Metabolomic Profiling Reveals Synchronized Accumulation of Biomass and Bioactive Flavonoids During In Vitro Development of *Anoectochilus roxburghii*

Shuangbin Fu[1,2], Yanping Yang[1,2], Zhiwei Kong[1,2], and Zhen Ying[1,2]

[1]Zhejiang Institute of Subtropical Crops, Wenzhou, Zhejiang Province 325005, China
[2]Innovation Center of Chinese Medicinal Crops, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang Province 310021, China

February 14, 2026

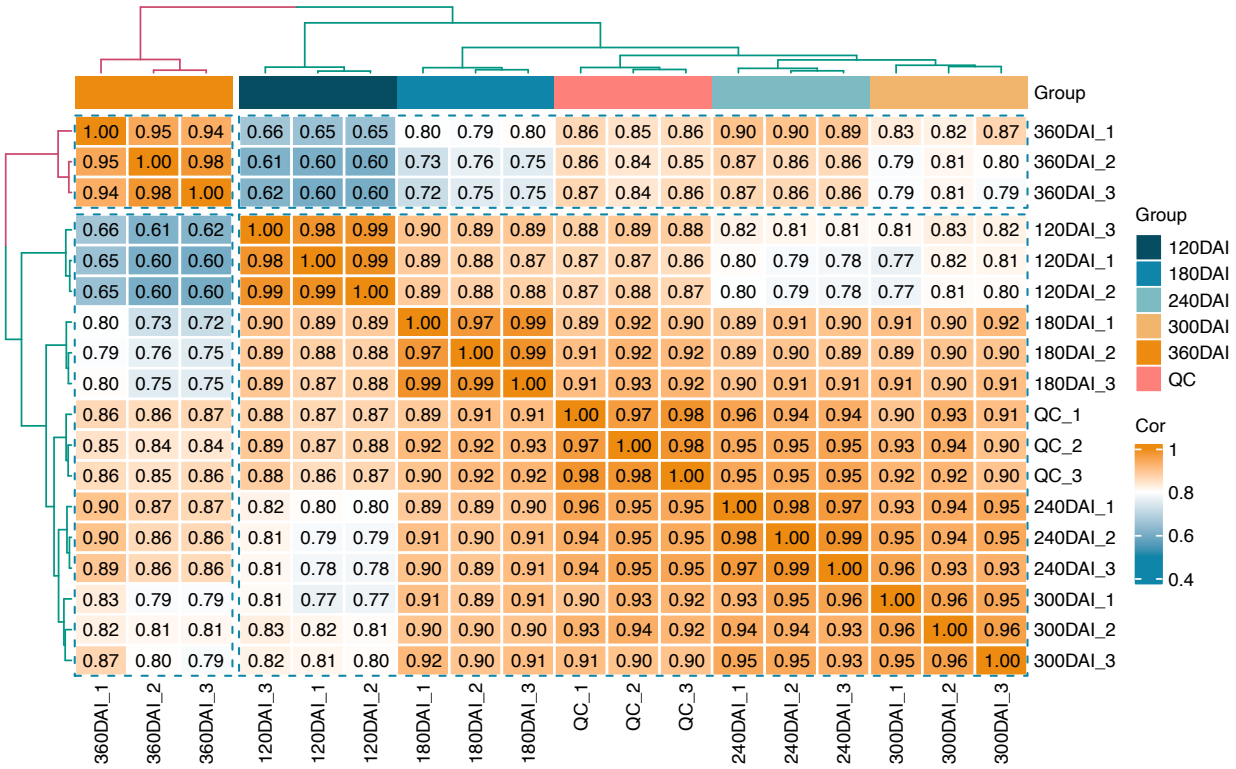
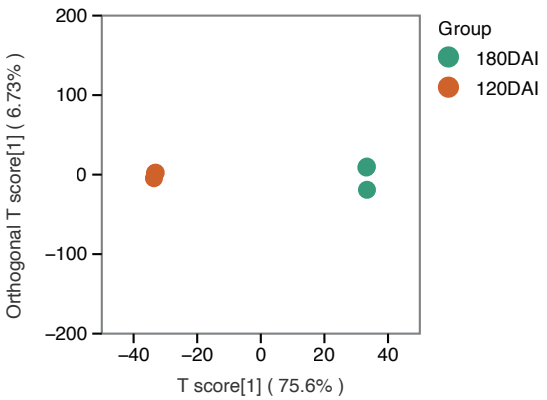
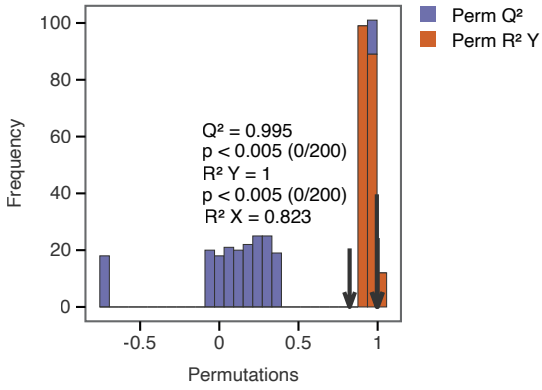
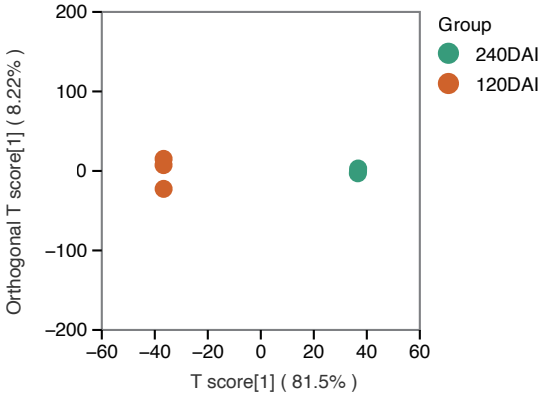
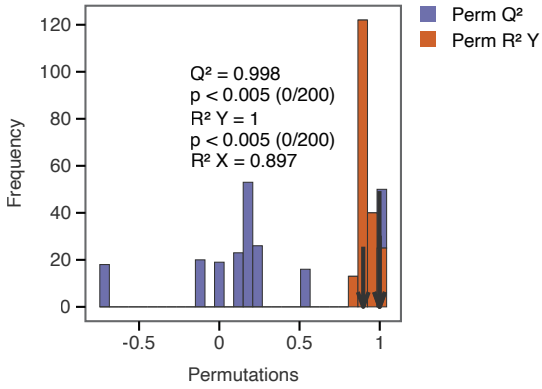


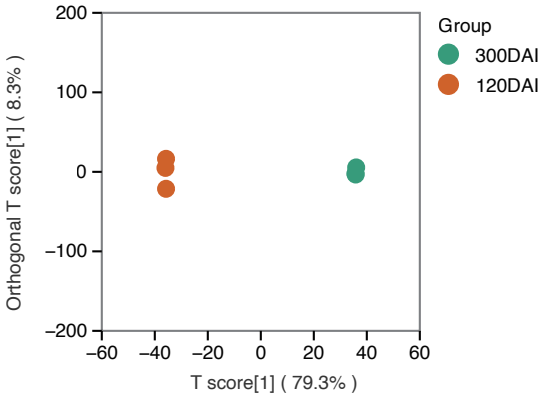
Figure S1: Pearson correlation analysis of samples across different stages.

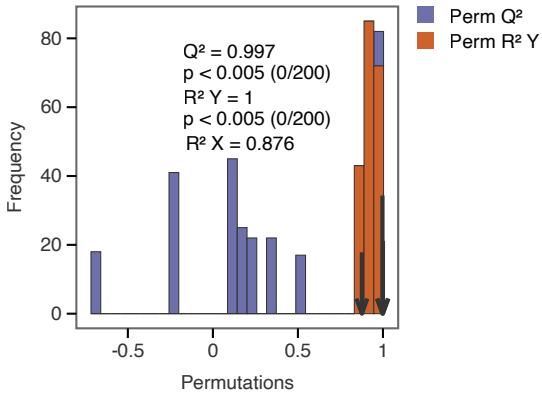

Group
180DAI
120DAI
Orthogonal T score[1] (6.73%)
T score[1] (75.6%)

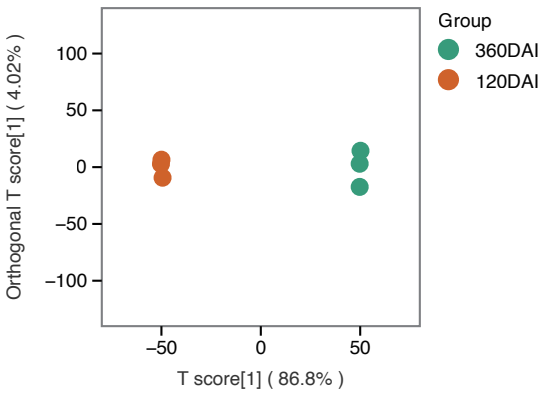

Perm Q²
Perm R² Y
Q² = 0.995
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.823
Frequency
Permutations

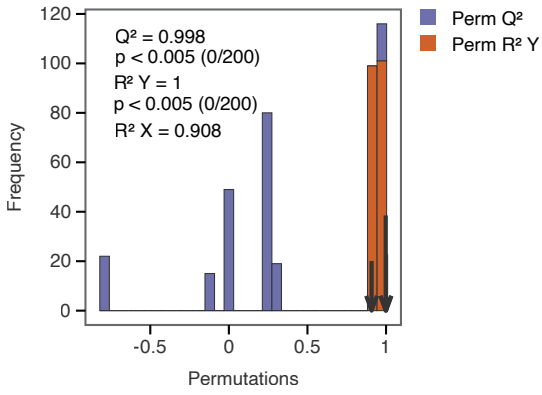

Group
240DAI
120DAI
Orthogonal T score[1] (8.22%)
T score[1] (81.5%)

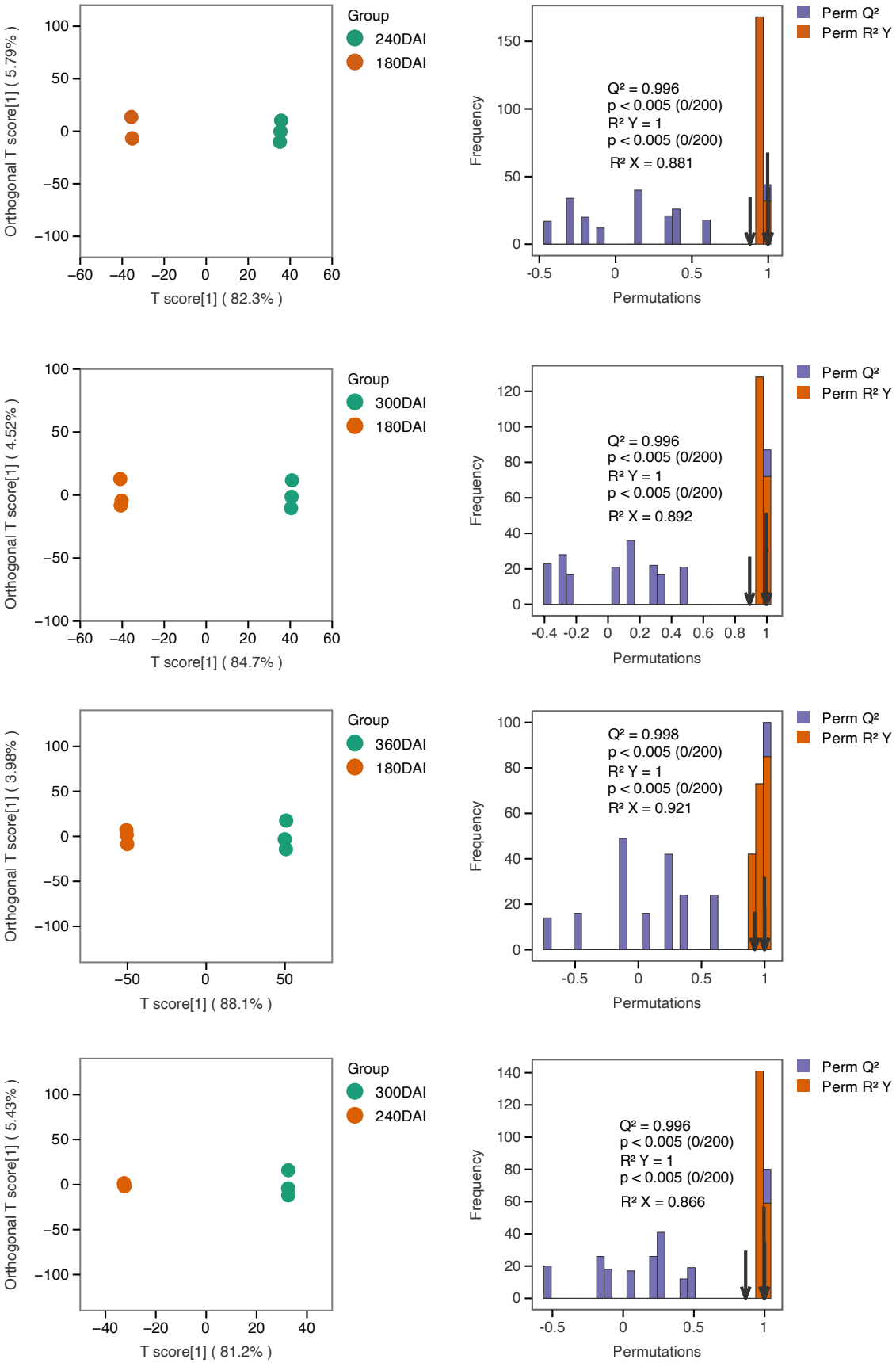

Perm Q²
Perm R² Y
Q² = 0.998
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.897
Frequency
Permutations


Group
300DAI
120DAI
Orthogonal T score[1] (8.3%)
T score[1] (79.3%)


Perm Q²
Perm R² Y
Q² = 0.997
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.876
Frequency
Permutations


Group
360DAI
120DAI
Orthogonal T score[1] (4.02%)
T score[1] (86.8%)


Perm Q²
Perm R² Y
Q² = 0.998
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.908
Frequency
Permutations


Group
240DAI
180DAI
Orthogonal T score[1] (5.79%)
T score[1] (82.3%)
Perm Q²
Perm R² Y
Q² = 0.996
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.881
Frequency
Permutations
Group
300DAI
180DAI
Orthogonal T score[1] (4.52%)
T score[1] (84.7%)
Perm Q²
Perm R² Y
Q² = 0.996
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.892
Frequency
Permutations
Group
360DAI
180DAI
Orthogonal T score[1] (3.98%)
T score[1] (88.1%)
Perm Q²
Perm R² Y
Q² = 0.998
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.921
Frequency
Permutations
Group
300DAI
240DAI
Orthogonal T score[1] (5.43%)
T score[1] (81.2%)
Perm Q²
Perm R² Y
Q² = 0.996
p < 0.005 (0/200)
R² Y = 1
p < 0.005 (0/200)
R² X = 0.866
Frequency
Permutations

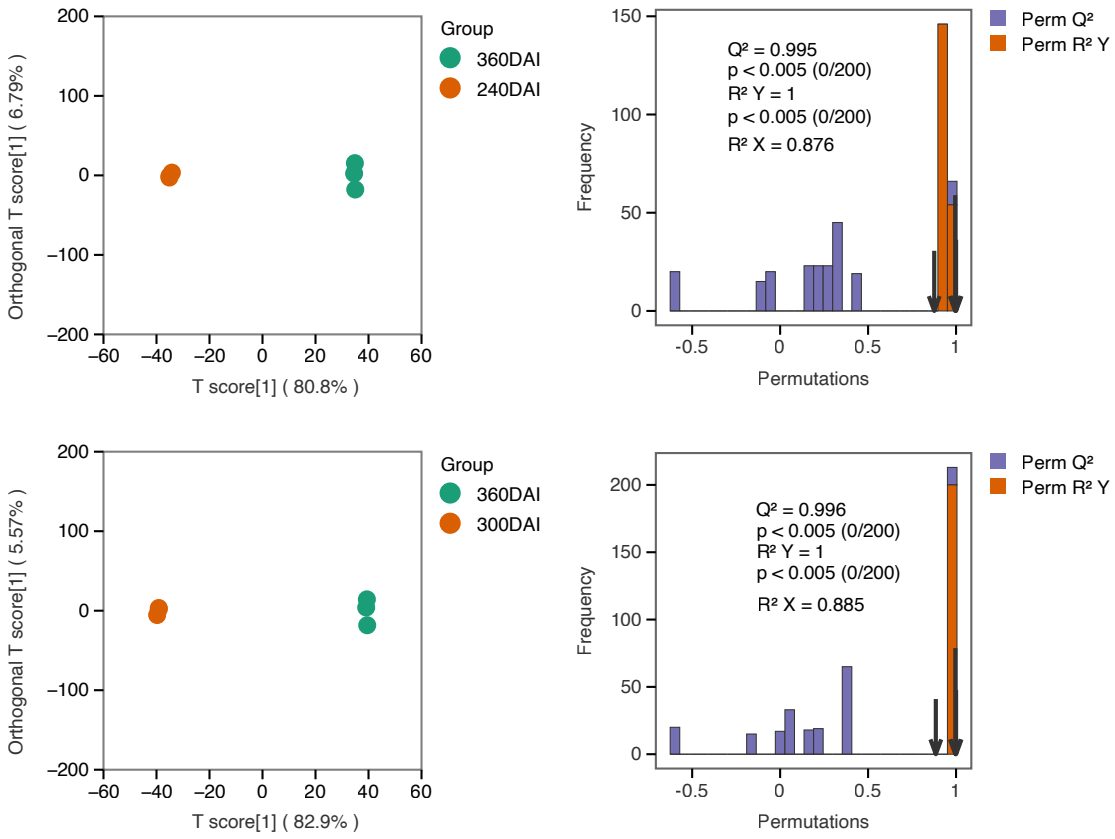


Figure S2: The score plots of OPLS-DA pairwise comparisons of differential metabolites and corresponding model validation. R^2 X and R^2 Y represent the explanatory power of the model for the X and Y matrices, respectively, while Q^2 reflects the model's predictive ability. The closer these three indicators are to 1, the more stable and reliable the model is. A Q^2 value greater than 0.5 indicates a valid model, and a Q^2 value greater than 0.9 indicates an excellent model.

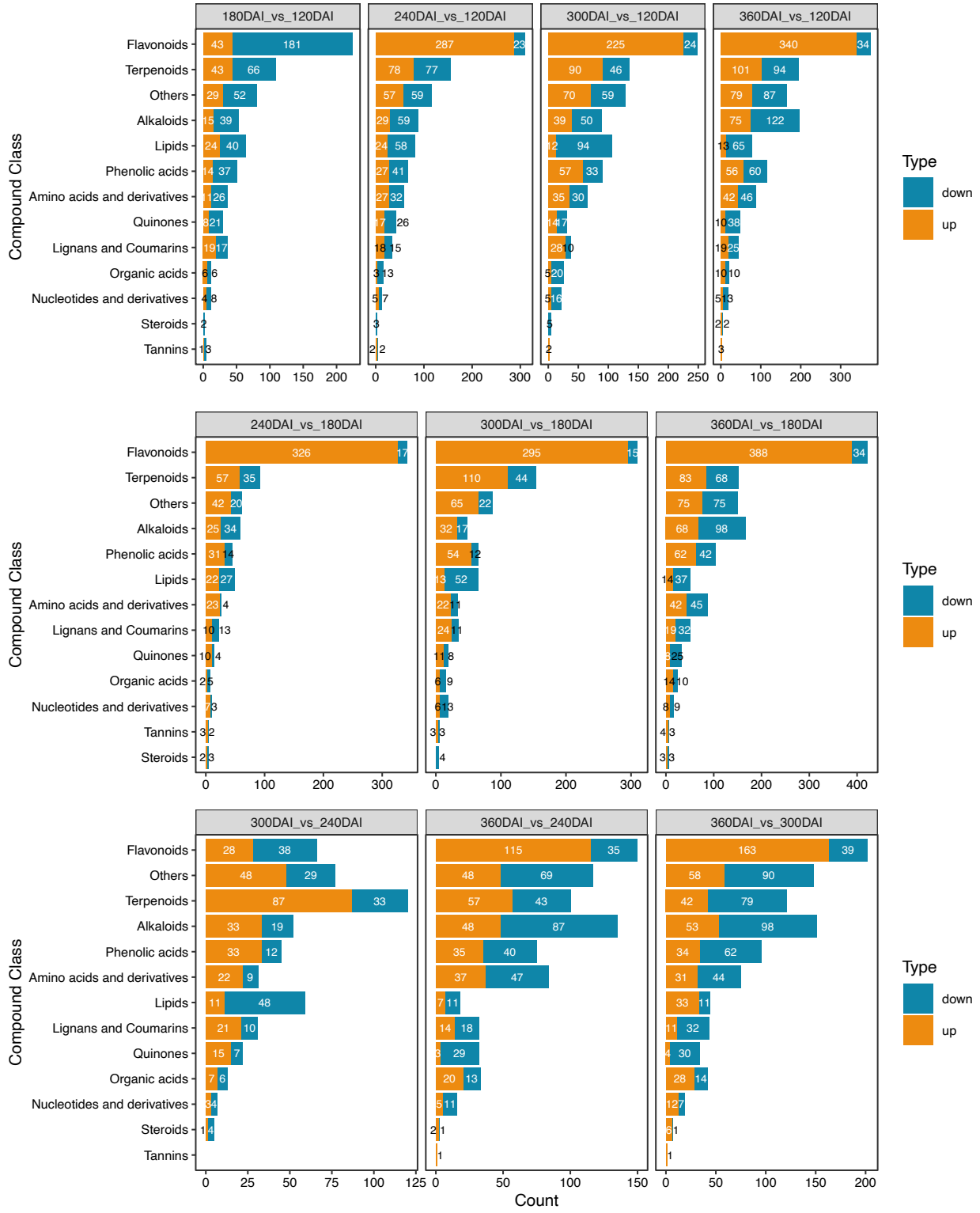


Figure S3: Statistical classification of DAMs across multiple comparison groups.

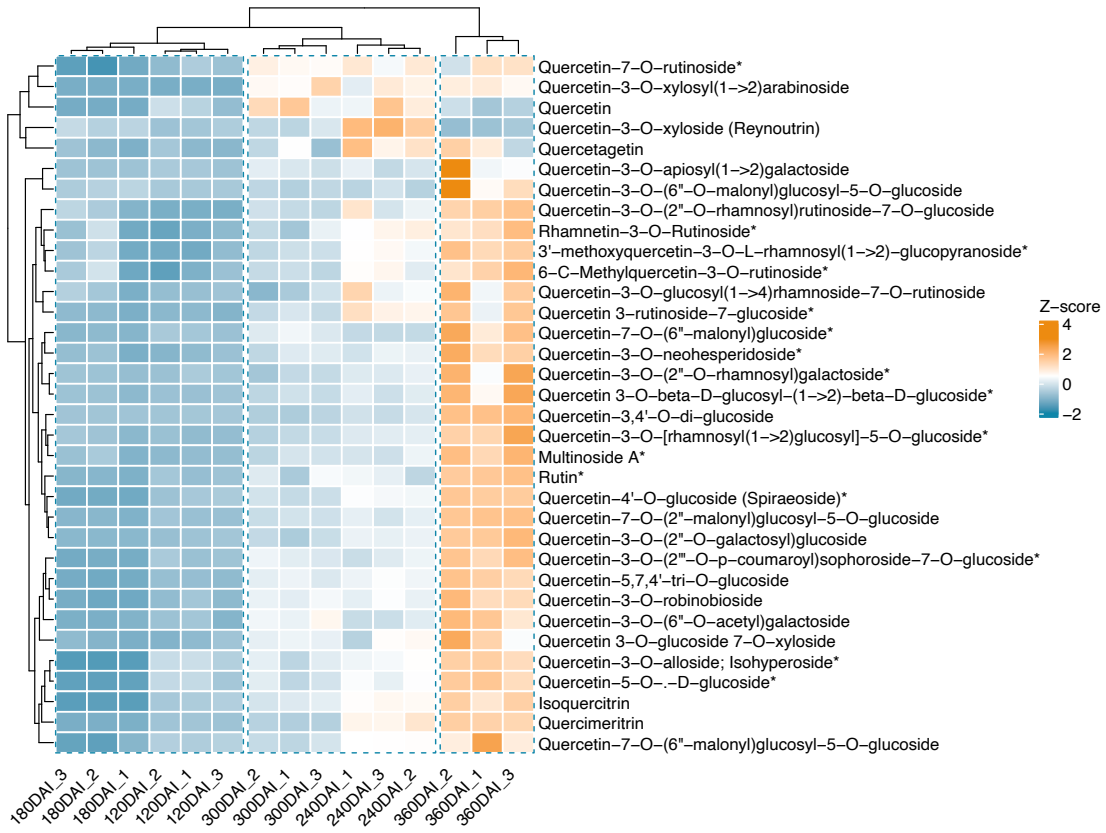


Figure S4: DAMs enriched in biosynthesis of quercetin aglycones I and II pathways across five stages. The color scale represents the Z-score normalized expression values by row. Columns are sorted by the average of the columns.

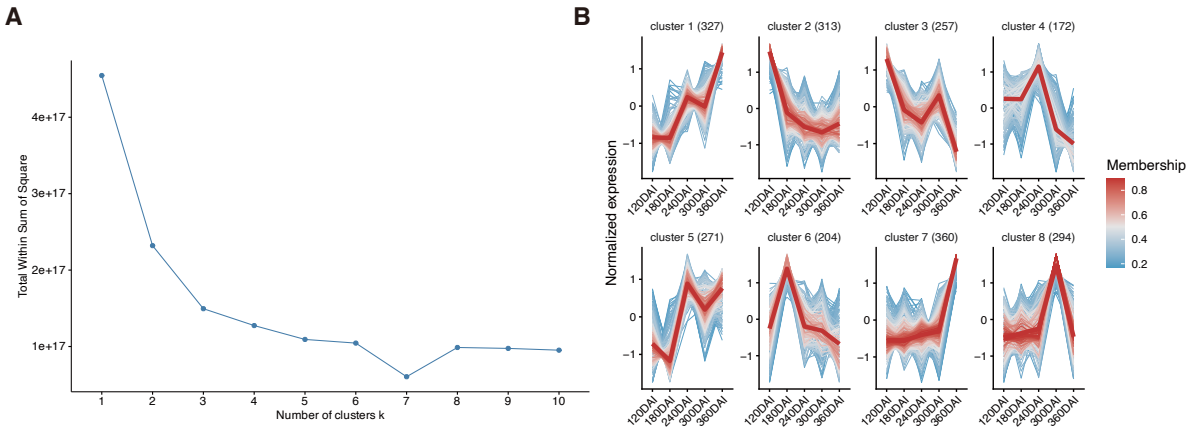


Figure S5: Mfuzz clustering Analysis. (A) The total within sum square value for different cluster numbers. (B) Mfuzz clustering and expression trend analysis of metabolites. This figure illustrates the grouping of metabolites based on their temporal accumulation patterns across five stages (120 DAI to 360 DAI).

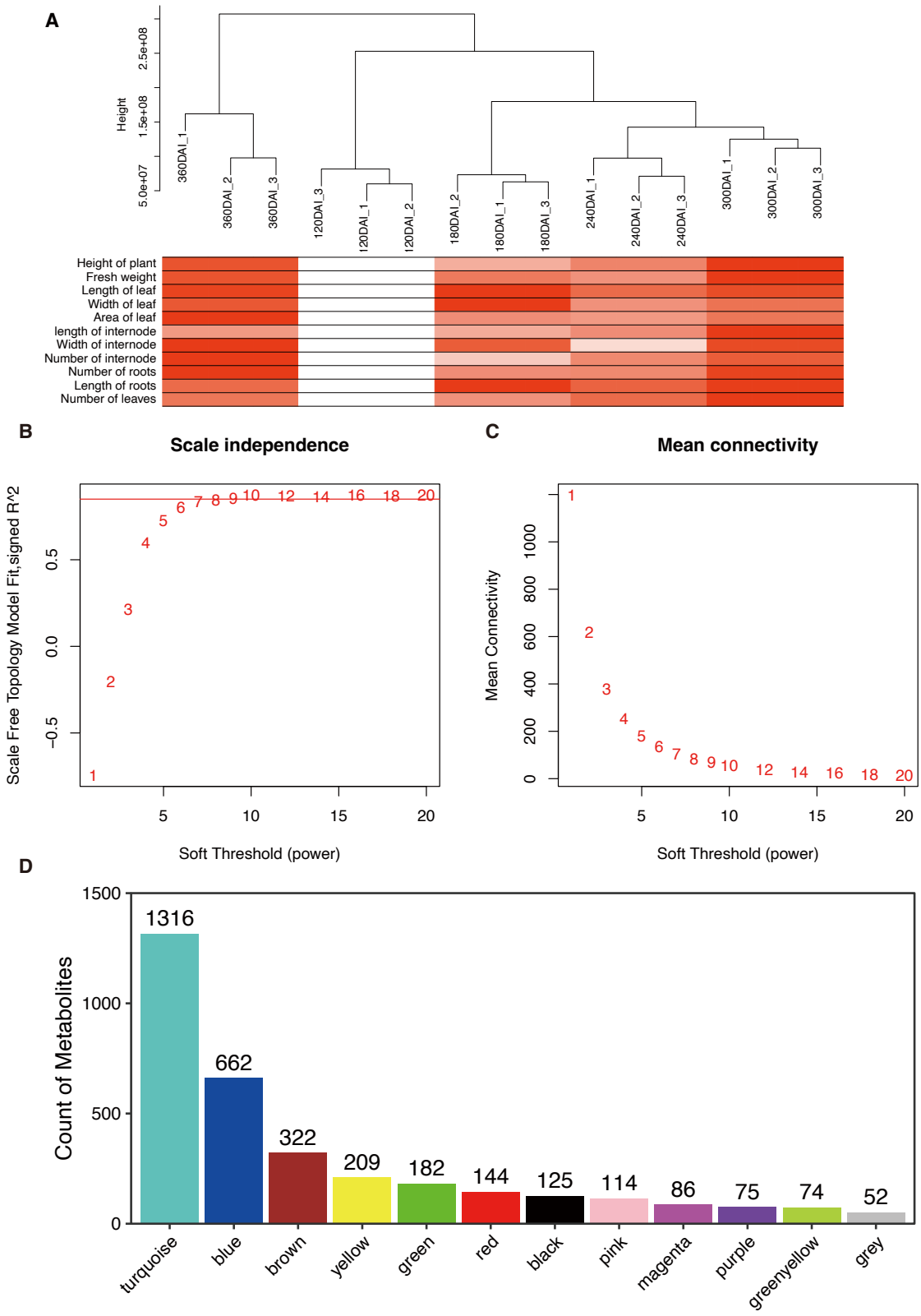


Figure S6: WGCNA analysis of all metabolites. (A) Clustering dendrogram of samples and heatmap of phenotypic traits. The color intensity in the heatmap represents the standardized values of each trait. (B) Scale independence of soft threshold. (C) Mean connectivity of soft threshold. (D) The count of metabolites of different modules.

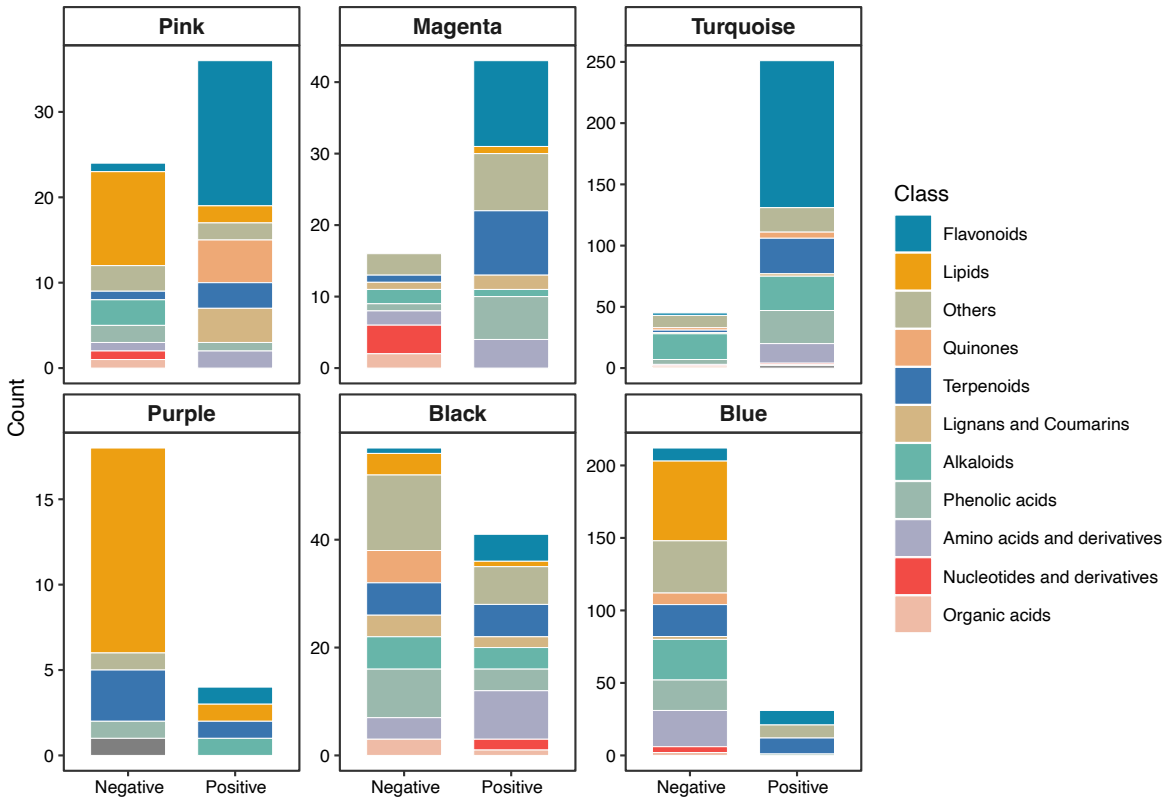


Figure S7: Classification of metabolites within specific correlation modules. Positive/Negative shows the count of metabolite classes that are positively or negatively correlated with the module's eigengene or associated trait.

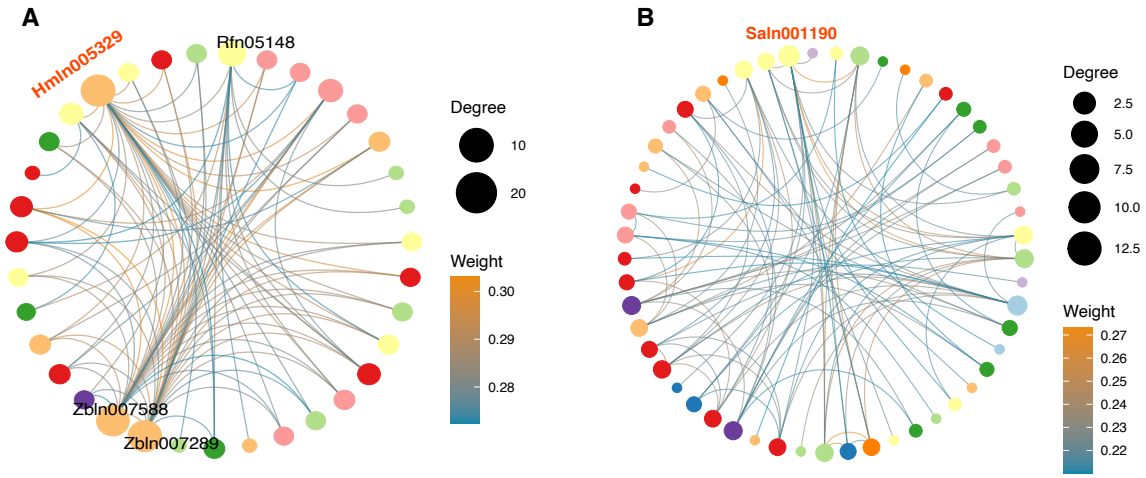
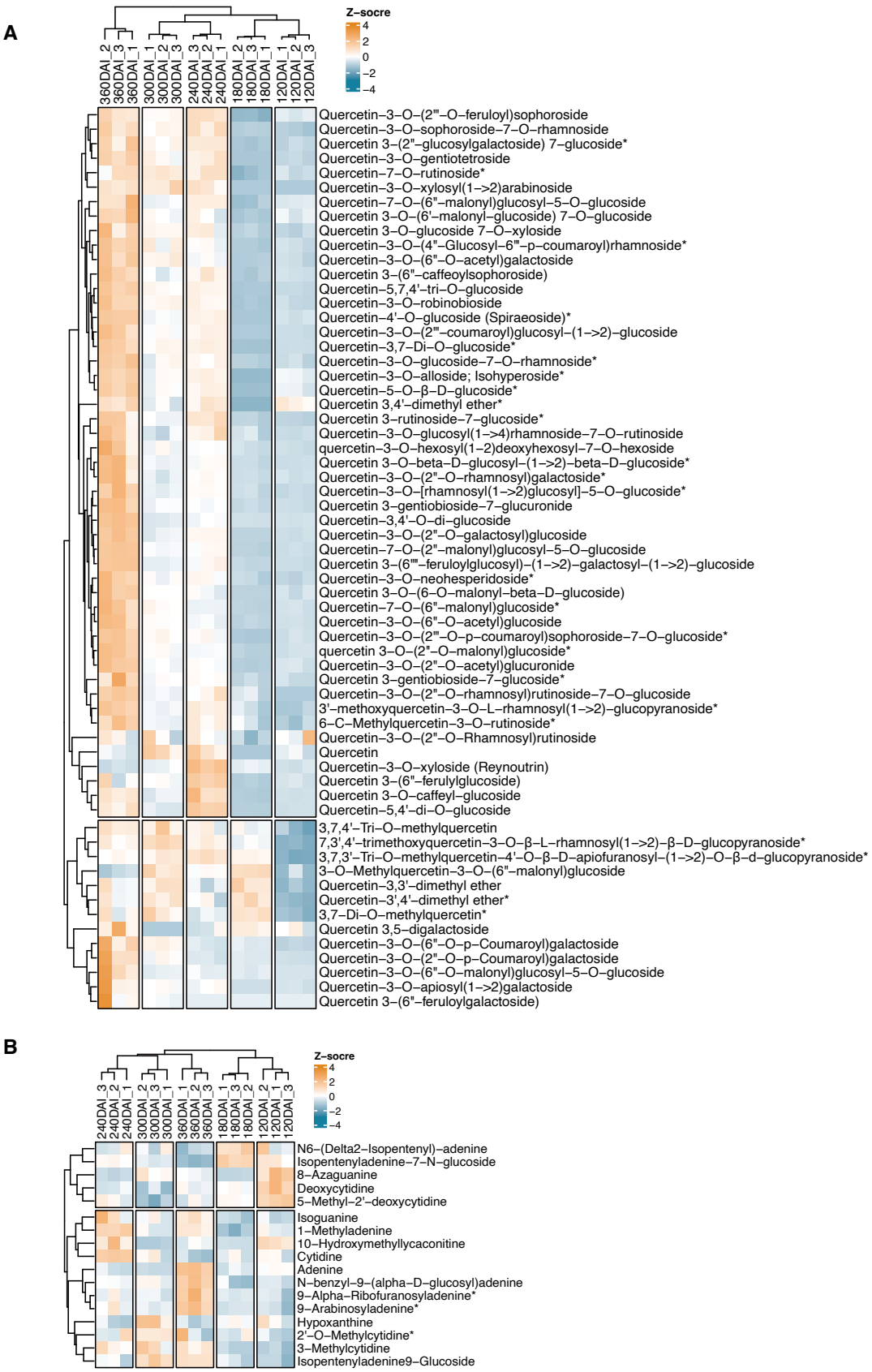
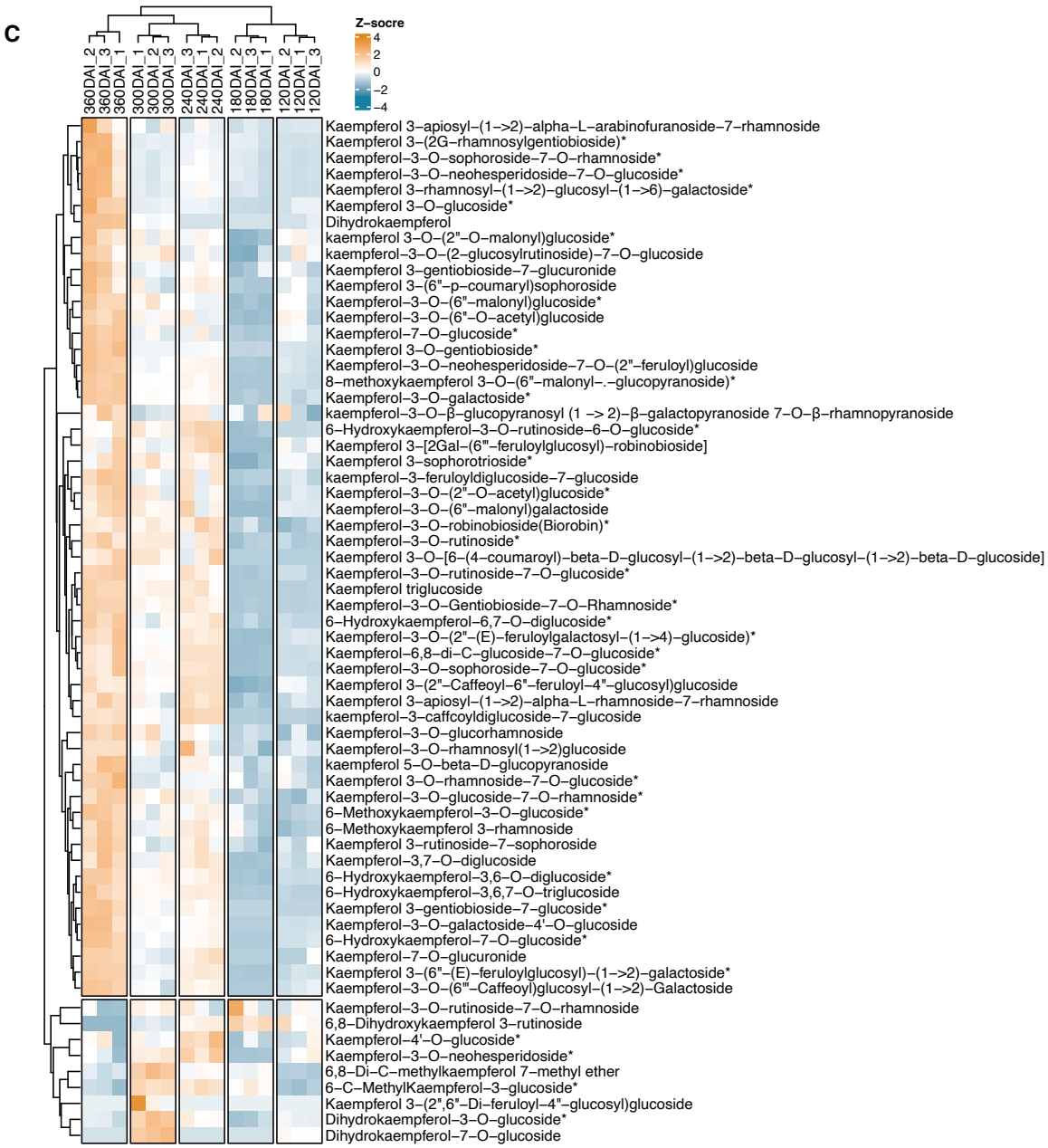


Figure S8: (A) Turquoise and (B) purple modules


A
Z-socre
4
2
0
−2
−4
360DAI_2
360DAI_3
360DAI_1
300DAI_1
300DAI_2
300DAI_3
240DAI_3
240DAI_2
240DAI_1
180DAI_2
180DAI_3
180DAI_1
120DAI_1
120DAI_2
120DAI_3
Quercetin−3−O−(2'''−O−feruloyl)sophoroside
Quercetin−3−O−sophoroside−7−O−rhamnoside
Quercetin 3−(2''−glucosylgalactoside) 7−glucoside*
Quercetin−3−O−gentiotetroside
Quercetin−7−O−rutinoside*
Quercetin−3−O−xylosyl(1−>2)arabinoside
Quercetin−7−O−(6''−malonyl)glucosyl−5−O−glucoside
Quercetin 3−O−(6'−malonyl−glucoside) 7−O−glucoside
Quercetin 3−O−glucoside 7−O−xyloside
Quercetin−3−O−(4''−Glucosyl−6'''−p−coumaroyl)rhamnoside*
Quercetin−3−O−(6''−O−acetyl)galactoside
Quercetin 3−(6''−caffeoylsophoroside)
Quercetin−5,7,4'−tri−O−glucoside
Quercetin−3−O−robinobioside
Quercetin−4'−O−glucoside (Spiraeoside)*
Quercetin−3−O−(2'''−coumaroyl)glucosyl−(1−>2)−glucoside
Quercetin−3,7−Di−O−glucoside*
Quercetin−3−O−glucoside−7−O−rhamnoside*
Quercetin−3−O−alloside; Isohyperoside*
Quercetin−5−O−β−D−glucoside*
Quercetin 3,4'−dimethyl ether*
Quercetin 3−rutinoside−7−glucoside*
Quercetin−3−O−glucosyl(1−>4)rhamnoside−7−O−rutinoside
quercetin−3−O−hexosyl(1−2)deoxyhexosyl−7−O−hexoside
Quercetin 3−O−beta−D−glucosyl−(1−>2)−beta−D−glucoside*
Quercetin−3−O−(2''−O−rhamnosyl)galactoside*
Quercetin−3−O−[rhamnosyl(1−>2)glucosyl]−5−O−glucoside*
Quercetin 3−gentiobioside−7−glucuronide
Quercetin−3,4'−O−di−glucoside
Quercetin−3−O−(2''−O−galactosyl)glucoside
Quercetin−7−O−(2''−malonyl)glucosyl−5−O−glucoside
Quercetin 3−(6'''−feruloylglucosyl)−(1−>2)−galactosyl−(1−>2)−glucoside
Quercetin−3−O−neohesperidoside*
Quercetin 3−O−(6−O−malonyl−beta−D−glucoside)
Quercetin−7−O−(6''−malonyl)glucoside*
Quercetin−3−O−(6''−O−acetyl)glucoside
Quercetin−3−O−(2'''−O−p−coumaroyl)sophoroside−7−O−glucoside*
quercetin 3−O−(2''−O−malonyl)glucoside*
Quercetin−3−O−(2''−O−acetyl)glucuronide
Quercetin 3−gentiobioside−7−glucoside*
Quercetin−3−O−(2''−O−rhamnosyl)rutinoside−7−O−glucoside
3'−methoxyquercetin−3−O−L−rhamnosyl(1−>2)−glucopyranoside*
6−C−Methylquercetin−3−O−rutinoside*
Quercetin−3−O−(2''−O−Rhamnosyl)rutinoside
Quercetin
Quercetin−3−O−xyloside (Reynoutrin)
Quercetin 3−(6''−ferulylglucoside)
Quercetin 3−O−caffeyl−glucoside
Quercetin−5,4'−di−O−glucoside
3,7,4'−Tri−O−methylquercetin
7,3',4'−trimethoxyquercetin−3−O−β−L−rhamnosyl(1−>2)−β−D−glucopyranoside*
3,7,3'−Tri−O−methylquercetin−4'−O−β−D−apiofuranosyl−(1−>2)−O−β−d−glucopyranoside*
3−O−Methylquercetin−3−O−(6''−malonyl)glucoside
Quercetin−3,3'−dimethyl ether
Quercetin−3',4'−dimethyl ether*
3,7−Di−O−methylquercetin*
Quercetin 3,5−digalactoside
Quercetin−3−O−(6''−O−p−Coumaroyl)galactoside
Quercetin−3−O−(2''−O−p−Coumaroyl)galactoside
Quercetin−3−O−(6''−O−malonyl)glucosyl−5−O−glucoside
Quercetin−3−O−apiosyl(1−>2)galactoside
Quercetin 3−(6''−feruloylgalactoside)
B
Z-socre
4
2
0
−2
−4
240DAI_3
240DAI_2
240DAI_1
300DAI_2
300DAI_3
300DAI_1
360DAI_1
360DAI_2
360DAI_3
180DAI_1
180DAI_3
180DAI_2
120DAI_2
120DAI_1
120DAI_3
N6−(Delta2−Isopentenyl)−adenine
Isopentenyladenine−7−N−glucoside
8−Azaguanine
Deoxycytidine
5−Methyl−2'−deoxycytidine
Isoguanine
1−Methyladenine
10−Hydroxymethyllycaconitine
Cytidine
Adenine
N−benzyl−9−(alpha−D−glucosyl)adenine
9−Alpha−Ribofuranosyladenine*
9−Arabinosyladenine*
Hypoxanthine
2'−O−Methylcytidine*
3−Methylcytidine
Isopentenyladenine9−Glucoside


C
Z−socre
4
2
0
−2
−4
360DAI_2
360DAI_3
360DAI_1
300DAI_1
300DAI_2
300DAI_3
240DAI_3
240DAI_1
240DAI_2
180DAI_2
180DAI_3
180DAI_1
120DAI_2
120DAI_1
120DAI_3
Kaempferol 3−apiosyl−(1−>2)−alpha−L−arabinofuranoside−7−rhamnoside
Kaempferol 3−(2G−rhamnosylgentiobioside)*
Kaempferol−3−O−sophoroside−7−O−rhamnoside*
Kaempferol−3−O−neohesperidoside−7−O−glucoside*
Kaempferol 3−rhamnosyl−(1−>2)−glucosyl−(1−>6)−galactoside*
Kaempferol 3−O−glucoside*
Dihydrokaempferol
kaempferol 3−O−(2"−O−malonyl)glucoside*
kaempferol−3−O−(2−glucosylrutinoside)−7−O−glucoside
Kaempferol 3−gentiobioside−7−glucuronide
Kaempferol 3−(6"−p−coumaryl)sophoroside
Kaempferol−3−O−(6"−malonyl)glucoside*
Kaempferol−3−O−(6"−O−acetyl)glucoside
Kaempferol−7−O−glucoside*
Kaempferol 3−O−gentiobioside*
Kaempferol−3−O−neohesperidoside−7−O−(2"−feruloyl)glucoside
8−methoxykaempferol 3−O−(6"−malonyl−.−glucopyranoside)*
Kaempferol−3−O−galactoside*
kaempferol−3−O−β−glucopyranosyl (1 −> 2)−β−galactopyranoside 7−O−β−rhamnopyranoside
6−Hydroxykaempferol−3−O−rutinoside−6−O−glucoside*
Kaempferol 3−[2Gal−(6'"−feruloylglucosyl)−robinobioside]
Kaempferol 3−sophorotrioside*
kaempferol−3−feruloyldiglucoside−7−glucoside
Kaempferol−3−O−(2"−O−acetyl)glucoside*
Kaempferol−3−O−(6"−malonyl)galactoside
Kaempferol−3−O−robinobioside(Biorobin)*
Kaempferol−3−O−rutinoside*
Kaempferol 3−O−[6−(4−coumaroyl)−beta−D−glucosyl−(1−>2)−beta−D−glucosyl−(1−>2)−beta−D−glucoside]
Kaempferol−3−O−rutinoside−7−O−glucoside*
Kaempferol triglucoside
Kaempferol−3−O−Gentiobioside−7−O−Rhamnoside*
6−Hydroxykaempferol−6,7−O−diglucoside*
Kaempferol−3−O−(2"−(E)−feruloylgalactosyl−(1−>4)−glucoside)*
Kaempferol−6,8−di−C−glucoside−7−O−glucoside*
Kaempferol−3−O−sophoroside−7−O−glucoside*
Kaempferol 3−(2"−Caffeoyl−6"−feruloyl−4"−glucosyl)glucoside
Kaempferol 3−apiosyl−(1−>2)−alpha−L−rhamnoside−7−rhamnoside
kaempferol−3−caffcoyldiglucoside−7−glucoside
Kaempferol−3−O−glucorhamnoside
Kaempferol−3−O−rhamnosyl(1−>2)glucoside
kaempferol 5−O−beta−D−glucopyranoside
Kaempferol 3−O−rhamnoside−7−O−glucoside*
Kaempferol−3−O−glucoside−7−O−rhamnoside*
6−Methoxykaempferol−3−O−glucoside*
6−Methoxykaempferol 3−rhamnoside
Kaempferol 3−rutinoside−7−sophoroside
Kaempferol−3,7−O−diglucoside
6−Hydroxykaempferol−3,6−O−diglucoside*
6−Hydroxykaempferol−3,6,7−O−triglucoside
Kaempferol 3−gentiobioside−7−glucoside*
Kaempferol−3−O−galactoside−4'−O−glucoside
6−Hydroxykaempferol−7−O−glucoside*
Kaempferol−7−O−glucuronide
Kaempferol 3−(6"−(E)−feruloylglucosyl)−(1−>2)−galactoside*
Kaempferol−3−O−(6'"−Caffeoyl)glucosyl−(1−>2)−Galactoside
Kaempferol−3−O−rutinoside−7−O−rhamnoside
6,8−Dihydroxykaempferol 3−rutinoside
Kaempferol−4'−O−glucoside*
Kaempferol−3−O−neohesperidoside*
6,8−Di−C−methylkaempferol 7−methyl ether
6−C−MethylKaempferol−3−glucoside*
Kaempferol 3−(2",6"−Di−feruloyl−4"−glucosyl)glucoside
Dihydrokaempferol−3−O−glucoside*
Dihydrokaempferol−7−O−glucoside
D
Z−socre
4
2
0
−2
−4
120DAI_1
120DAI_2
120DAI_3
180DAI_1
180DAI_3
180DAI_2
300DAI_1
300DAI_3
300DAI_2
240DAI_3
240DAI_2
240DAI_1
360DAI_2
360DAI_3
360DAI_1
Rhamnazin 3−O−β−D−glucopyranoside(Flavoyadorinin A)*
Rhamnazin 3−rutinoside*
rhamnazin−3−O−β−D−apiosyl−(1−>2)−O−β−D−glucoside
Glucosyl−rhamnazin−3−O−β−D−glucoside*
Rhamnosyl−Rhamnazin−3−O−β−D−glucoside

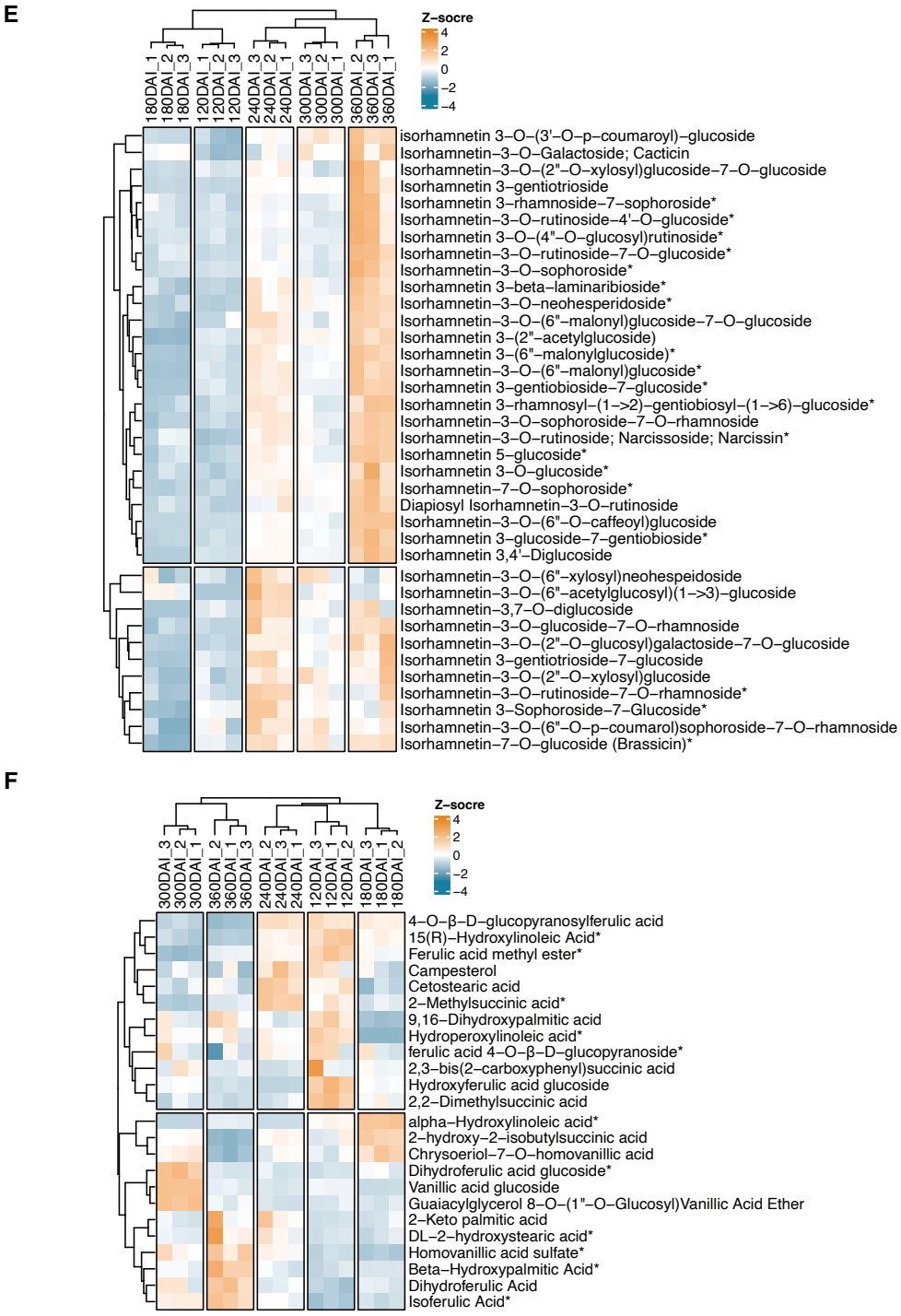


Figure S9: Heatmap of flavonoid glycosides, organic acids and volatile compounds, and steroids based on Z-score normalization across five stages. (A) Quercetin, (B) steroids, (C) kaempferol, (D) rhamnazin, (E) isorhamnetin, and (F) organic acids and volatile compounds.