Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Plant Sciences

ISSN 1816-4951
DOI: 10.3923/jps.2020.54.63

Research Article
Callus Induction and Establishment of Cell Suspension Culture of Cumin (*Cuminum cyminum* L.)

[1]S. Suthar Ramchandra, [2]P.N. Bhatt and [2]D.P. Bhatt

[1]Department of Biotechnology, P.S. Science and H.D. Patel arts College, Kadi-382517, KSV University, Gandhinagar, Gujarat, India
[2]Sun Agrigenetics Pvt. Ltd, Vadodara, India

Abstract

Background and Objective: Resistance to diseases is an important step for the establishment of a genetic transformation system in plants. In current research, callus induction and establishment of cell suspension culture were investigated in *Cuminum cyminum*. **Materials and Methods:** Callus inducted from hypocotyls segments. The experiment was arranged in a factorial experiment with 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin (KIN) different concentrations (0, 0.1, 1 and 10 µM) on MS medium. Callus was inducted 95.6% on MS medium supplements with 2, 4-D 0.1 and KIN 10 µM L^{-1}. The cell suspension culture was established from the callus of Cumin GC-4. Using different concentrations and combinations of 2,4-D and kinetin the growth patterns of cell suspension cultures were examined during a range of culture periods (0, 7, 14, 21 and 28 days). **Results:** The growth rates of cells were initially slow, but as the culture proceeded, they increased significantly and accumulated great amounts of biomass over a period of 28 days. **Conclusion:** Friable callus production is important in establishment of good quality of cell suspension. Researcher can take benefit from its investigation to manipulation genetically.

Key words: Cumin, callus culture, suspension culture, hypocotyls, genetic transformation, accumulation, biomass

Citation: S. Suthar Ramchandra, P.N. Bhatt and D.P. Bhatt, 2020. Callus induction and establishment of cell suspension culture of cumin (*Cuminum cyminum* L.). J. Plant Sci., 15: 54-63.

Corresponding Author: S. Suthar Ramchandra, Department of Biotechnology, P.S. Science, H.D. Patel arts College, Kadi-382517, KSV University, Gandhinagar, Gujarat, India

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cumin (*Cuminum cyminum*) is a member of Apiaceae family. Cumin is originally cultivated in Iran, India, Pakistan, Egypt, Turkey and the Mediterranean region. India is a large producer and an earlier growing season than Syria, but 90% of national production is consumed internally. Callus tissue is an essential material in plant cell culture systems. When it is introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a cell suspension culture. Such cells are, in theory, totipotent and should also have a potential to synthesize any of the compounds normally associated with the intact plant[1]. As new cells are formed they are dispersed into the liquid medium and become clusters and aggregates. Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture. Thus, cell suspension offers advantages when rapid cell division or many cell generations are desired or when a more uniform treatment application is required[2].

The techniques of plant cell culture facilitate the rapid production of variant cell lines. These variant cell lines are useful for research into the genetics and biochemistry of plant cells and also in biotechnology for the production of new plant varieties and secondary metabolites. Rapidly growing, fine suspension cultures or friable calluses are generally the most suitable for selection purposes. Where it is possible to regenerate plants from variant cells, selection techniques have potential for the production of crop varieties with new characteristics. Cell suspensions have also proven to be excellent starting materials for the isolation of protoplasts to be used in a wide range of applications including cell fusion and genetic manipulation[3].

Callus and suspension culture were reported earlier investigators with use of different plant growth regulators. Callus induction and shoot regeneration were investigated onto B5 medium containing 1.0 mg L^{-1} BAP, 0.2 mg L^{-1} NAA and 0.4 mg L^{-1} IAA with best result in Cumin[4]. The BAP is strongly recommended to initiates callus production and indirect regeneration pathways in cumin[5]. Organogenesis of cumin were reported in MS media with supplement TDZ plant hormone concentrations 0.5 and 0.1 and 0.5 mg L^{-1} 2,4-D[6,7].

Friable callus were obtained difficult in Cumin and further use in cell suspension. Presented study focus very well line of attack to achieve friable callus in good amount with a reduction of time. It used in protoplast isolation and fusion to acquire hybrid plants and genetically modifications and Establishment of Resistance variety.

MATERIALS AND METHODS

Study area: Seeds of GC-4 (Gujarat Cumin-4) were collected from Center for Research on Seed Spices. S.D. Agricultural University, Jagudan Gujarat, India. Research was carried out at plant tissue culture lab, established under potential for excellence, UGC grant. Research was conceded to solve wilt problem in Cumin and done in month of January, 2015 and 2016.

Surface sterilization of seeds: Seeds were first pre treated with 1% aqueous bavistin solution (Fungicide) for 15 min. Then they were soaked for 5 min in 20% solution of commercial bleach. Sterilization was done by using 0.1% $HgCl_2$ solution for 3 min and finally washed 4-6 times with auto-claved distilled water. Sterilized seeds were placed in sterile Petri dishes lined with moist filter papers and allowed to germinate. Individual Petri dishes were wrapped with parafilm to maintain it free from contamination and incubated under dark at 26°C. Almost 5-7 days after emergence, 4-6 mm long hypocotyls and epicotyls segments were cut from the seedling. These were incubated for the generation of callus.

Media optimization: A factorial experiment using total of 16 combinations for induction of callus from hypocotyls explants were done. The Callus Induction Medium (CIM) consisted of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin (KIN) plant growth regulators at four concentrations ranging from 0, 0.1, 1 and 10 µM in the MS basal medium. The callus obtained from the hypocotyls was sub-cultured every 3 weeks in the respective medium for 4-6 cycles and maintained under fluorescent light (16 h/day).

Establishment of cell suspension culture: The establishments of Cumin cell suspension cultures were from three month old white/creamy friable callus as obtained. Callus obtained from hypocotyls of GC-4 on solid MS medium supplemented with 0.1 µM 2,4-D and 10 µM KIN were used for culture initiation. Approximately 1 g fresh weight of friable callus, which was still in its active growth phase was placed in 250 mL flasks containing 25 mL liquid MS medium[8] supplemented with 0.1 µM 2,4-D in combinations with 10 µM KIN. The flasks were placed on rotary shaker at 25°C under a 16 h photoperiod and agitated at 90 rpm. Sub-cultures were performed bi-weekly for the first 1 month and later at weekly intervals. For this the entire old medium was replaced by an equal volume of fresh medium by carefully decanting the top layer without agitating

and addition of fresh medium. Only small aggregates were decanted at every sub-culture. The ratio of cells to medium was 1:3 (v/v) in culture flasks.

Growth measurements

Viable cell count: Cell viability was determined by using trypan blue stain method. A small aliquot of cell suspension was mixed with 0.4% aqueous trypan blue and immediately observed under the microscope[9] viable cells extrude the dye and hence, are not blue. Dead cells absorb the dye and appear blue.

Cell number count: For measuring the increase in ratio of cells, cells were calculated (3×10^5 cells mL^{-1}) by using hemocytometer. To count the number of cells in Cell Suspension Culture (CSCs), these are treated with chromium trioxide. One volume of suspension cell culture were added to 4 volumes of 12% (w/v) aqueous chromium trioxide and heated at 70°C until the cells were stained and plasmolysed. The number of cells counted under microscope.

Packed Cell Volume (PCV): To calculate PCV, 10 mL of cell suspension culture were transferred in graduated conical centrifuge tubes then centrifugation at 2000xg for 5 min using a swing-out rotor. The cells were allowed to settle down (Pellet) and PCV was calculated. To study growth kinetics of cells in suspension cultures, a known number of cells need to be inoculated and this has to be uniform throughout the various replicates. The cell numbers can be established by calculating the ratio between number of cells and its packed cell volume. For this, a known volume of cell suspension was taken in replicates and transferred into graduated conical centrifuge tubes followed by centrifugation at 2000xg for 5 min using a swing-out rotor. The packed cell volume was determined as an average of several replicates and the number of cells counted using a haemocytometer. Thus, any volume used from this suspension could give total number of initial cells in the inoculums. For experimental purpose, different cell volumes (1, 2, 4 and 8 mL of 2 month old cell suspension) were taken and centrifuged as above. The cells that settled down (pellet) in each case were cultured in 10 mL of liquid MS medium supplemented with 0.1 μM 2,4-D, 10 μM KIN, 30 g L^{-1} sucrose, with pH 5.8 in a 100 mL flask on a rotary shaker at 110 rpm in fluorescent light (16 h/day) at 25-27°C. There were three replicates of 1, 2, 4 and 8 mL aliquots of cell suspension tested for 28 days. From these cultures, the PCV, fresh weights of the cells and cell number were determined at 0, 7, 14, 21 and 28 days of culture incubation.

Fresh weight: The cells that settled down (pellet) were cultured in 10 mL of liquid MS medium supplemented with 0.1 μM 2,4-D, 10 μM KIN, 30 g L^{-1} sucrose, with pH 5.8 in a 100 mL flask on a rotary shaker at 110 rpm in fluorescent light (16 h/day) at 25-27°C. From these cultures, the PCV and fresh weights of the cells were determined at 0, 7, 14, 21 and 28 days of cultures. Fresh weights were determined by collecting cells from pellet. Pellet was filtered and washed with water to remove the medium, drained under vacuum and weighted. There were three replicates of each treatment tested.

Statistical analysis: Experiments were repeated 3 times by using a complete randomized block design. Analysis of variance was carried out and differences between the means of the treatments were determined by Duncan's Multiple Range test at p-value of 0.05 and 0.01. The analysis of variance and means were carried out with the online statistical program, using Proc ANOVA and Proc Mean procedures. Doubling time and growth rates of CSCs were calculated and plot exponential regression graph by Doubling-Time 1.0 software. The population standard deviation was measured the variability of data in a population.

RESULTS AND DISCUSSION

Seed germination: When the seeds of Cumin GC-4 were placed for germination on moist filter paper only, they germinated within 4-7 days under dark condition. This was based on an earlier study[10]. Prior to this, other investigators had reported that seed germination of cumin required MS media with addition of gibberellic acid and increased concentration upto 2 mg L^{-1} for better result. Seed gemination were observed upto 75% and break seed dormancy[11]. In the present study, as high as 92% germination was achieved without having to grow the seeds on MS media with GA3, so MS media was omitted in this study (Fig. 1, 2).

Callus culture: In the presence of kinetin with 2,4-D and BAP with NAA in the callus induction medium, higher percentage of the explants produced callus. The hypocotyls segments

Fig. 1: Germinated Cumin seeds (between 4-7days)

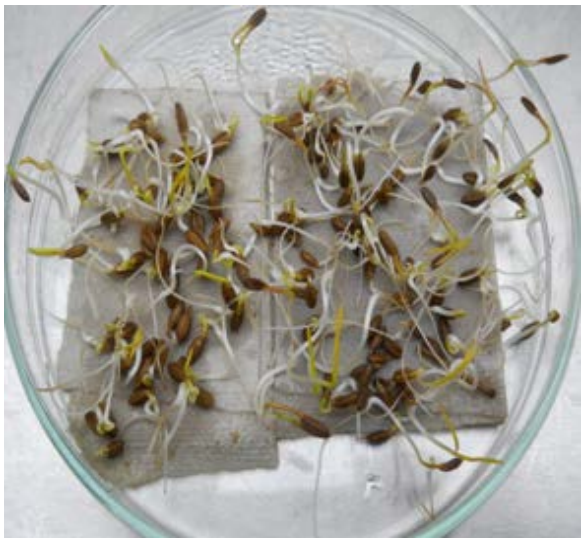


Fig. 2: Germinated Cumin seeds on filter paper after 7 days

(Fig. 3, 4), in contrast to the epicotyls explants were more responsive to the tested combinations of 2,4-D and kinetin (Fig. 5). As observed in case for callus induction and maintenance of other Apiaceae such as; fennel (*Foeniculum vulgare* Mill.), Cumin callus were more proliferative with kinetin and 2,4-D containing medium. Hypocotyls formed a great mass of callus during 3-6 weeks of the incubation on the Callus Induction Medium, CIM-14 having 0.1 µM 2, 4-D and 10 µM kinetin (Fig. 6, 7 and Table 1). On the other hand, only limited marginal callus was initiated from the epicotyls explants.

The callus obtained from the epicotyls explants, however, was comparable to the hypocotyls derived callus in the proliferation upon a sub-culture for an additional passage on fresh induction/maintenance medium. The callus from both explants was creamy to greenish creamy and soft/friable with smooth surface (Fig. 6). Similar to the present research also noticed that hypocotyls responded better than other Cumin seedling explants for callus production and proliferation with 95%. Earlier reporters were stated that 0.1 µM KIN and10 µM

Table 1: Callus initiation in Cumin from hypocotyls and epicotyls

Plant growth regulators (µM)		Callus initiation frequency (%)	
2,4-D	KIN	Hypocotyls	Epicotyls
0.0	0.0	0	0
0.1	0.0	20	18
1.0	0.0	43	40
10.0	0.0	62	56
0.0	0.1	31	34
0.1	0.1	49	55
1.0	0.1	59	56
10.0	0.1	62	58
0.0	1.0	41	43
0.1	1.0	61	58
1.0	1.0	62	62
10.0	1.0	71	68
0.0	10.0	79	76
0.1	10.0	95*	90
1.0	10.0	89	89
10.0	10.0	90	93

Table 2: Analysis of variance for callus initiation from hypocotyls percentage in Cumin (*Cuminum cyminum* L.)

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares
Replications	2	1.167	0.583
Treatments	16	30360.146	2024.010
Error	30	20.167	0.672
Total	48	-	-

Table 3: Comparison of treatment means with critical difference (0.05)

Treatment numbers	T 14	T 16	T 15	T 13	T 12	T 4	T 11	T 8	T 10
Treatment average	95	90	89	79	71	62	62	62	61
Critical Difference (CD) compared	a	b	b	c	d	e	e	e	e

a: 100-95, b: 94-89, c: 88-79, d: 78-71, e: 72-65

Fig. 3: Hypocotyls segments of Cumin

Fig. 5: Callus induced on CIM-14 with 0.1 µM 2,4-D and 10 µM Kinetin (after 14 days)

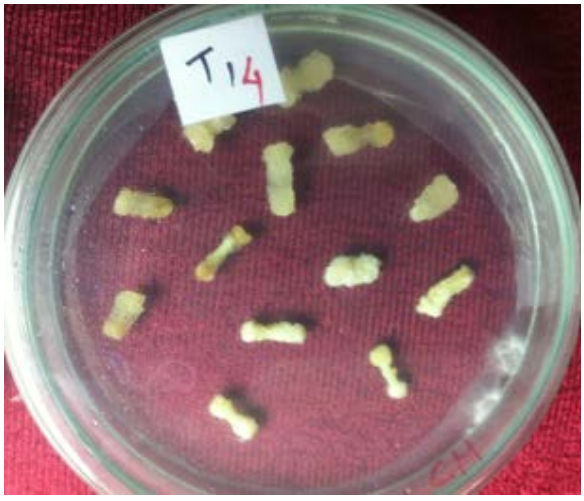


Fig. 4: Callusing explants on CIM after 7 days of inoculation

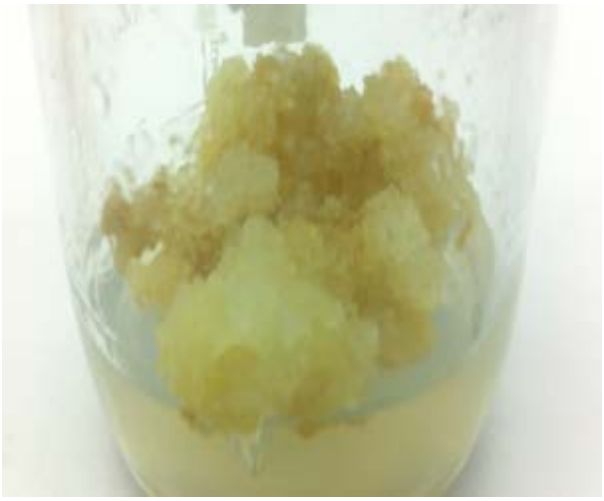


Fig. 6: Sub-culture of callus on CIM-14 with 0.1 µM 2,4-D and 10 µM Kinetin (after 28 days)

2,4-D growth regulators initiated cell suspension in Cumin in liquid medium[12]. Most of investigators used 4 µM 2,4-D alone or plus 2 or 4 µM KIN on Cumin studies[13-16].

Analysis of variance in a total 16 treatments was observed with different concentrations of 2,4-D and KIN (Table 2). Coefficient of variation is 1.423. Treatments were found to be significant at 1 and 5% level of significance Critical Difference (CD) (0.01) = 1.841 CD (0.05) = 1.367. Treatment-14 (CIM-14) was selected for further experiments (Table 3).

Treatment average: Analysis of variance in a total of 16 treatments was observed with different concentrations of 2, 4-D and KIN for epicotyls. Most significant were a, b, c, d and e shown in Table 4. Treatment average 95 in treatment No. 14 was best combination of hormones to produced superior amount of callus in Cumin. Coefficient of variation was 1.105.

Treatment was found significant at 1 and 5% level. CD (0.01) = 1.383 and CD (0.05) = 1.027.

Sub-culture: The CIM-14 (Callus Induction Medium) gave very profuse callus proliferation compared to other combinations. The callus was creamy to greenish white in color and very friable, which is most appropriate for initiation of cell suspension cultures (Table 5). As a result, CIM-14 was

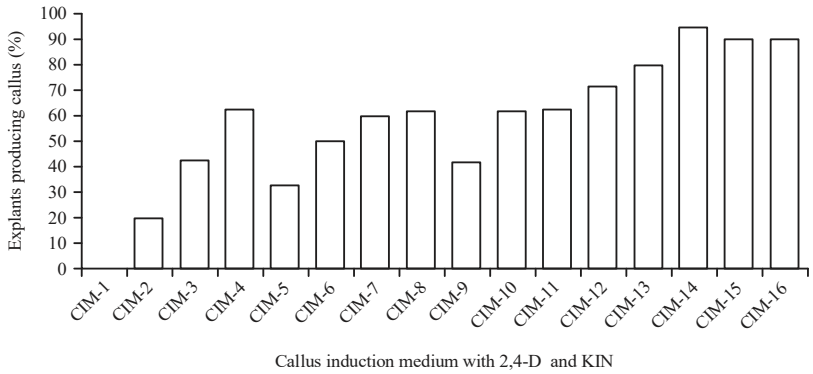


Fig. 7: Callus induction from hypocotyls

Table 4: Analysis of variance for callus initiation from epicotyls percentage in Cumin (*Cuminum cyminum* L.)

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares
Replications	2	2.625	1.313
Treatments	16	28917.000	1927.800
Error	30	11.375	0.379
Total	48	-	-

Table 5: Characterization of Cumin callus induced from hypocotyls

Callus induction medium	2,4-D (μM)	KIN (μM)	Characterization
CIM-1	0.0	0.0	---
CIM-2	0.1	0.0	Friable creamy
CIM-3	1.0	0.0	Friable creamy
CIM-4	10.0	0.0	Friable creamy
CIM-5	0.0	0.1	Compact greenish
CIM-6	0.1	0.1	Friable creamy
CIM-7	1.0	0.1	Compact greenish
CIM-8	10.0	0.1	Friable creamy
CIM-9	0.0	1.0	Compact greenish
CIM-10	0.1	1.0	Friable greenish
CIM-11	1.0	1.0	Friable creamy
CIM-12	10.0	1.0	Friable brown
CIM-13	0.0	10.0	Compact greenish
*CIM-14	0.1	10.0	*Friable greenish
CIM-15	1.0	10.0	Friable greenish
CIM-16	10.0	10.0	Friable brown

CIM: Callus induction medium, *Selected CIM-14 for subsequent experiments

Table 6: Growth kinetics of Cumin cell suspension cultures

	Aliquot (1 mL)		Aliquot (2 mL)		Aliquot (4 mL)		Aliquot (8 mL)	
Days	PCV	Fresh weight (mg)	PCV	Fresh weight (mg)	PCV	Fresh weight (mg)	PCV	Fresh weight (mg)
0	0.41	4.0	0.83	9.0	0.96	10.2	1.46	14.3
7	0.56	6.2	0.95	10.1	1.21	12.4	1.56	15.0
14	0.72	8.2	1.20	12.2	1.25	12.5	1.67	16.2
21	0.95	10.1	1.23	12.1	1.42	14.0	1.97	18.0
28	1.20	13.2	1.28	12.7	1.56	16.3	2.10	20.0
Total increase after 28 days	0.79	9.2	0.45	3.7	0.60	6.1	0.64	5.7
Initial cell number (cells mL^{-1})	3×10^5		6×10^5		1.1×10^6		2.2×10^6	
Growth rate	0.038		0.0154		0.0157		0.0139	
Doubling time (day)	0.75		1.86		1.66		2.22	

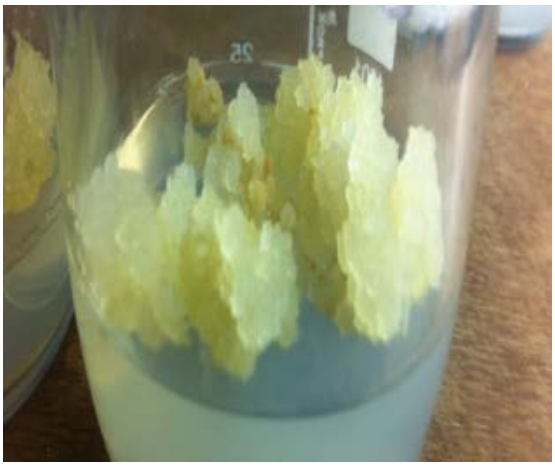


Fig. 8: Friable callus of cumin

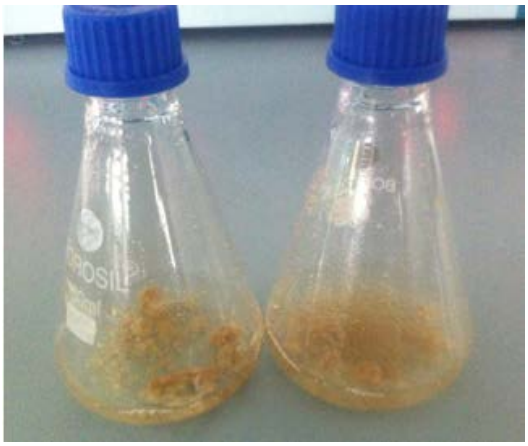


Fig. 9: Cell suspension culture in 0.1 µM 2, 4-D and 10 µM KIN medium

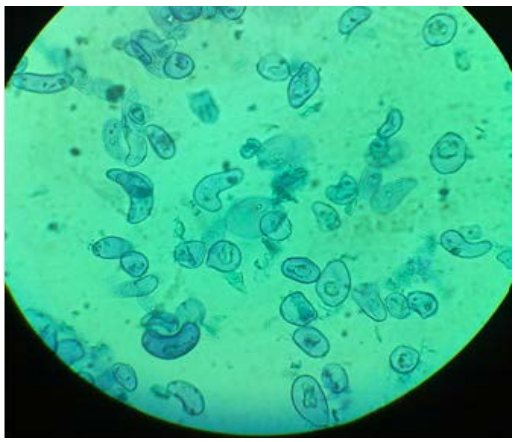


Fig. 10: Cumin cells suspension under inverted microscope stained for viability (Non-viable cells appear dark blue)

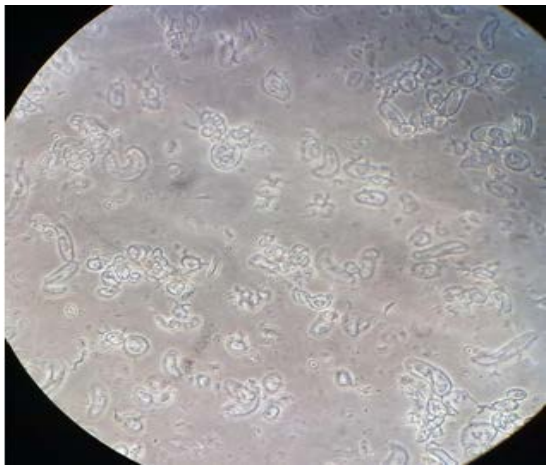


Fig. 11: Cumin cells suspension under inverted microscope stained for viability (viable cells remain unstained)

selected from all the 16 combinations tried for sub-culture and the callus were maintained on this medium for all subsequent experiments.

Cell suspension culture: The establishment of Cell Suspension Culture (CSC) was only possible by using friable callus. The CSC was initiated from single cells, callus clump and friable calli (Fig. 8, 9). At an early stage of CSC, large, elongated and highly vacuolated cells with thick cell wall were observed (Fig. 10, 11). During the subsequent frequent sub-cultures, they were stepwise eliminated. Extrusion of dye leaving cells unstained indicated viability, the ratio of which gradually increase to unviable blue cells in cell cultures over successive cycles. With repeated bi-weekly/weekly sub-cultures, it was possible to achieve a suspension consisting of small cell aggregates. The growth dynamics of CSCs was determined using small cells aliquots (1, 2, 4 and 8 mL) from 2 months old CSCs which were inoculated in fresh cell culture medium. It consisted of MS+0.1 µM 2,4-D and 10 µM KIN. Each cell suspension consisted of a total volume of 10 mL and aliquots with cell density of 3×10^5 cells mL^{-1} were added to the cell culture medium (Fig. 12). Four replicates were observed for the experiment.

To establish a well growing cell suspension culture it is necessary to inoculate at least 10^4 cells mL^{-1}, 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.

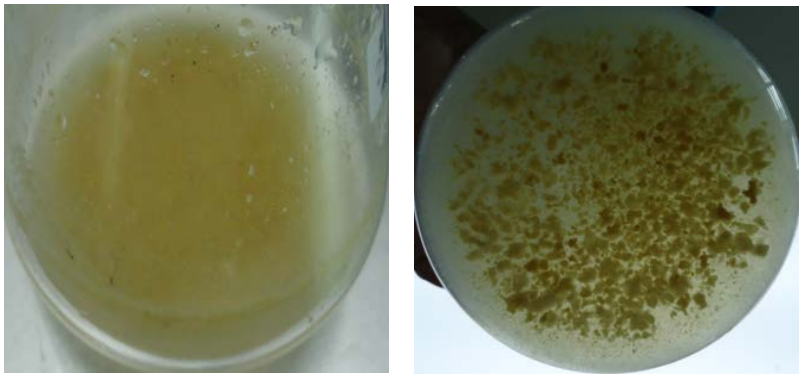
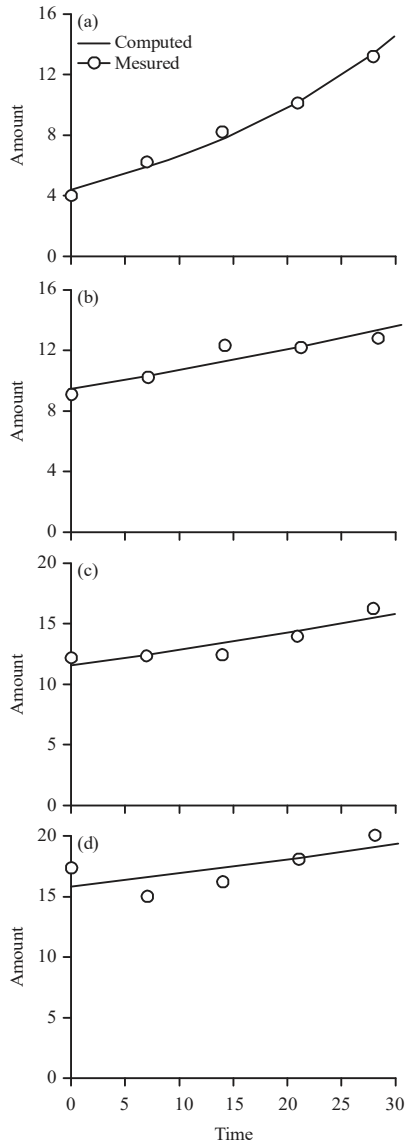


Fig. 12: Cell suspension culture in 0.1 µM 2,4-D, 10 µM KIN after 2 months



A cell density of 3×10^5 cells
Doubling time (Days) = 0.75 = ln (2)/0.0393
Growth rate = 0.0393
Growth rate = Number of doublings that occur per unit of time
Equation: Amount = $4.4715\times e^{0.0393*time}$
At t = 0, calculated cell concentration = 4.4715

B cell density of 6×10^5 cells
Doubling time (Days) = 1.86 = ln (2)/0.012
Growth rate = 0.0154
Growth rate = Number of doublings that occur per unit of time
Equation: Amount = $9.4316\times e^{0.012*time}$
At t = 0, calculated cell concentration = 9.4316

C cell density of 1.1×10^6 cells
Doubling time (Days) = 1.66 = ln (2)/0.0132
Growth rate = 0.0157
Growth rate = Number of doublings that occur per unit of time
Equation: Amount=$11.9646\times e^{0.0132*time}$
At t = 0, calculated cell concentration = 11.9646

D cell density of 2.2×10^6 cells
Doubling time (Days) = 2.22 = ln (2)/0.0068
Growth rate = 0.0139
Growth rate = Number of doublings that occur per unit of time
Equation: amount = $15.7344\times e^{0.0068*time}$
At t = 0, calculated cell concentration = 15.7344

Fig. 13(a-d): Detail of doubling time, growth rate and cell concentration calculated by software for different cell density of CSCs of Cumin

The growth dynamic of CSCs was determined at various cell densities on CIM-14 medium containing 0.1 μM 2,4-D and 10 μM KIN lacking agar. The data on PCV and fresh weight measured every week indicated that the cells grew and multiplied rapidly with a doubling time of 0.75 days. Elongated and divided cells were observed more in 21 days.

Fresh weight and PCV of the cells increased nearly 3 folds and a cell doubling time of 0.75 and growth rate of 0.038 with initial inoculum density of 3×10^5 cells mL^{-1} (Table 6) with a doubling time of 1.86 days was calculated with 2 folds increased fresh weight mean 11.16% from 6×10^5 cell mL^{-1}, growth rate 0.0154 was calculated during 28 days. With an increase in the initial inoculums density, the growth rate decreases and there is a corresponding increase in the doubling time to the extent that with 8 fold increase of initial inoculum, the growth rate fall three fold (0.0139) and the doubling time goes up three fold (2.22 days) as shown in Table 6 and Fig. 13a-d. Earlier, investigator reported a doubling time of 2.0-2.2 days for Cumin cell suspension in B5 medium[15] and also reported 2-2.5, 4.7 and 3-6 days doubling time respectively in wheat cell cultures[17-19]. In present study, the minimum doubling time was as short as 0.75 and maximum was 2.22 days as shown by Ahmed *et al.*[15]. These differences could be due to the difference in medium composition (here MS medium was used instead of B5) or the initial cell density of the suspension.

In this study, it described callus culture and the establishment of cell suspension cultures from white and friable callus, which was previously obtained from hypocotyls segments of cumin GC-4. Using different concentrations and combinations of the cytokinin KIN and auxin 2,4-D (0.0, 0.1. 1.0 and 10 μM) the growth patterns of the cultures were examined during a range of culture durations. The growth rates of cells were initially slow, but as the culture proceeded, they increased significantly and accumulated great amounts of biomass over a period of 28 days. Medium containing high kin (10 μM L^{-1}) and 2,4-D (0.1 μM L^{-1}) induced higher rates of cell division than the medium containing low KIN (0.1 μM L^{-1}) and 2,4-D (10 μM L^{-1}) or the control. After selection of small aggregates and single cell culture at 15 days intervals for 2 months, homogeneous and light yellow CSCs, composed of single and small cells aggregates were established. The optimized culture conditions obtained from the present study will then be used in resistance cell selection against pathogen toxin experiments. Investigated study promotes genetic manipulation.

CONCLUSION

The MS basal medium with 1.0 μM 2,4-D and 10 μM Kinetin was found most suitable for high callus induction from epicotyls and hypocotyls as explants of Cumin. Cell suspension cultures were derived from these calli by transfer to liquid MS medium with 1.0 μM 2,4-D and 10 μM Kinetin. The information is imbibed in this research article indicate the role of 2,4-D and kinetin in differentiations of cumin.

SIGNIFICANCE STATEMENT

Epicotyls and hypocotyls of Cumin were good sources of callus induction. Friable callus production is important to establishment of good quality of cell suspension. Researcher can take benefit from its investigation to manipulation genetically.

ACKNOWLEDGMENT

The authors wish to acknowledge the principal Dr. Ajay Gor and staff of the Department of Biotechnology, P.S. Science and H.D. Patel Arts College, Kadi, India, for granting us access to their equipments.

REFERENCES

1. Allan, E., 1991. Plant Cell Culture. In: Plant Cell and Tissue Culture, Stafford, A. and G. Warren (Eds.). Wiley Publishers, Chichester, UK., ISBN-13: 9780471932611, pp: 1-23.
2. Phillips, G.C., J.F. Hubstenberger and E.E. Hansen, 1995. Plant Regeneration by Organogenesis from Callus and Cell Suspension Cultures. In: Plant Cell, Tissue and Organ Culture: Fundamental Methods, Gamborg, O.L. and G.C. Phillips (Eds.). Springer, Berlin, Germany, ISBN: 978-3-642-79048-5, pp: 67-79.
3. Hall, R.D., 1991. The Initiation and Maintenance of Plant Cell Suspension Cultures. In: Plant Tissue Culture Manual, Lindsey, K. (Ed.). Springer, Berlin, Germany, ISBN: 978-94-011-7658-3, pp: 45-65.
4. Ebrahimie, E., A.A. Habashi, B. Ghareyazie, M. Ghannadha and M. Mohammadie, 2003. A rapid and efficient method for regeneration of plantlets from embryo explants of cumin (*Cuminum cyminum*). Plant Cell Tissue Org. Cult., 75: 19-25.
5. Ebrahimie, E., A.A. Habashi, M. Mohammadie-Dehcheshmeh, M. Ghannadha, B. Ghareyazie and B. Yazdi-Amadi, 2006. Direct shoot regeneration from mature embryo as a rapid and genotype-independent pathway in tissue culture of heterogeneous diverse sets of cumin (*Cuminum cyminum* L.) genotypes. *In vitro* Cell. Dev. Biol.-Plant, 42: 455-460.

6. Gupta, D., 2013. Studies on biochemical markers associated with regeneration potential in *Cuminum cyminum* L. Res. J. Chem. Environ. Sci., 1: 63-65.
7. Gupta, D. and S. Bhargava, 2001. Thidiazuron induced regeneration in *Cuminum cyminum* L. J. Plant Biochem. Biotechnol., 10: 61-62.
8. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.
9. Freshney, R.I., 1987. Culture of Animal Cells: A Manual of Basic Technique. 2nd Edn., Alan R. Liss Inc., New York, USA., ISBN-13: 9780845142417, pp: 117.
10. Suthar, R.S., D.P. Bhatt and P.N. Bhatt, 2014. Effect of culture filtrate of *Fusarium equiseti* on seed germination and seedling growth of cumin (*Cuminum cyminum*). Indian Phytopathol., 67: 193-194.
11. Mann, R., K. Agarwal, C. Singh, V. Aeri and R. Nema, 2008. Adventitious shoot proliferation from aseptically germinated seedlings of *Cuminum cyminum*. Pharmacogn. Mag., 4: 132-137.
12. Dave, A. and A. Batra, 1995. Role of protein metabolism constituents in somatic embryo formation in cumin. Indian J. Plant Physiol., 38: 25-27.
13. Tawfik, A.A. and G. Noga, 2001. Adventitious shoot proliferation from hypocotyl and internodal stem explants of cumin. Plant Cell Tissue Organ Cult., 66: 141-147.
14. Tawfik, A.A. and G. Noga, 2002. Cumin regeneration from seedling derived embryogenic callus in response to amended Kinetin. Plant Cell Tissue Org. Cult., 69: 35-40.
15. Ahmed, K.Z., M.K.A. Aly, S.K. Ahmed, J.A.T. da Silva and H.M. Kamel, 2011. Establishment of embryogenic cell suspension culture and plant regeneration of Egyptian cumin (*Cuminum cyminum* L.). Funct. Plant Sci. Biotechnol., 5: 83-90.
16. Kahrizi, D. and J. Soorni, 2013. Study on shoot regeneration and somatic embryogenesis in cumin (*Cuminum cyminum* L.) landraces. Biharean Biol., 7: 37-41.
17. Redway, F.A., V. Vasil, D. Lu and I.K. Vasil, 1990. Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.). Theoret. Applied Genet., 79: 609-617.
18. Ahmed, K.Z. and F. Sagi, 1993. Culture of and fertile plant regeneration from regenerable embryogenic suspension cell-derived protoplasts of wheat (*Triticum aestivum* L.). Plant Cell Rep., 12: 175-179.
19. Qiao, Y.M., M. Cattaneo, F. Locatelli and E. Lupotto, 1992. Plant regeneration from long term suspension culture-derived protoplasts of hexaploid wheat (*Triticum aestivum* L.). Plant Cell Rep., 11: 262-265.