Development and Increasing the Efficiency of Hybrid Macapuno Coconuts Tissue Culture in Thailand

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Abstract

The embryo culture of hybrid macapuno coconuts takes 12-16 months from growing the embryo until developing into a plantlet and the production efficiency of seedlings is still low.The Department of Agriculture has therefore developed and increased the efficiency of hybrid macapuno tissue culture. The objective is to obtain propagation technology by developing embryo culture techniques to increase the number of coconut plantlets to meet the needs of farmers. The research was conducted from 2018-2021 at the Horticulture Research Institute, Bangkok and Chumphon Horticultural Research Center. The result showed that medium and embryo placement characteristics affected embryo germination in the dark, and appropriate formulations for seedling development in five varieties, NamHom x Kathi (NHK), Malayan Dwarf x Kathi (RDK), Thungkled x Kathi (TKK), West African Tall x Kathi (WAK) and Malayan Yellow Dwarf x Kathi (YDK). The research found that 5 varieties of hybrid Kathi coconut embryo germination in the solid medium were better than in liquid mediums. After culturing for 8 weeks in the dark, when they were sub-cultured in a modified Y3 solid medium and transferred to the light, it was found that the percentage of embryos development cultured in both solid and medium were better than those taken from embryos cultured in liquid medium.

For suitable formulations for the development of seedlings of 5 cultivars of hybrid macapuno coconut, from an 11-month-old embryo, it was found that the formula developed by the embryo was the most complete seedling of varieties of Malayan Dwarf x Kathi (RDK), Thungkled x Kathi (TKK), and NamHom x Kathi (NHK), These are MS solid medium with 2,4-D 1 mg L-1or B2 in the dark and B2 or Y3 modified liquid food in the light.West African Tall x Kathi (WAK) and Malayan Yellow Dwarf x Kathi (YDK), is solid and liquid medium B2 in dark and bright places.

Observing the effect of coconut aging and culture medium cut in half of the shoot to the plant of Chumphon 84-2 hybrid macapuno coconut, it was found that the number of shoot halves of the embryo can develop to shoot formation within 2 months. The piece of shoot halves of every fruiting age that were cultured on MS medium with 0.4 mg L-1 IBA and 3.2 mg L-1 kinetin had a higher percentage of seedling development than those cultured on modified Y3 medium. The piece of shoot halves of the fruiting age of 10 and 11 months cultured on both culture mediums had a higher percentage of seedling development than at 9 months. The piece of shoot halves of fruiting aged 10 months that cultured on MS medium with 0.4 mg L-1 IBA and 3.2 mg L-1 kinetin resulted in the highest seedling development.

Keywords – Hybrid Macapuno Coconuts, medium, Placement, Halves, Embryo

Introduction

Coconut (*Cocos nucifera L.*) is a major Thai economic crop. At present, the major growing areas are only in the south, especially in Prachuap Khiri Khan, Chumphon, and Surat Thani provinces. During the period, 2008–2013, the productive area and yield decreased with age and plant conditions, because in most of the area, the coconut plantations were very old. In 2010, the coconut pest outbreak and the drought during the relatively dry weather was suitable for the infestation of such insects. As a result,

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the coconut yield became less. The shortage of raw materials led to high price per fruit. As a result, the demand for good coconut varieties incread among the farmers while the government was having only insufficient production capacity.

Embryo culture is a technique that has been practiced by breeders for a long time. The key benefit is helping the embryos of plants that cross-species or cross-genus and become sterile to grow into a complete plant. Kathi coconut cannot germinate in nature, so the embryo rescue technique was used. But the efficiency of seedling production is still low. Using the Kathi coconut embryo rescue technique, the zygotic embryos were successfully cultured in several laboratories. (Ashburner, 1991, Assy-Bah, 1989, Karunaratne et al., 2009, Rillo and Paloma, 1990) In Thailand, Somchai et al. (2008) successfully made Kathi coconut embryo culture and this technique is currently used as a good Kathi coconut production system by the Department of Agriculture. The result showed that medium and embryo placement characteristics affected 5 varieties, NamHom x Kathi (NHK), Malayan Dwarf x Kathi (RDK), Thungkled x Kathi (TKK), West African Tall x Kathi (WAK), and Malayan Yellow Dwarf x Kathi (YDK), of hybrid Kathi coconut embryo germination.

In addition to trying to increase the number of seedlings produced from a single zygotic coconut embryo by somatic embryo genesis, propagation efficiency can also be increased by developing higher embryo culture techniques and this percentage can be increased up to 95%. The development of techniques at each stage of embryo culture, germination, suitable recipes for each stage of development, and increasing the number of new shoots from a single embryo, etc., are the purposes of this activity.

Materials & Methods

Medium and Embryo Placement Characteristics affected 5 varieties of Hybrid Kathi Coconut Embryo Germination

Embryos of 5 varieties of hybrid Kathi coconut:NamHom x Kathi (NHK), Malayan Dwarf x Kathi (RDK), Thungkled x Kathi (TKK), West African Tall x Kathi (WAK) and Malayan Yellow Dwarf x Kathi (YDK),11 months old fruit, were isolated in Suratthani Seed Research and Development Center Tha Chana District, Suratthani 88170. They were shaken in 70% alcohol for 5 min. followed by 15 and 10% Clorox solution for 15 and 10 min. and then washed with sterile distilled water 3 times in a laminar airflow station.

The experiment used a completely randomized design with 3 treatments, consisting of hybrid Kathi coconut 5 varieties' embryo with 11 months fruiting age and culture medium with Embryo Placement Characteristics; modified Y3 liquid medium (Parinda, 2018) (Figure 1A), modified Y3 solid medium with placed upward (Figure 1B) and Murashige and Skoog (MS) solid medium with the addition of 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mg L-1(Orathai, 2019) placed upward. Each embryo was cultured in the dark and taken after 8 weeks. The number of embryo's germination and development were observed and recorded every 2 weeks, 2 months after culturing.

Figure 1. Embryo in liquid medium (A)and solid medium placed upward (B)

Eight weeks later when shoots started growing, they were sub-cultured in a modified Y3 solid medium and transferred to the light under the illumination of cool-white, fluorescent tubes of about 37 µmol m-2 s -1 for 16 h/day photoperiods, 25+2 °C about for 8 weeks.The number of shoots and plantlet development were observed and recorded every 2 weeks for 2 months.

Effect of Appropriate Medium on Propagation of 5 Varieties Macapuno Using Plant Tissue Culture Technique

Completely randomized design (CRD) experiments were planned with 5 iterations. (the size of the experimental unit. (experimental unit) 10 vials (embryos) per experimental procedure). 5 varieties of macapuno were selected and the embryos contained in the varieties were cultured under sterile conditions.

Step 1: Induction of embryo germination to form roots and shoots. (Figure 2)

Peel the coconut. Use a knife to open the coconut shell and divide it to half. Use the device to cut the

coconut flesh around the embryo into a square shape. After that, the embryo is bleached and disinfected and washed three times with distilled water.Use a dark slice to remove the embryo from the macapuno. It is carried out in a sterile cabinet. The prepared coconut embryos were cultured with a synthetic medium in sterilized bottles. The 3 mediums are Y3 media, MS with 2,4-D, and B2. The macapuno embryo culture bottles were placed in a dark room with a temperature of 25-27 °C.

Figure 2. Sterilization of endosperm, dissection, and inoculation on the media.

Step 2: Embryo development in the bright room

The seedlings with shoots and roots were taken from the dark room and transferred to solid media Y3 and B2 media and was placed in a room with light 14 hours a day and with temperature 25-30 ° C. Embryos were cultured for 12 weeks, the pulp was removed, and sub-cultured by placing in the original solid media and placed in a bright room. Embryos were cultured for 16 weeks, sub-cultured solid medium to liquid medium. using the original recipe and sub-cultured every month. Seedling survival rate and plant height were recorded.

The effect of coconut aging and culture medium with cut in half of shoot to plant of Chumphon 84-2 hybrid macapuno coconut

The experiment design has a completely randomized design (CRD) with 6 treatments, consisting of the age of fruit being 9, 10, and 11 months (Figure 3A – C)and culture medium namely modified Eeuwens medium (Y3) (Parinda, 2018) and Murashige and Skoog (MS) medium supplemented with 0.4 mg L-1Indole-3-butyric acid (IBA) and 3.2 mg L-1 kinetin (referred from Sisunandar *et al.*, 2015). The fruiting aged 9, 10, and 11 months were selected. The embryo was cultured on a modified Y3 solid medium in the dark condition for 2 months to develop into the germination stage (Figure 4A). Cut in half of shoot (Figure 4B) were cultured on modified Y3 and MS medium supplemented with 0.4 mg L-1 IBA and 3.2 mg L-1kinetin in the light condition, light Intensity 4,000-5,000 Lux and photo period 12 hours per day, for 2 months. The percentage of embryo development in the light was recorded.

Figure 3. Chumphon 84-2 Hybrid Macapuno Coconutembryo, fruit age 9 months (A), 10 months (B) and 11 months (C)

Figure 4. The embryo begins to germinate after 2 months and is ready to be halved (A) The pieces of halves (B) 2 halves of the embryo after 2 months of culture (C).

Results and Discussion

Effect of medium and Embryo orientation on germination.

To study the effect of Medium and Embryo Placement Characteristics on the germination percentage in the dark, the germination rate of solid medium ranged from 69.0 to 99.7 percent while

Treatment	Embryo germinationin the dark (percent)				
	NHK	RDK[1/]	TKK[1/]	WAK	YDK
modified Y3 liquid medium	51.0	53.3 b	50.7 b	60.0	72.3
modified Y3 solid medium placed upward	86.7	74.0 ab	86.7 a	69.0	74.0
MS solid medium with 2, 4-D 1 mgl-1 placed upward	80.0	86.7 a	99.7 a	93.3	82.3
C.V. (%)	44.8	20.0	18.5	29.5	22.7

[1/] The averages in the same column that follow with the same letter were not statistical difference at a 95% confidence level by DMRT

Table 1.Embryo germination percentage of five varieties of hybrid Kathi coconut after 8 weeks of culturing inmedium with embryo placement characteristics in the dark.

the liquid culture was 50.7 - 72.3 percent, embryo germination in a solid medium was better than in liquid mediums. The hybrid coconuts of TKK cultivars grown in both solid mediums, were significantly had greater germination than in the modified Y3 liquid medium (Table 1).

It was found that the hybrid coconuts of RDK, TKK, WAK, and YDK cultivars which were grown in Murashige and Skoog (MS) solid medium with the addition of 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mgl-1, showed that the germination percentage (86.7, 99.7, 93.3 and 82.3%) was better than in the modified Y3 solid medium (74, 86.4, 69 and 74%). Also, MS solid medium with the addition of 2,4-D 1 mgl-1was found longer shoots than the modified Y3 solid medium (Figure 5). Eight weeks later when shoots started growing, they were sub-cultured in a modified Y3 solid medium and

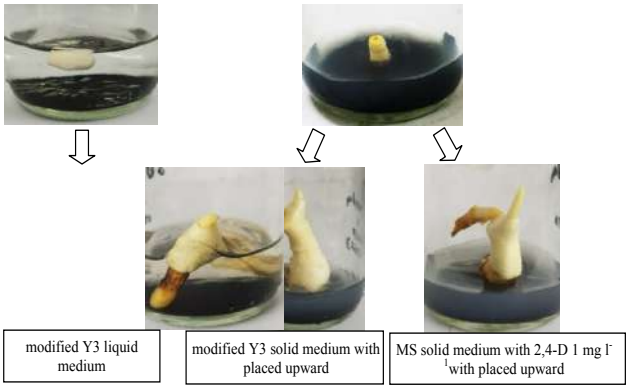

Figure 5. Embryo germination of hybrid Kathi coconut after 8 weeks of culturing in medium with embryo placement

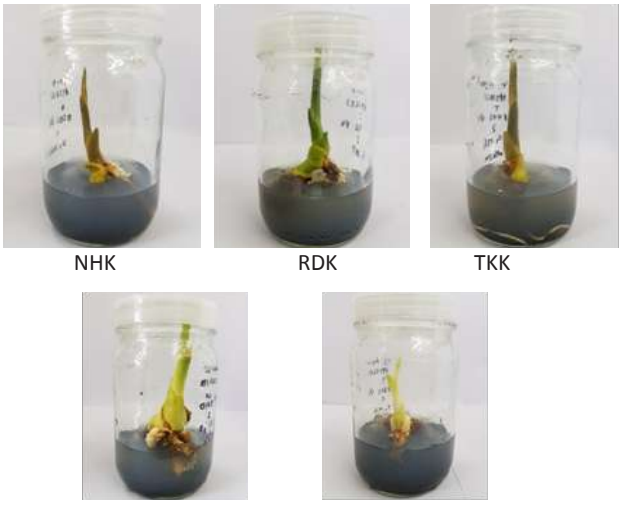

Figure 6 . Embryo development percent of five varieties of hybrid Kathi coconut after 8 weeks of sub-culturing in modified Y3 solid medium and transferring to the light.

transferred to the light. It was found that embryos from the modified Y3 liquid medium had only 28.7 - 53.3 percent of developed plantlets. while embryos from solid medium culture modified Y3 and MS added 2,4-D 1 mgl-1 place upward had 62.7 - 99.7 percent to developed plantlets. (Figure 6)

In the hybrid coconuts of NHK and TKK cultivars from the solid mediums, there was significantly greater plantlet development than in the modified Y3 liquid medium (Table 2).

To study the effect of Medium and Embryo Placement Characteristics on the percent

Treatment	Embryo germination in the light (percent)				
	NHK	RDK[1/]	TKK[1/]	WAK	YDK
modified Y3 liquid medium	28.7 b	45.0	35.7 b	53.3 b	45.3
modified Y3 solid medium placed upward	80.0 a	62.7	86.7 a	69.0 ab	62.7
MS solid medium with 2, 4-D 1 mgl-1 placed upward	73.3 a	86.7	99.7 a	93.3 a	82.3
C.V. (%)	35.5	34.8	27.8	27.5	30.6

[1/] The averages in the same column that follow with the same letter were not statistical difference at a 95% confidence level by DMRT

Table 2. Embryo development percentage of five varieties hybrid Kathi coconut, from a cultured medium with embryo placement characteristics in the dark, after 8 weeks of sub-culturing in modified Y3 solid medium and transferring to the light.

germination in the dark, it was found that 5 varieties of hybrid Kathi coconut embryo germination in the solid medium was better than in liquid mediums (Table 1). After culturing for 8 weeks in the dark, when they were sub-cultured in a modified Y3 solid medium and transferred to the light, it was found that the percentage of embryos development which were cultured in both solid mediums are better than from those which were cultured in liquid medium (Table 2). In accordance with Pech Y Ake *et al.* (2004) studies enhanced aerobic respiration improves *In Vitro* coconut embryo germination and culture. Germination of Malayan Green Dwarf (MGD) coconut embryos was tested in liquid and solid medium. It was found that the percentage of germination increased when the embryo was fed on a solid medium, especially when the embryo with the

micropyle side is placed upward. Causing exposure to the air inside the bottle and embryo proliferation is inhibited when the ambient atmosphere is replaced by N2 or when anaerobic respiration inhibitors are added to the medium. The result showed that embryo proliferation requiring aerobic respiration and germination in an upward position will result in better seedling development. In conclusion, medium and embryo placement characteristics affected 5 varieties of hybrid Kathi coconut embryo Germination, it was found that the embryos cultured in solid medium in the dark showed the best embryo germination and development to plantlet.

Effect of Appropriate Medium on Propagation of 5 Varieties Macapuno Using Plant Tissue Culture Technique

After culturing the embryos of 5 macapuno strains for 19 weeks, it was found that the sprouts had an elongation of shoots and increased development of the main root and branch roots. NHK macapuno hybrids grown on MS (dark)/Y3 (light) (Treatment 3) and B2 (dark)/ Y3 (light) (Treatment 5) mediums were able to develop into seedlings. However, from the embryo culture of RDK macapuno varieties, it was found that MS (dark)/B2 (light) (Treatment 4) and B2 (dark)/Y3 (light) diets (Treatment 5) were able to develop the most mature seedlings at 70 percent. The embryo culture of TKK macapuno varieties showed that MS (dark)/Y3 (light) medium, Treatment 3 and B2 (dark) medium/B2 (Bright) (Treatment 6) was able to develop into mature seedlings at 70 percent. WAK and YDK hybrid macapuno were found the B2 (dark)/ B2 (light) (Treatment 6) were able to develop into seedlings, the most complete, 70 and 80 percent, respectively (Table 3).

The NHK macapuno embryo hybrid varieties were cultured for 32 weeks in dark and light conditions.

Media and conditions	Embryo development (%)				
	NHK	RDK	TKK	WAK	YDK
Treatment 1 Y3 (dark room) /Y3 (lightroom) (control)	70	30	40	30	70
Treatment 2 Y3 (dark room)/B2 (lightroom)	40	60	60	20	50
Treatment 3 MS (dark room)/Y3 (lightroom)	80	50	70	30	40
Treatment 4 MS (dark room)/ B2 (lightroom)	40	70	60	30	50
Treatment 5 B2 (dark room)/ Y3 (lightroom)	80	70	60	40	60
Treatment 6 B2 (dark room)/ B2 (lightroom)	60	60	70	70	80

Table 3. Effects of culture media and conditions on embryo development of 5 varieties of macapuno at 19 weeks of culture under dark condition and transferred to light condition.

There were no statistically significant differences. The mean plant height was 7.6-14.5 cm (Table 4), RDK species had an average plant height of 5.8-11.0 cm (Table 4), but TKK species showed that the mean

Table 4 Effects of culture media and conditions on shoot length of five macapuno hybrid lines at 32 weeks of culture

Treatment	Shoot length (cm.)				
	NHK1/	RDK	TKK1/	WAK1/	YDK1/
Treatment 1 Y3 (dark room)/ Y3 (lightroom) (control)	8.7 b	11	7.0b	10.0 ab	6.0ab
Treatment 2 Y3 (dark room)/ B2 (lightroom)	14.5 a	9.3	13.3 a	17.0 a	5.3b
Treatment 3 MS (dark room)/ Y3 (lightroom)	11.0 ab	8.9	5.8 b	3.0 c	13.3 ab
Treatment 4 MS (dark room)/ B2 (lightroom)	9.8 ab	9.8	13.2 a	9.7 b	0
Treatment 5 B2 (dark room)/ Y3 (lightroom)	11.7 ab	5.8	8.7 b	12.5 ab	7.2 ab
Treatment 6 B2 (dark room)/ B2 (lightroom)	7.6 b	8.4	8.3 b	6.5bc	13.5 ab
C.V. (%)	22.66	32.84	23.41	22.26	46.66

[1/] The averages in the same column that follow with the same letter were not significantly different at a 95% confidence level by DMRT

Table 4. Effects of culture media and conditions on shoot length of five macapuno hybrid lines at 32 weeks of culture

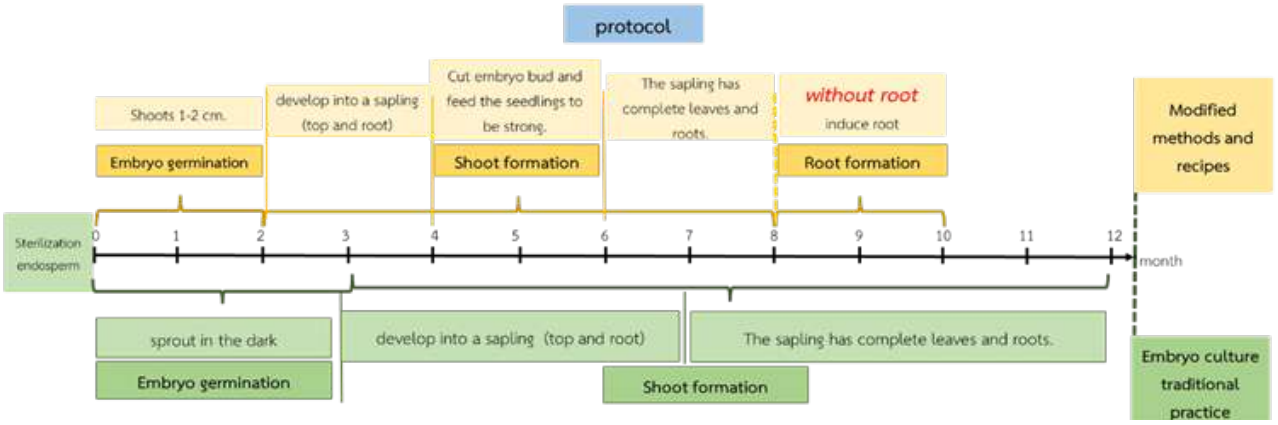

Figure 7. Comparison of the time period of the traditional practice of macapuno embryo culture and modified methods.

plant height was significantly different. Method 2 and Method 4 had the highest mean plant height at 13.3 cm and 13.2 cm, respectively, and Treatment 3 had the lowest mean plant height at 5.8 cm (Table 4), while in the WAK and YDK strains,it was found that the plant height in each treatment was not significantly different. The average plant height is 4.5-17.0 centimeters and 5.3-13.5 centimeters, respectively. (Table 4)

The culture study of 5 varieties of hybrid macapuno embryos was carried out in a single embryo culture. It will be a variety that is harvested at the same time. But each set received different environmental factors, each time of mating, which affects the integrity of the variety due to inbreeding between different parents or some bunches obtained from the same father and mother, not simultaneous maturation according to physiological characteristics. Coconut is a plant with gradual blooming of female flowers which takes about 7-15 days until they are completely bloomed. (Tippayaet *al.*, 2021). The experiment showed that the formula affected shoot formation in the dark and the development of roots in the light. Embryos fed on the B2 formula showed better growth prospects. Due to the micronutrient elements concentration and growth factor,the B2 formula was 10 times higher than that of the Y3 formula and from embryo culture development in the dark, the development in the bright room, and the complete seedling development in each batch. The reduction in development percentage may depend on the management of the parent plot. Fertility and management within the laboratory and from experimental modification of methods and recipes, shoot emergence, and seedling development until the seedlings complete with leaves and roots take 8 months, which can reduce the embryo culture duration from 12 months in traditional culture (Figure7).

The effect of coconut aging and culture medium with cut in half of shoot to plant of Chumphon 84-2 hybrid macapuno coconut.

Feasibility study of double shoots from a single zygotic embryo of Chumphon 84-2 Hybrid Macapuno Coconut (1 embryo can be cut in half to make two pieces. If it can develop into a whole shoot (Figure 4C), it will give two plants or 200% embryo. Observation of the development of new shoots from embryo halves with fruit maturity at 9, 10, and 11 months in a modified Y3 solid medium(Parinda, 2018) and Murashige and Skoog (MS) medium supplemented with 0.4 mg L-1 Indole-3-butyric acid (IBA) and 3.2 mg L-1 kinetin (referred from Sisunandar *et al.*, 2015). It is found that using shoots from embryos with fruit maturity at 10 and 11 months in Murashige and Skoog (MS) medium supplemented with 0.4 mg L-1 Indole-3-butyric acid (IBA) and 3.2 mg L-1 kinetin, makes the percentage of development of a new shoot higher than the Y3 medium.The use of shoots from 9-month-old embryos showed the lowest percentage of new shoot development in both mediums (123.3 and 133.3%) compared to 10 and 11 months of fruiting (Table 5). Somchai (2003) mentioned that coconut embryo culture would be successful depending on the important component that the embryo aging must be between 10-11 months. Sisunandar *et al.* reported used dwarf kopyor-type coconuts isolated from 11-month-old fruit.

Table 5. Embryo development percent of shoot halves of coconut fruiting age at 9, 10, and 11 months after 2 months

Fruiting age	Medium		% Embryo development in the light1/
9 months	modified Y3		123.3 b
9 months	MS with0.4 mg L^{-1} Kinetin	IBAand3.2 mg L^{-1} IBA	133.3 ab
10 months	modified Y3		144.3 ab
10 months	MS with0.4 mg L^{-1} Kinetin	IBAand3.2 mg L^{-1} IBA	161.5 a
11 months	modified Y3		138.3 ab
11 months	MS with0.4 mg L-1 Kinetin	IBA and 3.2 mg L^{-1} IBA	150.0 ab
C.V. (%)			15.7

1/ The averages in the same column that follow with the same letter were not statistical difference at 95% confidence level by DMRT

culturing in medium in the light

The effect of the medium on the development of the embryo is a new shoot by culturing in comparison with both mediums. It is found that the percentage of embryo development at 9-11 months of age fruit on MS medium added 0.4 mg/L IBA plus 3.2 mg/L Kinetin higher when raised on a modified Y3 medium. In accordance with Sisunandar *et al.* (2015), the best protocol is to first incise the germinated embryos at the meristem site, followed by splitting the embryo into two after four weeks of culture and then recovering the embryos in Murashige and Skoog (MS) medium supplemented with 2 µM IBA and 15 µM kinetin. Results from the addition of growth regulators IBA and Kinetin, which are substances in the auxin and cytokinin group assists cell division and budding/new shoots in multi-vegetative culture. Shou *et al.* (2008) reported that the maximum number of shoots was induced from lotus bud explants on MS medium containing agar, sucrose, and BA added with NAA similar to Noraini *et al.* (2014). In addition, Jala (2012) reported that shoot tips of *Curcuma longa L.* were given the highest average number of new shoots when cultured on MS medium supplemented with NAA and BA. The Y3 medium is a specific formula for coconut tissue culture (Somchai, 2003) without the addition of growth regulators. Therefore, the study induced new shoots in the modified Y3 formula with growth regulators. In the embryo halves, the shoot must be cut in half length wise into two equal parts and multiple halves that resulted in the need to cut off the root part (Figure 8A). When cultured in a Y3 liquid medium, the sapling develops into a mature plant and has 2-3 leaves, but is unable to take root(Figure 8B). Therefore, it is necessary to study the root-inducing formula (Figure 8C), which is another experiment in the research project on the development and efficiency of coconut tissue culture (It is not mentioned in this report).

Figure 8. Characteristics of halved fragments (A) embryo

halves No root development (B) and root emergence of embryo halves when cultured in Root medium (C).

Conclusion

1. Medium and embryo placement characteristics affected 5 varieties of hybrid Kathi coconut embryo germination. It was found that the embryos cultured in a solid medium in the dark showed the best embryo germination and development to plantlet.

2. Different varieties of macapuno hybrids affect the response to different recipes. The suitable medium for propagating NHK macapuno hybrids were MS formula with 2, 4-D or B2 formula in the dark and Y3 formula in the lightroom. RDK is MS formulation in the dark and B2 liquid medium in the lightroom. The embryo develops at most 70 percent of the seedling maturity, and the TKK, WAK, and YDK strains are B2 formula in the dark and B2 liquid medium in the lightroom.

3. Embryo incision can be applied to produce double seedlings of Hybrid Macapuno Coconuts. The best protocol is to first incise the germinated embryos at the meristem site, followed by cutting half the embryo into two and then recovering the embryos in Murashige and Skoog (MS) medium supplemented with 2 µM IBA and 15 µM kinetin.

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Retirement

Shri M A Sebastian, Administrative Officer retired from the services of Coconut Development Board on 31st May 2023 after serving the Board for more than 38 years.