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Micropropagation of *Neolamarckia macrophylla* (Roxb.) Bosser to support *ex situ* conservation and breeding: multiplication, rooting, and acclimatization

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Abstract

This research aimed at obtaining the appropriate media for *in vitro* multiplication, rooting, and acclimatization, as well as seedling quality, for the development of red jabon through tissue culture technique. Two experimental designs were used in this study: a factorial design for multiplication and rooting stages and a completely randomised design for acclimatization stage and seedling quality. The results showed that Murashige and Skoog (MS) medium, Woody Plant Medium (WPM), and Driver Kuniyuki Walnut (DKW) medium responded well during the multiplication stage. *Neolamarckia macrophylla* plantlets from WPM had the highest percentage of survival rate. The appropriate acclimatization medium for *N. macrophylla* is compost and sand (4:3). The sturdiness of stem and seedling quality index of the two-month-old seedling has already fulfilled the requirement for replanting to the field. High multiplication frequency ensures the efficiency of this protocol. This tissue culture method can be applied for the large-scale production and sustainable conservation of *N. macrophylla*, a vulnerable species for agroforestry of coastal regions for windbreak and soil reclamations.

Key words: genetic conservation, *in vitro*, jabon merah, seedling, WPM.

Introduction

Jabon merah (*Neolamarckia macrophylla* (Roxb.) Bosser) is a fast-growing tree, currently a mainstay of the timber industry, including plywood, lamina wood, and other timber industries that have favourable cultivation prospects for the community (Hal-

awane et al. 2011, Qalbi et al. 2019). However, it is still cultivated using conventional techniques; therefore, its production is unable to fulfill the needs of *N. macrophylla* seedlings (Marzuki et al. 2016). Moreover, the shortage of seedlings might also be caused by limited fruiting season, varying offspring traits, and low seed quality produced (Nursyamsi 2010, Taiyeb and Baharuddin 2017). One effort to overcome the problem of seed supply shortage can be made through tissue culture techniques. Tissue culture is a widely used technique (Yelnititis 2014) and could also be used to promote conservation purposes. Conservation actions include *ex situ* conservation in gene banks and collections stored in the form of seeds, *in vitro* plant tissue, and calluses that have not been differentiated in liquid nitrogen (cryopreservation) (Herawan and Leksono 2018a).

Tissue culture techniques start from the preparation of plant material/explants to stages in the laboratory (induction, multiplication, and rooting stages), to acclimatization and planting in the field; these processes are vital in the production of tissue culture seedlings (Herawan and Leksono 2018a). Growing media is a major factor in the propagation of tissue culture and has a profound effect on the growth and development of explants and the seeds produced (Tuhuteru et al. 2012). Rathwell et al. (2016) revealed that there are three basic media for tissue culture that are often used for woody plants: Driver Kuniyuki Walnut (DKW) medium, Murashige and Skoog (MS) medium, and Woody Plant Medium (WPM). Growth regulators, which are non-nutritional organic compounds, are other essential factors to encourage, inhibit, or qualitatively change the physiological processes of plants if added in low concentrations (Khan et al. 2011).

Research related of *N. macrophylla* tissue culture has not been conducted much. Some previous studies used basic MS media. The study by Djumat (2014) found that multiplication in shoot buds did not occur, but multiplication in axillary shoot explants occurred, with the highest number of shots at 1 $mg \cdot L^{-1}$ 6-benzylaminopurine (BAP). The best treatment to induce shoots and leaf formation was a combination of 4 $mg \cdot L^{-1}$ BAP concentration and 0.3 $mg \cdot L^{-1}$ Indole Acetic Acid (IAA) (Marzuki et al. 2016), whereas the best treatment to induce callus from red leaf leaves was a combination of 0.3 $mg \cdot L^{-1}$ Naphthaleneacetic Acid (NAA) and 4.4 $mg \cdot L^{-1}$ BAP (Gusmiaty et al. 2015), and the highest shoot and leaf induction were observed at 1.5 $mg \cdot L^{-1}$ BAP and 0.1 $mg \cdot L^{-1}$ indole-3-butyric acids (IBA) (Taiyeb and Baharuddin 2017). Since previous studies only tested specific media or growth regulators, it is, thus, necessary to conduct exploratory research to compare several tissue culture media and various concentrations of growth regulators. Therefore, this study aims to compare some basic tissue culture media (i.e. MS media, WPM, and DKW media), growth regulators (i.e. BAP and IBA), and acclimatization media to obtain a suitable method of *N. macrophylla* tissue culture techniques from multiplication to acclimatization stage.

Materials and Methods

Place and time of research

The present study was conducted at the Laboratory of Tissue Culture of 2nd Regional of Seed/Seedling Forest Tree (BPTH Wilayah II, South Sulawesi, Indonesia).

Tools and materials of research

The plant materials used were the sterile apical buds of 2-month-old plantlets of *N. macrophylla*. MS media, WPM, and DKW media were evaluated in the present study. The other materials used were agar, BAP, IBA, sterile distilled water, 70% alcohol, 96% alcohol, 0.1 N HCl, 0.1 N NaOH, sand, compost, and husk charcoal.

Methodology

This research was conducted in four stages: multiplication, root formation, acclimatization, and seedling quality observation.

Multiplication

This stage was conducted with a factorial design. The first factor was basic media consisting of three levels (MS media (A0), WPM (A1), and DKW media (A2)). The second was BAP concentration consisting of nine levels (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 mg·L^{-1}). The experiment was repeated eight times, thus totalling in 216 experimental plants. The observation was conducted for eight weeks. The parameters observed include time of initial shoot growth, number of shoots, height of the shoot, number of leaves, and conditions of culture.

Root formation

In this stage, the factors' basic media consisted of three levels (MS media (A0), WPM (A1), and DKW media (A2)), and IBA concentrations consisted of nine levels (0 (b0), 0.25 (b1), 0.5 (b2), 0.75 (b3), 1 (b4), 1.25 (b5), 1.5 (b6), 1.75 (b7), and 2 (b8) mg·L^{-1} BAP), were also considered. The experiment was repeated five times at each media level, resulting in 135 experimental units.

There are five plants in the observation conducted for six weeks with parameters: time of root growth, number and length of roots.

Acclimatization

In the acclimatization stage, the materials used were compost, rice husk, compost + sand (4:3), compost + sand + rice husk (1:1:1), and compost + rice husk (2:1). For each basic media (MS media, WPM, and DKW media), each combination of acclimatization media and basic media was repeated five times at each medium acclimatization, resulting in 75 experimental units. The observation was conducted for four weeks. The observation parameter was the percentage of survival.

Physical quality of seeds

To measure the quality of seedlings, height, diameter, dry root weight, dry leave weight, and dry stem weight at the age of 0 months or right after seedling removal to polybag, 1 month, 2 months, and 3 months with a sample of five seeds each were measured. Formula (1) was used to calculate the index (Dickson et al. 1960).

$$SQI = \frac{TW}{\frac{H}{RCD} + \frac{SW}{RW}}, \quad (1)$$

where: *SQI* is seedling quality index, *TW* is total weight (g per plant), *H* is height (cm), *RCD* is root collar diameter (mm), *SW* is shoot weight (g per plant), *RW* is root weight (g per plant).

Data analysis

The data obtained from observations in the analysis of variance and comparative

tests between treatments were analysed using Statistical Product and Service Solution 22.0 program, and if the treatment had a significant effect, Duncan's multiple range test (DMRT) with a 95% confidence level was conducted.

Results and Discussion

Multiplication

Multiplication is the process of multiplying prospective plants by planting explants in the media. The results of the analysis of variance at the multiplication stage are as follows.

Day after planting (DAP)

The analysis of results on the various basic media showed a very significant effect (p-value = 0.000), BAP concentration was significantly affected (p-value = 0.050), and the interaction between the basic media and BAP concentration had no significant effect (p-value = 0.149) on DAP.

The results of further tests on basic media using DMRT with 95% confidence level showed that WPM and MS media were not significantly different, whereas DKW media was significantly different. WPM responds the fastest, with a mean value of 7.46 DAP, and DKW media responds the slowest, with a mean value of 10.19 DAP (Fig. 1).

The results of further tests of BAP concentration using DMRT with a confidence level of 95% showed that 1 mg·L^{-1} BAP concentration was significantly different from other BAP concentrations and responded most quickly at 6.5 DAP. It is

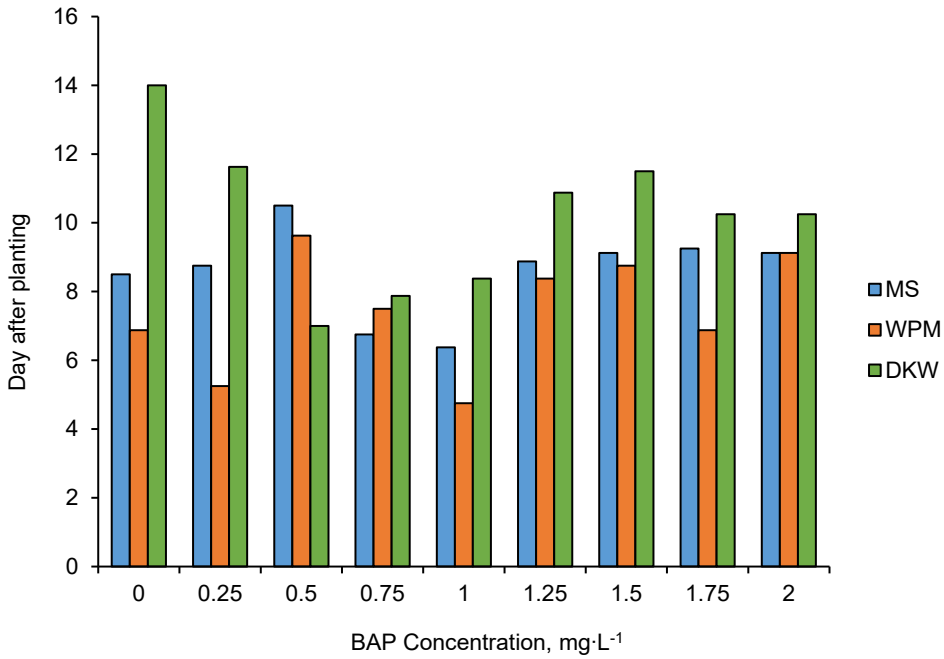

Fig. 1. Days needed for the explant to grow a new shoot after planting in different BAP concentration and planting media.

suspected that the endogenous cytokinins found in the explants have been able to encourage the formation of shoots and leaves; it requires 1 mg·L^{-1} BAP cytokinins to form buds and leaves of *N. macrophylla* explants. The results of this study were different from those of Putriana et al. (2019), in which MS basic media without addition of growth regulators was used and responded more quickly in comparison with the addition of growth regulators. The interaction between the basic media and BAP concentration had no significant effect, which meant that the basic media of MS, WPM, and DKW with addition of 0–2 mg·L^{-1} BAP were not significantly different even though the addition of 1 mg·L^{-1} BAP produced better result. In addition, the previous study demonstrated that WPM media provided better multiplication for *Syzygium densiflorum* Wall. ex Wight & Arn. due to its potential in organogenesis process.

Number of leaves

The analysis of results on the various basic media showed no significant effect (*p*-value = 0.195), BAP concentration had a very significant effect (*p*-value = 0.000), and the interaction between the basic media and BAP concentration had a very significant effect (*p*-value = 0.000) on the number of leaves.

The results of further tests of BAP concentration of DMRT with a confidence level of 95 % showed that 1 mg·L^{-1} BAP concentration produced the highest number of leaves with a mean value of 10.42 leaves, which was not significantly different from the addition of 1.5 mg·L^{-1} BAP, with a mean value of 10.17 leaves. The interaction between DKW basic media and 0.75 mg·L^{-1} BAP concentration produced a mean value of 13 leaves; however, the result was not significantly different from the interaction between MS basic media and 1 mg·L^{-1} BAP concentration, with a mean value 10.75 leaves. WPM basic media and 2 mg·L^{-1} BAP concentration produced a mean value of 12 leaves. It is assumed that DKW basic media, which already contains 52.1 µM·L^{-1} nitrogen (N) and 198.0 µM·L^{-1} manganese (Mn) was sufficient to increase leaf growth; thus, the addition of 0.75 mg·L^{-1} BAP was able to produce more leaves compared with MS basic media and 1 mg·L^{-1} BAP and WPM basic media and 2 mg·L^{-1} BAP (Fig. 2).

Number of shoots

The analysis of results of various basic media showed no significant effect (*p*-value = 0.260), BAP concentration had a very significant effect (*p*-value = 0.000), and the interaction between basic media and BAP concentration also had a very significant effect on the number of shoots (*p*-value = 0.000).

The results of further tests of BAP concentration using DMRT with a confidence level of 95 % showed that the addition of 1.5 mg·L^{-1} BAP produced the highest number of shoots with a mean value of 2.46 shoots. The average value of shoots produced on the media with the addition of BAP was higher by 2 times than in the media without addition of BAP.

This is in line with the previous study conducted by Widiastoety et al. (1997) which concluded that BAP administration in the media can stimulate the formation and multiplication of shoots from explants (tissue pieces). BAP was predicted to stimulate RNA synthesis and protein in various tissues which can further encourage cell division (Yu et al. 2019). In this case, BAP played a role as exogenous factor which supported the shoot produc-

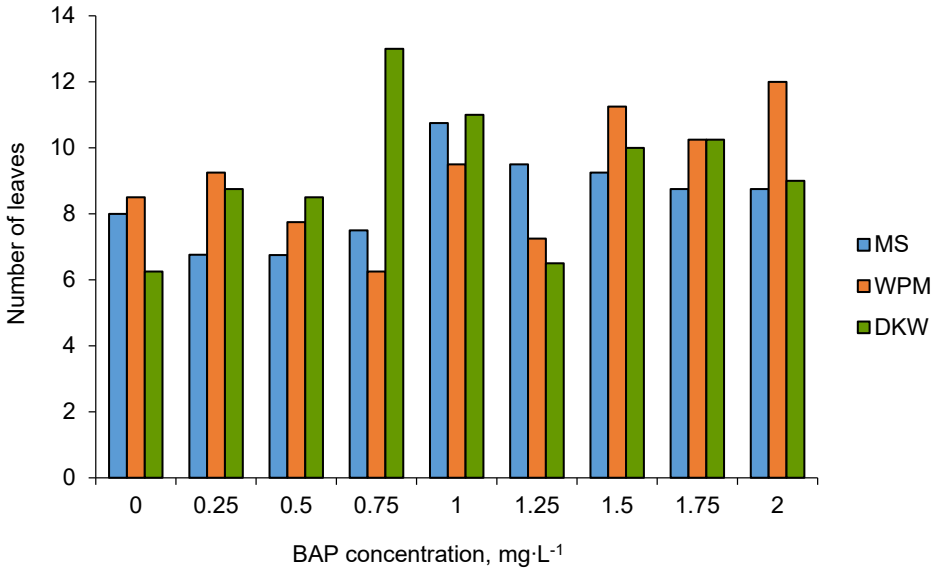

Fig. 2. Number of leaves appeared after 8 weeks of planting with various BAP concentrations and different media.

tion as the endogenous. It is strengthened by Gunawan (1992) that stated the interaction between exogenous and endogenous growth regulators determines the direction of culture development.

The interaction between WPM basic media and 2 mg·L^{-1} BAP produced the highest number of shoots, which were three buds. However, it was not significantly different from the interactions between WPM basic media and 1.5 mg·L^{-1} BAP, MS basic media and 1 mg·L^{-1} BAP, MS basic media and 1.5 mg·L^{-1} BAP, DKW basic media and 0.75 mg·L^{-1} BAP, and DKW basic media and 1 mg·L^{-1} BAP. This is due to the role of N and phosphorus (P) contained in MS media, WPM, and DKW media. WPM had 14.8 µM·L^{-1} N and 1.3 µM·L^{-1} P contents, which were lower than in MS and DKW media. Thus, it requires BAP greater than 2 mg·L^{-1} in comparison with MS and DKW media (with around 0.75–1.5 mg·L^{-1}) to produce shoots on *N. macrophylla* explants (Fig. 3).

Shoot height

The analysis of results of various basic media had no significant effect (p-value = 0.173), BAP concentration had a very significant effect (p-value = 0.000), and the interaction between basic media and BAP concentration also had a very significant effect (p-value = 0.000) on shoot height.

The results of further tests of BAP concentration using DMRT with a 95% confidence level showed that the shoot height was 3.9 cm higher without BAP in comparison with the addition of any BAP concentration. MS basic media without addition of BAP gave the best results, with a median value of 5.56 cm, and was not significantly different from DKW basic media with 0.75 mg·L^{-1} BAP. It is estimated that the planted explants produce endogenous auxin with a high enough concentration without BAP; thus, the cell elongation process occurs. On the other hand, with the addition of BAP, the activity of endogenous

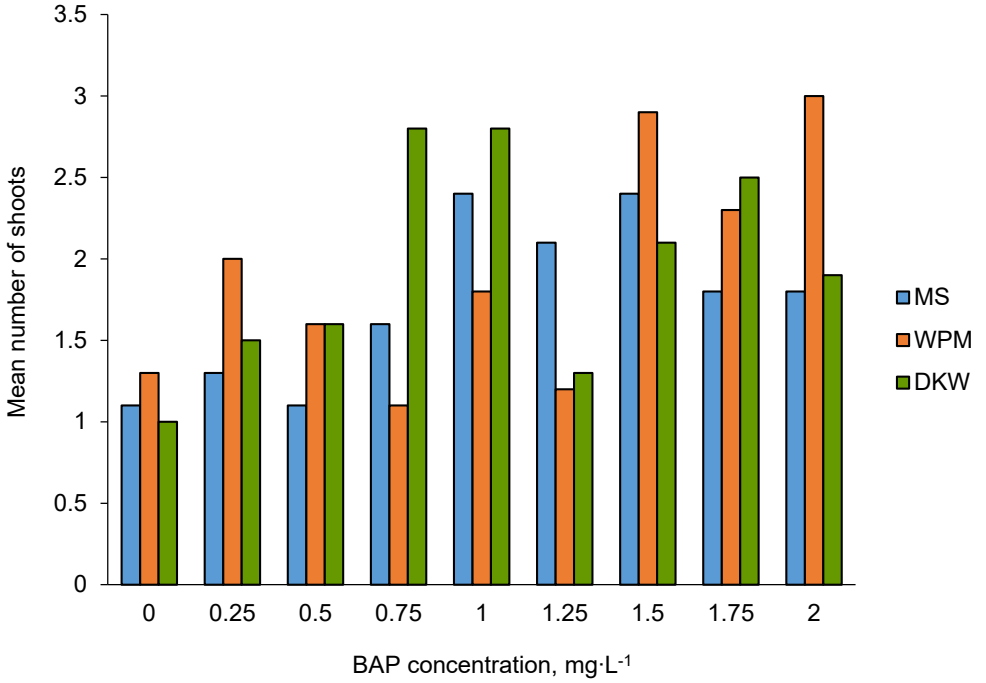

Fig. 3. Number of shoots after 8 weeks of planting with various BAP concentrations and different media.

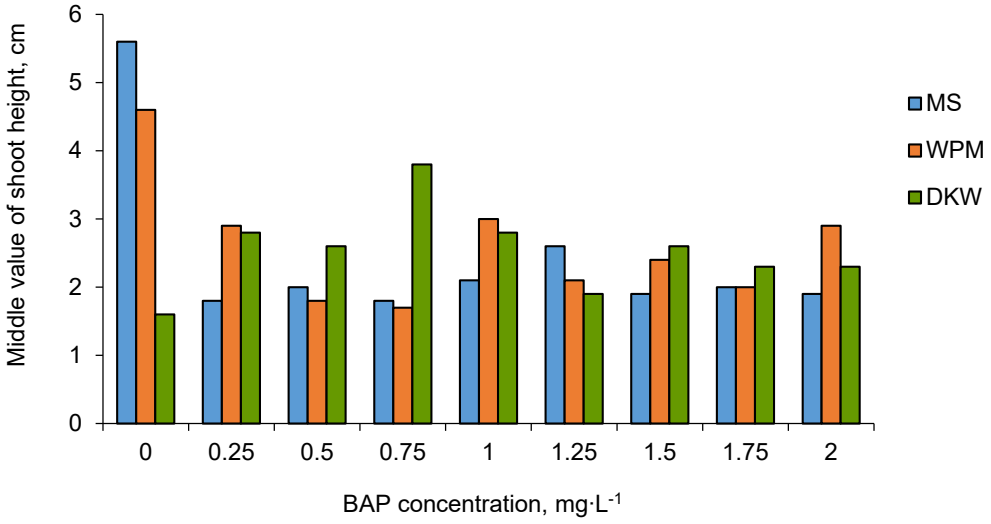

Fig. 4. Shoot height of explants after 8 weeks of planting with various BAP concentrations and different media.

auxin is inhibited because of the presence of exogenous cytokinins (Fig. 4).

Colour and callus of the shoot

The leaves and callus were observed to be formed at the bottom of the plantlet stem. The basic media of MS and WPM were similar in colour, which was dark green, whereas the basic media of DKW was light green. On the basic media of MS and DKW, callus was not found in the lower part of the plantlet stem, but in the basic media of WPM, solid callus was found at the bottom. The callus formed in this research was green and dense. It is called a proliferative callus, it can grow but is unable to develop toward organogenesis or embryogenesis (Fig. 5).

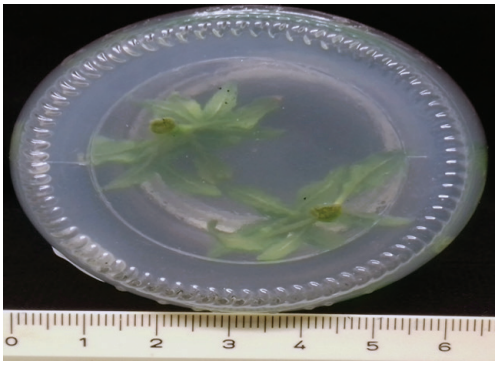

Fig. 5. Solid callus found at the bottom of the plantlet stem on WPM media.

Rooting

The rooting stage happens when the buds are transferred to the root induction medium, which contains hormones for root formation.

Day after rooting induction

The analysis of results of various basic media had a very significant effect (p-value = 0.000), BAP concentration had a very significant effect (p-value = 0.001), and the interaction between the basic media and BAP concentration had a very significant effect (p-value = 0.000) on DAP.

The results of further tests of basic media of MS, WPM, and DKW using DMRT with a 95% confidence level showed significant difference in DAP. WPM basic media responds faster at 7.16 DAP compared with MS and DKW basic media, because the concentration of macronutrients in WPM basic media was lower than that in MS and DKW basic media. The high N content in MS (30 $\mu M \cdot L^{-1}$) and DKW (26.05 $\mu M \cdot L^{-1}$) media compared with N content in WPM (7.4 $\mu M \cdot L^{-1}$) media caused the inhibition of root induction (Fig. 6).

The analysis of results of further BAP concentration using DMRT with a 95% confidence level showed that 2 $mg \cdot L^{-1}$ IBA concentration had a faster root formation, which was 7.13 DAP, whereas 0 and 1.25 $mg \cdot L^{-1}$ IBA showed a significant result and had the slowest root formation, which was 10 DAP.

The interactions between WPM basic media and 0.75 $mg \cdot L^{-1}$ IBA and between WPM basic media and 1.5 $mg \cdot L^{-1}$ IBA resulted in a faster root formation at 5.6 DAP, and DKW basic media and without the addition of IBA resulted in the slowest root formation at 13.2 DAP. The result of this study is in line with that of Yunita and Nugraha (2021), who showed that IAA, IBA, and NAA treatments were to obtain the appropriate type and concentration of auxin for *in vitro* induction of *Alternanthera reineckii* Griseb. roots.

Number of roots

The analysis of results of various basic media had a very significant effect (p-value = 0.003), BAP concentration had

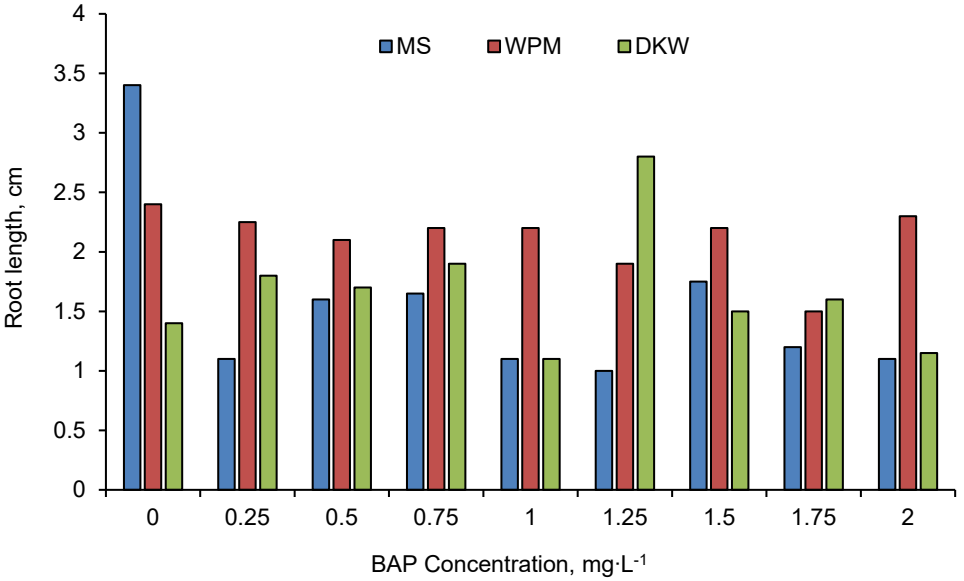

Fig. 6. DAP for explants producing roots by various IBA concentrations and different media.

a very significant effect (*p*-value = 0.000), and the interaction between basic media and BAP concentration had no significant effect (*p*-value = 0.054) on the number of roots.

The results of further tests of the basic media using DMRT with a 95% confidence level showed that the number of roots formed in WPM was 50.7, which was more than MS and DKW media. This is due to the sufficient and balanced nutrients in WPM needed by plantlets to grow.

The results of the further analysis of IBA concentration using DMRT with a 95% confidence level showed that the number of roots produced at 0.75 mg·L^{-1} IBA concentration was 61, which is significantly different from other concentrations. These results were presumed by the ratio of auxin which was higher than that of organogenesis cytokinin. Hence, it tended to lead the root formation. This finding was in line with the study conducted by Kristina and Syahid (2012).

Length of the root

The results of the analysis of various basic media had a very significant effect (*p*-value = 0.000), IBA concentration had a very significant effect (*p*-value = 0.000), and the interaction between basic media and IBA concentration had a very significant effect (*p*-value = 0.000) on root length.

The results of further basic test analysis using DMRT with a 95% confidence level of root length in WPM significantly differed from MS and DKW basic media. Further test results of IBA concentrations using DMRT with a 95% confidence level on addition of IBA with a concentration of 0.25–2 mg·L^{-1} showed a significant result. The treatment without IBA showed the longest root length, which was 2.43 cm, whereas the shortest root length with IBA concentration treatment (1.75 mg·L^{-1}) was 1.43 cm. The treatment without IBA produced the longest root length, but not the highest number of roots; this is due to the unavail-

ability of optimum auxin in *N. macrophylla* plantlets. It requires exogenous auxin to produce a greater number of roots. When the higher concentrations of IBA level were given, the lower the average length value produced. These results are in line with that of Ak et al. (2021); giving high concentrations of IBA will inhibit root lengthening, whereas lower concentrations produce longer roots in 'Garnem' culture (Fig. 7).

Shape and callus of the root

The root media without addition of IBA formed fiber roots, whereas the addition of IBA initially formed callus at the bottom of the stem. Besides, the leaves touched the root media, and this happened to MS and DKW rooting media. Rooting in WPM is not followed by callus formation. The appearance of roots turned out to have a different pattern on MS and DKW media: they felt soft, and there was callus. On the other hand, on WPM, the root extraction looked larger and felt rough, and there was no callus because IBA played a vital role in the process of cell division and enlargement, especially at the beginning of root formation. The concentration of IBA absorbed by the plant will affect the cell division.

Callus formed on MS and DKW media had crumb texture (friable) with white colour. Although the callus formed in this study had been able to form roots that need to be developed further with various combinations of auxin and cytokinin growth regulators; thus, callus becomes embryogenic. This is in line with the study of Gusmiaty et al. (2015), in which it was found that the use of MS media with addition of 0.3 mg·L^{-1} NAA and 4.4 mg·L^{-1} BAP formed callus with friable texture and varying colours on the explants of *N. macrophylla* leaves (Fig. 8).

Acclimatization

The analysis of results on acclimatization variety of media treatments had a significant effect (p-value = 0.021); basic me-

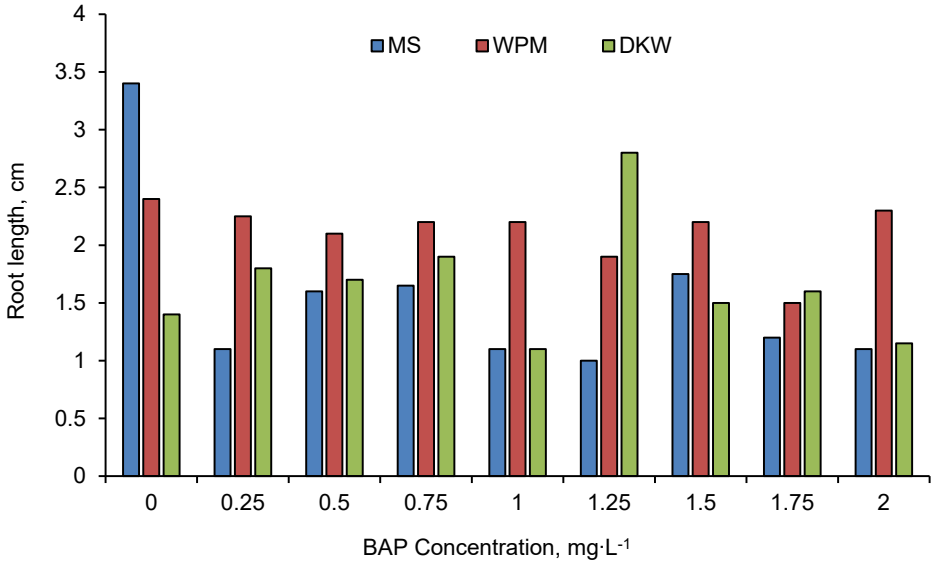

Fig. 7. Root length after 6 weeks in different rooting media with various BAP concentrations.

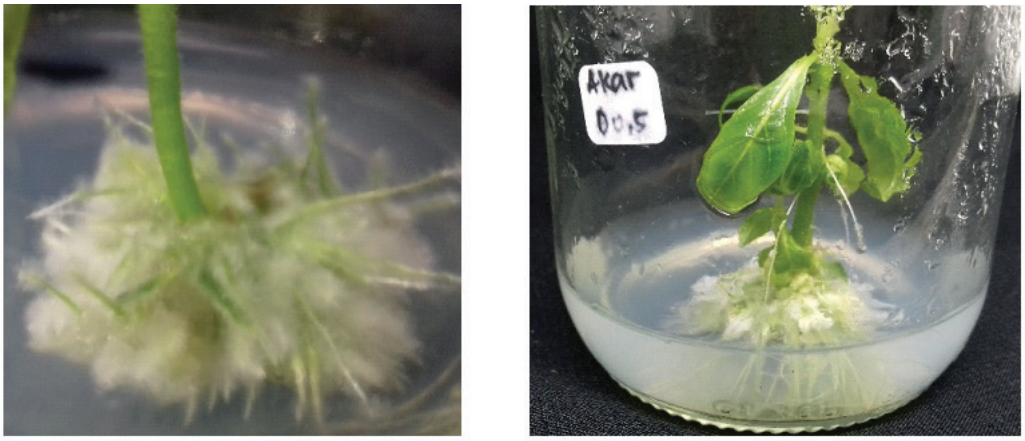

Fig. 8. Callus formed in the MS medium (left) and DKW medium (right).

dia treatment of tissue culture had a very significant effect on the percentage of living plantlet in the acclimatization media (*p*-value = 0.000) (Table 1). A further test showed that acclimatisation in compost + sand media produced 93.3 % survival rate of the explant, which was not significantly different from only compost media and compost + rice husk + sand media. It was due to the addition of sand to the loose structure of the media (Fig. 9). The aeration and drainage of the planting media were also good, and plant roots can grow circulation optimally, and the availability of adequate air is needed by root cells to breathe.

The use of single husk charcoal acclimatization media resulted in the lowest percentage survival, which was 13.3 %, and it was not significantly different from the compost and husk charcoal media. The low percentage survival can be seen from the high mortality of plantlets during acclimatization. The root system produced in *in vitro* culture contributes to a high percentage of viability at the acclimatization stage to the green house or field. Therefore, the selection of substrate materials needs to be done carefully. This can be caused by an adaptive process that is not carried out gradually, as in *Thalictrum foliolosum* (Mishra et al. 2020).

The results of further test analysis of tissue culture basic media treatment using DMRT with a confidence level of 95 % showed that the survival rate of WPM basic media was 74 %, which was not significantly different from DKW media. Meanwhile, Thengane et al. (2006) reported that the successful acclimatization

Table 1. Average survival percentage of seedlings in various acclimatization media.

Acclimatization medium	Average survival, %	DMRT (95% confidence level)
Compost	86.7	a
Rice husk	13.3	b
Compost + sand (4:3)	93.3	a
Compost + rice husk + sand (1:1:1)	80.0	a
Compost + rice husk (2:1)	36.7	b

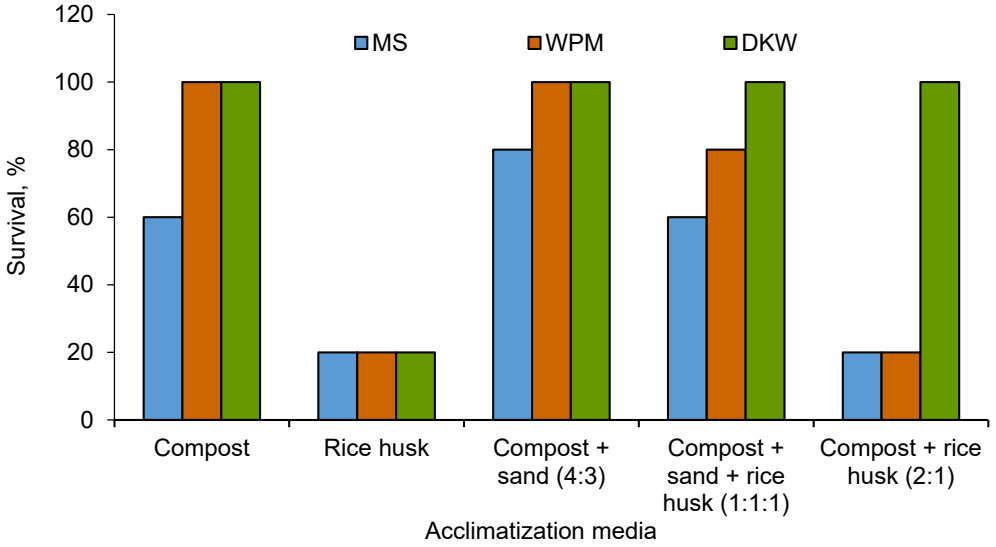

Fig. 9. Survival percentage of seedlings in various acclimatization media taken from various basic media from the rooting stage.

(up to 80 %) of *Calophyllum inophyllum* L. was due to WPM. Also, Kruczek et al. (2021) showed that acclimatization of two cultivars of goji regenerated on the medium with chitosan were successfully acclimatized and established in the mixture of 90 % peat and 10 % perlite under field conditions. Hence, these findings could be a further alternative as acclimatization media.

Physical quality of seeds

The analysis of results on the variety of sturdiness of seeds showed a significant effect (*p*-value = 0.029), the root:shoot ratio (RPA) (*p*-value = 0.030) was significantly affected, and the seed quality index (IMB) had a very significant effect (*p*-value = 0.008). The results of further sturdiness tests on RPA and IMB using DMRT with a 95% confidence level showed that seed sturdiness increases with seedling age. The sturdiness of seedlings at 2 (4.698) and 3 (4.886) months were not significantly different but was very significantly different from seedlings at 1 month (3.522). An optimum sturdiness value is 4–5 (Febrianti et al. 2013). Furthermore, the results of this study had met the optimum sturdiness criteria. Root:shoot ratio (RSR) is a valuable selection criterion that can be used for phytoremediation and associated phytotechnologies (Rogers et al. 2019).

The results of RPA follow-up test at 2 (2.232) and 3 (3.980) months were not significantly different. However, it showed a very significant difference at 1 month (3.0480). The lower RSR generally results in higher plant survival and adaptability (Komala et al. 2008). Mindawati et al. (2005) stated that seeds with RPA value of 2–5 would be ready to be moved to the field. A high total dry weight will affect the seed quality index (SQI); this means that the higher the total dry weight, the higher the BMI in a treatment (Bayau 2018). Those criteria could be used as the quality information for seeds. Plants with SQI greater than 0.09 will have high survival in

the field. Furthermore, the analysis of result of the seed quality index in this study showed that all age level treatments would be able to grow in the field because BMI value was greater than 0.09. The tissue culture method can be used in the *ex situ* conservation of *N. macrophylla* in the form of plantlets and can be further developed for breeding activities and as conservation efforts.

The use of MS, WPM, and DKW basic media responded well to the multiplication stage. WPM and 1 mg·L^{-1} BAP responded faster to the multiplication stage, DKW and 0.75 mg·L^{-1} BAP produced the greatest number of leaves, WPM and 2 mg·L^{-1} BAP produced the highest number of shoots, and MS without addition of BAP produced the highest shoot height.

The use of MS, WPM, and DKW rooting media was able to induce root formation. Several previous studies (Batti et al. 2020, Pandey et al. 2022) got the similar results to this present study. WPM rooting media was better than MS and DKW media (Fadladeen et al. 2021). WPM and 0.75 mg·L^{-1} IBA as a rooting media and WPM and 1.5 mg·L^{-1} IBA responded faster to root formation, WPM and 0.75 mg·L^{-1} IBA produced the highest number of roots, and MS without addition of IBA as a rooting media produced the longest roots. The appropriate acclimatization media for *N. macrophylla* were compost and sand (4:3) and red jabon plantlets originating from WPM, which produced the highest percentage of survival. By the age of 2 months, the physical quality of *N. macrophylla* seedlings (height, diameter, sturdiness, RSR, and SQI) meets the requirements to be planted in the field. In this research, the effects of media on plant growth showed a good result and a protocol is necessary to optimize the efficient micropropagation of *N. macrophylla*.

Conclusions

In this study, an effective micropropagation system for *N. macrophylla* has been developed by using three media, including DKW, WPM, and MS. Those media produced equally effective results. At the age of two months, physical characteristics of *N. macrophylla* seedlings (height, diameter, sturdiness, shoot ratio, and seed quality index) met the requirements for field planting. Hence, these findings are promising to be applied in the conservation fields.

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