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Somatic Embryogenesis: An Alternative for Propagating Selected Highland Clone of *Artemisia annua* L. of Vietnam Origin

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Abstract
Artemisinin, currently used in malaria treatment therapy, is produced in *Artemisia annua* L. Conventionally, *A. annua* is propagated via seeds which result in variation in plant quality and production of artemisinin content. Various *in vitro* culture techniques have been used for production of *A. annua* plantlets. In the present study, somatic embryogenesis protocol was successfully established for *A. annua* of Highland clone of Vietnam origin. Somatic embryos of *A. annua* could be induced from the leaf explants on Murashige and Skoog (MS) medium supplemented with 0.5 mgL^{-1} BAP (6-Benzylaminopurine) and 0.5 mgL^{-1} NAA (1-napthaleneacetic acid), 3% sucrose and 0.5 mgL^{-1} casein hydrolysate (CH). Proliferation of embryogenic calli was enhanced in MS medium added with 0.1- 2.0 mgL^{-1} 2,4-dichlorophenoxy-acetic acid (2, 4-D) and 0.5 mgL^{-1} casein hydrolysate (CH). The somatic embryos after culturing onto MS medium supplemented with 0.5 mgL^{-1} BAP developed into shoots. Plantlets were then generated after rooting the micro-shoots in MS medium supplemented with 1.0 mgL^{-1} IBA. The somatic embryos derived plantlets produced flowers and bore fruits and seeds two months earlier in tropical climates as compared to cool environment.
Keywords: artemisinin, somatic embryos, embryogenic callus, leaf explants

1. Introduction

Malaria is affecting many parts of the world. About 5% of the world's population is suffering from this disease (Baldi & Dixit, 2008). Three-quarters of the fatalities are found among children younger than five years old (Bell et al, 2005). Most of the malaria is caused by *Plasmodium fulciparum*. Currently, the most effective drug against this disease is artemisinin and is highly recommended by the World Health Organization (WHO) to combat against the chloroquine-resistant strains of Plasmodium species.

Artemisinin, a sesquiterpene lactone, is extracted mainly from *A. annua* L. (Asteraceae), a temperate medicinal plant. *A. annua* has been used in traditional medicine in China for the treatment of fever and as anti-malarial herb for nearly 2000 years (Mueller *et al.,* 2000). It was also reported to be effective for the treatment of skin diseases and used as natural herbicide (Liu *et al.,* 1998). Artemisinin and its derivatives were also found to have antitumor activities (Crespo-Ortiz & Wei, 2012).

A. annua has been conventionally propagated by seeds. This has resulted in high variation in the production artemisinin content due to location and different environmental factors (Huang *et.al* 2010). *In vitro* culture techniques have been used as the alternatives for the mass propagation of *A. annua* plantlets (Janarthanam et al., 2012: Mathur and Kumar, 1996; Elhag et al., 1992). However plants regeneration via somatic embryogenesis technique has not been reported in *A. annua*. Hence the present study reports the protocol for the regeneration of *A. annua* plantlets from the somatic embryos derived from the leaf explants

2. Materials and Methods

2.1 Plant materials

The seeds of the Highland clone of *A. Annua,* supplied by the Institute of Tropical Biology (ITB), Ho Chi Minh City, Vietnam, were germinated to obtain the *in vitro* seedlings. The seeds were surface-sterilized with 10% (v/v) Clorox® containing 5.3% sodium hypochlorite and 2-3 drops of Tween 20 for 20 minutes for the first stage followed by 10% (v/v) Clorox® with 2-3 drops of Tween 20 for another 10 minutes at the second stage. The seeds were rinsed twice with sterile distilled water at each stage. The cleansed seeds were then cultured onto Murashige and Skoog (MS) medium (1962) gelled with 8 gL^{-1} agar (Algas, Chile) without any plant growth regulator for 8 weeks. The leaves of the eight weeks old seedlings were used as explants for the induction of pre-embryogenic

callus.

2.2 Induction of pre-embryogenic callus

The leaf (0.5 cm x 0.5 cm) explants were transferred to MS basal medium supplemented with combination of naphthalene acetic acid (NAA) (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mgL^{-1}) and 6-benzylaminopurine (BAP) (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mgL^{-1}) plus 3% (w/v) sucrose, 0.5 mgL^{-1} Casein hydrolysate (CH) and 8 gL^{-1} agar (Algas, Chile). Six experimental units were used for each combination medium in a 6 x 6 factorial block design. Fresh biomass of the embryogenic callus formed was determined after 40 days of inoculation. The morphology of the pre-embryogenic callus was observed at every two days interval. The quantitative data was analyzed using 2-Way ANOVA followed by mean comparison using Tukey HSD at $p \leq 0.05$.

2.3 Induction of somatic embryos

The pre-embryogenic callus (0.5 g) was transferred to MS medium supplemented with 2,4-dichlorophenoxy-acetic acid (2-4D) (0, 0.1- 2.0 mgL^{-1}). The formation of somatic embryos was observed every week and the total number was recorded after three weeks of culture. The data was analyzed using 1-Way ANOVA followed by mean comparison using Tukey HSD at $p \leq 0.05$.

2.4 Plant Regeneration

The somatic embryos with the callus mass (0.5 - 0.6 g) were transferred onto MS medium supplemented with 0.5 mgL^{-1} BAP, the shoot induction medium. The number of shoots formed from each inoculated mass was determined after three weeks of culture. The micro-shoots formed were separated individually and transferred onto MS medium with 1.0 mgL^{-1} indolebutyric acid (IBA) for rooting.

2.5 Acclimatization of the regenerated plantlets

Plantlets with well developed roots were removed from the rooting medium and washed gently under running tap water to remove the agar. The cleansed plantlets were planted in plastic pots containing mixture of top soil and sand (1:1). The soil mixture was sterilized by autoclaving (Tommy autoclave, Japan) for 20 min at 121°C and 1.2 kg cm^{-2} pressure. Each of the potted plantlet was covered with plastic bag to ensure high humidity and the plantlets were watered every day. They were maintained at the growth chamber at 25°C. After two weeks of acclimatization, the plastic bags were removed and the plantlets were placed in the greenhouse. The percentage of survived plantlets and the morphology of the plants were observed after one month of acclimatization in the greenhouse.

2.6 Culture condition

All the culture media were adjusted to pH 5.7-5.8 before adding gelling agent (agar Algas Chile) and autoclaved (Tommy autoclave, Japan) at 121 °C and 1.2 kg cm^{-2} pressure for 11 min. All the cultures were maintained in a culture room at 25±2°C with 16 h photoperiod and a light intensity of 100 µmol/m^2/s provided by the cool-white fluorescent tubes.

3. Results and Discussion

The aim of this study was to regenerate plantlets of *A. annua* Highland clone of Vietnam origin via indirect somatic embryogenesis technique. This was achieved via several stages. The first stage involved the establishment of embryogenic callus from the leaf explants of seed-derived *in vitro* seedlings. When the leaf explants of Highland clone of *A. annua* were cultured on MS medium supplemented with 0.5 mgL^{-1} casein hydrolysate, BAP and NAA, the embryogenic calli started to form at the cut edges after two weeks of culture. Results obtained indicated that MS medium without any plant growth regulator could not induce the formation of the embryogenic callus. The presence of BAP without NAA in the MS medium also did not induce the formation of embryogenic callus from the leaf explants. The addition of NAA without BAP into the culture medium was sufficient for induction of embryogenic callus and it was found that the amount of embryogenic callus formed was positively correlated with the amount of NAA supplemented into the culture medium. The addition of BAP as low as 0.5 mg L^{-1}combined with NAA (0.5 – 3.0 mg L^{-1}) could enhance the formation of embryogenic callus between two to four folds. However, increasing amount BAP did not accelerate the production of embryogenic callus. Based on the biomass of the embryogenic callus, MS medium supplemented with 0.5 mg L^{-1} BAP and 1.5 mg L^{-1} NAA could be used for the induction of embryogenic callus from the leaf explants of *A. annua* of Highland clone (Table 1). The pre-embryogenic callus formed was green and compact in nature. This study hence indicated that cytokinin is required for enhancing the formation of embryogenic callus of *A. annua*. However, Aslam et al. (2006) reported that culture medium enriched with combination of 2,4-D and BAP could induce the formation of embryogenic callus from the leaf explants of *Artemisia scorpia,* another species of *Artemesia* genus.

At the second stage of embryogenesis process, somatic embryos normally can be induced for most plant species from the embryogenic callus when transferred onto basic culture medium without any plant growth regulator. The actively growing green compact embryogenic callus of *A. Annua* did not lead to the formation of embryos as normally expected when it was transferred to the modified MS medium without 2,4-D. Instead the embryogenic calli turned brown and became necrotic after seven days of culture. However, the embryogenic calli that were transferred onto the culture medium containing 2, 4-D produced globular embryos after 14-21 days of sub-culturing (Fig. 1A). MS medium supplemented with 0.5 mg L^{-1} casein hydrolysate and 0.5 mg L^{-1} 2,4-D was found to be the best medium for the induction of globular embryos from the embryogenic callus of *A. annua* with an average of 7.3 ± 0.6 embryos formed from the 3.0 ± 0.9 g of embryogenic callus after three weeks of culture (Table 2). Zhang *et al.* (2000) also found that the presence of 2, 4-D in the culture medium was necessary for the induction of somatic embryos in cotton. In the present study, casein hydrolysate was also added into the culture medium and it was used as nitrogen nutrient source for the induction of embryo formation from the embryogenic callus of *A. Annua.* Yuan et al. (2011) also reported that increase formation of embryos from the embryogenic callus of *Catharanthus roseus* was due to the addition of casein hydrolysate in the culture medium as it acted as the organic source of nitrogen. Nikam et al (2009) also stated that number of embryos of *Tribulus terrestris* increased with the addition of casein hydrolysate in the culture medium. But Chen and Chang (2002) reported that peptone was more effective as compared to casein hydrolysate in promoting embryo formation of Oncidium 'Gower Ramsey'.

At subsequent stage, BAP was required to be added into the MS medium for germination of the somatic embryos of *A. annua* to form shoots. The germination of embryos was obtained after two weeks of culturing in the regeneration medium, MS containing 0.5 mg L^{-1} BAP, and an average of 7.6 ± 0.6 of somatic embryos were germinated into shoots from 0.5 g of embryogenic callus (Figure 1B). Sharrma *et al*. (2010) also reported MS supplemented with 0.5 mg L^{-1} BAP supported highest shoot regeneration from somatic embryos of *Withania somnifera* L. Dunal. Rooting of the regenerated shoots was best achieved by culturing them on MS medium supplemented with 1.0 mg/L IBA and an average of 4.3 ± 0.5 roots were developed after two weeks of culture (Figure 1C). Optimum rooting response using IBA has also been reported for several medicinal plants including *Oenothera* spp. (Gyves et al., 2001) and *Mucuna pruriens* (Faisal et al., 2006).

Table 1 Effect of plant growth regulators NAA (0 - 3.0 mgL^{-1}) and BAP (0 – 2 mgL^{-1}) on pre-embryogenic callus induction (g) from the leaf explants of *Artemesia annua* Highland clones

BAP (mgL^{-1})	NAA (mgL^{-1})					
	0	0.5	1.0	1.5	2.0	3.0
0	0.00± 0.00 a	2.80±0.57 abc	1.40±0.07 ab	2.96±0.10 abcd	5.895± 0.16 cdef	6.75± 0.26 cdefgh
0.5	0.00 ± 0.00 a	8.79±0.63 efghijk	9.94±0.43 ghijklm	10.8± 0.66 jklmn	11.14± 0.77 jklmn	10.535±0.86 hijklmn
1.0	0.00 ± 0.00 a	8.73±0.55 efghijk	6.98±0.32 defghi	11.18±0.99 jklmn	11.30± 0.57 jklmn	9.65±0.36 fghijkl
1.5	0.00 ± 0.00 a	9.88± 0.20 fghijklm	13.35±0.79 jklmn	13.47±0.64 lmn	14.3±0.92 n	12.45± 0.44 klmn
2.0	0.00 ± 0.00 a	8.39± 0.62 efghij	13.69± 0.28 mn	13.77±0.31 mn	7.96± 0.69 efghij	10.85± 0.49 ijklmn
3.0	0.00 ± 0.00 a	9.89± 0.57 fghijklm	6.21± 0.9 cdefg	6.51± 0.49 cdefgh	4.92± 0.66 bcde	5.26± 0.58 bcde

Mean values followed by different alphabets were significantly different (Tukey HSD, $p \leq 0.05$)

Table 2 Effect of 2-4, D (0 – 2 mgL^{-1}) on production of embryogenic callus (g) and development of somatic embryos after three weeks of culture

2-4, D (mg L^{-1})	Mean fresh weight (g)	Mean No. of globular embryos
0.0	0.0	0.0
0.1	1.23 ± 0.06 a	5.5 ± 0.4 a
0.5	3.0 ± 0.29b	7.3 ± 0.6 b
1.0	1.56 ± 0.16 a	4 ± 0.4 a
2.0	1.31 ± 0.14 a	4.8 ±0.9 a

The regenerated plants were transplanted to small plastic pots containing soil and sand mixture and cover with plastic bags during acclimatization. After two weeks, the acclimatized plantlets were transferred to the greenhouse. Only 40% of the acclimatized plantlets survived after four weeks in the green house. The low survival percentage might be due to *A annua* being a temperate or high altitute plant was sensitive to the tropical climate of Malaysia. The survived plantlets of *A. annua* continued to grow and started to produce flowers and fruits after four months (Figure 1D). Normal flowering period was 6-8 months after transplantation on selected sites in China, Vietnam, Kenya and United Republic of Tanzani (WHO, 2006). The earlier flowering of the plantlets could be due to longer time exposure to the sunlight and hot temperature as Malaysia is a tropical country. Marches et al, (2002) also reported that variations in the flowering behaviour under the same photoperiod and temperature condition is depending on genotype and geographical origin.

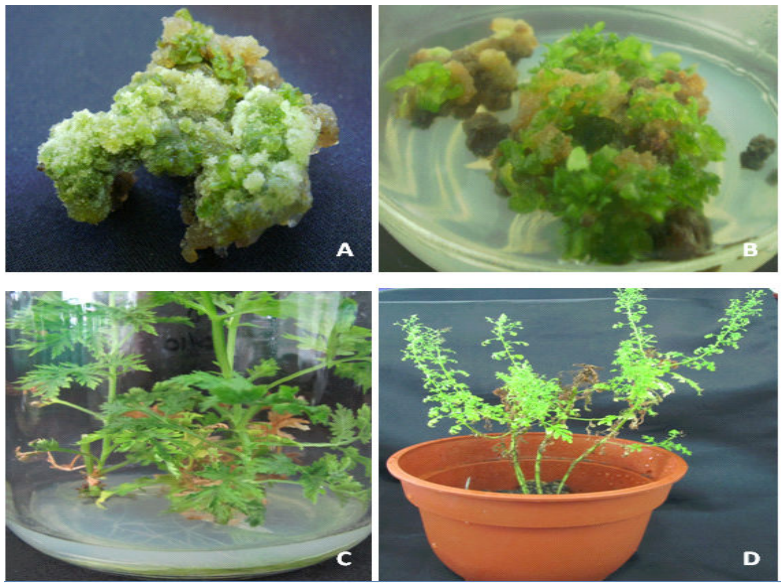

Figure 1: Plant regeneration of *Artemisia annua* via indirect somatic embryo formation **A.** Highland somatic embryos on MS supplemented with 0.5 mg/L 2, 4-D + 0.5mg/L CH ; **B**. Shoot formation on MS supplemented with 0.5 mg/L BAP; **C**. Complete plantlet on rooting medium, MS supplemented with 1.0 mg/L IBA; **D**. Acclimatized plantlet after 4 months with flowers and fruits.

4. Conclusion

Artemisia annua L. plantlets were able to regenerate via indirect embryogenesis method with the formation of somatic embryos from the pre-embryogenic callus. The induced somatioc embryos germinated to form normal plants and able to grow until mature stage producing flowers and seeds.

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