ANALYSIS OF KEY DIFFERENTIAL METABOLITES IN SUNFLOWER AFTER DOWNY MILDEW INFECTION

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ABSTRACT

The sunflower inbred line 33G was used as the experimental material, which was planted in the downy mildew disease nursery and the normal field, respectively, to examine changes in metabolites differences in metabolic pathways, and the mechanism of regulation of metabolic pathways in the process of sunflower susceptible to downy mildew. At the seedling stage, six biological replicates were collected from the leaves of diseased plants in the disease nursery and non-diseased plants in the normal field, respectively (S33G in the disease nursery and R33G in the normal field). The alterations in metabolites and metabolic pathways in susceptible and normal plants were studied by using LC/MS technology. The results demonstrate that in the S33G-R33G comparison group, 679 differentially expressed metabolites are screened, with 294 up-regulated metabolites and 385 down-regulated metabolites, and the differential metabolites are enriched to 58 metabolic pathways. Alkaloids, fatty acids, flavonoids, terpenoids, and polyketones are the most up-regulated differential metabolites, while lipids and lipid molecules, organic oxygen compounds, organic acids and derivatives, and other compounds are the most down-regulated differential metabolites. By comparison, it is discovered that arachidonic acid metabolism, diterpene biosynthesis, purine metabolism, oxidative phosphorylation, α-linolenic acid metabolism, citrate cycle (TCA cycle), nicotinate and nicotinamide metabolic pathways are considerably activated.

Keywords: sunflower, downy mildew, metabolites, metabolic pathway, function analysis.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the world's fourth most significant oil crop, behind palm oil, soybean oil, and rapeseed oil, with roughly 90% of cultivated sunflowers being oil types (Hladni, 2016). Sunflower is one of the main oil crops in northern China. The current sunflower planting area in China is around 66.7×10^4 ha. Sunflower downy mildew is one of the most common diseases of sunflower production in the world caused by *Plasmopara halstedii* (Farl.) Berl. & deToni. There are eight physiological races of the pathogen, which can be spread through seeds, soil, disease leftovers, and wind. Sunflower downy mildew is a systemic infectious disease that is most hazardous from seed germination to the appearance of the first pair of genuine leaves. When the seedling's root system is infected, it causes seedling growth retardation, leaf chlorosis, and the production of white spores on the backs of the leaves; when the disc is infected, the disc shrinks and becomes unfruitful (Qi et al., 2020), or it causes a loss of plant yield and a sharp decline in quality (Molinero-Ruiz et al., 2003; Jiang et al., 2012). In China, sunflower downy mildew was originally reported by Liu Tiruo in 1963, and later in major sunflower-producing areas across the country. Currently, the effectiveness of applying fungicides to treat seeds as a preventative step is not optimum. Downy mildew resistance in sunflower is governed by *PI* single gene dominance, according to research (Zimmer and Kinman, 1972; Miller and Gulya, 1987, 1991; Tan et al., 1992; Molinero-Ruiz et al., 2003). As a result, if possible, resistant hybridization is an efficient technique of controlling downy mildew. Furthermore, several investigations have discovered that when plants are exposed to biological stress, they initiate autoimmune

responses like as the manufacture of disease-resistant metabolites or the production of signal molecules that govern metabolic processes. Efforts to improve the application method of plant defense inducers may result in improved disease control (Li et al., 2021). Metabolomics, also known as metabolite analysis, can detect and screen metabolites with important biological significance and significant differences by comparing the differences in metabolic patterns between two or more groups of biological samples, and further study the metabolic processes and change mechanism of organisms (Nicholson et al., 1999; Fiehn et al., 2000; Weckwerth and Fiehn, 2002; Ma et al., 2010; Jan and Ahmad, 2019). Metabolites are the final products of cellular regulation and can be regarded as the final response of biological systems to genetic or environmental changes (Ma et al., 2012). It can be utilized as a quantitative trait as well as a biomarker to predict crop phenotypes (Xu et al., 2021). The response of sunflower to downy mildew infection is hypersensitivity, a resistant host response to pathogen attacks (Virányi, 1980; Mouzeyar et al., 1993). According to Herbette et al. (2003), glutathione peroxidase was involved in sunflower hypersensitivity and was linked to the activation of the *hsr203Jlike* gene (Radwan et al., 2005). Chaki et al. (2009) showed that the pathogen of downy mildew caused nitrosative stress in sensitive sunflowers, but not in resistant sunflowers. Researchers have been working to locate resistance genes in sunflower genomes and apply them to plant breeding since the 1990s. Gentzbittel et al. (1998) showed that at least three *NBS*-like loci were located in reported downy mildew resistance locations, according to probes obtained by homologous cloning. Radwan et al. (2004) employed the entire CC-NBS-LLR sequence to carry out PCR markers on the *PI5/PI8* locus, as well as SSR markers to map the *PI_{ARG}* gene, with amazing results. Pankovic et al. (2007) created two enzyme-digested amplified polymorphic sequence markers (CAPS) that were completely co-segregated with the *PI6* gene and resistant to downy mildew 730. CAPS markers were found to be beneficial for marker-assisted selection of the 730 genotype of sunflower downy mildew resistance. In the genetic research of sunflower resistance to downy mildew, De Labrouhe et al. (2005) found that polymerization, alternation, or mixing of a few important genes could extend the effective control time of downy mildew. The induced resistance of sunflower to downy mildew was greatly boosted after treatment with benzothiadiazole. Microscopic observation of the treated tissue revealed a distinctly characteristic shift in the host's infection response, similar to the plant resistance mediated by the *PI* gene after the downy mildew infection (Bán et al., 2004). Treatment of sensitive sunflower varieties with chitosan can early trigger and increase the defense-related response to downy mildew, according to Nandeeshkumar et al. (2008). The sunflower inbred line 33G was utilized as the material in this study, and LC-MS analysis technology was used to analyze the metabolomics of downy mildew-susceptible and normal leaves, as well as to investigate changes in the metabolic group level following downy mildew infection. It provides a theoretical reference for the in-depth consideration of the interaction mechanism between sunflower and downy mildew, as well as the anticipation and control of downy mildew, through the analysis of differential metabolites and metabolic pathways.

MATERIAL AND METHODS

Experimental materials: the test materials were sunflower inbred line 33G, provided from Inner Mongolia Agricultural University and sown in a downy mildew disease nursery and a normal test field during 2020. After the materials were naturally infected, six biologically repeated leaf samples were obtained respectively from the disease nursery and the normal test field on July 8, rinsed with sterile water, treated with liquid nitrogen, and stored at -80°C for further use. Six non-susceptible repeated samples (control group, CK) were numbered as R33G1, R33G2, R33G3, R33G4, R33G5, and R33G6.

Six susceptible repeated samples (treatment group) were numbered as S33G1, S33G2, S33G3, S33G4, S33G5, and S33G6. Water, methanol, acetonitrile, and formic acid were purchased from CNW Technologies GmbH (Düsseldorf, Germany). L-2-chlorophenylalanine was from Shanghai Hengchuang Bio-technology Co. Ltd. (Shanghai, China). LysoPC17: 0 was purchased from Avanti, USA. All chemicals and solvents were analytical or HPLC grade.

Pre-processing of the samples: 80 mg accurately weighed sample was added with internal standard (L-2-chlorophenylalanine, 0.3 mg/mL; Lyso PC17:0, 0.01 mg/mL, all methanol configuration) 20 μL each and 1 mL mixture of methanol and water (7/3, vol/vol) were added to each sample. The samples were then pre-cooled for 2 minutes at -20°C. Then grinded at 60 HZ for 2 min, and ultrasonicated in ice-water bath for 30 min after vortexed, then placed at -20°C for 20 min. Samples were centrifuged at 13000 rpm, 4°C for 10 min. A freezing concentration centrifugal dryer was used to dry 300 μL of supernatant into an LC-MS injection vial. Each sample received 400μL of methanol and water (1/4, vol/vol), vortexed for 30 s, sonicated for 3 min, and then stored at -20°C for 2 hours. The samples were centrifuged for 10 minutes at 13000 rpm and 4°C. The supernatants (150 μL) from each tube were collected using crystal syringes, filtered through 0.22 μm microfilters, and transferred to LC-MS injection vials with foot-lined tubes for analysis.

Liquid chromatography-mass spectrometry analysis conditions: the analytical instrument in this experiment is an LC/MS system comprised of an ACQUITY UPLC system coupled with an AB Triple TOF 5600 System. The analytical conditions were as follows, UPLC: column, ACQUITY UPLC BEH C18 (1.7 μm, 100 mm×2.1 mm); The column oven was set to 45°C; the mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile/methanol (2/3, v/v) with 0.1% formic acid. The flow velocity was set as 0.4 mL per minute; The injection volume was 2 μL. The sample mass spectrometry signal was collected in both positive and negative ion scanning modes using an ESI ion source. The quality control (QC) samples were prepared by mixing aliquots of all samples in equal volume to be a pooled sample. The volume of QC is the same as that of the samples and all extraction reagents are pre-cooled at -20°C before use. Table 1 shows the elution gradients, while Table 2 shows the mass spectrometry parameters.

Table 1. Elution gradient

time / time	A%	B%
0	95	5
2	75	25
9	0	100
13	0	100
13.1	95	5
16	95	5

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Table 2. Mass spectrometry parameter

Parameter	Positive ions	Negative ions
Nebulizer Gas (GS1, PSI)	50	50
Auxiliary Gas (GS2, PSI)	50	50
Curtain Gas (CUR, PSI)	35	35
Ion Source Temperature (℃)	550	550
Ion Spray Voltage (V)	5500	-4500
Declustering Potential (DP, V)	80	-80
Mass Scan Range (TOF MS scan)	50-1000	50-1000
Collision Energy (TOF MS scan, eV)	10	-10
Mass Scan Range (Product Ion scan)	50-1000	50-1000
Collision Energy (Product Ion scan, eV)	30	-30

Data analysis: the Progenesis QIv2.3 software was used to analyze the collected LC-MS raw data on a qualitative and relative quantitative level, as well as perform standardized pretreatment. The pretreatment of the experimental portion, the sample on the machine, and the mass spectrometry system's stability were all studied and evaluated using QC samples. Then, for the metabolic group data of sunflower samples (R33G and S33G), unsupervised principal component analysis (PCA) was performed to observe the overall distribution of the samples and the stability of the entire analysis process. And then, the supervised partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) was carried out to distinguish the overall differences in the metabolic profiles among experimental groups, as well as a combination of multi-dimensional analysis and single-dimensional analysis to screen the differential metabolites between groups. In OPLS-DA analysis, the criteria of screening are VIP>1 (Variable importance in the projection, VIP) of the first principal component of the OPLS-DA model, and the p-value<0.05. Finally, results of metabolic pathway enrichment were obtained using data from the KEGG database. The application database is the KEGG database (website: http://www.genome.jp/KEGG/pathway.html). The Human Metabolome Database (HMDB, website: http://www.hmdb.ca/). METLIN primary data (website: https://metlin. scripps.edu/).

RESULTS AND DISCUSSION

Quantitative statistics of substance peaks and metabolites: the raw data were gathered using UNIFI1.8.1 software and processed using Progenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK) for baseline filtering, peak recognition, integration, retention time correction, peak alignment, and normalization. It were removed the ion peaks with missing values (0 value)>50% in the group from the retrieved data and was replaced the 0 value with half of the minimum value. The qualitative compounds were screened based on the score of the qualitative results of the compounds. The screening standards were 30 points (60 points), with scores below 30 being erroneous qualitative results and deleted. Finally, the positive and negative ion data were combined into a data matrix table that contained all of the information collected from the original data and could be utilized for further analysis. Figure 3 shows the substance peaks and metabolites statistics that were measured. As can be observed, there are a total of 11034 substance peaks, with 5735 in negative ion mode and 5299 in positive ion mode (Figure 1). There are 6542 total metabolites, with 2958 in negative ion mode and 3584 in positive ion mode.
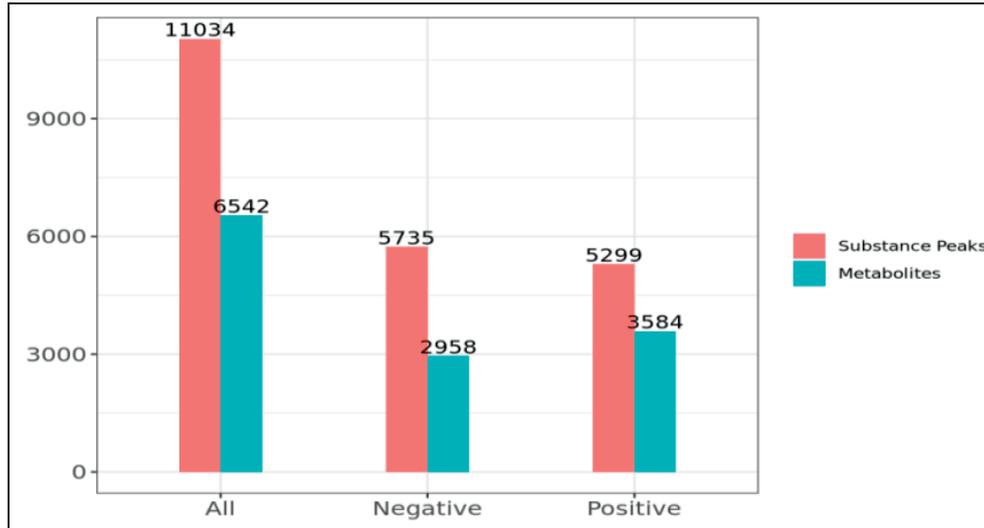
Figure 1. Statistical chart of substance peaks and metabolites

OPLS-DA analysis of susceptible and normal leaves: OPLS-DA (orthogonal partial least squares discriminant analysis) is a supervised statistical method for discriminant analysis. This method is modified based on PLS-DA. The analytical ability and efficacy of the model can be increased by filtering out the noise that is extraneous to the classification information, and the contrasts between different groups within the model can be highlighted to their full potential. The OPLS-DA model of the susceptible group (group S) - the normal group (group R) was built using SIMCA-P software on the measured data of oil sunflower leaves in the susceptible and normal leaves. Figure 2 depicts the model's results. In the OPLS-DA score diagram, the two groups of S-R samples are concentrated respectively, and the two sets of data points are significantly distinguished in space and have significant differences, as shown in Figure 2.

To avoid model overfitting, the study used 7-fold cross-validation and 200 response permutation testing (RPT) to assess the model's quality. The fitting verification results reveal that the obtained parameters $Q2<0$, suggesting that the right prediction samples are obtained and that the OPLS-DA model can effectively identify the largest difference between the control and treatment groups, allowing for further study (Figure 3).
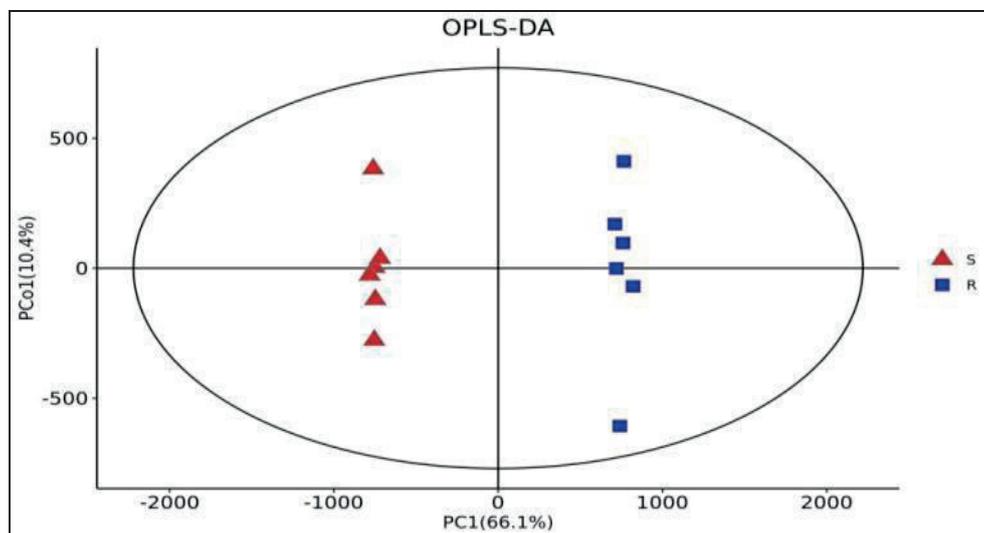

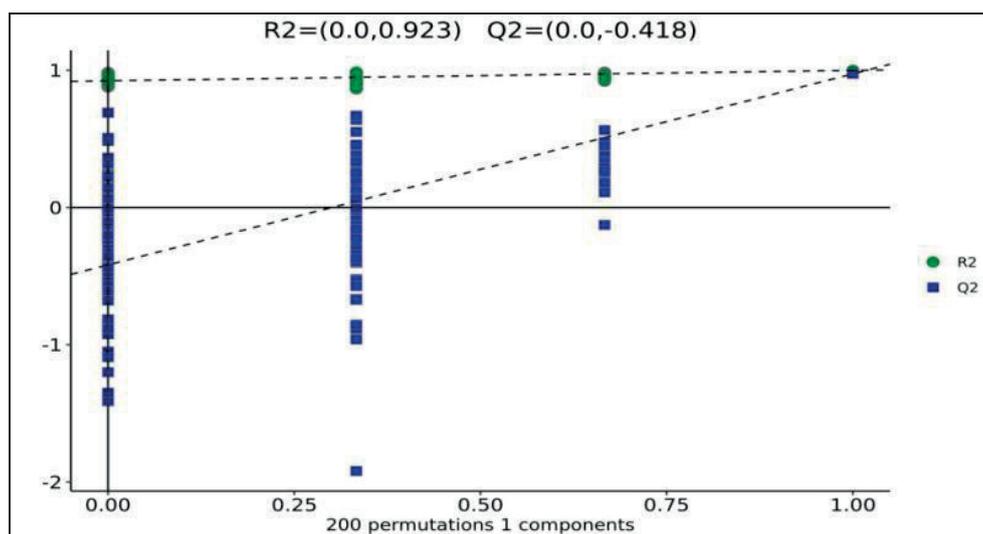*Figure 2.* OPLS-DA score plot between susceptible and normal leaves

Figure 3. Over-fitting validation of susceptible and normal leaves OPLS-DA model

Screening of differential metabolites: multi-dimensional analysis (OPLS-DA) and single-dimensional analysis (t-test) were used to screen differential metabolites between groups in order to accurately screen and acquire differential metabolites. The OPLS-DA analysis' variable important in projection (VIP) was utilized to determine the influence intensity and explanatory power of each metabolite's expression pattern on the classification and discrimination of each set of samples, and the differential metabolites with biological importance were mined. After then, a t-test was done to verify if the differences in metabolites between groups were significant. VIP>1 in the first principal component of the OPLS-DA model and the p-value<0.05 in the t-test were used as screening criteria. The heat map of the screening results is shown in Figure 4. The sample name is shown by the abscissa, and the differential metabolites are represented by the ordinate. The color scale goes from blue to red, indicating low to high expression abundance of the differential metabolites. The redder the hue, the higher the expression abundance of the differential metabolites. The screening results revealed that the S33G-R33G comparison group obtained a total of 679 metabolites in both positive and negative ion modes, with 294 up-regulated and 385 down-regulated. This means that the expression of 294 metabolites was substantially higher in susceptible leaves than in normal leaves, whereas the expression of 385 metabolites was considerably higher in normal leaves than in susceptible leaves. Table 3 lists the top 50 metabolites by VIP ranking. It can be seen that there are 17 up-regulated metabolites and 33 down-regulated metabolites in positive and negative ion modes. Alkaloids, fatty acids, flavonoids, terpenoids, and polyketides were the most up-regulated differential metabolites, while lipids and lipid molecules, organic oxygen compounds, organic acids, and derivatives were the most down-regulated differential metabolites (Table 3).
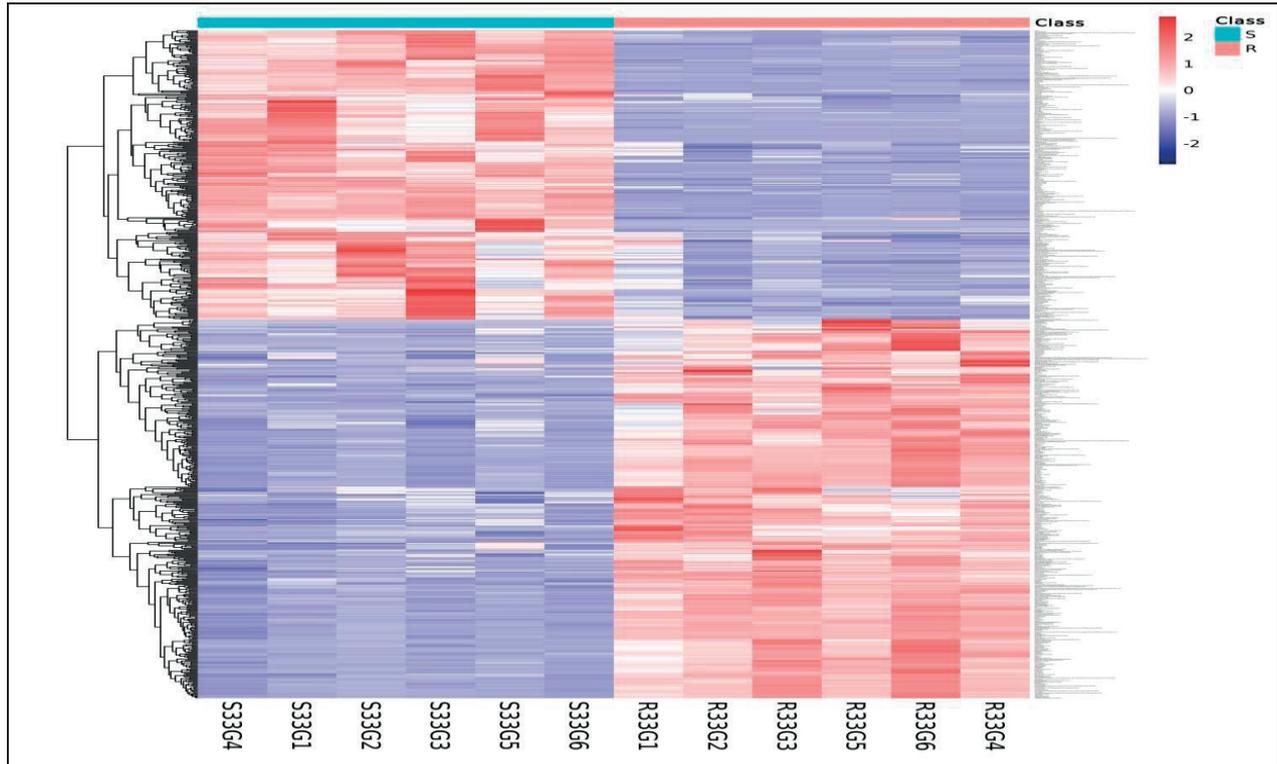
Figure 4. Cluster analysis diagram

Table 3. The top 50 differential metabolites in VIP value

Metabolites (metabolites)	Retention time (min)	VIP	P-value	$\log_2$(FC)	FC (Fold of S/R difference)	Compound ID
Scorzoside	4.494	14.000	6.33E-08	-1.707	0.306	HMDB0040713
Pteroside D	4.482	13.219	2.99E-08	-1.756	0.296	HMDB0036603
3-O-Methylniveusin A	4.482	12.880	2.39E-06	-1.648	0.319	HMDB0039084
Gibberellin A39	3.512	12.210	3.53E-06	-1.611	0.327	HMDB0035045
(1E,4Z,6a,8b,10a)-8-(2-Methylbutanoyloxy)-10,15-dihydroxy-3-oxo-1,4,11(13)-germacratrien-12,6-olide	3.756	10.956	3.50E-09	-1.962	0.257	HMDB0041002
Acetyl tributyl citrate	**8.003**	**10.592**	**8.78E-04**	**0.473**	**1.388**	**89746**
Niveusin C	4.385	10.582	6.58E-08	-1.481	0.358	HMDB0034474
2-HoTrE	**6.433**	**10.508**	**3.43E-04**	**1.078**	**2.112**	**LMFA02000183**
Triethylamine	**9.195**	**10.499**	**1.19E-02**	**0.549**	**1.463**	**70265**
3,4,5-trihydroxy-6-{[(2Z)-6-hydroxy-2-(phenylmethylidene)heptyl]oxy}oxane-2-carboxylic acid	3.584	10.474	7.55E-09	-1.637	0.322	HMDB0126534
4,5-Dihydroniveusin A	3.599	7.795	4.74E-09	-1.639	0.321	HMDB0032844
alpha-Peroxyachifolide	3.971	7.616	3.89E-04	-1.173	0.444	HMDB0039575
Pteroside P	3.959	7.597	6.69E-07	-1.455	0.365	HMDB0036608
Hydroxyisonobilin	4.424	7.431	1.20E-06	-1.485	0.357	HMDB0034475
Molephantinin	4.482	7.423	2.59E-08	-1.815	0.284	67765
3,4,5-trihydroxy-6-(2-hydroxy-6-methoxyphenoxy) oxane-2-carboxylic acid	**1.344**	**7.178**	**9.76E-08**	**3.539**	**11.625**	**HMDB0128037**
Stearidonic acid	**6.433**	**7.106**	**4.17E-05**	**1.094**	**2.134**	**LMFA01030357**
3-O-Methylrimiterol glucuronide	3.597	6.920	1.31E-08	-1.181	0.441	2337
Fastigilin C	3.584	6.897	2.46E-08	-1.839	0.279	67710

Metabolites (metabolites)	Retention time (min)	VIP	P-value	log$_2$(FC)	FC (Fold of S/R difference)	Compound ID
Triphenyl phosphate	**6.709**	**6.783**	**3.87E-03**	**0.425**	**1.343**	**69897**
Hydroxygaleon	4.385	6.705	1.34E-07	-1.544	0.343	HMDB0031535
Troglitazone quinone	3.944	6.639	6.76E-07	-1.041	0.486	2974
9R,10S-EpOME	**6.920**	**6.533**	**1.86E-05**	**0.726**	**1.654**	**43441**
gamma-CEHC Glc	3.857	6.414	4.68E-08	-1.604	0.329	LMPR02020053
Licoricesaponin E2	4.482	6.066	2.72E-06	-2.963	0.128	HMDB0038719
PRIEURIANIN	3.597	6.064	4.92E-06	-2.083	0.236	43870
Artonin P	**1.488**	**6.053**	**6.85E-13**	**2.974**	**7.857**	**LMPK12111511**
Dibutyl phthalate	**7.384**	**6.052**	**5.22E-03**	**0.396**	**1.315**	**69881**
Glycyrrhisoflavanone	**7.725**	**5.947**	**1.70E-03**	**0.570**	**1.484**	**LMPK12050506**
PE (0:0/18:3(6Z,9Z,12Z))	3.256	5.934	1.93E-03	-1.466	0.362	LMGP02050042
Ro 31-8220	3.525	5.752	1.39E-06	-1.604	0.329	45540
Perilloside B	**8.003**	**5.716**	**3.76E-04**	**0.488**	**1.403**	**HMDB0040465**
2-O-(beta-D-galactopyranosyl-(1->6)-beta-D-galactopyranosyl) 2S,3R-dihydroxytridecanoic acid	9.030	5.618	2.80E-03	-2.338	0.198	LMFA13010049
9-hydroxy-10E,14Z-octadecadien-12-ynoic acid	**6.751**	**5.600**	**8.55E-05**	**1.542**	**2.912**	**LMFA02000192**
Gibberellin A66	4.808	5.549	9.62E-06	-2.070	0.238	HMDB0036900
[4-(3-hydroxybut-1-en-1-yl)-2-methoxyphenyl] oxidanesulfonic acid	3.227	5.509	8.73E-03	-1.649	0.319	HMDB0135698
Isocolumbin	3.971	5.474	7.24E-04	-1.202	0.435	HMDB0036837
9-OxoODE	**7.222**	**5.468**	**4.48E-04**	**1.632**	**3.100**	**35860**
Pteroside B	3.850	5.430	1.47E-08	-1.610	0.328	HMDB0030760
Gluconic acid	**0.628**	**5.417**	**2.44E-06**	**1.092**	**2.132**	**345**
Cajaisoflavone	3.597	5.356	1.73E-09	-1.655	0.318	LMPK12050308
Amygdalin	3.683	5.344	5.40E-07	-1.400	0.379	63626
3,4,5-trihydroxy-6- [(14-hydroxy-3-methyl-1,7-dioxo-3,4,5,6,7,8,9,10-octahydro-1H-2-benzoxacyclotetradecin-16-yl) oxy]oxane-2-carboxylic acid	3.197	5.229	1.24E-04	-1.453	0.365	HMDB0134196
2,5-didehydro-D-gluconic acid	**0.830**	**5.127**	**3.24E-06**	**0.795**	**1.735**	**LMFA01050471**
Cynaroside A	3.683	5.126	4.89E-07	-1.523	0.348	HMDB0040532
4,8,14-trihydroxy-17-methoxy-2-oxatricyclo[13.2.2.1³,] icosa-1(17),3,5,7(20),15,18-hexaen-10-one	3.527	5.114	5.39E-07	-1.895	0.269	HMDB0133425
Absindiol	3.109	4.681	2.67E-07	-1.740	0.299	HMDB0033229
Casegravol isovalerate	3.956	4.674	5.78E-06	-1.246	0.422	HMDB0039034
Cellopentaose	**0.824**	**4.593**	**4.34E-08**	**7.494**	**180.320**	**63207**
Maltohexaose	**0.85883**	**4.57 9**	**2.1E-05**	**9.14218**	**565.028**	**62889**

The red fonts and bold in the table indicate significantly up-regulated differential metabolites, and the unmarked red fonts indicate significantly down-regulated differential metabolites.

Metabolic pathway enrichment and key differential metabolites analysis: differential metabolites pathway enrichment analysis aids in determining the mechanism of metabolic pathway variations in distinct samples. In this study, the metabolic pathways of differential metabolites were enriched based on the KEGG database (https://www.kegg.jp/). In the S and R groups, there were 58 metabolic pathways annotated, of which 7 were enriched to metabolic pathways with an apparent p-value less than 0.05. The ordinate of the metabolic pathway enrichment diagram was log10 (p-value), while the abscissa was the metabolic pathway name (Figure 5). The obviousness of the metabolic pathway's enrichment was measured by the p-value in the metabolic pathway. The red line

represented a p-value of 0.01, whereas the blue line represented a p-value of 0.05. The signal pathway was obvious when the top of the column was higher than the blue line. The seven metabolic pathways involved in the differential metabolites caused by normal and susceptible leaves were arachidonic acid metabolism, diterpene biosynthesis, purine metabolism, oxidative phosphorylation, α-linolenic acid metabolism, citric acid cycle (TCA cycle), nicotinic acid and nicotinamide metabolism, as shown in Figure 5, with arachidonic acid metabolism, diterpene biosynthesis, and purine metabolism reaching an extremely significant level (p=0.01) among them. 17 important differential metabolites were screened and assigned to the seven metabolic pathways based on the conditions that the fold-change threshold>1.5 or fold-change threshold<0.5, VIP>1, p<0.05 were met at the same time, as shown in Table 4.

It can be seen from Table 4 that there are four key differential metabolites involved in the arachidonic acid metabolic pathway, namely delta-12-PGJ2, LTD4, 12-keto-10,11,14,15-tetrahydro-LTB4, and leukotriene F4, among which delta-12-PGJ2 and LTD4 are up-regulated, while 12-keto-10,11,14,15-tetrahydro-LTB4 and leukotriene F4 are down-regulated. Gibberellin A34, Gibberellin A29, Gibberellin A8-catabolite, Gibberellin A1, and ent-Copalyl diphosphate

were the five differential metabolites implicated in diterpenoid biosynthesis, with ent-Copalyl diphosphate being up-regulated and the others down-regulated. Guanine and Adenine, two important differential metabolites in purine metabolism, were both up-regulated. Succinic acid, a major differential metabolite involved in the oxidative phosphorylation pathway, was up-regulated. There were three differential metabolites in the α-linolenic acid metabolic pathway, namely 9 (S) -HOTrE, Stearidonic acid, and alpha-Linolenic acid were all up-regulated; there were two differential metabolites in the citrate cycle (TCA cycle), Citric acid and Succinic acid, were all up-regulated. Nicotinate D-ribonucleoside, Trigonelline, and Succinic acid were three important differential metabolites involved in the metabolic pathways of nicotinate acid and nicotinamide, and they were all up-regulated. Succinic acid was found to be involved in oxidative phosphorylation, citrate cycle (TCA cycle), nicotinic acid, and nicotinamide metabolic pathways, as well as being up-regulated in these three metabolic pathways. In conclusion, 17 major differential metabolites annotated to seven metabolic pathways may be involved in sunflower resistance to downy mildew pathogen infection or may be the metabolites of plant cell damage.
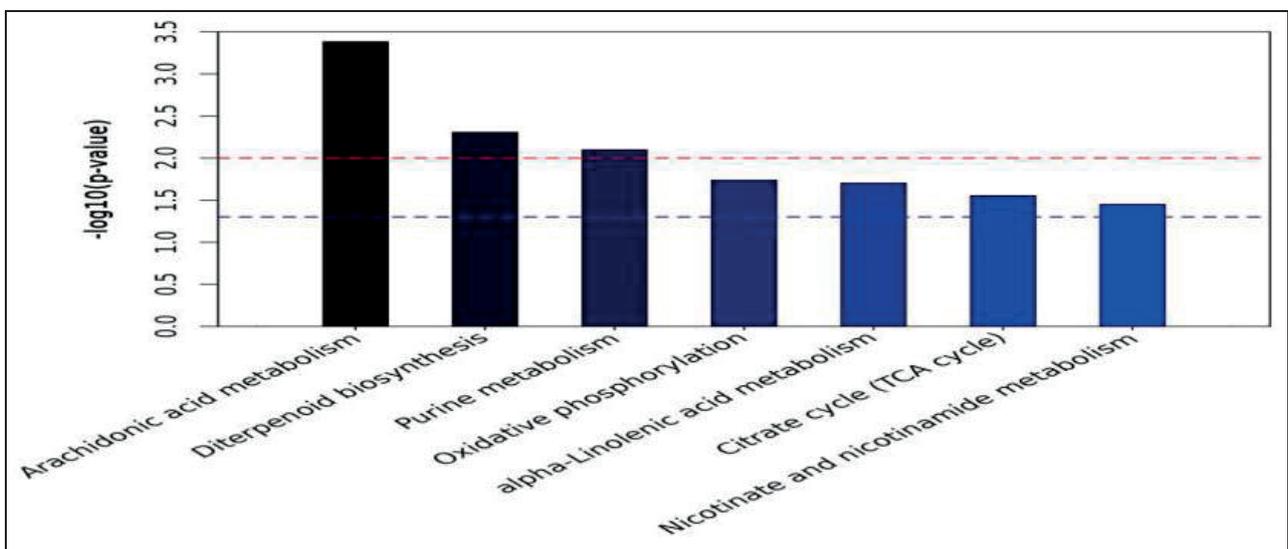
The red line indicates p<0.01, and the blue line indicates p<0.05. When the top of the column exceeds the red line, the signal pathway represented by it has a very significant difference, and when it exceeds the blue line, the signal pathway represented by it has a significant difference.

Figure 5. Pathway analysis of S-R DEMs

Table 4. Key metabolites involved in 7 metabolic pathways that were significantly enriched

| Pathway	Key Metabolites	KEGG	FC	VIP	ID Annotation	P-value
Arachidonic acid metabolism	delta-12-PGJ2	C05958	2.7 3	1.0446	han00590	0.000001
	LTD4	C05951	2.5 6	1.0404	han00590	0.000011
	12-keto-10,11,14,15-tetrahydro-LTB4	C02165	0.36	1.1389	han00590	0.000925
	leukotriene F4	C06462	0.1 4	1.0395	han00590	0.000006
Diterpenoid biosynthesis	Gibberellin A34	C11868	0.2 9	1.8187	han00904	0.000000
	Gibberellin A29	C06096	0.24	1.4752	han00904	0.000001
	Gibberellin A8-catabolite	C11870	0.15	2.6046	han00904	0.000150
	Gibberellin A1	C00859	0.12	1.3936	han00904	0.000021
	ent-Copalyl diphosphate	C06089	20.39	1.4080	han00904	0.000007
Purine metabolism	Guanine	C00242	2.5 8	1.1594	han00230	0.037951
	Adenine	C00147	1.9 4	3.8547	han00230	0.000002
Oxidative phosphorylation	Succinic acid	C00042	1.55	1.5697	han00190	0.000004
Alpha-linolenic acid metabolism	9(S)-HOTrE	C16326	3. 20	4.464	han00592	0.000839
	Stearidonic acid	C16300	2.1 3	7.1059	han00592	0.000042
	alpha-Linolenic acid	C06427	1. 60	3.2742	han00592	0.000630
Citrate Cycle (TCA Cycle)	Citric acid	C00158	1.6 7	1.2102	han00020	0.000618
	Succinic acid	C00042	1.55	1.5697	han00020	0.000004
Nicotinate and nicotinamide metabolism	Nicotinate D-ribonucleoside	C05841	2.4 3	1.078	han00760	0.001304
	Trigonelline	C01004	2. 20	1.1213	han00760	0.006337
	Succinic acid	C00042	1.55	1.5697	han00760	0.000004

In the table, FC>1.5 is the key differential metabolites that up-regulated significantly, and FC<0.5 is the key differential metabolites that down-regulated significantly.

***Functional analysis of key differential metabolites*:** in this study, the enantio-cobayl diphosphate and succinic acid of the S group were found to be significantly up-regulated compared with the R group, indicating that the expression of enantio-cobayl diphosphate and succinic acid in susceptible leaves was significantly higher than that in normal leaves.

Furthermore, according to the physiological indexes determined in this study, the oxalic acid level in susceptible leaves was 2.0384 mg/g, which was considerably greater (p=0.05) than that in normal leaves (1.3173 mg/g) (Figure 6A). The ent-Copalyl diphosphate is located at the beginning of oxalic acid anabolism, indicating that the significant increase in oxalic acid content in susceptible leaves may be related to the up-regulated expression of ent-Copalyl diphosphate and succinic acid, as shown in the metabolic pathway of oxalic acid synthesis (Figure 7).

The differential metabolite L-arginine phosphate was significantly down-regulated, showing that L-arginine phosphate expression was significantly higher in normal leaves than in susceptible leaves. At the same time, the glutathione level in normal leaves was 58.67 ng/ml, which was considerably higher (p=0.05) than that in susceptible leaves (50.56 ng/ml) according to the physiological indicators of downy mildew assessed in this study (Figure 6B). L-arginine phosphate was discovered to be involved in glutathione production in studies. As a substrate, L-arginine phosphate creates glutathione (Figure 7), implying that the rise in glutathione concentration may be linked to the down-regulation of L-arginine phosphoric acid in normal leaves. Reduced glutathione has been proven in studies to have a significant role in maintaining the integrity of plant cell membrane structure and avoiding membrane lipid peroxidation induced by free radical accumulation during stress.
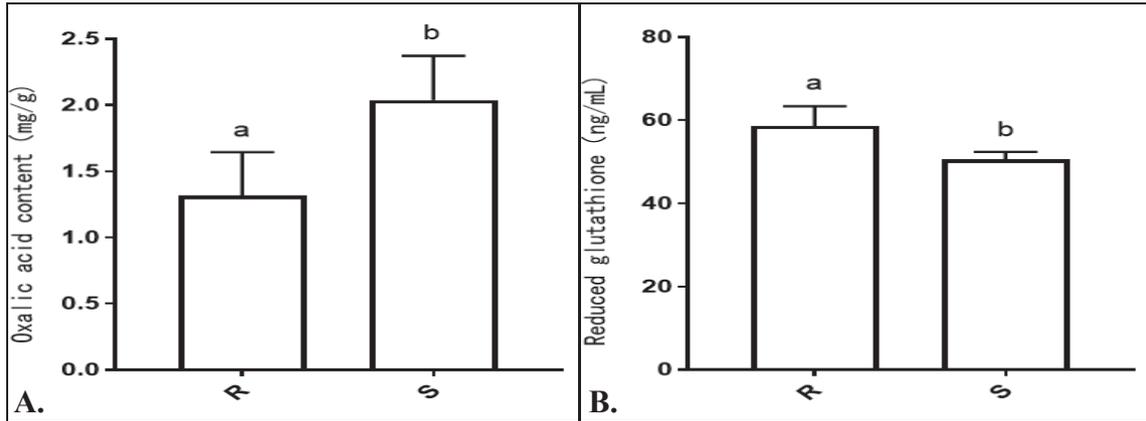
The value in the figure is the mean value of standard deviation, the error bars represent the standard deviation of 6 biological replicates, and the lowercase letters indicate that the difference in t test was statistically significant ($p<0.05$).

Figure 6. Contents of oxalic acid and reduced glutathione in normal and susceptible leaves.
Contents of oxalic acid in normal and susceptible leaves (A).
Contents of reduced glutathione in normal and susceptible leaves (B).
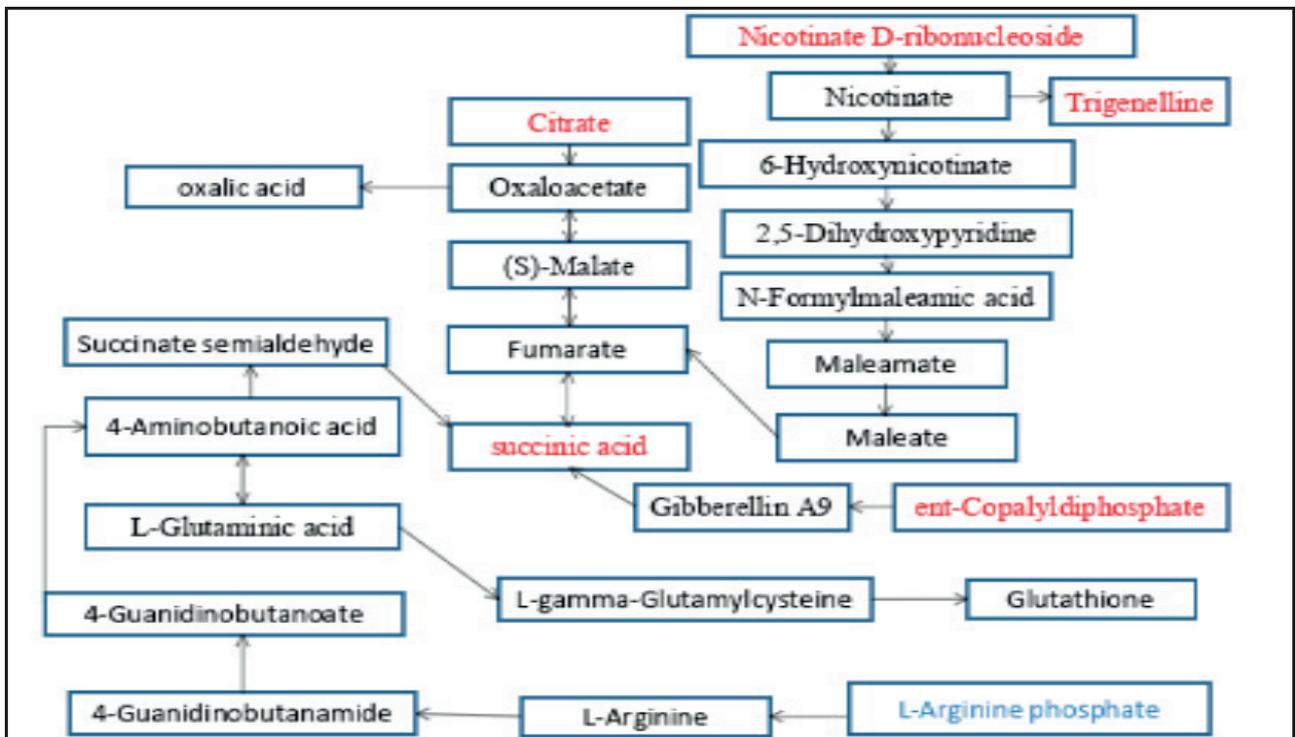
Red fonts are up-regulated substances in the pathway and blue fonts are down-regulated substances in the pathway

Figure 7. Biosynthesis metabolic pathway diagram of oxalic acid and glutathione

Downy mildew is one of the major diseases threatening sunflower production around the world (Zhang et al., 2017). It is a fungal soil-borne disease caused by the Hols uniaxial enzyme of obligate parasite flagella submens. Plants' roots, stems, blooms, and fruits can all be infected by the fungus. Hols uniaxial enzyme is a pathogen with thick-walled oospores that can live in soil for years. Oospores can create zoospores after germination, which then release zoospores to infect germinated seedlings. Plants become infected throughout their bodies, and the majority of infected plants eventually die (Harveson et al., 2016). As a result, it is critical and challenging to strengthen research into the prevention and control of downy mildew, as well as to find key genes and metabolites that can combat downy mildew. Zhang et al. *(*2017) successfully transferred

the first downy mildew resistant gene from wild sunflower into edible sunflower, which was a big breakthrough in downy mildew resistance sunflower breeding.

For a long time, studies on sunflower downy mildew have mostly concentrated on biology, host-pathogen relationships, and disease control, with studies on metabolites associated with the illness being rare. A variety of differential metabolites were screened with normal and susceptible leaves as test materials before and after the sunflower was infected by downy mildew in this study, which should be related to the tendency of sunflower resistance to downy mildew or disease aggravation. Except for the differential metabolites enriched in differential metabolic pathways mentioned above, the content of gallic catechin, a phenolic compound involved in flavonoid biosynthesis, was down-regulated among the screened significant differential metabolites, indicating that gallic catechin expression in normal leaves was significantly higher than that in susceptible leaves. Dai et al. (1995) discovered that resistant grape callus had higher levels of gallic catechin derivatives than sensitive callus, proving that phenolic compounds gallic catechin derivatives had certain antifungal activity against downy mildew. Gallic catechin derivatives' antibacterial activity may help to protect against downy mildew infection by directly damaging fungi metabolism or causing hyphae to lose their natural flexibility. Oxalic acid is an important disease-related metabolite that can effectively regulate plant-pathogen interactions. Malenčić et al., (2004) investigated the effects of various oxalic acid concentrations on the antioxidant system of sunflower inbred lines. The findings revealed that oxalic acid can increase the activity of antioxidant enzymes in plants and enhance disease resistance. Reduced glutathione functions as an antioxidant, regulating cellular redox state and maintaining intracellular homeostasis. Gallego et al. (2005) discovered that high cadmium stress significantly enhanced membrane lipid peroxidation and protein peroxidation in plant calluses. Reduced glutathione played a leading role in the

adaptive response to cadmium stress and helped to maintain a redox state in physiological conditions. The increased content of L-arginine phosphate in normal leaves, as a substrate for the synthesis of reduced glutathione, may increase the expression of reduced glutathione in normal leaves, enhance the antioxidant mechanism of sunflower under stress, and maintain the balance of redox state in plants, allowing sunflower to resist downy mildew infection.

Salicylic acid is an important plant hormone that not only governs flowering growth, gender differentiation, stomatal movement, and photoperiod but also is a sufficient and necessary factor to induce pathogen defense responses (Raskin, 1992). Salicylic acid-mediated plant defense is an important defense route for plants to resist pathogens that are biotrophic or semi-trophic (Palmer et al., 2017). After identifying the invading pathogens, salicylic acid can not only induce local defense responses but also promote systemic acquired resistance (An and Mou, 2011). By inducing systemic defense responses such as the synthesis of pathogen-associated PR proteins, phytoalexin, and enhanced cell wall thickness, systemic acquired resistance can protect plants from further pathogen colonization. The verticillium wilt resistance *GhGPA* gene isolated from cotton also plays an important role in verticillium wilt resistance by inducing salicylic acid and jasmonic acid signaling pathways and regulating the production of reactive oxygen species (Chen et al., 2021). In addiction, the phenylalanine ammonialyase pathway, which converts phenylalanine to transcinnamic acid, then to benzoic acid, and finally to salicylic acid, can also synthesize salicylic acid (Mauch-Mani and Slusarenko, 1996). 4-hydroxybenzoic acid was significantly up-regulated among the screened differential metabolites, indicating that the content of 4-hydroxybenzoic acid in susceptible leaves was significantly higher than that in normal leaves. The transformation of 4-hydroxybenzoic acid into salicylic acid is speculated to be the cause of the decrease in 4-hydroxybenzoic acid content in normal

leaves. Salicylic acid is overexpressed in plants, resulting in disease resistance.

β-aminobutyric acid (BABA) and potassium phosphite can induce downy mildew resistance in lettuce and protect it against infection, and this protection is both systematic and long-term. The synthesis of a series of compounds involved in the resistance mechanism, such as plant disease-related proteins (PR), phytoalexins, or nitric oxide, could be the mechanism of action (Pajot et al., 2001). Pearl millet treated with β-aminobutyric acid can diminish downy mildew infection on seedlings, reduce disease severity, and the induced protective effect is long-lasting and effective. Other researches have shown that is closely linked β-aminobutyric acid's protective impact to the accumulation of defense-related proteins including phenylalanine ammonialyase, peroxidase, β-1,3-glucanase, and cell wall hydroxyproline-rich glycoprotein (HRGP) (Shailasree et al., 2007). According to studies by Slaughter et al. (2008), BABA treatment causes plants to accumulate pterostilbene, a phytoalexin that does not exist in plants but can effectively interfere with the flow of zoospores and the development of general hyphae. The differential metabolite 5-aminovaleric acid, which is the same non-protein amino acid as β-aminobutyric acid and is a methylene homologue of γ-aminobutyric acid (GABA), is significantly down-regulated in this study, indicating that the content of this metabolite in normal leaves is significantly higher than that in susceptible leaves. Many studies have reported the resistance of β-aminobutyric acid to downy mildew, therefore it's possible that higher 5-aminovalerate expression in normal leaves may be linked to downy mildew resistance, but no relevant research has been published yet, so further research is needed.

CONCLUSIONS

In summary, through the metabolomics analysis of sunflower leaf samples in different treatment groups (susceptible group and normal group), a total of 679 significant differential metabolites were screened out, which were related to the process of sunflower downy mildew, including 294 up-regulated differential metabolites, mainly alkaloids, fatty acids, flavonoids, terpenoids, and polyketides. A total of 385 differential metabolites were down-regulated, mainly including lipids and lipid molecules, organic oxygen compounds, organic acids, and derivatives. A total of 58 metabolic pathways were enriched in differential metabolites, of which 7 were significantly activated, including arachidonic acid metabolism, diterpene biosynthesis, purine metabolism, oxidative phosphorylation, α-linolenic acid metabolism, citrate cycle (TCA cycle), nicotinate and nicotinamide metabolic pathways.

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