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Effects of BA and NAA on plant regeneration of neck orange (*Citrus reticulate* Blanco)

Hansuek, S.[1*], Liamnimitr, N.[1] and Khawniam, T.[2]

[1]Faculty of Agriculture, Rajamangala University of Thechnology Srivijaya Nakhon Sri Thammarat Saiyai Campus, Nakhon Sri Thammarat Province, Thailand, 80110; [2]Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla Province, Thailand, 90112.

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Abstract Neck orange (*Citrus reticulate* Blanco) is native orange that very popular in southern of Thailand. It had health benefits and also used in many industries of citrus processing. There are expansion of the plantation area to increase the production of sufficient to demand of consumers. The branches are currently not enough to cultivation. This study showed the effect of benzyladenine (BA) and naphthalene acetic acid (NAA) to develop a new plant of Neck orange. The seeds of orange were surface sterilized with sodium hypochlorite and culturing on Murashige and Skoog (MS) medium supplemented with BA (0-2 mg/L) and NAA (0-1 mg/L), 3% sucrose, with and without 0.2% activated charcoal for 6 weeks. The result revealed that MS free medium (without plant growth regulators) gave the highest percentage of germination (83.93 percent). The highest of Shoot length (3.17 cm) were obtain from MS medium containing 0.5 mg/L BA adding activated charcoal, whereas MS medium with 2.0 mg/L BA gave the highest number of shoot (3.33 shoots/seed) and MS medium with 0.5 mg/L NAA, 0.5 mg/L BA adding activated charcoal gave the highest root length (21.30 cm).

Keywords: Neck Orange, plant growth regulators, plant regeneration

Introduction

Neck orange (*Citrus reticulata* Blanco) is an economic crop in southern of Thailand. It is very popular in Chana district and another district of Songkhla province and expanded to other areas such as Yala, Nakhon Si Thammarat and Chumphon province (Sujit, 2017). Plants propagation of the species has sexual (seed) and asexual (vegetative plant) such as cuttings, etc., also have tissue culture that can propagate faster and more. The explants are cultured on sterile media (synthesis) and controlled environment such as temperature. Parts of the plant can grow and develop to new complete plantlet (Jantaratin, 2013). The area of citrus plantation was changed to other economic crops, causing the area

[*]**Corresponding Author:** Hansuek, S. ; **Email:** sakulrat_s@hotmail.co.th

of citrus to decrease until it became extinct, while the market was in high demand but yield was not enough. The rapid spread of citrus to the death of a large number of plants until lost from native area by disease virus and greening bacterium which is a major producer of citrus (George *et al*., 2008). Moreover, plant regeneration by tissue culture methods and genetic transformation has been shown to be a powerful strategy for cultivar improvement (khan *et al*., 2009). Thus, this experiment showed an efficient protocol for *in vitro* plantlet is successful micropropagation or genetic transformation procedure. The plants have a capacity to generate whole plant from any cell/explant (Perez-Torner*et al*., 2010; Tallon *et al*., 2013). It also relates to type of plant growth regulators (PGRs) such as an auxin and cytokinins ratio to development of the tissue culture (Te-chato and Nooduang, 2003; Nooduang, 1998; Kaweeta, 1998). Savita *et al*. (2011) reported that tissue culture of lemon by seed and then callus induction from cotyledons on MS medium 2.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.75 mg/L benzyladenine (BA) gave the highest callus induction (83%). Shoot induction on MS supplemented with 3.0 mg/L BA gave the highest shoot induction (87.50%) and root induction (100%) obtains from MS supplemented with 2 mg/L NAA. Plant regeneration of Mexican lime and Mandarin from stem culture of seedlings on MS medium supplemented with 3.0 mg/L BA and 0.5 mg/L NAA gave the highest shoot induction at 96% and 88%, respectively (Perez and Alejo, 1997). Shawkat and Mirza (2006) reported that tissue culture from root, leaf, cotyledon and stem of lemon seedlings on MS medium supplemented with 1.5 mg/L NAA showed that stem culture gave the highest callus induction (92%), shoot induction (70%) on MS medium supplemented with 3.0 mg/L BA and transfer to MS medium supplemented with 0.5 mg/L NAA gave the highest root induction (70%). The aims of this study were to evaluate the possibility of *in vitro* plant regeneration of neck orange from seed and effect of plant growth regulator.

Materials and methods

Fruit of neck orange (*Citrus reticulate* Blanco) were collected from orchard in Chana district, Songkhla province, Thailand (Fig. 1A). Seeds (Fig. 1B) were washed with under running tap water. The seeds were sterilized by immersion in 70% ethanol for 30 to 45 seconds and then immersed in 20% of sodium hypochlorite together with1-2 drops of Tween-20 for 20 minutes, respectively. The seeds were rinsing 3 times with sterilized distilled water under laminar air flow. Then cut the seed coat and culture on MS medium supplemented with various concentrations (0, 0.5, 1.0, 1.5 and 2.0 mg/L) of BA and (0, 0.5, and 1.0 mg/L) of NAA, 3% sucrose, with and without 0.2%

activated charcoal. The pH of MS medium was adjusted to 5.7 with 1 N NaOH or HCl and autoclaved at 121 °C with for 15 min. After that the cultures were kept under controlled environment at 27±2 °C under a 14-h photoperiod of 25 μmol/m^2s^1 irradiance provided by cool white fluorescent. The emergence of shoot number and percentage of germination were recorded. The experiment with factorial in completely randomized design (CRD) with 10 replicates was performed. Percentage of germination, number of shoot, shoots and root length were recorded after culturing for 6 weeks. Data were analyzed by ANOVA. Means were separated by using Duncan's multiple range tests (DMRT) at the 0.01 of probability. The F-test showed significant differences among means.

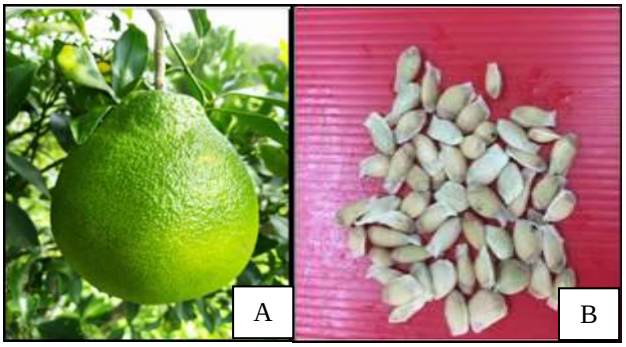

Figure 1. Characteristic of neck orange (*Citrus reticulata* Blanco) (A) and their seed (B)

Results

Seeds were placed on MS medium containing BA either alone or combination with NAA for seed germination. The faster response was recorded on explant cultured on media supplemented with only BA. After 1 week of culture, the seed explants swelled after two weeks and adventitious shoots were developed in all disinfectant. The development of germinated neck orange after cultured for 6 weeks was showed in Fig. 2.

After cultured for 6 weeks, the results showed that MS medium without plant growth regulator and activated charcoal gave the highest percentage of germination at 83.93%, while the addition of activated charcoal gave the highest germination at 80.08% (Table 1). Media containing only BA gave the higher germination than BA and NAA combination. Root occurred in activated charcoal and the characteristics of neck orange germination on MS free medium with and without activated charcoal showed in fig.3.

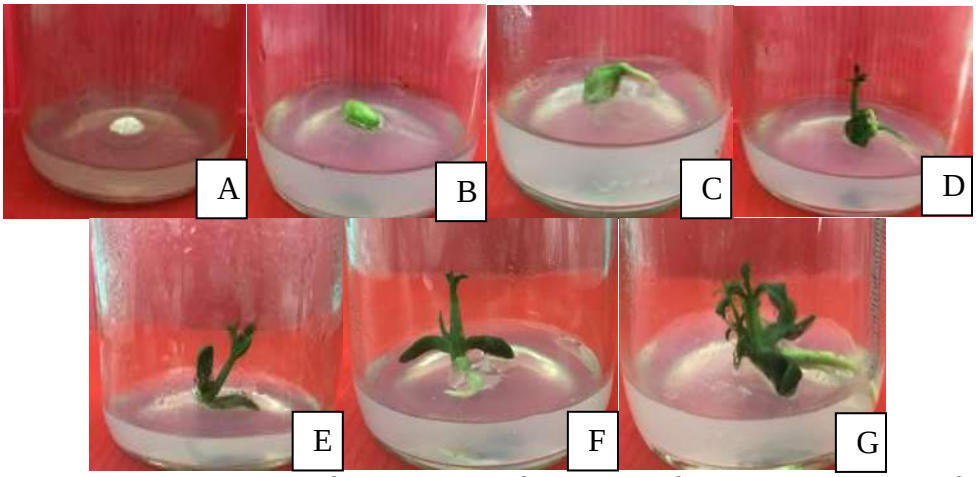

Figure 2. Development of neck orange from seed after culturing on MS free medium without activated charcoal for 6 weeks (bar = 0.5 cm). A: initiation culture B: after cultured for 1 week C: after cultured for 2 weeks, D: after cultured for 3 weeks E: after cultured for 4 weeks F: after cultured for 5 weeks G: after cultured for 6 weeks

Number of shoots, MS medium with 2.0 mg/L BA gave the highest number of shoots (3.33 shoots per seed) (Table 2), while MS medium containing 2.0 mg/L BA adding activated charcoal gave the number of shoots (1.22 shoots per seed). In this study clear that BA at the concentration of 2.0 mg/L gave the affect effecting on number of shoot. Number of shoot occurred on different various concentration of BA and NAA showed in fig.4.

For shoot length, the grater shoot length were obtain from MS medium supplemented with 0.5 mg/L BA adding activated charcoal (3.17 cm.), while MS free medium and without activated charcoal gave the shoot length (2.88 cm.) (Table 3). Overall, the shoot lengths were greater in treatment adding activated charcoal than without activated charcoal (Fig. 5). In this result showed that plant growth regulator combination with activated charcoal gave the effect on shoot length.

It is clears that the experiment data to MS medium supplemented with 0.5 mg/L BA, 0.5 mg/L NAA and activated charcoal gave the highest root length (21.30 cm) (Table 4), while the MS medium with 0.5 mg/L BA and 1.0 mg/L NAA gave the root length (8.22 cm) (Fig. 6). But the increase NAA did not promote the root length: there were downward trends of 1.46 to 3.61 cm, and root was more slender. The root obtained from media with activated charcoal was small and long, besides it appeared that white and healthy. On the other hand, Media without activated charcoal was big, thick and short.

Table 1. Germination percentage of neck orange after cultured on MS medium supplemented with various concentration of BA and NAA for 6 weeks

Culture Medium	Percentage of germination (%)	
	activated charcoal	without activated charcoal
1. MS Free	80.08a	83.93a
2. MS+0.5BA	62.45g	62.97h
3. MS+1.0BA	75.33c	76.16c
4. MS+1.5BA	62.67g	66.87g
5. MS+2.0BA	78.55b	80.53b
6. MS+0.5 NAA+0.5BA	71.57e	77.11d
7. MS+0.5NAA+1.0BA	44.36k	44.85J
8. MS+0.5NAA+1.5BA	69.67f	70.83f
9. MS+0.5 NAA+2.0BA	73.33d	75.67cd
10. MS+1.0NAA+0.5BA	60.64h	66.77g
11. MS+1.0NAA+1.0BA	45.53j	50.51i
12. MS+1.0NAA+1.5BA	54.97i	66.88g
13. MS+1.0NAA+2.0BA	70.30f	71.63e
F-test	**	**
C.V. (%)	10.46	7.05

Mean followed by the same letter within the column are not significantly different at P≤0.01 according to DMRT.

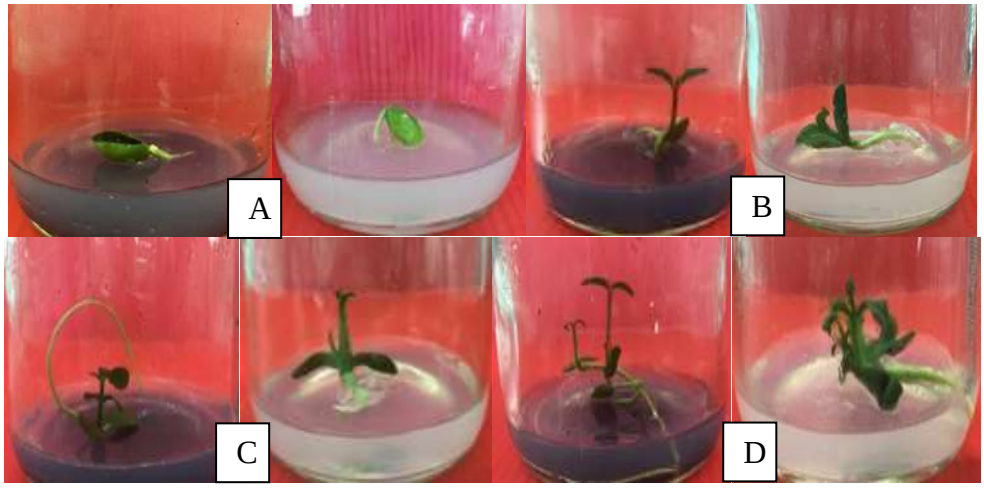

Figure 3. Germination of neck orange on MS medium without plant growth regulator, with and without activated charcoal for 6 weeks (bar = 0.5 cm). A: after cultured for 2 weeks B: after cultured for 4 weeks, C: after cultured for 5 weeks, D: after cultured for 6 weeks

Table 2. Shoots number of neck orange after cultured on MS medium supplemented with various concentration of BA and NAA for 6 weeks

Culture Media	No. of shoot (shoot/seed)	
	activated charcoal	without activated charcoal
1. MS Free	1.00c	1.33e
2. MS+0.5BA	1.22abc	1.44e
3. MS+1.0BA	1.33ab	2.56cd
4. MS+1.5BA	1.44ab	1.44e
5. MS+2.0BA	1.22abc	3.33a
6. MS+0.5 NAA+0.5BA	1.11bc	2.44d
7. MS+0.5NAA+1.0BA	1.11bc	1.56e
8. MS+0.5NAA+1.5BA	1.11bc	2.67bcd
9. MS+0.5 NAA+2.0BA	1.0c	3.00ab
10. MS+1.0NAA+0.5BA	1.22abc	2.78bcd
11. MS+1.0NAA+1.0BA	1.33abc	2.89bc
12. MS+1.0NAA+1.5BA	1.22abc	2.89bc
13. MS+1.0NAA+2.0BA	1.56a	3.00ab
F-test	**	**
CV.(%)	13.59	18.39

Mean followed by the same letter within the column are not significantly different at P≤0.01 according to DMRT.

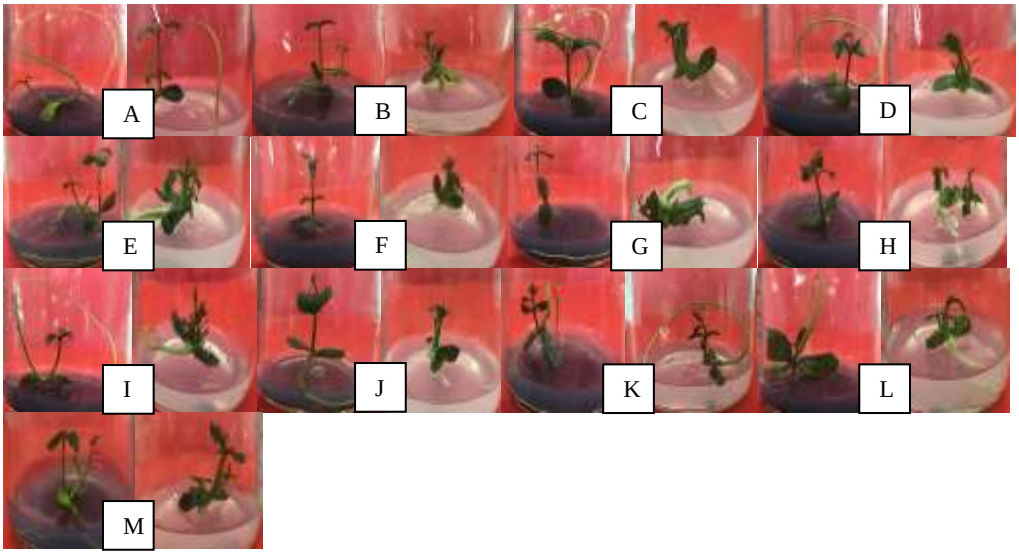

Figure 4. Shoot number of neck orange after cultured on MS medium supplemented with various concentration of BA and NAA with and without activated charcoal for 6 week (bar= 0.6 cm). (A) MS free (B) MS+0.5 mg/L BA (C) MS+1.0 mg/L BA (D) MS+1.5 mg/L BA (E) MS+2.0 mg/L BA (F) MS+0.5 mg/L NAA+0.5 mg/L BA (G) MS+0.5 mg/L NAA+1.0 mg/L BA (H) MS+0.5 mg/L NAA+1.5 mg/L BA (I) MS+0.5 mg/L NAA+2.0 mg/L BA (J) MS+1.0 mg/L NAA+0.5 mg/L BA (K) MS+1.0 mg/L NAA+1.0 mg/L BA (L) MS+1.0 mg/L NAA+1.5 mg/L BA (M) MS+1.0 mg/L NAA+2.0 mg/L BA

Table 3. Shoot length of neck orange after cultured on MS medium supplemented with various concentrations of BA and NAA for 6 weeks

Culture Media	**Shoot length (cm.)**	
	activated charcoal	**without activated charcoal**
1. MS Free	2.26e	2.88a
2. MS+0.5BA	3.17a	1.87c
3. MS+1.0BA	2.26e	2.17b
4. MS+1.5BA	2.67c	1.31h
5. MS+2.0BA	2.50d	1.67e
6. MS+0.5 NAA+0.5BA	2.70bc	1.76d
7. MS+0.5NAA+1.0BA	2.48d	1.50e
8. MS+0.5NAA+1.5BA	2.32e	1.91c
9. MS+0.5 NAA+2.0BA	2.80b	1.57f
10. MS+1.0NAA+0.5BA	2.78bc	1.72de
11. MS+1.0NAA+1.0BA	2.50d	1.29h
12. MS+1.0NAA+1.5BA	2.72bc	1.40g
13. MS+1.0NAA+2.0BA	2.47d	1.20i
F-test	**	**
C.V. (%)	5.20	5.39

Mean followed by the same letter within the column are not significantly different at P≤0.01 according to DMRT.

Table 4. Root length of neck orange after cultured on MS medium supplemented with various concentration of BA and NAA for 6 weeks

Culture Media	Root length (cm.)	
	activated charcoal	without activated charcoal
1. MS Free	17.80ef	4.24d
2. MS+0.5BA	19.13c	2.24i
3. MS+1.0BA	17.11g	7.21b
4. MS+1.5BA	17.83e	5.84c
5. MS+2.0BA	19.70b	2.74g
6. MS+0.5 NAA+0.5BA	21.30a	2.95f
7. MS+0.5NAA+1.0BA	5.80k	2.31hi
8. MS+0.5NAA+1.5BA	13.10h	1.76k
9. MS+0.5 NAA+2.0BA	6.50i	3.61e
10. MS+1.0NAA+0.5BA	17.60f	8.22a
11. MS+1.0NAA+1.0BA	18.19d	2.04j
12. MS+1.0NAA+1.5BA	17.20g	1.46l
13. MS+1.0NAA+2.0BA	6.10j	2.39h
F-test	**	**
C.V. (%)	6.59	9.82

Mean followed by the same letter within the column are not significantly different at P≤0.01 according to DMRT.

Figure 6. Characteristics of neck orange roots after cultured on MS medium supplemented with various concentration of BA and NAA with (A) and without (B) activated charcoal for 6 week (bar = 2 cm)

Discussion

Plant growth regulators, auxin and cytokinin, alone or in combination, are important role in development of plant regeneration of neck orange. In addition, different plant growth regulators provide different plant responses. On the other hand, seed germination can be proliferated in MS medium without plant growth regulator, while the addition of plant growth regulator this observation is supported the number of shoot, shoots and root length according to Savita *et al*. (2011) reported that lime seed were cultured on MS medium containing 2.0 mg/L 2,4-D and 0.75 mg/L BA gave the highest percentage of callus (83%) follow by transferred to shoots induction on MS medium supplemented with BA 3.0 mg/L gave the shoot ratio (87.50%) and then subculture on MS supplemented with 2.0 mg/L NAA for root induction gave the root (100%). The result showed that different varieties of plant gave the different respond on plant regeneration. Techaipiyawat (2001) reported that the processing of germinated seeds BA had accumulated within the cell in high doses; BA stimulates cell division, lateral growth and trunk growth. It helps to move the food from the roots to the shoots, so a little BA is enough for germination. In

addition, adventitious roots can develop from alternate tissues under certain conditions. When treated with exogenous auxin and cytokine. According to Datta *et al*. (2007) reported that *in vitro* clonal propagation of *Jatropha curcas* L gave the rate of root induction on MS medium supplemented with 1.0 μM IBA in 2–3 weeks from nodal explants gave the elongation of roots (8.7 ± 1.35 cm) was obtained.

The effects of PGRs on the number of citrus shoots are the high concentration gave the high number of shoots. However, the highest percentage of rooting obtained in the higher concentrations of auxins because they are a powerful growth hormone produced naturally by plants, the root plant showed a weak growth after transferring to soil when compared the root plant without plant growth regulators or low-concentration of auxin.

It concluded that MS medium without plant growth regulator and activated charcoal gave the highest percentage of germination (83.93%). MS medium supplemented with 2.0 mg/L BA gave the highest number of shoots (3.33 shoots per seed). MS medium supplemented with BA 0.5 mg/L adding activated charcoal gave the highest shoot length (3.17 cm.). MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L NAA with activated charcoal gave the highest root length (21.30 cm.)

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