

Screening Nos-*bar* gene of Transgenic Rice Callus (*Oryza Sativa* L.) Containing Antihypertensive Gene Encoding Gg-7pAH

Ridho Rizkiantoro

Graduate School of Biotechnology,
University of Jember, Indonesia

Tri Agus Siswoyo

The Center of Excellence on Crop Industrial Biotechnology
(PUI-PT BioTIn), University of Jember, Indonesia

ABSTRACT

Peptides derived from food protein have the potential to become antihypertensive agents with relatively few adverse side effects. The Gg-7pAH gene encoding antihypertensive is a gene containing the Cys-Met-Try-Leu-Ala-Ser-Gly (CMYLASG) heptapeptide, angiotensin-converting enzyme (ACE) inhibitor peptide from *Melinjo* (*Gnetum gnemon* L) seeds. This study aimed to investigate the expression of the tandem repeat heptapeptide (10x peptides, Gg7-pAH) and Nos-bar genes through callus resistance to phosphinothricin (PPT) in 4 different phases of callus: co-cultivation, elimination, screening, and regeneration. The Gg7-pAH gene was constructed in the *pGreen II 0229* vector containing the Nos-bar gene and Gg7-pAH gene under the control of the *Glu4* promoter and transformed into rice callus using an *Agrobacterium tumefaciens* vector. PPT was used in the screening at precise concentrations of 0, 20, 50, and 100 ppm. The screening efficiency was then evaluated by calculating the putative callus. As a result, 37 putative callus were developed with a concentration of 0 ppm PPT, 36 putative callus with a concentration of 20 ppm PPT, and none at 50 and 100 ppm PPT. The Gg-7pAH gene was identified by PCR analysis in putatively transgenic rice callus. According to PCR data, a positive callus with an antihypertensive gene insertion was detected at four phases: media, co-cultivation, elimination, screening, and regeneration. These findings suggest that the recombinant Gg-7pAH gene, which is transformed into rice plants to produce rice plants that can treat hypertension, has some potential.

Keywords: recombinant Gg-7pAH, transformation, heptapeptide, hypertension, *A. tumefaciens*.

INTRODUCTION

In Indonesia, Rice (*Oryza sativa* L.) is the primary source of staple foods. Because rice contains carbohydrates, protein, lipids, vitamins, and other essential minerals for the body, rice plants can be employed as nutraceutical and pharmaceutical ingredients (1). Due to Indonesia's high

rice consumption in food, there are chances to use rice as an alternative to hypertension medications. One opportunity to use them is to add ACE inhibitory peptides derived from *melinjo* seed isolates, which can be used to improve the quality of rice plants so that they can be alternative medications and can prevent hypertension. According to previous studies, the protein content in *melinjo* has high antioxidant, antidiabetic, and antihypertensive activity (2). Gen transformation is a technique for inserting a foreign gene into plants by isolating an organism into the plant genome. It can be used to improve the quality of rice plants. The antihypertensive gene derived from the *melinjo* protein will be transformed into the rice plant using *Agrobacterium tumefaciens* (3).

Gene transformation is the process of combining the desired target gene with the host plant gene to alter and enhance the traits of the host plant. Several techniques are used to introduce genes into host cells during the construction of genetically modified plants, including the microprojectile transfer method and the bombardment particle method; both require highly sophisticated tools. Bacterial intermediaries are another alternative for gene transformation. *Agrobacterium tumefaciens* has the benefit of more effectively and highly transferring a single gene without the need for sophisticated instruments. The heptapeptide gene was cloned and expressed as the recombinant protein Gg-7pAH in earlier research; the peptides are probably attached to the active substrate and induced ACE-inhibitor activity, and the results demonstrated that the Gg-7pAH recombinant protein could inhibit ACE [6].

The recombinant gene, which is resistant to the herbicide (phosphinothricin), has been inserted into the vector *pGreenII* with a selectable marker *nos-bar*. To distinguish between cells, tissues, or plants that have successfully transformed and those that have not, calluses infected by *A. tumefaciens* must be screened. Herbicide resistance brought on by the *bar* gene, including Basta and bialafos. L-phosphinothricin (PPT) is the active ingredient of basta non-selective herbicide used for weed control. The enzyme glutamine synthetase, sometimes known as GS, converts ammonia from glutamine to glutamate. Phosphinothricin is a structural analog of glutamate that binds to GS [4]. The bialaphos gene, also known as the basta resistance gene (*bar*), which encodes the phosphinothricin acetyl transferase enzyme, is generally chosen to produce transgenic plants. Plants are able to detoxify PPT since PAT activities exist. The development of putative rice plants was impacted by the herbicide or PPT screening procedure on rice callus transformed with Gg-7pAH.

METHODOLOGY

Callus Induction

Callus explants for transformation were obtained from milled rice seeds. Rice seeds from the short MS (Merah Sertani) variety were peeled and placed into a falcon containing sodium hypochlorite (NaClO) solution and 1:1 (v/v) aquadest. Peeling off the rice seeds is followed by washing and five rounds of sterilization with sterile distilled water to clean the rice seeds. The seeds were then dried in the air for five minutes using filter paper. The dried seed explants were then cultivated for 14 days on solid MS media containing ZPT 2.4D at a concentration of 1.5 ppm. 14-day-old calluses that have grown and been incubated are prepared for transformation [5].

Transformation and Co-cultivation Rice Callus

The *Agrobacterium tumefaciens* LBA4404 cells carrying binary plasmid pGreenII 0229 and *A. tumefaciens* concentration is 0.8 at OD₆₀₀. The *A. tumefaciens* was cultivated in 50 ml of liquid YEP media, 5 µl of the antibiotic kanamycin was added, and the mixture was then shaken at 150 rpm for 24 hours at room temperature. Infection was carried out by immersing the callus in MS solution with *A. tumefaciens* pellets added for 5 minutes while applying gentle pressure to the callus using tweezers. After that, the infected callus will be cultured on solid MS media with Acetosyringon 100 ppm added. The explants were incubated for three days in the dark to grow *A. tumefaciens* (7).

Screening Rice Callus Putative Transgenic

The screening phase involved transferring the callus from the co-cultivation medium to the screening medium. The screening was carried out at four treatment levels using graded PPT concentrations. *A. tumefaciens*-infected callus was transferred to MS media with the addition of 1.5 mg/L 2,4-D, 0 mg/L PPT, and 300 mg/L Cefotaxime for the initial treatment (SC-0). The callus was subcultured in both treatments on a screening medium (SC-20) containing MS medium, 1.5 mg/L 2,4-D, 20 mg/L PPT, and 300 mg/L Cefotaxime. In the third treatment, calluses were screened on media (SC-50) MS, 1.5 mg/L 2,4-D, 50 mg/L PPT, and 300 mg/L Cefotaxime. Calluses were evaluated on media (SC-100) MS, 1.5 mg/L 2,4-D, 50 mg/L PPT, and 300 mg/L Cefotaxime in the fourth treatment. Calluses were grown on a screening medium for 14 days and subcultured on a regeneration medium to grow plantlets (8).

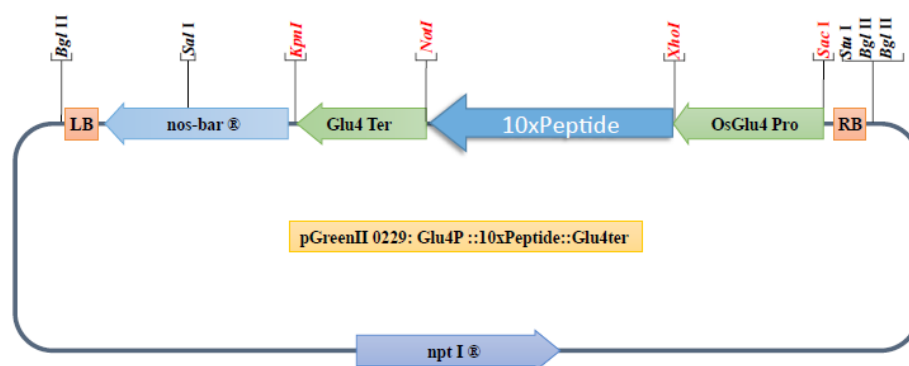


Figure 1. Construction map of the plasmid pGreenII 0229 10xPeptide. LB: Left border, RB: Right Border, nos: nopaline synthetase gene, Glu4 Ter: Gluteline 4 Terminator, 10xPeptide Gnetum gnemon, OsGlu4 Pro: Gluteline 4 Promoter.

Callus Regeneration

The regeneration phase is intended to grow green spots and which will later grow into shoots/plantlets. This phase is carried out on calluses that have passed the selection phase. Callus explants were grown on MS media with ZPT kinetin and NAA at 2 mg/L and 0.2 mg/L, respectively. Callus was planted to form plantlets. By subculturing the callus every two weeks, the callus receives maximum nutrition for the formation of plantlets [9].

Table 1. Composition of the hormone on medium for rice callus

Medium	Abbreviation	composition
Callus Induction medium	IM	30g/L sucrose, 4.4 g/L MS basal, 1 g/L Casein hydrolysate, 1.5 mg/L 2,4D.
Solid co-cultivation medium	CM	30g/L sucrose, 4.4 g/L MS basal, 1 g/L Casein hydrolysate, 1.5 mg/L 2,4D and 100 mg/L Acetosyringon
Recovery medium	RM	30g/L sucrose, 4.4 g/L MS basal, 1 g/L Casein hydrolysate, 1.5 mg/L 2,4D and 300 mg/L Cefotaxime
Selection medium	SM	30g/L sucrose, 4.4 g/L MS basal, 1 g/L Casein hydrolysate, 1.5 mg/L 2,4D, 200 mg/L Cefotaxime and 0/20/50/100 mg/L PPT
Regeneration medium	REM	30g/L sucrose, 4.4 g/L MS basal, 1 g/L Casein hydrolysate, 2 mg/L Kinetin, 100 mg/L Cefotaxime
Shooting medium	SM	30g/L sucrose, 4.4 g/L MS basal, 1 g/L Casein hydrolysate, 2 mg/L Kinetin 0.5 mg/L NAA and 100 mg/L Cefotaxime

Analysis PCR of Transgenic Callus

Plant DNA was extracted by pulverizing 0.1-0.2 grams of rice leaves with liquid nitrogen at the KIT Gene All GenEX Plant in Seoul, South Korea. PCR was tested with a 25 µL reaction volume containing 100 ng of template DNA, 10µM of each dNTP, 1 unit of Taq DNA Polymerase enzyme in buffer solution, and 0.2µM of each. The synthesized primer sequences were 5'CTC GAG ATG AAG TGT ATG ATC TTG CTT CTG GA-3' and 5'-GCG GCC GCT TAC TTT CCA GAA GCA AGA TA CA-3'. The 35 cycles of PCR were performed with predenaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 40 seconds, elongation at 72°C for 1 minute, and final extension at 72°C for 5 minutes. The PCR results were then electrophoresed using a 1.5 % agarose gel containing 3 µL Ethidium Bromide at 100 volts for 25 minutes. The DNA marker was 1 kb of PROMEGA ladder DNA in 3 µL. The electrophoresis results are then viewed in the gel documentation system.

RESULTS AND DISCUSSION

Callus Induction

The research began with the formation of embryogenic rice callus. Grown on induction media, induced rice seeds will begin to form callus three days after planting (HST) because the explants will show swelling and rapid cell proliferation 3-4 days after culture (10). The callus is derived from the scutellum, a layer of embryonic cells (11). Callus will experience callus volume development at the age of 14 HST. The cells found in the rice callus will undergo cell division and increase in volume to form a crumbly callus texture following the development of the rice callus (Figure 3.1).

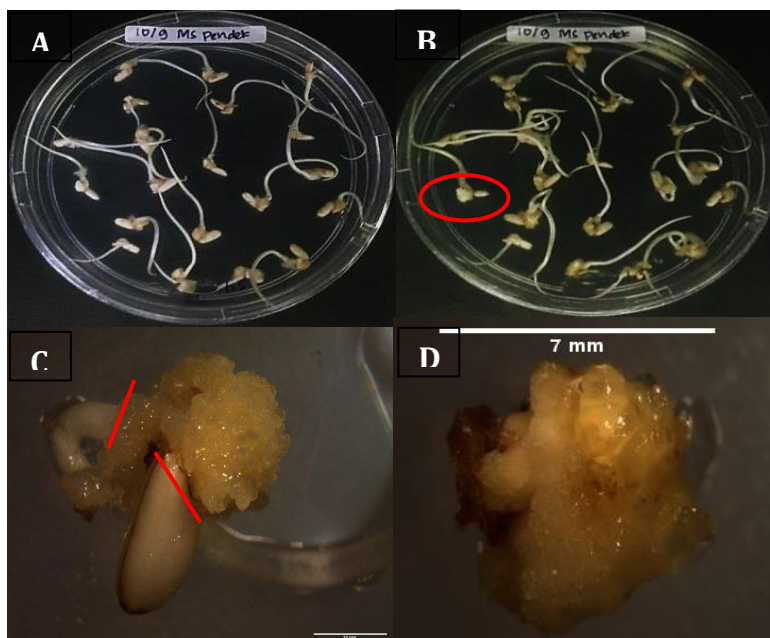


Figure 3.1 Callus growth at seven days old (A) Callus growth at 14 days old, seeds bud formation, and friable callus (B). Callus, aged 14 days, was removed from the buds (C). 14 days of callus have been removed (D).

After 14 days, a callus explant will appear, which is then removed from the bud. This is done to reduce the inhibition of callus development caused by the division of nutrients between the shoots and the callus. Maximizing the growth of callus explants and accelerating the infection process can be accomplished by severing embryos in the callus [12]. Furthermore, callus can be used for transformation. The transformation was carried out using callus, grown for 14 days on solid MS media, and *A. tumefaciens*, which had the Gg-7pAH gene inserted as a transformation intermediate. Callus induction is an important step in the process of obtaining callus explants. Callus is a group of undifferentiated cells that develop irregularly [14]. The callus used is embryogenic. The embryogenic callus has a desiccated, white, creamy callus structure and a crumb structure (13).

Transformation and Co-cultivation Rice callus

A. tumefaciens containing the antihypertensive protein-containing plasmid pGreenII 0229 was utilized to infect rice callus. The callus will be incubated for three days in the co-cultivation medium containing Acetosyringon to maximize the infection process during the co-cultivation phase.

Table 2. The efficiency of transformation by *Agrobacterium tumefaciens* of transgenic rice callus

Experiment	Number of callus	Co-cultivated	Over Growth	Browning	Texture	Color
1	65	63	2	0	Friable	Yellow
2	80	73	6	1	Friable	Yellow
3	80	80	0	0	Friable	Yellow

There are two types of callus growth in co-cultivation media: healthy callus growth and callus growth characterized by proliferation or excessive bacterial growth. Good callus growth in the co-cultivation phase is characterized by growth in the callus and experiencing embryogenic development. The absence of *Agrobacterium* contamination allows the callus to develop normally. In contrast, *Agrobacterium* contaminants impede the growth of a callus with excessive growth, resulting in the callus's death.

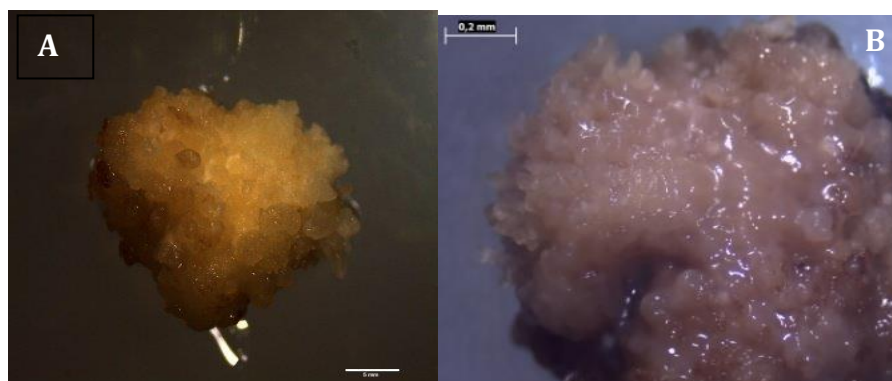


Figure 3.2 Callus after infection, which is in good condition and has overgrowth. Callus in good condition (A). callus experienced bacterial overgrowth/Overgrowth (B).

Naturally, damaged plant cells will produce acetosyringone, an *Agrobacterium* attractant. AS will then increase the vir gene and produce the vir protein that transfers T-DNA into the plant genome (16). An infected or transformed callus will then enter the co-cultivation stage to allow *A. tumefaciens*, which carries the Gg-7pAH gene, sufficient time to grow and be properly inserted. Callus was grown on solid media containing Acetosyringone. According to Karthikeyan[15], adding acetosyringone can optimize T-DNA transfer in an explant, which is anticipated to increase the efficacy of *A. tumefaciens* infection. Calluses that experience overgrowth will be resqueued using antibiotics. Cefotaxime antibiotics can inhibit bacterial growth while remaining safe for target gene networks (17). After being cleansed with cefotaxime, calluses are replanted on solid media. There is a possibility that the callus will continue to overgrow even if it has been washed using cefotaxime because cefotaxime only slows the development of bacteria and does not kill or stop it. Therefore, cefotaxime antibiotics must be administered later on. Therefore, cefotaxime antibiotics must be administered later on.

Screening on Transgenic Rice Callus

After three days of incubation and co-cultivation, the callus will be subcultured on screening media. Screening or selection media contain antibiotics that select explants not inserted with the target gene. The herbicide PPT was used as a selective agent in this investigation. Fourteen days were devoted to the selection procedure. The inserted callus will grow well, with noticeable callus growth and the appearance of a green spot callus.

Table 3. Screening phosphinothricin on transgenic rice callus

Screening treatment	Number of callus	Overgrowth	Browning	Callus passed the selection	Callus on regeneration media	Callus green spot
SC-0	40	2	1	38	38	37
SC-20	65	9	14	42	42	34
SC-50	60	16	18	26	26	0
SC-100	51	14	26	10	10	0

Calluses that have undergone three days of cultivation will then reach the screening phase. Screening or selection is conducted to determine whether callus explants can survive certain antibiotics or compounds. PPT as herbicides are residues of the natural antibiotic bialaphos found in microbes that inhibit glutamine (18). PPT herbicide was used at the screening stage because the plasmid contained the bar gene resistant to PPT herbicide. Herbicide-resistant genes are the most critical selectable marker gene in the development of transgenic crops; they have been widely used for nearly 20 years and are one of the marker gene options in transforming rice plants (4).

Explants that this gene has inserted will have resistance to PPT herbicides. Callus explants that survive the screening phase for 14 days will continue to develop, and either greening or a green spot will emerge on the callus. Greenspot is a callus that has formed chlorophyll under the influence of cytokinins; the callus will produce small green lumps (nodules) known as green spots [19] and is also an indication that the callus is still growing and will form new shoots (20). The *bar gene* confers herbicide resistance and is a widely used selectable marker gene for generating transgenic plants. Rice callus shows resistance to phosphinothricin in a medium containing 20 ppm phosphinothricin, whereas callus cannot survive in a medium containing concentration greater than 20 ppm. The effectiveness of antibiotic selection depends on plant species, cultivar types, and tissue types [18].

PCR Analysis on Transgenic Rice Callus

In this study, we analyzed the presence of target genes using PCR at several callus stages. We selected the callus during the co-cultivation, elimination, and regeneration phases. The probability of successful PCR in the co-cultivation phase is high because the *Agrobacterium* is in its infection phase. There is a possibility that DNA from *Agrobacterium* may also be isolated. During the elimination phase, cefotaxime antibiotics are added to the media to reduce or stop the growth of *Agrobacterium*, so it is extremely unlikely that DNA from the bacteria will be isolated. During the regeneration phase, the callus is free of *Agrobacterium*, so no DNA from the bacteria is isolated.

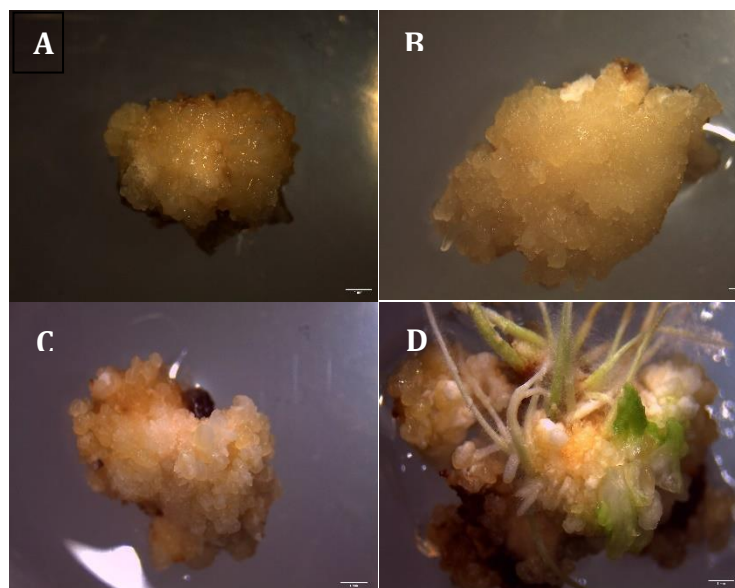


Fig 4. Visualization of selected callus from four phases: Co-cultivation (A), Elimination (B), Screening (C) and Regeneration (D)

The putative transgenic callus was screened for transgene integration by PCR using a specific primer. The amplified product 210bp indicated positive transgene insertion in each putative transgenic line. PCR analysis was carried out on the callus at various phases; during the co-cultivation phase, several callus were sampled. PCR results showed that the callus showed positive results of the gene being inserted into the callus cells, as indicated by the bands at 210bp, which were identical to the plasmid control.

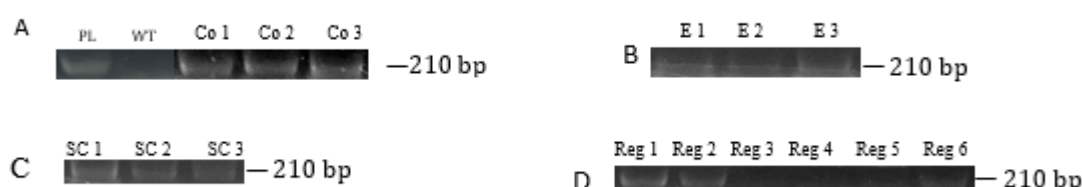


Fig 4. PCR analysis of four different phases of transgenic callus lines.

Using the primers XhoI and NotI, genomic DNA was amplified. Figure (A) depicts the PCR results from the positive control and wild type and the PCR results from the co-cultivation phase of transgenic callus. Figure B shows the PCR results of a transgenic callus during the elimination phase. (C) represents the PCR results of the transgenic callus during the screening phase, while (D) represents the PCR results of the transgenic callus during the regeneration phase. Co, E, SC, and Reg are phase codes, and 1-6 are callus numbers.

This study evaluated four samples: callus samples from the co-cultivation, elimination, screening, and regeneration phases. There were different results across the four phases. The presence of bands of size 210bp in the co-cultivation, elimination, and screening phases of the

PCR electrophoresis indicated that the Gg-7pAH gene had been inserted into the callus. In the regeneration phase of the six callus samples examined, three callus samples produced bands at 210 bp, and three callus samples did not. At the co-cultivation stage, all samples tested produced bands at 210bp. During this phase, it is likely that all callus samples tested will also produce bands at 210bp, as the callus at this stage was still filled with *Agrobacterium*, which was deliberately grown to contaminate PCR results with DNA fragments originating from bacterial DNA [22]. Calluses in the screening and regeneration phases are unlikely to be contaminated with bacterial DNA because, in the screening phase, the callus has escaped the herbicide Phosphinothricintrichin and cefotaxime media used to inhibit bacterial growth in explants so that callus overgrowth does not occur (21). Callus in the regeneration phase has greater gene stability than in other phases. The results indicate that some calluses do not insert target genes, indicating that in the regeneration phase, there is no growing *agrobacterium*, there are calluses that pass to the regeneration phase, but no target gene could be due to the presence of an escape callus or the callus does not touch the screening media ideally.

CONCLUSION

Screening of transgenic rice callus containing the herbicide resistance gene phosphinothricintrichin/nos-bar can be carried out by providing a concentration of 20 mg/L Phosphinothricintrichin (PPT). Based on the PCR results from four different callus phases, regeneration is the phase where the callus can already be PCR, so there is no bacterial DNA contamination of the PCR results.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Research, Technology and Higher Education, Indonesia.

References

- [1] Pradipta, S., Siswoyo, T. A., & Ubaidillah, M. (2023). Nutraceuticals and bioactive properties of local Java pigmented rice. *Biodiversitas*, 24(1), 571–582. <https://doi.org/10.13057/biodiv/d240166>
- [2] Siswoyo, Tri Agus, et al. "Isolation and Characterization of Antioxidant Protein Fractions from Melinjo (*Gnetum Gnemon*) Seeds." *Journal of Agricultural and Food Chemistry*, vol. 59, no. 10, 2011, pp. 5648–56, doi:10.1021/jf2000647. <https://pubs.acs.org/doi/abs/10.1021/jf2000647>
- [3] Kumar, Krishan, et al. "Genetically Modified Crops: Current Status and Future Prospects." *Planta*, vol. 251, no. 4, Springer Berlin Heidelberg, 2020, pp. 1–27, <https://doi.org/10.1007/s00425-020-03372-8>.
- [4] Tian, Y. S., Xu, J., Zhao, W., Xing, X. J., Fu, X. Y., Peng, R. H., & Yao, Q. H. (2015). Identification of a phosphinothricin-resistant mutant of rice glutamine synthetase using DNA shuffling. *Scientific Reports*, 5(September), 1–11. <https://doi.org/10.1038/srep15495>
- [5] Pathania, S., & Sandhu, J. S. (2021). Effect of 2,4-D on embryogenic callus induction and evaluation of g418 on growth inhibition in rice calli. *Agricultural Research Journal*, 58(1), 18–22. <https://doi.org/10.5958/2395-146X.2021.00002.8>

-
- [6] Yuliana, Akbar, R. R., & Siswoyo, T. A. (2019). Expression of recombinant angiotensin I-converting enzyme-inhibitory heptapeptide gene in *Escherichia coli* BL21. *Current Topics in Peptide and Protein Research*, 20, 81–89.
<http://www.researchtrends.net/tia/abstract.asp?in=0&vn=20&tid=26&aid=6450&pub=2019&type=3>
- [7] Wang, X., Zhou, F., Liu, J., Liu, W., Zhang, S., Li, D., Song, J., Wang, R., & Yang, Y. (2021). Establishment of efficient callus genetic transformation system for *Pyrus armeniacaefolia*. *Scientia Horticulturae*, 289(December 2020), 110429. <https://doi.org/10.1016/j.scienta.2021.110429>
<https://doi.org/10.1016/j.scienta.2021.110429>
- [8] Al, Hertfordshire, et al. *Art%3A10.1023%2FA%3A1009689906936.Pdf*. 1999, pp. 255–62.
<https://link.springer.com/article/10.1023/A:1009689906936>
- [9] Binte Mostafiz, Suraiya, and Alina Wagiran. “Efficient Callus Induction and Regeneration in Selected Indica Rice.” *Agronomy*, vol. 8, no. 5, 2018, doi:10.3390/agronomy8050077
<https://doi.org/10.3390/agronomy8050077>
- [10] Ahmadpour R., Nasser Z., Rasool A. Z and Parisa S. 2016. Efficient In Vitro Somatic Embryogenesis and Plant Regeneration from Mature and Immature Embryos of Wheat (*Triticum aestivum* L.) *Brazilian Archives of Biology and Technology*, vol 59: e17160288.
<https://doi.org/10.1590/1678-4324-2016160288>
- [11] He, G.Y., P.A. Lazzer. 2001. Improvement of somatic embryogenesis and plant regeneration from durum wheat (*Triticum turgidum* var durum Derf) scutellum and inflorescence culture. *Euphytica*. 119 (3)369-376. <https://link.springer.com/article/10.1023/A:1017587628995>
- [12] Hiei, Y. and Komari T. 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.* 3 824-834 10.1038/nprot.2008.
<https://doi.org/10.1038/nprot.2008.46>
- [13] Hoque ME, Ali MS, Karim NH (2007) Embryogenic Callus Induction and Regeneration of Elite Bangladeshi Indica Rice Cultivars. *Plant Tiss Cult Biotechnol*. 17: 65-70. doi: 10.3329/ptcb.v17i1.1122
<https://doi.org/10.3329/ptcb.v17i1.1122>
- [14] Ikeuchi, M., Sugimoto, K., and Iwase, A. 2013. Plant Callus: Mechanism of Induction and Repression. *The plant cell*, vol. 25: 3159-317. <https://doi.org/10.1105/tpc.113.116053>
- [15] Karthikeyan, A., Shilpha, j., Karutha Pandian, S., and Ramesh, M. 2012. *Agrobacterium*-mediated transformation of indica rice cv. ADT 43. *Plant Cell Tiss Organ Cult*. 109: 153-165.
<https://doi.org/10.1007/s11240-011-0083-8>
- [16] Agrawal, Shreni, and Esha Rami. “A Review: *Agrobacterium*-Mediated Gene Transformation to Increase Plant Productivity.” *The Journal of Phytopharmacology*, vol. 11, no. 2, 2022, pp. 111–17, doi:10.31254/phyto.2022.11211 <https://doi.org/10.31254/phyto.2022.11211>
- [17] Kant, Sashi, et al. “A Novel STK1-Targeted Small-Molecule as an ‘Antibiotic Resistance Breaker’ against Multidrug-Resistant *Staphylococcus Aureus*.” *Scientific Reports*, vol. 7, no. 1, Springer US, 2017, pp. 1–19, doi:10.1038/s41598-017-05314-z. <https://link.springer.com/content/pdf/10.1038/s41598-017-05314-z.pdf>
- [18] [18] Cui, Y., Liu, Z., Li, Y., Zhou, F., Chen, H., & Lin, Y. (2016). Application of a novel phosphinothricin N-acetyltransferase (RePAT) gene in developing glufosinate-resistant rice. *Scientific Reports*, 6(February).
<https://doi.org/10.1038/srep21259>
-

- [19] Santosa dan Nursandi. 2001. *Kultur Jaringan Tanaman*. UMM Pres Malang.
- [20] Maftuchah dan Loedin, I.H.S. 2000. Seleksi InVitro Tanaman Lada Untuk Ketahanan Terhadap Penyakit Busuk Pangkal Batang. *Jurnal Agrobiogen* 1(1): 13-19 DOI: 10.21082/jbio.v1n1.2005.p13-19
- [21] Rojo, Fernando Perez, et al. "An Improved Protocol for Agrobacterium-Mediated Transformation in Subterranean Clover (*Trifolium subterraneum* L.)." *International Journal of Molecular Sciences*, vol. 22, no. 8, 2021, doi:10.3390/ijms22084181. <https://doi.org/10.3390/ijms22084181>
- [22] Grahn, Niclas, et al. "Identification of Mixed Bacterial DNA Contamination in Broad-Range PCR Amplification of 16S rDNA V1 and V3 Variable Regions by Pyrosequencing of Cloned Amplicons." *FEMS Microbiology Letters*, vol. 219, no. 1, 2003, pp. 87-91, doi:10.1016/S0378. [https://doi.org/10.1016/S0378-1097\(02\)01190-4](https://doi.org/10.1016/S0378-1097(02)01190-4)