Improved callus induction from immature seed of Indonesian wild banana (*Musa acuminata* ssp. malaccensis and rutilifes) for gene editing materials

Diyah Martanti[1], *Tri* Handayani[1], *Jelia Carolina* Talahatu[2], *Alfia Annur Aini* Azizi[3], *Carla Frieda* Pantouw[3], *Aviv* Andriyani[3], *Enny* Sudarmonowati [3], *Bernadetta Rina* Hastilestari[3*]

[1]Research Center for Applied Botany, National Research and Innovation Agency of Indonesia
[2]Calvin Technology Institute
[3]Research Center for Genetic Engineering, National Research and Innovation Agency of Indonesia

Abstract. As a country rich in biodiversity, Indonesia possesses large numbers of wild bananas as valuable germplasm which are potential for banana breeding as they are rich of genetic variability for disease resistance, stress tolerance and other beneficial characteristics. Conventional breeding is hampered by low fertility which has caused inefficiency in producing improved varieties. Tissue culture has been applied to harness banana genetic improvement to produce massive banana plants that are identical to their parents which callus could also be used as materials for modern genetic engineering. This paper aims at investigating the response of several *M. acuminata* subspecies i.e. ssp. malaccensis and ssp. rutilifes in differ. Calli of these subspesies were induced from immature seeds that were inoculated using modified macronutrient and plant growth regulator. The research was designed using a completely randomized design with two factors, modified macro substances (Murashige and Skoog - MS and modified Gamborg's B5 - BDS) and modification of growth regulators combination, including 2,4D, NAA, IAA and BA. Different macro elements led to different percentage of callus formed on *Musa acuminata* seeds. Ratio of callus production of var. malaccensis was higher on BDS media (67.49%) than on MS media (58.17%). In contrast, that of seeds of *Musa acuminata* var. rutilifes was higher on MS media (67.34%) than BDS media (65.29%). Growth regulator composition and concentration were also critical as media containing 2,4D (1 mg/L) + NAA (1 mg/L) and IAA (1 mg/L) were better than a combination of 2,4D (1 mg/L) + NAA (1 mg/L) and BA (1 mg/L) in callus induction in both *Musa acuminata* subspecies malaccensis and rutilifes (68.14% and 68.42% respectively). Meanwhile, the growth regulator treatment combination of (2,4D (1 mg/L) + NAA (1 mg/L) and IAA (1 mg/L)) has induced 68.14% calli and 68.42% of spp, malaccensis and rutilifes respectively. Therefore, for propagating banana ssp. malaccensis and rutilifes as source of materials for genetic transformation using gene editing, BDS media containing (2,4D (1 mg/L) + NAA (1 mg/L) and IAA (1 mg/L)) will be used. Embryogenic callus as the source of protoplasts would be the

* Corresponding author: bern003@brin.go.id

best regeneration procedure of transformed gene edited wild banana in the near future.

1 Introduction

Banana (*Musa* spp. L.) is one of global agricultural products, following rice, wheat, and cassava that contribute significantly to global food security [1]. The main banana producers include India, China, Indonesia, Brazil, Ecuador, the Philippines, Guatemala, Colombia, Angola and India yielding 30,808,000 tons [2]. Bananas are especially popular in developing countries due to their low cost and high nutritional value. Bananas are rich in carbohydrates, proteins, minerals (such as potassium, calcium, magnesium, and manganese), and vitamins, including vitamins A, C, and B6 [3].

Besides the fruit, other useful parts of the banana plant, such as the peel, pseudostem, rhizome, leaves, and more, can be utilized in various industries like agriculture, food, textiles, and others [4][5][6]. There are over 1000 banana varieties cultivated worldwide, but the Cavendish variety is considered one of the most important commercial varieties [7][8]. Banana cultivation can significantly enhance the global bioeconomy based on agriculture.

According [1], bananas are generally propagated conventionally using suckers growing from the rhizome, producing about 2 to 3 shoots per year from one banana plant, not all of which are productive. There is a significant gap between the demand and supply of healthy planting materials [9] in order to cope with climate change [10]. However, banana production has been threatened by various climate factors and pathogens such as bacteria, viruses, fungi, and nematodes [7].

In order to increase banana quality and yield, breeding to get better cultivars is necessary. Conventional breeding is quite challenging because cultivated banana plants have much reduced fertile seeds compared to wild banana plants [11]. Crossing cultivated banana is difficult because of crossing blocks due to the small number of fertile seeds. This crossing block is possible because the ancestors of subspecies and species of banana plants differed in genome structure due to large reciprocal translocations [12]. This translocation infertility has involved at least 7 of the 11 banana plant chromosomes in the species *Musa acuminata* [13]. These differences hinder the process of crossing over in the meiosis stage, as a result of alleles carrying desirable traits are difficult to separate from alleles carrying undesirable traits [14].

Wild bananas cultivars have more fertile seeds compared to cultivated ones, the prior cultivars are potential for pre-breeding as they may have superior traits such as productivity, fruit flavor and stress resistance. The pyramiding of desired traits conventionally requires hybridization, but due to differences in chromosome structures [13][15], the resulting hybrids may be sterile [16].

This challenge can be overcome by advanced technology. The development of biotechnology has shed a light to provide better cultivar with more quickly and with more precision [17]. The most current technology, genome editing (GE), has been developed to make targeted changes to multiple nucleotides in target genes. This method requires optimized tissue culture systems in which DNA is delivered into the cell plants regenerated through the tissue culture [18]. In addition, in the tissue culture, different concentrations and ratios of hormones like auxin and cytokinin need to be examined for increasing the regeneration probability of plants from the calli [19]. Tissue culture propagation generally involves specific media compositions and growth regulators to achieve the targeted production results. Selected tissues are grown in selected media under controlled conditions which induce totipotency of a single plant cell to designate full genome through cell division with this cell totipotency, cell is able to regenerate a whole plant [20].

The aim of this study is to investigate the response of wild *M. acuminata* subspecies ssp. malaccensis and ssp. rutilifes to different concentration of growth regulators 2.4-D, α-Naphtalene acetic acid (NAA), Indole-3-acetic acid (IAA), 6-benzylaminopurine (BA) and picloram. The developed protocols will be beneficial for plant regeneration that can be used for developing superior cultivar through genetic engineering.

2 Material and methods

2.1 Time and place

The experiment was conducted at National Research and Innovation Institute of Indonesia (BRIN), KST (Integrated Science Area) Soekarno-Cibinong, from May to December 2023.

2.2 Plant material

The plant materials in this research were banana ssp. malaccensis and ssp. rutilifes. While Both varieties are wild banana with AA genome. These varieties were planted at the banana collection garden of BRIN. Banana ssp. malaccensis was collected from West Java Indonesia, with local name Cau Kole [21]. Meanwhile, var. Rutilifes derived from East Java [22].

The research was designed using a completely randomized design with two factors, namely modification of macro substances, (Murashige and Skoog – MS and modified Gamborg`s B5 - BDS) and modification of growth regulators using 2,4D, NAA, IAA and BA. Besides, the pH of the media is adjusted into 5.8.

2.3 Embryogenic Calli cultures of wild banana ssp. malaccensis and ssp. rutilifes

2.3.1 The used explants came from immature embryos of self-pollinated wild banana varieties, namely Musa acuminata ssp. malaccensis and Musa acuminata ssp. rutilifes.

Immature seeds were removed from fruit 2 months after pollination. These seeds were then washed with running water and brought into laminar air flow. In laminar air flow, the seeds were sterilized with 96% ethanol for 1 minute, followed by soaking in 10% sodium hypochlorite solution for 20 minutes, then rinsed twice with sterile water. The seeds were cut vertically, then the embryos were removed and placed in media with modified macronutrients and growth regulators with 2% w/v agar (Table 1). Cultures were maintained in the dark at 28 ^{0}C and subcultured every four weeks. Observations made included characterization of the callus formed and its percentage, as well as the interaction of callus formation with the treatments tested.

Table 1. Media used in wild banana seed callus induction

Media	**2,4D (1 mg/L) + NAA (1 mg/L) + IAA (1 mg/l)**	**2,4D (1 mg/l) + NAA (1 mg/L) + BA (0.5 mg/l)**
BDS	BDS4	BDS9
MS	60MS4	60MS9

2.3.2 Explant from the banana flower of cultivated banana cultivars : M. accuminata cv. Giant Cavendish and cv. Raja

The growths of wild bananas calli will be compared to those of the cultivated ones. The explants were taken from banana flower of *M. accuminata* cv. Giant Cavendish and cv. Raja from the Banana Germplasm Garden, Yogyakarta. The flower was peeled, cut until about 10 cm and sterilized using 96% ethanol for 5 minutes, then they were placed in the media listed in table 2. Characterization of the formed callus and calli emergence percentage were observed.

Table 2. Media used for callus induction from Cavendish and Raja banana flowers

Media	Growth Regulator
DP1	2,4D (2 mg/L)
DP2	Picoram (2 mg/L)
DP3	2,4D (2 mg/L) + Picloram (2 mg/L)
DP4	2,4 D (2 mg/L) + Picloram (4 mg/L)
DP5	2,4D (4 mg/L) + Picloram (2 mg/L)
DP6	2,4 D (4 mg/L) + Picloram (4 mg/L)

3 Result and Discussion

Seeds of wild banana plants were taken as explant source as reproductive tissue or organ have lower contaminant levels [23]. The explants were grown in tissue culture medium. This media consists of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, and some gelling agents in case of solid medium [24]. Explant grown in Media with macronutrient MS and BDS are presented in Figure 1.

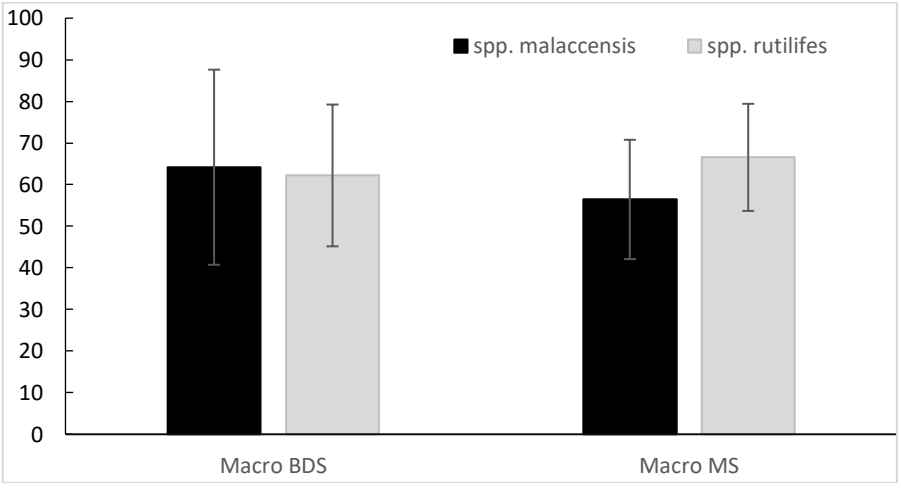

Fig. 1. Effect of macronutrient of BDS media and MS media on the calli emergence in *M. accuminata* spp. malaccensis and spp. rutilifes

In this experiment, callus formation began at around four weeks after planting the explant. Formation of calli began with the development of swollen part of tissue. Those swollen part were embryogenic callus with compact non embryogenic and fibrous calli. The efficiency of calli production was calculated based on the percentage of calli emergence (Figure 1).

ANOVA test showed that there was no significant effect of macronutrient BDS and MS on the calli emergence in ssp. malaccencis and ssp. rutilifes. Macro BDS has induced 64.18 ± 23.45 % calli emergence in ssp. malaccensis and 62.20 ± 17.04 % in ssp rutilifes; Meanwhile, macro MS affected 56.43 ± 14.34 % of calli emergence in ssp. malaccencis and 66.55 ± 12.87 % in ssp. rutilifes. These results indicated that ssp. malaccensis and rutilifes seeds have better germinated in macro BDS and macro-MS respectively. Following subcultured callus on the same media showed more proembryo formed.

Regeneration of plant calli are also influenced by various factors such as growth regulators. In this experiment, applied combination of growth regulators (Table1). Media with 2,4D (1 mg/L) + NAA (1 mg/L) and IAA (1 mg/L) were better at inducing *Musa acuminata* ssp. callus. malaccensis and rutilifes, namely 68.14% and 68.42% respectively compared to media containing 2,4D (1 mg/L) + NAA (1 mg/L) and BA (1 mg/L) (Figure 2). Growth regulator combination is essential to induce callus and might be different among *Musa acuminata* ssp. Although 2,4 -D is the common growth regulator, it has been reported that combination 2,4 D with other growth regulator succeed to induce banana callus [25][26][27]. Beside growth regulator used, callus formation also depends on its concentration in the media and exposure time [28]. This is in line with [29] who has mentioned that a high concentration of cytokinin can accelerate shoot growth with 6 ppm Kinetin treatment being the best treatment with a shoot emergence time of 9.25 days in Kepok banana explants. A kinetin concentration of 2.5 mg/l is the right concentration and can help in cell division so that shoot formation in Barangan banana shoot explants. *M. acuminata* ssp. malaccensis has shown not very good shoot proliferation in the MS Media added with 2 mg/L 6-benzylaminopurine (BAP) compared to *M. gracilis* [30].

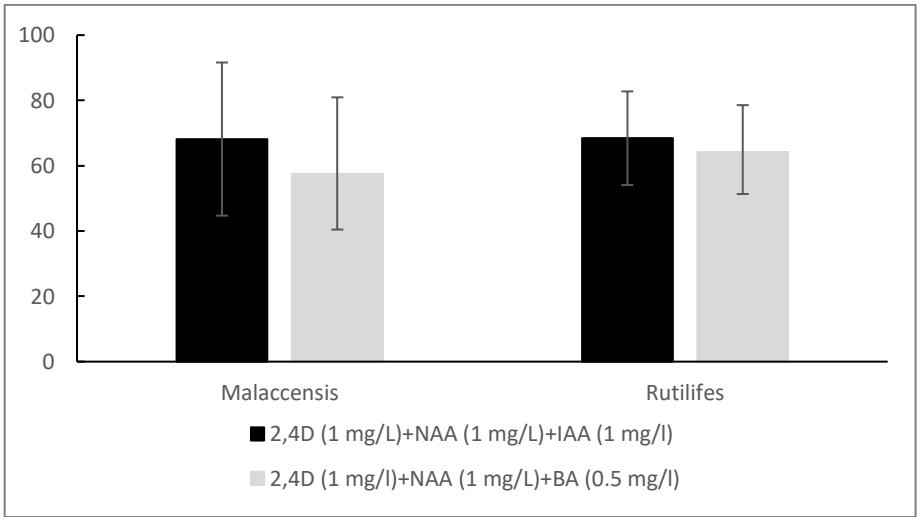

Fig. 2. Effect of plant growth regulator on the calli emergence in *Musa accuminata* spp. malaccensis and spp. rutilifes

The callus formed from this embryo has a compact texture, yellowish white color, and in some parts of the callus it has the shape of small dots (Figure 3). These small dots will later form embryonic cells. These calli were formed around 4 months after culture on the treatment media. The colour might change to brown and cease to grow because the calli has reached their optimum cell division, lack of nutrients or physiological cessation [31][32], reveal this decrease in growth is due to the callus reaching optimum cell division, its physiological degradation and/or lack of nutrients.

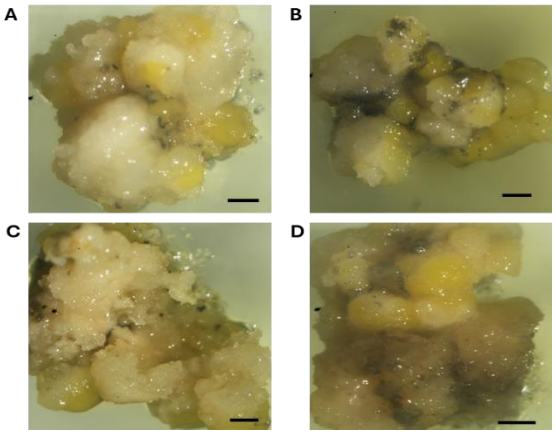

Fig. 3. Callus of A) *Musa acuminata* ssp. *malaccensis* in media makro BDS (B) *Musa acuminata* ssp. *malaccensis* in media makro MS (C) *Musa acuminata* ssp. *rutilifes* in media makro BDS (D) *Musa acuminata* ssp. *rutilifes* in media makro MS

Meanwhile, in the cultivated cultivars ssp. Giant Cavendish and ssp. Raja, DP1 media with 2,4D (2 mg/L) has induced 100 % callus (Table 3). This media is recommended for the callus induction of these cultivars. It indicated that application of 2.4 D (2mg/L) has been effective for inducing calli for these cultivars. These responses are genotype specific, *M. Acuminata var. Kluei Namwa* (ABB group) has shown high embryogenesis from the shoot apex of plants when this cultivar was grown in MS medium added with picloram (8 mg/l), but it has shown no callus when they were grown in MS Medium added with 2,4 D [11].

Table 3. Percentage of growing callus in different treatment media

Code	Treatment media	Percentage of growing callus	
		ssp. Giant Cavendish	ssp. Raja
DP1	2,4D (2 mg/L)	100	100
DP2	Picoram (2 mg/L)	71,43	90
DP3	2,4D (2 mg/L) + Picloram (2 mg/L)	55,56	70,59
DP4	2,4 D (2 mg/L) + Picloram (4 mg/L)	60	41,67
DP5	2,4D (4 mg/L) + Picloram (2 mg/L)	92,31	70
DP6	2,4 D (4 mg/L) + Picloram (4 mg/L)	58,33	92,86

Figure 4 shows the callus formation of Musa acuminata var. Giant Cavendish and var. Raja after four weeks on the treatment media. The calli of var. Giant Cavendish look like grey cotton, while calli of var. Raja look compact and solid.

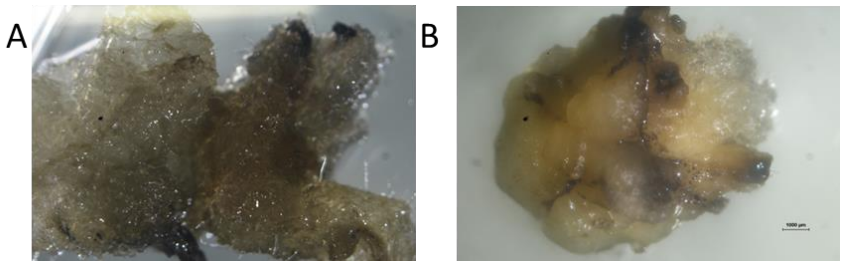

Fig. 4. Callus of A) *Musa acuminata* ssp. Giant Cavendish (B) *Musa acuminata* ssp. Raja

4 Conclusion

Embryogenic callus propagation has been tested in the Indonesian wild bananas, ssp malaccencis and ssp. rutilifes, as well as cultivated bananas ssp. Giant Cavendish and ssp. Raja. Application of BA 0.5 mg/l , NAA 1 mg/l and 2,4 D 1 mgl/l to MS medium could induce embryogenic calli propagation of ssp. malaccencis and rutilifes. Meanwhile, the cultivated varieties could effectively propagate in MS media added with 2,4 D (2 mg/L). The ability to produce embryogenic callus is essential for initiating the cassava regeneration system through embryogenesis. This result will be beneficial developing better cultivar through genome editing.

Reference

1. L. Tripathi, V. O. Ntui, and J. N. Tripathi, CRISPR/Cas9-based genome editing of banana for disease resistance, Curr. Opin. Plant Biol., **56**, 118–126, Aug. (2020), doi: 10.1016/j.pbi.2020.05.003.
2. J. O. Ighalo and A. Adeniyi, Thermodynamic modelling and temperature sensitivity analysis of banana (*Musa* spp.) waste pyrolysis, Appl. Sci., **1**, 1086, (2019), doi: 10.1007/s42452-019-1147-3.
3. M. Ranjha, S. Irfan, M. Nadeem, and S. Mahmood, A Comprehensive Review on Nutritional Value, Medicinal Uses, and Processing of Banana, Food Rev. Int., **38**, 199–225, (2022), doi: 10.1080/87559129.2020.1725890.
4. A. Adeniyi, S. Adeoye, J. O. Ighalo, and D. Onifade, FEA of effective elastic properties of banana fiber- reinforced polystyrene composite, Mech. Adv. Mater. Struct., (2020), doi: 10.1080/15376494.2020.1712628.
5. A. Adeniyi, J. O. Ighalo, and D. Onifade, Banana and plantain fiber-reinforced polymer composites, J. Polym. Eng., **39**, (2019), doi: 10.1515/polyeng-2019-0085.
6. O. Akatwijuka, M. Gepreel, A. Abdel-Mawgood, M. Yamamoto, Y. Saito, and A. Hassanin, Overview of banana cellulosic fibers: agro-biomass potential, fiber extraction, properties, and sustainable applications, Biomass Convers. Biorefinery, **14**, (2022), doi: 10.1007/s13399-022-02819-0.
7. J. N. Tripathi, V. O. Ntui, M. Ron, S. K. Muiruri, A. Britt, and L. Tripathi, CRISPR/Cas9 editing of endogenous banana streak virus in the B genome of *Musa* spp. overcomes a major challenge in banana breeding, Commun. Biol., **2**, 46, Jan. (2019), doi: 10.1038/s42003-019-0288-7.
8. R. Thangavelu et al., Identification of sources resistant to a virulent *Fusarium* wilt strain (VCG 0124) infecting Cavendish bananas, Sci. Rep., **11**, 3183, Feb. (2021), doi: 10.1038/s41598-021-82666-7.

9. K. Jacobsen et al., Seed degeneration of banana planting materials: strategies for improved farmer access to healthy seed, Plant Pathol., **68**, 207–228, Feb. (2019), doi: 10.1111/ppa.12958.
10. B. R. Hastilestari, Pengaruh cekaman panas terhadap daun stroberi (*Fragaria* L. Elsanta)," Jul. (2015). doi: 10.13057/psnmbi/m010435.
11. R. R. R. Annisa, A. Setiaji, and A. B. Sasongko, *In Vitro* Shoot Induction of *Musa* acuminata cv. Mas Kirana, Quagga J. Pendidik. dan Biol., **13**, 34, (2020), doi: 10.25134/quagga.v13i1.3431.
12. G. Martin et al., Chromosome reciprocal translocations have accompanied subspecies evolution in bananas, Plant J., **104**, 1698–1711, Dec. (2020), doi: 10.1111/tpj.15031.
13. K. Shepherd, Cytogenetics of the genus Musa. (1999). [Online]. Available: http://musalit.inibap.org/pdf/IN990087_en.pdf
14. M. Pillay, Classical genetics and traditional breeding in *Musa*, in Genetics, Genomics, and Breeding of Bananas, (2012), pp. 34–55. doi: 10.1201/b11776.
15. F. Ahmad, N. M. Martawi, Y. S. Poerba, H. de Jong, H. Schouten, and G. H. J. Kema, Genetic mapping of *Fusarium* wilt resistance in a wild banana *Musa acuminata* ssp. malaccensis accession, Theor. Appl. Genet., **133**, 3409–3418, (2020), doi: 10.1007/s00122-020-03677-y.
16. S. Chang, Y. Yen, I. Miyajima, and K. Huang, The Efficiency of Hybridization and Seed Production in *Musa* spp, J. Fac. Agric. Kyushu Univ., **64,** 169–176, Sep. (2019), doi: 10.5109/2339043.
17. M. Fendiyanto, D. Maysha, and B. Hastilestari, In-silico Gene Editing of LCYB in *Musa acuminata* and Its Functional Analysis Related to Lycopene Beta-Cyclase Pathway, IOP Conf. Ser. Earth Environ. Sci., **1255**, 12055, Jul. (2023), doi: 10.1088/1755-1315/1255/1/012055.
18. R. G. Birch, PLANT TRANSFORMATION: Problems and Strategies for Practical Application, Annu. Rev. Plant Physiol. Plant Mol. Biol., **48**, 297–326, Jun. (1997), doi: 10.1146/annurev.arplant.48.1.297.
19. N. Karimi, Effect of Different Growth Regulators on Callus Induction and Plant Regeneration of Satureja species, Annu. Res. Rev. Biol., **4**, 2646–2654, Jan. (2014), doi: 10.9734/ARRB/2014/7938.
20. T. A. Thorpe, History of plant tissue culture, Mol. Biotechnol., **37**, 169–180, Sep. (2007), doi: 10.1007/s12033-007-0031-3.
21. W. Kurniajati, F. Ahmad, D. Martanti, Herlina, W. Witjaksono, and Y. Poerba, Hybridization compatibility of wild and cultivated bananas (*Musa* spp.), (2024), p. 80032. doi: 10.1063/5.0187594.
22. Y. Poerba, T. Handayani, F. Ahmad, W. Witjaksono, and D. Martanti, Deskripsi Pisang: Koleksi Pusat Penelitian Biologi LIPI. (2018).
23. B. Panis, M. Nagel, and I. Van den houwe, Challenges and Prospects for the Conservation of Crop Genetic Resources in Field Genebanks, in In Vitro Collections and/or in Liquid Nitrogen, Plants, **9**, p. 1634, Nov. (2020), doi: 10.3390/plants9121634.
24. T. Murashige and F. Skoog, A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures, Physiol. Plant., **15**, 473–497, Jul. (1962), doi: 10.1111/j.1399-3054.1962.tb08052.x.
25. Uma, Subbaraya, et al. Somatic embryogenesis as a tool for reproduction of genetically stable plants in banana and confirmatory field trials. Plant Cell, Tissue and Organ Culture (PCTOC), **147**, pp.181–188, June(2021 https://doi.org/10.1007/s11240-021-

02108-0

26. Latunra, Andi Ilham. Induksi kalus pisang barangan merah *Musa acuminata* Colla dengan kombinasi hormon 2, 4-D dan Bap secara in vitro. Jurnal Ilmu alam dan lingkungan, **8**, 53-61 (2017).
27. P. Karintanyakit, et al. The impact of genome and 2, 4-d on callus induction from immature male flowers of seven banana cultivars. International Symposium on Tropical and Subtropical Fruits **1024**, 253-255 (2011)
28. B. Fazeli-Nasab, L. Shahraki-Mojahed, M. Hassanzadeh, and F. Bidarnamani, Investigation of Antimicrobial Activity of Medicinal Plant Extracts on *Bacillus cereus* Isolated from Soil, Gene, Cell Tissue, In Press, (2021), doi: 10.5812/gct.115133.
29. Yoyon Riono., Zat pengatur tumbuh kinetin untuk pertumbuhan sub kultur pisang barangan (*Mussa paradisiaca* L) dengan metode kultur jaringan, J. AGRO INDRAGIRI, **4**, 22–33, Jan. (1970), doi: 10.32520/jai.v4i1.1049.
30. N. H. Baruddin and F. Kayat, Development of Malaysian Wild Bananas Seed progenies, *Musa acuminata* ssp. malaccensis and *Musa gracilis*., J. Trop. Resour. Sustain. Sci., **3**, 247–251, Dec. (2015), doi: 10.47253/jtrss.v3i1.696.
31. F. Yelli, A. Titin, S. D. Utomo, and A. Pathak, Somatic embryogenesis in two cassava (*Manihot esculenta* Crantz) genotypes, Not. Bot. Horti Agrobot. Cluj-Napoca, **51**, 13039, Feb. (2023), doi: 10.15835/nbha51113039.
32. A. Azizi et al., Embryogenic callus induction of Indonesian Cassava (Menti and Adira 4) on different picloram concentrations, IOP Conf. Ser. Earth Environ. Sci., **1255**, 12053, (2023), doi: 10.1088/1755-1315/1255/1/012053.