Expression of Proteins Related to *Phytophthora capsici* Tolerance in Black Pepper (*Piper nigrum L.*)

**Ton Trang Anh**, Ton Bao Linh, Nguyen Vu Phong, Thi Lan Thanh Bien, To Thi Nha Tram and Le Dinh Don

1Department of Biotechnology, Nong Lam University Ho Chi Minh City, Vietnam

*Corresponding Author: tontranganh@hcmuat.edu.vn*

---

**Abstract**—Vietnam is the number one black pepper exporter in the world. However, pepper production in Vietnam is facing challenges from natural disasters and soil-borne diseases, especially *Phytophthora* foot rot. Pepper leaves from five black pepper cultivars commonly grown in Vietnam were inoculated with *Phytophthora capsici*. The activities of pathogenesis-related proteins such as chitinase, β-1, 3-glucanase and phenylalanine ammonia lyase (PAL) significantly increased in the infected leaves compared to the healthy leaves. An SDS-PAGE analysis revealed the expression of two polypeptides with molecular masses of approximately 30 and <10 kDa in the *P. capsici*-infected leaves after 2 and 3 days following inoculation, respectively. Furthermore, western blot analysis confirmed the presence of a 34 kDa band corresponding to β-1, 3-glucanase in protein extracts from the infected leaves. This study suggests the involvement of β-1, 3-glucanase and related enzymes in the defense response of black pepper to fungal pathogens.

**Keywords**—Black Pepper, Chitinase, β-1, 3-glucanase, *Phytophthora capsici*.

---

**I. INTRODUCTION**

Vietnam is currently the world’s largest producer and exporter of black pepper (*Piper nigrum L.*), supplying 50% of the world’s black pepper [1]. However, black pepper production in Vietnam has been challenged by natural disasters and plant diseases. The most common and devastating disease affecting Vietnam’s pepper areas is foot rot or quick death caused by *Phytophthora capsici* [2] This fungal strain is one of the most important phytopathogens in the world because it causes significant decreases in, or total losses of pepper crops. In some cases, the mortality rate of pepper vines was 100%. *Phytophthora* foot rot is rapidly spread and difficult to control. Plants infected by this pathogen die within 2-3 weeks in the rain, and adjacent plants may be infected within one or two months [3].

It has been reported that plant defense responses against invading phytopathogens (*e.g.*, bacteria, fungi, or viruses) include the accumulation of a number of specific proteins known as pathogenesis related (PR) proteins [4]. They are low molecular weight heatstable proteins (6-43 kDa) that are selectively extractable and stable at low pH ~ 3 [5]. However, little is known about the tolerance mechanisms of Vietnam’s pepper cultivars during pathogen infection.

In this study, the induction of PR proteins of *P. capsici* was evaluated in five commonly grown Vietnamese pepper cultivars. The results provide useful insights into the tolerance mechanisms of pepper to fungal disease, which is important information for black pepper breeding strategies focused on *Phytophthora* resistance.

---

**II. MATERIALS AND METHODS**

**A. Preparation of Inoculum, Plant Growth and Inoculation**

Mycelium from a single colony of *P. capsici*, grown in carrot agar plates for 4 days, was inoculated into 15 mL of carrot broth medium and incubated at 27°C for 48 h in the dark. Sporangium formation was observed under a light microscope (10X). Plates with a high concentration of sporangia were incubated at 4 °C for 1 h, then illuminated with an incandescent bulb for 30 min. Zoospores were collected by filtration and suspended in sterile distilled water at a concentration of 10^5 zoospores/mL [6]. The third and fourth black pepper leaves from *ex vivo* grown plants of the varieties Vinh Linh, Trau, Se, Se Xanh and Kuching were used in this study. The leaves were surface sterilized by washing in sterile water and soaking in 70% alcohol for 30 s and then again washed 2-3 times with sterile water. For inoculation, wounds were made on the adaxial midribs of the pepper leaves using a sterile needle and inoculated with 40 μL of *P. capsici* zoospore suspension. The infected leaves were then inoculated at 27°C in the dark for 5 days. Wounded leaves inoculated with autoclaved distilled water were used as controls.

**B. Total Protein Extraction**

Total leaf protein was extracted according to a previous study as in [7]. In brief, 0.5 g of leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. An extraction buffer was added to the leaf powder at a ratio of 1:2 (w/v). The buffer consisted of Tris (pH 8.0), 0.001% PMSF, 0.05 M cysteine-HCL, 10% PVP, 5% ascorbic acid, and 0.001% β-mercaptoethanol. The mixture was then centrifuged at 12,000 rpm for 5 min to collect the supernatant. The resulting supernatant was combined with Tris-phenol at a 1:1 (v/v) ratio, briefly vortexed, and centrifuged at 12,000 rpm for 5 min prior to adding 0.1 M ammonium sulfate (1:1, v/v). The mixture was incubated overnight at ~20°C and then centrifuged at 12,000 rpm for 5 min. The pellet was collected and washed twice with 80% acetone and 70% ethanol, and then resuspended in TE buffer containing 0.001% PMSF. Protein concentration was measured using the Lowry assay with BSA as a protein standard. The concentration of BSA ranged from 0.02 - 0.1 mg/mL. The absorbance at 660 nm was recorded [8].

**C. Enzyme assays**

**Chitinase activity** - Chitinase was extracted from black pepper leaves using the method described in [8]. The activity of chitinase was determined by quantitative estimation of N-acetylglucosamine produced from a reaction between chitinase and colloidal chitin as...
mentioned in [9] with minor modifications. Each reaction consisted of 0.4 mL of enzyme solution and 0.4 mL of 1% colloidal chitin in 1.2 mL of acetate buffer. Reactions were incubated for 30 min at 30°C, stoped by adding 0.4 mL of 1% NaOH and boiled for 3 min. These mixtures were subsequently centrifuged at 4,000 rpm for 5 min. The amount of reducing sugars released in the supernatant was measured based on a method that uses dinitrosalicilic (DNS) acid reagent, and the absorbance at 540 nm was measured [10].

**Glucanase activity** - Glucanase extraction from black pepper leaves was performed using a method as in [7]. Assays for β-1, 3-glucanase activity were based on the release of reducing sugar (glucose) from glucan as described by [11]. For each assay, enzyme extract (0.3 mL) was incubated with 0.3 mL of 1% glucan in 0.9 mL of acetate buffer at 35°C for 30 min. The reaction was stopped by boiling for 3 min. The mixture was centrifuged at 5,000 rpm for 5 min. The amount of reducing sugar released was measured using the DNS method [10].

**Phenylalanine ammonia lyase (PAL) activity** - PAL extraction from black pepper leaves was performed following a method by Nazeem et al. [7]. PAL activity was measured using the method suggested by Sadasivam and Manikam [12] with slight modifications. Briefly, 1 mL of L-phenylalanine solution was added to a mixture containing 0.5 mL of borate buffer, 0.2 mL of enzyme solution and 1.3 mL of distilled water. The mixture was incubated for 1 h at room temperature. The reaction was stopped by adding 0.5 mL of trichloroacetic acid and the absorbance at 290 nm was measured.

**SDS-PAGE and western blot analysis** - For SDS-PAGE analysis, 50 µg of total protein was mixed with loading buffer (1:1, v/v) and boiled for 5 min, then loaded in each well on a 15% SDS polyacrylamide gel. Electrophoresis was started at 40 V to move the protein through the stacking gel and into the separating gel, then run at 80 V. The gel was stained with Coomassie Brilliant Blue for 1 h to observe the protein profiles.

For western blotting, the protein that was separated on the SDS-PAGE gel was electro blotted on to a PVDF membrane (0.45 µm) following the procedure of Sambrook et al. [13]. The membrane was probed with PR2-GLU 1-class 1 β-1, 3-glucanase (1:500, v/v), followed by goat-rabbit IgG, HRP conjugate (1:25000, v/v). For detection, the membrane was incubated with ECL substrate and exposed to X-ray film.

**D. Data Analysis**

Experiments were performed in triplicate. The results are expressed as the mean ± SD (error bars) of three independent experiments. Statistically significant differences (p<0.01) were determined using Statistical Analysis Systems 9.1 (SAS 9.1).

### III. RESULTS & DISCUSSION

Changes during the time-course in the activities of chitinase, β-1, 3-glucanase and PAL in black pepper leaves inoculated with *P. capsici* are shown in Figure 1. In general, the *P. capsici* inoculum increased the activities of these enzymes in the infected leaves compared to the healthy controls. The increase in enzyme activities differed between pepper varieties (p<0.01). The highest chitinase activities (Fig. 1A) were recorded at: 3 days post-inoculation (dpi) in the cultivar Vinh Linh (1.56 ± 0.05 µg NAG/mg protein/min), 5 dpi in Trau pepper (3.00 ± 0.10 µg NAG/mg protein/min), 1 dpi in Se (0.81 ± 0.03 µg NAG/mg protein/min), 4 dpi in Se Xanh (1.63 ± 0.09 µg NAG/mg protein/min), and 5 dpi in Kuching (1.72 ± 0.04 µg NAG/mg protein/min). A similar increasing trend was observed for β-1, 3-glucanase activity (Fig. 1B), except that the highest activity in the Se variety was reached at 3 dpi (2.98 ± 0.18 µg glucose/mg protein/min).

Many previous studies have demonstrated that the accumulation of PR proteins such as chitinase and β-1, 3-glucanase in plants is a defense mechanism in response to pathogen attack [7], [14] – [16]. The expression of these proteins is an important factor contributing to phytopathogen tolerance in plants [17]. The results of this study suggest that the pathogen infection might induce the expression of chitinase and β-1, 3-glucanase. This is consistent with a previous report by Lee et al. [18] that showed an increase in chitinase and β-1, 3-glucanase activities in *P. capsici*-treated leaves after 6 days of infection. Moreover, the increased level of chitinase [19] and activity of β-1, 3 glucanase [7] in *P. capsici* inoculated *P. nigrum* leaves after 48 h and 3 days, respectively, was reported. A higher level of β-1, 3 glucanase activity in the tolerant ‘Kalluvally’ (1.70 µmol/mg protein/10 min.) compared to the susceptible ‘Panniyur-1’ (0.262 µmol/mg protein/10 min.) was recorded by Nazeem et al. [7], while the chitinase activity of black pepper was found to be 40% higher than that of *P. colubrinum* under the same conditions [19]. The resistant genotype *P. colubrinum* possessed high β-1, 3 glucanase activity (>1.5 µmol/mg protein/10 min.), but it did not show any significant variation following infection [7].
Chitinase and β-1, 3-glucanase are known to inhibit the growth of fungi by lysing fungal cell walls [20]. Antifungal activity of chitinases can be synergistically enhanced by β-1, 3-glucanase, both in vitro and in vivo [30]. β-1, 3-glucanases hydrolyzes the (1, 3)-β-D-glucosidic linkages in (1, 3)-β-glucans present in the fungal cell wall and chitinase reacts to a natural homopolymer of β-1, 4-linked N-acetyl-D-glucosamine (NAG) residues [21]. The differences in the expression of chitinase and β-1, 3-glucanase might be due to variations in _P. capsici_ resistance among the pepper genotypes in terms of the timing and magnitude of the defense response [22].

PAL activity (Fig. 1C) did not change significantly after inoculation except in the Trau (0.275 ± 0.07 µg cinnamic acid/mg protein/min) and Kuching (0.405 ± 0.07 µg cinnamic acid/mg protein/min) varieties. PAL is a key enzyme in the phenylpropanoid biosynthetic pathway that plays important roles in flavonoid production [23]. The activity of this enzyme is associated with the biosynthesis of defense-related metabolites, such as phytoalexins, phenols, lignins and salicylic acid [24]. Increases in PAL activity have been reported in plants under different types of stress [25]-[27]. However, in this study, the activity of PAL in infected leaves showed subtle change in the pepper varieties and significant increase in the Trau and Kuching varieties after 2 dpi. This result is consistent with the report by Zhang _et al._ [22] that there was no change in PAL activity in the _Capsicum annuum_ cultivars CM334 and PBC602 before or after inoculation, but a significant increase and peak in activity between 2 and 4 dpi. The
enhanced PAL activity might result from a hypersensitive response that occurs in many plants following wounding or infection [7], which causes the increased expression of related genes under pathogen stress, or different resistance mechanisms among pepper varieties. PAL involves in synthesis of salicylic acid which then activates expression of PR genes [28]. Delayed activity of chitinase and β-1, 3-glucanase up to the fifth day in Trau and Kuching may relate to the elevated activity of PAL at 2 dpi (Fig.1). The results of this study support the roles of chitinase, β-1, 3-glucanase and PAL in the suppression of *P. capsici* disease in *P. nigrum*. Previous studies have confirmed a significant role of β-1, 3-glucanase and PAL, but suggested less involvement from chitinase in the tolerance of black pepper to *Phytophthora* infection [7], [22]. It was also reported that chitinase and PAL are usually coexpressed with, and can induce β-1, 3-glucanase expression in pathogenesis-related responses [7], [29], [30]. This suggests that the interaction of PR proteins is important in defending against pathogen infection.

An SDS-PAGE analysis of total protein from *P. capsici* infected pepper leaves is shown in Figure 2. A protein of 30 kDa was observed in infected leaves of Vinh Linh and Se Xanh at 2 dpi (Fig. 2A). At 3 dpi, this protein was found in all infected varieties (Figure 2B) and was not found in the corresponding controls. However, by 4 dpi, the 30 kDa protein was not found in infected Vinh Linh (Figure 2C) and only Trau and Kuching peppers expressed this protein at 5 dpi (Figure 2D). In addition, a polypeptide <10 kDa was also observed in the infected pepper leaves at 3 and 4 dpi (Figure 2B and 2C). Western blot analysis (Figure 3) showed the presence of a 34 kDa protein in both infected and healthy leaves, which immunologically reacted to a PR2-GLU I-class I β-1,3-glucanase. The lowest expression of β-1, 3-glucanase was observed in the Se variety showing only a weak protein band on the membrane (Figure 3D). Relatively stable expression was found in the Vinh Linh (Figure 3A) and Se Xanh (Figure 3B) varieties, while the expression of β-1, 3-glucanase was clearly increased in Trau and Kuching (Figure 3C and 3E) after infection.

The expression of these proteins was consistent with the changes in enzyme activities, particularly β-1, 3-glucanase (Figure 1). At 2 dpi, there was a significant increase in β-1, 3-glucanase activity in Vinh Linh and Se Xanh varieties along with the presence of the 30 kDa protein, but the activity rapidly decreased to the control level at 4 and 5 dpi when the protein band was not observed in these varieties. Additionally, in Trau and Kuching varieties, increased β-1, 3-glucanase activity was accompanied by the presence of the 30 kDa protein from 3 to 5 dpi. The presence of the <10 kDa protein was found at 3 and 4 dpi when the activities of most enzymes were elevated compared to the controls. Consistent with these results, Nazeeem et al. [7] detected a <10 kDa protein (8 kDa) in black pepper *P. capsici*-infected leaves after 3 days of infection. These results indicate the involvement of 30 kDa and <10 kDa proteins in the defense mechanisms induced by *P. capsici* inoculation. The Western blot analysis (Figure 3) also revealed that the expression of β-1, 3-glucanase was stronger in the infected leaves compared to the healthy leaves, which again confirms the role of glucanase in response to pathogen infection in plants.

In conclusion, the present study demonstrates the important roles of chitinase, β-1, 3-glucanase and PAL in the defense response of black pepper against foot rot disease caused by *Phytophthora*. The increases in the activities of these enzymes in pepper leaves upon *P. capsici* infection and the positive results in the SDS-PAGE and western blot analyses confirmed the involvement of β-1, 3-glucanase and related enzymes in black pepper’s tolerance to pathogens. Molecular mechanisms leading to the expression of these genes in black pepper should be studied further to gain a better understanding of the cultivar-specific responses to *Phytophthora* species.

**ACKNOWLEDGMENT**

The authors are thankful to the Ministry of Education and Training (MOET, Vietnam) for funding this study and Seed Labs (USA) for the financial support on completion of this article.

**REFERENCES**


**AUTHOR’S PROFILE**

**First author Ton Trang Anh**

She was born on February 2nd 1973 in Quang Ngai province, Vietnam. She completed Master degree in Crop Science in 2010 at Nong Lam University Ho Chi Minh city, and currently is a lecturer at Department of Biotechnology, Nong Lam University Ho Chi Minh city. Her research interest focuses on plant micropropagation and plant pathology. Email: tontranganh@hcmuaf.edu.vn

**Ms. Ton Bao Linh**

Lecturer at Department of Biotechnology, Nong Lam University Ho Chi Minh city, Linh Trung Ward, Thu Duc District, Ho Chi Minh city, Vietnam. Her research focuses plant-microbes interactions and genetic engineering in plants. Email: tonbaolinh@hcmuaf.edu.vn

**Dr. Bien Thi Lan Thanh**

She completed her PhD on Environmental Microorganism at Ehime University, Japan in 2017, and is currently a lecturer at Department of Biotechnology, Nong Lam University Ho Chi Minh city, Linh Trung Ward, Thu Duc District, Ho Chi Minh city, Vietnam. Email: bienthanhlan@hcmuaf.edu.vn

**Assoc. Prof. Le Dinh Don**

Head of Department of Biotechnology, Nong Lam University Ho Chi Minh city, Linh Trung Ward, Thu Duc District, Ho Chi Minh city, Vietnam. His research focuses on crop science and phytopathology. Email: ledinhdon@hcmuaf.edu.vn