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First report of original-strain Intarachit pineapple conservation in Thailand

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Abstract The shoot introduction of Intarachit pineapples was optimized by determining the hormone combination of culture media. The results showed that shoot buds on crown meristem had elongated, and the primary leaves had opened gradually after two weeks of culture. At week four, Intarachitdaeng in MS with 4 mg/l BA provided the highest average length of shoots at 7.46±0.94 mm, while Intarachitkow in MS with 2 mg/l BA provided the highest average length of shoots at 7.05±0.33 mm. The numbers of shoots per piece (1/8 of crown meristem), MS with 4 mg/l BA showed highest average numbers of shoots at 3.33 and 3.67 shoots in Intarachitdaeng and Intarachitkow, respectively. After eight weeks, the average shoot length was 3-4 cm with leaves fully open. MS was used as root-inducing media. After rooting in hormone-free MS for 4 weeks, roots were successfully grown and then transferred to planting material. Following these steps, one crown of Intarachitdaeng and Intarachitkow can be produced for 26.64 and 29.36 shoots respectively.

Keywords: Intarachit pineapple, Tissue culture, Conservation, Chachoengsao

Introduction

Pineapple (*Ananas comosus*) is one of the significant tropical Bromeliads consumed globally. The pineapple cultivated area and market have been widely increasing because of its attractive flavor and nutritional values, such as vitamins (A, C, B1 and B6), fiber, copper, manganese and several minerals (Mohd Ali *et al*., 2020; Kader *et al*., 2010). Pineapple is mostly cultivated in tropical zones due to the temperate climate and rainfall distribution (Shamsudin *et al*., 2020). In 2017, Thailand ranked fourth of the most pineapple producers worldwide with 2153.18 metric tons of pineapple after Costa Rica (3056.45 metric tons), Philippines (2671.71 metric tons) and Brazil (2253.90 metric tons) (Statista, 2020). Pineapple can be catagorized into five groups namely Abacaxi, Cayenne, Maipure or Perolera, Queen, and Spanish, based on the morphology including length and shape of the leaves, weight, shape, texture and taste of the fruits (Py *et al*., 1987). For the aforementioned categories, Cayenne, Queen, and Spanish

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are farmed in Thailand (Popluechai *et al*., 2007). Pineapple cultivation areas have been divided into geographical regions: northern, northeastern, central, and southern parts. Each geographical region produces many pineapple cultivars with various colors, shapes, sizes, and flavors (Hemung *et al*., 2022). Pattavia, Phuket, Nang Lae and Phu Lae are well-known cultivars for producing and delivering to pineapple industry (Sirimuangmoon, 2021).

Intarachit is also one of the famous cultivars in the past (Sirimuangmoon, 2021; Dittakan *et al*., 2008). This is the oldest cultivar in Thailand, mainly cultivated in Chachoengsao province, the central part of Thailand (Siam agro-food industry public company limited, 2023). As a report in 2007, Intarachit was separated into 2 cultivars namely Intarachitdaeng (red Intarachit) and Intarachitkow (white Intarachit). These two cultivars could be morphologically classified to be members of the Spanish. The Spanish cultivars yield small fruits with oval or cylindrical shape, golden-yellow flesh with low sugar and acidity. After RAPD analysis technique, it was revealed that Intarachitdaeng and Intarachitkow have similarity coefficient of 0.963, it means that they are very closely related to each ther (Popluechai *et al*., 2007).

After a severe flooding in 2011 in Thailand, there was a few Intarachit-cultivated areas left in only Paknam subdistrict, Bangkhla district, Chachoengsao. At present, both Intarachitdaeng and Intarachitkow were endangered cultivars. It might be because of several reasons, for example, the urban society became a new value and replaced an agricultural society, some popular cultivars were selected to produced, some GMO cultivars were proper for canning than the old ones, and normal pineapple propagation takes long a time before budding, blossoming, flowering, and fruiting around 15-18 months (Vanijajiva, 2012).

To plant and to grow pineapple, *in vivo* propagation is easy but the multiplication rate is low and not enough for cultivar conservation (Be and Debergh, 2006). Tissue culture or *in vitro* propagation is one of the effective ways to increase the multiplication rate of pineapples. The establishment of shoot cultures in solid media and induction of roots using bioreactor can produced 6,000-8,000 shoots from the two starting shoots in less than 6 months. High effectiveness of bioreactor method could produce much higher root induction than traditional way, but it also took much higher in cost of production at more than 35% (Firoozabady and Gutterson, 2003).

The aim of this research was to conserve the original pineapple cultivars, Intarachitdaeng and Intarachitkow, using tissue culture techniques with lower price *in vitro* propagation. The solid media was varied to find the best media that induce highest number of shoots, then root induction was examined in MS solid

media to lower cost of production and easy to follow by farmer who interested in Intarachit *in vitro* propagation.

Materials and methods

Time and place of research

This research was conducted in year 2022-2023 at Faculty of Science and Technology, Rajabhat Rajanagarindra University, Bangkhla district, Chachoengsao.

Experimental design

The experimental design used a completely randomized design: 6 varied media were tested, while experimental units were crown meristems of two pineapple (*Ananas comosus* L.) cultivars, Intarachitdaeng and Intarachitkow. Experiments were run in triplicate.

Media preparation

Two hormones were used in this research, BA (Benzyl adenine) as cytokinin and NAA (Naphthaleneacetic acid) as auxin, for shoot multiplication. There were 6 varied media applied in this experiment as shown in table 1. Sucrose was added at 30 g/l and agar 8 g/l was added for solidification. Then, the pH of these media was adjusted to 5.7 before autoclaving.

Table 1. Six varied of hormone combination of MS solidified culture media

Media formula	BA (mg/l)	NAA (mg/l)
I	-	-
II	2	-
III	4	-
IV	8	-
V	1	0.2
VI	2	0.1

Sample collection

Crown meristems of pineapple Intarachitdaeng and Intarachitkow were collected from Paknam subdistrict, Bangkhla district, Chachoengsao, Thailand. The stems of crowns were prepared by removing all leaves by hand. Then the surface was initially washed by commercial detergent for 10 min and washed in water and exposed to the air overnight (Figure 1).

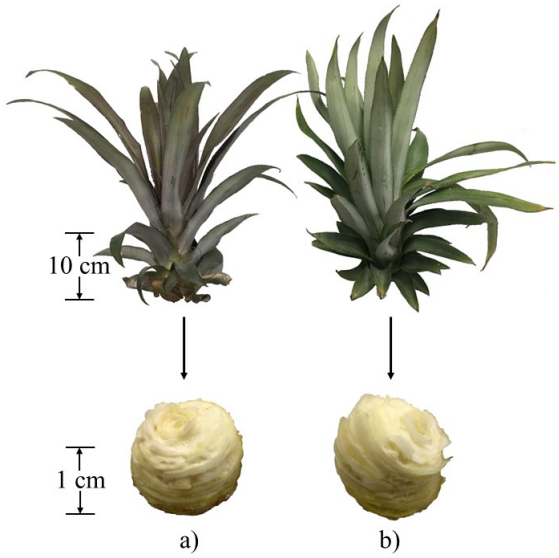


Figure 1. The stems of crowns of pineapple samples. a) Intarachitdaeng b) Intarachitkow

Shooting in vitro

Before *in vitro* cultured, the stems of crown were surfaced sterilized with 10% clorox for 10 min, 5% clorox for 5 min and triple washed with sterile water. Each sample was vertically cut for divided into 8 pieces equally (Figure 2). Then samples were cultured in six formulas of MS medium in triplicate. The crown meristem with tiny shoots were maintained in same media for 8 weeks and subcultured at four-week intervals before rooting in the subsequent experiment. Cultures were incubated at 25 °C under fluorescent light 16 h/day. The shoot formations in six varied media were compared.

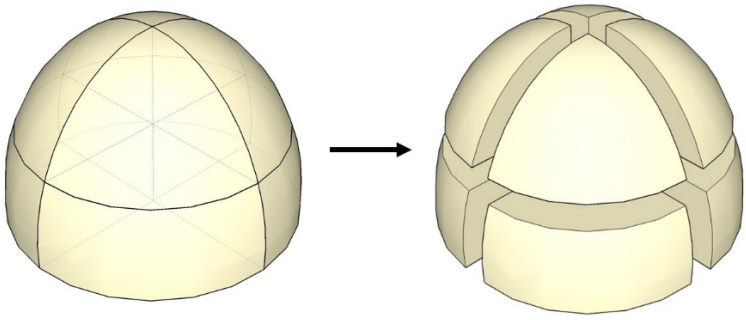


Figure 2. Pineapple crowns were divided into 8 pieces equally

Rooting in vitro

The crown shoots were used as explants. The explants were cut longitudinally with a single shoot bud on a small portion of the basal stem (approximately 5-7 mm^2). Each section was placed aseptically on MS to induce root formation (applied form Be and Debergh, 2006). All cultures were incubated at 25 °C under fluorescent light 16 h/day as before. After 4 weeks, complete pineapple plantlets with roots were produced and transferred to planting material.

Growing in planting material

Planting material was prepared by soaked peat moss in water for 5 min before use. Then filled the soaked peat moss in the container and made a hole for putting the roots of plantlet in. Each plantlet was planted with receptacle which totally wrapped by transparent plastic bag for a week for keeping moisture, before growing in natural air in the next week.

Results

Morphology comparison of Intarachitdaeng and Intarachitkow

It was found that Intarachitdaeng and Intarachitkow pineapples have remarkably similar morphologies including shape of stems and leafs. The only distinction is the leaf color, Intarachitdaeng has green leaves with red pigments dispersed while Intarachitkow has only green pigment throughout the leaves (Figure 3a). The fruits of both cultivars are small, Intarachitdaeng has cylindrical shape, some of them have long base of crown (part of fruit between flesh and leaf) with orange color when ripening, while Intarachitkow has oval shape with bright yellow color (Figure 3b). The ripening flesh of both cultivars were orangish-yellow (Figure 3c). Their taste was remarkably similar, less sweet and less sour.

Shooting comparison between media

The increase of shoot length was tested in comparison of six different hormone combinations in solid media as shown in table 1. After two weeks of culture initiation, the shoot buds on crown meristem had elongated, the primary leaves had opened gradually. After four weeks, the mean of length and number of shoots between six combinations of media were compared and were shown in Table 2. Intarachitdaeng in formula I and V showed highest average length of

shoots at 7.46±0.94 and 4.93±0.93 mm, respectively. Simultaneously, Intarachitkow in formula II, V and III showed highest average length of shoots at 7.05±0.33, 6.50±1.32 and 5.50±1.65 mm, respectively.

Concerning the numbers of shoots, both Intarachitdaeng and Intarachitkow in formula III showed highest average numbers of shoots at 3.33±0.58 and 3.67±1.15 shoots per piece (1/8) of pineapple crown, respectively.

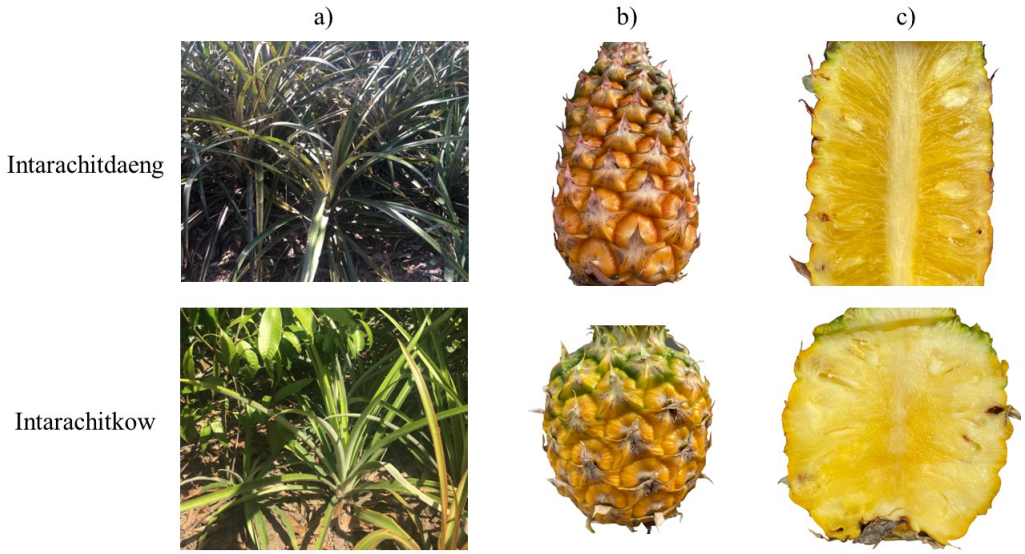


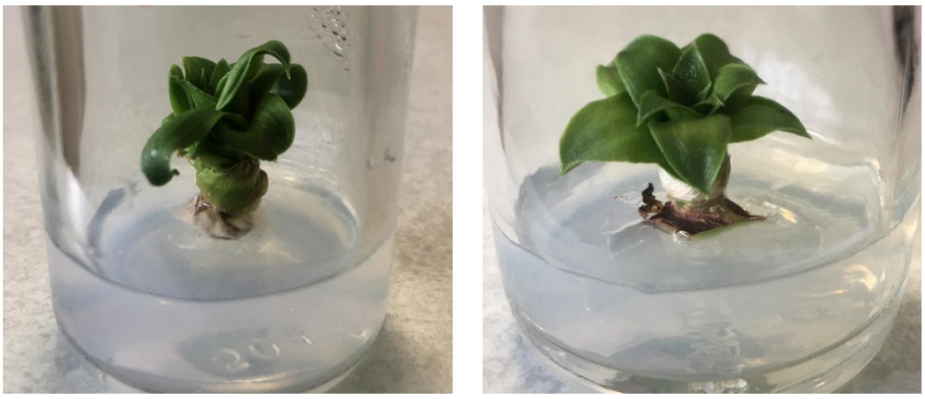
Figure 3. The difference between Intarachitdaeng and Intarachitkow collected from Paknam subdistrict, Bangkhla district, Chachoengsao province, Thailand. a) stem and leaf, b) fruit shape and color and c) flesh texture

Table 2. Shooting comparison of six media in triplicate

Media formula	Intarachitdaeng		Intarachitkow	
	Length of shoots (mm)	Number of shoots (shoots)	Length of shoots (mm)	Number of shoots (shoots)
I	2.83±0.29^{cd}	2.00±0.00^{bc}	4.33±0.42^{bc}	1.33±0.58^{b}
II	2.17±0.29^{d}	1.33±1.58^{c}	7.05±0.33^{a}	2.67±1.15^{ab}
III	7.46±0.94^{a}	3.33±0.58^{a}	5.50±1.65^{abc}	3.67±1.15^{a}
IV	3.67±0.29^{c}	1.33±0.58^{c}	3.20±1.74^{c}	3.00±1.00^{ab}
V	4.93±0.93^{b}	2.00±1.00^{bc}	6.50±1.32^{ab}	1.67±0.58^{b}
VI	2.39±0.35^{d}	3.00±0.00^{ab}	3.33±1.04^{c}	1.33±0.58^{b}

Note: Means marked with the same superscript letter in a column were not statistically different ($p<0.05$) using Duncan's Multiple Range Test.

After eight weeks, the average shoot length was around 3-4 cm with open fully leaves. These shoots were individually transferred to MS solid media without hormones to induce the roots (Figure 4).



a) b)

Figure 4. Transferred shoots with small crown base into MS media a) Intarachitdaeng and b) Intarachitkow

Rooting in MS and plantlet growing in planting material

After four weeks of culture in MS, roots were formed continuously and ready to transfer to plant material, soaked peat moss and perlite (50:50), and planted in cleaned environment. However, these plantlets were vulnerable to dehydration. Thus, they were grown under cling wrap of transparent plastic bag with few tiny holes for the first few weeks to maintain air temperature and humidity. After 8 weeks, Intarachit pineapple plants were transplanted into soil and changed to bigger planting pot every period of 8 weeks. The Intarachit plants after growing in planting material in 8 weeks were demonstrated in figure 5.

a) b)

Figure 6. Pineapple Intarachit plants after growing planting material for 8 weeks a) Intarachitdaeng and b) Intarachitkow

Discussion

In the media preparation step, 30 mg/l sucrose was added into every media formula. According to the research in 2006, Be and Debergh tried an experiment to test the effects of sucrose content. They found that there was no affect performance between 3-4% sucrose and there was no correlation between sugar content and condition of shoot regeneration.

Firoozabady and Gutterson (2003) cultured crown-tip meristems isolated from the crown of pineapple fruits in MS in 2 weeks and then transferred to MS with 2 mg/l each of BA and NAA. They found that the leaf bases around the crowns regenerated shoots directly, without callus production, within 4–5 weeks. Similarly, I found that crown meristem regenerated shoots continuously within 3-5 weeks after they were cultured in varied MS media.

For shoot induction, samples in MS with 4 mg/l BA provided the better results (both in average length of shoots and average number of shoots) than the formula added both BA and NAA. These results conformed to the previous work reported that only BA in culture media did higher yield of pineapple shoots than media with hormone combination of BA and IBA or NAA (Be and Debergh, 2006).

For root regeneration, crown meristem with 3-4 cm shoots that open fully leaves were transferred to MS solid media without hormones to induce the roots. After four weeks of culture in MS, roots were formed continuously. This result conformed to Be and Debergh' s work (2006). They found that pineapple plantlets are easily rooted in hormone-free media and plantlets over 95% survived under environmental conditions after transferred to plant material. Moreover, this method was decreased the cost price by 35% compared with the periodic immersion bioreactor (PIB) (Firoozabady and Gutterson, 2003).

In conclusion, this research successfully conserved by micropropagation of Intarachit pineapple in cost-reduced way. First, in the early stages of pineapple production, micropropagation using MS with 4 mg/l BA as shooting culture media provided the highest number and length of shoots after 4 weeks. The rooting by cultured in hormone-free MS provided the good result, and then growing in peatmoss planting material also appropriate. Following this methodology, one piece of crown sample (1/8 of pineapple crown) produced 3.33 shoots of Intarachitdaeng and 3.67 shoots of Intarachitkow. Then, one crown of pineapple can be produced 26.64 and 29.36 shoots of Intarachitdaeng and Intarachitkow, respectively. These shoots can also be matured to be planlets in 3 months (12 weeks) before they are transferred into proper planting material. Afterwards, this study provides the knowledge of pineapple micropropagation in practical and time-saving methods that are uncomplicated to follow by farmers interested in pineapple *in vitro* propagation.

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