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Studies on *in-vitro*propagation in glory lily (*Gloriosasuperba*L.)

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ABSTRACT

Glory lily (GloriosasuperbaL.) is one of the important medicinal plants which has recently attained commercial importance. The medicinal value of glory lily is attributed to the presence of important alkaloids namely, Colchicine and Gloriosine. The cultivation of this medicinal plant has been widespread in the states of Tamil Nadu and Goa. The drug extracted from the seeds is biter, pungent and astringent in taste. The drug has so far been obtained from collection of tubers from wild sources and due to indiscriminate exploitation, the supply has been affected badly. Due to large scale cultivation of this medicinal plant, the demand for the seed tubers has increased considerably. In order to meet out the demand, the necessity for large scale multiplication of tubers through in-vitro techniques was thought of and an experiment was conducted in the Tissue Culture Lab at the Department of Horticulture.The results revealed that among the explants tried for callus induction, shoot tips performed better than nodal segments. Surface sterilization of shoot tips with 0.5 per cent mercuric chloride for 2 minutes resulted in maximum survival of explants with the least contamination. Highest culture response for callusing was obtained when MS medium was supplemented with NAA @ 0.5 mg l^{-1} + BAP @ 2.0 mg l^{-1}in a shortest duration of 43.16 days. Highest culture response for shoot regeneration was observed in MS medium supplemented with 3.0 mg l^{-1} of BAP which also resulted in earlier shoot regeneration with higher number of lengthier shoots. Highest culture response for root regeneration was obtained in the MS medium supplemented with 2.0 mg l^{-1} of IBA. Maximum culture response with respect to direct organogenesis of shoot tip was obtained in the treatment combination of IBA 2.0 mg l^{-1} + BAP 4.0 mg l^{-1} which also resulted in earliest direct organogenesis (43.99 days), whereas BAP @ 3 mg l^{-1} took the longest duration of 80.74 days.

Keywords: Glory lily, in-vitro techniques, explants, sterilization, MS medium, shoot, root, regeneration.

INTRODUCTION

India is endowed with a rich wealth of medicinal plants, which have contributed to the development of ancient Indian materiamedica. In one of the earliest treatises on Indian medicine, The CharakSamhita (1000 B.C.) had recorded the use of over 340 drugs of plant origin. Most of the medicinal plants are gathered from their wild habitats to meet the increasing demand. However, of late, their availability from natural sources is dwindling due to increased diversion of land for non-agricultural uses, rapid urbanization and indiscriminate deforestation. Hence, medicinal plants deserve special attention for extensive cultivation on a commercial scale. Among others, the Glory lily (*Gloriosasuperba*L.) has attracted large scale cultivation in recent times in the states of Tamil Nadu and Goa.The drug has so far been obtained from collection from wild sources but of late, the indiscriminate exploitation of tubers has affected its supply badly. This has necessiated establishment of commercial plantations to meet the demand of pharmaceutical industries. Being a newly domesticated crop, appropriate production technologies for this medicinal plant have not yet been standardized on a scientific footing.Seeds and tubers are being used for propagation of Gloriosa. Propagation through tubers are preferred as the seedlings take a longer time to come to flowering and yield. The tubers, however exhibit wide variation for their size and weight. The plants obtained from larger tubers may be expected to grow vigorously with higher production of flowers and pods (Farooqi and Sreeramu, 2001). On the other hand, the plants from very tiny tubers may affect the growth adversely which may even fail to flower in the first year. On the other hand, non-availability of sufficient quantity of large size tubers may also pose problem.Tissue culture techniques for rapid mass clonal propagation of horticultural crops have assumed greater importance in the past few decades. Clonal multiplication through conventional methods of asexual propagation may not suffice to meet out the increasing demand for propagating materials.

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Micropropagation is specially useful for those crops, wherein corms and tubers are used for propagation due to their slow multiplication rates. *In-vitro* methods of propagation will help to produce large number of genuine planting materials relatively at a shorter span of time. Hence, a trial was conducted to standardize ideal explants and chemicals for sterilization for higher success, ideal nutrient media plus growth hormone combination to maximise shoot and root generation and to optimise culture response and duration for direct organogenesis using glory lily as test crops.

MATERIALS AND METHODS

The experiment was conducted at the Tissue Culture Laboratory of Department of Horticulture in a completely randomised design (CRD).The plants of *Gloriosasuperba* generated from healthy tubers obtained from Sooriyamanal village of Jayamkondamtalukof Tamil Nadu were utilized for the study. Healthy and disease free plants maintained in a greenhouse aseptic condition at the Department of Horticulture were utilized as a source for explant collection.

Type of explants: Shoot tip: 1-1.5 cm length with one or two primordiaand Nodal segment: 1-1.5 cm length were used in this study.

Nutrient media: Major and minor nutrients and vitamins in the present study were used as prescribed by Murashige and Skoog (1962). The composition of the MS media is presented in Table 1. Analytical grade nutrients and chemicals were obtained from Hi-media Chemicals, Mumbai. The required compounds such as agar (8 g l^{-1}) and sucrose (30 g l^{-1}) were added to the media. Glass double distilled water was used for the entire study.

Preparation of media: The basal MS medium at 4.2 g was dissolved in a small quantity of double distilled water. To this, 8 g l^{-1} agar dissolved in boiling water, 30 g l^{-1} sucrose and 150 ml of deproteinised coconut water were added and the final pH was adjusted to 5.6 to 5.8 by using 0.1 N NaOH or 0.1 N Hcl. The media was homogenized by following the procedure of Butenko (1967) and 5 ml of the medium was transferred to clear culture tubes of 25 × 150 mm size. The culture tubes containing media were plugged with sterilized non-absorbant cotton wool (Bhojwani and Bhatnagar, 1974). The tubes with media were then autoclaved at 15 psi at 121 °C for 20 minutes (Dodds and Roberts, 1982).

Preparation of growth regulators

i) Auxins: 100 mg in each of Indole-3 Acetic acid (IAA) and Naphthalene Acetic Acid (NAA) were dissolved in 2-3 ml of ethanol, warmed and gradually diluted to 100 ml using double distilled water. It was further diluted to the desired concentrations.

ii) Cytokinins: 100 mg in each of Benzyl amino purine (BAP) and kinetin were weighed and dissolved using few drops of 0.1 N HCl, gently warmed and diluted to 100 ml with double distilled water. These were then serially diluted to the desired concentrations.

iii) Coconut water: Tender coconut water was filtered through muslin cloth and boiled for 10 minutes to denature and precipitate the proteins. It was then filtered through Whatman No.1 filter paper after cooling to ambient temperature. The filtrate was collected in a reagent bottle and stored at 5 °C. The coconut water at 150 ml (15 % v/v) was utilized for preparing 1 litre of media.

Isolation of explants

i) Shoot tips: Shoot tips were collected from 30-35 days old 'stock-plants' and were thoroughly washed with 0.1 per cent teepol followed by washing thrice with sterilized distilled water. The explants were then surface sterilized with 70 per cent ethanol for 30 seconds followed by washing with sterilized distilled water thrice. The shoot tips of size 1.0 to 1.5 cm length possessing 1 or 2 primordia were excised aseptically.

ii) Nodal segments: Nodal segments of 1.0 to 1.5 cm length were taken from 30-35 days old 'stock-plants'. The excised nodal segments were thoroughly washed in running tap water and in detergent solution followed by washing with sterile distilled water thrice. Then, these were surface sterilized with 70 per cent ethanol for 30-40 seconds followed by washing with sterilized distilled water thrice to completely wash off the alcohol.

Sterilization of explants

Surface sterilization of the explants was done using mercuric chloride at various concentrationsnamely Mercuric chloride @ 0.1 % for 1 min. (T_1), Mercuric chloride @ 0.1 % for 2 min. (T_2), Mercuric chloride @ 0.5 % for 1 min. (T_3) and Mercuric chloride @ 0.5 % for 2 min. (T_4),

Culture of explants

i) Callus culture: Callus culture was induced from shoot tips and nodal segments in MS medium supplemented with IAA, NAA and BAP at various concentrationsviz., i) MS basal (control), ii) IAA (0, 0.1, 0.5, 1.0 mg l^{-1}), iii) NAA (0, 0.1, 0.5, 1.0 mg l^{-1}) and iv) BAP (0, 1.0, 2.0, 3.0 mg l^{-1}). The other treatments include various combinations of the above auxins and cytokinin totaling 16 treatments. The cultures were incubated at 25 $\pm$ 2 °C with 16/8 hours light/ dark cycle.

ii) Shoot regeneration: In order to standardize the growth regulators for shoot regeneration, a total of seven treatment combinations were tried. i) MS basal (control), ii) MS + BAP (1.0, 2.0, 3.0 mg l^{-1}) and iii) MS + kinetin (1.0, 2.0, 3.0 mg l^{-1}).

iii) Root regeneration: A total of seven treatment combinations were triedviz., i) MS basal (control), ii) MS + IAA (1.0, 1.5, 2.0 mg l^{-1}) and iii) MS + IBA (1.0, 1.5, 2.0 mg l^{-1}).

iv) Direct organogenesis: For direct organogenesis, the shoot tips were inoculated in MS medium supplemented with IBA and BAP with a total of 16 treatment combinations viz., i) MS basal (control), ii) MS + IBA (0, 1.0, 1.5, 2.0 mg l^{-1}) and MS + BAP (0, 3, 4, 5 mg l^{-1}). Twenty culture tubes were maintained in each treatment and the cultures were exposed to a light/ dark cycle of 16/ 8 hrs. The temperature of the culture room was maintained at 25 $\pm$ 2 °C.

Observations recorded

Name of experiment	Observations recorded
Type of explants Sterilization of explants	Percentage of contamination, Percentage of survival
Callus culture	Percentage of culture response Duration for callusing (days)
Shoot regeneration	Percentage of culture response Duration of shoot regeneration (days) Number of shoots, Length of shoot
Root regeneration	Percentage of culture response Duration for root regeneration (days) Number of roots, Length of root
Direct organogenesis	Percentage of culture response Duration for direct organogenesis (days)

Statistical analysis: Statistical analysis was carried out as per procedures outlined by Panse and Sukhatme (1978). Wherever the treatment effects were significant, Critical differences (CD) at 5 per cent probability level were computed for effective comparison of treatments.

RESULTS AND DISCUSSION

The results of the investigation on *in-vitro* techniques in glory lily are discussed here under.

Table 1: Composition of Murashige and Skoog's medium (Murashige and Skoog, 1962)

Constituents	Molecular weight	Amount (mg l^{-1})
I. Macro nutrients		
Ammonium nitrate ($NH_4 NO_3$)	80.04	1650.00
Potassium nitrate (KNO_3)	101.11	1900.00
Calcium chloride ($CaCl_2 2H_2O$)	147.02	440.00
Magnesium sulphate ($MgSO_4 7H_2O$)	246.47	370.00
Potassium hydrogen phosphate ($KH_2 PO_4$)	136.09	170.00
II. Micro nutrients		
Potassium iodide (KI)	166.01	0.83
Boric acid (H_3BO_3)	61.83	6.20
Manganese sulphate ($MnSO_4 4H_2O$)	223.01	22.30
Zinc sulphate ($ZnSO_4 7H_2O$)	287.54	8.60
Sodium molybdate ($Na_2 MoO_4 2H_2O$)	241.68	0.03
Copper sulphate ($CuSO_4 5H_2O$)	249.68	0.03
Cobalt chloride ($CoCl_2 6H_2O$)	237.95	0.03
III. Iron EDTA		
Iron sulphate ($FeSO_4 7H_2O$)	278.30	27.80
Sodium EDTA (Na_2-EDTA $2H_2O$)	372.25	37.30
IV. Organic nutrients		
Myo-inositol	180.16	100.00
Nicotinic acid	123.11	0.50
Pyridoxine HCl	205.64	0.50
Thiamine HCl	337.29	0.10
Glycine	75.07	2.00

Sterilization of explants

The results of the present study clearly indicated the effectiveness of mercuric chloride ($HgCl_2$) as a surface sterilant at 0.5 per cent concentration treated for two minutes. The level of contamination seemed to have influenced by the incubation time, with lesser the time, greater was the level of contamination (Table 2). Custers and Bergervoet (1994) found that when higher dose of mercuric chloride was used with prolonged exposure, the degree of contamination in the explants of *Gloriosasuperba* was reduced. Kanimozhi (2002) observed that mercuric chloride of 0.5 per cent exposed for 2 minutes was highly effective and these results support the findings of the present study.

Table 2: Effect of mercuric chloride on contamination and survival (%) of different explants in glory lily

Treatments			Shoot tip		Nodal segment	
T. No.	$HgCl_2$ (%)	Period of exposure (min.)	Contamination (%)	Survival (%)	Contamination (%)	Survival (%)
T_1	0.1	1.0	86.85	11.32	90.63	7.36
T_2	0.1	2.0	75.96	26.03	87.88	14.11
T_3	0.5	1.0	67.03	30.96	80.56	17.43
T_4	0.5	2.0	44.93	56.97	78.92	23.08
	Grand mean		68.69	31.32	84.50	15.49
	SE_D		1.21	0.61	1.30	0.74
	CD (p=0.05)		2.43	1.24	2.61	1.55

Suitability of explants

Among the explants tried in the present study, shoot tips responded significantly to a concentration of0.5 per cent of mercuric chloride with an exposure of 2 minutes compared to the nodal segments. Moreover, the level of contamination was lesser in shoot tips as compared to nodal segments. The concentration of surface sterilants seemed to differ depending upon crop species and parts of plants used (Rao and Selvarajan, 1982). Dixon (1985) opined that besides the concentration and period of exposure, the incubation condition will also aid in

reducing the contamination. In *Gloriosasuperba* L., Samarajeewa *et al.* (1993) observed that apical buds and nodal segments separated from shoot tips resulted in successful production of plantlets. Kanimozhi (2002) reported that shoot tips gave better culture response than nodal segments which support the results of the present study.

Table 3: Effect of IAA + BAP on callusability (per cent) and duration of callusing (days) of shoot tip

Treatment	IAA (mg l^{-1})	BAP (mg l^{-1})	Culture response (%)	Duration of callusing (days)
T_1	0.0	0.0	0.00	0.00
T_2	0.1	0.0	2.82	66.72
T_3	0.5	0.0	9.54	62.81
T_4	1.0	0.0	3.81	66.01
T_5	0.0	1.0	12.27	69.81
T_6	0.1	1.0	16.61	64.13
T_7	0.5	1.0	29.17	56.02
T_8	1.0	1.0	22.33	60.65
T_9	0.0	2.0	19.57	60.09
T_{10}	0.1	2.0	22.58	56.15
T_{11}	0.5	2.0	34.84	50.02
T_{12}	1.0	2.0	29.89	50.07
T_{13}	0.0	3.0	14.50	65.00
T_{14}	0.1	3.0	19.16	57.89
T_{15}	0.5	3.0	30.98	54.00
T_{16}	1.0	3.0	25.13	53.81
	Grand mean		18.33	55.82
	SED		0.54	0.52
	CD (p=0.05)		1.09	1.06

Callusability of explants

The type of explants and the plant growth regulators used are the important factors that determine the callusability of explants. Callus can be induced in numerous plant organs and tissues. The success of *in-vitro* culture is greatly dependent on the use of appropriate growth regulators in optimum concentration for desired effect (Krikorian, 1982). Auxin and cytokinins could be used to promote differential growth of embryogenic and non- embryogeniccalli and accelerate callus growth.

Table 4: Effect of NAA + BAP on callusability (per cent) and duration of callusing (days) of shoot tip

Treatment	NAA (mg l^{-1})	BAP (mg l^{-1})	Culture response (%)	Duration of callusing (days)
T_1	0.0	0.0	0.0	0.00
T_2	0.1	0.0	2.92	65.87
T_3	0.5	0.0	8.77	56.54
T_4	1.0	0.0	4.96	60.63
T_5	0.0	1.0	13.01	66.43
T_6	0.1	1.0	17.91	61.06
T_7	0.5	1.0	22.07	52.33
T_8	1.0	1.0	27.04	54.24
T_9	0.0	2.0	22.07	57.58
T_{10}	0.1	2.0	24.74	50.49
T_{11}	0.5	2.0	40.14	43.16
T_{12}	1.0	2.0	35.89	48.17
T_{13}	0.0	3.0	18.60	59.90
T_{14}	0.1	3.0	20.30	53.07
T_{15}	0.5	3.0	35.13	48.06
T_{16}	1.0	3.0	20.89	52.17
	Grand mean		19.65	51.86
	SED		0.59	0.58
	CD (p=0.05)		1.19	1.17

Wainwright (1988) reportedthat in a culture medium, when the concentrations of cytokinins in relation toauxins are intermediate, the tissue will grow as an un-organized mass of callus. In the present study, the culture response for callus induction from shoot tip was higher in the combination of MS medium supplemented with NAA @ 0.5 mg l^{-1} + BAP @ 2.0 mg l^{-1} when compared to the combination of IAA @ 0.5 mg l^{-1} + BAP @ 2.0 mg l^{-1}. Besides, callus induction was earlier with 43.16 days in NAA -0.5 mg l^{-1} + BAP -2.0 mg l^{-1} as compared to 50.02 days in IAA – 0.5 mg l^{-1} + BAP -2.0 mg l^{-1}. With the exclusion of growth regulators, no callus was observed. This is in conformity with the findings of Neelam Sharma *et al.* (1991) in *Coleus forskohlii* who reported that callus growth hardly occurred in the basal media alone (Table 3 and 4). Successful attempts have been made on callus induction and proliferation by the use of various concentrations of auxin and cytokinins in *Gloriosasuperba* L. by Samarajeeva *et al.* (1993) and Kanimozhi (2002) which corroborate the results obtained in the present study.

Sai Ram Reddy *et al.* (1998) and Hema (2000) in *Gymnemasylvestre*; Kulneet Kaur *et al.* (1996) in *Catharanthusroseus* and Manickam *et al.* (2000) in Indian ginseng have demonstrated successful callus induction through appropriate combination of auxins and cytokinins.

Table 5: Effect of growth regulators on shoot regeneration from shoot tip callus

Treatment (mg l^{-1})	Culture response (%)	Duration for shoot regeneration (days)	Number of shoots	Length of shoot (cm)
T_1 – MS alone (basal)	0.00	0.00	0.00	0.00
T_2 – MS + BAP 1.0	39.86	34.13	0.76	3.11
T_3 – MS + BAP 2.0	50.28	32.21	1.22	4.16
T_4 – MS + BAP 3.0	51.30	24.06	1.40	4.50
T_5 – MS + Kn 1.0	38.14	38.14	0.42	2.92
T_6 – MS + Kn 2.0	44.18	26.21	1.13	3.88
T_7 – MS + Kn 3.0	42.20	34.13	0.98	3.39
Grand mean	38.00	26.98	0.84	3.14
SED	0.65	0.86	0.04	0.14
CD (p=0.05)	1.30	1.72	0.08	0.28

Callus regeneration

The ratio of auxin and cytokinin used decides the efficiency of callusability and organogenesis (Kohlenback, 1977). A high level of auxin in relation to cytokinin in general favours root formation, whereas, an increase in the level of cytokinin than auxinfavours root formation.In the present study, highest culture response for shoot regeneration was obtained in MS medium supplemented with 3.0 mg l^{-1} of BAP when compared to kinetin. The above combination resulted in earlier shoot regeneration (24.06 days) with higher number of lengthier shoots when compared to a longer duration (38.14 days) taken by the combined treatment with kinetin (Table 5).

Table 6: Effect of growth regulators on root regeneration from shoot tip callus

Treatment (mg l^{-1})	Culture response (%)	Duration for root regeneration (days)	Number of roots	Length of root (cm)
T_1 – MS alone (basal)	0.00	0.00	0.00	0.00
T_2 – MS + IAA 1.0	20.23	56.10	0.41	0.52
T_3 – MS + IAA 1.5	30.02	49.30	1.20	1.24
T_4 – MS + IAA 2.0	24.21	50.37	0.71	0.94
T_5 – MS + IBA 1.0	23.09	55.14	0.63	0.78
T_6 – MS + IBA 1.5	30.34	44.50	1.42	1.42
T_7 – MS + IBA 2.0	36.11	43.15	1.85	1.74
Grand mean	23.43	42.65	0.89	0.95
SED	0.67	0.66	0.03	0.03
CD (p=0.05)	1.35	1.33	0.07	0.07

The culture response for root regeneration in the present study indicated the usefulness of IBA @ 2.0 mg l^{-1} which gave highest culture response, besides producing higher number of lengthier roots as compared to IAA (1 mg l^{-1}) which showed least promise in relation to the above parameters (Table 6). The rapid utilization of carbohydrates as stimulated by auxin may be responsible for the growth and secondary differentiation of tissues and organs.The results of the present study are in conformity with the earlier findings of Samarajeeva*et al.* (1993) and Kamimozhi (2002) in *Gloriosasuperba* L.; Rani *et al.*, (1999) in ashwagandha; KulneetKaur *et al.* (1996) in *Catharanthusroseus*.

Table 7: Effect of growth regulators on direct organogenesis from shoot tip

Treatment	IBA (mg l^{-1})	BAP (mg l^{-1})	Culture response (%)	Duration (days)
T_1	0.0	0.0	0.00	0.0
T_2	1.0	0.0	24.10	79.85
T_3	1.5	0.0	28.11	80.74
T_4	2.0	0.0	28.82	73.99
T_5	0.0	3.0	23.03	83.11
T_6	1.0	3.0	29.98	70.91
T_7	1.5	3.0	31.86	69.99
T_8	2.0	3.0	36.86	58.79
T_9	0.0	4.0	24.16	81.00
T_{10}	1.0	4.0	43.19	51.99
T_{11}	1.5	4.0	47.19	47.90
T_{12}	2.0	4.0	53.07	43.99
T_{13}	0.0	5.0	29.97	65.63
T_{14}	1.0	5.0	45.05	48.61
T_{15}	1.5	5.0	46.73	50.06
T_{16}	2.0	5.0	40.02	55.03
	Grand mean		33.26	60.10
	SED		0.55	0.64
	CD (p=0.05)		1.10	1.28

Direct organogenesis

In the present study, direct organogenesis from shoot tip has been successfully obtained with varied concentration and combination of growth regulators like IBA and BAP. Maximum culture response relatively in a shorter duration (44 days) was obtained in the treatment combination of IBA - 2.0 mg l^{-1} + BAP 4.0 mg l^{-1} when compared to BAP @ 3 mg l^{-1} alone which took a longer duration of 80.74 days (Table 7). Similar results have been obtained by Kanimozhi (2002) in *Gloriosasuperba* and Adinpunya Mitra *et al.* (1998) in *Catharanthusroseus*.To sum up, the results revealed that among the explants tried for callus induction, shoot tips performed better than nodal segments. Besides they found to be less contaminated as compared to nodal segments.Surface sterilization of shoot tips with 0.5 per cent mercuric chloride for 2 minutes resulted in maximum survival of explants with the least contamination. Highest culture response for callusing was obtained when MS medium was supplemented with 0.5 mg l^{-1} of IAA + 2 mg l^{-1} of BAP.Among the treatment combination of NAA and BAP, NAA @ 0.5 mg l^{-1} + BAP @ 2.0 mg l^{-1} recorded the highest callusability in a shortest duration of 43.16 days.Highest culture response for shoot regeneration was observed in MS medium supplemented with 3.0 mg l^{-1} of BAP as against the least response in MS medium supplemented with kinetin @ 1.0 mg l^{-1}.Earlier shoot regeneration in 24.06 days was obtained when MS medium was supplemented with 3.0 mg l^{-1} of BAP as against the longer duration (38.14 days) taken by kinetin @ 1.0 mg l^{-1}.The number and length of shoot were maximum in the combination of MS medium supplemented with 3.0 mg l^{-1} of BAP. Maximum root regeneration from the shoot tip callus was obtained in the MS medium supplemented with 2.0 mg l^{-1} of IBA with a least duration of 43.15 days. The number of roots and root length were maximum in the combination of MS media with IBA @ 2.0 mg l^{-1}.

Maximum culture response with respect to direct organogenesis of shoot tip was obtained with treatment combination of IBA 2.0 mg l^{-1} + BAP 4.0 mg l^{-1}.

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