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Research Article **Plant Tissue Culture**

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OPTIMIZATION OF CALLUS AND CELL SUSPENSION CULTURE OF CAPSICUM ANNUM L

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ABSTRACT

Tissue culture optimization may play an important role to optimize the cell growth of chilly. Plant produced through *in vitro* techniques are true to type and are free from diseases. The present investigations were carried out to standardize surface sterilization of explants, suitable explants type for culture establishment, growth regulators for callus formation and to determine cell growth. It was observed that water socked blotting paper gave the maximum seed germination. In all the combination of plant growth regulators the highest number of callus formation was observed in growth regulators 2, 4-D (0.1 µM/L) and BAP (0.1 µM/L) containing MS medium. Cells grew and multiplied rapidly with a doubling time of 0.72 days. Elongated and divided cells were observed more in 21 days. Fresh weight and PCV% of the cells increased nearly 3 folds with 8.64% and 0.77% mean of growth. In the present study, this method was used to develop a growth curve for chilly suspension culture identifying the onset and duration of the various growth phases, growth rate and doubling time.

KEYWORDS: Callus, cell suspension, growth rate and doubling time

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1. INTRODUCTION

Chilies are the dried ripe fruits of *Capsicum annum L*. Comes under the family of *Solanaceae, which* has about 90 genera and 2000 species.[1] This family includes tobacco a commercial, cash crop, and important vegetables like Tomato, Brinjal, Potato *etc.* Chilies are cultivated mainly in tropical and subtropical countries *viz.*, India, China, Africa, Japan, Mexico, Turkey, USA *etc.* The nutrition value of *Capsicum* is high and considered as an excellent source of vitamins A, B-complex, E and C (ascorbic acid) that latter is seven times more than orange[2.] *Capsicum annum L*. is most cultivable in India and other countries of the world. Chillies contain steam volatile oil, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fibre and mineral elements.They are widely used in foods, drugs, and cosmetics because of their nutritional value, flavor, aroma, texture, pungency and color. Like other crops, the production of chili pepper is affected by biotic and abiotic factors that reduce its crop quality and yield[3]. The search for biotechnological alternatives that may increase the production of this Solanaceae member is necessary[3, 4]. In vitro plant regeneration of chilli has been achieved via protoplast, hypocotyls, cotyledons, young leaves, direct somatic embryogenesis and shoot organogenesis from seedling explants of different chilli cultivars [5, 6, 7]. However, these procedures failed or to be modified when they were used to regenerate plants from other pepper varieties. Thus, the strong influence is pepper variety in regeneration from various explants and this makes it necessary to optimize in vitro propagation protocols for the specific cultivars [8]. Low differentiation frequency, difficulty in shoot elongation, and low repeatability are also main barriers to the development of pepper gene engineering [9]. '*In vitro'* culture has often been acknowledged as one of the more, promising avenues in plant improvement. Suspension culture can be used to produce true - to - type plants for both agronomic and research purposes.

2. MATERIALS AND METHODS

Experimental Materials

Chilli seeds GCH-1 (*Capsicum annuum* L.) used for initiation of experiments were collected from spice centre of Jagudan for the present study.

Plant Materials

For investigation, the stored seeds of Chilli were used to get the explants.

Explants Source

Hypocotyls and epicotyls were used as an explant of chilli [10] cultivar for "Tissue Culture Optimization for Chill.

Plant Growth Regulators

The following plant growth regulators were used in different experiments at different concentrations 2, 4-Dichlorophenoxy Acetic Acid (2, 4-D) and Benzylaminopurine (BAP)

Culture Vessels

Glass bottles and test-tubes were used as culture vessel in experiments. Bottles were properly cleaned and oven dried before use.

Media

In the present investigation Murashige and Skoog, 1962 (MS) [11], media were used as basal salt medium during different studies. Detailed composition of these media is given in Table- 1.

Table 3.1
Composition of basal medium (Murashige and Skoog, 1962).

Constituent	Concentration (mgl^{-1})
Macro elements	
Ammonium nitrate	1650
Potassium nitrate	1900
Calcium chloride.2H2O	440
Magnesium sulphate.7H2O	370
Potassium dihydrogen phosphate	170
Micro elements	
Boric acid	6.2
Potassium iodide	0.83
Sodium molybdate	0.25
Cobalt chloride.6H2O	0.025
Manganese sulphate.4H2O	22.3
Zinc sulphate.7H2O	8.6
Copper sulphate.5H2O	0.025
Ferric sulphate	27.8
Na2EDTA	37.3
Vitamins	
Nicotinic acid	0.5
Pyridoxine hydrochloric acid	0.5
Thiamine hydrochloric acid	0.1
Glycine	2
Myo inositol	100

Surface Sterilization

Seeds were washed with tap water for 5 - 10 minutes to remove surface contamination and then treated with distilled water mixed with Tween 20. Later they were surface sterilized by immersion fungicide (Bavistin) (1 gm/lit) for 30 minutes with vigorous shaking. Seeds were then rinsed three times with autoclaved sterilized water in laminar air flow cabinet to remove minor amounts of fungicide. The seed was then sterilized with 0.1% Mercury chloride ($HgCl_2$) in laminar air flow cabinet for 3 minutes then rinsed 3 times with autoclaved distilled water in a laminar air flow cabinet to remove minor amounts of disinfection liquid[12].

In Vitro Seed Germination

Sterilized seeds were then placed in Petri dishes containing water soaked blotting paper for germination. 30 seeds were placed in each Petri dish. The Petri dishes were then incubated in an incubation room till the germination of seeds.

Inoculation of Explants into Medium

From 6 days old seedlings, hypocotyls and epicotyls explants were excised aseptically and 10 segments were cultured on MS medium, supplemented with growth regulators 2, 4-D and BAP. In this factorial design, 2,4-D levels 0.1 and 0.2 µM / liter and BAP levels 0.1 and 0.2 µM /liter were tested in 4 different treatment combinations for callus induction.

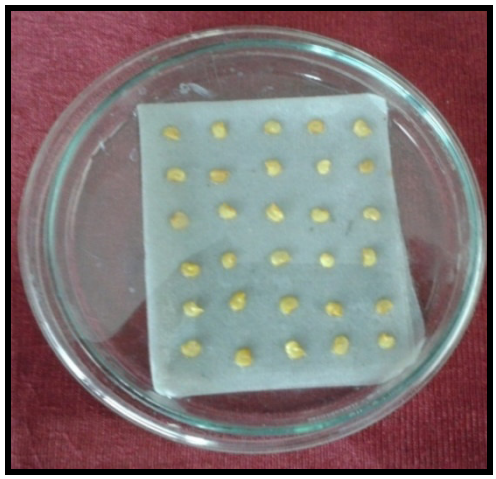

Figure 1
Inoculation of chilly seeds into water socked blotting paper for germination.Inoculation of Explants into Medium

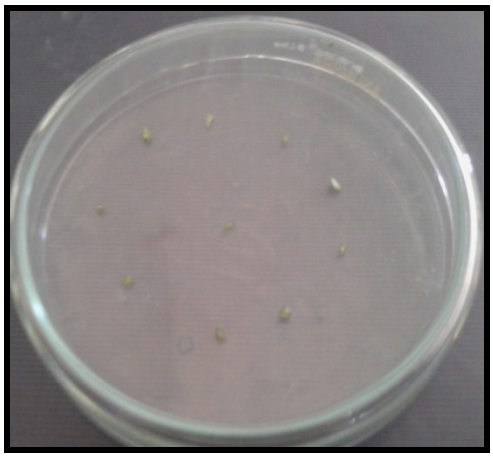

Figure 2
(A) Inoculation of hypocotyls explants on MS medium supplemented with 0.1 µM/L 2, 4-D and 0.1 µM/L BAP (B) Inoculation of Epicotyls explants on MS medium supplemented with 0.1 µM/L 2,4-D and 0.1 µM/L BAP

Callus Induction

Two explants *viz.,* hypocotyls and epicotyls were cultured on MS medium supplemented with different growth regulators and incubated in both dark and light condition for callus induction. Observations were recorded on days to callus initiation, number of explants responding, treatment and quantity of callus induced, type of callus induced (Friable, white, brown, compact), color of callus, visual callus quality.

Subculture

Calli were sub cultured monthly on the same fresh solid MS medium supplemented with 2,4-D and BAP (0.1 and 0.1µM). The first subculture was incubated at 25°C in the dark and the following two-three subcultures were kept in fluorescent light (16h/day).

Cell Suspension Cultures (CSCs)

The establishments of chilli cell suspension cultures were from three month old white brown friable callus. Callus obtained from hypocotyls of GCH-1 on solid MS medium supplemented with 0.1 µM/l 2, 4-D and 0.1 µM/l BAP was used for culture initiation. Approximately 1 g fresh weight of friable callus, which was still in its active growth phase, was placed in 150 ml flasks containing 50 ml liquid MS medium[5] supplemented with 0.1 µM/l 2, 4-D in combinations with 0.1µM/l BAP. The flasks were placed on the rotary shaker at 25-27°C

under a 16 h photoperiod and agitated at 100 rpm

3. RESULTS AND DISCUSSION

In vitro Seed Germination

Several Systems for regeneration of Capsicum has been reported [5, 6]. Therefore, Effects of explants source on in vitro propagation were evaluated in terms of regeneration.

The highest percentage (90 %) of seed germination was observed in Petri plates containing water socked blotting paper after 6 days (Table-2 & Figure-3).

Callus Induction

Hypocotyls explants cultured on MS media supplemented with different concentration of 2, 4-D in combination with different concentration of BAP. Among the hormone combinations, callus formation potentiality of hypocotyls was the highest (86.7 %) in 0.1 µM/L 2, 4-D + 0.1 µM/L BAP. The Lower percentage of callus induction was obtained in Treatment T4 53.34% (Table-3 & Figure-4)

Establishment of Cell Suspension Culture

Friable callus of chilly were transferred into liquid MS media with supplemented with 0.1 µM/l 2, 4-D and 0.1µM/l BAP for an establishment of cell suspension culture. An early stage of cell suspension culture has large elongated and large vacuolated cells with thick cell wall were observed. They were stepwise eliminated by subculture. Selection of fine aggregates continuous to attempt by weekly subcultures. The growth dynamic of cell suspension cultures was determinate. Aliquots were $3x10^5$ cells per ml inoculated in 50 ml of liquid MS with 0.1 µM/l 2, 4-D and 0.1µM/l BAP.

Table 2
Percentage of seed germination

No.	No. of Seeds inoculated	No. of Seeds germinated	Percentage of seeds germination %
1	30	27	90
2	30	25	80.33
3	30	24	80

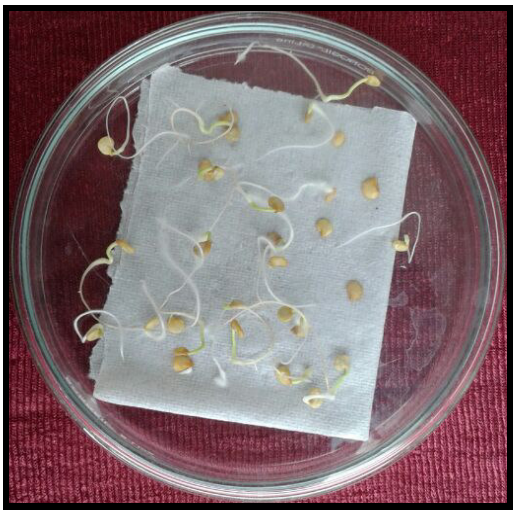

Figure 3
Seed germination on water socked blotting paper after 6 days

Table 3
Percentage of callus induction from hypocotyls

Treatment	2,4-D (µM/L)	BAP (µM/L)	No. of hypocotyls inoculated	No. of hypocotyls showing callus	Percentage %
T1	0.1	0.1	15	13	86.7
T2	0.1	0.2	15	11	73.34
T3	0.2	0.1	15	9	60
T4	0.2	0.2	15	8	53.34

Figure 4
Callus formations from hypocotyl on MS medium supplemented with 0.1 µM/L 2, 4- D and 0.1µM/L BAP after 21 days

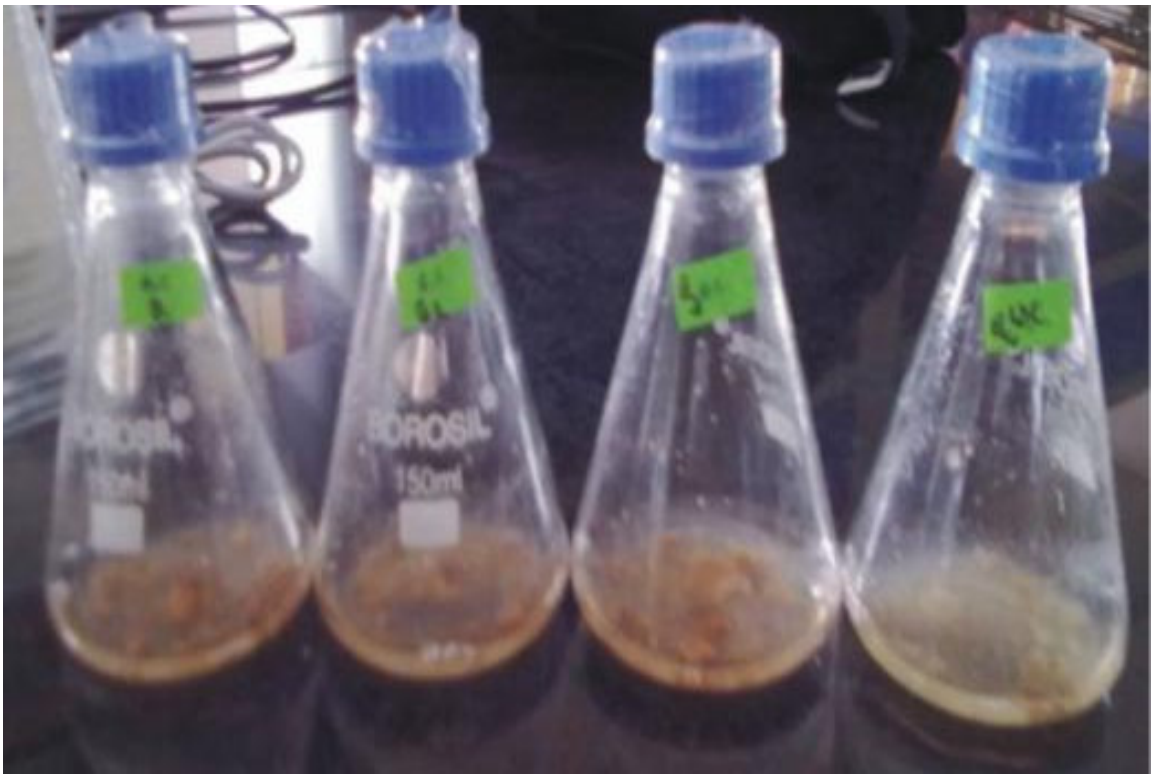

Figure 5
Cell suspension culture in MS basal medium supplemented with 0.1 µM/L 2, 4-D and 0.1µM/L BAP

Determination of Growth

Cell growth in suspensions was measured on the basis of fresh weight and PCV[13] measurements which provide the best estimate of cell doubling time. 10 ml of aliquot of cultures were removed from each flask and measured PCV, fresh weight at the end of the 0, 7, 14, 21 days. Routine measurement of fresh weight was done for study of growth kinetics. Growth rate was determined.

Growth Kinetics of CSCs

To establish a well growing cell suspension culture it is necessary, to inoculate at least 10^4 cells/ml, otherwise the cells may not divide. This value depends, however, also on the aggregate size. The aggregate size has an influence on natural product formation as well. The reason why plant cells need such relatively high cell densities for undergoing division surely is that they lose hormones and/or vitamins and nutrients to the surrounding medium. Growth dynamic was determined when cells were grown in MS medium containing 0.1 µM/l 2,4D and 0.1µM/l BAP on a rotary shaker at 100 rpm in fluorescent light (16h/day) with an initial cell density of $3x10^5$ cells per ml. The growth dynamic of CSCs was determined at various cells/ml. Fine aggregates of 1ml aliquot CSCs were centrifuged and measured PCV, fresh weight at every week. Cells grew and multiplied rapidly with a doubling time of 0.72 days. Elongated and divided cells were observed more in 21 days. Fresh weight and PCV% of the cells increased nearly 3 folds with 8.64% and 0.77% mean of growth.

Table 4

The growth of chilli cell suspension, expressed in PCV % and fresh weight

Day	Cell suspension culture	
	PCV%	Fresh weight (mg)
0	0.41	4.0
7	0.56	6.2
14	0.72	8.2
21	0.95	10.1
Mean	0.77	8.64
SD	0.317	3.427
Initial cell No. (cells/ml)	$3x10^5$	
Growth rate	0.038	
Doubling time (day)	0.72	

CONCLUSION

For the present study, we recommend to collect explants from 8-15 days old seeding. For callus formation, MS media supplemented with 0.1 µM/L 2, 4-D and 0.1 µM/L BAP was the best. Friable, white, brown and compact callus was obtained. Plant cells have been maintained as suspension cultures can grow exponentially, probably because in such suspension cultures the majority of cells remain capable of cell division. The present research was successful to obtain cell suspension culture and determine the growth of chilli using the PCV and fresh weight. Using PCV method, different phases, growth rate and doubling time were obtained and using fresh weight, growth kinetic was measured.

ABBREVIATIONS

BAP**:** 6-Benzylaminopurine;
2,4D: 2,4-Dichlorophenoxyacetic acid;
MS: Murashige and Skoog medium.
CSC: Cell suspension culture,
PCV: Packed Cell Volume

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