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RESEARCH ARTICLE

Agrobacterium rhizogenes mediated hairy root induction for increased Colchicine content in *Gloriosa superba* L.

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Abstract

Gloriosa superba L. a medicinally important monocot, member of the family Liliaceae, was investigated for callusing, direct regeneration and induction of hairy root using soil bacterium *Agrobacterium rhizogenes*. Friable callus was initiated from corms on basal MS medium amended with 2,4-D (1 mg L^{-1}) and kinetin (1 mg L^{-1}). Callus generated from such effective combinations were co-cultivated with the bacterium *in vitro*. Infection was facilitated by acetosyringone (AS) (20 mg L^{-1}) supplemented during co-cultivation. Extensive hairy roots were induced from the callus within 15-18 d. These roots were then established on MS basal broth medium. Alkaloids like colchicine and colchicoside were extracted from hairy roots and compared with authentic standard colchicines in HPLC. Statistical media optimization was employed to select effective media composition. The findings indicated that nitrogen, inoculum and ferric ions have the highest f value that influenced colchicine production (22.7 mM) and hairy root growth.

Keywords: *Gloriosa superba*, *Agrobacterium rhizogenes*, acetosyringone, alkaloids, colchicine**.**

Introduction

Gloriosa superba L. a liliaceous monocot plant is valued for colchicine and other tropolone alkaloids (Gupta, 1982). It is used as cure for a number of diseases (Finnie and Staden, 1991). Its mode of multiplication is by corm division and the rate is very low. Corms are thermogenic, abortifacient, alexteric, antipyretic and contain two important alkaloids namely colchicine ($C_{22}H_{25}O_6N$) and colchicoside ($C_{27}H_{33}O_{11}N$) (Evans *et al.,* 1981). Colchicine and related compounds generally exert antimitotic properties, which interfere with microtubule dependent cell function and irreversibly bind to tubulin because; colchicine itself is too toxic for human use as an antitumor drug. Demecolcine, trimethylcolchicine acid methyl ester, 2-demethyl and 3-demethylthiocolchicine has been evaluated as anti-leukaemia agents. Findings on 3-demethylthiocolchicine showed to be a broad spectrum antitumor agent of some promise (Wagner *et al.,* 1988). Numerous studies have focused on the production of commercially important secondary metabolites from *in vitro* cultures, especially callus and cell suspension cultures of various plants (Banerjee *et al.,* 1993; Drewes and Van Staden, 1995). Infection of plants with a soil borne bacterium *Agrobacterium rhizogenes*, induces the formation of proliferative and multi-branched adventitious roots at the site of infection (Chilton *et al.,* 1982), called “hairy roots”. For a long period of time, however, since monocotyledonous plants such as Liliaceous ornamentals had not been included in the host range of *Agrobacterium*, their genetic transformation had been carried out exclusively via direct gene transfer. However, transformed monocotyledonous plants can be produced via *Agrobacterium*-mediated gene transfer (Delbreil *et al*., 1993; Eady *et al*., 2000). Though, a lot of work has been done on induction of transformed hairy roots from various plants of different genera, there are no reports on induction and establishment of hairy roots in *Gloriosa superba* L. In the present study, we are reporting a successful induction and establishment of hairy roots in *Gloriosa superba* L. and its colchicine content.

Materials and methods

Induction of callus: Corms of *G. superba* L. were collected from the Botanical Garden of Loyola College, Chennai. Fresh sprouts were excised from these corms and washed thoroughly under running tap water for 15 min and were surface sterilized by soaking in 0.2% mercuric chloride for 5 min. The sprouts were then washed twice with sterile distilled water and dissected under aseptic conditions and inoculated on basal medium consisting of MS (Murashige and Skoog, 1962) salts, vitamins, 2.5% sucrose and 0.8% agar. Basal medium was supplemented with 2,4-D (1 mg L^{-1}) and kinetin (1 mg L^{-1}) induced the friable organogenic callus (Sivakumar and Krishnamurthy, 2000). Multiple tubers were also induced in MS medium supplemented with (BAP and NAA): combinations. Both callus and multiple tubers were used as explants for transformation using *Agrobacterium rhizogenes*.

Bacterial strain: Wild type strain of *A. rhizogenes* (MTCC 2364) obtained from microbial type culture collection (MTCC), Chandigarh, India was used for infecting the plant material. Yeast Extract Peptone (YEP) medium comprising sucrose 5.0, peptone 5.0, yeast extract 5.0 and $NaCl_2$ g L^{-1} was used for growth and maintenance of the bacterial strain.

Induction and establishment of hairy root culture: For transformation studies, 48 h old culture of *A. rhizogenes* was first centrifuged at 3000 rpm for 5 min and the resultant cell suspension was resuspended in 5 mL of sterile MS medium. This suspension was used for infecting the plant tissues.

Co-cultivation: Agrobacterium cells were resuspended into MS based liquid inoculation media with or without 10, 20, 50, 100 mg L^{-1} of acetosyringone (AS). The friable organogenic calli of *G. superba* were immersed into the inoculation medium containing bacterial cells for 1, 5, 30 or 60 min and then blotted dry on sterile filter paper. The calli were then transferred onto MS-based co-cultivation medium containing 20 mg L^{-1} as devoid of phytohormones. Then, these cultures were incubated in the dark for two days at 25±1°C.

Proliferation: After two days of co-cultivation of callus and bacterial cells, the explants were removed and washed thoroughly with sterile MS medium containing 1% mannitol and 250 mg L^{-1} cefotaxime. Washed explants were blot dried with sterile filter paper and placed on proliferation medium MS amended with 250 mg L^{-1} cefotaxime and then incubated in the dark for 15-21 d at 25±1°C. After initiation of hairy roots, the fast growing root tips were sub-cultured and maintained on solid MS medium with 250 mg L^{-1} cefotaxime. Various growth characteristics of established root clones were observed for two months.

Alkaloid extraction and HPLC analysis: The technique for colchicine extraction from hairy roots was followed as described by Hamerslag (1950) and Walaszek *et al.* (1952). Twenty grams of hairy roots were washed thoroughly with sterile distilled water, blotted dry followed by freeze drying and then extracted using 200 mL methanol under low temperature (10°C) overnight. The homogenate was centrifuged at 4000 rpm for 5 min. The methanolic extract was evaporated to dryness and the residue was re-dissolved in 50 mL distilled water. The aqueous extract was then centrifuged at 10,000 rpm for 5 min. The supernatant was partitioned twice against petroleum ether, once in diethyl ether and then washed five times with equal volumes of chloroform which was retained and evaporated to dryness. The residue was re-dissolved in methanol and filtered through a 0.45 µm millipore filter. Quantitative determination of the alkaloids was carried out by reverse phase-HPLC using a Luna C_{18} column (125 x 4.6 mm, 5 µm particle size). The mobile phase was gradient of water and acetonitrile. The flow rate was maintained at 1 mL/min, with UV detection at 350 nm (Poutaraud and Girardin, 2002).

Statistical design for optimization of colchicines: Based on the preliminary experiments, parameters such as carbohydrates, nitrates, inoculum, tyrosine, phenylalanine, ferric sulphate, calcium chloride, potassium hydrogen phosphate, benzyl amino purine, inositol and pH conditions were selected for Plackett Burmann design (Plackett and Burmann, 1946). High (+) and low (-) concentrations for the different nutrients were selected on the basis of experimental results (Table 1). Eleven experiments were formulated with 11 parameters and response made in terms of dry hairy root tissue weight and colchicine content. The design was developed using Design Expert software version 5.0.9 (State Ease Corporation, USA). The hairy root culture in the above formulated medium was incubated at dark with 25±°C. After 25 d of inoculation, the hairy roots were collected, shade dried and weighed for biomass for each treatment. The dried tissue were powdered and used for extraction and quantification of colchicine using HPLC.

Results and discussion

In MS medium supplemented with 2,4-D and kinetin each 1 mg/L responded prolific callus production using corms as explants. This is in accordance with the findings of Sivakumar and Krishnamurthy (2000). In order to induce regeneration from the 2,4-D derived callus, MS medium with BAP and NAA combination concentrations were attempted. The callus doubled in size about every 12 d, provided it was routinely sub-cultured according to Finnie and Staden (1989) and Pierick (1976). The calli derived from the cormlets were sub-cultured to the MS medium supplemented with series of BAP and NAA combination at low concentrations (1 mg/L each). Multiple tuber formation was observed at MS with BAP (4.5 µM) and NAA (10.7 µM). Direct regeneration of shoot was observed at kinetin (4.6 µM) and IBA (4.9 µM) (Table 1).

Hussey (1982) stated that in the families of Liliaceae, Iridaceae and Amaryllidaceae, the induction of direct adventitious shoots from explants *in vitro* is the principle method of propagation. Similarly, Sivakumar and Krishnamoorthy (2000) reported that there is no direct shoot organogenesis in *Gloriosa*. In our study, explants cultured in medium supplemented with kinetin and IBA did not result in callus formation, but direct shoot induction was achieved with kinetin (4.6 µM) and IBA (4.9 µM). This mode of regeneration is generally as genetically stable as axillary meristem propagation. During co-cultivation for hairy root production, a thin film of bacteria on explant surface and a thick growth along the callus was observed within 24 h. Explant calli were then transferred to MS-based solid medium amended with AS 20 mg L^{-1} and 250 mg L^{-1} of cefotaxime (Fig. 1).

Table 1. Effect of different concentrations of plant growth regulators with MS medium on the development of callus, multiple tuber formation and direct shoot induction.

Plant hormones and its concentrations in µM	Number of multiple tubers formed	Number of direct shoot induced
BAP (4.5) + NAA (5.3)	-	-
BAP (6.7) + NAA (5.3)	-	-
BAP (9) + NAA (5.3)	-	-
BAP (4.5) + NAA (7.9)	3	-
BAP (6.7) + NAA (7.9)	4	-
BAP (9) + NAA (7.9)	4	-
BAP (4.5) + NAA (10.7)	**18**	-
BAP (6.7) + NAA (10.7)	15	-
BAP (9) + NAA (10.7)	12	-
Kn (2.3) + IBA (4.9)	-	-
Kn (3.4) + IBA (4.9)	-	1
Kn (4.6) + IBA (4.9)	-	2
Kn (2.3) + IBA (7.3)	-	1
Kn (3.4) + IBA (7.3)	-	-
Kn (4.6) + IBA (7.3)	-	-
Kn (2.3) + IBA (9.4)	-	-
Kn (3.4) + IBA (9.4)	-	-
Kn (4.6) + IBA (9.4)	-	-

Fig. 1A. *In vitro* callus formation; B. Multiple tubers; C. Shoot induction; D. Plantlet (*Gloriosa superba*).

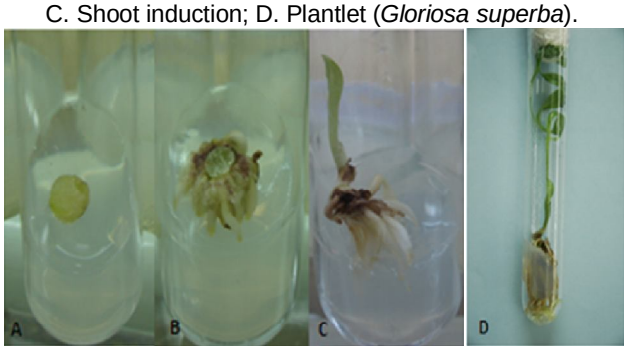
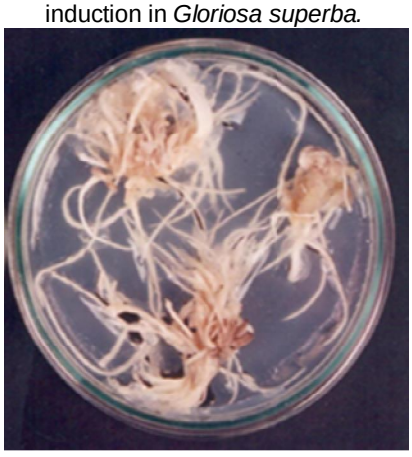


Fig. 2. *Agrobacterium rhizogenes* mediated hairy root induction in *Gloriosa superba.*



There were no signs of necrosis; rather they remained healthy and swelled to certain extent. After 15 d on proliferation medium, emergence of very small hairy roots was observed on the callus which continued to grow well (Fig. 2).

The frequency of emergence of hairy roots was found to be 30%. During early stages of the root growth, the increase in number of hairy root branches was almost logarithmic. The extensive branching was due to the presence of many meristems, which accounted for high growth rates of hairy roots in culture. This particular characteristic was observed to be the most common for many plants (Tepfer, 1990; Gelvin, 1990). Several reports are available on the production of secondary metabolites through hairy root cultures from the medicinal plants viz. *Saussurea medusa* for jaceosidin (Dexin *et al.,* 2004), *Coleus forskohlii* for rosmarinic acid (Wei *et al.,* 2005), *Rauvolfia micrantha* for ajmalicine and ajmaline (Sudha *et al*., 2003). These hairy root clones when cut and maintained on proliferation medium showed some typical features of hairy root syndrome i.e. plagiotropic growth, hormone independence and extensive lateral branching in agreement with a previous report on *Solanum nigrum* (Subroto *et al*., 2001).

Plagiotropism of hairy roots is a phenomenon common with hairy roots and is infact, reported as one of the readily observed characteristic during *A. rhizogenes* mediated transformation (Gelvin, 1990). Organogenic and embryogenic calli have generally been found to have high proliferation and regeneration abilities (Suzuki *et al*., 2001) and have been successfully utilized as a target material for *Agrobacterium*-mediated production of transgenic plants in both monocot (Delbreil *et al*., 1993) and dicotyledonous plant species (Nakano *et al*., 1994). AS treatment drastically increased the transformation efficiency whereas explant calli untreated with AS does not produce hairy roots, but remains friable and latter start producing shoot primordia.

Table 2. Design of the trial experiments by Plackett-Burmann (1946) method for optimization of colchicine production by hairy root cultures in *Gloriosa superba*.

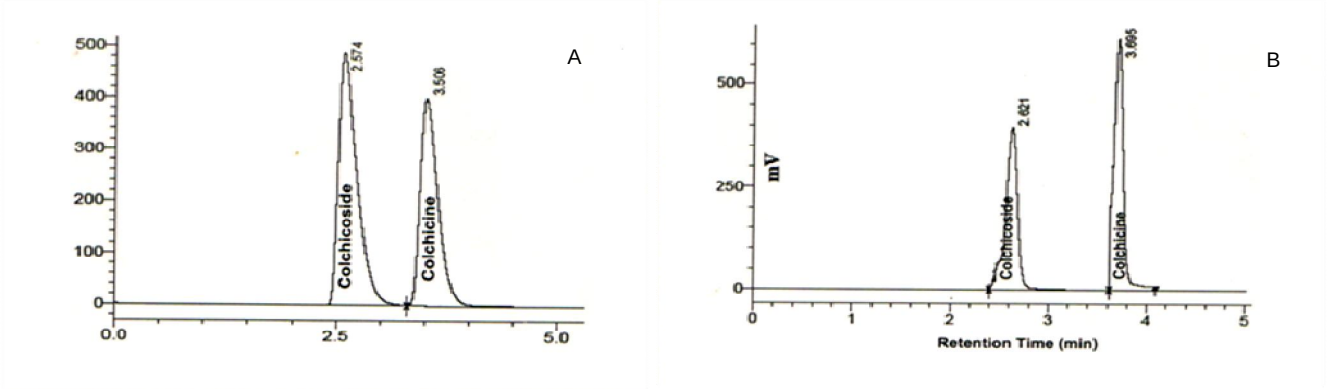
Sucrose (%)	Nitrate (mM)	Inoculum (g/L)	Tyrosine (µM)	Phenyl alanine (µM)	$FeSO_4$ (mM)	$CaCl_2$ (mM)	KH_2PO_4 (mM)	pH	BAP (mg/L)	Inositol (mg/L)	Response	
L:1 H:4	L:20 H:60	L:2 H:8	L:20 H:40	L:20 H:40	L:1 H:4	L:4 H:8	L:5 H:2	L:5.5 H:5.8	L:1 H:2	L:97 H:100	Colchine (mM)	DW (mg)
4	20	8	20	20	0.1	8	2.0	5.8	1	100	17.6	500
4	60	2	40	20	0.1	4	2.0	5.8	2	97	7.23	780
1	60	8	20	40	0.1	4	0.5	5.8	2	100	9.48	360
4	20	8	40	20	0.4	4	0.5	5.5	2	100	11.0	375
4	60	2	40	40	0.1	8	0.5	5.5	1	100	14.8	600
4	60	8	20	40	0.4	4	2.0	5.5	1	97	12.2	320
1	60	8	40	20	0.4	8	0.5	5.8	1	97	10.8	700
1	20	8	40	40	0.1	8	2.0	5.5	2	97	**22.7**	450
1	20	2	40	40	0.4	4	2.0	5.8	1	100	5.73	730
4	20	2	20	40	0.4	8	0.5	5.8	2	97	20.2	**880**
1	60	2	20	20	0.4	8	2.0	5.5	2	100	11.3	200
1	20	2	20	20	0.1	4	0.5	5.5	1	97	9.26	736

L-Low; H-High; DW-Dry weight of hairy root biomass.

AS is a phenolic compound activating *vir* genes of *Agrobacterium* and production of AS has been demonstrated to be low or lacking in monocotyledonous plant tissues (Usami *et al*., 1987). Therefore, AS treatment has so far been found to be effective for *Agrobacterium*-mediated transformation of several monocotyledonous species. The effectiveness of the AS treatment was also confirmed for *G. superba* L. Attempts were made for the establishments of these hairy roots on large scale in shake flasks. For initial 48 h, the clones remain intact in 25 mL medium in 100 mL conical flask and started to grow. Then the clones, were cut into pieces and inoculated in 100 mL of MS liquid medium with 250 mg L^{-1} of cefotaxime. The growth attained maximum within 15 d. Statistical design has long been adopted for the optimization of microbial fermentation process and its application is gaining importance for plant cell culture process recently, particularly in the optimization of growth and product formation (Sung and Hung, 2000) and also in suspension culture process (Das *et al*., 2001). Plankett–Burman design was adopted to select the most significant effectors among carbohydrates, nitrates, inoculum, tyrosine, phenylalanine, ferric sulphate, calcium chloride, potassium hydrogen phosphate, pH, BAP and inositol concentrations (Table 2). From the data analysis and the corresponding statistical f-value for the effect of the above mentioned selected nutrients on response of hairy root biomass (growth) and colchicines content.

It is apparent that nitrogen, inoculums and ferric ions have the highest f-value. Highest f-value for nitrates indicated that it had maximum positive influence on colchicines formation and hairy root biomass. Prakash and Srivastava (2005) reported that glucose, nitrate, calcium chloride and inoculums have positive t-value and representing 99 and 96% validity of the predicted models for growth and azdiractin production respectively in suspension culture of *Azadiracta indica.* Hayashi *et al*. (1988) reported that the levels of colchicines in *Colchicum autumnale* markedly decreased in the medium containing 2,4-D. This is why auxins are commonly added to the medium for callus induction but they are added at very low concentrations or omitted for production of metabolites. Whereas, cytokinins have been reported to stimulate alkaloid synthesis in cultures from different plants (Arvy *et al*., 1994; Hara *et al*., 1994). Sivakumar *et al*. (2005) revealed that various organic nutrients play critical role during *Panax ginseng* hairy root growth, biomass and ginsenoside production in large scale bioreactors. Our results correlated with the above findings that various organic and inorganic nutrients influence the colchicines production in the hairy root cultures of *Gloriosa superba*. Extraction and separation of alkaloids were made from the hairy roots. Extraction of colchicine using petroleum ether and chloroform was equally effective as that of soxhlet extraction which was found to be more reliable and most efficient method for extraction and separation of cochicine.

Fig. 3A. Colchicine and cochicoside standard HPLC chromatogram; B. Colchicine and cochicoside HPLC chromatogram of *Gloriosa superba* hairy root culture.



TLC of the authentic standard (SDFine) and the plant extracts showed that colchicine separated into three major UV detectable spots. The Rf value of authentic colchicine occurred at 0.65, whereas extracts showed 0.59, 0.67 and 0.76. The other zones observed from the separation of the standard colchicine represent the major breakdown products of colchicine. The results of TLC (not shown) observed are in accordance with Finne and Staden (1991). The HPLC profile of the hairy root culture is represented in Fig. 3. The retention time of colchicine contents of the hairy root culture was compared with the standard colchicine and colchicoside (Sigma). In the HPLC profile, the RT value showed similar peak for both standard and the extract of hairy root culture. Further investigations are needed to identify the molecular structure of compounds and large scale production of secondary metabolites in bioreactors from the hairy roots of *G. superba* L.

Conclusion

In this study, we have demonstrated for the first time an efficient *Agrobacterium rhizogenes* mediated transformation of a monocot plant, *Gloriosa superba* L. Using this standardized protocol, hairy roots can be harvested in large scale, in turn large quantities of important alkaloids can be produced in future investigations.

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