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Micropropagation of Asam Karanda (*Carissa carandas* Linn)

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ABSTRACT

An efficient micropropagation protocol was developed for *C. carandas* using nodal segment as the explants. Murashige and Skoog medium (MS), supplemented with 2.0 – 4.0 mg/L Benzylaminopurine (BA), induced an average of 3.6 - 5.5 shoots/explants but the number of multiple shoots formed was not significantly different. However, the addition of IBA combined with BA had shown a reducing trend in the number of shoot being formed. The MS medium supplemented with 2.0 mg/L 3-Indolebutyric acid (IBA) was found to be effective for rooting of micro-shoots. After the acclimatization process, 84% ± 4.3% of the *in vitro* plantlets survived but there was a reduction in the number of survived plantlets (59 ± 3.7%) after 4 weeks of incubation in the green house condition.

Keywords: Acclimatization, nodal segment, shoot multiplication, root formation

INTRODUCTION

Carissa carandas Linn. (Family – Apocynaceae) is an important medicinal perennial shrub growing to a height of 2 to 3 meters. The species is native to India and distributed in Sri Lanka, Indonesia, Malaysia, Myanmar and Pakistan (Hegde *et al.,* 2009). The fruit are traditionally used in the treatments of malaria, epilepsy, nerve disorder, relieve of pain and headache, fever, blood purifier, myopatic spams, dog bite, cough, colds, itches and leprosy (Rahmatullah *et al.,* 2009; Warrier *et al.,* 1993). The ripe fruit are used as appetizer for the prevention of scurvy and in the treatments of anorexia, burning sensation, skin diseases and pruritus. Meanwhile, the roots are used as anthelmintic, stomachic and antiscorbutic agents and for the treatments of intestinal worm, scabies and pruritus (Warrier *et al.,* 1993). The green fruit of *C. carandas* are also used for making pickles in India (Sturock, 1959).

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The plant extract of *C. carandas* has been reported to possess cardioprotective, antipyretic and antiviral activities (Dhawan & Patnaik, 1985; Taylor *et al.,* 1996; Rajasekaran *et al.,* 1999). It is also a potential source of anthocyanin and used as a natural colouring agent for products that require mild processing treatment and low temperature storage (Iyer &Dubash, 1993).

Conventionally, *C. carandas* can be propagated through seeds, cuttings, grafting, air layering, and stooling (Misra & Jaiswal, 1993; Misra & Singh, 1990; Tyagi *et al.,* 1999). However, these methods are season-specific and require a long time to propagate. In the recent years, there has been an increased interest in the use of *in vitro* culture technique as a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora & Bhojwani, 1989; Sharma *et al.*, 1991; Sudha & Seeni, 1994). Attempt has been made for the micropropagation of *C. carandas* by using shoot tips as explants (Rai & Misra, 2005) and our experiences on shoot culture involved severe contamination problem and difficulty in establishing aseptic cultures. Hence, the present study reports on the establishment of an efficient micropropagation protocol of *C. carandas* using nodal segment as the explants.

MATERIALS AND METHODS

Establishment of Aseptic Seedlings

Mature fruit of *C. carandas* were collected from Gelugor area, Penang, Malaysia. They were washed with a combination of detergent and Clorox®, a commercial bleach containing 5.3% Sodium hypochlorite, and also rinsed under running tap water for 30 minutes to remove any soil contaminants. Two different methods of surface sterilization were used. The fruit were immersed in 95% ethanol for 2 sec and then flamed for 10 sec. This process was repeated twice. In another method, the fruit were immersed with continuous agitation in 20% Clorox® for 20 minutes, followed by rinsing them three times with sterile distilled water. The fruit were subsequently surface-sterilized with 10 % Clorox® solution for 10 minutes, followed by rinsing them three times with sterile distilled water.

The seeds were removed from the fruits and inoculated in 350 ml culture bottle (with 5 seeds in each culture bottle) containing basic Murashige and Skoog (1962) medium (MS) for germination. Seven replicates (experimental units) were used for each sterilization treatment and the experiment was repeated four times. The percentages of seed germination and survival were determined after 4 weeks of culture and the data were analyzed using Student t-test at $p \leq 0.05$. The best sterilization method was selected for subsequent experiments.

The nodal segments of the six–week old seedlings were used as the explants for the subsequent studies. All the cultures were maintained at 25 ± 2°C in a culture room with a continuous lighting provided from cool white fluorescent tubes at 35µmol m^{-2} s^{-1}.

Induction of Multiple Shoot Formation

The nodal segments (1.0 cm) of the aseptic seedlings were inoculated in a 350 ml culture bottle containing MS medium which was supplemented with different concentrations of Benzylaminopurine (BA) (0.0, 1.0, 2.0, 3.0 and 4.0 mg/ L) and 3-Indolebutyric acid (IBA) (0, 1, and 2 mg/L). A total of five nodal segments were inoculated into each culture vessel and three vessels were used for each treatment combination using 5 x 3 factorial with a complete randomized design. The numbers of shoots and roots regenerated from each explant were recorded after 8 weeks of culture. The data for each parameter were analyzed using One-Way ANOVA, and this was followed by mean comparison using Tukey test at $p \leq 0.05$, with the aid of SPSS ver. 17.

In vitro Rooting of Micro-shoots

Multiple shoots of *C. carandas* were separated into single shoot and were sub-cultured on MS medium supplemented with 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L of IBA. In more specific, five shoots were cultured into each culture vessel and each treatment was done in three replicates with a complete randomized design. The number of roots produced from each micro-shoot was recorded after six weeks of culture and the data were analyzed using the One-Way ANOVA, followed by the mean comparison using Tukey test at $p \leq 0.05$.

Acclimatization

The eight-week old rooted *in vitro* plantlets of *C. carandas* with shoot height of 3-5 cm were removed from the culture bottle and washed thoroughly under running tap water before they were transplanted into a plastic tray (30 cm x 20 cm) containing a mixture of top soil, organic soil and sand (1:1:1). The trays were covered with a plastic sheet to create a relative humidity of 80- 90%, and they were then placed in the green house with a day temperature of 28 ± 2ºC and a night temperature of 24 ± 2ºC. After one week, the plants were transferred into poly-bags and the percentage of the surviving plantlets was recorded after four weeks in the plant house condition. The plantlets were watered with tap water twice a day (morning and evening).

RESULTS AND DISCUSSION

The results obtained in this study indicated that 100% of the aseptic seeds could be established by flaming mature fruit or

TABLE 1
The establishment of the aseptic seedlings of *C. carandas* and their germination percentage

Sterilization Technique	Aseptic seeds (%)	Seed Germination (%) ± s.e
Double surface-sterilization with Clorox®	100 a	81.1 ± 5.5 b
Immersed in 95% ethanol and flame.	100 a	81.1 ± 4.9 b

Mean values within the same column followed by same alphabet are not significantly different (Tukey's HSD test, $p \leq 0.05$)

double surface-sterilization with Clorox®. More than 80% of the aseptic seeds of *C. carandas,* which were germinated using either of methods of sterilization, were found to be insignificantly different (Table 1).

The results also revealed that the shoot proliferation of *C. carandas* was affected by the addition of plant growth regulator auxin and cytokinin such as BA and IBA. The MS medium, which was supplemented with different concentrations of BA, induced multiple shoot formations from the nodal segments of *C. carandas*. The results also indicated that as the amount of BA added into the medium increased, the number of shoots produced would also increase. The MS medium supplemented with 2 – 4.0 mg/L BA induced an average of 3.6-5.5 shoots/explants but the number of the multiple shoots formed was not significantly different. However, the addition of IBA (1.0 and 2.0 mg/L) with the presence of BA showed a reducing trend in the number of the shoots formed from each explant (Table 2). Gomes *et al.* (2010) also found that the addition of NAA into the MS medium in combination with different types of cytokinin was unable to increase the number of shoots of *Arbutus unedo*. Since the MS medium supplemented with 2.0 mg/L BA was sufficient for the induction of multiple

TABLE 2
The effects of BA and IBA supplemented into the MS medium on the *in vitro* growth of *C. carandas* after 8 weeks of culture

Treatment (mg/L)	Number of shoots (n ± s.e)	Length of shoots (cm ± s.e)	Number of roots (n ± s.e)
0 IBA 0 BA	1.6 ± 0.2 cd	0.9 ± 0.1 b	0 ± 0 b
0 IBA 1 BA	2.3 ± 0.2 bcd	2.8 ± 0.7 ab	0 ± 0 b
0 IBA 2 BA	3.6 ± 0.6 abcd	3.3 ± 0.5 a	0 ± 0 b
0 IBA 3 BA	4.2 ± 0.7 ab	2.8 ± 0.5 ab	0 ± 0 b
0 IBA 4 BA	5.5 ± 0.9 a	2.5 ± 0.2 ab	0 ± 0 b
1 IBA 0 BA	1.3 ± 0.2 d	2.2 ± 0.7 ab	2.6 ± 1.2 b
1 IBA 1 BA	2.1 ± 0.4 bcd	3.5 ± 0.4 a	0 ± 0 b
1 IBA 2 BA	3.1 ± 0.7 bcd	3.1 ± 0.3 a	0 ± 0 b
1 IBA 3 BA	2.6 ± 0.3 bcd	2.1 ± 0.2 ab	0 ± 0 b
1 IBA 4 BA	3.7 ± 0.5 abc	1.8 ± 0.3 ab	0 ± 0 b
2 IBA 0 BA	1.3 ± 0.2 d	2.9 ± 0.6 ab	6.6 ± 1.9 a
2 IBA 1 BA	1.5 ± 0.6 cd	1.7 ± 0.5 ab	0 ± 0 b
2 IBA 2 BA	2.7 ± 0.4 bcd	2.3 ± 0.3 ab	0 ± 0 b
2 IBA 3 BA	2.0 ± 0.5 bcd	1.9 ± 0.6 ab	0 ± 0 b
2 IBA 4 BA	3.6 ± 0.6 abcd	2.6 ± 0.3 ab	0 ± 0 b

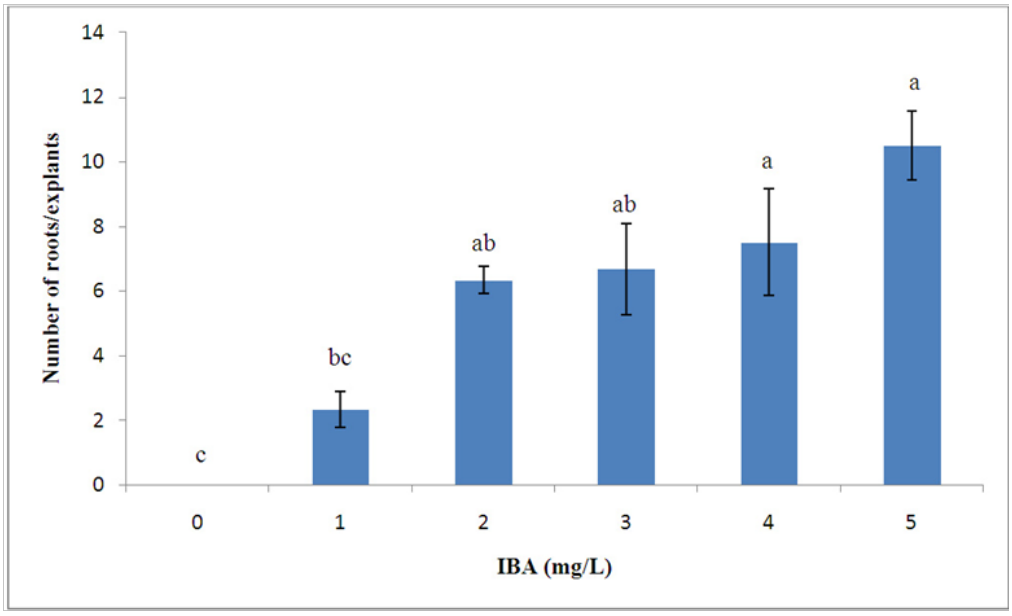
Mean values within the same column, followed by same alphabet, are not significantly different (Tukey's HSD test, $p \leq 0.05$).

shoots formation, it was used as the shoot proliferation medium for mass production of *C. carandas*. Rai and Misra (2005) also found that the presence of only cytokinin (13.33 µM BA) in the MS medium could induce the formation of high number of shoots (12.5 shoot/explants) in *C. carandas* but using shoot tips as the explants. Hence, this finding indicates that the requirement of plant growth regulators is dependent upon the type of explants, genotype and plant species. For instance, Oliveira *et al.* (2010) reported that different plant growth regulators gave different effects on the shoot growth of *Melaleuca alternifolia*.

The results obtained in the current study indicated that the MS medium supplemented with different concentrations of BA and IBA did not affect the height of the shoots. There was a high variation in the shoot height when they were cultured in the BA and IBA combination treatment. Meanwhile, a reasonable good shoot height (3.5 cm) was obtained in the MS medium supplemented with 1.0 mg/L IBA and 1.0 mg/L BA and MS plus 1.0 mg/L IBA and 2.0 mg/L BA (3.1 cm), as shown in Table 2.

The addition of BA in the medium inhibited the root formation in *C. carandas* and callus, and they appeared at the base of

Fig.1: Callus appeared at the base of the micro-shoots of *C. carandas* cultured on the MS medium containing 2.0 mg/L BA



Mean values with the same alphabet are not significantly different (Tukey's HSD test, $p \leq 0.05$)

Fig.2: The effects of different concentrations of IBA supplemented in the MS Medium on the rooting of *C. carandas*

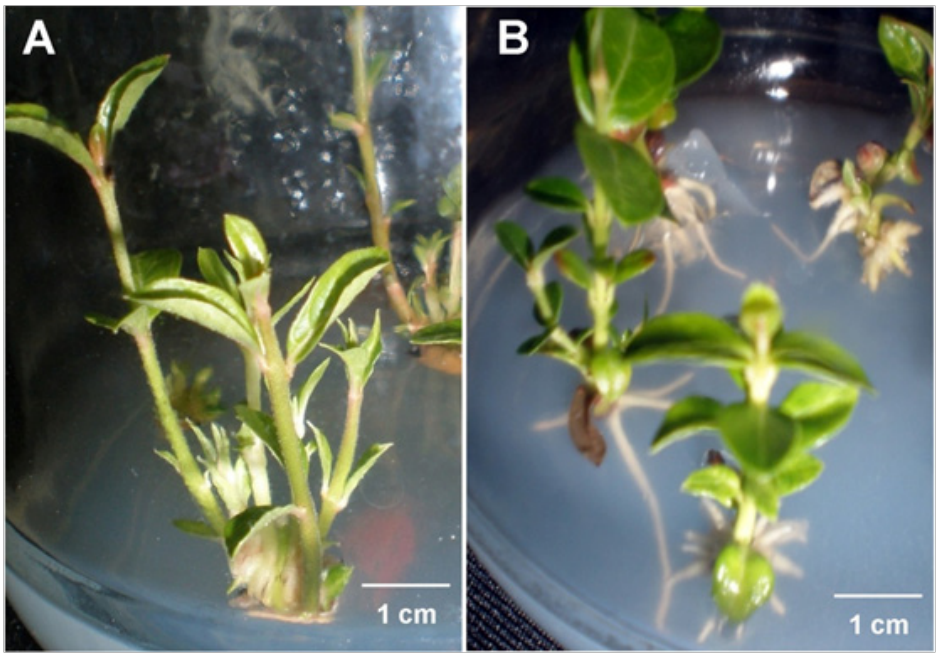


Fig.3: *In vitro* cultures of *C. Carandas;* (A) Multiple shoots on the MS medium supplemented with 3.0 mg/L BA (B) The rooting of micro-shoots on the MS medium supplemented with 2.0 mg/L IBA.

the micro-shoots when cultured in the MS medium containing different concentrations of BA (Fig.1). A similar result was observed for *Dendrobium candidum*, whereby the addition of BA inhibited root formation (Wang *et al.,* 1997). On the other hand, the addition of IBA was found to be effective for the root induction in *C. carandas*. MS medium supplemented with 2 mg/L IBA inducing the highest number of roots (6.6 roots/ shoot) (Table 2). Additional study indicated that the addition of more than 2.0 mg/L (2- 5.0 mg/L) of IBA into the MS medium induced the roots but not significantly different in the number of roots produced (Fig.2). Therefore, MS supplemented with 2 mg/L IBA was chosen as the rooting medium for the micro-shoots separated from the multiple shoots (Fig.3A) for the production of *in vitro* plantlets (Fig.3B).

The *in vitro* plantlets of *C. carandas* could survive well (84 ± 4.3%) when they were acclimatized in the plastic tray covered with transparent plastic sheet. However, only 59 ± 3.7% of the plantlets survived after four weeks of being transferred to the poly-bags and placed in the plant house. These results indicate that plantlets cannot adapt well with the outside environment. Low humidity and full exposure to light may be the main cause for the lower survival rate of *C. carandas* plantlets under green house condition. Nonetheless, the plantlets that survived did not show any morphological abnormalities (Fig.4).

Fig.4: Four-week old acclimatized plantlets of *C. carandas*

CONCLUSION

The present *in vitro* propagation protocol using MS medium supplemented with 3.0 mg/L BA was found to be effective for the mass multiplication of *C. carandas* shoots. However, the addition of 2.0 mg/L IBA into the culture medium was required for the rooting of the micro-shoots. The established *in vitro* propagation protocol could be used as an alternative for the propagation of *C. carandas* plantlets.

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