RESULTS AND DISCUSSION

Glory lily (G. superba L.) is a very important medicinal plant; it is tuberous and herbaceous climber in nature. Due to the presence of commercially important alkaloids colchicine, it is continuously harvested from the forests thus now declared threatened, like critically vulnerable (Ministry of Environment, Govt. of India) and endangered (Chhattisgarh Medicinal Plant Board, Raipur).

The present experimentation is basic objective for the optimization of efficient protocol for in vitro micropropagation via microtuberization technique. Three different explants-apical shoot tips, nodal segments and meristematic zone of underground tubers were used to initiate culture on MS medium which containing with different concentration of PGRs. For microtuberization were standardized on different sucrose levels, PGR and other adjuvant etc. Results were analysed by ANOVA using recorded data during entire studies, i.e. percent of bud break, number and length of shoots per explants, also numbers and lengths of microtubers. Colchicine quantification was done for in vitro developed microtuber and wild tubers by HPLC.

In vitro propagation of G. superba L.

Surface disinfestations of aerial shoot explants: apical shoot tips and nodal segments:

Both the aerial shoot explants apical shoot tips and nodal segments were thoroughly washed with running tap water and treated with Tween-80, 0.1% for 10-15 min., consecutively treated with fungicides and antibiotic solutions: 0.1% Bavistin (as a broad spectrum systemic fungicide), 2.5% Dithane M-45 (contact fungicide) and
0.1% Amoxyll (antibiotic) for 15-20 min respectively. These surface disinfestations of explants is a played a major role for successful culture establishment. After this fungicide and antibiotic treatment, both the explants types were subjected to quick dip in 70% ethanol, and then dipped in 0.1% aqueous HgCl₂ for different durations (2, 5, 10 and 15 minutes), in LAF. Now surface disinfested explants were rinsed with sterilized distil water for 3 to 4 times to remove HgCl₂ and then cut to final size approx 1 cm long, then inoculated onto explant establishment medium. Explants treated with 0.1% HgCl₂ for 2 min had 70% contamination; which was reduced with the increase in the treatment duration. Twenty percent and 5% explants were contaminated with the treatment of 0.1% HgCl₂ for 5 and 10 minutes, respectively. And the best zero contamination was recorded with the 15 min duration of 0.1% HgCl₂ treatment (Table1, Fig. 1).

Sivakumar and Krishnamurthy (2000) used 0.1% of mercuric chloride for 2-3 minutes for surface sterilization of apical shoot tip explants of G. superba L. and produced contamination free cultures. Hassan and Roy (2005) treated with 0.1% HgCl₂ for 5 min. of terminal shoot tips and stem nodes explant of G. superba L. They successfully established culture on initiation medium. Madhavan and Joseph (2010) subjected to 0.1 % HgCl₂ for 10 minute to surface sterilization of nodal and leaf explants of G. superba L. and contamination free culture established. Ade and Rai (2011) carried out surface sterilized of delicate explant (leaf base and axillary buds) of G. superba L. by using 0.2 % HgCl₂ for 3 minutes and reduced the contamination.

**Surface disinfestations of underground tuber explant:**

The third explant type, meristematic region of underground tubers were washed thoroughly in running tap water to remove soil particles. These explants were
also received the similar treatment of Tween 80, fungicide and antibiotic. In LAF, these explants were dipped in 70% ethanol and then treated with 0.1% and 0.2% HgCl$_2$ solution for different durations; 0.1% concentration with the maximum duration i.e., for 15 min was able to reduced contamination at 45% only, for these underground tuber explants (Table 2, Figs. 2-3).

It was also reported by Selvarasu and Kandhasamy (2012) that the herbaceous parts of \textit{G. superba} L. were treated with minor concentration of sterilants with lesser period’s exposure, the soft tissues were not affected. They only overcome the contamination rate (8.00%) when treated with 0.1% mercuric chloride for 60 second of tuber explants. Similarly, Sivakumar and Krishnamurthy (2000) treated with 0.1% of mercuric chloride for 2-3 minutes for surface sterilization of non dormant corm explants of \textit{G. superba} L. Yadav \textit{et al.} (2012) used 0.1 \% HgCl$_2$ for surface sterilization of underground tuber part of \textit{G. superba} L. They obtained maximum survival percent (90\%) and also overcome to contamination rate (10\%). Therefore 0.2\% HgCl$_2$ solution was tested; its 5 and 10 minutes durations were reduced contamination 20\% and 4\%, respectively; and zero contamination and complete survival was observed with 0.2\% HgCl$_2$ treatment for 15 min. duration. Ade and Rai (2011) reported that the 0.2 \% of HgCl$_2$ for 3 minutes effective for of tuber explants of \textit{G. superba} L. and overcome the contamination rate.

\textbf{Culture Initiation}

In the present study, there were 3 types of explants: apical shoot tips, nodal segments and meristematic regions of underground tubers were inoculated on MS medium which was supplemented with different concentration of plant growth regulator.
**Apical shoot tip explants:**

Shoot tip explants exhibited 70% bud break response on PGR free basal MS medium and also induced single shoot per culture with 3.7 cm ± 0.3 length and 1.9 ± 0.2 nodes per explant were recorded. The explants in medium containing 0.5 mgL⁻¹ BA, exhibited 90% bud break induced single shoot 6.3 cm ± 0.4 long with 3.3 ± 0.2 nodes per explant. Level at 2.0 mgL⁻¹ BA also induced 90% bud break with single shoot only from each explants culture with reduced shoot length 3.9 cm ± 0.2 and node number 2.2 ± 0.2. While MS supplemented with 4.0 mgL⁻¹ BA, induced slightly reduced bud break (80%) with single shoot per explant and significantly shorter shoot 2.9 cm ± 0.2 only. Further reduced only 60% bud break and shorter shoot 3.0 cm ± 0.1 long were observed on MS medium containing 6.0 mgL⁻¹ BA. Thus the maximum bud break 90%, significantly longest shoot with highest nodes per explant was obtained at 0.5 mgL⁻¹ BA, in MS medium (Table 3, Figs. 4-6 and Plate1).

Also noticed that although the apical shoot tips showed bud break response but each explant always produced only a single shoot and moreover which were never branched. Hassan and Roy (2005) did not found shoot induction in MS basal medium even after four weeks culture of *G. superba* L. But they found two or three shoots of *G. superba* L. within three-four weeks after inoculation in MS medium with BA alone, but the number of shoots increased gradually, when cultured on MS medium with 1.5 mgL⁻¹ BAP with 0.2 mgL⁻¹ NAA with 15 % coconut water and 2 gL⁻¹ activated charcoal. Sivakumar & Krishnamurthy (2000) achieved the maximum number of shoot from apical shoot tip explants of *G. superba* L. presence of MS medium containing with 9.84 µM 2ip and 2.32 Kin µM. Sivakumar & Krishnamurthy (2004b) reported organogenesis of this species. Ghosh *et al.* (2007) reported
micropropagation of *G. superba* L. In the present studies also indicate that the percentage of bud breaks and shoot length declined with increase in concentration of BA beyond the best combination of MS medium (0.5 mgL\(^{-1}\)) BA. Similar reported by Venkatachalam *et al.* (2012) the reduction number of shoot length of *G. superba* L. on higher than the (2 mgL\(^{-1}\) BA) of tuber explant.

**Nodal explants:**

The nodal explants were also inoculated on MS media supplemented with various concentration of BA (0.0, 0.5, 2.0, 4.0 or 6.0 mgL\(^{-1}\)). Nodal explants failed to produce shoots *in vitro*, in any case. Thus, the nodal segments (axillary meristems) were not able to produce shoots *in vitro*, even when the apical meristem was absent and exogenous cytokinin was present. So, few reports are available for micropropagation techniques of nodal segment as explant of *G. superba* L. (Table 4, and Plate 2).

Hassan and Roy (2005) reported the stem nodes along with single apical bud explants of *G. superba* L. inoculated on zero PGR MS medium, didn’t give response for the bud break or shoot formation. But presence of MS medium containing with 1.5 mgL\(^{-1}\) BA and 0.2 mgL\(^{-1}\) NAA, recorded the 93% of shoots forming.

**Underground Tuber explants:**

The third explant of *G. superba* L. is meristematic zone of underground tuber, inoculated on MS medium with different levels of PGR (0.0, 2.0 and 4.0 mgL\(^{-1}\) BA). This explant produced shoot as well as microtubers also. The underground tuber explant exhibited best shoot production (70%) in the presence of 2 mgL\(^{-1}\) BA in MS medium and furthermore induced single longest shoot 3 cm ± 0.3 and number of
node 2.4 ± 0.2 were recorded. In addition, significantly maximum number of microtuber 6.1 ± 0.3 with significantly longest 1.6 cm ± 0.07 in size. Explants on PGR free MS basal medium also showed 70% bud break response, but the shoot length, microtuber number and length were significantly lower than the response recorded with 2 mgL⁻¹ BA. When BA level was enhanced up to 4 mgL⁻¹, reduction in bud break 50% was observed. Similarly microtuber number 4.0 ± 0.3 and size 0.9 cm ± 0.05 were also reduced significantly (Table 5, Figs. 7-11, and Plate 3).

Venkatachalam et al. (2012) obtained the maximum shoot regeneration (60 ± 0.57) and highest number (3.4 ± 0.45) of shoots from per culture in the presence of MS medium which containing with 2 mgL⁻¹ BA from tuber explants of G. superba L. Finnie and Van Staden (1989) reported that a concentration ratio of 10:1 (cytokinin: auxin) both with BA and Kinetin, tuber explants produced only multiple shoot. Similarly, Sivakumar and Krishnamurthy (2004b) obtained the greatest number of shoots was recorded on MS medium supplemented with 4 mgL⁻¹ BA and 0.5 mgL⁻¹ Kin. Madhavan and Joseph (2011) obtained shoot initiation and multiplication optimum in MS medium which containing with 2 mgL⁻¹ Kin and 1 mgL⁻¹ NAA from tuber explants of G. superba L.

**In vitro response of the 3 explants types:**

Thus all the three explants responded differently in vitro. The nodal explants were not able to produce shoot at any case. Both shoot tip and underground tuber explants were able to produce shoots. Shoot tip explants showed better bud break response (90%) than tuber explants (70%). However in both the cases only a single as well as unbranched shoot elongated from apical meristems. Again the length of the shoot form shoot tip explant was bigger (6.3 cm), > double than tuber explants’ shoot
(3 cm). But the major difference was the formation of microtubers also in case of the underground tuber explants apart from the production of shoot. These in vitro microtubers were not produced in case of aerial shoot tip and nodal explants (Table 6).

**Shoot proliferation in subculture:**

After the growth of 4 weeks, shoots those elongated from all the three explants were sub cultured on fresh medium for further shoot production. These shoots were divided into nodes and apical tips and then transferred to fresh medium for further shoot production. All the shoot tips were produced shoot and all the in vitro regenerated nodes were again failed to produced shoot in any concentration of BA (0, 0.5, 2, 4 or 6 mgL$^{-1}$). Although the shoot tips of in vitro grown shoots were able to produce shoot during subculture. But always only a single shoot regenerated again, during the subculture stage also; as recorded in the explant establishment experiments (Table 7-8).

**Rooting:**

*In vitro* regenerated shoots from apical meristems were subjected rooting using auxin treatment in $\frac{1}{2}$ strength MS medium, in order to produce complete plant. For rooting, auxins IBA, NAA and IAA in different concentrations (0.0, 0.5, 1.0, 2.0 or 4.0 mgL$^{-1}$) were used; but fail to induce roots in shoots (Table 9). Although rooting in regenerated shoots was not achieved in the present studied. But reports are there on *in vitro* rooting of this species. Probably in those reports, first the microtuber was formed and then the root induced from that microtuber. As observed in the microtuber
experiment in this present study. Thus there was no root induction directly from shoots. Roots produced always from microtubers only.

Gopinath and Arumugam (2012) found maximum numbers of (96 ± 1.22) root initiation of *G. superba* L. from rhizome callus on MS medium with BA (8.0 mgL⁻¹); (1.0 mgL⁻¹ GA₃ and 1.0 mgL⁻¹ NAA along with 2 gL⁻¹ of activated charcoal. Selvarasu and Kandhasamy (2012) reported *G. superba* L. tuber node explants to produce maximum number of root in the MS medium supplemented with 1.0 mgL⁻¹ IAA and 0.5 mgL⁻¹ IBA. Madhavan and Joshep (2010) achieved 80% of root induction in regenerated shootlets of *G. superba* L. on half strength of MS medium containing with 3 mgL⁻¹ NAA. Yadav *et al.* (2012) induced the root from underground tubers explant in half MS with 1.0 mgL⁻¹ IBA and 0.5 mgL⁻¹ NAA. Ade and Rai (2011) obtained rooted tuber induction of *G. superba* L. from nodal segment on MS medium supplemented with 3 mgL⁻¹ NAA and 1 mgL⁻¹ TDZ (1, 2, 3-thidiazol-5-yl-urea).

**Callus induction:**

For callus induction different explants were taken- leaves, internodes, petiole, etc. These explants were surface disinfested similarly as aerial shoot explants. Further inoculated on MS medium supplemented with different concentrations of 2, 4-D (0, 0.5, 1 or 2 mgL⁻¹) and NAA (0, 0.5, 1 or 2 mgL⁻¹) and combination of Kin, 2, 4-D and NAA in various concentration. However, in the present studied, callus was not induced either by any single treatment or in combination from any explant (Table 10). Few researchers are reported callus induction from dormant and nondormant corm buds of *G. superba* L. Sivakumar and Krishnamurthy (2004b) achieved the 98% of callus induction from non dormant corms of *G. superba* L., presence on MS medium with combination of 2,4-D and Kin. in the range of 0.452-4.65 µM.
Microtuberization:

*G. superba* L. is a having naturally programmed behaviour of this plant that the absence of shoot proliferation capacity via nodal meristems; in bud culture method always a single shoot was regenerated during *in vitro* propagation. A very strong apical dominance is present in *G. superba* L. like other tropical climbers, and any damage to apical shoot tip resulted in the death of complete plant because this plant is not able to produce branches before flowering. For herbivorous tolerance, activation of dormant axillary buds after the apical shoot damage is an important adaption. Huhta *et al.* (2000) observed that *Gentianella campestris* was able to recover from damage even if the damage was high up to 75%.

This strong apical dominance in *G. superba* L. was also recorded during its *in vitro* culture when only the apical bud was produced shoot; the nodal meristems were unable to produced shoots even when the apical shoot tip was absent and exogenous cytokinin was present in the medium. While, the *in vitro* release of apical dominance and axillary buds growth using cytokinin in the culture medium, was reported in *Alstroemeria* (Pumisutapon *et al.*, 2011) and in *Rosa hybrid* (Kapchina-Toteva *et al.* 2000). But in *G. superba* L. apical dominance was not released *in vitro*; hence the microtuber production approach was adopted for its *in vitro* culture using the underground tuber explants.

Therefore, different strategy was designed for the *in vitro* regeneration of this medicinal plant. The established cultures from underground tuber meristem explants were subjected to microtuberization on higher concentrations of sugar and with manipulation of PGR. Rest of the 2 explants (shoot tips and nodes) were failed to induce microtubers.
Effect of Sucrose level:

These underground tuber meristems have tendency to produce microtubers, as mentioned above, these explants produced microtubers in the culture establishment medium. These microtubers were again sub cultured to optimize the *in vitro* tuber production. In the present study MS basal medium with various concentration of sucrose 60, 90 or 120 g/L (Standard is 30 g/L sucrose in MS) were used. MS medium without any PGR but sucrose at 60 g/L level induced maximum number of microtubers 2.4 ± 0.3 per culture. While MS medium supplemented with 90 g/L sucrose, 1.9 ± 0.3 microtubers per culture were produced. MS medium containing with maximum 120 g/L sucrose, only 1.2 ± 0.2 microtubers per culture were induced with reduced size 0.9 cm ± 0.03 (Table 11, Figs. 12-13 and Plate 4).

Previously, Jha *et al.* (2005) reported the MS basal medium was the most effective in inducing for *in vitro* tubers (six tubers in 24 week) from tuber explants as compared to medium supplemented with growth regulators. Yadav *et al.* (2012) reported that the sucrose seems to be the most essential stimulus for *in vitro* microtuberization of *G. superba* L. They used lower concentration of sucrose (2%, 3% and 4%) for *in vitro* tuberization. Sivakumar *et al.* (2003b) were used MS medium with B₅ vitamins, 6% sucrose supplemented with cytokinin and Ancymidol; reported multiple shoot formation and a single corm formation, *in vitro* at the base of each shoot of *G. superba* L. Similarly, Ghosh *et al.* (2007) were used MS medium with normal sucrose level (3%) without any PGR; and observed three *in vitro* tubers per culture in 12 weeks, of this plant. Present report showed faster production of microtubers.
Effect of BA:

To increase the number of microtubers per culture effect of BA at different concentrations were tested on MS medium containing standardized 60 g/L sucrose, in the previous experiment. Microtuber production was increased with the increase level of BA in the medium. MS basal medium induced 2.4 ± 0.3 microtubers per culture. Lowest BA level, 0.5 mgL⁻¹, slightly enhanced the microtuber production 3 ± 0.2 per culture. Significant increase in microtuber production was observed 4.5 ± 0.3, 4.8 ± 0.4 and 5.3 ± 0.4 per culture, in presence of 1, 2 and 4 mgL⁻¹ BA, respectively.

Therefore, the best cytokinin concentration was optimized in MS medium containing 60 g/L sucrose. Highest numbers of microtubers, 9.8 ± 0.8 per culture were obtained in presence of 8 mgL⁻¹ BA and containing 60 g/L sucrose in MS medium (Table 12, Figs. 14-15 and Plates 5-6). This studied supported by Yadav et al. (2012), found the large number of microtuber from tuber explants of *G. superba* L. on MS medium containing with 2.0 mgL⁻¹ BA. Also reported that the microtuber of *G. superba* L. were initially light green in colour but later turned to yellowish brown colour. The microtuber look like a bulb structures, it’s further elongated to form very small V-shaped structures, after 4 weeks in culture. Finnie and Van Staden (1989) reported that mini tubers were similar to normal tuber of *G. superba* L.

Anirudha (2011) used the B₅ medium with various concentrations of BA and NAA for multiple shoot production from microtuber explant of *G. superba* L. Ghosh *et al.* (2007) used different medium i.e. MS, B₅ and SH for *in vitro* tuberization of *G. superba* L. They found that the 8.8 cm on B₅; 9.1 cm on SH; and 11.0 cm long shoot in MS medium from tuber explant, after six weeks of culture. Similar results were reported by Selvarasu and Kandhasamy (2012) that they found the MS medium
supplement with 5 mgL\(^{-1}\) BA and 1.0 mgL\(^{-1}\) NAA recorded the highest response for primary tuber (100%) and secondary tuber formation (100%). Yadav et al. (2012) observed the various factor of microtuber formation such as cytokinin is the most valuable phytohormes; it’s generally involved in the regulation organ formation by promoting cell division. Consequently, BA has been one of the most prominent successfully applied cytokinin for \textit{in vitro} tuberization. A positive effect on size and weight of microtubers formation such as sucrose and photoperiod has been observed.

Previously, Sivakumar et al. (2003b) were used MS medium with B\(_5\) vitamins, 6% sucrose supplemented with cytokinin and Ancymidol; reported shoot formation and a single corm formation, \textit{in vitro} at the base of each shoot of \textit{G. superba} L. Similarly, Ghosh et al. (2007) were used MS medium with normal sucrose level (3%) without any PGR; and observed three \textit{in vitro} tubers per culture in 12 weeks, of this plant. Present report showed faster production of microtuber (9.8 ± 0.8 per culture).

**Effect of citric acid and PVP:**

After the maximum induction of microtubers in 8 mgL\(^{-1}\) BA; cultures were transferred to lower level of BA 2 mgL\(^{-1}\) in MS with 60 g/L sucrose, for further growth of microtubers and shoot production. However, during the subculture for microtuber production blackening of tubers was observed and cultures died subsequently. To overcome this problem, mixture of antioxidant and absorbent was also added in the medium, citric acid 100 mgL\(^{-1}\) and PVP 1 gL\(^{-1}\), respectively (Table 13, Fig. 16 and Plate 7). This strategy was also adopted for \textit{Cleistanthus collinus} cultures by Quraishi and Mishra (1998), to solve the similar problem of blackening and phenolic leaching.
After the 8 weeks of culture on this modified medium- MS with 60 g/L sucrose, 2 mgL\(^{-1}\) BA, 100 mgL\(^{-1}\) citric acid and 1 gL\(^{-1}\) PVP, 70% bigger microtubers produced shoots; they were separated and subjected to in vitro acclimatization. Through microtuberization techniques, produced the large number of microtubers, within a short period time. Ghosh et al. (2007) successfully achieved 128 in vitro tubers per culture per years whereas each tuber produces 2 tubers in vivo.

Sivakumar et al. (2003a) reported that the rate of microtuber formation can be increased, were added in various PGR in liquid MS medium. Arumugam and Gopinath (2012) were carried out histological studies by using confocal microscope to study the developmental stages of corm bud of *G. superba* L. and the initiation of microrhizome. They described that the morphological anatomical structure of in vitro raised tuber resembled on in vivo plants. Shoot developed from bigger size microtuber further subjected to in vitro acclimatization. Rooting as well as acclimatization of *G. superba* L. microtubers was carried out in vitro at single step combined with the good survival rate.

**In vitro acclimatization:**

At these stage microtubers produced roots also without any auxin and the shoot became healthier. The production of plantlet with regenerated rooting under in vitro approaches is a valuable step for successful establishment of plant on coco pit as well as soil. Therefore, in vitro regenerated microtubers with shoots were hardened in vitro successfully, for 8 weeks and transferred aseptically in to vessels filled which were filled with carrier coco-pit and irrigated with inorganic MS salts only (Table 14, Plate 8).
Finally, microtuber produced roots without any auxin and the shoot appeared healthy. In present study, the *in vitro* acclimatized plantlet with functional roots and photo synthetically active leaves were successfully transferred to green house with 95% survival rate after 4 weeks.

Similar report has been given by Jha *et al.* (2005) that the *in vitro* regenerated tubers were developed into healthy green shoots within 6 days of initiation and rooted in basal medium with variation of size; that depends on the strength of inorganic component used in the medium. Gopinath and Arumugam (2012) reported that rooted shoots were transferred into small plastic tray, which contain vermi compost, sand and red soil in the ratio of 1:2:2 and kept in the mist house, later regenerated plantlets were hardened in green house.

*Ex vitro transfer under shade net:*

Because the last *in vitro* experiments were carried out on coco-pit as carrier containing MS inorganic salts only. Therefore, the *in vitro* regenerated microtubers were already subjected to *in vitro* hardening; as the medium was lacking of organic supplements therefore the shoots were photo-synthetically active, also the roots were functional due to the development of root hairs because they were grown in coco-pit. Moreover, in the absence of organic supplements there was no contamination at all (Table 14, Fig. 17 and Plates 9-10). Hence, the *in vitro* acclimatized plantlets with functional roots and photo-synthetically active leaves were successfully transferred to plastic pots under green shade net with 90% survival and further transfer to field. Gopinath and Arumugam (2012) successfully hardened to regenerated plant of *G. superba* L. and acclimatized 90% of plantlet was survival under natural condition after transplantation.
Colchicine content in tubers and microtubers of *G. superba* L.:  

In the present studies, HPLC analysis carried out of methanolic extracts samples (tubers directly from field area, *in vitro* developed microtubers; subcultures microtubers and *in vitro* developed plantlet later hardening in fields’ tubers) showed the presence of colchicine. The concentration of colchicine in sample against to standards colchicine was calculated by formula. These showed varied concentration of colchicine in difference samples. Also, sharp symmetrical peaks were recorded for individual samples. The colchicine concentration were recorded of wild tubers sample: 0.36%, *in vitro* developed 1 month old microtubers: 0.08%; *in vitro* developed 3 month old microtubers: 0.11% and tuber from acclimatized tissue culture raised plants (6 month old): 0.22%. In the present experiment no elicitor was used *in vitro* (Table 15, Figs. 18-22 and Plate 11).  

Also *in vitro* microtubers were small and few months old. Similarly acclimatized plantlet tuber was only 6 months old; which may be the possible region of lesser colchicine content. But as visible that the colchicine content is increasing with the growing size and age. It’s a first time reported for *G. superba* L. for during to various developmental stages and performed the quantitative estimation of colchicine.  

Previous reports on microtuber production of this plant by Ghosh *et al.* (2007) and Sivakumar *et al.* (2003b), the colchicine content present in the regenerated microtubers were not examined. In the present study HPLC analysis was done, the regenerated microtubers were contained lower percentage of colchicine than the field plant tubers but they were much smaller and younger than the natural one. Also the
in vitro raised tubers were showing increasing pattern for colchicine content, as they were became bigger and older.

Nautiyal (2011) obtained 0.35% yield with 97% of chromatographic purity of colchicine sample. Gupta et al. (2005) found colchicine content 0.37% in tuber and 0.75% in seeds of G. superba L., which were growing in mid hill zone of the Himachal Pradesh. Similar reported by Jha et al. (2005) the in vitro generated tubers were found to accumulated 0.03% colchicine, when analyzed after 6 months in culture. Thus the tissue culture raised plants have lesser content of colchicine. However, field grown tubers were bigger in size 10-15 cm long and more than 2 years old. Moreover the colchicine content was recorded increasing with the growing size and age. This is supported by Jha et al. (2005) according to them the amount of colchicine recorded in the tissue culture of Colchicum and Gloriosa were 10-25 times lower than those found in plants grown in in vivo approaches. The quantification of colchicine is influenced by geographical region; some elicitor may be biotic or abiotic which are directly regulating the synthesis pathway.

Field study of G. superba L. in natural habitat:

Field work was carried out at forest near Rajnandgaon district of Chhattisgarh state; to study the life cycle and threats facing by the plant at its habitat. Monsoon reaches in the second half of the June month; the tubers of G. superba L. sprout to form aerial climbing shoots. Tubers are ‘V’ or ‘L’ shaped (Plate 12) and each tuber sprouted up to 2 or 3 shoots only from different meristematic zones present on it. Each shoot attained approx 4 - 5 meters in length and not branched until flower buds appeared.
The shoots have strong apical dominance, only 3 or 4 branches emerged that too after the appearance of flower buds. Solitary flower buds appeared from leaf axis; looked pale green in colour. Flowers strikingly changed their colour as they matured; from pale to dark red within 20 days (Plates 13-14). We were observed different pollinators of the plant, from butterfly to humming bird (Plate 15). Seeds dispersal taken placed as capsules busted in three parts (Plate 12). At the end of November as temperature drops, the climber tended to dried up; tubers as well as seeds remained dormant up to next rainy season.

Throughout the observation period (2009-2012), we have recorded to total 430 plants (Table 16). G. superba L. is susceptible for disease and pests. Pests attacked recorded on 20% plants especially at juvenile stage at study site. Lily moths (Polytela gloriosae) were observed as major pest of the plant G. superba L. its caterpillars eaten all leaves of infected plants (Plates16-17); some plants were also found infected by leaf blight disease (Plate18) at study site.

Maiti et al. (2007) reported that fungus Alternaria alternata caused leaf blight disease in G. superba L. Insect attack was fatal for all the infected juvenile plants, once its apical bud was damaged; the shoots were not able to grow further and subsequently died because axillary meristems were not able to produce new shoots. Similarly 35% plants were severely damaged by cattle grazing and if there apical part was damaged there was no further growth of the plant; as it happened in case of the pest attack (Plate18). In this manner species is vulnerable during its vegetative growth. Since G. superba L. is a valuable medicinal plant; reports are also there that the tubers have been collected from forest. Also its beautiful flowers have been collected during the tribal festivals.
Similar to other tropical climbers, *G. superba* L. is also highly sensitive to damage due to that it possesses strong apical dominance, and there is no branching from axillary buds until flowering. Therefore, the damage to the apical portion of the shoot was fatal for it. Soft juvenile shoots were susceptible to insect pests. Cattle browsing were recorded highly injurious and caused mass destruction, particularly because of the plant exists in the boundaries of the forests where the sunlight is sufficient; but which is also the cattle browsing area (Plate18). No recovery of the damaged population in both the cases; due to its inability to sprout from axillary meristems.

Ramawat & Goyal (2008) reported that in developing countries, besides tribal, who collect forest products for their livelihood, traders collect plant products illegally, so *G. superba* L. plant pushed to threaten category. Although cultivation of *G. superba* L. has been started in South India, to provide material for commercial uses; but the production is not sufficient (Sivakumar et al., 2003b). Further addition to the problem that the remaining seeds exhibited poor germination; resulted in low regeneration of plant in next monsoon season in the study site. Poor seed germination in *G. superba* L. is also mentioned by Sivakumar et al. (2003a) and Sivakumar & Krishnamurthy (2002). Thus, besides the poor seed germination and susceptibility to pests and diseases; cattle browsing and tuber collection are the major threats faced by the species at its habitat.

**Bio-control of Pest Polytela gloriosae:**

**Effect of Ocimum sanctum:**

Attack of pest *Polytela gloriosae* is fatal for the plant. Early instars of *P. gloriosae* feeds on leaves and later instars feed voraciously leaving only the hard
stem of the plant and complete devastation; and damaged apical shoot tip never recovered and subsequently died (Plate 18).

The *Ocimum sanctum* herb is used antibacterial, antifungal, antimicrobial and insecticidal activities. Aqueous and ethanol extraction of *O. sanctum* herb sprayed weekly on *G. superba* L. plants, were protected from insect attack. Prabhu *et al.* (2009) tested essential oils of *Ocimum gratissimum* protected 74% of the test-material against the maize weevil population after 4 days. A direct application of the *O. gratissimum* on the test insects was found to be 85.7% by knock down effect. Similarly, there was no pest attack on those *G. superba* L. plants which were grown with the *O. sanctum* plants (Table 17-18). Ogunnika (2007) also reported insect protection by inter cropping and mix cropping of main crop *Mahhot esculenta* with pesticidal plant *O. gratissimum*.

**Effect of different plant leaves extracts:**

Eggs of *P. gloriosae* were collected from lower surface of infected leaves and also allowed to rearing of instars for further bioassay in plastic beaker covered with porous polyethylene sheet. Various concentrations (12.5%, 25%, 50%, 66.7%, 75% and 100%) of plant leaves extract (*Pongamia pinnata*, *Azadirachta indica*, *Calotropis gigantea*, *Thevetia nerifolia*, *Cassia tora* and *Eucalyptus lanceolatus* etc.), were used to control insect attack. The highest mortality of larvae was recorded by extract (100%) of *A. indica* within shortest time span: 1 minute. Lowest biopesticidal efficiency recorded for *C. gigantea*, for 100% concentration exhibited 17.66% mortality rate (Table 19, Fig. 23-29 and Plate 19). Alam *et al.* (2009) seen the insecticidal activity of root bark of *C. gigantea* against to *Tribolium castaneum* insect. Kumar *et al.* (2012) observed the larvicidal, repellent and ovicidal properties of
aqueous leaves extract of *C. gigantea* against *Culex gelidues* and *Culex tritaeniorhynchus*. Amerasan *et al.* (2012) mentioned the adulticidal and repellent activities of leaves of *C. tora* against *Culex quinquefasciatus*. Subbalakshmi *et al.* (2012) used *A. indica* to control different pest such as for brown plant hopper, black bug, ear head bud and bacterial leaf blight. Sharma *et al.* (2012) used seed cake of *P. pinnata* to control termites.

**Experiments on induction of nodal meristems of *G. superba* L.:**

In the departmental nursery, shoots tips of *G. superba* L. were removed to observe the revival from the damage; and none of the 100 decapitated shoots were recovered including those which were treated with cytokinin (BA); and subsequently all the decapitated plants were died. Thus inability to recover via axillary buds from damage due to cattle browsing and pests along with the tuber collection are the major threats facing by *G. superba* L. at its natural habitat (Table 20 and Plate 18).

Activation of dormant lateral buds after the apical damage is important adaptation for herbivorous tolerance. Huhta *et al.* (2000) observed that herb *Gentianella campestris* was able to recover from damage by grazing, even if the damage was high up to 75%. Turnbull *et al.* (1997) reported that either shoot decapitation or application of cytokinin stimulated rapid bud growth in chickpea. But in case of *G. superba*, there was no activation of lateral buds when apical shoot tip was removed moreover the exogenous cytokinin was also present. Thus, present studied reveals that developed of efficient (9.8 ± 0.8 microtubers per culture) micropropagation technique for plantlets production. Field studied reveals the major plant damaging factors in natural habitat. The poor seed germination, susceptibility to pest; cattle browsing and tuber collection are the major threats.