Efficient Protocol of Micropropagation, and Organogenesis of *Euphorbia pulcherrima* Willd. Plants via Stem and Leaf Segments

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Abstract— *A highly efficient plant propagation protocol was developed for Euphorbia pulcherrima Willd. using nodal shoot segments. Different concentrations of 6-Benzylaminopurine (BAP) singly or in combination with both GA_3 and adenine sulfate. Use of BAP at 0.5 mg/l combination with 20 mg/l adenine sulfate induced the highest frequency (100%) of shoot induction as well as maximum number of shoots per explant (10.6) followed by using the combination of 1.5 mg/l BAP with 0.5 mg/l GA_3 which create 6.76 shoot /explants, while lower number of shoot/explants (5.88) was recorded in MS medium supplemented with 0.5 mg/l BAP and the proliferated shoot clumps were further multiplied and elongated. The highest rooting frequency (77.8%) as well as highest number of roots (4.9) was observed in full strength MS medium supplemented with 1.0 mg/l Indole-acetic acid (IAA). In order to study the effects of organs culture (stem and leaf) in vitro culture of poinsettia, shoot regeneration frequently was 100% when the stem explants were placed on MS medium containing 1.0 mg/l BAP combination with 0.2 mg/l of NAA. Highest number of shoots per explant (6.95) was recorded followed by (5.83) shoots per explants in MS medium containing BAP 0.5 mg/l combination with GA_3 0.1 mg/l. Shoot regeneration from Leaf explants reached 77.73% when the leaf explants were placed on MS medium containing BAP 1.0 mg/l combination with 0.2 mg/l of IAA and highest number of shoots per explant (3.00) was recorded. Regenerated plantlets were acclimatized successfully in the green house and finally transplanted in the pots.*

Keywords— Euphorbia pulcherrima, Micropropagation, shoot tips, Organogenesis.

I. INTRODUCTION

Poinsettia plants (*Euphorbia pulcherrima* Willd). belonging to the *Euphorbia*ceae family and is number one of the flowering potted plant in the United States include trees, shrubs, herbs but are rarely woody climbers ,this plant is an important winter holiday symbol in many parts of the world. (Clarke *et al*.,2008). About 2000 species are within the larger genera *Euphorbia* and is native of Central United States of America, this fast growing plant is one of the most common growing shrubs throughout the world (Zokian, 2011).

This shrub can be conventionally propagated by seeds and cuttings, the seeds lose their viability on storage while propagation through cuttings is seasonal. In addition that these cuttings takes around six - eight weeks n root formation. These methods of clonal propagation are not sufficient to meet the demand of this ornamental shrub during the Christmas period (Jasrai *et al*., 2003).

Few studies on micropropagation through axillary bud proliferation and organogenesis of poinsettia have been reported (Jasrai *et al*., 2003 ; Uchida *et al*., 2004; Toma and Mizory 2011; Bidarigh *et al*., 2012; Bidarigh and Azarpour 2013). The development of *in vitro* protocols for plant regeneration through either organogenesis or somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation and genetic transformation that are an alternative efficient strategy to implement the improvement of poinsettia (Vila *et al*., 2003). This study was initiated to find the best protocol for clonal propagation and focused to regenerate through direct regeneration method from stem and leaf explants of *Euphorbia pulcherrima* via plant tissue culture technique with presence of different plant growth regulators, as well as for reduction the cost of production and enhance rapid multiplication.

II. MATERIALS AND METHODS

This investigation was carried out at plant biotechnology laboratories of Scientific Research Center in the faculty of Science, University of Duhok, Kurdistan Region of Iraq during the period from June 2013- February 2015. 3-4 cm. in length terminal cuttings of *Euphorbia pulcherrima* Willd were collected and used as a source of plant material. The cuttings were thoroughly washed with liquid soap under running tap water for 1 hour. The leaves were removed acropetally to expose the youngest leaf pairs. They were surface sterilized by immersion them in a 250 ml beaker containing 3:1 (v/v) commercial bleach

(5% sodium hypochlorite) and water containing few drops of Tween -20 surfactant. The beaker was then placed under vacuum for 15 minutes to dislodge any possible air bubbles in the explants. After sterilization was achieved, the sterilant was decanted and the explants were rinsed 4 times with sterilized distilled water to remove the harmful effects of the NaOCl. The process were carried out within the confines of a laminar-air-flow hood, the last pair of leaf were removed using a dissecting microscope, Epical dome with 2-3 pairs of leaf primordial (50-70µm) of the shoot tips were excised and transferred to nutrient medium .

Murashige and Skoog (1962) inorganic salts media supplemented with (mg/l): thiamine HCL. (0.4), inositol (100), BAP (0.5) and sucrose (30,000) were used as basal medium. The pH of the medium was adjusted to 5.7 with 1N HCl or NaOH and solidified with 0.7% agar. The medium was dispensed into 250 ml Mason jars at the rate of 25 ml per jar. The nutrient media was sterilized by autoclaving for 15 min. at 121C^{o} and 1.04 kg/cm^{2}. After solidification, the explants were inoculated at a rate of 3 explants /jar. The cultures were incubated in the culture room at 24 ± 1C^{o} under 16 hours daily exposure to 1000 lux from cool white florescent lamps.

In order to study the effect of different cytokinins on bud proliferation, the cytokinins Benzyl amino purine (BAP) were tested at 0.0, 0. 5, 1.0, 1.5 and 2.0 mg/l levels alone or combinations with (0.2 or 0.5) mg/l GA_3 or (0, 20, 40,60,80) mg/l of adenine sulfites . Four replicates of single nodal segments of shoot tips were cultured in tested media per each concentration for 45 days. The numbers of initiated shoots were calculated and their length was measured.

On the other hand, a new protocol were tested for improve direct organogenesis via 1 cm length stem cuttings cultured on MS medium supplemented with BAP (0.5, 1.0, 1.5) mg/l combination with (0.1, 0.2, 0.5) mg/l from GA_3 or NAA. Excised leaf discs (1×1 cm^{2}) from *in vitro* plants were used for direct organogenesis cultured on MS medium supplemented with BAP (0.5, 1.0, 1.5) mg/l combination with (0.1, 0.2, 0.5) mg/l IAA. After 45 days of incubation organogenesis percentage, branch number/ explants, average shoots length and leaves number/explants was calculated.

Similarly, the effect of auxin on rooting of individual shoots was investigated. Indole -3-acetic acid (IAA), indole-1-butyric acid (IBA) and naphthaleneacetic acid (NAA) were tested at 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l concentrations. 1-2 cm in length of shoots, were cultured at a rate of 3 shoots / jar for each concentration and 4 replicates per each concentration. After 45 days of incubation under previous environmental conditions, Rooting percentage, number of roots and their length were recorded.

Finally, for acclimatization stage, a quite number of successfully rooted plantlets were removed from culture vessels and their roots were washed with distilled water and immersed in Benlate fungicide (0.1% for 10 min.). They were transferred to pots containing a steam sterilized soil mix (peatmoss+ loam+ Styrofoam 1:1:0.5, v:v:v) under tightly controlled atmosphere of the greenhouse.

III. RESULTS AND DISCUSSION

Effects of cytokinins on shoot multiplication.

A new shoots have been emerged after six weeks of Euphorbia incubation from shoot tips on cytokinin - subsidized medium. Table (1) declares the effect of BAP on shoot proliferation, highest shoot number was attained at 0.5 mg/l BAP concentration and was significantly higher than other treatments except 1.5 mg/l treatment and the number of shoots reached its highest level at 0.5 mg/l treatment (5.887 shoots/ explants) (Fig. 1,A), followed by the next highest number of shoots which was attained at 1.5 mg/l (5.267 shoots/explant) although the difference between these concentration was not significant. .On the other hand, inclusion of 1.0 mg/l of BAP in the culture medium causes a reduction on shoot development in to only 3.887. Similarly, another significant reduction was identified through the high BAP concentration (2.0 mg/l) While, the least shoot number was obtained in a medium devoid of BAP and it was significantly different with all other treatments.

Table (1).Effect of {BAP,(BAP + GA_3) and BAP + adenine sulfites)} on shoots multiplication in Euphorbia after six weeks in culture

BAP Concentrations (mg/l)	Number of shoots/ explant	Mean length of shoots (cm)	Number of leaves/ explant
0.0	1.00 c	2.667 a	7.333 b
0.5	5.887 a	0.943 b	12.447 a
1.0	3.887 b	0.903 b	11.890 a
1.5	5.267 a	0.823 b	11.557 a
2.0	3.780 b	0.463 c	8.887 b
BA+ GA_3 (mg/l)			
0.0+ 0.0	1.200 b	0.633 a	6.167 d
0.5+ 0.2	6.200 a	0.733 a	26.633 a
1.0+ 0.2	5.967 a	0.500 a	18.967 b
1.5+ 0.5	6.767 a	0.667 a	19.200 b

2.0+ 0.5	6.733 a	0.667 a	14.933 c
BAP+ Adenine Sulfate (mg/l)			
0.5+ 0.0	4.633 b	1.283 b	13.733 c
0.5+ 20.0	10.600 a	1.297 b	28.000 a
0.5+ 40.0	4.867 b	1.717 b	11.400 c
0.5+ 60.0	1.433 c	1.433 b	18.067 b
0.5+ 80.0	4.167 b	2.407 a	11.277 c

Different letters within columns parts represent significant differences according to Duncan's multiple range test at 5% level.

The effect of BAP on shoot length is illustrated in Table (1). In general, the length of the shoot was reduced with different concentrations of BAP. Control treatment shows the highest length average (2.667cm), which was reduced significantly by increasing BAP concentration but this reduction wasn't significant among BAP levels except 2.0 mg/l concentration which record the least length of shoots (0.463 cm).

The highest number of leaves per explant (12.447) was recorded in 0.5 mg/l treatment, which was significantly reduced to only 7.333 leaves/ explant for the control treatment. These results are in agreement with those published by (Bidarigh *et al* 2013 ; Toma and Al-Mizory 2011 ; Pickens *et al*. 2005). The reasons behind the positive role of BA on multiplication stage might be due to cytokinins great role in releasing lateral buds from the dominance of terminal buds without need to remove the apical bud via promoting the formation of xylem tissues of buds which will facilitate the transport of water and nutrients leading to lateral bud growth as well as, the important role of cytokinins in increasing the synthesis of RNA, proteins and enzymes inside the cell which enhance bud growth as well (Mohammed and Al-Younis, 1991)

The combination between BA and GA_3 showed a similar effect on shoot multiplication as that observed with BAP, however high number of shoots was obtained (Table 1).

The data reveals that the highest shoot number (6.767 shoots) was achieved at 0.5 mg/l GA_3 with 1.5 or 2.0 mg/l BA (fig. 1, C). All concentrations differed significantly from the rest of phytohormones concentration when compared with control treatment, but not between them. Furthermore, the results illustrate that presence of BAP and GA_3 in the culture medium, caused no insignificant increased in shoot length when compared to the free of cytokinin medium. Whereas, the addition of 0.5 mg/l BA + 0.2 mg/l GA_3 gave the highest number of leaves per explant (26.633) significantly when compared to the control (6.167) and other protocols.

Several researchers have reported that the combination between BA and GA_3 have a synergistic effect (Bidarigh and Azarpour 2013). The synergistic effect produced by two plant hormones together is greater than the sum of the individual parts. Likewise, combined BA and GA induce lateral shoot formation when applied during active shoot growth, effective in increasing stem length, bract size and counteracting early or excessive growth regulator application in Poinsettia plants (Odula, 2011).

Table (1) also shows the effect of 0.5 mg/l BAP with different concentrations from adenine sulfate on the multiplication of *Euphorbia* shoots.

The number of branches per explant was highly influenced by the addition of adenine sulfate to nutrient medium. The highest number of branches (10.600) was achieved from the addition of 20.0 mg/l adenine sulfate (Fig. 1, B). While the lowest number of branches per explant (1.433) was recorded in 60mg/l adenine sulfate treatment. A direct proportional correlation can be found between the adenine sulfate concentration and the mean length of branches, since adenine sulfate caused a remarkable increased in the mean length of branches as compared to the control treatment from 1.283 cm (control) to 2.407 cm (0.5 mg/l BA+ 80.0 mg/l adenine sulfate). The highest number of leaves per explant was recorded in 20 mg/l adenine sulfate (28.00) leaves/ explant, which was significantly reduced to only 11.277 leaves/ explant when 80.0 mg/l adenine sulfate was added to the nutrient medium. Therefore, it was obvious that the combination of adenine sulfate and BAP induce better shoot formation. This observations was similarly reported by (Nandagopal and Ranjitha Kumari , 2006 ; Medza Mve *et al*. 2010). Who demonstrated that adenine has same of cytokinin activity and adding it to the culture medium will improve growth or reinforce the response normally as cytokinin action attributed to. In this sense, adenine stimulates somatic embryogenesis and caulogenesis, enhances the growth of isolated meristem tips, induces the proliferation of axillary shoots in shoot cultures and promotes adventitious shoot formation indirectly from calli or directly from explants (Andres and Arias 2010).

Direct organogenesis form stem

An efficient protocol has been developed for direct shoot organogenesis from stem and leaf explants in *Euphorbia* plant . Shoot regeneration frequently was 100% (Table 2) via obtaining 2-5 shoots per explants. When the stem explants were placed on media containing BAP (0.5, 1.0 and 1.5 mg/l) combine with GA_3 (0.1, 0.2 and 0.5 mg/l). The highest number of shoots per explant (5.833) and (5.60) was recorded in (0.5 mg/l BAP + 0.1 mg/l GA_3)

and (1.5 mg/l BAP + 0.5 mg/l GA_3) which differed significantly from the other treatment (Fig. 1, D). On the other hand, BAP at 0.5 and 1.0 mg/l combination with 0.1 and 0.2 GA_3 significantly increase the mean length of shoots as compared to the other treatment which produced the longest shoots (1.133 cm). The highest number of leaves per explant (17.067) was recorded in 0.5 mg/l BAP + 0.1mg/l GA_3, and was significantly reduced to (9.733) leaves/ explant when 1.5 mg/l BAP + 0.5 mg/l GA_3 was added to the nutrient medium. Concerning the role of GA_3 in organogenesis, Bele *et al*. (2012) reported that the leaf discs cultured in MS medium fortified with TDZ and GA_3 proved superior for plant regeneration via somatic embryogenesis of sandalwood plant. While Mookkan (2015) mentioned that the combination of L-Glutamine with BA and GA_3 will significantly increase the shoot proliferation from cotyledonary node explant of *Cucurbita pepo* L. This behavior may be due to the tissue system which apparently depending on the endogenous levels of the hormones present in the tissue. Moreover, the formations of adventitious organs depend on the reactivation of genes concerned with the organogenic phase of development (Bele et al. 2012).

Table (2). Effects of different concentrations of (BA + GA_3) and (BAP + NAA) in stem organogenesis of Euphorbia plant.

BA+ GA_3 (mg/l)	Organogenesis Percentage (%)	Number of shoots/ explant	Mean length of shoots (cm)	Number of leaves/ explant
0.5+ 0.1	100	5.833 a	1.133 a	17.067 a
1.0+ 0.2	100	2.867 b	0.933 a	9.867 b
1.5+ 0.5	100	5.600 a	0.643 b	9.733 b
BA+ NAA (mg/l)				
0.5+ 0.1	100	5.200 a	0.887 a	16.533 a
1.0+ 0.2	100	6.950 a	1.033 a	12.667 b
1.5+ 0.5	77.800	4.167 b	0.460 b	7.600 c

Different letters within columns parts represent significant differences according to Duncan's multiple range test at 5% level.

Analysis variance results shown that the highest organogenesis percentage 100% was recorded in 0.5 and 1.0 mg/l BAP combination with 0.1 and 0.2 NAA mg/l , whereas the least organogenesis percentage 77.8 % was found in 1.5 mg/l BAP +0.5 mg/l NAA treatment respectively (Table 2). Although both of the number of shoots and their high length reached its highest level 6.95 and 1.033 respectively (Fig. 1, E) at 1.0 mg/l BAP + 0.2 mg/l NAA treatment, and the next highest number of shoots was attained at 0.5 mg/l BAP +0.1 mg/l NAA (5.200 shoots/explant) despite of non significant differences between these concentration. The highest number of leaves was attained at 0.5 mg/l BAP +0.1 mg/l NAA (16.533 leaves /explant) and significantly differs from other treatments. On the other hand, inclusion of 1.5 mg/l of BAP+0.5 mg/l NAA in the culture medium significantly reduced the number of shoots, mean length of shoots and number of leaves / explants.

The observation indicates that BAP and NAA play crucial roles in organogenesis. These results are in agreement with those published by Devendra and Srinivas, (2011) who reports that the multiple shoot induction was achieved using various concentrations of Kin and BAP alone or in combination with NAA in *Crotalaria retusa* L..The addition of NAA into the medium considerably enhanced multiple shoot induction. Furthermore, it was clear that a cytokinin is essential for multiple shoot induction from nodal cuttings and the combination of these plant growth regulators induced axillary bud sprouting at a higher frequency than the presence of BAP alone.

Direct organogenesis form leaf explants

Organogenesis was induced directly without any intervening callus phase from leaf explants on MS medium supplemented with BA and IAA (Table 3). Highest organogenesis percentage (77.73%) was recorded in the 1.0 mg/l BAP combination with 0.2mg/l IAA which produced 3.0 shoots / explants (Fig. 1, F) followed by 1.5 mg/l BAP +0.5 mg/l NAA 44.40% with 2.83 shoots / explants respectively although the difference between these concentrations was not significant. The high mean length of shoots 0.690 cm was recorded in medium contains 1.0 mg/l BAP + 0.2 mg/l IAA significantly when compared with other treatments**.** The highest number of leaves per explant was obtained in 1.0 mg/l BAP +0.2 mg/l IAA treatment (3.833). These results are in agreement with those published by Martin *et al*., (2005) who clarify the reduction effect of Kin on *in vitro* morphogenesis of mesophyll cells of *Euphorbia nivulia*. (Shekhawat, 2013) reported that the combination between BA and IAA induced the highest frequency of shoots from nodal shoot segments in *Euphorbia hirta* L. Also, it was found by (Kondamudi *et al.* 2009) that the combination of BA with NAA and IAA had positive effect on morphogenesis.

Table(3).Effects of different concentrations of BAP and IAA in leaf organogenesis of Euphorbia plant.

BAP+ IAA (mg/l)	Organogenesis Percentage (%)	Number of shoots/ explant	Mean length of shoots (cm)	Number of leaves/ explant
0.5+ 0.1	0.0	0.0 b	0.0 c	0.0 b
1.0+ 0.2	77.733	3.000 a	0.690 a	3.833 a
1.5+ 0.5	44.400	2.833 a	0.233 b	3.333 a

Different letters within columns represent significant differences according to Duncan's multiple range test at 5% level.

Effects of auxins on rooting

Following shoot multiplication, several experiments were conducted to stimulate root initiation in excised shoots. The results revealed that the three investigated auxins varied in their activity on root formation. In general the results indicate that IAA was the most active auxin that increase rooting percentage (77%), followed by IBA (33%) whereas the least rooting percentage (11%) was found in NAA treatment.

Table (4). Shows the optimal concentration of IAA for root induction, the results reveals that each shoot creates 4.977 roots as an average. (Fig. 1, G) and it was significantly different than the control. A comparable number of roots were also developed in other IAA levels, although there weren't significant differences observed between them. Inverse response has been existed in root length with presence of IAA (Fig.1, H). In spite of non significant differences, the best root length was achieved at control treatment followed by 0.5 mg/l IAA treatment.

Table(4). Effect of different auxins concentrations on in vitro rooting of Euphorbia pulcherrima plant

Auxin	Conc. mg/l	Rooting Percentage%	Number of roots/ explant	Mean length of roots(cm)
IAA	0.00	44.433	3.333 b	5.050 a
	0.50	66.667	4.867 a	4.867 a
	1.00	77.800	4.977 a	2.333 c
	1.50	77.733	4.167 a	4.167 b
	2.00	77.800	4.867 a	3.833 c
IBA	0.00	44.767	4.167 a	1.633 d
	0.50	22.200	4.333 a	4.333 b
	1.00	33.300	2.333 b	7.233 a
	1.50	22.200	1.000 c	3.900 b
	2.00	33.300	2.667 b	2.500 c
NAA	0.00	22.200	2.667 b	2.533 a
	0.50	11.100	3.667 a	1.333 b
	1.00	11.100	0.667 c	0.333 c
	1.50	0.0	0.0 d	0.0 d
	2.00	0.0	0.0 d	0.0 d

Different letters within columns represent significant differences according to Duncan's multiple range test at 5% level.

Regarding the roots number, 0.5 mg/l from IBA caused insignificant increment in this parameter when compared to control treatment (Table 4). However, the rest levels induce rooting but less than the control treatment. On the other hand, root length increased significantly with rising IBA concentrations who reach its maximum value at the 1.0 mg/l IBA level (7.233 cm) when compared to the control and other treatments.

The effects of different NAA concentration on the shoots rooting of *Euphorbia* plant are shown in (Table 4). It is quite clear from the results that the addition of NAA was not significantly affective in raising the number of roots per explant as compared to the auxin-free medium (control) except the highest number of roots per explant (3.667) which was recorded when 0.5 mg/l NAA was added. On the other hand, NAA significantly reduced the average length of roots as compared to the control that was creates the longest roots (2.533 cm).

From the previous observations, the results revealed that the three tested auxins (IBA, IAA, and NAA) stimulate root initiation in (*Euphorbia pulcherrima* Willd). However, rooting extension varied according to auxin type and concentration, IAA was the most effective auxin, followed by IBA and NAA. These results indicated that the presence of auxins had positive influences on rhizogenesis in poinsettia *in vitro* due to its effects in increase the cell wall elasticity and stimulates cell elongation followed by adventitious root formation (Damiano *et. al*., 2007).

The regenerated shoots were rooted on a medium containing 1.0 mg/l IAA and then transferred to the greenhouse (Fig.1, J).

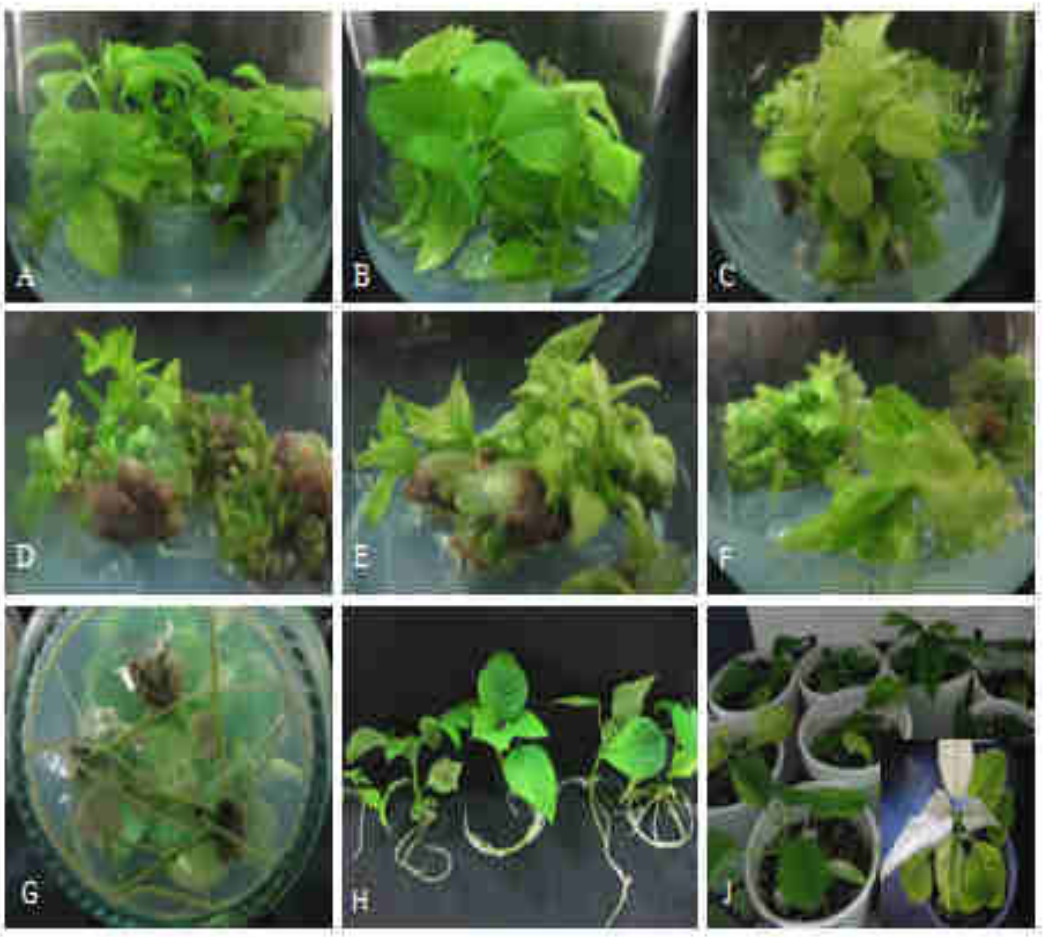

Fig.1:Shows different stages of micropropagation and organogenesis of Euphorbia pulcherrima Willd. Plants

A. Multiple shoots was observed in MS medium + 0.5 mg/l BA

B. Multiple shoots was observed in MS medium +0.5 mg/l BA+ 20 mg/l adenine sulfite

C. Multiple shoots was observed in MS medium + 1.5 mg/l BA+ 0.5 mg/l GA_3

D. Plant regeneration from stem segments in MS medium + 0.5 mg/l BA+ 0.1 mg/l GA_3

E. Plant regeneration from stem segments in MS medium + 1.0 mg/l BA+ 0.2 mg/l NAA.

F. Plant regeneration from leaf segments in MS medium + 1.0 mg/l BA+ 0.2 mg/l IAA.

G. Best roots formation in MS medium supplements with 1.0 mg/l IAA.

H. Formation of root in MS medium supplements with different concentration of IAA.

J. Acclimatized plantlet ready for transplantation in the field.

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