Cacao (*Theobroma cacao* L.) somatic embryos development under heat stress condition

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**Abstract.** The study aimed to evaluate the effect of heat stress on the developments of cacao somatic embryos under *in vitro* condition. Somatic embryos were induced from petal explants of ICCRI 4 cacao clone using DKW medium with the addition of 2.4-D and kinetin. Mature stage of cotyledonary somatic embryos were cultured on a germination medium and incubated at temperatures of 25 (control), 35, 40, 45 and 35 followed by 45°C with sub-cultured on the same medium monthly. The results showed cacao somatic embryos were turned brown and suffered death after 16, 5 and 3 days of incubation at temperatures of 40, 35-45 and 45°C, respectively, while somatic embryos cultured at temperatures of 25 and 35°C could develop to form normal plantlets. The temperature of 40-45°C might be the lethal temperature for cacao somatic embryos. The results of this study gave illustration how heat stress affected cacao embryo development and could be used as a base information for developing an *in vitro* selection method for heat stress tolerance in cacao.

**1. Introduction**

Intergovernmental Panel on Climate Change (IPCC) projected a range of 1.1°C to 6.4°C increase in global mean temperature from 1990 to 2100 (1). High temperature stress, as an impact of global warming, is now considered to be one of the major abiotic stresses for restricting crop production and a critical factor for plant growth and productivity. Changes in temperature affect the crop yield mainly through disruptions of phenological development (2) and pollination process (3).

High temperature stress affects not only annual crops but also perennial plants (4). It has been reported that perennial cropping systems are less adaptable and more susceptible to damage caused by climate change (2). Among the plants that are vulnerable to climate change e.g. cacao, in which the production of cacao is predominantly influenced (5). In India, climate change made the peak flowering time of cacao changed (2). High temperature is expected to hit cacao plants into stress condition in the future, and the maximum temperature during the dry season will be more important than the availability of water in cacao plants (6). Cacao grows well in the average annual temperature range from 22 to 25 °C with a minimum temperature of 21°C and a maximum of 32 °C (FAO *In 6*). Temperatures below
or above the temperature threshold will inhibit photosynthesis and other metabolic processes in cacao. This condition should be considered in the selection of cocoa germplasm tolerant to maximum high temperatures, in addition to tolerance to the drought and diseases. Assembling high temperature tolerant varieties of cacao is one strategy to maintain the level of cacao production in the future (7).

Studies regarding genetic variation in the response of heat stress in tree crops are still limited, therefore it is very important to explore the existing variations within species to improve heat stress tolerance in economically important tree species (3) including cacao. In recent years, tissue culture has become the preferred alternative method for developing tolerant plants to abiotic and biotic stresses through induction of somaclonal variations followed by in vitro selection. The advantage of this technique are timeless and space, and potentially important for the development of various stress-tolerant crop plants (8). High temperature will affect plant developments, therefore it is important to understanding plants respond to the environmental conditions (4).

2. Materials and Methods
The research was conducted at Tissue Culture and Plant Breeding Laboratory of Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), Indonesia and Horticultural Science Laboratory of Agriculture College, Ibaraki University, Japan in 2017. Somatic embryos were induced from petal explants of ICCRI 4 cacao clone according to (9). Explants were firstly cultured on DKW basal salt medium with addition of 2,4-D (2 mg/l) and kinetin (0.5 mg/l) for primary callus induction. After two weeks explants were transferred into secondary callus growth medium containing WPM basal salt medium with the addition of 2,4-D (2 mg/l) and kinetin (0.125 mg/l). Then, two weeks later callus were transferred into embryo development medium containing DKW basal salt medium without plant growth regulator and sub cultured into the same medium every 3 weeks until the somatic embryo were formed. The mature cotyledonary stage of somatic embryos were then cultured onto germination medium (9) and incubated at different temperatures according to the treatments, i.e. 25°C (control), 35°C, 40°C, 45°C and 35-45°C for about 2 months with sub-culturing onto the same medium monthly. In the treatment of 35-45°C, somatic embryos were cultured and incubated firstly at 35°C for 3 days as a hardening and acclimation treatment, and then subsequently transferred into temperature of 45°C. The observations were made every day on the morphological appearance of the embryos. After two months of incubation all of somatic embryos were transferred into control temperature.

3. Results and Discussions
Somatic embryos incubated at temperatures of 25°C and 35°C grown normally and develop to forming shoots and roots. Initiation of shoot primordia has begun at third week of incubation (Table 1), and more clearly visible at week 5th. The same phenomenon was also shown in the formation of root primordia (Figures 1E and F). After 6 weeks of incubation, somatic embryos cultured at 25°C and 35°C were undergo forming young leaves (Figure 1G) indicated that the activity of cell division and differentiation occurred properly. However, the forming leaves appearance at incubation temperatures of 35°C remained pale and almost without pigment after 2 months of incubation (Figure 1H) as compared to those of forming leaves at 25°C temperature (Figure 1I), indicated that there was an inhibition in chlorophyll synthesis and other pigments or chlorosis (chlorophyll degradation). Cacao plants found to be tolerant to the field temperature of about 10°C (minimum) and 38°C (maximum), with annually temperature of about 20-27°C (FAO In 6). Therefore 35°C is still in the range of threshold temperature for cacao. However, exposed continuously to 35°C had caused high stress to the cacao plantlets and inhibited the synthesis of certain metabolites such as chlorophyll. Discoloration is one of the symptoms that commonly occur in plants due to high temperature stress. Pruacan (Pimpinella pruatajan Molk.) plantlets which were exposed to high temperature stress (32°C) during in vitro condition shown chlorosis symptoms, and death at prolonged time (Ajijah, unpublished). The ability of plants to
maintain chlorophyll persistence in high temperature stress conditions has also been used as an indicator of resistance of potato during in vitro selection to high temperature, and resulted new variants tolerant to high temperatures (10). In Pruacan, a plant species which prefer to be grown at a high altitude with optimum temperature between 18-21°C, chlorosis occurred at 32°C during in vitro culture (Ajijah, unpublished). However, symptoms of heat stress occurs varies according to the plant species and its natural environment (12).

According to (11), in general, heat stress occurred at temperature about 10-15 ºC above the optimum condition for the plant growth and development. As optimum temperature for cacao growth and development is 22-25ºC (FAO In 6), incubation of cacao plantlets for two months in temperature condition of 35ºC resulted in heat stress of plantlet which indicated by chlorosis. Based on this result, expose cacao plantlet to 35ºC temperature for 2 month might be used for in vitro selection of cacao somatic embryos. However, further study is needed to establish the method. In this study, the synthesis of chlorophyll is recovered after the plantlets were transferred into control temperature (Figure 2I).

Tabel 1. Developmental stages of somatic embryos under various incubation temperatures

| Incubation temperatures | Incubation period of somatic embryo |
|-------------------------|-----------------------------------|
|                         | 1 week | 2 weeks | 3 weeks | 4 weeks | 5 weeks | 6 weeks | 7 weeks | 8 weeks |
| 25 ºC                   | Embryo developed | Embryo developed | Initiation of shoot and root primordia | root and shoot primordia developed | Shoot primordia more clear | Leaves formation | Leaves and root growth | Leaves and root growth |
| 35 ºC                   | Embryo developed | Embryo developed | Initiation shoot and root primordia | Initiation of root and shoot primordia | Shoot primordium more clear | Leaves formation | Leaves and root growth | Leaves and root growth |
| 40 ºC                   | Embryo not developed | Embryo not developed | Not developed | Not developed | Not developed | Not developed | Not developed | Not developed |
| 45 ºC                   | Embryo death | Embryo death | Death | Death | Death | Death | Death | Death |
| 35-45 ºC                | Embryo death | Embryo death | Death | Death | Death | Death | Death | Death |
Figure 1. Developmental stages of cacao somatic embryos under various incubation temperatures: Somatic embryo at first day of incubation at 40°C (A), somatic embryo was turning brown and suffered death after 16 days of incubation at 40°C (B), 5 days at 35-45°C (C) and 3 days at 45°C (D). The Somatic embryo could germinate to form a primordia of apical shoot and root after 5 weeks of incubation at 25°C (E) and 35°C (F). The formation of leaf 6 weeks after incubation(G) and 2 moths (H) of incubation at 35°C. Germinated embryo incubated at 35°C, could develop to form planlet with normal leaves (I) after transferred into control temperature. rp = root primordia, sp = shoot primordia, yl = young leaf.

Incubation at 40°C, the somatic embryo still survive up to days 15th of incubation but did not develop further, and death at the 16th day of incubation, likewise the somatic embryo incubated at 45°C and 35-45°C successive deaths on days 3rd and 5th after incubation, respectively (Table 1 and Figure 1B, C, D). In this study, temperatures of 40°C for 16 days, 35-45°C for 5 days and 45°C for 3 days found to be lethal condition or caused death in cacao somatic embryos development. Although, hardening at 35°C for 3 days can extend the life phase of somatic embryos 2 days longer (from 3 days to 5 days) when
exposed to a lethal temperature of 45°C (Table 1), but finally the embryos death after 5 days. This indicated that 45°C temperature is too high and has exceeded the tolerance limit for cacao somatic embryos.

Maximum temperature is the most correlated climate factor to the yields of cacao plants followed by minimum temperature (7). Optimum temperature allows plant growth and physiological processes were occurred maximally, with maximum and minimum temperatures as one of the limiting factors. When the temperature or other environmental factors exceed the limit there will be stress condition occur which resulting the changes in metabolism and growth and development functions. These changes can be reversible or even irreversible and may lead to the death of the organism, depending on the intensity and duration of the stressfull conditions (12). In this study te 40°C and 45°C temperatures has exceeded the maximum condition that can made cacao plants tolerant to heat stress in the field with temperature of 38°C, that can cause damage and death to somatic embryo cells after a few days of incubation. If the tolerance limit and adaptation of plants to stress are exceeded, there will be a change in the plant at the molecular, physiological and cellular levels, which can cause negative effects to the plant growth and development and may trigger plant death. At a very high heat stress, cell damage and death can occur within minutes due to a very fast protein denaturation, but at moderate heat stress, damage and death of the cells can be observed after a few hours or days (11). In this study 40°C and 45°C temperatures gave moderate stress to cacao somatic embryos. However, the time at which the onset of symptoms of heat stress occurs, varies according to the plant species and its natural environment (12). When embryogenic callus of Pruacan were incubated at 32°C, the percentage of explants formed shoots and the average number of shoots per explant increased until the 2nd month but did not increase in the 3rd month (stagnant), while the average survival rate dropped dramatically from 80% in the first month to less than 20% and only around 10% in the 2nd and 3rd month (13). According to Howarth et al 2005 In (14) and (11) direct injuries due to high temperatures stress included protein denaturation and aggregation and increased fluidity of membrane lipids, while indirect or slower heat injuries included inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity. Membrane disintegration can cause leakage of ions and solutes which can be resulted in cell death. This condition is thought to cause death of cacao somatic embryos incubated at 40°C and 45°C temperatures.

Although the results of this study gave an illustration on how somatic embryos and cacao plantlets responded to the future temperature and provide an illustration for the development of in vitro selection method for high temperature stresses in cacao, further study is needed to ascertain at what temperature the in vitro selection for high temperature resistance in cacao will be carried out. The temperature of 35°C can be used for in vitro selection based on the ability of the plantlet to maintain chlorophyll persistence with a longer period, the temperature of 40°C can be used for in vitro selection based on the ability of the embryo to survive in several days (chronic), while the 45°C can be used for provide acute stress (heat shock). Further research is worth to be fulfilled to find out which one is more effective to be used in in vitro selection method for cacao.

4. Conclusion

The incubation temperature of 35°C, 40°C and 45°C resulted in stress condition for cacao somatic embryos. At 35 ºC incubation temperature, embryo-forming young leaves shows chlorosis symptoms after 2 months, while at 40°C and 45°C temperatures, somatic embryos turning death after 16 and 3 days of incubation, respectively. Therefore, the incubation temperature of 40°C and 45°C could be the lethal temperatures for in vitro selection in developing heat tolerant cacao somatic embryos, according to its ability to be survive within several days (chronic), while the 45°C can be used for providing acute stress (heat shock). Further research is worth to be fulfilled to find out the more effective method in determining in vitro selection method for high temperature stress in cacao.
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