Priming Effect of Thiamine on the Enhancement of Induced Resistance to the Plant Disease *Phytophthora Nicotianae* in Tobacco

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Abstract

Induced resistance by elicitors is considered to be an eco-friendly strategy to stimulate plant defense 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.To date, the mechanisms by which VB1 provides protection against plant disease have yet to be fully elucidated, expecially no reports about VB1 treatment influenced the development of *Phytophthora nicotianae* in plant. Tobacco black shank (TBS) caused by *P. nicotianae* is destructive to almost all tobacco cultivars and is widespread in many tobacco-growing countries. In the present study, the priming effect of VB1 on tobacco against the disease *P. nicotianae* and its biochemical and molecular impact on plant defense mechanisms were evaluated. Base on the effect of VB1 on mycelial growth and zoospore formation, the appropriate VB1 treatment was used in protecting tobacco against *P. nicotianae.* For VB1 pretreatment, tobacco exhibited a significant reduction in disease severity. Consistent with the occurrence of induced resistance, the pronounced increase in H_2O_2 level, phenylalanine ammonia lyase (PAL) and peroxidase (POD) activities were observed. For defense reactions, VB1 promoted the increases of H_2O_2, SA and lignin contents. Moreover, the expressions of PR1, PR5, NPR1, PAL, CM1, H1N1 and EFE26 were induced by VB1, which also involved in defense reactions. Our findings indicate that the priming effect of VB1 may partially depend on the production of the callose deposition, H_2O_2 accumulation, and hormone SA production.

Introduction

Black shank, caused by *Phytophthora nicotianae*, is among the most widespread and damaging diseases of cultivated tobacco (*Nicotiana tabacum*) worldwide[1]. The pathogen infects roots, stems, and leaves at any stage of plant growth, resulting in 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[2,3]. The traditional control strategies, including crop rotation, fungicide applications, and the use of resistant cultivars, are not sufficient to control this soil-borne disease[4]. Fungicides become less and less unwelcome although being used in most cases nowadays, because of its long time existing in the field and lead to resistant pathogen strains[5]. Some recent studies showed several years of monoculture plant could recruit growth-promoting rhizobacteria (PGPR) for preventing the losses caused by soil-borne pathogens. However, this discovery is not practical for preventing the soil-borne pathogens because of some economic reasons[4]. Thus, it is urgent to explore more efficient and sustainable control methods for this soil-borne diseases.

The plant defense system usually consists of preexisting physical and chemical barriers as well as inducible defense responses[6]. Some environmental stimuli, such as the attack of avirulent pathogens, colonization of beneficial microbes, and application of chemicals, can activate the plant defense systems against specific biotic stresses (e.g., fungi, bacteria, viruses and nematodes)[7]. The so-called priming could be a more environmentally friendly control method[7]. Priming is a unique physiological state that enables plant cells to respond to a biotic or abiotic stress in a more rapid and robust manner than non-

primed cells. Priming is also one of the most efficient types of induced resistance because the metabolic investment of the plant is reduced compared with constitutive defence activation[7]. After inducing by the application of low levels of so-called “priming agents”, the plant defence responses could be strongly activated by pathogen or insect invasion. Some chemical activators, such as phytoalexins, can induce plant strong defense responses, including strengthening of cell walls, production of antifungal proteins, and enhanced accumulation of salicylic acid (SA) et al[8]. Nowadays, many chemical inducers, such as SA, SA analog benzothiadiazole (BTH), and β-aminobutyric acid (BABA), had been proved for inducing plant disease resistance[9].

VB1 is a water-soluble B-complex vitamin that is produced in plants and microbes[10]. Many studies have documented the beneficial roles of VB1 in enhancing plant disease (including bacterial wilt) resistance[11,12,13]. For example, it had been found an increase on the basal level of resistance of rice plants supplied with VB1 when infected by the blast fungus *Magnaporthe grisea* 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[11]. After treatment with VB1 on pearl millet seed, lipoxygenase (LOX) gene expression could be induced rapidly, which resulted in significant disease protection against downy mildew disease[14]. Although VB1 has been found to induce resistance to different pathogens, the molecular mechanisms underlying this have not been completely elucidated[15], especially *Phytophthora* infection has not been tested previously.

Because of the protective activity of VB1 in inducing plant priming against disease, we hypothesized that VB1 might have the potential to activate the plant disease resistance against *P. nicotianae* infections in VB1 non-accumulator plant tobacco. So, the related experiments were conducted, using *N. tabacum* (tobacco) and *P. nicotianae* interaction system, to evaluate the potential function and investigate the related underlying mechanisms of VB1 in inducing plant disease resistance. For the outcome of the study, we speculated that VB1 could activate the tobacco plant disease resistance against *P. nicotianae* through enhancing the lignin deposition, H_2O_2 accumulation, and SA accumulation.

Materials And Methods

Plant material and inoculum preparation

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

P. nicotianae strain maintained in our laboratory were 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 darkness for 15 days standby[16,17].

Effect of VB1 at different concentrations on mycelial growth and sporangium production of *P. nicotianae* in vitro

VB1 stock solution was prepared in sterile distilled water, and the solution was filtered through a microfiltration membrane. The effect of VB1 on mycelial growth of *P.nicotianae* was evaluated on PDA plates according to the method of Zhang et al[18,19]. A 7-mm *P. nicotianae* agar disk from actively growing 15-day mycelium of the pathogen was transferred into a new PDA plate with different concentrations of VB1 (0, 1, 2, 5, 10, 20 and 50 mM). The mycelial disk was placed in the center of the plate (diameter 90 mm). After 15 days of incubation at 28℃, the colony diameters were measured by the cross method. Each of the VB1 concentrations was replicated on four plates and the experiment was repeated thrice.

According to the reported method[20], with slight modifications, the effect of VB1 on the sporangia of *P. nicotianae* was studied. Briefly, 0.1% KNO_3 was used to prepare induction solutions containing different concentrations of VB1 (0, 1, 2, 5, 10, 20, 50 mM), and 7 mM agar of *P.nicotianae* agar disk from actively growing 15-day mycelium was transferred to a petri dish supplemented with induction solution (10ML), followed by culturing at 28°C for 48h. In an aseptic operating environment, the agar medium at the lower part of the bacterial disk was cut off in parallel to make the thickness about 1 mm, and placed on the slide to observe the number of sporangia under a 10x20 optical microscope, and take pictures and record. All treatments consisted of four replicates and the experiment was repeated thrice. By studying the effect of VB1 on the mycelial growth and sporangium production of *P. nicotianae*, an optimal VB1 induction concentration was screened for the next experiments.

Induction of 'HD' tobacco resistance against *P. nicotianae* by VB1

Tobacco seedlings of uniform size were sprayed with either distilled water (DW) or VB1 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 triplicates 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, respectively[21].

Three days after treatment of either DW or VB1(20mM) on the leaves, *P. nicotianae* was inoculated using the stem base trauma inoculation method of mycelium blocks (cut the base 2mm wound of tobacco stem with a scalpknife, inoculated with 5g *P.nicotianae* per plant, and then moisturized with sterile cotton)[22]. The inoculated seedlings were then maintained in a greenhouse. Experimental design and inoculation with *P.nicotianae*, two months old tobacco seedlings were treated as follows: (a)control:distilled water treated/distilled water treated (DW); (b)distilled water treated/inoculated with *P.nicotianae* (DW+*P.nic*); (c)VB1 treated/distilled water treated(VB1+DW); (d)VB1 treated/inoculated with *P.nicotianae*(VB1+*P.nic*). Six plants were used for each treatment. The experiment was repeated three times. Leaves were collected from plants at different intervals (1, 5, 10 dpi) and immediately frozen in liquid nitrogen, and stored at -80℃ 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 protein assay kit, H_2O_2 content Assay kit, CAT Assay Kit, POD Assay Kit, PAL Assay Kit (Suzhou Greys Biological Technology Co., Ltd, Suzhou, China) , respectively, following the protocol provided by the manufacturer.

Leaf samples (0.1g fresh weight) were ground in a chilled mortar with liquid nitrogen, and then homogenized with 1 mL of cold 0.1 M Tris-HCl buffer, pH 7.0 containing 0.25% (v/v) triton-X and 3% (w/v) polyvinylpolypyrrolidone (PVPP). The extracts were then centrifuged at 12,000 rpm for 10 min at 4℃, and the supernatants were used for determining H_2O_2 content, enzyme activities and total protein content.

Protein and H_2O_2 content

The protein content was determined by the biuret method. In a strong alkaline solution, the biuret and $CuSO_4$ form a purple complex. The color of the purple complex was proportional to the protein. The absorbance value was detected at a wavelength of 540nm. For H_2O_2 content assay, frozen sample (0.1g) was ground and homogenized with 1mL of chilled 100% acetone and then centrifuged at 10,000 rpm for 10 min at 4℃. By measuring the absorbance of the titanium-peroxide complex at 415 nm. The absorbance values were calibrated against a standard curve and expressed as μmol per gram of fresh weight (μmol g^{-1} FW).

CAT, POD and PAL activities

For CAT activity assay, the sample was treated with excess hydrogen peroxide, and the absorbance of the remaining H_2O_2 was measured at 510nm. One unit of CAT activity was defined as 1 μmol of H_2O_2 used in 1 min. The activity was represented as units per gram fresh weight (U g^{-1} FW). According to the reaction principle of peroxidase catalyzed by H_2O_2, the activity of peroxidase was determined by monitoring the increase in absorbance at 470 nm. One unit of POD activity was defined as the amount of enzyme that provided a change of 0.5 in absorbance per min per gram fresh weight (U g^{-1} FW). For PAL activity assay, according to PAL-catalyzed cleavage of L-phenylalanine into trans-cinnamic acid and ammonia, The maximum absorption value of trans-cinnamic acid was 290nm, and the PAL activity is calculated by measuring the increase rate of absorbance value. One unit was defined as the amount of enzyme that caused an increase in absorbance for 0.05. PAL activity was identified as units per gram fresh weight (U g^{-1} FW).

Total phenolic content and lignin detection

Leaf samples (0.1 g fresh weight) were frozen immediately in liquid nitrogen, ground to a fifine powder with a chilled mortar and pestle and then homogenized with 1.5 mL of 60% ethanol. The homogenate was centrifuged at 12,000 rpm for 10min at room temperature. The total phenol content of the extract

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

SA and scopoletin measurements

The contents of SA and scopoletin in the leaf of tobacco seedlings were measured by high performance liquid chromatography (HPLC). SA content was determined according to previous studies[23,24], With some little modifcations. Leaf samples (0.2 g fresh weight) were ground to a fine powder with mortar and pestle and then homogenized with 1 mL of 70% methanol, Extracted overnight at 4°C. After centrifugation at 8000g for 10min, the supernatant was extracted with 0.5ml 70% methanol for two hours. After centrifugation, the supernatant was extracted, combined with twice supernatant. The supernatant was adjusted with 50% (w/v) trichloroacetic acid (TCA) to produce a final concentration of 5% (w/v) TCA, and subsequently filtrated through a 0.45 μm membrane.

Scopoletin content was determined according to the method of Lerat et al[25], Leaf samples of 0.1g fresh weight were ground into a fine powder with a mortar and pestle, and then dissolved in a flask with 20mL 50% methanol. The mixture was ultrasonically extracted at room temperature for 20 min, then centrifuged at 3000 rpm for 5 min and filtered through a 0.45μm aqueous phase membrane.

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

Analysis of genes expression by quantitative real-time PCR

Transcription of defense-related genes was determined, and the expression levels of PR protein, SA pathway, ET pathway and HR pathway genes listed in Table 1 were detected[26]. Total RNA was extracted with MagenHiPure HP Plant RNA Mini Kit (R4165-02, Magen, China) from tobacco leaf tissue. The RNA samples were measured for the quality and quantity by measuring a ratio of 260/280 nm absorption and their integrity was evaluated by visualizing the bands on a 1% agarose gel electrophoresis. cDNA for RT-

qPCR was synthesized from 2 ug 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 3 s and 60℃ for 30 s and then by a melting curve cycle. The threshold period (CT) and melting curve of each gene were analyzed. The relative mRNA amount was calculated by the $2^{-\triangle\triangle Ct}$ method. Three biological replicates were performed for each experiment.

Statistical analysis

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

Results

In vitro inhibition of mycelial growth and zoospore inhibition of *P. nicotianae* by VB1

Inhibition of mycelial growth started at 1 mM and reached 93% inhibition with 50 mM of VB1 (Fig. 1c). The EC50 value was calculated as 18 mM. The number of zoospores released was significantly lower for all VB1 treatments (Fig. 1b). The EC50 value for zoospore inhibition was determined as 1.9 mM, about ten times lower than the inhibition of mycelial growth.

Effect of VB1 priming on H_2O_2 content, total protein content, CAT and POD activities, total phenolic content, SA content, scopoletin content and lignin content in tobacco leaves after *P. nicotianae* challenge

Priming tobacco leaves with 20 mM VB1 before *P. nicotianae* inoculation (VB1 + *P. nic*) caused a significant increase in H_2O_2 content, total protein content, SA content, scopoletin content and lignin content, compared to the control (VB1 + DW). However, inoculation with *P. nicotianae* (DW + *P. nic*) slightly affected their levels, including H_2O_2 content, total protein content, CAT and POD activities, total phenolic content, SA content, and lignin content, as compared to the control (DW) (Fig. 2).

For POD and total phenolic content, the activity was not significantly increased after triggering with *P. nicotianae* compared to the control (VB1 + DW). Differently, the treatment with VB1 (VB1 + DW) resulted in an increase of scopoletin content in tobacco leaves as compared to the control (DW) (Fig. 2).

Effect of VB1 on kinetics of H_2O_2 content, CAT, POD and PAL activities, lignin contents in tobacco leaves

The effect of SA on the activities of H_2O_2 content was investigated in tobacco leaves after treatment with 20 mM VB1 over a time-course. The results revealed that the VB1 treatment caused a accumulation of H_2O_2 in leaf at 6 h till 72 h, then declined thereafter, compared to the control (DW). CAT activity was slightly increased from 6–24 h, subsequently remained at the same level of the control until120 h. For POD, the activity was continuously increased at 6 h till 120 h and reached the highest level at 120 h when

compared to the control (DW). CAT activity was continuously increased at 6–24 h, culminated at 24h and abruptly decreased at 24–120 h when compared to the control (DW). Lignin content remained at the same level of the control from 24–72 h, suggested that VB1 could not induce lignin deposition in leaves (Fig. 3).

Effect of VB1 on PR1, PR5, NPR1, PAL, CM1, H1N1, and EFE26 expressions in tobacco leaves

To investigate the effect of VB1 on tobacco defense gene expression, the expressions of PR1, PR5, NPR1, PAL, CM1, H1N1, and EFE26 were determined by qRT-PCR. The expression of PR1 was slightly induced at 6 h till 24 h and greatly induced at 72 h compared to control, then the expression was rapidly declined at 120 h, while up the level of control. For PAL, the expression was induced at 6 h and 24 h, respectively, in the VB1-treated plants, then the expression reached the highest level at 12 h. Subsequently, the expression decreased and still up the level of control. For NPR1, the expression was up-regulated at 72 h, thereafter the expression was suppressed to remain same as the level of control. For PAL, the expression was significantly up-regulated at 6 h and then the expression decreased and remained the same at 24 h when compared to the control plants. Subsequently, the expression was induced at 72 h and 120 h, respectively. For CM1, the expression was significantly up-regulated at 6 h and reached the highest level at 72 h, thereafter the expression was decreased, while up the level of control at 120h. The expression of H1N1 was induced 6 h, in the VB1-treated plants, then the expression decreased at 24 h, while still higher than control. The expression increased again at 72 h and 120 h, respectively. The expression of EFE26 reached the highest level at 6 h, thereafter the expression was decreased. However, compared to the level of control, the expression of EFE26 was still higher induced at 24 h till 120 h (Fig. 4).

Effect of VB1 on PR1, PR5, NPR1, PAL, CM1, H1N1, and EFE26 expressions in tobacco leaves

To determine the transcript levels of disease-related genes involved in VB1 induced resistance, we used quantitative real-time PCR to analyze the expression of PR1, PR5, NPR1, PAL, CM1, H1N1, and EFE26 genes in tobacco leaves during *P. nicotianae* infection. Compared with the control, 20 mM VB1 application induced significant increase in PR1and PR5 transcripts at 1 dpi, respectively. However, the different treatments triggered enhanced gene expression at various time points. Transcript levels of PR genes in leaves treated with VB1 and inoculated with *P. nicotianae* increased earlier and higher than other treatments. The expression profile of PR1 and PR5 exhibited a similar pattern in the pathogen-inoculated leaves that increased with the time after inoculation (Fig. 5).

As shown in Fig. 5, VB1 treatment led to the up-regulation of PAL, NPR1 and CM1 at 1 dpi, 10 dpi and 1 dpi respectively, and inoculation with *P. nicotianae* did not significantly affect their transcript levels. Compared with VB1 treatment, *P. nicotianae* inoculation up-regulated the expression of NPR1 at 3 dpi significantly after VB1 priming. At 1 and 10 dpi, VB1 treatment induced stronger expression of H1N1 gene leaves, compared with *P. nicotianae* inoculation. For EFE26, *P. nicotianae* inoculation up-regulated its expression significantly after VB1 priming at 3 dpi, compared with VB1 treatment only(Fig. 5).

Discussion

P. *nicotianae* is one of the significant destructive plant pathogens that severely threaten tobacco cultivation[27]. Application of fungicides for disease control is now well known to have adverse environmental and health effects[28]. SAR induced by different biological and chemical agents provides safe, long-lasting and efficient resistance against a broad spectrum of pathogens[7]. Besides some well-known chemicals (e.g. BTH, INA) used to induce SAR in plants, some vitamins are now emphasized as novel SAR inducers. Although many studies have focused on the role of VB1 on plant–microbe Interactions[11,12,13], the mechanistic basis and regulation of VB1-mediated disease resistance are still poorly understood. Especially, there is less study on the potential of VB1 to induce the plant immunity against the oomycete pathogens. Therefore, in the present study, the role of VB1 on plant–*P. nicotianae* interactions and the underlying mechanisms was investigated using the *P. nicotianae* and tobacco interaction system.

In this study, we found that the pretreatment of 20 mM VB1 provided significant protection in tobacco against *P. nicotianae* infection. The biochemical changes of tobacco treated either with VB1 alone or VB1 prior to subsequent inoculation with *P. nicotianae* were then investigated. Vitro assays proved that mycelial growth was inhibited in a dose dependent manner and the EC50 value was calculated as 2.4 mM of VB1. Surprisingly, growth of *P. nicotianae* was not inhibited totally, even at the highest VB1 concentrations, possibly indicating that the *P. nicotianae* isolate was able to detoxify VB1 to a certain amount. Besides affecting mycelia growth, the effect of VB1 on the viability of zoospores were also evaluated. At doses ranging from 1 to 50 mM VB1, the percentage of inhibition on *P. nicotianae* zoospore in the VB1 incubated group (24.6% ± 97.2%) was significantly different from that in the water-incubated control group (4.3% ± 0.4%) (Fig. 1). These data show that VB1 has direct toxic effect on *P. nicotianae* at the investigated doses. This results were firstly reported about VB1 effect on *P. nicotianae* growth, at least showing *P. nicotianae* isolates isolated by us were sensitive to VB1.

The rapid accumulation of ROS is one of the earliest responses when plant be attacked by pathogen at attacking site. The rising ROS can participate in the orchestration of hypersensitive response and be used to destroy invading pathogens[29]. 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[30]. H_2O_2 could also induce the expression of genes encoding proteins involved in defensive and antioxidant processes, which had be reported by some studies as as a diffusible selective signal[31]. Compared to the mock-treated tobacco plants, the H_2O_2 accumulation were significantly increased in the VB1 treated tobacco plants with the *P. nicotianae* inoculation, indicating a priming effect mediated by VB1 on the *P. nicotianae* induced oxidative burst (Fig. 2).

Lignin is a polymer with a high–molecular weight, and it consists of β-(1,4)-glucan[32]. Lignin deposition could be seen as a strengthen of plant cell wall. It is also a judging method widely used to evaluate the plant PTI[33], which is the first-level protection of plants and be activated via the perception and recognition

by the pattern recognition receptors of plants[34]. In this study, the results showed that significantly higher levels of lignin were present in VB1 amended plants than in non-amended plants (Fig. 2), indicating a strengthened cell wall in the VB1 treated tobacco plants, which might contribute to the VB1 induced plant disease resistance. Our data showed that H_2O_2 was enhanced in tobacco pretreated with VB1 before inoculation with *P. nicotianae* (Fig. 2), which might resulted in the formation of lignin, as previous studies reported that increased production of H_2O_2 leads to polymerization of monolignols by peroxidase and subsequent lignin formation[35]. Certainly, this speculation need further studies in the future.

It is known that plant cells could be hurted by an excess H_2O_2 level. The plant must employ some mechanism to detoxify excess H_2O_2, such as antioxidant enzymes system, of which CAT is a famous antioxidant enzymes[36]. Our data showed that the activity change of CAT was according with H_2O_2 concentration (Fig. 2), which may imply CAT's degrading function on H_2O_2 at relatively high H_2O_2 concentration. The fact that massive accumulation of H_2O_2 caused only less damage during the pathogen-induced oxidative burst might be due to the induction of CAT activities. Plant secondary metabolites are important for plant defense responses against pathogens and herbivores, which could be synthesized by different regulatory enzymes[37]. POD is just one of the key regulatory enzymes for the biosynthesis of a variety of secondary metabolites, including scopoletin in plants[38]. Our current study also showed the increase of scopoletin content in the tobacco treated with *P. nicotianae* after VB1 primed (Fig. 2). Scopoletin is a kind of polyphenyl compounds and could be involved in plant defenses[39]. We speculate that the VB1 pretreatment caused a consequent chain of defense responses, including increasing activity of POD, and subsequently resulted in the induced scopoletin, for resisting against *P. nicotianae* infection in tobacco .

As an abiotic elicitor, VB1 may affect tobacco by itself. So its effect on tobacco was also investigated solely. VB1 application could induce a increase in the levels of CAT and POD activities (Fig. 3) and a change of H_2O_2 accumulation (Fig. 3). CAT and POD are considered as main antioxidant systems to protect cells against oxidative damage[40]. An excess H_2O_2 level is also harmful to plant cells and must simultaneously be detoxified by antioxidant enzymes, such as CAT and POD[36]. Our data showed that the activity of CAT and POD pretreated with VB1 before inoculation with *P. nicotianae*, which have resulted from the degrading function of CAT and POD at relatively high H_2O_2 concentration, as CAT and POD had been considered as main antioxidant systems to protect cells against oxidative damage[40]. As mentioned above, an excess H_2O_2 level is also harmful to plant cells and must simultaneously be detoxified by antioxidant enzymes[36]. Our results also revealed a significant increase in activity of PAL with a concomitant increase and decrease in lignin content (Fig. 3). The enhancement of PAL and lignin in VB1-treated tobacco indicated the reinforcement of a cell wall' physical barrier to restrict the penetration of pathogen[41].

The response of defense-related genes to exogenously applied VB1 was examined in tobacco leaves. Our results showed that the expression of PR1, PR5, NPR1, PAL, CM1, H1N1, and EFE26 genes was

significantly induced by VB1 (Fig. 3). Chemical elicitors have been reported to induce resistance against fungal pathogens infection in crops by the stimulating PR gene expression[42]. The increased expression of PR1, PR5 and others is widely accepted as a hallmark of plant defense induction[43]. Our results suggest that VB1-induced expression of PR genes might be contributing to VB1 conferred resistance against *P. nicotianae* in tobacco. The NPR1 regulator mediatd SAR to a broad spectrum of plant pathogens, by activating the defense genes (PRs) in SAR, including PR-1 and PR-5 by virtue of their particular structures and functions[44,45]. Here, results showed that VB1 treatment enhanced the expression of NPR1,PR1, and PR5 in tobacco leaves to conferred resistance against *P. nicotianae*, although is reaction is dependent on days after *P. nicotianae* infection (Fig. 4,5). EFE26, H1N1 and CM1 were ET biosynthesis and signaling marker genes[46], our results also showed that tobacco resistance against *P. nicotianae* had relationship with their expression.

We also observed a significant up-regulation of PAL gene(Fig. 4) which was correlated with the increase of PAL activity (Fig. 3) by VB1 treatment. The results also showed a significant induction of PAL gene (Fig. 4) and PAL activity (Fig. 3) as well as an enhanced amount of lignin (Fig. 2) in VB1-treated tobacco leaves. As mentioned above, PAL is considered to a key enzyme in catalyzing various phenylpropanoid defense metabolites, such as lignin. Furthermore, it had been proved that PAL worked as a rate-limiting enzyme in the phenylpropanoid pathway[47]. So we can concluded the cell wall reinforcement by accumulation of lignin involves in supporting the mechanical resistance to pathogen penetration[39,48], which may attributed to the increasing expression of PAL gene.

From our result mentioned above, we suggest that the VB1' function in inducing resistance in tobacco against *P. nicotianae* infection may attribute to induce resistance gene expressions, which lead to the bio synthesis of defense-related enzymes and secondary metabolites.

Declarations

Author Contributions:

Tao Liu and Xianwen Ye conceived and designed the experiments. Tian Suohui and Chen Yanping performed the experiment. Zi Shuhui and Mei Jian analyzed the data. Wang Zhijiang revised the paper. All authors read and approved the final manuscript.

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Conflicts of interest:

The authors declare no conflict of interest.

Statement:

The use of plants parts in the present study complies with international,national and/or institutional guidelines for plants.

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Table

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

Figures

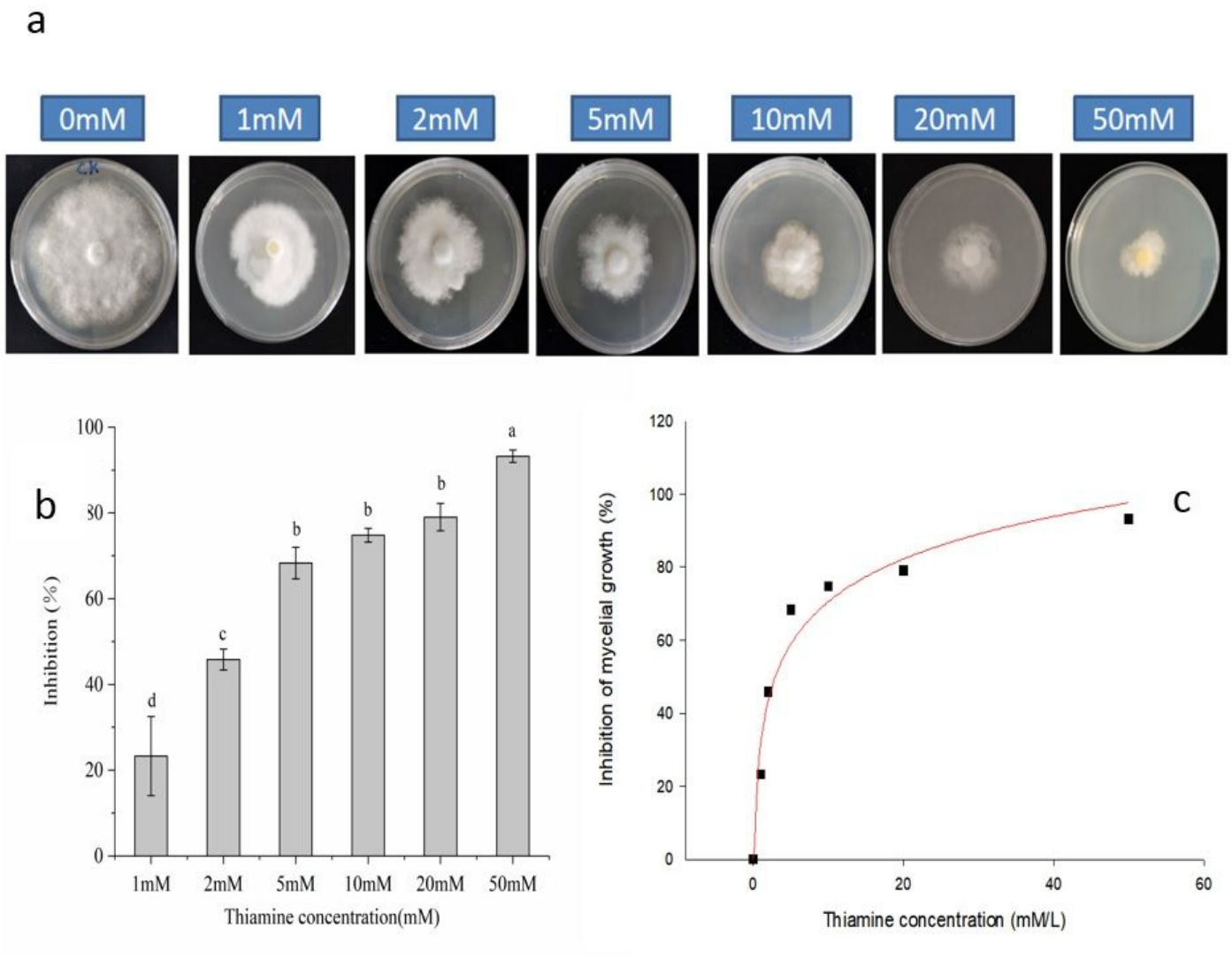

Figure 1

Effect of VB1 on in vitro growth and zoospore production of P. nicotianae. (a) P. nicotianae cultures in Petri dishes illustrating the inhibition of mycelial radial growth with increasing VB1 concentrations. The mycelial colonies were six days old. (b) Inhibition of P. nicotianae zoospore production at different VB1 concentrations. (c) Inhibition of P. nicotianae mycelial growth using different VB1 concentrations. Trend-lines were fitted using a logarithmic function. These assays were repeated three times showing similar results. n= 5 for each assay.

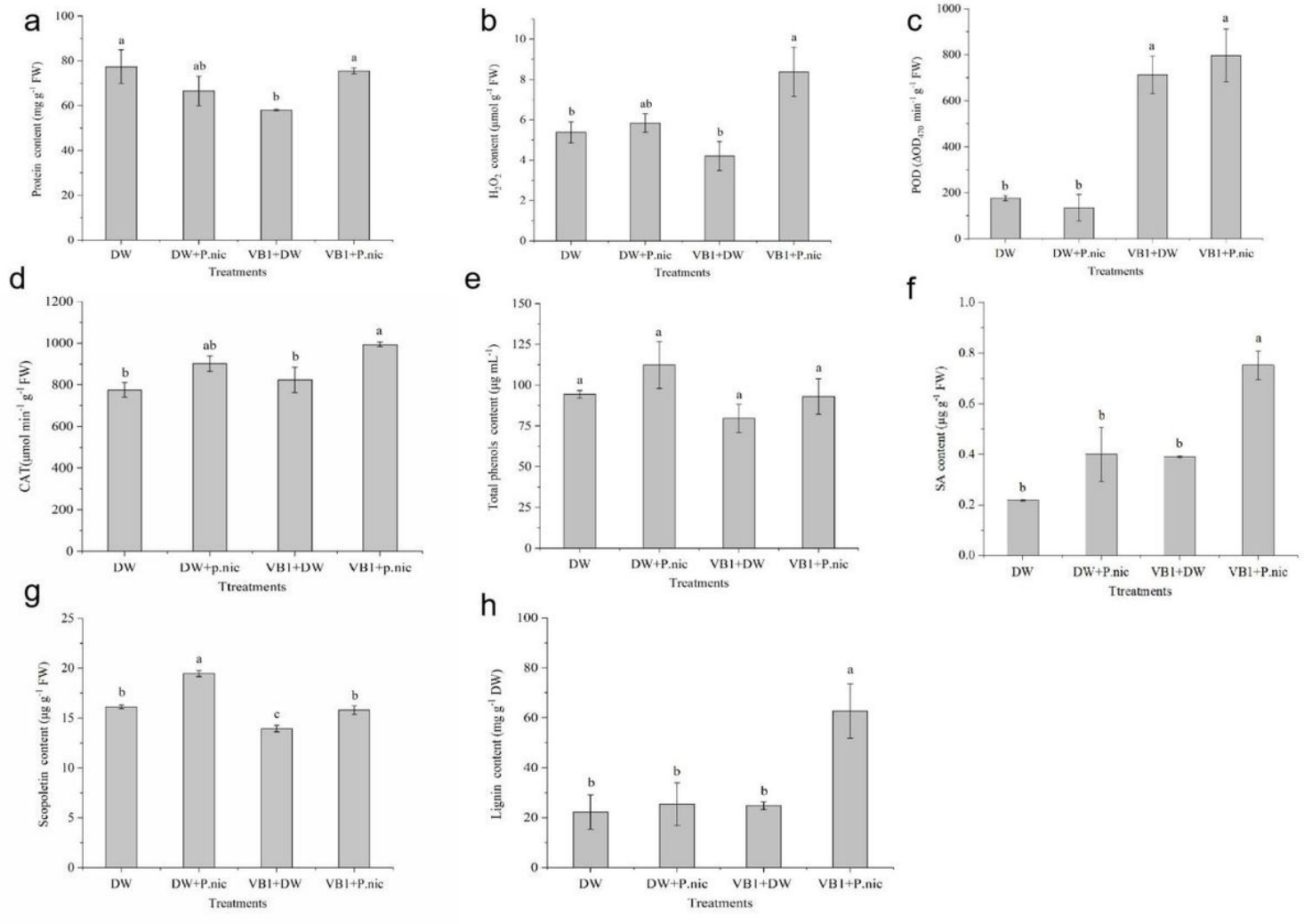

Figure 2

Effect of exogenous VB1 pretreatment on total protein content, H2O2 content, POD and CAT activities, total phenolic content, endogenous SA content, scopoletin content and lignin content in tobacco leaves after inoculation with P. nicotianae (P. nic). The leaves of tobacco were sprayed with either distilled water (DW) or VB1. After treatment, leaves were subsequently treated with either DW or P. nicotianae zoospore suspensions at 1 × 105 zoospores mL−1. After 1 day, the leaf samples were collected for determining (a) total protein content; (b) H2O2 content; (c) POD activity; (d) CAT activity staining; (e) total phenolic content; (f) endogenous SA content; (g) scopoletin content; (h) lignin content; The columns and vertical bars represent mean ± standard errors (SE) of three independent replicates of 10 leaves. Different letters represent significant differences, according to Tukey's HSD test at $p \leq 0.05$.

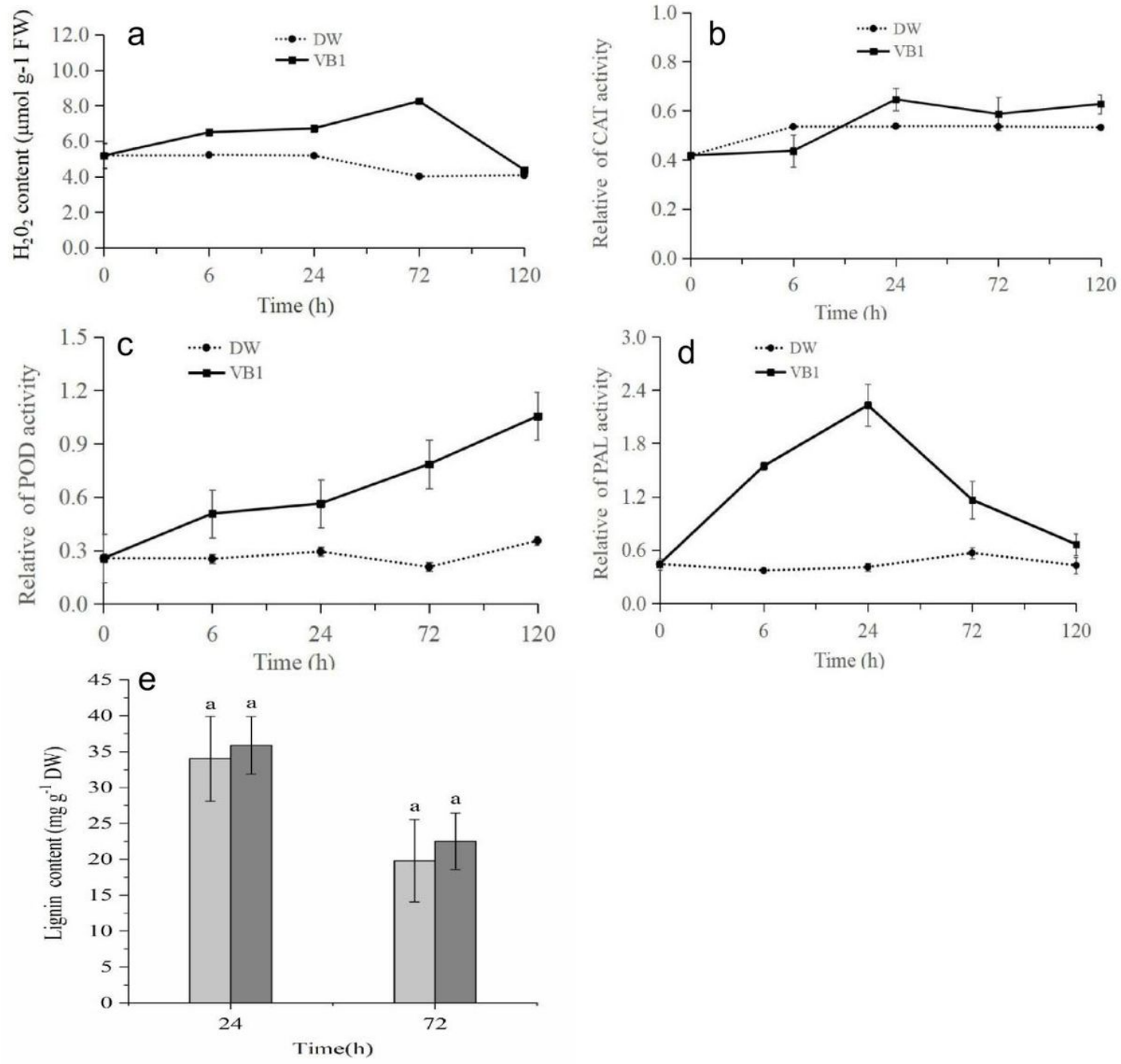

Figure 3

The effect of VB1 on (a)H2O2 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 VB1 and harvested at different points of time (6, 24, 72 and 120 h) for enzyme activity measurements, H2O2 content and lignin content. All data represent mean ± SE of three biological replicates of 10 leaves. Different letters represent significant differences, according to Tukey's HSD test at $p \leq 0.05$.

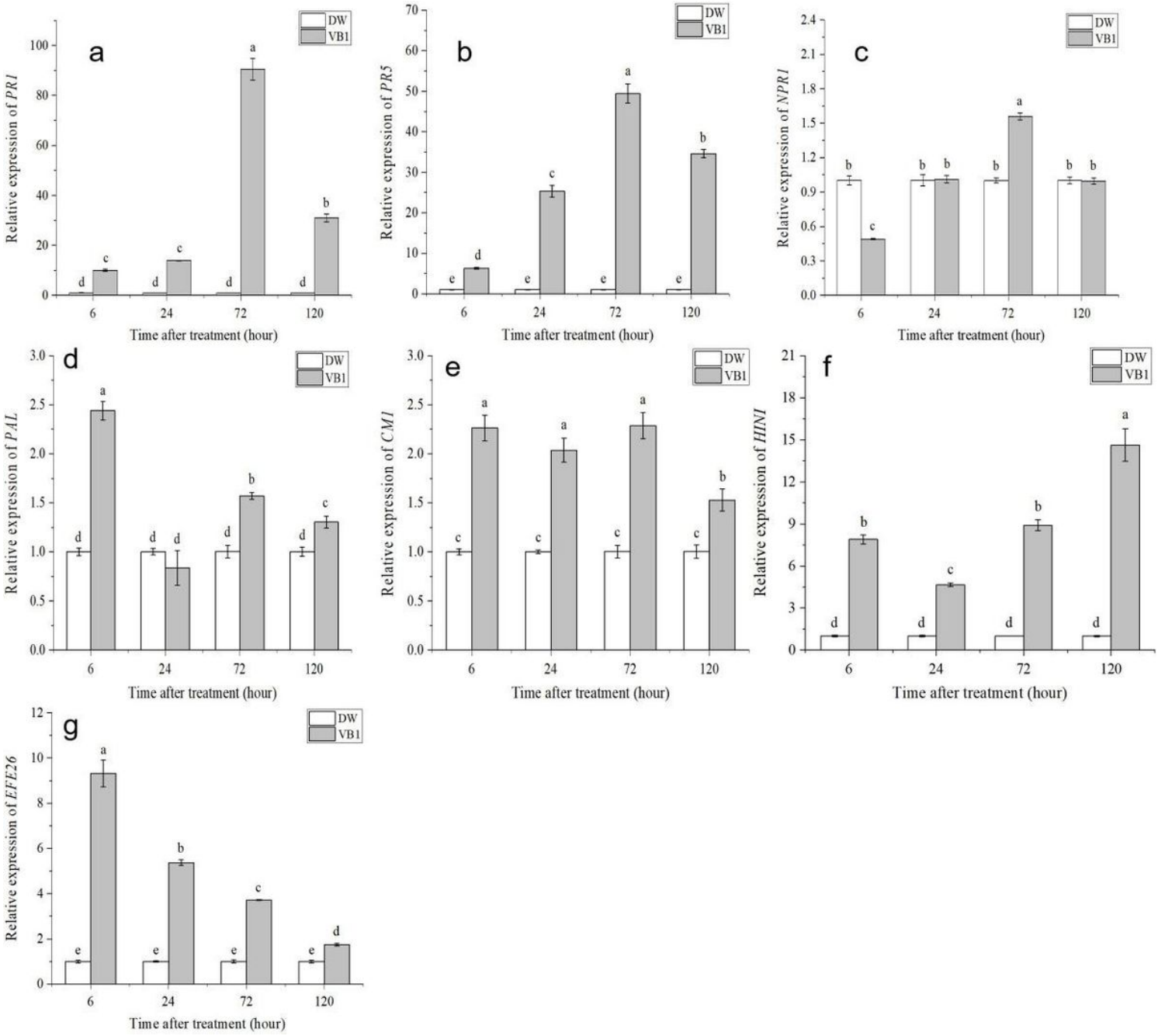

Figure 4

Effect of VB1 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 VB1. 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. All data show the average of three replications. Error bars indicate standard errors. Different letters represent significant differences, according to Tukey's HSD test at $p \leq 0.05$.

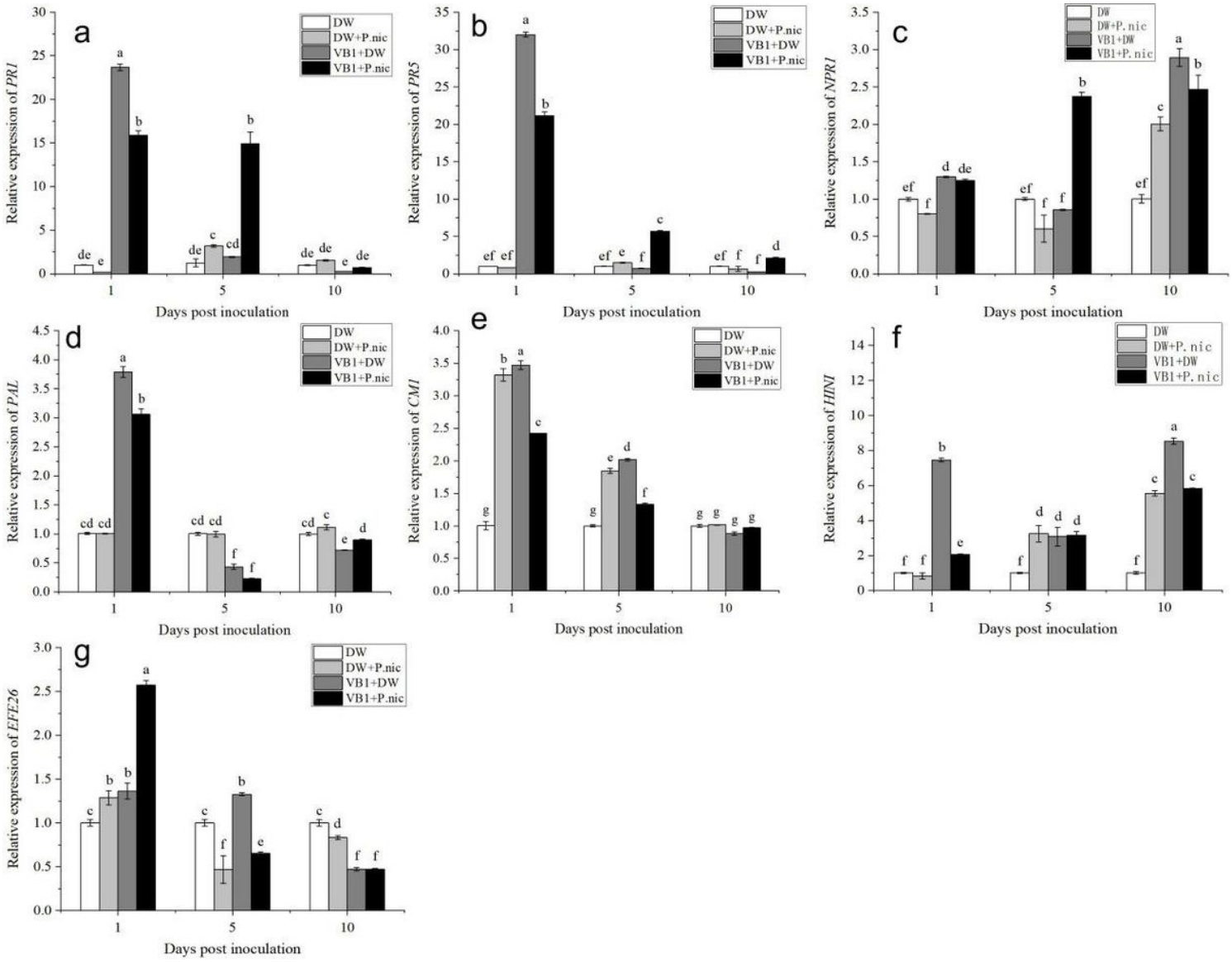

Figure 5

Effect of VB1 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. Data represent the mean with three biological replicates. Different letters indicate significant differences at each time point ($P < 0.05$). Error bars represent standard errors.