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In vitro Multiple Shooting, Root Tuberization and Evaluation of Anti-inflammatory Properties of *Gloriosa superba*

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Abstract

Gloriosa superba is an important medicinal as well as ornamental plant belongs to the family Liliaceae. The plant is the source of many secondary metabolites like colchicine, colchicoside, gloriosine, chelidonic acid, β sitosterol, luteolin etc. The plant is used to treat various diseases like cancer, gout, scrofula and act as antipyretic, antihelmintic and antiabortive. Due to excessive use of the plant for diverse medicinal purposes the species is threatened in the wild. The main aim of the present study was to evaluate the effect of M S medium with different hormonal concentrations on multiple shoot*ing* and *in vitro* root tuberization in *Gloriosa superba*. The medicinal properties like anti-inflammatory effect of *in vitro* root tubers was also evaluated and compared with roots grown in natural habitat. The results indicated that MS medium supplemented with NAA (0.5 mgl^{-1}) and BAP (2.5 mgl^{-1}) promoted the formation of the maximum number of shooting within 3 weeks from the node explants with mean shoot number 11±0.02 and shoot length of 10.2±0.02 cm. The results also revealed that maximum *in vitro* root tuberization was observed in the M S medium supplemented with NAA (2.5 mgl^{-1}) and BAP (0.5 mgl^{-1}). The evaluation of anti-inflammatory properties of *in vitro* root tubers showed significant effect compared to *in vivo* root tuber. Due to overexploitation for its diverse medicinal applications, *Gloriosa superba* has been threatened and also there is an urgent need to conserve the plant by biotechnological approaches like tissue culture. *In vitro* shooting and rooting helps to conserve the plant and avoid derooting of the plant for medicinal purposes.

Key words: Anti-inflammation, Colchicine, *In vitro* root Tuber, M S medium, Multiple Shooting

Introduction

Micropropagation is one of the aseptic method for the production of large number of plantlets within a short period of time. It is the process of isolating explants from a plant and grown aseptically for indefinite period on a semi defined or defined nutrient medium (Ignacimuthu, 1997). Murashige and Skoog's medium is commonly used for plant tissue culture studies (Murashige & Skoog, 1962). Micropropagation is a commercially viable method for the propagation of several medicinal plants which are on the verge of extinction due to the over exploitation.

Gloriosa superba L. is one of the major climbing medicinal herb belongs to the family Liliaceae. The plant has large red and yellow flowers with wavy edges and leaves with coiled tips (Baloda, 2002; Shanmugam, 2009). All the plant parts have medicinal potential due to the presence of various phytochemical constituents such as colchicine, gloriosine, chelidonic acid etc. The plant has 'V' shaped root tuber with many secondary

metabolites and is used for the treatment of various disorders and ailments like asthma, cancer, gout, ulcer, leprosy etc (Ghani, 1998). Due to the over exploitation and the unscientific collection, the plant is becoming threatened and present study is planned to induce multiple shooting and root tubers of this valuable medicinal plant *Gloriosa superba* L. in *in vitro* condition with various concentrations of plant growth regulators, and comparative evaluation of anti-inflammatory properties of *in vivo* and *in vitro* tubers.

Materials and Methods

The plant *Gloriosa superba* was collected from the garden of University College, Thiruvananthapuram. The leaf and node were taken as explants for the *in vitro* propagation. The explants were cut into small pieces and washed thoroughly under tap water followed by teepol. Then the explants were rinsed with running water for the removal of remnants of teepol. Finally the explants were dipped in mercuric chloride solution for 8 minutes and washed thrice in double distilled water before inoculation (Ashok *et al*., 2011). Murashige and Skoog's medium was used for the *in vitro* culture of *Gloriosa superba* L. The explants were inoculated on MS medium supplemented with different concentrations of auxins in combination with cytokinin for shoot induction and *in vitro* root tuberization. MS medium with NAA, BAP, IBA, IAA and Kinetin (0.5- 2.5 mgl^{-1}) was used for shoot induction and NAA, BAP, IBA, IAA and 2, 4- D (0.5- 2.5 mgl^{-1}) was used for inducing roots. The *in vitro* regenerated root tubers were sub cultured on the M S medium with NAA, 2, 4- D and IAA for growth and the elongation (Ashok *et al*., 2011). The mean number, frequency and length of *in vitro* shoots and roots were measured and statistically analyzed.

The anti-inflammatory activity of *in vivo* and *in vitro* root tubers were evaluated using Cyclooxygenase (COX) and Lipoxygenase (LOX) inhibition assay. The dried powdered tuber was defatted with petroleum ether and further extracted with methanol for 48- 72 hours in a soxhlet apparatus. This methanol extract was used for the evaluation of anti-inflammatory potential of *Gloriosa superba* tuber by cycloxygenase inhibition assay and 5-lipoxygenase inhibition assay.

Lymphocyte culture preparation:

Human Platelet Lysate (HPL) culture was prepared in the medium RPMI (Roswell Park Memorial Institute) 1640 and then supplemented with 20% heat inactivated Foetal Bovine Serum (FBS) and 20% antibiotics (Penicillin). The culture was filtered in completely aseptic conditions followed by addition of fresh plasma at a concentration of $1x10^6$ cells ml^{-1} and incubated for 72 hours followed by addition of 1µl of Lipopolysaccharides and kept for 24 hours incubation. The sample and the standard (Aspirin) were added in the concentration of 100µgml^{-1}, 500µgml^{-1} and 1000 µg ml^{-1} respectively. Then the culture was allowed to incubate for 24 hours and then centrifuged at 6000 rpm for 10 minutes. Then 200µl of cell lysis buffer (1MTris HCl, 0.25M EDTA, 2M NaCl, 0.5% Triton) was added and incubated for 30 minutes at 4°C. Finally the pellet with a small amount of supernatant was collected and used for the assay (Copeland *et al*., 1994).

Cycloxygenase Assay inhibition (COX assay):

In COX assay arachidonic acid was added to the pellet and incubated at 37ºC. Then 0.2ml of 10% TCA in 1N HCl was mixed and heated in a boiling water bath for 20 min followed by adding 0.2ml of thiobarbituric acid (TBA), cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632nm for COX activity (Copeland *et al*., 1994).

5-lipoxygenase Assay inhibition (LOX assay):

For LOX assay 70mg of linoleic acid and tween 20 was dissolved in 4ml of oxygen free water, followed by sufficient amount of 0.5N NaOH to yield a clear solution and then made up to 25ml using oxygen free water. This was divided into 0.5ml portions and flushed with nitrogen gas before closing and kept frozen until needed. The reaction was carried out in a quartz cuvette at 25°C with 1cm light path. The increase in OD was measured in 234nm (Reddanna *et al*., 1990).

% inhibition was calculated using the formula: (C-T/ C) ×100

(C =Optical density of control, T = Optical density of Test)

Results and Discussion

In the present study maximum (98±0.08 %) multiple shooting of *Gloriosa superba* L. were observed on the MS basal medium supplemented with 2.5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA within 3 weeks from the nodes with maximum mean shoot number 11±0.02 and shoot length 10.2±0.02 cm (Table 1, Fig 1). The result also indicated that the M S medium with 1.0 mgl^{-1} NAA and 2.0 mgl^{-1} BAP induced 90±0.01%, 1.5 mgl^{-1} NAA and 1.5 mgl^{-1} BAP induced 85±007 %, and 0.5 mgl^{-1} BAP alone induced 85±0.38 % of multiple shooting within 3 weeks of inoculation. Multiple shoot formation in *Gloriosa superba* using tuber as

explants were reported (Ravindra & Mahendra, 2011). The previous results indicated that MS medium supplemented with 2, 4-D and BAP induced maximum number of shoots within 5 weeks. *In vitro* shoot initiation from apical shoot buds and meristems of *Gloriosa superba* within five weeks was also reported (Ashok *et al*., 2011). Venkatachalam *et al*.,(2012) reported the *in vitro* shoot induction in *Gloriosa superba* and the result revealed that 80.2% of shoot induction was noticed in the MS medium with BAP and Kinetin within 3 weeks of inoculation. Micropropagation of *Gloriosa superba* from non-dormant apical buds within 35 days of inoculation was also reported by Sivakumar and Krishnamurthy (2000). Sayeed and Shyamal (2005) reported the addition of coconut water and activated charcoal enhances the production of *in vitro* shoots of *Gloriosa superba* on the M S medium fortified with BAP +NAA. But in the present study efficient shoot induction (98%) were noticed in NAA (0.5 mgl^{-1}) + BAP (2.5 mgl^{-1}) without the additional supplements.

The present study also indicated that maximum (98±0.06 %) *in vitro* tuberization were observed on the M S medium supplemented with 2.5 mgl^{-1} NAA and 0.5 mgl^{-1} BAP within 3 weeks of inoculation from nodes with maximum mean root number 26±0.33 and root length 1.3±0.11cm. MS medium with 0.5 mgl^{-1} NAA induced 90±1.01%, 1.0 mgl^{-1} induced 86±0.01%, 2.0 mgl^{-1} NAA and 1.0 mgl^{-1} BAP induced 84±0.02%, 1.5 mgl^{-1} NAA induced 83±0.32% of *in vitro* root tuberization within 3 weeks of inoculation (Table 2, Fig 2). The *in vitro* regenerated tubers were sub cultured and the results suggested that the root with maximum growth and elongation (2.5±0.09 cm) were noticed on the M S medium with NAA (Table2, Fig 3). The *in vitro* root tuberization in *Gloriosa superba* was reported by Selvarasu and Kandhasamy (2012) with 4.0 mgl^{-1} NAA and 1.0 mgl^{-1} BAP within 4 weeks of inoculation. Kuldeep *et al*., (2015) studied and reported the micropropagation of *Gloriosa superba* on M S medium at different hormonal concentrations. The previous report suggested that maximum shoot (76.6%) and root induction (66.6%) noticed in the MS medium within 6 weeks of inoculation. The *in vitro* tuberization and the quantification of colchicine from *Gloriosa superba* were also investigated by Chandrawanshi (2015). But in the present study maximum *in vitro* roots were established within 3 weeks and also the sub cultured roots were showed maximum number and length which could be used for the conservation of this endangered plant.

The anti-inflammatory activity of *in vitro* root tubers indicated significant percentage of inhibition when compared with the root tubers under natural habitat. In COX inhibition assay percentage of inhibition were found to be 1.97±0.22%, 23.42±0.01%, 37.47±0.02% and in 5-LOX inhibition assay 40.09±0.11%, 64.66±1.01%, 71.21±0.02% for the sample concentration 100μgml^{-1}, 500μgml^{-1}, 1000μgml^{-1}respectively. In the 5-LOX assay the *in vitro* root tuber showed highest percentage of inhibition which is nearly comparable to the standard aspirin. When compared with the root tuber from natural habitat, the *in vitro* root tuber showed nearly closest percentage of inhibition to standard in both inhibition assay (Table 3, Fig 5). The COX has its own two isoforms namely COX 1 and COX 2. The COX 2 is one of the major factor responsible for the inflammation by acceleration of the cell growth and inhibition of apoptosis. The LOX have the ability to the conversion of arachidonic acid to the leukotrienes. The leukotrienes are the major proinflammatory mediators produced within the leucocytes by the oxidation of arachidonic acid by 5-LOX. Both the action of COX-2 and leukotrienes responsible for the inflammation (Lewis *et al*., 1990) can be successfully controlled by root tubers of *Gloriosa superba*. The anti-inflammatory activity of *Gloriosa superba* in carrageenan induced animal models was reported by Abhishek *et al*., (2011). The previous result suggested that both the aqueous and methanol extracts of *Gloriosa superba* showed good anti-inflammatory activity against carrageenan induced rat's edema. The present study confirmed that since both the *in vitro* as well as *in vivo* root tubers showed highly significant anti-inflammatory potentialities the *in vitro* root tubers could be used for the development of an anti-inflammatory drug without the uprooting of plant. The present study could be helpful for the rapid *in vitro* propagation and the conservation of this highly important medicinal plant.

Acclimatization

The regenerated plantlets of *Gloriosa superba* were transferred to the small pots containing the sterilized soil and covered with polyethylene bags for 2 weeks and finally acclimatized in the field (Fig 4).

Conclusion

Plant tissue culture is one of the important techniques for the multiplication of genetically identical copies of medicinal and ornamental plants. In the present study different hormonal combinations in MS medium suitable for multiple shooting and induction of *in vitro* root tubers

with anti-inflammatory potentialities of *Gloriosa superba* were identified. It can be concluded that *in vitro* propagation technique is a powerful tool for the germplasm conservation of this threatened medicinal plant as tissue culture is a rapid process for the mass propagation of *Gloriosa superba* L. Evaluation of anti-inflammatory properties of *in vitro* regenerated root tubers will be helpful for the conservation of the plant and in future identification as well as production of possible drug leads through *in vitro* secondary metabolite production.

Table 1
Multiple Shoot induction in Gloriosa superba L.

Hormonal Concentration (*mgl^{-1})					Explant	FSI (%)	MSN	MSL(cm)
NAA	IBA	IAA	KIN	BAP				
-	-	-	0.5	-	N	50±0.01***	7±0.01***	3±0.01**
-	-	-	1.0	-	N	45±0.02***	6±0.021***	2.4±0.33**
-	-	-	1.5	-	N	31±0.22***	5±1.02***	3.2±1.00***
-	-	-	2.0	-	N	24±0.26***	5±0.33***	4.6±1.02***
-	-	-	2.5	-	N	18±0.1***	4±0.11***	7.1±0.06***
-	-	-	-	0.5	L	85±0.38***	5±0.02***	8.3±0.06***
-	-	-	-	1.0	N	77±0.11***	6±0.33***	5±0.33***
-	-	-	-	1.5	L	64±0.04***	2±0.01**	6.1±0.03***
-	-	-	-	2.0	N	53±0.02***	4±0.01***	2.8±1.03**
-	-	-	-	2.5	N	51±0.01***	3±1.01***	5±0.01***
0.5	-	-	-	2.5	N	**98±0.08*** **	**11±0.02*** **	**10.2±0.02*** **
1.0	-	-	-	2.0	N	90±0.01***	9±0.01***	9.3±0.02***
1.5	-	-	-	1.5	N	85±007***	6±0.01***	7.2±0.12***
2.0	-	-	-	1.0	N	81±0.62***	9±0.21***	8.6±0.39***
2.5	-	-	-	0.5	N	75±0.01***	10±0.38***	5.2±0.32***
-	0.5	-	-	2.5	N	50±0.10***	8±0.32***	4.6±0.21***
-	1.0	-	-	2.0	L	47±0.02***	7±0.21***	5.3±0.16***
-	1.5	-	-	1.5	N	49±0.03***	7±0.22***	4.7±0.01***
-	2.0	-	-	1.0	N	42±0.02***	5±0.01***	8.3±0.02***
-	2.5	-	-	0.5	N	38±0.02***	5±0.01***	8.9±0.03***
-	-	0.5	-	2.5	N	72±0.04***	6±0.04***	8.1±0.01***
-	-	1.0	-	2.0	L	41±0.10***	9±0.02***	3.7±0.01***
-	-	1.5	-	1.5	L	29±1.02***	8±0.42***	3.9±0.01***
-	-	2.0	-	1.0	N	17±0.41**	5±1.00***	7.5±1.05***
-	-	2.5	-	0.5	N	11±1.00 ns	3±0.28***	3.5±0.02***

*mgl^{-1}– Milligram/litre, FSI– Frequency of Shoot Induction, MSN- Mean Shoot Number, MSL- Mean Shoot Length, L – Leaf, N – Node, Each value represents the ± SD of triplicate measurements and superscript represent level of significance comparing to the control, *significant at $p<0.05$, **significant at $p<0.01$, ***significant at $P< 0.001$, ns - not significant at $P>0.05$ (according to Tukey-Kramer Multiple Comparisons Test)

Table 2
***In vitro* Root induction in *Gloriosa superba* L.**

Hormonal Concentration *(mgl^{-1})					Explant	FRI (%)	MRN	MRL (cm)	After subculture
NAA	IBA	IAA	2,4-D	BAP					
0.5	-	-	-	-	N	90±1.01***	21±0.01***	0.9±0.01***	1.1±0.01
1.0	-	-	-	-	N	86±0.01***	14±0.02***	1.2±0.02***	1.6±0.02
1.5	-	-	-	-	N	83±0.32***	16±0.03***	1.0±1.01***	1.9±0.11
2.0	-	-	-	-	N	79±0.11***	8±0.02***	0.5±1.00***	**2.5±0.01**
2.5	-	-	-	-	L	75±0.21***	6±0.31***	0.4±0.04***	1.7±1.10
-	0.5	-	-	-	N	69±0.02***	7±0.26***	0.7±0.01***	0.9±0.21
-	1.0	-	-	-	L	62±0.07***	4±0.18**	0.8±0.04***	1.4±0.33
-	1.5	-	-	-	N	47±1.01***	8±0.22***	0.6±0.01***	1.0±0.03
-	2.0	-	-	-	N	31±0.03***	9±0.33***	0.5±0.31***	1.1±0.23
-	2.5	-	-	-	N	22±0.01***	6±0.11***	0.7±0.21***	1.0±0.01
-	-	0.5	-	-	N	5±0.02 ^{ns}	4±0.01**	0.6±0.33***	0.8±0.02
-	-	1.0	-	-	L	71±0.10***	12±0.01***	0.6±0.01***	0.9±0.04
-	-	1.5	-	-	L	42±0.17***	3±0.02**	0.5±0.03***	1.2±0.26
-	-	2.0	-	-	N	37±0.08***	6±1.00***	0.8±0.11***	1.4±0.01
-	-	2.5	-	-	N	21±0.16***	5±0.13**	0.8±0.04***	1.5±0.21
-	-	-	0.5	-	N	17±0.01**	4±0.36**	0.6±0.01***	0.6±0.03
-	-	-	1.0	-	N	64±1.02***	10±0.28***	0.6±0.11***	0.8±0.01
-	-	-	1.5	-	L	62±0.09***	9±0.44***	0.5±0.03***	1.1±0.11
-	-	-	2.0	-	L	68±0.01***	9±0.22***	0.7±0.02***	1.0±0.33
-	-	-	2.5	-	L	27±1.03***	6±0.11***	0.6±0.01***	0.9±0.01
0.5	-	-	-	2.5	N	68±0.01***	13±0.33***	0.4±0.44***	0.7±0.43
1.0	-	-	-	2.0	L	75±1.02***	17±0.11***	0.7±0.22***	1.5±0.01
1.5	-	-	-	1.5	N	72±1.01***	15±0.22***	0.6±0.11***	1.3±0.04
2.0	-	-	-	1.0	N	84±0.02***	19±0.01***	0.5±0.31***	0.9±0.33
2.5				0.5	N	**98±0.06*****	26±0.33***	1.3±0.11***	1.9±0.01

*mgl^{-1} – milligram/litre, FRI– Frequency of Root Induction, MRN- Mean Root Number, MRL- Mean Root Length, L – Leaf, N – Node, Each value represents the ± SD of triplicate measurements and superscript represent level of significance comparing to the control, *significant at $p<0.05$, **significant at $p<0.01$, ***significant at $P< 0.001$, ns - not significant at $P>0.05$ (according to Tukey-Kramer Multiple Comparisons Test)

Table 3
Comparison between Anti Inflammatory Activity of *in vitro* and in vivo Root Tubers of *Gloriosa superba* L.

Sample Concentration *(μgml^{-1})	Percentage of Inhibition			
Standard drug (100μgml^{-1})	COX		5-LOX	
	95.34±0.21***		96.38±0.02***	
Root extracts	*In vitro* root tuber		*In vivo* root tuber	
	COX	5-LOX	COX	5-LOX
100μgml^{-1}	1.97±0.22^{ns}	40.09±0.11***	3.38±0.10^{ns}	49.23±0.01***
500μgml^{-1}	23.42±0.01**	64.66±1.01***	26.27±0.12***	76.92±0.14***
1000μgml^{-1}	37.47±0.02***	71.21±0.02***	43.22±0.01***	84.61±0.02***

*(μgml^{-1}) – Microgram / milliliter. Each value represents the ± SD of triplicate measurements and superscript represent level of significance comparing to the control, * significant at p<0.05, ** significant at p<0.01, ***significant at P< 0.001, ns - not significant at P>0.05 (according to Tukey-Kramer Multiple Comparisons Test)

Figure 1: Multiple Shoot Induction in *Gloriosa superba* L.

Figure 2: *In vitro* Tuberous Root induction in *Gloriosa superba* L.

Figure 3: The elongated *in vitro* tubers of *Gloriosa superba* L.

Figure 4: Acclimatization of *Gloriosa superba* Plantlets

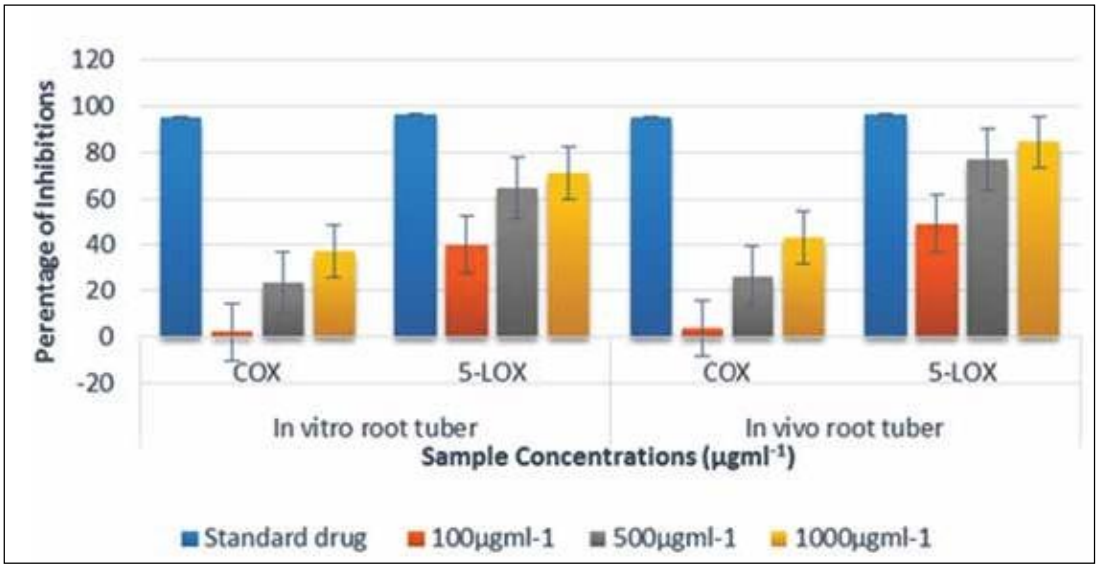

Figure 5: Anti Inflammatory Activity of *in vitro* and *in vivo* Root Tubers of *Gloriosa superba* L.

Legends

Table 1: Multiple Shoot induction in *Gloriosa superba* L.

Table 2: *In vitro* Root induction in *Gloriosa superba* L.

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