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Indirect organogenesis and *in vitro* layering of *Acmella calva* (DC.) R.K. Jansen. from various explants

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An effective method for indirect organogenesis and *in vitro* layering of *Acmella calva* (DC.) R.K.Jansen. have been developed using leaf, internode and nodal explants. The highest of 100% callusing was observed on MS medium containing 3.0 µM BAP in combination with 2.0 µM NAA from leaf and nodal explants. The hard, green and well developed calli were transferred to MS medium with different concentrations of BAP for regeneration. The shoots were harvested at frequent interval. A maximum of 25 shoots/callus was harvested from leaf callus at 5.0 µM BAP in 3rd harvest. The regenerated shoots were rooted in quarter and half strength MS medium with different concentrations of IAA, IBA and NAA (1.0 – 5.0 µM) individually. The well rooted plantlets were transferred to plastic cups containing vermiculite for hardening and subsequently transferred to soil condition. Survival rate of plantlets was found to be 100%.

Key words: Asteraceae, callus formation, medicinal herb, shoots regeneration, toothache plant

Introduction

Acmella calva (DC.) R.K.Jansen is a rare medicinal flowering herb that belongs to the family Asteraceae. The herb is commonly familiar as toothache plant or paracress as the leaves and flower heads contain an analgesic agent spilanthol. It is native to the tropics of Brazil (Caius, 1998). All parts of the plant are acrid but the flowers are by far the most pungent. The pungent flavour of paracress is due to an unsaturated alkamide, spilanthol, which reaches its highest concentration (1%) in flowers (Ramsewak *et al*., 1999).Worldwide the flower heads are powdered and used to treat toothache, throat and gum infections (Gasquet, 1993). The flower heads have been well documented for its uses as spices, antiseptic, anti-bacterial, anti-fungal, anti-malarial and as remedy for flu, cough, rabies diseases and tuberculosis (Burkill, 1966; Oliver-

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Bever, 1986; Di Stasi *et al*., 1994; Akah and Ekekwe, 1995; Singh, 1995; Storey and Salem, 1997). The plant is said to be a popular remedy for stammering in children in western India. The plant is further recommended as a cure for dysentery, rheumatism and to enhance the immune system (Gasquet, 1993). The principle or most active biomolecule is an antiseptic alkaloid, (2E, 6Z, 8E)-deca-2, 6, 8-trienoic acid N-isobutyl amide, commonly known as spilanthol. Due to this compound, the plant has immense application in pharmaceuticals, food and health and body care products. Besides, it is also known to possess antimicrobial (Fabry *et al*., 1996, 1998), larvicidal (Ramsewak *et al*., 1999; Saraf and Dixit, 2002; Pandey *et al*., 2007) and insecticidal (Borges-Del-Castillo *et al*., 1984) properties.

Due to its multifold uses, the plant is being overexploited by the local population as well as pharmaceutical companies. Today, much research has been done on the chemical analysis and structure determination of pungent alkamides. To date, only brief reports are available on callusing and *in vitro* regeneration of shoots but no report on rooting. The present investigation deals with the study of the development of an effective protocol for indirect organogenesis and *in vitro* layering of *A. calva* using leaf, internode and nodal explants which serve as a source of raw material to meet the needs of the society and for manufacturing of drugs and also in drug research.

Materials and methods

Plant material

Acmella calva (DC.) R.K.Jansen is grown in the Herbal Garden of Department of Botany, Holy Cross College, Tiruchirapalli, and Tamil Nadu. A voucher specimen of the plant is deposited in the Rapinat Herbarium and Center for Molecular Systematics, St.Joseph's College (Campus), Tiruchirappalli, Tamil Nadu. The shoots of the experimental material of about 2 cm with 2 or 3 nodes were collected from garden grown plants and kept in running tap water for 30 minutes. These shoots were treated with 0.1% bavistin for 15 min. and washed 3–4 times using distilled water. Then they were taken into laminar air flow chamber where they were surface sterilized with 0.1% (w/v) mercuric chloride for 3 min. and rinsed 3s–4 times with sterile distilled water. The leaf, inter node and nodal segments were excised and used as explants for inoculation.

Preparation of culture medium

The wounded explants of leaf, internode and node were excised and transferred to the combination of Murashige and Skoog (Murashige and Skoog, 1962) basal medium + 3.0 μM BAP + different concentrations (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 μM) of NAA and MS basal medium with 3.0 μM BAP + different concentrations (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 μM) of IAA for callus induction. Fully matured calli were transferred to MS medium with different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0) of BAP for regeneration. The well developed, regenerated shoots were excised and transferred to quarter and half strength MS medium with various concentrations (1.0, 2.0, 3.0, 4.0, and 5.0 μM) of IBA, NAA and IAA individually for root formation. The nodal explants were transferred to MS basal medium without hormones for *in vitro* layering.

Hardening and acclimatization

Rooted plantlets were washed with distilled water to remove the adhering culture medium and transferred individually to plastic cups containing sterilized vermiculite. The plantlets were covered with perforated plastic film to prevent desiccation and to maintain humidity and were incubated under controlled condition for hardening. The hardened plantlets were transferred to pot containing sand: soil: cow dung mixture (1:1:1) to a shaded area in the green house condition.

Culture condition

The cultures were incubated at 24±2°C under 2000 Lux intensity provided by white fluorescent lamp for 16 hrs photoperiod. In all experiments, 20 replicates were used and each experiment was repeated thrice. All the results were statistically analyzed.

Statistical analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment was replicated thrice. Observations were recorded on the percentage of response, number of shoots per explants and number of roots per shoot. Means and standard deviations were calculated for each treatment. The data means ± SD of at least three different experiments were represented.

Results and discussion

Callus induction

The leaf, internode and nodal explants of *A. calva* inoculated on callusing medium began to swell after 5 days of inoculation. Callus initiation was observed at the cut end of the explants after 8 days of culture (Fig. 1e, i). Then proliferation of the calli was noticed throughout the surface of explants. Of these different concentrations, the maximum of 100% response was observed at 2.0 µM NAA with 3.0 µM BAP from leaf explants (Fig. 1b). It was followed by nodal explants (Fig. 1j), in which 86% of response was recorded. The internode explants showed maximum response (85%) at 4.0 µM NAA with 3.0 µM BAP (Fig. 1f). The calli were dark green, hard, compact and organogenic on MS medium with NAA and BAP. Whereas, the IAA combination with BAP produced pale green, friable calli, in which the response was less effective when compared with NAA combination (Table 1). The increasing concentrations of auxin may have negative response with reference to organogenic callus formation. In the present study, BAP with NAA was found to be more effective than BAP with IAA for callus induction. The highest of 100% callusing was observed from leaf explant. However, the previous report on the same plant showed 95% callusing on same medium (Senthilkumar *et al*., 2007). Similar findings were recorded from the leaf explants of *Saussurea obvallata* (Dhar and Joshi, 2005), *Phyllanthus niruri* (Karthikayan *et al*., 2008), *Scorparia dulcis* (Sayeed Hassen *et al*., 2008) and *Tinospora cardifolia* (Afshan and Nag, 2008).

Of these different explants, the leaf explants showed the maximum response. Our results are in accordance with the report of Velayutham *et al*. (2006) in *Cichorium intybus*. Callus induction was documented to be due to endogenous growth regulator in explants and application of exogenous growth regulator combination, along with other growth factor added to the medium (Butenko, 1984; Gamburg, 1990).

Auxins, cytokinin and auxin-cytokinin interaction are usually considered to be the most important aspects for regulating growth and organized development in plant tissue and organ culture, as these two classes of growth regulators are generally required by plants (Evans *et al*., 1981). In the present investigation, variations also existed between different types of explants in their ability to form callus. Among the three explants tested (leaf, internode and node), leaf explants produced considerably more callus than the other explants. These results showed that the nature of explants is an important factor in determining the rate of success in such tissue culture experiments. This also indicates that levels of endogenous hormones or their sensitivity might vary between organs. Similar finding was reported in *Anthurium scherzerianum*

(Geier, 1986). He stated that the easiest way to get organogenic callus is by using leaf explants.

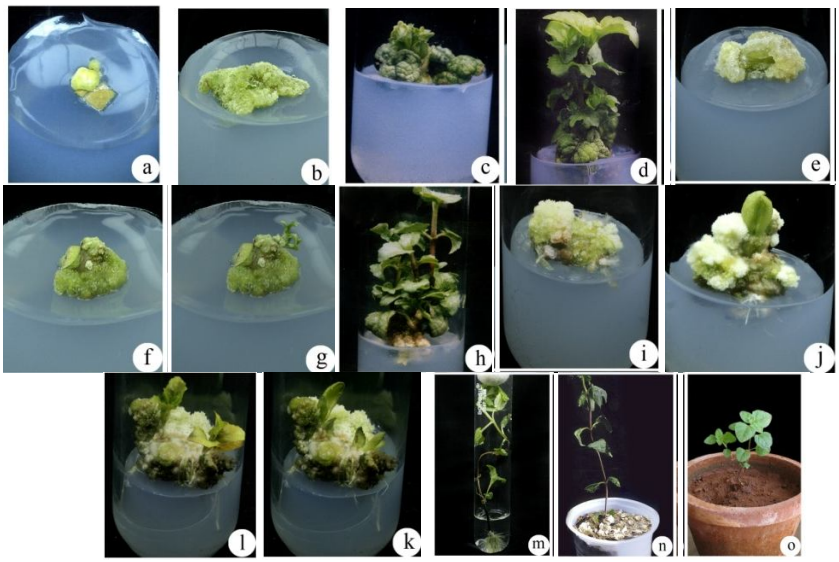

Fig. 1. Indirect organogenesis of *A. calva* using leaf, internode and nodal explants.
a, Swelling of callus from leaf explants, b, Maturation of callus from leaf explants, c, Initiation of shoot buds from leaf derived callus, d, Proliferation of shoots from leaf derived callus, e, Initiation of callus from internode explants, f, Maturation of callus from internode explants, g, Initiation of shoot buds from internode derived callus, h, Proliferation of shoots from internode derived callus, i, Swelling of callus from nodal explants, j, Maturation of callus from nodal explants, k, Initiation of shoot buds from nodal derived callus, l, Proliferation of shoots from nodal derived callus, m, Rooting of shoot, n, Hardening of plantlet, o, Acclimatization of plantlet.

Regeneration of shoots

The well developed, green organogenic calli began to produce shoot buds after 14 days of inoculation (Fig. 1k, g & k). Then proliferation of shoots was observed all over the surface of callus. The elongated shoots were harvested at frequent interval. The number of shoots increased upto 3rd harvest and then gradually declined. A maximum of 25 shoots were observed at 5.0 µM BAP in 3rd harvest from leaf explants (Table 2; Fig. 1d). It was followed by nodal explant, in which 19 shoots/callus (Fig. 1l) were noticed in the 3rd harvest. On the whole, there were 35 shoots /callus after 21 days of culture. The least number of shoots were noticed in internodal explants (Fig. 1h). The average length of shoots was 3-7 cm. The present results indicated that the increasing

concentration of BAP was proved to be the best for regeneration of shoots. Synergistic effect of cytokinins on organogenesis from callus was reported earlier (Sajuan and Claveria, 1995; Manisha Thakur *et al*., 2001; Hiren *et al*., 2004; Vasantha and Shivanna, 2004). According to them, the reason for effectiveness of BAP may lie in its ability to stimulate the plant tissue to metabolize the natural endogenous hormones or could induce the production of natural hormones system for the induction of shoot organogenesis.

In the present study, calli produced from leaf explants with the addition of BAP showed the highest number of shoots (35 shoots/callus) after 21 days of culture. The same plant produced the maximum of 25 shoots from leaf callus only after 12 weeks of culture (Senthilkumar *et al*., 2007). In our study, BAP alone was sufficient for proliferation of regenerated shoots from callus. Conversely, BAP + IAA combination showed the best response in micropropagation on the same plant (Chandra *et al*., 2007).

Table 1. Effect of BAP (3.0 µM) with different concentrations (1.0-6.0 µM) of NAA and IAA on callus induction of *Acmella calva* (DC.) R.K.Jansen.

Hormone concentration (µM)			Percentage of callusing		
BAP	**NAA**	**IAA**	**Leaf**	**Internode**	**Node**
3	1	-	60	42	78
3	2	-	100	59	100
3	3	-	80	75	81
3	4	-	65	85	73
3	5	-	49	61	51
3	6	-	45	56	48
3	-	1	59	21	41
3	-	2	86	45	74
3	-	3	73	63	89
3	-	4	54	80	67
3	-	5	35	59	35
3		6	33	55	34

Table 2. Effect of different concentrations (1.0 - 7.0 µM) of BAP on shoot regeneration from callus of *Acmella calva* (DC.) R.K.Jansen.

Explants	Hor. Con. BAP	% of response	No. of shoots in each harvest/explant				Length of shoots
			1st	2nd	3rd	4th	
Leaf	1	78	3.42	6.0	6.7	3.2	3.4
	2	69	2.8	5.2	8.2	2.1	3.5
	3	84	4.5	8.0	11.3	2.3	4.7
	4	75	2.4	7.3	14.8	1.2	4.5
	5	100	5.3	11.1	21.8	4.8	6.3
	6	59	5.2	8.0	15.3	3.4	4.2
	7	62	6.0	9.1	7.1	3.2	4.0
Internode	1	62	2.6	5.2	2.8	2.5	3.0
	2	73	2.4	4.1	3.9	1.4	3.7
	3	71	1.5	3.1	6.3	1.2	4.5
	4	65	2.3	4.2	6.5	2.1	4.1
	5	81	4.3	7.3	12.0	3.1	5.5
	6	63	3.0	6.1	6.9	2.0	4.9
	7	74	2.5	5.0	6.0	1.7	4.4
Node	1	75	1.4	3.0	6.5	1.2	5.4
	2	86	3.8	7.0	9.5	3.0	4.7
	3	90	4.0	7.0	15.5	2.2	6.0
	4	100	5.1	8.2	19.1	3.4	6.4
	5	88	2.6	6.4	12.4	3.2	5.2
	6	75	2.1	4.9	7.3	1.8	4.2
	7	70	1.2	2.2	5.5	1.4	3.2

Values are Mean±SD of ten replicates per treatment and repeated three times.
Harvest was done at 15 days of interval.

Rooting of regenerated shoots

The well developed elongated shoots of about 3 cm length were excised and transferred to quarter and half strength MS medium supplemented with different concentrations of IBA, NAA and IAA individually. Initiation of the roots was recorded after 5 days of inoculation from the cut end of the shoot. Among the different strength of medium used, the best response was observed on both quarter and half strength MS medium. In which 100% response was noticed at 4.0 µM IBA (Fig. 1m). It was followed by 2.0 µM IAA (92%). The quarter and half strength MS medium with IBA was proved to be more effective than NAA and IAA in root formation. The frequency of root formation and the number of roots/shoot increased with the increase in IBA concentrations from 2-4 µM (Table 3). The maximum number of 55 roots was

noticed at 4.0 μM of IBA and was found significant statistically. On NAA supplemented MS medium, though a large number of roots were formed, they were very slender and thin in size and were found not suitable for survival of shoots.

Of the different strength of media used (quarter and half strength MS medium) in the present study, quarter strength was proved to be the best for rhizogenesis. On the contrary, in *Acmella calva* (Shanthi and Anne Xavier, 2006) and in *Centella asiatica* (George *et al*., 2004) the rhizogenesis was reported on half strength MS medium only. Among the different auxins experimented in our study, IBA was found to be more effective in lateral root induction than the other two auxins and IBA generated many healthy roots. The effectiveness of IBA over NAA and IAA on rhizogenesis has been reported in the same plant only with half strength MS medium (Saritha *et al*., 2004; Saritha and Naidu, 2008; Panday *et al*., 2007) and in several other medicinal taxa such as *Saussurea obvallata* (Joshim and Dhar, 2003), *Holostemma ada-Kothen* Schult (Martin, 2002), *Hemidesmus indicus* (Sreekumar *et al*., 2000) and *Chorophytum borivilianum* (Purohit *et al*., 1994). According to Zolman *et al*. (2000), IBA is more effective than other auxins in lateral root induction, thus generating healthy roots.

Table 3. Effect of different concentrations (1.0-5.0 μM)) of auxins (IBA, NAA and IAA) on root induction from regenerated shoots of *Acmella calva* (DC.) R.K.Jansen.

Hor.con	Percentage of rooting		No. of roots/shoot	
	¼ strength	½ strength	¼ strength	½ strength
1IBA	66	90	34.6	28.6
2IBA	88	85	23.4	30.0
3IBA	90	90	37.0	45.2
4IBA	100	98	55.4	46.0
5IBA	80	90	17.4	24.4
1IBA	78	80	28.2	22.7
2IBA	92	90	46.4	20.2
3IBA	90	86	51.4	29.4
4IBA	89	83	11.0	18.8
5IBA	90	89	12.0	19.0
1IBA	90	90	30.0	16.0
2IBA	98	95	27.4	18.6
3IBA	86	84	28.8	16.1
4IBA	88	80	23.6	15.3
5IBA	78	70	20.2	12.3

Values are Mean±SD of ten replicates per treatment and repeated three times.

In vitro layering

Layering is a process by which a part of shoot and root is made to develop into an individual plant in a hormone free medium. The nodal explants inoculated on basal MS medium started to produce shoot buds after 5 days of inoculation (Fig. 2a). Small roots were visible from the basal part of the regenerated shoot after 11 days of culture on MS basal medium without hormones (Fig. 2b, c). The response was 100% after 25 days of incubation and the shoots attained the height of 3.0–7.0 cm length with roots. A maximum of 3 shoots/explant was recorded (Table 4; Fig. 2d). All the regenerated shoots were green and healthy in appearance. After 30 days, the fully developed plantlets were transferred to vermiculite for hardening (Fig. 1e).

This study showed that the nodal explants of *A. calva* have the potential to produce number of plantlets (3 shoots/explant) in the hormone free basal medium. It indicates that this is the easiest method to produce this valuable medicinal herb without any costly hormonal requirements. Our findings are similar to the report of Salgado-Gareiglia *et al*. (1996) in *Acmella oppositifolia*.

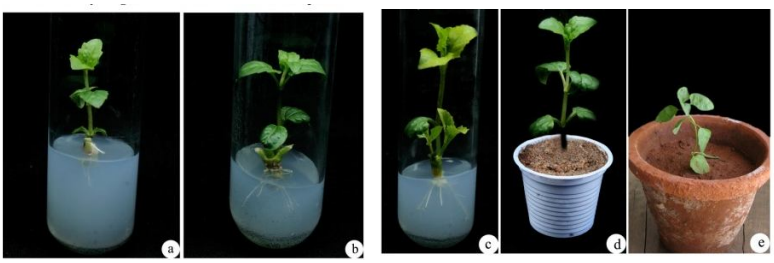

Fig. 2. *In vitro* layering of *A. calva* from nodal explants.
a = formation of shoot from nodal explants, b = simultaneous formation of shoot and roots from nodal explants, c = maturation of shoot and roots from nodal explants, d = proliferation of shoots from nodal explants, e = hardening of *in vitro* derived plantlet.

Table 4. *In vitro* layering of *Acmella calva* (DC.) R.K.Jansen.

No. of days for culture of roots/explant	Average No. of shoots/explant	Average No
7	1.0	0
15	1.2	1.4
22	1.5	1.8
30	1.9	2.2

Values are Mean±SD of ten replicates per treatment and repeated three times.

Hardening and acclimatization of in vitro derived plants

The *in vitro* rooted plantlets of *A. calva* obtained from indirect organogenesis and *in vitro* layering were washed with distilled water. After washing, the rooted plantlets were successfully transferred to plastic cups containing sterilized vermiculite. After two weeks, the shoots showed elongation up to 10 cm long and were transferred to pot containing sand: soil: cow dung mixture (1:1:1) to a shaded area in the green house condition. The survival rate was 100% (Fig. 1n, 2f). Hundred percentage survival rates were documented by Velayutham *et al*. (2006). All the hardened plantlets were successfully acclimatized under soil condition (Fig. 1o). The plantlets did not show any morphological abnormalities in field condition.

It is concluded from the present study that the protocol devised for indirect organogenesis of callus from the leaf, internode and nodal explants was found to be highly effective and suitable for this plant. It was also found to be successful for *in vitro* layering of *A. calva*. The protocol can be exploited for *in vitro* production of plantlets on a large scale. Of the three explants used, the callus obtained from leaf explants showed a maximum number of shoots in our study. Hormone free MS medium could produce plantlets from nodal explants much effectively in *in vitro* layering.

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