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Effect of Chitosan Elicitor Addition and Elicitation Duration on the Growth and Antioxidant Activity of Shallot (*Allium ascalonicum* L.) Callus

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Abstract

Shallot (Allium ascalonicum L.) contains various active secondary metabolites such as phenolics, flavonoids, quercetin, saponins, and anthocyanins, which function as natural antioxidants to protect the body from free radicals. Callus culture with the addition of elicitors is an effective method to produce shallot callus with enhanced secondary metabolite content compared to the original plant through manipulation of biosynthetic pathways. This study aimed to determine the most optimal combination of chitosan concentration and elicitation duration to enhance the growth and antioxidant activity of shallot bulb callus in vitro. A Completely Randomized Design (CRD) was used with 12 treatment combinations (chitosan at 50, 100, 150, and 200 mg/L for 2, 4, and 6 days) and 1 control treatment. The results showed that the elicitation combination had no significant effect on fresh weight but significantly affected dry weight and antioxidant activity of the callus. The control treatment was the most optimal for callus growth, yielding the highest average biomass (0.07 grams). Meanwhile, the 2 days + 200 mg/L combination treatment was the most optimal for increasing the antioxidant activity of the callus, producing the highest inhibition value (35.65%). Callus morphology in all treatments exhibited compact texture and varied shades of yellow. This study demonstrates that chitosan elicitor is effective in enhancing the antioxidant activity of A. ascalonicum callus and is therefore recommended for further phytochemical and biotechnological development.

1. INTRODUCTION

Shallot (*Allium ascalonicum* L.) is one of the horticultural crop commodities that has high economic value so that it becomes a leading commodity for the agricultural sector. The public generally utilizes shallot bulbs as seasoning to enhance flavor in cooking as well as traditional medicine to help overcome various health disorders [1]. Shallot bulbs contain secondary metabolite compounds including phenolics, flavonoids, quercetin, kaempferol, alkaloids, steroids, tannins, saponins, anthocyanins, and catechins [2, 3]. These various compounds have a variety of benefits, among them as antioxidants, antibacterial, anti-inflammatory, anticancer, antihypertensive, and antidiabetic [4]. A number of secondary metabolite compounds contained in shallot bulbs such as phenolic compounds, flavonoids, and quercetin have natural antioxidant activity which plays an important role in protecting the body from the negative impact of free radicals [5].

Antioxidants are compounds that are needed in small amounts but have an important role in reducing or inhibiting cell damage caused by oxidative stress. Their mechanism of action involves inhibition of oxidative chain reactions through stabilization of the formed free radicals [6]. Naturally, the human body is able to produce its own antioxidants, but the production capacity is still very limited. Therefore, additional intake of antioxidant compounds from outside the body is needed to maintain the body's balance from the effects of free radicals [7]. One source of natural antioxidants that is very potential is plants, because they contain many secondary metabolite compounds that have antioxidant activity [8].

Plants naturally produce secondary metabolite compounds as a defense response against biotic and abiotic stress [9]. However, the amount is still very low, which is only around ≤ 1% of the total content of the plant [10]. The production of secondary metabolites is influenced by two main factors, namely genetics and environmental conditions that vary [11]. Conventional plant propagation methods have a number of weaknesses, among them are susceptibility to pathogens, dependence on chemical fertilizers, heavy metal contamination, and environmental instability. These factors can inhibit the productivity and quality of the secondary metabolites produced [12].

Callus culture with elicitor addition is an effective approach to produce shallot callus with high-quality secondary metabolite content consistently, without being influenced by environmental conditions [13]. Callus culture has totipotency ability, because it contains all genetic information so that it is able to synthesize secondary metabolites. In addition, callus culture can produce secondary metabolite derivatives that are superior compared to the original plant through manipulation of the biosynthesis pathway [14]. The elicitation process begins when the elicitor interacts with receptors on the cell membrane, then will trigger a series of signal transductions. This process will activate gene expression and change the metabolic pathway, thus triggering an increase in secondary metabolite production [15].

Types of elicitors that are often used to stimulate the production of secondary metabolites include jasmonic acid, salicylic acid, and methyl jasmonate. These elicitors are effective, but their prices are relatively high [16]. As a more affordable and easily obtainable alternative, chitosan becomes a promising option because it has similar effectiveness. Chitosan is a biotic elicitor known to be biodegradable, biocompatible, and non-toxic [17]. Considering the cost and its safety for plants, chitosan can be considered an appropriate choice as an elicitor to increase the production of secondary metabolites [18].

Previous research has proven the effectiveness of chitosan as an elicitor. Application of 50 mg/L chitosan with a duration of 5 days was effective in increasing phenolic and flavonoid content in *Iberis amara*

L. [19]. Meanwhile, application of 200 mg/L chitosan with a duration of 72 hours was effective in increasing biomass, phenolic and flavonoid content in *Decalepis salicifolia* [20]. In addition, application of 100 mg/L chitosan with a duration of 7 days was effective in increasing saponin content in *Talinum paniculatum* [21].

Chitosan elicitation has indeed been studied in various plants, but its application on shallots to increase callus growth (texture, color, fresh weight, and dry weight) and antioxidant activity content has not been reported. This study aims to determine the optimal chitosan concentration and elicitation duration to increase growth and antioxidant activity of shallot bulb callus in vitro.

2. MATERIALS AND METHODS

The study was conducted at the Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Semarang State University. This study was an experimental type of research with a Completely Randomized Design (CRD) of two factors. The first factor was chitosan concentration with 4 levels of treatment (50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L), while the second factor was elicitation duration with 3 levels of treatment (2 days, 4 days, and 6 days). The experimental units consisted of 12 elicitation treatment combinations and 1 control treatment, each with four replications.

Media Preparation

The shallot callus induction medium consisted of instant MS, sugar, myo-inositol, solidifying agent, and plant growth regulators BAP 2 mg/L + Picloram 3 mg/L. Based on the previous study related to shallot callus, this combination of BAP and picloram was proven to produce the best shallot callus growth [22]. Before sterilization, the pH of the medium was adjusted to 6 using NaOH or HCl, then sterilized using an autoclave at a temperature of 121°C with 2 atm pressure for 20 minutes.

Explant Sterilization

The part of the shallot used as explant was the bulb. Sterilization began with washing under running water for 30 minutes, then soaking in antiseptic solution 10%, bactericide 0.1 g/L, and fungicide 0.1 g/L, each for 50 minutes. Next, sterilization was carried out inside a Laminar Air Flow (LAF) cabinet, where the explant was soaked in 100% and 70% sodium hypochlorite, each for 10 minutes. At the end of each sterilization stage, the explants were rinsed with sterile distilled water for 3–5 minutes.

Callus Induction

The explant planting process began by peeling the outermost layers of the bulb until reaching the innermost part to remove tissue damaged due to exposure to sterilizing agents. The explants were then incised to stimulate callus formation and planted on callus induction medium, with each bottle containing 1 piece of explant. The explants were incubated for 30 days at a temperature of 20–25°C, humidity of 52–58%, photoperiod of 16 hours light and 8 hours dark using an LED lamp with an intensity of 2000 lux.

Elicitor Preparation

The chitosan used was obtained from Sigma Aldrich with medium molecular weight and degree of deacetylation of 75–88%. Chitosan is not soluble in water or at neutral pH, therefore 1% acetic acid was used as the solvent. A stock solution of 1000 mg/L was prepared by dissolving 200 mg of chitosan in 20 ml of 1% acetic acid, then adding sterile distilled water until the volume reached 200 ml. This stock solution was then diluted into four different concentrations to be used as elicitor, namely 50, 100, 150, and 200 mg/L.

Subculture and Elicitation

Explants that had been incubated for 30 days, then subcultured into the same medium as the induction medium. The subcultured callus was re-incubated for 25 days until the callus reached the stationary growth phase. Determination of the callus growth phase

based on initial optimization was carried out by weighing the fresh weight of the callus every 5 days over a period of 35 days. Subcultured callus aged 25 days was taken in the amount of ±1 gram and transferred to new medium for elicitation. Elicitation was carried out by adding chitosan solution (50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L), each as much as 15 ml to the surface of the culture medium. The elicited callus was then incubated for 2, 4, and 6 days. Meanwhile, cultures that were not given a combination of chitosan and elicitation duration were used as control treatment.

Observation

Observation was conducted on callus harvested on the 2nd, 4th, and 6th day after the elicitation process. The observed parameters included morphology of texture, color, fresh weight, dry weight, and antioxidant activity of the callus. Callus texture was observed visually to identify whether the callus was compact or friable, while callus color was determined using a reference from the Munsell Color Chart. Fresh weight measurement was carried out by weighing the callus immediately after harvesting. Meanwhile, to obtain dry weight, the callus was dried in an oven at 50°C for four days until a constant dry weight was obtained. Drying was carried out at 50°C to prevent damage to secondary metabolite compounds [23]. A drying temperature that is too high can cause the decomposition of polyphenol compounds, resulting in a decrease [24]. The dried callus was used for analysis of antioxidant activity content.

Preparation of Extract Solution, DPPH, and Blank

The callus extraction process began by weighing 2 grams of dried callus, which was then ground using a mortar until a finer callus powder was obtained. Next, the callus powder was macerated using 20 mL of 96% ethanol for 24 hours. The extract was then filtered and evaporated using a rotary evaporator until a thick callus extract was obtained. The DPPH solution was prepared by weighing 5 mg of DPPH powder and dissolving it in 50 mL of 96% ethanol, stored in a closed container. Meanwhile, the blank solution used as a control was prepared by mixing 1 mL of DPPH solution with 3 mL of 96% ethanol and incubating for 30 minutes.

Determining Antioxidant Activities

The test was carried out by mixing 3 mL of each sample solution with 1 mL of DPPH solution and then homogenizing it. The mixture was subsequently incubated at 37°C for 30 minutes. DPPH has a purple color, when it reacts with antioxidant compounds, it turns yellow accompanied by a decrease in absorbance value. Absorbance measurement was performed using a UV-Vis spectrophotometer at wavelength of 517 nm. The percentage of antioxidant activity content could be calculated using the following formula:

$$\%\ \text{inhibition} = \frac{\text{ab control} - \text{ab sample}}{\text{ab control}} \times 100\%$$

Data Analysis

The data from observation of callus texture, color, and antioxidant activity were analyzed descriptively. Meanwhile, quantitative data in the form of fresh weight and dry weight were statistically analyzed using IBM SPSS Statistics 26, with significance ($p < 0.05$). The data were first tested for normality using the Kolmogorov-Smirnov test and for homogeneity using the Levene test. If the data were normally distributed and homogeneous, the analysis continued with Two-way ANOVA and DMRT test. However, if the data were not normal or not homogeneous, the Kruskal-Wallis test was performed and followed by the Post-Hoc Dunn test if there were significant differences. The fresh and dry weight showed data that were normal but not homogeneous, therefore the Kruskal-Wallis test was used.

3. RESULTS and DISCUSSION

Callus Morphology

The effect of the combination of chitosan concentration and elicitation duration on the growth of shallot callus was observed through changes in texture and color of the callus before and after elicitation. Before elicitation, the callus had a compact texture and yellowish-white color, similar to the control treatment. Until the end of observation, all elicitation treatments showed callus texture that remained compact. The morphological appearance of callus from the control and elicitation treatment is presented in Figure 1. and Figure 2

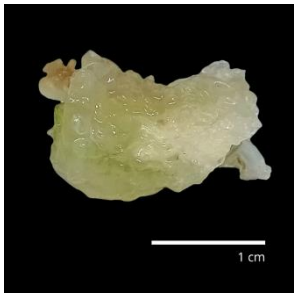

Figure 1 Morphology of *A. ascalonicum* callus in control treatmen

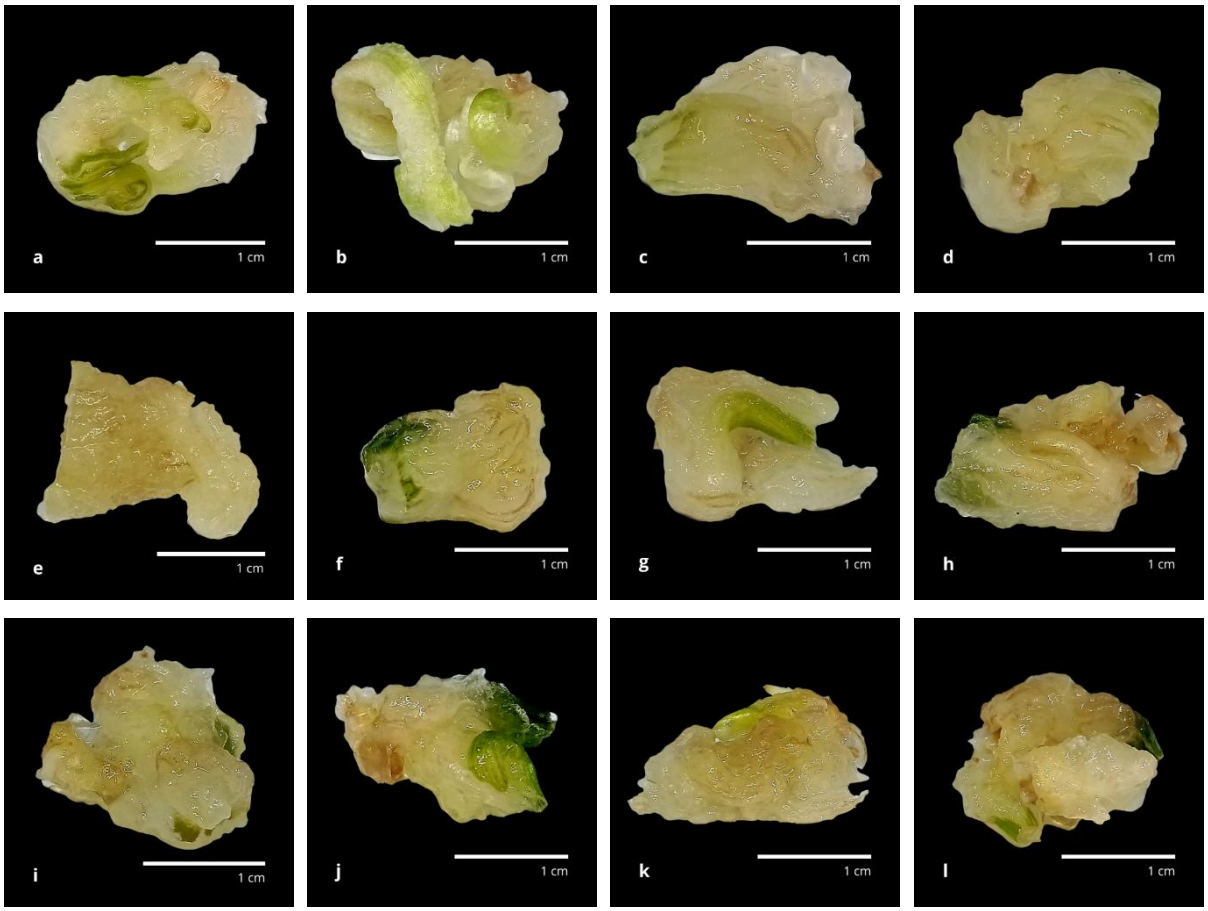

Figure 2 Morphology of A. ascalonicum callus after elicitation (a) 2D50, (b) 2D100, (c) 2D150, (d) 2D200, (e) 4D50, (f) 4D100, (g) 4D150, (h) 4D200, (i) 6D50, (j) 6D100, (k) 6D150, (l) 6D200

Table 1. Morphology of texture and color of *A. ascalonicum* callus

Treatment		Texture	Color and Code (Munsell Color Chart)
Duration (Day)	Chitosan (mg/L)		
0	0	Compact	Yellow - 7.5Y 8/6
2	50	Compact	Yellow - 7.5Y 8/4
	100	Compact	Yellow - 7.5Y 7/4
	150	Compact	Yellow - 7.5 Y 6/4
	200	Compact	Yellow - 7.5 Y 6/4
4	50	Compact	Yellow - 7.5Y 7/6
	100	Compact	Yellow - 7.5Y 6/6
	150	Compact	Yellow - 7.5Y 6/6
	200	Compact	Yellow - 5Y 6/6
6	50	Compact	Yellow - 7.5Y 6/6
	100	Compact	Yellow - 5Y 7/4
	150	Compact	Yellow - 5Y 6/6
	200	Compact	Yellow - 5Y 5/6

Color assessment was carried out using the Munsell system. The Munsell color code consists of three components: Hue, Value, and Chroma (HVC). Hue refers to the color designation, such as red (R), yellow (Y), green (G), yellow-green (YG), and so on. Value indicates the lightness or darkness of a color, determined on a scale from 0/ to 8/. The higher the value, the lighter the color. Chroma reflects the intensity or purity of the color, usually ranging from 2 to 14. The higher the chroma, the more intense the color. The callus colors are presented in Table 1.

The elicitation treatment did not affect texture, but it affected color. After elicitation, the callus showed color changes varying from yellow to brownish. This color change became darker along with the increase in chitosan concentration and elicitation duration, as shown in Table 1 and Figure 2. The treatments 4D200 (4 days + 200 mg/L), 6D100 (6 days + 100 mg/L), 6D150 (6 days + 150 mg/L), and 6D200 (6 days + 200 mg/L) affected the callus color to become the darkest, as indicated by their lower value scores on the Munsell scale compared to the other treatments.

Callus Biomass

The effect of the combination of chitosan concentration and elicitation duration on the growth of shallot callus was also observed through the measurement of fresh and dry weight. The data were analyzed using the Kruskal-Wallis test due to non-homogeneity. The analysis results showed that the fresh weight of the callus was not significantly different among treatments ($p = 0.57 > 0.05$), while the dry weight showed significant differences ($p = 0.00 < 0.05$). The data from the analysis of fresh and dry weight are presented in Table 2.

The elicitation treatment did not give a significant effect on fresh weight, but it was known that the highest average fresh weight of callus was found in the 2D200 treatment (2 days + 200 mg/L) at 1.37 grams, followed by the 2D100 treatment (2 days + 100 mg/L) at 1.25 grams. Meanwhile, the lowest average fresh weight of callus was found in the 6D200 treatment (6 days + 200 mg/L) at 1.06 grams. Although the differences were not statistically significant, the 2-day elicitation treatment consistently showed a higher average fresh weight of callus than the other treatments.

The results of the analysis of dry weight of callus showed that elicitation treatment gave a significant effect. However, all elicitation treatments decreased the dry weight of callus compared to the control treatment. The highest average dry weight of callus was found in the control treatment (0 days + 0 mg/L) at 0.07 grams, followed by the 2D200 treatment (2 days + 200 mg/L) at 0.06 grams. Meanwhile, the lowest average dry weight of callus was found in the 6D150 treatment (6 days + 150 mg/L) at 0.03 grams. Callus dry weight is a more effective parameter for measuring biomass, as it more accurately represents the actual cell mass. In other words, the control treatment is the most optimal treatment for increasing callus biomass content.

Table 2. Average biomass of *A. ascalonicum* callus

Treatment		Fresh weight (gram)	Dry weight (gram)
Duration (day)	**Chitosan (mg/L)**		
0	0	1.173 ± 0.195^{ns}	0.077 ± 0.007^{a}
2	50	1.170 ± 0.119^{ns}	0.054 ± 0.003^{bcdef}
	100	1.251 ± 0.148^{ns}	0.064 ± 0.019^{abc}
	150	1.219 ± 0.327^{ns}	0.059 ± 0.004^{bcd}
	200	1.373 ± 0.189^{ns}	0.066 ± 0.014^{ab}
4	50	1.165 ± 0.095^{ns}	0.058 ± 0.006^{bcde}
	100	1.152 ± 0.169^{ns}	0.051 ± 0.005^{cdefg}
	150	1.067 ± 0.204^{ns}	0.042 ± 0.008^{fg}
	200	1.132 ± 0.053^{ns}	0.048 ± 0.009^{defg}
6	50	1.087 ± 0.107^{ns}	0.045 ± 0.004^{efg}
	100	1.118 ± 0.141^{ns}	0.043 ± 0.008^{fg}
	150	1.121 ± 0.175^{ns}	0.039 ± 0.002^{g}
	200	1.066 ± 0.030^{ns}	0.040 ± 0.007^{fg}
Average		**1.161 ± 0.167**	**0.053 ± 0.013**

Note: The same superscript letters indicate no significant difference between treatments, whereas different letters indicate a significant difference. The symbol "ns" (not significant) indicates that the treatment had no significant effect

Antioxidant Activities

The effect of the combination of chitosan concentration and elicitation duration on antioxidant activity content was analyzed using the DPPH method based on the percentage of inhibition. The % inhibition value reflects the extent to which antioxidant compounds are able to neutralize free radicals. The higher the % inhibition value of a sample, the greater its antioxidant activity content. The data on the antioxidant activity content of shallot callus are presented in Table 3.

The results of the DPPH test showed that the highest antioxidant activity content was found in the 2D200 treatment (2 days + 200 mg/L) with an inhibition value of 35.65%, followed by the 2D150 treatment (2 days + 150 mg/L) with an inhibition value of 30.88%, 2D100 (2 days + 100 mg/L) with an inhibition value of 29.80%, and 2D50 (2 days + 50 mg/L) with an inhibition value of 21.36%. Meanwhile, the lowest antioxidant activity was found in the 6D150 treatment (6 days + 150 mg/L) with an inhibition value of 1.09%.

Based on the analysis results, the antioxidant activity content in this study was not significantly affected by chitosan concentration, but was influenced by elicitation duration, in line with the results of callus biomass measurements. The antioxidant

activity content in all 2-day elicitation duration treatments increased compared to the control. In the 2-day duration treatment, the higher the concentration of chitosan elicitor applied, the higher the resulting antioxidant activity. However, in the 4-day and 6-day duration treatments, higher chitosan concentrations tended to result in lower antioxidant activity.

Table 3. Antioxidant assay results: % inhibition of *A. ascalonicum* callus

Treatment		**A_{517nm}**	**A_{517nm}**	**Corrected A_{517nm}**	**Average**	**% Inhibition**
Duration (day)	**Chitosan (mg/L)**	**Dpph**	**fk**			
Blank		0.735	0.000	0.735	0,735	**0.00**
0	0	0,596	0.017	0,579	0,579	**21.22**
2	50	0,588	0.010	0,578	0,578	**21.36**
	100	0,528	0.012	0,516	0,516	**29.80**
	150	0,515	0.007	0,508	0,508	**30.88**
	200	0,483	0.010	0,473	0,473	**35.65**
4	50	0,628	0.002	0,626	0,626	**14.83**
	100	0,696	0.000	0,696	0,696	**5.31**
	150	0,724	0.000	0,724	0,724	**1.50**
	200	0,717	0.003	0,714	0,714	**2.86**
6	50	0,709	0.012	0,697	0,697	**5.17**
	100	0,666	0.005	0,661	0,661	**10.07**
	150	0,729	0.002	0,727	0,727	**1.09**
	200	0,702	0.009	0,693	0,693	**5.71**

Note: Sample measurements were conducted at a concentration of 1% b/v or 1 g/100 mL

4. Discussion

Callus Morphology

The combination treatment of chitosan concentration and elicitation duration did not affect callus texture but did influence callus color. After the elicitation treatment, the callus maintained a compact texture similar to that before elicitation. Compact callus is characterized by being dense, firm, and difficult to separate, which is generally caused by cell wall thickening or lignification, resulting in a rigid and hardened callus tissue [25]. Callus with compact texture tends to accumulate higher levels of secondary metabolites and thus has the potential to be used as a source of bioactive compounds [26]. The upper surface of the callus that was in direct contact with the medium and elicitor appeared moist. This condition is commonly observed during elicitation, as the tissue surface in direct contact with the medium and elicitor actively participates in nutrient absorption, which is then transported to the inner tissues for plant growth and development [27].

The callus experienced a color change to yellowish brown after elicitation treatment. This change is closely related to cell aging and the accumulation of secondary metabolites such as phenolics and flavonoids [28, 29]. Such color changes may also serve as an indicator of intensified secondary metabolism activity. These findings are consistent with research conducted in *Pelargonium graveolens*, which reported that higher concentrations of chitosan tend to produce darker-colored callus with dense texture and higher content of

phenolic and flavonoid compounds [30]. However, it should be noted that callus browning does not always indicate increased secondary metabolite content. Color change can also result from enzymatic oxidation of phenolic compounds into quinones when stress occur [31].

The change in callus color to brown indicates the occurrence of enzymatic reactions toward phenolic compounds, generally triggered by stress exposure, in this case stress exposure induced by chitosan and the elicitation duration given [28]. Chitosan acts as an elicitor that triggers the activation of molecular signaling pathways in wounded tissue through the increase of phenylpropanoid pathway activity to produce phenolic compounds. Phenolic compounds produced from this pathway will undergo oxidation reactions by the enzyme polyphenol oxidase (PPO) to form quinones. Quinone compounds will undergo non-enzymatic polymerization so that they produce brown pigment on the surface of callus that is given stress [32].

The PPO enzyme and phenols are located in different cell compartments when in normal tissue conditions and not experiencing damage. The PPO enzyme is located in plastids, while phenols are stored in vacuoles. Because of this location difference, oxidation reactions of phenols by PPO cannot take place directly in healthy and intact tissue conditions. Oxidation reactions of phenols by PPO can only occur when there is disturbance to the membrane or cell permeability, for example due to wounds and the giving of stress precursors in the form of elicitation which causes phenolic compounds to be able to exit toward the cytosol and then react with PPO [33]. The oxidation of phenolic compounds into quinones causes the loss of hydroxyl groups that can donate electrons to scavenge free radicals, resulting in a decrease in antioxidant activity [34].

Callus Biomass

The treatment combining chitosan concentration and elicitation duration did not have a significant effect on fresh weight, but it did have a significant effect on the dry weight of the callus. The non-significant result for fresh weight may be due to the elicitation duration being too short, preventing the callus from having enough time to absorb nutrients from the new medium and respond effectively to the elicitor signals. Although no significant difference was observed, the 2-day elicitation duration tended to increase the fresh weight of the callus. This may indicate an initial, temporary biostimulation effect, during which the metabolic process of nutrient absorption from the medium remains active and is not yet affected by elicitor-induced stress. The highest average observed in the 2-day treatment still falls within a wide data variability range, which makes it insufficient to produce a statistically significant difference. Callus with higher average fresh weight is generally associated with higher water and carbohydrate content [35].

The addition of chitosan as an elicitor can accelerate the increase in fresh weight by enhancing the absorption of water and essential nutrients that help maintain the cell's osmotic pressure balance [36]. An increase in fresh weight is also linked to ongoing processes of cell division and elongation. Chitosan contains amine groups and auxin hormones that support these cell division and elongation processes [37]. However, to achieve optimal callus biomass accumulation, the addition of chitosan must also be adjusted to the appropriate concentration and elicitation duration. Based on the results of this study, the 2-day elicitation treatment tended to increase the fresh weight of the callus compared to the control.

Callus dry weight is a more accurate parameter for assessing callus growth because its value is no longer influenced by water content, unlike fresh weight. The results of the study showed that elicitation treatment led to a decrease in callus dry weight compared to the control treatment. The control treatment

yielded the highest average dry weight. This is in line with the findings related to *T. paniculatum* callus, which showed that the addition of chitosan and variations in elicitation duration generally led to a decrease in the dry weight compared to the control group [38].

The differing patterns between fresh and dry weight are due to variation in the callus's ability to absorb and retain water. In the control treatment, the callus did not come into direct contact with the elicitor through immersion. In contrast, under elicitation treatment, the callus was in direct contact with the elicitor through immersion for various durations, which increased the callus's capacity to absorb and retain water. Higher water uptake in the elicitation treatments resulted in lower dry weight, due to a greater initial water content, which in turn led to more water being lost during the drying process. Meanwhile, in the control treatment, the callus could only absorb a small amount of water from the medium without immersion, so the amount of water lost during drying was also relatively lower [39].

The higher the chitosan concentration and the longer the elicitation duration, the more it reduces callus biomass accumulation. This is because higher elicitor concentration with longer exposure duration induces greater stress on the callus, thus inhibiting growth [16]. The decrease in callus biomass following elicitation is caused by competition for precursors between primary and secondary metabolic pathways, which inhibits the activity of the enzyme IPP isomerase that plays an important role in cell multiplication [40]. In addition, chitosan as an elicitor functions to stimulate the increase of secondary metabolites, causing the metabolic energy and resources in the callus to be directed more toward producing secondary metabolites than increasing cell mass [41].

Antioxidant Activities

The treatment combining chitosan concentration and elicitation duration had an effect on the antioxidant activity content of shallot callus. Based on the results of the study, the 2-day duration treatment showed higher antioxidant activity compared to the control. In this study, elicitation duration had a more significant effect than chitosan concentration on the antioxidant activity content of the shallot callus. This is in line with the findings in *Celosia argentea* callus, which showed that variations in chitosan concentration did not have a significant effect on secondary metabolite levels [42].

The increase in antioxidant activity in the callus may be caused by elicitor exposure for a certain duration that induces eustress, which stimulates the production of secondary metabolites and activates antioxidant enzymes. However, elicitor exposure and elicitation duration that exceed a plant's tolerance threshold may cause metabolic damage leading to cell death due to excessive stress [18, 43]. In this case, the tolerance limit for chitosan elicitor application in *A. ascalonicum* callus is 2 days. Additionally, each plant species has a certain maximum limit in synthesizing specific secondary metabolites. When secondary metabolite accumulation reaches this maximum threshold, the plant responds by activating enzymes through feedback regulation to degrade the already-formed secondary metabolite [44].

The mechanism of elicitor action involves a series of biochemical and physiological reactions within the cell as a plant defense mechanism against stress resulting from elicitor application [43]. The plant's response to stress begins when the elicitor binds to a specific receptor located on the plasma membrane of the plant [45]. The signal transduction process from the elicitor received by the receptor involves Ca^{2+} ions as secondary messengers. The level of Ca^{2+} ions in the cytoplasm will increase due to the influx of Ca^{2+} from outside the cell and the release of Ca^{2+} from the internal reserves of the cell [46].

The change in ion concentration will activate various signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, ion channels, and G-proteins. The activation of these pathways will stimulate the enzyme NADPH oxidase, which functions to transfer electrons from NADPH to oxygen, thus producing superoxide anions (O_2^-), which play a role in the formation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) [47]. ROS play a role in activating genes involved in the biosynthetic pathways of secondary metabolites such as phenolics, flavonoids, alkaloids, terpenoids, and others that possess antioxidant properties as part of the plant's defense mechanism [48].

Antioxidant compounds have an important role in neutralizing the effects of free radicals that arise due to exposure to stress precursors, thereby protecting the cell from oxidative damage [49]. The contact between the elicitor and specific receptors requires an optimal duration for the plant to be able to produce secondary metabolites and maximum antioxidant activity [50]. This sufficient contact duration allows the plant to respond to stimuli effectively. The most effective elicitation treatment in this study was 2D200 (2 days + 200 mg/L), because in this combination the plant was able to maintain a balance between eustress and distress, thereby stimulating the biosynthesis of secondary metabolites that resulted in the highest antioxidant activity.

5. Conclusion

Elicitation treatment did not affect the texture but influenced the color of the callus, which tended to darken with increasing concentration and duration of elicitation. Based on the results of this study, new insights were gained regarding the most optimal elicitation combination to enhance the growth and antioxidant activity of shallot callus. The control treatment was the most effective in promoting callus growth, producing the highest biomass (0.07 grams), while the 2-day + 200 mg/L treatment was the most optimal for enhancing antioxidant activity, with the highest inhibition percentage (35.65%). The best-growing callus does not necessarily yield the highest secondary metabolite content, possibly due to experiencing lower stress pressure. Further research is recommended to optimize variations in elicitor concentration and longer exposure durations to maximize the yield of other secondary metabolite compounds.

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