Australasian Plant Pathology (2022) 51:231–243
https://doi.org/10.1007/s13313-021-00848-3

ORIGINAL RESEARCH ARTICLE

Thiamine induces resistance in tobacco against black shank

Tian Suohui[1] · Chen Yanping[1] · Zi Shuhui[1] · Li Zhihua[2] · Jin Honggang[2] · Liu Jun[2] · Liu Tao[1]

Received: 2 August 2021 / Accepted: 27 December 2021 / Published online: 18 January 2022

Abstract

Induced resistance by elicitors is considered to be an eco-friendly strategy to stimulate plant defence against pathogen attack. Thiamine (vitamin B1, VB1) can act as a plant defence trigger or priming agent, leading to a rapid counterattack on pathogen invasion, but the underlying molecular mechanisms have not yet been fully elucidated. In the present study, the priming effect of thiamine on tobacco against the disease *Phytophthora nicotianae* and its biochemical and molecular impact on plant defence mechanisms, as well as the *in vitro* inhibitory effect of thiamine on *P. nicotianae*, were evaluated. The results showed that the mycelial growth and sporangium production of *P. nicotianae* were inhibited by thiamine in a dose-dependent manner. After thiamine pretreatment, the resistance of tobacco plants to *P. nicotianae* was enhanced, and the severity of tobacco related disease was significantly reduced. In tobacco plants stimulated by thiamine, H_2O_2 accumulation and catalase (CAT) and peroxidase (POD) and phenylalanine ammonia lyase (PAL) activity levels were enhanced, and seven defence-related genes were upregulated in the plant leaves in order to avoid anthropomorphising plant responses to pathogen attack. Overall, this study demonstrates that thiamine effectively induces resistance against *P. nicotianae* in tobacco under greenhouse-controlled conditions through a dual mode of action involving direct antifungal activity and induction of host defence mechanisms. It is suggested that thiamine may be an attractive alternative to chemical fungicides in tobacco plant disease management.

Keywords Defence response · Induced resistance · Thiamine · *P. nicotianae*

Introduction

Black shank, caused by *Phytophthora nicotianae*, is among the most widespread and damaging diseases of cultivated tobacco (*Nicotiana tabacum*) worldwide (Csinos et al. 1999). The pathogens infect plants at any stage of their growth, with the field stage being the most damaging, causing root rot, stem lesions, leaf necrosis and plant death. Disease damage can spread rapidly under conditions of high temperature (23 °C–28 °C) and high soil moisture, causing serious yield losses (Gallup et al. 2018; Vontimitta et al. 2012). Traditional control strategies, including crop rotation, fungicide applications, and the use of resistant cultivars, are not sufficient to control this soil-borne disease (Haas et al. 2005). Fungicides have become increasingly unwelcome, although they are currently used in most cases because of their long time in the field, and can lead to resistant pathogen strains (Aguiar et al. 2017). Some recent studies have shown that several years of monoculture plants can recruit growth-promoting rhizobacteria (PGPR) to prevent losses caused by soil-borne pathogens. However, this discovery is not practical for preventing soil-borne pathogens for certain economic reasons (Haas et al. 2005). Thus, it is urgent to explore more efficient and sustainable control methods for these soil-borne diseases.

The plant defence system usually consists of preexisting physical and chemical barriers as well as inducible defence responses (Jackson et al. 1996). A plant's successful defence against invading pathogens depends on early recognition of the pathogens and initiation of the appropriate signalling processes to activate a multicascade defence response

Liu Jun and Liu Tao equally contributed to this work

✉ Tian Suohui

✉ Liu Jun

✉ Liu Tao
yantao618@126.com

[1] College of Agriculture and Biotechnology, Yunnan Agricultural University, Kunming 650201, China

[2] Technology Centre of Hongyun Honghe Tobacco Group, Kunming 650201, Yunnan, China

(Ryals et al. 1996; Meng et al. 2013). Local or systemic two-layer defence mechanisms produce systemic acquired immune resistance (SAR) to produce resistance to protect themselves against pathogen invasion (Dempsey et al. 1997; Bigeard et al. 2015). Similarly, upon perception of certain stimuli, such as the colonization of beneficial microorganisms and the application of chemical substances, plants can activate their own defence system and enhance their defence ability against future pathogens and pests, namely induced resistance (IR) (De Kesel et al. 2021). A number of chemical inducers are used for IR stimulation, such as SA, benzothiadiazole (BTH), and β-aminobutyric acid (BABA), have been proven to induce plant disease resistance (Barilli et al. 2010; Frąckowiak et al. 2019). Inducers restrict pathogen development by activating host celldefence responses, including oxidative burst, lignin and callose deposition, and a complex signal transduction network (SA JA and ET) (Ryals et al. 1996; Thomma et al. 1998; Kunkel et al. 2002). The IR phenotype is associated with both direct induction of defence responses, and primed defence responses (Wilkinson et al. 2019; Balmer et al. 2015). Direct defensive responses are defensive responses that are locally or systematically induced in plants when exposed to IR stimuli, and these responses can be detected prior to any challenge (Mauch-Mani et al. 2017; Conrath et al. 2002; Van Hulten et al. 2006). In the priming defence response, the plant's defence system is not directly affected by IR stimulation but can be activated in late challenge (Mauch-Mani et al. 2017). Studies have shown that a low concentration of MeJA (10 µmol/L) triggers a priming defence mechanism, while a higher concentration of MeJA (50 or 100 µmol/L) directly activates defence responses, thus enhancing disease resistance in grape berries (Wang et al. 2015). Protecting plants by inducible resistance mechanisms may be a more environmentally friendly control method. The metabolic investment of the plant is reduced compared to constructional defence activation (Conrath et al. 2006).

Thiamine is a water-soluble B-complex vitamin that is produced in plants and microbes (Burrows et al. 2000). Many studies have documented the beneficial roles of thiamine in enhancing plant disease resistance (including to bacterial wilt) (Ahn et al. 2005, 2007; Jung et al. 2003). It has been shown that thiamine can directly inhibit the activity of *Plasmopara viticola in vitro* (Boubakri et al. 2012). Some early experiments demonstrated that thiamine can activate PR-1 gene expression in tobacco and stimulate resistance to TMV in a SA-dependent manner (Malamy et al. 1996). Subsequent investigations revealed that thiamine primes the pathogen-induced expression of PR-1 and PAL as well as callose deposition and an oxidative burst associated with the HR in Arabidopsis (Ahn et al. 2007). When infected by the blast fungus *Magnaporthe grisea*, the basic resistance level of rice plants supplied with thiamine was increased, which was attributed to the formation of a physical barrier that prevented or slowed fungal penetration and stronger and more rapid pathogenesis-related (PR) gene expression and activity of protein kinase C (Ahn et al. 2005). Recent studies have shown that thiamine induces rice to inhibit nematode infection by enhancing lignin production mediated by H_2O_2 and phenylpropanol (Huang et al. 2016). Although thiamine has been found to induce resistance to different pathogens, the molecular mechanisms underlying this have not been completely elucidated (Asensi-Fabado et al. 2010), especially *Phytophthora* infection, which has not been tested previously.

Because of the protective activity of thiamine in inducing plant priming against disease, we hypothesized that thiamine might have the potential to activate plant disease resistance against *P. nicotianae* infections in the thiamine nonaccumulator plant tobacco. Therefore, related experiments were conducted using *N. tabacum* (tobacco) to investigate the related underlying mechanisms of thiamine in inducing plant disease resistance. We monitored the content of H_2O_2 and SA and the activity of defence enzymes in tobacco leaves. In addition, we also examined the expression of a set of pathogenesis-related genes in SA defence signalling pathways, the ET pathway and the HR pathway, or encoding the proteins participating in antifungal defence by quantitative real-time PCR (RT–qPCR).

Materials and methods

Plant material and inoculum preparation

The tobacco cultivar Honghua Dajinyuan (HD), which is highly sensitive to black shank, was used as the material. The seeds (gifted by tobacco companies) were sown in seedling trays, and the seedlings were transferred to larger pots (diameter = 30 cm) at the 5–6 true-leaved stage, keeping one seedling per pot, and grown in a greenhouse (25 ± 3℃), Yunnan Agricultural University, Kunming, Yunnan Province, China. The plantlets were used within two weeks of transplanting.

The *P. nicotianae* strain maintained in our laboratory was routinely recultured on potato dextrose agar (PDA: 200 g of potato, 20 g of glucose and 15 g of agar in 1000 ml water) at 27 °C in the dark for 15 days (Huang et al. 2015; Gallup et al. 2018).

Effect of thiamine at different concentrations on mycelial growth and sporangium production of *P. nicotianae in vitro*

Thiamine stock solution was prepared in sterile distilled water, and the solution was filtered through a microfiltration

membrane(0.45 μm). The effect of thiamine on the mycelial growth of *P. nicotianae* was evaluated on PDA plates according to the method of Zhang et al. (2018; 2020). Mycelial plugs (3 mm diameter) from actively growing the pathogen for 15 days were transferred into a new PDA plate with different concentrations of thiamine (0, 1, 2, 5, 10, 20 and 50 mM). The mycelial disk was placed in the centre of the plate (diameter 90 mm). After 15 days of incubation at 28 °C, the colony diameters were measured by the perpendicular diameters, took the mean of diameters measured at right angles to each other, and the inhibition rate was calculated. Each of the thiamine concentrations was replicated on four plates, and the experiment was repeated three times.

According to the reported method of Dalio et al. (2014), with slight modifications, the effect of thiamine on the sporangia of *P. nicotianae* was studied. Briefly, 0.1% KNO_3 was used to prepare induction solutions containing different concentrations of thiamine (0, 1, 2, 5, 10, 20, 50 mM), and a 7-mm *P. nicotianae* agar disk from actively growing 15-day mycelium was transferred to a petri dish supplemented with induction solution (10 mL), followed by culturing at 28 °C for 48 h. In an aseptic operating environment, the agar medium at the lower part of the fungal mycelia disk was cut off in parallel to make the thickness approximately 1 mm and was placed on the slide to observe the number of sporangia under a 10 × 20 optical microscope, and pictures were taken and recorded. All treatments consisted of four replicates, and the experiment was repeated three times.

Induction of 'HD' tobacco resistance against *P. nicotianae* by thiamine

Thiamine Treatment: Four- to five-leaf tobacco seedlings were uniformly sprayed with either distilled water (DW) or thiamine at concentrations of 20 mM. After that, the treated seedlings in each treatment group were separately covered with plastic bags to maintain high humidity and incubated in a climate-controlled room. A second spray was given seven days later. Each treatment was conducted in triplicate, and each replicate contained 10 plants. Leaves were detached from the same layer of plants for assays at 0, 6, 24, 72 and 120 h (Deenamo et al. 2018).

P. nicotianae inoculation: Three days after the second spray of distilled water (DW) or thiamine (20 mM) on the leaves, *P. nicotianae* was inoculated according to the method of Keller (Sullivan et al. 2005). Mycelial plugs (7 mm diameter) from actively growing the pathogen for 15 days were placed on the base surface of the petiole that was scratched with the blade first (the 4th extended leaf from top to bottom) and then moisturized with sterile cotton. After inoculation, the seedlings were kept in a plastic bag with high humidity and placed in a greenhouse. Tobacco stalk symptoms were evaluated at 0, 2, 6, 8, 10 and 15 dpi. At 15 dpi, the morphological indexes were measured, and the disease condition was recorded and classified by plant. The disease severity was based on the degree of spread of disease spots at the stem base and leaf wilting. The disease classification standard and investigation method were carried out according to the provisions of the tobacco industry standard YC/T 39–1996 of the People's Republic of China. Ten plants were used for each treatment. The experiment was repeated three times. Leaves were collected from plants at different intervals (1, 5, 10 dpi), immediately frozen in liquid nitrogen, and stored at -80 °C until use for enzyme assays and gene expression analysis.

Protein extraction, H_2O_2 content and enzyme activity assays

Protein content, H_2O_2 content, and the activities of main antioxidant enzymes, including CAT, POD and PAL, were determined using a protein assay kit, an H_2O_2 content assay kit, a CAT activity assay kit, a POD activity detection kit, and a PAL activity assay kit (Suzhou Greys Biological Technology Co., Ltd, Suzhou, China), respectively.

Total phenolic content and lignin detection

Leaf samples (0.1 g fresh weight) were ground to a fine powder in liquid nitrogen with a frozen mortar and pestle and then homogenized with 1.5 mL of 60% ethanol. The homogenate was centrifuged at 12,000 rpm for 10 min at room temperature. The total phenol content of the extract was determined by the Folin phenolic method (Blainski et al. 2013). Under alkaline conditions, the phenolic substances reduced tungstomolybdic acid to produce blue compounds. The absorbance value was read at 760 nm to determine the total phenol content. The absorbance values were calibrated against a standard curve and expressed as μg per millilitre (μg ML^{-1}). For the determination of lignin content, the leaf samples (1.5 mg dry weight) were ground into a fine powder with a mortar and pestle, homogenized with 1.5 mL of 80% ethanol and centrifuged at 12,000 rpm for 10 min at room temperature. The total phenolic content of the extract was determined by the acetylation method (Moreira-Vilar et al. 2014), and the phenol hydroxyl group in the lignin was acetylated. The absorbance value was read at 280 nm to determine the lignin content. The absorbance values were calibrated against a standard curve and expressed as milligrams per gram of dry weight (mg g^{-1} DW).

SA and scopoletin measurements

The contents of SA and scopoletin in the leaves of tobacco seedlings were measured by high-performance liquid chromatography (HPLC). The SA content was determined as

described in previous studies (Wen et al. 2005; Drzewiecka et al. 2012) with some slight modifications. Leaf samples (0.2 g fresh weight) were ground to a fine powder in liquid nitrogen with a frozen mortar and pestle and then homogenized with 1 mL of 70% methanol and extracted overnight at 4 °C. After the supernatant was centrifuged at 8000 × g for 10 min, it was subsequently filtered through a 0.45-μm membrane.

Scopoletin content was determined according to the method of Lerat et al. (2009). Leaf samples of 0.1 g fresh weight were ground into a fine powder with a mortar and pestle and then dissolved in a flask with 20 mL of 50% methanol. The mixture was ultrasonically extracted at room temperature for 20 min, centrifuged at 3000 rpm for 5 min and filtered through a 0.45-μm aqueous phase membrane.

Chromatographic separation was performed on a C18 reverse-phase column (250 mm × 4.6 mm, 5 μm) using a Jingdao LC-20AT high-performance liquid chromatograph. The compound in the sample (10 μL) was separated in a mobile phase containing methanol and 0.1% acetic acid water. The flow rate was 0.8 mL min^{-1}, the column temperature was controlled at 35 °C, and the retention time was 40 min. The SA UV detection wavelength was 306 nm, and the scopoletin detection wavelength was 340 nm. Each sample was subjected to HPLC with three independent replicates.

Analysis of gene expression by quantitative real-time PCR

Transcription of defence-related genes was determined, and the expression levels of SA pathway, ET pathway and HR pathway genes listed in Table 1 were detected (Guo et al. 2020). Total RNA was extracted from tobacco leaf tissue with a MagenHiPure HP Plant RNA Mini Kit (R4165-02, Magen, China). The RNA samples were measured for quality and quantity by measuring the ratio of 260/280 nm absorption, and their integrity was evaluated by visualizing the bands following electrophoresis on a 1% agarose gel. cDNA for RT–qPCR was synthesized from 2 μg of total RNA using the ABM 5 × All-in-One RT MasterMix (with AccuRT Genomic DNA Removal Kit) kit according to the manufacturer's instructions.

The PCR conditions were as follows: an initial incubation at 95℃ for 3 min, followed by 45 cycles of 95℃ for 10 s and 60℃ for 30 s and then by a melting curve cycle. The threshold period (CT) and melting curve of each gene were analysed. The relative mRNA amount was calculated by the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were performed for each experiment.

Statistical analysis

All of the data were analysed using SPSS 20.0. Significant differences between each experimental value between treatments were analysed at $p < 0.05$ by Student's t-test. Data are presented as means ± SE. The graphs were generated using Origin 2018.

Results

In vitro inhibition of mycelial growth and sporangium production of *P. nicotianae* by thiamine

The results of *in vitro* inhibition test showed that the mycelial growth and sporangium production of *P. nicotianae* were inhibited by thiamine in a dose-dependent manner. Inhibition of mycelial growth started at 1 mm/L and reached 93% inhibition with 50 mm/L thiamine (Fig. 1a, b). The number of sporangia released was significantly lower for all thiamine treatments (Fig. 1c). These data indicated that thiamine has a significant inhibitory effect on *P. nicotianae in vitro*.

Induced resistance of thiamine-pretreated tobacco against *P. nicotianae*

The tobacco leaves were sprayed with 20 mM thiamine, and there was no negative effect on tobacco growth during

Table 1 Primer pairs used for qRT-PCR

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
β-Actin	ATGCCTATGTGGGTGACGAAG	TCTGTTGGCCTTAGGGTTGAG
PR1	TTCTCTTTTCACAAATGCCTTC	CACCTGAGTATAGTGTCCACAC
PR5	GCTTCCCCTTTTATGCCTTC	CCTGGGTTCACGTTAATGCT
NPR1	ACATCAGCGGAAGCAGTAG	GTCGGCGAAGTAGTCAAAC
PAL	CGATAGACTTGAGGCATTTGG	TCAGTGGGTAGTTGGCGATG
CM1	TACCATTACTATTCGTCCCCTT	AGCCGTGAAACCCATCCA
HINI	CGACCTAACAAAGTCAAGTTCTACG	CTCTATCTCCCAATAAAACCAAGC
EFE26	CGGACGCTGGTGGCATAAT	CAACAAGAGCTGGTGCTGGATA

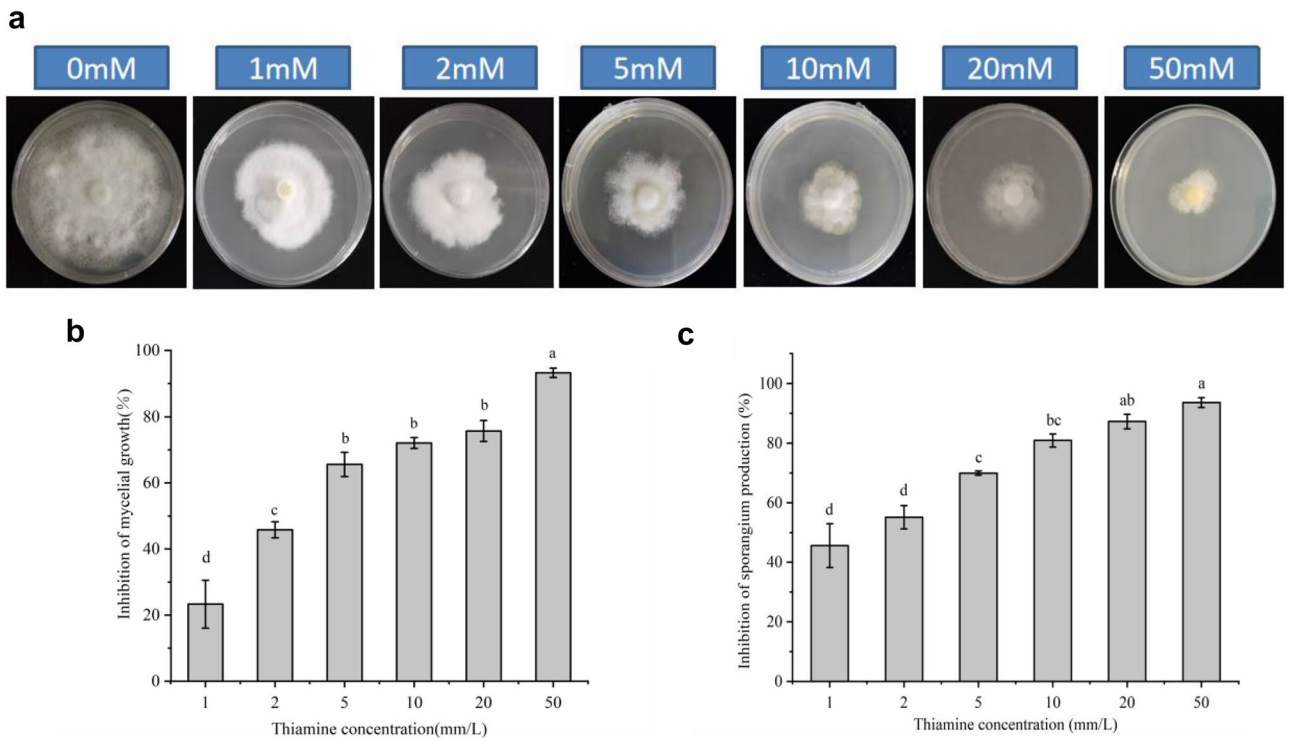

Fig. 1 Effect of thiamine on *in vitro* growth and sporangium production of *P. nicotianae* **a** *P. nicotianae* cultures in Petri dishes illustrating the inhibition of mycelial radial growth with increasing thiamine concentrations. The mycelial colonies were 15 days old. **b** Inhibition of *P. nicotianae* mycelial growth using different thiamine concentrations. **c** Inhibition of *P. nicotianae* sporangium production at different thiamine concentrations. These assays were repeated three times showing similar results. Bars show means ± SE ($n = 12$). Values with the same letter are not significantly different at $P < 0.05$

the whole experimental period (Table 2). However, the tobacco leaves were slightly damaged in the early stage of thiamine treatment and gradually returned to normal in the later stage.

At 2 dpi, small black and necrotic lesions were observed on the stems inoculated with the pathogen. In contrast, 20 mM thiamine pretreatment significantly inhibited the growth of *P. nicotianae* on infected stems, reduced the area of disease patches to varying degrees (Fig. 2a), and significantly reduced the severity of the disease by 37% (Fig. 2b). The results indicated that thiamine pretreatment could effectively inhibit *P. nicotianae* growth on tobacco stalk.

Effects of thiamine priming on H_2O_2 content, lignin content, CAT and POD activities, total protein content, and total phenolic content in tobacco leaves after *P. nicotianae* challenge.

The leaves were collected at 5 dpi, and the test results are shown in Fig. 3. H_2O_2 did not change with thiamine or inoculation wih *P. nicotianae* alone, but increased significantly with both thiamine and *P. nicotianae* together (Fig. 3a). There were similar results with lignin content (Fig. 3b). By contrast, POD activity did not change with *P. nicotianae* alone but increased significantly with thiamine, to the same

Table 2 Effects of different treatments on tobacco morphology indexes

Treatments	Plant height (cm)	Stem circumference (cm)	Maximum leaf length (cm)	Maximum leaf width (cm)
DW	55.00 ± 1.47 a	6.15 ± 0.29 a	55.25 ± 2.18 a	22.87 ± 0.83 a
DW + *P.nic*	42.00 ± 2.42 b	5.30 ± 0.27 b	46.50 ± 2.38 b	19.63 ± 1.25 b
Thiamine + DW	52.50 ± 1.19 a	5.98 ± 0.16 ab	54.38 + 2.08 a	24.00 ± 0.91 a
Thiamine + *P.nic*	54.38 + 2.08 a	5.85 ± 0.16 ab	52.25 + 1.25 ab	21.88 ± 0.43 ab

Morphological indexes were determined at 15dpi. Values are mean ± SE, Different letters represents a significant difference between treatments ($P < 0.05$)

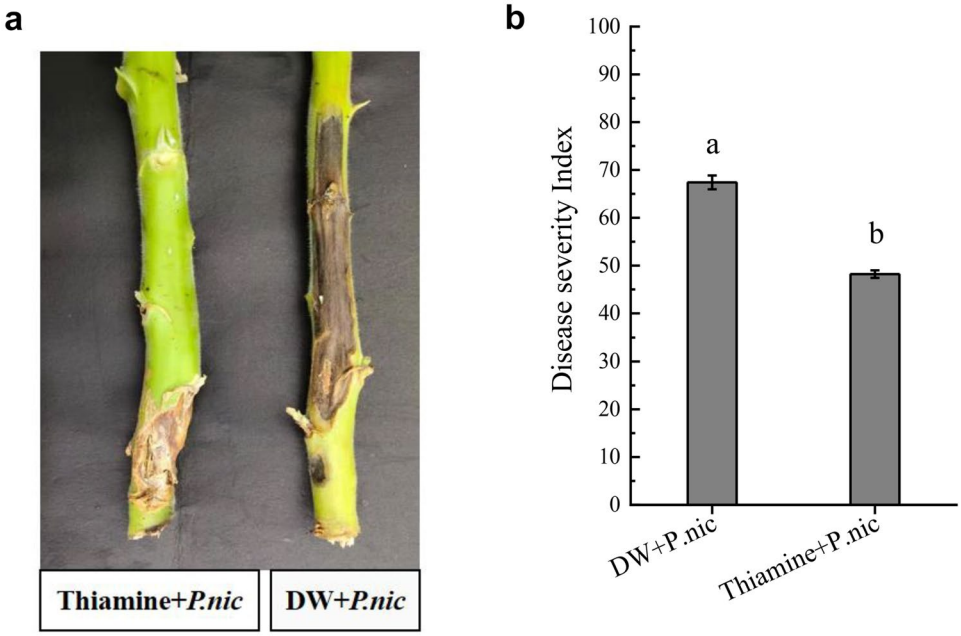

Fig. 2 Effect of exogenous thiamine treatment on tobacco against *P. nicotianae*. **a** Disease symptom **b** disease severity (%) of tobacco leaves pretreated with either DW or 20 mM thiamine for 3 day prior to subsequent inoculation with *P. nicotianae* at 15 dpi. Bars show means ± SE ($n = 30$). Different letters indicate significant diferences among treatments ($P < 0.05$; Duncan's multiple-range test)

extent, alone and with *P. nicotianae* (Fig. 3c). CAT activity did not change with thiamine alone but showed small but significant increases with *P. nicotianae*, alone and with thiamine (Fig. 3d). Protein content did not change either when inoculated with *P. nicotianae* alone or with thiamine, but significantly reduced protein content with thiamine

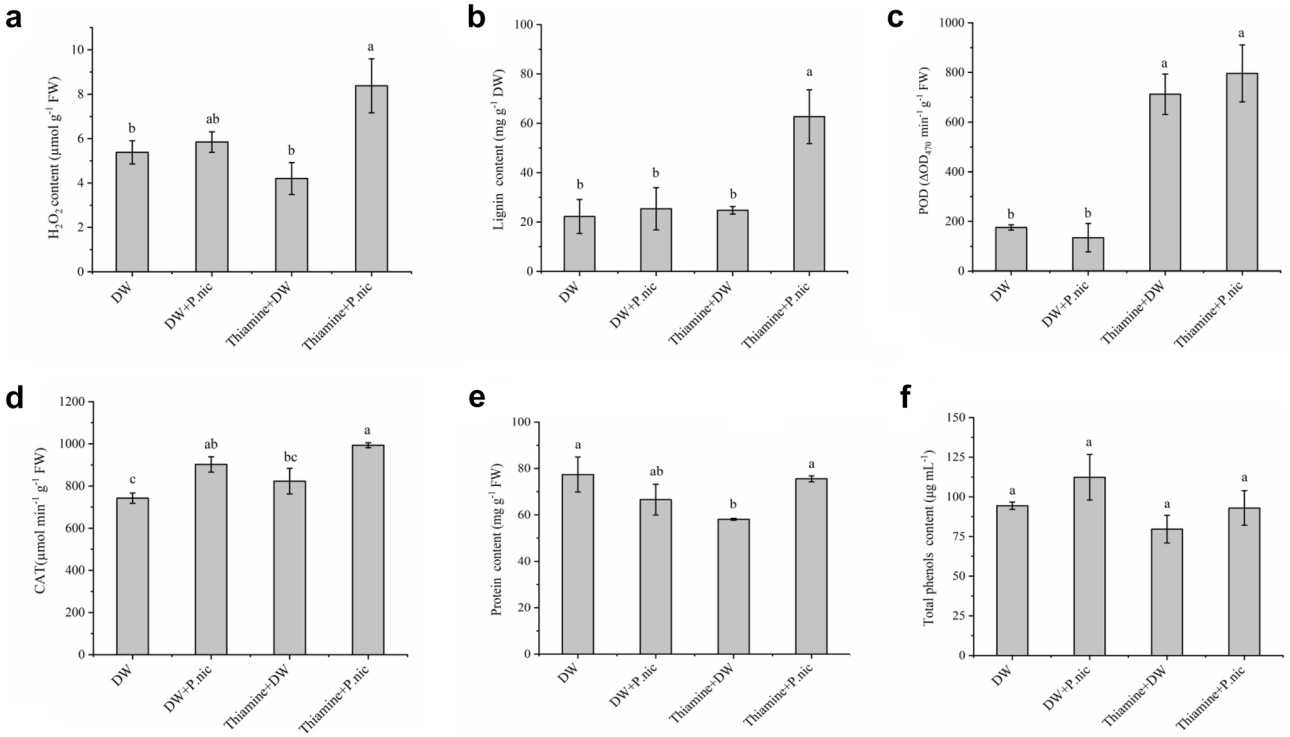

Fig. 3 Effect of exogenous thiamine pretreatment on H_2O_2 content, lignin content, POD and CAT activities, total phenolic content and total protein content in tobacco leaves after inoculation with *P. nicotianae* (*P. nic*). The leaves of tobacco were sprayed with either distilled water (DW) or Thiamine. After treatment, leaves were subsequently treated with either DW or *P. nicotianae*. After 5 day, the leaf samples were collected for determining **a** H_2O_2 content; **b** lignin content; **c** POD activity; **d** CAT activity staining; **e** protein content; **f** total phenolic content; Bars show means ± SE ($n = 4$). Different letters indicate significant diferences among treatments ($P < 0.05$; Duncan's multiple-range test)

alone(Fig. 3e). Total phenol content did not change with thiamine or inoculation with *P. nicotianae* (Fig. 3f).

Effects of thiamine priming on salicylic acid and scopoletin contents in tobacco leaves after *P. nicotianae* challenge

The treated leaves were collected at 5dpi, and the contents of endogenous SA and Scp were determined. The results showed that the content of SA did not change with thiamine or inoculation with P. nicotianae alone, but but increased significantly with both thiamine and P. nicotianae together (Fig. 4a). In addition,

thiamine alone decreased SCP content, but inoculation with P. nicotianae increased it, with and without thiamine(Fig. 4b).

Effects of thiamine on the kinetics of H_2O_2 content, defence enzyme activities and lignin contents in tobacco leaves

The results revealed that thiamine treatment caused an accumulation of H_2O_2 in leaves at 6 h until 72 h, which was significantly higher than the control (DW), and then gradually decreased (Fig. 5a). CAT activity was slightly increased from 6–24 h, peaked at 24 h (1.54-fold) and subsequently remained at the same level until 120 h, and was significantly higher than the control within 24–120 h (Fig. 5b). POD activity continuously increased at 6 h until 120 h and reached its highest level at 120 h (4.11-fold), and was significantly higher than that of the control (DW) within 6 to 120 h (Fig. 5c). PAL activity increased continuously from 6 to 24 h, peaked at 24 h (5.03-fold), and then decreased gradually, and was significantly higher than that of the control (DW) in 6–72 h (Fig. 5d). At 24 h and 72 h, the lignin contents remained at the same level as the control (Fig. 5e), indicating that thiamine did not induce lignin deposition in tobacco leaves.

Effects of thiamine on *PR1*, *PR5*, *NPR1*, *PAL*, *CM1*, *H1N1*, and *EFE26* expression in tobacco leaves

To investigate the effects of thiamine on the expression of tobacco defence genes, the expression levels of SA pathway (*PR1*, *PR5*, *NPR1*, *PAL*, *CM1*), ET pathway (*EFE26*) and HR pathway (*H1N1*) genes were determined by qRT–PCR. The expression of *PR1* was slightly induced at 6 h until 24 h and greatly induced by 90.49-fold and 30.92-fold at 72 h and 120 h, respectively (Fig. 6a). The expression of *PR5* increased gradually from 6 to 72 h, reached a maximum at 72 h (39.4-fold), and then decreased but was still higher than that of the control (Fig. 6b). For *NPR1*, the expression was downregulated at 6 h and increased by 1.55-fold at 72 h. After 72 h, the expression was inhibited to maintain the same level as the control (Fig. 6c). For *PAL*, the expression was significantly upregulated by 2.44-fold at 6 h and remained the same at 24 h when compared to the control plants. Subsequently, the expression was increased by 1.53-fold and 1.30-fold at 72 h and 120 h, respectively (Fig. 6d). The expression of *CM1* was significantly upregulated at

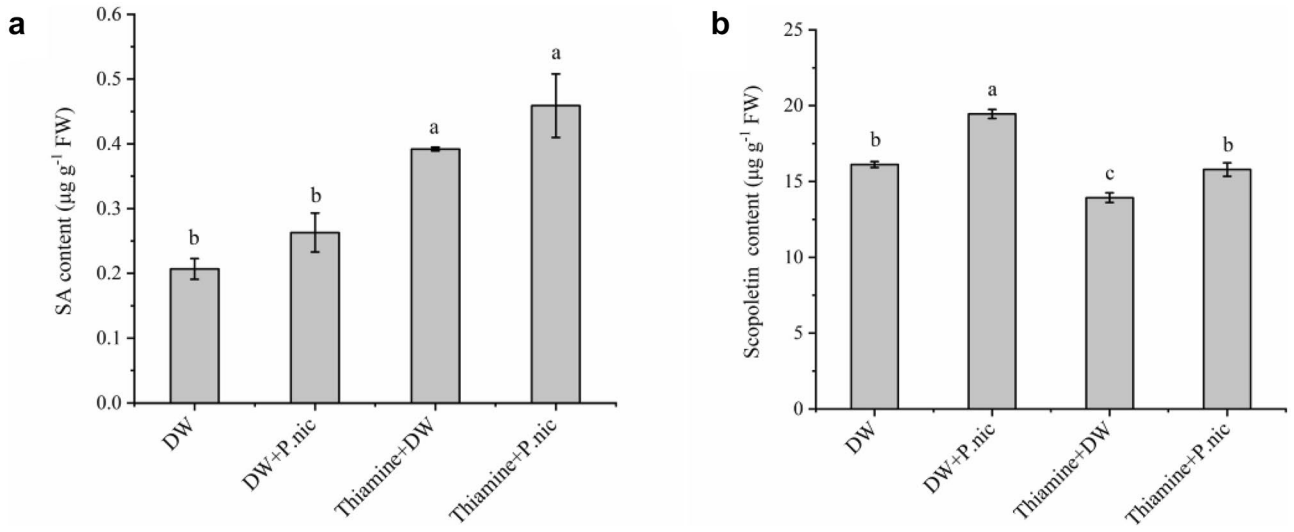

Fig. 4 Effect of exogenous thiamine pretreatment on salicylic acid content and scopoletin contents in tobacco leaves after inoculation with *P. nicotianae* (*P. nic*). The leaves of tobacco were sprayed with either distilled water (DW) or Thiamine. After treatment, leaves were subsequently treated with either DW or *P. nicotianae*. After 5 day, the leaf samples were collected for determining **a** SA content; **b** scopoletin content; Bars show means ± SE ($n = 4$). Different letters indicate significant diferences among treatments ($P < 0.05$; Duncan's multiple-range test)

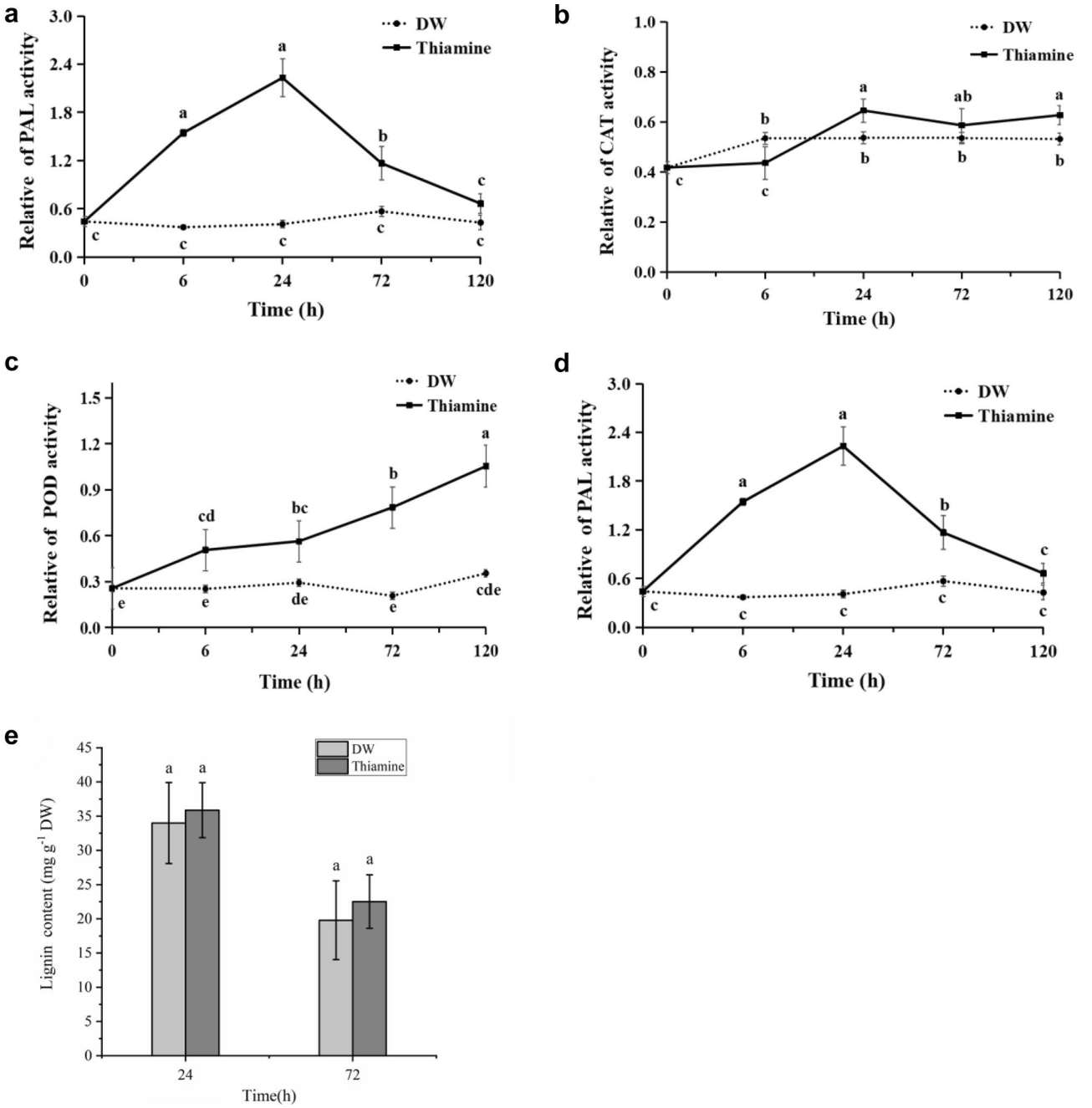

Fig. 5 The effect of Thiamine on **a** H_2O_2 content; **b** CAT activities; **c** POD activities; **d** PAL activities and **e** lignin contents in tobacco leaves. The leaves were sprayed with either distilled water (DW) as control or 20 mM Thiamine and harvested at different points of time (6, 24, 72 and 120 h) for enzyme activity measurements, H_2O_2 content and lignin content. Bars show means ± SE (n = 4). Different letters indicate significant diferences among treatments ($P < 0.05$; Duncan's multiple-range test)

6–72 h, then the expression decreased, but still higher than that of the control (Fig. 6e). In thiamine-treated plants, the expression of *H1N1* increased at 6 h and decreased at 24 h but was still higher than that in the control. The expression increased again by 8.92- and 14.62-fold at 72 h and 120 h, respectively (Fig. 6f). The expression of *EFE26* reached the highest level at 6 h (9.13-fold), after which the expression decreased. However, compared to the level of the control, the expression of *EFE26* was still higher at 24 h until 120 h (Fig. 6g). These data indicated that 20 mM thiamine treatment resulted in significant upregulation the expression levels of genes related to the SA, ET and HR pathways.

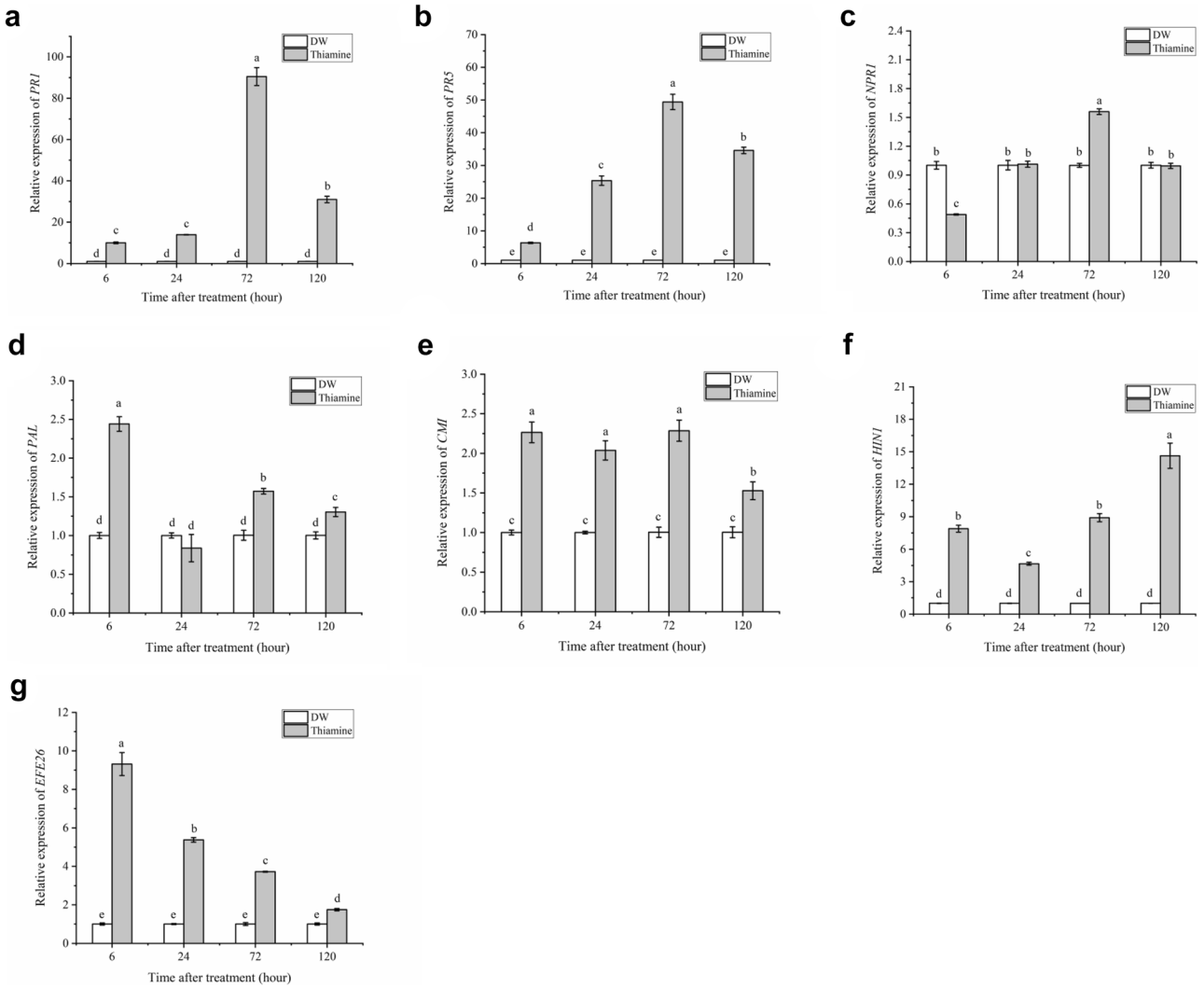

Fig. 6 Effect of Thiamine on transcript abundant of **a** *PR1*; **b** *PR5*; **c** *NPR1*; **d** *PAL*; **e** *CM1*; **f** *H1N1* and **g** *EFE26* genes in tobacco leaves. The leaves were sprayed with either distilled water or 20 mM Thiamine. qRT-PCR were taken at various time points (6, 24, 72 and 120 h). The expression levels of genes were expressed as a relative transcript fold change to their controls. Bars show means ± SE ($n = 4$). Different letters indicate significant diferences among treatments ($P < 0.05$; Duncan's multiple-range test)

Effects of thiamine priming on defence genes in tobacco leaves after *P. nicotianae* challenge

The expression profiles of *PR1* and *PR5* exhibited similar patterns in the pathogen-inoculated leaves. Compared with the control, with thiamine alone application induced 23.69- and 32.01-fold increases in *PR1* and *PR5* transcripts at 1 dpi, respectively. However, the different treatments triggered enhanced gene expression at various time points. The transcript levels of PR genes in leaves treated with thiamine and inoculated with *P. nicotianae* increased earlier and higher than other treatments. At 5 dpi, an additive effect was observed with both thiamine and P. nicotianae together, and the expression levels of *PR1* and *PR5* were increased by 14.91- and 5.70-fold compared with thiamine alone, respectively (Fig. 7a, b).

The expression levels of *PAL*, *NPR1* and *CM1* were up-regulated at 1 dpi 10 dpi and 1 dpi with thiamine alone, respectively, and inoculation with *P. nicotianae* did not significantly affect their transcript levels. At 5 dpi, the expression of *NPR1* was significantly upregulated (2.37-fold) after inoculation with *P. nicotianae* compared with thiamine alone(Fig. 7c-e). At 1 and 10 dpi, the expression of the *H1N1* gene were up-regulated with thiamine alone. At 10 dpi, all treatments had significantly higher expression than the control (Fig. 7f). For *EFE26*, an additive effect was observed with both thiamine and *P. nicotianae* together at 1 dpi, with a 2.57-fold increase in *EFE26* expression compared to thiamine alone (Fig. 7g).

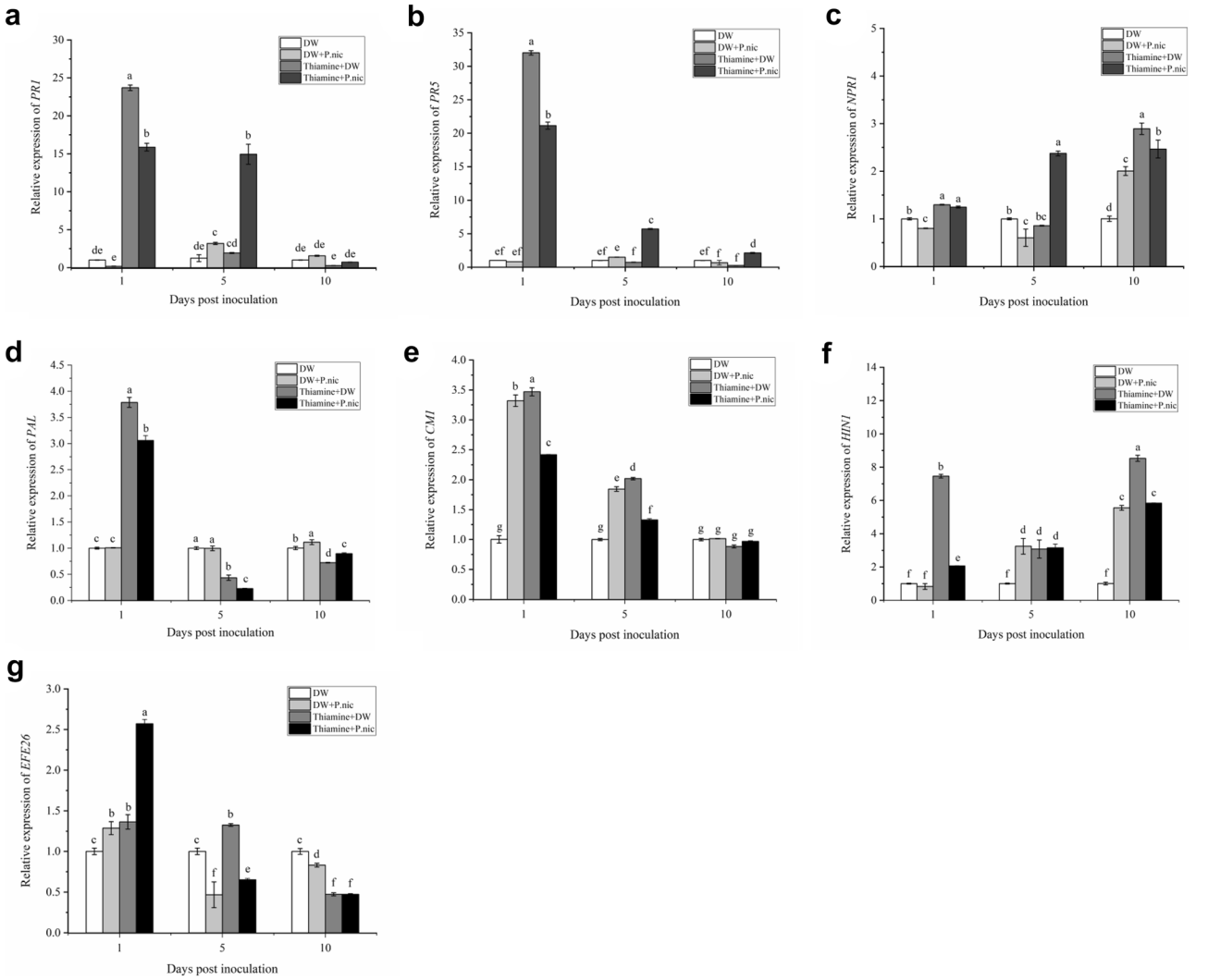

Fig. 7 Effect of Thiamine on transcript abundance of **a** *PR1*; **b** *PR5*; **c** *NPR1*; **d** *PAL*; **e** *CM1*; **f** *H1N1* and **g** *EFE26* genes in tobacco leaves after inoculation with *P. nicotianae*. Total RNA was extracted from leaf tissues taken at different time points, converted to cDNA, and subjected to quantitative real-time PCR. Bars show means ± SE ($n = 4$). Different letters indicate significant diferences among treatments ($P < 0.05$; Duncan's multiple-range test)

Discussion

In this study, we found that pretreatment with 20 mM thiamine could effectively improve tobacco resistance to *P. nicotianae* without toxic effects, indicating that 20 mM thiamine was a safe concentration range for tobacco cultivation. Previous studies have shown that 30 mM thiamine can protect grapes from *Plasmopara viticola* (Boubakri et al. 2012), and 50 mM thiamine can replace carbendazim as a systematic fungicide to effectively control sheath blight disease in rice (Bahuguna et al. 2012), indicating that thiamine meets the requirements of being an activator of plant-induced resistance and is a systematic broad-spectrum persistent drug resistance inducer, which can be further developed and used.

It has been reported that thiamine can inhibit the growth of *Plasmopara viticola* and grey mould *in vitro* (Boubakri et al. 2012; Hong et al. 2016). Our results demonstrated that the mycelial growth and sporangium production of *P. nicotianae* were inhibited by thiamine in a dose-dependent manner (Fig. 1). Surprisingly, the growth of *P. nicotianae* was not completely inhibited, even at the highest thiamine concentrations, possibly indicating that the *P. nicotianae* isolate was able to detoxify thiamine to a certain amount. These results were first reported about the effect of thiamine on *P. nicotianae* growth, at least showing that *P. nicotianae* isolates isolated by us were sensitive to thiamine.

The rapid accumulation of ROS is one of the earliest responses when plants are attacked by pathogens at attack sites. Increasing ROS can participate in the orchestration of

the hypersensitive response and can be used to destroy invading pathogens (Bastas et al. 2014). H_2O_2 is a stable intermediate of ROS and has been shown to inhibit the viability of diverse microbial pathogens, and its oxidative potential contributes to plant wall strengthening during plant-pathogen interactions (Wu et al. 1997). H_2O_2 could also induce the expression of genes encoding proteins involved in defensive and antioxidant processes and has been reported by some studies as a diffusible selective signal (Bhattacharjee 2005). Compared to the inoculated with *P. nicotianae* without thiamine pretreatment tobacco plants, H_2O_2 accumulation was significantly increased in the thiamine-treated tobacco plants with *P. nicotianae* inoculation, indicating a priming effect mediated by thiamine on the *P. nicotianae*-induced oxidative burst (Fig. 3a). Ahn et al. also reported thiamine-induced H_2O_2 generation in Arabidopsis plants but only following pathogen invasion. These results suggest that H_2O_2 plays a key role in thiamine signal transduction, leading to the induction of defence responses that ultimately inhibit the pathogen.

Lignin is a high-molecular-weight polymer that consists of β-(1,4)-glucan (Luna et al. 2011). Lignin deposition could be seen as a way to strengthen the plant cell wall. It is also an evaluation method widely used to judge plant-triggered immunity (PTI) (Bittel and Robatzek 2007). It is well known that monolignols are efficiently polymerized by peroxidase with H_2O_2 consumption, leading to lignin formation (Pauwels et al. 2008). In noninoculated plants, no significant differences in lignin levels were measured in thiamine-treated versus nontreated plants (Fig. 5e). However, significantly higher lignin levels were measured at 5 dpi in thiamine-treated plants that were inoculated than in those that were inoculated but not treated, indicating that thiamine can induce cell wall lignification after pathogen challenge (Fig. 3b). This result is consistent with the result reported by Huang et al. (2016).

It is known that plant cells can be injured by excess H_2O_2. Thus, plants must employ some mechanism to detoxify excess H_2O_2, such as the antioxidant enzyme system, of which CAT is a well-known member (Wojtaszek 1997). Our data showed that the activity change of CAT was according to H_2O_2 concentration (Fig. 3), which may indicate CAT's degrading function on H_2O_2 at relatively high H_2O_2 concentrations. The fact that massive accumulation of H_2O_2 caused less damage during the pathogen-induced oxidative burst might be due to the induction of CAT activities. CAT and POD are considered the main antioxidant systems to protect cells against oxidative damage (De Gara et al. 2003). Thiamine application could induce increases in CAT and POD activity levels (Figs. 3 and 5). Studies have shown that thiamine can increase H_2O_2 and lignin contents and the activity of defence compounds (Boubakri et al. 2012; Huang et al. 2016; Bahuguna et al. 2012). Our results also showed that thiamine preconditioning induced a series of defence responses, including H_2O_2 accumulation, and significantly increased CAT, POD and PAL activity and SA content (Figs. 3 and 4), which in turn induced lignin production and increased the physical barrier of the cell wall to restrict the penetration of pathogens.

The response of defence-related genes to exogenously applied thiamine was examined in tobacco leaves. Our results showed that the expression of the PR1, PR5, NPR1, PAL, CM1, H1N1, and EFE26 genes was significantly induced by thiamine (Figs. 6 and 7). Chemical elicitors have been reported to induce resistance against fungal pathogen infection in crops by stimulating PR gene expression (Yu et al. 2014). The increased expression of PR1, PR5 and others is widely accepted as a hallmark of plant defence induction (Aćimović et al. 2015). In addition, the PR1, PR5, NPR1, PAL, and CM1 genes mediated the defence response through the SA pathway. The results showed that the thiamine-induced expression of the PR gene might be related to the tobacco resistance conferred on it, and the PR gene was rapidly activated at 24 h (Fig. 6). The NPR1 regulator mediates SAR to a broad spectrum of plant pathogens by activating defence genes (PRs) in SAR, including PR-1 and PR-5, by virtue of their particular structures and functions (Mach et al. 2015; Yang et al. 2018). Here, the results showed that thiamine treatment enhanced the expression of NPR1, PR1, and PR5 in tobacco leaves to confer resistance against *P. nicotianae,* although the reaction was dependent on the days after *P. nicotianae* infection (Fig. 7). EFE26, H1N1 and CM1 are ET biosynthesis and signalling marker genes (Chen et al. 2003), and our results also showed that tobacco resistance against *P. nicotianae* was related to their expression. We also observed a significant upregulation of the PAL gene (Fig. 6), which was correlated with the increase in PAL activity (Fig. 5) by thiamine treatment. As mentioned above, PAL is considered to be a key enzyme in catalysing various phenylpropanoid defence metabolites, such as lignin. Furthermore, it has been proven that PAL functions as a rate-limiting enzyme in the phenylpropanoid pathway (Kolahi et al. 2013). Therefore, we can conclude that cell wall reinforcement by the accumulation of lignin supports mechanical resistance to pathogen penetration, which may be attributed to the increased expression of the PAL gene.

The IR phenotype is associated with both direct induction of defence responses, which can be transient or long-lasting, and primed defence responses (Wilkinson et al. 2019; Balmer et al. 2015). Ahn et al. (2007). reported for the first time that thiamine induced resistance in Arabidopsis against *P. syringae* through priming defence responses. Boubakri et al. (2012). showed that thiamine effectively induces resistance against *P. viticola* in grapevine through a dual mode of action involving direct antifungal activity and induction of host defence

mechanisms. Studies have also shown that inducers can trigger priming defence mechanisms at low concentrations and directly activate defence responses at high concentrations (Wang et al. 2015; van Hulten et al. 2006). Our data showed that 20 mM thiamine could directly inhibit the activity of *P. nicotianae*, and regardless of pathogen infection, thiamine could induce the accumulation of a large amount of H_20_2 in tobacco that would be expected to increase the activity of defence-related enzymes and the expression of related defence genes. These defence genes were upregulated or downregulated to different degrees during subsequent *P. nicotianae* infection. It was suggested that thiamine acted as an inducer in tobacco, inducing a direct defence response in tobacco, and may initiate a defence response to the subsequent attack of *P. nicotianae* through the SA, ET and HR pathways.

In conclusion, the findings of this study clearly demonstrate that thiamine can effectively induce resistance against *P. nicotianae* in tobacco under glasshouse-controlled conditions through a dual mode of action involving direct antifungal activity and induction of host defence mechanisms. The thiamine-induced defence response included the generation of H_2O_2, lignin deposition, defence enzyme activity enhancement and the expression of a number of defence-related genes (SA pathway, ET pathway and HR pathway). These results suggest that thiamine can be used in tobacco cultivation to test its effectiveness under field conditions.

Acknowledgements This work was supported by Yunnan Tobacco Company Science and Technology Plan Project (Number: 2020530000242026).

Author contribution Tao Liu and Jun Liu conceived and designed the experiments. Tian Suohui and Chen Yanping performed the experiments. Zi Shuhui and Li Zhihua analysed the data. Jin Honggang revised the paper. All authors read and approved the final manuscript.

Declarations

Conflicts of interest The authors declare no conflicts of interest.

References

Aguiar TR, Bortolozo FR, Filho EF, Parron LM, Luz LD, Brito AG, Ferreira MT (2017) Fate of selected agrochemicals in a tropical karst aquifer: A five-year study. Groundw Sustain Dev 5:187–192

Ahn IP, Kim S, Lee YH (2005) Vitamin B1 functions as an activator of plant disease resistance. Plant Physiol 138(3):1505–1515

Ahn IP, Kim S, Lee YH, Suh SC (2007) Vitamin B1-induced priming is dependent on hydrogen peroxide and the NPR1 gene in Arabidopsis. Plant Physiol 143(2):838–848

Asensi-Fabado MA, Munné-Bosch S (2010) Vitamins in plants: occurrence, biosynthesis and antioxidant function. Trends Plant Sci 15(10):582–592

Aćimović SG, Zeng Q, McGhee GC, Sundin GW, Wise JC (2015) Control of fire blight (Erwinia amylovora) on apple trees with trunk-injected plant resistance inducers and antibiotics and assessment of induction of athogenesis-related protein genes. Front Plant Sci 6:16

Bahuguna RN, Joshi R, Shukla A, Pandey M, Kumar J (2012) Thiamine primed defense provides reliable alternative to systemic fungicide carbendazim against sheath blight disease in rice (Oryza sativa L.). Plant Physiol Biochem 57:159-167

Balmer A, Pastor V, Gamir J, Flors V, Mauch-Mani B (2015) The "prime-ome": towards a holistic approach to priming. Trends Plant Sci 20(7):443–452

Barilli E, Sillero JC, Rubiales D (2010) Induction of systemic acquired resistance in pea against rust (*Uromyces pisi*) by exogenous application of biotic and abiotic inducers. J Phytopathol 158(1):30–34

Bastas KK (2014) Importance of reactive oxygen species in plants-pathogens interactions. Selcuk J Agric Food Sci 28(1):11–21

Burrows RJ, Byrne KL, Meacock PA (2000) Isolation and characterization of Saccharomyces cerevisiae mutants with derepressed thiamine gene expression. Yeast (chichester, England) 16(16):1497–1508

Blainski A, Lopes GC, de Mello JC (2013) Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from Limonium brasiliense L. Molecules 18(6):6852–6865

Boubakri H, Wahab MA, Chong J, Bertsch C, Mliki A, Soustre-Gacougnolle I (2012) Thiamine induced resistance to Plasmopara viticola in grapevine and elicited host-defense responses, including HR like-cell death. Plant Physiol Biochem 57:120–133

Bhattacharjee S (2005) Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transducation in plants. Curr Sci 89:1113–1121

Bigeard J, Colcombet J, Hirt H (2015) Signaling mechanisms in pattern-triggered immunity (PTI). Mol Plant 8(4):521–539

Bittel P, Robatzek S (2007) Microbe-associated molecular patterns (MAMPs) probe plant immunity. Curr Opin Plant Biol 10(4):335–341

Csinos AS (1999) Stem and Root Resistance to Tobacco Black Shank. Plant Dis 83(8):777–780

Conrath U, Beckers GJ, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA, Pieterse CM, Poinssot B, Pozo MJ, Pugin A, Schaffrath U, Ton J, Wendehenne D, Zimmerli L, Mauch-Mani B (2006) Priming: getting ready for battle. Molec Plant-Microbe Interact: MPMI 19(10):1062–1071

Conrath U, Pieterse CM, Mauch-Mani B (2002) Priming in plant-pathogen interactions. Trends Plant Sci 7(5):210–216

Chen N, Goodwin PH, Hsiang T (2003) The role of ethylene during the infection of Nicotiana tabacum by Colletotrichum destructivum. J Exp Bot 54(392):2449–2456

Dempsey DA, Shah J, Klessig DF (1997) Salicylic acid and disease resistance in plants. Crit Rev Plant Sci 18(4):547–575

De Kesel J, Conrath U, Flors V, Luna E, Mageroy MH, Mauch-Mani B, Pastor V, Pozo MJ, Pieterse C, Ton J, Kyndt T (2021) The Induced Resistance Lexicon: Do's and Don'ts. Trends Plant Sci 26(7):685–691

Dalio RJ, Fleischmann F, Humez M, Osswald W (2014) Phosphite protects Fagus sylvatica seedlings towards *Phytophthora plurivora* via local toxicity, priming and facilitation of pathogen recognition. PloS One 9(1):e87860

Deenamo N, Kuyyogsuy A, Khompatara K, Chanwun T, Ekchaweng K, Churngchow N (2018) Salicylic Acid Induces Resistance in Rubber Tree against *Phytophthora palmivora*. Int J Mol Sci 19(7):1883

Drzewiecka K, Borowiak K, Bandurska H, Golinski P (2012) Salicylic acid-a potential biomarker of tobacco Bel-W3 cell death developed as a response to ground level ozone under ambient conditions. Acta Biol Hung 63(2):231–249

De Gara L, de Pinto MC, Tommasi F (2003) The antioxidant systems vis-à-vis reactive oxygen species during plant-pathogen interaction. Plant Physiol Biochem 41(10):863–870

Frąckowiak P, Pospieszny H, Smiglak M, Obrępalska-Stęplowska A (2019) Assessment of the Efficacy and Mode of Action of Benzo(1,2,3)-Thiadiazole-7-Carbothioic Acid S-Methyl Ester (BTH) and Its Derivatives in Plant Protection Against Viral Disease. Int J Mol Sci 20(7):1598

Gallup CA, McCorkle KL, Ivors KL, Shew D (2018) Characterization of the Black Shank Pathogen, *Phytophthora nicotianae*, Across North Carolina Tobacco Production Areas. Plant Dis 102(6):1108–1114

Guo W, Yan H, Ren X, Tang R, Sun Y, Wang Y, Feng J (2020) Berberine induces resistance against tobacco mosaic virus in tobacco. Pest Manag Sci 76(5):1804–1813

Haas D, Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3(4):307–319

Huang WK, Ji HL, Gheysen G, Kyndt T (2016) Thiamine-induced priming against root-knot nematode infection in rice involves lignification and hydrogen peroxide generation. Mol Plant Pathol 17(4):614–624

Huang Y, Ma L, Fang DH, Xi JQ, Zhu ML, Mo MH, Zhang KQ, Ji YP (2015) Isolation and characterisation of rhizosphere bacteria active against *Meloidogyne incognita*, *Phytophthora nicotianae* and the root knot-black shank complex in tobacco. Pest Manag Sci 71(3):415–422

Hong JK, Kim HJ, Jung H, Yang HJ, Kim DH, Sung CH, Park CJ, Chang SW (2016) Differential Control Efficacies of Vitamin Treatments against Bacterial Wilt and Grey Mould Diseases in Tomato Plants. Plant Pathol J 32(5):469–480

Jackson AO, Taylor CB (1996) Plant-microbe interactions: life and death at the interface. Plant Cell 8(10):1651–1668

Jung IL, Kim IG (2003) Thiamine protects against paraquat-induced damage: scavenging activity of reactive oxygen species. Environ Toxicol Pharmacol 15(1):19–26

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol 5(4):325–331

Kolahi M, Jonoubi P, Majd A, Tabandeh MR, Hashemitabar M (2013) Differential expression of phenylalanine ammonia-lyase in different tissues of sugarcane (*Saccharum officinarum* L.) during development. Bioresources 8(4):4912–4922

Lerat S, Babana AH, El Oirdi M, El Hadrami A, Daayf F, Beaudoin N, Bouarab K, Beaulieu C (2009) Streptomyces scabiei and its toxin thaxtomin A induce scopoletin biosynthesis in tobacco and *Arabidopsis thaliana*. Plant Cell Rep 28(12):1895–1903

Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: a multifaceted plant defense response. Mol Plant 24(2):183–193

Meng X, Zhang S (2013) MAPK cascades in plant disease resistance signaling. Annu Rev Phytopathol 51:245–266

Mauch-Mani B, Baccelli I, Luna E, Flors V (2017) Defense Priming: An Adaptive Part of Induced Resistance. Annu Rev Plant Biol 68:485–512

Malamy J, Sanchez-Casas P, Hennig J, Guo A, Klessig DF (1996) Dissection of the salicylic acid signaling pathway in tobacco. Mole Plant-Microbe Interact: MPMI (USA) 9:474–482

Moreira-Vilar FC, Siqueira-Soares Rde C, Finger-Teixeira A et al (2014) The acetyl bromide method is faster, simpler and presents best recovery of lignin in different herbaceous tissues than Klason and thioglycolic acid methods. PLoS One 9(10):e110000

Mach J (2015) Phosphorylation and Nuclear Localization of NPR1 in Systemic Acquired Resistance. Plant Cell 27(12):3291

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc Natl Acad Sci USA 105(4):1380–1385

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic Acquired Resistance Plant Cell 8(10):1809

Sullivan MJ, Melton TA, Shew HD (2005) Fitness of Races 0 and 1 of *Phytophthora parasitica var. nicotianae*. Plant Dis 89(11):1220–1228

Thomma BP, Eggermont K, Penninckx IA, Mauch-Mani B, Vogelsang R, Cammue BP, Broekaert WF (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci 95(25):15107–15111

Vontimitta V, Lewis RS (2012) Mapping of quantitative trait loci affecting resistance to *Phytophthora nicotianae* in tobacco (*Nicotiana tabacum* L.) line Beinhart-1000. Mol Breed 29(1):89–98

Van Hulten M, Pelser M, van Loon LC, Pieterse CM, Ton J (2006) Costs and benefits of priming for defense in Arabidopsis. Proc Natl Acad Sci USA 103(14):5602–5607

Wang K, Liao Y, Kan J, Han L, Zheng Y (2015) Response of direct or priming defense against Botrytis cinerea to methyl jasmonate treatment at different concentrations in grape berries. Int J Food Microbiol 194:32–39

Wen D, Li C, Di H, Liao Y, Liu H (2005) A universal HPLC method for the determination of phenolic acids in compound herbal medicines. J Agric Food Chem 53(17):6624–6629

Wilkinson SW, Magerøy MH, López Sánchez A, Smith LM, Furci L, Cotton T, Krokene P, Ton J (2019) Surviving in a Hostile World: Plant Strategies to Resist Pests and Diseases. Annual review of phytopathology 57:505–529

Wu G, Shortt BJ, Lawrence EB, Leon J, Fitzsimmons KC, Levine EB, Raskin I, Shah DM (1997) Activation of Host Defense Mechanisms by Elevated Production of H_2O_2 in Transgenic Plants. Plant Physiol 115(2):427–435

Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem J 322(Pt 3):681–692

Yu C, Zeng LZ, Sheng K, Chen FX, Zhou T, Zheng XD, Yu T (2014) γ-Aminobutyric acid induces resistance against Penicillium expansum by priming of defence responses in pear fruit. Food Chem 159:29–37

Yang G, Tang L, Gong Y, Xie J, Fu Y, Jiang D, Li G, Collinge DB, Chen W, Cheng J (2018) A cerato-platanin protein SsCP1 targets plant PR1 and contributes to virulence of Sclerotinia sclerotiorum. New Phytol 217(2):739–755

Zhang S, Liu S, Zhang J, Reiter R, Wang Y, Qiu D, Luo X, Khalid A, Wang H, Feng L, Lin Z, Ren M (2018) Synergistic anti-oomycete effect of melatonin with a biofungicide against oomycetic black shank disease. J Pineal Res 65(2):e12492

Zhang C, Feng C, Zheng Y, Wang J, Wang F (2020) Root Exudates Metabolic Profiling Suggests Distinct Defense Mechanisms Between Resistant and Susceptible Tobacco Cultivars Against Black Shank Disease. Front Plant Sci 11:559775