In vitro shoot induction of BesNO H 382 tobacco using benzyl amino purine

Dyah Nuning Erawati[1*], *Putri* Deviah[1], *Sepdian Luri* Asmono[1], *Eko Hadi* Cahyono[2], and *Harits Ramadhan* Fajriant[3]

[1]Agricultural Production Department, Politeknik Negeri Jember, Mastrip PO Box 164 Jember, Indonesia
[2]Tissue Culture Laboratory, Politeknik Negeri Jember, Mastrip PO Box 164 Jember, Indonesia
[3]Agriculture Department, Jember University, Kalimantan 37 Jember, Indonesia

Abstract. Besuki Na Oogst (BesNO) H 382 tobacco is a superior commodity intended for export as a raw material for cigar production in the international market from Jember Indonesia. The development of its seed development through tissue culture has advantages such as the ability to produce healthy and uniform seeds, pathogen-free and regardless of the season. This study aimed to determine the optimal concentration of benzyl amino purine (BAP) for in vitro shoot induction. The research using a Completely Randomized Design with four levels of BAP concentration ranging from 0 to 3 ppm. The findings showed that BAP concentrations between 1 and 3 ppm significantly influenced shoot induction of BesNO H 382 tobacco. Among these treatments, 3 ppm BAP was identified as the most effective concentration, with shoot emergence observed at 8.40 days, an explant weight of 4.44 g, and an average of 30 shoots per explant at 56 days after inoculation. These results provide valuable insights into the optimization of plant growth regulators from the Cytokinin group for optimal shoot induction of BesNO H382 tobacco which is still rarely reported with a new modification of the explant sterilization technique.

1 Introduction

Tobacco plants play a vital role in the economy and have high economic value as a source of income for farmers and a source of foreign exchange that supports the tobacco agribusiness and agro-industry in Indonesia. Tobacco export volume in 2023 was 3,028,537.00 kg, with a foreign exchange value of US$ 31,947,248.88.

The types of tobacco used in the cigar industry are often referred to as Besuki cigar tobacco or Besuki Na Oogst (BesNO) [1]. Besuki Na Oogst (BesNO) tobacco has several varieties, one of which is H 382. This variety produces elastic, thin leaves with a good aroma. This plant also has the advantage of being resistant to pests and diseases. H 382 is the most

* Corresponding author: dyah_nuning_e@polije.ac.id

commonly for cigar production. This variety has long and wide leaves, fine veins, and good quality, making it a favourite among farmers and international buyers for its superior qualities. H 382 also produces a thicker krosok than other BesNO varieties, making it suitable for use as a cigar wrapper (dekblad). The H382 variety has a broad market, making it easier for farmers to market their crops. Furthermore, climate conditions also significantly influence the availability of seeds for planting [2].

Tobacco plants are usually cultivated conventionally using seeds for propagation. However, seed propagation often results in heterogeneous genetic traits among the offspring, as tobacco is capable of self- and cross-pollination, leading to genetic characteristics that differ from the parent plant. In addition, seed-based cultivation is highly susceptible to disease. Therefore, tissue culture is carried out to overcome this problem by providing quality planting material.

Tobacco development through tissue culture techniques can be achieved using tobacco leaves as explants grown with plant growth regulators. Obstacles to the level of growth capacity of explants can be overcome by adding plant hormones, which play a greater role in directing explant growth. In vitro culture techniques, cell division and shoot proliferation are influenced by cytokinin-based plant growth regulators. By adding cytokinin to tissue culture media, more than one shoot will emerge (proliferation) allowing the formation of multiple shoots. However, at high concentrations, plant growth regulators may inhibit shoot proliferation and root formation.

Several studies on tobacco tissue culture have reported varying results. Erawati et al. (2024) [3] reported that adding BAP at a concentration of 3 ppm induced up to 42 shoots. The fastest shoot emergence occurred at 10 days after inoculation, and produced the highest shoot with an average weight of 4.7 gram in Kasturi Mawar tobacco. Rahayu and Asmono (2019) [4] showed different results because the addition of 2 ppm of the growth regulator BAP combined with 0.5 ppm of IAA to tobacco tissue cultures of the Prancak 95 variety produced 34.25 shoots. The unequal response of tobacco explants was reported by Shinde et al. (2019) [5], that 55% of tobacco leaf callus induction was successful on MS + 0.1 ppm NAA + 1.0 ppm BAP media. Meanwhile, the MS + 1.0 ppm NAA + 1.0 ppm BAP treatment had the highest number of shoots with an average of 10 shoots/explant.

Therefore, this study aims to determine the optimal BAP concentration for in vitro induction of BesNO H 382 tobacco shoots, so the preparation of uniform tobacco seeds that are not dependent on the planting season can be achieved through the development of plant propagation innovations using tissue culture techniques.

2 Materials and Methods

The trial was conducted from March to November 2024 at the Tissue Culture Laboratory of Politeknik Negeri Jember Indonesia with a latitude 8°09'35.1"S longitude 113°43'27.2"E. The trial followed a Completely Randomized Design (CRD), consisting of with 4 treatment levels and 5 repetitions. The treatment involved additional of Benzyl Amino Purine (BAP) to Murashige-Skoog media consisting of several concentrations: 0 ppm BAP; 1 ppm BAP; 2 ppm BAP and 3 ppm BAP. Data were analysed using the F test, further analysis with Least Significant Difference (LSD) test for further comparison.

2.1 Preparation room and equipment

The preparation process began with sterilize the room using a 10% disinfectant solution to mop the floor and clean the incubation rack. Laminar Air Flow sterilization using 70% alcohol. Sterilization of the inoculation room and incubation room using 1% formalin for 24

hours. Equipment preparation involved washing all glassware and inoculation tools with dish soap, followed by thorough rinsing with running water until clean. Sterilize glassware using an oven for 120 minutes at 160ºC. Sterilize planting tools and metal tools with the autoclave, then all sterile tools are put into a storage cabinet [6].

2.2 Preparation of culture medium

The culture medium was prepared based on the Murashige and Skoog (MS) formulation, consisting of inorganic and organic nutrients, vitamins, 30 g of sucrose, and 8 g of agar per liter, with the pH adjusted to 5.8. Benzyl Amino Purine (BAP) was added according to the treatment concentration. The medium was sterilized in an autoclave at 121°C for 30 minutes and subsequently stored on a media storage rack [7].

2.3 Preparation of explants

Sterilization of explants began by washing H 382 tobacco leaves under running water and soaking the tobacco leaves in a 20% Tween solution for 5 minutes, then rinsing with distilled water. The leaves were then soaked in a fungicide and bactericide solution for 30 minutes, followed by immersion in 96% alcohol for 1 minute and in 10% Clorox (sodium hypochlorite) solution for 5 minutes and rinse in sterile distilled water 3 times. Leaf segments measuring approximately 1 cm^2 were then cut and inoculated into culture bottles containing the prepared medium. The inoculated explants were subsequently maintained in an incubation room [3]. A new modification to the explant sterilization technique by adding 50 minutes of soaking time in fungicide and bactericide solutions.

2.4 Observation

The observed parameters included: a) Shoot formation time (days after inoculation) – recorded daily by noting the appearance of the first shoots on each explant with daily observations. The rate of shoot formation was expressed as the number of days after inoculation until the appearance of the first shoots. Observations continued until all treatments had formed shoots; b) Number of shoots (shoots per explant) – determined by counting shoots that reached a minimum height of 0.5 cm on explants that successfully formed shoots at 56 days after inoculation; c) Fresh weight of explants (grams per explant) – measured by weighing explants from each treatment at 56 days after inoculation; and d) Qualitative observation of explant growth and development – carried out descriptively to document morphological changes during the culture period.

3 Results and discussion

3.1 Shoot formation

The shoot formation parameter indicates the explant's ability to initiate its first shoot. Shoot development was observed daily until all explants cultured on Murashige and Skoog (MS) medium with different BAP concentrations developed shoots and exhibited green coloration. The appearance of shoots was recorded in days after inoculation (DAI).

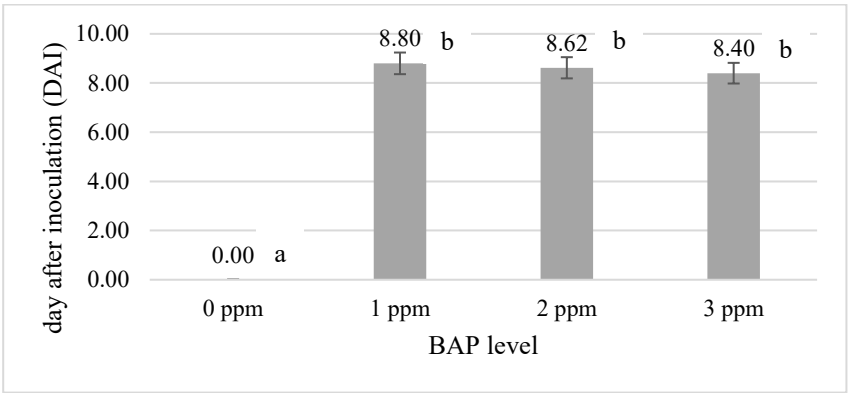

Fig. 1. Rate shoot formation of BesNO H 382 explant

Figure 1. shows based on the results of the LSD test at the 0.01 level, that the addition of BAP to BesNO H 382 tobacco produced the same effect on the rate of shoot emergence among different BAP concentrations at 8 days after inoculation. However, a significant difference was observed in explants cultured on MS basal medium without BAP, which exhibited a lower ability to form shoots. Shoot initiation occurred at the incision scars and on the parts of the explants in direct contact with the medium. The appearance of shoots was indicated by the formation of green swellings, followed by the development of needle-like shoots. The use of the plant growth regulator BAP at concentrations of 1–3 ppm was found to be optimal for stimulating shoot formation in BesNO H 382 explants. BAP, a type of cytokinin, plays a crucial role in promoting cell division and inducing shoot development. Within this concentration range, BAP effectively accelerates the initiation and emergence of shoots.

Young tobacco leaves, when used as explants, will grow and regenerate more quickly because young leaves have actively dividing cells with less complex cell walls than older leaves, making them easier to modify. The culture medium also plays a significant role in the success of tissue culture, as it provides the essential nutrients required for explant growth, such as those supplied by MS medium. However, when explants were cultured on MS medium without the addition of cytokinin, shoot formation did not occur due to the absence of this essential growth regulator.

The addition of the growth regulator BAP to the culture medium serves to supply cytokinin, which function as key stimulants of cell division. According to Rustam et al. (2020) [8], BAP is classified as an aromatic cytokinin with high physiological activity. Its molecular structure contains saturated double bonds, which contribute to its stability and resistance to oxidation. These properties enhance its effectiveness in promoting shoot induction, callus formation, and the development of shoots and leaves in in vitro culture systems.

3.2 Explant weight

Figure 2. shows that the addition of 1 ppm BAP resulted in the highest explant weight for BesNO H 382, with an average of 5.52 grams. This value was not significantly different from those obtained with 2 ppm and 3 ppm BAP treatments based on the results of the LSD test at the 0.01 level. These results indicate that the application of BAP significantly influences explant biomass accumulation. The fresh weight of explants is affected by BAP due to cell wall thickening and high metabolic activity during cell division and enlargement. In contrast, explants grown without BAP exhibited the lowest weight. The absence of BAP led to a

hormonal imbalance in the culture medium, thereby inhibiting optimal explant growth and development. This finding is consistent with the report of Nurani et al. (2020) [9], which stated that the addition of BAP can accelerate cell division and promote in vitro shoot proliferation.

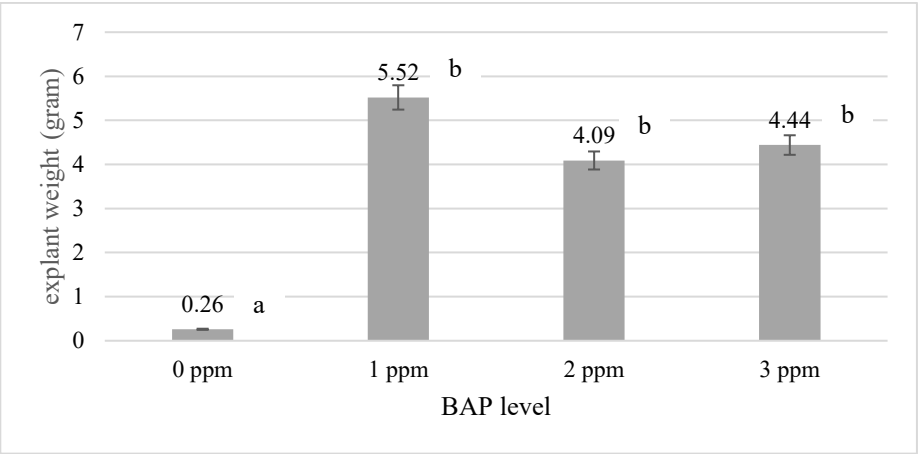

Fig. 2. Rate of explant weight increase during shoot induction of BesNO H 382 tobacco

The endogenous hormones present in the explants were insufficient to induce shoot growth, resulting in the lowest fresh weight observed in the 0 ppm BAP treatment. However, Yu et al. (2023) [10] reported that in vitro tobacco development is associated with increased activity in the GS/GOGAT cycle, which enhances energy production but simultaneously reduces photosynthetic activity and carbon metabolism.

The BesNO H 382 explants grown on MS medium without the addition of BAP showed no visible callus or shoot growth. Endogenous and exogenous hormones alone are often insufficient to efficiently stimulate growth, thereby inhibiting callus and shoot formation. Explant weight is strongly influenced by the explant's ability to produce multiple shoots, as BAP plays a crucial role in stimulating cell division and promoting shoot formation in tobacco explants.

3.3 Number of shoots

The number of shoots is a key parameter in in vitro tissue culture, as it reflects the effectiveness of the applied concentration of the growth regulator BAP. Observing shoot number provides insight into the explant's response to cytokinin treatment. In this study, shoots were considered countable when they reached a minimum height of 0.5 cm.

Figure 3. shows that the highest average number of shoots was obtained at the 3 ppm BAP concentration, producing an average of 30.00 shoots. This suggests that 3 ppm BAP can be considered an optimal concentration for shoot induction in BesNO H 382. The 1 ppm and 2 ppm BAP treatments showed no significant difference in their effects on shoot formation. Increasing cytokinin concentrations enhances callus differentiation and promotes shoot formation. Shoot induction, which refers to the initiation and development of shoots, is strongly influenced by the presence of plant growth regulators. Among these, cytokinin are particularly effective because they stimulate cell division and organogenesis. This finding provides clear evidence that BAP effectively induces shoot formation and stimulates the regeneration of BesNO H 382 explants through tissue culture techniques.

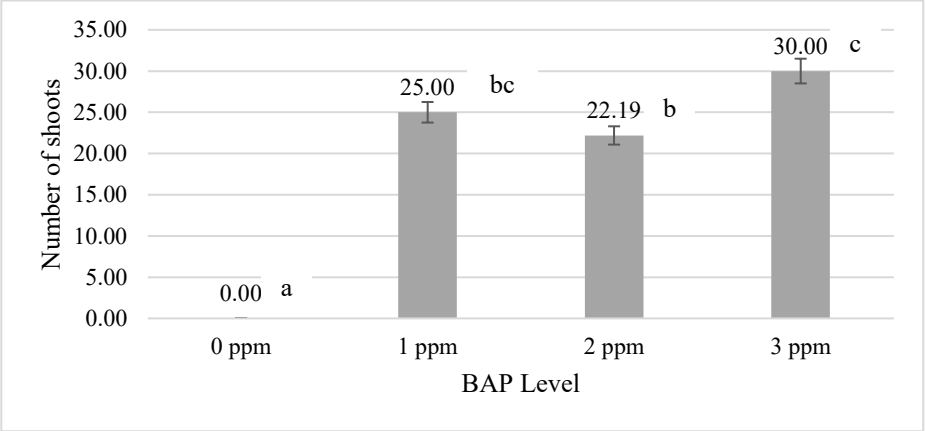

Fig. 3. Rate of Shoot Induction in BesNO H 382 Using BAP

Explants cultured without the addition of BAP did not exhibit any shoot growth, although they appeared slightly swollen compared to their normal size. This condition indicates a deficiency of growth hormones in the medium. BAP functions as a key stimulator of shoot formation. Cytokinin such as BAP can stimulate shoot growth, promote cell division, and enhance shoot initiation. Similarly, Fatikhasari et al. (2021) [11] stated that BAP is a synthetic cytokinin capable of promoting cell division, growth, and overall plant development. The addition of cytokinin accelerates shoot emergence and increases shoot number and plant survival rates through the action of BAP. However, Muhammed et al. (2021) [12] reported different results, showing that the highest number of shoots was obtained on MS medium supplemented with 0.2 mg L^{-1} NAA and 4.0 mg L^{-1} BAP. The greatest callus fresh weight was observed at 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP, while the best shoot regeneration in the TAPM 26 tobacco variety occurred with MS + 3.0 mg L^{-1} NAA + 1.0 mg L^{-1} BAP.

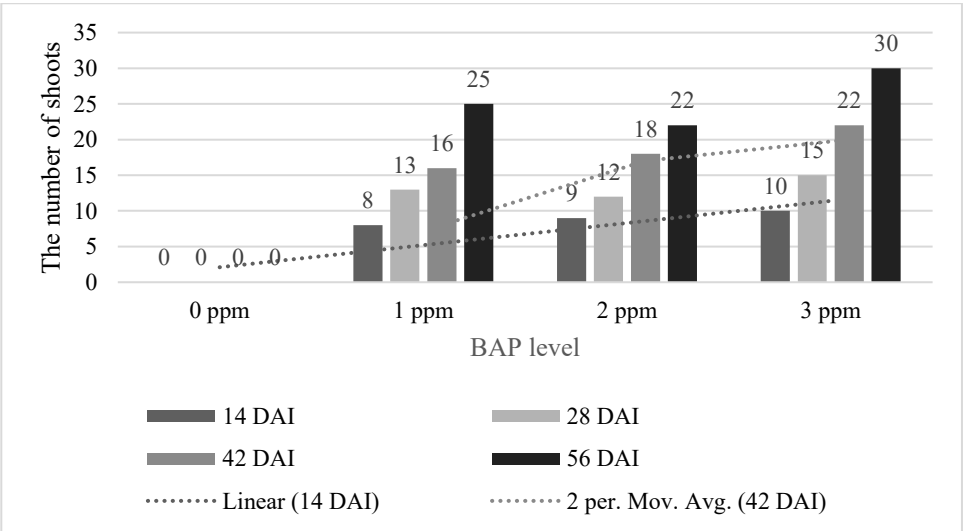

Fig. 4. Growth rate of the shoots number in BesNO H 382 explants

Based on Figure 4. a linear relationship was observed between BAP concentration and the number of shoots 14 days after inoculation (DAI). This trend indicates that the number of

shoots in BesNO H 382 explants increased linearly with higher BAP concentrations. The moving average trend line at 42 DAI also showed a consistent linear increase in shoot number throughout the culture period, reflecting stable and progressive growth under BAP treatment. However, the concentration of BAP added to the MS medium must be carefully optimized, as plant growth regulators are required only in very small amounts to achieve effective morphogenesis.

3.4 Growth and Development of Explants

The growth and development of explants in forming tobacco shoots is a primary indicator of shoot induction, , as tobacco leaf tissues do not naturally produce shoots immediately. An explant must grow and develop to form shoots. For shoot formation to occur, the explant must first undergo growth and developmental processes. Growth refers to measurable physical changes, including increases in size, number, and weight of explants, whereas development involves cellular differentiation, organ formation, and transitions in the explant's physiological phase.

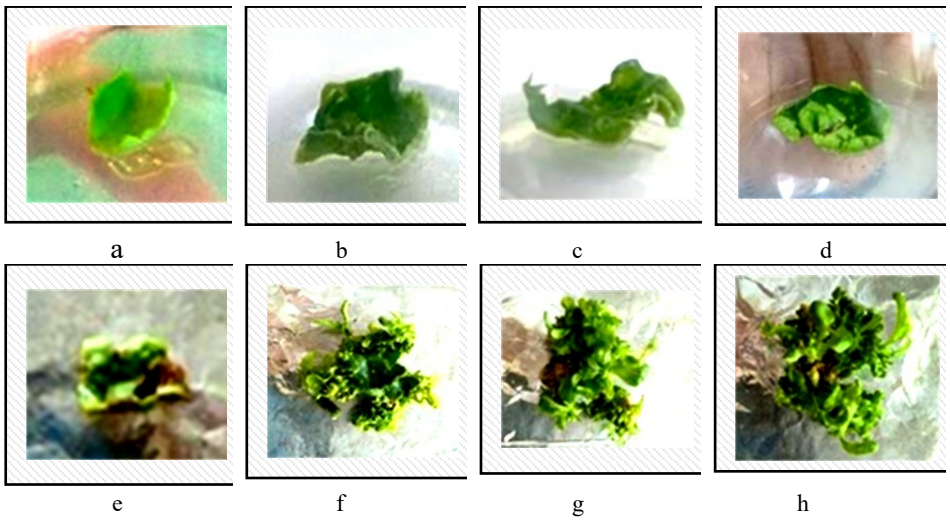

Fig. 5. Comparison of the growth and development of BesNO H 382 explants treated with 0–3 ppm BAP, observed at 14 days after inoculation (a–d) and 56 days after inoculation (e–h).

Figure 5. (a–d) illustrates the growth of BesNO H 382 explants 14 days after inoculation. Explants cultured on MS medium without BAP (0 ppm) exhibited only swelling, with no visible shoot formation (Fig. 5a). In contrast, explants treated with 1–3 ppm BAP showed callus formation followed by successful shoot induction (Fig. 5b, 5c, and 5d). The emergence of shoots represents a developmental response triggered by wounding during inoculation and the influence of growth regulators in the culture medium, enabling the explant to regenerate into a complete plant. Shoot formation thus serves as a crucial indicator of success in tissue culture propagation.

Figure 5. (e–h) illustrates the growth of BesNO H 382 explants 56 days after inoculation. Explants cultured without BAP exhibited no shoot formation, and the tips of the explants showed necrosis, appearing dry and brownish (Fig. 5e). This indicates that tobacco leaf explants are unable to initiate bud formation in the absence of BAP, as the endogenous phytohormone levels are insufficient for shoot induction, even when grown on nutrient-rich MS medium. In contrast, H 382 explants treated with 1–3 ppm BAP (Fig. 5f, 5g, and 5h) showed a positive response, with progressively enhanced shoot growth and development

corresponding to increasing BAP concentrations. Explants supplemented with 3 ppm BAP (Fig. 5h) produced the highest number of shoots, with several already undergoing organogenesis to form leaves. Similarly, Hussein et al. (2020) [13] emphasized the importance of optimizing BAP concentrations to enhance the growth and regeneration of *Nicotiana tabacum* explants.

The success of BesNO H 382 tobacco shoot induction with the addition of BAP is also supported by the low risk of explant contamination. Explant contamination can be reduced because the work stages in the explant sterilization process follow strict aseptic procedures according to the Standard Operating Procedures (SOP) at the Tissue Culture Laboratory of Politeknik Negeri Jember, Indonesia.

4 Conclusion

The tissue culture of BesNO H 382 using BAP for shoot induction proved effective at concentrations between 1–3 ppm, which significantly influenced shoot formation. Among the tested treatments, 3 ppm BAP was identified as the optimal concentration for the growth and development of H 382 tobacco explants, producing shoot emergence at an average of 8.40 days, an explant weight of 4.44 g, and 30 shoots at 56 days after inoculation.

The advantages of tissue culture propagation of BesNO H 382 include the production of clonal, uniform, and disease-free tobacco seedlings. The novelty in explant sterilization techniques further guarantees the successful growth and development of explants into plantlets.

These findings provide valuable insights for in vitro propagation and improvement of H 382 tobacco, which is still rarely reported. However, further research is required to evaluate the response of H 382 explants to BAP concentrations above 3 ppm to determine the upper threshold for optimal shoot induction.

References

1. N. Eurika & A.I Hapsari, An analysis of Na Oogst tobacco as biology learning resource. J Biologi dan Pembelajaran Biologi 2 (2) (2017) https://doi.org/10.32528/bioma.v2i2.824
2. Soetriono, E. Solihanani, F.E Zulan, N. Inayatin, N. Susanti and Q. Zuniana, Agribisnis tembakau besuki na oogst (Surya Pena Gemilang, 2014)
3. D.N Erawati, I.A Nikmatin, H.R Fajrianto and S.L Asmono, Technology of shoot induction culture tobacco through the addition of benzyl amino purine. J of Agricultural Industry and Food Technology **1** (1) (2024) https://doi.org/10.37148/jaiftech.v1i1.7
4. E.M Rahayu & S.L Asmono, Respon pertumbuhan eksplan daun tembakau (*Nicotiana tabacum* L.) Prancak-95 pada beberapa konsentrasi sukrosa dan BAP secara in vitro. Agriprima: J of Applied Agricultural Sciences **3** (2) (2019) https://doi.org/10.25047/agriprima.v3i2.328
5. S.S Shinde, S.B Sanap, A.M Patil, N.A Shinde, A.A Daspute and D.K Sarode, In vitro regeneration of *Nicotiana tabacum* L. from callus culture. *The Pharma Innovation Journal* **10** (12) (2019)
6. D.N Erawati, I. Wardati, S. Humaida, Y. Mawaddah, A. Ikanafi'ah and W.M Ryana, Shoots multiplication of vanilla (*Vanilla planifolia*) with benzyl amino purine and kinetin modification. IOP Conf. Series: Earth and Environmental Science 672 (2021) https://doi.org/10.1088/1755-1315/672/1/012007

7. S. Tyub, S.A Dar, I.M Lone, A.H Mir and A.N Kamili, A robust in-vitro protocol for shoot multiplication of *Echinacea angustifolia*. Current Plant Biology **2** (2021) https://doi.org/10.1016/j.cpb.2021.100221
8. Rustam, R. Syamsuddin, E. Soekandarsih E and D.D Trijuno, Study of the use of growth regulators (BAP) on shoot formation and absolute growth of seaweed (*Kappaphycus alvarezii*, Doty.) Prosiding Simposium Nasional VII Kelautan dan Perikanan (2020)
9. A. Nuraini, E. Aprilia, Murgayanti and A.P Wulandari, Pengaruh konsentrasi Benzylaminopurine terhadap pertumbuhan eksplan tunas aksilar rami klon lokal Wonosobo secara in vitro. Jurnal Kultivasi **21** (2) (2020)
10. J. Yu, X. Wang, Q. Yuan, J. Shi, J. Cai, Z. Li and H. Ma, Elucidating the impact of in vitro cultivation on *Nicotiana tabacum* metabolism through combined in silico modeling and Front. Plant Sci. **14**:1281348 (2023) https://doi.org/10.3389/fpls.2023.1281348
11. N.N Fatikhasari, Karno and B.A Kristanto, Effect of diameter lower stem and BAP (Benzyl Amino Purin) hormone on the success of top grafting *Sapodilla*. Agrosains: Jurnal Penelitian Agronomi **23** (1) (2021) http://dx.doi.org/10.20961/agsjpa.v23i1.44696
12. K.T Muhammed, M.A Javel, F. Huyop and Y. Kaya, Plant tissue culture of *Nicotiana tabacum* cv. TAPM 26 and its minimum inhibition against herbicide-Dalapon MANAS Journal of Engineering **9** (1) (2021) https://doi.org/10.51354/mjen.839516
13. N.M Hussein, Y.Kaya and F.Z Huyop, An easy and reliable method for establishment and maintenance of tissue cultures of *Nicotiana tabacum* cv. TAPM 26International Journal of Science Letters **2** (2) (2020) https://doi.org/10.38058/ijsl.764947