Developing *in vitro* selection methods to high temperature stress in pruacan and cacao

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Abstract. Increasing in the earth's surface temperature due to global warming threatens the survival of plants in the sub-tropics and in the tropics areas. Looking for plant varieties that can adapt to the threat of global warming impacts is very important at this time. The availability of effective and efficient selection methods are needed to get it. This study aimed to develop an in vitro selection method for resistance to high temperature stress in two tropical plants: Pruacan (*Pimpinella pruatjan* Molk.) and Cacao (*Theobroma cacao* L.). The in vitro selection methods were developed by testing the embryogenic culture of pruacan and cacao somatic embryos development under various temperature incubations. The two plants showed a different level of tolerance to high-temperature stress. Temperatures of 35 °C for two months did not cause the death of cacao somatic embryos, but the temperature of 32 °C for 2 and 3 months had caused the death of pruacan somatic embryos by 80 and 90%, respectively. The 100% death of cacao somatic embryos occurred at 40 °C and 45 °C on days 16 and 5 of incubation, respectively. The in vitro selection for high-temperature stress in pruacan and cacao plants were at 32 °C and between 40-45 °C respectively. Developing *in vitro* selection methods in plant species is needed to face the challenges of climate change in the future.

1. Introduction

The increase in the earth's surface temperature (global warming) has threatened living things on earth, even in their natural habitat. In crops, high-temperature stress results in disturbances in various phases of growth and development, which reduces productivity mainly through disruptions of phenological development [1] and pollination process [2]. High temperature stress threatens not only subtropical but also tropical crops. Pruacan (*Pimpinella pruatjan* Molk.) and cacao (*Theobroma cacao* L.) are two tropical plants that have different habitats. Pruacan is an Indonesian medicinal plant that naturally spreads in the highlands at an altitude of 1,800 – 3,500 meter above sea level (m asl), however, at this time, they are only found in the Dieng Plateau at an altitude of 1,850-2,050 m asl with a temperature of 15-21 °C [3]. Meanwhile, cacao is the only plant that produces chocolate that grows well in the average annual temperature 22 to 25 °C with a minimum temperature 21 °C and a maximum 32 °C [4]. Perennial crops are known to have less adaptation and more susceptible to climate change [1], including cacao [5]. High-temperature stress, especially the maximum temperature during the dry season, is predicted to threaten cacao in the future, besides drought and disease [4]. How the two plants tolerate high-temperature stress at the in vitro level will be discussed in this paper.

Screening of tolerant germplasm is an important step to anticipate the impact of global warming and in such circumstances, an effective and efficient method of selection is indispensable. In vitro
selection using tissue culture technique has advantages and is a cost-effective technique for developing plants tolerant to biotic and abiotic stresses [6]. Therefore, developing an in vitro selection method to high-temperature stress in plants is needed. This study aimed to develop an in vitro selection method of two tropical plants with different natural habitats, namely pruacan and cacao. The development of in vitro regeneration methods and optimization of the temperature range for in vitro selection in both plants will be discussed in this paper.

2. Materials and methods
Before developing an in vitro selection method, developing an in vitro regeneration method through tissue culture is necessary. In vitro regeneration methods for pruacan and cacao were carried out through indirect somatic embryogenesis [7][8]. Indirect somatic embryogenesis includes callus induction, somatic embryo formation, and embryo conversion to form plantlets. Pruacan callus was induced from leaf explants using Murashige and Skoog (MS) media with the addition of 2 mg/l 2,4-D and 0.5 mg/l picloram (Figure 1A). Meanwhile, embryogenic callus formation (Figure 1B), somatic embryo formation (Figure 1C-D), and plantlet formation (Figure 1E) were carried out on the Kuniyuki Walnut Driver medium (DKW) with the addition of 5 mg/l IBA in the lighting conditions with TL lamps [7].

![Figure 1](image1.png)

Figure 1. Pruacan regeneration through indirect somatic embryogenesis: callus induction from leaf explants (A), embryogenic callus formation (B), somatic embryo formation (C, D), and embryo conversion to form plantlets (E).

Cacao callus was induced from petal explants or staminodes from flower buds on DKW medium with the addition of 2 mg/l 2,4-D and 0.125-0.5 mg/l kinetin (Figures 2 A, B, and C). Somatic embryo formation (Figure 2 D-E) and conversion of somatic embryos to plantlets (Figure 2F) were carried out on DKW medium without growth regulators [8].
Development of an in vitro selection method for resistance to high-temperature stress in pruacan begins with testing the growth and development of embryogenic callus at three levels of incubation temperature, namely 17.3 ± 0.5 °C (control), 23.3 ± 2.1 °C (temperature 2), and 32.8 ± 1.7 °C (temperature 3) [7]. Observations were made on the variable fresh weight addition, the percentage of explants forming plantlets, the number of plantlets per explant, and the mortality rate of explants at the age of 1, 2, and 3 months after incubation. While the temperature optimization for in vitro selection of cacao was initiated by testing the growth and development of the cotyledonal stage of somatic embryos at three incubation temperature levels, namely 25 °C (control), 35 °C, 40 °C, and 45 °C. The observation was made on the appearance and the survival rate of somatic embryos.

3. Results and discussion
The growth and development of pruacan embryogenic culture were significantly inhibited at 32.8 ± 1.7 °C. Increased mean fresh weight of explants, percentage of plantlets formation, number of plantlets per explant, and percentage of explants survival at 32.8 ± 1.7 °C was significantly lower compared to that at control temperature and 23.3 ± 2.1 °C at culture age 1, 2, and 3 months. Meanwhile, 23.3 ± 2.1 °C produced growth and development, which were not significantly different from the control except after three months of culture. Temperature 32.8 ± 1.7 was also a lethal temperature for pruacan embryogenic culture resulting in 11.1, 81.2, and 87.5% culture mortality at 1, 2, and 3 months of age, respectively (Figure 3) [7]. Figure 4 shows the development of pruacan embryogenic cultures at each incubation temperature at cultures aged 1, 2, and 3 months.
Figure 3. Effect of high-temperature stress on the growth and development of pruacan embryogenic culture: the addition of fresh explant weight (A), the percentage of explants forming plantlets (B), the number of plantlets per explant (C), and the percentage of live explants (D) (Source: Ajijah et al 2010 [7]).

Figure 4. Development of pruacan embryogenic cultures aged 1, 2, and 3 months at three incubation temperature levels.
Temperatures 40°C and 45°C resulted in 100% mortality of cacao somatic embryos on days 16 and 5 after incubation, respectively (Figure 5B and Figure 5C). While temperature 35°C did not cause death in cacao somatic embryos, somatic embryos could grow and develop to form shoots and roots at that temperature (Figure 5D). However, the continuous exposure of the somatic embryos to temperature 35°C for two months with subcultures every one month, resulted in pale-colored shoots and young leaves indicating inhibition of chlorophyll synthesis or chlorophyll degradation (chlorosis) (Figure 5E) [9]. Chlorosis also occurred in pruacan cultures, which were incubated at 32.8 ± 1.7°C (Figure 4). Chlorosis in plants occurs in high-temperature stress conditions and can be used as a selection criterion for high-temperature stress resistance [10].

From this study on those two plants, it can be seen that the two plants showed different tolerance levels to high-temperature stress under in vitro conditions. Cacao had a higher tolerance to higher-temperature stress (35°C) than pruacan, which had died at 32°C. The difference in response between the two plants was probably due to the difference in the developmental stage of the explants used, namely embryogenic callus in pruacan and somatic embryo in cacao and natural habitats of the two plants. In vitro selection for resistance to high-temperature stress based on the mortality rate of explants in pruacan can be done using embryogenic callus explants at a temperature of 32°C with an incubation duration of 3 months, while in vitro selection on cocoa plants can be done using somatic embryo explants at a temperature range of 40-45°C with optimization in the incubation period. In vitro selection for the nature of tolerance to high-temperature stress in cacao plants can also be done based on the ability to maintain (persistence) chlorophyll at 35°C with an incubation period of 2 months.

4. Conclusion
Developing in vitro selection methods for resistance to high temperature stress and other biotic and abiotic stresses is needed to face climate change challenges in the future, including an increase in the earth's surface temperature. The development of selection methods for high-temperature stress should be carried out for each species according to the species' characteristics. In vitro selection to high temperature in pruacan plants can be done at a temperature of 32°C with an incubation duration of 3 months, while in cacao plants, it can be done at a temperature range of 40-45°C with optimization in the incubation period, or was done based on the ability to maintain (persistence) chlorophyll at 35°C with an incubation period of two months.
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