Biocatalysis and Agricultural Biotechnology 18 (2019) 101030

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Influence of exogenous polyamines and plant growth regulators on high frequency *in vitro* mass propagation of *Gloriosa superba* L. and its colchicine content

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ARTICLE INFO

Keywords:
Gloriosa superba
Non dormant corm bud
Polyamines
Mass propagation
HPLC

ABSTRACT

An efficient *in vitro* mass propagation technique was successfully developed for *Gloriosa superba* L. using callus induced from non-dormant corm buds. Sulfuric acid treatment was optimized to break seed dormancy which resulted in high frequency germination. Proficient callus induction and proliferation was observed when the explants were grown on modified MS (mMS) medium supplemented with 2,4-D (1.5 mg l^{-1}) and Kin (1.0 mg l^{-1}). The combination of BA (1.5 mg l^{-1}) and NAA (0.6 mg l^{-1}) induced highest multiple shoot formation frequency. When the medium was supplemented with putrescine (15 mg l^{-1}) the explants responded with highest multiplication frequencies of *in vitro* shoots. The regenerated shoots showed superlative response for *in vitro* rooting on mMS medium fortified with IBA (1.0 mg l^{-1}), IAA (0.6 mg l^{-1}) and 15% coconut water. Colchicine content of both field grown and *in vitro* raised plants were compared using HPLC and it was found that leaf samples from field grown plants showed 0.00241 mg/ml colchicine compared to methanolic extract from Spermidine, Spermine and Putrescine treated leaf samples which showed relatively higher concentrations of colchicine (0.00265 mg/ml, 0.00356 mg/ml and 0.00569 mg/ml) respectively. Putrescine treated *in vitro* plants showed only a marginal increase in colchicine content compared to spermine and spermidine treated plants. This established protocol will help in both pharmaceutical and conservation aspects of *G. superba* to meet its commercial demands in the future.

1. Introduction

Gloriosa superba, a semi woody herbaceous climber is native to tropical Africa and is now grown widely in many parts of tropical Asia including India, Burma, Malaysia and Srilanka. *G. superba* is commercially valuable due to its high medicinal properties and its wide use in Siddha, Ayerveda and Unani system of medicines. High value therapeutic compounds from different parts of the plant have been harvested among which seeds and root tubers contain valuable alkaloids. Colchicine and gloriosin are the major constituents of *G. superba*. Generally the amount of colchicines present in the corms is around 0.3%, and varies among different geographical locations and species (Jana and Shekhawat, 2010). The roots and tubers of *G. superba* have been widely used in traditional Indian medicine for the treatment of gout, rheumatic arthritis, snake and insect bites, intermittent fevers, wounds, anti-fertility, gonorrhea, leprosy, piles, debility, dyspepsia, flatulence, hemorrhoids, helminthiasis, inflammations and cancer (Ambasta, 1986; Warrier et al., 1995; Pulliah, 2002; Ade and Rai, 2009). It also helps in inducing labor pain and expulsion of placenta (Evans et al., 1981; Arumugam and Gopinath, 2012). The pharmaceutical demand for *G. Superba* has increased considerably due to its high medicinal value. It is imperative to establish a mass propagation system through *in vitro* culture technology in order to balance the demand and overexploitation of the plant.

In vitro regeneration of *G. superba* is very tedious and time consuming. Several factors influence the *in vitro* regeneration efficiencies of *G. superba* such as genotype, explant, media constituents, growth regulators, gelling agent, light intensity, photoperiod and temperature (Reed, 1999; Ghimire et al., 2010; Vinoth et al., 2011). Due to overexploitation, this medicinally important plant species was included

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https://doi.org/10.1016/j.bcab.2019.101030
Received 9 November 2018; Received in revised form 2 February 2019; Accepted 3 February 2019
Available online 05 February 2019
1878-8181/

under the endangered category in Red Data Book (Sivakumar and Krishnamurthy, 2000; Jana and Shekhawat, 2010) which led to several conservation efforts towards re-establishing this valuable species. Since the plant has a very low seed set ratio (Mamtha et al., 1993), vegetative propagation is the most preferred method of propagation for *G. superba* in horticulture. But the growth of this plant is relatively slow when compared to other propagation methods (Kranse, 1986). The production of corms and tubers is considered a complex developmental process, greatly influenced by genetic, environmental and physiological factors (Ewing and Struk, 1992; Villafranca et al., 1998). *In vitro* propagation is one of the most reliable and efficient technique to mass propogate plant species. Paramasivam and Arumugam (1991) reported that major problems towards cultivation of *G.superba* is the scarcity of planting material and lack of information on seed dormancy breaking techniques. *In vitro* regeneration using various explants of *G. superba* has been reported earlier including shoot cuttings and nodal explants (Somani et al., 1989; Samarajeewa et al., 1993; Custers and Bergervoet, 1994), shoot tips, nodes, internodes, leaves, flowers, pedicels and tubers, (Sivakumar and Krishnamurthy, 2000, 2002 and 2004), corms from non-dormant and dormant buds (Sivakumar et al., 2003), production of embryoids from leaf tissue (Finnie and Van Staden, 1994; Sivakumar et al., 2003; Sivakumar and Krishnamurthy, 2004), micropropagation from young sprout (Hassan and Roy, 2005). Gopinath and Arumugam (2012) developed a protocol for multiple shoot formation with callus derived from rhizome explant.

Recently polyamines viz. spermidine, spermine and putrescine has been recognized and used as PGR's (plant growth regulators) (Liu et al., 2007; Kusano et al., 2008; Farooq et al., 2009; Joshi et al., 2010; Sivanandhan et al., 2011). Polyamines (PAs) play a vital role in several plant growth and development processes including cell division, morphogenesis, flower initiation, pollen tube extension and senescence (Bagni and Tassoni, 2001). Coueé et al. (2004) elucidated the role of PA's in lateral and adventitious root formation. The role of endogenous PA's during initial stages of adventitious rooting (Chiancone et al., 2006), *in vitro* organogenesis, somatic embryogenesis in many plant species have been reported (Mengoli et al., 1989; Faure et al., 1991; Kevers et al., 2002; Rajesh et al., 2003; Bertoldi et al., 2004; Sivanandhan et al., 2011). Jawahar et al. (2018) reported enhanced colchicine accumulation from morphogenic cultures of *G.superba* by feeding different combinations and concentrations of different elicitors and precursor molecules. Similarly, Mahendran et al. (2018) reported that various elicitors like yeast extract, casein hydrolysate and salicylic acid proved to be most effective for stimulating the enhanced production of colchicine in cell suspension cultures of *G. superba.* An efficient and rapid mass propagation protocol is imperative to meet the commercial demand of the pharma industries and to conserve the plant from the extinction. In the present study, we investigated the effects of polyamines (spermidine, spermine and putrescine) and plant growth regulators for efficient *in vitro* mass propagation of *G. superba* using callus derived from corm bud explants. Further, the colchicine content from field grown and *in vitro* plants was analyzed using High Performance Liquid Chromatography (HPLC).

2. Materials and methods

2.1. In vitro and field grown seed germination

Seeds of *G. superba* were collected from experimental garden, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. To break the dormancy, seeds were treated with sulfuric acid (10–100%) for 2 min and rinsed in running tap water overnight (Sivakumar and Krishnamurthy, 2004). The seeds were then washed with 1% Teepol for 10 min and kept in running tap water for 30 min. The seeds were then disinfected with mercuric chloride (0.1%) for 10 min followed by rinsing 5 times with sterile distilled water 5 min each and treated with 1 mg l^{-1} GA_3 (Gibberellic acid) overnight in an Orbitek platform shaker set at 220 RPM (Sivakumar and Krishnamurthy, 2004). After 12 h, a set of seeds were placed in paper cups containing sterile red soil, sand and vermiculite (2:1:1). Another set of seeds were washed thrice with half strength modified MS (mMS) medium consisting of MS salts and B_5 vitamins for 3 min (Murashige and Skoog, 1962; Gamborg et al., 1968) to provide an acclimatized germination environment after the acid scarification and hormonal treatment. The seeds were inoculated onto mMS medium supplemented with 1 mg l^{-1} GA_3, 4 mg l^{-1} BA (Benzyl Adenine), 1% sucrose. pH of the medium was adjusted to 5.8 and solidified with 0.8% agar (Sivakumar and Krishnamurthy, 2004). The Plant growth regulators were added to the medium prior to autoclaving at 121 °C for 15 min.

2.2. Corm germination

The field grown non dormant corms were washed thoroughly with 5% (v/v) Teepol solution and fungicide (0.1% Bavistin) (BASF India Ltd., Mumbai) for 30 min followed by sterilization with mercuric chloride (0.1%) for 30 min (Ghosh et al., 2007), and subsequently rinsed five times with sterile distilled water. The sterilized corm buds were inoculated onto mMS medium containing MS salts, B_5 vitamins and 3% sucrose. pH of the medium was adjusted to 5.8 and solidified with 0.8% (w/v) agar prior to autoclaving at 121 °C for 15 min. All cultures were maintained at 24 ± 2 °C, 16 h photoperiod under light intensity (36 μmol $m^{-2}s^{-1}$) provided by cool white fluorescent lamps. About 5.8 mm of sprouted buds were excised from the germinated non dormant corm and used for callus induction experiments.

2.3. Callus induction and proliferation

The non-dormant corm buds were cultured on slants with mMS medium containing 0.5–2.5 mg l^{-1} 2,4,D (2,4-Dichlorophenoxyacetic acid), 0.5–2.5 mg l^{-1} Kin (Kinetin), 3% sucrose. pH of the medium was adjusted to 5.8 and solidified with 0.8% agar (Sivakumar and Krishnamurthy, 2004). After 4 weeks of culture, the best responding concentration and the fresh weight of the callus were noted. The calli were routinely subcultured at two weeks interval. 20 explants were tested per treatment and each experiment was repeated thrice.

2.4. Influence of plant growth regulators and polyamines on multiple shoot induction

To test the influence of PGR's and polyamines (PAs) on multiple shoot formation and elongation the experiments were divided into two sets. In one set of experiments, green, compact organogenic calli was cultured in MS medium supplemented with diverse concentrations and combinations of PGR's; [0.5–2.5 mg l^{-1} BA (6-Benzyl adenine); 0.2–1.0 mg l^{-1} NAA (Naphthalene acetic acid), 0.2–1.0 mg l^{-1} Kin (Kinetin)]. To increase the efficiency of multiple shoot induction, polyamines (spermidine, spermine, and putrescine) were tested at different concentrations (5–25 mg l^{-1}). The polyamines were tested in combination with best responding concentrations of BA + NAA. Twenty calli were taken for each experiment and repeated thrice for each concentration. The mean number of shoots per callus and mean shoot length was scored after 4 weeks of culture.

2.5. Rooting of elongated shoots

The elongated shoots (about 4 cm) were excised from the shoot clumps and transferred to ½ strength mMS medium supplemented with 0.5–2.5 mg l^{-1} IBA (Indole-3-butyric acid), 0.2–1.0 mg l^{-1} IAA (Indole-3-acetic acid). 5–25% of coconut water (CW) was used along with the best responding concentration of IBA + IAA. 20 shoots were tested for each experiment and the experiment was repeated thrice. The number of roots per shoot and root lengths were determined after 4 weeks of culture.

2.6. Hardening of in vitro raised plantlets

Plantlets with well-developed roots were gently washed in tap water to remove the media adhered and then transferred to paper cups containing sterilized red soil, sand and vermiculated (2:1:1) mixture and soaked with half strength MS salts. Each cup was covered with a polythene bag to maintain humidity and acclimatized in artificial plant growth chamber. The acclimatized plants were then transferred to pots containing 2:1 mixture of soil and vermiculite and grown under greenhouse condition.

2.7. Extraction of colchicine for HPLC analysis

Leaf samples were taken from both field grown and *in vitro* regenerated plants. Samples were shade dried and ground to fine powder. Powdered plant material (1 g) was extracted with 10 ml methanol under sonication. The methanolic extract was evaporated at room temperature for 1 week and then the sample was redissolved in 1 ml methanol. The solution was then centrifuged (Eppendorf, USA) at 7000 RPM for 20 min. The resulting supernatant was filtered through 0.22 μm membrane filter. The filtered samples were analyzed by HPLC (Waters, Australia) on a reverse phase C-18 (5.0 µm particle size) 250 × 4.6 mm column. The mobile phase was methanol/water (50:50 *v/v*) mixture supplied at the rate of 0.8 ml/min, temperature set at 25 °C during the analysis. The absorbance range was set at 210–400 nm.The analytical HPLC was performed using Empower 2 software following isocratic method. Colchicine was detected by monitoring absorbance at 350 nm (Basak et al., 2012) using a Photo Diode Array (PDA) detector. The retention time (RT) of Colchicine in the crude methanolic extract was compared with Colchicine standard (Sigma Aldrich, India).

2.8. Estimation of colchicine

Colchicine content was estimated in leaves and tuberous roots collected from both field grown and *in vitro* raised plants and calculated using the formulae (Siva et al., 2015a, b)

$$\text{Sample concentration} = \frac{\text{Sample area}}{\text{Mean STD Area}} \times \frac{\text{STD Weight}}{\text{STD Dilution}} \times \frac{\text{Sample Dilution}}{\text{Sample Weight}}$$

2.9. Statistical analysis

Data on organogenic callus induction, multiple shoot induction, proliferation and rooting of elongated shoots were recorded after 4 weeks. All data were statistically analyzed using analysis of variance (ANOVA). Data presented as mean ± standard error. The mean separations were carried out using Duncan's multiple range tests and significance was determined at 5% level (SPSS 17).

3. Results

3.1. Effect of sulfuric acid treatment on seed germination

G. superba seeds were treated with different concentrations of sulfuric acid (10–100%). Among the tested concentrations, 70% sulfuric acid treatment was found to be effective in breaking seed dormancy. It was found that above 70%, the sulfuric acid treatment damaged the seeds and resulted in germination failure. After GA_3 treatment for 12 h, the seeds were allowed to germinate both at field and *in vitro* conditions. One set of seeds were placed in plastic pots and another set of seeds were inoculated on to ½ strength mMS medium supplemented with GA_3 (1 mg l^{-1}) and BA (4 mg l^{-1}). Between the two conditions evaluated, maximum percentage of seed germination was observed at field grown condition (85.2%) compared to *in vitro* condition (62.4%) (Table 1). It was also observed that during *in vitro* germination, seeds secreted phenolic compounds in the inoculated medium that reduced the frequency of seed germination.

Table 1
Effect of Sulfuric acid treatment (2 min) on seed germination.

Sulfuric acid (%)	Number of seeds	Germination (%)	
		Field grown	*In vitro*
Control[a]	50	–	–
10	50	11.2 ± 0.22^{i}	4.8 ± 0.15^{i}
20	50	17.2 ± 0.18^{g}	7.4 ± 0.22^{g}
30	50	31.2 ± 0.20^{f}	13.6 ± 0.16^{f}
40	50	43.0 ± 0.18^{d}	24.8 ± 0.18^{d}
50	50	55.4 ± 0.18^{c}	35.4 ± 0.22^{c}
60	50	67.2 ± 0.22^{b}	51.2 ± 0.20^{b}
70	50	85.2 ± 0.20^{a}	62.4 ± 0.18^{a}
80	50	35.4 ± 0.18^{e}	17.6 ± 0.22^{e}
90	50	13.4 ± 0.22^{h}	5.2 ± 0.15^{h}
100	50	5.2 ± 0.20^{j}	2.4 ± 0.18^{j}

Data was collected after 15 days. Values represent the mean ± S.E of three replications. The data was analyzed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).
[a] Control – Seeds without Sulfuric acid treatments.

3.2. Callus induction from corm bud explant

In vitro and field grown non dormant corm buds about 5.8 mm were inoculated onto the medium containing different concentrations and combination of auxins and cytokinins. The medium fortified with 1.5 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} Kin induced greenish yellow compact callus after 15 days of culture. The highest percentage of callus induction (85%) was obtained on mMS medium supplemented with 2,4-D (1.5 mg l^{-1}) and Kin (1.0 mg l^{-1}) (Table 2). Induced calli were

Table 2
Effect of 2,4-D and kinetin on callus induction from non dormant corm bud explants.

Plant growth regulators (mgl^{-1})		Callus induction (%)
2,4-D	Kin	
Control[a]		
–	0.5	20.4 ± 0.16^{e}
–	1.0	34.7 ± 0.15^{c}
–	1.5	54.8 ± 0.18^{a}
–	2.0	41.5 ± 0.12^{b}
–	2.5	31.6 ± 0.16^{d}
0.5	–	29.6 ± 0.16^{e}
1.0	–	40.5 ± 0.20^{c}
1.5	–	64.6 ± 0.16^{a}
2.0	–	50.6 ± 0.22^{b}
2.5	–	39.6 ± 0.16^{d}
0.5	1.0	39.9 ± 0.18^{e}
1.0	1.0	64.2 ± 0.22^{c}
1.5	1.0	84.9 ± 0.20^{a}
2.0	1.0	69.8 ± 0.16^{b}
2.5	1.0	53.8 ± 0.20^{d}

The medium was supplemented with various concentrations of 2,4-D and Kin. Data was collected after 4 weeks of culture. Values represent the mean ± S.E of three replications. The data was analyzed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).
[a] Control – MS modified medium without Plant Growth Regulators.

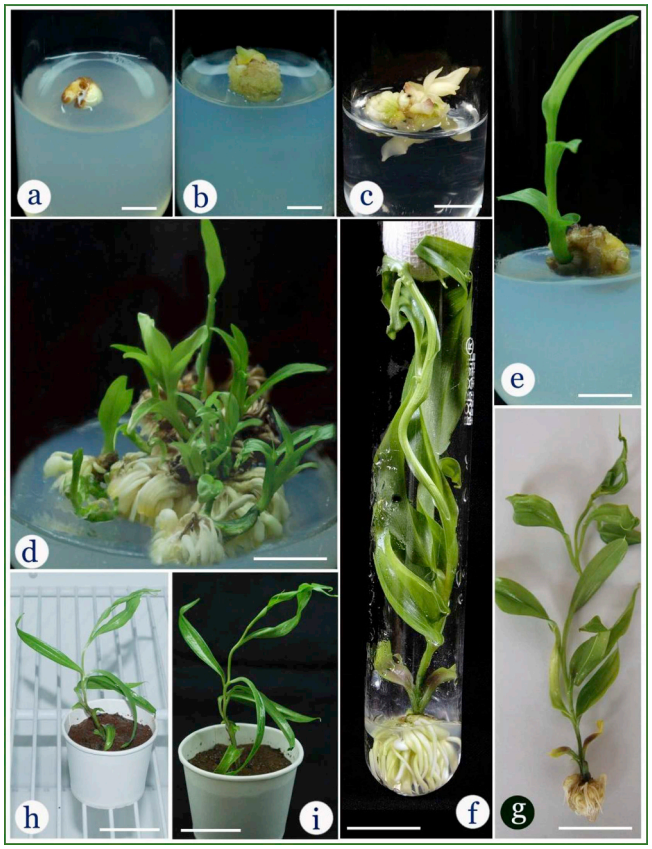

Fig. 1. Indirect organogenesis of *Gloriosa superba* L. using non dormant corm bud explant. a) Non dormant corm bud explant, b) Callus proliferation in the mMS medium fortified with 1.5 mg l^{-1} 2,4-D and 0.9 mg l^{-1} kin, c) Multiple shoot initiation in the medium supplemented with 1.5 mg l^{-1} BA and 0.6 mg l^{-1} NAA, d) Multiple shoot proliferation of *Gloriosa superba* in the mMS medium supplemented with 1.5 mg l^{-1} BA, 0.6 mg l^{-1} NAA and 15 mg l^{-1} Putrescine, e) Excised elongated shoot, f) & g) Rooted plantlet in the half strength mMS medium containing 1.5 mg l^{-1} IBA, 0.6 mg l^{-1} IAA and 15% coconut water, h) Hardened plantlet in plant growth chamber, i) Acclimatized plantlet at greenhouse conditions.

proliferated on subculture medium containing the same hormonal combination. Non organogenic calli were excised and removed at every subculture.

3.3. Multiple shoot induction

Proliferating green compact organogenic calli were transferred to shoot induction medium supplemented with different concentrations of 0.5–2.5 mg l^{-1} BA (Fig. 1c). The medium fortified with 1.5 mg l^{-1} BA induced best response with 4.6 shoots/callus and 1.7 cm shoot length. To increase the frequency of multiple shoot induction BA (1.5 mg l^{-1}) was tested in combination with 0.2–1.0 mg l^{-1} Kin and 0.2–1.0 mg l^{-1} NAA. Among the two PGR combination tested, BA (1.5 mg l^{-1}) and NAA (0.6 mg l^{-1}) induced maximum number of shoots (22.6 shoots/callus) with shoot length of 2.2 cm. Comparatively, the medium fortified with BA (1.5 mg l^{-1}) and Kin (0.8 mg l^{-1}) induced 11.8 shoots/callus with 1.8 cm shoot length (Table 3).

3.4. Influence of polyamines on multiple shoot induction

Polyamines (spermidine, spermine and Putrescine) were tested along with BA (1.5 mg l^{-1}) + NAA (0.6 mg l^{-1}) to assert their influence on multiple shoot induction and proliferation. It was observed that secretion of a gum like substance from the inoculated explants in the

Table 3
Effect of plant growth regulators on multiple shoot induction from non-dormant corm bud derived organogenic callus.

Plant growth regulators (mg l^{-1})			Mean number of shoots per responsive callus	Mean shoot length (cm)
BA	Kin	NAA		
Control[a]	-	-	-	-
0.5	-	-	1.2 ± 0.13^{e}	0.7 ± 0.01^{d}
1.0	-	-	3.4 ± 0.13^{b}	1.1 ± 0.01^{c}
1.5	-	-	4.6 ± 0.22^{a}	1.7 ± 0.02^{a}
2.0	-	-	2.6 ± 0.22^{c}	1.2 ± 0.06^{b}
2.5	-	-	1.5 ± 0.16^{d}	0.5 ± 0.02^{e}
1.5	0.2	-	5.5 ± 0.16^{d}	0.8 ± 0.02^{d}
1.5	0.4	-	8.6 ± 0.16^{c}	1.5 ± 0.02^{b}
1.5	0.6	-	9.4 ± 0.21^{b}	1.2 ± 0.02^{c}
1.5	0.8	-	11.8 ± 0.24^{a}	1.8 ± 0.01^{a}
1.5	1.0	-	4.0 ± 0.14^{e}	0.8 ± 0.02^{d}
1.5	-	0.2	7.8 ± 0.20^{e}	0.8 ± 0.02^{e}
1.5	-	0.4	11.8 ± 0.24^{d}	1.2 ± 0.02^{d}
1.5	-	0.6	22.6 ± 0.26^{a}	2.2 ± 0.02^{a}
1.5	-	0.8	17.8 ± 0.20^{b}	2.0 ± 0.02^{b}
1.5	-	1.0	13.6 ± 0.16^{c}	1.6 ± 0.04^{c}

The medium was supplemented with various concentration of BA in combination with Kin and NAA. Data was collected after 4 weeks of culture. Values represent the mean ± S.E of three replications. The data was analyzed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

[a] Control – MS modified medium without Plant Growth Regulators.

medium favored the initiation of shoots and the immediate transfer of the culture in multiple shoot induction medium gave high frequency response. After 2 weeks of culture, the shoots turned white and gradually became greenish on the medium supplemented with polyamines. The white textured shoot bud initiation for each explant derived callus was distinguished prior to the green shoot initiation and number of shoots was thus recorded. Among three polyamines tested, putrescine (15 mg l^{-1}) showed best response with a mean number of 87.5 shoot buds per callus with shoot length of 3.2 cm (Table 4) (Fig. 1d). Spermine (10 mg l^{-1}) produced 67.4 mean number of shoot buds with 1.8 cm shoot length. On the other hand, medium fortified with Spermidine (20 mg l^{-1}) induced 46.5 mean number of shoot buds with 1.3 cm shoot length. These results suggest that polyamines along with PGR's play a positive role in multiple shoot induction.

3.5. Rooting of elongated shoots

The elongated shoots were excised from the multiple shoots and inoculated in the rooting medium containing different auxins and CW (Fig. 1e). Initially, elongated shoots were inoculated into mMS medium supplemented with IBA. Among different concentrations (0–2.5 mg l^{-1}) of IBA tested, 1 mg l^{-1} IBA showed best response with 7.9 mean number of roots/shoot with root length of 1.2 cm. IBA (1 mg l^{-1}) was tested in combination with 0.2–1.0 mg l^{-1} IAA to increase the frequency of root formation. As hardening of *in vitro* derived plantlets with poorly developed roots reduces the survival rate, it is important to achieve a well-developed root system at *in vitro* condition. The medium fortified with 1.0 mg l^{-1} IBA and 0.6 mg l^{-1} IAA induced 15.9 mean numbers of roots/shoot with root lengths of 1.4 cm. The best concentrations of IBA and IAA combinations were tested with 5–25% of CW to increase number of roots in elongated shoots. High frequency root formation was observed on mMS medium fortified with 1.0 mg l^{-1} IBA, 0.6 mg l^{-1} IAA and 15% of CW in which 27.9 mean numbers of roots/shoot were induced with root lengths of 2.3 cm (Table 5), (Fig. 1f).

Table 4
: Influence of polyamines in combination with BA (1.5 mg l^{-1}) + NAA (0.6 mg l^{-1}) on multiple shoot proliferation from non-dormant corm bud derived organogenic callus.

Polyamines (mg l^{-1})	Mean number of shoots per responsive callus	Mean shoot length (cm)
Control[a]	22.8 ± 0.15	0.6 ± 0.10
Spermidine		
5	23.2 ± 0.16^{e}	0.6 ± 0.01^{e}
10	27.9 ± 0.17^{d}	0.8 ± 0.02^{d}
15	32.2 ± 0.20^{c}	1.2 ± 0.02^{b}
20	46.5 ± 0.22^{a}	1.3 ± 0.02^{a}
25	35.0 ± 0.21^{b}	1.0 ± 0.03^{c}
Spermine		
5	58.1 ± 0.17^{b}	1.2 ± 0.02^{b}
10	67.4 ± 0.22^{a}	1.8 ± 0.02^{a}
15	53.0 ± 0.14^{c}	0.9 ± 0.02^{c}
20	45.9 ± 0.17^{d}	0.8 ± 0.02^{d}
25	32.8 ± 0.20^{e}	0.6 ± 0.02^{e}
Putrescine		
5	66.0 ± 0.21^{c}	2.2 ± 0.02^{c}
10	83.0 ± 0.21^{b}	2.8 ± 0.01^{b}
15	87.5 ± 0.22^{a}	3.2 ± 0.01^{a}
20	52.0 ± 0.21^{d}	1.8 ± 0.02^{d}
25	41.9 ± 0.17^{e}	1.2 ± 0.02^{e}

The control medium was supplemented with BA (1.5 mg l^{-1}), NAA (0.6 mg l^{-1}). Different concentration (5–25 mg l^{-1}) of polyamines; Spermidine, Spermine and Putrescine were tested. Data was collected after 4 weeks of culture. Values represent the mean ± S.E of three replications. The data was analyzed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

[a] Control – MS modified medium with Plant Growth Regulators (BA 1.5 mg l^{-1}, NAA 0.6 mg l^{-1}).

Table 5
Effect of auxins and coconut water on root induction from elongated shoots.

Plant growth regulators (mg l^{-1})		CW (%)	Mean number of roots per responsive shoots	Mean root length (cm)
IBA	IAA			
Control[a]	-	-	-	-
0.5	-	-	3.9 ± 0.17^{c}	0.5 ± 0.01^{d}
1.0	-	-	7.9 ± 0.17^{a}	1.2 ± 0.01^{a}
1.5	-	-	5.4 ± 0.22^{b}	1.0 ± 0.01^{b}
2.0	-	-	3.8 ± 0.13^{cd}	0.8 ± 0.01^{c}
2.5	-	-	3.3 ± 0.15^{d}	0.3 ± 0.02^{e}
1.0	0.2	-	7.9 ± 0.17^{d}	0.8 ± 0.01^{c}
1.0	0.4	-	10.4 ± 0.22^{c}	0.7 ± 0.01^{d}
1.0	0.6	-	15.9 ± 0.17^{a}	1.4 ± 0.01^{a}
1.0	0.8	-	12.4 ± 0.16^{b}	1.2 ± 0.01^{b}
1.0	1.0	-	7.7 ± 0.15^{de}	0.4 ± 0.01^{e}
1.0	0.6	5	16.6 ± 0.22^{d}	0.8 ± 0.01^{d}
		10	22.2 ± 0.20^{b}	1.5 ± 0.01^{b}
		15	27.9 ± 0.17^{a}	2.3 ± 0.02^{a}
		20	17.8 ± 0.20^{c}	1.2 ± 0.02^{c}
		25	13.8 ± 0.24^{e}	0.6 ± 0.01^{e}

CW— Coconut water.
The medium was supplemented with various concentrations of IBA and IAA, the best responding concentrations were tested in combination with coconut water (5–25%). Data was collected after 4 weeks of culture. Values represent the mean ± S.E of three replications. The data was analyzed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

[a] Control – MS modified medium without Plant Growth Regulators.

3.6. Plantlet hardening

The *in vitro* raised plantlets were taken out from the culture tubes and the roots were washed in tap water to remove the adhered media (Fig. 1g). The plantlets were hardened in a paper cup containing red soil, sand and vermicomposite (2:1:1) (Fig. 1h). The platelets were covered with polyethylene bags and placed in a growth chamber maintained at 25 ± 2 °C for 10 days. The hardened plantlets showed 90% survival rate. After 10 days, polythene bags were removed and the plantlets were transferred to get acclimatized in the green house condition (Fig. 1i).

3.7. HPLC analysis for colchicine content

Colchicine content was evaluated from both naturally grown plants and polyamines treated *in vitro* regenerated plants of *G. superba*. Methanolic extract of leaf samples collected from spermidine (20 mg l^{-1}), spermine (10 mg l^{-1}) and putrescine (15 mg l^{-1}) treated *in vitro* regenerated plants were taken for the analysis. HPLC chromatogram of colchicine standard showed a single peak at RT 3.424 (Fig. 2A). HPLC analysis of colchicine content in leaf samples derived from naturally grown plants was 0.00241 mg/ml (Fig. 2B). HPLC analysis of methanolic extract of Spermidine treated leaf samples showed a concentration of 0.00265 mg/ml colchicine (Fig. 2C). Leaf samples collected from Spermine treated *in vitro* plant had a concentration of 0.00356 mg/ml (Fig. 2D). For Putrescine treated leaf sample the estimated colchicine concentration was 0.00569 mg/ml. From these results, it can be inferred that Putrescine treated *in vitro* plants showed a minimal increase in colchicine concentration than spermine and spermidine treated plants (Fig. 2E) and supplementation of polyamines influenced the colchicine concentration among the *in vitro* raised plants compared to that of field raised/naturally grown plants. Further, HPLC analysis of colchicine from field grown tuberous root had a colchicine concentration of 0.00266 mg/ml (Fig. 2F). Coconut water treated *in vitro* tuberous root showed colchicine concentration of 0.00282 mg/ml (Fig. 2G). The above results suggested that the colchicine content of field grown and *in vitro* tuberous roots did not vary significantly.

4. Discussion

The propagation of *G. superba* is usually done using corm and seeds, but the plant exhibits poor seed germination and low regeneration frequencies naturally (Ade and Rai, 2009). Various disadvantages in the conventional method of propagation include 50% of the yield has to be set aside for raising the next generation, transmittance of soil-borne diseases between crops, from one location to another and during the storage period (2–3 month) between harvest and the raising of next crop (Mrudul et al., 2001). Developing an efficient system to multiply the plant through *in vitro* mass propagation technique is essential to conserve this medicinally valuable species. In a natural habitat, the seed coats of physically dormant seeds of certain plant species become permeable to water via repeated cycles of heat and cold. During tissue culture studies the hard covering layers of embryo can be leached out using chemical treatments (Bewley and Black, 1994). Kher and Nataraj (2015) suggested that depending on the type of dormancy the treatment methods vary which include gibberllic acid treatment, chilling, hot water treatment, water soaking and acid treatments. Further they suggested that in order to break the mechanical seed dormancy, it is necessary to treat seeds with acid. Naveen and Tarar (1988) reported that seed germination of *G. superba* is poor and vegetative propagation was hindered by low tuber numbers. In the present study, treatment of seeds with 70% sulfuric acid for 2 min was found to be optimal to break seed dormancy resulting in 86% germination. This observation complies with the results obtained by Venudevan et al. (2010) where acid scarification of seeds with concentrated H_2SO_4 for 2 min improved seed germination to 52%. During *in vitro* experiments, GA_3 supplemented

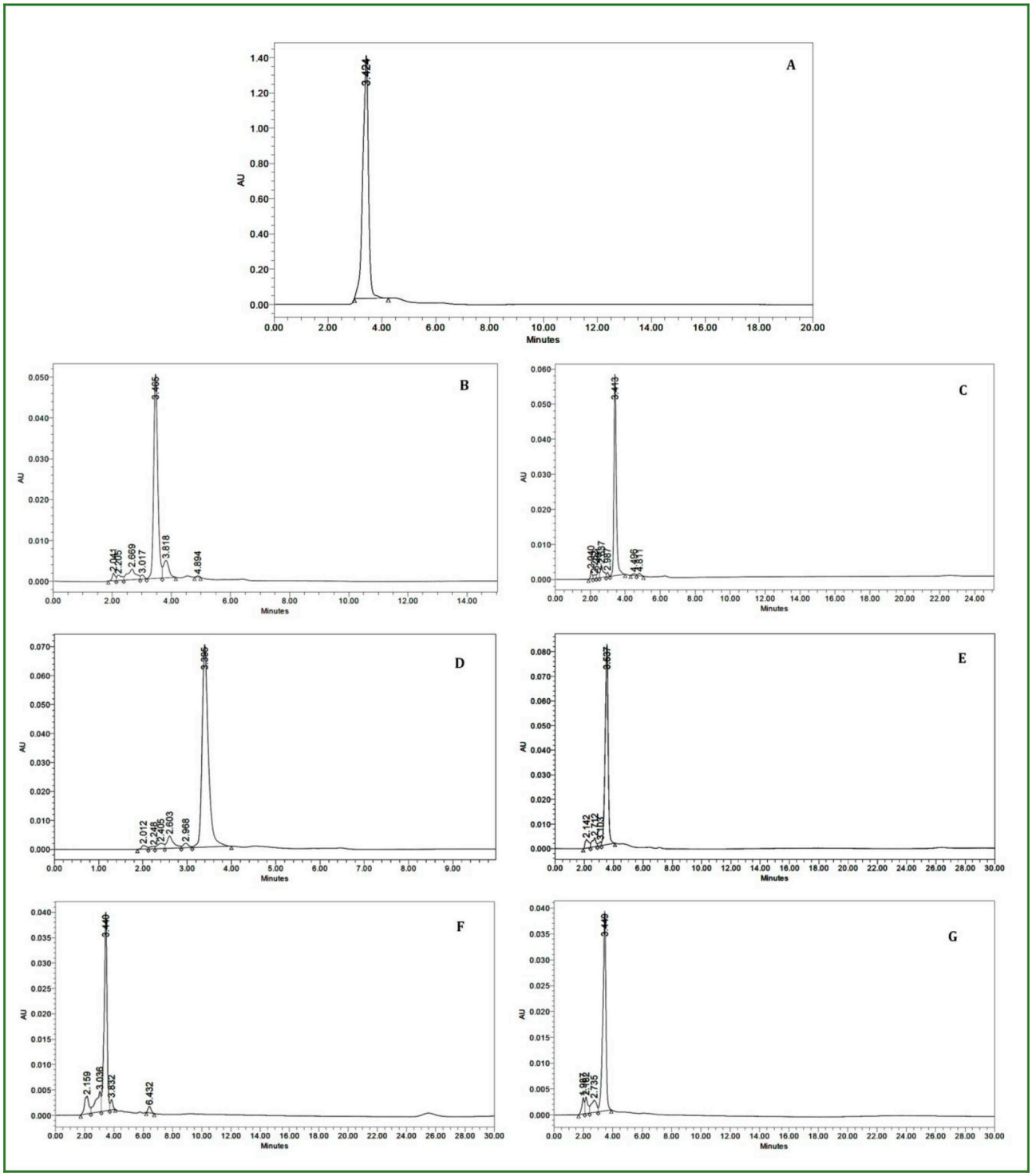

Fig. 2. HPLC analysis of colchicine in methanolic extracts of *in vitro* regenerated plants and naturally grown plants of *G. superba* (L.). A) Colchicine standard, B) Leaf sample from naturally grown plant, C) *In vitro* plant treated with Spermidine, D) *In vitro* plant treated with Spermine, E) *In vitro* plant treated with Putrescine, F) Tuberous root from naturally grown plant, G) Tuberous root from *in vitro* plant treated with 10% CW.

medium accelerated the germination of *G. superba* seeds (Sivakumar and Krishnamurthy, 2004). Contrastingly hot water and various growth hormone treatments have also been tried to break the seed dormancy in *G.superba* (Anandhi, 2017). Concentrated sulfuric acid has been used for softening of hard seed coats thus breaking the seed dormancy and higher seed germination percentages have been reported previously (Purohit et al., 2015). Wochok (1981) reported that seed dormancy is one of the major difficulty encountered towards conservation of threatened and endangered plant species. In our study, field grown seeds treated with H_2SO_4 showed high percentage of germination when compared to seeds inoculated *in vitro*.

Finn and Van Staden (1994) achieved higher rates of organogenic callus in 2,4-D (1.5 mg l^{-1}) and Kin (0.9 mg l^{-1}) and maximum callus proliferation with 2, 4-D (1 mg l^{-1}) and Kin (1 mg l^{-1}). Multiple shoot induction was achieved from non-dormant corm bud explants with BAP (7.77 μM) + ADS (5.44 μM) and from shoot tip explant on 2iP

(8.61 μM) + Kin (2.32 μM) by Sivakumar and Krishnamurthy (2000). Manju et al. (2010) developed organogenic callus from internodal explants with a combination of 2, 4-D (3.0 mg l^{-1}) and BAP (0.5 mg l^{-1}) and observed shoot initiation on MS medium supplemented with Kin (2.0 mg l^{-1}) and 2, 4-D (1.0 mg l^{-1}). Gopinath and Arumugam (2012) obtained yellowish textured callus with 99.40% callus initiation efficiency from rhizome explants on medium containing 2,4-D (1.0 mg l^{-1}) + IAA (0.5 mg l^{-1}) and obtained multiple shoot production with 93.40 mean number of shoots from rooted micro rhizomes on ½ MS medium supplemented with Kin (1.0 mg l^{-1}) + BAP (1.5 mg l^{-1}) + 20% CW. Gopinath et al. (2014) also reported yellowish callus formation from root explants with 2,4-D (2.0 mg l^{-1}) + IAA (1.0 mg l^{-1}) + NAA 0.75 (mg l^{-1}) and production of dark greenish shoots on ½ MS medium containing BAP (3.0 mg l^{-1}), IBA (1.0 mg l^{-1}) and IPA (0.75 mg l^{-1}). In our study, 22.6 shoots per callus was obtained with BA (1.5 mg l^{-1}) and NAA (0.6 mg l^{-1}). These results are in accordance with that of Ade and Rai (2009) who reported higher shoots per callus on MS basal medium fortified with 1.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA. Similar to our results, Custers and Bergervoet (1994) reported that addition BA (1 mg l^{-1}) at minimal concentrations improved plant growth, whereas high levels of BA (10 mg l^{-1}) induced proliferation of multiple shoots.

To accelerate the frequency of multiple shoot induction, PGR's were tested in combination with polyamines. Recently, polyamines viz. spermidine, spermine, and putrescine have been used as plant growth regulators which play a vital role as secondary messengers in signaling pathways (Liu et al., 2007; Kusano et al., 2008; Farooq et al., 2009). PAs have been reported to facilitate the accumulation of several economically important secondary metabolites like alkaloids and phenolic compounds (Bassard et al., 2010; Shoji and Hashimoto, 2015). Polyamines (spermidine, spermine and putrescine) when supplemented to the medium, induced multiple shoots in a positive manner compared to control. Sivakumar and Krishnamurthy (2004) reported that medium fortified with BA along with Adenine Sulfate (ADS) and Sodium Citrate resulted in inducing 13.2 shoots per callus culture. In our study, we found that medium supplemented with BAP (1.5 mg l^{-1}), NAA (0.6 mg l^{-1}) and 15 mg l^{-1} putrescine induced maximum shoot buds (87.5). Our results clearly indicate that polyamines play a vital role in enhancing multiple shoot production in combination with PGR's. There are several reports that confirm the crucial role of polyamines during somatic embryogenesis in species like *Brassica campestris*, *Perkinensis* (Chi et al., 1994), *Oryza sativa* (Rajam, 1997), *Vitis vinifera* (Bertoldi et al., 2004), *Daucus carota* (Takeda et al., 2002) and *Solanum melongena* (Yadav and Rajam, 1998). Exogenous PAs were earlier reported to induce cell division and increase plant regeneration in cell cultures (Steiner et al., 2007) and several organogenetic processes have also been correlated with their endogenous concentration (Francisco et al., 2008; Viu et al., 2009; Joshi et al., 2010). Ravinder Singh et al. (2011) reported that plants regenerated via callus or suspension cell cultures comprises of polyploidy or mixploid cells which are genetically unstable, whereas plant regeneration through direct adventitious shoot organogenesis is genetically stable. When the objective is species conservation, effective tools for the micropropagation of plants involve callus induction and tissue proliferation, especially in case of endemism or seed dormancy (Ayan and Cirak, 2006). In the present study, the number of multiple shoots induced for non-dormant corm bud explants derived calli was comparatively higher than direct or any other regeneration methods reported earlier. The effect of PA's on plant growth and developmental processes is well studied and in addition to that these PA's affect plants' secondary metabolite production. Though there is no common precursor between the colcichine and the exogenous polyamines supplied there was enhanced production of the metabolites, which could mainly due to increased biomass accumulation. Further, Mader and Hanke (1997) perceived that the phenyl propanoid pathway provide conjugation partners for the polyamines and their activity is correlated with that of regulatory machinery of plant growth. The PA's might have a direct impact on the production of metabolites or by indirectly involving in plant growth. For instance, Bais et al. (2000) reported that addition of polyamines putrescine and spermidine enhanced the yield of secondary metabolites betalaine and thiophene in *Alternaria panax* and *Cylindrocarpon destructans* in *Panax quinquefolius* (L.) (Yu et al., 2016). A wide range of plant secondary metabolites can be metabolized from PAs through unelucidated pathways (reviewed by Mustafavi et al., 2018). Orabi et al. (2015) exogenously applied spermine and found enhanced on growth and essential oil accumulation in lemongrass. Rooting of *In vitro* propagated shoots was best achieved with IBA (1.0 mg l^{-1}) and IAA (0.6 mg l^{-1}) along with 15% CW in which 27.9 number of roots per shoot was produced. Addition of CW into the media enhanced shoot growth and development in medicinal plants propagated *in vitro* (Tefera and Wannakrairoj, 2004). *In vitro* experiments have demonstrated that, CW in combination with synthetic auxins such as IBA and NAA can be used for shoot induction and multiplication (Loc et al., 2005). In contrast to the above reports, the present study showed that CW along with auxins can also be used to induce root formation from elongated shoots. The *in vitro* rooting activity of CW is due to the presence of plant growth regulatory substance (IAA) in the extract (Agampodi and Jayawardena, 2009).

HPLC analysis of methanolic extracts from polyamines treated leaves and coconut water treated tuberous roots revealed the presence of colchicine at varying concentrations. Finnie and Staden (1994) reported the presence of colchicine content in leaves (0.05 mg), young leaves (2.36 mg) and adult leaves (0.87 mg) of *G. superba*. Analytical HPLC experiments of polyamines treated leaves revealed that putrescine treated leaves showed high colchicine content (0.00569 mg/ml) compared to other two polyamines (spermidine, spermine) treated *in vitro* plants and naturally grown plants. Our results are in accordance with the earlier study reported in *Withania somnifera* by Sivanandhan et al. (2011) in which quantity of withanolides was comparatively higher in polyamines treated *in vitro* plants than that of field-grown plants.

5. Conclusion

An efficient *in vitro* mass propagation protocol for *G. superba* using corm bud explant was developed in the present study. 70% sulfuric acid treatment was found to be optimum for breaking the seed dormancy and GA_3 treatments resulted in higher germination percentages. Polyamines in combination with plant growth regulators enhanced multiple shoot induction and our results clearly indicated the promoting effect of CW in rooting of elongated shoots. HPLC analysis of leaves and tuberous roots from *in vitro* regenerated plants showed higher concentrations of colchicine than the naturally-grown plants. This variation could be attributed to the culture conditions and composition of growth medium and addition of polyamines. The protocol established here is highly efficient for mass propagation of *G. superba* and provide a valuable strategy towards restoration of this plant species. Further, studies on *in vitro* grown *G. superba* species can be carried out using the protocol developed in this study to understand and analyze the metabolic composition, profiling of the plants with high medicinal value.

Acknowledgments

Prof. N. Jayabalan, UGC-BSR Faculty Fellow is thankful to University Grants Commission (UGC), New Delhi, India. The authors are grateful to Department of Plant Science, Bharathidasan University, Tiruchirappalli for providing HPLC facility. Dr. S. Sivakumar thanks National Post-Doctoral Fellowship (Sanction Order No: PDF/2016/002258), Science and Engineering Research Board, Department of Science and Technology, Government of India. Mr.S. Sathish acknowledges Indian Council for Medical Research (No.3/1/2/102/2018-Nut) for fellowship support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101030.

Author contributions

SS carried out the experiments. SS and SS prepared the manuscript. GS, GP, MV and SV contributed substantially in experimental analysis and discussion. TS, RS and NJ mobilized funds and critically evaluated the manuscript.

Conflicts of interest

All authors read, approved the manuscript and declare that there is no conflict of interest.

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