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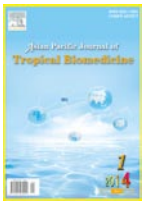

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In vitro callus induction and plantlet regeneration of *Achyranthes aspera* L., a high value medicinal plant

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PEER REVIEW

Peer reviewer

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Comments

Both science and fairness of this paper are correct.

Dr. Rezuanul Islam, Associate Professor, Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia-7003, Bangladesh.

Comments

In this study, an effort has been made for *in vitro* callus induction and micropropagation for medicinally important plant *A. aspera*. The data presented in results and discussion of this work is interesting.

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ABSTRACT

Objective: To study callus induction from different explants (internode, leaf, root) and *in vitro* plantlets propagation from medicinally important plant *Achyranthes aspera* L.

Methods: Sterilized explants were prepared by using 0.1% $HgCl_2$ and 0.5% Bavistin and callus was obtained when cultured onto Murashige Skoog's (MS) medium by using different concentrations and combination of 2,4-D, NAA, BAP, IAA, IBA with 3% sucrose and 0.8% agar. Induced callus was immediately transferred to MS medium containing at different concentrations of phytohormones for shootlets and rootlets induction respectively.

Results: Sterilization treatment of 0.1% $HgCl_2$ for 2-3 min and Bavistin 0.5% for 10-12 min showed the highest percentage of asepsis and survival rate. Maximum induction of callus was obtained from a combination of 2.0 mg/L 2,4-D and 0.5 mg/L NAA from leaf. Highest shootlets number (4.83±0.17) and length (3.8±0.16) cm were observed on full strength MS medium when fortified with BAP 4.0 mg/L and KIN 0.5 mg/L. Concerted efforts of BAP 2.0 mg/L and NAA 0.5 mg/L on full strength MS medium showed highest leaf number (6.77±0.94). *In vitro* raised shoots were allowed to root on different strengths of MS medium fortified with IAA and IBA at different concentrations. Experimentally, 3.0 mg/L IBA was enabled to induce maximum rootlets number (10.0±9.82) on full strength MS medium. Afterwards, regenerated shoots with well developed roots were successfully subjected to hardening process and were acclimatized. The survived plantlets showed 66.67% survival frequency without any morphological abnormality.

Conclusions: The results demonstrated that different explants were good source of callus induction, morphology analysis as well as indirect plantlets regeneration.

KEYWORDS

Achyranthes aspera, Callus induction, Plantlets propagation, Murashige Skoog's, Micropropagation

1. Introduction

Achyranthes aspera L. (Amaranthaceae) (*A. aspera*) locally termed as Apang (in Bengali) is one of the plant used for medicinal purposes. It is an erect, annual herb which is a common plant found throughout Bangladesh, India, tropical Asia and other parts of the world as weed[1]. The major form of healthcare in the rural parts of developing countries is traditional medicine, which is mostly plant-based. *A. aspera* has diverse medicinal uses in the folk medicinal system[2]. Along with the utilization in traditional medicine by local practitioners and healers, this plant also reportedly showed diverse pharmacological properties including antimicrobial[3,4], lavicidal, anti cancer, antipyretic, cardiovascular agent, immunostimulant, hypoglycaemic, antioxidant, anti asthmatic, anti obesity, anti snake venom, anti plant pathogen, anti-depressant, anti-dandruff, anthelmintic, and wound healing[1,5-12].

Production of calli form from fragments of steams, leaves and roots are mainly carried out to determine the

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culture conditions required by the explants to survive and grow, study cell development, exploit products coming from primary and secondary metabolism and obtain cell suspention in propagation. It can also pave the way for isolating economically valuable phytochemicals, which can avoid collecting plant materials from natural sources[13,14]. In the areas of plant biotechnology, callus and cell culture carries a special role for producing medicinal and bioactive compounds in large-scale from plants. Phytochemicals are serving as a major source of pharmaceuticals, flavors, agrochemicals, colors, biopesticides and food additives. Phytochemical screening of *A. aspera* showed that it contains alkaloids, flavonoids, tannins, anthrapuinones, saponins, glycosides and volatile oils[15].

Callus culture of *A. aspera* has been previously reported only by using leaf explants but there is no systematic study on this plant by using different explants and *in vitro* plant regeneration. From a medicinal point of view, the importance of this plant and exploitation will lead to a decline in its quantity. Therefore the present investigation is working on developing its *in vitro* regeneration protocol.

2. Materials and methods

2.1. Collection of plant material

The plants were collected from Atomic Energy Research Establishment campus and grown in the medicinal plant nursery of Plant Biotechnology Division of National Institute of Biotechnology, Savar, Dhaka, Bangladesh. Plant was taxonomically identified by Bangladesh National Herbarium.

2.2. Surface sterilization

Both *in virto* and *ex vitro* explants were selected as culture explants. *Ex vitro* explants were washed thoroughly under running tap water for 20 min to remove the traces of dust, *etc*. followed by treatment with Bavistin at different concentrations followed by treatment with 10% teepol/tween-20 for 2 min. Then the explants were sterilized in 70% ethanol for 45 seconds and finally with 0.1% $HgCl_2$ for 2-3 min and washed 3-4 times with sterile double distilled water.

2.3. Culture medium

Young leaf, internodal segments and root [(1-2) cm] were inoculated on Murashige Skoog's (MS) medium containing 3% sucrose and gelled with 0.8% agar supplemented with various concentrations of auxins such as 2,4-D, NAA, IAA and cytokinin BAP, and for shoot and root initiation BAP, KIN, NAA, IBA and IAA were used. The pH of the medium was adjusted to 5.8 before being gelled with agar and autoclaved for 20 min at 121 °C for 15 lbs pressure.

2.4. Culture conditions

The growth room conditions maintained for *in vitro* cultures were (25±2) °C and 60%-70% relative humidity, and the light intensity is 3 000 lux with a photoperiod of 16 h light and 8 h dark. Each experiment was conducted at least thrice with 10 replicates per treatment.

2.5. Sub culturing

Sub culturing was carried out at regular intervals. Visual observations of the cultures were taken for every transfer and the effects of different treatments were quantified on the basis of percentage of cultures showing response.

2.6. Data recording and analysis

The percentage of survival explants and creating calli, its structure, color, texture, percentage of response and initiation time were determined for 4 weeks and tabulated for detailed study. The data pertaining to frequency of callus induction per culture was subjected to standard deviation and mean separation was carried out by using computer software.

3. Resutls

3.1. Ex vitro explants sterilization

The frequency of survival explants and callogenic response of explants varied with concentration of Bavistin, $HgCl_2$ and sterilization time. Concentration of 0.1% $HgCl_2$ for 2-3 min and Bavistin 0.5% for 10-12 min showed the highest percentage (94.60%) of survival rate of Internodal explants and 76.07%, 40.20% on leaf and root explants respectively. Concentration of 0.1% $HgCl_2$ for 4-5 min showed the highest percentage of microbial contamination free but lower percentage of explants survived. It was observed that when the explants were sterilized with 0.1% $HgCl_2$ for 5 min, nearly 100% of microbes are free from explants but the mortality rate of explants were high up to 81% to 87% (Table 1).

Table 1
Effect of sterilants on surface sterilization of explants of *A. aspera*.

Serial number	Sterilants	Time	Percentage of survival (%)		
			Leaf	Internod	Root
S1	Bavistin 0.5% $HgCl_2$ 0.1% Alcohol 70%	10-12 min 4-5 min 45 seconds	14.33	19.25	13.43
S2	Bavistin 0.5% $HgCl_2$ 0.1% Alcohol 70%	10-12 min 3-4 min 45 seconds	30.19	60.76	20.17
S3	Bavistin 0.5% $HgCl_2$ 0.1% Alcohol 70%	10-12 min 2-3 min 45 seconds	76.07	94.60	40.20

3.2. Effect of 2,4-D

Within 3 weeks of culture period, 100% callogenic response was achieved when the medium was fortified with 2,4-D at different concentrations for all types of explants leaves, internodes and roots (Table 2). Leaf explants showed 100% callogenic response at 2.0 mg/L and it formed brownish loose callus. Internodal and root explants were best at concentration 2.0 mg/L, but both were less percentage and callogenic response than leaf explants. Each responded explant with a concentration range of 0.5 mg/L to 5.0 mg/L. Results from this experiment revealed that all types of explants responded not equally in every medium composition.

3.3. Synergistic effect of 2,4-D and BAP

Purple colored callus appeared when the internodal explants were cultured in MS medium supplemented with combinations of 2,4-D and NAA. Fastest callogenic response was observed when 2,4-D (2.0 mg/L) and BAP (0.5 mg/L) was added to MS medium (Table 3). The noticeable characteristic of the responded callus was variation of texture in callus along with changes of hormonal combinations. It varied from compact to friable and brownish to purple.

3.4. Synergistic effect of 2,4-D and NAA

Only medium combinations of both 2,4-D and NAA

Table 2

Effect of 2,4-D (mg/L) in MS medium for callus induction of *A. aspera*.

MS+2,4-D	Source explants	No. of explants inoculated	Response percentage (%)	Intensity of callus formation	Time for response (d)	Color	Texture
0.5	Leaf	10	60	+	12	Brownish	Friable
	Internodal	10	60	+	14	Purple	Friable
	Root	10	20	+	17	Off white	Loose
1.0	Leaf	10	90	+++	9	Green	Soft
	Internodal	10	80	+++	12	Purple	Friable
	Root	10	20	+	17	White	Loose
2.0	Leaf	10	100	+++	8	Brownish	Loose
	Internodal	10	80	+++	12	Purple	Friable
	Root	10	20	+	17	White	Loose
3.0	Leaf	10	70	++	8	Brownish	Friable
	Internodal	10	60	++	13	Purple	Friable
	Root	10	10	+	18	Pale	Friable and wet
4.0	Leaf	10	70	++	9	Brownish	Compact
	Internodal	10	60	+	13	Purple	Friable
	Root	10	10	+	18	White	Loose
5.0	Leaf	10	50	+	11	Brownish	Compact
	Internodal	10	40	+	13	Purple	Friable
	Root	10	10	+	18	Off white	Friable

Observation: Profuse callus: +++, Moderate callus: ++, Poor callus: +, No response: -.

Table 3

Effect of MS+(2,4-D+BAP) (mg/L) in MS medium for callus induction of *A. aspera*.

MS+(2,4-D+BAP)	Source explants	No. of explants inoculated	Response percentage (%)	Intensity of callus formation	Time for response (d)	Color	Texture
1.0+0.2	Leaf	5	60	++	11	Brownish	compact
	Internodal	5	60	+	14	Purple	Friable
	Root	5	10	+	20	Off white	Friable
1.5+0.5	Leaf	5	80	+++	11	Brownish	compact
	Internodal	5	60	++	14	Purple	Friable
	Root	5	10	+	20	White	Friable
2.0+0.5	Leaf	5	100	++++	9	Brownish	Friable
	Internodal	5	80	+++	13	Copious purple	Friable
	Root	5	30	++	18	White	Friable
3.0+0.5	Leaf	5	100	++++	9	Brownish	Soft
	Internodal	5	70	++	13	Purple	Friable
	Root	5	30	++	18	White	Friable
4.0+0.5	Leaf	5	100	++++	9	Profuse, brownish	Friable
	Internodal	5	60	++	13	Brownish	Friable
	Root	5	20	+	22	Brownish	Friable
5.0+0.5	Leaf	5	60	++	10	Brownish	compact
	Internodal	5	60	+	16	Brownish	Friable
	Root	5	10	+	22	White	Friable

Observation: Profuse callus: +++, Moderate callus: + +, Poor callus: +, No response: -.

formed better calli. The texture was loose with wet surface. Callus growth was not prominently increased or showed variations along with the change of concentration. The best medium found from these synergistic experiments was 2,4-D (2.0 mg/L) and NAA (0.5 mg/L) fortified MS medium (Table 4).

3.5. Synergistic effect of 2,4-D and IAA

Good callogenic response was found in MS+2,4-D+IAA (1.5+0.5) mg/L. All treatments of 2,4-D with NAA resulted in formation of loose and compact callus with light green, dark brown and pale color in the medium composition as described in Table 5. Root explants were ineffective when the hormonal concentration were low at concentration.

3.6. Synergistic effect of 2,4-D and IBA

Quick callus growth was obtained with concentrations at (1.5+0.5) mg/L and (2.0+0.5) mg/L. Good callogenic response was found in MS+2,4-D+IBA (1.5+0.5) mg/L. All treatments of 2,4-D with IBA resulted in formation of friable and loose callus with off white color in the medium composition as described in Table 6. No further morphogenic response was observed when the cultures were maintained up to 8 weeks in the medium.

3.7. Indirect shoot organogenesis from callus

Vegetative plant parts especially leaf and nodal parts

Table 4

Effect of MS+2,4-D+NAA (mg/L) in MS medium for callus induction of *A. aspera*.

MS+2,4-D+NAA	Source explants	No. of explants inoculated	Response percentage (%)	Intensity of callus formation	Time for response (d)	Color	Texture
1.0+0.2	Leaf	5	60	++	13	Greenish	Compact,Friable
	Internodal	5	40	+	14	Greenish white	Friable
	Root	5	20	+	20	White	Loose, wet
1.5+0.5	Leaf	5	70	+++	10	Light green	Compact,Friable
	Internodal	5	60	+	14	Greenish white	Friable
	Root	5	20	+	20	White	Loose, wet
2.0+0.5	Leaf	5	100	++++	10	Luxuriant greenish	Friable
	Internodal	5	80	+++	13	Purple	Friable
	Root	5	30	++	20	White	Friable, wet
3.0+0.5	Leaf	5	100	+++++	10	Luxuriant greenish	Friable
	Internodal	5	80	++	13	Purple	Friable
	Root	5	30	+	21	Pale	Friable, wet
4.0+0.5	Leaf	5	100	++++	10	Luxuriant greenish	Friable
	Internodal	5	80	+++	13	purple	Friable
	Root	5	20	++	21	Pale	Friable, wet
5.0+0.5	Leaf	5	80	+++	12	Greenish white	Friable
	Internodal	5	80	+	13	purple	Friable
	Root	5	20	+	21	Pale	Loose, wet

Observation: Profuse callus: +++, Moderate callus: + +, Poor callus: +, No response: -.

Table 5

Effect of MS+2,4-D+IAA (mg/L) in MS medium for callus induction of *A. aspera*.

MS+2,4-D+IAA	Source explants	No. of explants inoculated	Response percentage (%)	Intensity of callus formation	Time for response (d)	Color	Texture
1.0+0.2	Leaf	5	50	++	14	Brownish green	Compact
	Internodal	5	20	+	17	Brownish	Compact
	Root	5	0	-	-	-	-
1.5+0.5	Leaf	5	70	+++	10	Light green	soft
	Internodal	5	50	++	15	Brownish	Soft
	Root	5	20	+	20	Pale	Loose, wet
2.0+0.5	Leaf	5	70	+++	12	Light green	soft
	Internodal	5	50	++	15	Brownish	soft
	Root	5	20	+	20	Pale	Loose, wet
3.0+0.5	Leaf	5	60	+++	13	Brownish	Compact
	Internodal	5	40	++	16	Brownish	soft
	Root	5	10	+	20	Pale	Loose, wet
4.0+0.5	Leaf	5	40	++	13	Dark brown	Compact
	Internodal	5	40	++	16	Brownish	Soft
	Root	5	10	+	20	Pale	Loose, wet
5.0+0.5	Leaf	5	40	++	13	Dark brown	Compact
	Internodal	5	20	+	18	Brownish	Soft
	Root	5	10	+	22	Pale	Loose, wet

Observation: Profuse callus: +++, Moderate callus: + +, Poor callus: +, No response: -.

Table 6

Effect of MS+2,4-D+IBA (mg/L) in MS medium for callus induction of *A. aspera*.

MS+2,4-D+IBA	Source explants	No. of explants inoculated	Response percentage (%)	Intensity of callus formation	Time for response (d)	Color	Texture
1.0+0.2	Leaf	5	60	++	16	Light green	Soft
	Internodal	5	50	++	18	White	Friable
	Root	5	10	+	27	Off white	Loose, wet
1.5+0.5	Leaf	5	80	+++	10	Light green	Soft
	Internodal	5	60	++	13	White	Friable
	Root	5	20	+	20	Off white	Loose, wet
2.0+0.5	Leaf	5	80	+ + +	11	Light green	soft
	Internodal	5	60	+ +	13	Bownish	Friable
	Root	5	20	+	22	Off white	loose
3.0+0.5	Leaf	5	80	+ + +	13	Brownish	Friable
	Internodal	5	40	+	18	Bownish	Friable
	Root	5	20	+	28	Off white	Friable
4.0+0.5	Leaf	5	70	++	13	Bownish	loose
	Internodal	5	30	+	18	White	Friable
	Root	5	–	–	–	–	–

Observation: Profuse callus: +++, Moderate callus: + +, Poor callus: +, No response: –.

are desirable explants for *in vitro* improvement because of regeneration from these explants would preserve the genetic homozygosity of the parent genotype. The presence of cytokinin along with auxin is necessary for indirect adventitious shoot induction. The induction of callus and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators such as BAP, KIN and NAA in the culture medium. Among the growth regulators tested, BAP+NAA (2/0.5 mg/L) induced maximum frequency of shoot regeneration. The maximum shoot number (4.83±0.17) was observed when it was grown on the MS medium with BAP+KIN (4/0.5 mg/L). But the maximum number of leaves (6.77±0.94) was observed in BAP+NAA (2/0.5 mg/L) (Table 7). The minimum regeneration frequency and shoot length [(1.4±0.40) cm] was noted at BAP+1/2 MS (5.0 mg/L). In the present study BAP alone and along with NAA exhibited better morphogenesis. *In vitro* derived shoots with a length of 2-4 cm were excised and transferred to MS medium supplemented with different concentrations of auxins such as IBA and NAA (1.0-4.0) mg/L. In all the concentrations tried, exogenous supply of auxins favoured the root formation and root primordial appeared between 5-6 d of inoculation. The minimum shoot length (3.23±0.19) cm was observed at IBA (1.0 mg/L). High rooting frequency (82%) with highest number of roots (10.0±9.82) was obtained in IBA (3.0 mg/L). The maximum root length [(4.67±0.94) cm] was observed at IBA (4.0 mg/L) (Table 8). Higher concentrations of IBA and NAA showed the organogenic callus formation (Figure 1).

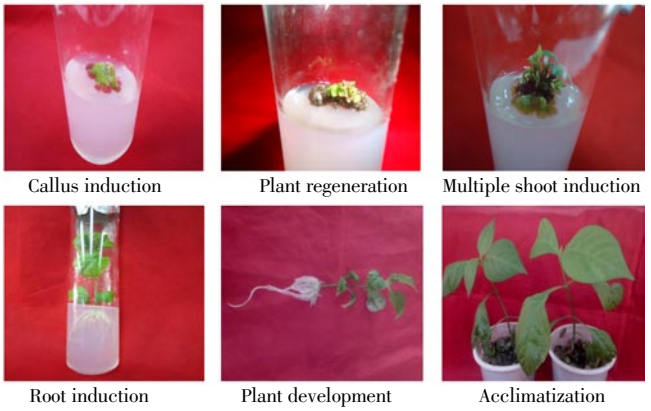

Figure 1. The organogenic callus formation.

Table 7

Indirect shoot organogenesis from callus obtained through leaf and Internodal explants.

Plant growth regulator	Concentration (mg/L)	Regeneration Frequency (%)	Mean number of shoots	Number of leaves	Mean Shoot length (cm)
BAP+KIN+MS	1/0.5	–	–	–	–
BAP+KIN+MS	2/0.5	15	1.73±0.20	2.67±0.94	2.60±0.29
BAP+KIN+MS	3/0.5	20	3.97±0.12	6.00±1.63	3.70±0.25
BAP+KIN+MS	4/0.5	25	4.83±0.17	6.67±1.88	3.80±0.16
BAP+KIN+1/2 MS	1/0.5	–	–	–	–
BAP+KIN+1/2 MS	2/0.5	10	1.30±0.29	2.00±0	2.90±0.08
BAP+KIN+1/2 MS	3/0.5	10	1.23±0.17	3.00±0.82	1.90±0.08
BAP+NAA+MS	2/0.5	32	4.00±0.08	6.77±0.94	2.90±0.22
BAP+NAA+MS	3/0.5	25	2.93±0.17	5.33±1.08	3.00±0.24
BAP+NAA+MS	4/0.5	30	2.83±0.54	5.00±2.16	2.90±0.12
BAP+NAA+MS	6/0.5	–	–	–	–
BAP+1/2 MS	1.0	–	–	–	–
BAP+1/2 MS	3.0	10	1.60±0.29	2.00±0	1.80±1.12
BAP+1/2 MS	5.0	8	0.93±0.25	2.00±0	1.40±0.40

Observation: After 4 weeks; Values are mean±SD; –: No result.

Table 8

Root organogenesis of *in vitro* derived shoot lets in MS medium supplemented with various concentrationsof auxins such as IBA and NAA.

Plant growth regulator	Concentration (mg/L)	Days for root induction	Frequency of root formation (%)	Mean number of roots/shoot	Mean length of root (cm)	Mean length of shoot (cm)	Callus
NAA	1.0	14-15	60	5.67±0.47	3.67±0.47	5.00±0.00	-
	2.0	10-11	64	7.00±0.81	2.67±0.47	3.87±0.19	-
	3.0	10-11	70	8.70±2.62	3.00±0.00	3.57±0.48	-
	4.0	7-8	74	9.00±2.16	2.67±0.47	4.37±0.45	+
IBA	1.0	13-14	70	5.70±0.47	2.00±0.82	3.23±0.19	-
	2.0	10-11	67	8.67±2.62	3.67±0.47	3.97±0.41	-
	3.0	7-8	82	10.00±9.82	3.67±0.47	4.29±0.17	+
	4.0	5-6	82	9.00±2.92	4.67±0.94	5.33±0.94	+

Observation: After 3 weeks; values are mean±SD; +: callus; -: No result.

4. Discussion

High period of exposure with $HgCl_2$ leads to the browning of explants and death. Our results were in tantamount to Johnson *et al*[16]. They reported that surface sterilization exceeding 5 min was lethal to explants, whereas the present study showed that above 3 min of surface sterilization was lethal to all of the tested varieties of *A. aspera*.

2,4-D along with BAP, IBA and IAA were observed to be potent hormonal combination for profuse callus production from leaf, internodal and root explants, in which light green to dark green, brownish to pale colour, compact to fragile callus were formed. The effectiveness of 2,4-D and in combination with BAP, IBA, IAA were mostly effective on leaf explants and similar callusing response was noted in this plant. Internodal explants also showed about same result as leaf whereas root was negligible as an explant. The effectiveness of 2,4-D and in combination with cytokinins in inducing callus might be due to their role in DNA synthesis and mitosis[17].

Though 2,4-D is a synthetic auxin, its role in callus induction was particularly observed in our experiment. Efficacy of exogenous 2,4-D has also been reported with other medicinal plants. Results described by Mungole *et al.* and Hassan *et al.* were also in agreement with our result for using this synthetic plant growth regulator in the culture medium for *Ipomoea obscura*, *Withania somnifera*, *Cardiospermum halicacabum* Linn. and *Abrus precatorius* respectively[18,19]. We have noted that the effect of 2,4-D with NAA, IBA and IAA have showed potential efficiency as a synthetic plant hormone to generate callus from leaf explants. Synergism of 2,4-D and NAA and *Cercis chinensis*. Synergism of 2,4-D and BAP found by the authors also agree with results obtained by Nikolaeva *et al*[20].

Callus tissue is good source of genetic variability and adventitious shoot formation. In the present study BAP along with KIN exhibited better morphogenesis. Indirect shoot regeneration through callus phase obtained from leaf explants was earlier reported in many plants like *Spilanthes acmella* and *Justicia gendarussa*[21,22]. Root formation and plant regeneration with IBA has been reported by Agastian *et al*[22,23].

In conclusion, the following points may mention the effectiveness of callus induction and plant regeneration depends on the type of used growth regulators and explants source. The characteristics of the callus (color and texture) depend on culture medium and the protocol of callus induction of *A. aspera* opens new vista that could facilitate phytochemical production and extraction of pharmaceuticals from callus without harvesting the plant itself.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

A. aspera is used in various traditional systems of medicines all over the world for the treatment of various diseases. This article contains wide spectrum researches and the authors have done well to collect and compile data which include current researches from all over the world. The scientific data in this article are novel and promising.

Research frontiers

Studies indicate that different explants were a good source of callus induction, morphology analysis as well as

indirect plantlets regeneration.

Related reports

Gnanaraj *et al*. (2012) reported that nodal buds are alternative, reproducible and dependable explants for clonal propagation of *A. aspera* and *A. bidentata*.

Innovations and breakthroughs

Data shows that different explants were a good source of callus induction, morphology analysis as well as indirect plantlets regeneration.

Applications

The protocol of callus induction of *A. aspera* opens new vistas that could facilitate phytochemical production and extraction of pharmaceuticals from callus without harvesting the plant itself.

Peer review

In this study, an effort has been made for *in vitro* callus induction and micropropagation for medicinally important plant *A. aspera*. The data presented in results and dicussion of this work is interesting.

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