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RESEARCH ARTICLE

Efficient regeneration from cotyledonary leaf explants of a medicinal herb *Corynandra chelidonii* var. *pallae* (Angiosperms: Cleomaceae)

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Abstract

Corynandra chelidonii var. *pallae* (Angisperms: Eudicots: Malvids) is a rare, endemic, and medicinally useful herb with gradually declining natural populations over the years. It was chosen for *in-vitro* regeneration in this study. An efficient protocol was developed using cotyledonary leaf explants from 10 to12 day-old *in-vitro* grown seedlings. The explants were inoculated on a shoot induction medium containing different concentrations with a combination of BAP, IAA, NAA, and coconut water. High efficiency shoot regeneration (21.62 ± 0.35^{a}) was achieved in cotyledonary leaf with MS medium supplemented with 2.0 mg/l BAP 0.2 mg/l NAA + 10% coconut water. The response obtained was highest in shoot elongation on MS medium with 2.0mg/l GA3 and 0.5 mg/l TDZ, with a 5.78 cm average shoot length. The MS medium supplemented with 1.0 mg/l IBA was found the best for root regeneration from *in-vitro* developed shoots. The regenerated plantlets with the well-developed shoot and root system have acclimatized successfully. The above mentioned protocol could be successfully used for conservation and for large-scale multiplication; and is the first of its kind in this taxon.

Keywords: *Corynandra chelidonii*, Leaf, Direct regeneration, Coconut water, Thidiazuron.

Introduction

The medicinal and aromatic plants play prime role in the socio-cultural, spiritual, and healthcare needs of rural folk and ethnic people of the world. In emerging and developing countries, a large section of the population still relies on traditional medicine systems. Besides, more and more global people are turning towards complementary and alternative therapies, resulting in a manifold increase in the demand for medicinal plants and their products in the developed world.

The Cleomaceae (Rosids, Eudicots, Angiospermae) of the order Brassicales are a widespread family in the tropics with several known medical species. The genus *Cleome* L., with its segregate genera, are claimed to offer medicinal and economic benefits. Examples are the studies on antidiabetic plant *Cleome droserifolia* (Hegazy and Fadl-Allah 1995), anthelminthic, autotoxicity' and antibacterial activities of aqueous and ethanolic extracts of *Cleome* species (Perumalsamy and Raja 1996; Khafagi 1998), and antibacterial activity of 16 ethnomedicinal plant species including *Corynandra viscosa* (*Cleome gynandra*) (Samy *et al*. 1999; Vijayakumar *et al*. 2005). *Corynandra* Schard. ex Spreng. is now an accepted segregate genus from *Cleome* L. (Cochrane and Iltis 2014). Accordingly, the common tropical species like *Cleome felina* L.f, *Cleome chelidonii* L.f. and *Cleome viscosa* L. all now belong to genus *Corynandra. C. chelidonii* is generally known to be used to treat headache, dysentery, colic, otitis and rheumatism (Kirtikar and Basu 1991). Recently, it was established that *Coynandra chelidonii* var. *pallae* (C.S.Reddy & V.S.Raju) V.S.Raju is distinct taxon known for its medicinal and economic uses (Sirangi *et al.* 2020). It was originally described as a new variety under *Cleome* from the littoral waters of Pakhal reservoir (Pakhal Forest Reserve), Warangal district, northern Telangana, India (Reddy and Raju 2001). This herb is locally called *'adavi avalu'*, and the seeds are used as a condiment, like mustard. This taxon has ethnomedicinal, economic and ecological uses. *Corynandra chelidonii* and *C. viscosa* contain glucocleomin and glucocapparin. *Cleome chelidonii* produced glucosinolates

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from callus suspension cultures (Songsak and Lockwood 2004). Micropropagation of endemic taxon *Corynandra chelidonii* var. *pallae* (Cleomaceae) through nodal explants was carried out by Srirangi *et al.* (2021).

Corynandra chelidonii var. *pallae* grows in littoral zone of water bodies with 3–5–6 foliate leaves, pink flowers, and 2–3 inch pods. It is endemic and not widespread, with intermittent sterility and low seed set (Srirangi *et al.* 2020). Its populations are on a steady decline due to the gradual disappearance of lentic water bodies, with the land-use shift. Therefore, it was contemplated to do micropropagation through *in-vitro* techniques in order to multiply and conserve the germplasm of this medicinally important taxon.

Materials and Methods

Plant Material

The specimens and seeds of *Corynandra chelidonii* var. *pallae* were collected from the districts of Warangal Rural and Mahabubabad (Pakhal RF, Pakhal tank), northern Telangana, India (17.9526°N; 80.0060°E). The taxon was identified and authenticated by Professor Vatsavaya S. Raju, Plant Systematics Laboratory, Kakatiya University, Warangal. The voucher specimens were deposited in KUW (Kakatiya University Herbarium, Warangal, India). The seeds were allowed to germinate in the laboratory. The cotyledonary leaf explants were collected from 10-12 day old aseptically grown seedlings and used for culture studies.

In-vitro seed germination

The seeds were surface sterilized with 0.1 % (w/v) HgCl2 for 5 minutes and thoroughly washed thrice with sterile distilled water. These seeds were inoculated on MS (Murashige and Skoog 1962) medium with different concentrations (15–30g/L) of sucrose without growth regulators. The pH of the medium was adjusted to 5.7 with either 0.1 N HCl or 0.1N NaOH before the addition of agar. The medium was solidified with 0.8% (W/V) Difco bacto-agar and later dispensed into different culture bottles/vessels to be autoclaved at 121°C under 15 lbs pressure for 15-20 minutes. The seeds were gently inoculated on the medium. Different methods were employed to break seed dormancy (pre-heating at 35-40°C temperature; pre-chilling at 0-50°C temperature for various periods; and the paper boat technique) but all procedures were ineffective. Each replicate with 50 seeds were soaked in 1-5 mg/ml concentration of GA3. The first replicate was kept under 24 hr dark, second at 20/4 LD and third at 6/18 LD, and fourth at 18/6 LD photoperiod. All these cultures were incubated at 25 ± 2°C temperature and light intensity of 40 µ mol/m^2/ s^2 by white fluorescent tubes.

Plant Regeneration from Cotyledonary Leaf Explants

To optimize the culture medium for high-frequency shoot regeneration, *in-vitro* grown cotyledonary leaf explants were excised (0.5-1.0 cm) from seedlings, these were cultured on Murashige and Skoog's basal medium supplemented with the various concentrations of BAP, Kin, TDZ, IAA, NAA (mg/l) and coconut water, prepared for each combination of plant growth regulators (Table 1) to achieve high-frequency plant regeneration.

The culture media used were adjusted to 5.6 to 5.8 pH by using 1N NaOH and HCl before adding agar-agar. The media were poured into culture vessels and sterilized at 15 lb pressure for 20 min in an autoclave. The explants were evaluated for a mean number of shoots per explants, and mean shoot length (cm) after inoculation. The culture vessels were kept in the culture room at 25 ± 20^0 C under a 16/8 h (LD) photoperiod, with cool white fluorescent lamps (40 µ mol/m^2/ s^2 with 70-80% humidity.

Shoot Elongation from *in-vitro* developed shoots

The *in-vitro* raised shoots from cotyledonary leaf were sub-cultured on shoot elongation media containing different concentrations of GA_3 (1-3) mg/l in combination with various concentrations of BAP, Kin and TDZ.

Root formation from *in-vitro* developed shoots

Regenerated shoots (about 2 to 3 cm long) obtained from cotyledonary leaf, excised, and cultured separately on full and half strength MS medium supplemented with various concentrations auxins IAA and IBA for root induction to achieve complete plantlets. After the root regeneration, root induction (%), the mean number of roots per explant and root length (cm) were calculated.

Acclimatization of regenerated plantlets

The regenerated plantlets carefully removed from the culture tubes, were washed gently under tap water to remove traces of adhering medium for few minutes to avoid initial wilting while transferring to soil in the sterilized coco peat-containing pots. The pots were covered with polyethylene bags in order to maintain optimum relative humidity under greenhouse conditions. The plants showed initial signs of establishment in pots in 10 to 12 days enabling initiation of new leaves. It was time to remove the polyethylene bags temporarily for a few hours daily. The plants were finally transferred to earthen pots containing soil, peat, and vermin-compost in a 1:1:1 ratio. The survival percentage of the hardened plants was recorded after four weeks of transfer to the pots.

Data Analysis

Each treatment consisted of at least 15 tubes with cotyledonary leaf explant. Each experiment was repeated thrice. The data recorded were as number of shoots and mean number of roots and their respective lengths. The mean values of the repeated experiments were compared by DMRT test at 5% level of significance using statistical software SPSS Ver.23.

Table 1: Response of cotyledonary leaf explant of *C. chelidonii* var. *pallae* on MS medium optimized with different concentration of BAP, Kin and TDZ alone and in combination with NAA, IAA and coconut water for multiple shoot induction.

Concentration of PGR (mg/l)						*Cotyledonary leaf explant*	
BAP	*KN*	*TDZ*	*IAA*	*NAA*	*Coconut water (%)*	*Mean no. of shoots/explants ± SE*	*Mean Shoot Length (cm) ± SE*
0.5	-	-	-	-	-	1.15 ± 0.24^{f}	1.12 ± 0.0614^{dc}
1.0	-	-	-	-	-	1.23 ± 0.23^{g}	1.13 ± 0.042^{de}
1.5	-	-	-	-	-	1.46 ± 0.14^{f}	1.08 ± 0.0119^{d}
2.0	-	-	-	-	-	2.23 ± 0.12^{d}	1.16 ± 0.080^{bcd}
2.0	-	-	0.5	-	-	3.54 ± 0.21^{de}	1.23 ± 0.078^{cd}
2.0	-	-	1.0	-	-	2.85 ± 0.22^{e}	1.02 ± 0.089^{e}
2.0	-	-	1.5	-	-	1.85 ± 0.19^{ef}	1.16 ± 0.023^{cd}
2.0	-	-	2.0	-	-	1.38 ± 0.14^{f}	1.09 ± 0.026^{bcd}
2.0	-	-	-	0.2	-	12.08 ± 0.23^{b}	1.99 ± 0.044^{b}
2.0	-	-	-	0.5	-	10.85 ± 0.31^{b}	1.65 ± 0.0291^{bc}
2.0	-	-	-	1.0	-	8.62 ± 0.21^{b}	1.21 ± 0.035^{cd}
2.0	-	-	-	1.5	-	7.85 ± 0.19^{b}	1.09 ± 0.028^{bcd}
2.0	-	-	-	0.2	10	21.62 ± 0.35^{a}	3.43 ± 0.042^{a}
2.0	-	-	-	0.5	10	20.85 ± 0.39^{a}	2.70 ± 0.038^{a}
2.0	-	-	-	1.0	10	16.46 ± 0.40^{a}	1.75 ± 0.026^{a}
2.0	-	-	-	1..5	10	13.23 ± 0.33^{a}	1.29 ± 0.034^{ab}
-	0.5	-	-	-	-	1.62 ± 0.29^{f}	1.01 ± 0.010^{d}
-	1.0	-	-	-	-	1.92 ± 0.21^{f}	1.27 ± 0.106^{de}
-	1.5	-	-	-	-	1.69 ± 0.20^{f}	1.13 ± 0.061^{cd}
-	2.0	-	-	-	-	1.46 ± 0.18^{ef}	1.01 ± 0.010^{d}
-	1.0	-	0.5	-	-	3.62 ± 0.18^{cde}	1.23 ± 0.078^{cd}
-	1.0	-	1.0	-	-	5.08 ± 0.26^{c}	1.17 ± 0.0652^{cd}
-	1.0	-	1.5	-	-	6.46 ± 0.14^{c}	1.31 ± 0.095^{cd}
-	1.0	-	2.0	-	-	4.62 ± 0.18^{c}	1.08 ± 0.029^{bcd}
-	1.0	-	-	0.2	-	4.31 ± 0.20^{c}	1.08 ± 0.024^{cd}
-	1.0	-	-	0.5	-	3.62 ± 0.18^{d}	1.84 ± 0.080^{ed}
-	1.0	-	-	1.0	-	2.92 ± 0.21^{d}	1.13 ± 0.030^{cd}
-	1.0	-	-	1.5	-	2.00 ± 0.19^{de}	1.05 ± 0.021^{cd}
-	-	0.5	-	-	-	1.38 ± 0.18^{f}	1.34 ± 0.119^{c}
-	-	1.0	-	-	-	1.15 ± 0.19^{g}	1.43 ± 0.10^{cd}
-	-	1.5	-	-	-	1.23 ± 0.20^{f}	1.39 ± 0.097^{bc}
-	-	2.0	-	-	-	1.38 ± 0.14^{f}	1.27 ± 0.106^{cba}
-	-	0.5	0.5	-	-	3.15 ± 0.19^{e}	1.86 ± 0.063^{b}
-	-	0.5	1.0	-	-	4.08 ± 0.17^{d}	1.76 ± 0.055^{c}
-	-	0.5	1.5	-	-	2.85 ± 0.24^{d}	1.61 ± 0.049^{ab}
-	-	0.5	2.0	-	-	2.31 ± 0.17^{d}	1.39 ± 0.064^{a}
-	-	0.5	-	0.2	-	4.15 ± 0.19^{cd}	2.07 ± 0.210^{b}
-	-	0.5	-	0.5	-	3.54 ± 0.18^{d}	1.84 ± 0.222^{b}
-	-	0.5	-	1.0	-	$2.38 \pm 0.14d^{e}$	1.30 ± 0.208^{cd}
-	-	0.5	-	1.5	-	2.00 ± 0.16^{de}	1.00 ± 0.161^{d}

The values are mean ± SE from 13 replicates per treatment. Means followed by different letters within PGR alone and combinations are significantly different from each other (p = 0.05). Comparison is by Duncan's multiple range tests. Data were recorded after four weeks of culture.

Results and Discussion

In-vitro seed germination

Each replicate with 50 seeds of four replicates were maintained. The seeds were soaked in 1–5 mg/l con of GA3. Then, these seeds were kept under 24 hr dark (replicate 1), at 20/4 LD (replicate 2), 6/18 LD (replicate 3) and 18/6 LD (replicate 4) photoperiods. The fourth replicate at 2mg/l GA3 showed highest percentage of seed germination - Fig. 1 A (Sirangi and Ragan 2017). Very low seed germination was observed with the paper bridge method.

Effect of hormones on cotyledonary leaf explants

For *in-vitro* shoot development from cotyledonary leaf explants, different concentrations and combinations of BAP, Kin, TDZ with IAA, NAA and coconut water were used. The cotyledonary leaf explants showed swelling at margins after 6–8 days of inoculation. Shoot initiation was observed between 20 and 30 days. The use of cotyledon explants for *in-vitro* plant regeneration has many advantages. A large number of cotyledons can be obtained by *in-vitro* seed germination, in a short period and at any time throughout

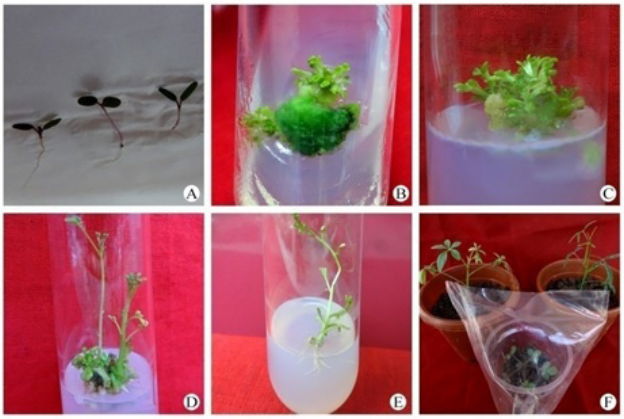

Figure 1: *In-vitro* cotyledonary leaf cultures in *Corynandra chelidonii* var. *pallae*. A. Seed germination *in-vitro* on MS+ 2 mg/l GA_3 with 18/6 LD photoperiod; B & C. Shoot buds formed after four weeks of subcultures on MS medium supplemented with 2.0 mg/ml BAP + 0.2 mg/l NAA + 10% coconut water; D. Shoot elongation on MS medium with 2.0 mg/l GA_3 and 0.5 mg/l TDZ; E. Number of roots initiated on half-strength MS basal medium with 1 mg/l IBA; F. Hardening.

the year without or very low microbial contamination. Moreover, cotyledons have been shown to possess high morphogenetic potential (Fazekas *et al.* 1986; George and Rao 1980; Rogozinska and Drozdowska 1980). Cotyledon explants show higher regeneration ability in comparison to hypocotyls and roots (Kamal *et al.* 2007). When 2.0 mg/l BAP alone was used, it helped to initiate vary few number of shoots regeneration (2.23 ± 0.12) (Table 1). The BAP alone or with other plant growth regulators, is the most effective inducer for explant proliferation in hazelnut (Diaz-Sala *et al.* 1990). When 2mg BAP fortified with 0.5 mg/l IAA was used, the mean number of shoots was 3.54 ± 0.21^{de} (Table 1). Rios-Ramirez *et al.* (2017) asses the dosages of BAP and IAA on the formation of new adventitious shoots in *Agave angustifolia*.

Shoot proliferation was achieved after 15-day cultures on MS medium supplemented with IAA–Kin (Osuna *et al.* 2006). The same results were obtained in the current study when 1.0 mg/l Kin +1.5 mg/l IAA used, with a mean number of shoots of 6.46 ± 0.14 (Table 1). Similar results were observed in *Crambe orientalis* on MS medium containing 0.50 µM NAA combined with 2.20 µM BAP (Ozdemir *et al.* 2017). The best shoot regeneration from cotyledon explants was found on MS medium supplemented with 0.2 mg/l BAP+0.5 mg/l NAA+10% coconut water as the maximum mean number of shoots (21.61 ± 0.34) with shoot length (3.43 ± 0.04) per explant was obtained (Table 1 and Fig. 1B, C). Hypocotyl and cotyledon explants were tested for regeneration in *Brassica* cultivar (Gerszberg *et al.* 2015). Coconut water (5%) and sucrose (3%) promoted the maximum number of shoots with best shoot lengths (Baskaran and Jayabalan 2005). The coconut water alone is not normally sufficient to promote satisfactory multiplication, but addition of coconut water, BAP and GA_3 promotes shoot elongation and proliferation in *Corylus avellana* L. (Diaz-Sala *et al.* 1990)

Table 2: Effect of MS medium supplemented with GA_3 and cytokinins in shoot elongation.

MS medium with Plant Growth Regulators (mg/l)				*Cotyledonary Leaf*	
GA_3	*BAP*	*KIN*	*TDZ*	*No. of shoots (Mean ± S.E)*	*Shoot length(cm) (Mean ± S.E)*
0.5	-	-	-	NR	NR
1.0	-	-	-	1.00 ± 0.00	2.13 ± 0.032^{a}
1.5	-	-	-	1.00 ± 0.00	1.74 ± 0.026^{b}
2.0	-	-	-	1.00 ± 0.00	1.38 ± 0.051^{b}
0.5	2.0	-	-	NR	NR
1.0	2.0	-	-	1.00 ± 0.00	1.63 ± 0.043^{b}
1.5	2.0	-	-	1.00 ± 0.00	1.14 ± 0.029^{c}
2.0	2.0	-	-	1.00 ± 0.00	0.67 ± 0.042^{c}
0.5	-	1.0	-	NR	NR
1.0	-	1.0	-	NR	NR
1.5	-	1.0	-	NR	NR
2.0	-	1.0	-	NR	NR
0.5	-	-	0.5	NR	NR
1.0	-	-	0.5	1.00 ± 0.00	1.58 ± 0.052^{b}
1.5	-	-	0.5	1.00 ± 0.00	2.26 ± 0.028^{a}
2.0	-	-	0.5	1.00 ± 0.00	5.78 ± 0.018^{a}

NR = No Response. The values are mean ± SE from 13 replicates per treatment. Means followed by different letters within PGR alone and combinations are significantly different from each other (p=0.05). Comparison is by Duncan's multiple range tests. Data were recorded after four weeks of culture.

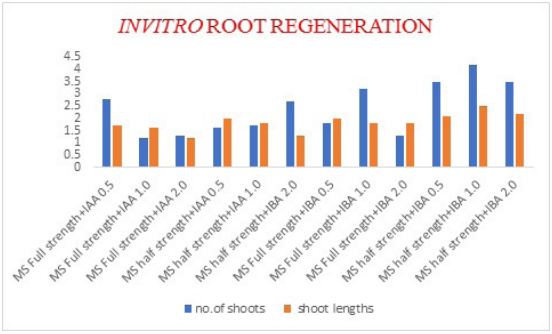

Figure 2: Effect of type of auxins and MS strength *in-vitro* rooting of shoots derived from cotyledonary leaf explants of *C. chelidonii* var. *pallae*.

Shoot elongation

The *in-vitro* raised shoots from cotyledonary leaf of *C. chelidonii* var. *pallae* were examined for shoot elongation, as tested by the growth regulators with different combinations and concentrations. The MS medium with GA_3 is known for shoots elongation (Deore and Johnson 2008). Highest response was achieved on MS medium fortified with 1.0 mg/l GA_3 alone; it promoted shoot length of 2.13 cm with cotyledonary leaf explants. Further increase in the concentration of GA_3 did not promote shoot length. The MS

medium with 1.0 mg/lGA_3, combined with 2.0 mg/lBAP, has shown a moderate response with four weeks of culture. On the other, the MS medium with various concentrations of GA_3 with Kin failed in shoot elongation, whereas MS medium with 2.0 mg/l GA_3 + 0.5 mg/l TDZ shown an appreciable high response shoot elongation was 5.78 cm (Table 2 and Fig. 1D).

For shoot elongation, TDZ was most favourable in combination with GA_3. The MS medium supplemented with GA_3 (2.0mg/l) and TDZ (0.5 mg/l) supported more shoot elongation than the BAP+GA_3 combination or GA_3 alone. GA_3 favoured a better response for shoot elongation cucurbits like *Momordica charantia* (Thiruvengadam *et al.* 2010). In *Corynandra viscosa*, the isolated microshoots were placed on shoot elongation medium fortified with 0.3 mg/l TDZ and 0.1 mg/l GA_3. It, maximized growth after two weeks of culture (Vijayakumar *et al.* 2014).

Root formation from *in-vitro* developed shoots

Elongated shoots (about 2 to 3 cm long) obtained from cotyledonary leaf carefully excised and cultured on MS medium augmented with various concentrations of auxins IAA and IBA. Root initiation started after 10–12 days of inoculation. The mean number of roots (4.41 ± 0.287) and mean root length (2.54 ± 0.007) were maximum on MS half-strength basal medium containing 1 mg/ml IBA (Figs. 1E and 2). Developed roots were observed after 20 days. IBA induced root formation was reported in several plants *Corallocarpus epigaeus* (Vemula *et al.* 2019), *Artemisia vulgaris* (Jogam *et al.* 2020) and *Origanum majorana* (Sandhya *et al.* 2021).

Hardening of regenerated plantlets

In-vitro plantlets of *Corynandra chelidonii* var. *pallae* were taken out from the culture tubes and transferred to pots containing sterilized coco peat for acclimatization. After four weeks plantlets acclimatized and showed signs of establishment and development of new leaves. The survival was 85% (Fig. 2) with the plantlets having normal morphology (Fig. 1F)

Conclusion

This is the first report on organogenesis in *Corynandra chelidonii*. The protocol is efficient and can be used for large-scale propagation to help conserve the taxon on one hand and the genetic transformation studies for metabolic engineering, on the other.

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