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# Indirect organogenesis in milkweed (*Calotropis procera*) from mature zygotic embryo explants

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**Abstract**

Milkweed (*Calotropis procera*) is a valuable medicinal plant which grows in many regions of Iran. Its significant medicinal properties have made it an important crop which is cultivated commercially. This plant is propagated from seeds as well as root and shoot cuttings. Due to problems in the usage of these reproduction methods, new propagation methods such as tissue culturing should be developed. This study was aimed at obtaining appropriate concentrations of plant hormones for indirect organogenesis of milkweed. The experiment was arranged in a completely randomized design (CRD) with 3 replications. The effects of various concentrations of (2,4-dichlorophenoxyacetic acid) 2,4-D (0.1, 0.5, 1, 2 and 3 mg/l) were studied in terms of callus induction and shoot regeneration on an MS based medium supplemented with BA (benzyl amino purine) and NAA (naphthalene acetic acid) at the same concentration. Mature embryos were used as explants and morphological traits such as embryo size, callus size, number and size of shoots and roots were recorded. The results showed that 2,4-D significantly increased the size of cultured embryos ($P < 0.05$). The largest embryo volume was observed in cultures treated with 3 mg/l 2,4-D. The highest callusing was recorded in 2 mg/l 2,4-D. The effects of BA and NAA concentrations on shoot regeneration were significant and the highest values were observed for a combination of 1 mg/l BA and 2 mg/l NAA. 1 mg/l IBA (Indole 3-butyric acid) was able to induce the highest number of better quality roots and shoots.

**Key words:** milkweed, BA, NAA, callus formation and rooting

**Abbreviations:**

MS – Murashige and Skoog
2,4-D – 2,4-dichlorophenoxyacetic acid
IBA – indole 3-butyric acid
NAA – naphthalene acetic acid
BA – benzyl amino purine

## Introduction

Milkweed (*Caltropis procera*) is a species of flowering plant belonging to the Apocynaceae family native to North Africa, Indochina, Western Asia, South Asia, and Tropical Africa (Grace, 2006). Common names for the plant include the apple of Sodom, Sodom apple, Stabragh, Kapok tree, King's crown, rubber bush, or rubber tree, while native people named this plant Karak, Estabragh or Bit apple. Milkweed is distributed across a large portion of Iran, including Southern Khorasan, Sistan and Baluchestan, Khuzestan and Hormozgan provinces. Milkweed has some desired agricultural characteristics, for instance drought and high temperature resistance, and salt tolerance. For a long time, native people used this plant for healing diseases. Latex extracted from milkweed shows anthelmintic (Igbal et al., 2005), anti-inflammatory (Alencar et al., 2006), antioxidant (Chavda et al., 2010), and anti-cancer activities (Magalhães et al., 2010). The large number of compounds obtained from milkweed include cardenolide glycosides such as proceraside, calotropin and frugoside with anti-cancer activity confirmed by research study (Van Quaquebeke et al., 2005). As a result of medicinal qualities, this plant is highly valuable for the pharmaceutical industry (Iqbal et al., 2005). Moreover, based on some studies, hydrocarbonic compounds from latex extraction from milk-

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weed can be used as a new source for renewable energy production (Erdman et al., 1981). Recently, due to its anti-cancer activity there is an increased interest in laboratory research on this plant material.

Natural regeneration of milkweed occurs through seeds; however, as a result of breeding, seedlings are extremely heterozygous. Tissue cultures, used as an alternative method, play an important role in the rapid propagation of medicinal plants. They may also be used to enhance the amount of secondary metabolites obtained from milkweed by mass propagation of this plant. Leakage of phenolic compounds to tissue culture media that causes browning and growth suppression of explants is one of the important problems occurring during micropropagation of milkweed-type plants. To overcome this problem, explants with the lowest latex content must be used. Immature embryos and hypocotyls are good sources for micropropagation of these plants due to their high totipotency and low latex content. Some researchers have obtained these types of explants from various plants such as Euphorbia (Kengar et al., 2015), Araujia (Torné et al., 1997), Thlophora (Lee et al., 1982), Hevea (Wilson et al., 1975), *Vigna mungo* (Srivastava et al., 2011) and *Tylophora indica* (Rao et al., 1970). In this research, we studied the effects of plant growth regulators on milkweed tissue cultures and on the optimization of the micropropagation of this plant.

## Materials and methods

### *Plant material*

Healthy milkweed plants growing around Jiroft city, in Kerman province, Iran, were used as a seed source. Fruits without any physiological disorders or disease symptoms were collected and kept at 28°C. After transfer to the laboratory, fruits were opened and before use healthy seeds were cleaned of silk fibers.

### *Embryo germination and explant production*

Healthy embryos of *C. procera* were extracted from seeds. First, seeds were washed with tap water, and then embedded in sterile water for 24 hours. In the next step, seeds were sterilized with 70% ethanol for 1 minute and thereafter embedded in 2.5% sodium chloride with 0.2% Tween 20 for 30 min, followed by 3 rinses in water. Finally, seed covers were carefully removed and embryos were placed in distilled water. Five to 7 embryos were cultured in glass flasks containing 25 ml of MS macro and micro-nutrients and vitamins (Merck), supplemented with 0.1, 0.5, 1, 2 or 3 mg/l 2,4-D (Sigma).

### *Callus induction*

Hypocotyls were produced from germinated embryos after 45 days of cultivation and were used for callus production. Hypocotyls were dissected to 3 mm sections and transferred into an MS medium with the following modifications: ammonium nitrate (2000 mg/l), potassium nitrate (2400 mg/l), calcium chloride (600 mg/l), thiamin (4 mg/l) and myo-inositol (300 mg/l), sucrose (30 g/l), 3% activated charcoal (pH 5.7 ± 0.2) and supplemented with 0.1, 0.5, 1, 2 and 3 mg/l NAA. Explants were maintained in the dark at 26°C for 30 days. Each flask with 5 to 7 explants was considered to be 1 replicate, and for each experiment 5 replicates were performed. Hypocotyl explants were cultured on an MS medium supplemented with different NAA concentrations. Callus induction commenced with enlargement of the exposed surfaces and cut regions of the hypocotyls after about 25 days. The induced calli were subcultured every 30 days and subculturing was performed 4 times.

### *Shoot induction and proliferation*

Callus segments of about 150 to 200 mg were cultured on a shoot production medium containing MS salts and vitamins supplemented with different combinations of NAA (0.1, 0.5, 1, 2 and 3 mg/l) and BA (0.1, 0.5, 1, 2 and 3 mg/l). Culture vessels were kept at 16/8 light and dark with cool white fluorescent lamps at 16-18 $\mu mol/m^2/s$ light intensity. The culture room temperature was kept at 26°C.

### *Root induction and acclimation*

The induced shoots were transferred to a rooting medium containing ½ MS salts either with or without IBA (indole 3-butyric acid) at 0.1, 0.5, 1, 2 and 3 mg/l and after 30-days, the number of roots per explant, root length and root weights were recorded. Rooted plantlets were taken out from culture tubes and washed thoroughly with tap water to remove any remaining culture medium from the plants. The washed plantlets were cultured in 40:60 (v/v) sterilized peat moss and sand, respectively, in a glasshouse with 85% RH (relative humidity) at 25°C.

***Statistical analysis***

Statistical analyses were performed using SPSS software, version 22. Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made with the least significant difference (LSD) test calculated at the $P < 0.05$ confidence level.

## Results and discussion

***Effect of 2,4-D on growth and size of embryos***

The analysis of variation showed that 2,4-D had a significant effect on increasing the size of embryos and hypocotyl production. Embryo enlargement occurred longitudinally. No significant increase in the traverse section of explants was observed. After 15 days, hypocotyls were produced on lateral sections of explants. Among the different 2,4-D concentrations tested, 3 mg/l exhibited the best results and gave maximum sizes of hypocotyls. About 40% of embryos did not germinate and were discarded; healthy and greenish-enlarged hypocotyls were selected to perform callus induction. The use of mature zygotic embryos as initial explants has been reported in many studies performed by various researchers. For example, Ismael Rocha and coworkers (2015) used mature embryo explants to micropropagate passion fruit (*Passiflora edulis* Sims). In another study, immature embryos were used for passion fruit micropropagation without the addition of plant growth regulators (Ferreira et al., 2015). Soorni and Kahrizi (2015) indicated that in spite of the technical ease of callus production from seeds, faster callus production was obtained using mature embryos instead of seed explants. In this study, embryos were successfully used to perform shoot organogenesis and somatic embryogenesis.

***Effect of PGRs on callus induction***

An MS medium containing 2 mg/l NAA induced an average of 4.5 g callus per explant, and we concluded that this was the best medium for callus production (Fig. 1). In fact, with NAA concentration increasing to 2 mg/l we observed increased callus production and this treatment gave globular light green calli. With a further increase in NAA concentration, callus mass decreased and we observed the formation of soft watery calli, light brown in color. These calli were slow-growing and their regeneration was not observed on a shoot induction

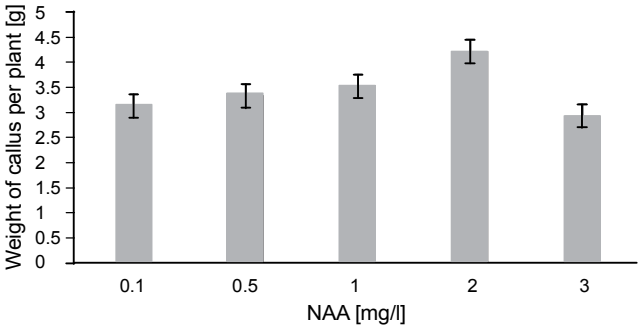


**Fig. 1.** Effect of NAA concentration on callus induction and proliferation in milkweed

medium. Various responses to different concentrations of auxins have previously been reported in other studies (Cavusoglu et al., 2011). Kengar and Paratkar (2015) studied the effect of 2,4-D concentration on indirect organogenesis of *Ruta graveolens*. L. In this study, a maximum callus proliferation was achieved with the addition of 4.2 μM 2,4-D. In another study, Sirvastava and Pandy (2011) obtained a maximum callus proliferation on an MS medium supplemented with 6 μM of this plant growth regulator (PGR). Since plants are sources of various secondary metabolites used in many fields such as pharmaceuticals, agrochemicals, food additives, biopesticides, flavors, fragrances and colors, callus induction is a useful approach for the large-scale production of secondary metabolites (Abyari et al., 2016).

***Effect of NAA and BA on shoot induction and proliferation***

To identify the optimum balance between NAA and BA for shoot induction and proliferation, different combinations of these 2 plant growth regulators were tested. Shoot induction was not observed on an MS medium without PGRs. The effect of interaction between NAA and BA concentrations was significant at $P < 0.01$ (Table 1) and it was observed that BA at 1 mg/l in combination with 2 mg/l NAA resulted in better shoot regeneration and the highest amount of shoot formation per explant (Fig. 2D). Similar results were reported by Kengar and Paratkar (2015). They used petiole and internodal segments of stems for callus induction and then used these calli for shoot regeneration. Also, Uzun and coworkers (2014) studied shoot regeneration in the endemic plant *Muscari muscarimi* Medik. and they reported similar results. The authors obtained the highest number of shoots per explant on a (MS) medium supple-

**Table 1.** The influence of different PGRs on shoot induction and proliferation in milkweed. Statistical analysis was performed using analysis of variance (ANOVA)

| Source | Type II sum of squares | df | Mean square |
|---|---|---|---|
| NAA | 40.894 | 4 | 10.224* |
| BA | 21.458 | 4 | 5.365** |
| NAA*BA | 17.239 | 16 | 1.077** |
| Error | 0.793 | 50 | 0.016 |
| CV | 3% | 35% | 16% |

* Significant at ($P < 0.05$) and ** ($P < 0.01$)

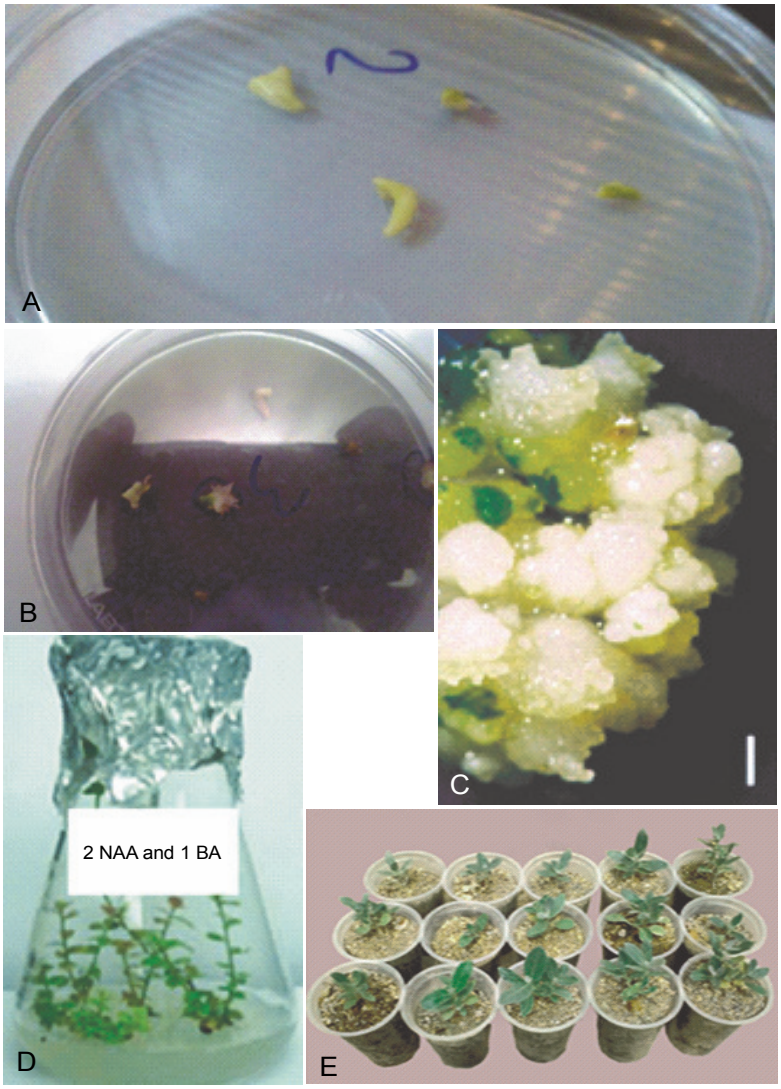


**Fig. 2.** Micropropagation stages of milkweed: A) enlarged embryo after 10-days of culture in MS with 3 mg/l 2,4-D, B) callus induction, C) calli produced from hypocotyl segments, D) shoot induction and proliferation in MS with 2 mg/l NAA and 1 mg/l BA, and E) 3-month-old plants grown in a greenhouse

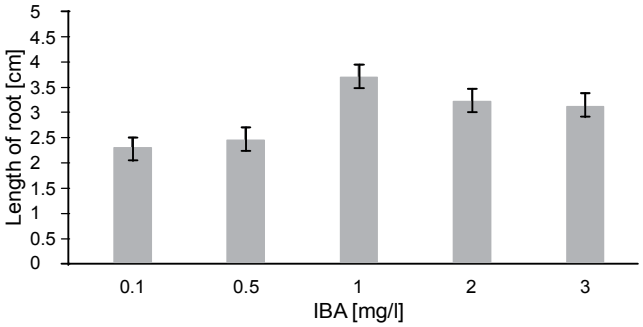


**Fig. 3.** Effect of IBA concentration on root induction

mented with 0.5 mg/l thidiazuron (TDZ) plus 0.5 mg/l (NAA) and 1 mg/l TDZ plus 0.5 mg/l NAA. One important step in any micropropagation study is shoot induction and proliferation. A tissue culture technique must produce the maximal number of plantlets in the shortest possible time. Several factors affect shoot induction and proliferation rates in plants; therefore, the hormonal balance between auxins and cytokinins is the most important factor. In tissue culture experiments, cytokinins appear to be necessary for cell division (Susheela et al., 2016).

***Effect of IBA on root induction***

The effect of IBA on rooting was significant and the best results were observed when the medium was supplemented with 1 mg/l of IBA. Although rooting occurred in ½MS medium (control) without any PGR, the best response was obtained on an MS medium supplemented with IBA. Rooting was observed in controls but when compared to the roots developed on a medium supplemented with 1 mg/l IBA, it turned out to be weaker. Increasing the concentrations of IBA on the rooting medium from 1 to 3 mg/l had no significant effect on root length or root mass production, thus it is not recommended to use more than 1 mg/l NAA in a rooting medium (Fig. 3). Other researchers have reported similar results in other plant species. For example, Roy and coworkers (2000) evaluated the effect of different concentrations of IAA and IBA on *Calotropis gigantean* rooting and reported that root mass increased with an increase in IAA concentration from 0 (control) to 2.45 μM, on a medium additionally supplemented with 0.49 μM IBA. Above that IAA concentration, the formation of healthy roots was decreased. The effect of IBA and NAA on the rooting of *Dendrobium nobile* var. Emma White was evaluated in a study by Asghar and coworkers (2011). These authors used both NAA and IBA at 0.5, 1, 1.5, 2, 2.5 and 3 mg/l and reported the higher rooting percentage, maximum number of roots and the greatest root length was achieved by MS medium supplemented with 1.5 mg/l NAA or 2 mg/l IAA.

## Conclusions

A system to regenerate *C. procera* from cotyledon segments derived from a mature zygotic embryo was established for the first time. Zygotic embryos are suitable for micropropagation of this plant. This protocol can be used to produce secondary metabolites from a callus or for large scale commercial propagation. This protocol may permit *in vitro* conservation of this important species.

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