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In Vitro Propagation of an Endangered Medicinal Plant Curculigo Orchioides Gaertn.

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ABSTRACT

The herbal drug has been in use for the treatment of various diseases since ancient times. 75-80% of the whole population in developing countries depends on the medicinal plants for their primary health care. *Curculigo orchioides* commonly known as 'Nilappana kizhangu' is an endangered medicinal plant and as such requires be conserving and domesticating. It is extensively utilized as a nutritive tonic for strength, vigour, and vitality. The main objective of the study is for developing a standard protocol for *in vitro* propagation of *C. orchioides* through rhizome disc. The multiple shoots development was observed directly on the rhizome proximal disc at *in vitro* condition on MS medium enhanced with different concentration of auxin and cytokinin. The sixty days old developed shoots were excised, subjected to vertical cuts (two to three) and inoculated into MS medium supplemented with a combination of BAP (4.44 –13.32 µM), Kn (2.32-4.65 µM) and IAA (2.85-5.71 µM) for multiple shoot induction. The optimum number of shoots (9.08±0.47) developed from a combination of BAP (11.10 µM), Kn (2.32 µM) and IAA (5.71 µM) supplemented medium, 90±8.66% of shoot response and the average height was 3.28±0.47 cm on the 60th day of observation in medium containing 8.88 µM of BAP, 4.65 µM of Kn and 5.71 µM of IAA. The rooting efficiency of shoots (100%), as well as the best root number per shoot (14±0.21) and length (11.92 ± 0.29 cm), were obtained on MS media supplemented with 10.72 µM of NAA supplemented MS medium.

1. introduction

The medicinal plant has been in use for the treatment of various diseases since ancient times. The practice of transmitting healing knowledge from generation to generation has enabled the consistency of application of given herbs for specific ailments. According to WHO report, herbal medicine is still the mainstay of therapy for about 75-80% of the whole population in developing countries for primary health care [1]. The herbal drug is because of better cultural acceptability, affordability, compatibility with fewer side effects. The validation of the folkloric claims of these medicinal plants will provide a scientific basis for the conservation of tropical therapeutic resources, the deployment of the beneficial ones as a phytomedicine in the primary health care and the development of potential bioactive constituents. These could provide novel compounds or precursors in drug development, and utilization of isolated compounds as investigative, evaluative, and other research tools in drug development and testing processes. The plant kingdom thus represents an enormous reservoir of pharmacologically valuable substance to be discovered [2]. About 25% of the prescribed medicines in the market are of plant origin, and some 120 plants derived metabolites from about 90 plant species used in modern therapy. Of the about 4,00,000 plant species on the Earth, only a few percentages have been phytochemically investigated, and the fraction submitted to pharmacological screening is even lower [3]. *Curculigo orchioides* Gaertn. an endangered medicinal herb belonging to the family Hypoxidaceae. The species is a stem less perennial herb of therapeutic importance and a native of India. "Kali musli" is reported to have hypoglycemic, spasmolytic and anticancer potential. The rhizomes are washed, freed from roots,

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and sliced; the slices dried in the shade. Usually, the dried slices are powdered, and small amounts of powder are mixed in a glass of milk with sugar or used to prepare a decoction for drinking. The rhizome extract shows hypoglycemic, spasmolytic, and anticancer activities. The powdered rhizome applied to cuts is said to stop bleeding and dry up wounds. The rhizome also prescribed for the treatment of piles, jaundice, asthma, diarrhea [4] and on pimples [5]. It also used as an antioxidant [6], spermatogenic [7], hepatoprotective [8], immunostimulant [9], anticancer [10]. antibacterial [11], antiosteoporotic [12], and hypoglycaemic [13]. Trade in herbal is growing in volume. It is estimated that the value of global trade in medicinal plants is around the US $ 800 million per year. China, with exports of over 1,20,000 tons per annum and India with some 32,000 tons per annum, dominate the international market. The botanical market, inclusive of herbs and medicinal plants, in the USA, was estimated approximately at the US $ 1.6 billion per annum. It was determined that Europe, annually, imports about 4,00,000 tons of medicinal plants with an average market value of US $ one billion from Africa and Asia [14]. While the demand for herbal is growing, some of them are increasingly being threatened in their natural habitats. For meeting future needs the cultivation of medicinal plants and tissue culture methods have to be encouraged. The tissue culture methods for rapid multiplication of *C. orchioides* are highly advantageous to meet the commercial demand and to conserve valuable endangered plants [15],[16]. Such research offers vast potentialities for large-scale clonal propagation, obtaining disease free-plant materials, and producing biologically active compounds for pharmaceutical use [17].

2. Materials and Methods

Plant material

Curculigo orchioides Gaertn. Fruct. 1: 63. t. 16. f. 11. 1788; Hook. f. Fl. Brit. India 6: 279. 1892; Fischer in Gamble, Fl. Pres. Madras 3: 1050. 1957 (repr. ed.). *C. malabarica* Wight, Ic. t. 2043A. 1853.



Fig. 1: *Curculigo orchioides* Gaertn.

Explant source

C. orchioides collected from Cuddalore District, Tamil Nadu, India. The plant specimen confirmed by Dr. P. Jayaraman, Director, Plant Anatomical Research Centre, Chennai, Tamil Nadu, India. The plants raised in pots containing a mixture of soil and farmyard manure (FYM) in the ratio of 2:1 at the botanical gardens of J.J. College of Arts and Science, Pudukkottai, Tamil Nadu, India.

Pre-treatment of mother plant

The mother plant maintained in a poly house. The pots were watered once in alternate days. Two to three days before harvesting the explants (Rhizome and leaves) of the mother plant pretreated with 0.1 % Bavistin + 0.1 % Dithane M-45.

Collection of explants

Small disease free rhizome collected from 4-6 months grown plants, cut into 1.5 - 2.0 cm, segments of proximal discs from rhizome used as explants to the induction of multiple shoots.

Sterilization of explants

Explants were clean thoroughly under running tap water for 10 min then washed in an agitated solution of 2% (v/v) Tween 20 (a mild detergent) for 10 minutes and washed with tap water. Then the explants were treated with a fungicide (1 g/l of bavistin) and bactericide (100 mg/l of streptomycin) for 2 hrs. (Proximal discs). Finally, the explants cleaned with double distilled water for clearing the fungicide and bactericide on the explant. The treated explants transferred into the Laminar Air Flow chamber for further sterilization. Then the explants were submerged in 70% ethanol for 60 seconds, followed by rinsing in HgCl2 (0.1%) for 5 minutes and washed thrice with sterile distilled water. After surface sterilization, both ends of the explant were cut and trimmed to 1 cm size.

Preparation of stock solution

MS Medium used in these studies [18]. The stock solutions of micro and macronutrients, iron chelates, and vitamins from different media prepared separately. The stock of iron stored in an amber color bottle.

Preparation of growth hormones

The growth regulators were dissolved independently in respective solvents. The molecular weight of each auxin such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) was individually dissolved in 0.1 N NaOH and diluted with 100 ml sterile distilled water to make the stock solution in such a way that ml represented 10 M of each. Similarly, the molecular weight of cytokinin such as 6-benzylaminopurine (BAP), and kinetin (Kn) were dissolved in 0.1 N HCl and diluted with 100 ml sterile distilled water to make the stock solution (10 M). All the hormones stored under refrigeration. These hormones were thermostable at 121˚C and to be added before autoclaving the medium [19],[20].

Preparation of culture medium

The working tissue culture medium (Concentration µM) was prepared from the stock solutions and made up to the required quantity using double distilled water with the addition of growth regulators depending on the nature of the experiment. The glassware and other accessories were sterilized at 180ºC for two hours using a hot air oven. The MS medium adjusted to pH 5.8 and 0.8% agar added. The medium was boiled up to milky appearance for dissolving the agar in the medium. About 50 ml of

the medium was dispersed in each culture bottle and sealed with a clean wrap cover before autoclaving at 121°C for 18 minutes under pressure of 15 Psi. The mediums in culture bottles were left to cool in the culture room until use. Under laminar air flow cabinet the surface sterilized explants were inoculated aseptically in the MS medium supplemented with various concentrations (µM) of phytohormones alone or in combinations such as 6-Benzylaminopurine (BAP), Kinetin (Kn), Indole-3-butyric acid (IBA), Naphthaleneacetic acid (NAA) and Indole-3-acetic acid (IAA). 0.5 g/l of activated charcoal powder added into the nutrient medium. The addition of activated charcoal to culture medium may be promoted or inhibit *in vitro* growth of the plant, and it depends on species and types of tissues used. The effects of activated charcoal may be attributed to establishing adsorption of undesirable/inhibitory substances; a darkened environment; adsorption of growth regulators and other organic compounds, or the releases of growth promoting substances present in or adsorbed by activated charcoal.

Effects of hormone strength on regeneration

The plant was regenerated directly from the explant by the influence of hormone.

Shoot regeneration from proximal rhizome discs

The MS medium supplemented with different concentrations and combinations of BAP (2.22-8.88 µM) for shoot regeneration. The proximal rhizome discs inoculated into the medium. The cultures incubated under the dark condition for three days; then fluorescent lights with 1500-2000 lux for 10 hours up to 10 days. Finally, the lighting duration increased to 14 hrs. from 11^{th} day onwards. The culture room maintained with a temperature of 25 ± 1°C and 60±10 relative humidity during shoot regeneration and scalp induction. The data recorded on the 10^{th}, 30^{th}, 45^{th}, & 60^{th} day of cultivation.

Sub-culture and shoot multiplication

The developed shoots were excised, subjected to vertical cuts (two to three) and then transferred to MS medium addition with a combination of BAP (4.44 – 13.32 µM), Kn (2.32- 4.65 µM) and IAA (2.85-5.71 µM) for multiple shoot induction.

Shoot elongation of multiple shoot*s*.

The MS medium was supplemented with different concentrations BAP (4.44- 11.10µM) in combinations with IAA (2.85 – 8.56 µM) for shoot initiation. The sterilized shoot tip inoculated into the culture medium.

The concentration of hormones for *in vitro* rooting

For root induction, *in vitro* - developed shoots were excised and cultured on MS medium supplemented with in combination of different concentrations of auxin (2.69-13.43 µM of NAA/2.46-12.30 µM of IBA/ 2.85 – 11.42 µM of IAA). The cultures grown for 60 days; then the data were recorded on 10^{th},30^{th}, 45^{th}, & 60^{th},day of observation.

3. Results and Discussion

The surface sterilized proximal rhizome discs as explants were cultured on MS basal medium supplemented with different concentrations of BAP (2.22-8.88 µM) combine with IBA (2.46-7.38 µM) respectively. 40% of the explants produced shoots with an average of 20 explants with three replicates. Initially, there was no shoot initiation on the 10^{th} and 30^{th} day of observations. The shoot initiation observed on the 45^{th} day of view in BAP in combination with IBA (Fig.2.B).

Influence of BAP and IBA for shoot initiation from proximal rhizome disc

The shoot growth performance, number, and length varied among the proliferated shoots. In the 60^{th} day the maximum number of the shoot (4.0±0.29) and shoot length was 2.9±0.30cm observed on MS medium supplemented with 11.10 µM of BAP and 4.92 µM of IBA (Table – 1) (Fig.2.B). The BAP (4.44-8.88 µM) combine with IBA (2.46-4.92 µM) supplemented medium to show the expression of multiple shoots. The shooting range between 0.96±0.43 - 4.0±0.29 and their shoot length was 0.32±0.05- 2.9±0.30. The shoot induction was weak and also the media turn to black on BAP (2.22 – 11.10 µM) combine with a higher concentration of IBA (7.38 µM) supplemented growth media (Table - 1).

Table-1: Effects of a combination of cytokinin (BAP) and auxin (IBA) on *in vitro* shoot proliferation from proximal rhizome discs segments of *C. orchioides*

Plant growth regulators µM		Number of explants	% of response	Number of shoot proliferation				Shoot length on the 60th day
BAP	IBA			10th day	30th day	45th day	60th day	
2.22	2.46	20	-	-	-	-	-	-
4.44	2.46	20	24.35±1.35	-	-		1.04±0.37	0.32±0.05
6.66	2.46	20	25.33±2.01	-	-	1.06±0.30	1.0±0.38	0.45±0.06
8.88	2.46	20	29.56±0.97	-	-	1.0±0.41	1.06±0.40	0.46±0.05
11.10	2.46	20	-	-	-	-	-	-
2.22	4.92	20	-	-	-	-	-	-
4.44	4.92	20	20.02±1.04	-	-	0.92±.34	1.88±0.39	1.16±0.25
6.66	4.92	20	24.75±1.15	-	-	0.94±0.50	0.96±0.43	1.1±0.212
8.88	4.92	20	20.80±1.57	-	-	1.1±0.30	0.98±0.32	1.96±0.43
11.10	4.92	20	31.10±1.50	-	-	1.96±0.28	4.0±0.29	2.90±0.30
2.22	7.38	20	-	-	-	-	-	-
4.44	7.38	20	22.10±2.01	-	-	-	1.08±0.47	0.34±0.05
6.66	7.38	20	-	-	-	-	Media turn to brown	
8.88	7.38	20	-	-	-	-	Media turn to brown	
11.10	7.38	20	-	-	-	-	Media turn to brown	

Sub-culture - shoot multiplication from initiated shoot

The sixty days old developed shoots were excised, subjected to vertical cuts (two to three) and inoculated into MS medium supplemented with a combination of BAP (4.44 –13.32 µM), Kn (2.32- 4.65 µM) and IAA (2.85-5.71 µM) for multiple shoot induction (Table – 2). The optimum number of shoots (9.08±0.47) developed from a combination of BAP (11.10 µM), Kn (2.32 µM) and IAA (5.71 µM) (Fig-2.C) supplemented medium, 90±8.66% of shoot response and the average height was 3.28±0.47 cm followed by 7.9±0.21 number of shoot with height of 3.9±0.21 cm on the 60th day of observation in medium containing 8.88 µM of BAP, 4.65 µM of Kn and 5.71 µM of IAA. Multiple shoot number increased (6.96±0.43), and also the height of the shoot (6.1±0.30) at higher concentrations of BAP (13.32 µM) and IAA (5.71 µM) supplemented medium on the 60th day of observation, but the medium turned into black colour. The multiple shoot base presence a bit of callus. The callus was arresting the initiation of root from the base of the shoot. So, the 60 days old shoot need to culture another multiple shoot induction media for preventing callus induction.

Table-2: The effect of different concentration of cytokinin (BAP & Kn) and auxin (IAA) for shoot multiplication from the initiated shoot of *C. orchioides*

Plant growth regulators (µM)			Number of explants	% of response	Number of shoot proliferation				Shoot length on the 60th day
BAP	Kn	IAA			10th day	30th day	45th day	60th day	
4.44	2.32	2.85	20	61.1±1.05	-	1.0±0.31	1.0±0.31	1.0±0.31	5.5±0.31
6.66	2.32	2.85	20	83.15±2.07	-	4.0±0.41	4.0±0.41	4.0±0.41	6.0±0.41
8.88	2.32	2.85	20	90.16±1.8	-	2.92±0.34	3.92±0.34	4.92±0.34	4.22±0.34
11.10	2.32	2.85	20	81.30±1.33	-	2.94±0.50	6.94±0.50	6.94±0.50	4.44±0.50
13.32	2.32	2.85	20	91.34±1.63		5.1±0.30	7.1±0.30	7.1±0.30	4.9±0.30
4.44	4.65	2.85	20	91.12±1.04	-	1.1±0.35	1.1±0.35	1.1±0.355	5.1±0.35
6.66	4.65	2.85	20	90.10±2.11	-	3.04±0.37	3.04±0.37	3.04±0.37	1.54±0.37
8.88	4.65	2.85	20	75.66±5.13	-	0.96±0.28	1.96±0.28	4.96±0.28	1.96±0.28
11.10	4.65	2.85	20	91.11±2.00	-	2.05±0.36	3.06±0.40	4.06±0.40	6.06±0.40
13.32	4.65	2.85	20	83.45±2.54	-	1.9±0.35	2.88±0.39	3.88±0.39	5.88±0.39
4.44	2.32	5.71	20	69.45±1.69	-	3.0±0.29	4.0±0.29	4.0±0.29	3.0±0.29
6.66	2.32	5.71	20	63.47±1.78	-	3.95±0.36	5.95±0.36	5.95±0.36	2.45±0.36
8.88	2.32	5.71	20	91.15±2.31		2.3±0.28	4.07±0.40	7.07±0.40	1.87±0.40
11.10	2.32	5.71	**20**	92±5.29	-	5.08±0.47	7.08±0.47	9.08±0.47	3.28±0.47
13.32	2.32	5.71	20	90.75±1.72		5.88±0.39	6.88±0.39	6.88±0.39	5.88±0.39
4.44	4.65	5.71	20	52.42±1.75	-	3.91±0.40	4.92±0.40	4.92±0.40	3.32±0.40
6.66	4.65	5.71	20	91.10±2.01	-	4.16±0.25	5.16±0.25	7.16±0.25	3.66±0.25
8.88	4.65	5.71	20	90.56±2.33	-	3.9±0.21	5.9±0.21	7.9±0.212	3.9±0.21
11.10	4.65	5.71	20	91.45±1.94	-	4.96±0.43	4.96±0.43	6.96±0.43	5.46±0.43
13.32	4.65	5.71	20	89.58±1.41	-	4.17±0.29	5.1±0.30	7.1±0.30	6.1±0.30

Multiple shoots induction and shoots elongation of regenerated shoot

The sixty days old regenerated shoots cut crossly and inoculated on MS basal medium supplemented with different concentrations of BAP (4.44- 11.10 µM) in combination with IAA (2.85 & 8.56 µM), (Table – 3). 75.66±5.13 - 92±5.29 % of the explants produced shoots with an average of 20 explants with three replicates. Initially, there was no shoot initiation on the 10th day of observation. The shoot regeneration was observed on the 30th day of inspection in all the growth regulator supplemented MS media, but the shoot number and length varied among the samples (Fig.2.D). In the 60th day, the maximum number of shoot (11.02±0.41) and shoot length was 3.04±0.29 cm observed on MS medium supplemented with 8.88 µM BAP and 5.71 µM of IAA (Table – 3; Fig.2.D) followed by the medium containing 11.10 µM BAP and 5.71 µM of IAA in which the shoot number was 10.04±0.36 and shoot height 4.0±0.22 cm and 8.88 µM of BAP

and 2.85 µM of IAA in which the shoot number was 10.04±0.38 and shoot height was 3.02±0.25 cm. The shoot height was an increase (4.41±0.31) in MS medium supplemented with 8.88 µM of BAP and 8.56 µM IAA, but the shoot number decreased (4.95±0.35).

Table – 3. The influence of BAP and IAA on *in vitro* shoot proliferation from shoot tip of

C. orchioides

Plant growth regulators µM		Number of explants	% of response	Number of shoot proliferation				Shoot length on the 60th day
BAP	IAA			10th day	30th day	45th day	60th day	
4.44	2.85	20	79.82±2.01	-	3.16±0.48	4.1±0.17	6.40±0.40	2.05±0.18
6.66	2.85	20	81.34±1.75	-	3.18±0.48	4.2±0.34	6.25±0.43	2.46±0.30
8.88	2.85	20	92.45±0.98	-	4.16±0.38	6.08±0.22	10.0±0.38	3.02±0.25
11.10	2.85	20	74.26±1.04	-	4.03±0.5	7.08±0.34	8.20±0.40	2.78±0.23
4.44	5.71	20	79.52±1.05	-	5.03±0.49	6.15±0.43	6.06±0.33	3.06±0.32
6.66	5.71	20	82.86±0.98	-	6.18±0.28	7.14±0.42	8.12±0.27	2.92±0.26
8.88	5.71	20	91.75±1.75	-	7.2±0.43	9.07±0.36	11.02±0.41	3.04±0.29
11.10	5.71	20	93.47±2.14	-	9.18±0.50	9.96±0.41	10.04±0.36	4.0±0.22
4.44	8.56	20	90.15±2.91	-	5.31±0.33	8.11±0.38	10.03±0.32	3.46±0.25
6.66	8.56	20	82.46±1.73	-	5.1±0.26	6.14±0.42	6.04±0.36	4.3±0.26
8.88	8.56	20	72.37±2.66	-	5.1±0.26	5.21±0.43	4.95±0.35	4.41±0.31
11.10	8.56	20	80.10±1.33	-	3.26±0.26	3.23±0.46	3.03±0.32	3.38±0.30

The multiple shoot induction was very low (3.03±0.32) in the MS medium supplemented high concentration of 11.10 µM of BAP and 8.56 µM of IAA (Table – 3). The BAP combine with IAA supplemented MS medium initiated multiple shoot induction in all the measured concentration from the 60-day old regenerated shoot, but these combinations are ineffective to initiative any multiple shoots from proximal rhizome disc directly. The multiple shoot range between 3.03±0.32 (11.10 µM of BAP combine with 8.56 µM of IAA) and 11.02±0.41(8.88 µM of BAP combine with 5.71 µM of IAA) and shoot length is between 2.05±0.18 and 4.41±0.31 cm at 60 days of incubation.

In vitro production of roots

The sixty days old regenerated shoots were transferred into MS media supplemented with a combination of auxin such as NAA (0.27 & 2.69-13.43 µM)/IBA (2.46-12.30 µM)/IAA (2.85-14.27 µM) (Table - 4). There was no root initiation observed on the 10th day. But the induction of root growth from the shoot was observed on the 30th, 45th & 60th day of observations. The root number and length were noted.

The efficiency of root development from regenerated shoots by the influence of cytokinin (BAP)

and auxin (IBA, NAA, and IAA) combinations

The BAP (2.22 µM) combine with various concentrations of NAA (2.69-13.43 µM), IBA (2.46- 12.30 µM) and IAA (2.85-14.27 µM) supplemented in MS medium individually for the root initiation and development. The concentration of auxin and their types were responsible for the predominant role in the initiation and developments of the root *in vitro* condition. The rooting efficiency of shoots (100%), as well as the best root number per shoot (8.25±0.31) and length (3.05 ± 0.17 cm), was obtained on MS medium (Table - 4). & Fig.2.E) supplemented with 2.22 µM of BAP and 10.74 µM of NAA followed by 2.22 µM of BAP and 8.06 µM of NAA supplemented MS medium (7.58±0.44 number with 2.40±0.36 length) and 2.22 µM of BAP with 9.84 µM of IBA supplemented MS medium (7.1±0.35 number with 6.40±0.35 length) on 60th day**.** The root initiation observed on the 45th day of observation, but no root found on 10th & 30th day. The root development was weak in the combination of 2.22 µM of BAP,

and 2.85-11.42 µM of IAA supplemented medium, the root number ranged between 4.10±0.49 and 2.85±0.3 with the length of 1.98±0.33 – 1.25±0.35 (Table - 4). In this observation NAA combine with BAP has influenced the formation of the number of roots (5.65±0.36 - 8.25±0.31) but the root length is minimum (1.65±0.46 - 3.05±0.17) compare to IBA combine with BAP (root number 5.1±0.40 - 7.1±0.35 and root length was between 2.75±0.26 and 6.40±0.35. The rooting efficiency of the shoots should be reduced in the IAA combination with BAP supplemented medium, the number of roots initiation observed the range between 2.85±0.34 - 4.10±0.45 and the length was 1.25±0.35 - 1.98±0.33 cm on the 60th day of observation. The BAP combine with IBA, NAA, and IAA supplemented to MS medium individually for root initiation and development. The rooting efficiency was better in the combination of BAP with NAA compare to IBA and IAA for the number and length of roots per shoot. In this study is indicated that the combination of BAP and NAA is the best for root initiation from the shoot at *in vitro* conditions.

Table - 4: The concentration of cytokinin (BAP) and auxin (IBA, NAA & IAA) for rooting of *in vitro* - derived shoots of *C. orchioides*

Plant growth regulators µM				Number of shoots cultured	% of response	Number of roots per shoot on the 45th day	Number of roots per shoot on the 60th day	Root length (cm)
BAP	IBA	NAA	IAA					
2.22	2.46	-	-	40	86.3±3.21	4.42±0.16	5.1±0.40	2.75±0.26
2.22	4.92	-	-	40	89.6±2.51	4.51±0.31	5.87±0.38	3.50±0.24
2.22	7.38	-	-	40	90.3±2.50	5.21±0.24	6.55±0.50	4.93±0.40
2.22	9.84	-	-	40	80.6±3.05	6.5±0.15	7.1±0.35	6.40±0.53
2.22	-	0.27	-	40	87.7±3.05	4.11±0.41	4.73±0.23	2.72±0.21
2.22	-	2.69	-	40	89.3±3.05	5.1±0.42	5.65±0.36	1.65±0.46
2.22	-	5.37	-	40	94.6±1.52	5.3±0.23	6.95±0.42	2.10±0.46
2.22	-	8.06	-	40	97.6±2.51	6.3±.12	7.58±0.44	2.40±0.36
2.22	-	10.74	-	40	86.0±3.60	6.1±.62	8.25±0.31	3.05±0.17
2.22	-	-	2,85	40	80.6±3.05	2.6±0.14	3.12±0.40	1.25±0.35
2.22	-	-	5.71	40	89.3±3.04	3.4±0.12	4.10±0.45	1.48±0.46
2.22	-	-	8.56	40	85.0±2.0	2.45±0.33	2.90±0.24	1.68±0.37
2.22	-	-	11.42	40	82.0±3.6	2.12±0.42	2.85±0.34	1.98±0.33

Hardening of the regenerated plant

The hardening process was carried out by transferring rooted plantlets on plastic pots 8.0 cm in diameter containing a mixture of sterilized coco pith and sand in the ratio 5:1 for primary hardening (Fig.2.F&G). The plastic pots were kept in covered polythene packs for a week in incubation at 25±2^{o}C for 16 h photoperiods. After a month, they were transferred to another poly bags containing an equal quantity of red soil, sand and farmyard manure (1:1:1) for secondary hardening and kept in the greenhouse for the one-month duration. After one month of incubation, the tuberous roots were observed on the shoot and also reduced the number of the fine root (Fig.2.H). Then the hardening plants were transferred to the field. The combination of BAP and Kn are ineffective at a different concentration level in this study. The present result is controversy to an earlier report of [21],[22] said that the proximal rhizome discs are optimal for high-frequency multiple shoot formation than shoot tip and distal rhizome disc, synergistic effect of 4.44 µM of 6-benzylaminopurine (BAP) and 4.65 µM of Kinetin (Kn) on the regeneration of shoot buds from proximal rhizome disc.

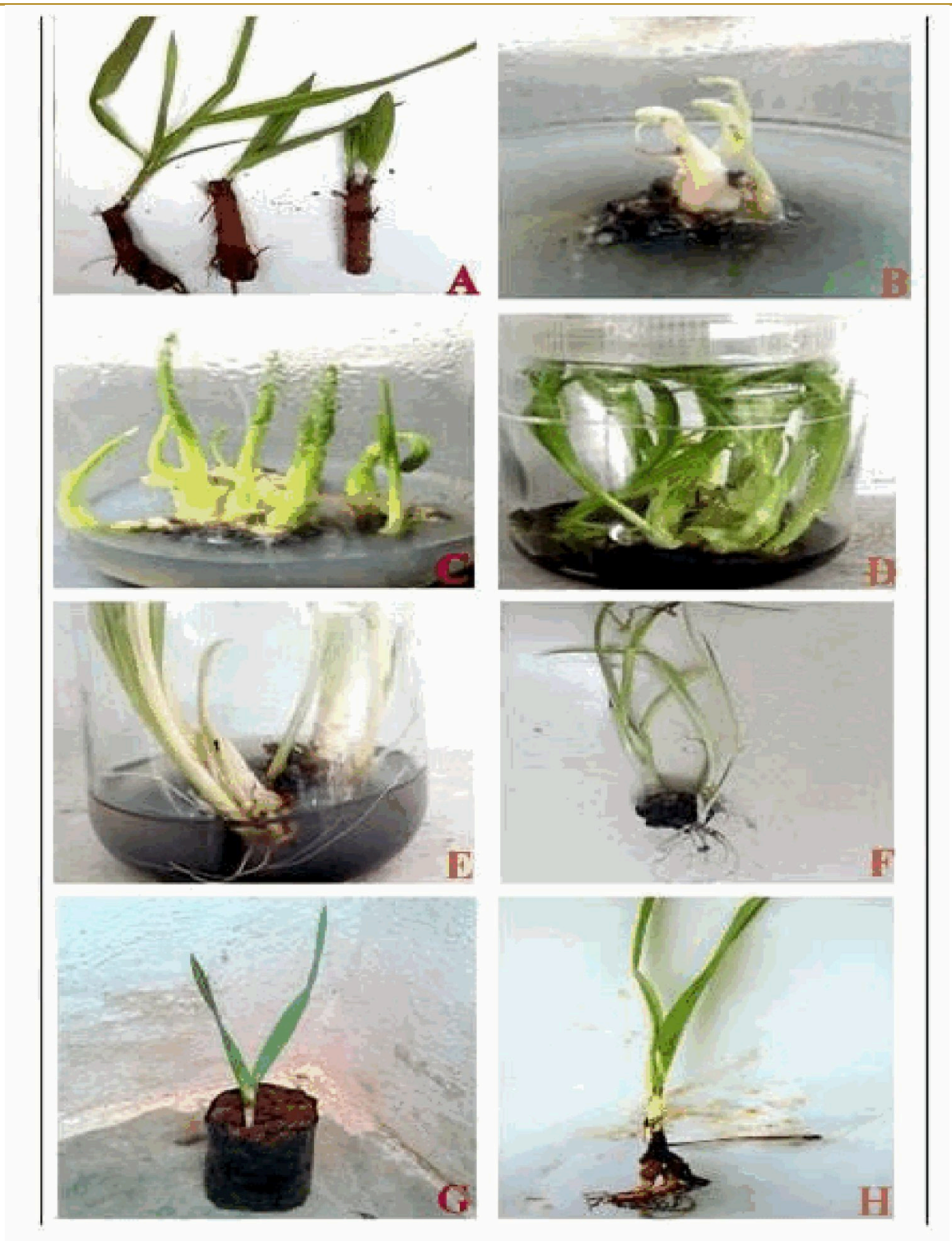


Fig. 2. Micropropagation of *C. orchioides*

B. Shoot induction from rhizome disc

A. Healthy plant collected from field

C. Multiple shoot induction from sub culture **D.** Shoot elongation

E. Root formation in rooting media **F.** Regenerated plant with roots

G. Regenerated plant into a poly bag for **H.** Hardening plant lets having a bunch of

hardening under semi-shade net. rhizome with roots

The cytokinin such as BAP combine with NAA supplemented MS media does not express any shoot initiation on proximal rhizome disc up to the 60th day of observation. The combination of BAP and NAA are ineffective at a different concentration level of this study. The BAP mostly involve in the formation of shoot and NAA is responsible for root initiation, but it combines is ineffective for induction of shoot from the proximal disc. This study is controversy in the earlier report of [23] observed that BAP & NAA combine with Kn supplemented medium involved

the shoot initiation and multiplication. The micropropagation protocol is an efficient method for improving the *in vitro* multiplication rate, genetic identity, stability, successful acclimatization, and establishment of tissue culture raised plantlets on the soil. The technique is also cost-effective [24], [25]. The BAP is an extremely useful plant growth regulator for multiplication of shoots from explants at *in vitro* conditions [26], [27]. The present study positively correlated with the early study that the shoot initiation and shoot length were best found on MS media fortified with a high concentration of BAP (13.32 µM) and 0.57 µM of IAA [28]. Similarly, the high concentrations of BAP (6.66 & 8.88 µM) decrease the shoot multiplication rate in *C. orchioides* [29],[30] and mulberry [31]**.** [21] found that the combination of 6-benzylaminopurine (BAP – 4.44 µM) and kinetin (Kn – 4.65 µM) on MS medium leads to synergistic effect for the better regeneration of shoot buds from proximal rhizome disc than distal rhizome disc and shoot tip. Similarly, BAP (2.22 – 4.44 µM) alone or combined with Kinetic (2.32 – 9.29 µM)/NAA (2.69 – 10.74 µM)/IBA (2.46 – 9.84 µM) supplemented on MS medium induce better multiple shoots from the proximal rhizome discs [11]. The equal concentration of Kn and BAP has capable of producing the maximum number of shoots multiplication from *C. orchioides* [32]. The major problems were faced during tissue culture establishment of *C. orchioides* rhizome because the exudation of the phenolic compound from the cut ends of the rhizome disc which is spreader in the culture medium. It interfered the growth of the plant and led to be caused wounds on the surface of the explant and also contamination of the culture medium [33]. The problem solved by the addition of activated charcoal to the culture medium and to stop the phenolic exudation [29].

4. Conclusion

In this study, reveal that the combination of BAP and IBA supplemented medium involved only the minimum shoot initiation in initial stages, but the BAP, Kn, and IAA fortified medium effectively influences the induction of several multiple shoots on the subculture of *C. orchioides* rhizome shoot. Similarly, BAP and IAA supplemented medium responsible for better shoot elongation. Thus, the present study concludes that multiplication of several regenerated plants from the rhizome disc of *C. orchioides* required subsequent of two subculture studies.

Conflict of Interest

Authors have no conflict of interest.

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