Metabolic analysis preliminarily reveals the defensive mechanism of coronatine to Mulberry Bacterial Blight caused by *Pseudomonas syringae* pv. *mori* in mulberry seedlings

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Coronatine (COR) can enhance resistance to *P. syringae* pv. *mori* in the mulberry to avoid the outbreak of Mulberry Bacterial Blight. In order to analyze the defensive mechanism, metabolic responses of mulberry leaves to COR were examined by gas chromatography-mass spectrometry and biochemical analysis. Antimicrobial activities of metabolites against *P. syringae in vitro* were also detected by colorimetric assay. Metabolic profiles revealed that 0.01 μM COR induced changes of various metabolites including organic acids, sugars, alcohols, ketones, phenols, hydrocarbons, and nitrogen or sulfur compounds. In particular, sugars, organic acids, phenols, flavonoids, and nitrogen or sulfur compounds increased significantly in the COR-treated leaves. Biochemical analysis also confirmed above findings. 1H-indole-3-acetic acid and abscisic acid were increased to regulate the signaling pathways; meanwhile, hydrogen peroxide and nitric oxide were induced to participate in the metabolic changes as signaling molecules. Concurrently, the metabolites showed antimicrobial activities against *P. syringae*. These results indicated that COR could increase contents of antimicrobial compounds by modulating the metabolic process to resist the pathogens entering into the plant tissues and prevent the outbreak of Mulberry Bacterial Blight.

Keywords: mulberry; metabolite; coronatine; *P. syringae*; plant immunity inducer; antimicrobial activity.

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Abbreviations: COR: Coronatine; PAMPs: Pathogen-associated molecular patterns; PRRs: Pattern recognition receptors; PTI: PAMP-triggered immunity; ETI: Effector-triggered immunity; JA: Jasmonic acid; Pn: Net photosynthesis rate; Gs: Stomatal conductance; Ci: Intercellular CO_2 concentration; Trmmol: Transpiration rate; RuBisCO: Ribulose-1, 5-bisphosphate carboxylase/oxygenase; ABA: Abscisic acid; IAA: Indole acetic acid; DCFH-DA: 2′, 7′-Dichlorofluorescin diacetate; DAF-FMDA: 3-Amino, 4-aminomethyl-2', 7'-difluorescein, diacetate; NOS: Nitric oxide synthetase; NR: Nitrate reductase; L-NAME: N-nitro-L-arginine methyl ester; NO: Nitric oxide.

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Introduction

Mulberry is one of the economic plants and has been widely distributed all over the world. Mulberry is considered insensitive to drought, high or low temperature, pH value, and salinity of the soil; but pathogens infection is recognized as the most devastatingly environmental stress [1]. Mulberry Bacterial Blight is the most common bacterial disease caused by *P. syringae* pv. *Mori*, which could reduce yield by about 60% owing to the damaged leaves and stems [1].

In nature, plants are usually in close contact with numerous microbial pathogens. But few pathogens can infect plants because of the

existence of plants' immune system. There are two consecutive stages, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), involved in the plant's immune system. When pathogens float on the plants, pathogen-associated molecular patterns (PAMPs) on the surface of pathogens are detected by pattern recognition receptors (PRRs) present at the plant's cell surface to induce PTI [2]. To successfully colonize in plants, bacterial pathogens have evolved to acquire a variety of virulence factors, such as coronatine (COR), to overcome PTI and further induce ETI [2]. Depending on ETI, the infected plants are induced to form a new resistance to the pathogen itself or other pathogens. Plant immunity inducers don't show antimicrobial activity *in vitro*, but could activate plant defense responses to cause the resistance to pathogens [3]. As an immunity inducer, COR has been proved to improve the resistance to microbial pathogens by inducing the production of some small molecules in rice and soybean [4]. In our previous studies [5], COR (2.5 μM to 5.0 μM) had the ability to enhance the resistance of adult mulberry to *P. syringae* pv. *mori* and prevent the outbreak of Mulberry Bacterial Blight.

COR, a structural and functional mimic of jasmonates, is a non-host-specific phytotoxin produced by several pathovars of *P. syringae* [6, 7] and has received more attention in recent years as phytohormone. The existing literatures have indicated that COR could alleviate or activate a series of responses in plants. COR can inhibit root elongation, tendril coiling, senescence, and induce leaf chlorosis [8-11]. Additionally, COR can also stimulate anthocyanin production, auxin synthesis, and ethylene emission [10]. But it is more noticeable that COR has been confirmed to activate the JA-responsive genes, with the involvement of some signaling molecules such as hydrogen peroxide, nitric oxide, and Ca^{2+}, to induce the production of secondary metabolites such as flavonoids, volatiles, nicotines, and alkaloids [8-12]. For example, COR could improve the production of pterocarpans in soybean [4], diterpene lactone A in rice [13], glucosinolate and phenylpropanoid in Arabidopsis [14], and taxane in Taxus cell cultures [15]. It is well known that many secondary metabolites have the abilities to kill or inhibit the microorganisms [16, 17]. But few studies focused on how COR modulated the metabolic process and whether COR alleviated or enhanced adverse effects of pathogens infection in mulberry.

The objective of this work, therefore, was to evaluate the effects of exogenously applied COR on mulberry and its roles in counteracting *P. syringae* pv. *mori*. It was hypothesized that COR improved resistance to pathogens by modulating the metabolic process to induce the changes of metabolites. For these purposes, we investigated the changes of metabolites by using gas chromatography-mass spectrometry (GC-MS) technique. We also investigated content changes of some metabolites, activity changes of some enzymes, and antimicrobial activity of metabolites *in vitro* to verify the hypothesis. The findings of this research may elucidate the mechanism of mulberry-COR interactions and assist in the application of COR to mulberry as an immunity inducer.

Material and methods

Plant materials, growth conditions, and treatments

Mulberry seeds (cultivar *Feng-Chi-Sang*) were provided by the Sericulture Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). Seeds were surface-sterilized in 5% (v/v) NaClO for 15 min and rinsed thrice with distilled water. The seeds were transferred to Petri dishes with two sheets of filter papers moistened with distilled water, and were germinated in an incubator at 28±2°C. After 8 days, the germinated seeds were sown in round pots (10 cm diameter × 15 cm depth) containing nutrient soil. The pots were arranged in a greenhouse under natural conditions. After 3 months, seedlings at the five- or six-leaved stages were used for the experiments. During

the growth period, no stresses were applied on the plants.

COR (Sigma-Aldrich, St. Louis, MO, USA) was diluted to appropriate concentration with distilled water and sprayed evenly on the mulberry leaves. The plants were then kept in a growth chamber under the following conditions: a 12 h/12 h light/dark photoperiod, 28±2°C/22± 2°C day/night temperature, approximate 70% relative humidity, and 350 µmol m^2/s photon flux density. To avoid the loss of function as the result of evaporation, plants were covered with clear plastic boxes during the first 24 h. The control group was treated with distilled water.

Pathogenic material

M4-13 (*P. syringae*, isolated and identified in our own laboratory, host pathogen of mulberry) was used as bacterial pathogen. The pathogen was grown at 37°C (120 rpm) in KB medium (Peptone 20 g, glycerin 10 ml, K_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, H_2O 1,000 ml) for 24 h for antimicrobial activities.

Extraction and derivatization of metabolites

Mulberry leaf metabolites were extracted and derived according to Gai *et al*. [18] with slight modifications. Samples were washed three times with ultra-pure water and dried naturally. After ground with liquid nitrogen, the 500 mg powders were suspended completely in 5 ml methanol with 100 µl ribitol (0.2% [w/v] in water, as internal standard). After 15 min at 70°C, the mixture was centrifuged for 5 min at 14,000 g. Supernatant was transferred into a new tube with 3 ml water and 1.5 ml chloroform added. The mixture was shaken thoroughly and centrifuged for 10 min at 4,000 g. The methanol/water phase was collected and vacuum freeze-dried. Subsequently, the residue was re-dissolved in 10 µl dimethyl sulfoxide and derivatized in 80 µl methoxyamine hydrochloride (2% [m/v] in pyridine) for 90 min at 35°C. 80 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide was added to resulting mixture and was incubated at 40°C for 30 min.

GC-MS analysis

Using a Tr-5MS (30 m × 0.25 mm × 0.25 µm) fused silica capillary column (Thermo ITQ1100 GC-MS), 1 µl of the derivatization solution was added through splitless injection. The GC conditions were used according to Gai *et al*. [18] with slight modifications. The injection temperature was set at 230°C, and the ion source was adjusted to 200°C. Helium was used as carrier gas at a flow rate of 1 ml/min. The column temperature was initially kept at 100°C for 2 min, followed by 3°C/min oven temperature ramp to 185°C. The temperature was then increased to 192°C at 0.5°C/min, held for 2 min, and then increased to 245°C at 10°C/min. The final temperature was increased to 280°C at 3°C/min and held for 15 min. The MS conditions were described as follows: EI source, ion source temperature of 230°C; interface temperature of 280°C; electron energy, 70 eV; solvent delay, 5.5 min; and scan range, 33-600 amu.

Retention time and mass spectral library (Wiley Library) for automatic peak of metabolite derivatives were analyzed. Identification of metabolites was conducted by comparison with authentic standards described by Gai *et al*. [18] and Roessner *et al*. [19]. The relative contents of metabolites were calculated by the method of peak areas using ribitol as internal standard.

Photosynthetic activity measurement

Net photosynthesis rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Trmmol) of mulberry leaves were measured using a portable photosynthesis system (Lci, England) at 10:00 under natural conditions. Sunlight was abundant during the experimental days.

Measurement of starch and soluble sugar contents

Starch content was measured using the iodine-starch chromogenic method. Sample powder was suspended completely in 80% $Ca(NO_3)_2$, and extracted in boiling water for 5 min. After

centrifugation at 5,000 rpm for 10 min, the supernatants were collected to measure the starch content. Supernatant (10 ml) was added into 2 ml iodine solution (0.5%, w/v), and placed at room temperature for 15 min. After centrifugation at 5,000 rpm for 10 min, the precipitate was washed twice with 5% $Ca(NO_3)_2$ containing 0.001% iodine, and then, was dissolved in 10 ml NaOH (0.1 M). The liquid was mixed with 10 ml iodine solution and 2 ml hydrochloric acid (0.1 M), and was diluted to 50 ml with water. Absorbance was determined at 590 nm.

Soluble sugar content was extracted and analyzed using the method developed by Dahech *et al*. [20]. Sample powder was suspended (1 g) in 5 ml distilled water and placed in boiling water for 30 min. After centrifugation at 5,000 g for 10 min, supernatant was collected to determine the soluble sugar content. After mixing 2 ml of the supernatant with 1 ml phenol solution (6%, w/v), 5 ml of 100% sulfuric acid was added. Absorbance was recorded at 490 nm after 20 min. The concentration was determined with glucose as standard and was calculated on the fresh weight basis.

Determination of RuBisCO and amylase activities

Ribulose-1,5-bisphosphate carboxylase/oxygen-ase (RuBisCO, EC 4.1.1.39) was extracted using the procedure of Gong and Zhang [21]. Samples were homogenized with extraction buffer (40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.25 mM EDTA, 5 mM β-mercaptoethanol). The crude homogenates were centrifuged at 14,000 rpm (4°C) for 15 min. The supernatants were used to measure the RuBisCO activities by using the Plant RuBisCO ELISA Kit (Shanghai Jianglai Biological Technology Co., Ltd., China).

Leaves were homogenized with distilled water and were placed at 25°C for 20 min. After centrifugation at 5,000 rpm for 10 min, the supernatants were diluted to 50 ml with water for amylase activity assay. α-amylase (EC3.2.1.1) and β-amylase (EC3.2.1.2) assays were performed using 3,5-dinitrosalicylic acid reagent following the method of Doehlert *et al*. [22]. Amylase activity was defined as the amount of maltose produced from soluble starch per minute per gram of fresh weight.

Measurement of organic acid content

Organic acid content was measured by alkali titration method [23]. Samples were homogenized with distilled water, and placed at 80°C for 30 min. To ensure effective extraction, the homogenates were shaken once every 5 min. After centrifugation at 5,000 rpm for 20 min, the precipitates were extracted repeatedly for three times. Supernatants were diluted to 50 ml with water for determination of organic acid content. Organic acids were titrated with NaOH (0.01 M) and were calculated using malic acid as the standard.

Measurement of abscisic acid (ABA) and indole acetic acid (IAA) contents

Samples were homogenized with 5 ml methanol and were stored at 4°C for 4 h. After centrifugation at 10,000 g (4°C) for 20 min, the supernatants were collected to analyze the ABA and IAA contents. Contents were measured using Plant Hormone ELISA Kits (ABA and IAA) (Shanghai Tongwei Biological Technology Co., Ltd., China) according to the manufacturer's instructions.

Analysis of amino acid and soluble protein contents

Leaf samples were homogenized with chloroform-ethanol-water (5:12:3, v/v/v) mixture and were placed at -20°C for 24 h. Supernatants were collected after centrifugation at 10,000 rpm at 4°C for 20 min to detect the free amino acid content. The total free amino acids were analyzed using ninhydrin reagent [23].

Soluble protein was extracted using phosphate buffer (pH 7.0, 50 mM). After centrifugation at 10,000 rpm (4°C) for 20 min, supernatants were collected to measure the soluble protein content. Protein content was determined

according to Bradford [24] with bovine serum albumin as standard.

Determination of phenols and flavonoids contents

The method for extraction and quantification of total phenols and flavonoids was as described by Ibrahim *et al*. [25] with slight modifications. Sample powder was extracted with 80% methanol in a tube. After wrapped with a layer of tinfoil to avoid photooxidation, the tube was incubated for 24 h (25°C, 150 rpm). After centrifugation at 10,000 rpm (4°C) for 20 min, supernatant was used for the quantification of total phenols and flavonoids. Total phenols content was determined by Folin-Ciocalteu reagent. Extract (0.3 ml) was mixed with Folin-Ciocalteu reagent (0.3 ml) and was placed at 25°C for 5 min before adding 1M Na_2CO_3 solution (0.4 ml). After 10 min, 1 ml distilled water was added into the mixture. Absorbance was measured at 725 nm after 1 h. The results were expressed with catechol as standard. For total flavonoids determination, extract (1 ml) was mixed with 60% methanol (1 ml) and 5% $NaNO_3$ (0.5 ml). After 5 min, 10% $Al(NO)_3$ (0.5 ml) was added followed by adding 4% NaOH (4 ml). The mixture was diluted to 10 ml with 60% methanol and was measured at 510 nm with rutin as standard.

Hydrogen peroxide (H_2O_2) assay

H_2O_2 was detected using the fluorescence indicator [26]. Leaves were soaked in TRIS-MES buffer (0.25 M, pH 7.2) for 30 min in the dark. Subsequently, 2',7'-dichlorofluorescin diacetate (DCFH-DA, Beyotime Institute of Biotechnology, China) was added to the final concentration about 50 mM. After 10 min, the leaves were washed five times with TRIS-MES buffer and were observed with the fluorescence microscope (Leica DM1000). Cells appeared red without H_2O_2 existence and green-yellow with H_2O_2 presence.

Nitric oxide (NO) and enzyme assays

NO fluorescence was detected by the method of Lü *et al*. [27]. Leaves were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, pH 6.15) containing 10 mM 3-amino,4-aminomethyl-2',7'-difluorescein, diacetate (DAF-FMDA, Beyotime Institute of Biotechnology, China) at 28°C in the dark. After 2 h, the leaves were rinsed five times with MES-KCl buffer and were observed with the fluorescence microscope (Leica DM1000).

Leaves were homogenized with 0.9% NaCl containing 0.5 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride. After the crude homogenates were centrifuged at 10,000 rpm for 20 min (4°C), supernatants were collected to detect nitric oxide synthetase (NOS, EC 1.14.13.39) activities using the Total Nitric Oxide Synthase assay kit (Nanjing Jiancheng Bioengineering Institute, China). Protein content was determined according to the method of Bradford [24] with bovine serum albumin as standard.

Nitrate reductase (NR, EC 1.7.1.3) was assayed using the naphthylamine-sulfanilic acid method [23]. Samples were dipped into the reaction mixture of 0.1 M phosphate buffer (pH 7.0) and 0.2 M KNO_3 (1:1, v/v) for 30 min. NO_3^-, penetrating into the leaf tissues, was oxidized to NO_2^- by NR. NO_2^- reacted with naphthylamine and sulfanilic acid to produce the red material. NO_2^- concentration was monitored by absorbance at 520 nm with $NaNO_2$ as standard. NR activity was defined as the amount of NO_2^- produced from NO_3^- per hour per gram of fresh weight.

Antimicrobial activity of mulberry leaf metabolites

Antimicrobial activities of the metabolites were evaluated by spectrophotometer. Mulberry leaf metabolites were extracted by the method described previously, and the residues were diluted with distilled water. After 2 ml bacterial cell suspension (OD_{600} = 1) was added into 100 ml sterile KB medium, metabolite diluents filtered with microporous filters (0.22 μm) were poured into the medium for the final

concentrations of 2 mg/ml and 1 mg/ml. Sterile water was used as control. The flasks were incubated at 37°C for 24 h in an incubator (120 rpm). During the incubation, mediums were collected at different time points to determine the absorbance at 600 nm. Antimicrobial activities were evaluated by comparing the values of absorbance.

Statistical analysis

Data were analyzed with SPSS software, and the means were separated using Duncan's multiple-range tests. The symbols of "*"and "**" indicated significant difference at $p<0.05$ and $p<0.01$, respectively.

Results

Choice of coronatine concentration

In our previous experiments [28], the function of COR showed dual characters in mulberry seedlings. High concentration COR (>1 μM) induced chlorosis, decreased chlorophyll content, and inhibited the growth; but low concentration COR exhibited the opposite effects. According to the chlorosis and the resistance to *P*. *syringae* pv. *mori*, 0.01 μM COR showed perfect effect on the five- or six-leaved mulberry seedlings (Figure 1). Therefore, 0.01 μM COR was selected to treat the mulberry seedlings in our research. Because the treated mulberry showed resistance to *P*. *syringa*e pv. *mori* after 7 days, we chose the six-day-treated leaves as samples.

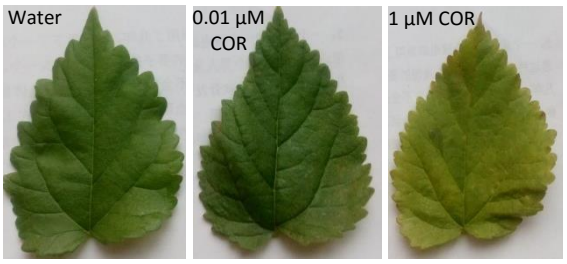

Figure 1. Mulberry symptoms with COR treatments.

Metabolic response of mulberry to coronatine

In our experiment, a total of 87 metabolites were identified in all the samples. The identified metabolites included 18 organic acids; 8 alcohols, ketones, and phenols; 11 esters and lactones; 10 hydrocarbons; 17 sugars; 12 sugar acids and sugar alcohols; and 11 nitrogen or sulfur compounds (Table 1). Nine metabolites including 5 organic acids, 1 ketone, 1 lactone, 1 hydrocarbon, and 1 sugar acid were detected in the COR-treated samples, whereas 8 metabolites including 1 organic acid, 1 lactone, 1 hydrocarbon, 1 sugar, 2 sugar acids, sugar alcohols, and 2 nitrogen or sulfur compounds were detected in the water-treated samples. Fifty out of the 87 metabolites increased while thirty-seven metabolites decreased in the COR-treated leaves. Notably, COR treatment resulted in obvious accumulation of organic acids, sugars, and nitrogen or sulfur compounds.

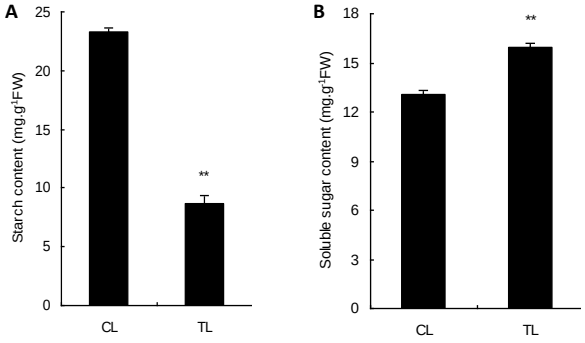

Figure 2. Effects of COR treatment on starch and soluble sugar contents. (A) Effect of COR treatment on starch content; (B) Effect of COR treatment on soluble sugar content. (CL: water-treated leaves; TL: COR-treated leaves; FW: fresh weight).

Coronatine increases sugar content

Metabolic analysis showed that the overall level of sugars increased in the COR-treated leaves (Table 1). To further confirm the result, starch and soluble sugar contents were measured by spectrophotometry. Our data showed that starch and soluble sugar contents in the COR-treated leaves were 62.8% lower ($p<0.01$) and 18.3% ($p<0.01$) higher than those in the water-treated leaves, respectively (Figure 2). COR had

Table 1. Identified metabolites of mulberry leaf extracts in GC-MS.

Metabolites	Fold change (log_2 TL/CL)[a, b]	Metabolites	Fold change (log_2 TL/CL)[a, b]
Organic acids **(18)**			
2-Butenedioic acid	−0.18	Malic acid	1.29
Nonadecanoic acid	0.28	Methylmaleic acid	-1.26
Octadecanoic acid	−1.02	2-Pyrrolidone-5-carboxylic acid	0.31
Glyceric acid	0.39	Acetic acid	*
Hexanedioic acid	0.03	Acrylic acid	*
Pentanedioic acid	0.65	Benzoic acid	*
Propanedioic acid	2.04	Hexadecanoic acid	*
Ascorbic acid	3.09	Nonanoic acid	*
Butanoic acid	−0.82	4-Hydroxy-3,5-dimethoxy-cinnamic acid	**
Alcohols, ketones, phenols **(8)**			
Glycerol	0.95	2-Aminoethanol	−0.59
Inositol	−1.05	Cyclooctene-1,2-diol	-0.06
Pyrocatechol	1.74	2-Butene-1,4-diol	2.18
2-Methyl-1,2-propanediol	-1.05	Androstan-17-one	*
Esters, lactones **(11)**			
3-Deoxy-2-C-(hydroxymethyl) tetronic acid, 1,4-lactone	−1.65	Sulphurous acid, hexyl octyl ester	-0.38
Allonic acid, lactone	1.04	Ribonic acid gamma-lactone	2.16
Gulonic acid lactone	−0.48	Talonic acid-1,4-lactone	-1.52
Isocitric acid lactone	−0.71	Glucuronolactone	*
Isopropenyl succinate	0.09	Gluconic acid -Lactone	**
Ethanedioic acid, 2-ethylhexyl pentyl ester	−2.15		
Hydrocarbons **(10)**			
Butanal	1.75	4,6-Dimethyldodecane	1.27
cyclohexene	2.12	5-Methylundecane	−0.09
4-Methyldodecane	−1.63	Heptadecane	0.92
Tetradecane	-0.62	3-methyl-pentacosane	*
6,7-Dihydroxy-1-oxotetrahydronaphthalene	-1.05	3,3-Dimethyl-4-methylene-1,2-dismethoxy-cyclopentene	**
Sugars **(17)**			
D-Erythrose	1.89	Levoglucosan	1.20
D- Pentose	−0.50	Lyxose	0.97
D-Ribose	0.22	D-Galactose	3.77
D-Xylopyranose	−1.25	D-Xylose	0.18
D-Glucose	0.05	Glucopyranose	0.25
Lactose	1.03	α-D-Glucopyranoside	2.14
Fructose	0.06	6-Deoxy-beta-L-galactopyranose	-1.58
Sucrose	1.37	2-Deoxy-ribose	**
Arabinose	2.07		
Sugar acids, Sugar alcohols **(12)**			
D-Ribonic acid	−0.28	Xylonic acid	0.93
D-Gluconic acid	2.13	Mannonic acid	-1.19
D-Mannitol	1.20	Erythritol	0.05

Threitol	−1.25	D-Gulonic acid	*
Xylitol	−0.39	Arabinonic acid	**
Glucitol	-0.18	Hexonic acid	**
Nitrogen or sulfur compounds **(11)**			
1H-Indole-3-carboxaldehyde	0.77	Putrescine	-0.09
3-methyl-1H-Indole	−1.07	Pyruvic acid oxime	1.53
1H-Indole-3-acetic acid	3.13	1-Formylpiperidine	4.72
3-Indole acrylic acid	-0.84	1H-Indole-2,3-dione, 7-isobutyl	**
Methanamine	0.38	2-Piperidinecarboxylic acid	**
1H-Indole-3-acetonitrile	0.58		

[a]The quotient of mean relative response ratio from COR-treated leaves (TL) and mean relative response ratio from water-treated leaves (CL) is given using a logarithmic scale.

[b]The quotient values < 0 represent a decrease in metabolite levels in the COR-treated leaves compared with water-treated ones. The quotient values > 0 represent an increase.

* indicates the metabolites detected in the COR-treated samples or those below the detection limit for the water-treated samples.

** indicates metabolites that were detected in the water-treated samples but were below the detection limit for the COR-treated samples.

different effects on Pn, Gs, Ci, and Trmmol. COR-treated leaves exhibited higher Pn (28.0%), Gs (33.5%), Trmmol (27.4%), and lower Ci (6.8%) compared to water-treated leaves ($p<0.05$) (Figure 3). Moreover, COR increased RuBisCO activity and promoted the chlorophyll synthesis (Data not shown). RuBisCO activity and chlorophyll content in COR-treated leaves increased 13.1% and 12.5%, respectively. However, the increased photosynthetic capacity was insufficient to explain the reduced starch content. To investigate this inconsistency, the amylase activities in the samples were measured. Results showed a significant increase by 21.5% of the amylase (α-amylase +β-amylase) activities in the COR-treated leaves ($p<0.01$), and suggested that COR accelerated photosynthetic capacity and starch degradation to increase soluble sugars content in mulberry.

Coronatine affects organic acid content

Metabolic data showed both quantities and types of organic acids in the leaves had significant changes. Among the organic acids detected by GC-MS, only 2-butenedioic acid, octadecanoic acid, butanoic acid, methylmaleic acid, and 4-hydroxy-3,5-dimethoxy-cinnamic acid decreased, and other 13 organic acids increased at different degree in the COR-treated leaves. Acetic acid, acrylic acid, benzoic acid, hexadecanoic acid, and nonanoic acid were detected only in the COR-treated leaves (Table 1). Meanwhile, the total organic acid content significantly increased by 49.8% ($p<0.01$) in the COR-treated leaves by spectrophotometric assay.

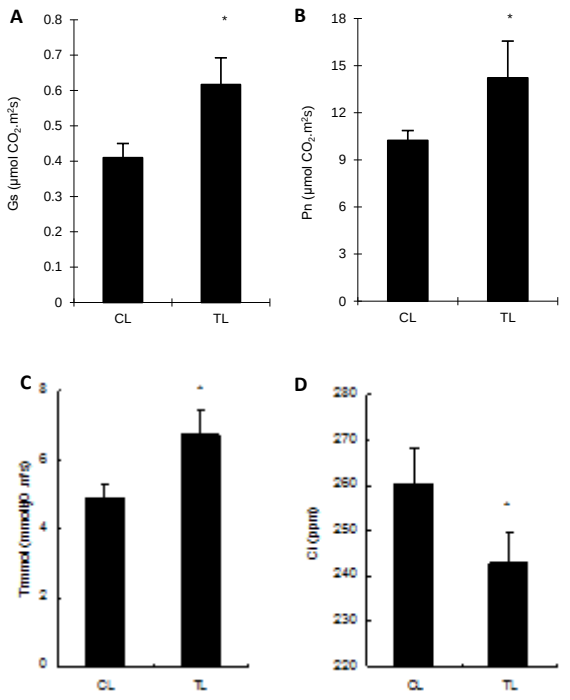

Figure 3. Effects of COR treatment on leaf photosynthetic characteristics. (A) Effect of COR treatment on Pn. (B) Effect of COR treatment on Gs. (C) Effect of COR treatment on Trmmol. (D) Effect of COR treatment on Ci.

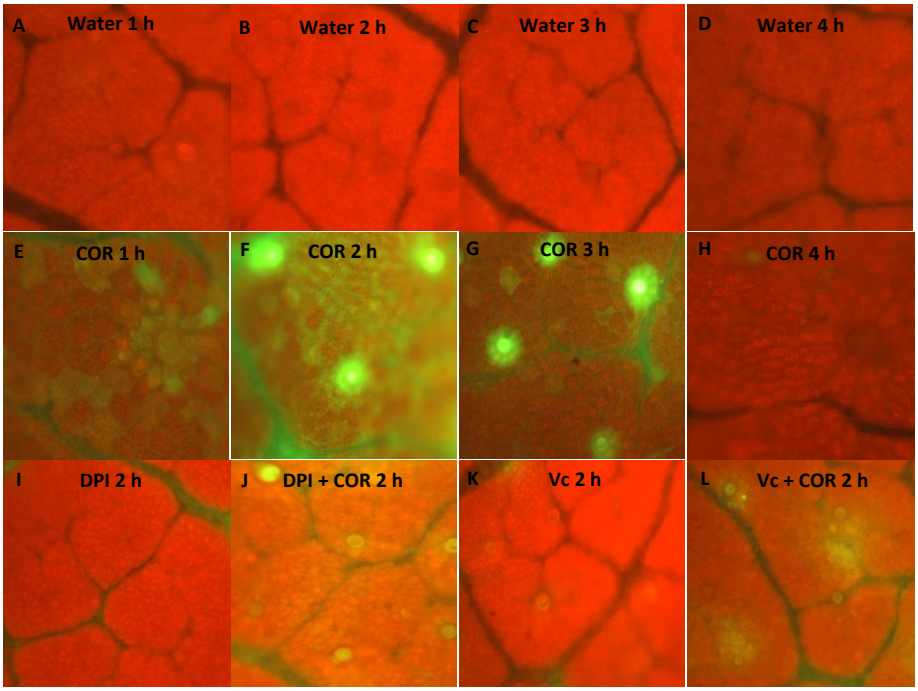

Figure 4. Fluorescence images of hydrogen peroxide (H_2O_2). DPI: 20 μM; Vc: 20 mM. Magnification: 10×40.

Coronatine affects protein metabolism

To explore the protein metabolism in the mulberry leaves, contents of total free amino acid and soluble protein were analyzed using ninhydrin colorimetric method and spectrophotometry, respectively. Data showed a decrease by 6.3% of free amino acid content and a significant increase by 15.3% ($p<0.01$) of soluble protein content in the COR-treated leaves. This suggested that 0.01 μM COR can influence the protein metabolism in mulberry.

Coronatine changes phytohormone content

In our metabolic data, a significant increase of 1H-indole-3-acetic acid (IAA) and its analogs occurred evidently in the COR-treated leaves (Table 1). The measurement of IAA content also confirmed the changes. The IAA content sharply increased by 60.9% ($p<0.01$). To investigate whether other phytohormones were involved in the response to COR, ABA content was also analyzed. Results showed that ABA level was 40.6% higher ($p<0.05$) in the COR-treated leaves; but the ratio of IAA and ABA also increased by 32.4%.

Coronatine induces accumulation of phenols and flavonoids

The contents of total phenols and flavonoids were influenced by COR. Total phenols and flavonoids contents were higher in the COR-treated leaves by 16.9% and 27.1%, respectively, than it in the control leaves ($p<0.01$). This showed that COR treatment could induce accumulation of phenols and flavonoids in mulberry.

Coronatine induces accumulation of H_2O_2 and NO

Accumulation of H_2O_2 and NO occurs at the early stage when plants are stimulated by external factors; thus, these compounds were detected within 12 h. Figure 4 showed the green-yellow fluorescence resulting from the reaction of fluorescence indicator with H_2O_2. Stronger fluorescence appeared from 1 h to 3 h in the COR-treated leaves (Figure 4: E, F, G), and the peak occurred at 2 h (Figure 4F), while no obvious changes were observed in the water-treated leaves (Figure 4: A, B, C, D). Diphenyleneiodonium chloride (DPI) inhibits the

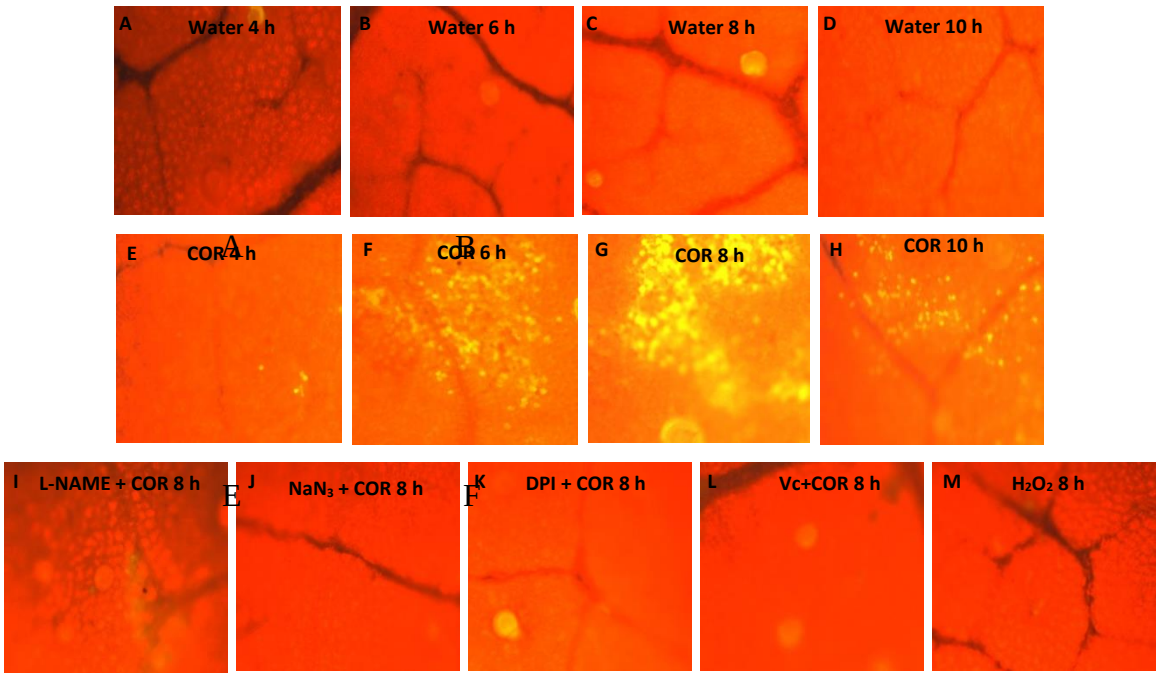

Figure 5. Fluorescence images of nitric oxide (NO). DPI: 20 μM; Vc: 20 mM; L-NAME: 0.05 mM; NaN_3: 0.2 mM; H_2O_2: 20 mM. Magnification: 10×40.

activity of NADPH oxidase associated with the generation of H_2O_2, whereas vitamin C (Vc) is the scavenger to H_2O_2. When DPI and Vc cooperated with COR to treat the mulberries, the fluorescence weakened at 2 h (Figure 4: J, L). These findings were consistent with the measurement of H_2O_2 content (data not shown). Analysis of NO content revealed the accumulation from 6 h to 8 h in COR-treated leaves (data not shown). Fluorescence experiments also showed the same results (Figure 5: E, F, G, H). N-nitro-L-arginine methyl ester (L-NAME) is the inhibitor of NOS, and NaN_3 can inhibit NR activity. When L-NAME and NaN_3 cooperated with COR to treat the mulberries, an obviously weak fluorescence at 8 h was observed (Figure 5: I, J). These results indicated that NO was produced via the NOS and NR pathways in mulberry. The tests of NOS and NR activities also confirmed the results (Figure 6). When DPI and Vc treated the mulberry with COR, NO fluorescence weakened (Figure 5: K, L). NO fluorescence was detected if H_2O_2 alone worked on the plant (Figure 5: M). Therefore, accumulation of H_2O_2 and NO occurred in the COR-treated leaves, but H_2O_2 appeared prior to NO.

Mulberry leaf metabolites inhibit bacterial pathogens

Mulberry leaf metabolites showed antimicrobial activities *in vitro* against bacterial pathogen M4-13 (Figure 7). The metabolites showed that antibacterial activities which expressed the absorbance values were lower than those of the control group, but the antibacterial effects in the COR-treated leaves were superior to those in the water-treated ones. Meanwhile, the high concentration metabolites were beneficial to bacteriostasis. The effect of 2 mg/ml metabolites was stronger than that of 1 mg/ml metabolites. The results obtained from the antimicrobial properties indicated that the metabolites in the COR-treated leaves inhibited the microbial growth more effectively.

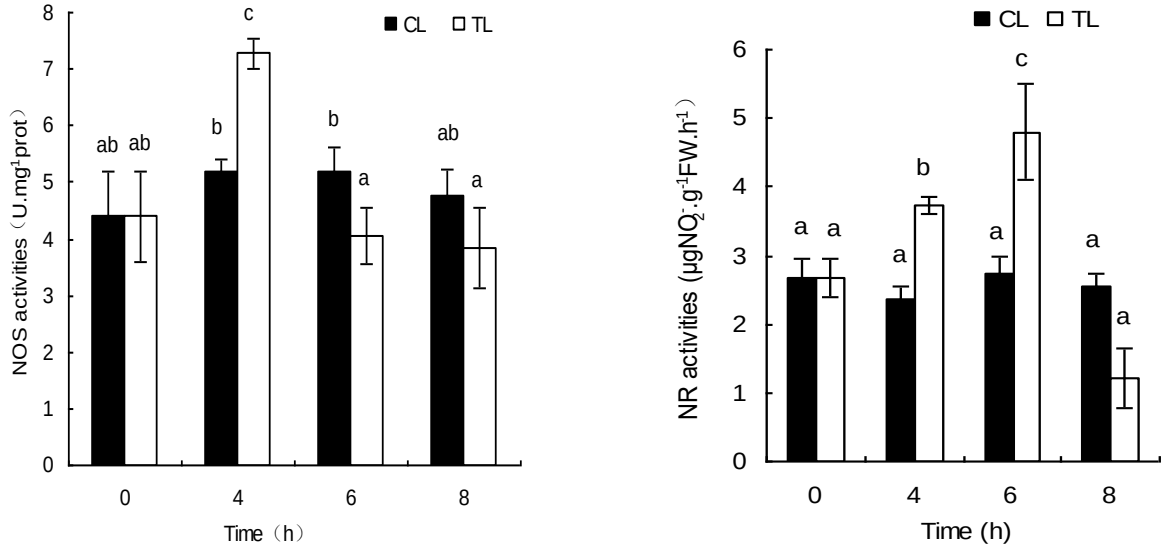

Figure 6. Activities of nitric oxide synthase (NOS) and nitrate reductase (NR). Bars with different letters (a, b, etc.) indicate significant differences at $p<0.05$ by Duncan's multiple range tests.

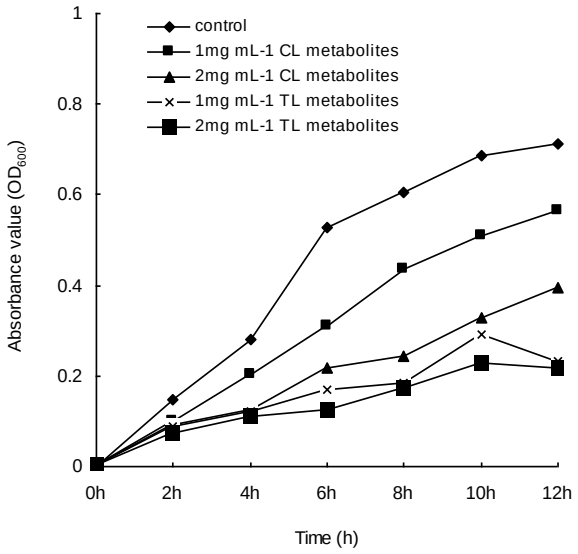

Figure 7. Antibacterial activities of mulberry leaf metabolites on M4-13.

Discussion

In higher plants, the leaves are the important assimilating organs where various biosynthetic reactions are performed to synthesize abundant metabolites. Meanwhile, the component of metabolites is influenced by multiple factors, such as stress, nutrition, developmental stage, and bacterial or viral infections [29-31]. In our data, about 87 metabolites were detected in the mulberry leaves, and the component of metabolites was also different in the water-treated and COR-treated leaves (Table 1). In addition to sugars and organic acids which increased evidently, dozens of alcohols, ketones, phenols, esters, lactones, hydrocarbons, and nitrogen or sulphur compounds originated from diverse metabolism pathways were also

detected and demonstrated changes at various levels. These results indicated that COR could regulate the metabolic processes of mulberry to change the content and component of metabolites. Once bacterial pathogens enter the plant tissues through stomata or wounds on the leaf surface, they colonize in the intercellular spaces and internal leaf tissues [32]. Bacterial pathogens in plants can also be transported over the body through the phloem. Therefore, metabolites have close contact with bacterial pathogens and directly affect them in plants. Some metabolites had been illustrated to have antibacterial or bactericidal activities, for example, organic acids to *Bacillus subtilis* and *B. licheniformis* [33], ketones in *Phaleria macrocarpa* (Scheff.) boerl fruit to *Staphylococcus aureu* [16], and nitrogen or sulfur compounds in *Tinospora capillipes* to *Colletotrichum gloeosporioides* and *Mycosphaerella sentina* [17]. Our results also indicated that the metabolites of mulberry seedling leaves inhibited the growth of M4-13, and COR treatment enhanced the antimicrobial effect of metabolites.

Sugars are the major products of photosynthesis and the important metabolites in most plants [34]. Detecting the sugars changes and the enzyme activities involved in the photosynthesis can help us to understand the effects of COR on the metabolic process in mulberry. After COR-treatment, soluble sugars accumulated in mulberry seedlings (Table 1 and Figure 2: B). COR-treated plants maintained higher Pn, Gs, Trmmol (Figure 3), chlorophyll content and RuBisCO activity, which helped the enhancement of assimilation rate and led to higher sugars accumulation. These results were consistent with ones observed in soybean [35] and wheat [36] treated with COR. COR induced the accumulation of starch in purple-fleshed sweetpotato [37], but we noted that COR-treated mulberry leaves had lower starch content (Figure 2: A). However, the decrease was not in contradiction with high photosynthetic capacity, because the amylase activities increased in the COR-treated leaves. Along with the improvement of photosynthetic performance, amylase activities accelerated the degradation of starch and led to the accumulation of more soluble sugars in the COR-treated leaves. Instead of antibacterial effect, sugars provided the carbon source for the growth of microorganisms. However, sugars are the basic materials for the synthesis of other metabolites. Amino acids, organic acids, and lipids are transformed from different carbon skeleton compounds derived from TCA cycle; meanwhile, secondary metabolites including alkaloids, auxins, phenols, flavonoids, lignin, and chlorogenic acid could be synthesized via different secondary metabolism pathways from sugars [38-40]. The increase of sugar content in the COR-treated mulberry leaves prepared for the accumulation of other metabolites.

Organic acids are widely distributed to perform antimicrobial, antioxidant, and other bioactivities in plants [41-42]. Among the organic acids detected in mulberry leaves, thirteen of them exhibited increase in the COR-treated plants, and five of them (acetic acid, acrylic acid, benzoic acid, hexadecanoic acid, and nonanoic acid) were absent in the water-treated samples (Table 1). Previous studies confirmed that hexanedioic acid [43], hexadecanoic acid [44], propanedioic acid [45], benzoic acid [46], acetic acid [46], malic acid [47], and acrylic acid [48] showed antimicrobial activities *in vitro*, which indicated that COR could induce the generation or accumulation of organic acids to improve the resistance to *P. syringae* in mulberry.

In fact, plant immunity is the hormone regulation which shows the antagonistical or synergistical interaction of each other via the SA-JA-ET backbone [49]. COR carries out its multiple contributions by mimicking JA-Ile to activate JA-signaling pathway and suppresses SA-signaling pathway [12]. In the COR-mediated process, other hormones are also involved. COR induced the tendril coiling of *Bryonia dioica* by increasing IAA content [50], and the expression of genes response to salt stress in cotton seedlings by enhancing the IAA, ABA, and GA contents [51].

Our metabolic data showed that IAA content increased in the COR-treated leaves (Table 1), which was consistent with the results obtained with Plant Hormone ELISA kits. IAA is the major endogenous auxin which signaling pathway represses SA signaling pathway to coordinate the function of COR [49]. Meanwhile, IAA is involved in many aspects related to plant growth [52]. The strong growth vigor benefited the synthesis and conversion of different metabolites. Tryptophan, the substrate of IAA, derives from sugars via shikimic acid pathway [50] and serves as the precursor of many secondary metabolites involved in enhancing the plant defense, such as benzoxazinoids, indole glucosinolates, and phytoalexin camalexin [12]. Besides the change of IAA content, high level of ABA in the COR-treated leaves was also found with the ratio of IAA and ABA increased. It was reported that high sugar concentration might lead to the increase of intracellular ABA [53], which may be the reason why the level of ABA was high in the COR-treated leaves. It had been well known that ABA was involved in the signaling pathways to regulate many aspects of plants associated with the abiotic (e.g., cold, drought, hot), and biotic stresses (e.g., pathogen) [54]. Accumulated studies had shown that ABA treatment resulted in the increased resistance to *P. syringae* [55]. Furthermore, ABA signaling and SA signaling antagonized each other from biosynthesis to intermediate components of the signal transduction pathways through ABA-SA crosstalk [49]. Additionally, ABA application could enhance the production of some secondary metabolites including total phenols and flavonoids [25, 56]. Conceivably, COR increased the contents of IAA and ABA to regulate the signaling pathway and promote the accumulation of antimicrobial metabolites.

COR could enhance the soluble protein content in rice, wheat, and peanut [10, 57]. In plants, most of the soluble proteins were the enzymes involved in various metabolisms. The significant increase of soluble protein content in the COR-treated mulberry leaves helped to catalyze the biosynthesis and bioconversion of metabolites. Total free amino acid content reduced in the COR-treated leaves because amino acids participated in the metabolism pathways to synthesize other metabolites. Flavonoids and phenols were the abundant secondary metabolites in the mulberry leaves which contents were positively related to sugars content [25]. Flavonoids and phenols also exhibited antimicrobial, antioxidant, and anti-inflammatory effects *in vitro* [16]. Therefore, the increase of those factors may be one reason that COR enhanced resistance to pathogen in the mulberry.

Numerous studies have confirmed H_2O_2 and NO are multifunctional signaling molecules to participate in signal transduction for plant resistance [58]. In most cases, H_2O_2 and NO induce each other. In our study, H_2O_2 accumulation induced by COR stimulated the production of NO. NO has emerged as a key factor in the signaling network leading to the biosynthesis of secondary metabolites [59], including terpenes [60], alkaloid [61], flavonoids [62], and phenols [63]. Therefore, the accumulation of H_2O_2 and NO was considered an inevitable effect of COR during the synthesis and conversion of metabolites.

In conclusion, COR induced metabolites against *P. syringae* pv. *mori* in mulberry by regulating the metabolism pathways. COR promoted the photosynthetic capacity to accumulate more sugars to provide raw materials for the synthesis of other organic compounds. Organic acids, flavonoids, phenols, and other secondary metabolites were significantly induced by COR to show antimicrobial effects. As signaling molecules, H_2O_2 and NO were produced to participate in the regulation, and IAA and ABA were also induced to regulate the process.

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