International Journal of Agricultural Technology 2023 Vol .19(4):1541-1552
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

In vitro propagation of *Musa acuminata* (AAA group) cv. 'Kluai Nak'

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Hansuek, S., Liamnimitr, N. and Khawniam, T. (2023). *In vitro* propagation of *Musa acuminata* (AAA group) cv. 'Kluai Nak'. International Journal of Agricultural Technology 19(4):1541-1552.

Abstract *In vitro* propagation of *Musa acuminata* (AAA group) cv. 'Kluai Nak' was studied on 4 factors such as sterilization method, shoot induction, root induction and acclimatization. Shoot explants were sterilization with 30% sodium hypochlorite for 30 minutes gave the highest survival rate (97.50%). For the shoot induction and multiplication, the result found that adding 4 mg/L BA on culture medium gave the highest number of shoots (3.20 shoots/explant) and number of leaves (9.20 leaves/explant), while culture medium without BA gave the highest height (5.90 cm). After the shoot multiplication, the root induction carried out by adding 2.5 mg/L NAA without AC gave the highest number of roots (25.80 roots/plant) but it was shot root, not complete plantlet and healthy plant for growth so the next experiment was chosen the plant from culture medium supplemented with 1.5 mg/L NAA and 0.2% activated charcoal that gave the number of roots (17.00 roots/plant) and root length (4.07 cm). After the root induction, the plantlet was successfully acclimatization in the combination of rice husk mixed potting soil that found to produce the highest percentage of plantlet survival rate with 90% and the plant height with 8.40 cm that obtained after 4 weeks of acclimatization.

Keywords: Plant regeneration, *Musa acuminata*, Tissue culture

Introduction

Kluai Nak is a fruit that found in the southern of Thailand and Thai-Myanmar border provinces such as Tak and Kanchanaburi province (Silayoi, 2015). It is benefits and nutritious, such as being used as food for infants or old people who have difficulty eating and used to eat during the day instead of other types of food. It is contains fiber, which helps in the digestive system and tannins can help reduce diarrhea, etc., including high anti-oxidants (Technology chaoban, 2021). There are also has medicinal properties, for example, young leaves can be applied to reduce swelling of the abscess and banana blossom can be eaten to nourish milk and reduce blood sugar. Mature fruit contains serotonin, which has a mild laxative

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effect and immature fruit contains substances that stimulate the cells lining the stomach to secrete substances to coat the stomach. They are 22% carbohydrates and contains minerals, pectin, vitamin A, B and C, which can resist bacteria and fungi (Selakorn *et al*., 2017).

At present, in addition to eating mature fruit also play an important role in exports, for example, as an ornamental plant (Srisa-ard, 2021), which requires high-quality, high-yielding and large-volume to respond market demand. There is propagating by suckers, farmers often face problems with diseases and insects, because sometimes the cultivars that are planted may have disease or insect outbreaks attached to them. In particular, viral diseases that are spread in phloem and xylem, causing shoots or stems to halt their growth. When it is planted, it will cause an outbreak or spread the infection during the flowering and fruiting stages, causing damage to the banana yield (Agricultural Promotion and Development Center Suphanburi Province (cultivated plant varieties), 2021). Natural propagation, resulting shoots are often infected with viruses.

Plant tissue culture technique is in vitro propagation that propagules are cloned from single plant tissue. The advantages of tissue culture are large numbers of plants in a short period of time, clean, sterile and consistently sized plants suitable for export production (Srisa-ard, 2021). Moreover, this technique is very useful for plant conservation of endangered plant species. For these advantages, there are more and more bananas propagating by tissue culture methods. (Department of Agriculture, 2013). There are several types of bananas that have been successfully grown from tissue culture, such as: Namwa Mali-Ong (Dechpala *et al*., 2019), Kluai Hin (Tudses *et al.*, 2019; Wamaedeesa and Deramae, 2011), Kluai Chang (Patchanun and Suraninpong, 2014), Cavendish Dwarf (Kalimuthu *et al*., 2007), etc. There are several factors contributing to the success of tissue culture such as: biological factors such as plant species and explant, the physical factors are sterilization methods and explant preparation methods (Edirisinghe *et al*., 2013) and chemical factors such as plant growth regulators, especially the cytokinin group, such as 6-benzylaminopurine (BA) and Thidiazuron (TDZ), etc.

Sterilization of banana, Kluai Nak shoots (complete shoots about 25-30 cm high) were washed with water. Then cut the top and peel off 2-3 leaves, then trim to a size of 5 cubic cm. Shoot explants were washed in washing detergent and dipped in 70 percent alcohol sterilization for 2 minutes. Then, sterilization with sodium hypochlorite at 15 and 5 percent concentrations for 10 and 5 minutes, respectively (Selakorn *et al*., 2017). While Namwa Mali-Ong shoots (complete plant and disease-free, height 25 cm.) were washed thoroughly with tap water. These shoots were cut to a size of 5-6 cm and surface sterilized by soaking in 70% alcohol for 30 seconds, then transferring to a 15% CloroxTM solution (NaOCl 0.9%), adding 2 drops of Tween-20 for 20 minutes, followed by 5% Clorox

solution (NaOCl 0.3%) with 2 drops of Tween-20 for 5 minutes, finally washed in distilled water sterile for 3 times before culturing (Dechpala *et al*., 2019). It can be seen that they are many steps and cumbersome.

In terms of plant growth regulators, It was found that BA was the most effective growth regulator for shoot inducing, perfect and strong plantlet in many banana species such as: *Musa sapientum* L. (Agricultural Promotion and Development Center Suphanburi Province (cultivated plant varieties), 2021), Kluai Leb Mu Nang (Rattanapan and Yeetchan, 2016), *Musa acuminata* "Cavendish" (Heedchim and Te-chato, 2017), Cavendish Dwarf (Kalimuthu *et al*., 2007), etc. Kluai Hin were cultured on MS medium supplemented with 5 mg/L BA and 3% sucrose gave the highest percentage of multiple shoot (58.35%) and number of shoot (3.25%) (Muangkaewngam, 2014). Kluai Sa and Kluai Leb Mue Nang were cultured on the same culture medium of Kluai Hin, it was able to induce maximum shoots of 4.9 and 5.5 shoots, respectively. (Srangsam and Kanchanapoom, 2007).

For root induction, most plants use auxin, especially NAA, to produce strong, healthy and complete roots before acclimatization. Study of NAA in combination with activated carbon, also known as activated charcoal, for root induction has not been reported, including potting medium for acclimatization before planting in the ground.

Therefore, the aims were to find the appropriate method for sterilization, to find the BA concentration for suitable on shoot induction, to find the NAA concentration and activated carbon on root induction and to find the potting mediums for acclimatization.

Materials and methods

Four factors affecting on the development of new plants from tissue culture of *Musa acuminata* (AAA group) cv. 'Kluai Nak' were studied such as sterilization method, shoot induction, root induction and acclimatization.

Experiment 1 appropriate method for sterilization

Fresh shoots were obtained from the farmer garden in Nakhon Si Thammarat province, Thailand. The shoots (approximately 10-20 cm) were cut and bring to the laboratory for sterilization processes. The sterilizations were conducted by washing the shoot with running tap water to eliminate the soils and contaminations. These shoots were peeled off to the size of 5 cm long consisting of shoot tip and immersed in 70 percent alcohol for 1 minute. Then, these explants were transferred to sodium hypochlorite at different concentration (10 15 20 25 and 30 percent), added 2-3 drops of Tween-20 and shaken for 3 periods (15 20 and 30 minutes). Finally, these explants were rinsed with sterile water for 3 times and trimmed to size of

0.5-1 cm in the laminar flow cabinet. The final size of shoots was cultured on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L BA, 3% sucrose, 0.75% agar and adjust pH to 5.7 with KOH and HCl. The cultures were kept in the culture room under condition 27±1 ℃, 14 hrs./day and 25 μmol/sq.m./sec. After 1 month of incubation, the black tissues were removed. The percentage sterility of explant was recorded for each sterilization method.

Experiment 2 concentration of BA suitable on shoot induction

For shoot induction, shoots were cultured on 6 culture medium of basal medium, MS medium supplemented with BA concentrations of 0 1 2 3 4 and 5 mg/L, all culture medium were supplemented with 3% sucrose, 0.75% agar and adjust pH to 5.7. All cultures were kept in the culture room under 27±1 °C, 14 hrs./day and 25 μmol/sq.m./sec. The number of shoots, height and leaf number were recorded after 3 months of culturing.

Experiment 3 concentration of NAA and activated carbon on root induction

For root induction, healthy plantlet at 3 months old, which were maintained on the best result in experiment 2, were used. Shoots were cultured on 7 culture medium of basal medium, MS medium supplemented with NAA at the concentrations of 0 0.5 1 1.5 2 2.5 and 3 mg/L, all culture medium were supplemented with 3% sucrose, 0.75% agar, adjust pH to 5.7 and with or without 0.2% activated charcoal (AC). The number of roots and root length were recorded after 3 months of culturing.

Experiment 4 the potting mediums for acclimatization

The acclimatization was conducted by transferring the healthy plantlet with least 4 leaves, height 7 cm and the well root (washed in a betadine solution for 10 minutes) into potting soil. The potting soil used was combination of 1) rice husk mixed potting soil (1:1), 2) sand mixed potting soil (1:1) and 3) potting soil. Finally, plantlets were cover with plastic and kept under the greenhouse with 70% shade. The survival rates were recorded after 1 month of transferring to potting mediums.

Data analysis

All experiments were conducted in completely randomized design with 10 replications and 3 explants for each replication. The analysis of variance (ANOVA) was used for data analysis and Duncan's Multiple Test

(DMRT) was used for mean separation. The analysis was performed using Statistics Package for Social Sciences (SPSS version 11.0).

Results

The appropriate methods for sterilization

Sterilization of Kluai Nak shoots with 30% sodium hypochlorite solution for 30 minutes gave the best result for cleaning and survival rate (97.50%) (Table 1). After 4 weeks of culturing, the external leaf primordial turned green and red (Figure 1B) which were initiation white (Figure 1A) but different percentage of survival rate depend on sodium hypochlorite concentration. The explant size also increased and blackening was observed at the explant base and due to secretion of phenolic compounds. The shoot multiplication was not found in all treatment after culturing for 4 weeks.

Table 1. Effect of Sodium hypochlorite concentration on percentage of explant survival rate after sterility and culturing on MS supplemented with 0.5 mg/L BA for 4 weeks

Concentrations of SH	Survival rate (%)			Average^{A} $_{\text{Concentrations of SH}}$
	Sterilization time			
	15 min	20 min	30 min	
10%	20.00	23.00	30.00	24.33E
15%	32.50	35.00	40.00	35.83D
20%	47.50	49.00	55.00	50.50C
25%	60.00	62.00	75.00	65.67B
30%	75.00	78.00	97.50	83.50A
Average^{B} $_{\text{Survival rate}}$	47.00C	49.40B	59.50A	
A (concentrations of SH)	**			
B (sterilization time)	**			
A:B (interaction)	**			
CV.(%)	17.46			

SH = Sodium hypochlorite, * Significant differences at $P \leq 0.05$, ** Significant differences at $P \leq 0.01$, Means values followed by the same letter within each column are not significantly different according to DMRT.

Concentration of BA suitable on shoot induction

The shoots from the best of experiment 1 were transferred to MS medium supplemented with various concentrations of BA. The number of shoot, height and number of leaf were significantly affected by different BA concentration. It was observed that after 1 week of culturing, the shoots began to respond to culture medium with new shoots (Figure 1C). After 3 months of culturing, MS medium supplemented with 4mg/L BA gave the maximum number of shoots (3.20 shoots/explant) and the number of leaves (9.20 leaves/explant) (Table 2). While, MS-free medium gave the highest

height (5.90 cm). The shoot multiplication after culturing for 3 months as shown in Figure 1D and shoot height as shown in Figure 2.

Table 2. Effect of BA concentration on shoot induction after culturing for 3 months

Concentration of BA	No. of shoots (shoots/explant)	height (cm.)	No. of leaves (leaves/explant)
1. MS Free	1.80^{c}	5.90^{a}	5.60^{bc}
2. MS + 1 mg/L BA	1.80^{c}	3.06^{b}	7.40^{a}
3. MS + 2 mg/L BA	1.80^{c}	3.18^{b}	3.80^{c}
4. MS + 3 mg/L BA	2.20^{b}	2.12^{d}	4.00^{c}
5. MS + 4 mg/L BA	3.20^{a}	2.18^{d}	9.20^{a}
6. MS + 5 mg/L BA	1.80^{c}	2.24^{cd}	4.00^{c}
F-test	*	**	**
CV. (%)	34.77	4.85	12.00

* Significant differences at $P \leq 0.05$, ** Significant differences at $P \leq 0.01$, Means values followed by the same letter within each column are not significantly different according to DMRT.

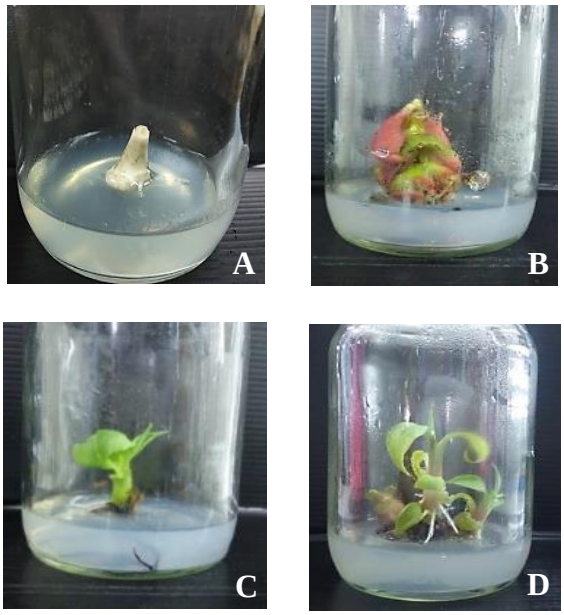

Figure 1. Shoot development after culturing on MS supplemented with 4 mg/L BA. Shoot initiation (A), 1 month (B), 2 months (C) and 3 months of culturing (D)

Figure 2. Characteristics of shoot and shoot height after culturing on MS free (A) MS supplemented with 0.5 mg/L BA (B) MS supplemented with 1 mg/L BA (C) MS supplemented with 1.5 mg/L BA (D) MS supplemented with 2 mg/L BA (E) MS supplemented with 2.5 mg/L BA (F) for 3 months

Concentration of NAA and activated carbon on root induction

Base on the previous experiment, the best medium was found to be the most suitable medium for *Musa acuminata* (AAA group) cv. 'Kluai Nak'. In this experiment, the roots were appeared by using different concentrations of NAA and AC (Table 3). After 4 weeks of inocutions, all treatment began to form roots were recorded at 100%. The treatment of 2.5 mg/L of NAA significantly different the highest percentage of root induction (100%) and the average number of roots (18.40 roots/plant) (Table 3) followed by treatment of 3.0 and 1.5 mg/L of NAA with 12.50 and 10.70 roots/plant, respectively. Effect of AC on number of roots, it was found that the culture medium with AC gave the better than culture medium without AC at 10.49 and 8.91 roots/plant, respectively. For interactions between the culture media and AC found that both factors had a statistically significant effect on the number of roots ($p \leq 0.01$). The root characteristic after culturing on different concentration of NAA with AC as shown in Figure 3A and without AC as shown in Figure 3B. It was concluded that MS medium supplemented with 2.5 mg/L NAA without AC gave the highest number of roots (25.80 roots/plant) (Table 3) but it was shot root, not complete plantlet and healthy plant for growth so the next experiment was chosen the number of roots from MS medium supplemented with 1.5 mg/L NAA (17.00 roots/plant).

Table 3. Effects of NAA concentration and AC on number of roots after culturing for 3 months

Concentration of NAA	No. of roots (roots/plant)		Average^{A} concentration of NAA
	With AC	Without AC	
1. MS Free	9.80^{d}	1.20^{g}	5.50D
2. MS + 0.5 mg/L NAA	7.40^{e}	3.0^{0fg}	5.20D
3. MS + 1.0 mg/L NAA	7.80^{e}	4.20^{f}	6.00D
4. MS + 1.5 mg/L NAA	17.00^{b}	4.40^{f}	10.70C
5. MS + 2.0 mg/L NAA	11.40^{c}	7.80^{e}	9.60C
6. MS + 2.5 mg/L NAA	11.00^{c}	25.80^{a}	18.40A
7. MS + 3.0 mg/L NAA	9.00^{d}	16.00^{b}	12.50B
Average^{B} with or without AC	10.49A	8.91B	
A (concentration of NAA)	**		
B (with or without AC)	**		
A:B (interaction)	**		
CV.(%)	10.55		

A = Average number of roots from NAA concentration, B = Average number of roots from medium with or without AC, ** Significant differences at P ≤ 0.01, Means values followed by the same letter within each column are not significantly different according to DMRT.

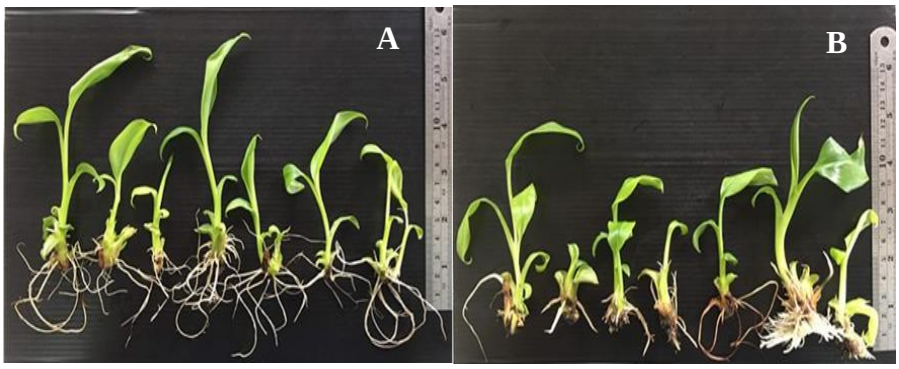

Figure 3. Characteristic of root after culturing on MS supplemented with 2.5 mg/L NAA with 0.2% AC and without 0.2% AC (B) for 3 months

The specific combination of NAA and AC significantly affected the number of roots and root length. Based on visual observationthe and the result of this experiment, the roots that grew from the shoots that were cultured on MS medium supplemented with 2 and 3 mg/L NAA gave the highest average of root length at 3.97 and 3.77 cm, respectively. They are look the best roots grown from other media. Effect of AC on root length, it was found that the culture medium with AC gave the better than culture medium without AC at 4.45 and 2.27 cm, respectively. The interaction of culture media and AC was found that both factors had a statistically significant effect on root length ($p \leq 0.01$) (Table 4). It was concluded that MS medium supplemented with 3.0 mg/L NAA with AC gave the highest root length (5.96 cm).

Table 4. Effects of NAA concentrations and AC on root length after culturing for 3 months

Concentration of NAA	Root length (cm.)		Average^{A} $_{\text{concentration of NAA}}$
	With AC	Without AC	
1. MS Free	5.22^{b}	2.23^{f}	3.72AB
2. MS + 0.5 mg/L NAA	3.92^{d}	2.10^{f}	3.01C
3. MS + 1.0 mg/L NAA	3.73^{d}	1.19^{h}	2.46D
4. MS + 1.5 mg/L NAA	4.07^{cd}	2.20^{f}	3.13C
5. MS + 2.0 mg/L NAA	3.81^{d}	4.09^{cd}	3.95A
6. MS + 2.5 mg/L NAA	4.46^{c}	2.56^{e}	3.51B
7. MS + 3.0 mg/L NAA	5.96^{a}	1.58^{g}	3.77A
Average^{B} $_{\text{with or without AC}}$	4.45A	2.27B	
A (concentration of NAA)	**		
B (with or without AC)	**		
A:B (interaction)	**		
CV.(%)	7.52		

A = Average of root length from NAA concentration, B = Average of root length from medium with or without AC, ** Significant differences at $P \leq 0.01$, Means values followed by the same letter within each column are not significantly different according to DMRT.

The potting mediums for acclimatization

This step is an important in micropropagation of commercially important plants. After the root induction step, the complete plantlet and healthy plant with 5 leave and more than 5 cm of plant height, more than 3 cm of root length and normal morphology were acclimatized on different potting mediums in greenhouse. After 4 weeks of acclimatization, among the combination of rice husk mixed potting soil significantly produce the highest percentage of plantlet survival rate with 90% and the plant height with 8.40 cm (Table 5) follow by sand mixed potting soil (70% and 6.10 cm, respectively) and potting soil (40% and 5.10 cm, respectively) (Table 5). The acclimatization of plantlet was shown in Figure 4.

Table 5. Effect of different potting mediums on survival rate and high after acclimatization for 4 weeks

Medium	Survival rate (%)	Plant height (cm.)
Rice husk mixed potting soil (1:1)	90^{a}	8.40^{a}
Sand mixed potting soil (1:1)	70^{b}	6.10^{b}
Potting soil	40^{c}	5.10^{c}
F-test	**	**
CV.(%)	16.33	22.86

** Significant differences at $P \leq 0.01$, Means values followed by the same letter within each column are not significantly different according to DMRT.

Figure 4. Plantlet after acclimatization on different potting mediums start from plantlet initiation (A) after 1 (B) and 3 months (C)

Discussion

The result of our study showed that the proper methods for sterilization was 30 percent sodium hypochlorite solution for 30 min because the experimental period was during the rainy season and causing the use of a high concentration of sodium hypochlorite and a longer time than other sterility methods of banana. It is also a method of disinfection that is convenient and the process is not complicated different from Dechpala *et al*. (2019) found that surface sterilization with 10% sodium hypochlorite for 20 min, then transferred to 5% sodium hypochlorite for 10 min gave the best method for sterility of Namwa Mali-Ong. Shoots were trimming to be small before sterilization that causing the apical meristem to be exposed to disinfectant and resulting the slow shoot or not response to growth. While trim the shoot to be large and used concentration or duration time of disinfectant, next trimmed to a small size before culture, it was found that the shoot gave a faster response and grow better. However different variety of banana may respond differently to sterilization method, survival and response to cultured medium.

For shoot induction, the new shoots were culture on added BA formed new shoots sooner than those in control (BA free). This result also indicates that BA was the most superior for shoot induction and shoot multiplication. The finding was agreement with Muangkaewngam (2014), which found that BA was the most effective plant growth regulator for shoot induction of Kluai Hin. The suitability of full strength MS medium might be a sufficient amount of nutrients to stimulate the plant height because in apical meristem has the cytokinin group.

For root induction, combination of NAA and AC on root induction was not reported. but in this result shown that the activated charcoal helps to filter the light, allowing less light to pass into the agar medium so makes the light conditions similar to those in the ground thus making the roots grow better. In addition, the porosity of activated charcoal absorb various wastes from tissue released to agar medium. NAA stimulates root growth, promote

cell division and enlargement of cells so this combination was the most effective on root induction and root length, same as Tudses *et al*. (2019), which found that NAA only 1 mg/L gave maximum percentage of root induction (71.43±18.44) and the addition of 0.2% AC showed greater root length than medium without AC.

The efficiency of rice husk mixed potting soil as a potting medium for acclimatization of *Musa acuminata* (AAA group) cv. 'Kluai Nak' whereas Tudses *et al*. (2019) using peat moss as potting medium gave the probability of the highest survival rate of Namwa Mali-Ong. Therefore, different cultivars of banana had the effected on sterilization method, shoot induction, root induction and acclimatization.

Acknowledgments

The authors would like to thank RUTS Fund for support this research fund and Fruit and Perennial Research Unit Faculty of Agriculture Rajamangala University of Technology Srivijaya Nakhon Si Thammarat Campus for the research site.

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(Received: 20 September 2022, accepted: 30 April 2023)