Identification and Functional Verification of Differences in Phenolic Compounds Between Resistant and Susceptible *Populus* Species

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ABSTRACT

Poplar canker, mainly caused by *Botryosphaeria* species, is a serious disease that has resulted in the reduced productivity and death of poplar worldwide. Different *Populus* species have varied resistance levels to poplar canker; however, whether phenolic compounds in poplar are involved in this resistance remains uncertain. Here, we determined the concentrations of phenolic compounds and their antifungal activities in canker-resistant *P. tomentosa* and canker-susceptible *P. beijingensis*. We identified 29 phenolic compounds with significantly different concentrations between the two species. Salicylic acid (SA), tremuloidin, salicin, and poplin were dominant in *P. tomentosa*, while benzoic acid (BA) and catechol were dominant in *P. beijingensis*. These six phenolic compounds were further tested for antifungal activities. SA, BA, and

catechol showed significant antifungal activities against *Botryosphaeria dothidea*, while the other three compounds showed no activity. As BA or catechol can be converted in plants to SA, which is a remote signaling molecule that plays an important role in plant defenses, we presumed that the resistance of *P. tomentosa* was initiated by SA, while the susceptibility of *P. beijingensis* resulted from the absence of SA. Further studies are required to confirm this hypothesis. These results provide a foundation for future research on the disease-resistance mechanisms of poplar.

Keywords: antifungal activity, *Botryosphaeria*, phenolic compounds, *Populus tomentosa*

Poplar (*Populus* spp.) is a widespread and economically important wood resource in China (Zhao et al. 2009). However, poplar canker is a serious disease that can cause the reduced productivity, and even death, of poplar (Ren et al. 2013). The pathogens of poplar canker include *Xanthomonas*, *Botryosphaeria*, *Dothichiza*, *Coniothyrium*, *Valsa*, *Coryneum*, *Nectria*, and *Phoma* (Detmers 1923; Li et al. 2018; Steenackers et al. 1996; Zhang et al. 1999). Among them, *Botryosphaeria* species are the main causal agents of poplar canker in China and some other countries (Huang and Su 2003; Wu et al. 2001). *B. dothidea* has spread worldwide, and consequently, it has been intensively studied (Nazerian et al. 2019; Yang et al. 2019; Zhang et al. 2018; Zhai et al. 2019). The pathogen can cause the formation of cankers on a wide variety of trees, including grape, mango, olive, eucalyptus, maple, oak, and poplar (Phillips et al. 2005; Plan et al. 2002; Smith et al. 1996; Urbez-Torres et al. 2006).

In the “arms races” between pathogens and plants, plant secondary metabolites play important roles (Kliebenstein et al. 2005), and they can be generally divided into three groups: phenolics, terpenes, and nitrogen- and sulfur-containing compounds (Pagare et al. 2015). Plant phenolic compounds are ubiquitous in plant species (Lattanzio 2013) and are diversified chemicals having an aromatic ring with at least one OH group. They include simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins derived from phenylalanine and tyrosine (Naczek

and Shahidi 2004). In general, phenolic compounds function in protecting plants from fungi, bacteria, and herbivores (Li et al. 2011; Hansen et al. 2017).

Poplar species have high levels of total phenolic compounds, including phenolic acids, flavonoid aglycons and phenolic glycosides (Dudonné et al. 2011). These phenolic compounds can inhibit pathogen growth or transmit defense signals (Cueva et al. 2010). Salicylic acid (SA) plays a critical role during the remote communication of systemic acquired resistance in *P. tomentosa* Carr. (Li et al. 2018). Salicin is a phytotherapeutic precursor of SA known for its anti-inflammatory properties (Bonaterra et al. 2010). In *P. nigra*, phenolic acids (caffeic acid and coumaric acid) are the major antioxidant components (Dudonné et al. 2011).

The genus *Populus* belongs to the family Salicaceae and comprises more than 100 species. Based on the leaf and flower characteristics, *Populus* is divided into six sections: *Populus*, *Aigeiros*, *Tacamahaca*, *Leucoides*, *Turanga*, and *Abaso* (Tuskan et al. 2006). Different *P.* species have varied resistance levels to poplar canker. For example, *P. tomentosa* is resistant to canker pathogens, while *P. beijingensis* Hsu is highly susceptible to the pathogens like *Dothiorella gregaria* (Hu 1999). While the mechanisms behind the different resistance levels of poplars against canker pathogens have been investigated, it is unclear whether phenolic compounds are involved in the process (Dickmann et al. 2002; Hu 1992; Hui 2000). Thus, we separated and identified the secondary metabolic compounds using high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR), respectively. Besides, we tested the antifungal activity levels of the phenolic compounds and found substantially different concentrations of them in *P. tomentosa* and *P. beijingensis*. Our work lays a foundation for further studies on the canker pathogen-resistance mechanisms of poplar.

MATERIALS AND METHODS

Poplar materials and growing conditions. Two poplar cultivars, one resistant (*P. tomentosa*) and one susceptible (*P.*

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beijingensis) were grown on a compost substrate in plastic pots in a greenhouse, in a 10 to 14 h light at 22 to 30°C/10 to 14 h dark at 20 to 25°C cycle with a fluorescent illumination of less than 30,000 lx and 50 to 75% relative humidity.

Extraction and measurement of phenolic compounds from *P. tomentosa* and *P. beijingensis*. Every 100 mm above the base of 1-year poplar, a 10 mm chip was chosen for sampling until nonlignified tissue was encountered at the top of the trees. Five saplings were used as repeats. The poplar chips were frozen in liquid nitrogen and then dried in a -40°C freeze-drier for 24 h. The dried twigs were ground into a powder in a mortar containing liquid nitrogen. Next, 2.0 g of poplar powder was transferred into a 150-ml glass conical flask and 50 ml of petroleum ether was added. The flask was shaken in an ultrasonic bath for 2 h and stirred overnight. The solution was filtered by 0.45-μm filter membrane (Pall, U.S.A.) and the remaining solid residue was dried at room temperature. Next, 50 ml of 50% methanol was added to the residue for a heated reflux-based extraction. After 2 h, the methanol extract was concentrated to 5 ml in a rotary evaporator and diluted in 25 ml of methanol. Finally, the methanol extract was passed through a 0.45-μm filter membrane. The filtered extract solution was stored in a -20°C refrigerator for the HPLC.

Reversed-phase HPLC was used for phenolic compound measurement. For chromatographic separation, a Sunfire C18 column (4.6 mm × 250 mm, 5 μm, Waters Corporation, U.S.A.) was used and maintained at 30°C for better retention time stability. The mobile phase consisted of 55% methanol/45% water for catechol, 4-hydroxybenzoic acid, catechin, gallic acid, coumalic acid, caffeic acid, ferulic acid, and chlorogenic acid (standard chemicals, Sigma,

China, >98% purity). Each of the standard chemicals was prepared for 5, 50, 500, and 5,000 ng/μl to make standard curves. Based on the peak area of standard chemicals and target extracts, we measured the phenolic compounds in poplar. The mobile phase consisted of 57% methanol/43% water for phenol, benzoic acid (BA), SA, and cinnamic acid. The mobile phase consisted of 60% methanol/40% water for quercetin, morin hydrate, rutin, isorhamnetin, and kaempferol. The flow rate was maintained at 0.8 ml/min, and the injection volume was 20 μl.

Extraction and isolation of phenolic compounds without standard chemicals from *P. tomentosa* and *P. beijingensis*. The powdered samples of *P. tomentosa* and *P. beijingensis* were degreased with petroleum ether (60 to 90°C) three times (90 min per degreasing), and the residues were refluxed with 70% ethanol twice (90 min per reflux reaction). The pooled extracts were concentrated, and the residues obtained were extracted sequentially with equal volumes of petroleum ether, dichloromethane, n-butanol, and ethyl acetate three times. The combined extracts were concentrated and separated on a silica gel eluted with a double-volume of dichloromethane/methanol (100:0, 96:4, 92:8, 88:12, 84:16, 80:20, 70:30, 60:40, 50:50, and 0:100) and a C18 reverse phase silica gel eluted with H₂O/ethanol (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, and 0:100). Finally, the purity levels of the eluted fractions were determined using a Thermo Scientific Surveyor plus HPLC (Phenomenex Kinetex C18, 100 mm × 4.6 mm, 2.6 μm) and separated using a semipreparative HPLC column (9.4 mm × 250 mm, 5 mm, XDB-C18). The detect temperature was 30°C. A linear gradient elution of 0.3% acetic acid (deionized water as solvent) (A) and acetonitrile (B, used as diluent of A) was used. The

Fig. 1. Main differences in phenolic compounds between *Populus tomentosa* and *P. beijingensis*, as assessed by high-performance liquid chromatography, at 254 and 320 nm detection wavelengths.

TABLE 1. Concentrations of phenolic compounds in *Populus beijingensis* and *P. tomentosa*^a

Compounds (mg/kg)	CAS number	Phenolic compounds	<i>P. tomentosa</i>	<i>P. beijingensis</i>
Phenolic compounds with standard chemicals	500-05-0	Coumalic acid	3.10 ± 0.30	23.40 ± 1.80**
	65-85-0	Benzoic acid	103.50	1,972.40 ± 188.00***
	149-91-7	Gallic acid	61.30 ± 1.20*	30.60 ± 0.90
	1135-24-6	Ferulic acid	6.40 ± 0.90	8.10 ± 1.10
	154-23-4	Catechin	86.10 ± 2.10**	20.60 ± 0.80
	99-96-7	4-Hydroxybenzoic acid	39.30 ± 3.50	52.70 ± 0.50
	120-80-9	Catechol	281.80 ± 16.70	1,560.60 ± 86.70***
	108-95-2	Phenol	650.30 ± 20.40	576.80 ± 39.00
	153-18-4	Rutin	85.40 ± 3.5	121.30 ± 4.50*
	207-542-9	Morin hydrate	0.90 ± 0.10	58.60 ± 0.60**
	331-39-5	Caffeic acid	1.54 ± 0.10***	nd
	327-97-9	Chlorogenic acid	9.80 ± 0.20***	nd
	69-72-7	Salicylic acid	2,585.50 ± 110.90***	nd
	621-82-9	Cinnamic acid	58.10 ± 2.00***	nd
	117-39-5	Quercetin	nd	0.80 ± 0.10***
	480-19-3	Isorhamnetin	nd	6.20 ± 0.10***
	520-18-3	Kaempferol	nd	0.10***
Phenolic glycoside compounds without standard chemicals	A1		1,035.20 ± 44.08**	725.19 ± 30.88
	A2		7,768.88 ± 330.80***	nd
	A3		1,121.72 ± 47.76***	nd
	A4		96.50 ± 4.10**	34.78 ± 2.43
	A5		10,342.00 ± 480.36***	nd
	B1		78.68 ± 5.26***	nd
	B2		190.47 ± 8.11***	nd
	B3		255.06 ± 20.36***	nd
	B4		941.15 ± 30.26***	nd
	B5		654.08 ± 27.85***	nd
	B6		298.39 ± 12.71***	nd
	B7		1,099.00 ± 46.59***	nd
	B8		153.79 ± 6.55**	58.57 ± 6.35
	B9		136.82 ± 5.85***	nd
	B10		2,220.14 ± 94.53***	nd

^a nd indicates compound not detected. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

Fig. 2. Speculative structures of phenolic compounds A1 to A5 without standard chemicals assessed by high-resolution mass spectrometry. Words in red or gray indicate compounds that we could purchase before antifungal experiments.

gradient was programmed as follows: 0 to 5 min: 95 to 90% (A), 5 to 25 min: 90 to 82% (A), 25 to 40 min: 82 to 82% (A), 40 to 65 min: 82 to 30% (A), 65 to 75 min: 30 to 30% (A). Then, the column was reequilibrated using the initial condition for 10 min before the next injection. The sample injection volume was 1 μ l. The UV spectra were recorded between 200 and 400 nm, and the detection wavelength was set at 254, 270, and 320 nm. The solvent flow rate was 0.8 ml/min, with 30% of the eluent being split into the inlet of the mass spectrometer (Thermo Scientific LTQ FT, U.S.A.). Ultra-high-purity helium was used as the collision gas, and high-purity N₂ was used as the nebulizing gas. The optimized parameters in the positive ion mode were as follows: ion spray voltage, 3.5 kV; capillary temperature, 260°C; capillary voltage, 49 V; and tube lens, 125 V.

Identification of the phenolic compounds without standard chemicals in *P. tomentosa* and *P. beijingensis* using HPLC-MS/MS and NMR. The chromatographic separation was performed using a Phenomenex Kinetex C18 column (100 mm $\times$ 4.6 mm, 2.6 μ m). HPLC methods were as described above. Mass spectra were recorded in a mass range of m/z 100 to 800, full-scan mode was adopted to obtain high-resolution mass spectrometry (HR-MS) data and multistage mass spectrometry data, but the data-dependent scan mode was set to collision-induced dissociation on preset ions in the mass list. The strongest ion peaks in last spectrum were in MS³. Collision energy was set to 35 V. Varian NMR system 600M (¹H 599.693 MHz and ¹³C 150.808 MHz) was used to identify the purified compounds. The solvent was dimethyl sulfoxide (DMSO) of deuterium (Cambridge Isotope Laboratory [CIL], U.S.A., purity $\geq$ 99.8%), and the test temperature was 298 K.

Antifungal effects of the main phenolic compounds of *P. tomentosa* and *P. beijingensis* on *B. dothidea*. To explore the functions of differential phenolic compounds between the disease-resistant *P. tomentosa* and the susceptible *P. beijingensis* infected by poplar canker, we selected six main phenolic compounds with high contents and large concentration differences between the two species to test their antifungal effects. Nystatin was used as the positive control, and sterile water was used as the blank control. In the experiment, potato dextrose agar in petri dishes was supplemented with five concentrations (0.001, 0.01, 0.1, 1.0, and 10 mM dissolved in DMSO) of the six phenolic compounds to cultivate *B. dothidea* (Zhou et al. 2019). The crisscross method was used to measure the growth rates of the hyphae. The phenolic compounds used were SA, BA, catechol, salicin, tremuloidin, and poplin. All the experiments were performed with five replicates.

Data analysis. The differences of the phenolic compounds between *P. tomentosa* and *P. beijingensis* were valued using unpaired two-tailed Student's *t* test and the differences in the growth rate of *B. dothidea* subjected to different chemicals were analyzed by a one-way analysis of variance with Tukey's test using SPSS 18.0 software (Green and Salkind 2010).

RESULTS

Main phenolic compounds in *P. tomentosa* and *P. beijingensis* using HPLC. Two detection wavelengths (254 and 320 nm) were used to detect the peaks of extracted phenolic compounds between *P. tomentosa* and *P. beijingensis* using HPLC. In *P. tomentosa*, there were 40 peaks, while in *P. beijingensis*, there were 48 peaks.

Fig. 3. High-resolution mass spectrometry data of crude extracts and purified A1 to A5 of *Populus tomentosa* and *P. beijingensis*.

Eleven peaks had the same peak time in extracts from both species, although the peak areas were different. Thus, 29 and 37 specific peaks were found in *P. tomentosa* and *P. beijingensis*, respectively (Fig. 1).

To quantify the different phenolic compounds in *P. tomentosa* and *P. beijingensis*, 17 phenolic standard chemicals were used. In *P. tomentosa*, 14 phenolic compounds (coumalic acid, BA, gallic acid, ferulic acid, catechin, 4-hydroxybenzoic acid, catechol, phenol, rutin, morin hydrate, caffeic acid, chlorogenic acid, SA, and cinnamic acid, but not quercetin, isorhamnetin, or kaempferol) were identified. Of the 14 phenolic compounds, SA had the greatest concentration at $2,585.50 \pm 110.90$ mg/kg. In *P. beijingensis*, caffeic acid, chlorogenic acid, SA, and cinnamic acid were not detected, while BA and catechol had the greatest concentrations at $1,972.40 \pm 188.00$ mg/kg and $1,560.60 \pm 86.70$ mg/kg, respectively. Compared with the phenolic compounds in *P. beijingensis*, the levels of gallic acid, catechin, caffeic acid, chlorogenic acid, SA, and cinnamic acid in *P. tomentosa* were significantly greater. In addition, the levels of coumalic acid, BA, catechol, rutin, morin hydrate, quercetin, isorhamnetin, and kaempferol in *P. tomentosa* were significantly lower than in *P. beijingensis*. Among the phenolic compounds, the

BA and catechin contents in *P. beijingensis* and the SA content in *P. tomentosa* were dominant (Table 1).

There were 15 phenolic compounds without standard chemicals that mainly existed in *P. tomentosa* named A1 to A5 and B1 to B10. Based on their peak areas, compared with phenols, their relative contents were calculated (Table 1). A2, A3, A5, B1 to B7, B9, and B10 were identified in *P. tomentosa* but not in *P. beijingensis*. The relative contents of A2 and A5 were greatest among all the detected compounds. In addition, the A1, A4, and B8 contents in *P. tomentosa* were higher than in *P. beijingensis*.

Identification of A1 to A5 and B1 to B10 by HPLC-MS/MS and NMR. In total, 15 phenolic compounds without standard chemicals were found in *P. tomentosa* and *P. beijingensis* (Table 1). Based on the NMR analysis, the structures of A1 to A5 were identified (Fig. 2 and Supplementary Table S1). A1 was salicin, m/z : 309.09457 $[M+Na]^+$, $C_{13}H_{18}O_7$, containing a β -D-glucose and a benzyl alcohol. A2 was 2'-O-acetylsalicylic acid, m/z : 489.13681 $[M+Na]^+$, $C_{22}H_{26}O_{11}$. A3 was tremuloidin, m/z : 413.12033 $[M+Na]^+$, $C_{20}H_{22}O_8$. A4 was populin, m/z : 413.12033 $[M+Na]^+$, $C_{20}H_{22}O_8$. A5 was tremulacin, m/z : 551.15216 $[M+Na]^+$, $C_{27}H_{28}O_{11}$. In addition, we predicted the molecular formulae of B1 to B10 and the structures of

Fig. 4. Growth rate of *Botryosphaeria dothidea* treated with different chemicals for 5 days. Six phenolic compounds found in *Populus tomentosa* or *P. beijingensis* were selected. Dimethyl sulfoxide (DMSO) was used as the solvent. Nystatin was used as the positive control. Error bars represent $\pm$ standard error of the mean. * $P < 0.05$; ** $P < 0.01$.

B1 to B7, with the greatest mass accuracy of less than 3 ppm of HR-MS (Supplementary Table S2 and Supplementary Fig. S1).

We separated A1 to A5 from the 15 phenolic compounds using a semipreparative HPLC column. To confirm the five compounds of A1 to A5, we compared the five isolated compounds to the purified compounds and crude extracts of *P. tomentosa* and *P. beijingensis*. The retention times of the five isolated compounds were consistent with the metabolites from poplar, with a relative drift of less than 5%. The maximum wavelengths of the ultraviolet absorptions of the

five isolated compounds were consistent with those of metabolites from poplar, and the differences were less than 3 nm. The HR-MS data of $[M+H]^+$ and the isotope measurement errors of molecular formulae were less than 2 ppm. The Ms^2 data of the five isolated compounds were consistent with those of metabolites from poplar. The retention times were the same, the maximum absorption wavelength were similarly less than 3 nm, the errors of isotopic peak were less than 2 ppm and the strength errors of the main fragment peaks were less than 30% (Fig. 3 and Supplementary Table S3).

Fig. 5. Growth culture dishes of *Botryosphaeria dothidea* treated with different chemicals after 5 days.

These results confirmed the identities of A1 to A5 derived from *P. tomentosa* or *P. beijingensis*.

Antifungal effects of the main phenolic compounds in *P. tomentosa* and *P. beijingensis* on *B. dothidea*. To determine which phenolic compounds were responsible for the resistance of *P. tomentosa* to poplar canker, we selected the dominant phenolic compounds (SA, BA, catechol, A1 salicin, A3 tremuloidin, and A4 populin) in *P. tomentosa* or *P. beijingensis* and tested their antifungal effects on *B. dothidea*.

The growth rates of *B. dothidea* against varying concentrations of the different compounds were calculated. As the concentrations of SA, catechol, or BA increased, the growth rate of *B. dothidea* decreased. There were no significant differences between 0.001 mM of SA, 0.001 and 0.01 mM of catechol and BA, compared with the control, having a growth rate of 6.2 mm/day. The antifungal effects of 10 mM SA, catechol, and BA were greatest, resulting in fungal growth rates of 1.71, 1.05, and 1.21 mm/day, respectively. In addition, the growth rate of *B. dothidea* against 0.01 mM SA was lower than that of the control, indicating an antifungal effect on *B. dothidea*. However, tremuloidin, salicin, and poplin at the tested concentrations did not show significant antifungal effects on *B. dothidea* compared with the control. The most effective antifungal compound, nystatin, which is used as a drug, showed antifungal effects on *B. dothidea* at 0.001, 0.01, 0.1, 1.0, and 10 mM concentrations. The solvent DMSO had no effect on the growth rate of *B. dothidea* (Figs. 4 and 5).

DISCUSSION

Phenolic compounds play important roles in plant defenses against disease (Bhattacharya et al. 2010; Mandal et al. 2010). For example, the phenolic compounds lignin, callose, and melanin are physical barriers to pathogen invasion and quickly accumulate after pathogen invasion (Bird 1988; Lattanzio et al. 2006). Most of these compounds inhibit spore germination and the mycelial growth or toxin production of pathogens (Tassou et al. 1991; Tranter et al. 1993; Yahyazadeh et al. 2008). Here, we determined the concentrations of phenolic compounds in canker-resistant *P. tomentosa* and canker-susceptible *P. beijingensis* and tested their antifungal activities. In total, 14 of the 17 known standard phenolic compounds had significantly different concentrations in the two plants. Among them, SA (2,585.50 mg/kg) was dominant in *P. tomentosa*, while BA (1,972.40 mg/kg) and catechol (1,560.60 mg/kg) were dominant in *P. beijingensis*. Furthermore, we found 15 phenolic compounds, which were unusual for getting their standard chemicals (A1 to A5 and B1 to B10) with higher contents in *P. tomentosa* compared with that in *P. beijingensis*. The structures of A1 to A5 were confirmed, and A1 salicin, A3 tremuloidin, and A4 populin were purchased or synthesized. In addition, the structures of B1 to B7 were predicted (Supplementary Fig. S1). In the antifungal tests, SA (dominant in *P. tomentosa*) and BA and catechol (dominant in *P. beijingensis*) showed significant antifungal activities against *B. dothidea*. However, the other three compounds, tremuloidin, salicin, and poplin, had no antifungal activities. SA is necessary for activating plant resistance and plays an important role in plant defense mechanisms (Casano et al. 2001; Chen et al. 1993; Li et al. 2018; Zhang et al. 2011). Our previous study showed that it might be converted to methyl-SA at infected sites and transported as a remote signaling molecule to uninfected sites, where it is converted to SA for systemic acquired resistance (Li et al. 2018). The structures of BA and catechol are similar to that of SA and could interconvert in plants (Klämbt 1962; Tsukamura 1965). Consequently, the resistance of *P. tomentosa* could result from the presence of SA, while the susceptibility of *P. beijingensis* could result from the lack of SA being converted from BA or catechol.

Phenolic glycosides (PGs) have a wide variety of physiological activities such as antibacterial, anti-inflammation, antitumor,

antivirus, and antioxidation. At present, there are multiple research teams working on the extraction, separation, structural identification, and synthesis of PGs, as well as measurements of PGs biological activities, but the focus is on medicinal applications, such as the antitumor and antivirus activities of PGs (Bermejo et al. 2002; Martin-Nizard et al. 2003; Wang et al. 1996a, b). In our studies, based on the density of poplar (0.4 g/cm³) (Zhu et al. 1993), the concentrations of tremuloidin, salicin, and poplin could be converted to 3.21, 2.96, and 0.27 mM, respectively. At these concentrations, none of the chemicals showed antifungal activities to *B. dothidea*. However, many PGs have not been separated or synthesized, such as A5 (10,342.00 mg/kg). Thus, more research is necessary to determine the functions of these compounds.

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