EFFECT OF COMBINATION NAPHTHALENE ACETIC ACID (NAA) AND 6-BENZYLAMINOPURINE (BAP) ON IN VITRO MICROPROPAGATION OF RICE (ORYZA SATIVA L.) CV. MR269

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ABSTRACT

Rice (Oryza sativa L.) is among the most significant food plants globally, with 90% of the total production are from Asia. MR269 variety is high-yielding and resistant to many diseases. However, there are limited study on the in vitro technique for the optimum plant propagation. Tissue culture technique is an efficient approach to improve the rice regeneration by optimization of plant growth regulators. This study is carried out to observe the effect of combination of NAA and BAP hormone and identify the best combination of NAA and BAP hormone for in vitro micropropagation of rice (Oryza sativa L.) cultivar MR269 towards the height of plantlets, number of shoots, number of roots, length of roots, and fresh weight. Five different combinations of BAP (1.0, 2.0, 3.0, 4.0, 5.0 mg/L) and NAA (0.1, 0.2, 0.3, 0.4, 0.5 mg/L) observed after 4 weeks. Significant results obtained between the parameters where maximum number of roots (15.25 ± 1.89), length of roots (1.8 ± 0.25cm) and height of plantlets (13.88 ± 0.47cm) were obtained in MS media supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. The highest number of shoots (7.5 ± 0.29) and fresh weight (0.21 ± 0.05g) were observed in MS medium supplemented with 3.0 mg/L BAP + 0.3 mg/L NAA and 5.0 mg/L BAP + 0.5 mg/L NAA respectively. The best combination of hormone for the efficient of rice regeneration was in the MS supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA.

Keywords: *Rice, Oryza sativa, MR269, NAA, BAP*

INTRODUCTION

Rice (*Oryza sativa* L.) belongs to the *Poaceae* family, is the primary source and diet portion for more than half of world's population (Yaqoob et al., 2021)(Goswami et al., 2022)(Britannica, 2023; Lu et al., 2020; Nurulhuda et al., 2022). Rice is a staple crop and grown in more than 100 countries with 90% of the total global production are from Asia (Abdul Rahman et al., 2019; Fukagawa & Ziska, 2019; Kayaa & Karakütük, 2018; Kim Rutledge et al., 2022). It is the third largest crop in the world (FAO, 2020; Lisa, 2021). Malaysia has produced 1.68 million metric tons of rice in 2021 (Statista, 2023). As the expected increasing population to nearly 10 billion until 2050, the production of the rice yield must be increasing 70% in order to meet and fulfill the needs of future demands (Ranganathan et al., 2018; Van et al., 2021). On average, Malaysians consume approximately 74kg of rice per year, making rice as the staple food for Malaysian (NST, 2022). Therefore, it is highly significant to focus on the yield and crop improvement of the plant.

MR269 variety is a promising and newly developed rice cultivar that exhibits adaptability to a wide range of growing regions in Malaysia, introduced by the Malaysian Agricultural Research and Development Institute (MARDI) in 2012 (Bernama, 2020; MARDI, 2012; Othman et al., 2012). It is a hybrid of two P347 and Y1362 breeds carried out in the season of 2000/2001 with maturity of about 104 to 113 days after sowing and optimum plant height can reach until 76 to 88 cm for the whole regeneration (Razak et al., 2020). MR269 has been cultivated to be resistant to leaf blast disease, moderate resistance to bacterial warming and bacterial leaf temperatures, and viral redness (MARDI, 2016; Misman & Zakaria, 2019). It is a non-fragrant and high-yielding rice variety (Lau et al., 2017; Nurulhuda et al., 2022; Sedgwick, 2014) The average of rice variety MR269 yield showed a nine percent improvement over MR219. The maximum production of MR269 was increasing to 9.9 tonnes compared to MR219 which was recorded producing 7.5 tonnes (Agnes Tugong, 2022; ISAAA, 2012).

Farmers are expected to use MR269, so that rice production yield can increase rapidly to meet the country's target of subsistence and domestic consumption needs (BusinessToday, 2021; Hashim et al., 2022; Sunian et al., 2022). Globally, conventional method used in the cultivation of rice plants has many negative impacts to the production yield of rice grains (Riyo, 2019). Poor agronomic managements practices and lack of nutrients application in the cultivation of rice are the main common factors that cause the low yield of rice production (Hanafi *et al*., 2009). Plant tissue culture, known as micropropagation in commercial context is a technique that has been extensively employed to provide year-round production (Ghosh et al., 2021). Micropropagation enables the large-scale propagation of multiple plants, resulting in its wide use in research and commerce (Soumare et al., 2021). Tissue culture or *in vitro* propagation technique can be defined as the growth and maintenance of plant tissues or cells in sterile and nutritional supportive condition under aseptic conditions. *In vitro* propagation method is a most efficient alternative for high production in short time with high rate of germination (Abiri et al., 2020).

Main keys to ensure success of tissue culture and regeneration of plantlets in tissue culture technique depends on the sources of explant, culture media composition and culture aseptic condition. Therefore, understanding the plant-regeneration mechanism promotes the use of effective protocols for plant micropropagation (Long et al., 2022). Plant growth regulators (PGRs) play a pivotal role in plant life as they coordinate and regulate many physiological processes governing crop growth and yield (Hussain et al., 2021). The combination of different amounts of hormones mostly enhances the impact of differences in rice propagation (*Oryza sativa* L.). Therefore, this study is carried out to observe the effects of different combinations of NAA and BAP hormone and to determine the optimum combination of NAA and BAP on *in vitro* micropropagation of rice (*Oryza sativa* L.) cv. MR269 via direct organogenesis using matured seeds as explants. The results of this study will provide valuable insights on improving approaches for optimizing the plant development and yield of this substantial crop by utilizing the *in vitro* technology.

RESEARCH METHODOLOGY

Healthy seeds of MR269 rice cultivar were obtained from the Malaysian Agricultural Research and Development Institute (MARDI). The outer layer of the seed (husk or hull) was removed through a dehusking process and incubated in oven for 35˚C for 15 minutes to break seed dormancy. The seeds were surface sterilized with 50% sodium hypochlorite and for 2-3 minutes and soaked in 70% absolute ethanol for 2 minutes. The seeds were then rinsed using sterile deionized distilled water for a few times and were kept on sterilized tissue papers to dry excess remaining water. The sterilized seeds germinated on Murashige and Skoog (MS) basal medium supplemented with 3% sucrose and 0.8% agar, pH 5.8, with each medium containing different hormone concentrations shown in Table 1.

Table 1. Different BAP and NAA combination concentration for *in vitro* culture of (*Oryza sativa* L.) cv. MR269

	BAP (mg/L)	**NAA (mg/L)**
MS	0 (control)	
	1.0	0.1
	2.0	0.2
	3.0	0.3
	4.0	0.4
	5.0	0.5

The cultures were then incubated at 25 ± 2°C with a 16-hour photoperiod (1000 lux) and observed after 4 weeks. Several parameters were observed, comprising the number of leaves, plant height, number of roots, length of roots, and fresh weight. These parameters were chosen based on their relevance to the micropropagation process and their potential impact on the growth and development of the rice plant. The experimental design that has been used for this observation is RCBD (Randomized Complete Block Design). All data were then analyzed through ANOVA Analysis of Variance using Statistical Package of Social Science (SPSS) Version 22 and Microsoft Excel 2019. The mean values of each parameter were calculated and compared using Turkey's multiple comparison test to access the significant difference $p < 0.005$ between treatments.

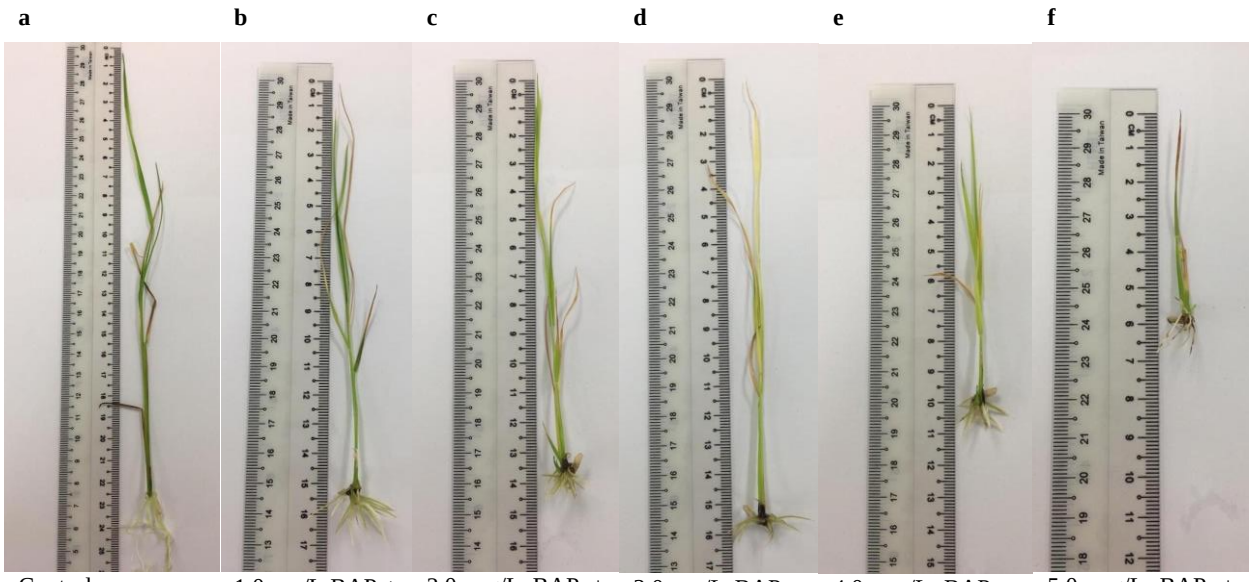
RESULTS AND DISCUSSION

Effect of BAP and NAA hormone combinations on *in vitro* propagation of rice (*Oryza sativa* L.) cv. MR269 was obtained from MS media supplemented with (0.0 - 5.0 mg/L) BAP and (0.0 - 0.5 mg/L) NAA. Observation was made after 4 weeks of the culture period. There are four (4) main parameters which include the height of the plant, the number of leaves, the production of roots, and the fresh weight. *In vitro* propagation technique is affected by several important conditions during culturing including the concentration of plant growth regulators in the media, the types of explants used and plant genotype (Kaur et al., 2022).

REGENERATION RESPONSE OF RICE (*Oryza sativa* L.) CV. MR269 ON PLANT HEIGHT

Plant heights were measured from the root of the plant to the highest point of growth, that is the tip of the highest leaf or shoot. Ruler was placed in a vertical line from the base of the plant to the tip, and the measurement was recorded. In the present study, rice seed germination occurred within two days. The embryonic shoot started emerged from the seeds and followed by roots started to grow on day 3. Germination of healthy seeds takes around 3-5 days (USDA, 2019).

Figure 1. Regeneration of rice (*Oryza sativa* L.) cv. MR269 derived after 4 weeks from MS basal medium supplemented with different combinations of BAP and NAA hormone.



The germination of seeds rice to the whole plant through direct somatic embryogenesis. Somatic embryogenesis has been found to occur in two processes that are directly and indirectly. Direct embryogenesis and organogenesis occurred when there was no proliferation and unorganized callus occurred during the development of the first shoot. Figure 1 shows the observation after 4 weeks on the regeneration of rice (*Oryza sativa* L.) cv. MR269 from MS basal medium supplemented with different combinations of BAP and NAA hormone.

Figure 2. Effect of different concentrations of BAP (0 - 5.0) mg/L and NAA (0 - 0.5) mg/L on the plant height of rice (*Oryza sativa* L.) cv. MR269

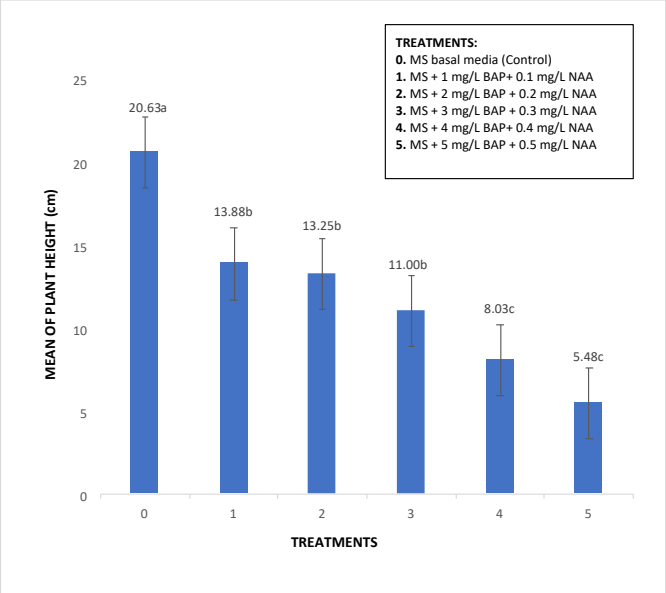


Figure 2 shows the mean of the plant height when treated with different concentrations of BAP (0 - 5.0) mg/L and NAA (0 - 0.5) mg/L. Notably, the highest plant height (20.63 ± 0.75 cm) are recorded in the control group which are the untreated plants. Significant variations in plant height were observed among the different treatment groups. The treatment that yielded the highest mean of plant height (13.88 ± 0.47 cm) was the one supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. Similar to a study by Saleh et al. (2019) on *Oryza sativa* L. cv. MRQ 74, they found that 0.1 mg/L BAP in combination with 0.1 mg/L NAA to be the best combination that produces highest plant height (Saleh et al., 2019).

In addition, 1 mg/L BAP in a study by Rodrigues et al. (2023) presented maximum height (14.2 cm), similar to the control (Rodrigues et al., 2023). Hussein et al. (2020) has also observed maximum plant height with 1 mg/L BAP hormone concentration (Hussein et al., 2020). Combination of NAA (0.5 mg/L) and BAP (5.0 mg/L) at the highest concentration shows the minimum ability (5.48 ± 0.16 cm) for plant height. Lowest plant height observed in the highest combination because BAP in higher concentration causes shoot bud retardation as the presence of excess hormone causes toxicity to the plant (Azizan, 2017).

Therefore, cytokinin-auxin ratio is crucial to induce the morphogenic response for *in vitro* plants (Sulaiman et al., 2020). This result can be attributed to the specific effects of these hormones on shoot growth, with BAP promoting cell division and lateral shoot development, and NAA contributing to overall plant height through synergistic effects. These findings align with previous research on the role of BAP and NAA in promoting plant height, highlighting the importance of hormonal balance in optimizing growth conditions. Phytohormones such as auxin and cytokinin have been concluded to be the most external factors that influenced somatic embryogenesis.

REGENERATION RESPONSE OF RICE (*Oryza sativa* L.) CV. MR269 ON NUMBER OF LEAVES

Figure 3. Effect of different concentrations of BAP (0 – 5.0) mg/L and NAA (0-0.5) mg/L on the number of leaves of rice (*Oryza sativa* L.) cv. MR269

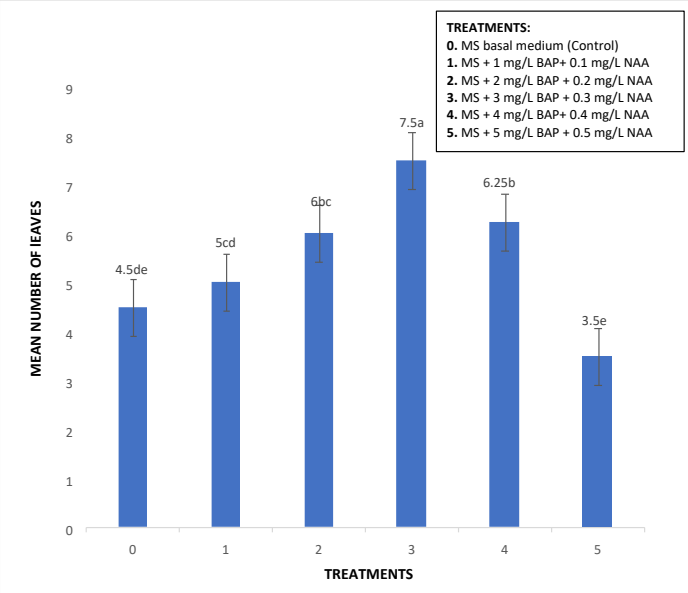


Figure 3 indicates the effect of different concentration of NAA and BAP on the production of leaves by each plantlet. Maximum mean number of leaves was observed in MS media supplemented with 3.0 mg/L BAP and 0.3 mg/L NAA (7.5 ± 0.29). Besides, the number of leaves observed in all treatments was not significantly different from each other. Generally, the higher concentration of BAP can induce more leaves or shoot formation. Wijaya et al., (2022) and Trunjaruen et al., (2020) also found that BAP of 3 mg/L concentration is the optimal treatment for leaves production (Trunjaruen et al., 2020; Wijaya et al., 2022)

Generally, the higher concentration of BAP can induce more leaves or shoot formation. The results from a previous study revealed that the highest regeneration of leaves obtained in rice variety MR269 was in MS medium supplemented with 3.0 mg/L BAP and 0.1 mg/L NAA (Che Radziah *et al*., 2016). Auxin and cytokinin promote many different processes in the development of plants and other environmental feedbacks. The two plant regulators hormone, BAP and NAA play a crucial role to control the organization, initiation, and maintenance of meristem cells. Meristem cells can be differentiated to develop new tissues and organs of plant embryonic development. In shoot proliferation and organogenesis, phytohormone cytokinin positively induces the division in the shoot stem cell region (Masekesa et al., 2016).

REGENERATION RESPONSE OF RICE (*Oryza sativa* L.) CV. MR269 ON THE PRODUCTION OF ROOTS

Figure 4 shows the production of roots of rice (*Oryza sativa* L.) cv. MR269 treated with different concentrations of BAP (0 - 5.0) mg/L and NAA (0 - 0.5) mg/L. Roots produced from the treated seeds appear to be thick and short while the control groups are more fibrous looking and appear to be longer.

Figure 4. Effect of different concentrations of BAP (0 - 5.0) mg/L and NAA (0 - 0.5) mg/L on the production of roots of rice (*Oryza sativa* L.) cv. MR269

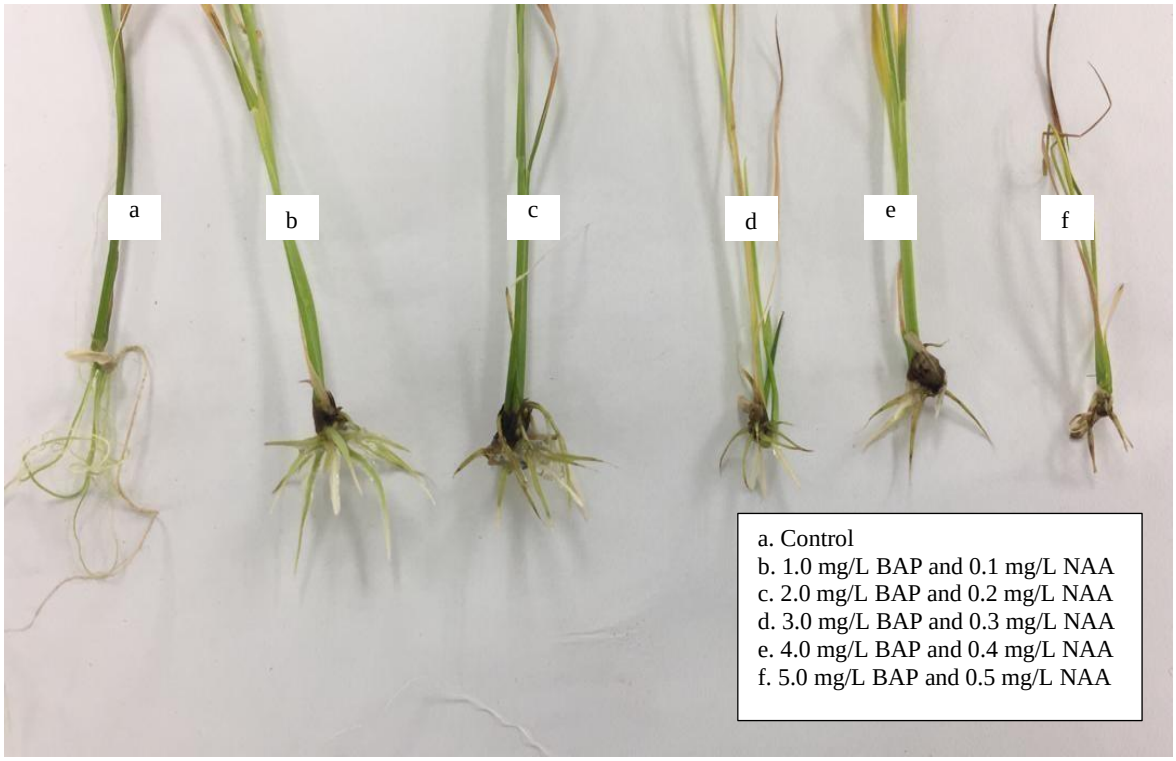
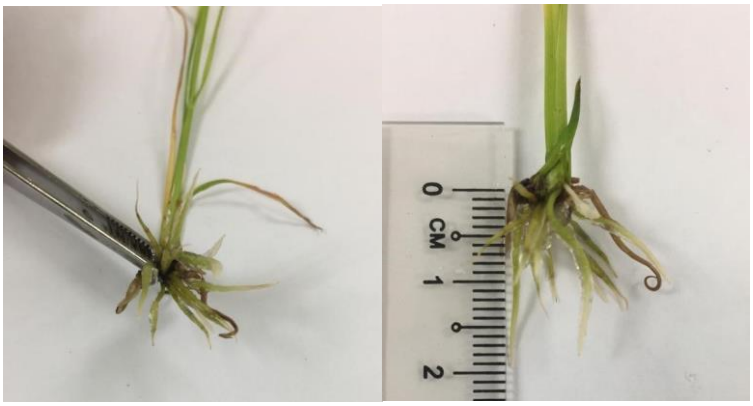
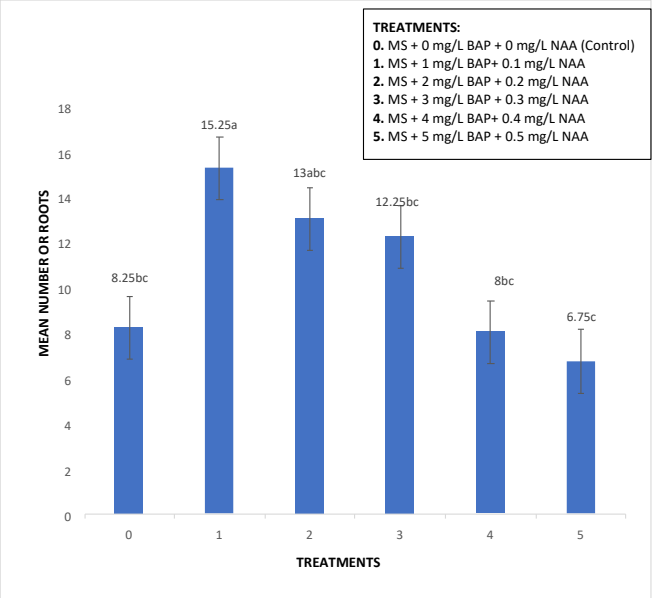


Figure 5. The maximum number of roots (left) and the length of roots (right) of *Oryza sativa* L. cv MR269 in basal MS medium supplemented with 1.0 mg/L of BAP and 0.1 mg/L of NAA.



Based on the results, the highest root production was obtained in the MR269 rice variety in MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. The highest number of roots (15.25 ± 1.89) and the length of the roots (1.8 ± 0.24 cm) were observed in the same treatments. The structure of the roots is also different between the treatments, as shown in FIGURE. Banu et al. (2021) observed significantly high number of root by MS + 1 mg/l BAP (Banu et al., 2021).

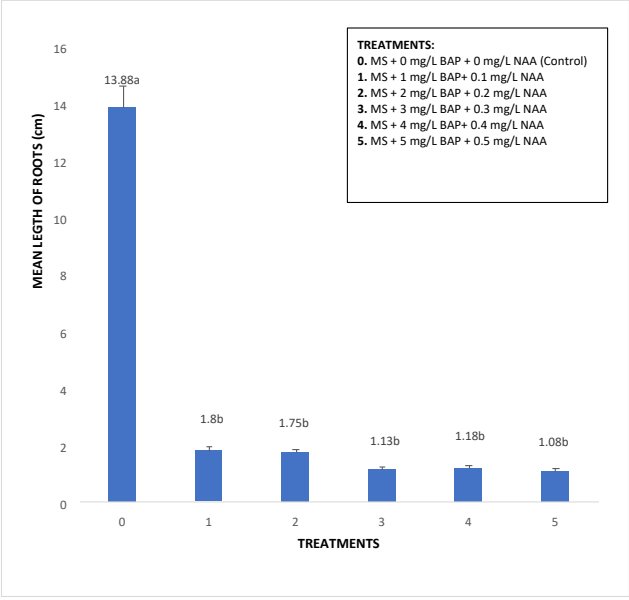
Figure 6. Effect of different concentration of BAP (0 - 5.0) mg/L and NAA (0 - 0.5) mg/L on the number of roots of rice (*Oryza sativa* L.) cv. MR269



Plant hormones are important to regulate the plant response, sending external signals into physiological characteristics (Wong et al., 2023). Based on the figure, the formation of roots in the untreated medium, which act as control in this study, was obtained as a lateral root structure and in the media treated with hormones, adventitious roots were formed. The main root-promoting hormone is auxin, which can promote the formation of adventitious roots and is associated with the development of other various rooting processes (Sosnowski et al., 2023).

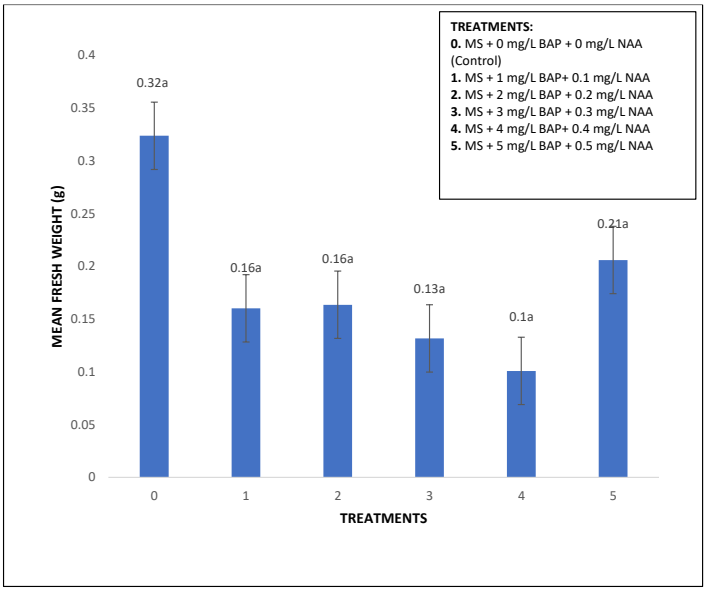
The combination of auxin and cytokinin interacts with each other to regulate the development and growth in plants including the development of lateral roots. Based on the previous study by Le, (2009) established that the role of these two hormones, auxin and cytokinin, is a main factor *in vitro* organogenesis.

Figure 7. Effect of different concentrations of BAP (0 – 5.0) mg/L and NAA (0-0.5) mg/L on the number of roots of rice (*Oryza sativa* L.) cv. MR269



REGENERATION RESPONSE OF RICE (*Oryza sativa* L.) CV. MR269 in FRESH WEIGHT

Figure 8. Effect of different concentrations of BAP (0 – 5.0) mg/L and NAA (0-0.5) mg/L on the fresh weight of rice (*Oryza sativa* L.) cv. MR269



Referring to Figure 8, the highest mean of fresh weight was revealed in the treatment 5 (MS + 5.0 mg/L BAP + 0.5 mg/L NAA) which was 0.206 g. The lowest mean for fresh weight was revealed in MS medium treated with 4 mg/L BAP and 0.4 mg/L NAA. As in Figure 8, it showed that the fresh weight was not consistent with the increasing concentration of NAA and BAP. Therefore, having a high regeneration capacity in the propagation of plants is extremely important.

The high capacity can be obtained when the good types and suitable concentration of plant hormones applied in cell culture systems can significantly affect the morphogenesis and growth of plant cell. According to Sun (2019), stated that the not consistent responses toward the treatment were affected by the nutrient uptake, transport and stability of hormones and gene activation. There is also a tendency to include the liquid weight during the measurement of the fresh weight of the plantlets.

CONCLUSION

The proper protocols on conducting regeneration and the combination of hormones NAA and BAP at the different concentration were play important roles to ensure the success of the study. The types of explants used, matured seeds, also lead to the success of the research. The results obtained that the basal MS medium treated with the combination of hormones BAP and NAA enhances the regeneration of rice (*Oryza sativa* L.) cv. MR269. The best combination hormone for the length of roots (1.80 ± 0.24) and number of roots (15.25 ± 1.89) and the height of plantlets (13.88 ± 0.47) were observed in MS media supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. The largest number of shoots (7.50 ± 0.29) and the highest fresh weight (0.21 ± 0.05) were observed in MS media supplemented with 3.0 mg/L BAP + 0.3 mg/L NAA and 5.0 mg/L BAP + 0.5 mg/L NAA respectively.

In summary, it is recommended that MS supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA was the most potential combination of hormone for the efficient of rice regeneration MR269. *In vitro* propagation is the most efficient technique to enhance the high production and quality of rice (*Oryza sativa* L.). In other hand, even though, *in vitro* seed germination and plant regeneration from germinated rice seeds were tried in the various medium treated with various hormone composition, the best germination was found in the MS medium treated without any hormone (Puhan & Siddiq, 2013). Culture media conditions also the one factor that play a main role in regeneration process other than genotype.

Findings from this study also suggest that the cytokinin-auxin ratio in the growth media significantly influences various aspects of MR269 rice growth and development in vitro. A balanced or higher cytokinin-auxin ratio tends to promote shoot elongation, increased leaf production, and fresh weight, while specific combinations of cytokinin and auxin concentrations are optimal for root development. These observations align with the well-established roles of cytokinins and auxins in plant growth and development and highlight the importance of fine-tuning the hormonal balance for desired outcomes in tissue culture experiments.

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