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Effect of MS and L2 Medium on Callusing and Regeneration From Nodal Explants of *Asclepias curassavica*- (L).

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Abbreviations

BAP – Benzyl aminopurine
KN – Kinetin
IAA – Indole acetic acid
IBA – Indole butyric acid
NAA – Naphthalene acetic acid
2, 4-D – 2, 4- dichloro phenoxy acetic acid
GA_3 – Gibberellic acid

Abstract

The present study demonstrates an efficient protocol for *in vitro* mass propagation of *A. curassavica* through direct as well as indirect regeneration. The effect of MS and L2 media supplemented with various concentrations and combinations of growth regulators has been studied. The growth regulators used include BAP and kinetin (cytokinins) and IAA, IBA, NAA and 2, 4-D (auxins). MS media proved better for callusing than L2 media and callus mediated shoot organogenesis was obtained by sub culturing the organogenic callus obtained from leaf explants on MS + 2 mg/L NAA + 1mg/L BAP+0.5 mg/L KN on to a fresh medium fortified with 1 mg/L BAP+ 0.1 mg/L GA_3 lacking NAA. Among the two different media (MS and L_2) tested, L_2 medium proved superior to MS medium in terms of shoot multiplication and shoot length. Nodal explants showed better organogenic response than shoot tip explants. Among nodal explants basal nodal explants produced more number of shoots than terminal nodes for shoot morphogenesis. Among the two different cytokinins tested in MS media BAP proved better than KN for improving shoot number and shoot length in combination with different auxins. Among the two different cytokinins tested in L_2 media KN proved better than BAP for improving shoot number and shoot length either individually or in combination with different auxins. Highest number of shoots was obtained from nodal explants cultured on L_2 media containing 3 mg/L KN in combination with IAA. Hence L2 media proved to be effective for organogenesis while MS yielded better callusing and can be employed for *in vitro* mass propagation of *A. curassavica.*

Keywords: - Asclepiadaceae, callus, micropropagation, organogenesis

Introduction

Asclepias curassavica (L.) (Milk weed) is an erect, evergreen sub shrub belonging to the family Asclepiadaceae. The family comprises more than 250 genera and 3,000 species, of which 43 genera and 243 species are present in India. Resinoid (galitoxin), the toxic principle in poisonous species is found in the milky latex of its stem. Root extracts of the plant are widely used in South America as an emetic and laxative to induce vomiting. A decoction of the plant is used as an abortifacient. Roots are known as 'Pleurisy root" and used as an expectorant for pneumonia, lung problems, employed to treat warts, fever, etc. Milk weeds are known to contain cytotoxic glycosides. Though many medicinally important plants of Asclepiadaceae are known, little tissue culture studies have been accomplished so far making the family endemic, endangered and vulnerable taxa.

Callus culture offers many advantages as a model system for several biological investigations. The callus model of plant flowering was proposed by Chailakhyan *et al.*, (1975) and from then on have been used widely in various physiological and related studies by Weinstein *et al.*, 1962 and Vesins *et al.*, 1972 in the genus Rosa and by Altman *et al.*, 1982. Venkateswara *et al.*, (1987) isolated cryptosin, a new steroidal glycoside, which is found to be cardioactive, from cultured tissue of *Cryptolepis buchanani*. *In vitro* multiple shoot formation from nodal explant was reported in *Decalepis hamiltonii* by Anitha and Pullaiah (2002). Komalavalli and Rao (1997) reported maximum number of shoots from mature nodal explants of *Gymnema elegans*. Lee *et al.*, (1982) cultured apical shoot tips of *Asclepias erosa* to obtain greatest number of shoots. Morphogenetic investigations of different explants of *Asclepias rotundifolia* were

carried out by Tideman and Hawker (1983). Various researchers have carried out extensive morphogenetic and suspension culture studies in *Asclepias tuberosea*. Researchers have shown interest in latex producing plants with considerable amount of low molecular weight hydrocarbons that could be used as substitutes for fossil fuels. Direct shoot organogenesis was also reported from an economically significant genus Asclepias by Mante *et al.,* (1989). Snyder (1955) initiated tissue culture studies in Asclepiadaceae. Majority of *in vitro* morphogenic studies have been conducted in *Hemidesmus indicus*, an Ascepiad.

Materials and methods

In the present investigation, *A. curassavica* plants and seeds were collected from Tirumala hills, Tirupathi, A.P., during March, 2005. The seeds were germinated in the garden, Department of Biotechnology, S.V. University, Tirupathi, A.P. Actively growing 1-2 year old healthy plants were collected from Tirumala hills and planted. Pods of *A. curassavica* were collected and germinated in garden during the period of June – October, 2005. After leaf excision, the cuttings were dissected into shoot tip, terminal node and basal mature single noded bits. All the explants were initially washed in running tap water for 30 minutes, then in 1% Teepol for 5 minutes followed by continuous washing in double distilled water for 5 times. Surface decontamination was performed by passing through 0.1% $HgCl_2$ (w/v) for 5 minutes. The optimal concentration of the sterilant was determined after several initial trials; surface sterilization was followed by 5-6 rinses in sterile distilled water. The cut ends of the explant were further trimmed. Then the explants were blotted on sterile filter paper discs. Initial experiments were designed for the selection of most desirable explants and optimal concentrations of cytokinins and auxins. MS medium and L_2 medium individually supplemented with different concentrations of cytokinins (BAP and KN) (0.5 – 4.0 mg/l) alone and in combination with auxins (IAA, IBA and NAA) (0.5mg/l) and explants from mature plants and young juvenile seedlings were inoculated. Explants showing better regenerative response were selected and the results were statistically analyzed and used for further experiments and analysis. In continuation, experiments were carried out with MS medium and L_2 medium supplemented with various cytokinins alone (BAP and KN), and in combination with auxins (NAA, IAA, IBA).

Results and discussion

The characteristic trait of a callus is that the unorganized mass has localized regions of meristematic activity (Meristemoids) and rudimentary cambial regions with zones of vascular differentiation which have the potential to produce normal shoots, roots and embryoids that ultimately form plantlets (Dodds and Roberts, 1982). Moreover, callus is a good source of genetic variability and adventitious shoot formation and so is used in the production of cell suspension culture which are used extensively for *in vitro* secondary metabolite production, enzyme extraction, and somatic embryogenesis.

In the present study, among the different explants tested, percent of response and intensity of callus formed were more with leaf explants followed by inter nodal explants. Calli produced from leaf explants with 2, 4-D at 2 mg/L were compact, brown colored and embryogenic in nature (Table 1). The significance of 2, 4-D embryogenic callus initiation had been emphasized by Patnaik *et al.,* (1997), Sagare *et al.,* (1995) and Tiwari *et al.,* (1998). The calli from leaf explants cultured on MS medium fortified with NAA (0.5 and 1 mg/L) were nodular and fragile. The highest intensity of calli formed were observed in MS medium containing 0.5 mg/L 2,4-D + 1.5 mg/L NAA + 0.5 mg/L BAP and are nodular and fragile in nature (Table 2). Calli produced from leaf explants with IAA (2.5 mg/L) were dark brown coloured and compact. The other auxin IBA did not induce any callusing response with the leaf explant tested. This may be attributed due to its thermolabile nature and similar response was observed by Prakash (2001) in *Pimpinella tirupatiensis* and Chakradhar (2004) in *Wattakaka volubilis*. Calli with varied morphology with respect to different auxins were reported by Kulkarni *et al.,* (2001), Ramesh and Padhya (1990) and Morini (2000).

The green patches of organogenic calli containing shoot buds further developed into shoots when sub cultured onto a fresh medium with 1 mg/L BAP, lacking NAA. Elongation of shoots occurred on sub culturing to fresh medium with 1 mg/L BAP and 0.1 mg/L GA_3. GA_3 improved elongation of shoot buds which was earlier reported by Arora and Bhojwani (1989), Pattnaik and Chand (1996), Sahoo *et al.,* (1997) and Vasanth *et al.,* (2002). The nodular callus developed shoots from deeper zone of its tissue when transferred to the medium fortified with 1 mg/L BAP + 0.1 mg/L GA_3. In general GA_3 was found to exert inhibitory action at bud induction stage due to its suppression on meristemoid formation (Thorpe and Meier, 1973 and Rubulo *et al.,* 1984), but is necessary for shoot bud development (Jarret and Hasegawa, 1981).

The decisive factor controlling organogenesis in tissue culture is the balance of cytokinins and auxins as envisaged by Chalupa (1987), Lakshmisita and Sankara Rao (1984) and Sinha and Mallick (1991). In this study, the leaf explants were cultured with different auxins at their optimum concentration in combination with BAP. The organogenic calli with differentiated shoot buds were formed from leaf explants at 1 mg/L NAA +

0.5 mg/L BAP + 0.5 mg/L KN. The organogenic callus formed on this medium was brown coloured and compact in nature. In the present investigation, different seedling and mature explants like axillary buds, shoot tips were inoculated on MS and L_2 media containing 3% sucrose and 1 mg/L cytokinins. The efficiency of the media was determined by percentage and nature of response i.e., number of shoots and their length. Among the two media screened, L_2 medium proved better than MS medium (Table 3). Axillary buds on L_2 medium developed better than MS medium yielding an average of 5 shoots per explant with 80 per cent of shoot regeneration. All explants inoculated on L_2 medium responded well with healthy shoots. Shoots developed on this medium were thin, light green in colour with less number of leaves. The response of any tissue *in vitro* is attributed to the composition of the medium besides other factors and balance between growth hormones (Mc Car dell and Frett, 1990). The effect of type of media was studied previously by Hogue and Arima (2003), Narula *et al.*, (2001), Suri *et al.*, (1998) and Heble *et al.*, (1974). For successful micropropagation the buds of apical (shoot tip) and axillary (nodal) explants were preferred as these buds have entire rudimentary vegetative shoot and offer no risk of obtaining cuttings different from mother plant than does the classical propagation in the green house (Anuradha and Pullaiah, 1992). The present study showed that cytokinins are indispensable for sprouting of axillary buds as stated by Hussey (1986) and Purohit and Dave (1996).

Shoot tip explants produced single long unbranched shoots at all concentrations of cytokinins alone and in combination with various auxins, which indicate the strong apical dominant nature of the plant. Production of axillary shoot by BAP and inhibition of its elongation simultaneously were observed as reported by Baraldi *et al.*, (1988). He suggested that shoot proliferation by this cytokinin was promoted by a low energy phytochrome response. In conditions which does not induce shoot proliferation i.e., dark, low fluency far red light, BAP inhibits shoot elongation. However, shoot tip explants were found to produce single long shoot in all responsive concentrations of BAP.

The highest number of shoots with maximum shoot length and frequency of shoot regeneration were obtained from KN than BAP when employed individually. Hence BAP was considered as second best cytokinin for axillary shoot proliferation in *A. curassavica* both qualitatively and quantitatively. Variation in the activity of different cytokinins in inducing multiple shoots can be explained by two ways – a) Their varied uptake rate reported in different genomes (Blakesley and Lenton, 1987a and b) Differential translocation rates to meristematic regions and metabolic processes after absorption, in which the cytokinin may be degraded or conjugated with sugars or amino acids present in the plant, to form biologically inert compounds as reported by Tran Tranh Van and Trinh (1990) and Kaminek (1992).

The superiority of KN over BAP in multiple shoot production has been illustrated among other Asclepiadaceans, *H. indicus* (Patnaik and Debata, 1996), *Tylophora indica* (Faisal and Anis, 2003) and in *Coleus forskohlii* (Sharma *et al.*,, 1991). The enhanced response with KN containing media can be attributed to its specificity of mechanism of action in preventing auxin synthesis. Kinetin is said to block the auxin stimulated synthesis of a polypeptide i.e., β-1, 3- glucanase enzyme (Lee, 1974). The regenerated plantlets obtained with BAP were stunted and had short internodes with relatively small leaves. Unlike BAP treated shoots, the shoots which developed on KN supplemented medium had normal dark green and broad leaves with long internodal space and showed improved elongation and growth. The best shoot quality in KN containing medium had been reported by Pierik *et al.*, in *Gerbera jamesonii* (1982). Among the two different media tested, L_2 medium fortified with BAP 2 mg/L was found to be the best medium for shoot sprouting, number and length followed by MS medium in *A. curassavica* (Table 4 and 5). The shoot buds sprouted on MS medium showed only limited number and growth even if they were maintained for longer period. Thus the degree of growth and differentiation varied considerably with the media composition. This may be because L2 medium has higher levels of Ca^{2+}, Mg^{2+} and SO_4^{2-} than MS media and since nitrogen is a significant constituent; its deficiency would inhibit plant growth.

Conclusions

Major success of micropropagation depends on successful acclimatization of *in vitro* rooted plantlets. Among the two different media (MS and L_2) tested, L_2 medium proved better than MS medium in terms of shoot multiplication and shoot length. Nodal explants showed better organogenic response than shoot tip explants. Among nodal explants, basal nodal explants produced more number of shoots than terminal nodes for shoot morphogenesis. Among the two different cytokinins tested in MS media, BAP proved better than KN for improving shoot number and shoot length in combination with different auxins. Among the two different cytokinins tested in L_2 media, KN proved better than BAP for improving shoot number and shoot length either individually or in combination with different auxins. Highest number of shoots obtained from nodal explants cultured on L_2 media containing 3 mg/L KN in combination with IAA. Thus an efficient protocol

for the micro propagation of *A. curassavica* has been established which would offer a great promise for future studies in the endemic medicinal plant.

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Table 1. Influence of MS and L_2 media supplemented with different auxins on callus induction from leaf explants of *A. curassavica* with 2 mg/L auxins. Observations: After 4 weeks.

Plant growth regulators mg/L		Medium	Intensity of callus formed	Fresh weight (mg) Mean ± SE	Dry weight (mg) Mean ± SE
2, 4 - D	2.0	MS	+++	860.00±1.03	30.00±0.98
	2.0	L_2	++	760.00±1.02	28.40±0.86
NAA	2.0	MS	++	730.00±2.19	26.40±0.80
	2.0	L_2	++	700.00±0.00	24.40±0.80
IAA	2.0	MS	++	550.00±0.03	20.00±0.00
	2.0	L_2	+	450.00±0.00	18.00±0.00

+ - Moderate, ++ - High, +++ - Intense callus formation.

Table 2. Effect of different concentrations of auxins alone and in combination with BAP in MS media on callus induction from leaf explants of *A. curassavica.* Observations made after 4 weeks.

Concentrations of auxins (mg/l)		Cytoki-nins (mg/l)	Frequency of response (%)	Intensity of callus formed	Nature of response
2,4 – D	NAA	BAP			
0.5	0.5	0.1	30	+	Yellow
0.5	1.1	0.25	40	++	Pale yellow, Nodular
0.5	1.5	0.5	65	+++	Nodular, Fragile
0.5	2.0	0.1	40	+	Brown, Compact
1.0	-	0.25	30	+	Brown, Compact
1.5	-	0.5	40	+	Brown, Compact
2.0	-	-	30	++	Brown coloured, Compact
0.5	2.5	-	40	+	Yellowish, Fragile
1.0	2.5	-	45	++	Nodular, Fragile
1.5	2.5	-	40	+	Nodular, Fragile

+ - Moderate, ++ - High, +++ - Intense callus formation.

Table 3. Effect of different auxins in combination with cytokinins in L2 media for the induction of organogenic callus from leaf derived callus of *A. curassavica.* Observations: After 4 weeks.

Auxins 1mg/l	BAP mg/L	KN mg/L	Intensity of morphogenic callus formed	Nature of response
	0.1	0.1	+	Brown Coloured, Embryogenic
2,4 – D	0.5	0.5	++	Greenish Yellow, Organogenic
	1.0	0.1	++	Pale Yellow.
	0.1	0.1	+	Pale Yellow, Nodular.
NAA	0.5	0.5	+++	Brown Coloured, Compact & Organogenic
	1.0	1.0	+++	Brown Coloured
	0.1	0.1	++	Whitish Green
IAA	0.5	0.5	+++	Whitish Green, Crystalline
	1.0	1.0	++	Light Green, Crystalline.

+ - Moderate, ++ - High, +++ - Intense callus formation.

Table 4. Effect of KN and BA individually on direct shoot regeneration from nodal explants of *A. curassavica* in MS media. Observations: After 4 weeks. (The results are mean (SE) of 20 independent determinations).

Cytokinins mg/L		Frequency of shoot regeneration (%)	No. of shoots/explant Mean ± SE	Length of shoots (cm) Mean ± SE
KN	BA			
0.5	-	60	1.49 ± 0.30	4.5 ± 0.6
1.0	-	60	1.30 ± 1.10	4.18 ± 1.08
2.0	-	75	1.52 ± 0.32	4.8 ± 0.7
3.0	-	85	1.62 ± 0.3	3.50 ± 0.08

4.0	-	80	1.16 ± 0.16	3.51 ± 0.13
-	0.5	55	1.33 ± 0.21	3.05±0.39
-	1.0	60	1.4 ± 0.24	4.78±0.82
-	2.0	65	1.4 ± 0.24	4.14±0.37
-	3.0	60	1.33±0.21	4.1±0.36
-	4.0	60	1.33±0.21	2.7±0.38

Cytokinins mg/l		Frequency of shoot regeneration (%)	No. of shoots /explant Mean ± SE	Length of shoots (cm) Mean ± SE
KN	BA			
0.5	-	60	1.4 ± 0.24	2.99 ± 0.45
1.0	-	65	1.4 ± 0.24	4.7 ± 0.79
2.0	-	60	1.4 ± 0.24	2.56 ± 0.13
3.0	-	70	1.6 ± 0.24	3.13 ± 0.23
4.0	-	65	1.4 ± 0.24	3.22 ± 0.23
-	0.5	60	1.4 ± 0.244	2.64 ± 0.172
-	1.0	60	1.4 ± 0.244	2.86 ± 0.206
-	2.0	65	1.6 ± 0.244	3.48 ± 0.115
-	3.0	70	2.0 ± 0.316	3.66 ± 0.150
-	4.0	60	1.4 ± 0.244	2.7 ± 0.109

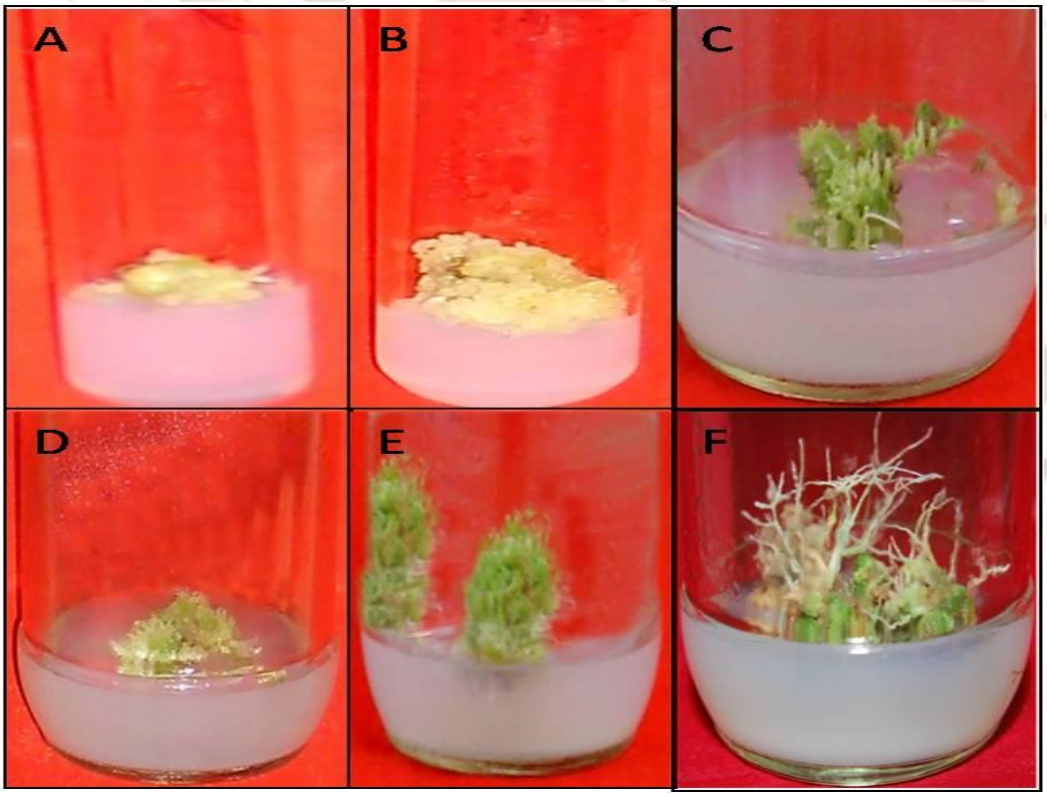

Figure 1. Callus induction from leaf explants of *A .curassavica* on MS and L_2 medium with different concentrations and combination of plant growth regulators A. brown and compact callus formed from leaf explant B. nodular and fragile callus formed from leaf explants C. greenish and organogenic callus formed from initial callus upon subculture D. Greenish yellow and organogenic callus formed from leaf explants of *A .curassavica* on L_2 medium E. Light green and crystalline callus formed from leaf explants of *A .curassavica* on L_2 medium D. whitish green and crystalline with nodular branches formed from initial callus upon subculture on L_2 medium

Figure 2: Direct shoot regeneration from nodal explants of *Asclepias curassavica* on MS and L_2 media supplemented with different combinations and concentration of growth regulators A. direct shoot initiation from nodal explants on MS media with KN (1.0 mg/l) B. shoot multiplication on MS media supplemented with KN (3.0 mg/l) C. elongated shoots multiplied in MS media after frequent subculture D. multiple shoot formation from nodal explants on L_2 media supplemented with BA (3.0 mg/l) E. initiation of rhizogenesis from well developed shoots on MS media F. well rooted plantlets ready for hardening