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AUXINS AND PHYSICO-CHEMICAL FACTORS INFLUENCED CALLUS FORMATION IN *ACHYRANTHES ASPERA* L. LEAF EXPLANT

S. Ramakrishnan [1], K. Nandhini [2], A. Porkalai [2], S. Allirani [2], A. Subathra [2], R. Ilakkia [2], P. Jayalakshmi [2], P. Kalaivani [2]*

Address(es):
[1]Department of Botany, Tagore Arts College, Puducherry- 605 008. Tel +91 94861 61500.
[2]Department of Plant Science, Bharathidasan Govt. College for Women (Autonomous), (Affiliated to Pondicherry University), Puducherry- 605 003.

*Corresponding author: ramkilinn@yahoo.co.in

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ABSTRACT

Callus induction from leaf explant of *Achyranthes aspera* L. had been achieved using Murashige and Skoog (MS) medium supplemented with various plant growth regulators under light and dark incubation conditions. Callusing potential on B5 medium had also been evaluated. Our results showed that Kinetin (KIN) was inefficient in callus formation. Leaf explant inoculated on MS+2,4-D (1 mg/L) or MS+2,4,5-T (7 and 8 mg/L) media and incubated under light produced good callus. But colour of the callus formed in 2,4-D medium became brown after 2 weeks. Better callusing response was seen in MS+2,4,5-T (8 mg/L) incubated under dark. Subculture of callus initiated using MS+2,4,5-T (8 mg/L) under light condition onto MS medium without any plant growth regulator, BM (basal medium) under dark incubation resulted in yellow green, fleshy and soft callus without browning. B5 medium was not good in callus formation under any incubation conditions.

Keywords: Leaf explant, callus, MS medium, B5 medium, 2,4-D, 2,4,5-T, NAA, IBA, BAP, KIN

INTRODUCTION

Vast numbers of plants grow as weeds are reported to have medicinal properties through ethnobotanical knowledge and scientific investigations. According to World Health Organization (**WHO, 1993**) more than 80% of the world's population depend on traditional herbal medicines for their primary health care. One such plant used in traditional medicine is *Achyranthes aspera* L. (Family: Amaranthaceae). It is commonly referred as "Prickly chaff flower" and in Tamil it is known as "*Nayuruvi*".

Various parts of this plant have been used by indigenous people of Tamil Nadu, Puducherry and other parts of Indian sub-continent for the treatment of asthma and cough. It is pungent, antiphlegmatic, antiperiodic, diuretic, purgative and laxative, useful in oedema, dropsy and piles, boils and eruptions of skin etc (**Srivastav *et al.*, 2011**). Crushed plant boiled in water is used in pneumonia and infusion of the root is a mild astringent in bowel complaints. The flowering spikes or seeds, ground and made into a paste with water, are applied externally for snake bite and reptile poison, used in night blindness and cutaneous diseases (**Nadkarni, 2009**). **Bafna and Mishra (2004)** reported hepatoprotective activity of methanolic extract from aerial parts of the plant.

Seeds of *A. aspera* have secondary metabolites like saponins A and B, oleanolic acid, amino acids and hentriacontane, 10-tricosanone, 10-octacosanone and 4-tritriacontanone. Root extracts contain ecdysterone and a new aliphatic acid, *n*-hexacos-14-enoic acid. Water soluble alkaloids "achyranthine" (**Neogi *et al.*, 1970**) and betaine (**Kapoor and Singh, 1966**) have been reported in this plant. Achyranthine has been used for the dilation of blood vessels, lowering of blood pressure, depression of heart, in addition to increase the rate, amplitude of respiration and anti-inflammatory activity (**Gokhale *et al.*, 2002**).

Some attempts have been made to regenerate this plant through plant tissue culture techniques using different explants: leaf, node, internode and root (**Kayani *et al.*, 2008**; **Gnanaraj *et al.*, 2012**; **Senthilmanickam *et al.*, 2012; Sen *et al.*, 2013; Sen *et al.*, 2014**). Experiments on callusing potential of *A. aspera* leaf explant using 2,4-D, NAA, IAA, IBA, BAP, KIN and Zeatin have been reported (**Kayani *et al.*, 2008; Sen *et al.*, 2014**). Their results show that callus formation from leaf was optimum in MS medium (**Murashige and Skoog, 1962**) containing 2,4-D+NAA or 2,4-D+BAP. None of the earlier studies in this plants employed B5 medium, 2,4,5-T and dark incubation condition to validate the callusing potential of leaf explant.

Hence, our study was carried out to document the callusing potential of leaf explant of *A. aspera* using various auxins (including 2,4,5-T) and cytokinins, and influence of B5 medium (**Gamborg *et al.*, 1968**) and dark incubation.

MATERIAL AND METHODS

Collection of plant material

Fresh leaves (fourth leaf from shoot apex) of *A. aspera* were collected from the campus of Bharathidasan Government College for Women (Autonomous), Puducherry. They were washed thoroughly with tap water followed by distilled water.

Surface sterilization of explant

Leaves were surface sterilized inside the laminar air-flow chamber using 0.1 % (10 min) of fungicide, ZEN (contains 50 % carbendazim) procured from PASIC, Puducherry. Then, explants were rinsed with sterile distilled water (1 min) and immersed in 0.05 % mercuric chloride solution ($HgCl_2$) for 5 min. Finally they were subjected to three rinses with sterile distilled water (3 min each).

Inoculation and incubation

The surface sterilized leaf explants containing midvein were trimmed (0.5 cm^2) in laminar air flow chamber and inoculated (adaxial side facing the medium) aseptically in culture tubes containing MS or B5 medium augmented with or without plant growth regulators (PGRs). The PGRs used were: auxins- 2,4-D (2,4-dichlorophenoxy acetic acid), 2,4,5-T (2,4,5-trichlorophenoxy acetic acid), IBA (Indole-3-butyric acid), NAA (*α*-naphthalene acetic acid) and cytokinins-BAP (*6*-benzyl aminopurine), KIN (kinetin). Media were gelled with 0.8 % (w/v) agar after setting the pH at 5.7. All the chemicals were procured from HI-MEDIA (Mumbai, India).

The cultures were incubated at 25±1° C under cool fluorescent white light (3000 lux 12 h light/day photoperiod). The calli induced were split into small pieces (0.5 cm dia.) and subcultured onto media containing different PGR(s) after a specified period (2 to 3 weeks). Again the cultures were incubated under the same physical conditions (in light or dark incubation).

RESULTS AND DISCUSSION

Callus induction by cytokinins

Callus induction was performed using four different concentrations of BAP and KIN in MS medium (Tab 1). The MS basal medium (BM) did not result in any appreciable level of callus induction. Despite callus (non friable) formation at all concentrations of BAP, 0.5 and 2 mg/L showed more callus induction (50 %) (Fig 1A). However, callus induced only at cut ends of explant and the callus did not grow further. Meanwhile roots were produced from end of mid vein in low concentration (0.5 mg/L). All the calli were pale green in colour. However, none of the calli developed further and they became brown in colour. Unlike BAP, KIN did not result in callus formation. Roots were formed on explant cultured on 1 and 2 mg/L KIN. Both cytokinins tested for the culture of *A. aspera* leaf explant were not helpful either in callus formation or plant regeneration even after 2 months of culture (Fig 1B).

Table 1 Potential of BAP and KIN supplemented MS medium on callus induction from leaf explant of *A. aspera*

Sl. No.	PGRs (mg/L)	No. of explants inoculated	Callus formation (%)	Colour (after 2 weeks)	Root formation in cultures
1	BM	8	12.5±1.2	Yellowish	1
2	BAP (0.5)	8	50±3.0	Whitish green	2
3	BAP (1)	8	25±1.0	Whitish green	--
4	BAP (2)	8	50±2.3	Whitish green	--
5	BAP (3)	8	25±5.1	Whitish green	--
6	KIN (0.5)	8	--	--	--
7	KIN (1)	8	--	--	2
8	KIN (2)	8	--	--	1
9	KIN (3)	8	--	--	--

Legend: -- indicates no response. The experiment was repeated once.

Cytokinins are reported to have caulogenic properties, callus was produced in leaf explant of *Acalypha indica* on KIN supplemented medium and subsequently plant regenerated when callus subcultured onto low concentration of the same PGR (**Ponmany *et al*., 2010**). In this study both BAP and KIN did not result in appreciable level of callus formation.

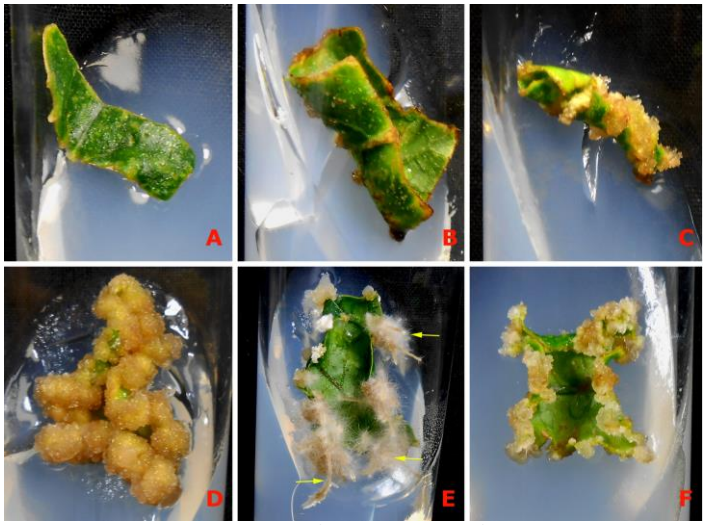

Figure 1 (A-F) Callus induction of *A. aspera* L. leaf explant on MS medium added with different PGRs: 3 mg/L BAP (A); 3 mg/L KIN (B); 4 mg/L NAA (C); 1 mg/L 2,4-D (D); 1 mg/L 2,4,5-T (E); (F) 8 mg/L 2,4,5-T. (Arrows indicate roots. All cultures are 3 weeks old except (B) which is 6 weeks old).

Callus induction by auxins

Four different auxins at different concentrations were used for induction of callus formation: 2,4-D, 2,4,5-T, IBA and NAA. Indole-3-butyric acid (IBA) was not useful in callus formation. All concentrations tested resulted in root formation (Tab 2). Only at 2 mg/L a sign of callus initiation occurred. All leaf explants were rolled with localized bulging. Long term incubation of cultures in light did not result in callus development. They all turned yellow and subsequently to brown colour.

Leaf explant was also cultured in NAA augmented MS medium (Tab 2). There was 100% callus formation in 1, 2 and 4 mg/L NAA treatments (Fig 1C). Roots were also produced in all concentrations. The calli were yellowish green and fleshy in nature. Quantity of callus produced in 4 mg/L was more. All calli changed their colour to brown after a month. NAA was relatively better than IBA.

Table 2 Callus induction in leaf explant on MS medium supplemented with IBA and NAA media.

Sl. No.	PGRs (mg/L)	No. of explants inoculated	Callus formation (%)	Colour (after 2 weeks)	Root formation in cultures
1	IBA (1)	8	--	--	2
2	IBA (2)	8	12.5±4.2	Whitish green	5
3	IBA (3)	8	--	--	1
4	IBA (4)	8	--	--	1
5	NAA (1)	8	100±0	Pale yellow	3
6	NAA (2)	8	100±0	Pale yellow	4
7	NAA (3)	8	75±0.9	Pale yellow	3
8	NAA (4)	8	100±0	Yellowish green	2

Legend: -- indicates no response. The experiment was repeated once.

Earlier study on stem explant of *A. aspera* also showed callus formation (up to 60 %) in MS medium containing 4 mg/L NAA (**Senthilmanickam *et al*., 2012**). Same PGR could produce callus in leaf explant in our study with 100% efficiency. This could be due to the type of explant type.

2,4-D is commonly used PGR in tissue culture studies for callus induction and somatic embryogenesis (**Milivojević *et al*., 2005**). Hence, it was selected in 10 different concentrations. Callus (non friable) induction was observed in all the concentrations (Fig. 1D). Callus induction decreased in proportion to increasing concentrations of 2,4-D. There was 100 % callusing in low concentrations (Tab. 3). Morphology of the calli was fleshy, soft and yellowish green. None of the cultures produced roots. But the colour started to become brown from second week of their formation.

Callus induction using 2,4-D has been reported from various explants of different plant species (**Ramakrishnan and Kulandaivelu, 2007; Kayani *et al*., 2008**). Our result proved that good amount of callus can be produced from *A. aspera* leaf explant using MS medium supplemented with 2,4-D even at low concentration (1 mg/L). Callusing of leaf explant of *A. aspera* using 2,4-D alone and in combination with NAA or BAP by **Kayani *et al*.** (**2008**) confirms this result that 2,4-D can be used for callus induction in this plant. However, they stated that the best callus induction could be obtained with 2,4-D (1 and 2 mg/L) + NAA (0.5 mg/L). 2,4-D is also suitable for the callus induction from *Oryza sativa* seed (**Ramakrishnan and Kulandaivelu, 2007**).

Table 3 Effect of 2,4-D and 2,4,5-T supplemented MS medium on leaf explant.

Sl. No.	PGRs (mg/L)	No. of explants inoculated	Callus formation (%)	Colour (after 2 weeks)	Root formation in cultures
1	2,4-D (1)	8	100±0	Pale brown	--
2	2,4-D (2)	8	100±0	Pale brown	--
3	2,4-D (3)	8	100±0	Pale brown	--
4	2,4-D (4)	8	100±0	Pale brown	--
5	2,4-D (5)	8	100±0	Pale brown	--
6	2,4-D (6)	8	25±3.2	Pale brown	--
7	2,4-D (7)	8	50±3.0	Pale brown	--
8	2,4-D (8)	8	50±5.0	Pale brown	--
9	2,4-D (9)	8	50±4.4	Pale brown	--
10	2,4-D (10)	8	12.5±0.7	Pale brown	--
11	2,4,5-T (1)	8	85.71±3.1	Whitish green	4
12	2,4,5-T (2)	8	85.71±2.8	Whitish green	4
13	2,4,5-T (3)	8	100±0	Whitish green	7
14	2,4,5-T (4)	8	100±0	Whitish green	2
15	2,4,5-T (5)	8	100±0	Whitish green	2
16	2,4,5-T (6)	8	100±0	Whitish green	4
17	2,4,5-T (7)	8	100±0	Whitish green	--
18	2,4,5-T (8)	8	100±0	Whitish green	--
19	2,4,5-T (9)	8	100±0	Whitish green	3
20	2,4,5-T (10)	8	85.71±2.7	Whitish green	2

Legend: -- indicates no response. The experiment was repeated twice. Results are average of eight replicates.

Second set of explant was inoculated on various concentrations of 2,4,5-T (1 to 10 mg/L) (Table 3). Like 2,4-D, the callus production occurred in all concentrations of 2,4,5-T. Maximum callusing potential was found in 3 to 9 mg/L^{-1}. Unlike 2,4-D, the 2,4,5-T media resulted not only in non friable callus induction but also adventitious root formation in most cultures (Fig. 1E).

The calli formed were pale green or yellowish green in colour and became brown after 6 weeks. Callus initiation began from cut ends of explant. Among the concentrations only 7 and 8 mg/L resulted in 100% callus (non friable) formation without root formation (Fig. 1F). For further studies 2,4,5-T at 8 mg/L was selected.

Very often good amount of callus has been produced from explant incubated in dark. Callus induction by a particular hormone at particular concentration may

vary due to type of culture media. The 2,4,5-T (8 mg/L) was used either in B5 or MS medium for the evaluation of callus initiation under light and dark conditions. Dark incubation of B5+2,4,5-T and MS+2,4,5-T showed better response in callus induction than their respective medium under light condition (Fig. 2 A-D). Among two different culture media, MS medium was better in callus formation. Moreover, the callus size was larger in MS medium under dark incubation (Fig. 2D). Light incubation produced callus as well as roots (Fig. 2C).

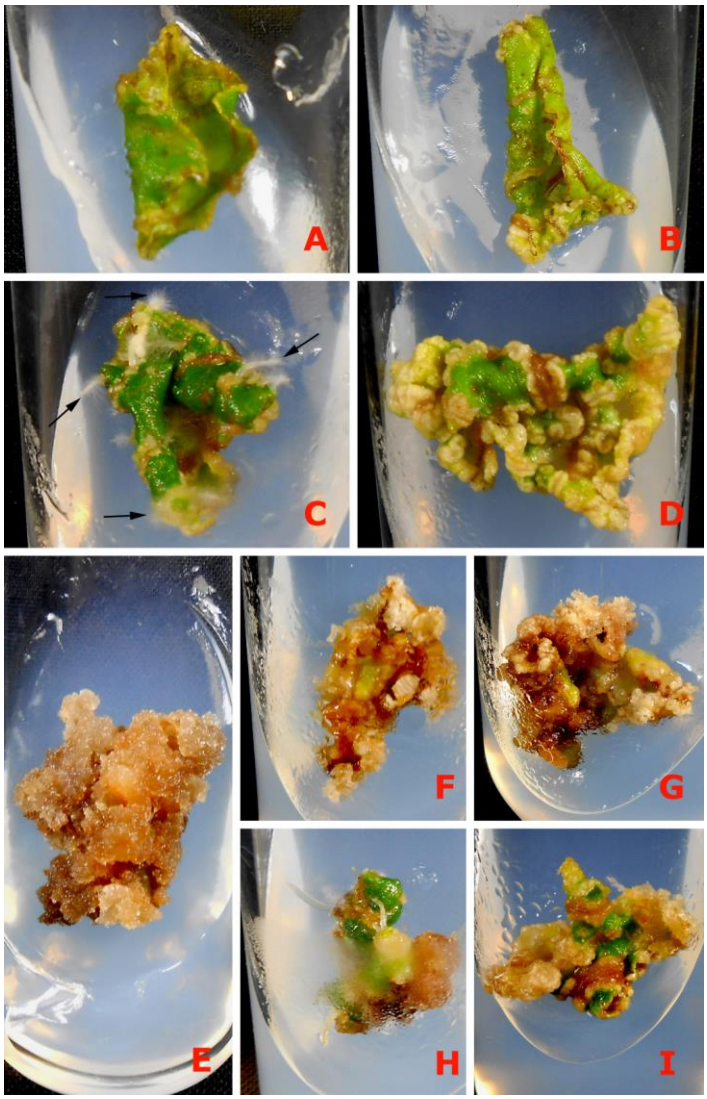

Figure 2 (A-I) Morphology of calli formed on different media and incubation conditions: B5+8 mg/L 2,4,5-T +light (A); Above culture in dark (B); Light+ MS+8 mg/L 2,4,5-T (C); Above culture in dark (D). Subculture of calli: MS+1 mg/L 2,4-D+light to MS+1 mg/L 2,4-D+1 mg/L BAP (E); MS+8 mg/L 2,4,5T+light to MS+5 mg/L BAP (F); Dark grown above culture to MS+5 mg/L BAP (G); MS+8 mg/L 2,4,5T+light to MS+BM (H); Dark grown above culture to BM (I). (Arrows indicate roots).

For callus induction 2,4,5-T had been used in garlic (**Nagasawa and Finer, 1988)** and *Hemidesmus indicus* (**Shanmugapriya and Sivakumar, 2011**). Our result with 2,4,5-T is similar to these results.

Subculture of 2,4-D and 2,4,5-T induced calli

Proliferation and survival of the leaf explants derived calli were analysed by subculturing them into second medium supplemented with 2,4-D + BAP (both at 1 mg/L).

Table 4 Subculture of calli (three weeks old) formed in 2,4-D media onto 2,4-D+BAP supplemented MS media

PGRs (mg/L)		Callus colour	Callus development (week)			
1st culture	2nd culture		First	Second	Third	Fourth
2,4-D (1) 2,4-D (2) 2,4-D (3) 2,4-D (4) 2,4-D (5) 2,4-D (6) 2,4-D (7) 2,4-D (8) 2,4-D (9) 2,4-D (10)	2,4-D (1)+ BAP (1)	Brown	No change (0)	Callus size increased (+)	Callus size increased (++)	Callus size increased (+++)

Legend: (0)- no change, (+)- small size, (++)- moderate size, (+++)- large size

The calli induced with MS added with various concentrations of 2,4-D were subcultured on MS+2,4-D (1 mg/L)+BAP (1 mg/L) media (Tab 4). There was no sign of any development in the calli after one week. From second week of subculture they started to grow and size increased.

Calli formed under dark and light incubations in MS+ 2,4,5-T (8 mg/L) medium was subcultured. The cultures were incubated under light. Callus size increased in second week and become brown in third week. In addition to callus growth, roots were also formed in some cultures. The callus produced in dark and transferred to MS+BAP (5 mg/L) or BM showed better growth (Fig 2G&I). Callus formed on BM was pale yellowish green and callus developed on MS+BAP (5 mg/L) after subculture was brownish yellow. Current study showed that the yellowish colour and luxuriant growth of the calli resulted when calli induced in MS medium under dark and subcultured on to BM.

CONCLUSION

Results of this present study have expanded the knowledge on callus formation from leaf explant of *A. aspera*. It was found that both KIN and IBA were inefficient in callus induction. MS medium added with 2,4-D (1 mg/L) or 2,4,5-T (7 and 8 mg/L) and incubated under light resulted in good callus initiation. However, colour of the callus formed in 2,4-D medium turned brown after 2 weeks. Better callusing response was seen in MS+2,4,5-T (8 mg/L) incubated under dark. The above callus subcultured onto basal medium and incubated under light resulted in yellow green, fleshy and soft callus. B5+2,4,5-T (8 mg/L) medium did not result in good callus formation under any incubation condition. Inoculation of leaf explant on MS medium added with 2,4,5-T (8 mg/L) under dark and subcultured onto BM under light was suitable for callus culture of *A. aspera*.

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