Advanced *in vitro* Embryo Rescue Techniques for Improving Plantlet Development of Sternospermocapic (Black Opal) Grapes

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Abstract

The Black Opal grape cultivar (*Vitis vinifera* L.), a stenospermocarpic seedless variety, has been extensively cultivated in Thailand. However, a recent surge in disease has critically threatened yield production in these seedless cultivars. This study explores the efficacy of advanced in vitro embryo rescue techniques and plantlet development to produce resistant progeny. Three primary factors were examined: berry sampling age (1 - 4 months after flowering, MAF), BA supplementation (0 0.5 1 and 1.5 mg/L) in MS media, and temperature conditions (4°C and room temperature). Results demonstrated that a cold treatment at 4°C for 7 days notably stimulated embryo development compared to room temperature and extended berry shelf life. The highest germination rates were observed in embryos from berries at 3 MAF cultured in 1.0 - 1.5 mg/L BA media, with embryo formation, germination, and plantlet development rates ranging from 13.3 - 33.3%, 6.7 - 20.0%, and 6.7 - 20.0%, respectively. While temperature did not significantly impact embryo development, it effectively prolonged berry shelf life. Optimal embryo formation rates were achieved at 3 MAF with 1.0 and 0.5 mg/L BA, at 40% and 33.3%, respectively. For plantlet development, 0.2 mg/L BA in WPM media was most effective in supporting the progression of embryos into plantlets.

Keywords: Somatic embryogenesis, in vitro culture, seedless grape, embryo rescue

Introduction

Parthenocarpy of seedless berries is the most common species of seedless grapes seen in vineyards; they develop without seeds through anthesis without fertilization. The second variety of berries is stenospermocarpy, meaning they fertilize but abort the embryo while the berry develops via a seed trace (Stout, 1936; Liu *et al*., 2003). The genotype is determined by the efficiency of embryo recovery, the size of the seed trace that remains in the berry, and the presence of endosperm inside the ovules or some dryness. Many seedless cultivars of this type are widely grown and used due to their adaptability to a wide range of climates, vigorous growth, high yield, small to medium berry size, good quality, and good flavor. Consumers around the world prefer these cultivars for table grapes (fresh fruit) and raisins, leading to increasing demand every year. Despite this, genotypes always support embryo rescue, but factors such as berry age sampling, plant growth regulators (PGR), and storage temperature play an important role. The age of grape berries after the flowering date determines whether they are immature or mature and indicates the need for embryo rescue before abortion. Embryo culture requires

specific plant hormones in the inoculation media, which also affects the ratio of cytokinin to auxin (Li *et al*., 2015). The Black Opal cultivar (*Vitis vinifera* L.) is a stenospermocarpic grape. It is famous for its high nutritional value and is one of the most important cash crops worldwide, as well as being a main production crop in Thailand for a long time. Seedless grapes are particularly susceptible to fungal diseases, especially in tropical regions with high moisture and temperature. Currently, Thailand is facing a serious problem with disease, greatly decreasing productivity. This is considered a serious crisis for this cultivar. For plantlet development, somatic embryogenesis, or embryo rescue from immature embryos from the berry age of stenospermocarpic grapes, is a highly efficient technique, and improving the protocol is important for breeding programs.

In recent years, *in vitro* somatic embryogenesis and embryo rescue techniques from stenospermocarpy have been reported to improve the efficiency of hybrid breeding technology. Hence, embryo rescue is useful for hybridizing seedless grapes (Tian and Wang, 2008; Li *et al*., 2018; Li *et al*., 2020). The proportion of seedless progeny obtained from the embryo rescue technique is 85% (Agüero *et al*., 1995), making it a frequently used method to obtain new hybrid progeny. However, researchers invest a lot of time in improving the embryo rescue technique by using immature embryos from the berry age of seedless grape cultivars via *in vitro* culture on MS media. There are still some challenging factors to develop, including genotype, sampling dates, composition of embryo needs, plant growth regulators (PGR), and culture conditions (Chatbanyong and Torregrosa, 2015; Jiao *et al*., 2018). Nowadays, embryo rescue is one of the important techniques in tissue culture and is also a very popular breeding method. In this field, genotype and MS media have been found to play crucial roles in the success of this technique to enhance germination to a useful level (Ramming *et al*., 1990; Tian and Wang, 2008). Research details the development of techniques to facilitate the high production of progeny from the early and immature stages of grapes, performed in many countries such as Israel (Ramming *et al*., 1990) and China (Tian and Wang, 2008; Li *et al*., 2014; Li *et al*., 2018; Li *et al*., 2020).

This study aimed to establish the optimum zygotic embryo stage from varying ages of berries, BA concentration in media, and treated zygotic embryo storage in cold storage to improve embryo rescue techniques and plantlet development of stenospermocarpic grapes. It was expected that appropriate embryo rescue techniques and plantlet development protocols will be obtained. However, there were still problems in embryo rescue techniques, such as low survival rates and complicated operations. Other research has indicated that during the embryo formation stage, cytokinin such as BA is often added to the media to stimulate embryo germination (Gray *et al*., 1990; Bharathy *et al*., 2005). Moreover, embryos require additional stimuli from cold temperatures and PGR to improve germination. Chilling appropriately provides the external force necessary for embryo development (Aguero *et al*., 1995). This study investigates suitable PGRs, particularly cytokinin, and its concentration, although most previous experiments used auxins or a mixture of other PGRs and cold temperatures before dissecting embryos.

Materials and Methods

The plant materials were 7-year-old Black Opal grapevines grown under a plastic roof at the Pomology Division Farm, Faculty of Agricultural Production, Maejo University, located in Chiang Mai province, and at a contract farming orchard in Chiang Rai province, Thailand. The stages of the embryos were investigated, with berries sampled and harvested at intervals of 1, 2, 3, and 4 months after flowering (MAF). Seedless berries from each month were separated and kept at room temperature (26°C) and cold temperature (4°C) for 7 days. The experimental design was a factorial in Completely Randomized Design (Factorial in CRD), with 16 treatments and 3 replications, each consisting of 40 seed traces, focusing on three factors: berries storage (room temperature and cold temperature), the sampling time of embryos from varying ages of berries (1 - 4 MAF), and BA concentrations in MS media (0, 0.5, 1.0, and 1.5 mg/L).

Embryo cultivation on the media involved the following steps: the berries were disinfected with bleach for 30 seconds, then soaked in 10% and 5% (w/v) NaClO containing 0.1% Tween 20 for 10 minutes, and washed with sterilized distilled water two times under aseptic conditions until all bubbles disappeared. For embryo cultivation, ovules were transferred to initiation culture media variants, including MS media (Murashige and Skoog, 1962) with 0, 0.5, 1, and 1.5 mg/L BA, 0.3 g/L activated carbon (AC), 30 g/L sucrose, and 6.2 g/L agar. After 2 months, the embryos were dissected from the ovules and cultured in WPM media with 0.2 mg/L BA, 30 g/L sucrose, 0.15 g/L AC, and 6.0 g/L AC in a glass container. Finally, the embryos germinated and were transferred to suitable media containing half-strength MS salts plus 2 mg/L NAA for root elongation.

All media were adjusted to pH 5.6 with 0.1 M NaOH or 0.1 M HCl, then autoclaved at 121°C for 25 minutes and cooled to room temperature. The embryos were grown in a culture room at 25±2°C, 100% relative humidity, and 40 µmol/m-2/s of white light. The steps of embryogenesis are displayed in Figure 1, where the embryo is divided into 4 phases: globular-shaped, heart-shaped, torpedo-shaped, and cotyledon-shaped. Regarding ovule appearance after culture, there was no embryo formation inside the ovule if the embryo was milky white or ivory, and developed inside the ovule. In the final stage, half-strength MS was applied with NAA to stimulate rooting and transplantation for plantlet development. Embryo formation was divided and calculated into three stages: 1) embryo formation rate (%), 2) embryo germination rate (%), and 3) plantlet development (%) (Figure 2). In the first phase, full-strength MS media were applied for embryo formation to supply ovules with the necessary nutrients for growth, with the addition of different BA concentrations to stimulate embryo development. In the second phase, WPM was applied for embryo germination and BA to provide the necessary nutrients for embryo culture. During embryo formation, as in the torpedo-shaped stage, the embryo develops through 4 stages. The third phase, plantlet development, was the most important step before transplanting, requiring careful attention to proper operation and cultivation.

Data analysis: All experiments were repeated three times, and the data were expressed as the means ± standard deviation (SD) and analyzed by ANOVA. The treatment means were tested for differences from the controls by LSD, at $p \leq 0.01$ and 0.05.

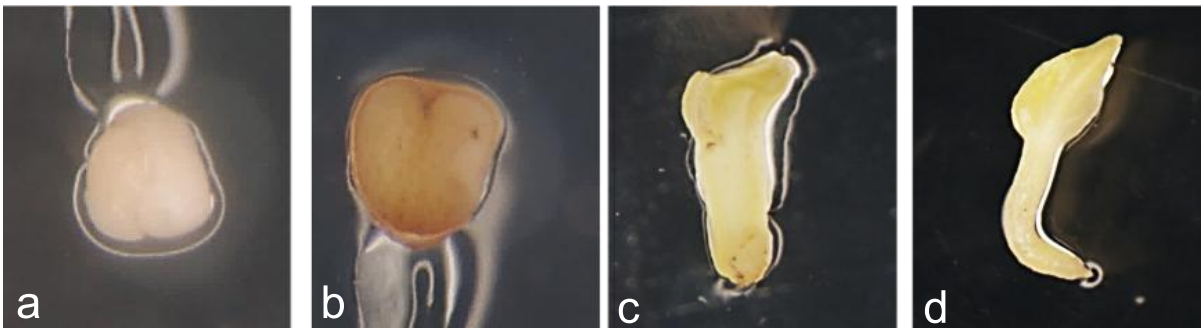

Figure 1 The four stages of embryo development include (a) globular, (b) heart, (c) torpedo, and (d) cotyledon-shaped

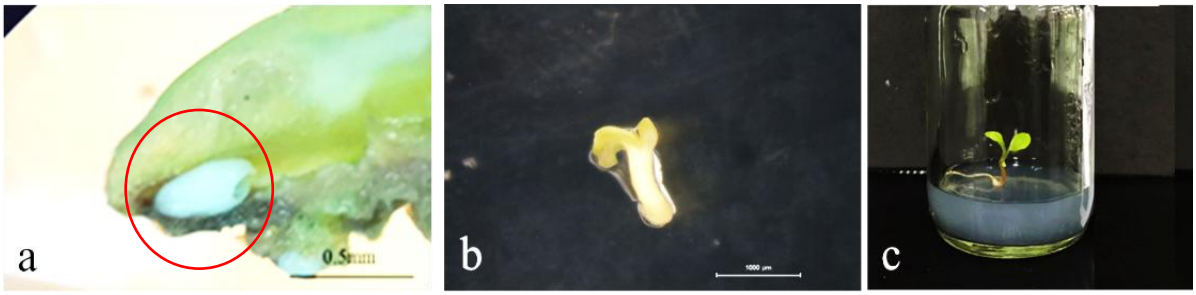

Figure 2 Calculation index of each stage: (a) embryo formation (red circle); Bar = 0.5 mm, (b) embryo germination; Bar=1.0 mm, and (c) plantlet development

Results and Discussion

The embryo rescue technique for embryo and plantlet development depends on many factors that influence embryo germination. These factors include PGR, sampling time of embryo stages (berry age or harvesting time; MAF), and storage conditions (room and cold temperature), although genotype is also important in embryo development. It was found that all concentrations of BA did not significantly affect embryo and plantlet development. The sampling time had a significant effect, with the highest embryo development rate from berries aged at 3 MAFs, yielding 13.3 - 33.3% embryo formation, 6.7 - 20.0% embryo germination, and 6.7 - 20.0% plantlet development. This was followed by 2 MAFs, with 16.4 - 28.9% embryo formation, 10.9 - 20.0% embryo germination, and 3.6 - 8.9% plantlet development. At 1 MAF, embryos did not form in the early stage of berries, with only 10.8 - 15.31% embryo formation, 4.6 - 5.3% embryo germination, and 1.3 - 3.5% plantlet development. At 4 MAFs, all samples were aborted due to late sampling and ripening. There was no interaction or effect of BA concentration and sampling time on embryo development (Table 1). Optimal embryo rescue is limited by immaturity before and over-maturity after this time, leading to natural abortion.

The Black Opal in this research presented the optimum sampling time at 3 MAFs, followed by 2 MAFs, and had a suitable time for embryo rescue. The 4 MAF was the important one to know the limit of embryo survival. Climate and planting methods, which differ from temperate areas, contribute to this unpredictability. Therefore, further research testing is needed to determine the best culture dates for Black Opal and other cultivars, as the optimum

culture dates and season influence embryo maturity (Pommer *et al*., 1995). Although no direct evidence proved that the small trace caused difficult embryo growth, seed trace size did not affect embryo development (Stout, 1936). The small size and difficult operations could warrant further investigation. Most studies have revealed that the sampling time of ovules within 40 to 70 days after the flowering period is crucial, with too early or too late sampling seriously influencing results. Liu *et al*. (2003) indicated that the best berry collection time is 43 to 70 days for stenospermocarpic seedless grapes and suggested that weak embryo growth may be linked to integument browning. Early cultivation was immature and unstable, making it difficult to establish on the growth media and even to dissect ovules. Late-stage embryos were also tough to culture, often aborted before cultivation, leading to low formation rates (Xu *et al*., 2008).

The sampling time of embryos from the berry age of the Black Opal seedless grape at 1 MAF resulted in a hard seed coat and presented no embryo growth due to the difficulty of operating with very small seeds or seed traces. At 4 MAFs, contamination was caused by the gradual maturation of berries, which become juicy and have increased sugar content. The size of the seed trace may cause different survival rates in embryo rescue (Bouquet and Davis, 1989; Li *et al*., 2020). Another factor is that the seed coat produces secondary metabolites (integument browning), which retard embryo formation (Liu *et al*., 2003). These substances, such as tannins, phenols, or other toxic compounds, accumulate in the media through the ovule envelopment, potentially leading to embryo abortion or underdevelopment *in vitro* (Valdez, 2005).

When comparing temperature stimulation and sampling time, the embryo rescue from berry age (MAF) and BA concentration on embryo and plantlet development of the 'Black Opal' grape showed that storage conditions (room and cold temperatures) had no significant difference in effect on embryo development; however, they could extend the shelf life of berries. Embryo development was significantly affected by berry age at the time of sampling in both 0.5 and 1.0 mg/L BA cultures. The highest rates of embryo formation and development were observed at 3 MAFs with 1.0 and 0.5 mg/L BA, resulting in 40.0% embryo formation, 33.3% embryo development in cold temperature, 33.3% embryo formation, and 20.0% in room temperature, indicating the optimum conditions. There were no significant differences at 2 MAFs, with 33.3% embryo formation and 18.0% embryo development in cold temperatures, respectively.

Table 1 Effect of BA and sampling time (embryo from berry age) on embryo germination of Black Opal grape at room temperature

BA concentration (mg/L) (A)	Sampling time (MAF) (B)	Embryo formation (%)	Embryo germination (%)	Plantlet development (%)
0	1	10.8bc	4.6bc	0.0c
	2	23.6ab	16.4a	3.6c
	3	20.0ab	20.0a	20.0a
	4	0.0c	0.0c	0.0c
0.5	1	12.5b	6.3bc	1.3c
	2	28.9a	17.8a	8.9bc
	3	33.3a	20.0a	6.7bc
	4	0.0c	0.0c	0.0c
1.0	1	15.3b	9.4b	3.5c
	2	26.0a	10.0b	4.0c
	3	13.3b	6.7bc	6.7bc
	4	0.0c	0.0c	0.0c
1.5	1	13.3b	5.3bc	2.7c
	2	16.4b	10.9b	3.6c
	3	33.3a	16.7a	13.3ab
	4	0.0c	0.0c	0.0c
BA concentration (A)		ns	ns	ns
Sampling time, embryo of berry age (B)		**	*	*
A x B		ns	ns	ns
CV. (%)		75.2	81.3	119.8

Remarks: * / **/ ns represent statistical significance at 0.05, 0.01 and not significant, respectively

Means within columns followed by different letters are significant at alpha 0.05

However, there were highly significant differences in the effects of other treatments, with rare occurrences of embryo formation and significant differences in embryo development. At 4 MAFs, embryos did not form at all. Embryos are very small and difficult to dissect if the sampling time is too early, leading to abortion. There were no interactions or effects on embryo formation. Plantlet development showed no significant differences across all treatments, and there were no interaction effects between BA concentration and sampling times on embryo and plantlet development (Table 2). The temperature conditions for the seedless grape berries, kept at both room (26°C) and cold (4°C) temperatures during the sampling time, significantly influenced embryo formation. As berries aged to 4 MAFs, the embryos were cultured in 0.5 and 1.0 ml/L BA media. The embryos of the Black Opal grape were stored at 4°C for 7 days and compared with those stored at room temperature (26°C). Both embryo and plantlet development rates at 1 and 4 MAFs gradually decreased, with some embryos showing malformations leading to physiological termination or abnormality. Although the two factors-storage condition and the sampling time of embryos from berry age-showed no interaction effect on embryo formation, other research has suggested that embryo development might require sufficient cold temperature duration. Some scholars attempted to keep embryos at cold temperatures for several months, but there was still no effect on embryo rescue (Emershad and Ramming, 1984; Gray *et al*., 1990). Sundouri *et al*. (2014) reported that the seeds of stone fruit could still germinate within 60 to 75 days after crossing if they were rescued, regardless of low temperature. However, the sampling harvest time of the embryo may determine the efficiency of embryo rescue, and cold treatment or the addition of plant hormones can indirectly improve embryo survival. Other scholars also reported that chilling ovules for 45 days before germination could facilitate embryo formation (Ramming, 1990).

This experiment does not show that adding BA to media at different temperatures can improve the berry's shelf life or affect embryo development, but there are still some relevant suggestions from other studies. Previous research has demonstrated that embryos preserved at low temperatures during the cultured phase and the addition of BA in the cultured media had the best effect on embryo rescue (Aili *et al*., 2002). Ramming (1990) indicated that embryos stratified at 0.5°C for 3 months, with various sucrose levels added, showed an improved survival rate.

However, the three factors of berry age, cold temperature, and BA concentrations are scarcely effective in promoting embryo development in this study. Plantlet development of the Black Opal at cold temperatures showed that some of the plantlets developed from embryos survived until transplanting. It is assumed that cold temperature and BA may have affected the subsequent growth of embryos. The growth conditions of the 'Black Opal' plantlets cultured from 2 and 3 MAFs embryos at room and cold temperatures with 0.5 mg/L BA media for 4 months (Figure 3), and the plantlet development at 1 - 3 months after transplantation in the media for 3 months (Figure 4). Previous studies proposed that chilling stimulates embryo germination and can also extend the shelf life of seedless berries and embryos. The most common cause of a low embryo germination rate is embryo dormancy or abnormal growth.

Table 2 Comparison of temperature conditions, sampling time, and BA on embryo and plantlet development of 'Black Opal' seedless grape (3 months after transplanting)

Storage condition (A)	Sampling time (MAF); (B)	BA Concentration (mg/L); (C)	Embryo formation (%)	Embryo germination (%)	Plantlet development (%)
Room temperature	1	0.5	12.5b	6.3bc	1.25
		1.0	15.3b	9.4bc	3.5
	2	0.5	28.9a	17.8a	8.9
		1.0	29.0a	18.0a	8.9
	3	0.5	33.3a	20.0a	6.7
		1.0	13.3b	6.7bc	6.7
	4	0.5	0.0c	0.0c	0.0
		1.0	0.0c	0.0c	0.0
Cold temperature	1	0.5	4.6bc	1.5c	0.0
		1.0	11.4bc	10.0b	1.4
	2	0.5	30.0a	10.0b	3.3
		1.0	16.0b	12.0b	4.0
	3	0.5	20.0ab	6.7bc	6.7
		1.0	40.0a	33.3a	0.0
	4	0.5	0.0c	0.0c	0.0
		1.0	0.0c	0.0c	0.0
Storage condition (A)			ns	ns	ns
Sampling time (MAF) (B)			**	*	ns
Concentration of BA (C)			ns	ns	ns
A x B			ns	ns	ns
A x C			ns	ns	ns
B x C			ns	ns	ns
A x B x C			ns	ns	ns
CV. (%)			72.5	143.8	118.5

Remarks: * / **/ ns represent statistical significance at 0.05, 0.01, and not significant, respectively.
Means within columns followed by different letters are significant at alpha 0.05

Emershad and Ramming (1984) reported that embryos cultivated at 1.5ºC for 30 days before ovule excision did not grow and became necrotic. Scholars later repeated a similar study, placing embryos in cold stratification at 4ºC for 6 weeks, but found no significant differences (Gray *et al*., 1990). Papers have proposed that chilling, which is intended to stimulate embryo germination, can also extend the shelf life of seedless berries by storing them in a refrigerator after harvest from the grapevine. The most common causes of low embryo germination rates are embryo dormancy or abnormal growth. However, room and cold temperatures had no significant effect on the embryo germination rate, but they can postpone the experiment dates. During this study, small traces were found in 'Black Opal,' which could be a significant reason for our research results. Unfortunately, this aspect was not the focus of this experiment.

This technique will accelerate the development of the immature zygotic embryo from seedless grape cultivars, allowing for the rapid production of resistant grape plantlets. In commercial grape cultivars, many factors are known to influence embryo growth, including genotypes, sampling dates, plant growth regulators (PGR), and transplantation.

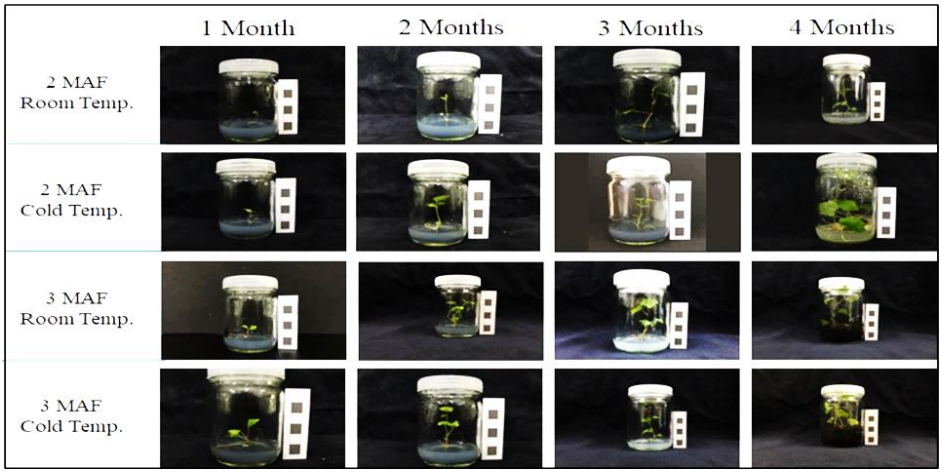

Figure 3 The 'Black Opal' grape plantlets from 2 and 3 MAF embryos were cultured on BA media for 1 - 4 months

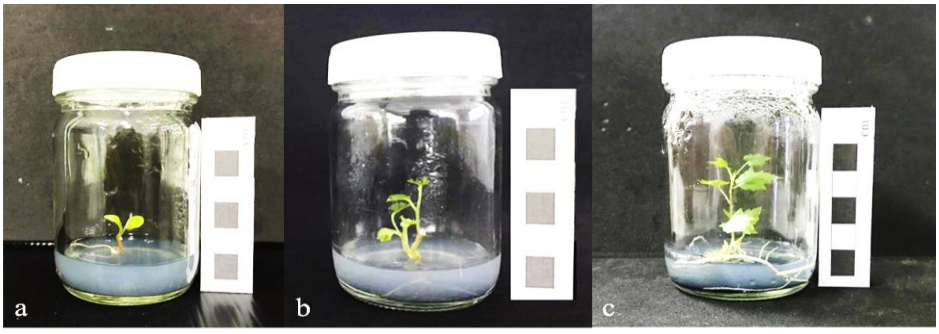

Figure 4 The plantlet development at a) 1 month, b) 2 months, and c) 3 months after transplantation

Conclusion

The optimum sampling time for embryo rescue of the Black Opal seedless grape is 3 months after flowering. At this age, the largest number of embryos was formed, with 40.0% embryo formation and 33.3% embryo development in cold temperatures. There was no significant effect at room temperature, with 33.3% embryo formation and 20.0% embryo development. There were no significant differences at 2 MAFs, with 33.3% embryo formation and 18.0% embryo development in cold temperatures.

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