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ARTICLE

In vitro mass propagation protocol for an ornamental banana: explant sterilization to plantlet acclimatization

Protocolo de propagação massal *in vitro* de bananeira ornamental: da esterilização do explante à aclimatização de plântulas

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Abstract: The study investigates the critical factors in establishing an ornamental banana's *in vitro* mass propagation protocol (*Musa spp*. 'Pink Nono') based on four important experiments. In the first experiment, testing three sterilization methods by applying varied-disinfectant agents revealed that high optimal clean shoots up to 92% with low contamination and 86% potential growth were proved by using a combination of streptomycin sulfate, and benomyl, 70% alcohol, 2.5% sodium, 0.1% $HgCl_2$ hypochlorite, and reducing sucker size. In the second experiment, immersing the shoot tips in 0.1, 0.2, and 0.3 mg L^{-1} thidiazuron (TDZ) for an hour and culturing them in MS medium supplemented with 1, 2, and 3 mg L^{-1} N6-benzyl amino purine (BAP) proved that immersing shoots in 0.1 mg L^{-1} TDZ and then culturing them on MS medium containing 3 mg L^{-1} BAP resulted in high axillary shoots per explant up to 30.6 shoots. In the third experiment, 0.0, 0.5, and 1.0 g L^{-1} activated charcoal (AC) and mg L^{-1} α-naphthalene acetic acid (NAA) for root formation were tested, and it found that MS medium supplemented with 0.5 g L^{-1} AC and 1.0 mg L^{-1} NAA was optimal for root formation with 9.4 roots per shoot. Finally, three acclimatization media were researched, and it established that the high plantlet survivability of up to 93% was recorded in a combination of burned-rice husk, soil, organic manure, and volcanic sand (1:1:1:1, v/v/v/v) for treated plantlets previously. These insights provide a valuable protocol for producing high-quality planting materials efficiently.
Keywords: aseptic culture, initiation, multiplication, *Musa spp*. 'Pink Nono', plant growth regulator, shoot-tips.

Resumo: O estudo investiga os fatores críticos no estabelecimento de um protocolo de propagação massal *in vitro* de bananeira ornamental (*Musa spp*. 'Pink Nono') com base em quatro experimentos. No primeiro experimento, a avaliação de três métodos de esterilização utilizando diferentes agentes desinfetantes revelou que a combinação de sulfato de estreptomicina, benomil, álcool a 70%, hipoclorito de sódio a 2,5%, $HgCl_2$ a 0,1% com a redução do tamanho do explante resultou em até 92% de brotações limpas, com baixa contaminação e 86% de potencial de crescimento. No segundo experimento, a imersão das pontas dos brotos em 0,1, 0,2 e 0,3 mg L^{-1} de tidiazuron (TDZ) por uma hora e o cultivo em meio MS suplementado com 1, 2 e 3 mg L^{-1} de N6-benzil aminopurina (BAP) comprovou que a imersão dos brotos em 0,1 mg L^{-1} de TDZ e, em seguida, o cultivo em meio MS contendo 3 mg L^{-1} de BAP promove até 30,6 brotos axilares por explante. No terceiro experimento, foram testados 0,0, 0,5 e 1,0 g L^{-1} de carvão ativado (AC) e mg L^{-1} de ácido α-naftalenoacético (ANA) para a formação radicular, e verificou-se que o meio MS suplementado com 0,5 g L^{-1} de AC e 1,0 mg L^{-1} de ANA propiciou a formação de 9,4 raízes por planta. Por fim, três meios de aclimatização foram pesquisados e estabeleceram que a alta capacidade de sobrevivência de plântulas de até 93% foi registrada em uma combinação de casca de arroz queimado, solo, adubo orgânico e areia vulcânica (1:1:1:1, v/v/v/v) para plântulas tratadas anteriormente. Esses insights fornecem um protocolo valioso para a produção eficiente de materiais de plantio de alta qualidade.
Palavras-chave: brotações, cultura asséptica, iniciação, multiplicação, *Musa spp*. 'Pink Nono', regulador de crescimento vegetal.

Introduction

Musa spp. 'Nono', the variegated red plant, is an ornamental banana originating from Duma in the Autonomous Region of Bougainville (AROB) of Papua New Guinea (Sardos et al., 2018). The plant had an accession number AROB006. Nono 1 is its genomic composition, and breast is its meaning name. It has edible fruits, but their fruits are not tasty (Sardos et al., 2018). The banana was recently introduced as an ornamental plant in the Indonesian market. The leaves of the *Musa spp.* 'Nono' exhibits a remarkable reddish-purplish hue with exquisite variegation and displays a diverse array of multi-colored patterns on each leaf. Initial molecular study suggests that *Musa spp.* 'Nono' shares the same genetic cluster as wild-type *Musa acuminata* subsp hybrids. *banksii* × *M. schizocarpa* and was classified as AS, similar to AROB001 ''Flower Banana'', AROB006 and AROB041 ''Glenda's Red'' (Sardos et al., 2018). The three ornamental bananas and the 28 other accessions form a coherent block in the middle of cluster XI in the diversity tree of core subset accessions with more or less evident links to diploid AA cultivars (Christelová et al., 2017). Although there is no existing data on the demand of the *Musa spp*. 'Pink Nono', increasing its popularity among ornamental plant hobbyists, leads to increasing demand for prime seedlings of plants.

Traditionally, *Musa spp*, involving *Musa spp*. 'Pink Nono' is generally propagated vegetatively by separating the suckers, which grow from lateral buds originating from corms (Kelta et al., 2018; Sivakumar and Visalakshi, 2021; Hansuek et al., 2023). However, this method is labor, cost, and time-consuming and results in low number and quality planting materials and more critical duo to the method can express transmission of pests, diseases, and poor preservation of original plant genetic materials (Kelta et al., 2018; Malemba et al., 2021). To avoid these problems, applying micropropagation as an alternative and viable technology to produce and supply high-quality planting materials of the banana free from any pests and diseases is important addressed (Malemba et al., 2021; Sivakumar and Visalakshi, 2021). The *in vitro* mass propagation started from initiation, proliferation, root formation to plantlet acclimatization, was successfully established on varied types of bananas and cultivars such as Grand Nine (AAA) (Ali et al., 2019), Elakki (AB) (Selvakumar and Parasurama, 2020), Barraganete, Curare, and Poovan (Musa AAB) (Sivakumar and Visalakshi, 2021; Quiñonez et al., 2021) and all were edible bananas. However, so far, there are several published works on ornamental banana.

Several *in vitro* mass propagation protocols of ornamental bananas were reported previously. Rashid et al. (2012) successfully developed *in*

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vitro mass propagation via axillary shoot proliferation of ornamental plant *Musa beccarii* using suckers and male buds as explant sources and BAP as main plant growth regulator (PGR). Micropropagation protocol through axillary proliferation for exotic banana (*Musa spp*.) Yangambi uses a sucker as an explant source and tests individual BAP or in combination with indole-3-acetic acid (IAA) (Qamar et al., 2015). Micropropagation protocol for an ornamental banana *Musa acuminata* var. *zebrina* (Van Houtte *ex* Planch.) Nasution was established by using shoot-tip explants. The effect of different concentrations (0, 2.3, 3.4, 4.5, and 6.8 mg L^{-1}) of BAP with and without 0.2 mg L^{-1} NAA supplemented to Murashige and Skoog (MS) medium was evaluated for *in vitro* shoot multiplication (Noor et al., 2017). A simple and efficient protocol developed for plantlet regeneration through *in vitro* somatic embryogenesis in three ornamental bananas, i.e. *Musa laterita, Musa beccarii,* and *Musa velutina* using immature male flower buds as explants (Natarajan et al., 2020). *In vitro* ornamental bananas of 'Little Prince' and 'Truly Tiny' were cultured under different light sources (Vendrame et al., 2022). However, until now, there has been no report on the in vitro mass propagation protocol of *Musa spp*. 'Pink Nono'.

The current research was carried out primarily to explore the potential of establishing an *in vitro* mass propagation protocol of *Musa spp*. 'Pink Nono' using shoot tips as explant sources. The research was initiated by determining the optimal sterilization method, followed by establishing axillary shoot initiation, proliferation, and root formation until plantlet acclimatization. Via this research, it was expected that premium planting materials for developing the ornamental banana commercially could be proved.

Materials and Methods

Planting materials, disinfection, explant preparation, and culture incubation

This research was conducted in the tissue culture laboratory at Kp. Cilastari, Ds. Alam Indah, Ciwidey Subdistrict, Bandung Regency, West Java province-Indonesia. The explants of *Musa spp*. 'Pink Nono' was obtained from a Ciwidey Nursery in Cibiru Subdistrict, Bandung Regency, West Java-Indonesia. The donor plants were planted in a mixture media of soil, organic manure, and burned rice husk (1:1:1, v/v/v), watered regularly, and fertilized with 5 g pot^{-1} plant once a month. Healthy small sword suckers with 0.3 - 0.5 kg fresh weight, 10 - 15 cm in height, and 10-15 cm in diameter were harvested from a 3-year-old mother plant and used as explant sources.

Explant preparation and pre-sterilization

The sheaths of suckers were peeled off gradually, sheath by sheath involving their leaves and roots removed with a clean knife. Afterward, the suckers were trimmed to 1.5 - 2.0 cm in length and 2 - 3 cm in diameter. The explants were placed into a container and put under tap water for two minutes to remove any remaining debris, then subjected to a 1% Sunlight solution and rinsed with clean water several times until no shop solution existed.

Culture incubation

All cultures in each experiment were incubated in the incubation room for a 16-h photoperiod under a cool fluorescent lamp with 13 µmol m^{-2} s^{1} light intensity at 24 ± 1 °C and 70% relative humidity.

Establishing sterilization method for in vitro culture of suckers

Three sterilization methods were tested to obtain an aseptic culture of shoots (Table 1). The experiments were arranged in a completely randomized design (CRD) with 9 replications. Each treatment consisted of seven bottles, and each bottle contained three explants. After final preparation, the shoot tips were cultured in an initiation medium perpendicular to the medium. The initiation medium was full-strength MS medium plant growth regulator (PGR) free supplemented with 2.5% (w/v) sucrose. The medium pH media were adjusted to 5.8 ± 0.1 before adding 11 grams of agar (Dolphin, agar) for a one-liter medium. The solution media were then put on the stove till boiled, poured into jam bottles (Ø 6 cm in diameter and 9 cm in height), 25 mL media in each bottle, and closed with transparent plastics. The bottles containing culture media were sterilized in an autoclave at 121 ºC and 15 psi pressure for 20 minutes. The total number of contaminated and clean explants and shoot growth potential was recorded 8 weeks after culture.

Table 1. Sterilization methods tested in the research

Nº.	Sterilization method (SM)	Description
1.	SM-1	The washed suckers were surface sterilized by soaking in 70% alcohol for five minutes, followed by immersing in 2.5% sodium hypochlorite with a few droplets of Tween 20 for five minutes, then rinsed 5 times (Each time was 2 minutes) with sterile water accompanied by manual shaking. Then sterilized suckers were trimmed to 1 – 1.5 cm in length of shoot tips. Finally, the treated shoot-tips were planted in the initiation medium.
2.	SM-2	The washed suckers were surface sterilized by soaking in 70% alcohol for five minutes, followed by immersing in 2.5% sodium hypochlorite with a few droplets of Tween 20 for five minutes, rinsed 5 times (Each time was 2 minutes) with sterile water accompanied by manual shaking. Then sterilized suckers were trimmed to 1 - 1.5 cm in length of shoot tip, then soaked in 0.1% $HgCl_2$ solution for 4 minutes. The treated shoot tips were then rinsed 5 times (Each time was 2 minutes) with sterile water and manual shaking. Finally, the treated shoot-tips were planted in the initiation medium.
3.	SM-3	The washed suckers were soaked in streptomycin sulphate solution (1 g L^{-1}) and benomyl fungicide (1 g L^{-1}) for 1 hour and put on a shaker at 100 rpm speed. The treated suckers were surface sterilized by soaking them in 70% alcohol for five minutes, followed by five minutes of 2.5% sodium hypochlorite solution with a few droplets of Tween 20. Rinsed 5 times (Each time was 2 minutes) with sterile water and manual shaking. Then sterilized suckers were trimmed to 1.0 - 1.5 cm in length of shoot-tips, immersed in 0.1% $HgCl_2$ solution for 4 minutes. Rinsed 5 times (Each time was 2 minutes) with sterile water and manual shaking. Finally, the treated shoot tips were planted in the MS Initiation medium.

Axillary shoot initiation and proliferation

Clean shoot tips derived from the sterilization stage were subcultured in the initiation culture media. The shoot tip explants were immersed in different levels of TDZ (T, Duchefa Biochemie, Haarlem, The Netherland) from 0.1, 0.2, and 0.3 mg L^{-1} for an hour in an Erlenmeyer flask (100 mL). The treated shoot tips were then cultured on MS medium in different concentrations of BAP (B, Duchefa Biochemie, Haarlem, The Netherlands) from 1, 2, and 3 mg L^{-1}. The cultures were then incubated in an incubation room for 6 weeks. After 6 weeks of the first incubation period, the whole regenerated shoots were subcultured in the same medium for the next 6 weeks for the second and third incubation periods. The factorial experiment was arranged in CRD with 4 replications. Immersing explants in the different concentrations of T was the first factor, and various concentrations of B were the second factor. Each treatment contained 6

bottles. Each bottle contained 4 shoots. Variable observed in the step were: (1) height of shoots (cm), (2) number of axillary shoots shoot^{-1}, and (3) multiplication rate (MR) of axillary shoots calculated by formula:

$$Multiplication\ rate\ (MR) = \frac{Number\ of\ axillary\ shoots\ at\ the\ end\ of\ culture}{Number\ of\ axillary\ shoots\ at\ initial\ culture} \qquad (Eq.1)$$

Each culture period was recorded and measured 6 weeks after culture.

Root induction

Root induction was carried out by splitting axillary shoots individually and gently using a tissue culture blade. The individual shoots were then cultured on rooting medium, MS medium containing different concentrations of AC from 0.0, 0.5, and 1.0 g L^{-1} as the first factor, combined with NAA of 0.0, 0.5, and 1.0 mg L^{-1} as the second factor. The factorial experiment was arranged in CRD with 4 replications. Each treatment contained 6 bottles. Each bottle contained 4 shoots. The variables observed in this experiment were (1) diameter of plantlets (mm), (2) number of roots per shoot, and (3) root length (cm). All variables were recorded and measured 8 weeks after culture initiation.

Plant Acclimatization

The rooted shoot (± 8 cm in height with 3 - 4 leaves) was carefully taken out from the jars using a blunt pinset. The plants were washed under tap water to remove the remaining agar attaching to the roots. The plantlets were soaked in fungicide solution (1 g L^{-1} propineb 70%) for 1 hour. The treated plantlets were planted in seedling trays (50 holes) containing different acclimatization media, (1) burned-rice husk, (2) burned-rice husk and cocopeat (2:1, v/v), and (3) burned-rice husk, soil, organic manure, and volcanic fine sand (2:1:1:1, v/v/v/v). The media were watered sufficiently. The experiment was arranged in CRD with 9 replications. Each treatment consisted of 50 plantlets. The plastic trays were then covered with transparent plastic for 8 weeks. The trays with planted plantlets were placed in a glass house under a reduced light intensity area. After 8 weeks incubation period, the survival and growth performances of the plants were measured and repotted individually in pots containing similar media. Variable observed in the acclimatization stage were (1) a percentage of plantlet survivability (%) was calculated by formula:

$$Plantlet\ survivability = \frac{Number\ of\ survive\ plantlets}{Number\ of\ plantlets\ acclimatized} \qquad (Eq.2)$$

(2) height of plants (cm), (3) Stem diameter (mm), and (4) Number of leaves per plant.

Data analysis

Data collected from all experiments were analyzed using analysis of variance (ANOVA) and utilized SmartstatXL V.3.6.5.4 Professional to processes. Significant differences between means were further analyzed using the Tukey test, $p = 0.05$.

Results

Establishing sterilization method for in vitro culture of suckers

Establishing an *in vitro* mass propagation protocol for *Musa spp*. 'Pink Nono' was initiated by finding the optimal sterilization method. Combining 1 g L^{-1} streptomycin sulphate and 1 g L^{-1} benomyl treatment for an hour, followed by immersing prepared suckers in 70% alcohol for five minutes, 2.5% sodium hypochlorite solution with a few droplets of Tween 20 for five minutes, reducing sucker size to 1.0 - 1.5 cm in length, and soaking them in 0.1% $HgCl_2$ solution for 4 minutes, then rinsing shoot tips with sterile water (SM-3) resulted in the highest percentage of clean explant up to 92% with contamination explants as low as 8% and percentage of potential growth of explant as high as 85% (Fig. 1A, 1B and 1C; Fig. 2A). The result confirmed that combination of disinfection agents utilized in SM-3 had highest power to reduced and eliminated contaminants both bacteria and fungi which can inhibit and reduce explant growth potential. The SM-3 also showed significant differences from other methods ($p = 0.05$). While SM-1 performed the lowest result.

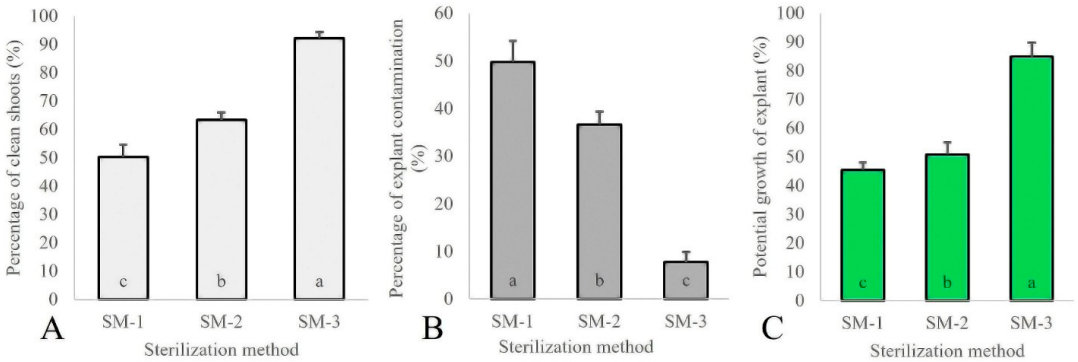

Fig. 1. The effect of three different sterilization methods on the percentage of explant-free contamination (A), percentage of explant contamination (B), and potential growth of explant in initial stage micropropagation of *Musa spp*. 'Pink Nono'. Histograms followed by different letters are significantly different based on Tukey at $p = 0.05$.

Shoot initiation and multiplication

The combination application of T immersion and B gave significant interaction effects on shoot initiation, and multiplication. All combination treatments gradually increased the height and number of shoots shoot tip^{-1}. A lower concentration of T and B produced higher shoots with a lower number of shoots shoot tip^{-1} (Fig. 3A and 3B), while increasing their concentrations declined the height of shoots but increased the number of shoots (Fig. 3A and 3B). The highest shoot height was noted on immersing suckers in T0.1 and culturing on MS medium B1. The highest number of shoots was recorded on immersing suckers in T0.1 and culturing on MS medium adding B3 (Fig. 3B). Other combinations generally resulted in lower number of shoots, with the lowest combination treatment on T0.1-B1.

Based on the MR of shoots of observation, the MR of shoots increased from initial to the first subculture and reduced thereafter. In the first subculture, the highest MR of shoots with as high as 2.57 was noted on T0.3-B1 (Fig. 2B). The second-best treatment combination was recorded on T0.3-B1 with 2.36 MR of shoots with the lowest MR of shoots (1.96) on T0.2-B2. In the second subculture, high MR of shoots was performed by T0.1-B1, with the lowest one on T0.2-B2 (Fig. 3C).

Fig. 2. *Musa spp*. 'Pink Nono' at different stages of micropropagation. A. Clean shoot-tip explant on MS initiation medium 8 weeks after planting in MS initiation medium, B. Early stages of shoot proliferation in the first subculture period after immersing the shoots in 0.1 mg L^{-1} TDZ for an hour and culturing on MS medium containing 3 mg L^{-1} BAP, C. Adventitious shoot formation at the end of the first subculture period after soaking the shoots in 0.1 mg L^{-1} TDZ for an hour and culturing on MS medium supplemented with 2 mg L^{-1} BAP, D. Regenerated shoots in the end of the third subculture period after dipping the shoots in 0.1 mg L^{-1} TDZ for an hour and culturing on MS medium fortified by 3 mg L^{-1} BAP, E. Early plantlet elongation on MS medium supplemented with AC 0.5 g L^{-1} and NAA 1 mg L^{-1} 4 weeks after culture, (F-G) Root formation on single plantlet in a jam bottle on MS medium supplemented with AC 0.5 g L^{-1} and NAA 1 mg L^{-1} 8 weeks after culture, H. Newly formed leaves from acclimatized plantlets on a mixture of husk charcoal, soil, manure, and volcanic fine sand (2:1:1:1, v/v/v/v) 4 weeks after planting, (I). Performance of *Musa spp*. 'Pink Nono' plants 5 months after acclimatization.

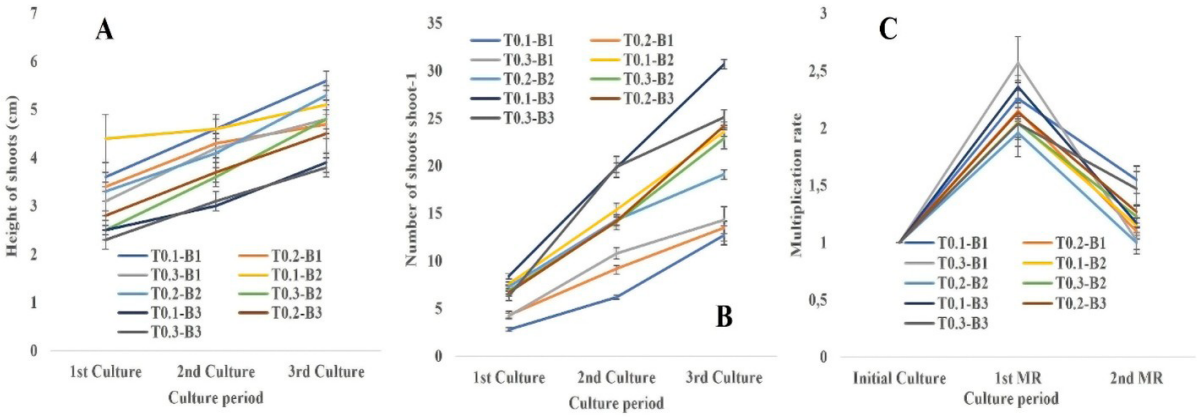

Fig. 3. Initial shoot initiation and multiplication under periodical subcultures on micropropagation of *Musa spp*. 'Pink Nono'. A. Gradual growth of shoot height from first culture to third culture. B. Number of shoots explant^{-1} under periodical subcultures on micropropagation of *Musa spp*. 'Pink Nono'. C. Multiplication rate of shoots from initial culture to first subculture, from first to second subculture on micropropagation of *Musa spp*. 'Pink Nono'.

In the initiation stage of axillary shoots, initial formation of axillary shoots was observed 3 – 4 weeks after culture initiation. The initial shoots continually grew and increased in height and number of shoots. Immersing shoot tips in T0.1 for an hour, followed culturing on MS medium containing B3 successfully regenerated the highest number of axillary shoots shoot^{-1}, 8.4 shoots in the first culture (Fig. 2B), 19.8 shoots in the second culture, and 30.6 shoots in the third culture (Fig. 2C, and 3C). The T0.1-B3 treatment produced lower shoot heights, with the highest average shoots shoot^{-1} up to 17.1 shoots, though it had lower MR shoots (1.76) (Table 2). Other combination treatments with more axillary shoots were noted in T0.1-B2, and the lowest result was recorded on T0.1-B1.

Table 2. Interaction effect of immersing shoot tips in different concentrations of TDZ and culturing them on MS Medium containing various concentrations of BAP on axillary shoot formation and its growth.

TDZ (mg L^{-1}) (T)	BAP (mg L^{-1}) (B)	Average shoot height (cm)	The average number of shoots shoot tip^{-1}	Average MR of shoot
0.1	1	4.2 ± 0.13 b	6.2 ± 0.28 g	1.92 ± 0.08 a
0.2	1	3.8 ± 0.11 cd	7.9 ± 0.25 f	1.63 ± 0.11 cd
0.3	1	3.7 ± 0.13 d	8.6 ± 0.34 e	1.79 ± 0.13 b
0.1	2	4.3 ± 0.13 a	13.6 ± 0.28 b	1.60 ± 0.05 d
0.2	2	3.9 ± 0.14 c	12.0 ± 0.26 d	1.48 ± 0.04 e
0.3	2	3.4 ± 0.17 e	12.7 ± 0.46 c	1.64 ± 0.10 cd
0.1	3	2.9 ± 0.11 f	17.1 ± 0.25 a	1.76 ± 0.04 b
0.2	3	3.3 ± 0.19 e	13.0 ± 0.22 c	1.70 ± 0.03 bc
0.3	3	2.8 ± 0.13 f	12.7 ± 0.37 c	1.76 ± 0.10 b

Means followed by the same letter in the same column are not significantly different based on Tukey, $p = 0.05$. Values reflect the means and standard errors of cultured explants, with n = 24 for each treatment.

Root Induction

Splitting regenerated axillary shoots followed by subculturing them on rooting media, initiation of root formation generally noted 2 – 4 weeks after culture initiation. The initial roots continually grew and increased in number and size (Fig. 2E). Application of different concentrations of AC in combination with NAA exhibited a significant interaction effect on root formation and growth. A higher application of AC reduced root formation; however, higher root formation was noted with a higher concentration of NAA. Combination treatment of 1 g L^{-1} AC and 0.5 mg L^{-1} NAA significantly affected root formation and its growth (Table 3). The combination regenerated well rooted shoots with 8.8 cm height of shoots, 4.8 mm stem diameter, 9.4 roots $shoot^{-1}$, and 6.2 cm root length (Fig. 2F and 2G). The combination improved root formation capacity up to 76% for the height of plantlets, 51% for stem diameter, 84% for root number, and 129% for root length. The second-best combination was noted on 1 g L^{-1} AC with 1 mg L^{-1} NAA, while the lowest results were recorded on the MS medium with no addition of AC and NAA.

Table 3. The effect of different concentrations of AC and NAA on *in vitro* rooting of *Musa spp*. 'Pink Nono' shoots 8 weeks after planting.

Treatment		Height of plantlets (cm)	Stem diameter (mm)	Number of roots $shoot^{-1}$	Root length (cm)
AC (g L^{-1})	NAA (mg L^{-1})				
0	0	5.0 ± 0.34 d	1.67 ± 0.05 e	5.1 ± 0.40 e	2.8 ± 0.18 g
0.5	0	6.4 ± 0.44 bc	2.14 ± 0.07 c	6.0 ± 0.32 d	4.5 ± 0.53 de
1	0	6.5 ± 0.39 bc	2.27 ± 0.10 b	6.6 ± 0.26 c	5.2 ± 0.47 bc
0	0.5	6.4 ± 0.38 bc	1.88 ± 0.12 d	5.7 ± 0.38 d	3.4 ± 0.64 fg
0.5	0.5	6.7 ± 0.27 b	2.18 ± 0.13 bc	8.3 ± 0.26 b	4.8 ± 0.60 cd
1	0.5	8.8 ± 0.43 a	2.52 ± 0.09 a	9.4 ± 0.44 a	6.4 ± 0.46 a
0	1	6.4 ± 0.27 bc	1.97 ± 0.11 d	6.5 ± 0.47 c	3.9 ± 0.45 ef
0.5	1	6.2 ± 0.22 c	2.24 ± 0.11 bc	5.8 ± 0.50 d	5.0 ± 0.49 cd
1	1	8.5 ± 0.40 a	2.40 ± 0.13 a	9.3 ± 0.42 a	5.8 ± 0.85 ab

Means followed by the same letter in the same column are not significantly different based on Tukey, $p = 0.05$. Values reflect the means and standard errors of cultured explants, with n = 24 for each treatment.

Plantlet acclimatization

In the acclimatization stage, three acclimatization media process gave significant effect on survivability and growth of plantlets (Fig. 2F). Utilizing a combination of burned-rice husk, soil, organic manure, and volcanic fine sand (1:1:1:1, v/v/v/v) accompanied by washing the plantlets under tap water to remove the remaining agar attached to roots, immersing in fungicide solution, and covering transparent plastic was the most suitable media and process for plantlet acclimatization. The combination media induced high survivability of plantlets up to 93% with 9.7 cm in height, 7.1 mm stem diameter, and 7.2 leaves $plant^{-1}$ (Table 4). The second-best media was burned rice husk and cocopeat; the lowest results on burned rice husk. In the *in vitro* performances, all shoots and plantlets were in variegated performances (Fig. 2H). They had no pink colour, but 5 months after acclimatizing plants individually (Fig. 2I), the pink colour on leaves appeared gradually and became more apparent in the next month.

Table 4. Effects of three acclimatization media on plantlet survivability and growth of *Musa spp*. 'Pink Nono' plantlets eight weeks after planting.

Acclimatization medium	Plant survivability (%)	Height of plants (cm)	Stem diameter (mm)	Number of leaves plant^{-1}
Burned rice-husk	84.2 ± 3.1 b	5.1 ± 0.28 c	4.3 ± 0.21 c	4.5 ± 0.47 c
Burned rice husk: cocopeat (2:1)	86.4 ± 3.4 b	6.4 ± 0.30 b	4.6 ± 0.26 b	4.7 ± 0.49 b
Burned rice husk: soil: organic manure: volcanic sand (2:1:1:1)	93.3 ± 2.0 a	9.7 ± 0.49 a	7.1 ± 0.34 a	7.2 ± 0.72 a

Means followed by the same letter in the same column are not significantly different based on Tukey, $p = 0.05$. Values reflect the means and standard errors of cultured explants, with n = 50 for each treatment.

Discussion

In this present study, an in vitro mass propagation protocol for *Musa spp*. 'Pink Nono' was successfully established, starting with determining the sterilization method, initiating and multiplying axillary shoots, rooting, and acclimatizing plantlets.

Sterilization of explants in obtaining an aseptic culture is an important step in initial tissue culture works due to microbial contamination (Shukla et al., 2020; Sivanesan et al., 2021; Yadav et al., 2021; Hassen et al., 2022). Microbial contamination, such as filamentous fungi, yeasts, bacteria, viruses, and viroids, is one of the most serious problems in plant cell and tissue culture. The contaminants generally grow faster than explant cultured, release phytotoxic substances that have a high effect on the culture process and media, increase culture mortality, and reduce shoot proliferation and root formation (Shukla et al., 2020; Kapadia and Patel, 2021; Sivanesan et al., 2021; Hassen et al., 2022). Application of disinfectant agents (mercury chloride, sodium chlorite, sodium hypochlorite, ethanol, hydrogen peroxide, silver nitrate, antibiotic, etc) that frequently cause explant browning, necrosis, high toxic effect, and reduce morphogenesis capacity of explants is needed to avoid serious losses in this works (Shukla et al., 2020; Kapadia and Patel, 2021; Yadav et al., 2021). A suitable combination of several disinfectant agents resulted in the optimal growth response of explants cultured. Though the application of antibiotics had negative and phytotoxic effects on the morphogenesis and growth of explants, the application of antibiotics in combination with fungicide, ethanol, sodium hypochlorite, and mercuric chloride (SM-3) was successfully applied in this research. Such combination with varied concentration and period application was also reported in *Alocasia* species (Rachmawati et al., 2025), chloramphenicol (500 mg L^{-1}) and Rifampicin (500 mg L^{-1}) for 30 min, 10% NaOCl for 10 min followed by 0.1% $HgCl_2$ for 10 min and rinsing with distilled water for 'Grand Nine' variety (Talla et al., 2022).

Successful in obtaining aseptic culture in *vitro* will then proceed to shoot or embryo initiation to multiplication. In this stage, choosing optimal combination of suitable basal medium, combination and/or concentration of PGR, carbon source, gelling agent, additive material, amino acid, etcare important addressed (Kelta et al., 2018; Deo et al., 2019; Sivakumar and Visalakshi, 2021; Khaskheli et al., 2021; Talla et al., 2022). Kelta et al. (2018) successfully used MS medium supplemented with the combination of 1.0 mg L^{-1} BAP with 0.5 mg L^{-1} kinetin for shoot initiation of Ethiopian banana '*Poyo'* and '*Giant'*, and 2.5 mg L^{-1} BAP + 2.5 mg L^{-1} kin and 2 mg L^{-1} BAP + 2 mg L^{-1} Kin for its proliferation. MS medium supplemented with 4 mg L^{-1} BAP + 0.5 mg L^{-1} IAA was utilized for shoot initiation of *Musa sp*. 'Patakpura', and 4 mg L^{-1} BAP + 0.5 mg L^{-1} IAA + 0.25 mg L^{-1} NAA for multiplication (Deo et al., 2019), MS medium with 4.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA + 30 g sucrose for initiation and proliferation of *M. acuminata* (Khaskheli et al., 2021), MS medium containing 3 mg L^{-1} BAP and 0.5 mg L^{-1} TDZ for initiation and proliferation of Poovan variety (Sivakumar and Visalakshi, 2021), MS medium with 4 mg L^{-1} BA under dark conditions for 3 weeks resulted in 12.2 shoots per explant of 'Grand Naine', and Shoot Bud Multiplication Medium (SBMM) fortified with 2 mg L^{-1} BA and 20 mg L^{-1} adenine sulfate-induced highest shoot buds (15.0 ± 0.75) in 3 weeks and showed consistent till seven passages (Talla et al., 2022). In the research, immersing shoots in 0.1 mg L^{-1} TDZ for an hour, followed by culture and subculturing axillary shoots periodically on MS medium containing 3 mg L^{-1} BAP was the most suitable pre-treatment and culture medium for initiation and multiplication of axillary shoots from 8.4 to 30.6 shoots.

Well plantlet preparation gives significant effect on acclimatization results. Successful shoot rooting can be carried out by reducing medium strength from full to half strength (Kumari et al., 2020), using PGR free medium, adding AC (Talla et al., 2022; Hansuek et al., 2023), applying auxin (IAA, indole-3-butyric acid (IBA), and NAA) individually or in combination to cytokinine (Kelta et al., 2018; Deo et al., 2019; Sivakumar and Visalakshi, 2021; Talla et al., 2022). In the research, well-rooted shoots with up to 9.4 roots per shoot were established on MS medium supplemented with 1 mg L^{-1} NAA and 0.5 g L^{-1} AC. In another research, MS medium containing 2 mg L^{-1} Kin, 1 mg L^{-1}, and 3 g L^{-1} AC was the optimal medium for 'Grand Nine' shoot rooting (12.7 roots shoot^{-1}) (Talla et al., 2022), optimal rooted shoots (17 roots plant^{-1}) for *M. acuminata* 'Kluai Nak' were proved on MS medium fortified by 1.5 mg L^{-1} NAA and 2 g L^{-1} AC (Hansuek et al., 2023). The results confirmed that a combination of auxin and AC was suitable for root formation of *in vitro* banana shoots.

Transferring *in vitro* plantlets to *ex vitro* conditions is frequently affected by plantlet condition and preparation, acclimatization media, and acclimatization process (Deo et al., 2019; Sivakumar and Visalakshi, 2021; Hansuek et al., 2023). Well plantlet preparation and hardening improve morphology, anatomy, and physiology of plantlets, utilize suitable acclimatization media (Noor et al., 2017; Natarajan et al., 2020), and optimal process (Khatab and Youssef, 2018; Natarajan et al., 2020; Hansuek et al., 2023). High survivability of banana plantlets of exotic bananas was established by shifting *in vitro* plantlets from the growth room to the greenhouse and culturing plantlets in polythene bags containing a mixture of garden soil and humus (1:1, v/v) (Qamar et al., 2015), hardening plantlets in a greenhouse for 30 days with spray irrigation twice a day, washing roots gently under running tap water to remove the medium, and transferring the plantlets to pots containing peat moss and clay soil with a ratio of 1:1 (v/v) (Khatab and Youssef, 2018), hardening plantlets in greenhouse conditions, taking plantlets from culture vessel, washing rooted plantlets thoroughly in sterile water to remove the traces of medium and transferring them to small cups and later to pots of 8 cm diameter containing sterilized pot mixture of sand: red soil: farmyard manure (FYM) (1:1:1, v/v/v) (Natarajan et al., 2020), transferring the healthy plantlets of *M. acuminata* 'Kluai Nak', washing in a betadine solution, culturing them into a mixture of rice husk and soil, covering plantlets plastic, and keeping under the greenhouse with 70% shade (Hansuek et al., 2023). In the research, high plantlet survivability was established by washing it under tap water, immersing in fungicide solution, culturing on mixture of burned-rice husk, soil, organic manure, and volcanic fine sand, and covering them with transparent plastic for two months.

Conclusions

In vitro mass propagation protocol for *Musa spp.* 'Pink Nono' was successfully established. The optimal aseptic culture was established by the application of a disinfectant combination of 1 g L^{-1} streptomycin sulfate and benomyl, 70% alcohol, 2.5% sodium, 0.1% $HgCl_2$ hypochlorite, and reduction of sucker size. High initiation and multiplication of axillary shoots was proved by immersing shoots in 0.1 mg L^{-1} TDZ and then culturing on MS medium containing 3 mg L^{-1} BAP. Well-rooted shoots were determined on MS medium supplemented with 0.5 g L^{-1} AC and 1.0 mg L^{-1} NAA. The high survivability of plantlets was verified by culturing processed plantlets by removing remaining agar, immersing them in fungicide solution, culturing on burned-rice husk, soil, organic manure, and volcanic fine sand (1:1:1:1, v/v/v/v), and covering transparent plastic.

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Author Contribution

ASS: Conceptualization, Methodology, Investigation, Formal Analysis, Validation, Writing – Original Draft, Writing – Review & Editing. **TSM**: Conceptualization, Methodology, Validation, Writing – Review & Editing. **FR**: Methodology, Investigation, Writing – Review & Editing. **SR**: Investigation, Validation, And Writing – Review & Editing. **BW**: Conceptualization, Methodology, Formal Analysis, Validation, Writing – Original Draft, Writing – Review & Editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

Data will be made available upon request to the authors.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that the use of AI and AI-assisted technologies was not applied in the writing process.

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