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Research Journal of Biotechnology

Publisher:
Dr. Jyoti Garg
Address:
Sector AG/80, Vijay Nagar, A.B. Road, Indore, 452010, INDIA

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Chitosan improving Growth in Chili (*Capsicum annuum* L.) Plants and acting through Distinct Gene Regulation between Cultivars

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Abstract
Chili is one of the most cultivated vegetables globally with a wide market potential due to the development in the food and pharmacy industries. A chili cultivation method must comply with good agricultural practices to reduce toxicity from chemical substances. Chitosan is a promising organic alternative to chemical fertilizer and pesticides because its elicitation activity is known to improve growth and resistance in plants. This research documented an improvement in growth parameters in chitosan-treated chili plants such as height increase, number of leaves and chlorophyll content. The regulation of gene expression was also investigated in CM334, C15080, LABA F1 and LADO F1 cultivars treated with chitosan. Seven regulated genes FC >|2| in CM334 and C15080 are involved in protein folding, sugar and protein metabolisms.

These genes were also regulated in LABA F1 and LADO F1 but in different patterns as shown by their relative expression level to a ubiquitin encoding gene. Thus, this study suggests that chitosan improves the growth of chili plants, but the molecular response is distinctive in each cultivar. However, the mode of chitosan-regulated gene expression in all cultivars indicates a correlation to the condition of stress and changes in metabolisms.

Keywords: Chitosan, heat shock protein, sugar metabolism, protein metabolism, elicitor.

Introduction

Red chili pepper (*Capsicum annuum L.*) is one of the most cultivated horticulture commodities due to its important role in the food industry globally[37]. Chili infuses dishes with its spiciness from capsaicin and also provides many other nutritional contents such as fiber, calcium, phosphorus, vitamins A and C, essential oils and flavonoids[24,32]. The annual demand for chili is constantly increasing along with the growth of the population and the development of non-food industries that require chili as raw materials such as in drugs[10,24,31].

The total consumption of chili in Indonesia in 2013 was 1 billion kg with 90% of it being traded as a fresh vegetable commodity[31]. As a consequence, chili cultivation methods are required to be in accordance with good agricultural practices. One of the requirements is the assurance of food security by minimizing the use of chemical fertilizers and/or pesticides and switching to the application of manure/compost and organic pesticides, among other approaches. This method is also considered more economical because the raw materials for organic alternatives often come from agriculture, husbandry, fishery, or forestry industry waste.

The use of organic materials is expected to also increase the value of chili in the international markets. Furthermore, the application of organic fertilizer and/or pesticides promotes the restoration of soil fertility because they provide nutrients to support microbial communities[3].

One of the natural materials that has been tested often in agriculture since the 1980s is chitosan. This chitin derivative compound is the biopolymer that makes up fungal cell walls, insects' exoskeleton and crustaceans' shells. Chitosan is nontoxic and biodegradable and it has been demonstrated to play several roles in various horticultural commodities including promoting growth (stimulants); protecting food products from pests such as fungi, bacteria and viruses (antipathogens); inducing tolerance in plants to biotic and abiotic stress (elicitor) and delaying fruit ripening (edible coating)[19,20,26,38].

The activity of chitosan as a regulator of plant growth has been reported in ornamental plants where the addition of 1% chitosan into growth media increases fresh and dry weights when compared with the control[22]. Chitosan also increases the morphological and biochemical characteristics of green bean plants including height, number of branches, number of leaves, leaf area, dry weight, chlorophyll content, photosynthesis and nitrate reductase[27]. Chitosan induction in influencing growth including morphological and biochemical characteristics was also reported in coffee, chili, tomato, cucumber, corn, orchid, soybean and rice plants.

The precise mode of chitosan in influencing plant growth and resistance to pathogens is not yet well explained. However, some references refer to its proposed role in modulating the diversity of microbiota in the soil that promotes plant growth[22] and in increasing the absorption of nutrients by plants[2]. Chitosan increases rice growth at the germination stage by influencing the expression of genes related to photosynthesis, carbon metabolism, developmental processes and other genes related to signal transduction in cells[5].

Therefore, this study investigated gene regulation related to stress and metabolism and performed morphophysiological observations on chitosan-treated chili plants. The results are expected to be an additional reference for the application of chitosan as a growth stimulator in chili plants.

Material and Methods

Plant, Chitosan and Foliar Treatment: Selected cultivars LABA F1 and LADO F1 were provided by PT East West Seed, Indonesia. Seeds were germinated in moistened cotton paper at room temperature for six days. Germinated seeds (±6 days) were then transplanted into growth medium containing a mixture of soil, husk and cocopeat (ratio = 1:1:1) in a polybag (d = 10 cm). Plants were watered once a day and fertilizer (GrowMore™) was applied ever three days when the plants were 14 to 35 days old. Experiments were conducted in a screen house with a temperature range of 29 °C to 30 °C, relative air humidity of 50% to 70% and 12/12 photoperiod.

Chitosan stock solution 1% (w/v) was prepared by dissolving chitosan powder (food grade, 85% to 89% deacetylation degree, PT Biotech Surindo, Cirebon, Indonesia) in 0.7% (v/v) acetate acid. The stock was made by stirring overnight at ±500 rpm speed and gradual pH adjustment (3M NaOH) to pH 6.4. The stock was then diluted into a concentration of 1000 µg mL^{-1} by adding distilled water. The chitosan solution was applied to the leaves by using foliar spraying techniques weekly from day 14 to 35 days after germination. Control plants were grown at the same time and sprayed with distilled water.

Growth parameters: During the four weeks of treatment, growth parameters such as the plant height increase, leaf number and chlorophyll content were measured. Plant height was measured from the stem that emerged from the soil up to the highest tip of the shoot by using a ruler (cm). Leaf number was determined by hand-counting and chlorophyll content was measured by using a SPAD-502 Plus Chlorophyll Meter (Konica Minolta, USA). The measurement data of growth parameters were then analyzed statistically using independent t-test methods with IBM SPSS Statistics 23.0©[11]. Significance and mean differences between treatment and control group were adjusted with a *P*-value < 0.05.

RNA-seq data analysis, gene ontology and primer design: The transcripts of chitosan-treated chili plants from resistant (CM334) and susceptible (C15080) cultivars were first sequenced. The available data were then analyzed to obtain unique genes related to chitosan treatment. The quality of the raw data sequence reads was checked using FastQC version 0.11.5[29]. Clean reads that were sequenced were then aligned against genome reference pepper v.1.55[14]. Sequential process including transcript assembly and gene quantification, were conducted by using a protocol from Trapnell et al.[33]

On the basis of the gene quantification data, the transcripts were then clustered by the expression gene value with a cut-off FC > |2|) between the chitosan and the control groups. Several overlapping genes between the two cultivars that clustered into induced and repressed genes related to chitosan treatment were chosen and relative expression to a ubiquitin gene (*CaUBI3*) was quantified in moderately resistant LABA F1 and LADO F1 cultivars. The differentially expressed genes (DEGs) were then visualized in Venn diagram[34].

The selected genes were then categorized based on gene ontology analysis and primers were designed for the selected genes by using the primer3 online program[1]. The quality and specificity of these primers were checked by using Clone Manager 9 demo program[30] and Primer-BLAST NCBI online program[21] respectively. The sequence of each primer is presented in table 1.

RNA isolation and cDNA synthesis: RNA total isolation from leaves was performed by using a PureLink™ RNA mini kit (Thermo Fisher Scientific Invitrogen, No. Catalog 12183018A). RNA total quantity and purity were determined by a spectrophotometry method (Eppendorf BioSpectrometer® kinetic) at 260/230 and 260/280 nm wavelengths. The RNA quality was visualized in 1.5% (w/v) agarose gel electrophoresis. The total RNA was then treated with DNAseI (Thermo Fisher Scientific) and cDNA synthesis was performed by using iScript™ cDNA Synthesis kit (Biorad).

Real-time reverse-transcription quantitative PCR validation of the DEGs: The expression levels of seven genes that were first chosen were validated by qRT-PCR. The PCR profile was set as follows: denaturation was performed first at 95 °C for 60 s followed by three-step amplification where the first cycle involved denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 50 s and then run for 40 cycles. The melting curve was made at the initial stage (60 °C, 30 s) and the final stage (97 °C, 1 s). The expression of the selected genes was then quantified relative to the expression of housekeeping gene *Capsicum annuum* ubiquitin 3 (*CaUBI3*) by using the Livak and Schmittgen method[18].

Results and Discussion

Improvement of growth parameters by chitosan application: The observed growth parameters include plant height, number of leaves and relative chlorophyll content, which are presented in table 2. The data suggest that chitosan-treated plants demonstrated superior growth characteristics compared with the control in both cultivars (LABA and LADO). Plant height increased significantly in the chitosan-treated plants than in the control. The mean height increase of the chili plants in the LADO and LABA cultivars was 10.71 and 5.88 cm respectively. The leaf number showed a higher average growth in the chitosan-treated plants than in the control in both cultivars.

However, statistically, the average number of leaves in the LABA cultivars did not differ significantly from the controls. By contrast, the average number of leaves in the LADO cultivars showed significant differences compared with the control treatment.

The physiological characteristics observed in this study were the relative chlorophyll content as measured by SPAD-502 Plus (Konica Minolta, USA); this measurement was conducted without damaging the leaf tissue for chlorophyll extraction. The average chlorophyll content in the chitosan-treated chili plants had a higher value in both cultivars compared with the controls. Statistically, the average value of the chitosan-treated plants was significantly different from that of the control. The average values of relative chlorophyll content by SPAD reading in the LABA and LADO cultivars were 38.78 and 38.51 unit leaf area respectively. On the basis of the observations of growth characteristics, the application of 1% (w/v) chitosan per week improved the plant height increase, number of leaves and chlorophyll content of chili plants.

Increased morphophysiological characteristics of chili plants F1 LABA and LADO F1 cultivars are also suggested by several references including our previous study on the CM334 and C15080 cultivars[7]. Chitosan is demonstrated to stimulate vegetable, seasonal and even annual plants such as coffee. According to El-Tanahy et al[6], the positive effect of chitosan on plant growth is due to the availability of amino acids in the polymer.

Degraded amino acids will provide an essential source of nitrogen for plant growth. This finding is in accordance with the study of Kumar[15] and Prashant and Taranthan[25] who found that chitosan polymers contain about 6.89% nitrogen. Orzali et al[23] revealed that chitosan can change the balance of the rhizosphere in the form of unfavorable effects for pathogenic microorganisms and supports beneficial microorganism activity. The given hypothesis is that with the addition of chitosan, chitinolytic microorganisms will augment their activities and could subsequently attack pathogenic fungal hyphae.

Table 1
Sequence of primers used in the analysis of relative gene expression by using qPCR method.

S.N.	Gene Name	Annealing temperature	Primer Sequences (5' → 3')	Fragment length (pb)
1	CaERD-6- F	58 °C	CCCAGAATCTCCAAGATGGC	649
	CaERD-6 R		AGTGTAAGAAACAGCCCACG	
2	CaSPI – F	58 °C	ACGAGTGGAGGTACGATAGG	204
	CaSPI – R		CTGTTGAGGAGGATCTTGGC	
3	CaPOR - F	58 °C	CGCCTGGTGTGACTACTAAC	312
	CaPOR - R		CAGCAGCATTAGCAACCAAC	
4	CaMGL - F	57 °C	GATGGATCTTCATCAAGGGTCTC	363
	CaMGL- R		GACATGAGGGTCTCGTAATAGC	
5	CaCHSP - F	59 °C	TGGGAGATAAGGTGCTTGC	209
	CaCHSP- R		ATCAGCACCAGGAGTAGGC	
6	CaCHSP2 - F	59 °C	TCGTATCAGTTTCGTATCGCAG	470
	CaCHSP2 - R		GTAACGGAAACAAGCAGTGAATG	
7	CaHSP1 - F	56 °C	CACTGCTGTTGAGCAACG	481
	CaHSP1 - R		ATGCACGTCAATGACCTTC	
Reference gene				
	CaUBI3 - F CaUBI3 - R		TCCATCTGCTCTCTGTTGACG CCCCAAGCACAATAAGACATTGT	201

Table 2
Measured growth parameters in control and chitosan-treated plants of LABA F1 and LADO F1 cultivars.

Cultivar	Treatment	Plant height (cm)		Leaf number		Chlorophyll content (unit)	
		Average	SD	Average	SD	Average	SD
LABA F1	Control	4.37[a]	0.79	4.00[a]	0.76	35.36[a]	3.04
	Chitosan	5.88[b]	0.90	4.60[a]	0.99	38.78[b]	1.56
	P_{sig}	0.000		0.08		0.005	
LADO F1	Control	7.19[a]	1.28	5.87[a]	0.64	36.43[a]	1.19
	Chitosan	10.71[b]	1.22	6.87[b]	0.74	38.51[b]	2.87
	P_{sig}	0.000		0.001		0.04	

a,b = significantly different at $P < 0.05$

In addition to affecting the soil microorganism community, chitosan, as a chelating agent, increases the availability of macro- and micronutrients for plants. A pH ±6.4 chitosan solution induces the formation of polycation, which can bind to anions through electrostatic interactions[8,9,25]. Jang et al[12] suggested that the cationic properties of chitosan can bind to NO_3^- and facilitates the absorption of minerals such as Ca^{2+}, Mg^{2+} and K^+. Increased availability of minerals in the soil allows higher absorption by plants. This finding is consistent with the observation that the leaves of plants treated with chitosan had higher N, P, K, Ca and Mg contents than the controls[35].

Physiological observation indicates that the amount of chlorophyll in plants is higher in the chitosan-treated plants than in the control. Chlorophyll is a photosynthetic apparatus requiring nitrogen and magnesium as its constituent essential molecules[35]. Increased absorption of nitrogen and magnesium may increase the amount of leaf chlorophyll. Physiological changes are also caused by cellular changes. The application of chitosan as fertilizer on orchid plants showed a change in the anatomy of chloroplasts, achieving a diameter greater than that of the control[17].

Modulation of selected genes in relation to chitosan treatments in both cultivars: RNA-seq data were generated from chili plant cultivars CM334 and C15080 from plants treated with chitosan and with distilled water as a control from previous research (data not shown). The average of mapped reads against genome references was 79% for var. CM334 and 75% for var. C15080 which were then used for subsequent analysis. A comparison indicated 258 unique transcripts of induced genes in CM334 chitosan-treated plants and 1017 in C15080, FC > |2|. A total of 203 unique transcripts in CM334 and 1142 in C15080 chitosan were suppressed under chitosan treatment, FC > |2| (Figure 1). Identical regulated transcripts in both cultivars were chosen for the subsequent analysis to visualize the modulation of gene expression. A set of seven genes was selected and was categorized based on their regulation: induced genes are *CaERD-6*, *CaSPI*, *CaPOR and CaMGL*; and repressed genes are *CaCHSP*, *CaCHSP2* and *CaHSP1*.

Gene ontology analysis showed that they are involved in biosynthesis or metabolism processes and defense responses (Table 3) with four of them being functional in chloroplast. We then analyzed the relative expression level of these genes in chitosan-treated plants of LABA F1 and LADO F1 cultivars to compare the means of regulation. Interestingly, the expression levels of these seven genes were higher than that of a ubiquitin gene (*CaUBI3*) (Figure 2). This result suggests that chili varieties have a distinctive response to chitosan application, thereby possibly stimulating physiological and/or morphological changes through different molecular pathways. Chitosan is expected to trigger a cascade of responses following its foliar application. This biopolymer is a well-known elicitor that is able to induce a defense response in plants[3] and is involved in initiating the narrowing of stomatal aperture[9]. Bittelli et al[4] confirmed that the width of the stomatal aperture was 0.92 µm ± 0.85 µm in the chitosan-treated plant and 1.82 µm ± 0.30 µm in the control. These results may be due to the ability of chitosan to mimic pathogen attack in which the first plant response is to narrow the stomatal aperture.

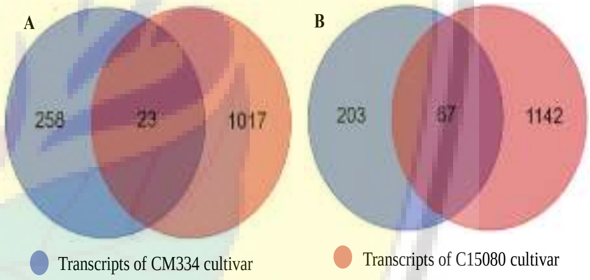

Figure 1: Venn diagram showing the number of induced (A) and repressed (B) transcripts in CM334 and C15080 cultivars. Overlapping areas represent the number of mutual regulated transcripts between the two cultivars

Table 3
Results of gene ontology analysis of the regulated genes in CM334 and C15080 cultivars with FC>|2|.

Sequence_ID	Gene Name	Description	GO Analysis	Location
XM_016686000.1	CaERD-6	PREDICTED: Capsicum annuum sugar transporer ERD6-like 16 (LOC107842239), mRNA	Sugar transporter	Plasma Membrane
XM_016708432.1	CaSPI	PREDICTED: Capsicum annuum serine protease inhibitor 1-like (LOC107862775), mRNA	Protein metabolism	Not Available
XM_016689752.1	CaPOR	PREDICTED: Capsicum annuum protochlorophyllide reductase-like (LOC107845435), mRNA	Chlorophyll biosynthesis	Chloroplast
XM_016709687.1	CaMGL	PREDICTED: Capsicum annuum methionine gamma-lyase-like (LOC107863655), mRNA	Isoleusin biosynthesis	Not Available
XM_016693464.1	CaCHSP	PREDICTED: Capsicum annuum stromal 70 kDa heat shock-related protein, chloroplastic-like (LOC107848686), mRNA	Protein maturation	Chloroplast
XM_016714448.1	CaCHSP2	PREDICTED: Capsicum annuum stromal 70 kDa heat shock-related protein, chloroplastic-like (LOC107867944), mRNA	Protein maturation	Chloroplast
XM_016708795.1	*CaHSP1*	PREDICTED: Capsicum annuum small heat shock protein, chloroplastic (LOC107863044), mRNA	Stress Response	Chloroplast

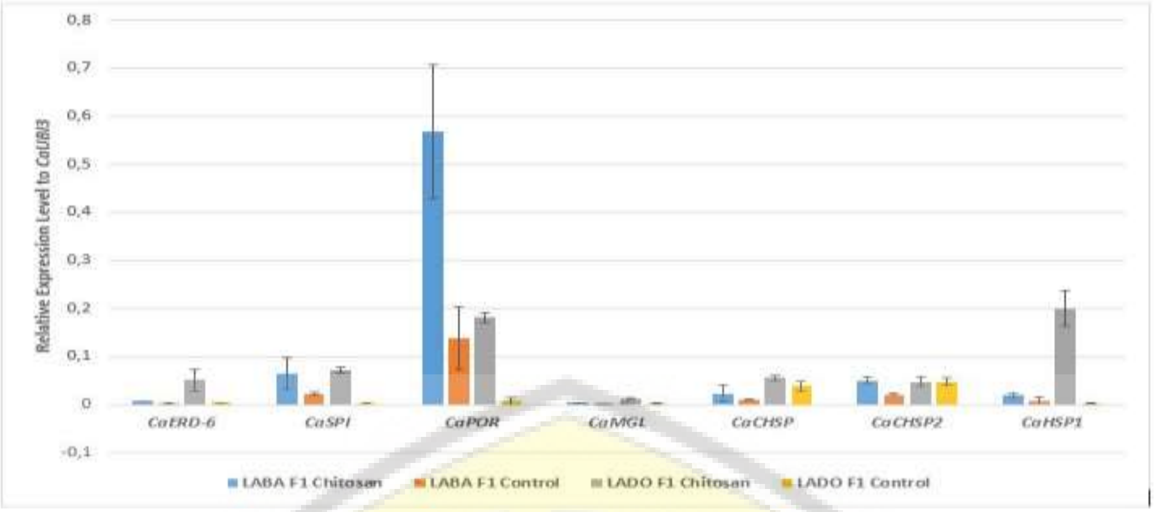

Figure 2: Expression of seven mutually regulated genes in CM334 and C15080 with FC>|2| relative to expression ubiquitin3 gene in LABA F1 and LADO F1 cultivars. The genes are involved in stress response and sugar and protein metabolisms

Contact between chitosan and the guard cell of the stomata is suggested to induce the synthesis of reactive oxygen species (ROS) such as H_2O_2. The ROS (H_2O_2) is then transduced into cells as a signal of pathogen attack[4]. Subsequently, ROS accumulation in the leaf tissues will induce the expression of transcription factors and then will trigger transcript reprogramming. We postulate that in this study, chitosan acts in a similar way as explained. In this study, chitosan is suggested to modulate the expression of two genes encoding chloroplastic heat shock-related protein (*CaCHSP* and *CaCHSP2*) and one gene encoding heat shock protein (*CaHSP1*). The changes in the expression of heat shock and its related protein indicate a stress condition that requires assistance to fold functional proteins. The modulated expression of *CaSPI* gene also emphasizes the presence of a stress condition. This gene code for serine protease inhibitor 1-like is involved in defense response and is also induced by chitosan[36]. A modulation related to primary metabolism was indicated by the changes in the expression of a gene that encodes sugar transporter ERD6-like 16 protein (*CaERD6*) and a gene in isoleucine biosynthesis (*CaMGL*, methionine gamma-lyase-like protein). In beet plant, the ERD-6 sugar transporter protein brings out sugars from vacuole as an energy source in stress conditions[13]. We also found a regulation of a gene that encodes light-dependent NADPH: protochlorophyllide oxyreductase (LPOR) protein (*CaPOR*) which plays an important role in chlorophyll biosynthesis[28]. Altogether, chitosan-induced genes in all tested cultivars are related to the condition of stress and metabolism changes.

Conclusion

This study demonstrates an improvement in measured growth parameters following chitosan application to chili plant cultivars LABA F1 and LADO F1. Transcript analysis in CM334 and C15080 cultivars showed regulation of a seven set of genes involved in biosynthesis or metabolism processes and defense responses. The data suggest that the molecular response to chitosan application in chili varieties is distinctive, further stimulating physiological and/or morphological changes through different molecular pathways, as revealed by the qPCR genes of identical genes in the LABA F1 and LADO F1 cultivars. However, the mode of chitosan-regulated gene expression in all cultivars indicates a correlation to the condition of stress and changes in metabolism.

Acknowledgement

This research was funded by the Southeast Asian Regional Center for Graduate Study and Research in Agriculture and Science and Education for Agriculture and Development research grant to FMD. The author (Kuswati) would like to thank the LPDP–Indonesia Endowment Fund for Education for the full funding during the postgraduate program in Institut Teknologi Bandung.

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(Received 25th July 2020, accepted 28th September 2020)