Jurnal Biologi Tropis

Original Research Paper

In vitro Propagation of Singgalang Cabbage (*Brassica oleracea* var. *capitata* L.) on Murashige and Skoog Modification Media for Preservation Purpose

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Article History
Received : Maret 25th, 2024
Revised : April 01th, 2024
Accepted : April 27th, 2024

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Abstract*:* Singgalang Cabbage is one of the local cabbage cultivated on the foothills of Mount Singgalang, West Sumatra. Recently, the existence of this cabbage has decreased, so conservation and preservation efforts need to be carried out. This research was conducted to develop method for *in vitro* propagation and preservation of singgalang cabbage. The Murashige and Skoog (MS) were used as basal media with two experimental stages, *i.e.*, shoot initiation with *6-Benzylaminopurine* (BAP), and root induction and plantlet preservation with modification of MS media. The nodal and shoots were used as explants. The results showed that increment of BAP concentrations gave a significant effect on shoot initiation after 60 days of treatment. MS media-enriched with BAP 2 mg/L gave significant increment of shoots (4 shoots/nodus) and leaves (11.67) numbers when compared to other treatments. For root induction and plantlet preservation, it was found that the earlier of root formation was observed in modification of MS media at ½ and ¼ strength. Meanwhile, MS media at ⅛ strength was observed to be better media for plant height increment (4.75 cm) when compared to other treatments. It was found that the plantlets survived and grew well after 120 days under *in vitro* condition.

Keywords: *6-Benzylaminopurine*, MS modification media, root induction, shoot initiation, singgalang cabbage.

Introduction

Cabbage (*Brassica* spp.) is a widely cultivated vegetable crop in Indonesia that is divided into two main types, namely annuals and biennial types. Annual cabbage is widely cultivated because it can flower in the tropics and produce seeds. This is in contrast to biennial cabbage, which cannot flower in the tropics, so the provision of seedlings only comes from shoot cuttings, which causes the quality of seedlings to decrease (Singh *et al*., 2010). Singgalang Cabbage (*Brassica oleracea* var. *capitata* L.), one of the local cabbages of West Sumatra, has been cultivated for a long time by farmers in the area around the slopes of Mount Singgalang. The three variants are Biaso, Batang Hitam, and Senggan (Afdi *et al*., 2005).

Singgalang cabbage has a distinctive flavor widely used in West Sumatra's culinary specialties, but its existence is decreasing due to declining of farmers to cultivate it. It was caused by the reason of less productivity of this vegetable when compared to introduced cultivars. On the other hand, according to (Rincon-Sanchez & Ruiz-Torres, 2018) local cultivars have higher potential than introduced cultivars, including being more adaptive to the local climate and tolerant of environmental stresses. In addition, the lack of farmers to cultivate singgalang cabbage is also caused by the retention of pests and diseases and the conversion of land functions for annual cabbage planting. Therefore, efforts to maintain the existence of the singgalang cabbage variant to exist as a typical vegetable of West Sumatra are

significant to be attempted, one of which is through tissue culture techniques and *in vitro* storage so that its existence will be maintained in the Future.

Tissue culture techniques for mass propagation and preservation purposes have been widely developed, including in cabbage species (Alawadi *et al*., 2019; Gambhir *et al*., 2017; Gerszberg *et al*., 2015; Pavlović *et al*., 2010; Srikanth *et al*., 2016). Gerszberg *et al*. (2015) used Murashige-Skoog (MS) basal medium with the addition of 6-*Benzylaminopurin* (BAP) in shoot propagation of eight *B. oleracea* var. *capitata* cultivars. Rahman *et al*. (2021) also used BAP to regenerate shoots *in vitro* on MS media for *B. oleracea* var. *italica* plants. The range of BAP used in the above research commonly from 1-5 mg/L.

In germplasm storage or preservation, tissue culture techniques play a pivotal role in the conservation of genetic diversity through the use of growth inhibition technique (Chauhan *et al*., 2019; Gianní & Sottile, 2015; Trejgell *et al*., 2015). The preservation of genetic diversity is further facilitated by minimal growth strategies. In the context of *in vitro* preservation utilizing minimal growth techniques, this entails the reduction of incubation temperatures and the modification or manipulation of the culture medium, thereby altering the availability of nutrients (Chen & Dribnenki, 2004).

Explant with roots is important in preservation of plant material genetics. Research conducted by Ghanbar *et al*. (2016) and *Islam et al*. (2017), found that the use of MS ½ is better for root induction. Before, Azad et al. (2005) found that increasing the MS strength was better for root formation, while Dhavala & Rathore (2010) found that MS ½ is the most effective medium for root induction.

Based on the description above, research on *in vitro* propagation of singgalang cabbage (*B. oleracea var. capitata*) needs to be done. Given the limited planting area and the declining farmers interest in cultivation of this vegetable, the existence of this cabbage will be reduced or even lost if there is no effort to propagate and preserve it. The tissue culture technique is an alternative way to maintain its existence as a local vegetable with high cultural value for the Minangkabau tribe in West Sumatra.

Materials and Methods

Time and place

This research was conducted in the Laboratory of Plant Physiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas. It was held from February to May 2023.

Plant material

The singgalang cabbage variant used in this experiment was Biaso variant. The samples were collected in the foothills of mount Singgalang. The shoots were cultured in MS media to get nodal free contaminated explants (Figure 1).

Figure 1. Source of singgalang cabbage explants used in this experiment. The explants were grown in MS media without growth regulator for approximately 4-6 weeks in each sub-culture cycle.

Experiment design

This research was conducted in an experimental method using a completely randomized design (CRD) consisting of four treatments and six replications. The experiment was conducted in two stages, *i.e.*:

1. *In vitro* shoot initiation from nodal explants. In this stage, the nodal were planted in MS media supplemented with 6-*Benzileaminopurine* (BAP) as follows:
   A= MS as a control (without BAP)
   B= MS + 1 mg/L BAP
   C= MS + 2 mg/L BAP
   D= MS + 3 mg/L BAP
2. *In vitro* root induction from shoot explants. In this stage, the shoots were planted in modification of MS media as follows:
   A= MS full strength (MS)
   B= MS half strength (MS ½)
   C= MS a quarter strength (MS ¼)

D= MS one-eighth strength (MS ⅛)

Research procedure

Sterilization of equipment

Glass bottles, tweezers, scalpel handles, and a Petri dish were soaked in commercial bleaching solution for one day. All equipment was washed and rinsed with running tap water then dried in the oven before sterilized using autoclave (17.5 psi and 121°C for 15 min).

Preparation of MS media stock solution

In this experiment, five stock of MS media solutions were prepared containing macronutrient, micronutrient, iron sources, vitamins, and myo-inositol. Their solubilities were 10x concentration for macronutrients, 50 times concentration for micronutrient, iron sources, and vitamins, and 20x concentration for myo-inositol based on MS media recipe by Murashige and Skoog (1962).

Preparation of treatment media

a. Shoot initiation media using BAP.

The treatment media for shoot initiation were MS media supplemented with BAP at the concentration of 0-3 mg/L. For each treatment media, BAP stock solution (100 mg/L) was pipetted based on concentration mentioned above. The pH of media was adjusted at 5.8-6 using pH meter. For one liter of MS media, 30 g sucrose and 7 g agar were added before boiled and poured into culture bottles. The bottles were tightly closed with a sterilized bottle cap. All treatment media were sterilized using an autoclave (17.5 psi 121°C for 15 min). The sterilized media were kept in the culture room for 3-7 days before being used to check for contamination.

b. Root induction media using modification of MS concentration.

The modification of MS concentration was used for root induction media. The concentration of MS media was modified from full strength to one-eighth strength (MS ⅛). After preparation of each stock solution based on concentration treatments, the procedure was similar to preparation of shoot initiation media, except for using of BAP stock solution.

Planting explants into treatment media and growth maintenance in growth room

The explants used for treatment consist of nodal and shoots. Nodal was used as explants for shoot initiation, while shoots for root induction. Nodal explants were planted into MS media supplemented with BAP, while shoot explants were planted into modification of MS media. All explants were cultured in treatment media, sealed with transparent parafilm before kept in growth room. The culture bottles were placed in the growth room for maintaining the explants growth. The growth room was set under photoperiodism 12HL/12HD with temperature 25±1ºC. The cultured bottles were kept under this condition until 60 days for shoot initiation and 30 to 120 days for root induction and plantlet preservation.

Parameter of observation and data analysis

The parameters were observed in this experiment including 1) shoot initiation - the first day of shoot emergence, number of leaves, number of shoots, shoot length, and root formation, 2) root induction and plantlet preservation - plantlet height, first day of root emergence, number of primary roots, root length, and plantlet morphology. All data were collected and categorized into qualitative and quantitative data. Qualitative data were analyzed descriptively, while quantitative data were analyzed using Analysis of variant (ANOVA) at $p < 0.05$. If the ANOVA was significantly difference between each treatment, the data were then analyzed using Duncan's New Multiple Range Test (DNMRT) at $p<0.05$.

Result and Discussion

In vitro Shoot Initiation

Average of first day shoot emergence

Table 1 shows that the average of first day shoot emergence is not significantly different between MS and MS media supplemented with BAP. It was shown that using MS media or the addition of BAP (plant growth regulator) did not affect the rapid emergence of a shoot on nodal explants. It was showed that BAP does not affect the time of shoot emergence. BAP is a synthetic cytokinin that induces shoot in the explant (*i.e.*, leaves cutting, nodal, and petiole)

and stimulates the growth of axillary and adventitious buds. In can be concluded that addition of BAP in MS media did not accelerate the emergence of shoot in this experiment by using nodal explants.

Table 1. The average of first day shoot emergence in nodal explants of singgalang cabbage on MS media supplemented with BAP (mg/L)

Treatment	First-day of shoot emergence (days after planting)
MS	6,83 ± 0,12 [a]
MS + 1 BAP	7,17 ± 0,03 [a]
MS + 2 BAP	7,50 ± 0,06 [a]
MS + 3 BAP	7,67 ± 0,07 [a]

Note: The values are means ± SE of 6 explants (n = 24). Numbers followed by the same letter indicate no significant difference on DNMRT at p<0.05.

Number and length of shoot

Based on the results in Table 2, the average number of shoots in MS media supplemented with BAP 2 mg/L has the highest shoots numbers when compared to other treatments. The high average number of shoots shows that BAP has an effect on initiation of new shoots in the meristematic area of nodus.

Number of leaves

Table 3 shows that the highest average number of leaves was found in the MS treatment with the addition of 2 mg. L^{-1} BAP, significantly different from the control treatment and other treatments. The high number of leaves is due to the large number of new shoots formed. Plant leaf growth is influenced by the number of axillary buds formed due to the interaction of exogenous growth regulators and endogenous growth regulators in the explants.

Table 2. The average of shoots numbers and shoot length in nodal explants of singgalang cabbage on MS media supplemented with BAP (mg/L).

Treatment	Number of shoots*	Length of shoot (mm)
MS	1,13 ± 0,08 [a]	3,82 ± 0,63 [a]
MS + 1 BAP	1,27 ± 0,08 [a]	3,64 ± 0,90 [a]
MS + 2 BAP	1,87 ± 0,30 [b]	3,76 ± 0,25 [a]
MS + 3 BAP	1,12 ± 0,12 [a]	2,37 ± 0,31 [a]

Note: The values are means ± SE of 6 explants (n = 24). Numbers followed by the same letter indicate no significant difference on DNMRT at p<0.05. *Data were transformed with $\sqrt{x}$ before statistically analysed.

Table 3. The average of leaves number in nodal explants of singgalang cabbage on MS media with the addition of BAP (mg/L)

Treatment	Number of leaves
MS	6,83 ± 0,94 [a]
MS + 1 BAP	6,83 ± 0,94 [a]
MS + 2 BAP	11,67 ± 0,98 [b]
MS + 3 BAP	5,33 ± 0,42 [a]

Note: The values are means ± SE of 6 explants (n = 24). Numbers followed by the same letter indicate no significant difference on DNMRT at p<0.05.

Observation of root formation

Based on Figure 2, the morphology of shoots produced from nodal explants shows that treatment B (MS+1 BAP) is the explant with the most root formations compared to other treatments. This treatment shows that the addition of BAP is able to form roots. Table 4 shows that the treatment media with the addition of BAP affects the formation of roots in singgalang cabbage node explants. The roots that appeared in the MS treatment were longer than those with the addition of BAP.

In vitro Root Induction and Plantlet Preservation

Height of plantlet

Based on table 5 shows that the ⅛ MS treatment has a high growth of (4.74 cm) which is significantly different compared to other treatments. In this study, the lower the concentration in the media showed the higher the plants formed. seen in MS media as a control has a plantlet height (1.99 cm) is the lowest plantlet height growth compared to other explants.

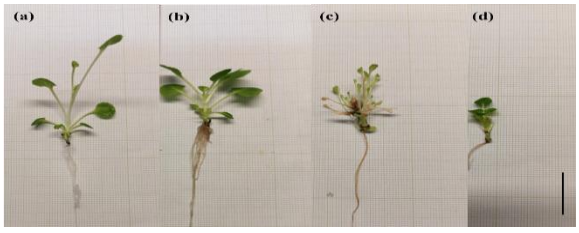

Figure 1. Morphology of shoot produced from nodal explants in (a) MS media, and MS media supplemented with (b) 1 mg/L BAP, (c) 2 mg/L BAP, and (d) 3 mg/L BAP.

Table 4. The average of root formation in nodal explants of singgalang cabbage on MS media with the addition of BAP (mg/L)

Treatment	Presence/absence of root formation (%)
MS	(50.00)
MS + 1 BAP	(66.67)
MS + 2 BAP	(16.67)
MS + 3 BAP	(33.33)

Table 5. The average of plantlet height on modification of MS media 30 days after planting

Treatment	Height of plantlet (cm)
MS	1,99 ± 0,30 [a]
MS ½	2,72 ± 0,82 [a]
MS ¼	2,63 ± 0,60 [a]
MS ⅛	4,74 ± 0,80 [b]

Note: The values are means ± SE of 6 explants (n = 24). Numbers followed by the same letter indicate no significant difference on DNMRT at p<0.05.

Average of first day root emergence

Based on table 6 shows that the average days to appear roots on ⅛ MS is significantly different from the other treatments. In the treatment it can be seen that the lower the concentration of media given, the longer it takes to appear roots on singgalang cabbage. This difference can be caused by low nutrients in the tissue and endogenous cytokinin so that plants are not optimal enough in root formation.

Table 6. The average of first day root emergence on modification of MS media 30 days after planting.

Treatment	First day of root emergence
MS	7,50 ± 0,19 [a]
MS ½	8,17 ± 0,03 [ab]
MS ¼	8,17 ± 0,03 [ab]
MS ⅛	10,50 ± 0,21 [b]

Note: The values are means ± SE of 6 explants (n = 24). Numbers followed by the same letter indicate no significant difference on DNMRT at p<0.05.

Number and length of roots

The results in Table 7 show that the average number of roots is not significantly different between the control treatment and other treatments. The roots produced in both media treatments did not affect the average number of roots. Judging from the results of the study, the lower the concentration of media given, the lower the number of roots produced by the explants. This is because the endogenous hormones in the explants are more focused on the formation and elongation of shoot height rather than root formation.

Table 7. The average of root numbers and length on modification of MS media 30 days after planting

Treatment	Number of roots	Root length (mm)
MS	5,16 ± 1,35 [a]	72,50 ± 0,63 [a]
MS ½	5,83 ± 1,07 [a]	86,33 ± 0,90 [a]
MS ¼	5,00 ± 0,77 [a]	70,83 ± 0,25 [a]
MS ⅛	4,00 ± 0,51 [a]	61,67 ± 0,31 [a]

Note: The values are means ± SE of 6 explants (n = 24). Numbers followed by the same letter indicate no significant difference on DNMRT at p<0.05.

Growth of plantlet after preservation in rooting media

Figure 3. shows the appearance of singgalang cabbage plantlets after being stored in MS modification media for 120 days. It can be seen that reducing the strength of MS media (from ½ to ⅛ did not affect the growth of the plantlets. The growth of plantlet in MS modification media is better when compared to MS full strength. It can be concluded that reducing the concentration of MS media is still able to support the growth of plantlet in the media for preservation purpose.

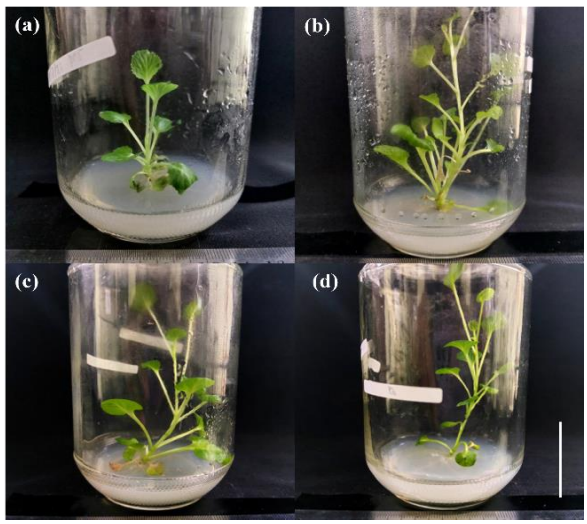

Figure 3. Growth of singgalang cabbage 120 days after planting in (a) MS (b) MS ½ (c) MS ¼, and (d) MS ⅛.

Discussion

Micropropagation of cabbage varieties for conservation and preservation purposes

The plant tissue culture system allows the aseptic propagation of plant material environments with high multiplication rates (Sharma *et al*., 2018). Plant tissue culture technique has been reported as an effective tool to conserve many plant species, especially of tropical origin (Engelmann, 2011). For the short- and mid-term conservations, various techniques have been developed, which not only results in slow growth of the cultures but also prolongs the time interval between two subcultures (Cordeiro *et al*., 2014). *In vitro* culture under slow-growth conditions is supposed to be the most effective method of plant germplasm conservation. The use of this approach is aimed at slowing down the growth of cultures and prolonging the interval between two successive transfers (Cordeiro *et al*., 2014).

Tissue culture techniques for mass propagation purposes have been widely developed, including in cabbage species (Pavlovic *et al*., 2010; Gerszberg *et al*., 2015). In most Brassica species, the success of in vitro regeneration is mostly dependent on the genotype and the influence of plant growth regulators (Ravanfar *et al*., 2009). The addition of cytokinin and auxins would enhance shoot multiplication in many species (Pierik, 1997; Razdan, 2003; Thorpe, 2007; George et al., 2008).

It was explained before that the use of MS media for shoot propagation and root induction have been widely performed in *Brassica* species (Gerszberg *et al*., 2015; Pavlovic *et al*., 2010; Rahman *et al*., 2021; Farooq *et al*., 2023; Kaminska & Sliwinska, 2023). The use of growth regulator is common in the tissue culture of *Brassica* species (for review, see Ravandar *et al*., 2017; Gerszberg, 2018). Conservation of genetic sources of *Brassica* species has been an important part of the research to preserve and produce new varieties or clones of the species for improving their ability to cope with climate change. Preservation by tissue culture technique is now developed by researcher beside the use of seeds in *Brassica* species (Hammer *et al*., 2018; Subramanian *et al*., 2023).

The role of cytokinin in shoot initiation

Cytokinin represents a class of growth regulators comprising small quantities of non-nutritive organic compounds that can either support, inhibit, or modify various physiological processes within plants. The overarching function of growth regulators, broadly speaking, is to induce and facilitate morphogenesis across cell, tissue, and organ cultures (Nisak *et al*., 2012). The efficacy of a tissue culture technique is contingent upon the judicious use of growth regulators. In tissue culture, cytokinin plays a pivotal role by fostering cell division in the utilized explants and promoting shoot development. Within the realm of *in vitro* plant propagation, cytokinin is strategically employed to mitigate apical dormancy and enhance the branching of lateral shoots emanating from axillary shoots. Cytokinin serves to stimulate shoot formation, influence cell metabolism, and activate dormant cells, with their principal function being the promotion of cell division.

According to Badriah *et al*. (1998), cytokinin affects shoot initiation. The most commonly used type of cytokinin is BAP because of its high effectiveness (Yusnita, 2003). BAP is one of the cytokinin containing adenine, which is active in induction shoot formation (Sutriana *et al.*, 2014) and can work effectively in induction cell division and shoot multiplication in plants (Azis *et al.*, 2017). Research by Ravantar *et al.* (2011), showed that adventitious shoots can be regenerated for plant propagation *in vitro* as produced in *B. oleracea* sub-sp. *Green Marvel*. Farzinebrahim *et al*. (2012) have regenerated and propagated *B. oleracea* var. *italica in vitro* where MS media supplemented with 1 mg/L BAP and 1.5 mg/L indole-3-butyric acid (IBA) gave the highest number of formations of new shoots.

The role of MS media modification in root induction and *in vitro* preservation of plantlet

Media modification is one of the important steps for rooting induction of the shoot produced *in vitro*. Many experiments conducted by researchers chose to reduce concentration of basal media composition for improving root induction and addition some of

growth regulator from auxin group as also applied in *Brassica* species. Alam *et al*. (2008) used MS ½ for root induction of five oilseed Brassica species. This media was supplemented with low concentration of IBA and NAA. Basak *et al*. (2012), also used MS ½ for rooting process of mustard shoots produced by *in vitro* technique. Attaya *et al*. (2017), using MS and MS ½ to evaluate the ability of shoot to produce roots for canola varieties *in vitro*.

In vitro culture is an effective method for *ex situ* conservation of plant genetic diversity, allowing rapid propagation from minimal plant material and exerting little impact on wild populations. Two types of *in vitro* preservation methods are employed in tissue culture: a) growth inhibition and b) cryopreservation. The first method is utilized for the medium-term preservation of genetic resources (from several months to several years), while the second method is employed for long-term preservation extending over decades or more (Day & Stacey, 2007). The development of *in vitro* slow-growth storage methods has emerged as a viable alternative for the medium-term preservation of fruit germplasm (Neveen & Bekheet, 2008). The goal of medium-term storage is to extend the duration between subcultures by reducing growth. This objective can be achieved through the application of various strategies, such as modifying environmental conditions, changing culture media, using growth inhibitors, low temperature, and osmotic regulators (Kameswara, 2004; Chauhan *et al*., 2019).

Slow-growth retention via *in vitro* culture has been documented across a wide spectrum of species (Maqsood & Muhammad, 2010; Engelmann, 2011). To ensure the integrity of *in vitro* cultures, regular subculturing under standard conditions is essential to reduce the risk of contamination and safeguard stock material from potential damage (Niino & Arizaga, 2015). Modification of media composition is another way to enhance plant preservation through tissue culture. This technique is commonly used by reducing the basal media concentration without causing deficiency symptoms in the explants that growth *in vitro* (Ashrafi *et al*., 2009; Vahdati *et al*., 2009; (Khas *et al*., 2020).

Conclusions

Based on the results explained above, it was concluded that (1) The use of MS media with the addition of 2 BAP significantly enhances shoot initiation, resulting in increased numbers of shoots and leaves in singgalang cabbage (2) MS modified media did not show a significant effect on root induction and growth, which was significantly in MS 1/8 and plantlet can be preserved for 12 weeks in these treatments media.

Acknowledgements

This research was funded by SIMBELMAWA DIKTI with Program Kreatifitas Mahasiswa- Riset (PKM-RE) 2022. Thanks to Zainal S.P. for laboratory assistance during the research. Thanks also to Dr. Zozy Aneloi Noli, Suwirmen, MS and Dr. Nurainas for critical discussion of this article.

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