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Plant Regeneration in *Amorphophallus muelleri* Blume. through Organogenic

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Abstract. Porang (*Amorphophallus muelleri* Blume.) usually used to functional food raw materials, caused its high glucomant content. Limitation of conventional propagation both vegetative and generative affected to decreasing of porang production to fulfil market demand. To solve the problem, plant tissue culture technique was chosen to rapid propagate bulbils of porang. So, the aim of this research to determine the combination and concentration of Plant growth Regulators (PGRs), such as 6-Benzylaminopurine (BAP), Naphthaleneacetic Acid (NAA), and Thidiazuron (TDZ) for quality of callus, also growth of callus and shoots for rapid propagation of porang, cause PGRs are one of factors that affect the success of in vitro propagation. Experimental design of this research was completely randomized designed with statistical analysis using analysis of variance (Anova) one way. Difference combination and concentration of cytokinin and auxin as manipulation variables, which several parameters growth of callus, shoots, and roots was observed to indicate the rapid organogenic of porang. The optimal shoot induction medium was Murashige and Skoog (MS) media with 5.0 mg.L^{-1} BAP and 0.2 mg.L^{-1} NAA on which number of shoots and shoot height reached 15±1.8 and (7.2±1.86) cm. After shoot formation stage, the proper media for rooting stage was determined, which was Murashige and Skoog (MS) media with 1.0 mg.L^{-1} NAA resulted the rooting rate reached 100% and roots appear earlier. Optimal combination and concentration of PGRs on micropropagation of *Amorphophallus muelleri* Blume. from bulbils explant should be known to increase the production of porang, which can fulfil the market demand.

Keywords: bulbils, plant regeneration, rooting stage.

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INTRODUCTION

Porang (*Amorphophallus muelleri*) is a group of Araceae family, easy to growth at tropical regions with low intensity of light, such as forest, along of river, garden, and others (Reflini, 2017). Porang usually used to functional food raw materials, caused its high glucomant content, which glucomant is polysaccharide compounds of the type of hemicellulose of a nature hydrocolloid, water soluble, low calorie, and gluten free (Ibrahim, 2019). In addition, porang also used to cosmetics, pharmacy industry, and others. Bulbils is the part of porang, which is very useful in industry. Bulbils of porang have dormant stage at specific time.

Conventional propagation of porang can use bulbils (vegetative) or seeds (generative). Vegetative propagation of porang using bulbils take a long times, caused one bulbil only produced one plant (R. Sari & Suhartati, 2015), whereas generative propagation would produce many variety genetic of porang and not always available. Moreover, bulbils of porang a bit expensive in large quantities, which takes a long time that caused very few available seedlings (Anturida & Azrianingsih, 2015). So, the production bulbils of porang was low and can not fulfil the market demand. To solve the problem, plant tissue culture technique was chosen to propagate bulbils of porang.

The plant tissue culture technique (in vitro propagation) is one of the plant propagation techniques, which only used a bit part of the mother plant and it will be regenerated into a whole plantlet in an aseptic and controlled environment (Nyoman Lidyawati & Nengah Suwastika, 2012). Other advantages of using in vitro propagation for porang are free to season, producing high quantities of porang in a relatively short time, good quality seedlings, no variety genetic, free disease, and others (Sulistiani & Yani, 2012). In vitro propagation of porang both through organogenesis or embryogenesis. Organogenesis is the stage of shoot formation from a bit part of the mother plant. Two ways of organogenesis are direct organogenesis and indirect organogenesis. Direct organogenesis does not go through the callus stage, whereas indirect organogenesis should be through the callus stage.

Factors that affect the success of in vitro propagation, such as composition of culture medium, type of culture medium, plant growth regulators (PGRs), sterile explants, and others. Plant growth regulators (PGRs) also can affect morphogenesis and growth of cultures, also can determine the success of in vitro plant regeneration (Ferziana et al., 2021). Two groups of plant growth regulators (PGRs) are cytokinin and auxin. Cytokinin play a role in cell cycle and cell division, which can affect the growth and development of plants (Liu et al., 2020), while auxin play a role in root formation and induce callus formation, caused of continuous cell division (Reflini, 2017). Several types of cytokinins that can be used in the culture medium, i.e. 6-Benzyl Amino Purine (BAP), thidiazuron (TDZ), kinetin, Benzeladenine (BA), and others. Auxin also have several types, such as Napthaleine Acetic Acid (NAA), Indole-3-Butyric Acid (IBA), Indole Acetic Acid (IAA), and others. The effect of plant growth regulators (PGRs) to the culture depends on the type or cultivar of explants (Liu et al., 2020). BAP is the most effective cytokinin to induce proliferation of in vitro shoots, which is usually used to increase percentage of shoot regeneration from callus. Meanwhile, addition of NAA as auxin in culture medium play a role for root formation and elongation of cells. In addition, TDZ is another type of cytokinin, Combination of auxin and cytokinin would be effective to induce shoot formation (N. Sari et al., 2014).

A combination of BAP and NAA successfully produced callus from *Taraxacum officinale* (L) Weber Ex. F.H. Wigg, which combination of 3.0 mg.L-1 BAP and 0.05 mg.L-1 NAA would give dark green callus (Martinez et al., 2021). Another research resulted in the heaviest callus formation from *Justicia gendarussa* with a combination of 1.5 mg.L-1 2,4 D and 2.0 mg.L-1 BAP (Wahyuni et al., 2017). Moreover, two heaviest callus formations from *Atropa acuminata* Royal ex Lindl, such as a combination of 1 mg.L-1 BAP and 1 mg.L-1 NAA, then a combination of 3 mg.L-1 BAP and 1 mg.L-1 NAA (Dar et al., 2021). According to Ferziana et al. (2021), about the regeneration of *Amorphophallus muelleri* Blume, shown that the optimal concentration of BAP to produce high numbers of shoots, which is added into Murashige-Skoog (MS) medium, i.e. 4 mg.L-1, 5 mg.L-1, and 6 mg.L-1, but through all aspect, such as number of budding, number of shoots, shoot height, and another efficiency they conclude that 2.0 mg.L-1 BAP was the optimal concentration for regeneration of *Amorphophallus mueleri* Blume. A combination of BAP and NAA can induce the elongation of shoots in a short time than only using BAP. The combination of 2 mg.L-1 BAP and 0.5 mg.L-1 NAA, then 4 mg.L-1 BAP and 0.5 mg.L-1 NAA can result in a high number of callus and shoots for *Amorphophallus paeoniifolius* (Isnaini & Novitasari, 2020). According to a few research, known that combination of BAP and NAA should be induced callus and shoot formation, which the combination of them could activate genes responsible for callus formation (Ikeuchi et al., 2013). Another PGR used in this research was TDZ as phenyl urea derivate, which is effective as a growth regulator to induce regeneration. The effect of TDZ would be different between plants depending on their genotype (Erland et al., 2020). In addition, this research used a Murashige-skoog (MS) medium, which is one of the media types, widely used for in vitro propagation.

Observed data for callus growth, such as color, structure, the texture of the callus, and diameter of explant with callus. A greenish callus is more likely, which indicates the callus is viable. Meanwhile, callus texture can indicate the quality of the callus. A variety of callus textures are compact, intermediate, and friable, as a response to PGRs (Aprilia et al., 2022). The friable texture is easy to separate, causing the high elasticity of the cell wall affected by auxin (Asmono & Sari, 2016). Each texture of the callus was favorable depending on the aim of the research, for example for suspension cells hence friable callus is more likely than a compact callus and others. Moreover, the number and height of shoots were also observed in this research to determine the effect of varying concentrations of BAP and NAA. So, this research aims to determine the optimal combination and concentration of BAP, NAA, and TDZ for the quality of callus, also the growth of callus and shoots for rapid propagation of *Amorphophallus muelleri* Blume. In vitro propagation for Porang can help society to produce high numbers of bulbils porang through organogenic with the rapid method, causing the conventional propagation of porang too slow. So, the high numbers of bulbils porang will help to fulfill the market demand.

METHODS

Porang (*Amorphophallus muelleri* Blume.) bulbils of this research were obtained from local farmers of porang, Klangoon, and Madiun. Then,

the PGRs in this research use BAP, NAA, and TDZ from Sigma Aldrich.

Culture Preparation

Bulbils of porang should be sterile before cultured into MS medium with different PGRs (BAP, NAA, and TDZ). In vitro propagation of porang using bulbils, which process of bulbils preparation using Laminar Air Flow (LAF) cabinet. Bulbils should be cutting into small part and cultured into medium. Minimize the size of bulbils can help to reduce the explant contamination. Treatment of different PGRs in this research can be shown below:

Table 1. Combination and Concentration of PGRs for Porang's Regeneration in This Research

Treatment Code	Combination of PGRs
4.0 BAP + 0.2 NAA	4.0 mg.L^{-1} BAP + 0.2 mg.L^{-1} NAA
4.0 BAP + 0.3 NAA	4.0 mg.L^{-1} BAP + 0.3 mg.L^{-1} NAA
5.0 BAP + 0.2 NAA	5.0 mg.L^{-1} BAP + 0.2 mg.L^{-1} NAA
5.0 BAP + 0.3NAA	5.0 mg.L^{-1} BAP + 0.3 mg.L^{-1} NAA
0.2 TDZ + 0.2 NAA	0.2 mg.L^{-1} TDZ + 0.2 mg.L^{-1} NAA
0.2 TDZ + 0.3 NAA	0.2 mg.L^{-1} TDZ + 0.3 mg.L^{-1} NAA
0.4 TDZ + 0.2 NAA	0.4 mg.L^{-1} TDZ + 0.2 mg.L^{-1} NAA
0.4 TDZ + 0.3 NAA	0.4 mg.L^{-1} TDZ + 0.3 mg.L^{-1} NAA

The first stage was callus formation with several observed variables, such as color, structure, and texture of callus, also the diameter of explants with callus. After the callus was formed, subculture into another medium to shoot induction, which had same combination of BAP or TDZ with NAA like in the first stage. For shoot induction stage, there were some observed variables, i.e. number of shoots and height of shoots. Then, the last stage was roots formation, which used two several types strength of MS media, such as half-strength and full-strength MS media with addition of 1.0 mg.L^{-1} NAA for each strength of medium. Then, incubated at blue light only during 3 weeks to stimulate roots formation and after that, incubated at white light until ready to acclimatization stage. Time of root appearance in days after planted and percentage of survival plantlet were observed for rooting stage.

Statistical Analysis

The experiment was designed in a completely randomized design with eight treatments. Analysis of data using one-way Analysis of Variance (ANOVA), which was followed by Duncan's Multiple Range Test (DMRT) at a 5% error level (a=0.05) in the case of significant difference was observed. Color, structure, texture of callus, and diameter of explant with callus, also the number and high of shoots were observed variables.

RESULTS AND DISCUSSION

Plant Growth Regulators (PGRs) are one of the factors that can affect the organogenesis of plants. Two groups of PGRs are auxin and cytokinin, which combination of them should be induced the formation of callus and shoots. Effect of a different combination of BAP or TDZ as cytokinin and NAA as auxin on callus formation can be shown on table 2 below:

Table 2. Effect of different cytokinin and auxin (combined) in varying concentration on in vitro response of cultured bulbil segments at 5 weeks

Plant Growth Regulator (mg.L^{-1})	Callus colour	Callus structure	Callus texture	Diameter of explant with callus (cm)
4.0 BAP + 0.2 NAA	Greenish white	nodular callus	compact	2.1 ± 0.14^{a}
4.0 BAP + 0.3 NAA	Greenish white	nodular callus	compact	2.2 ± 0.09^{ab}
5.0 BAP + 0.2 NAA	Greenish white	nodular callus	compact	2.5 ± 0.05^{cd}
5.0 BAP + 0.3NAA	Greenish white	nodular callus	compact	2.6 ± 0.08^{d}
0.2 TDZ + 0.2 NAA	Greenish white	nodular callus	compact	2.1 ± 0.18^{a}
0.2 TDZ + 0.3 NAA	Greenish white	nodular callus	compact	2.2 ± 0.35^{ab}
0.4 TDZ + 0.2 NAA	Greenish white	nodular callus	compact	2.3 ± 0.26^{abc}
0.4 TDZ + 0.3 NAA	Greenish white	nodular callus	compact	2.4 ± 0.31^{bcd}

Note: Values followed by the different letter in the same column were significantly different at $p < 0.05$ (α=5%) by DMRT test

According to table 2, shown that combination of each treatment produced the same characteristics of callus, which had greenish white colour with compact nodular callus. Greenish white color more likely than yellowish or brownish callus, to shoots formation. Another characteristics of callus was callus structure, which the structure was nodular callus indicate the callus would growth to form the shoots. The aim of this research not to produce of secondary metabolite or suspension cells, hence the compact callus also acceptable.

In addition, the two highest diameter of explant with callus resulted from combination of 5.0 mg.L^{-1} BAP + 0.3 mg.L^{-1} NAA and the second was 5.0 mg.L^{-1} BAP + 0.2 mg.L^{-1} NAA, with diameter of explant with callus was (2.6±0.08) cm and (2.5±0.05) cm, respectively (table 2.). However the proper ratio of cytokinin to auxin was required for callus induction. The lower cytokinin would stimulate root formation, whereas the higher cytokinin than auxin would stimulate callus formation (Fabiana Meijon Fadul, 2019).

Table 2 shown that BAP more effective than TDZ as cytokinin, which diameter of explant with callus resulted by combination of TDZ and NAA still lower than combination of BAP and NAA. Callus cells are resulted by a differentiation from parenchymal cells (Zhu, 2017), which BAP more effective on callus formation from explant than other cytokinin. According to Sjahril et al. (2015), callus cultured on combination of BAP, NAA, or 2iP with TDZ did not produce shoots, which the color of callus turning from green into brown after 90 days. So, that was indicated that TDZ inhibited the formation shoots of *Chrysanthemum*. Another research found that low concentration of TDZ (0.1 µM) did not give a significant effect at *Alstroemeria aurantiaca* cv. 'Rosita', but high concentration of TDZ (5.0 µM) was play role as inhibitor (Hutchinson et al., 2014).

Another observed parameters was shoot formation from bulbil's explant. Combination of cytokinin and auxin not only performed the formation of callus, but also the formation of shoots. Effect of different combination of cytokinin and auxin to shoot formation can be shown at table 3 below:

Table 3. Effect of different cytokinin and auxin (combined) in varying concentration to morphogenesis response of cultured bulbil segments at 8 weeks

Plant Growth Regulator (mg.L^{-1})	Plant regeneration (%)	Number of shoots	Shoots height (cm)
4.0 BAP + 0.2 NAA	100	11±2.4^{b}	8.4±1.25^{bc}
4.0 BAP + 0.3 NAA	100	9±3.2^{a}	9.5±2.43^{c}
5.0 BAP + 0.2 NAA	100	15±1.8^{c}	7.2±1.86^{ab}
5.0 BAP + 0.3 NAA	100	16±1.5^{c}	7.8±1.94^{b}
0.2 TDZ + 0.2 NAA	100	14±1.9^{c}	8.3±2.78^{bc}
0.2 TDZ + 0.3 NAA	100	15±1.2^{c}	7.4±2.45^{b}
0.4 TDZ + 0.2 NAA	100	16±2.7^{c}	7.5±1.69^{b}
0.4 TDZ + 0.3 NAA	100	16±1.4^{c}	6.1±1.48^{a}

Note: Values followed by the different letter in the same column were significantly different at $p < 0.05$ (α=5%) by DMRT test

Based on table 3, combination of cytokinin and auxin can resulted 100% of plant regeneration, which indicated by shoots formation, caused cytokinin and auxin play a role for cell division (Reflini, 2017). Moreover, cytokinin also helps to induced shoots formation(Liu et al., 2020). Although, single-use of BAP can induce shoot formation, but combination with NAA would be effective for plant regeneration (Goswami et al., 2016).

Ratio of cytokinin and auxin was different to resulted the highest number of shoots and highest shoot. Highest number of shoots can be resulted by combination of 5.0 mg.L^{-1} BAP + 0.3 mg.L^{-1} NAA with 16±1.5 shoots. According to Ferziana et al. (2021), the highest number of *Amorphophallus muelleri*'s shoots can performed with concentration of BAP were 4.0 mg.L^{-1}, 5.0 mg.L^{-1}, and 6.0 mg.L^{-1}. Addition of BAP also increase the callus formation and regeneration of callus to form shoots. BAP has a strong activity to stimulate shoot multiplication for in vitro propagation (Bariyyah & Istianingrum, 2021). Addition of NAA into BAP treatment would stimulate shoots formation, but the high concentration of NAA exceeding 3 mg.L^{-1} was found to inhibit shoot growth even in combination with BAP (Fabiana Meijon Fadul, 2019). In addition, the highest shoots stimulated by 4.0 mg.L^{-1} BAP + 0.3 mg.L^{-1} NAA at (9.5±2.43) cm.

BAP is one of the most effective cytokinin to induce proliferation of in vitro shoots, which is usually used to increase percentage of shoot regeneration from callus (N. Sari et al., 2014). Shoot formation also can induced by addition of TDZ, but not significantly different to addition of BAP (table 3).

Based on table 2 and table 3, it concluded that proper combination of cytokinin and auxin was 5.0 mg.L^{-1} BAP and 0.2 mg.L^{-1} NAA, which resulted the larger diameter of explant with callus, also can induce shoots formation in high enough value. Those combination was chosen, cause more efficient than another high concentration of BAP and NAA but give the same results or not significantly different.

After shoot formation stage, the adventitious shoots would be cultured into another media for roots induction. Murashige and Skoog (MS) media was commonly used for in vitro propagation. In this research, two strength of MS medium were observed, i.e. half-strength MS and full-strength MS or namely MS. Effect strength of MS medium for rooting stage can be shown at table 4 below:

Table 4. Effect of MS medium strength for rooting of adventitious shoots

MS Medium	Time of root appearance (days after shoot planted)	Survival plantlet (%)	Acclimatization survival rate (%)
Half-strength MS	12±1.25	100	87 (35/40)
Full-strength MS	7±1.85	100	90 (36/40)

MS media is rich both in macronutrients and micronutrients, also vitamins for in vitro propagation. So, different strengths of MS medium could affect the plant propagation in vitro (Rezali et al., 2017). Based on the research of Rahayu et al. (2015), decreasing the nutrient of in vitro media could affect the growing potency after subcultured. The highest number of *Carica pubescens* roots was 6.6 roots, which were cultured at full-strength MS media. But, the effectiveness of strength media to stimulate roots is also different depending on the genotype of plants.

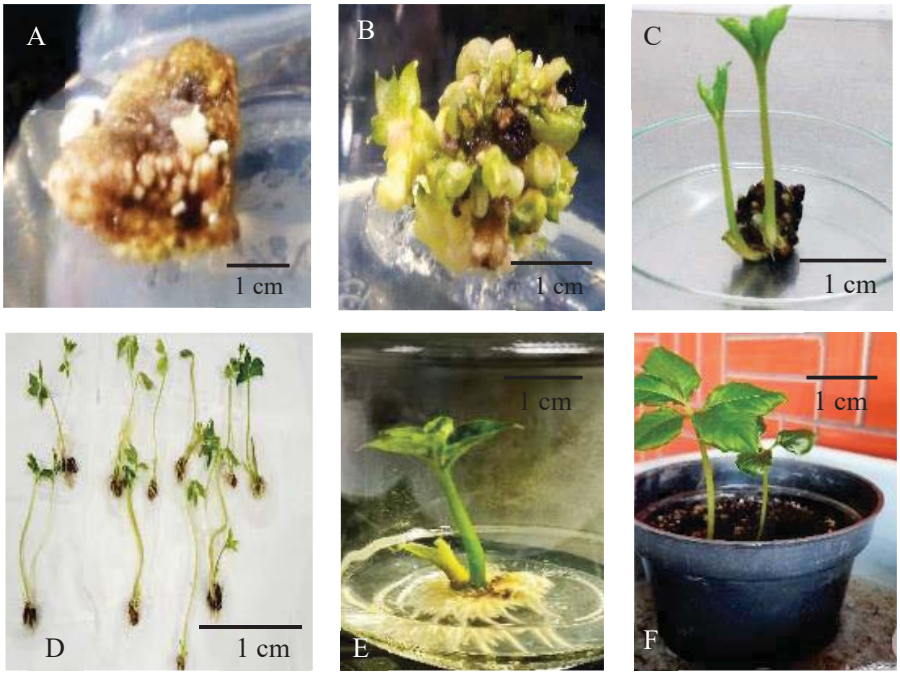

Figure 1. Plant regeneration of *Amorphophallus muelleri* Blume. (A)Callus induction from segments of bulbil; (B) Adventitious of shoot induction; (C) Shoot elongation; (D) Rooting induction; (E) Plantlet; (F) Transplanted plantlets for hardening

Plant regeneration of *Amorphophallus muelleri* Blume. from bulbils shown in figure 1. After the plantlet had some long roots, the plantlet was ready for acclimation into ex-vitro. Acclimatization is the stage to adapt the plantlet into ex vitro condition. Root induction is an essential process to determine the success of acclimatization (Ajadi et al., 2018). According to table 4, full-strength MS can induce roots faster than half-strength MS with an acclimatization survival rate of 90% more than half-strength MS treatment, i.e. 87%. Roots of bulbil porang were easy to stimulate, which almost in every treatment on the rooting stage can result in many roots, so the acclimatization survival rate can reach above 87%. So, the rooting stage of porang was a very crucial stage, which help porang to survive at ex vitro conditions until growth into high porang plants and produce bulbils to fulfill the market demand.

CONCLUSION

The proper combination of cytokinin and auxin for rapid propagation of *Amorphophallus muelleri* Blum. was 5.0 mg.L-1 BAP + 0.2 mg.L^{-1} NAA with greenish-white compact nodular callus and diameter of explant callus was (2.5±0.05) cm, also good for stimulating shoots formation with several shoots and shoot height, i.e. 15±1.8 shoots and (7.2±1.86) cm, respectively. Meanwhile, the optimal treatment for the rooting stage was MS media with 1.0 mg.L^{-1} NAA. For further research, the composition of acclimatization media or optimal treatment for plantlet porang should be determined to propagate porang at ex vitro conditions, which the plantlets already get from in vitro propagation.

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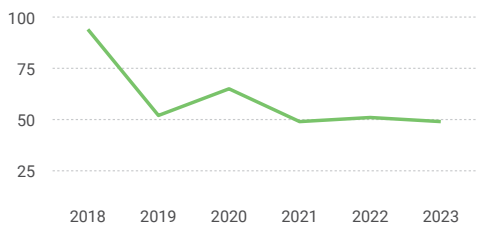

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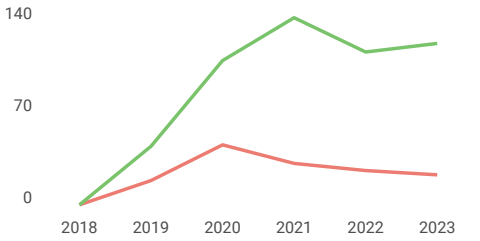

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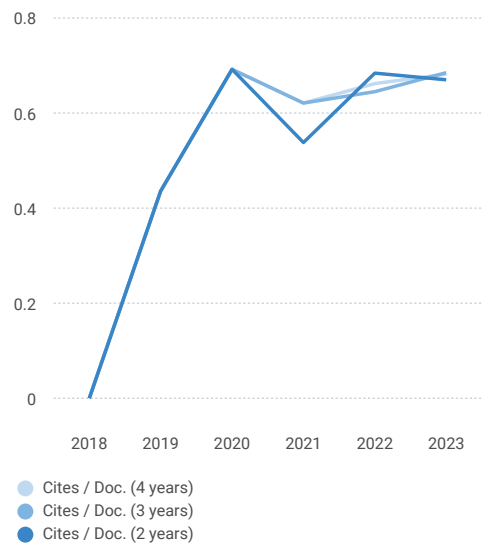

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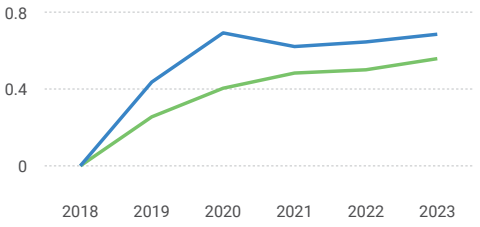

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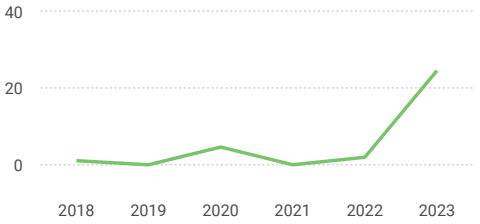

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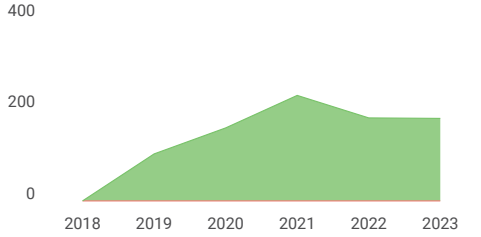

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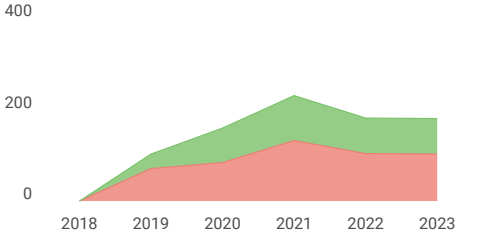

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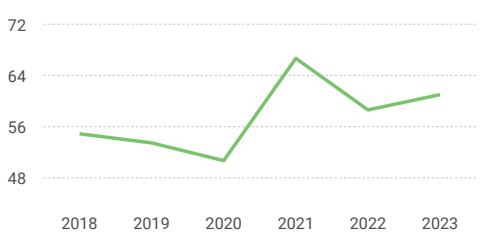

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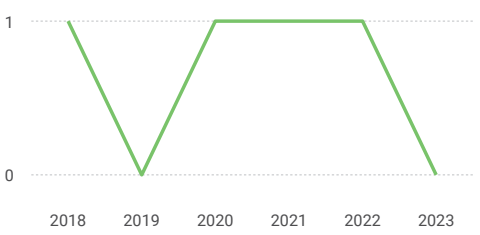

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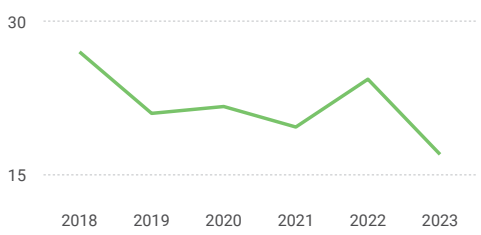

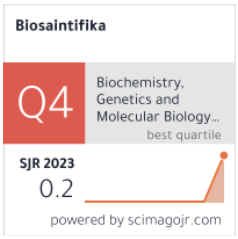

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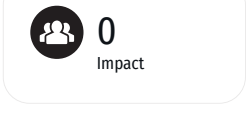

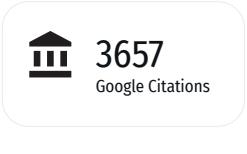

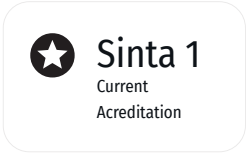

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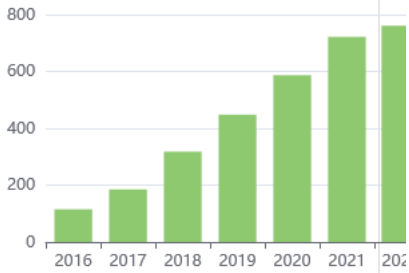

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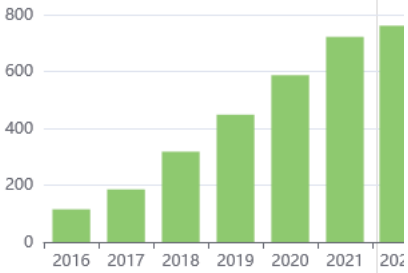

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