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The deleterious effects of chitosan application on red chili pepper growth and capsaicin biosynthesis under drought stress

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Abstract. *Aziz MA, Esyanti RR, Meitha K, Dwivany FM. 2022. The deleterious effects of chitosan application on red chili pepper growth and capsaicin biosynthesis under drought stress. Asian J Trop Biotechnol 19: 52-61.* The production of red chili pepper is often impeded by drought. Chitosan is considered as a promising alternative natural fertilizer and defense elicitor. This study aimed to investigate the effect of chitosan on red chili plant growth and capsaicin biosynthesis under drought stress. At the onset of the generative phase, the plant was subjected to 1 mg mL^{-1} chitosan, 50% drought, or chitosan-drought treatment. Observations were made on several growth parameters, yield, PAL expression, PAL activity, and capsaicin content. The results showed that chitosan-drought treatment significantly decreased plant growth and yield. The PAL gene expression was up-regulated around 129-fold higher than control, followed by increased PAL activity and capsaicin content about 1.22 and 1.39-fold higher than control, respectively. PAL activity and capsaicin content on chitosan-drought treated plants were lower than on individual chitosan applications. Therefore, we suggested that the double treatment of chitosan-drought might decrease red chili pepper production and capsaicin content. Interestingly, the individual chitosan treatment significantly increased PAL expression level, PAL activity, and capsaicin content compared to other treatments. Furthermore, it was suggested that chitosan might play a role in the red chili plant defense system involving capsaicin biosynthesis through the phenylpropanoid pathway.

Keywords: Capsaicin, *Capsicum annuum*, chitosan, drought, growth, PAL expression

INTRODUCTION

Red chili pepper (*Capsicum annuum* L.) is one of the most favored agricultural commodities with a high economic value. The popularity of the commodity is because of its various nutritional content such as protein, fat, fiber, minerals, vitamins A, B, and C, as well as capsaicin, a secondary metabolite known to provide heat sensation, increase body temperature and burn calories (The Indonesian Ministry of Agriculture 2015; Aziz et al. 2021). However, the fluctuating production of chili pepper causes a damaging impact on scarcity and market price escalation. In addition to pathogen infections, drought is major stress causing low productivity. According to the statistical data (The Indonesian Ministry of Agriculture 2016), it is indicated that a significant decrease in the chili harvested area due to the prolonged dry season, around 35.98% and 63.00%, occurred in 1987 and 1990, respectively.

In recent years, global climate change has been the most frequent abiotic stress (Khan et al. 2014). In brief, plants respond to water stress by regulating proline accumulation (Solichatun et al. 2022) and stomatal behavior mediated by abscisic acid (ABA) as the defense regulator. It plays a role in maintaining a plant's water capacity. However, this causes a reduction in photosynthesis, hampered plant growth and productivity (Iriti et al. 2009; Phimchan et al. 2012). Drought stress in chili plants might cause flowers and immature fruits to fall easily. In addition, the fruit tends to contain a higher level of capsaicin (Sung et al. 2005). Mahmood et al. (2021) added that flowering is one of the most sensitive stages of the chili plant affected by drought.

Capsaicin is one of the specific secondary metabolites of the phenol group in the genus of Capsicum, which is well known to cause pungency. Capsaicin is synthesized through the phenylpropanoid and fatty acid pathways. In addition to being responsible for pungency, capsaicin has many benefits in pharmaceutical properties, including anti-inflammatory, antioxidant, analgesic, and anti-carcinogenic (Gonzalez-Zamora et al. 2013). The biosynthesis pathway of capsaicin involves four main enzymes, including phenylalanine-ammonia-lyase (PAL), cinnamic acid-4-hydroxylase (C4H), p-coumaric acid-3-hydroxylase (C3H), and caffeic acid-o-methyltransferase (CAOMT). PAL regulates capsaicin production using phenylalanine as the main precursor. Capsaicinoid is synthesized from vanillylamine and isocapryl-CoA through capsaicinoid synthetase (CS) activity, then accumulated in the placenta (Sung et al. 2005). Various environmental conditions influence capsaicin biosynthesis. The higher level of capsaicin is one of the plant's biological responses in increasing resistance against pathogenic attacks. Veloso et al. (2013) proved that capsaicin could inhibit the growth

of *Verticillium dahliae* Kleb. by 33% and *Phytophthora capsici* Leonian by 63.7%. On the other hand, Solichatun et al. (2022) stated that the resistance increment of paprika (*C. annum*) against drought stress could be performed using the KCl seed priming technique.

Increasing plant growth and resistance using environmentally friendly technologies is continuously developed. This is by public awareness regarding the use of synthetic compounds causing any hazardous impacts on living things' sustainability. Chitosan is a natural compound of acetylated chitin derivatives that are easily decomposed (Mondal et al. 2013; Pichyangkura and Chadchawan 2015). The role of chitosan as a plant biostimulant is suggested through several mechanisms such as supplying the nitrogen (El-Tanahy et al. 2012), increasing macronutrient and micronutrient absorption (Chookhongkha et al. 2012; Jang et al. 2012), increasing the activity of many enzymes related to protein metabolisms and increasing nitrogen transport to leaves (Mondal et al. 2013). In addition, it is suggested that chitosan promotes plant resistance by increasing the secondary metabolism of phenolic compounds such as capsaicin (Khan et al. 2003; Aziz et al. 2021). However, the role of chitosan on plant growth and capsaicin accumulation under drought stress has not been thoroughly studied. Therefore, this study aimed to investigate the effect of chitosan on red chili plant growth and capsaicin biosynthesis under drought stress.

MATERIALS AND METHODS

Plant materials and treatments

This research was conducted at the greenhouse of the Institut Teknologi Bandung, Indonesia. This experiment was performed for ± 112 days, with the average photoperiod being 12 hours. This study uses the commercial red chili pepper (*C. annuum*) Laba cultivar from East-West Seed Indonesia Ltd. The cultivar is widely cultivated due to its superior defense system against pest and microbial infection and the ability to produce 18-20 tons of Ha^{-1}. The individual red chili pepper was grown in a plastic polybag with a 3 kg planting medium containing soil, husks, and organic fertilizer with the corresponding ratio (4:3:2, v:v:v). The plantlets were irrigated up to 100% field water capacity for the vegetative stages (7 weeks). At the onset of the generative phase, indicated by the emergence of flower buds, the plantlets were subjected to the treatments of chitosan (Chi), chitosan and drought (Chi-D), drought (D), and control (C) up to sixteen weeks of planting (n = 3 replications). Several growth parameters, including plant height and the number of leaves, followed by the number of flowers and fruits, were observed during the treatment (weeks 8 to 16). These were made to show the effect of chitosan on plant growth and yield under drought conditions. In addition, several environmental microclimate conditions, including pH, light intensity, humidity, and temperature, were also recorded.

A 1 mg mL^{-1} Chitosan solution, prepared as described in Esyanti et al. (2019) and Aziz et al. (2020), was sprayed once a week by foliar feeding technique to Chi and Chi-D treatments. Esyanti et al. (2019) stated that 1 mg mL^{-1} chitosan solution maintained the growth rate of the red chili plant cultivar. Drought treatment was applied to reach 50% of field water capacity by applying 500 mL of water (in two days) subjected to D and Chi-D treatments. Dorji et al. (2005) suggested that a 50% of water deficit is a feasible irrigation strategy to maintain plant growth performance. In addition, the control group (C) was irrigated with 100% field water capacity but not sprayed with any solutions. Fresh leaves and fruits were collected 55-60 days after flowering (harvesting period) (Sung et al. 2005). The fresh leaves from three plants were pooled into one sample and then used for RNA extraction to analyze PAL gene expression and PAL enzyme activity, while the fresh fruits were used for capsaicin content measurement.

RNA extraction and qPCR analysis

The total RNA was extracted from the leaves sample (50 mg) using the PureLinkTM RNA mini kit (Invitrogen). RNA quality was visualized in a 1.5% (w/v) agarose gel with gel-red as a coloring agent by using an electrophoresis instrument. Meanwhile, the quantity of RNA was measured using a nanodrop spectrophotometer (Eppendorf, biospectro-meter) to evaluate the purity of RNA and quantify RNA concentration. DNAse treatment was performed using DNAseI (Thermo Scientific), while cDNA synthesis was carried out using iScript cDNA Synthesis Kit (Bio-Rad) containing reverse transcriptase enzyme. In addition, the quality of cDNA was determined in a 2% agarose using CaUbi3 as the housekeeping gene by using the specific primer (Table 1). The Polymerase Chain Reaction (thermal cycler) protocol was performed as follows: 95°C 3 min, 95°C 30 s, 58°C 30 s, 72°C 1 min, 72°C 5 min (35 cycles).

Table 1. Primer for PAL and CaUbi3 genes

Primer	Accession number	Nucleotide (5' to 3')	Size (bp)
PAL	NM_001324603		283
Forward		TGGGCTTAATCTCATCAAGG	
Reverse		TAGGTTGAGCTGCAGGTATC	
CaUbi3	AY486137.1		201
Forward		TCCATCTGCTCTCTGTTGACG	
Reverse		CCCCAAGCACAATAAGACATTGT	

The PAL gene's amplification was performed using specific primers (Table 1). After being evaluated in a 2% agarose electrophoresis, sequencing was performed through Macrogen service, Korea. Further, the quantitative PCR was performed using instruments and software from MyGo Pro with the dye from Toyobo (Thunderbirds SybrqPCR Mix QPS-201). The quantification cycle (Cq) score was then used to quantify the expression level through a relative method using the corresponding formula (Livak and Schmittgen 2001).

PAL enzyme activity assay

Sung et al. (2005) adopted the PAL enzyme activity assay method with a few modifications. In brief, 0.5 gr of chili leaves were crushed using liquid nitrogen and added by a 3 mL protein extraction buffer. The samples were homogenized at high speed for 1 minute and then centrifuged at 7000 rpm for 40 minutes. The supernatant was then centrifuged at 10000 rpm for 10 minutes, then transferred to the vial bottle followed by adding 5 mL of 50 mM Tris-HCl buffer pH 8. The total protein was quantified by using the Bradford method. The absorbance was measured by using UV-Vis spectrophotometer at λ595 nm. Bovine Serine Albumin (BSA) was used as the standard solution at concentrations of 0, 1, 2.5, 5, 10, and 15 ppm.

PAL activity assay was carried out by mixing 1 mL of 100 mM Tris-HCl, 0.5 mL of 10 mM L-phenylalanine, 0.4 mL of sterile deionized water, and 0.1 mL protein extract. The mixture was incubated at room temperature for 1 hour. The reaction was ended by adding 0.5 mL of 6 M HCl. In addition, 7.5 mL of diethyl ether was added to bind the polar compound, and then the mixture was stored in a -20 freezer. After 2 phases were formed, diethyl ether was discarded. The frozen extract was dried using a freeze dryer, and the residue was dissolved using 3 mL of 50 mM NaOH. The absorbance was measured at λ290 nm. PAL activity was quantified by using cinnamic acid as a standard solution at concentrations of 0, 0.3125, 0.625, 1.25, and 2.5 ppm (Sung et al. 2005).

Capsaicin content assay

Capsaicin was extracted from the chili placenta by using the maceration method. The quantification method of capsaicin level was adopted from Sung et al. (2005) with a few modifications using HPLC (C-R7A Plus Chromatopac, Shimadzu). The placenta was immediately sampled from harvested chili fruits and dried at 60°C for 30 hours, then crushed and macerating tissue in 5 mL of 96% ethanol for 24 hours. Macerated tissue was then filtered in a 50 mL beaker glass and incubated at room temperature to evaporate ethanol. Finally, the dried filtrate was dissolved using 1 mL of 100% methanol and re-filtered using oil-free cotton into a 2 mL microtube for immediate assay or storage at 4°C.

A 2.5 µL of the extract was injected into the HPLC system. A mix of acetonitrile and aqua bides at a ratio of 6:4 (v:v) with a rated flow of 0.7 mL min^{-1} was used as the mobile phase. It runs for 12 min $sample^{-1}$ by using absorbance at λ280 nm. Before running, acetonitrile solution was filtered using a Polytetrafluoroethylene (PTFE) filter, while aqua bides used nitrocellulose membrane. Capsaicin standard (SIGMA, Lot #BCBJ2271V) solution at the concentration of 0, 125, 250, and 500 ppm was prepared by diluting from capsaicin powder.

Statistical analysis

The effect of chitosan, drought, and the interaction of both treatments towards the increase in height, PAL genes expression level, PAL enzyme activity, and the capsaicin content were analyzed by Two-way ANOVA performed using IBM SPSS statistics 22 software. In addition, the significant differences in the treatments were determined using the Tukey HSD test at $P < 0.05$ using the R studio 3.5.2 (32/64 bit) application.

RESULTS AND DISCUSSION

Chitosan application under drought conditions inhibited plant growth and productivity

The microclimate condition, pH, humidity, and temperature of the media and light intensity, humidity, and temperature of the environment, are shown in Table 2. There was no significant difference between the pH and temperature of the media in all treatments. However, the drought-treated Chi-D and D plants showed a lower media humidity at 43.44% and 46.56%, respectively. In addition, the average relative humidity was recorded at 78.96%, a light intensity of 10240 lux, and temperature ± 27.78°C.

To determine the effect of chitosan-drought (Chi-D) application on the red chili pepper plant, it is important to investigate the plant growth performance parameters such as height increment and the number of leaves described in Figures 1A and 1B. The similar performance in the vegetative phase determined the plant's growth variability. The identical growth parameters indicate it at week 8 for the number of leaves and flowers and at week 10 for the number of fruit (Figure 1). According to the difference between the plant height in weeks 8 and 16, it was found that the double treatment of chitosan and drought (Chi-D) caused the lowest increase in height significantly, which is 52.00 cm. in addition, the highest increase in height was recorded in the C group, 64.67cm, although was not significant to Chi and D treated plant which is 62.50 cm and 60.33 cm, respectively. Furthermore, we also documented an average decrease of ± 5 cm in drought-treated plants compared to the control.

The effect of chitosan application during drought stress conditions was also determined based on the number of leaves of each treated plant from weeks 8 to 16. All treatment groups showed an increment in the number of leaves from week 8 until 12, which tends to be constant until week 16. Overall, Chi-D treated plants possessed the least number of leaves from week 11, totaling 114.33 in week 16. Chi-D and D-treated plants showed a similar trend until week 16. The individual chitosan group (Chi) showed the highest number of leaves recorded in weeks 8 to13, then possessed the equal number with C groups until week 16. Chi-D and D treatments suppressed the number of leaves since week 11 compared to Chi and C groups.

Table 2. The microclimate condition recorded during the treatment

	Media			Environment		
	pH	**Humidity (%)**	**Temperature (°C)**	**Light Intensity (Lux)**	**Humidity (%)**	**Temperature (°C)**
Chi	6.66	63.61	27.44	10240	78.96	27.78
Chi-D	6.64	43.44	27.53			
D	6.63	46.56	27.59			
C	6.63	77.11	27.41			

Note: Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

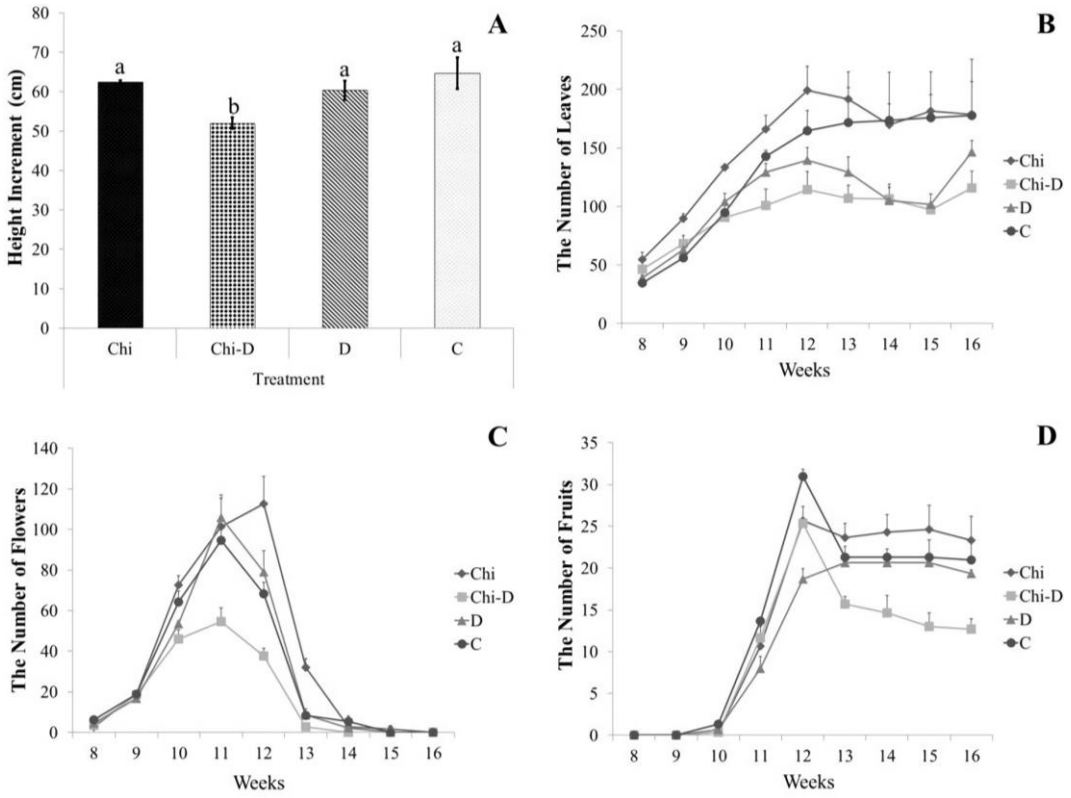

Figure 1. The growth performance of red chili pepper plants. The height increment was calculated from the difference at the start (week 8) and end (week 16). The number of leaves, flowers, and fruits was measured from the start (week 8) until the end (week 16) of treatment. The bars represent the mean of each growth parameter with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, $p < 0.05$. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

All treatments were given before the commencement of the generative phase. Throughout this phase, the number of flowers followed by the number of fruits was also recorded. The results showed that chitosan application during drought conditions (Chi-D) led to the least total of flowers. The number of flowers peaked in week 12 when treated with chitosan (Chi). A steep decrease was recorded starting in week 11 in almost all treatments, which was thought to be due to abscission and fruit development. An exponential fruit emergence was recorded in weeks 11-12. However, the immature fruits did not entirely reach the mature stage, with fruit abscised as early as week 12. Chi-treated plants were relatively able to maintain fruit numbers with the highest yield at the harvesting period in week 16, followed by C and D groups. Chi-D-treated plants could only produce the least mature fruits, although the total number of fruits in week 12 coincided with Chi-treated plants.

According to the results, all growth parameters showed a massive change in chitosan treatment under drought conditions. The significantly lowest height increment and the small number of flowers, leaves, and fruits were recorded in the double treatment of chitosan-drought (Chi-D). This follows the suggestion of Iriti et al. (2009) that combining chitosan and drought treatment in plants can cause a synergistic response in increasing plant defense response but result in stunted growth. Malerba and Cerena (2016) added that the effect of chitosan application on plant biological responses, such as resistance to stress and increased productivity depends on the chemical composition, application period, and application intensity.

Therefore, it is suggested that the application of 1 mg mL^{-1} chitosan in the generative phase of the chili plant has no significant effect on balancing the harmful effects of stress. Meanwhile, Bistgani et al. (2017) proved that the application of chitosan in 50% drought conditions caused a decrease in the fresh weight of thymus plants up to 29.98% and 5.60% along with the two times cultivation. However, the results contrast with the previous studies performed in *Ocimum basilicum* L. (Malekpoor et al. 2016) and *Ricinus communis* L. (Karimi et al. 2012) that chitosan application could enhance the physiological aspects under drought treatment.

The individual chitosan application could increase the number of leaves, flowers, and fruit. This is supported by Salachna and Zawadzinska (2014) showed that the application of chitosan significantly increases the plant height of the freesia plant by about 10 cm compared to control, accompanied by an increase in chlorophyll level. Furthermore, according to Dzung et al. (2011), chitosan could improve the function of the nitrogen source in forming leaf organs and the nitrogen can contribute to plant growth, such as forming new cells and cell elongation. In addition, applying chitosan can accelerate the flowering period and increase the number of flowers. Dzung et al. (2011) also stated that chitosan application could reduce the abscission rate of coffee up to 15.25% compared to control. This was by an increase in chlorophyll level, mineral accumulation, and plant growth. In addition, Malerba and Cerena (2016) showed that the application of chitosan could increase the productivity of various plants such as tomatoes, grapes, strawberries, and coffee.

A 50% drought stress could decrease plant growth and productivity. Him and Radhauane (2015) observed that a 50% drought caused a decrease in plant height up to 13-15%. Dorji et al. (2005) and Sung et al. (2005) stated that a 50% drought caused a reduction in the chili pepper fresh weight up to 34.7%, the number of fruits by 20%, and the fresh weight of fruit up to 22% compared to control. Zamljen et al. (2020) stated that 50% drought treatment on two different chilies species significantly decreases yield quantity caused by the abscised flower. Mahmood et al. (2021) added that drought treatment subjected to hot and sweet pepper at flowering and pod formation stages significantly decreases the flower survival percentage, the number of fruits per plant, and the fruit weight. Drought can stimulate the synthesis of jasmonic acid (JA) and suppress the action of gibberellic acid (GA), thus affecting impeded plant growth. In addition, plants respond to drought by activating abscisic acid (ABA), which can induce stomatal closure and reduce transpiration (Iriti et al. 2009). However, this also impacts CO_2 absorption, so the photosynthetic activity decreases, resulting in a degradation of plant growth and survival (Anjum et al. 2011).

The combination treatment of 1 mg mL^{-1} chitosan application and a 50% drought (Chi-D) caused a decrease in plant physiological characteristics followed by the yield compared to control. This is supported by the microclimate data indicating the lower humidity recorded at 46.56 and 43.44% for D and Chi-D, respectively. It was in line with the lack of water capacity due to the treatment. In addition, it was presumed that the chitosan-drought treatment caused plants to experience excessive stress, which can trigger the more closed stomata induced by reactive oxygen species (ROS) production (Pichyangkura and Chadchawan 2015). This assumption is in line with the investigation of Aziz et al. (2020) that the double treatment of chitosan and drought caused the highest stomatal closure compared to the drought and chitosan treatments separately as well as control. This phenomenon caused a decrease in photosynthetic efficiency due to the limited absorption of CO_2 and induced the abscission of main plant organs. Maser et al. (2018) explained that plants induced by either chitosan application or drought could promote higher Ca_{2+} accumulation in guard cells and the continuous release of K^+. This causes a decrease in the turgor pressure of the guard cells so that the stomata are closed.

Chitosan application during drought conditions increased PAL gene expression

To quantify the relative gene expression, the validation in every single step needs to be performed. According to the total RNA quality and quantity assay, there are 2 bands observed under UV light visualization with the sizes of 28s and 18s (Figure 2), by means that the total RNA was successfully extracted. The quantitative assay using a nanodrop spectrophotometer showed that the purity of RNA (ratios λ260 and λ280) ranged from 2.14 to 2.15, while the quantity ranged from 327.7 to 664.4 µg mL^{-1}. Based on the sequencing result for ± 260 bp of the PCR product, after being analyzed using the BLAST technique, a 97% identity value was confirmed with the CaPAL gene (accession number: NM_001324603) (Table 3). PAL gene was selected to analyze the influence of chitosan on plant defense response under drought stress. The gene was selected from the previous study that Phenylalanine ammonia-lyase (PAL) is considered one of the key enzymes on capsaicin biosynthesis coded by the PAL gene (Sung et al. 2005; Khan et al. 2014).

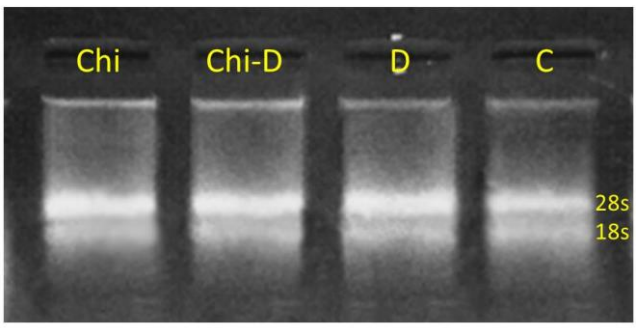

Figure 2. The total RNA quality and quantity assay

Table 3. BLAST result of PAL & CaUbi3 genes

PAL gene

Capsicum annuum phenylalanine ammonia-lyase-like (LOC107843092), mRNA
Sequence ID: **NM_001324603.1** Length: 2318 Number of Matches: 1
Range 1: 1564 to 1822

Score	Expect	Identities	Gaps	Strand	Frame
444 bits(240)	8e-121()	257/264(97%)	5/264(1%)	Plus/Minus	

Features:

```
Query  1     GCAGGTATCATCAGTCATAGGTCAAACAAGTATTCCCTGATCCACGACTCGAAGCAACTC  60
             ||||||||||||||| |||||| ||||||||||||||||| ||||||||||||||||||||
Sbjct  1822  GCAGGTATCATCAG-CATAGG-CAAACAAGTATTCCCTG-TCCACGACTCGAAGCAACTC  1766

Query  61    CTTTTCCGCAGAATCCTTGCTGGATGAAGTTCACCGTTAGCGCCCATTGTCAAAGTTCTC  120
             |||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1765  CTTTTC-GCAGAAT-CTTGCTGGATGAAGTTCACCGTTAGCGCCCATTGTCAAAGTTCTC  1708

Query  121   TTAGCTACTTGGCTGACTGTGTTCTTGACTGCATTCTTCAAGTTTTCTTCCAAGTGCCTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1707  TTAGCTACTTGGCTGACTGTGTTCTTGACTGCATTCTTCAAGTTTTCTTCCAAGTGCCTC  1648

Query  181   AAGTCTATAGCTTGGCAAAGAGCCACGAGATAGGTTGATGACATCAGCTTCAAGATGTCA  240
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1647  AAGTCTATAGCTTGGCAAAGCGCCACGAGATAGGTTGATGACATCAGCTTCAAGATGTCA  1588

Query  241   ACCGCCTCGGCTGTTTTCCTTGAT  264
             || ||||||||||||||||||||||
Sbjct  1587  ACTGCCTCGGCTGTTTTCCTTGAT  1564
```

CaUbi3 reference gene

Capsicum annuum ubiquitin-conjugating protein mRNA, complete cds
Sequence ID: **AY486137.1** Length: 578 Number of Matches: 1
Range 1: 276 to 444

Score	Expect	Identities	Gaps	Strand	Frame
287 bits(155)	8e-74()	165/169(98%)	3/169(1%)	Plus/Plus	

Features:

```
Query  1     CCAATGAT-CGCTCGTA-CAG-AATTGCTCACATGTACAAGACTGACAGGGCCAAATACG  57
             || ||||| ||||||||| ||| ||||||||||||||||||||||||||||||||||||
Sbjct  276   CCGATGATCCGCTCGTACCAGAAATTGCTCACATGTACAAGACTGACAGGGCCAAATACG  335

Query  58    AGACCACTGCTCGTAGCTGGACTCAGAAATATGCTATGGGATAATGGACAAAATCGTCTC  117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336   AGACCACTGCTCGTAGCTGGACTCAGAAATATGCTATGGGATAATGGACAAAATCGTCTC  395

Query  118   CAGGCATGTCTGGGACTTTTGCAACAACAATGTCTTATTGTGCTTGGGG  166
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396   CAGGCATGTCTGGGACTTTTGCAACAACAATGTCTTATTGTGCTTGGGG  444
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The target gene, PAL, was amplified using the specific primer (Table 1). As shown in Figure 3A, the particular bands of PAL and the housekeeping CaUBi3 gene described that both genes were successfully amplified. Since the specificity of primers with the size ± 300 bp for PAL and ± 200 bp for CaUbi3 was confirmed, the quantitative PCR was then performed. According to the investigation, we found that the expression level of the PAL gene was up-regulated in all treatments (Figure 3B). The relative expression level of the double treatment of chitosan and drought (Chi-D) was recorded as the highest at 129-fold higher than control, followed by chitosan (Chi) and drought (D) treatment at 73 and 4-fold higher than control, respectively. Based on the statistical analysis, the PAL expression level in each treatment was significantly different. Thus, we suggested that chitosan application (Chi) might increase the expression level of the PAL gene, and it was more pronounced when applied under drought conditions.

Besides being involved in plant growth, PAL is the important gene in synthesizing secondary metabolites, capsaicin, which acts as a plant defense system (Gonzalez-Zamora et al. 2013; Kim and Hwang 2014). The previous studies supported the up-regulation of PAL gene in all treatment groups and demonstrated the increment of PAL gene expression level 12-fold higher than control after 1 mg mL^{-1} chitosan application (Mejia-Teniente et al. 2013) and 3.61-fold higher after drought stress treatment (Khan et al. 2014). Drought could induce ABA and JA synthesis (Golldack et al. 2014). Meanwhile, the application of chitosan also induced similar signaling pathways involving H_2O_2, ABA, and JA in activating defense responses such as stomatal closure and several genes expression related to abiotic stress (Pichayangkura and Chadchawan 2015). Interestingly, both chitosan and drought activate a similar signaling pathway, so that chitosan and drought applied together could modulate the excessive defense responses, including the expression of resistance genes such as PAL.

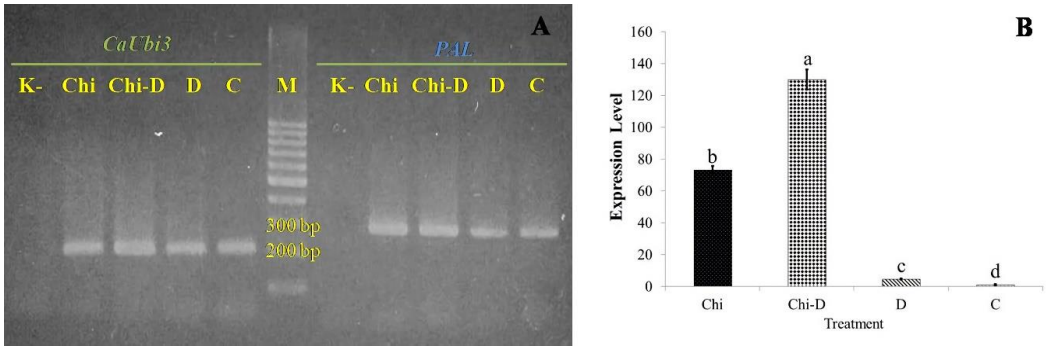

Figure 3. The visualization of PAL gene amplification coupled with CaUbi3 housekeeping gene (A) and the PAL gene expression level of red chili plant treated by chitosan application under drought stress condition (B). The bars represent the mean of PAL gene expression with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, $p < 0.05$. 35 PCR cycles. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control; K-: negative control; M: marker

Therefore, it is known that both drought and chitosan could enhance the expression level of the PAL gene, and the combination was suggested to increase the higher gene expression level than those treatments separately. This is in line with the finding of Ali et al. (2021) that demonstrated the up-regulation of strictosidine synthase (STR), deacetylvindoline-4-O-acetyltransferase (DAT), peroxidase 1 (PRX1) and geissoschizine synthase (GS) genes up to 5.6 folds on *Catharanthus roseus* (L.) G.Don under drought stress. However, the addition of 1% of chitosan nanoparticle found a higher expression level.

Chitosan application under drought conditions inhibited PAL enzyme activity

After being expressed, the PAL gene will be translated into the PAL enzyme (Sung et al. 2005). According to Figure 4, it was indicated that either chitosan application (Chi) or drought (D) treatment is suggested to increase PAL activity. Interestingly, although not significantly different, the double treatment of chitosan and drought (Chi-D) showed lower PAL activity than the Chi group. Chitosan and chitosan-drought treatment significantly increased the PAL activity at 1.35 and 1.22-fold higher than control, respectively, followed by the drought treatment group recorded at 1.03-fold higher than control. Thus, we suggested that chitosan application (Chi) could promote PAL activity, but the activity slightly decreased when applied under drought conditions (Chi-D).

Drought stress conditions could affect the activity of PAL as the first enzyme activated in the synthesis of capsaicin through the phenylpropanoid pathway (Sung et al. 2005). In Figure 4, it is indicated that 1 mg mL^{-1} chitosan (Chi) application could significantly increase PAL activity compared to the control. This is supported by Khan et al. (2003) that applying a chitosan oligomer could increase the activity of PAL in soybean leaves. It was suggested as an initial response to stress, either due to elicitation or pathogen infection. According to the observation, both Chi-D and D treatments could potentially enhance PAL activity compared to control. However, although experiencing the highest PAL expression level, PAL activity in the Chi-D treatment group tends to be lower than in the Chi treatment group. This is suggested as the consequence of the dramatic increase in ROS levels caused by the excessive stress experienced by the plant (Pichyangkura and Chadchawan 2015). This would lead to the oxidative damage of proteins, DNA, and lipids (Anjum et al. 2011). However, Ali et al. (2021) demonstrated that the application of 1% of chitosan nanoparticles under drought conditions could mitigate the reduced growth performance accompanied by the increment of proline accumulation and antioxidant activity such as catalase (CAT) and ascorbate peroxidase (APX) so that it reduced H2O2 and malondialdehyde (MDA) accumulation. In addition, PAL activity might fluctuate depending on the level of stress experienced by the plants. Khan et al. (2003) stated that PAL activity increased from the 24th to 36th hour after the chitosan treatment, then decreased when entering the 48th hour. Sung et al. (2005) added that a 50% drought caused PAL activity in the pericarp to increase at 1.18-fold higher than control at 40 days after flowering (DAF). Still, they decreased 0.9-fold lower at 50 DAF than the control, while the opposite response occurs in the placenta.

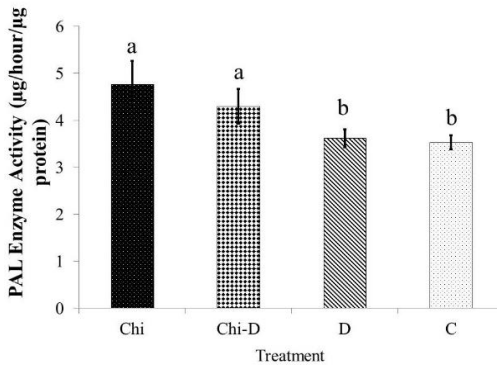

Figure 4. The PAL activity of red chili plant treated by chitosan application under drought stress condition. The bars represent the mean of PAL activity with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, $p < 0.05$. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

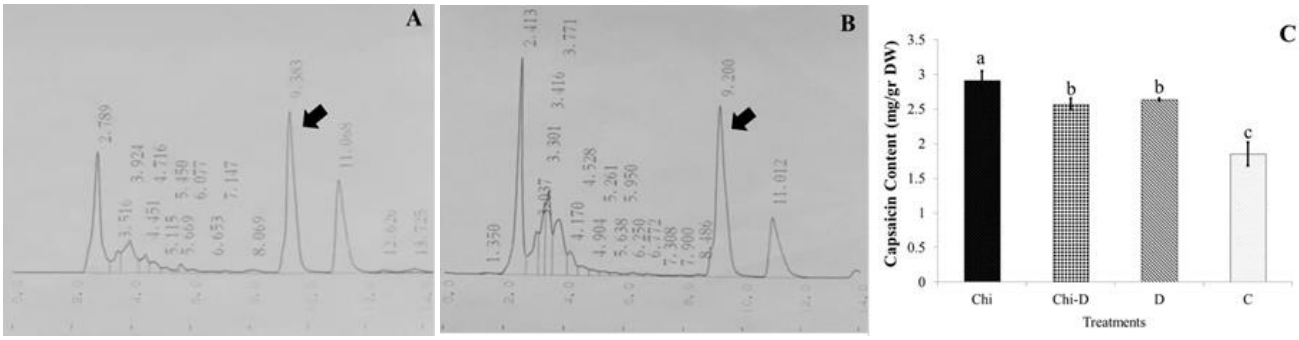

Figure 5. The chromatograms of capsaicin measurement using HPLC instrument in the 500 ppm capsaicin standard (A) and sample (B). The capsaicin content (C) of red chili plant treated by chitosan application under drought stress conditions. The bars represent the mean of capsaicin content with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, $p < 0.05$. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

Chitosan application under drought conditions decreases capsaicin accumulation

Capsaicin biosynthesis of chili pepper plants was the critical plant defense mechanism against pathogenic attack, so the regulation after chitosan application was essential to evaluate either under normal or drought conditions. Based on the chromatograms of capsaicin standard (Figure 5A) and sample (Figure 5B) obtained from the HPLC instrument, it was shown that the capsaicin compound peak was detected at the 9th minute. Regarding this study (Figure 5C), we suggested that either chitosan application (Chi) or drought treatment (D) could enhance capsaicin content significantly. Chitosan application showed significant capsaicin content enhancement up to 1.57-fold higher than control, followed by the drought treatment at 1.42-fold higher than control. Surprisingly, the capsaicin content at chitosan-drought (Chi-D) treatment increased only 1.39-fold higher than control, although not significant compared to D treatment. In addition, we suggested that chitosan application under drought conditions slightly decreases the capsaicin level compared to individual chitosan or drought treatment.

The level of PAL expression and PAL activity plays an important role in the biosynthesis regulation of secondary phenolic metabolites, capsaicin (Gonzalez-Zamora et al. 2013). Based on Figure 5, it is shown that capsaicin levels in Chi, Chi-D, and D treatment groups are significantly higher than control. In line with PAL activity, the individual chitosan application (Chi) was recorded to increase capsaicin levels compared to other treatment groups significantly. Khan et al. (2003) stated that the application of chitosan oligomer could increase soybean's total phenol content, which is estimated to be in accordance with the increase in PAL activity. Meanwhile, Sung et al. (2005) stated that a 50% drought could increase the level of capsaicin in the placenta up to 2.56-fold higher than control. Zamljen et al. (2020) stated that 50% drought treatment on *C. annum* caused higher capsaicin and dihydrocapsaicin content, while the higher concentration of ascorbic acid and total phenolic content were recorded at optimal irrigation. Ali et al. (2021) added that drought stress promotes the higher accumulation of alkaloid content, and a further increase was observed with the addition of 1% chitosan nanoparticle treatment.

However, the highest PAL expression in the Chi-D treatment group was not in line with the slight decrease in PAL activity, and the capsaicin level recorded was not significantly different from the D treatment group. This was suggested to support our previous assumption that plants experienced the excessive stress caused by the double treatments, which triggers the higher ROS level (Pichyangkura and Chadchawan 2015), as stated by Anjum et al. (2011) that a higher level of ROS might cause damage to plant cells through increased lipid peroxidation, protein degradation, and DNA degradation. It was suggested to contribute to the capsaicin degradation in the Chi-D group resulted in a lower amount of capsaicin than Chi and D groups. Jeeatid et al. (2017) stated that the appropriate water stress could significantly increase capsaicinoid content in some hot pepper species depending on each genetic variability. It was supported by Mahmood et al. (2021) that drought stress decreased reproductive growth parameters and pungency of pepper fruit as most of the plant energy was consumed in defense antioxidant molecules. However, several enzymes related to capsaicin biosynthesis, either from phenylpropanoid or fatty acid pathways, are also suggested to affect capsaicin accumulation. Therefore, this study needs to be continued to gain a more profound understanding of the responses of those enzymes after Chi-D treatment.

In conclusion, double treatment of chitosan-drought red chili plants significantly decreased the growth performance recorded in plant height increment, followed by the small number of leaves, flowers, and fruit. Therefore, chitosan application was suggested that could not alleviate plant growth under drought stress. The PAL expression level after chitosan-drought treatment was recorded up-regulated 129-fold higher than control. However, PAL activity and capsaicin content were decreased. In addition, the individual chitosan application significantly increases the PAL expression level, PAL activity, and capsaicin level compared to the control. Thus, we suggested that chitosan application might play a role in the defense system of red

chili pepper plants involving capsaicin biosynthesis through the phenylpropanoid pathway.

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