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Research Article

Regeneration of *Gardenia gummifera* Linn.f by using Cyanobacteria – A novel approach to tissue culture

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Abstract: Propagation of the medicinal plants by usage of different media and PGR's is laborious, cost-effective and is the possibility of genetic variation. In this study, a novel protocol was developed for propagation of medicinal plants with special reference of *Gardenia gummifera* Linn.f by using cyanobacteria instead of plant growth regulators. This protocol is useful in all aspects viz low cost, time and free from genetic variation. This technology is efficient as compared to normal tissue culture technique which is used for conservation from last of two decades.

Key words: Tissue culture, Cyanobacteria, *Gardenia gummifera.*

Introduction

Medicinal plants play the pivotal role in traditional healthcare as well as modern science. People use medicinal plant species for the sustenance of their traditional healthcare system by both logistically as well as economically. Due to inclination towards modern technology and over extraction of secondary metabolites from these plants has resulted in considerable depletion of the population of such species and some have become extinct and *Gardenia gummifera* is one among them.

Gardenia gummifera Linn.f (Dikamali) is an endangered (Firdoous *et al.,* 2010) medicinal plant belongs to family Rubiaceae and is indigenous to China where it is commonly called Zhi Zhi. Its leaves are simple, elliptical, oblong, 4-8 cm long and shinning. The flowers are solitary or in small clusters, white or pale yellow, 4-7 cm length and axillary). Dikamali is a resiniferous plant and it is one of the herbs mentioned in all ancient scriptures in Ayurveda. It has been reported to contain resin, steam volatile oil, a colouring matter, gardenin-A etc. (Mathuram *et al.,* 1998).

Resin of *G.gummifera* Linn.f has great medicinal value and is used for medicinal purposes externally as well as internally. Its exudate is used for neuropathy, anorexia, colic, obesity (Parvati menon, 2001) and is also found effective for wound, indigestion, constipation, abdominal distention, cough, fever (Venugopal, 2002; Jangde *et al.,* 2004). It is marketed in the form of tears or cakes and Unmadnashak Ghrita (UG) (Achliya *et al.,* 2004) which is an ayurvedic formulation. It can be helpful in treating digestive problems including dyspepsia and diarrhea or used as an astringent and expectorant for nervous conditions and spasms (Reddy *et al.,* 1977). The stem bark of *G.gummifera* Linn.f contains triterpenoids 5, 7, 3, 5-Tetrahydroxy-8, 4-demethoxy flavone (Reddy *et al.,* 1977), and has the antifungal activity. Among the flavones identified hypolaetin - 8, 3, 4-tri-O-methyl ether is an extremely rare compound that has been reported only from the resin of *G.gummifera* Linn.f (Krishnamurti *et al.,* 1972) and from the leaf of *G.lucida* (another source of dikamali gum Kumari, 1989).

The application of tissue culture as a biotechnological tool in the conservation of threatened economic plants has gained tremendous impetus in the last two decades. Plant tissue culture is a key technique for plant germplasm conservation and can be a viable alternative to conventional propagation of slow growing species or species that produce recalcitrant or few viable seeds. Apart from conservation, this technique is low cost, rapid and eco-friendly. We are succeeded in developing such type of protocol for *Gardenia gummifera* Linn.f and would be applicable to other medicinal plants.

Materials and Methods

Stock culture

Stock cultures of *Anabaena aequalis* and *Nostoc muscorum* were taken from the CSIR-AMPRI, Bhopal. Isolation, purification and maintenance of axenic culture were carried out. These cultures of equal proportion along 20gm/l sucrose and 0.8gm/l agar were fortified to Chu's 10 medium[3] adjusting the pH between 5.6-5.8 before sterilization and autoclaved at 121^{0}C under 15 lb pressure for 15-20 minutes.

Plant material

Nodal segments containing buds of 5-6-year-old plants were collected from the Botanical Garden of Sagar University (M.P), India. For the surface sterilization, the explants first were washed

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thoroughly in running tap water for 30 minutes. After that they were again washed with liquid detergent (Dettol, Ranbaxy India) for 10 minutes with vigorous shaking. After washing with detergent explants were again washed with running tap water to remove any traces of detergent for 30 minutes. After that explants was shifted to the 1% v/v solution of Savlon (Himedia Labs Limited, India) for 1-2 minutes and inoculated into Chu's 10 medium. Callus of nodal explant was taken as initial material obtained during indirect organogensis. During *in vitro* technique inoculation of explant up to hardening needs five to six times transferring from basal media under sterile conditions, that increases chances of genetic diversity.

Results and Discussion

We develop protocol for *Gardenia gummifera* Linn. f for conservation via direct and indirect organogenesis by alternation of all the possible ways such as alternation in PGR's, macro and micronutrients, addition of additives. In this experiment we have taken callus of nodal explant, axenic culture of *Anabaena aequalis* and *Nostoc muscorum* and Chu's 10 medium. The aim of performing such experiment was only to develop protocol that should be rapid, cost-effective, free from genetic variation's and meet the escalating demand of this plant at commercial level. In one of our study, four types of media were used for developing protocol MS, B_5, LS and Whites and three types of explants were used nodal, leaf and petiole. Out of them MS media and nodal explant proves to be efficient throughout the protocol.

On perusal of data in previous study, when MS medium was supplemented with different concentration of PGR's viz BAP, Kn, IBA and IAA. These growth regulators affect the growth of explants and manipulation of concentration of PGRS leads the contamination and time consuming. But the nodal explant produces good amount of shoots with suitable shoot length. By fortifying MS media with BAP (2mg/l) and IBA (0.5mg/l) produces highest amount of shoots/explant (5.04 ± 0.2) with shoot length of (4.29 ± 0.2) were obtained from nodal explants (Table 1).

In B_5 media nodal explants produces (4.98 ± 0.1) shoots/explant with a shoot length of (4.02 ± 0.1), when media was supplemented with BAP (2mg/l) and IBA (0.5mg/l) (Table 2). Nodal explant are find excellent once for shoot production in white's media with (4.94 ± 0.9) shoots/explant with shoot length (3.89± 0.2) at the hormonal concentration of IBA (0.5mg/l) and BAP (2mg/l) in white's media (Table 3).

Similarly, in LS media nodal explants again shows good result at the same concentration as in above experiments, produces (3.93 ± 0.4) shoots/explant with shoot length of (2.21 ± 0.5) cm (Table 4). Apart from the above experiments, we use the cyanobacterial media for developing the protocol. The following stages are involved to develop the protocol for *in vitro* propagation of *Gardenia gummifera* Linn.f. By keeping the above facts in mind we choose callus obtained from nodal explant for entire study, it leads a better response in all experiments. During this entire study neither any macro or micronutrient nor any PGR's or additive were used.

Three approaches were emphasized for developing protocol.

1. Chu's 10 medium with cyanobacterial spp. *Anabaena aequalis* and *Nostoc muscorum* (1-6ml/l) + Surose (20gm/l) + Agar (0.8gm/l).
2. Chu's 10 medium with cyanobacterial spp. *Anabaena aequalis* and *Nostoc muscorum* (5-10ml/l) + Surose (25gm/l) + Agar (0.8gm/l).
3. Chu's 10 medium with cyanobacterial spp. *Anabaena aequalis* and *Nostoc muscorum* (10-15ml/l) + Surose (30gm/l) + Agar (0.8gm/l).

Approach 1:

In this approach callus was taken as initiation material for shoot proliferation. Chu's media was taken as basal media supplemented with sucrose 20mg/l, cyanobacteria spp. with equal proportion and agar 0.8mg/l. Total of six treatments were given to callus for shoot proliferation. Out of these treatments number 5th (Table 5) produces maximum numbers of shoots 6.37 ± 0.3 with shoot length 4.82 ± 0.2.

Approach 2:

In this approach again the same material was taken for further study, only the alternation in concentration of adulterants was done. Concentration of cyanobacteria spp. was increased (5-10ml/l) and sucrose concentration was hiked up to 25mg/l. interestingly, the number of shoots remains same, but the elongation of shoots occurred much more as compared to approach 1. The maximum length of shoots (Table 6) was obtained at treatment number five (5.05 ± 0.3).

Approach 3:

Similar, procedure was used as in approach 2, but the concentration of cyanobacterial spp. was increased from 10-15ml/l and sucrose up to 30mg/l. When the concentration of each cyanobacteria spp. 12ml/l was supplemented to Chu's 10 medium root formation was occurred. Maximum root percentage was obtained (85 ± 0.1). Total of six treatments was given and treatment number 3rd was found best for root formation, Table 7.

Figure 1: Proliferation in Chu's 10 medium fortified with cyanobacterial spp., sucrose and agar.
Figure 2: Elongation in Chu's 10 medium fortified with cyanobacterial spp., sucrose and agar

Figure 3: Proliferation, elongation and rooting in Chu's 10 medium fortified with cyanobacterial spp., sucrose and agar.
Figure 4: Rooting in Chu's 10 medium fortified with cyanobacterial spp., Sucrose and agar.

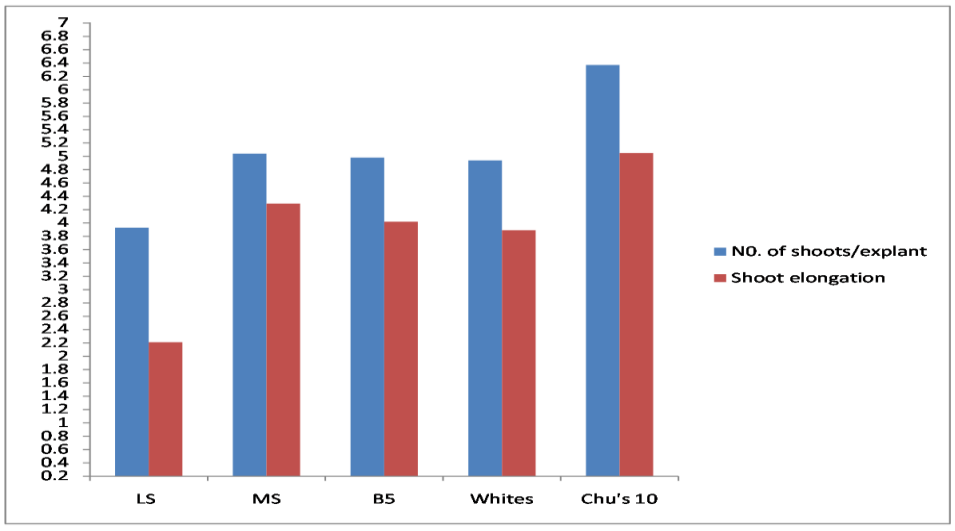

Figure 5: Comparative analysis of shoot formation and shoot elongation in different media.

Table 1: Shoot proliferation from nodal explant of *G. gummifera* Linn.f in culture media MS.

No. of Treatments	Type and concentration of PGR's (mg/l)				No. of shoots/explant (M±SE)	Average shoot length (M±SE)
	BAP	IBA	Kn	NAA		
1	1			0.5	2.12±0.7*	1.72±0.3*
2	1.5			0.5	3.97±0.5	1.96±0.5*
3	2			0.5	4.02±0.3	2.32±0.7*
4	2.5			0.5	3.82±0.5	1.92±0.3*
5	2			0.6	3.72±0.4	1.72±0.5*
6	1	0.5			2.18±0.9*	1.96±0.5*
7	1.5	0.5			2.82±0.1*	2.25±0.7*
8	2	0.5			5.04±0.2	4.29±0.2
9	2.5	0.5			4.03±0.3	3.92±0.5
10	2	0.6			3.37±0.2	3.78±0.1
11			1	0.5	1.17±0.6*	1.70±0.5*
12			1.5	0.5	2.94±0.3*	1.76±0.2*
13			2	0.5	3.95±0.5	1.98±0.2*
14			2.5	0.5	2.72±0.2*	1.77±0.6*
15			2	0.6	2.67±0.3*	1.71±0.3*
16		0.5	1		1.57±0.2*	1.74±0.3*
17		0.5	1.5		3.02±0.8	1.82±0.8*
18		0.5	2		3.98±0.2	2.33±0.3*
19		0.5	2.5		2.82±0.7*	1.96±0.5*
20		0.6	2		2.79±0.4*	1.99±0.2*

Values are M±SE of treatment repeated four times, each treatment having five replicates; F test; P*<0.05.

Table 2: Shoot proliferation from nodal explant of *G. gummifera* Linn.f in culture media B_5.

No. of Treatments	Type and concentration of PGR's (mg/l)				No. of shoots/explant (M± SE)	Average shoot length (M± SE)
	BAP	IBA	Kn	NAA		
1	1			0.5	2.10±0.3*	1.69±0.5*
2	1.5			0.5	3.90±0.2	1.92±0.3*
3	2			0.5	3.97±0.5	2.27±0.6*
4	2.5			0.5	3.79±0.2	1.86±0.9*
5	2			0.6	3.67±0.5	1.68±0.4*
6	1	0.5			2.13±0.2*	1.91±0.5*
7	1.5	0.5			2.78±0.4*	2.19±0.7*
8	2	0.5			4.98±0.1	4.02±0.1
9	2.5	0.5			3.97±0.5	3.87±0.5
10	2	0.6			3.39±0.5	3.73±0.3
11			1	0.5	1.19±0.2*	1.64±0.8*
12			1.5	0.5	2.96±0.3*	1.62±0.6*
13			2	0.5	3.05±0.2	1.89±0.2*
14			2.5	0.5	2.70±0.5*	1.73±0.1*
15			2	0.6	2.65±0.2*	1.66±0.3*
16		0.5	1		1.48±0.4*	1.71±0.2*
17		0.5	1.5		2.98±0.2*	1.79±0.4*
18		0.5	2		3.97±0.6	2.27±0.9*
19		0.5	2.5		2.84±0.2*	1.91±0.4*
20		0.6	2		2.80±0.3*	1.89±0.6*

Values are M±SE of treatment repeated four times, each treatment having five replicates; F test; P*<0.05

Table 3: Shoot proliferation from nodal explant of *G. gummifera* Linn.f in culture media Whites.

No. of Treatments	Type and concentration of PGR's (mg/l)				No. of shoots/ Explant (M± SE)	Average shoot length (M± SE)
	BAP	IBA	Kn	NAA		
1	1			0.5	2.8±0.4*	1.67±0.2*
2	1.5			0.5	3.87±0.2	1.80±0.2*
3	2			0.5	3.89±0.1	2.17±0.3*
4	2.5			0.5	3.65±0.7	1.79±0.5*
5	2			0.6	3.61±0.5	1.69±0.7*
6	1	0.5			2.13±0.1*	1.75±0.1*
7	1.5	0.5			2.49±0.5*	2.01±0.4*
8	2	0.5			4.94±0.9	3.89±0.2
9	2.5	0.5			3.91±0.2	3.81±0.8
10	2	0.6			3.33±0.4	3.79±0.9
11			1	0.5	1.27±0.5*	1.64±0.3*
12			1.5	0.5	2.89±0.6*	1.67±0.6*
13			2	0.5	3.67±0.9	1.81±0.7*
14			2.5	0.5	2.97±0.4*	1.78±0.1*
15			2	0.6	2.94±0.8*	1.77±0.2*
16		0.5	1		1.59±0.2*	1.73±0.4*
17		0.5	1.5		2.89±0.3*	1.75±0.5*
18		0.5	2		3.69±0.6	2.21±0.5*
19		0.5	2.5		2.92±0.2*	1.97±0.4*
20		0.6	2		2.88±0.9*	1.92±0.2*

Values are M±SE of treatment repeated four times, each treatment having five replicates; F test; P*<0.05

Table 4: Shoot proliferation from nodal explant of *G. gummifera* Linn.f in culture media LS.

No. of Treatments	Type and concentration of PGR's (mg/l)				No. of shoots/ Explant (M± SE)	Average shoot length (M± SE)
	BAP	IBA	Kn	NAA		
1	1			0.5	2.8±0.4	1.68±0.1*
2	1.5			0.5	3.89±0.5	1.82±0.2*
3	2			0.5	3.93±0.4	2.21±0.5*
4	2.5			0.5	3.67±0.2	1.83±0.7*
5	2			0.6	3.65±0.1	1.66±0.2*
6	1	0.5			2.11±0.3*	1.88±0.4*
7	1.5	0.5			2.55±0.5	2.09±0.3*
8	2	0.5			4.92±0.2	3.98±0.4
9	2.5	0.5			3.93±0.1	3.84±0.7
10	2	0.6			3.45±0.6	3.69±0.9
11			1	0.5	1.53±0.2*	1.74±0.7*
12			1.5	0.5	2.93±0.4	1.72±0.1*
13			2	0.5	2.98±0.6	1.83±0.2*
14			2.5	0.5	2.89±0.4	1.79±0.3*
15			2	0.6	2.81±0.2	1.73±0.2*
16		0.5	1		1.23±0.5*	1.77±0.5*
17		0.5	1.5		2.84±0.5	2.24±0.4*
18		0.5	2		3.89±0.1	2.34±0.7
19		0.5	2.5		2.66±0.2	1.97±0.2*
20		0.6	2		2.59±0.2	1.95±0.3*

Values are M±SE of treatment repeated four times, each treatment having five replicates.
F test; P*<0.05.

Table 5: Response of nodal callus of *G. gummifera* Linn.f in Chu's 10 media.

No. of Treatment	Adulterants in Media			No. of shoots/ explants (M±SE)	Shoot length (M±SE)
	Sucrose (g/l)	Agar (g/l)	C.C.P (ml/l)		
1			1	2.27±0.7	1.98±0.5
2			2	2.72±0.4	2.17±0.4
3			3	3.35±0.1	2.72±0.4
4			4	4.32±0.6	3.53±0.7
5	20	0.8	5	6.37±0.3	4.82±0.2
6			6	3.85±0.4	3.82±0.7

Values are M±SE of treatment repeated four times, each treatment having five replicates.
C.C.P- Concentration of each cyanobacteria spp. (1:1).

Table 6: Response of nodal callus of *G. gummifera* Linn.f in Chu's 10 media.

No. of Treatment	Adulterants in Media		C.C.P (ml/l)	No. of shoots/ Explants (M±SE)	Shoot length (M±SE)
	Sucrose (g/l)	Agar (g/l)			
1			5	6.29±0.2	4.89±0.2
2			6	6.25±0.4	4.97±0.5
3			7	6.23±0.2	4.99±0.1
4			8	6.17±0.2	5.05±0.3
5	25	0.8	9	6.11±0.4	5.03±0.7
6			10	6.7±0.2	4.98±0.3
7			11	6.2±0.9	4.96±0.1

Values are M±SE of treatment repeated four times, each treatment having five replicates.
C.C.P- Concentration of each cyanobacteria spp. (1:1)

Table 7: Response of nodal callus of *G. gummifera* Linn.f in Chu's 10 media.

No. of Treatment	Adulterants in Media			No. of shoots/explants (M±SE)	Shoot length (M±SE)	% of shoots produce roots (M±SE)
	Sucrose (g/l)	Agar (g/l)	C.C.P (ml/l)			
1			10	6.3±0.2	4.98±0.3	57±0.3
2			11	5.98±0.4	4.94±0.2	69±0.4
3			12	5.83±0.6	4.91±0.4	85±0.1
4			13	5.79±0.2	4.89±0.1	78±0.3
5	30	0.8	14	5.75±0.2	4.87±0.7	74±0.2
6			15	5.43±0.6	4.83±0.1	72±0.3

Values are M±SE of treatment repeated four times, each treatment having five replicates.
C.C.P- Concentration of each cyanobacteria spp. (1:1)

These rooted plantlets were successfully acclimatized and shows 100% survival rate. On perusal of the above results, Chu's 10 media supplemented with cyanobacteria spp. is one step media Figure 3 in which the *Gardenia gummifera* is well developed and meet to present demand and help to conservation of this endangered medicinal plant. During our study via direct organogenesis, indirect organogenesis and using cyanobacterial media for *in vitro* propagation of *Gardenia gummifera*. Cyanobacterial media shows promotive effect in shoot proliferation, shoot elongation and root induction.

Our results agree with Metting and Pyne (1986), cyanobacteria produce a variety of compounds including PGR's that could be used in invitro propagation. According to Zulpa *et al.,* (1979), Stirk

et al., (1999) and Sergeeva *et al.,* (2002) cyanobacteria synthesizes and liberate various number of plant growth regulators viz auxins, gibberellins, cytokinins, vitamins, polypeptides, aminoacids, which promote plant growth and development. This supports strongly our study that cyanobacteria consist of promoting factors viz organic additives, plant hormones and aminoacids.

Comparative analysis:

Throughout our work, we reach conclusion Chu's 10 supplemented with *Anabaena aequalis* and *Nostoc muscorum* was found best among all the medias not only regeneration of this plant, but also for shoot proliferation, shoot elongation and root induction Figure 5. This protocol not only meet the escalating demand at commercial level, but it is of low cost, ecofriendly and reliable.

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