Micropropagation of Java Cardamom (Amomum compactum)

Lukita Devy[1]*, Henti Rosdayanti[2], Hayat Khoiriyah[1], Tati Sukarnih[3], Karyanti[1], Winda Nawfetrias[1], Sasanti Widiarsih[4], Cheppy Syukur[1], Adi Setiadi[1], Yuda Purwana Roswanjaya[5]

[1]Research Center for Horticultural and Estate Crops, National Research and Innovation Agency (BRIN), Bogor, Indonesia.

[2]Research Center for Research Center for Plant Conservation, Botanic Gardens and Forestry, National Research and Innovation Agency, National Research and Innovation Agency (BRIN), Bogor, Indonesia.

[3]Directorate of Laboratory Management, Research Facilities, and Science and Technology Park, South Tangerang, Indonesia.

[4]Research Center for Radiation Process Technology, National Research and Innovation Agency (BRIN), Jakarta, Indonesia.

[5]Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Bogor, Indonesia.

*e-mail: lukita.devy@brin.go.id

Abstract. Java cardamom (Amomum compactum) is hardly propagated with rhizome without the mother plant. In vitro culture could overcome the problem through mass propagation for seedling production or other purposes such as genetic material for mutation breeding. The aim of the research was to generate protocol of establishing Java cardamom micropropagation. This research consisted of 4 aspects i.e. shoot induction of mother plant (without Plant Growth Regulator (PGR) and using PGR BAP (6-benzylaminopurine) 1000 ppm), explant origin selection (main stem, rhizome bud height >3 cm, rhizome bud height ≤3 cm and lateral rhizome), sterilization procedure establishment (4 methods differ in the use of detergent, HgCl2, Alcohol, NaOCl, Ethanol, Iodine and soaking time in fungicide and bactericide) and shoot multiplication (MS 0, MS 0 + BAP 1 ppm and MS 0 + BAP 1 ppm + NAA 1 ppm). Result showed the application of 1000 ppm BAP to mature plant could induce shoot emergence. The best explant source was rhizome bud that smaller or equal to 3 cm. The highest survival rate (71%) was recorded when explants disinfected with 70% alcohol for 30 seconds and 0.1 % mercuric chloride for 5 minutes. Java cardamom in vitro culture showed highest shoot multiplication rate in MS 0 + BAP 1 ppm medium (multiplier of 10 shoots/explant in 18 weeks). Keywords: explant, in vitro propagation, Plant Growth Regulator, rhizome, shoot multiplication

1. Introduction

Java cardamom (Amomum compactum) is one of Indonesian main spices crop. Cardamom is used as spices in food and beverage, perfumes, cosmetics, traditional medicine and pharmaceutical [1]. This plant has a broad range of pharmacological activities in traditional medicine such as antifungal, antibacterial, antioxidant, gastroprotective, anti-inflammatory, immunomodulatory, anti-cancer, anti-asthmatic, and acute renal failure [2, 3]. Most of the phytochemical constituents of this plant are flavonoids, saponins, essential oils, steroids and triterpenoids [3].

Splitting of rhizome suckers is the main method of Java cardamom propagation but this method required bulky plant materials because the rhizomes could not develop without its main mother plant so that it is necessary to use rhizome seedling that is mature enough to be planted in field condition. Seed propagation was not efficient enough due to its poor germination and slow seedling development. Therefore, micropropagation using tissue culture as mass propagation, true to type and

disease-free procedure is assumed to be one of the solutions for Java cardamom seedling production. Tissue cultured plants could also be used as genetic materials for breeding or plant transformation purposes.

In vitro plant culture is a technique of mass propagation of plants, elimination of plant diseases through meristematic tissue culture technique, plant conservation, and crop improvement through gene transfer. Plant culture technique consists of transferring various pieces of a plant and whole plant into a sterile nutrient medium so that they could multiply [4].

No universal medium for in vitro culture because plant species are specific to different components of the medium. Murashige and Skoog medium has been proved to be the most suitable medium for successful explant development in various plants and used extensively in the regeneration of the members of Zingiberacee [5]. Some research conducted to study the effect of various medium and Plant Growth Regulators to in vitro culture of several cardamom species [6, 7, 8, 9, 10]

Important aspects of successful micropropagation are explant selection, determination of effective sterilization procedure, culture condition and proper concentrations of plant growth regulators [5]. Therefore, the objective of this study was to develop a basic protocol for in vitro propagation of Java cardamom (Amomum compactum) because there is no specific procedure available to date for this cardamom type..

2. Methods

This research was conducted from April to September 2022 at LAPTIAB, Science and Technology Park BJ Habibie, National Research and Innovation Agency, South Tangerang, Banten, Indonesia. Java cardamom plant used in this study originated from Pamijahan, Bogor Regency, West Java, Indonesia. Stages of the research consisted of seedling treatment with Plant Growth Regulator (PGR), explant origin selection, explant sterilization and shoot multiplication.

Mature plant in polybag size 50 cm x 50 cm were treated with spraying 2 levels of PGR condition i.e. without PGR and with 1000 ppm BAP (6-benzylaminopurine) 50 mL. No PGR treatment was sprayed with tap water 50 mL. The research was arranged in randomized complete design with 5 replications and each experimental unit consisted of 2 plants. Observation conducted on the emergence of new rhizome shoots every 2 weeks.

Explant origin experiment consisted of 4 levels i.e. from K1 (main stem), K2 (rhizome bud height >3 cm), K3 (rhizome bud height ≤3 cm) and K4 (lateral rhizome) (Figure 1). Explant was planted in Murashige and Skoog (MS 0) medium [11] added with sucrose 3% (w/v). Research arranged in randomized complete design with 5 replications. Observation conducted on survival rate, bud number and explant condition every week.

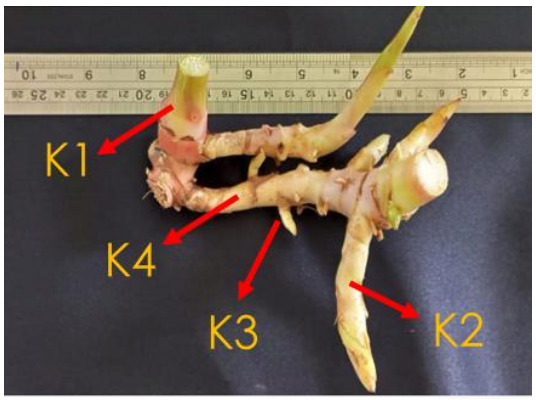

Figure 1. Parts of explant origin tested on Java cardamom micropropagation
(K1: main stem, K2: rhizome bud height >3 cm, K3: rhizome bud height ≤3 cm, K4: lateral rhizome)

Explant sterilization studied 4 procedures differed by the sterilant concentrations, methods, and exposure times (Table 1). Explants were originated from rhizome bud of mature plant. The procedure differences in detail were the use of detergent, $HgCl_2$, Alcohol, NaOCl, Ethanol, Iodine and soaking time in fungicide and bactericide. The first step in sterilization is to harvest the rhizome and clean it from soil and other debris under running tap water 15 min. Then the rhizome sheath was peeled off and continue to each sterilization procedure steps. In each end of one step, explant was washed 3 times with sterile water.

Shoot multiplication in vitro designed in randomized complete design with 6 replications. Treatments consisted of 3 levels i.e. MS 0; MS 0 + BAP 1 ppm and MS 0 + BAP 1 ppm + NAA (1-Naphtaleneacetic acid) 1 ppm. Observation conducted on shoot number, shoot height and root number.

For tissue culture, chemical used are Tween 20 (Sigma), Commercial Bleach-Bayclin (5.4 % NaClO), Alcohol (Merck), Ethanol (Merck), Mercuric Chloride $HgCl_2$ (Sigma), Iodine (Betadine), Commercial fungicide-Dithane M-45 80 WP (Mankozeb 80%), Commercial bactericide-Agrept 20 WP (Streptomycin sulphate 20%) and Commercial Iodine-Betadine (Povidone Iodine 10%). pH was adjusted to 5.7-5.8 using 1 N HCl or 1 N NaOH before autoclaving. All cultures were incubated in the growth room under 24 h light at 23±2 ºC and 60-70% relative humidity.

Data analysis performed with Analysis of Variance (ANOVA) and proceed with Duncan Multiple Range Test analysis if the ANOVA showed significant different. R Studio was used for the data analysis.

Table 1. Effect of sterilization treatment of Java cardamom in vitro

Method	Detergent	Fungicide 2 gL^{-1}, Bactericide 2 gL^{-1} and Tween-20 3 drops	$HgCl_2$ 0.1%	Cutting bud sheath	Alcohol 70%	NaOCl	Ethanol 70%	Iodine
1	Sterile water+ detergent + Tween-20 2 drops, shake 10 min	Soak 30 min, shake several times	Soak 15 min	Peel the sheath then cut 1-2 cm	-	-	-	-
2	Sterile water+ detergent, shake 10 min	Soak 30 min, shake several times	Soak 5 min	-	Soak 30 sec, shake several times	-	-	-
3	Sterile water+ detergent, shake 3 min	Soak 2 hours, shake several times	-	-	Soak 3 min	-NaOCl 10% 5 min -NaOCl 5% 10 min		Dip the wound into high concentrate Iodine
4	Sterile water+ detergent, shake 3 min	Soak 2 hours, shake several times	-	-	-	-NaOCl 10% 10 min -NaOCl 5% 10 min	Soak 3 min	Dip the wound into high concentrate Iodine

3. Results and Discussion

None of four experiments tested showed significant difference in ANOVA test. Therefore, the interpretation of the experiment conducted with descriptive statistic.

3.1. Seedling Shoot Induction

Tissue culture propagation used explant as its plant material and usually derived from young shoots for the successful sterilization. Therefore, mother plant need to produce many shoots to be used as explant. This research studied the effect of BAP spraying in the emergence of new shoots in mature Java cardamom plant.

One thousand ppm BAP 50 mL sprayed onto rhizomes above the ground. Meanwhile the control treated with tap water spraying. Result showed up to 6 weeks after spraying, plants treated with BAP 1000 ppm produce more shoots than control (Table 2). Buds from BAP treated plants were longer than the untreated ones (Figure 2) so that hopefully it will survive better after the sterilization process. This result in line with previous research which stated that the use of BAP could produce higher number and length of new shoots [7].

Table 2. Effect of BAP spraying on shoot induction of Java cardamom seedling

Plant growth regulator (PGR)	Weeks After Spraying				
	2	3	4	5	6
No PGR	0	0.42±0.15 (0-1)	0.25±0.18 (0-2)	0.25±0.18 (0-2)	0.42±0.19 (0-2)
BAP 1000 ppm	0.17±0.11 (0-1)	0.83±0.30 (0-3)	0.83±0.30 (0-3)	0.92±0.31 (0-3)	1.08±0.34 (0-3)

Note: Values are mean ± standard error and (minimum-maximum)

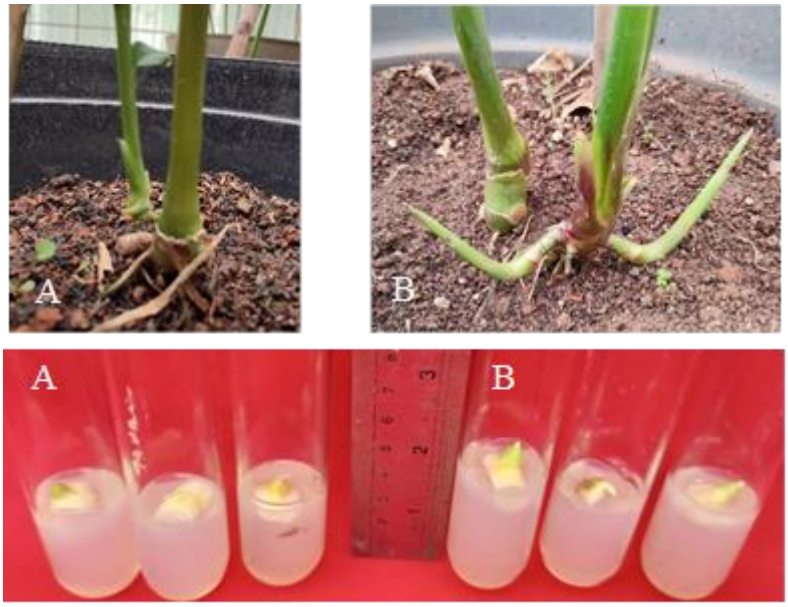

Figure 2. Effect of BAP application on shoot induction 10 weeks after spraying and in vitro growth of Java cardamom 1 week after planting (A: without BAP, B: BAP 1000 ppm spraying on mature plant)

3.2. Explant Origin

Selection of explant is one of important factor in micropropagation. Explant type, location and age estimated will affect their survival and growth [12]. This research tried to use the whole part of the rhizome and cut them into several parts i.e. the main stem, rhizome bud longer than 3 cm, buds smaller and equal to 3 cm and lateral rhizome (stolon) between shoots. Result showed buds smaller and equal to 3 cm performed the largest survival rate (100%) with highest shoot number up to 3 shoots at 4 weeks after planting (Table 3) and mostly the new shoots were fresh (Figure 3).

Smaller buds seem to have better ability to grow and more sterile than larger shoots so that the growth is better. It is probably due to its growth stage as investigated in a study on explant origin

differences of Ceratonia siliqua and concluded that the best was obtained with herbaceous explants taken from juvenile trees [13]. Young developmental stage has often been found to be more optimal for shoot regeneration than older stages, which may be explained by differences in anatomical and physiological properties [13]. Study on orchid with various explant origin showed each type could performed well, include the use of the rhizome part [12]

Table 3. Effect of different explant part source to survival rate and shoot number of Java Cardamom in vitro

Explant source	Weeks After Planting			
	1	2	3	4
			Survival rate (%)	
K1 (Main stem)	100	60	60	60
K2 (Shoot > 3 cm)	100	80	80	80
K3 (Shoot ≤ 3 cm)	100	100	100	100
K4 (Lateral rhizome)	100	100	60	60
			Shoot number*	
K1 (Main stem)	0	0	0	0
K2 (Shoot > 3 cm)	0	0	0.6 ± 0.89 (0-2)	0.6 ± 0.89 (0-2)
K3 (Shoot ≤ 3 cm)	0	0	0.8 ± 0.84 (0-2)	1.0 ± 1.22 (0-3)
K4 (Lateral rhizome)	0	0	0.2 ± 0.45 (0-1)	0.4 ± 0.55 (0-1)
			Explant Condition	
K1 (Main stem)	60% fresh; 40% pale	20% fresh; 40% browning; 20% died	60% browning; 40% died	60% browning; 40% died
K2 (Shoot > 3 cm)	60% fresh; 40% pale	60% fresh; 20% pale; 20% died	60% fresh; 20% pale; 20% died	60% fresh; 20% pale; 20% died
K3 (Shoot ≤ 3 cm)	60% fresh; 20% pale; 20% browning	60% fresh; 20% pale; 20% browning	60% fresh; 20% pale; 20% browning	60% fresh; 20% pale; 20% browning
K4 (Lateral rhizome)	20% fresh; 80% pale	20% fresh; 80% pale	60% pale; 40% died	60% pale; 40% died

*Note: Values are mean ± standard error and (minimum-maximum)

3.3. Sterilization Method

In tissue culture, new plants are grown from pieces of explant in a nutrient medium under sterile conditions. Contaminations from microorganism become a serious problem which could cause losses explant sources. These microorganisms include viruses, bacteria, fungi, yeast, etc. Nutrient media also good source for microbial growth. These microorganisms compete hard with the plant material for nutrient in the media [14]. Therefore, it is important that the explants must be free of any contaminants prior to the tissue culture since microorganisms may grow in the culture medium [15].

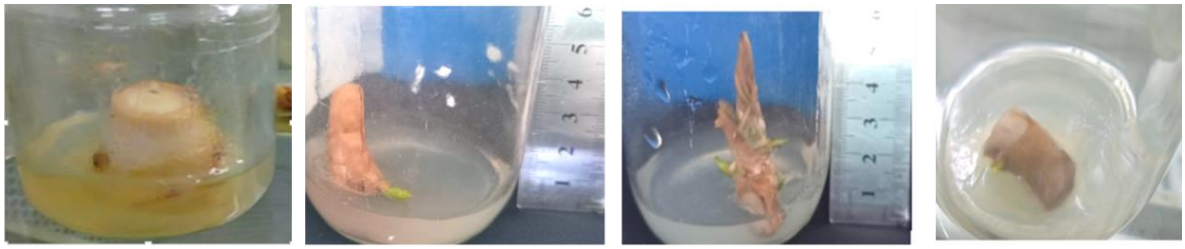

Figure 3. Effect of explant origin on Java cardamom growth at four weeks after planting

Some surface sterilizing materials were applied with different methods, concentrations and exposure times to determine the most efficient sterilization procedure (Table 1). Efficient sterilization procedure will be able to accelerate the success of explant establishment in tissue culture. The best survival rate revealed by method 2 meanwhile shoot emergence up to 4 weeks after planting showed the same number for all methods tested (Table 4). Explant treated with method 2 showed fresh explant compared to other treatments (Figure 4). It is concluded that method 2 is potential to be used as sterilization procedure for Java cardamom micropropagation in vitro.

Procedure in Method 2 started by washing Java cardamom rhizome from soil and debris under running tap water 15 min. Rhizome sheath from mature plant was peeled and immersed in water containing detergent on a shaker 10 min and then rinsed with running tap water. Explants then treated with solution of fungicide 2 gL^{-1}, bactericide 2 gL^{-1} and Tween-20 3 drops for 30 minutes, with several time shaking then rinsed with sterile water (x3) in a laminar air flow cabinet. Next treatment was immersing in alcohol 70% 30 sec and continue with $HgCl_2$ 0.1 % 5 min then rinsed with sterile water (x3). The explant was planted in MS 0 medium without PGR. Method 2 with $HgCl_2$ resulted in minimum contamination. That is probably because of the highly toxic properties of $HgCI_2$ to human, plant, and microorganism. $HgCl_2$ was used for surface sterilization in tissue culture and exposure to $HgCI_2$ decreased the survival rate [16]. However, the result of this study showed opposite trend where method 2 which contained $HgCl_2$ immersion resulted fresh green explant. Sathyanarayana and Varghese described that surface sterilization method depend on plant species, surface contaminant levels, growth environment, age, and explant origin [17]

Table 4. Effect of different sterilization method to survival rate and bud emergence of Java Cardamom in vitro

Sterilization Method	Weeks After Planting			
	1	2	3	4
			Survival (%)	
1	100	100	100	67
2	100	100	71	71
3	100	100	38	38
4	100	100	50	25
			Bud emergence per explant*	
1	0	0	0	1.0 (0-1)
2	0	0	1.0 (0-1)	1.0 (0-1)
3	0	0	1.0 (1)	1.0 (1)
4	0	0	0	1.0 (0-1)

*Note: Values are mean ± standard error and (minimum-maximum)

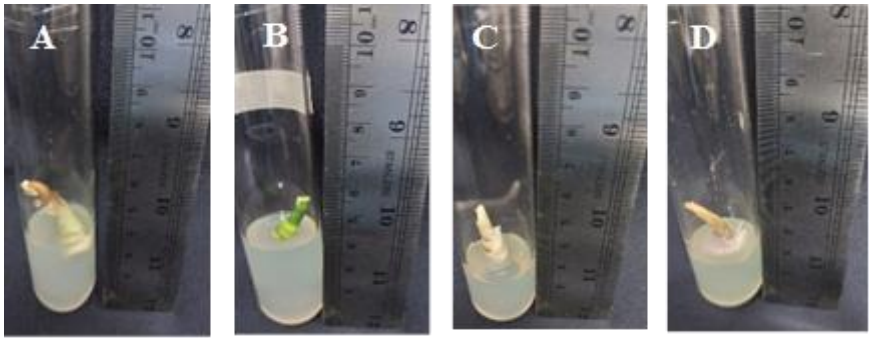

Figure 4. Effect of different sterilization procedure on Java cardamom explant performance at 4 weeks after sterilization (A: Method 1, B: Method 2, C: Method 3, D: Method 4)

3.4. Shoot multiplication

Stages in micropropagation or in vitro culture were explant surface sterilization, shoot multiplication, rooting, elongation and acclimatization [4]. Shoot multiplication usually used in vitro medium enriched with PGR. Cytokinin is one of PGR used for shoot multiplication while auxins are usually used for root initiation. Several studies showed the effect of BAP as one of cytokinin PGR on in vitro culture especially for shoot multiplication [7, 8, 9, 10].

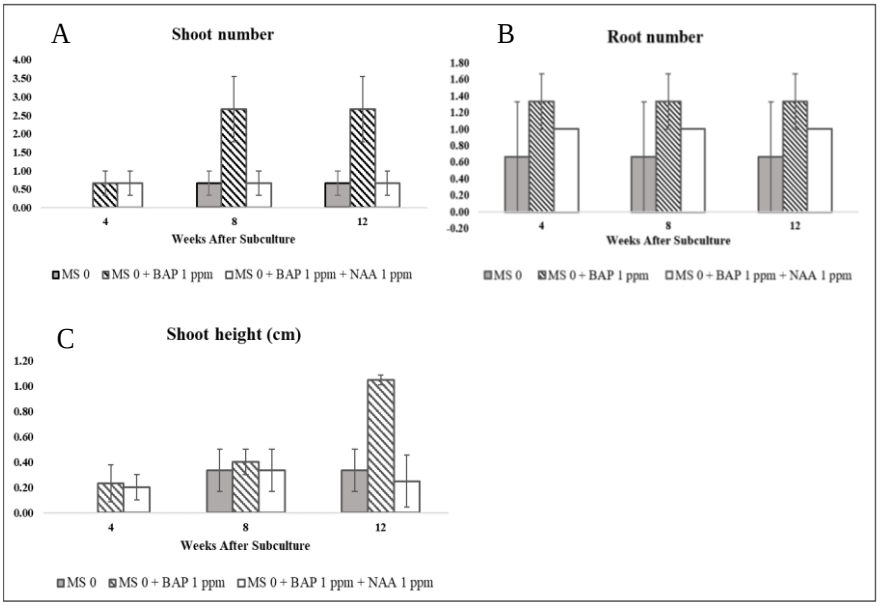

Figure 5. Effect of in vitro medium to Java cardamom growth

Medium MS 0 enriched with BAP 1 ppm showed the highest shoot number, root number and shoot height compared to other treatments up to 12 weeks after subculture (Figure 5). Plant Growth Regulator selection for micropropagation must considered hormone balance between shoot and root development. At early stage of Java cardamom tissue culture, MS 0 + BAP 1 ppm medium was more suitable for shoot induction and multiplication than MS 0 only or MS 0 + BAP 1 ppm + NAA 1 ppm (Figure 6). This result is similar with similar to Quyet et al 2020 who used MS 0 + BAP 1 mgL-1 which was the most suitable medium in the fast multiplication phase of cardamom (*A. tsaoko*), with a multiplier of 4.54 shoots/explant [7]. However these result is different with previous result on in vitro culture of large cardamom (*A. subulatum*) where MS supplemented with BAP and NAA is favorable for micropropagation [8] Meanwhile other research showed that explants grown in MS 0 + BAP 1 mgL^{-1} + IBA mgL^{-1} produced maximum root induction rate [9].

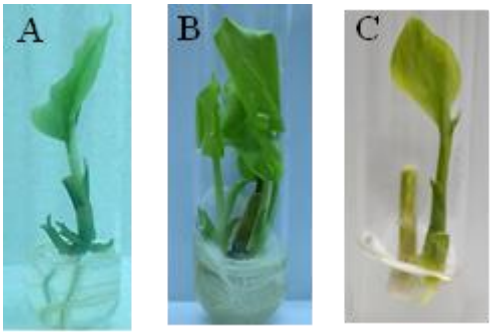

Figure 6. Effect of different in vitro medium to Java cardamom growth
(A: MS 0, B: MS 0 + BAP 1 ppm, C: MS 0 + BAP 1 ppm + NAA 1 ppm)

In this study, one explant planted on MS 0 + BAP 1 ppm medium was able to produce 4 new shoots at 8 weeks after planting and produced total of 10 new shoots from those 4 shoots at 18 weeks after planting (Figure 7). This means the shoots were increasing from 1 to 4 to 10 in 18 weeks. More subcultures are hopefully could increase more shoot emergence so that it could be used for in vitro breeding purposes or continue to the rooting, elongation and acclimatization of mass propagation.

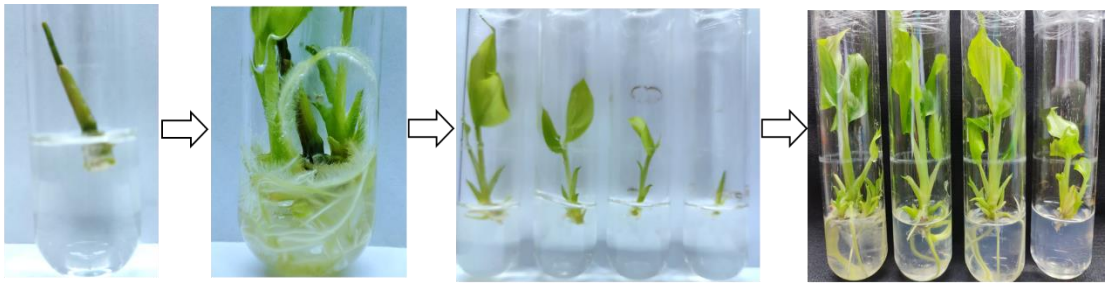

Figure 7. Explant development in medium MS 0 + BAP 1 ppm showed shoot multiplication

4. Conclusions

In vitro propagation as one of Java cardamom propagation method has established. Explant from small shoot less than 3 cm showed highest survival rate, BAP 1000 ppm spraying at seedling stage could induce shoot emergence, the best sterilization method established using 0.1 $HgCl_2$ 5 min and 70% alcohol 30 sec. Addition of BAP 1 ppm to MS 0 medium performed the best shoot multiplication in vitro. These protocols need further study to ensure the stability of the in vitro propagation procedure.

5. Acknowledgement

The authors are grateful to the Research Organization for Nuclear Energy (Project No: Rumah Program Organisasi Riset Tenaga Nuklir TR-019 Year 2022) for their kind financial support.

6. References

[1] Wolff X Y, Hartutiningsih 2015 Amomum compactum. PROSEA Spices

[2] Hartady T, Balia R L, Syamsunarno M R A A, Jasni S and Priosoeryanto B P 2020 Bioactivity of Amomum compactum Soland ex Maton (Java cardamom) as a natural antibacterial. Syst. Rev. Pharm. 11(9) 384-387

[3] Alkandahri M Y, Shafirany M Z, Rusdin A, Agustina L S, Pangaribuan F, Fitrianti F, ... and Mardiana L A 2021 Amomum compactum: A review of pharmacological studies. Plant Cell

Biotechnol Mol Biol 22 (33&34) 61-69

[4] Hartmann H T, Kester D E, Davies F T and Geneve R L 1997 Plant propagation: principles and practices (No. Ed. 6). Prentice-Hall Inc USA

[5] Bhattacharya M, Goyal A K and Mishra T 2014 In vitro regeneration of some lesser known medicinal Zingibers: a review. Biology of Useful Plants and Microbes 167-186 Ed: Arnab Sen Narosa Publishing House India

[6] Khuat Q V, Kalashnikova E A, Kirakosyan R N, Nguyen H T, Baranova E N and Khaliluev M R 2022. Improvement of In Vitro Seed Germination and Micropropagation of Amomum tsao-ko (Zingiberaceae Lindl.) Horticulturae 8 640

[7] Khuat Q V, Hai N T, Hai N T L, Hang P T T, Kirakosya R and Kalashnikova E 2021 Plant regeneration of Amomum tsaoko Crevost & Lemarié in vitro IOP Conf. Ser.: Earth Environ. Sci. 677 042065

[8] Purohit S, Nandi S K, Paul S, Tariq M and Palni L M S 2017 Micropropagation and genetic fidelity analysis in Amomum subulatum Roxb.: a commercially important Himalayan plant. J Appl. Res. Medic. Aromatic Plant 4 21-26

[9] Poudel K, Prasai H K and Shreshta J 2018 Micropropagation and acclimatization of large Cardamom (Amomum subulatum Roxb.) Türk Tarım ve Doğa Bilimleri Dergisi 5(3) 231-235

[10] Pradhan S, Pradhan S, Basistha B C and Subba K B 2014 In vitro micropropagation of Amomum subulatum (Zingiberaceae), a major traditional cash crop of Sikkim Himalaya Intl J Life Sci. Biotech. Pharma Res. 3(2) 169

[11] Murashige T, Skoog F 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 473-497

[12] Chugh S, Guha S and Rao I U 2009 Micropropagation of orchids: a review on the potential of different explants. Sci. Hort. 122(4) 507-520

[13] Naghmouchi, S, Khouja M L, Rejeb M N and Boussaid M 2008 Effect of growth regulators and explant origin on in vitro propagation of Ceratonia siliqua L. via cuttings. BASE 3(12) 251-258 http://popups.ulg.be/1780-4507/index.php?id=2708

[14] Odutayo O I, Oso R T, Akinyemi B O and Amusa N A 2004 Microbial contaminants of cultured Hibiscus cannabinus and Telfaria occidentalis tissues. Afric. J. Biotech. 3(9) 473-476

[15] Guma T B, Jane K, Justus O and Kariuki P N 2015 Standardization of in vitro sterilization and callus induction protocol for leaf explants of anchote: Coccinia abyssinica. Intl. J. Res. Dev. Pharm. Life Sci. 4(2) 1427-1433

[16] Danso K E, Azu E, Elegba W, Asumeng A, Amoatey H M and Klu G Y P 2011 Effective decontamination and subsequent plantlet regeneration of sugarcane (Saccharum officinarum L.) in vitro. Int. J. Integra. Bio. 11 90-96.

[17] Sathyanarayana B N and Varghese D B 2007 Plant tissue culture: practices and new experimental protocols. IK International Pvt Lt