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PRELIMINARY STUDIES OF REACTOR SYSTEM DESIGNED FOR CELL SUSPENSION CULTURE OF CHICKPEA (*Cicer aritenum)*

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ABSTRACT

The chickpea (*Cicer arietinum*) is an edible legume of the family Fabaceae, subfamily Faboideae. Chickpeas are high in protein, and one of the earliest cultivated vegetables. 7,500-year-old remains have been found in the Middle East.They were said to have medical uses such as increasing sperm and milk, provoking menstruation and urine and helping to treat kidney stones. Wild cicers were thought to be especially strong and helpful Chickpea is one of the most important leguminous, cool-season, food crops, cultivated prevalently in the Asian Pacific region. In spite of its nutritional importance, its area of cultivation has been low, with virtually no increase. Conventional breeding has resulted in several important improvements in this crop, and recent advances in biotechnology such as plant tissue culture and genetic transformation can significantly contribute to better sustainability of this important food crop.Protein provided mainly by the cotyledon, ranges in concentrations from about 17 to 40%. Protein content of chickpea can be improved by using tissue culture and genetic transformation technique.Recent advances in Biotechnology such as plant tissue culture can significantly contribute to better sustainability of this important food crop.Here, we describe an efficient cell suspension culture protocol for *Cicer artienum* using cotyledon, hypocotyl explants which results in high recovery of valuable cell nutrients in a relatively short period (90-100 days).The cells of *Cicer artienum* will be maintained in suspension cultures under constant shaking conditions. And then they will be transferred to the lab scale photobioreactors, for the production of bioactive compounds. The varying parameters and the enhancement factors are studied.

Key words: *Photobioreactor, cell suspension cultures,chickpea* (*Cicer arietinum*), *airliftbioreactor, callus of chickpea*

INTRODUCTION

The chickpea (Cicer arietinum) is the foremost grain legume of bangaladesh and India. Chickpea is one of the most important leguminous, cool-season, food crops, cultivated prevalently in the Asian Pacific region. In spite of its nutritional importance, its area of cultivation has been low, with virtually no increase. Chickpea (Cicer arietinum L.) is an ancient and major food legume crop cultivated in over 40 countries. In spite of its large demand, global yield of chickpea has not been increased markedly in the past few decades due to damage caused by several pathogens and susceptibility to abiotic stress. Conventional breeding has resulted in several important improvements in this crop, and recent advances in biotechnology such as plant tissue culture and genetic transformation can significantly contribute to better sustainability of this important food crop.The plant grows to between 20 and 50 cm high and has small feathery leaves on either side of the stem. One seedpod contains two or three peas. The flowers are white or sometimes reddish-blue. Chickpeas need a subtropical or tropical climate with more than 400 mm of annual rain. They can be grown in a temperate climate but yields will be much lower.Protein provided mainly by the cotyledon, ranges in concentrations from about 17 to 40%. Protein content of chickpea can be improved by using tissue culture and genetic transformation technique. Where they are a major source of protein in a mostly vegetarian culture.The chickpea (Cicer arietinum) an edible legume of the family Fabaceae, subfamily Faboideae. Chickpeas are high in protein and one of the earliest cultivated vegetables.This crop is significant source of protein,phosphorus,iron and

certain water-soluble vitamins.One hundred grams of mature boiled chickpeas contains 164 calories, 2.6 grams of fat, 7.6 grams of dietary fiber and 8.9 grams of protein. Recent advances in Biotechnology such as plant tissue culture can significantly contribute to better sustainability of this important food crop.Here, we describe an efficient cell suspension culture protocol for Cicer artienum using cotyledon, hypocotyl explants which results in high recovery of valuable cell nutrients in a relatively short period (90-100 days). [1]

MATERIALS AND METHODS

PLANT MATERIAL – Chickpea (Cicer arietinum) FIG:1

FIG:1 Chickpea (Cicer arietinum)

PREPARATION OF TISSUE CULTURE MEDIUM

The basic nutritional requirements of cultured plant cells as well as plants are very similar. However, the nutritional composition varies according to the cells, tissues, organs and protoplasts and also with respect to particular plant species. The appropriate composition of the medium largely determines the success of the culture.A wide variety of salt mixtures have been reported in various media. A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and other organic supplements. When referring to a particular medium, the intention is to identify only the salt composition unless otherwise specified. Any number and concentration of amino acids, vitamins, growth regulators and organic supplements can be added in an infinite variety of compositions to a given salt composition in order to achieve the desired results.

UNITS FOR SOLUTION PREPARATION

The concentration of a particular substance in the media can be expressed in various units that are as follows :

UNITS IN WEIGHT

It is represented as milligram per litre (mg/l)

10^{-6} = 1.0 mg/l or 1 part per million (ppm)

10^{-7} = 0.1 mg/l.

10^{-8} = 0.001 mg/l or 1 μg/l.

MOLAR CONCENTRATION

A molar solution (M) contains the same number of grams of substance as is given by molecular weight in total volume of one litre.

1 molar (M) = the molecular weight in g/l1

mM = the molecular weight in mg/l or 10^{-3} M

1 μM = the molecular weight in μg/l or 10^{-6} M or 10^{-3} mM.

CONVERSION FROM MILLI MOLAR (MM) TO MG/L

For example, molecular weight of auxin 2,4-D = 221.0

1M 2,4-D solution consists of 221.0 g per litre

1 mM 2,4-D solution consists of 0.221 g per litre = 221.0 mg per litre

1 μM 2,4-D solution consists of 0.000221 g/l = 0.221 mg/l

MEDIA COMPOSITION

The salt composition of Murashige and Skoog (1962) [2] nutrient medium, referred to as MS medium, is very widely used in different culture systems as it gives satisfactory results. But it must be remembered that it is not always the best medium. Generally, in all the media, the nutritional milieu consists of inorganic nutrients, carbon and energy sources, vitamins, growth regulators, and complex organic supplements.

It is desirable to choose a composition according to the knowledge of the physiology of species vis-a-vis mineral nutrition.(Table no.1[2]. The commercial MS medium which was obtained contained the nutritional components, agar and sucrose. But it was without the growth factors so it was added in the medium during preparation.The obtained medium was weighed and dissolved in the appropriate amount of water and it was heated for complete dissolution of agar. The medium when heating was strried continuously inorder to prevent charing of agar.After dissolving the required amount was dispensed into culture tubes and kept for autoclaving.After sterilization the medium was cooled to the room temperature and required amount of auxin and cytokynin was added from the preapred stock solutions. Medium was allowed to cool and solidify in the laminar airflow hood Then each vessel to show the precise medium and date of preparation. After cooling, the media containers are stored preferably at 4-10^{o}C but that is not absolutely necessary. Medium should be used after 3-4 days of preparation, so that it medium is not properly sterilized, contamination will start to appear.

TECHNIQUES

SEED GERMINATION (FIG:2 ,3 & 4)

The seeds were washed by submerging in water with a few drops of detergent in a beaker and then it is shake thoroughly with hand. Or the seeds can be wrapped in two layers of cheese cloth / muslin cloth / nylon pouch and then they can be washed with water. The seeds were submerged in 70% alcohol for 40 sec. And the alcohol is decanted. The seeds were transfer to a flask or beaker containing 20% commercial sodium hypochlorite solution for 20 min for surface sterilization. And it is rinsed 3 times with sterile distilled water.2-3 seeds were placed per culture vessel on the surface of B5medium (B5agar medium without growth regulators).The cultures were incubated at 25^{o}C under 16hrs photoperiod with – 1000 lux light intensity for 1-2 weeks. The seeds were observed for germination regularly.

Table:1 Preparation of stock solutions of Murashige and Skoog [2](MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrients (10x) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 . 7H_2O$	370	3700	
KH_2PO_4	170	1700	
Macronutrient (10x) Stock solution II			
$CaCl_2$ $2H_2O$	440	4400	100 ml
Micronutrients (100x) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 . 4H_2O$	22.3	2230	
$ZnSO_4 . 7H_2O$	8.6	860	
Kl	0.83	83	
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_45H_2O$	0.025	2.5	
$CoCl_2 . 6H_2O$	0.025	2.5	
Iron source			
Fe EDTA Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	4 ml
Sucrose	30,000	Added fresh	
Agar	8000	Added fresh	
pH 5.8			

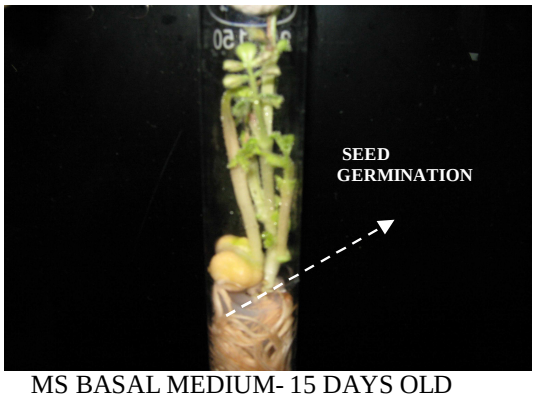
HALF SEED EXPLANTS PREPARATION

Disinfected seeds were soaked in sterile distilled water for about 4 h and a longitudinal cut along the hilum was made to separate the cotyledons, and the seed coat was removed. The embryonic axis found at the junctions of the hypocotyls and cotyledon was excised to obtain the half- seed explants. Half seed explants is an efficient source for the chickpea initiation and shoot regeneration. The half seed derived cotyledonary nodal callus could serve as an ideal starting material for developing an efficient chickpea Plant. (Sairam *et al*., 2003).(FIG:5)

FIG:2 SEED GERMINATION

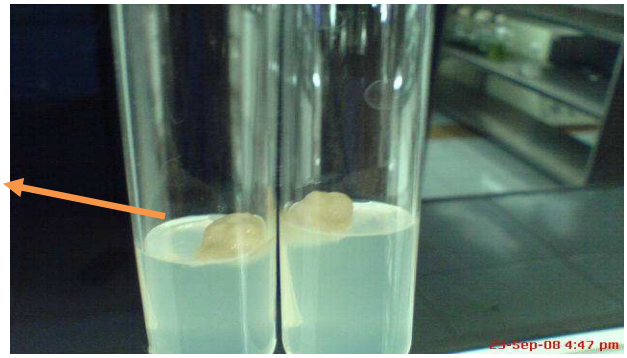
FIG: 3 PLAMULE DEVELOPED SEED

FIG: 4 GERMINATED SEED



MS BASAL MEDIUM- 15 DAYS OLD

FIG: 5 HALF SEED METHOD



B5 Medium- 2,4-D + BAP (13.5µm+13.3µm)

CALLUS INDUCTION

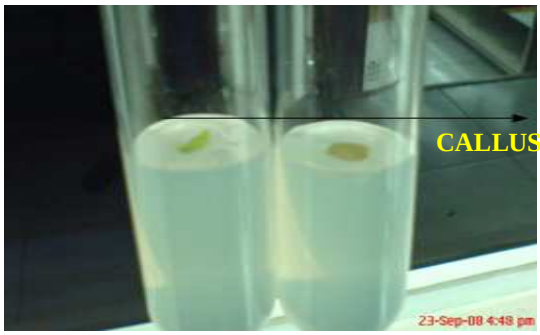
Plant production through organogenesis can be achieved by two modes: (i) Organogenesis through callus formation with de novo origin; and (ii) Emergence of adventitious organs directly from the explants Plant regeneration from a cultured explants involves the initiation of basal callus followed by shoot bud differentiation. Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. [7] However, explants with mitotically-active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants *in vitro* from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus. Callus may be serially sub cultured and grown for extended periods, but its composition and structure may change with time as certain cells are favored by the medium and come to dominate the culture. Callus tissue from different plants species may be different in structure and growth habit: white or colored, soft (watery) or hard, friable (easy to separate in to cells) or compact. The callus growth within a plant species is dependent on various factors such as the original position of the explants within the plant, and the growth conditions(table:2) . Although the callus remains unorganized, with increasing growth, some kinds of specialized cells may be formed again. Such

differentiation can appear to take place at random, but may be associated with centers of morphogenesis, which can give rise to organs such as roots, shoots and embryos. By varying the growth regulator levels, i.e. lowering the auxin and increasing the cytokinin concentration is traditionally performed to reduce shoot organogenesis from the callus. The next phase involves the induction of roots from the shoots developed. IAA or IBA auxins, either alone or in combination with a low concentration of cytokinin, are important in the induction of root primordial. Thus organ formation is determined by quantitative interaction, i.e. ratios rather than absolute concentrations of substances participating in growth and development.(FIG 6,7,8, 9 & 10).

TABLE:2 MEDIUM FOR CALLUS INDUCTION

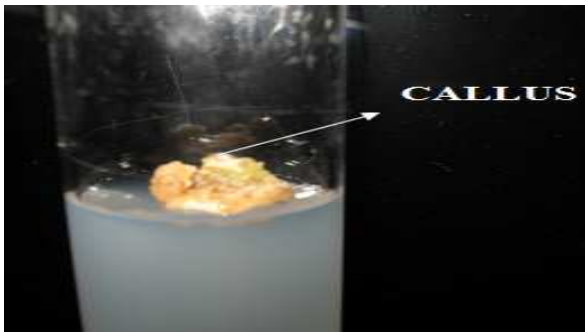
S.No	Explant	Medium	Hormone combination	Reference
1	Cotyledon	MS	2,4-D + Kinetin	R.Chandra,*et al.*, [7] Biologia Plantarum 40(3):337-343 1997-98.
2	Cotyledon	MS OR B5	2,4-D+BAP NAA+BAP	S. Huda, *et al.*, [1] Plant tissue cult.13(1):53-59 2003 (June)
3	Cotyledon Epicotyl Hypocotyl Leaflet	MS	2,4-D, NAA,BAP, IAA	Dipankar Chakraborti,*et al.*,[8] Plant Cell Tissue & organ culture (2006) 86:117-123.

FIG: 6 USED EXPLANTS- COTYLEDON, HYPOCOTYL.



MS Medium- 2,4-D + BAP (3mg/ml+3mg/ml)

FIG: 7 COTYLEDON DERIVED CALLUS

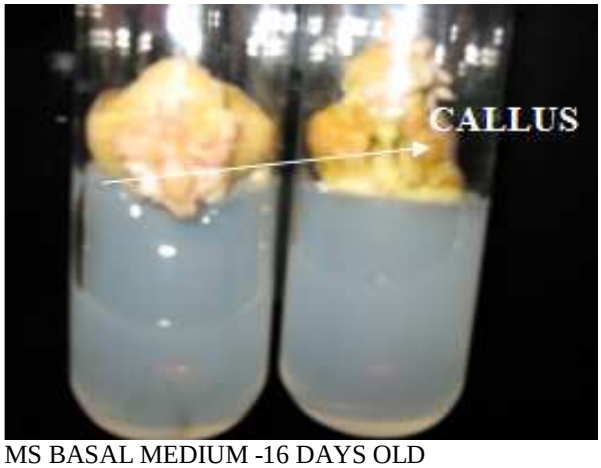


2,4-D (3mg/ml) + BAP (3mg/ml).
2,4-D (2mg/ml) + Kn (3mg/ml).
Days to callus initiation- 11 to 14 days.

FIG: 8 HYPOCOTYL DERIVED CALLUS

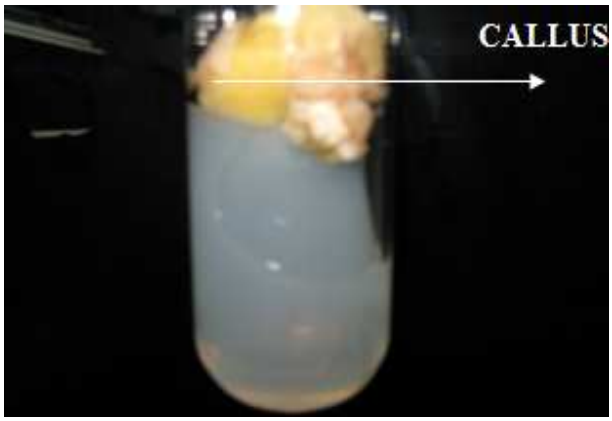
2,4-D (3mg/ml) + BAP (3mg/ml).
2,4-D (1mg/ml)
Days to callus initiation- 12 to 18 days.

FIG: 9 CALLUS DERIVED FROM SEED-I



MS BASAL MEDIUM -16 DAYS OLD

FIG: 10 CALLUS DERIVED FROM SEED-II



MS MEDIUM-2,4-D (3mg/ml) + BAP (3mg/ml).
12 DAYS OLD

CELL SUSPENSION CULTURE

When the callus is placed into a liquid medium (usually the same composition as the solid medium used for the callus culture,B5 MEDIA) and then agitated, single cells and/or small clumps of cells are released into the medium. Under the correct conditions, these released cells continue to grow and divide, eventually producing a cell-suspension culture. A relatively large inoculum should be used when initiating cell suspensions so that the released cell numbers build up quickly. Cell suspensions can be maintained relatively simply as batch cultures in conical flasks. They are continually cultured by repeated sub culturing into fresh medium. This results in dilution of the suspension and the initiation of another batch growth cycle. The batch growth-cycle parameters are determined for each cell-suspension culture. The following is the general methodology adopted here. First *in vitro* germination of seeds was performed.Collected the aseptically – germinated seedlings when the cotyledons are fully expanded and the epicotyls is beginning to emerge. Usually, this will occur when the seedlings are 2-week-old. Then the seedlings are placed on a sterile slide/Petri dish and the explants are prepared.Various explants can be used. But here seed explants were placed on the MS medium + (BAP 3mg/ml+2,4-D 3mg/ml) for callus induction. The cultures were kept in dark at 25°C. Callus was being produced in 3-4 weeks.Then small pieces of callus – 0.5g fresh weight were cut and sub cultured on the same fresh medium for proliferation.MS + (BAP 3mg/ml+2,4-D 3mg/ml) liquid medium without agar was prepared. For experimental purposes, 150ml medium in a 250ml Erlenmeyer flask is placed. The openings of the flasks were sterilized with the flame of a burner in the hood.Piece of a callus was transferred to a sterile Petri dish.The small pieces of the calli were transferred to the liquid media using forceps.The openings of the flask were flame-sterilized and then were cotton plugged. And then replicas of the samples were prepared.They were incubated on a gyratory shaker set at 125 rpm, and kept in a temperature – controlled room.Sub cultured every week. For the first few subcultures, a portion was removed and the spent medium was replaced with a fresh medium with the help of a large bore sterilized pipette. When the cell mass has nearly doubled, carefully the culture was split into two flasks with an equal volume of fresh medium. Then the incubation cycle was repeated.An aliquot from the flask was kept on a glass slide while maintaining sterile conditions, and the cells were counted.(FIG: 11)

FIG: 11 CELL SUSPENSION CULTURE

COUNTING OF CELLS

The cell number in very finely divided suspensions may be counted directly in a haemocytometer. However, most cultures usually contain aggregates and it is difficult to count the number of cells in each clump. Thus clumps are generally broken and then the cell number is counted.The haemocytometer was

placed and the center was focused and it has 25 squares.The cover slip was placed on top of it and 100µl of suspension was pipetted out into a sterile haemocytometer The cells were counted in 4 corner squares and the center one was counted.The number and the average is then counted.

BIOREACTOR DESIGN

The function of the fermenter or bioreactor is to provide a suitable environment in which an organism can efficiently produce a target product—the target product might be

· Cell biomass
· Metabolite
· Bioconversion Product

The sizes of the bioreactor can vary over several orders of magnitudes[3]. The microbial cell (few mm3), shake flask (100-1000 ml), laboratory fermenter (1 – 50 L), pilot scale (0.3 – 10m3) to plant scale (2 – 500 m3) are all examples of bioreactors.Whatever may be the size of the bioreactor, the conditions in the bioreactor have to be favorable so that living microorganisms can exhibit their activity (specific biochemical and microbial reactions) under defined conditions.The performance of any fermenter depends on the following key factors:

· Agitation rate
· Oxygentransfer
· PH
· Temperature
· Foam production

The general requirements of the bioreactor are as follows:

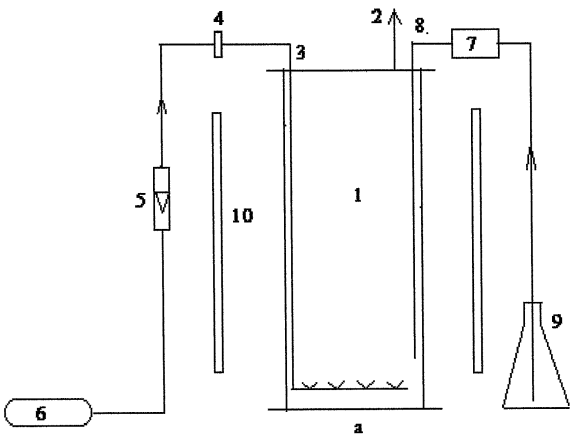
a. The design and construction of biochemical reactors must preclude foreign contamination (sterility). Furthermore, monoseptic conditions should be maintained during the fermentation and ensure containment.
b. Optimal mixing with low, uniform shear
c. Adequate mass transfer (oxygen)
d. Clearly defined flow conditions
e. Feeding of substrate with prevention of under or overdosing
f. Suspension of solids
g. Gentle heat transfer
h. Compliance with design requirements such as: ability to be sterilized; simple construction; simple measuring, control, regulating techniques; scaleup; flexibility; long term stability; compatibility with up-downstream processes; antifoaming.The other area of major importance in bioreactor design involves the bioreaction parameters, including:

• Controlled temperature
• Optimum pH
• Sufficient substrate (usually a carbon source), such as sugars, proteins and fats
• Water availability
• Salts for nutrition
• Vitamins
• Oxygen (for aerobic processes)
• Gas evolution and
• Product and byproduct removal.

For plant cell and tissue cultures, a variety of bioreactor types providing growth and expression of bioactive substances is available today. [4] Most of the plant cell suspension cultures and hairy roots used are grown in submerged stirred bioreactors, bubble columns, airlift reactors and their modifications, gas-phase bioreactors and their combinations, or socalled hybrid systems up to m3-range. Whereas low biomass and product level can be achieved in virtually any of these bioreactor types, an improved understanding of the manifold interactions between cultivated cells, product formation and the specific designs for different bioreactor types will help not only to enhance and sustain high productivity, but also to reduce process costs. The application of scalable low-cost or disposable bioreactors operating with cultivation bags made from plastic film will clearly contribute to additional savings in cost and will exploit the potential of plant cell-based bioprocessing.A mathematical model of the lumped parameter type was developed in order to analyze the water temperature in bioreactors for outdoor biomass production. A photobioreactor (PBR) water tank was employed to properly validate the model outcome by means of the experimental data and the main environmental parameters such as solar radiation, relative humidity, ambient temperature and wind

velocity.Solar radiation control is achieved by means of shading the water tank. The model allows modifying the aspect ratio of the bioreactor and studying the temperature variations, one of the variables of major concern in the growth of biomass, for different mesh shading and for any given day along the year. Hence, geometry selection and reactor design, coupled with the design of the shading device, can be chosen to reduce conventional energy usage and capitalize thermal exchange with the environment. Hence, optimization ofbioreactor design can be attained once the optimal conditions for commercial production are established for a particular biomass production [5]. Bioreactor designs currently in use are discussed with respect to specific operating parameters that can be varied to modulate cell growth and function in order to optimize product release and separation.Flow and mixing are recognized as key factors responsible for both the direct hydrodynamic effects on cell shape and function and flow induced changes in mass transfer of nutrients and metabolites(FIG:12).The integration of biosynthesis and separation is considered as a possible approach towards more efficient plant cell and tissue culture. [6]

FIG: 11 SIMPLE FLOW DIAGRAM OF PHOTOBIOREACTOR



1. Bioreactor 2. Air outlet 3. Air inlet 4. Air filter, 5. Flowmeter,6. air storage tank, 7. Peristaltic pump, 8. Sterile water inlet, 9. Sterile water bath, 10. 40W fluorescent lamp.

RESULTS AND DISCUSSION

Callus induction was observed onto MS and B5 media containing different concentrations and combination of 2,4-D, NAA, IAA, BAP and Kn within 8 – 14 days of incubation of cotyledon explants depending upon the concentration and combination of hormones. Callus induction was noticed in all media formulations. But there was a wide range of variation in percentage of callus formation and average fresh weight of callus. The highest percentage of callus induction (95) was observed on MS containing 3.0 mg/l 2,4-D and 3.0 mg/l BAP (Table 1). This kind of auxin (2,4-D) alone or in combination with cytokinin (BAP, Kn) 100% callus induction has been reported in the past by Panday and Ganopathy (1984) and Anil et al. (1986a and 1986b). Highest callus growth in terms of fresh weight (0.701 g) was observed in B5 medium fortified with 3.0 mg/l 2,4-D and 1.0 mg/l BAP. Colour of calli was mostly light brown to whitish green and light green. It was observed that only light green calli produced shoot buds. Proliferation of shoot buds was observed on MS + 3.0 mg/l 2,4-D + 1.0 mg/l BAP; MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP (Fig. 1A) and B5 + 3.0 mg/l 2,4-D + 3.0 mg/l BAP. The shoot buds first appeared as nodular growth within three - four weeks of culture and at the end of four weeks these nodular growth increased in size and produced leaf primordial Maximum number of shoot buds was obtained in MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP. Root formation was recorded on MS + 1.0 mg/l 2,4-D; MS + 3.0 mg/l 2,4-D; B5 + 1.0 mg/l 2,4-D;B5 + 3.0 mg/l 2,4-D and B5 + 5.0 mg/l 2,4-D.In the present investigation it was observed that 2,4-D without cytokinin could induce callus but for better proliferation auxin (2,4-D, NAA and IAA) and cytokinin (BAP, Kn) were required and it was also observed that 2,4-D alone promoted root formation.

CELL SUSPENSION CULTURE

The cell number in very finely divided suspensions was counted directly in a haemocytometer. However, most cultures usually contain aggregates and it is difficult to count the number of cells in each clump.

Thus clumps are generally broken and then the cell number is counted. The various parameters for growth are measured by fresh weight and dry weight method and spectrophotometer method. Recent advances in Biotechnology such as plant tissue culture can significantly contribute to better sustainability of this important food crop. Here, we describe an efficient cell suspension culture protocol for Cicer artienum using cotyledon, hypocotyl explants which results in high recovery of valuable cell nutrients in a relatively short period (90-100 days).

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