tryptophan, Phenylalanine |
| Flavone and flavonol biosynthesis | 2 | 0.013 | 0.42 | 0.15 | Quercitrin, quercetin rhamnoside, glucoside |
| Biosynthesis of secondary metabolites | 1 | 0.087 | 1.0 | 1.00 | 4-Coumarate |
| Isoquinoline alkaloid biosynthesis | 1 | 0.104 | 1.0 | 0.50 | Tyrosine |
| Phenylalanine metabolism | 1 | 0.183 | 1.0 | 0.47 | Phenylalanine |

*Statistical analyses are used to describe these pathways by the *p*-values and false discovery rate (FDR**) of the individual metabolites [19] (Liu *et al.*, 2019). The *p* value was set at < 0.1 and the FDR cut-off was < 0.5.

**Table S3.** Retention times and multiple reaction monitoring (MRM, MS/MS) data of the precursor ions, product ions, dwell times and collision energies of the standard compounds. Generated quantifier ions resulting from precursor-to-product ion transitions are highlighted in bold.

| Compound | Rt (min) | Precursor ions (*m*/*z*) | | Product ions (*m*/*z*) | | Dwell time (msec) | Q1 (V) | CE (V) | Q3 (V) |
|---|---|---|---|---|---|---|---|---|---|
| | | $[M+H]^+$ | $[M-H]^-$ | (+) Mode | (-) Mode | | | | |
| **Aglycones** | | | | | | | | | |
| Naringenin | 17.46 | 273.00 | 271.00 | **163.15*,** 153.05, 147.00 | 151.05, 119.10, 106.95 | 100.0 | -11.0, -11.0, -11.0 | -16.0, -23.0, -19.0 | -28.0, -14.0, -14.0 |
| Apigenin | 18.23 | 271.00 | 269.00 | **253.10*,** 239.20, 153.00 | 151.10, 117.05, 88.95 | 100.0 | -12.0, -10.0, -10.0 | -9.0, -9.0, -29.0 | -26.0, -16.0, -15.0 |
| Luteolin | 16.81 | 287.00 | 285.00 | **269.20*,** 246.10, 153.05 | 175.15, 151.15, 132.95 | 100.0 | -23.0, -11.0, -10.0 | -8.0, -10.0, -32.0 | -19.0, -26.0, -14.0 |
| **Mono-glycosylated derivatives** | | | | | | | | | |
| Apigetrin | 15.42 | 433.00 | 431.00 | **400.95*,** 294.95, 271.10 | 269.15, 268.10, 210.90 | 100.0 | -10.0, -10.0, -10.0 | -7.0, -22.0, -22.0 | -28.0, -23.0, -28.0 |
| Luteoloside | 10.83 | 449.00 | 447.00 | 417.15, **287.15*,** 153.10 | 379.25, 285.10, 284.15 | 100.0 | -13.0, -11.0, -11.0 | -9.0, -21.0, -54.0 | -21.0, -19.0, -29.0 |
| Vitexin | 9.23 | 433.00 | 431.00 | **415.10*,** 313.05, 283.00 | 341.20, 311.15, 283.05 | 100.0 | -10.0, -10.0, -14.0 | -20.0, -29.0, -33.0 | -20.0, -20.0, -12.0 |
| Iso-vitexin | 9.27 | 433.00 | 431.00 | 313.15, 283.05, **200.60*** | 341.00, 311.15, 283.10 | 100.0 | -10.0, -10.0, -17.0 | -25.0, -25.0, -45.0 | -21.0, -29.0, -16.0 |
| **Di-glycosylated derivatives** | | | | | | | | | |
| Vicenin-2 | 4.61 | 595.00 | 593.00 | **475.25*,** 379.20, 324.90 | 353.25, 383.25 | 100.0 | -20.0, -22.0, -22.0 | -17.0, -30.0, -35.0 | -16.0, -27.0, -22.0 |
| Vicenin-3 | 10.88 | 565.00 | 563.00 | **547.20*,** 457.25, 325.20 | 473.20, 383.05, 353.00 | 100.0 | -22.0, -20.0, -20.0 | -17.0, -19.0, -39.0 | -26.0, -16.0, -22.0 |
| **Internal Standard** D-Fluorophenylalanine | 1.54 | 184.00 | - | **138.15*,** 118.15, 91.15 | - | 100.0 | -12.0, -11.0, -10.0 | -14.0, -22.0, -30.0 | -26.0, -11.0, -17.0 |

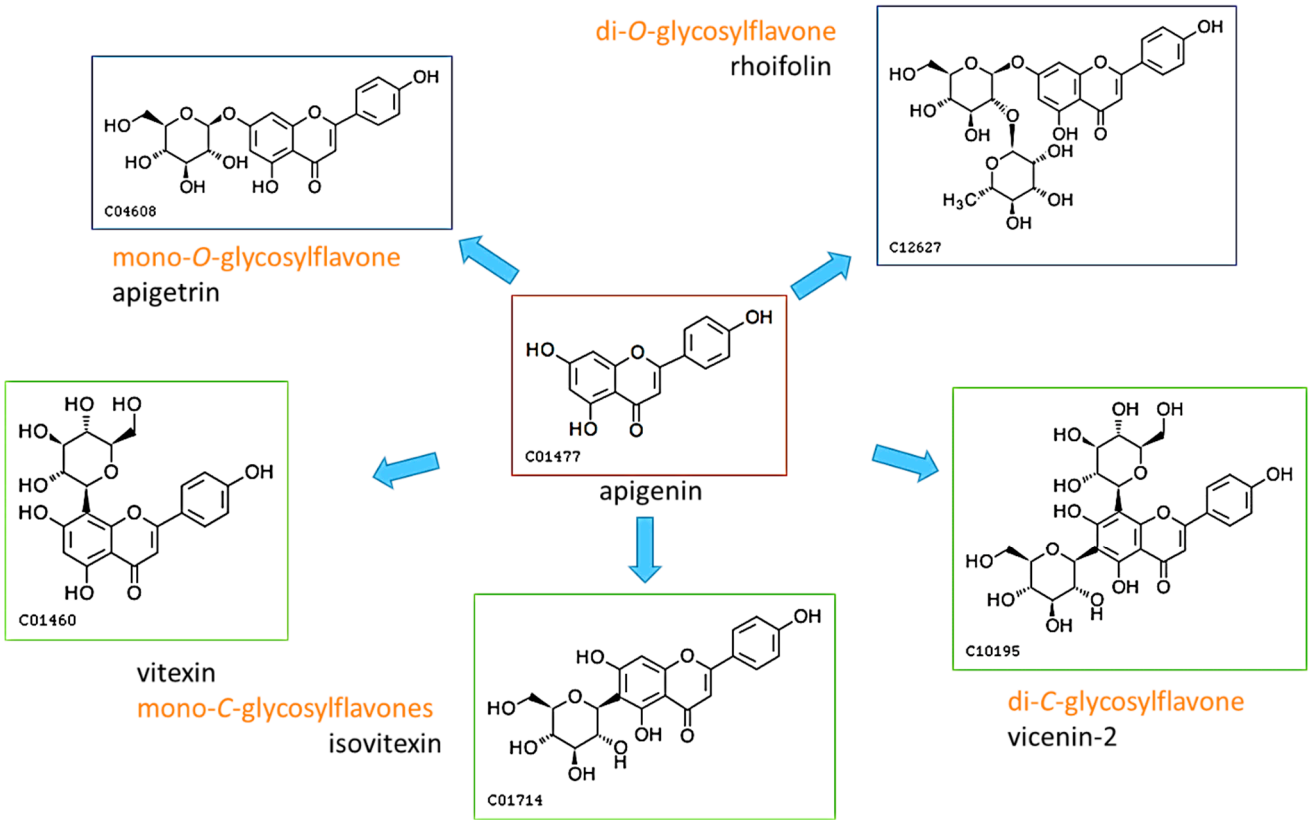
*Automated optimisation of MRM parameters was conducted in triplicate for each compound to determine the optimum ionisation polarity of targets. This was followed by optimisation of MRM transitions using LabSolutions software (Shimadzu, Kyoto, Japan). Analyses were performed on a triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. LC–MS/MS data were collected and processed by LabSolutions software. The MRM mode was used to quantify the analytes: the assay of investigated compounds was performed following three transitions per compound, the quantifier ions are highlighted in bold and the second and/or the third ones for confirmation.

**Table S4.** Standard curve equations and $R^2$ values of the standard compounds used for construction of calibration curves.

| Compound | Standard Curve Equation | Regression ($R^2$) |
|---|---|---|
| Apigenin | 2E+06x + 106218 | 0.998 |
| Apigetrin | 5E+06x + 782269 | 0.997 |
| Isovitexin | 3E+06x +82308 | 0.999 |
| Luteolin | 5E+06x + 36600 | 0.990 |
| Luteoloside | 4E+06x + 282335 | 0.998 |
| Naringenin | 3E+06x + 178602 | 0.999 |
| Vicenin-2 | 133667x + 145.48 | 0.997 |
| Vicenin-3 | 811914x - 20143 | 0.999 |
| Vitexin | 1E+06x + 8768.9 | 0.999 |
| D-Fluorophenylalanine (Internal standard) | 1E+06x + 190319 | 0.990 |

Each calibration curve for the standards included a working concentration range of 0.05 ppm – 5 ppm within which sample concentrations were obtained. The $R^2$ (regression) is a measure of the linearity of the standard curve by determining the correlation between the independent (x-axis) and dependent (y-axis) variables. The $R^2$ values of the calibration curves ranged from 0.990 to 0.999 which is a satisfactory indication of linearity.

**Figure S5.** Structures of the flavone, apigenin, and mono- and di-glycosylated derivatives, substituted at either O or C. The numbers in the rectangles refer to the unique KEGG identifiers. **Table 1** (main text) lists apigenin-8-*C*-glucoside (vitexin), apigenin-7-*O*-glucoside (apigetrin), apigenin-6-*C*-glucoside-8-*C*-xyloside (vicenin-3), apigenin-6-*C*-glucoside-8-*C*-glucoside (vicenin-2), apigenin 6-*C*-xyloside-8-*C*-glucoside (vicenin-1) and apigenin 7-*O*-neohesperidoside (rhoifolin).




(**Note**: Flavone *C*-glycosides differ from flavone *O*-conjugates in their biosynthetic origin, involving a F2H enzyme (flavanone 2 hydroxylase) that generates isomeric open-ring 2-hydroxyflavanones as substrates for *C*-glycosyl transferases (CGTs) and dehydratases (DHTs) that produce flavone 6- or 8-*C*-glycosides. While the initial steps of the pathway are constitutive, further glycosylation steps may be inducible [50] (Lam, L.P.Y., *et al*. 2023).