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In vitro regeneration and root induction from *Curculigo orchioides* Gaertn - A high valued medicinal plant

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Abstract

Studies on optimizing of disinfection protocol for *Curculigo orchioides* have not been reported so far. Here we report the effects of two commonly used surface disinfectants such as $HgCl_2$ (0.1%) and NaOCl (1%) for disinfecting rhizome and leaf explants of *C. orchioides* at different durations ranging from 1 - 15 min. Treatment with $HgCl_2$ (0.1%) for 15 min was found suitable for rhizome explants whereas leaf explants responded well for $HgCl_2$ (0.1%) treatment for 6 min. Experiment on shoot regeneration shown that 4.4 µM BAP was the optimum concentration for producing healthy shoots. Among the various combinations of auxin and cytokinins tested for root induction, highest percentage of rooting (76.6%) with maximum number of roots (40.4) was recorded when 4.4 µM BAP, 4.6 µM KN and 5.3 µM NAA were combined. The micropropagated plants were similar to the mother plants without any morphological variation. HPLC analysis of the regenerated roots revealed a phytochemical profile similar to that of the mother plant. This protocol can be used to generate elite planting material for large scale cultivation.

Keywords: *Curculigo orchioides, In vitro* regeneration, Medicinal plant, High Performance Liquid Chromatography

Introduction

Curculigo orchioides Gaertn. (Hypoxidaceae) is regarded as one of the important endangered medicinal herbs used as a key member of the *Dasapushpa*, an ayurvedic formulation used over many centuries in India. It is commonly distributed in Asian sub continents upto an altitude of 2300 m. This herb is strictly perennial in nature, grows about 30 cm in height with a short or elongated root-stock bearing a number of fleshy shorter lateral roots which are often black in color. This small geophilus perennial herb have been extensively used in indigenous system of medicine in India, Pakistan and China, for treating various diseases including jaundice, asthma and diarthrosis (Dhar *et al*., 1968), impotency (Chopra *et al*., 1956), and preventing bone loss (Cao *et al*,, 2008). Flavones, glycosides, steroids, saponins and triterpenoid are the major bioactive principles, present in *C. orchioides* (Misra *et al.,* 1984; Misra *et al*., 1990; Xu *et al*., 1992). However, *C. orchioides* has become endangered due to indiscriminate collection for the preparation of herbal medicines and depletion of natural habitat (Wala and Jasrai, 2003). Poor seed setting and germination (Suri *et al*., 1999) and high incidence of viral and bacterial diseases in rhizome (Dhenuka *et al*., 1999) become the major reasons for drastic reduction of population of this species.

Low toxicity and proven therapeutic effects of herbal medicines attracts the worldwide consumer which in turn increases the global demand for herbal medicines (Rajshekharan, 2002). *C. orchioides* is propagated through seeds but grows only during rainy season. To meet the ever increasing demand for the raw materials of *C. orchioides*, appropriate alternate strategies for plant regeneration and multiplication need to be developed. In view of this, both direct and indirect micropropagation protocols were developed for *C. orchioides* by many workers

(Augustine, 1997; Suri *et al*., 1999; Thomas and Jacob 2004 and Nagesh *et al*., 2008). However, optimization of surface sterilization was not dealt in any of the reports. Here, we report the efficacy of two widely used disinfectants such as NaOCl and $HgCl_2$ in surface sterilization of leaf and rhizome explants of *C. orchioides* and a reliable and reproducible *in vitro* regeneration protocol for rapid multiplication of *C. orchioides*. In addition, HPLC analysis was carried out with normal and micropropagated plantlets of *C. orchioides* for ensuring the medicinal properties of micropropagated plants.

Materials and Methods

Plant collection and maintenance

Well grown plants of *Curculigo orchioides* were collected from the forest of Wadakanchery Village in Thrissur District of Kerala and maintained in the green house at Sri Paramakalyani Centre for Environmental Sciences, Alwarkurichi, Manonmaniam Sundaranar University. There plants were used as source of explants throughout the study.

Media and culture condition

MS medium (Murashige and Skoog's, 1962) supplemented with sucrose (3%) become the basic media. The media was augmented with various growth regulators such as BAP, KN, 2, 4-D and NAA (Hi-media, Mumbai, India) in different concentrations ranging from (0.44 µM – 23.2 µM) depending upon the experimental design. A range of concentrations of $AgNO_3$ and PG (British Drug House, Chennai, India) was also used for optimizing the culture conditions for shoot proliferation. The pH of the media was adjusted to 5.8 before gelling with 0.7% agar (Hi-media, Mumbai, India). Molten medium was dispensed into 200 ml screw-capped glass jars and 25 x 150 mm culture tubes (Borosil, Chennai, India) and capped with cotton plugs before sterilization at 121°C for 20 min. All the cultures were maintained at 25 ± 2°C and kept under a 16 hrs photoperiod provided by cool white fluorescent tubes (Philips, Mumbai, India) with a light intensity of 40 µmol m^{-2}. The number of explants cultured in each treatment was varied from 20 - 30 depending upon the experimental design. The duration of culture was ranging from 30 - 120 days.

Efficacy of NaOCl and $HgCl_2$ on rhizome

Efficacy of two commonly employed surface disinfectants, namely NaOCl (1%) and $HgCl_2$ (0.1%) were tested to eliminate microbial contamination. The rhizomes of *C. orchioides* were surface sterilized with above disinfectants for various durations (3, 6, 9, 12 and 15 min) and were thoroughly rinsed with sterile distilled water for 4 to 5 times under aseptic condition. The explants were slightly trimmed, measuring about 1cm length and implanted vertically on MS medium augmented with 4.4 µM BAP. Explants cultured without surface sterilization formed the control for this experiment. A total of 150 explants were cultured with three replications, each with 50 explants. The recovery of explants without microbial contamination and browning was scored after 60 days of culture and data were subjected to statistical analysis.

Efficacy of NaOCl and $HgCl_2$ on leaf explant

A similar experiment was carried out with leaf explants of *C. orchioides* as described above. After disinfection with NaOCl (1%) and $HgCl_2$ (0.1%), the leaf explants were trimmed, measuring about 1 cm^2 under aseptic condition and cultured on MS medium augmented with 4.4 µM BAP. Explants cultured without surface sterilization formed the control for this experiment. A total of 150 explants were cultured with three replications, each with 50 explants. The recovery of explants without microbial contamination and browning was scored after 60 days of culture and the data were subjected to statistical analysis.

Effect of auxin and cytokinins on shoot proliferation in leaf explants

To optimize shoot proliferation, leaf explants of *C. orchioides* were inoculated on MS medium fortified with BAP and KN either alone or in combinations with 2, 4-D and NAA. Various concentrations of BAP (0.44 µM, 4.4 µM and 22.0 µM), KN (0.46 µM, 4.6 µM and 23.0 µM), 2, 4-D (4.5 µM) and NAA (5.3 µM) were used for this experiment. A total of 30 explants were used for each treatment with three replications. All the cultures were kept under diffused natural light. Explant cultured initially for 40 days at different concentrations were observed and recovered explants showing response were transferred to fresh media of same composition and maintained for 90 days. Data on number of bulbils formed and sprouting percentage of bulbils were recorded and analysed.

Effect of auxin and cytokinins on regenerations of adventitious roots

To optimize the development and regeneration of large number of microshoots from the rhizome explant of *C. orchioides,* explants were inoculated on MS medium supplemented with two different cytokinins (BAP and KN) in combinations with two of the commonly used auxin (2,4-D and NAA). Various concentrations of BAP (2.2 µM and 4.4 µM), KN (2.3 µM and 4.6 µM), 2, 4-D (5.0 µM) and NAA (5.3 µM) were used for this experiment. A total of 60 explants were used with three replications, each with 20 explants. Responding explants after 30 days of primary culture were transferred on to fresh media of the same composition and maintained for 90 days, bringing the total culture period to 120 days. Data on regeneration of shoots and root development was recorded and analysed.

High Performance Liquid Chromatography (HPLC)

Powder forms of various samples were prepared by following the general methods described for TLC analysis. These samples were mixed with methanol (1:20) and continuously stirred for four days and finally filtered through 0.2 µm membrane. HPLC was performed using Shimadzu (Japan) HPLC system equipped with a main column of analytical – shim-pack CLCOCTA DECYL SILANE (ODS-C18) (46 mm 10x25 cm) and guard column was shim-pack G-ODS (4 mm 10 x 1cm). Stationary phase of the HPLC was silica gel (reversed phase) and mobile phase was methanol (100% HPLC grade). The maintained column head pressure for all the samples was 125 kg + 1 cm^2. Hamilton Microlitre Syringe (Japan) was used to inject 20 µl samples into the column. The maintained flow rate was one ml per minute and run time was 15 minutes. The chromatogram was viewed and the peak was observed at 280 nm.

Statistical analysis

Majority of the experiments were analyzed with three replications. However, the number of explants for the treatment in various experiments was varying due to differences in final recovery of explants. The effect of the different treatments on various parameters was quantified and the level of significance was determined by the analysis of variance (ANOVA) using SPSS version 11.0 and level of differences between the treatments were assessed by Duncan's New Multiple Range Test (DMRT) at $P \leq 0.05$.

Results

Efficacy of NaOCl and $HgCl_2$ on disinfection of rhizome

Effect of different concentrations of NaOCl and $HgCl_2$ on disinfection of rhizome explants of *C. orchioides* is summarized in Table 1. High incidence of microbial contamination was observed in control and none of the explants could be recovered at the end of 40 days of initial culture. However, explants disinfected with NaOCl (1%) and $HgCl_2$ (0.1%) for different durations (3, 6, 9, 12 and 15 min) shown varying levels of recovery. It was observed that explants treated with NaOCl for 3 - 15 min and $HgCl_2$ for 3 - 9 min shown poor recovery of explants since many of the cultures were lost due to microbial contamination during initial culture period. Explants treated with NaOCl and $HgCl_2$ for shorter durations (3 - 6 min) recorded high incidence of microbial contamination and further increase in duration from 9 - 15 min improved the recovery of explants significantly. Browning of rhizomes explants increased with increases in duration of treatment probably due to longer exposure of rhizomes to both disinfectants.

In this experiment, disinfection of rhizome explants with NaOCl (1%) recorded lesser recovery of explants (5.17) even at longer duration of treatments (15 min). When rhizome explants were exposed to the same duration with $HgCl_2$ (0.1%), the recovery of rhizome explants was significantly improved (12.1). This experiment clearly shown that disinfection of rhizome explants with $HgCl_2$ (0.1%) for 12 - 15 min was more effective due to highest recovery of explants (Table 2). It was found that oxidation of rhizome tissues was noticed during disinfection as well as during primary culture of explants. The present experiment lead to optimization of disinfection of rhizome explants of *C. orchioides* for efficient initiation of primary culture.

In contrast, when rhizome was exposed to NaOCl (1%) for longer duration (15 min), maximum sprouting was achieved with 32.2%. Decrease in exposure of rhizome to NaOCl for 12, 9, 6 and 3 mins reduced the percentage of sprouting to 23.0%, 16.9%, 12.4% and 6.9% respectively. An

experiment carried out with $HgCl_2$ also showed similar results. Rhizome explants exposed $HgCl_2$ for shorter duration ranging from 3 - 9 min reduced the overall sprouting to 10.2%, 19.9% and 28.8% for 3, 6 and 9 min respectively. However, longer exposure of rhizome explants to $HgCl_2$ for 12 and 15 min showed 53.3% and 66.6% of sprouting. Comparative analysis on recovery and sprouting of rhizome explants revealed that $HgCl_2$ was more effective than NaOCl due to higher recovery and sprouting.

Table - 1: Effect of NaOCl (1%) and $HgCl_2$ (0.1%) on microbial contamination in rhizome of *C. orchioides.* Explants were cultured on MS medium supplemented with BAP (4.4 µM). Duration of culture 60 days.

Treatments (min)	Microbial contamination		Browning	Recovery	Sprouting (%)
	Bacteria	Fungi			
NaOCl					
00.0	13.66[a]	16.34[a]	0.00[f]	0.00[f]	00.00[f]
03.0	12.66[b]	14.50[b]	1.08[e]	1.75[e]	06.94[e]
06.0	11.50[c]	13.25[c]	2.66[d]	2.58[d]	12.47[d]
09.0	10.91[d]	12.33[d]	3.16[c]	3.59[c]	16.90[c]
12.0	09.75[e]	11.66[e]	4.25[b]	4.33[b]	23.02[b]
15.0	07.41[f]	10.33[f]	7.08[a]	5.17[a]	32.20[a]
$HgCl_2$					
00.0	16.16[a]	13.84[a]	0.00[f]	00.00[f]	00.00[f]
03.0	13.41[b]	11.50[b]	3.33[e]	01.75[e]	10.26[e]
06.0	11.50[c]	10.16[c]	4.00[d]	04.33[d]	19.97[d]
09.0	09.41[d]	08.16[d]	6.50[c]	05.93[c]	28.85[c]
12.0	05.91[e]	07.66[d]	7.16[b]	09.27[b]	53.30[b]
15.0	03.66[f]	05.41[e]	8.83[a]	12.10[a]	66.60[a]

Data represents the mean values of three replications, each with 30 explants. Mean values within each column followed by the same letter in superscripts are not differing significantly ($P \leq 0.05$: Duncun's New Multiple Range Test). Treatments of rhizome explants with NaOCl and $HgCl_2$ were indipendently analyzed.

Table 2: Effect of NaOCl (1%) and $HgCl_2$ (0.1%) on microbial contamination in leaf explants of *C. orchioides.* Explants were cultured on MS medium supplemented with BAP (4.4 µM). Duration of culture 60 days.

Treatments (min)	Microbial contamination		Browning	Recovery	Sprouting (%)
	Bacteria	Fungi			
NaOCL					
00.0	14.58[a]	15.41[a]	00.00[f]	00.00[f]	00.00[f]
03.0	12.08[b]	13.75[b]	01.58[e]	02.58[e]	13.29[e]
06.0	06.33[c]	11.41[c]	05.41[d]	06.84[d]	37.19[d]
09.0	05.33[d]	08.33[d]	08.58[c]	07.75[c]	42.19[c]
12.0	01.33[e]	02.33[e]	13.41[b]	12.92[b]	57.75[b]
15.0	00.00[f]	00.00[f]	16.50[a]	13.49[a]	68.91[a]
$HgCl_2$					
00.0	12.92[a]	17.08[a]	00.00[e]	00.00[d]	00.00[d]
03.0	03.83[b]	06.08[b]	04.83[d]	15.25[b]	57.19[b]
06.0	02.75[c]	04.41[c]	06.58[c]	16.25[a]	71.29[a]
09.0	00.00[d]	00.00[d]	26.08[b]	03.91[c]	16.35[c]
12.0	00.00[d]	00.00[d]	30.00[a]	00.00[d]	00.00[d]
15.0	00.00[d]	00.00[d]	30.00[a]	00.00[d]	00.00[d]

Data represents the mean values of three replications, each consist of 30 explants. Mean values within each column followed by the same letter in superscripts are not differing significantly ($P \leq 0.05$: Duncun's New Multiple Range Test). Treatments of leaf explants with NaOCl and $HgCl_2$ were indipendently analyzed.

Table 3: Effect of various growth regulators on regeneration of bulbils and microshoots from leaf explants of *C. orchioides*. Duration of culture 90 days.

Concentrations (μM)				Sprouting (%)	Length of longest shoot (cm)
BAP	KN	2, 4-D	NAA		
-	-	-	-	49.08[c]	08.3[c]
0.44	-	-	-	78.50[a]	11.5[a]
-	0.46	-	-	75.83[a]	11.0[a]
04.4	-	-	-	73.41[a]	11.5[a]
-	04.6	-	-	71.16[a]	10.2[b]
04.4	04.6	-	-	65.41[b]	08.6[c]
04.4	-	4.5	-	34.83[d]	11.2[a]
-	04.6	-	5.3	30.10[d]	04.0[f]
22.0	-	-	-	36.41[d]	01.2[g]
-	23.0	-	-	36.00[d]	01.6[g]
22.0	-	4.5	-	45.16[c]	08.5[c]
-	23.0	-	5.3	25.10[e]	04.5[f]
04.4	04.6	4.5	-	42.20[c]	07.5[d]
04.4	04.6	-	5.3	26.75[e]	05.5[e]

Data represents the mean values of three replications, each consist of 30 explants. Mean values within each column followed by the same letter in superscripts are not differing significantly (P≤ 0.05: Duncun's New Multiple Range Test).

Table -4: Effect of auxins and cytokinins on regeneration of *in vitro* rooting of shoots derived from rhizome of *C. orchioides* Duration of culture 90 days.

MEDIA (μM)				Rooting (%)	Days to initiate root	Nature of root
Cytokinin		Auxin				
BAP	KN	2,4-D	NAA			
-	-	-	-	52.50[b]	30-35	Linear root
2.2	2.3	4.5	-	31.66[d]	35-40	Linear root
2.2	2.3	-	5.3	44.16[c]	15-20	Smooth, brownish
4.4	4.6	-	5.3	76.66[a]	10-15	Smooth, whitish
-	2.3	4.5	-	52.50[b]	20-25	healthy root, whitish
2.2	-	4.5	-	16.66[f]	20-30	healthy root, whitish
4.4	4.6	4.5	-	18.75[e]	25-30	Linear root, whitish
2.2	2.3	4.5	5.3	52.50[b]	20-25	Healthy root, brownish

Data represents the mean values of three replications, each consist of 20 explants. Mean values within each column followed by the same letter in superscripts are not differing significantly (P≤ 0.05: Duncun's New Multiple Range Test).

Efficacy of NaOCl and $HgCl_2$ on disinfection of leaf tissues

Results on the efficacy of $HgCl_2$ and NaOCl on disinfection of leaf explants of *C. orchioides* are summarized in Table- 2. Bacterial and fungal contamination was reduced significantly with increase in duration of exposure of leaf explants to NaOCl (1%) and $HgCl_2$ (0.1%). In general, the overall recovery of explants was improved with leaf explants as compared to rhizome when disinfected with NaOCl (1%) and $HgCl_2$ (0.1%). In this experiment, disinfection of leaf explants with NaOCl (1%) for 15 min and $HgCl_2$ (0.1%) for 3-6 min shown the highest recovery of explants in the respective experiment (Table 3 and 4). This experiment also revealed that leaf explants of *C. orchioides* was very sensitive to $HgCl_2$ since longer exposure (15 min) of leaf tissues caused more browning (30.0). However,

reduction of exposure time to 6 min minimized the browning of explants significantly (6.58) with the highest percentage of sprouting (71.29%).

The overall response of explants could be correlated with duration of disinfection either with NaOCl or $HgCl_2$. It was observed that longer exposure of leaf tissues to NaOCl resulted in the highest frequency of sprouting (68.9%). Decrease in duration of treatment resulted in gradual reduction of sprouting from 57.75 % - 13.29% (Table 3). A similar experiment carried out with $HgCl_2$ for longer duration (12 and 15 min) resulted in no recovery due to highest rate of browning. However, the duration of exposure of leaf tissues to $HgCl_2$ could be optimized when leaf tissues were surface sterilized for 3 – 6 min. Of the various treatments, disinfection of leaf tissue with $HgCl_2$ (0.1%) for 6 min was more suitable. In the above experiments, shoot regeneration could be obtained from rhizome and leaf explants by optimizing the duration of disinfection with NaOCl (1%) and $HgCl_2$ (0.1%). The effect of surface sterilization of leaf and rhizome explants and their responses during subsequent culture was shown in Figure 1.

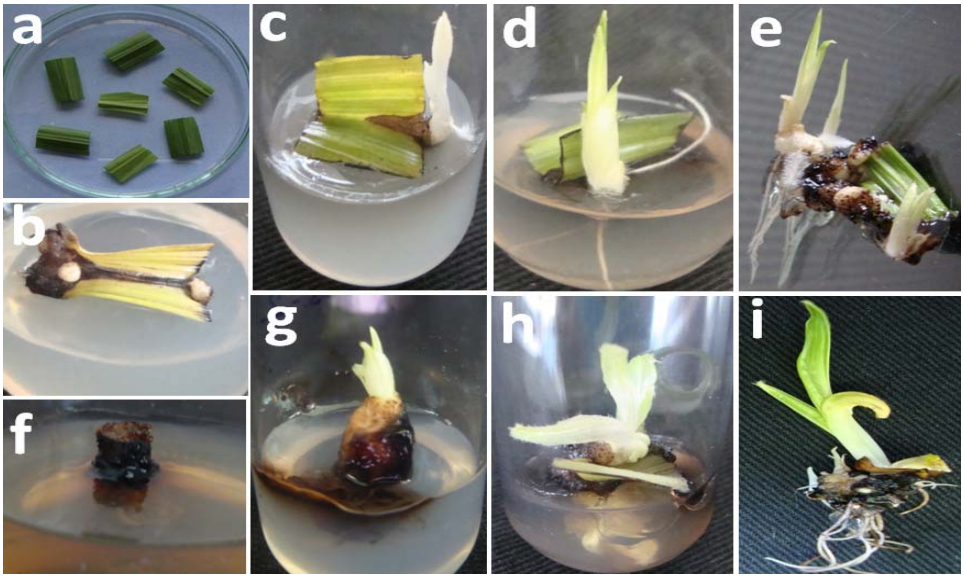

Fig. 1: Initiation of leaf and rhizome cultures after disinfection with 0.1% $HgCl_2$. a) fresh leaf segments before culture on nutrient media, b) browning of leaf explants due to longer exposure to $HgCl_2$, c) leaf explants showing regeneration of shoots after disinfection with 0.1% $HgCl_2$, d) direct shoot development from leaf explants, e) culture shown regeneration of bulbils with development of microshoots and roots, f) browning of rhizome explants due to longer exposure to $HgCl_2$, g) rhizome showing leaching of phenolic compounds into the medium, h) regeneration of shoots with freshly emerging leaves, i) development of plantlets with simultaneous induction of shoot and root system.

Table 5: Relative retention time and peak area of four common peaks of HPLC obtained from rhizome of normal and micropropagated plants of *C. orchioides.*

Peak No	Retention time			Relative peak area		
	Rhizome of normal plant	Rhizome of micropropagated plant	RSD (%)	Rhizome of normal plant	Rhizome of micropropagated plant	RSD (%)
1	2.993	2.727	6.58	095.949	037.276	62.2
2	3.223	3.213	0.22	389.892	123.082	73.5
3	3.510	3.527	0.34	079.842	029.959	65.2
4	4.267	4.270	0.05	010.611	001.527	105.8

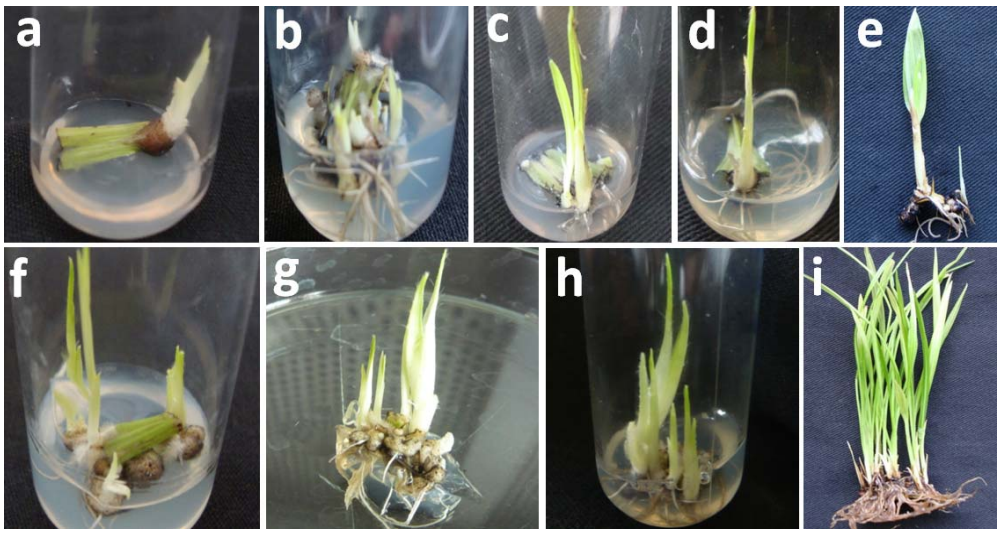

Fig. 2: Effect of auxin and cytokinins on regeneration of bulbils and microshoots from leaf explants of *C. orchioides*: a) response of leaf explants on MS basal medium (control), b) regeneration of multiple shoots with initiation of roots on MS medium containing 0.44 µM BAP, c) regeneration of long shoots in presence of 0.46 µM KN, d) induction on single shoot with many adventitious roots on medium containing 4.4 µM BAP and 4.5 µM 2,4-D, e) development of single shoot on medium containing 4.6 µM KN and 5.3 µM NAA, f) regeneration of multiple shoots in presence of 4.4 µM BAP and 4.6 µM KN, g - i) culture showing regeneration of bulbils and high frequency multiple shoots.

Effect of auxin and cytokinins on shoot regeneration

Leaf explant of *C. orchioides* was found to respond well within 2 - 3 weeks of initial culture with varying percentage of sprouting from 25.1% - 78.5% depending upon the concentrations and combinations of BAP, KN, 2, 4-D and NAA used in this experiment. Of the 13 combinations of media tested, highest percentage of sprouting was observed in presence of 0.44 µM BAP with 78%. Lowest percentage of response was observed when leaf explants were cultured in medium containing 23.0 µM BAP and 5.3 µM NAA with only 25.1%. Interestingly, explants cultured in MS basal medium shown 49% response during initial culture. But further growth and development was minimized due to lack of any growth regulators in the medium.

It was observed that multiple shoots regenerated from leaf explants on two different composition of medium containing 0.44 µM, 4.4 µM BAP and 0.46 µM KN had produced the longest shoots with 11.5 cm and 11.0 cm respectively. In these combinations, large number of healthy microshoots could be regenerated either from bulbils or directly from the leaf tissues. Increase in concentration of BAP and KN alone or in combinations with 2, 4-D and NAA was found to produce mostly single shoot per leaf explant. The highest concentrations of KN (23.0 µM) in combination with NAA (5.3 µM) and another combination with BAP (4.4 µM), KN (4.6 µM) and NAA (5.3 µM) tested in this experiment was found to reduce the response of explants with 25.1% and 26.7% respectively with poor regeneration of bulbils. This experiment clearly indicated that presence of BAP either at lower (0.44 µM) or higher (4.4 µM) concentrations and similarly KN at lower (0.46 µM) and higher (4.6 µM) concentrations were effective for regeneration of healthy microshoots from leaf tissues of *C. orchioides*. It was observed that higher concentration of KN at 23.0 µM and NAA at 5.3 µM was not effective unlike BAP and 2, 4-D. The effect of various treatments on shoot regeneration was shown in Figure 2.

Effect of auxin and cytokinins on regeneration of adventitious roots

The effect of auxin and cytokinins on induction of adventitious roots from the microshoots of *C. orchioides*. In control, about 52.5% of the microroots produced adventitious roots within 30

days of culture. However, presence of anyone of the auxin (2, 4-D and NAA) along with BAP and KN induced adventitious roots in varying percentages ranging from 16.66% - 76.66%. It was observed that microshoots cultured on medium containing BAP (4.4 µM), KN (4.6 µM) and NAA (5.3 µM) had induced the highest percentage of rooting with 76.66%. On this medium, most of the microshoots had produced roots within 10 – 15 days of culture. The days required for initiation of roots was varying from 10 – 35 days depending upon the presence of auxin and cytokinins. In other media, initiation of roots was considerably delayed. The number of roots produced by the shoots was varying from 7 – 40 depending upon the culture conditions. The highest number of roots per shoot (40.41) could be observed when microshoots were cultured on medium containing BAP (4.4 µM), KN (4.6 µM) and NAA (5.3 µM). In other media, the number of roots produced by the shoot was significantly reduced ranging from 10 – 25 roots per shoot. In this experiment, the roots produced by the microshoots were varying in morphology and color (Fig. 3). Most of the microshoots had produced smooth roots. These roots were either brownish or whitish in color. Adventitious roots produced by the most of the microshoots were healthier.

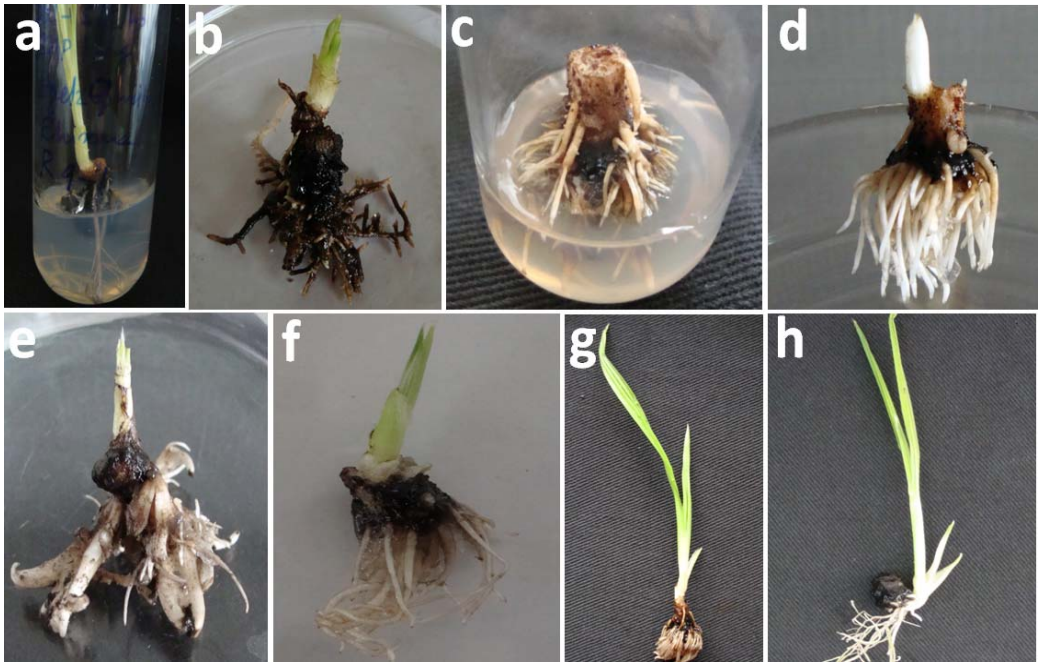

Fig. 3: Effect of auxin and cytokinins on regeneration of adventitious roots from rhizome explants of *C. orchioides*: a) rhizome explant showing the development of normal root on MS basal medium (control), b) rhizome showing simultaneous development of shoot with short brown color roots in medium containing 2.2 µM BAP, 2.3 µM KN and 4.5 µM 2,4-D, c & d) rhizome showing initiation of shoot with large number of whitish – brown shorter roots on medium containing 4.4 µM BAP, 4.6 µM KN and 5.3 µM NAA, e) initiation of shoots and roots on medium containing 4.4 µM BAP, 4.6 µM KN and 4.5 µM 2,4-D, f) regeneration of healthy single shoot with well developed roots on medium containing 2.2 µM BAP, 2.3 µM KN, 4.5 µM 2,4-D and 5.3 µM, g & h) well developed plantlets with good shoot and root system.

High performance liquid chromatography (HPLC)

Data on retention time and relative peak areas of four prominent peaks observed in the HPLC chromatogram from the rhizome extracts of normal and micropropagated plants of *C. orchioides* is summarized (Table 5). Four common and prominent peaks were seen in both rhizome extract of normal as well as micropropagated plants. The retention time of peak number 1, 2, 3 and 4 of normal plant was 2.993, 3.223, 3.510 and 4.267. A similar tendency of retention time was noticed with rhizome extract of micropropagated plants. The values of retention times between normal and micropropagated plants of *C. orchioides* were

very closer with their corresponding peaks. However, the highest RSD value was observed in peak 1. Whereas, peak number 2, 3 and 4 shown the lowest RSD values. Since the four peaks of RSD values between normal and micropropagated plants were much closer with less than 1%, the corresponding peaks between normal and micropropagated plants were phytochemically same. In this experiment, the peak areas of all the four compounds were varying with their RSD values. The four peaks obtained in the chromatogram of HPLC was appears to be more prominent in the rhizome extract of *C. orchioides.*

Table - 5: Relative retention time and peak area of four common peaks of HPLC obtained from rhizome of normal and micropropagated plants of *C. orchioides.*

Peak No	Retention time			Relative peak area		
	Rhizome of normal plant	Rhizome of micropropagated plant	RSD (%)	Rhizome of normal plant	Rhizome of micropropagated plant	RSD (%)
1	2.993	2.727	6.58	095.949	037.276	62.2
2	3.223	3.213	0.22	389.892	123.082	73.5
3	3.510	3.527	0.34	079.842	029.959	65.2
4	4.267	4.270	0.05	010.611	001.527	105.8

Discussion

This study, revealed optimum concentration of $HgCl_2$ (0.1%) for effective disinfection of rhizome and leaf explants. Explants disinfected with $HgCl_2$ (0.1%) for rhizome 15 min and leaf 3 min recorded considerable number of explants for establishment of aseptic culture. In general, $HgCl_2$ was reported to be an effective disinfectants and widely used in micropropagation of several medicinal plants such as *Curcuma zedoaria* (Stanly and Keng, 2007), *C. orchioides* (Nagesh *et al*., 2008), *Thalictrum dalzellii* (Sharanappa and Rai, 2011). Thus, the present study revealed that $HgCl_2$ is more effective than NaOCl for elimination of microbial contamination in rhizome and leaf explants of *C. orchioides* as reported in many plant species.

Experiments carried out for optimizing the concentrations of BAP, KN, 2, 4-D and NAA revealed that 0.44 µM, 4.4 µM of BAP and 0.46 µM, 4.6 µM of KN were effective for inducing the maximum number of bulbils with high frequency multiple shoots. Shoots regenerated in presence of BAP at 0.44 µM, 4.4 µM attained the length of 11.5 cm while the higher concentration of BAP and KN had induced shorter shoots with 1.2 cm and 1.6 cm respectively. Thus, BAP at 0.44 µM was more suitable than other concentration for inducing longer shoots in micropropagation of *C. orchioides*. However, the number of shoots obtained per leaf explant was very low even at higher concentration of various cytokinins either alone or in combination with auxin. In general, explants sourced from herbaceous plants are highly responsive to BAP and most of the cultures produced healthy shoots suitable for subsequent shoot proliferation in micropropagation (Debergh and Zimmerman, 1991). A number of reports are also available, where MS medium with different concentrations and combination of BAP and NAA were used for high frequency shoot regeneration from different explants of *Zingiber officinale* (Bhagyalakshmi and Singh, 1988; Ikeda and Tanabe, 1989).

Experiment carried out with cytokinins (BAP and KN) in combination with auxin (2, 4- D and NAA) for *in vitro* rooting of microshoots of *C. orchioides* had shown varying responses with regard to percentage of rooting and number of roots produced by a shoot. Presence of 4.4 µM BAP, 4.6 µM KN and 5.3 µM NAA had induced the highest percentage of rooting (76.6%), with maximum number of roots/shoot (40.4). All the seven combinations of media had induced healthy and longer roots with were either brownish or white in color. In *C. orchioides*, it was reported that emergence of roots occurred within 15 – 20 days and further incubation of one week lead to vigorous root growth with the development of maximum number of roots in presence of 0.53 µM of NAA (Wala and Jasrai, 2003). In *Curcuma longa*, microshoots produced from the rhizome were transferred from solid – liquid medium for enhancing the development of roots (Nadgauda *et al*, 1978). But, in the present experiment, large number of microshoots was

converted into complete plantlets with healthy root system without transfer of microshoots from solid to liquid medium, avoiding an addition step besides to reduce the cost of production of plantlets.

Comparative study of rhizomes obtained from normal and micropropagated plants of *C. orchioides* was carried out by performing HPLC finger prints. This experiment had shown four common and prominent peaks from the rhizome of normal and micropropagated plants. It was reported that if the relative standard deviation (RSD) of retention time of common peaks for different samples is less than 1%, these peaks can be referred to be the same compound. This analysis was already reported in *Erigeron breviscapus* (Lie *et al*., 2004; Liu *et al*., 2007). It was reported that if the RSD of relative peak area of common peaks is more than 10%, it can be referred as different compounds as previously reported in *E. breviscapus* (Liu *et al*., 2008). However, Akarasereenont *et al*., (2010) have reported that if the RSD of relative retention times of common peaks were 1% - 10%, the compounds can be considered as same phytochemically. Thus the four peaks noticed in the HPLC chromatogram of rhizome obtained from micropropagated and normal plants were considered as same phytochemically since the RSD values were less than 1%. To conclude, appropriate disinfectant and optimization of surface sterilization is expected to be helpful in large scale establishment of aseptic culture of *C. orchioides*. The present protocol for mass propagation of *C. orchioides* is reliable and reproducible. Chemical profile, generated from *in vitro* regenerated plantlets suggests that the micropropagated plants are phytochemically similar to that of normal plant.

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