Stem canker pathogen
Botryosphaeria dothidea inhibits poplar leaf photosynthesis in the early stage of inoculation

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Fungal pathogens can induce canker lesions, wilting, and even dieback in many species. Trees can suffer serious physiological effects from stem cankers. In this study, we investigated the effects of Botryosphaeria dothidea (B. dothidea) on Populus bolleana (P. bolleana) leaves photosynthesis and stomatal responses, when stems were inoculated with the pathogen. To provide experimental and theoretical basis for preventing poplar canker early. One-year-old poplar stems were inoculated with B. dothidea using an epidermal scraping method. In the early stage of B. dothidea inoculation (2–14 days post inoculation, dpi), the gas exchange, stomatal dynamics, hormone content, photosynthetic pigments content, chlorophyll fluorescence parameters, and non-structural carbohydrate (NSC) were evaluated to elucidate the pathophysiological mechanism of B. dothidea inhibiting photosynthesis. Compared with the control groups, B. dothidea notably inhibited the net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO2 concentration (Ci), transpiration rate (Tr), and other photosynthetic parameters of poplar leaves, but stomatal limit value (Lm) increased. Consistent with the above results, B. dothidea also reduced stomatal aperture and stomatal opening rate. In addition, B. dothidea not only remarkably reduced the content of photosynthetic pigments, but also decreased the maximum photochemical efficiency (ΦPSII), electron transfer efficiency (ETR), and photochemical quenching coefficient (qP). Furthermore, both chlorophyll and ΦPSII were positively correlated with Pn. In summary, the main reason for the abated Pn under stem canker pathogen was that B. dothidea not merely inhibited the stomatal opening, but hindered the conversion of light energy, electron transfer and light energy utilization of poplar leaves. In general, the lessened CO2 and Pn would reduce the synthesis of photosynthetic products. Whereas, sucrose and starch accumulated in poplar leaves, which may be due to the local damage caused by B. dothidea inoculation in phloem, hindering downward transport of these products.

KEYWORDS
poplar stem canker, fungal pathogens, Botryosphaeria dothidea, photosynthesis, stomatal closure, chlorophyll fluorescence
Introduction

Plant photosynthesis is continuously challenged by a variety of environmental stresses, including abiotic stresses such as drought, high temperature, salt, and UV radiation, as well as biotic stresses caused by fungal pathogens and pests (Major et al., 2010; Suzuki et al., 2014; Bernal et al., 2015; Gururani et al., 2015; Ruehr et al., 2016; Saiki et al., 2017; Fermín et al., 2018; Blackman et al., 2019; Savi et al., 2019). Tree canker is a kind of most serious tree bark disease occur mainly in the stems and branches which have various kinds of pathogens, wide distribution of host and complex symptoms (Slippers and Wingfield, 2007; Marsberg et al., 2017). The canker disease of royal poinciana (Delonix regia) can result from fungal pathogen Neoscytalidium dimidiatum attacking different parts of the plant under certain favorable conditions (Raish et al., 2020). Fungal pathogens cause bark blisters and sunken lesions on stems in by first destroying the phloem and cambium (Biggs et al., 1983; Mcpartland and Schoeneweiss, 1984; Czemmel et al., 2015). Canker caused by necrotrophic fungal pathogens is commonly forming necrotic lesions be limited to the regions around the infection sites and do not girdle the branches pathologically at once, however, canker lesions on saplings or cankers abundant and/or perennial persistently can girdle branches, which eventually lead to yellowing tree canopy withering, dieback distal to the canker region, and even death (Marsberg et al., 2017; Li et al., 2019; Xing et al., 2020). However, the mechanisms of stem canker inhibit photosynthesis in distal leaves need more in-depth investigation.

Fungal pathogen infection usually results in plant tissue necrosis, a significant reduction in net photosynthetic rate ($P_n$) and carbon assimilation, slower growth, and even death (Berger et al., 2007; Moradi and Ismail, 2007; Christian et al., 2008). Leaf pathogens can directly depress photosynthesis by regulating stomatal opening, decreasing gas exchange, impairing green tissues, damaging photosynthetic apparatus, and suppressing key enzyme activities (Gruber et al., 2012; Júniör et al., 2017; Fermín et al., 2018; Franziska et al., 2018). Globally, B. dothidea causes canker, dieback, shoot blight, and fruit rot (Marsberg et al., 2017). Studies have also shown that stem canker can remotely alter the photosynthetic characteristics of distal leaves (Rohrs-Richey et al., 2011; Cerqueira et al., 2017). Quambalaria coyrecup remarkably reduced Corymbia calophyta leaf net $P_n$ and stomatal conductance in the later stage of inoculation (Hossain et al., 2019). In the interaction between hazelnut (Corylus avellana L.) and the canker pathogen Anisogramma anomala, canopy branch blight occurs distal to the canker areas (Lachenbruch and Zhao, 2019). In the early stage of poplar inoculation with stem canker pathogen B. dothidea and Valsa sordida (V. sordida), net $P_n$ and stomatal conductance were prominently weakened (Li et al., 2019; Xing et al., 2020). The above remarks indicate that although plant canker occurs on stems and branches, it eventually affects the photosynthetic process of distal leaves. In their early stages, canker diseases are unclear in terms of their physiology.

Stomata can respond rapidly to environmental stress, regulating gas exchange, controlling CO$_2$ uptake and water loss by adjusting stomatal size, thereby affecting photosynthesis (Schroeder et al., 2001; Melotto et al., 2006; Lawson and Viallet-Chabrand, 2019). A part of the immune responses of plants is closing stomata against pathogenic microbes (McLachlan et al., 2014). Alternatively, persistent stomatal closure leads to the reduced CO$_2$ uptake, affecting plant photosynthetic productivity, eventually depleting carbohydrates in plant tissues (O’Grady et al., 2013). Pathogens also interfere with phytohormones metabolism (Bari and Jones, 2009). In one sense, hormone promote disease development, but they also participate in plant immune responses, which increases plant resistance (Bari and Jones, 2009; Verma et al., 2016; Kunkel and Harper, 2018). Abscisic acid (ABA), jasmonic acid (JA), and auxin (IAA) are involved in stomatal movement. Research has shown that stress promotes crosstalk between phytohormones (Bu et al., 2018). Necrotrophic pathogens produce a variety of hormone-like active substances during the infection period, which directly or indirectly affect hormone metabolism in plants (Yang et al., 2015).

Biotic stress can disrupt plant photosynthetic apparatus (Júniör et al., 2017). Chlorophyll fluorescence parameters can sensitively reflect the activity of the photosynthetic apparatus (Björkman and Demmig, 1987; Krause and Weis, 1991; Watling et al., 2000). The leaf maximum photochemical efficiency ($F_v/F_m$) was strikingly reduced in poplar trees infected by the leaf rust fungus, Melampsora medusae (Fermín et al., 2018). As well as leaf pathogens, stem pathogen, Seiridium cardinale, also inhibited leaf photosystem II (Muthuchelian et al., 2005). Botrytis cinerea obviously reduced strawberry leaf $F_v/F_m$ and chlorophyll index (Meng et al., 2020). In pecans infected with stem canker agents (Phomopsis spp.), the leaf chlorophyll fluorescence parameters declined with the extension of infection time (Guillermo et al., 2021).

In this study, we recruited the P. bolleana-B. dothidea interaction system as a research object. In the early stage of B. dothidea inoculation, the gas exchange, stomatal dynamics, hormone content, photosynthetic pigments content, and chlorophyll fluorescence parameters were measured. Through analyzing the effects of B. dothidea on leaf stomatal movement, light energy utilization, and electron transfer, the pathophysiological mechanism of poplar stem canker inhibiting the leaf photosynthesis was investigated, aiming to provide a theoretical basis on the pathogenic mechanism operating at the initial stage of poplar canker.
Materials and methods

Plant material, fungal pathogen, and inoculation

One-year-old *P. bolleana* clones from cuttings were planted in 5-L pots containing sand. Potted plants were placed within the greenhouse at Chinese Academy of Forestry and watered 2–3 times weekly at 20–25°C under 12-h daily photoperiod with 200–300 µmol m⁻²s⁻¹. The *P. bolleana* saplings were assigned to the two groups: inoculation with *B. dothidea* (Bd) and potato dextrose agar (CTR). *P. bolleana* leaves (4th–6th mature leaves from the top) were selected for the following measurements. Seven biological replicates in every group.

Stems infected with *B. dothidea* were sampled from *Populus hopeiensis* Hu et Chow in Yi County, Hebei Province, China. Infected regions were cut into small pieces and disinfested with 75% ethanol for 30–60 s, then transferred onto potato dextrose agar (PDA, 2.0% potato extract, 2.0% dextrose, and 1.5% agar; pH 6.0). Following hyphal tip purification, all isolates were plated on fresh PDA dishes for further analysis. A variety of isolates were tested for pathogenicity by inoculating stems on poplar saplings with mycelium. The most pathogenic isolate was identified (NCBI accession number: MK990559 for rRNA-ITs and MN025271 for EF1α gene) and stored at 4°C in Chinese academy of forestry.

Inoculation treatments were performed as following steps. Briefly, after cultivation on PDA for 7 days, the mycelium of *B. dothidea* was cut into strips with 1.2–1.5 cm in width and 2.5–3.0 cm in length, which were used to inoculate *P. bolleana* stems. PDA culture medium was used as control. The inoculation sites (30 cm above the sand surface) on *P. bolleana* stems were pre-sterilized with 75% ethanol, then the barks were scraped gently with blade. Taking care to avoid damaging the remaining phloem, cambial and xylem tissue during the scraping process. The scraped region was covered completely by strips of *B. dothidea* mycelium or PDA medium. Finally, all strips were wrapped with sterilized Parafilm™.

Photosynthetic parameters

Net $P_\text{et}$, stomatal conductance ($G_s$), intercellular CO₂ concentration ($C_i$), transpiration rate ($T_\text{r}$), and vapor pressure deficit (VPD) were measured using a Li-6400XT portable photosynthesis system (LI-COR, Lincoln, USA). The selected *P. bolleana* leaves were measured and recorded from 9:00 to 11:00 a.m. on 0, 2, 4, 6, 8, 10, 14 dpi. Stomatal limit value ($L_s$) and water use efficiency (WUE) were calculated using the following formulae:

$$L_s = 1 - C_i / G_s \text{ (CO}_2 \text{ concentration in the air)}$$

$$WUE = P_\text{et} / T_\text{r}$$

Stomatal movement

Stomatal density

The lower epidermis of *P. bolleana* leaves was examined by SEM (5136, TESCAN, Brno, CS) to capture images. 10–15 visual fields were selected randomly at the magnification of 1 kx and 2 kx, respectively. Stomatal density of 6 and 14 dpi was calculated as the number of stomata per unit leaf area (mm²).

Stomatal aperture and opening rate

*P. bolleana* leaves of 6 and 14 dpi were washed 3–4 times with PBS buffer. Preparation for the samples was carried out according to Zhu et al. (2019). The resulting samples were examined through scanning electron microscopy (SEM, 5136, TESCAN, Brno, CS). Image-Pro-Plus 6.0 software was used to measure the stomatal aperture and opening rate. The stomatal aperture was evaluated by measuring the width of the stomatal pore observed under the SEM.

Plant hormone content determination

*P. bolleana* leaves of 6 and 14 dpi were collected. All leaves were ground into powder in liquid nitrogen, then 0.3 g was taken and stored at 4°C for the following experiment. Plant endogenous hormone content was analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) according to Yang et al. (2001).

Photosynthetic pigments content

The acetone extraction and colorimetric assays were applied to determine the content of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car). *P. bolleana* leaves of 6 and 14 dpi were minced by scissors. A 0.2 g sample was accurately weighed and 20 mL of 80% acetone was added to extract photosynthetic pigments in the dark for 48 h. Absorbance was, respectively, read at 663, 645, and 470 nm using a spectrophotometer (Dynamax, CA, US). The following formulae were used for calculation of photosynthetic pigments content:

$$
\text{Chl a} = 12.21A_{663} - 2.81A_{645}
$$

$$
\text{Chl b} = 20.13A_{645} - 5.03A_{663}
$$

$$
\text{Car} = (1000A_{470} - 3.27 \text{ Chl a} - 104 \text{ Chl b}) / 229
$$

Pigments content (mg/g) = [pigments concentration (mg/L) × extracts volume (ml) × dilution multiple/sample quality(g)
**Chlorophyll fluorescence parameters**

Chlorophyll fluorescence parameters were measured with FMS-2 Pulse Modulated Fluorometer (FMS-2, Hansatech, UK). *P. bolleana* leaves of 0, 2, 4, 6, 8, 10, and 14 dpi were dark-adapted for 30 min, then the initial fluorescence ($F_0$), maximum fluorescence ($F_m$), and maximum photochemical efficiency of PSII ($F_v/F_m$) were determined in darkness. After light-adaptation for 30 min, the initial fluorescence ($F'_0$), maximum fluorescence ($F'_m$), steady-state fluorescence ($F_s$), and actual PSII efficiency ($\Phi_{PSII}$) were measured under irradiance. After that, $F_v/F_m$, $\Phi_{PSII}$, electron transfer rate (ETR), photochemical quenching coefficient ($q_p$), and non-photochemical quenching coefficient (NPQ) were calculated according to the following formulae:

$$F_v/F_m = (F_m - F_0) / F_m$$

$$\Phi_{PSII} = (F'_m - F_s) / F'_m$$

$$ETR = \Phi_{PSII} \times 0.5 \times 0.84$$

$$q_p = (F'_m - F_s) / (F'_m - F'_0)$$

$$NPQ = (F_m - F'_m) / F'_m$$

**Non-structural carbohydrate concentration assay**

*P. bolleana* leaves of 6 and 14 dpi were sampled and promptly frozen in liquid nitrogen and stored at −80°C until assay. The extraction and $C_i$ of starch and sucrose were measured using the Plant Sucrose Sugars Assay Kit and Soluble Starch Assay Kit (BC2465 and BC0705; Solarbio Life Sciences, Beijing, China) according to the manufacturer’s instructions.

**Statistical analysis**

All of the experimental data were subjected to SPSS version 17.0 for statistical tests and analyses. $P < 0.05$ was considered significant unless otherwise noted. All statistical data were presented as mean ± standard error. In this study, correlation analysis was performed on Chl, $\Phi_{PSII}$ and $P_n$, and linear regression fitting was performed. All fittings were tested by t-test ($P < 0.001$).
**Figures 2**

*Figure 2B* dothidea inoculation on water transportation in *P. bolleana* leaves. One-year old poplar stems were inoculated, respectively, by *B. dothidea* (Bd) or PDA (CTR).

(A) Transpiration rate (*Tₚ*), (B) vapor pressure deficit (VPD), and (C) water use efficiency (WUE) were measured at 0, 2, 4, 6, 8, 10, and 14 dpi. Data are presented as the mean of seven replicates. Error bars represent the standard error of the mean. Asterisks denote significant difference at *P* < 0.05 between treatments.

### Results

#### Photosynthetic parameters

The photosynthetic parameters were measured in leaves of *B. dothidea*-inoculated and control poplars. *Pₚ* and *Gₛ* declined rapidly in *B. dothidea* inoculation treatments (range: 2–14 dpi, Figures 1A,B). On the fourth day after *B. dothidea* inoculation, *P. bolleana* leaves displayed completely opposite trends in *Cᵢ* and *Lₛ* (Figures 1C,D). *Cᵢ* level declined after *B. dothidea* inoculation (range: 4–14 dpi), while *Lₛ* showed an increasing trend. These results suggested that the main reason for declined *Pₚ* after *B. dothidea* inoculation was stomatal restriction. In addition, compared with the control plants, *Tᵢ* of inoculated plants was markedly reduced, VPD increased, but WUE had no significant change, indicating that *B. dothidea* did not cause damage to the water transport in the early stage of inoculation (Figures 2A–C).

#### Stomatal behavior

At 6 and 14 dpi, leaves of *B. dothidea*-inoculated and control poplars showed any difference in stomatal density (Figure 3A), which may be associated with the detectable leaves having already matured. *B. dothidea* inoculation on poplar stem could inhibit stomatal opening of distal leaves at 6 and 14 dpi (Figures 4A,B,E,F). In controls, the stomata were almost completely opened (Figures 4C,D,G,H). Nevertheless, compared with control plants, *B. dothidea* inoculation reduced the width of the stomatal aperture by 76.1% at 6 dpi and 74.2% at 14 dpi (Figure 3B). Accordingly, the stomatal opening rate of *B. dothidea*-inoculated plants was also decreased by 39.6% at 6 dpi than in comparison with controls, 63.2% at 14 dpi (Figure 3C). The above results were combined with the declined *Gₛ* and increasing *Lₛ* (Figures 1B,D).

#### Variation in hormone

Stomatal closure is usually controlled by hormone. At 6 dpi, the content of ABA and JA-me in leaves of *B. dothidea*-inoculated plants had no significant difference with the controls (Figures 5A,B). Whereas, at 14 dpi, the level of ABA and JA-me in leaves of *B. dothidea*-inoculated plants, respectively, increased by nearly twofold and 24.6% in comparison with their controls (Figures 5A,B). In contrast with controls, *B. dothidea*-inoculated plants sustained higher content of IAA and zeatin (ZR) across the whole observation (Figures 5C,D). Compared with the control plants, IAA content increased by 30.6 and 28.9%, while ZR increased by 28.3 and 59.5% at 6 and 14 dpi, respectively (Figures 5C,D).

#### Photosynthetic pigments content

As a result of *B. dothidea* inoculation, chlorophyll content, fractional chlorophyll a, b, and carotenoids content were severely reduced in poplar leaves at different degrees (Figures 6A–D). At 6 dpi, in contrast with control leaves, *B. dothidea* inoculation caused a 42.4, 38.5, 41.8, 29.4, 12.2, and 16.3% decline in *Chl a*, *Chl b*, total chlorophyll (*Chl*), *Car*, chlorophyll a/b (*Chl a/b*) and total chlorophyll/carotenoids (*Chl/Car*), respectively (Figure 6). Compared to control leaves, *Chl* a content decreased by 32.2% at 14 dpi under *B. dothidea* inoculation, *Chl* b by 27.3%, *Chl* by 31.4%, *Car* by 14.3%, *Chl a/b* by 8.1%, and *Chl/Car* by 18.5% (Figure 6).

#### Evaluation of chlorophyll fluorescence characteristics

When poplar stems were inoculated with *B. dothidea*, the chlorophyll fluorescence parameters of leaves were seriously affected.
affected. Plants inoculated with *B. dothidea* experienced abrupt declines in $F_{v}/F_{m}$, $\Phi_{\text{PSII}}$, and ETR, which were consistently lower than those in control plants (Figures 7A–C). Additionally, *B. dothidea*-inoculated poplars had less $q_{p}$ than controls, except for 8 dpi (Figure 7D). The undifferentiated data at 8 dpi did not affect the whole variant trend of $q_{p}$. Compared with control plants, a higher NPQ was always observed after *B. dothidea* inoculation from the fourth day onward (Figure 7E).

**Correlation analysis in physiological indices**

According to the linear correlation performed, both Chl and $\Phi_{\text{PSII}}$ were positively correlated with $P_{n}$ throughout *B. dothidea* inoculation (Figure 8).

**Leaf non-structure carbohydrate content**

*B. dothidea*-inoculated plants showed similar trends in starch and sucrose content of distal leaves. At 6 dpi, *B. dothidea* inoculation plants contained less sucrose than controls (Figure 9A). With the prolonged inoculation time, more sucrose was accumulated in *B. dothidea*-inoculated plants at 14 dpi (Figure 9A). In *B. dothidea*-inoculated plants, starch content was 48.5 and 80.5% higher than in controls at 6 and 14 dpi, respectively (Figure 9B).

**Discussion**

**Photosynthetic rate**

Photosynthesis can negatively affect by leaf pathogens in several ways, including modulating stomatal opening, reducing gas exchange, damaging green tissues and photosynthetic apparatus (Gruber et al., 2012; Júnior et al., 2017; Fermín et al., 2018; Franziska et al., 2018). Additionally, stem canker fungi such as *Quambalaria coyrecup* and *Anisogramma anomala* are among those may alter the photosynthetic properties of distal leaves remotely on *Corymbia calophylla* and hazelnut (Hossain et al., 2019; Lachenbruch and Zhao, 2019). In previous studies, we had similar results that leaves in *B. dothidea*-inoculated and *V. sordida*-inoculated plants showed weakened $P_{n}$ and $G_{s}$ during the observation stages (Li et al., 2019; Xing et al., 2020). In the initial stage of stress, stomatal closure resulted in the reduction in carbon assimilation thus reducing net photosynthesis. This phenomenon is known as a stomatal limitation. The type of stomatal limitation is mainly determined by $C_{i}$ and $L_{s}$ (Farquhar and Sharkey, 1982). In this study, *B. dothidea* inoculation obviously declined $P_{n}$ and $G_{s}$ at early inoculation stages (Figures 1A,B). $C_{i}$ level drastically reduced at 2–14 dpi, while $L_{s}$ showed an opposite trend, indicating that $P_{n}$
FIGURE 5
B. dothidea inoculation on phytohormone content in P. bolleana leaves. One-year old poplar stems were inoculated, respectively, by B. dothidea (Bd) or PDA (CTR). Sample collection and phytohormone content measurement were performed at 6 and 14 dpi. (A) ABA content, (B) JA-me content, (C) IAA content, (D) ZR content. Each column is the mean of seven replicates. Error bars represent the standard error of the mean. Columns labeled with different letters (a–c) denote a significant difference ($P < 0.05$) between treatments.

FIGURE 6
B. dothidea inoculation on photosynthetic pigments in P. bolleana leaves. One-year old poplar stems were inoculated, respectively, by B. dothidea (Bd) or PDA (CTR). Sample collection and photosynthetic pigments measurement were performed at 6 and 14 dpi. (A) Chlorophyll a content (Chl a), (B) Chlorophyll b content (Chl b), (C) total Chlorophyll content (Chl), (D) Carotenoids content (Car), (E) Chlorophyll a/b ratio, (Chl a/b), (F) Chlorophyll/Carotenoids ratio, (Chl/Car). Each column is the mean of seven replicates. Error bars represent the standard error of the mean. Columns labeled with different letters (a–c) denote a significant difference ($P < 0.05$) between treatments.
is reduced by stomatal limitation at the early stage of inoculation (Figures 1C,D). Stem canker pathogens have been shown to infiltrate xylem tissues in histopathological studies (Biggs et al., 1983; Mcpartland and Schoeneweiss, 1984; Czemmel et al., 2015). Fungal pathogens also reduce plant hydraulic conductance and sap flow (Parke et al., 2007; Hossain et al., 2019). In this study, the reduced $G_s$ also dramatically reduced the $T_r$ when compared with control treatments (Figure 2A). Conversely, VPD was increased, and WUE had no significant change, indicating that *B. dothidea* inoculation did not cause severe damage to water transport during the early stage (Figures 2B,C).

**Stomatal closure**

Stomatal movement is affected by guard cells’ osmotic/turgor pressure, the latter reacting in response to
external and internal stimuli (Blatt, 2008). The stomata can adjust their size in response to environmental stress, which regulates gas exchange, CO$_2$ uptake and water loss (Schroeder et al., 2001; Melotto et al., 2006; Lawson and Vialet-Chabrand, 2019). Plants close their stomata to combat pathogenic microbes as part of their immune response (McLachlan et al., 2014). In this study, B. dothidea inoculation on poplar stems induced stomatal closure in distal leaves (Figure 4). Although B. dothidea inoculation caused remarkable reduction of stomatal aperture and opening rate, no obvious influence was detected on stomatal density (Figure 3), which may be related to the maturation of the detected leaves. These results were consistent with the decreased $G_s$ and $C_r$, as well as the increased $L_s$ (Figures 1B,C). Photosynthesis is also reduced due to this phenomenon, which confirms stomatal limitation during B. dothidea inoculation (Li et al., 2019; Xing et al., 2020). We speculated that B. dothidea inoculation reduced the stomatal apertures and stomatal opening rate, which in turn limited the $G_s$ subsequently caused a declined $P_n$.

Phytohormones metabolism can also be disrupted by pathogens (Bari and Jones, 2009). Hormones are considered part of the immune system of plants, and may play a role in disease development (Verma et al., 2016; Kunkel and Harper, 2018). Similar to ABA, JA-me can also induce stomatal closure. There has been evidence that stress can lead to crossovers of phytohormone in plant (Ku et al., 2018). ABA and JA-me exhibit phytohormones crosstalk in the pathway for stomatal closure (McLachlan et al., 2014). In this study, B. dothidea inoculation triggered stomatal closure at 6 and 14 dpi, but the level of ABA and JA-me only increased at 14 dpi (Figures 5A,B). In addition, IAA and ZR content in B. dothidea-inoculated plants were significantly higher than controls at 6 and 14 dpi (Figures 5C,D). It is generally believed that IAA and ZR contribute to the stomatal opening and inhibited ABA-induced stomatal closure (Blackman and Davies, 1984; Irving et al., 1992), which is in disagreement with our study. Pathogens that cause necrotroptosis produce diverse hormone-like substances and secondary metabolites, which disturb hormone metabolism and trigger other effects on distal tissues from the inoculation site (Spor and Dong, 2008; Andolfi et al., 2011; Yang et al., 2015; Fonseca et al., 2018). Our results showed that the stomatal closure induced by these active agents were not affected by IAA and ZR (Figures 3, 4). The asexual stage of B. dothidea is Fusicoccum aesculi, which belong to the same genus with Fusicoccum amygdale, the latter producing Fusicoccin stimulating stomatal opening (Squire and Mansfield, 2010). Therefore, it is possible that B. dothidea-induced stomatal closure is also associated with hormone-like toxins and secondary metabolites released by B. dothidea. Determination of these active substances and the underlying physiological mechanism require further study.

**Absorption and transformation of light energy**

Adversity stress may generate irreversible damage to plant cell structure and cause metabolic disorder (Flexas and Medrano, 2002). Plants are susceptible to biotic stress that can disrupt their photosynthetic apparatus (Júnior et al., 2017). The decreased photosynthetic pigments and chlorophyll fluorescence parameters were also in charge of the reduced $P_n$ in B. dothidea-inoculated plants (Figures 6–8). Environmental stress can affect the photosynthetic pigments metabolism of plants. Leaf pathogen infection can decline chlorophyll content, and hinder light energy absorption and electron transfer, thus suppressing the photosynthetic carbon assimilation (Mandal et al., 2009). Seiridium cardinal-infected cypress needles showed decreased content of chlorophyll and carotenoids (Muthuchelian et al., 2005). Compared to the control plants, poplar stems inoculated with B. dothidea had lower content of Chl $a$, Chl $b$, total Chl, and Car at 6 and 14 dpi in distal leaves (Figures 6A–D). In B. dothidea-inoculated plants, the chlorophyll $a/b$ ratio was reduced at 6 and 14 dpi, indicating Chl $b$ proportion increased in total Chl, which is conducive to efficient utilization of low light (Figure 6E). By absorbing
excess light energy and quenching active oxygen, carotenoids help protect the photosynthetic apparatus (Chi et al., 2015). It is possible that *B. dothidea*-induced quenching of active oxygen is partly responsible for the simultaneous decrease of Car and Chl/Car (Figures 6D,F). Meanwhile, Car's descent speed was much slower than Chl's degradation rate, which might play a crucial role in light protection (Figure 6F). Several lines of evidence indicate that *B. dothidea* accelerates the degradation of photosynthetic pigments or inhibits their synthesis. Inoculating poplar stems with *B. dothidea* reduced photosynthetic pigments in distal leaves, causing $P_n$ to decline. These results were further supported by the positive correlation between Chl and $P_n$ (Figure 8A).

Chlorophyll fluorescence can give a good indication of the activity of the photosynthetic apparatus (Björkman and Demmig, 1987; Krause and Weis, 1991; Watling et al., 2000). $F_v/F_m$ can reflect the energy conversion efficiency of PSII reaction center, and this parameter will drop under environmental stress (Neil, 2008). For example, *Melampsora medusae*, a fungus that causes leaf rust, caused a striking reduction of $F_v/F_m$ in poplar trees (Fermin et al., 2018). Besides the leaf pathogen, stem pathogens, such as *Seiridium cardinale*, also inhibited leaf photosystem II in cypress canker (Muthuchelian et al., 2005). In this context, as a result of *B. dothidea* inoculation on poplar stems, $F_v/F_m$ declined in distal leaves, which indicates that *B. dothidea* inhibited the conversion of light energy (Figure 7A). In response to adversity stress, both ETR and $\Phi_{PSII}$ were restrained at different levels, which affected photosynthetic carbon assimilation (Guo et al., 2016). In distal leaves, *B. dothidea* decreased $\Phi_{PSII}$ and ETR, which suggests that this fungus suppressed electron transport efficiency (Figures 7B,C). This conclusion was further supported by the positive correlation between $\Phi_{PSII}$ and $P_n$, indicating that the decrease in $P_n$ was due to the obstruction of photosynthetic electron transfer (Figure 8B). In PSII, $q_P$ and NPQ are commonly used to measure light energy utilization and dissipation. The $q_P$ of distal leaves was decreased across 2–14 dpi following *B. dothidea* inoculation (Figure 7D). However, NPQ was consistently higher than control across the detecting stage (Figure 7E). After *B. dothidea* inoculation, the distal leaves dissipated the excess light energy by heat dissipation to maintain the normal photosynthesis physiological process. According to these results, a decrease in photosynthetic pigments content and a blockage of electron transport were partially responsible for *B. dothidea* inoculation suppressing $P_n$.

As shown above, *B. dothidea*-inoculated on stems suppressed the activity of PSII reaction center of distal leaves, leading to reduction in light energy capture by antenna pigments, thereby declining light energy utilization. In response to the reduced photochemical efficiency, PSII accumulated a large amount of light energy. Leaf NPQ levels were increased to dissipate excess light energy, thereby protecting the photosynthetic apparatus.

**Carbohydrate content**

When fungi invade plant tissues, necrosis occurs, net $P_n$ and carbon assimilation are reduced (Berger et al., 2007; Christian et al., 2008). Photosynthesis produces starch and sucrose, which are widely considered energy sources for metabolism. As a non-reducing sugar, sucrose exhibits a high level of solubility, resistance to degradation, and low viscosity (Patrick et al., 2013). Due to these advantages, sucrose has become a major long-distance transport product in the phloem (Patrick et al., 2013). In response to environmental stress, the stomata can regulate gas exchange and control the absorption of CO$_2$ (Melotto et al., 2006; Lawson and Vialet-Chabrand, 2019). When *B. dothidea* was inoculated on poplar stems, stomatal closure of distal leaves occurred and CO$_2$ uptake was reduced (Figures 3, 4). In spite of this, persistent stomatal closure results in reduced CO$_2$ uptake for plants, affecting their photosynthetic productivity (O’Grady et al., 2013). In agreement with this conclusion, a reduction in photosynthetic pigments and chlorophyll fluorescence parameters resulted in a lower net $P_n$ (Figures 1A, 6–8). Photosynthetic products will be reduced by the above results. Nevertheless, *B. dothidea* inoculation on poplar stems reduced leaf sucrose content at 6 dpi, but by 14 dpi, leaf sucrose content rose (Figure 9A). Throughout the observation period, *B. dothidea* inoculation increased leaf starch levels (Figure 9B). Previously, we demonstrated that cankers disrupt the downward transport of photosynthetic products (Li et al., 2019; Xing et al., 2020). In brief, starch and soluble sugar content were higher in the above regions of *B. dothidea* inoculation sites than below at 20, 25, 30 dpi during long-term inoculation (Xing et al., 2020).

Phloem girdling involves removing all the phloem, causing more damage to the stem. Instead of phloem girdling, *B. dothidea* inoculation treatments in this study were just scraped the barks of inoculation sites gently without damaging the cambial, xylem, and most of the phloem tissue, resulting in less possibility switched to serious injury in short-term. Phloem girdling prevents the downward movement of starch, which builds up in leaves and inhibits photosynthesis through negative feedback (Paul and Peliny, 2003; Urban et al., 2004). Consequently, sucrose and starch accumulation in leaves might be due to the local phloem damage caused by *B. dothidea*, hindering the assimilates downwards (Figure 9). According to our results, $P_n$ was not affected by the negative feedback mechanism in the early stage of *B. dothidea* inoculation, but whether it works during the later stage remains to be verified.

**Conclusion**

In this study, *B. dothidea* inhibited the leaf photosynthesis in the early stage of inoculation (2–14 dpi). Physiological mechanisms are as follows, (i) Decreasing $G_i$, $C_i$, and
increasing $L_s$ following $B. dothidea$ inoculation, indicating that stomatal limitation was responsible for inhibiting $P_n$. We observed stomatal closure in leaves leading to reduction in CO$_2$ absorption, as expected. (ii) Inoculation of $B. dothidea$ on stems affected the metabolism of distal leaves’ photosynthetic pigments. Deficiencies in chlorophyll and carotenoids hindered light energy absorption, conversion and electron transfer. (iii) The decreased $F_v/F_m$, $\Phi_{PSII}$, ETR, $q_P$ and increased NPQ indicated that $B. dothidea$ could partially close PSII reaction center on distal leaves, reducing light energy capturing capacity by photosynthetic pigments, thereby declining light energy utilization. Photosynthetic pigments suppression was also reflected in the chlorophyll fluorescence parameters. In combination, the above findings listed in i, ii, and iii inhibited photosynthetic carbon assimilation, thereby reducing photosynthetic products. However, due to the local damage caused by inoculation in phloem prevented the downward transport of assimilation products, leading to an accumulation of sucrose and starch in leaves.

Data availability statement

The original contributions presented in this study are included in the article-supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

JX, JZ, and YZ conceived and designed the original research plans. YZ supervised the experiments. JX and ML performed most of the experiments. JL, WS, and PL provided technical assistance to JX and ML. JX designed the experiments and analyzed the data. JX conceived the project and wrote the article with contributions of all the authors. YZ and JZ supervised and complemented the writing. All authors have read and approved the manuscript.

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Melampsora medusae causes unusual damage to leaves and modifies carbohydrate metabolism in Grapevine.

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