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THE EFFECT OF AUXINS ON CALLUS INDUCTION IN *ACHYRANTHES ASPERA*

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Abstract

To induce *in vitro* callus induction, leaf and nodal stem explants of *Achyranthes aspera* were compared for their callusing response on MS (Murashige and Skoog, 1962) medium supplemented with NAA, IAA, IBA and 2,4-D at various concentrations. Best callus was derived from adaxial surface of leaf explants. MS medium supplemented with 2.0 mg/l 2,4-D was found suitable for callus induction. After subculture the colour of callus turned brown and the quantity of callus declined. Influence of charcoal was tested to stop browning of the callus. Influence of sucrose percentage on callus fresh weight and growth regulators combined with coconut milk was also tested.

Key words: Auxins, Callus induction, *Achyranthes aspera*, Browning, Fresh weights, Coconut milk

Introduction

Achyranthes is a small genus of some 15 species of stiff herbs (Manjunath, 1948), belongs to family Amaranthaceae. *A aspera* is a plant of medicinal importance. It is used as an abortifacient, alexipharmic, appetizer, stomachic (Memon *et al*., 1988) and possesses antidiabetic and antirheumatic properties. It is used beneficially in abdominal tumors (Purohit & Vyas, 2004).The interesting and immense valuable properties of the genus have prompted us to select *Achyranthes aspera*, which have been relatively less explored pharmaceutically and under *in vitro* conditions for micropropagation and for the presence of insecticide, phytoecdysone.

Tissue culture methods for some species of *Achyranthes* are available. Maximum callus growth was observed on MS medium supplemented with coconut milk, 2,4-D and Kinetin (Hikino *et al*., 1971). The best callus induction response from leaf explants of *A. aspera, Alternanthera sessilis, Guilleminea densa* was observed on MS medium supplemented with 1.0 mg/l 2, 4-D and 1.0 mg/l BA (Singh *et al.*, 2009). Callus obtained from *Alternanthera sessilis* on MS medium supplemented with 1.0 mg/l 2, 4-D and 1.0 mg/l BA successfully formed shoots on half MS medium with 1.0 mg/l IAA and 1.0 mg/l BA (Singh *et al.*, 2009). Callogenic response from leaf explants of *A. aspera* was also observed under the influence of different concentrations of 2, 4-D applied alone and in combinations with BA, NAA, IAA, IBA, Zeatin (Kayani *et al*., 2008).

Material and Methods

Plant materials: Plants of *Achyranthes aspera* were grown in the greenhouse of Department of Botany, University of Karachi, under moderate shade. The voucher specimen No 68506 (Herbarium, University of Karachi).

Explants: The explants of *Achyranthes aspera* used for inducing callus were young leaves (1-1.5 cm long and 1-1.2 cm wide), nodal and internodal segments (1-1.5 cm long) of stem.

Leaves were placed flat either touching abaxial or adaxial surface on the medium. Nodal and internodal explants were placed horizontally on the medium.

Surface sterilization: Surface sterilization of plant material was done with aqueous 0.1% mercuric chloride having 3-4 drops of tween-20 for 10 minutes with intermittent shaking and finally rinsed several times with sterile water. The explants were cut with a sterile scalpel in a laminar flow cabinet and were inoculated on the medium.

Culture medium and conditions of culture: Throughout this investigations basal MS medium (Murshige & Skoog, 1962) contained 30g/l sucrose and 0.6% Agar agar (Microbiologie Merck, USA) adjusted to PH 5.5-5.6 were used. Unless otherwise stated growth hormones were always added before autoclaving the medium which was done at 121˚C under 15Lbs. P.s.i cultures were incubated at 25 ± 2˚C under light: dark period (16:8 hrs).

Callus induction and growth: For callus induction and growth IAA, IBA, NAA, and 2, 4-D at various concentrations cultures were tested. Leaves (adaxial and abaxial surface), nodes and internodes were used.

Sub culturing: Sub culturing of induced callus was carried out every 22 days.

Browning of callus: To stop browning of the callus 2.0 mg/l 2, 4-D was combined with charcoal (0.2 g/l).

Influence of sucrose: For the determination of the influence of varying sucrose (3%, 6%, and 9%) with 2,4-D (2.0 mg/l) on fresh weights of callus induced and grown on actively growing callus (0.4g each) obtained from 2.0 mg/l 2, 4-D containing medium was used.

Influence of coconut milk: In order to increase callus growth, in separate experiments leaf explants were cultured on fresh media supplemented with varying combinations of growth hormones. First combination used was 2,4-D (2.0 mg/l) and coconut milk (0, 2.5, 5.0, 10, 15%). Other combinations were Kinetin (1.0 mg/l), coconut milk (10%) 2, 4-D (1.0, 2.0, 3.0, 4.0 mg/l).

Evaluation of results: Observations were made after specified time and effect of treatments was tested by analysis of standard deviation (± SD).

Results

Three main characteristics in the explants appeared within 2 weeks of culture were 1) browning 2) contamination with micro-organisms (bacteria and /or fungi) 3) change in colour to brown, whit or green. The explants which remained green were considered as established. On hormone free medium, callus was formed only on 2nd node but the extent was less. Leaves did not form callus in the absence of 2, 4-D.

There was no callus formation on leaf explants cultured on media containing 0.25 to 1.5 mg/l IAA for 20 days, however, in the presence of 2.0 mg/l IAA small quantity of transparent friable callus was formed at cut end. The roots were formed at all concentrations on edges of lamina, veins, midrib and petiole of the leaves. Nodal explants produced moderate amount of callus in few explants on media containing 0.25 mg/l IAA whereas very small quantity of callus was formed as the concentration was increased. Origin of callus at all concentrations of IAA was basically from cut ends and on the surface of stem segments .The regenerated callus was transparent.

In 28 days old cultures, media incorporated with IBA ranging from 0.25 to 2.0 mg/l showed no response of callus induction from leaves. Moderate roots formation was observed from cut ends of petiole of leaves when placed on media containing 0.25 and 1.5 mg/l of IBA. Nodal explants formed very little transparent callus on media incorporated with 0.25mg/l IBA and brown callus was formed at cut ends of petiole on medium containing 2.0 mg/l IBA. Number of explants forming shoots increased as the concentration of IBA was increased from 0.25 to 0.50 mg/l but on further increasing the concentration of IBA a decline in callus induction was observed. Few roots arose from callus and from cut ends of nodal explants under the influence of IBA.

NAA did not stimulate callus formation in the Nodal segments when present at low concentration, however, as the concentration was increased light brown callus was formed at 1.5 mg/l in 26 days culture. The callus formed under the influence of higher concentration of NAA was transparent and soft. It was formed either on whole surface, half surface or cut ends of the segments. The roots were formed from callus at 3.5 and 4.0 mg/l NAA. Leaf explants exhibited lesser extent of callus formation at low concentrations of NAA and complete inhibition at 1.0 mg/l. The extent of callus formation at higher concentrations was little better than at lower concentrations of NAA. Origin of callus was on the edges, midrib or the whole surface of leaf. 0.25, 3.0, 4.0, 4.5 mg/l induced transparent callus. Pale brown callus was formed at 0.5 mg/l, brownish white callus at 1.5 mg/l, however at 2.0 mg/l some calli were grey brown and few were transparent in colour. Creamish brown callus was formed at 2.5 mg/l and brownish grey at 3.5 mg/l. Roots were formed at all concentrations which developed either from petiole or on edges of leaf. Roots formed lateral roots.

Out the four auxins used, 2,4-D gave better results at all concentrations regarding duration of callus induction. Proliferation response of explants i.e. node, internode and leaf (abaxial side and adaxial side) are given in Table 1. Leaf explants gave higher percentage of callus induction as compared to other segments.

In 13 days old cultures transparent callus formation on the nodal explants occurred only in the presence of 3.5 mg/l 2, 4-D.Internodal segments formed callus at lower concentrations (0.1 to 1.5 mg/l). No callus formation occurred at higher concentrations except at 4.0 mg/l 2, 4-D where little quantity of transparent callus occurred. Cluster of roots arose from petiole in callused explants.

Leaf explants were cultured on MS medium in two positions either adaxial side touched the medium or abaxial side. When abaxial side was placed on medium fluctuation was observed in callus formation on leaf explants. White compact callus was formed in the presence of 0.1 mg/l 2, 4-D on lamina close to petiole, green compact callus was formed in the presence of 0.25 mg/l 2, 4-D on lower portion of leaf, creamish green friable callus at 0.5, 1.0 and 4.0 mg/l on whole surface or margins of leaf, creamish brown compact callus from 1.5 to 3.5 mg/l on veins, margins in some leaves and in others on the whole surface of leaf. When adaxial surface of lamina touched the medium at 0.1 mg/l white compact callus was formed on edges of leaf, at 0.25 mg/l green compact callus was formed on adaxial surface of leaf, at 0.5 mg/l creamish green on whole surface, at 1.5 mg/l brown callus was formed on 1/3 part of leaf, from 2.0 (Fig. 1A) to 4.0 mg/l creamish brown callus was formed on the whole surface, edges, midrib, veins of leaf. Cluster of roots were formed on petiole at 0.1, 0.25, 0.5 mg/l concentration of 2, 4-D. After 22 days roots were formed at higher concentrations starting from 2.5 mg/l. The adaxial side orientation had higher callus induction response on leaf explants than abaxial side.

Browning of callus: To overcome the problem of browning in callus, the callus obtained after 1st sub culturing on 2.0 mg/l 2, 4-D was transferred on control medium (2.0 mg/l 2,4-D) 2% grayish brown, 7% transparent and 14% brownish white callus was obtained on previous callus, however when transferred on the 0.2% g/l charcoal augmented medium there was no change in colour of callus.

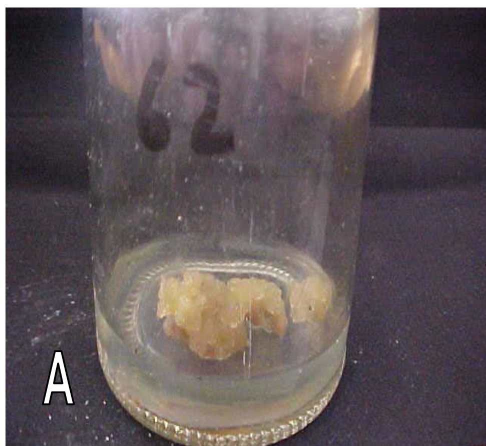
Influence of the addition of sucrose percentage on callus fresh weights: The callus (0.40 g) induced on 2.0 mg/l 2,4-D when was sub cultured on the media of same composition showed the highest fresh weight as compared to MS media supplemented with 2.0 mg/l 2,4-D but containing in 6% and 9% indicating an inhibitory effect of 6% and 9% sucrose (Table 2).

Sub culturing: After 1st subculture (22 days) the calli obtained on 2.0 mg/l 2, 4-D were not much in amount as compared to fresh culture (Fig. 1B) indicating a decrease in callus forming response and the colour was changed to brown. On further subculture again little quantity of dark brown callus was formed.

Table 1. Effect of 2, 4-D on callus induction in different explants of *A. aspera* after 13 days of culture.

MS medium+2, 4-D conc. (mg/l)	Explants	Explants forming callus (%)	Extent of callus formation	Roots formation	Place of callus formation
0.1	Is	40.0	+	-	Little portion
	Ns	0.00	-	-	
	Ab	50.0	+	-	Close to petiole
	Ad	20.0	+	*	Edges
0.25	Is	50.0	++	*	2/3 part of stem
	Ns	0.00	-	-	
	Ab	75.0	+	*	Lower portion
	Ad	40.0	+	*	Lower portion
0.5	Is	100	+	-	Half of stem
	Ns	0.00	-	-	
	Ab	100	+++	-	Whole surface
	Ad	100	+++	*	Whole surface
1.0	Is	85.71	+	-	Cut ends
	Ns	0.00	-	-	
	Ab	75.0	++	-	Edges or whole surface
	Ad	0.00	-	-	
1.5	Is	60.0	++	-	2/3 part of stem
	Ns	0.00	-	-	
	Ab	66.6	+++	-	2/3 part of stem
	Ad	20.0	+	-	1/3 part of stem
2.0	Is	0.00	-	-	
	Ns	0.00	-	-	
	Ab	85.71	+++	-	Whole surface or edges
	Ad	83.3	++++	-	Edges, midrib, vein, whole surface
2.5	Is	0.00	-	-	
	Ns	0.00	-	-	
	Ab	75.0	+++	-	Whole surface
	Ad	0.00	-	-	
3.0	Is	0.00	-	-	
	Ns	0.00	-	-	
	Ab	100	+++	-	Whole surface
	Ad	100	+++	-	Whole surface
3.5	Is	0.00	-	-	
	Ns	100	+	-	Little portion of stem
	Ab	62.5	+++	-	Veins or whole surface
	Ad	85.0	+++	-	Whole surface
4.0	Is	33.3	+	-	Scattered
	Ns	0.00	-	-	-
	Ab	66.6	+++	-	Whole surface
	Ad	57.14	+	-	Whole surface

Explant: Ns = Nodal stem, Is = Internodal stem, Ab = Abaxial side of leaf, Ad = Adaxial side of leaf
Proliferation of callus: + = Slow, ++ = Moderate, +++ = Good, ++++ =Excellent, - = Absent
Presence of Roots: * = Present, - = Absent
Data is average of 28 replicates



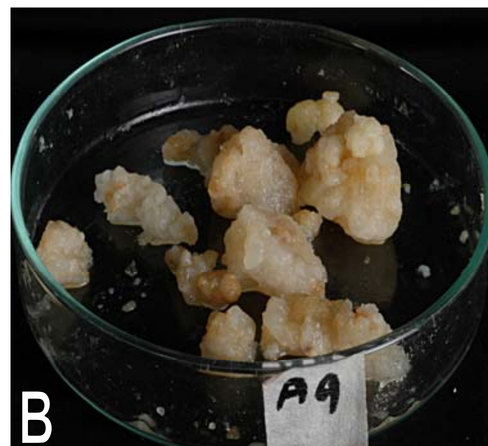


Fig. 1. Callus derived from leaf explants of *Achyranthes aspera* in MS medium containing 2.0 mg/l 2,4-D (A) after 13 days (B) after 22 days.

Table 2. Effect of different concentrations of sucrose on fresh weight of callus (0.4g) of *A. aspera* induced on 2.0 mg/l 2, 4-D containing 3%of sucrose after 22 days of subculture.

1st subculture medium composition		Fresh weight of callus (gm) after 1st subculture
2, 4-D conc. (mg/l)	Sucrose conc. (%)	
2.0	3	1.46 ± 0.67
2.0	6	0.76 ± 0.56
2.0	9	0.27 ± 0.19

Influence of growth regulators combined with coconut milk: Table 3 shows result of the when leaf explants were cultured on a GR-free MS medium very few explants showed callus formation but when leaf explants were cultured on a fresh MS medium supplemented with 2.0 mg/l 2, 4-D alone creamish brown callus was formed on the whole surface of leaf. When 2.5% level of coconut milk was added to 2.0 mg/l 2, 4-D there was no callus formation. Leaves became curved. Highest quantity of brown nodular callus was formed on margins of leaf as quantity of coconut milk was increased to 15% as compared to 5% and 10% level (Table 3).

Results of combination of 2, 4-D and Kinetin shows that very few leaves formed brown nodular callus on sides at 1.0 mg/l 2, 4-D and 1.0 mg/l Kinetin, very little nodular callus was formed on sides of leaf at 2.0 mg/l 2, 4-D and 1.0 mg/l Kinetin. Callus was formed on sides of leaf its amount was little more at 3.0 mg/l 2, 4-D and 1.0 mg/l Kinetin than previous one. There was no callus formation at 4.0 mg/l 2, 4-D and 1.0 mg/l Kinetin and leaves shrank. None of the combinations increased the callus induction (Table 3).

Table 3. Influence of growth regulators combined with different concentrations of coconut milk on *A. aspera* after 39 days of culture.

Plant growth regulators		Coconut milk (%)	Explants forming callus (%)	Extent of callus formation
2,4-D conc. (mg/l)	Kinetin conc. (mg/l)			
0.0	0.0	0.0	7.14	+
2.0	0.0	0.0	100	+++
2.0	0.0	2.5	0.0	-
2.0	0.0	5.0	33.3	+
2.0	0.0	10.0	50.0	+
2.0	0.0	15.0	100	+
1.0	1.0	10.0	14.2	+
2.0	1.0	10.0	100	+
3.0	1.0	10.0	100	++
4.0	1.0	10.0	0.0	-

Proliferation of callus: + = Slow, ++ = Moderate, +++ = Good, - = Absent
Data is average of 14 replicates

Discussion

The results confirm the great capacity of *Achyranthes aspera* to form callus. However, although callus can be obtained but not in large quantity. During the present study, from both stem and leaf callus initiation was found to be possible but best result was obtained from adaxial surface of leaf.

The present studies with *Achyranthes aspera* have confirmed that in this species, callus growth was much faster with 2,4-D (Table 1) than with NAA, IAA and IBA. Higher efficiency of 2, 4-D apparently depends perhaps on its greater availability in tissues. Jacobsen (1983) has reported that as 2, 4-D has high mobility and has only a restricted rate of oxidation and conjugation so it accumulates in tissue, whereas IAA and NAA consequently are present in the tissue as free auxin at much lower concentrations and have higher degradation rates and lower mobility. This hypothesis could justify the higher efficiency of 2, 4-D vis-a-vis other auxins. Many different concentrations and types of auxins have been used but as we have shown in present study for *Achyranthes aspera* 2,4-D (2.0 mg/l) is adequate for callus proliferation. Similarly Kayani *et al*., (2008) reported callogenic response from leaf explants of *A. aspera* and found 2,4-D at 1.0 mg/l and 2.0 mg/l produced light green and soft callus but at relatively lower (0.5 mg/l) and higher concentrations (3.0 and 4.0 mg/l), the callus morphology and callus mass changed.

Similarly, it has been noted in the present work, that although callus proliferates extremely well from the explants, in the first passage, during three subsequent passages the rate of growth declines rapidly to a low level and the colour of callus becomes dark brown showing necrosis. The calli used in the present experiments grew vigorously. This vigorous growth may have unable the calli to regenerate because the callus cultures that show exuberant growth are least conductive for regeneration as observed in stem callus of citrus (Chaturvedi *et al*., 1974; Hussain *et al.,* 2013). Furthermore the physical factors and genotype levels of endogenous growth hormones may have resulted to this non regeneration response of the calli (Narayanaswamy, 1977; Khan *et al.,* 2014).

Formation of quinones and oxidation of polyphenols results in browning of tissue and medium are highly toxic to plant tissues (Taji & William, 1996, Hussain *et al.,* 2011). In present work to overcome the problem of callus browning, in *Achyranthes aspera* 0.2% charcoal was used with 2.0 mg/l 2, 4-D but the problem was not solved. This result does not support Razan (1993). According to him activated charcoal at concentrations of 0.5-3% when added to the culture medium helps to reduce toxicity and permits unhindered cell growth or 0.2% level is low.

In plant tissue the most preferred carbon source is sucrose. In our experiment there was decrease in callus fresh weight as the sucrose concentration was increased from 3%. In *Allocasuarina verticillata* when sucrose

concentration was increased from 3% to 6% there was a decrease in weight of culture (Cao *et al.,* 1990). The decrease in fresh weight with increase in sucrose concentration may be interpreted due to decrease in osmolarity as reported by George (1993/1996).This may result a change in colour to brown.

In order to determine maximum yield of callus 2.0 mg/l 2, 4-D in MS medium was interacted with Kinetin and additive i.e., coconut milk but the callus was not best effective. This means there is no need of additional reduced nitrogen. This does not support the findings of Nakamura *et al.,* (1971) who reported the callus tissues was best effected when the MS medium was supplemented with 10% of coconut milk, 1.0 mg/l of 2, 4-D and 1.0 mg/l of Kinetin for *A. fauriei, A. japonica, A. japonica* var *hachijoensis, A. obtusifolia* and *A. rubrofusca* and with 10% of coconut milk, 4.0 mg/l of 2, 4-D and 1.0 mg/l of Kinetin for *A. longifolia*

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