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Rapid Micropropagation of *Curculigo orchioides* in Shake Flask Culture

R.K.Nema[a*], K.G.Ramawat[b] and J.M.Merillon[c]

[a]*S.D.College of Pharmacy & Vocational Studies, Muzaffarnagar*
[b]*Laboratory of Biomolecular Technology, College of Science, MLS University, Udaipur*
[c]*Faculte' des Sciences, Pharmaceutiques, Universite' de Bordeaux II, France*
**Corresponding Author: nema_pharmacy@yahoo.co.in*

ABSTRACT

Curculigo orchioides (Hypoxidaceae) is a monocot stemless perennial herb. It is an endangered plant species of medicinal importance. Efficiency of two different culture systems, viz., different gelling agents as static cultures and shake flask cultures were compared for the production of bulbils. A protocol for micropropagation of *C. orchioides* through direct bulbil formation from leaf explants in shake flask cultures has been standardized. Effect of different concentrations of sucrose and different sugars has also been studied. Plantlets developed *in vitro* were successfully transferred to soil with a high rate of survivability (90%) and were comparable to natural population in growth and vigour.

KEY WORDS: *Curculigo orchioides,* Micropropagation, Shake Flask Culture

INTRODUCTION

Tuberous roots of *Curculigo orchioides* (Hypoxidaceae) also called "Kali musli", are widely used as tonic for health, vigour and vitality due to the presence of flavanone glycosides and other steroidal saponin (1,2). Plant extract of *C. orchioides* showed hypoglycemic, spasmolytic and anticancer properties (3). The rhizome is also prescribed for the treatment of piles, jaundice, asthma, diarrhoea (4). Pharmacological studies in China showed several active effects of alcoholic extract such as adaptogenic, anti-inflammatory, anticonvulant, sedative, androgenic and immunopromotion activities (5).

The plant is naturally propagated through seeds and underground bulbils. Over-exploitation of the plant associated with poor seed set and germination has made it an endangered species. Efforts are needed to propagate this species on the commercial scale to meet the demand of pharmaceutical firms and to protect the existing natural population of the species. Plant tissue culture technique has become a powerful tool to develop micropropagation methods for such plants. Excised leaf explants from *in vitro* plantlets serve as aseptic reservoir of inoculum required for large-scale multiplication. Direct regeneration of embryos and bulbils from the leaf explants makes them an excellent system for such a purpose. Bulbil formation has gained considerable attention as a novel method for micropropagation due to easy in transportation, better survivability of germinated bulbils in soil, low cost, and rapid propagation. This study shows a method for large-scale propagation of *Curculigo orchioides* through direct bulbil formation from leaf explants in shake flask culture.

MATERIAL AND METHODS

Improvement in direct bulbils formation from leaf explants of *C. orchioides* for scale up technology has been achieved. Initially MS medium containing BA (0.1mg/l) and (IBA 0.1mg/l) was used (6). Young leaves of *C. orchioides* from aseptically maintained plantlets, raised through shoot proliferation from stem disc and leaf explants were used as source material (7). Leaf segments (5×10 mm) were aseptically transferred in the MS liquid and on MS solid medium containing various combinations and concentrations of auxins and cytokinins as per the design of the experiment. The Murashige and Skoog (1962) medium was gelled with different concentrations of different gelling agents and pH of the medium was adjusted to 5.8 with 0.5 N HCl or NaOH after incorporation of all ingredients of the medium and various concentrations and combinations of cytokinins and auxins.100 ml medium was dispensed in each conical flasks (250 ml Borosil). Flasks were plugged with nonabsorbent cotton and autoclaved for 20 minutes at 121^0 C at 1.05 kg cm^{-2}(8).

For the comparison of the efficiency of two different culture systems, *viz.*, different gelling agents as static cultures and shake flask cultures the appropriate concentration of different gelling agents was incorporated in MS medium supplemented with BA (0.1 mg/l) and IBA (0.1 mg/l). Following agents were used

viz., agar (8 g/l), agarose (7 g/l), phytogel (5 g/l), transfer gel (25 g/l) and husk of plantago (50 g/l). Ten leaf explants (5×10 mm) were transferred aseptically into each 250 ml conical flask containing 100 ml gelled medium and 20 leaf explants in 100 ml liquid medium.

To study the effect of density on regeneration potential, different number of the leaf explants *viz.*, 33, 50 and 100 (5×10mm) were aseptically transferred in 100 ml (250 ml 'Borosil') standard MS medium supplemented with BA (0.1mg/l) and IBA (0.1mg/l). The effect of different concentrations of sucrose and different sugars on the initiation and growth of bulbils in standard MS liquid medium supplemented with IBA (0.1 mg/l) and BA (0.1 mg/l) was studied by preparing the medium containing 2, 3, 4, 5 and 6 % of sucrose. MS liquid medium containing 3% fructose, maltose and glucose separately was also prepared and 50 explants were aseptically transferred in each 250 ml conical flasks containing 100 ml liquid medium. After 4-weeks of growth the spent medium was replaced with 100ml fresh media and incubated for another period of 4-weeks.

To study rhizogenesis, B_5 gelled medium with various concentrations and combinations of IAA (0.0, 0.1, 1.0, 10.0 mg/l) with or without IBA 0.1 mg/l was prepared. Ten germinated bulbils of uniform size were transferred in each flask containing 100 ml medium. After 8-weeks of growth, plantlets were planted in pots containing sterilized soil: vermiculite mixture (3:1 v/v). To maintain ambient humidity, pots were covered with transparent polythene bags and plants were irrigated with tap water (25 ml / pot) on alternate days. After one month's growth, plants were transferred to green house.

All cultures were incubated in the culture room at 26.0 ± 0.5^0C under white fluorescent light (Philips cool TL 36 W, 220 V) with a total irradiance of 36 µ mol. $m^{-2}s^{-l}$ for 16 hours photo-period and 55-60% relative humidity. Liquid cultures were agitated on a rotary shaker at 80 rpm (with 2" displacement from the central axis) and medium was replaced with its corresponding fresh medium after four weeks. Formations of bulbils were recorded at an interval of four weeks and eight weeks. The medium was replaced by fresh medium after four week.

RESULTS AND DISCUSSION

A method for rapid and high frequency multiplication of *C. orchioides* through bulbil formation has been developed by utilizing leaf explants grown in static and liquid media. Leaf-segment in the liquid medium supplemented with 0.1mg/l benzyl adenine (BA) and 0.1 mg/l indole butyric acid (IBA) produced maximum number of bulbils (7140 bulbils/l at 8-weeks growth, 100% explants response) in the medium. Higher number of bulbils per explants and shoots (germinating bulbils) per flask was recorded in liquid media with shorter initiation time and maximum percent response of explants as compared to gelled media. This was applicable to all gels irrespective of its type (Table:1). Maximum number of bulbils per explant was recorded in the liquid medium (Figure-1). Plantago husk was least effective for bulbils formation from the leaf explants. It is evident from the data that these bulbils started germinating during 6 to 8 weeks growth irrespective of gelled or liquid media (Figure-2).

Numbers of bulbils per explant and percent response were highest in one explants/two ml of medium (Table: 2). However, in terms of bulbils yield/liter of medium was maximum when one explant/one ml of medium was used because of high number of explants (Figure-3). Growth index (GI) was higher in former condition. Therefore, one explant/two ml of medium was used in further experiments. Sucrose is used as source of carbon but at higher concentration it also acts as osmoticum. The bulbils are storage organs full of starch. Therefore, increasing concentration of sucrose was incorporated in MS liquid medium to record effect on bulbils formation. At 4-weeks growth, standard concentration (30 g/l) was recorded optimal for % explant response (100%), bulbils (10.7 / explant), yield (5350 /l) and fresh weight of bulbils (90.7 g/l). However, maximum number, yield and fresh weight was recorded in explants grown in the medium supplemented with 50 g/l sucrose. Therefore it is evident that at higher concentration of sucrose, initial growth was slow. High concentration of sucrose (60 g/l) was slightly inhibitory to the bulbils formation (Table: 3). Among different sugars used as source of carbon at 3% concentration, fructose was the best source at 4-weeks and 8-weeks growth. Maximum number of bulbils per explant, yield per litre and fresh weight per litre were recorded in the medium supplemented with fructose followed by sucrose, maltose and glucose (Table: 4).

A number of roots and length of roots increased with increase in concentration of IAA in the rooting medium. However, improvement in rooting was observed when IAA and IBA both were used in combination of 0.1 mg/l each (~ 6 roots/bulbil). This may be due to synergistic effect of combined auxins (Table: 5; Figure-4). Bulbil formation has gained considerable attention as a novel method for

Table 1 Effects of different gelling agents on the formation of bulbils in MS liquid medium containing BA 0.1mg/l and IBA 0.1mg/l

Gelling agents	(g/l)	Response (%)	Initiation time (days)	Bulbils / Explant ±SD	Shoots / flask **6 weeks**	**8 weeks**
Agar	8.0	80	25	1.5 ± 0.71	2.1 ± 0.99	5.9 ± 1.66
Agarose	7.0	100	25	1.7 ± 0.82	12.6 ± 1.84	22.4 ± 2.41
Phytogel	5.0	50	28	0.8 ± 0.63	8.0 ± 1.49	10.9 ± 1.91
Transfer gel	25.0	100	28	1.6 ± 0.84	3.2 ±1.03	4.3 ± 1.16
Husk of Plantago	50.0	12	30	0.7 ± 0.67	0.6 ± 0.7	1.2 ± 1.03
Cotton bridge	--	100	25	3.7 ± 1.77	26.4 ± 2.91	34.6 ± 3.31
Liquid		100	18	11.4 ± 2.27	56.9 ± 5.93	84.6 ± 5.80

Readings are the average of 8 explants per flask per treatment.

Table 2 Effect of inoculums size on in vitro formation of bulbils from leaf explants in MS liquid medium supplemented with BA 0.1 mg/l and IBA 0.1 mg/l.

Inoculum Explant / 100 ml	Response (%)	4– weeks			8– weeks			GI
		Bulbils / Explant ±SD	Yield Bulbils/l	F.W. g/l	Bulbils / Explant ±SD	Yield Bulbils/l	F.W. g/l	
100	97	7.5 ± 0.2	7512	686	8.3 ± 1.33	8370	1105	0.61
50	100	10.6 ± 0.1	5277	525	14.4 ± 1.37	7140	886	0.69
33	89	7.3 ± 0.1	2422	136	7.5 ± 1.24	2499	188	0.38

Table 3 Effects of different concentrations of sucrose on the formation of bulbils in MS liquid medium containing BA 0.1 mg/l and IBA 0.1 mg/l

Sucrose (%)	Response (%)	4 – weeks			8 – weeks		
		Bulbils / Explant ±SD	Yield Bulbils/l	F.W. g/l	Bulbils / Explant ±SD	Yield Bulbils/l	F.W. g/l
0	50	1.3 ± 0.3	650	7.7	2.4 ± 0.4	1190	14.3
2	90	8.4 ± 0.9	4210	84.8	10.4 ± 1.0	5190	212.4
3	100	10.7 ± 1.0	5350	90.7	12.5 ± 0.9	6240	306.3
4	90	6.1 ± 0.7	3040	59.3	14.1 ± 0.6	7055	375.0
5	85	6.5 ± 0.9	3230	75.7	18.4 ± 0.9	9210	387.5
6	75	5.9 ± 0.8	2940	54.0	14.3 ± 0.9	7170	285.8

Table 4 Effects of different types of sugars on the formation of bulbils in MS liquid medium containing BA 0.1 mg/l and IBA 0.1 mg/l

Sugars (3 %)	Response (%)	4 – weeks			8 – weeks			GI
		Bulbils / Explant ±SD	Yield Bulbils/l	F.W. g/l	Bulbils / Explant ±SD	Yield Bulbils/l	F.W. g/l	
Sucrose	100	10.7 ± 1.0	5350	90.7	12.5 ± 0.9	6240	306.2	2.38
Fructose	100	12.7 ±1.0	6370	102.5	17.3 ± 0.9	8655	320.8	2.13
Maltose	80	3.9 ± 0.7	1940	37.7	6.5 ± 10.7	3225	133.7	2.54
Glucose	75	3.1 ± 0.7	1560	29.7	4.7 ± 0.7	2365	99.9	2.36

Readings are the average of 15 explants per 30 ml treatment.

Table 5 Rhizogenesis on B_5 medium

PGR (mg/l)		%	4-Weeks	
IBA	IAA	Rooting response	No. of roots ±SD	Root length mm ±SD
0.0	0.0	50	1.80 ± 0.8	9.83 ± 1.7
	0.1	100	3.60 ± 1.6	12.65 ± 2.1
	1.0	100	3.9 ± 1.2	18.47 ± 1.4
	10.0	80	4.7 ± 1.2	19.59 ± 1.9
0.1	0.0	80	3.0 ± 1.1	17.70 ± 1.4
	0.1	100	5.9 ± 1.5	43.38 ± 1.3
	1.0	100	2.6 ± 1.3	16.08 ± 1.6
	10.0	76	1.9 ± 0.9	10.54 ± 1.5

Figure:1.Leaf explants in MS liquid medium containing BA (0.1mg/l and IBA (0.1mg/l)
2.Multiplication of bulbils by formation of secondary bulbils in MS liquid medium.
3.Production of bulbils from leaf explants of C.orchioides in shake flask culture.
4.Plantlets formation on GA_3, supplimented medium in large culture vessel.

micropropagation due to ease of handling, storage, transport, exchange, better survivability of germinated bulbils in soil and low-cost rapid propagation. Methods for rapid multiplication of *C. orchioides* are highly desirable to meet the commercial demand and to conserve the valuable endangered plant. Plant tissue culture technique has become a powerful tool to develop micropropagation methods for such plants.

CONCLUSION

Subterranean parts pose serious problem of contamination and aerial parts in monocots are difficult material to regenerate (9,10). Improvement in direct bulbils formation from leaf explants of *C. orchioides* for scale up technology has been achieved. Initially MS medium containing BA (0.1mg/l) and (IBA 0.1mg/l) was used (6,7). Explants produce bulbils when grown on IBA and BA supplemented medium and in liquid medium irrespective of presence of different auxins as reported earlier (6). Attempts made for large-scale rapid multiplication of *C. orchioides* through leaf explants was successful.These results were sufficient to produce the bulbils in large numbers and allow their successful transfer in pots/field conditions for mass propagation. Field transfer was already evaluated in previous study (6,7). Therefore, plantlets were successfully transferred in field conditions without any problem.

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