Elicitation Method using *Saccharomyces cerevisiae* to Improve Bioactive content of *Morinda citrifolia* (Mengkudu)

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Abstract

An experiment about elicitation method used elicitors derived from *Saccharomyces cerevisiae* yeast to increased quinone and oxazole (alkaloid) of *Morinda citrifolia* callus, have been conducted. Objective of experiment was to increased concentration of secondary metabolite (bioactive compound) quinone and oxazole from *M.citrifolia* callus by elicitor derived from *S. cerevisiae*. Callus was induced from 1,5 month old seedling by culturing on solid Murashige & Skoog (MS) medium supplemented with 2,4-D (2.10^{-1} and 3.10^{-1} mg/L), aceptically. Callus was transfered into the same media and after 2 times subcultured, it was followed by elicitation with 0% (control), 2,5%, 5,0% and 7,5% (v/v) homogenate yeast derived from *S. cerevisiae*. Before elicitation process, growth and production curve have been made to certain the best time for elicitation. Product of elicitation was harvested on the second and fourth days after elicitation. Analysis of quinone and oxazole content used GCMS (Gas Cromathography Mass Spectrum). The result showed that quinone and oxazole content callus could be induced in MS medium supplemented with 2,4-D (2.10^{-1} and 3.10^{-1} mg/L). Addition elicitor from homogenate of *S. cerevisiae* on several concentration could be increased quinon and oxazole concentration of callus. The optimum concentration of elicitor *S. cerevisiae* wich induced highest concentration of quinone, was 5,0% , i.e. on second days after elicitation. Whilst optimum concentration of elicitor *S. cerevisiae* wich induced highest concentration of oxazole, was 2,5,0% , i.e. on second days after elicitation too. Increment of quinone and oxazole concentration was influence by concentration of elicitor and harvest time.

I. Introduction

One of the main source of natural medicines is plants. Plant bioactive material is usually secondary metabolite, a metabolite yielded from secondary metabolism. Conventionally, secondary metabolite can be directly extracted from plant's organ. But this method needs great scale plant cultivation. Beside that, extraction and isolation processes are expensive. Whereas sinthetic metabolite is expensive because the active structure is very complex (Balandrin & Klocke,1988). Several disadvantages of conventional method need to be accomplished by finding better method. Utilizing tissue culture method to produce secondary metabolite can be an alternative method because it

can accomplish the difficulties. Tissue culture method does not need excessive material, extensive area, the secondary metabolite can be produced continuously, and purification process is cheaper because tissue culture cells contains not much pigmen. Tissue culture, cell culture and callus (group of cells that have not organized and differentiated) culture are potential as secondary metabolite factory, including plant bioactive material.

Mantell & Smith (1993) stated that secondary metabolite contain in several cell culture and callus culture is relatively low, therefore in tissue culture is needed a method to improve secondary metabolite contain, included plant bioactive material. One of the method that about to be developed is elicitation method. Elicitation is a method to induce simultaneously phytoalexin forming, constitutive secondary metabolite and other secondary metabolites that is normally not accumulated (Barz *et al*., 1990). Elicitation method is developed based on natural phenomena that is plant defence mechanism against microbes attacks or other fear conditions. Elicitation can be conducted by adding abiotic or biotic elicitor. Biotic elicitor can be fungi or yeast. Many researches about elicitation successfully improve plant bioactive contains using fungi as elicitor. Purwianingsih (1997) has been successfully improved gosipol content doubly in *Gossypium hirasutum* callus after elicitor addition of fungi extract *Verticillium dahliae* dan *Rhizoctonia solani*. Gosipol content is also increased by fungi extract *Rhizopus arrhizus* addition (Hamdiyati, 1999). Several elicitation researches using yeast, especially *Saccharomyces cerevisiae*, also successfully improved plant bioactive content. Anthocyanin in *Daucus carota* cell culture has been successfully improved as much as 58% using cells extract of *S. cerevisiae* (Survanalatha *et al*., 1994). Beside that, carbohidrate fraction from yeast extract S. cerevisiae can also induce gliceolin synthesis until 200 µg/BK in *Glycine max* cell culture and barberin biosynthesis four times in *Thalictrum rugosum* culture (Funk *et al*., 1997). Among plants that are known as medicine material resources, nowadays *Morinda citrifolia* (mengkudu) has a very popular benefit because its bioactive content is already known. There is already identified more than 70 bioactive compound in mengkudu. The bioactive compounds are widely distributed in various organs, such as root, leaf and fruit. From these many secondary metabolite bioactive compounds, there are several important compounds such as antraquinon (quinon group), skopoletin (alkaloid group), ascorbic acid (vitamin), β-

carotein, l-arginin, proseronin and proseroninase-β that is a precursor of seronin (alkaloid group) (Wang *et al*., 2002).

Zenk *et al*. (1975 in Bajaj, 1988) have been successfully produced suspension cell culture of *M. citrifolia* which contained antraquinon using B5 medium with 2 mg/L NAA addition and B5 medium with 10-5M NAA addition. Tewtrakul *et al*. (1997) can grow callus that contain antraquinon from *M. citrifolia* leaves in Murashige & Skoog (MS) medium with 2,4-D and kinetin addition. Purwianingsih & Rani (2003) have found *M. citrifolia* callus that contain several secondary metabolite with leaf as tissue resource. Callus is successfully formed in MS medium with 2,4-D (2.10^{-1} until 3.10^{-1} mg/L) addition and in B5 medium with NAA (1.10^{-5} and 5.10^{-5} M) addition. Secondary metabolites that successfully identified from callus using GCMS are from alkaloid group, flavonoid group and phenolic group. Based on research background outlined above, this research has objection to improve bioactive content from quinon group (phenolic) and alkaloid group in callus culture of *Morinda citrifolia* using elicitation method and *S.cerevisiae* elicitor.

II. Materials & Methods

2.1. *Morinda citrifolia* callus preparation

To form callus, explant is derived from the leaf of 1.5 month *Morinda citrifolia* sprout grown from seed (gambar 2.1A & 2.1.B). Medium used is solid medium Murashige & Skoog (MS) with 2,4-D (2.10^{-1} and 3.10^{-1} mg/L) addition (Purwianingsih & Rani, 2003).

A B

Figure 4.1.A (*M.citrifolia* seed), B (1,5 months *M.citrifolia* sprout as explant resource).

2.2. Subculture and determination of callus subculture yield

Callus formed in induced medium, then transferred to similar medium after 1.5 months, or a part of explant covered with callus. The best subculture yields are based on the maximum growth of callus and the rest of explant is not visible anymore.

2.3. Growth curve and production curve of *Morinda citrifolia* L. callus

Callus from the first subculture were transferred to new medium. The increasing rate of callus weight was measured for 32 days with 4 days interval. Callus growth curve was made based on these data. Callus was also extracted using methanol, and bioactive compounds produced were measured using GCMS (Gas Chromatography Mass Spectrum). Data obtained were used to make production curve. Bioactive content was determined based on compound content percentage against the whole compound content in the sample. From growth curve and production curve were determined the best elicitation time.

2.4. *Saccharomyces cerevisiae* H. growth curve measurement

Two days pure culture of *Saccharomyces cerevisiae* H. was inoculated as much as two ose into 4 ml NaCl (2,56 x 10^7 sel/ml). After counting the number of cells, it was taken 3 ml of inocullum, inoculated into 50 ml GYEA and incubated at 30^oC without shaking. To make a complete *S. cerevisiae* H. growth curve, cells were harvested for 24 hours with 2 hours interval. The number of cells were counted using dilution plate count method duplo (Irdawati, 1999). Cell counting is started from 10^{-4} until 10^{-6} dilution. The number of cells that have already reached the initial stationer phase is used as elicitor material (Eilert *et al*., 1986).

2.5. Elicitor preparation

Saccharomyces cerevisiae culture at the initial stationer phase is used as elicitor resource, autoclaved and centrifuged. Pellet derived was washed, aquadest was added appropriate with the pellet volume and autoclaved. Homogenate was solubilized in sterile aquadest to reach the concentration of 0; 2,5; 5; 7,5 % (v/v) that will be used in the next step(Survanalatha *et al*., 1994).

2.6.Callus elicitation

Elicitation was conducted by adding 0.5 ml elicitor with certain concentration. Sterile aquadest was added in control callus. Elicitation yield harvesting was done on day- 0, 2, and 4. Then analysis of phenolic and alkaloid content were conducted using GCMS.

III. Result and discussion

3.1.Growth and development of *Morinda citrifolia* L. callus

Explant derived from leaf cut of 1.5 months *Morinda citrifolia* L seed sprout can grow into callus in MS medium with 3 x 10^{-1} mg/L 2,4-D addition. Callus was started to grow in seven days explant after induced in the medium (Figure 3.1).

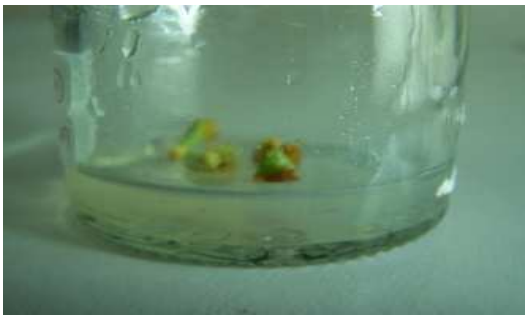

Figure 3.1. Callus forming after seven days culture

Callus formed soft and crumb texture, with brown colour and getting more brown along the increasing age of callus. Callus will develop continuously on the explant and reach maximum callus development on day-33, as seen on Figure 3.2.

Figure 3.2 Callus, that has already reached maximum growth for 33 days, has a compact structure and soft texture.

3.2. Correlation between *Morinda citrifolia* L callus growth and its bioactive content.

Callus growth and development data and phenolic and alkaloid analysis data using GCMS can be seen on Figure 3.3 and Figure 3.4. On Figure 3.3, it can be seen that quinon production is started since 0 day culture. Callus growth is very slow, as seen on relatively flat curve. Border between lag phase and log phase is not clear, but on day-12 faster growth of callus can be already seen. It indicated that cell has already adapted to its environment and nutrien used for primary metabolism. Maximum growth is reached on day-28. The increasing content of quinone is parallel with the callus growth since day-0 until day-20, after that the pattern is adverse. This result is corresponding to the opinion of Lindsey and Yeoman (1983), that there is a competitive precompound between primary metabolism and secondary metabolism pathways. If the primary metabolism pathway is active then the secondary metabolism pathway will be inhibited. Correlation pattern between callus growth and quinone content is included in pattern III, that secondary metabolite accumulation is parallel with the growth curve (Endress, 1994). Based on the correlation between callus growth and quinone content, elicitation time is determined at 12 days of callus age. On that age, callus has already adapted with its environment and the secondary metabolite quinone has not reached the maximum content, therefore it is provided that elicitor addition will stimulate the increasing quinone production.

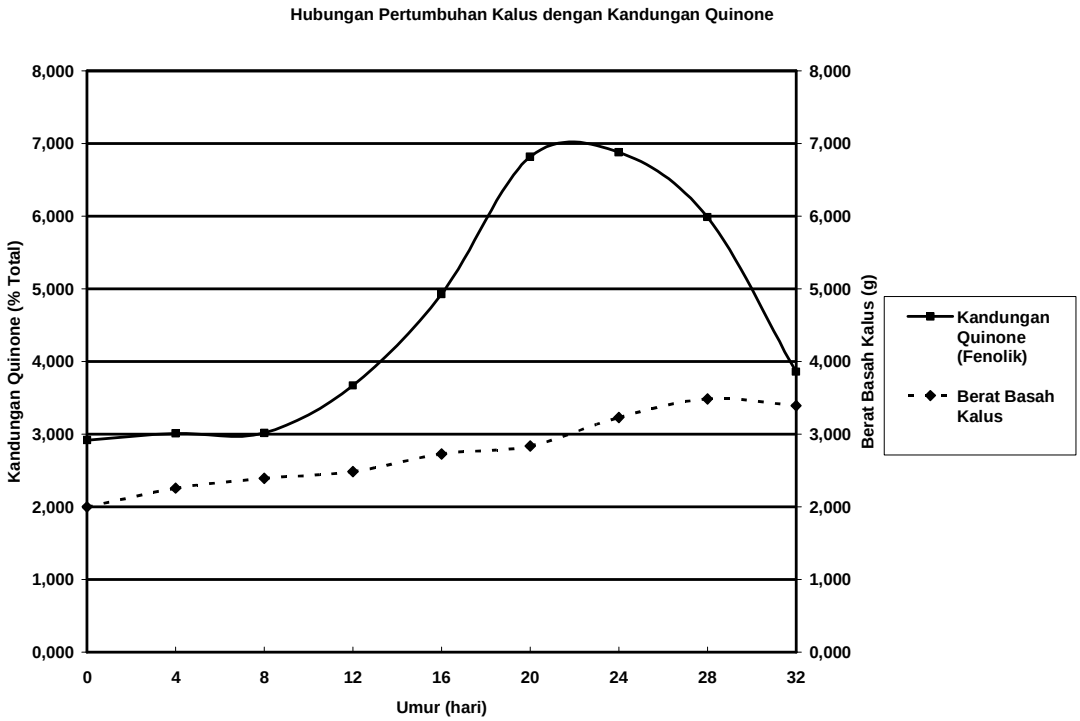

Figure 3.3. Correlation between callus growth and quinone content

On Figure 3.4 can be seen that oxazol production is started since 0 day culture. Callus growth is very slow, as seen on relatively flat curve. In the initial growth, nutrien is used more to form secondary metabolite. Border between lag phase and log phase is not clear, but on day-12 the callus growth is faster than before. It showed that cell has already adapted with its environment and nutrien is used for primary metabolism. Oxazol production on day-12 is still increase and maximum production of secondary metabolite has been reached on day-16. Whereas maximum growth has been reached on day-28. Correlation pattern between callus growth and oxazol production is incuded in pattern II, that secondary metabolite accumulation is happened after acceleration phase (Endress, 1994). Based on correlation between callus growth and oxazol content, the elicitation time is when callus age is 12 days. On that age, callus has already adapted with its environment and maximum oxazol content has not been reached, therefore it is provided that elicitor addition can stimulate the increasing of oxazol production.

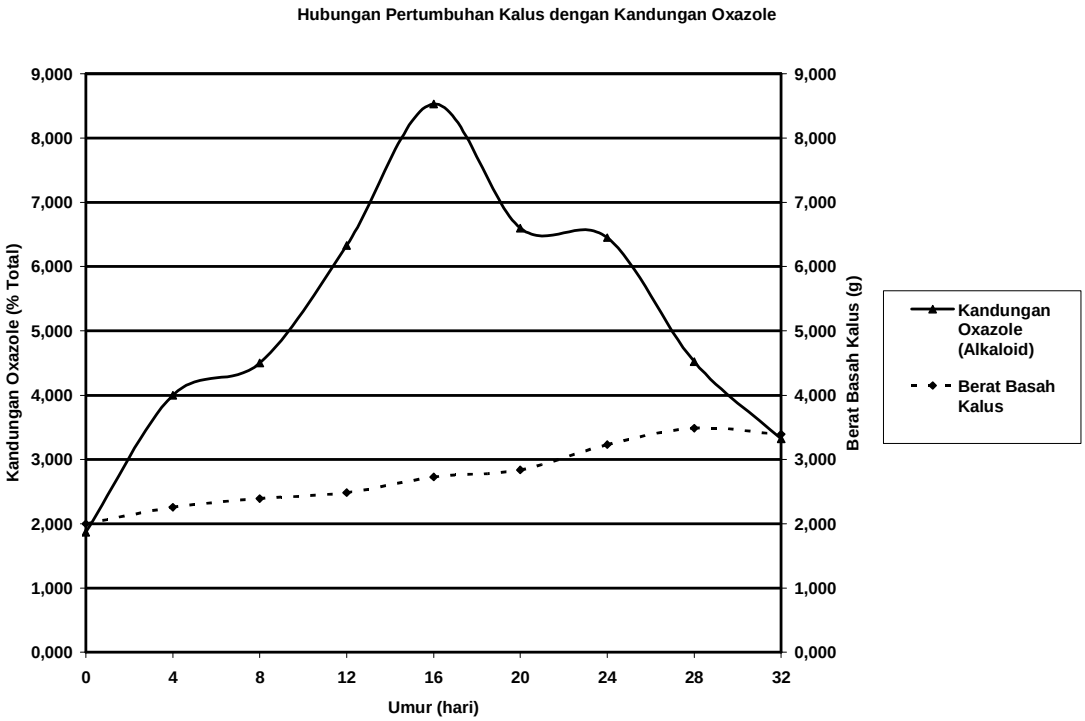

Figure 3.4. Correlation between callus growth and oxazol (alkaloid) content

3.3. *Saccharomyces cerevisiae* H. growth curve measurement

The growth curve of *S. cerevisiae* H., that is yielded for 24 hours with 2 hours interval, is showed on Figure 3.5

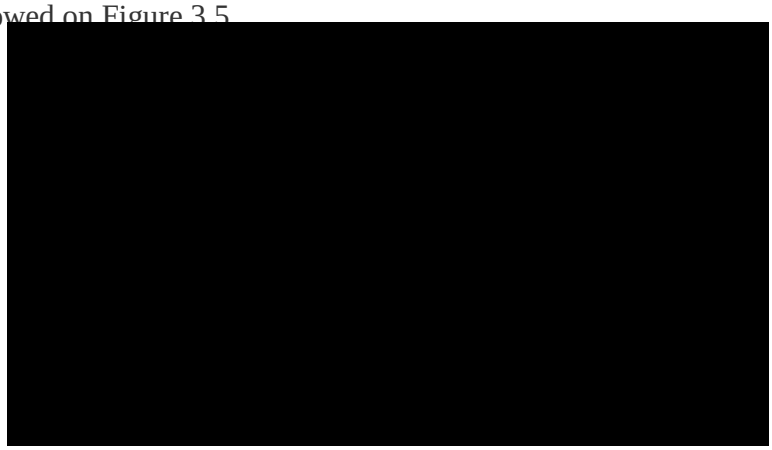

Figure 3.5. The growth curve of *S. cerevisiae* H

3.4. Elicitor preparation

Based on the yeast *S. cerevisiae* H. growth curve, maximum growth is in 16 hours culture, when cells have already reached initial stationer phase. It is the perfect time to yield the yeast culture as elicitor, because at maximum growth the cell wall component, that is usefull as elicitor, has already formed perfectly than before its maximum growth (de Wit & Roseboom, 1980). Vazquez-Flota *et al*. (1994) stated that if we use autoclaved yeast homogenate as elicitor, the response of plant cells to elicitor is directly correlated with the composition of yeast cell wall.

3.5. The effect of elicitation on quinone (phenolic) and oxazol (alkaloid) contents

M.citrifolia callus, that is elicited using *S.cerevisiae*, showed response on Figure 3.6.

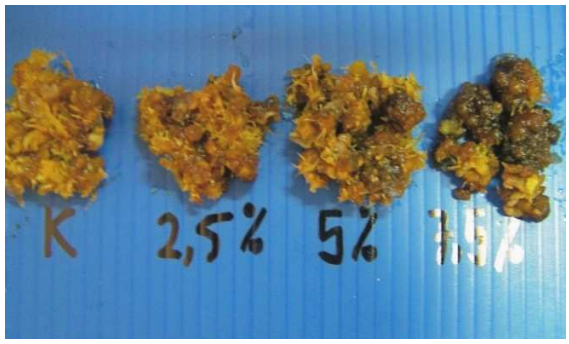

Figure 3.6. Control (K) callus & elicited callus.

From the figure, it can be seen that elicited callus is coloured more brown than unelicited callus. The brownish colour maybe a hypersensitive response that is showed by plant tissue after threatening with elicitor. This is correponding with Isaac (1992) that stated that threatened tissue will be brown and its growth will be inhibited. Isaac also stated that, in threatened cells, the accumulation of certain secondary metabolite will increase. Table 3.3. and Graphic 5.8. are showed as followed.

Table 3.1. The effect of *S.cerevisiae* elicitor addition in various concentration and yielding time on quinone content (%) in *M.citrifolia* callus culture.

Constr. (% v/v) **Day**	**0**	**2,5**	**5,0**	**7,5**
0	2,74 ± 0,43	2,74 ± 0,12	2,98 ± 0,08	3,04 ± 0,08
2	3,59 ± 0,15	7,24 ± 0,58	7,82 ± 1,21	3,24 ± 0,24
4	3,53 ± 0,33	4,61 ± 0,32	5,24 ± 0,28	2,54 ± 0,19

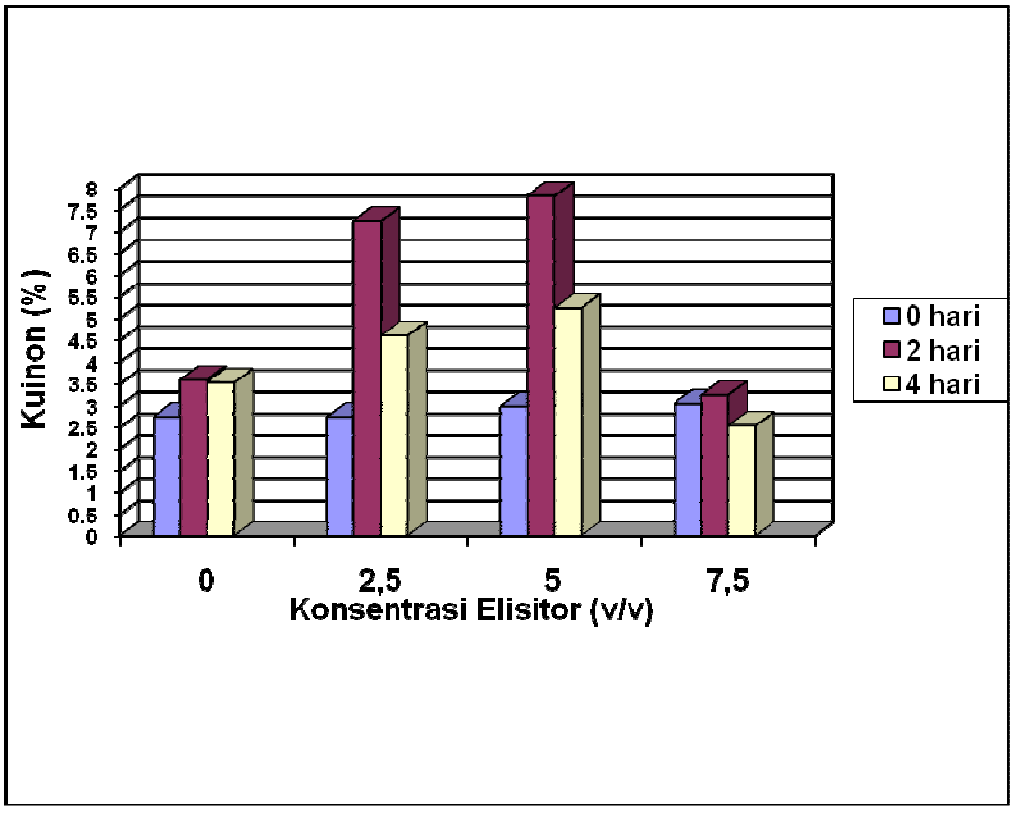

Figure 3.7. The effect of elicitor *S.cerevisiae* addition on various concentration and yielding time on quinone content in *M.citrifolia* callus culture.

Based on Table 3.1. and figure 3.7., the following things can be seen: On the day-0 elicitation, the effect of elicitor has not increased quinone content. Maybe because the contact between callus cells and elicitor component has not been occured. Therefore callus cells have not respond to elicitor in increasing the quinone content. Yoshikawa (1993) research supported the hypothesis that elicitor initiates physiological activity in plant cells through the interaction on receptor in plasma membrane of plant cells. This has maybe not happened on day-0, because there is no receptor interaction on plasma membrane. On 2-day and 4-day yielding time, elicitor addition can increase quinone content. Quinone content in treatment group (especially addition of 2,5% and 5% elicitor) is increase compared to control (0% elicitor). Whereas in 7.5% elicitor addition, both in 2-day or 4-day yielding time, quinone content is decrease compared to control. On 2-day yielding time, quinone content after elicitation with 7.5% is 3.23%, compared to 3.59% in control. Whereas in 4-day yielding time, quinone content after elicitation with 7.5% is 2.54% compared to 3.53% in control.

In treatment group using concentration of 7.5%, quinone content is decrease because the secondary metabolite is synthesized in a very short time. This is corresponding with Bell (1981), terpenoid in cotton is only synthesized in a very short time because it is metabolized by plant tissue as a less toxic compound. It is a factor that contributes to regulate secondary metabolite accumulation. Quinone content on day-2 is higher compared to day-4, especially when treated with concentration of elicitor 2.5% and 5.0%.

These data showed that yielding time affects quinone content. Corresponding with research conducted by Purwianingsih (1997) on *Gossypium hirsutum* callus, yielding time affects secondary metabolite gosipol content. It is maybe caused by the post binding effect (Ridge,1991), that is a different rate of cells capability to response certain signal.

Elicitation results showed that the highest increase content of quinone is reached in elicitor concentration of 5.0% on day-2 yielding time. The increasing content of quinone after elicitor addition is caused by the increasing enzyme synthesis belongs in

quinone synthesis. Isaac (1992) stated that elicitor can induce secondary metabolite accumulation in plant tissue by stimulating mRNA synthesis through the increasing transcription rate of engaged genes. As stated by Josst *et al*. (1995), cotton plant that is elicited by viable and autoclaved fungi *Verticillium dahliae*, showed an increasing number of mRNA to sinthesis 3-hydroxi-3-methyl-gutaril KoA reductase (HMGR). HMGR is an important enzyme in gosipol synthesis. Increasing activity of HMGR can increase mevalonic acid pool that is needed in gosipol synthesis.

3.6. The effect of elicitor *S. cerevisiae* addition on the increasing content of oxazol (alkaloid) in *M.citrifolia* callus culture.

According to Table 3.2 and Figure 3.8, it can be seen that on day-0 elicitation the effect of elicitor addition has not been seen on the increasing of oxazol content. It is maybe caused by the contact between callus cells and elicitor component have not been existed. Therefore callus cells have not respond to elicitor to induce elicitation and increase oxazol content.

Table 3.2. The effect of elicitor *S.cerevisiae* addition on various concentration and yielding time on oxazol content in *M.citrifolia* callus culture.

Const.. (% v/v) **Day**	**0**	**2,5**	**5,0**	**7,5**
0	3,29 ± 1,03	2,96 ± 0,66	2,68 ± 0,37	2,87 ± 0,50
2	5,66 ± 0,34	7,45 ± 0,38	4,51 ± 0,92	2,23 ± 0,29
4	6,98 ± 0,18	6,09 ± 0,15	3,96 ± 0,42	2,51 ± 0,39

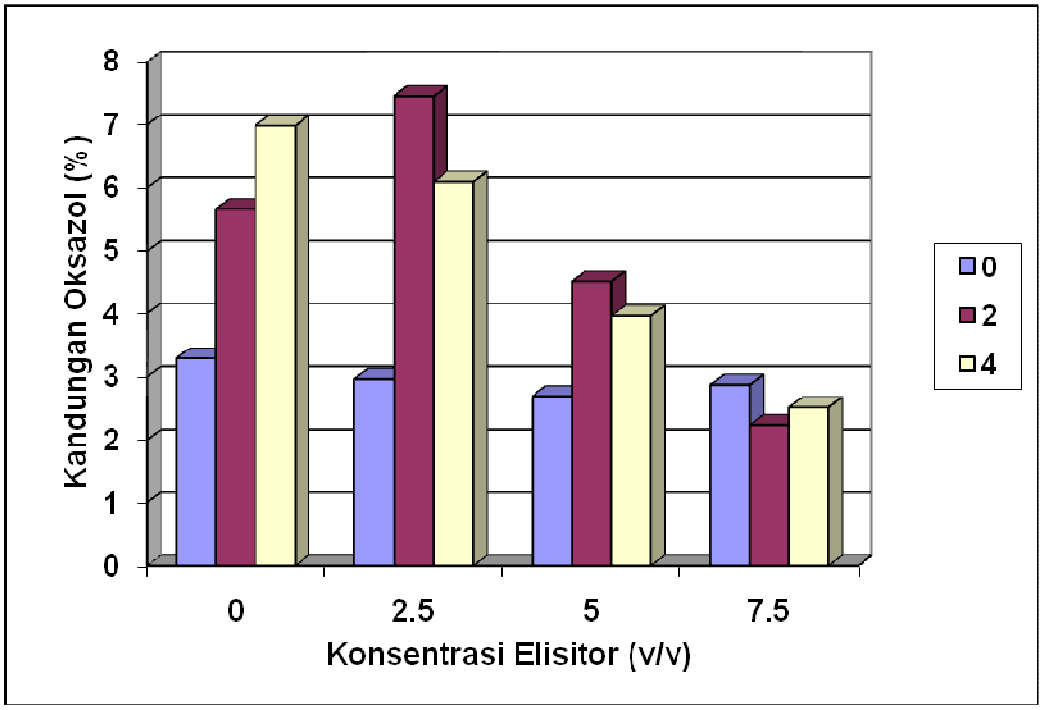

Figure 3.8. The effect of elicitor *S.cerevisiae* addition on various concentration and yielding time on oxazol content in *M.citrifolia* callus culture.

On day-2 yielding time, elicitor addition can be respond with the increasing content of oxazol. It can be seen from the increasing content of oxazol in treated group (2.5% elicitor addition) compared to control (0% elicitor). Whereas on callus added with 5.0% and 7.5% elicitor and yielded on day-2 and day-4, oxazol content is decrease compared to control. Only on day-2 yielding time and 2.5% elicitor concentration, oxazol content will increase. Whereas in other treated groups, there is no increasing content of oxazol. Elicitor concentration of 2.5% is an optimum concnetration that can affect oxazol production. It indicated that using several concentration of *S. cerevisiae* elicitor has not been effective enough to increase oxazol content. Hahn (1996) stated that cell wall component that act as elicitor in *S. cerevisiae* is glucan. Glucan active structure as elicitor is β-1,6-D glucopiranocil. From this research, it can be seen that the increasing content of oxazol by certain concentration of elicitor can be caused by the sensitivity of cells to effector, in this case elicitor is depend on its number of receptor (Ridge, 1991). Research conducted by Yoshikawa (1993) supported the hypothesis that elicitor initiates physiological activity in plant cells through the interaction of receptor on plasma

membrane of plant cells. Elicitation result showed that the increasing content of oxazol is happened after 2.5% addition of elicitor and day-2 yielding time. The increasing content of oxazol after elicitor addition is maybe caused by the increasing synthesis of enzymes that including in quinone synthesis. Isaac (1992) stated that elicitor can induce secondary metabolite accumulation in plant tissue by stimulating mRNA synthesis through the increasing rate of gene transcription. The results above showed that elicitor concentration and yielding time affect quinone and oxazol content in *M. citrifolia* callus culture. It is corresponding with statement of Buitelaar & Tramper (1991) that elicitation can be affected by elicitor concentration and yielding time, or contact time between elicitor and plant cells.

IV. Conclusion

1. Elicitor *Saccharomyces cerevisiae* is effective to increase bioactive quinone and oxazol content in *Morinda citrifolia* callus culture.
2. The highest quinone content is produced by *M. citrifolia* callus culture after elicitation with concentration of 5,0% and 2-day yielding time.
3. The highest oxazol content is produced by *M citrifolia* callus culture after elicitation with concentration of 2,5% and 2-day yielding time.
4. Concentration of *S. cerevisiae* elicitor and yielding time are factors that affected the increasing of quinone and oxazol contents in *M citrifolia* callus culture.

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