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# In vitro multiple shoot induction through axillary bud of *Asclepias curassavica L.* – A valuable medicinal plant

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***Abstract-*** Micropropagation of *Asclepias curassavica* L. a medicinal plant valued for its medicinal properties has been carried out using two different plant culture media, Murashige and Skoog (MS) and L2. L2 medium proved to be superior to MS medium in terms of shoot multiplication and shoot length. Nodal explants showed enhanced organogenic response than shoot tip explants. Basal nodal explants produced more number of shoots than terminal nodes for shoot morphogenesis. Among the two different cytokinins tested in MS media, 6-benzylaminopurine (BAP) proved better than Kinetin (KIN) for improving shoot number and shoot length in combination with different auxins. Among the two different cytokinins tested in L2 media, KIN 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 L2 media containing 3 mg/L KIN in combination with Indole-3-acetic acid (IAA). The microshoots developed through *in vitro* regeneration were rooted on full and half strength MS media containing IBA alone and in combination with KIN and the highest number of roots was observed on MS medium with Indole-3-butyric acid (IBA) 1 mg/L + 0.2 mg/L KIN.

***Index Terms-*** Asclepiadoideae, Micropropagation, Regeneration, Cytokinin

**Abbreviations**
BAP 6-benzylaminopurine
IAA Indole-3-acetic acid
IBA Indole-3-butyric acid
KIN Kinetin
L2 Phillips and Collins medium (1979)
MS Murashige and Skoog medium (1962)
NAA 1-Naphthalene-acetic acid

## I. INTRODUCTION

*Asclepias curassavica* (L.) (Tropical milkweed) is an erect, evergreen sub shrub belonging to the sub family *Asclepiadoideae*, family *Apocynaeceae* (Endress and Bruyns, 2000). The sub family *Asclepiadoideae* constitutes many medicinally important plants comprising more than 250 genera and 3,000 species, of which 43 genera and 243 species are present in India. In general, *Asclepiadoideae* plants are the source of cytotoxic and cardiac glycosides and contain highly valuable potential products for curing many diseases of animals and human beings. The plants of the family also contain esterified polyoxy pregnane glycosides, which show anticancer and antitumor activity. *In vitro* micropropagation offers the advantage of rapid multiplication of many medicinal plants with no limitations of growth seasons (Kalidas et al. 2008). Its root extracts are widely used as an emetic and laxative. A decoction of the plant is used as an abortifacient. Roots are of medicinal importance (termed 'Pleurisy root') and are used as an expectorant for pneumonia, lung problems, employed to treat warts, fever, to treat ringworm and to prevent bleeding. The estimated global trade in medicinal and aromatic plants was more than US$60 billion in 2000 and is anticipated to reach 5 trillion by 2050.

India and China are the world's leading exporters of medicinal and aromatic plant materials (Kumar, 2003). 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). Researchers have shown interest in latex producing plant containing considerable amount of low molecular weight hydrocarbons that might be used as a substitute for fossil fuels. Stem explants of *Calotropis procera* were cultured by Dhir et al. (1984). However, very few attempts have been made to propagate this plant either *in vitro* or *ex vitro*. In the present investigation, an efficient and reproducible protocol has been developed for rapid multiplication of *Asclepias curassavica* (L.) through axillary bud explants in order to conserve and preserve the germplasm of this rare species.

## II. METHODOLOGY

*In vitro* micro-propagation studies largely depend on the techniques adapted for the manipulation of plant cells, tissues or organs on artificial nutrient media under controlled and aseptic conditions. *Asclepias curassavica* plants and seeds were collected from Tirumala hills, Tirupati, Andhra Pradesh during March, 2005. The seeds were germinated in the experimental plots of Department of Biotechnology, S.V. University, Tirupati, Andhra Pradesh. Actively growing shoots with five to six nodes, young leaves, axillary buds, nodal segments were used as explants. The explants were washed under running tap water for 5-10 minutes, presoaked in liquid detergent (1% Tween 20) for 1 to 5 minutes and surface sterilized in 70% ethanol for 60 seconds and Mercuric chloride (0.1-1%) for 1-5 minutes. Then they were rinsed with sterile double distilled water for 4-5 times. Two different types of culture media were used for shoot proliferation

and regeneration - MS medium (Murashige and Skoog, 1962) and L2 medium (Phillips and Collins, 1979) individually supplemented with different concentrations of cytokinins (BAP and KIN) (0.5 to 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. In addition, antioxidants like activated charcoal and polyvinyl pyrrolidone (0.025%) were added to test the release of phenolic compounds. The culture medium was also supplemented with growth adjuvants – coconut milk, casein hydrolysate and ascorbic acid to determine their effect on shoot proliferation (10%). Both the MS and L2 media integrated with sucrose (3%, w/v) and were solidified with 0.8% agar. The pH was adjusted to 5.8 and sterilized by autoclaving at 15lbs pressure at $121^0$C for 15-20 minutes.

## III. RESULTS AND DISCUSSION

The choice of using type of media for the metabolic needs of the cultured cells and tissues is a major factor in success of plant regeneration. Each plant requires different quantities of inorganic and organic nutrients for its morphogenic response, so no single medium combination will give satisfactory results with all tissues used. Therefore to determine the suitable medium, in the present study, mature explants like axillary buds, shoot tips were inoculated on MS and L2 media containing 3% sucrose and 1 mg/L cytokinins. Among the two media screened L2 medium proved better than MS medium. Axillary buds on L2 medium developed better than MS medium. Axillary buds on L2 medium developed an average of 5 shoots per explant with 80 per cent shoot regeneration. All explants inoculated on L2 medium responded well with healthy shoots. Shoots developed on this medium were thin, light green in colour with less number of leaves. In the present investigation highest number of shoots with maximum shoot length and frequency of shoot regeneration were obtained from KIN than BAP when employed individually (Table 1 & 2). Hence, in the present study BAP was consider as next best cytokinin for axillary shoot proliferation of *A. curassavica* both qualitatively and quantitatively. The regenerants obtained with BAP were stunted and had short internodes with comparatively small leaves. Unlike BAP treated shoots, the shoots that developed on a KIN supplemented medium had normal dark green and broad leaves with long internodal space and showed better growth and elongation.

In our observations, the potentiality of shoot bud regeneration from nodal explants of *A. curassavica* was higher than that of shoot tip explants. Axillary shoot system which arises from the resident axillary meristems of the nodes and shoot tips were the best suited explants for *in vitro* culture system (Phillips and Collins, 1979; Campbell and Tomes, 1984). Among the two different media tested, the nodal explants for *A. curassavica*, L2 medium prepared with BAP 2 mg/L was found to be the excellent medium for shoot sprouting, number and length without callus formation followed by MS medium in *A. curassavica* (Juan et al. 2004). The shoot buds sprouted on MS medium showed only limited shoot number and growth even after extended period. Thus the quantity of growth and differentiation varied considerably with the media composition. L2 medium has a composition intermediate for $NO_3^-$, $NH_4^+$ and $K^+$ and it has higher levels of $Ca^{2+}$, $Mg^{2+}$ and $SO_4^{2-}$ than MS media (Schenk and Hildebrandt, 1972). Since nitrogen is a constituent, its deficiency inhibits plant growth. This is because the ratio of nutrients strongly influences the pH of the medium, which in turn determines the absorption of other nutrients (Schenk and Hildebrandt, 1972). Thus in the present investigation on *A. curassavica*, L2 medium proved to be the best balanced nutrition for growth (Juan et al. 2004).

A balance between auxins and cytokinins is prerequisite for the formation of either axillary bud or adventitious proliferation of shoots. In this study two different cytokinins (IBA & KIN) were tested with different auxins like NAA, IAA and IBA in both MS and L2 media, to assess their efficiency on shoot multiplication from nodal explants of *A. curassavica*. Among the various combinations of cytokinin and auxins tested, KIN (2 mg/L) + NAA (0.5 mg/L) produced a maximum No. of shoots (3.65) with mean shoot length of 3.25 cm. But maximum frequency of response was obtained with 2 mg/L BAP + 0.5 mg/L NAA with 82% of shoot regeneration capacity. An average of 1-2 shoots formed at lower concentrations of KIN (0.5 and 1.0 mg/L) with (0.5 mg/L) NAA. Shoot number increased excessively in 3 mg/L KIN along with 0.5 mg/L NAA. NAA at 1 mg/L produced basal callus from the basal cut end portion of the explant with all concentrations of KIN tested (0.5-4.0 mg/l). Shoot number was promoted in BAP amended media also in combination with NAA, than BAP alone, but inferior to KIN. A mean shoot number of 2.66 were observed at 3 mg/L BAP supplemented medium in combination of 0.5 mg/L NAA with mean frequency of 70%. Shoot length recorded at this concentration was 7.2 cm. Compact basal callus formation was observed from basal cut end portion of the explants at 2 mg/L BAP + 0.5 mg/L NAA. The leaf lamina size and inter-nodal space of shoots growing in BAP with NAA containing MS media increased slightly than in media with BAP alone. In combination with IAA, not much improved effect was observed on either shoot number or frequency of response. A maximum of shoots (1.5) were obtained at 2 mg/L KIN + 0.5 mg/L IAA with mean shoot length of 4.5 cm. Highest shoot length among all combinations of BAP containing media was observed with IAA combination. A maximum shoot length of 8.1 cm was observed at 3.0 mg/L BAP + 0.5 mg/L IAA containing medium. Inter nodal space was found more in IAA derived shoots.

Axillary shoots produced during direct and indirect organogenesis were made to root on transferring to separate media with varied concentrations of auxins alone and in combination with cytokinin. Two different strengths of MS media were scrutinized to know the effect of nutrients and carbohydrate concentration on root initiation, development and subsequent field establishment. The separated single shoots started to root after 2 weeks of inoculation from basal cut portion of the shoots on two different strengths of media were tested. The roots formed were of different kind i.e., morphology, number of roots produced were determined by the type of treatment under which they were produced. Roots induced on ½ MS medium with IBA (0.5mg/L+ 0.2 mg/L KIN) were long, white coloured and branched. Roots induced on Full strength MS medium with IBA (0.5 mg/L) + KIN (0.2 mg/L) were stout, chlorophyllous, thick and unbranched (Table 3). Thus our report provides an efficient, simple protocol for the micropropagation of *A.*

*curassavica.* This protocol facilitates the conservation and preservation of *A. curassavica*, an economically important medicinal plant.

## IV. CONCLUSION

*In vitro* multiple shoot induction through axillary bud for *A. curassavica* was established on MS and L2 media. L2 medium found to be superior to the MS medium in terms of shoot multiplication and shoot length. Nodal explants showed better organogenic response than other explants. Cytokinins tested in MS media, BAP performed superior than KIN for enhancing shoot number and shoot length in combination with different auxins, where as in L2 media amended with BAP and KIN showed opposite response, KIN proved better than BAP for improving shoot number and shoot length either individually or in combination with different auxins. Plantlets were successfully acclimatized to *ex vitro* conditions. For the first time, the present study reports the establishment of an effective micropropagation protocol for the mass production of *A. curassavica* plants contributing to the conservation of this endangered species in the wild. This protocol will be applied to several genotypes, and the plants produced in this study can be used for further biochemical or other studies or to develop long-term conservation of *A. curassavica*, an economically important medicinal plant.

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**Table 1:** Effect of KIN alone and in combination with different Auxins on MS medium for direct shoot regeneration from nodal explants of *A. curassavica*. Observations: After 4 weeks. (The results are mean (SE±) of 20 independent determinations)

| PGRs mg/L | | | | Frequency of shoot regeneration (%) | No. of shoots/explant Mean ± SE | Length of shoots (cm) Mean ± SE |
|---|---|---|---|---|---|---|
| KN | IAA | NAA | IBA | | | |
| 0.5 | 0.5 | - | - | 55 | 1.05 ± 0.2 | 3.91 ± 0.09 |
| 1.0 | 0.5 | - | - | 65 | 1.4 ± 2.5 | 3.35 ± 0.27 |
| 2.0 | 0.5 | - | - | 60 | 1.5 ± 0.30 | 4.5 ± 0.6 |
| 3.0 | 0.5 | - | - | 50 | 1.0 ± 0 | 3.9 ± 0.2 |
| 4.0 | 0.5 | - | - | 50 | 1.0 ± 0 | 1.14 ± 1.6 |
| 0.5 | - | 0.5 | - | 50 | 1.49 ± 0.30 | 3.9 ± 0.2 |
| 1.0 | - | 0.5 | - | 55 | 1.57 ± 0.25 | 3.35 ± 0.27 |
| 2.0 | - | 0.5 | - | 82 | 3.68 ± 0.18 | 3.28 ± 0.18 |
| 3.0 | - | 0.5 | - | 63 | 1.62 ± 0.31 | 2.72 ± 0.4 |
| 4.0 | - | 0.5 | - | 60 | 1.5 ± 0.28 | 7.0 ± 0.7 |
| 0.5 | - | - | 0.5 | 55 | 1.28 ± 0.18 | 4.37 ± 0.68 |
| 1.0 | - | - | 0.5 | 60 | 1.28 ± 0.19 | 4.71 ± 1.04 |
| 2.0 | - | - | 0.5 | 70 | 1.33 ± 0.21 | 1.28 ± 0.18 |
| 3.0 | - | - | 0.5 | 65 | 1.51 ± 0.25 | 5.65 ± 0.8 |
| 4.0 | - | - | 0.5 | 60 | 1.33 ± 0.21 | 1.28 ± 0.18 |

**Table 2:** Effect of BAP alone and in combination with different Auxins on MS medium for direct shoot regeneration from nodal explants of *A. curassavica*. Observations: After 4 weeks. (The results are mean (SE±) of 20 independent determinations)

| PGRs mg/l | | | | Frequency of shoot regeneration (%) | No. of shoots/explant Mean ± SE | Length of shoots (cm) Mean ± SE |
|---|---|---|---|---|---|---|
| BAP | NAA | IAA | IBA | | | |
| 0.5 | 0.5 | - | - | 55 | 1.28±0.18 | 3.64±0.35 |
| 1.0 | 0.5 | - | - | 60 | 1.4±0.24 | 3.50±0.41 |
| 2.0 | 0.5 | - | - | 82 | 1.44±0.33 | 4.81±0.38 |
| 3.0 | 0.5 | - | - | 70 | 2.66±0.33 | 7.23±0.43 |
| 4.0 | 0.5 | - | - | 60 | 1.2±0.20 | 6.07±0.49 |
| 0.5 | - | 0.5 | - | 55 | 1.33±0.21 | 4.10±0.30 |
| 1.0 | - | 0.5 | - | 65 | 1.57±0.29 | 5.89±0.22 |
| 2.0 | - | 0.5 | - | 70 | 2.0±0.30 | 5.20±0.92 |
| 3.0 | - | 0.5 | - | 70 | 2.66±0.61 | 8.10±0.90 |
| 4.0 | - | 0.5 | - | 60 | 1.33±0.21 | 4.85±0.31 |
| 0.5 | - | - | 0.5 | 55 | 1.4±0.21 | 2.91±0.26 |
| 1.0 | - | - | 0.5 | 60 | 2.14±0.40 | 4.75±0.09 |
| 2.0 | - | - | 0.5 | 65 | 2.16±0.30 | 6.44±0.21 |
| 3.0 | - | - | 0.5 | 70 | 2.66±0.55 | 5.46±0.76 |
| 4.0 | - | - | 0.5 | 70 | 2.42±0.36 | 6.44±0.21 |

**Table 3:** Effect of ½ strength MS medium supplemented with different concentrations of IBA alone and in combination with KIN on root induction. (The results are mean (SE±) of 20 independent determinations).

| IBA mg/L | KIN mg/L | Frequency of response (%) | No of roots/shoots Mean ± SE | Root length (cm) Mean± SE |
|---|---|---|---|---|
| 0.5 | - | 65 | 8.8 ± 0.21 | 2.40 ± 0.09 |
| 1.0 | - | 70 | 9.4 ± 0.29 | 2.82 ± 0.23 |
| 1.5 | - | 73 | 9.0 ± 0.00 | 2.86 ± 0.19 |
| 2.0 | - | 75 | 8.5 ± 1.25 | 3.0 ± 0.36 |
| 0.5 | 0.2 | 70 | 11.2 ± 0.71 | 2.8 ± 0.49 |
| 1.0 | 0.2 | 85 | 12.2 ± 0.53 | 3.07 ± 0.24 |
| 1.5 | 0.2 | 72 | 10.0 ± 0.00 | 2.62 ± 0.17 |
| 2.0 | 0.2 | 70 | 9.8 ± 1.08 | 5.05 ± 0.48 |
| 0.5 | 0.4 | 70 | 9.16 ± 0.82 | 3.30 ± 0.44 |
| 1.0 | 0.4 | 65 | 6.6 ± 0.53 | 3.03 ± 0.13 |

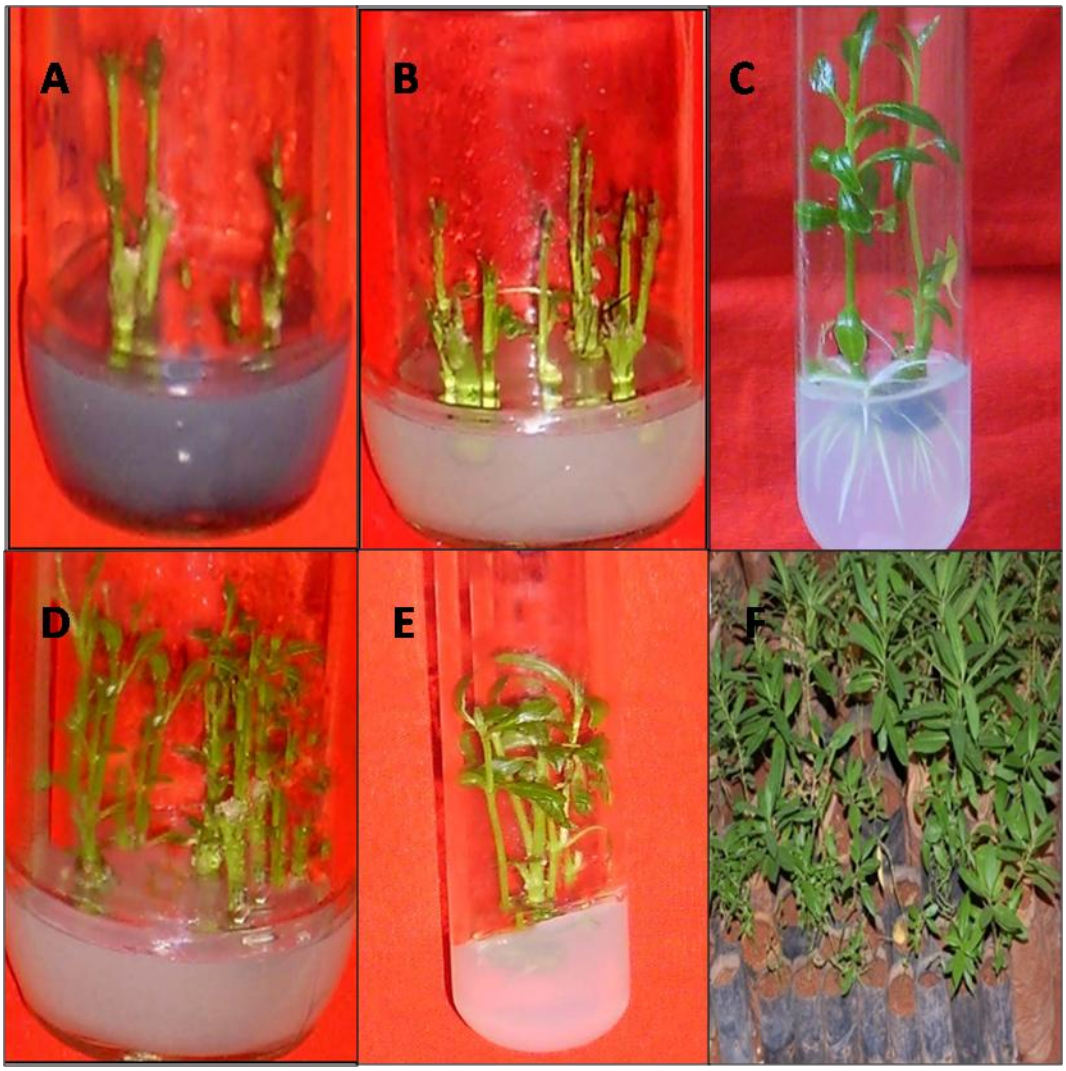


**Figure 1:** Direct shoot induction and Rhizogenesis of *A. curassavica* in MS and L2 media. **A**. Shoot bud initiation from axillary buds on MS media **B.** Shoot bud initiation from axillary bud culture on L2 media **C.** Rhizogenesis on ½ strength MS media **D.** Multiple shoot development after 5 weeks on MS media **E.** Multiple shoot development after 5 weeks on L2 media and **F.** Acclimatized plants growing in plastic pots.