

DEVELOPMENT OF AN APPROPRIATE PROTOCOL FOR MICROPROPAGATION OF *Calotropis gigantea* (L.) (WARA)

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ABSTRACT

Calotropis gigantea (Wara) is one of the least concerned native plant in Sri Lanka. Because of its high pharmaceutical application and other usage, this species has been over exploited and included in the Red list since year 2012. The present study was carried out to develop a protocol to produce healthy plantlets of *C. gigantea*. Murashige and Skoog medium (MS) (0, ¼, ½, ¾ and full MS) were tested to select the best medium for *in vitro* seed germination. To determine the best explant and 6-Benzyl amino purine (BAP) concentration for micro propagation, *in vitro* shoot tip, stem without node, stem with node, leaf and root were tested with different concentrations of BAP (0, 1 mg/l, 2 mg/l, 3 mg/l, 4 mg/l, 5 mg/l and 6 mg/l) and 0.1 mg/l NAA. Agar and Corn flour were used to gelatinize the medium. To determine the proper maturity level of plantlet, 1, 1 ½, 2, 2 ½ and 3 months old plants were tested. Then regenerated shoots were transferred to different IBA (Indole 3-butyric acid) concentrations (0, 0.5 mg/l, 1 mg/l and 2 mg/l) for rooting. Ten replicates per each treatment were used in every experiment. Data were analyzed according to 2-factor factorial Completely Randomized Design (CRD) using SAS statistical software. The medium with agar (without MS) showed the highest seed germination of 72% while the medium containing full MS showed the lowest seed germination of 34%. Significantly higher number of shoots (2.4) was observed from MS medium with 2 mg/l BAP + 0.1 NAA + Agar within 11days, showing 100% regeneration from stem with node. When corn flour was used instead of agar for the above medium, shoots regeneration was 2.7 within 17 days. One month old explant (stem with node) showed significantly higher number of shoots (2.0) whereas three month old explant showed lowest number of shoots (0.8). The highest number of roots (3.4) was observed from 1mg/l IBA +MS + Agar. However, with corn flour instead of agar, average number of roots was 4.4. Considering these observations, it could be concluded that Agar is suitable as a medium for seed germination. Stem with node is the best explant to produce large number of healthy shoots. The best BAP concentration for shoot regeneration was 2mg/l BAP+0.1mg/l NAA, while 1mg/l IBA was good for rooting. One month old explants were better than matured explants, and corn flour was better compared with agar in increasing the growth and development of *C. gigantea*.

Key Words: *Calotropis gigantea*, Micropropagation, MS Medium, BAP, NAA

INTRODUCTION

Calotropis gigantea (Apocynaceae) is drought resistant, salt tolerant plant grows on road sides and coastal area. There are two common species of *Calotropis*, viz. *Calotropis gigantea* (Linn.) and *Calotropis procera* (Ait.). *Calotropis gigantea* grows up to 8-10 m, with sessile leaves and white to purple unscented flowers, while *Calotropis procera* grows upto 3-6 m with sub sessile leaves and white to pink scented flowers. Among those two species,

Calotropis gigantea is native to Sri Lanka (Barberyn Ayurveda Resorts and University of Ruhuna, 2009). It has many uses such as; extract chemicals to use as medicine, floricultural industry as a potted plant, in landscaping, fiber production, mulching purposes, sweetmeat production, paper pulp production, component in gun powder and fireworks. Mosquito repellent efficacy of flower extracts of the species is already noted. (Dhivya *et al.*, 2013; Motaleb *et al.*, 2011).

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C. gigantea is a plant which has been used in several traditional medicines to treat various diseases from the ancient times. The latex, leaves, flowers, bark, and roots are also used to treat piles, eruptions on the body, asthma, enlargement of spleen and liver, dropsy and applied to painful joint swellings (Joseph *et al.*, 2013). *C. gigantea* species has been identified by Motaleb *et al.* (2011) as a threatened plant. Endangered plant species can be strongly related to human threats and environmental limiting factors, and understanding of these factors has been identified important to protect the vegetation of *C. gigantea* plant species. (Wei *et al.*, 2009). Though *C. gigantea* propagates by seeds and rooted cuttings, seed germination percentages at the normal environmental conditions have not been found.

Micro-propagation, using somatic embryo and shoot tip culture techniques assists many plant development programmes and increasingly these methods are being used for the conservation of endangered plant species. Though, *C. gigantea* is considered to be highly essential medicinal plant, tissue culture studies are very limited (Roy, 1986; Rathore *et al.*, 2012). In Sri Lanka history, there has not been any review of literature on this medicinally important plant.

Because of its high pharmaceutical application and other usage, plant species were over exploited and the conventional propagation methods cannot be used for mass propagation. Since *C. gigantea* has that much value, it is included in The national red list 2012 of Sri Lanka under list of angiosperms in Sri Lanka (Weerakoon and Wijesundara, 2012). Therefore, there should be a reliable conservation method and efficient way to produce large number of plants within short period of time for commercial level and to re-introduce regenerated plants into their natural habitats. The present study was carried out to find a protocol to produce healthy plantlets of *C. gigantea*.

The objectives of this study were to determine a suitable concentration of MS medium for *in vitro* seed germination, identify the best explant for shoot production, determine the best concentration of BAP for shoot regeneration, determine the proper age of the plantlets to collect the ex-plants, determine the best concentration of IBA for root induction, and determine effect of Agar and Corn flour for growth and development of shoots.

MATERIALS AND METHODS

To identify a suitable concentration of MS medium for *in vitro* seed germination

Mature fruits of *Calotropis gigantea* L. were collected from a healthy mother plant in Ratnapura District. After removing long silky hairs which can be seen at one end of the seeds, they were sun dried for 3-4 days.

Five concentrations of MS medium [Control-only Agar (Without MS), $\frac{1}{4}$ MS, $\frac{1}{2}$ MS, $\frac{3}{4}$ MS and Full MS] were prepared. The pH of the media was adjusted to 5.6-5.8 using HCl or KOH prior to adding Agar. The sundried seeds were washed by tap water for 30 min and then surface-sterilized using 70% ethanol for 1 min. After that, seeds were rinsed in 20% Clorox (NaOCl) for 20 min under laminar flow conditions. After surface sterilization, the seeds were thoroughly washed for several times with sterile (autoclaved) distilled water and they were scarified by sterilized knife. After that, seeds were transferred into 25 ml culture bottles with different concentration of MS medium *viz* Agar (without MS), $\frac{1}{4}$ MS, $\frac{1}{2}$ MS, $\frac{3}{4}$ MS and Full MS. Culture bottles were sealed with caps and maintained in growth room at 25 ± 5 °C, 25% RH under fluorescent illumination with 16 hours photo period. Number of days to seed germination and number of seedlings were recorded after 9 days. The emergence of hypocotyl with green color was considered as germinated.

To identify best ex-plant and concentration of BAP for *in vitro* shoot regeneration

The explants of *Calotropis gigantea* were obtained from the *in-vitro* established 2-2½ month old seedlings. On the basis of their physical performance and appearance, healthy plants were selected for *in-vitro* mortification. Shoot tips, stem with node, stem without node, leaves and roots were used as explants. Five explants were introduced to MS medium with different BAP concentrations (0-Control, 1, 2, 3, 4, 5 and 6 mg/l). Gelling agents, Agar and Corn flour were used to determine growth and development of explants. Two-factor factorial CRD was used for the statistical analysis.

The explants were cut into 0.5-1 cm parts and transferred to MS medium supplemented with hormone. Just after the inoculation, culture bottles were sealed tightly with caps. The cultures were incubated at 25±5 °C under 16 hour photo period.

Number of days to regenerate shoots, and number of shoots per explant were counted after four weeks.

To identify the proper age of the plantlets to get ex-plants

Different aged (1, 1 ½, 2, 2 ½ and 3 month) stem with node (0.5-1 cm) were established in MS medium supplemented with 0.1 mg/l NAA and 2 mg/l BAP. The cultures were incubated at 25±5 °C with 16 hour photo period. Number of days to regenerate shoots and number of shoots per explant were counted after four weeks. Completely randomized design with 10 replicates per each treatment was used.

To identify the best concentration of IBA for root induction

One month old, healthy, 2-3 cm height regenerated shoots were cultured in different IBA concentrations (0, 0.5, 1, 2 mg/l). The regenerated shoots were incubated at 25±5 °C with 16 hour photo period. Experiment was ar-

ranged in a CRD with 10 replicates per each treatment.

Number of days to regenerate roots and number of roots per shoot were counted after six weeks.

RESULTS AND DISCUSSION

Determination of suitable concentration of MS medium for *in vitro* seed germination

Germination percentage and number of days to germinate were evaluated after inoculation. In all treatments, the rate of germination was varied (Table 1). The highest seed germination (72 %) was observed in agar without MS within 6 days while in full MS medium seed germination percentage was lowest (34 %) (Table 1, Plate 1). It was also observed that the number of days to germinate was increased when increasing the concentration of MS basal medium.

According to Kone *et al.* (2015), seed germination is mainly affected by water availability, but not with the composition of the medium. Since the medium with Agar without MS has more available water, it showed highest seed germination percentage. Due to high concen-

Table 1. Effect of different concentrations of MS basal medium on *in vitro* seed germination of *Calotropis gigantea*

Treatment (Agar + MS medium)	Average no of days to germination	Percentage of seed germination
Agar (without MS)	6.6 ^c	72
¼ MS	6.8 ^c	65
½ MS	7.4 ^b	55
¾MS	8.1 ^a	48
Full MS	8.3 ^a	34

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT)

trates and less water availability in full MS medium, it showed the lowest seed germination percentage.

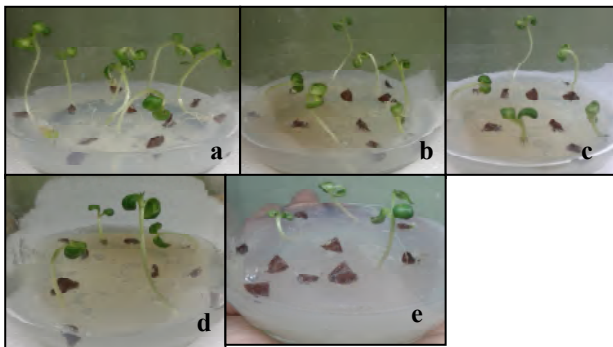


Plate 1: Effect of different concentration of MS medium for *in vitro* seed germination of *Calotropis gigantea*, a-Agar (without MS), b- $\frac{1}{4}$ MS+Agar, c- $\frac{1}{2}$ MS+Agar, d- $\frac{3}{4}$ MS+Agar, e- Full MS+Agar

Determination of the best explant and BAP concentration for shoot regeneration

The highest number of shoots (0.97) per explant was observed from stem with node (Table 2) while no shoots obtained from stem without node, leaf, and root explants. However, callus formation was observed from stem without node (2 mg/l BAP+0.1 mg/l NAA, Plate 2b).

Table 2. Effect of different concentrations of MS basal medium on *in vitro* seed germination of *Calotropis gigantea*

Treatment (Type of explants)	Average no. of shoots per ex-plant	Average no. of days to regenerate shoots
Shoot tip	0.32 ^b	26.42 ^b
Stem without node	0.00 ^c	-
Leaf	0.00 ^c	-
Root	0.00 ^c	-
Stem with node	1.02 ^a	22.8 ^c

Mean values with the same letter are not significantly different ($p < 0.05$), mean separation was done with Duncan's multiple range test (DMRT).

The medium gelled from corn flour, stem with node showed highest number of shoots (1.02) per explant while stem without node, leaf and root did not show any shoot formation.

However, in the same medium, leaf also showed callus formation in addition to stem without node (Plate 3).

Sharma (2009) reported that the petiole with nodal segments of *Cinnamomum tamala* Nees. & Ebrm were the best explant to produce large number of harvestable shoots than apical shoot, shoot with internodes and leaf. Maximum number of shoots induced from the nodal segment of *P. cineraria* in *in-vitro*

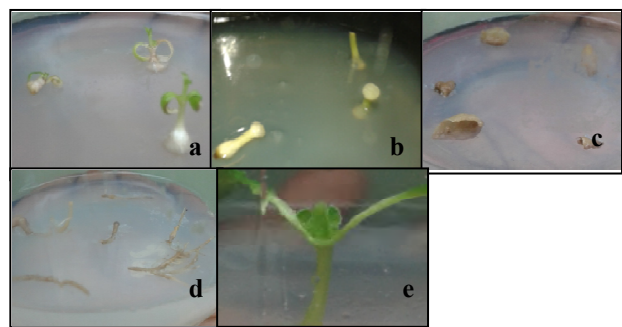


Plate 2: Effect of different explant types on shooting in the medium with Agar +MS+BAP+NAA, a-Shoot tip, b-Stem without node, c-Leaf, d-Roots, e-Stem with node

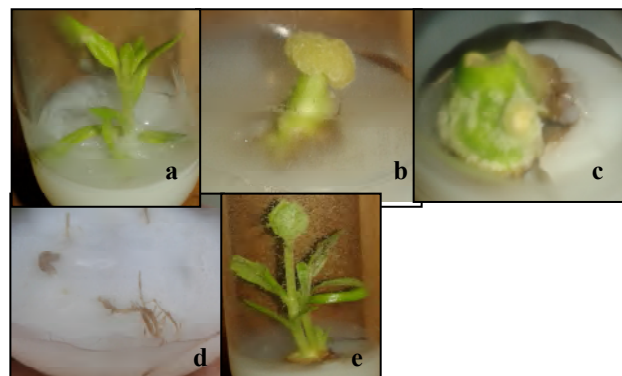


Plate 3: Effect of different explant types on shoot proliferation using Corn flour + MS+BAP+NAA, a-Shoot tip, b-Stem without node, c-Leaf, d-Roots, e-Stem with node

propagation was also observed by Shekhawat *et al.* (1993). Studies of Anis (2003) revealed that nodal explants were the best part for sprouting compared to shoot tips in the primary cultures of *Morus alba* L. Same results were observed from this study, where MS medium + Agar + 2 mg/l BAP + 0.1 mg/l NAA showed significantly higher number of shoots (2.4), within lowest number of days (11.4) showing highest regeneration (100 %) from nodal explants (Table 4, Plate 4).

Roy *et al.* (1990) also documented in his study on *Calotropis gigantean*, the best shoot formation and growth had been observed in 2 mg/l of BAP and 0.1mg/l of NAA. The highest shoot multiplication was observed for *Asclepias curassavica* on 2.0 mg/l BAP + 0.5 mg/l NAA by Reddy *et al.* (2012) and, for *Caralluma adscendens* it was on 2.24 mg/l BAP (Aruna *et al.*, 2009). These results were similar with present study as 2 mg/l BAP+0.1 mg/l NAA + corn flour, showed significantly higher number of shoots (2.7).

Table 3. Effect of explant type for Ten replicates per each treatment were used shooting of *Calotropis gigantea* in the medium with Corn flour without MS

Treatment (Type of explants)	Average no. of shoots per ex-plant	Average no. of days to regenerate shoots
Shoot tip	0.17 ^b	24.77 ^b
Stem without node	0.00 ^c	-
Leaf	0.00 ^c	-
Root	0.00 ^c	-
Stem with node	0.97 ^a	23.33 ^b

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT)

Table 4. Effect of different BAP concentration for shoot regeneration of *Calotropis gigantea* using Agar

BAP concentration (mg/l) (along with 0.1mg/l NAA)	Average no. of shoots per ex-plant	Average no. of days to regenerate	Regeneration Percentage
0 (Control -Without hormone)	-	-	-
1	0.7 ^b	22.3 ^a	50
2	2.4 ^a	11.4 ^b	100
3	0.8 ^b	22.4 ^a	70
4	0.7 ^b	21.1 ^a	70
5	0.8 ^b	19.5 ^a	60
6	0.4 ^b	25.7 ^a	40

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT).

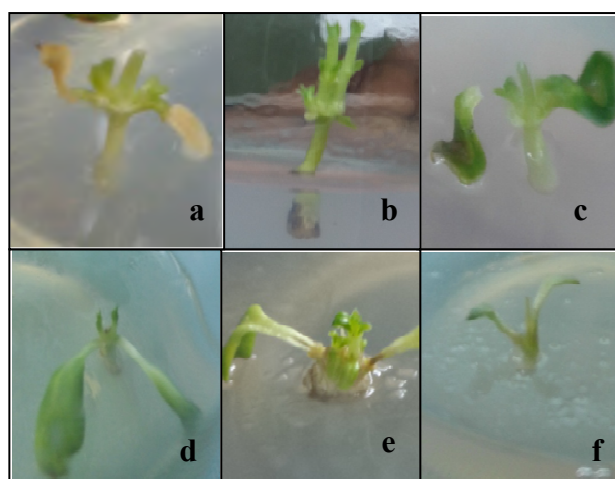


Plate 4: Effect of different BAP concentrations for shoot regeneration of *Calotropis gigantea* using Agar and 0.1mg/l NAA , a-control (without hormone), b-1mg/l BAP, c-2mg/l BAP+, d-3 mg/l BAP, e-4 mg/l BAP, f -5 mg/l BAP, g-6 mg/l BAP

Determination of the proper age of the plantlets to collect ex-plants

The highest number of shoots (2.0) per explant and the lowest number of days to regen-

Table 5. Effect of different BAP concentration for shoot regeneration of *Calotropis gigantea* using Agar

BAP concentration (mg/l) (along with 0.1mg/l NAA)	Average no. of shoots per explant	Average no. of days to regenerate	Regeneration Percent- age
0 (Control- Without hormone)	-	-	-
1	0.8 ^{bc}	22.8 ^{bc}	70
2	2.7 ^a	17.2 ^d	100
3	1.3 ^b	19.9 ^{cd}	80
4	0.8 ^{bc}	21.1 ^{cd}	80
5	0.4 ^{cd}	26.6 ^{ab}	40
6	0.1 ^d	29.2 ^a	10

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT).

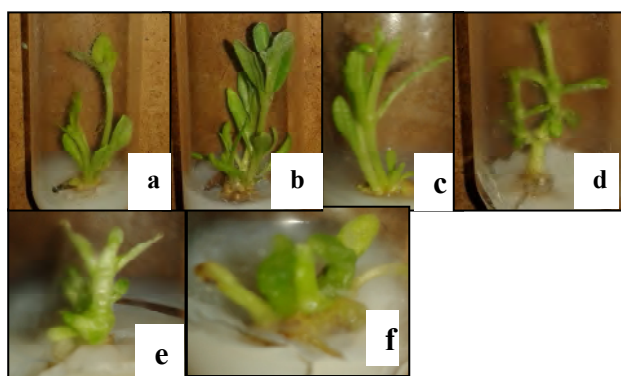


Plate 5: Effect of different BAP concentrations for shoot regeneration of *Calotropis gigantea* using Corn flour and 0.1mg/l NAA, a-control (without hormone), b-1mg/l BAP, c-2 mg/l BAP, d-3 mg/l BAP, e-4 mg/l BAP, f-5 mg/l BAP, g-6 mg/l BAP

eration was observed from 1 month old seedlings (15 days) (Table 6; Plate 6). However, lowest number of shoot formation per explants (0.8) was obtained in three month old seedlings.

The experiment was conducted by Yildiz (2003) for Flax (*Linum usitatissimum* L.) with different aged explants. It revealed that regeneration capacity of young plants was higher than old ones. When the organ used as explant source gets elder, regeneration capacity decreases.

Table 6. Effect of age of explant on shoot regeneration

Treatment (Month)	Average number of shoots per explant	Average number of days to regenerate
1	2.0 ^a	15.2 ^e
1 and ½	1.6 ^{ab}	20.6 ^d
2	1.4 ^{abc}	26.2 ^c
2 and ½	1.2 ^{bc}	29.2 ^b
3	0.8 ^c	33.6 ^a

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT).

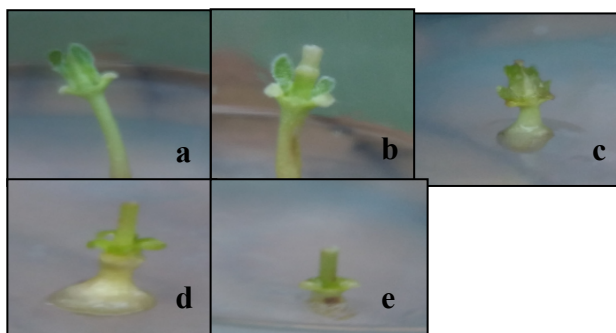


Plate 6: Effect of different age of explant for *in vitro* shoots regeneration after one month of establishment, a- 1 month old, b- 1 ½ month old, c- 2 month old, d- 2 ½ month old, e- 3 month old

But the MS with Corn flour showed significantly higher number of roots (4.4) within 12.4 days (Table 8; Plate 8).

Table 7. Effect of different IBA concentrations for root induction using Agar

Treatment (IBA mg/l)	Average no. of days to first rooting	No. of roots per shoot after one month	No. of roots per shoot after one & half month
0 (Control- Without hormone)	-	-	-
0.5	38.2 ^{ab}	0.5 ^a	0.6 ^b
1.0	30.8 ^b	1.6 ^a	3.4 ^a
2.0	44.1 ^a	0.4 ^a	0.4 ^b

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT).

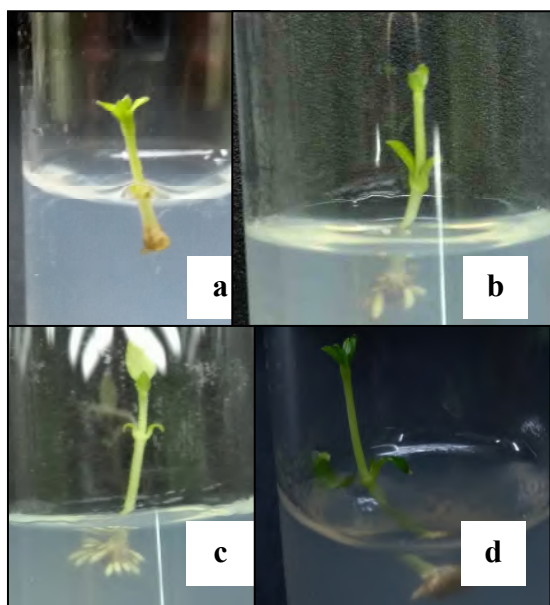


Plate 7: Effect of different IBA concentrations for *in vitro* root growth of *Calotropis gigantea* using Agar after 1 ½ months of establishment, a-0-control, b-0.5 mg/l IBA, c-1 mg/l IBA, d-2mg/l IBA

Table 8. Effect of different IBA concentrations on root induction using Corn flour

Treatment IBA mg/l	Average no. of days to first rooting	After one month no. of roots per shoot	After one & half month no. of roots per shoot
0 (Control without hormone)	-	-	-
0.5	21.0 ^b	1.0 ^b	2.4 ^b
1.0	12.4 ^c	3.4 ^a	4.4 ^a
2.0	29.4 ^a	0.2 ^b	0.6 ^c

Mean values with same letters are not significantly different ($p < 0.05$), mean separation was done by Duncan's multiple range test (DMRT).

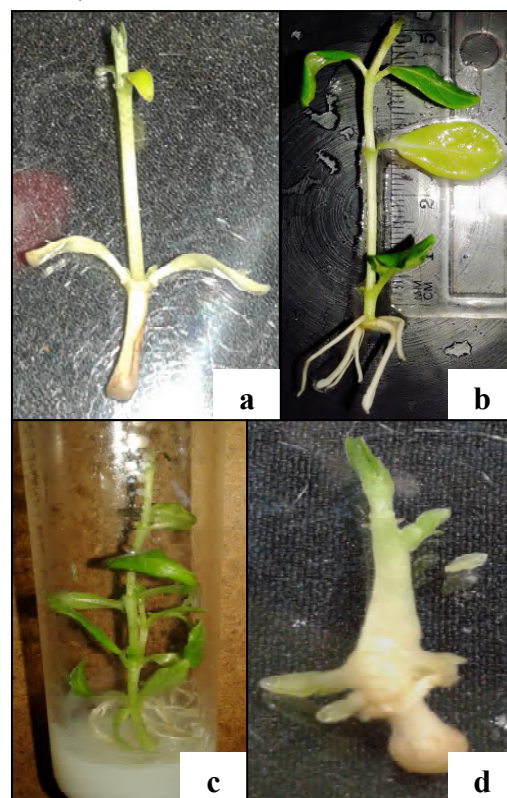


Plate 8: Effect of different IBA concentrations for *in vitro* root growth in *Calotropis gigantea* using Corn flour after 1 ½ months of establishment, a-0-control, b-0.5mg/l IBA, c-1mg/l IBA, d-2mg/l IBA

Determination of best concentration of IBA for root induction

The one and half month old shoots were used for rooting in different IBA concentrations. The results showed that there were significant differences (at 5% level) between treatments on rooting after one and half month (Table 7). MS medium with 1mg/l IBA, showed that highest number of roots per shoot (3.4) within lowest number of days (30.8) while 0.5mg/l IBA and 2 mg/l IBA showed lowest rooting 0.6 & 0.4 per shoot, respectively (Table 7; Plate 7).

CONCLUSION

For *in vitro* seed germination of *Calotropis gigantean*, the medium with agar without MS is the best. Stem with nodal explant were proved higher shoot proliferation at 2.0 mg/l BAP+0.1 mg/l NAA+ MS. One month maturity was the proper age to collect explants. The highest number of roots observed from MS medium supplemented with 1mg/l IBA. Corn flour is better than Agar in two ways; cost reduction and increase the growth and development of *Calotropis gigantea*. This protocol could be utilized for conservation and clonal propagation of *Calotropis gigantean*, an important medicinal plant.

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