https://doi.org/10.31925/farmacia.2022.1.13 **ORIGINAL ARTICLE**

ELICITATION OF ORGANOSULFUR BIOACTIVE COMPOUNDS WITH Fe^{3+} AND Zn^{2+} IN CELL SUSPENSION CULTURE OF SINGLE GARLIC (*ALLIUM SATIVUM* L.)

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Abstract

Single garlic (*Allium sativum* L.) is an herbal plant that is rich in organosulfur compounds and widely used for the treatment in health problems such as high blood pressure, hypercholesterolemia, atherosclerosis and cancer. This study aims to investigate the different concentrations effect of Fe^{3+} and Zn^{2+} elicitor (0, 0.1, 0.2, 0.3, 0.4, 0.5 mM, respectively) in single garlic cell suspension culture. The formation of suspension culture was done by culturing the callus in Murashige and Skoog liquid medium. The HPLC analysis revealed the presence of 30 organosulfur compounds. The Fe^{3+} and Zn^{2+} elicitors can increase the growth of cell suspension culture and production of organosulfur compounds. The highest of fresh weight, dry weight, settled cell volume, growth index were produced in the medium augmented by 0.5 mM Fe^{3+} and 0.5 mM Zn^{2+}, while organosulfur compounds increase with 0.3 mM Zn^{2+} (2-fold) and 0.5 mM Fe^{3+} (1.5-fold). The Fe^{3+} and Zn^{2+} metal ions are effective elicitors to increase the production of organosulfur bioactive compounds.

Rezumat

Usturoiul monobulb (*Allium sativum* L.) este o plantă bogată în compuși organosulfurici și este utilizat pe scară largă pentru tratamentul diferitelor probleme de sănătate, cum ar fi hipertensiunea arterială, hipercolesterolemia, ateroscleroza și cancerul. Acest studiu își propune să investigheze efectul concentrațiilor diferite ale elicitorilor Fe^{3+} și Zn^{2+} (0, 0,1, 0,2, 0,3, 0,4 și 0,5 mM) asupra unei suspensii celulare provenite de la *Allium sativum*. Suspensia a fost obținută prin cultivarea calusului în mediul lichid Murashige și Skoog. Analiza HPLC a relevat prezența a 30 de tipuri de compuși organosulfurici. Elicitorii Fe^{3+} și Zn^{2+} pot determina înmulțirea celulelor în suspensie și biosinteza de compuși organosulfurici. S-au determinat următorii parametrii: greutatea proaspătă și uscată, volumul celular și indicele de creștere. Concentrațiile de 0,5 mM Fe^{3+} și 0,5 mM Zn^{2+} au avut cel mai important efect asupra acestor parametri. Concentrația compușilor organosulfurici a crescut sub acțiunea Zn^{2+} 0,3 mM (de 2 ori) și respectiv Fe^{3+} 0,5 mM (de 1,5 ori). Ionii metalici Fe^{3+} și Zn^{2+} sunt elicitori eficienți pentru valorificarea producției de compuși organosulfurici bioactivi.

Keywords: cell suspension, metal elicitor, organosulfur compound, single garlic

Introduction

Single garlic (*Allium sativum L.*) has been widely used in medical fields [1]. The use of medicinal plants to treat various health problems have received considerable attention since ancient times [1] and currently have applied in numerous industries such as pharmaceutical, chemical, food and cosmetic [2]. As an herbal medication, single garlic plays a role in overcoming various types of health problems, including an antithrombotic, anti-hypertensive, antiatherosclerosis, antihyperhomocysteinemic [3, 4] and anticancer [5] effect. These benefits are due to the presence of organosulfur bioactive compounds [6, 7]. A recent study reported that all bioactive compounds encompass approximately 33 of these organosulfur compounds [8]. Organosulfur is a compound containing sulphur atoms that are bound to cyanate groups or carbon atoms in cyclic or non-cyclic configurations [9]. Some organosulfur compounds in garlic include alliin, allicin, diallyl (mono-, di-, tri-) sulphide, and vinyl dithiins [10]. In the biosynthesis of organosulfur compounds, alliin is parental from the formation of other groups of organosulfur compounds.

In vitro plant culture of cells and the tissue is an effective alternative technology for the production of secondary plant metabolites [11]. One type of cell cultures that can be used to produce plant bioactive compounds is cell suspension cultures accompanied by elicitation [12]. The use of elicitors, such as heavy metals, is one of the effective strategies in increasing the accumulation of bioactive compounds [13, 14]. Elicitor, as a signalling molecule, will activate signal

transduction, which mediates gene expression related to the biosynthesis of bioactive compounds [15].

Zn^{2+} elicitor can increase quercetin compound two times higher than control in callus culture of *Plucea lanceolata* [16], proline compound two times higher than control in suspension culture with *Withania somnifera* cell suspension [17], and xanthotoxin compound two times higher than controls in the *Ammi majus* callus culture [18]. Fe^{3+} elicitor can increase red pigment compound eight times higher than the control in *Carthamus tinctorius* cell suspension culture [19]. The main objective of this study is to evaluate the growth of cell suspension culture and the production of organosulfur bioactive compounds in a single garlic cell suspension culture (*Allium sativum* L.) with elicitation using Fe^{3+} and Zn^{2+} at different concentrations.

Materials and Methods

The plant materials used in this study was single garlic (*Allium sativum* L.) of Tawangmangu Baru variety obtained from Magelang, Central Java, Indonesia.

Callus induction and formation of cell suspension culture

The explants used in callus induction were single garlic crown. Explants were inoculated in Murashige and Skoog solid medium supplemented with growth regulators 0.3 ppm 2.4-dichlorophenoxyacetic acid and 0.5 ppm kinetin [20]. The callus culture was incubated with a 16 hour lighting period and a temperature of 25°C for six weeks. The formed callus was cultured separately for the formation of cell suspension culture. Cell suspension culture was grown by transferring 1 gram of callus into culture bottles containing 25 mL Murashige and Skoog liquid medium with the same growth regulators addition. Incubation was done by shaking using a shaker at 100 rpm and a temperature of 25°C. The subculture of cell suspension was done every two weeks by transferring 1 g of fresh cells into 25 mL of fresh Murashige and Skoog liquid medium.

Elicitation of cell suspension culture

For elicitation experiments, Fe^{3+} and Zn^{2+} were added to suspension culture medium in different concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM, respectively). The medium without an elicitor was used as a control. Observations and data recording of the cell suspension growth and organosulfur bioactive compounds accumulation were performed after two weeks. Triplicate flasks were used in all experiments.

Determination of cell suspension growth and organosulfur bioactive compounds content

The cell suspension growth was expressed as fresh cell weight, dry cell weight, Settled Cell Volume, and growth index. Measurement of fresh weight was done by weighing the cell pellets, which were first deposited employing centrifugation. Measurement of dry cell weight was done by drying cells in an oven at 50°C for 12 hours. The growth index was obtained by calculating the difference in the final weight and initial cell weight divided by the initial cell weight. Measurement of Settled Cell Volume is done by pouring the cell suspension into a measuring cup and allowed to stand for 1 h until all cells were deposited. Settled Cell Volume is the deposited cell volume fraction [21]. The levels of organosulfur compounds was obtained by extracting cell suspensions and analysing them using HPLC.

Assay of organosulfur bioactive compounds

For sample preparation, extraction of cell suspensions resulting from the treatment of Fe^{3+} and Zn^{2+} elicitation was begun by screening the cell suspensions until cell pellets were obtained. The cell pellets were macerated in 95% methanol at a ratio of 1:5. The solution was stirred until homogeneous and let stand for 24 hours at cold temperatures and in a closed bottle; furthermore, the solution was filtered to obtain the filtrate. The filtrate was then centrifuged at 5000 rpm for 20 min to take the supernatant. The supernatant obtained was further identified for the level of bioactive compounds using the HPLC technique based on retention time (RT) and mass-to-charge (*m/z*) corresponding to those in the LC-MS library. The presence of organosulfur bioactive compounds had been identified using the LC-MS technique previously described. Quantification of organosulfur compounds is obtained from the curve area of each organosulfur compound which is calculated using a linear regression equation from the standard. Determination of levels of organosulfur bioactive compounds in the supernatant was carried out using a Shimadzu HPLC apparatus model with the SPD 20-A UV-Vis type detector and a wavelength detector of 210 nm. Separation was performed on Shim-pack VP ODS column (150 x 4.6 mm, 5 µm), temperature of 40°C. The mobile phase used was 10 mM potassium dihydrogen phosphate:acetonitrile (1:1) (v/v) by the isocratic method. Fifty microliters of processed sample was injected into the HPLC.

Statistical Analysis

The experiments were analysed using a two-way ANOVA. The average value was compared using Duncan's Multiple Range Test at the 5% significance level ($p = 0.05$) using SPSS version 25 software.

Results and Discussion

Callus culture and single garlic cell suspension culture

A single garlic crown explants of the Tawangmangu Baru variety was used for callus induction. Friable and yellowish-white callus formed at 6 weeks after explant inoculation. The initiation of cell suspension culture was carried out by transferring callus into Murashige and Skoog liquid medium. At day 0, age suspension culture, the transferred callus was still in the form of cell aggregates with rather large granules. Cell aggregates slowly disintegrate to form clumps of cells that were pale yellow and became uniform at

two weeks of age. The settled cell volume method was used to measure the growth of cell suspensions, namely by recording the number of cell fractions that are deposited every three days for 24 days (Figure 1).

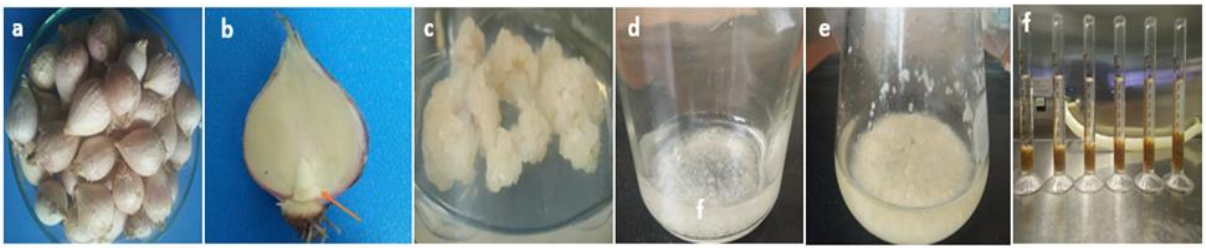

Figure 1.
Callus induction and cell suspension culture growth from single garlic crown explant; (a) Tawangmangu Baru varieties of single garlic, (b) crown explant, (c) single garlic callus, (d) cell suspension culture 0 weeks old, (d) Cell suspension culture two weeks old, (f) measurement of settled cell volume cell suspension

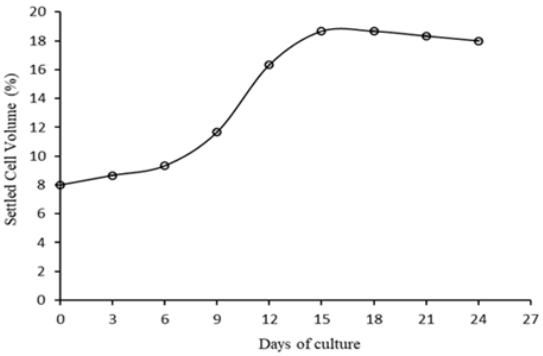

Figure 2.
Growth curve of single garlic cell suspension

The growth curve of single garlic cell suspension culture shows a sigmoid growth pattern. The pattern begins with the lag phase where in at this phase the cells begin to divide, then followed by an exponential phase which is characterized by the maximum cell division rate, and ends with a stationary phase where the cell stops dividing (Figure 2). The lag phase characterizes early cell suspension culture until the 6th day. The growth phase occurs from day 6 to day 15. On incubation on the 15th day, there was an almost 3-fold increase in cell fraction. The stationary phase occurs around the 15th to 18th days, and thereafter decreases gradually. Based on the growth curves obtained, subculture and the time required for elicitation treatment were carried out in the 2-week-old suspension culture, i.e., at the end of the exponential growth phase or before the culture entered the stationary phase. The subculture of cell suspensions needs to be carried out to keep cell suspension cultures in good condition because cultures tend to form cell groups into cell aggregates [22]. Measurement of cell suspension culture growth is essential to ensure the reproducibility of cell suspension culture [23].

The effect of Fe^{3+} and Zn^{2+} elicitation on the growth of single garlic cell suspension culture

The administration of Fe^{3+} and Zn^{2+} elicitors in the culture medium affected the growth of cell suspension culture, namely the fresh weight, dry weight, settled cell volume, and growth index of cell suspension culture, with the same increase pattern by all cell suspension growth parameters (Figure 3). The fresh weight of cells resulting from the administration of Fe^{3+} and Zn^{2+} elicitors increases with increasing elicitor concentration. The increase began at a concentration of 0.1 mM and more clearly visible at 0.2 mM to 0.5 mM. The administration of 0.5 mM Fe^{3+} and Zn^{2+} elicitors to culture media produced the highest cell fresh weight (2.66 g; 2.37 g, respectively). The Fe^{3+} elicitor was able to increase almost two times the cell's fresh weight, while the Zn^{2+} elicitor was able to increase 1.5 times the cell's fresh weight compared to the untreated medium (1.54 g). The dry weight of cells resulting from Fe^{3+} and Zn^{2+} elicitation also increases with increasing elicitor concentration. The Fe^{3+} 0.5 mM elicitor was able to increase the dry weight of cells 3-fold compared to controls, while the Zn^{2+} elicitor was able to increase the dry weight of cells almost 2.5-fold compared to controls.

Settled cell volume expresses the cell volume fraction in 30 mL of culture. In culture media without elicitation, the cell volume fraction was 8.5%. At the highest concentration, both of the elicitors give results the cell volume fraction 14.8% and 13.2% for addition of Fe^{3+} and Zn^{2+}, respectively. The growth index has increased with increasing elicitor concentration. The administration of Fe^{3+} elicitor at each treatment concentration resulted in a higher growth index compared to Zn^{2+}. At the highest concentration, the Fe^{3+} elicitor was able to increase 3-fold, while the Zn^{2+} elicitor was slightly lower at 2.5 times compared to the control.

Heavy metals such as zinc (Zn) and iron (Fe) are essential trace elements that are needed in various structural functions and biochemical processes in plants, including plant growth, oxidation-reduction reactions, electron transport, and many other metabolic processes [24]. Metal ions added to the medium are one of the factors that can influence cell or tissue growth and synthesis of secondary metabolites [25]. Giving metal as an elicitor in the medium influences cell division and

cell death [17]. Some critical factors that can influence the success of cell growth include concentration and duration of exposure from elicitors and culture age [26].

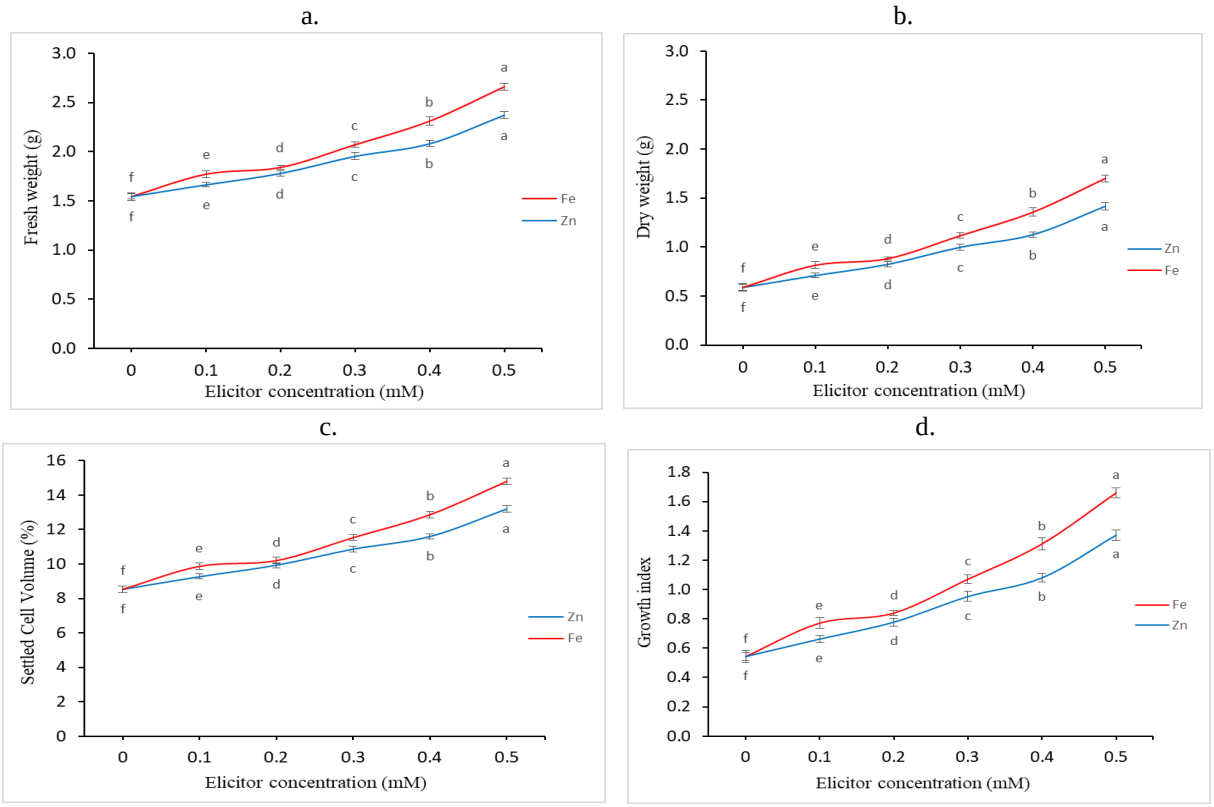

Figure 3.

The different concentrations effect of Fe^{3+} and Zn^{2+} on culture growth in cell suspension culture of single garlic. (a) fresh weight, (b) dry weight, (c) settled cell volume, (d) growth index

The effect of Fe^{3+} and Zn^{2+} elicitation on the increase of organosulfur bioactive compounds in a single garlic cell suspension

The levels of organosulfur bioactive compounds in single garlic cell suspension cultures were analysed using HPLC, and analysis results were obtained in the form of a signal chromatogram. The HPLC analysis revealed a similarity in the presence of 30 types of organosulfur compounds both in suspension culture without elicitation and with elicitation treatment. The difference is the compound levels as an effect of the treatment of different elicitor concentrations. Several compounds are the main organosulfur compounds, namely alliin, allicin, ajoene, allyl methyl disulfide, 2-vinyl 1,3-dithiin, 3-vinyl 1,2-dithiin, E1-propenyl allyl disulfide, 2-propenyl 1-propenyl disulfide, allyl propyl disulfide, allyl methyl trisulfide, allyl trisulfide and diallyl heptasulfide. Components and levels of organosulfur bioactive compounds in a single garlic cell suspension culture without elicitation are presented in Table I. The levels of several organosulfur compounds found in a single garlic cell suspension culture vary from 0.477 to 8.906 mg/g. Of all the organosulfur compounds detected, alliin, allicin and ajoene organosulfur compounds had higher levels compared to other organosulfur compounds, such as the dithiin group and the allyl sulphide group.

Table I

Components and levels of organosulfur bioactive compounds in a single garlic cell suspension culture

Peak Number	Retention Time (RT)	Mass-to-charge ratio (*m/z*)	Compounds	Quantity (mg/g)
1	1.155	74.0190	1,2-Epithiopropane	0.906
2	1.158	74.0190	Allyl mercaptan	2.442
3	1.203	93.9911	Dimethyl disupfide	0.551
4	1.318	112.0347	2,5-Dimethylthiophene	0.656
5	1.322	112.0347	3-Methyl 2-cyclopentene 1 thione	0.560
6	1.341	115.0456	Isobutyl isothiocyanate	0.661
7	1.348	116.0660	2,5-Dimethyltetrahydrothiophene	0.493
8	1.357	118.0816	Propyl disulfide	0.662
9	1.383	120.0067	1,3-Dithiane	0.963

Peak Number	Retention Time (RT)	Mass-to-charge ratio (*m/z*)	Compounds	Quantity (mg/g)
10	1.391	120.0067	Allyl methyl disulfide	1.062
11	1.461	125.9632	2,3,4-Trithiapentane	0.548
12	1.479	134.0024	1,2-Dimercaptocyclopentane	0.564
13	1.517	144.0067	2-Vinyl 1,3-dithiin	0.531
14	1.519	144.0067	3-Vinyl 1,2-dithiin	0.634
15	1.544	146.0224	E1-Propenyl allyl disulfide	0.747
16	1.547	146.0224	2-Propenyl 1-propenyl disulfide	0.477
17	1.587	148.0380	Allyl propyl disulfide	0.861
18	1.618	151.9788	Allyl methyl trisulfide	0.836
19	1.665	161.0510	Deoxyalliin	2.087
20	1.666	162.0173	Allyl 2-propenethiosulfinate	1.552
21	1.667	162.0173	Trans 1-propenyl allyl thiosulfinate	1283
22	1.669	162.0173	1-propenyl allyl thiosulfinate	1.207
23	1.671	162.0173	2-propene 1-sulfinothioic acid 1-propenyl ester	0.777
24	1.673	162.0173	Allicin	3.974
25	3.208	177.0460	Alliin	8.906
26	3.211	177.0460	Cycloalliin	2.317
27	3.505	177.9945	Allyl trisulfide	0.691
28	4.733	180.0101	3,5-Diethyl 1,2,4-trithiolane	0.681
29	7.935	234.0207	Ajoene	3.399
30	11.508	305.8827	Diallyl heptasulfide	1.115

The HPLC analysis of organosulfur compounds resulting from elicitation treatment is presented in a bar chart (Figure 4). The administration of Fe^{3+} and Zn^{2+} elicitors in culture media can increase the levels of organosulfur bioactive compounds. All types of organosulfur bioactive compounds produced by elicitation using Fe^{3+} have increased with increasing elicitor concentration (Figure 4a). The highest Fe^{3+} concentration (0.5 mM) can increase the highest organosulfur compound compared to the lower concentration. Levels of several organosulfur bioactive compounds produced at 0.5 mM Fe^{3+} elicitation include, alliin, allicin and ajoene (11.98, 5.35,4.57 mg/g, respectively) increased by about 1.5 times higher than with controls (8.90, 3.97 and 3.39 mg/g respectively). Other compounds are 2-propenyl 1-propenyl disulfide, 2-vinyl 1,3-dithiin, 3-vinyl 2,2-dithiin, allyl trisulfide, allyl methyl disulfide and diallyl heptasulfide also experienced the same increase compared to controls.

Zn^{2+} elicitor was able to increase levels of organosulfur compounds in cell suspension culture higher than Fe^{3+} elicitor. The levels of organosulfur bioactive compounds produced in the medium with the addition of 0.3 mM Zn^{2+}, among others, alliin, allicin and ajoene (respectively 17.11, 7.69 and 6.44 mg/g) increased about two times higher than the one with a medium without Zn^{2+} administration. At higher Zn^{2+} concentrations of 0.4 and 0.5 mM, the levels of organosulfur bioactive compounds decreased slightly (Figure 4b). Therefore, the optimum concentration of each elicitor in increasing levels of organosulfur bioactive compounds is different.

Cell suspension culture is an effective method in the production of bioactive compounds [27, 28]. The strategy for increasing the production of bioactive compounds can be by giving elicitors. Metal is one of the abiotic elicitors that can be used to enhance plant bioactive compounds. Fe^{3+} and Zn^{2+} metals have been shown to increase the production of bioactive compounds in some plants. The role of metal ions in enhancing bioactive compounds is influenced by several factors, including plant species, types and metal concentrations [29, 30]. Fe^{3+} elicitor can increase the production of betalain bioactive compounds two times higher in *Bougainvillea* cell culture with a concentration of 0.1 mM [31]. Zn^{2+} elicitor can increase the production of sulforaphane bioactive compounds three times higher in *Lepidium draba* cell cultures [32].

Stress caused by metal exposure will cause several chemicals, physiological and morphological changes in growth, respiration, photosynthesis, protein synthesis, lipid metabolism and changes in secondary metabolism [33]. Elicitors can be recognized by receptors to form elicitor-receptor complexes. Activated receptors will cause activation of effectors such as oxidative burst [25]. The activated effector will further increase the synthesis of signalling molecules. The occurrence of signalling molecules will activate genes for transcription factors, and subsequently, transcription factors will activate genes for enzymes that play a role in the biosynthetic pathway of bioactive compounds [34, 35].

Oxidative burst is a response that is often generated related to the pressure that can cause a variety of secondary responses [36]. Fe^{3+} and Zn^{2+} will activate the formation of Reactive Oxygen Species (ROS). In increasing ROS production, the Fe^{3+} metal will react directly through the Haber-Weiss reaction or the Fenton reaction, whereas the Zn^{2+} metal will first bind to other compounds that have active sulfhydryl (-SH) groups [37]. To overcome the excess production of ROS,

plant cells activate antioxidant enzymes such as non-enzymatic antioxidants, one of which is glutathione [38]. In the biosynthesis of organosulfur compounds, glutathione is a precursor in producing alliin. Alliin formed is parental for other organosulfur bioactive compounds [39]. With the induction of glutathione in the elicitation process of Fe^{3+} and Zn^{2-}, there will be an increase in organosulfur compounds.

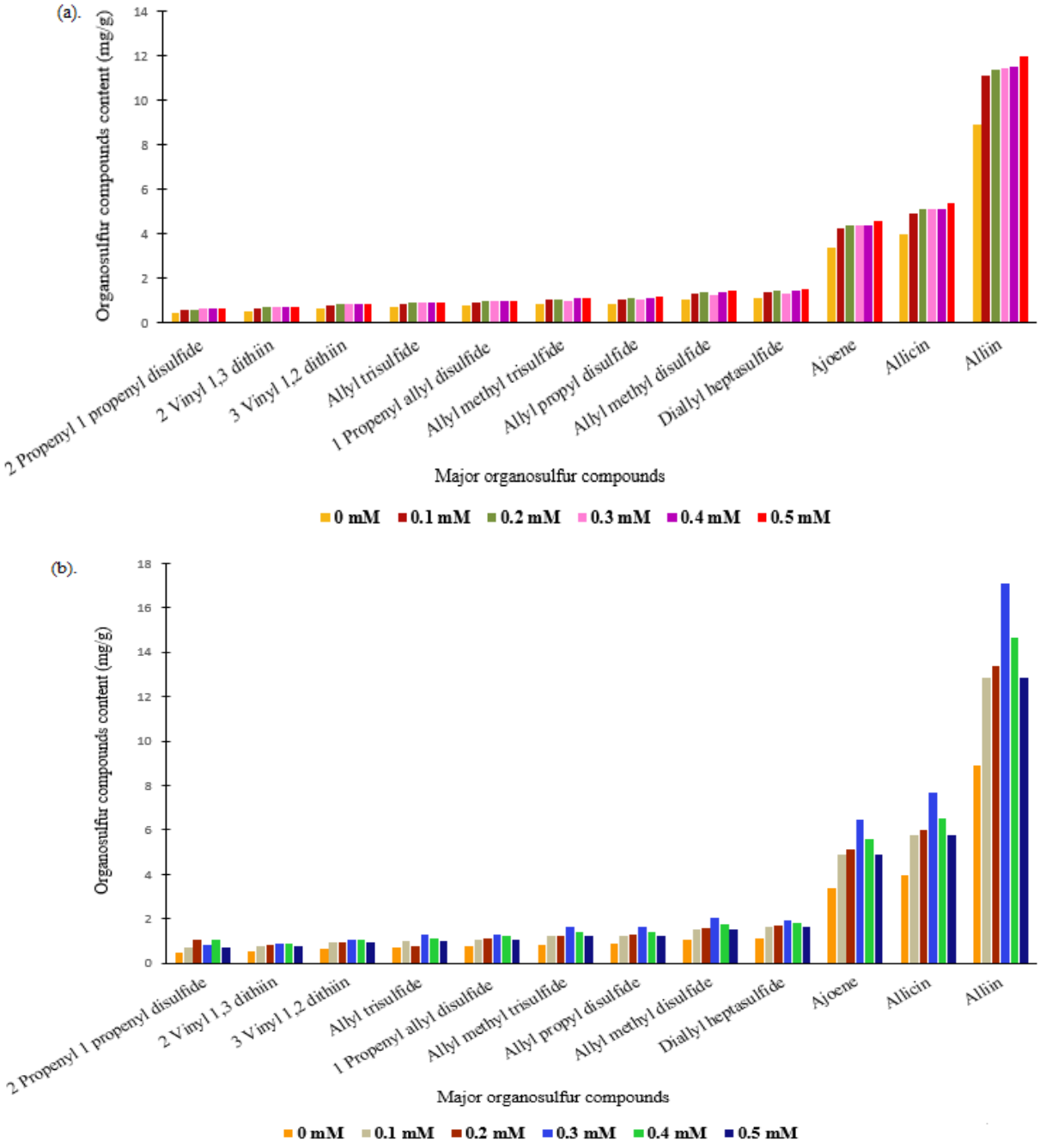

Figure 4.
Differences in concentration of Fe^{3+} and Zn^{2+} elicitors on organosulfur bioactive compounds content in single garlic suspension culture (a) Fe^{3+}, (b) Zn^{2+}

The levels of organosulfur bioactive compounds in cell suspension culture treated with Fe^{3+} increased with cell suspension growth. Slightly different compared to Fe^{3+}, the levels of organosulfur bioactive compounds in cell suspension cultures treated with Zn^{2+} increased with cell suspension growth, but at high concentrations, they experienced a slight decrease (Figure 5).

Secondary metabolite production does not always show a positive correlation with the maximum level of culture growth [40]. The difference in elicitor on growth patterns and metabolic activity will affect the production of proteins, photosynthetic pigments, sugars and non-protein thiols. These effects can arise due to the inhibition of various enzymes involved in the

biosynthesis of bioactive compounds through the disruption of substrate utilization [41]. Glutathione is included in the group of non-protein thiols and are precursors in the biosynthesis of organosulfur compounds. With the difference in elicitor, there will be a change in the formation of glutathione, so that it affects the abundance of organosulfur bioactive compounds production.

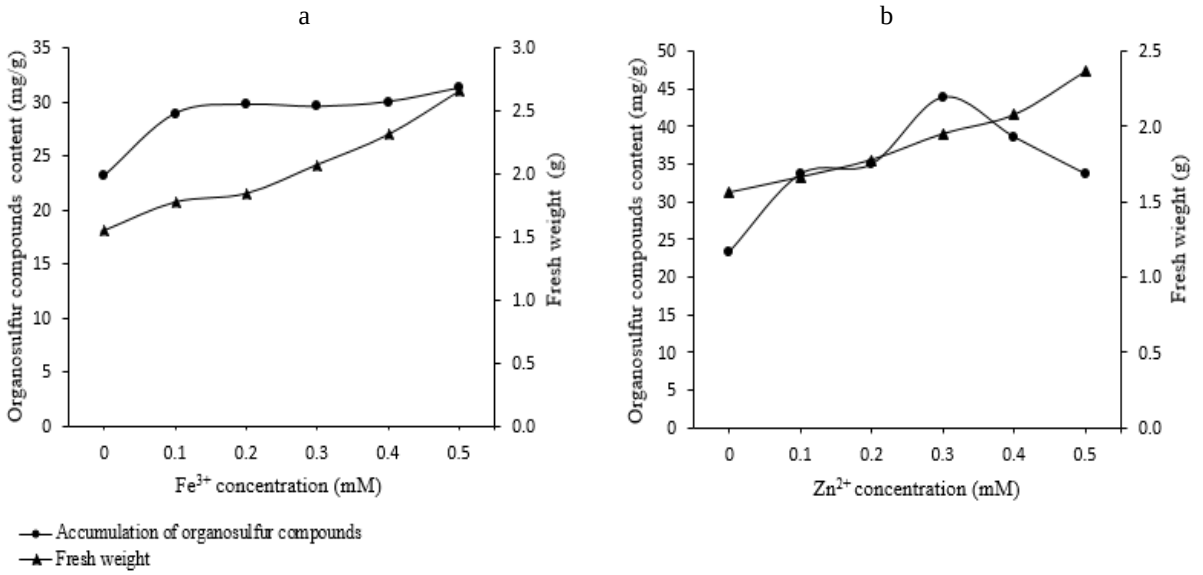

Figure 5.
Correlation between the accumulation of organosulfur bioactive compounds and cells fresh weight in cell suspension culture of single garlic (a) Fe^{3+}, (b) Zn^{2+}

Conclusions

The result of this study proved that elicitation using metal Fe^{3+} and Zn^{2-} was an effective method in increasing the production of organosulfur bioactive compounds in single garlic cell suspension cultures. Zn^{2+} elicitor increases levels of organosulfur bioactive compounds higher than Fe^{3+} and requires lower concentrations compared to Fe^{3+}. The organosulfur bioactive compounds produced in a single garlic cell suspension culture were: alliin, allicin, ajoene, allyl methyl disulfide, 2-vinyl 1,3-dithiin, 3-vinyl 1,2-dithiin, E1-propenyl allyl disulfide, 2-propenyl 1-propenyl disulfide, allyl propyl disulfide, allyl methyl trisulfide, allyl trisulfide and diallyl heptasulfide.

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Conflict of interest

The authors declare no conflict of interest.

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