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In Vitro Callus Induction in Ephedra Foliata Boiss.

Dr. Reena Rastogi
Asst. Professor Botany
R.B.A. Govt. Degree College Gajraulla, Amroha
Reenarastogi76@gmail.com

Dr. Sanjeev Kumar Srivas
Asst. Professor Botany
Govt. Mahila Degree College Badaun
Sanjeev_srivas@yahoo.com

Ephedra foliata is a medicinal plant belonging to the family Ephedraceae, Ephedrine is the main alkaloid in Ephedra which has different medicinal properties. However, the amount of Ephedrine in plant material is low and callus culture can be a way to increase the alkaloid content. The present study is based on in vitro callus induction on Ephedra foliata. The internodal portion of the stem was inoculated on Murashige and Skoog's basal medium supplemented with plant growth regulators and various combinations of Auxins (IAA, IPA, IBA, NAA, 2,4-D and 2,4,5-T) and Cytokinin (Kinetin) to study callus induction, growth and differentiation. Growth patterns of callus were also studied under the influence of growth hormones. 2,4-D was used in the concentration of 1.0 mg/l and 2.0 mg/l and 2,4,5-T was used in the concentration of 1.0 mg/l and 2.0 mg/l in the combination of Kinetin (0.5mg/l) to study callus initiation. Best callus initiation and growth were found on the medium MS±2-4D (2.0 mg/l+kn(0.05 mg/l) and on the medium MS±2,4D (4.0 mg/l)+Kn(1.0 mg/l).
KEYWORDS: Ephedra, Callus, hormones, culture

I. INTRODUCTION

Plant cell tissue culture and its development as a specialization through the establishment of true tissue culture, demonstration of plant cell totipotency including somatic embryogenesis, haploidy, and overcoming of sexual barriers through protoplast fusion helped usher in the era of genetic engineering. Most dicotyledonous annuals and foliage plants have been reported to regenerate in vitro and considerable success have been achieved in the whole plant regeneration from agronomically important monocots. Various techniques of plant tissue culture have proved to be useful in basic studies like growth, development, physiology, and biochemistry and acquired many practical applications in the field of agriculture and horticulture.

The history of plant tissue culture began more than 225 years ago. The discovery of callus formation was made by Heri-Louis Duhamel du Monceau (1756). Haberlandt's (1902)-" Father of Tissue Culture" was the first who predicted the phenomenon of totipotency. He cultured and isolated, fully differentiated cells in a nutrient medium containing Glucose, Peptone, and knop'sSalt Solution but he failed to achieve division in free cells. After Haberlandt, further progress in this respect was made by Muir (1953) and P.R. White (1939), working independently at different places.

In 1926, Went discovered growth hormones which were established by Kogl et al.(1934) as Indole acetic acid and Snow (1935) demonstrated that it stimulated cambial activity. In the medium when IAA was added, an undifferentiated mass was developed known as callus and this process is known as dedifferentiation. This callus has unlimited growth. This provided good material for morphologists to study the phenomenon of redifferentiation. Probably the most significant event leading to advancement in the next decade was the discovery of the nutritional quality of liquid endosperm extracted from coconut. Van Overbeek and his coworkers (1941) cultured successfully the excised embryo of Datura on a medium supplemented with coconut milk. In 1957, Skoog and miller proposed the concept of hormonal control of organ formation.

Different modified media have been devised by several workers from time to time. Some of the commonly used media are white medium (White,1963); Heller medium (Heller,1953); Murashige and Skoog's medium (Murashige and Skoog,1962); B_5 medium (Gombarg 1963); NT medium (Nagata and Takabe,1971), SH medium (Schenck and Hilderbrandt,1972). However, MS medium is preferred over other media.

With the advancement in tissue culture techniques, they have been widely exploited in some major areas of agriculture viz. (1) obtaining disease-free plants (2) rapid propagation of plants that are difficult to

propagate (3) somatic hybridization (4) genetic improvement (5) obtaining haploid plants from pollen for the breeding programs.

Nowadays the production of primary and secondary metabolites of economic value is one of the major objectives of plant cell culture scientists (Yamunda, 1984). It has been proposed that although many of these secondary metabolites are produced by intact plants grown in nature, there exists a great advantage when they can be obtained from plant cell culture under controlled conditions. Various chemicals like diosgenin, quinine, digoxin, codeine, scopolamine, vinblastine, vincristine, ephedrine, and many others were reported through tissue culture which is in great demand (Carew and Staba, 1965; Alfermann and Reinhard, 1978; Khanna et al, 1975; Mehta, 1987; Street, 1973; Tobata et al.,1978).

In India, very few laboratories are engaged in the production of secondary metabolites. The beginning of this work goes back to 1964 when Mitra and Kaul at NDRI, Lucknow showed the production of reserpine from *Rauwolfia serpentina* culture. Later on, work on various metabolites was carried out in other laboratories.

Ephedra is the only genus belonging to the family Ephedraceae under the class Gnetopsida. It comprises nearly 40 species widely distributed in the arid region of the New World and Old world from the Mediterranean and black sea shoes up to China including northern and north-eastern India. In India, the genus is represented by six species viz . *E. foliata, E. intermedia, E.gerardiana, E. saxatilies, E. nebrodensis and E. regelia*na. Present observation is based on the studies made *on Ephedra foliate.*

The young stem is green, hard, ribbed, glabrous and much branched. It is distinctly jointed, slender, and has long internodes. The branches are also green and photosynthetic. The leaves are deciduous, opposite or whorled, more or less connate basally and usually reduced to membranous sheaths. The scale leaves bear a bud each in their axils. All the species of Ephedraare dioecious and bear male and female reproductive organs or different plants. The male strobilus of Ephedra is regarded as a compound structure and arises in dusters from nodes of branches. Female strobilus usually arises at each node in the axil of scale leaves. In Ephedra the process of polyembryony is unique among the gymnosperms. But only one embryo can grow to maturity.

Various species of Ephedra are used as an important source of the alkaloids ephedrine and pseudoephedrine which may also be prepared by synthesis.

The therapeutic activity of Ephedra is due to the alkaloids ephedrine and pseudoephedrine. Ephedrine is a non-heterocyclic alkaloid. Ephedrine has a heavy pine-like aromatic odor and a strong astringent. On reduction, ephedrine yields the therapeutically useful deoxy-ephedrine. Synthetic product of ephedrine (di-ephedrine; M.P.76°) is marketed under the name, Racephedrine, dry extract can be used for the preparation of pure ephedrine (Ghose and Krishna, 1943). The major disease in which ephedrine has been helpful are:

Dilated eye pupils, increased blood pressure, in spinal anesthesia, heart block, bronchial asthma, hay fever, compulsive sleeping, drug poisoning and Myasthenia gravis.

Ephedrine in excessive doses causes nervousness, insomnia, headache, vestige, palpitation, sweating, nausea, vomiting, occasionally pericardial pain and sometimes dermatitis.

Most of the morphogenetic work in Ephedra has been done after the sixties. Sankhuls et al. (1967), Khanna et al. (1976), Ramwat and Arya (1979), Singh et al (1981), O'Dowd and Richardson (1993), Mamta Garla et al. (2011), Azra Ataei Azimi and Babak DelnavazHashemloian (2015), Joshi A.R. and Deokule S.S. (2019), are important researchers who worked on Ephedra. Ephedrine is the main alkaloid in Ephedra which has different medicinal properties however the amount of Ephedrine in plant materials is low and callus culture can be a way to increase the alkaloid content.

Keeping the facts in mind present study was done.

II. MATERIALS AND METHODS

The explants of Ephedra foliata were procured from the Botanical Garden, Chaudhary Charan Singh University, Meerut. The nodal and internodal explants were used for the callus initiation and further experiments.

CULTURE METHODS

Culture Medium

Murashige and Skoog's (1962) basal medium (BMS) was used to study the various morphogenetic phenomenon in Ephedra. Detailed composition of media used in experiments is given in Table 1.

a. Chemicals

The chemical used in experiments were research grade purity (Anola R) and were obtained from E. Mark (INDIA) Pvt. Ltd. and Sigma Chemical (U.S.A.)

b. Culture Vessels

100 ml Erlenmeyer flasks and 25 x 150 mm of Borosil brand were used in culture vessels. All culture vessels and glassware used in the preparation of the media and other purposes were cleaned with chromic acid and then washed in running tap water. Finally, the glassware was rinsed with double distilled water and dried in an oven at 180°C for the furthers

c. Sterilization of Glasswares.

Petri dishes were wrapped in aluminum foil and were sterilized in an autoclave at 15 lbs./inch2 (1.06 kg/cm^2) for 15 minutes at a temperature 121°C. Double distilled water in flasks were also sterilized in the same manner.

d. Preparation of Culture medium

The Composition of Murashige and Skoog's (1962) mediuma is given in Table-1

Table – 1. Composition of Murashige and Skoog's Medium (1962).
Stock solution number constituents' concentrations (mg/l)

S.No.	MS Medium supplemented with auxins (mgl^{-1})	Fresh weight (mg)
1	NH_4NO_3	1650
2	KNO_3	1900
3	H_3BO_3	6.2
4	DKH_2PO_4	170
5	$Na_2MoO_4.2H_2O$	0.25
6	KI	0.83
7	$COCl_2$.6H2O	440
8	$CaCl_2.6H_2O$	0.025
9	E $MgSO_4.4H_2O$	370
10	$MnSO_4.4H_2O$	22.3
11	$ZnSO_4.4H_2O$	8.6
12	CuSO4.5H2O	0.025
13	F Na EDTA	37.35
14	$FeSO_4.7H_2O$	27.82
15	G Thiamine HCL	0.1
16	Nicotinic acid	0.5
17	Pyridoxine HCI	0.5
18	Glycine	2

Meso-inositol-100 mg/l; Sucrose 20-30 gm/l; Agar -agar-8 mg/l.

Separate stock solutions like A, B, C, D, E, F, and G were prepared according to the composition of the nutrient medium in double distilled water. These stock solutions were stored in the refrigerator at 5-100°C and were used only for up to 1 month of their preparation. The solution was mixed in the required amount and the final volume of the medium was made after dissolving sucrose (2-4%).

Similarly, growth substances were prepared as a stock solution in a 1:1 ratio. For this 20 mg of growth, regulators were dissolved in 20 ml of double distilled water. The growth regulators were dissolved in 20 ml of double distilled water. The growth regulators used during the experiment were auxin (IAA, IPA, IBA, NAA, 2, 4-D, and 2, 4, 5-T) and cytokinin (Kinetin). The auxin was dissolved in a few drops of absolute alcohol whereas cytokinin was dissolved in a few drops of IN NaOH and the final volumes were made by adding double distilled water. Each growth regulator prepared was stored in dark at a low temperature in the refrigerator.

0.6% to 0.8% of Agar-Agar was used for solidification of the medium. Meso-inositol (100 mg/l) and sucrose (30 gm/l) as a carbon source were weighed separately and added to the medium.

e. The pH of the Medium

The pH of the medium was adjusted to 5.4-5.8 by using IN-HCL or IN-NaOH before autoclaving.

f. Sterilization of Medium

About 40 ml of culture medium was dispensed in a 100 ml Erlenmeyer flask. The culture flasks were plugged with non-absorbent cotton covered with paper/aluminum foil and kept in the autoclave at 15 lbs./inch-2 (1.06 kg/cm^2) for 15 minutes at 121°C temperature.

g. Sterilization

The working table of the laminar airflow chamber was cleaned with 9% alcohol before carrying out operations the cabinet as well as media vessels, sterilized petri plates, and all equipment like scalpel forceps spatula, etc.

were sterilized by flaming with 90% alcohol inside the laminar air flow chamber, when not in use, these equipment's were kept immersed in alcohol.
Both inoculation and incubation chambers were sterilized with fumigation every week from fumes obtained by potassium permanganate ($KMnO_4$) with formaldehyde or with the help of bromine gas. All inoculations and aseptic manipulations were carried out in a laminar airflow chamber.

h. Surface Sterilization of Explants
The stem nodal and internodal explants from field-raised plants were first washed with a teapot. They were then washed thoroughly for 1 hour in running tap water and finally rinsed with double distilled water.
In the case of Ephedra foliate, internode was cut into pieces of about 1 cm in size, then it was cut longitudinally into two halves. The sterilization is done by treating the piece with 0.1% $HgCl_2$ for 2-3 minutes. After surface sterilization explants were rinsed 5-6 times with sterilized double distilled water to remove the traces of mercuric chloride. All these sterilization processes were carried out in a laminar airflow cabinet under aseptic conditions.

i. Inoculation
After surface sterilization, internodal explants were kept in sterilized double distilled water in a Petri dish. During inoculation, the instruments used like scalpels, forceps, spatula, etc. were sterilized with 90% alcohol and followed by flaming on a spirit lamp and cooling. Now with the help of this sterilized equipment, explants were incubated into the flask containing nutritional medium supplemented with different growth regulators at various concentrations. The inoculation in culture vessels was carried out in laminar air flow under aseptic conditions.
j. Incubation
After the incubation procedure, the culture vessels were incubated in an incubation chamber under controlled conditions. The temperature of the culture room was adjusted to 25 ±2°C with the help of an air conditioner. The culture was exposed to 16 hrs. photoperiod providing a light intensity of about 1500 Lux.
k. Culture techniques
The cultures derived from the plant material were grown and maintained on semi-solid media; the callus was initiated within 30-40 days of incubation and soon extended all over the explant. The callus proliferated and a considerable amount of callus was formed after one month of induction.
l. Sub-culture techniques
The calli were regularly transferred after every 3-4 weeks in their experimental phase of growth on the fresh media. For subculture, the callus was cut into small pieces of almost equal size and transferred aseptically to other flasks containing fresh medium. The process of subculture was repeated until a large amount of callus tissue was obtained.

III. OBSERVATIONS AND RESULTS

The present observation is based on the studies made on Ephedra foliata. The stem, internodaland nodal explants were inoculated on MS basal medium supplemented with plant growth regulations to study callus induction, growth, and differentiation. The growth pattern of callus was also studied under the influence of growth hormones. The callus was harvested after 45 days of inoculation. The initial amount of callus inoculated was approximately 100 mg of fresh weight per flak of 100 ml capacity. The readings were taken for fresh weight and dry weight after 30 days, an average of calli of three flasks were taken. Dry weights were taken after drying the calli at 60°C for 48 hours.

Callus Initiation

The internodal explants were inoculated on MS medium supplemented with various combinations of auxins and cytokinin 2, 4-D was used in concentrations of 1.0 mg/l and 2.0 mg/l, and 2, 4, 5-T was used in the concentration of 1.0 mg/l and 2.0 mg/l in the combination of kinetin (0.5 mg/l) to study callus initation.

The initiation of callus was started by the swelling of explants and followed by the proliferation of cells. The subepidermal cells were stimulated to show hyper tropical growth. The epidermal cells were mostly peeled off. Inner cells were also involved in the formation of callus. Induction was found after 30-35 days of inoculation. A considerable amount of callus was formed after one month of induction. The color, texture, and amount of callus formed were different on different combination of growth regulators used.
Best callus initiation and growth were found on the medium MS +2, 4-D (2.0 mg/l) + Kn (0.05 mgL-1) and the medium MS +2, 4-D (4.0 mg/l) + Kn(1.0 mgL-1)
Generally, pale yellows of light brown, fragile and nodular callus were formed.

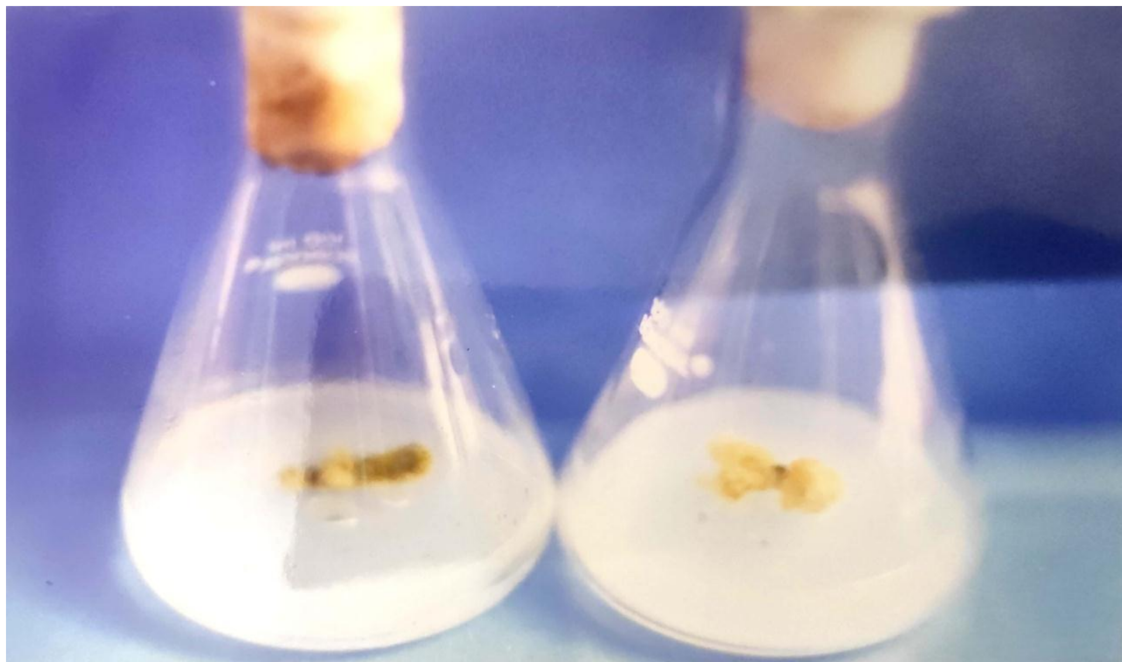

Figure A: Callus induction from internodal explants, cultured on MS medium supplemented with 2.0 mg/l 2,4-D along with 0.5 mg/l Kn.

The callus grew slowly up to three weeks, after that there was a rapid growth of callus in the fourth and fifth weeks, but again it slowed down.

The callus was subculture for its multiplication and the growth, was maintained on the media MS +2, 4-D (2-0 mg L^{-1}) + Kn (0.05 mgL^{-1}) on this medium yellowish white, fragile, and nodular callus was formed which later on get pigmentation and turned green.

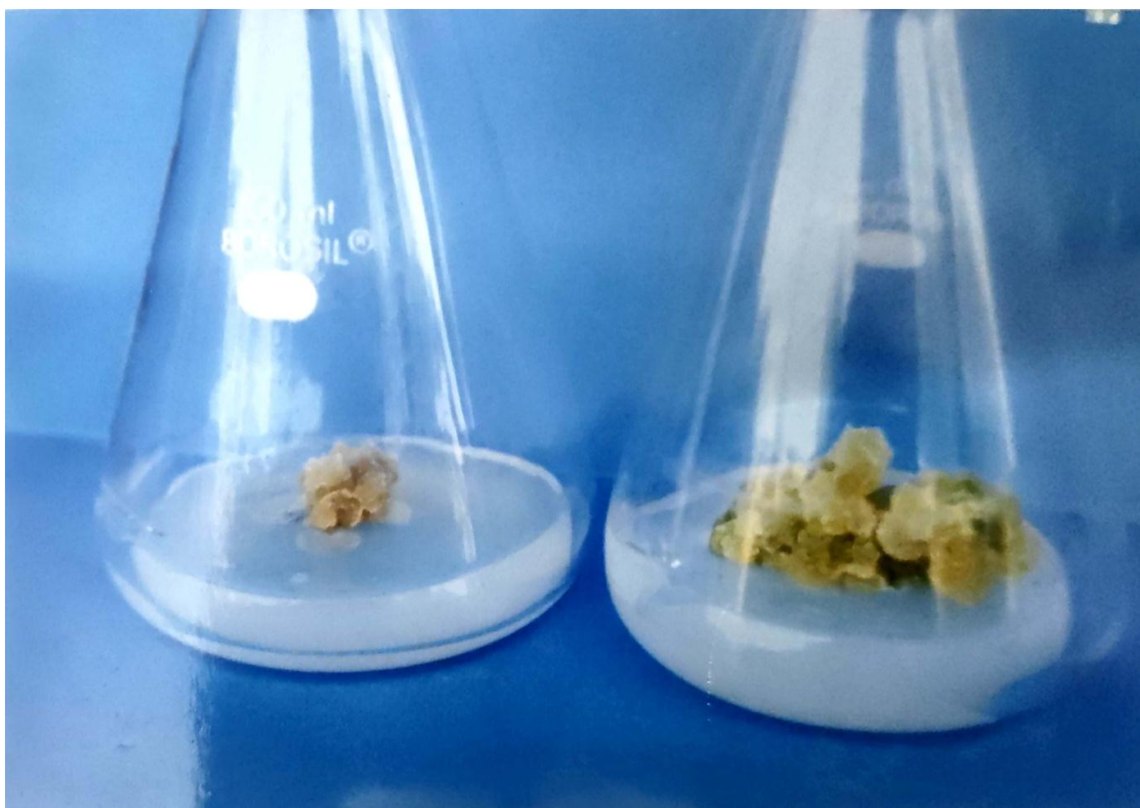

Figure B: Showing greenish yellow callus developed on MS medium supplemented with 2.0 mg/l 2,4-D along with 0.5 mg/l Kn.

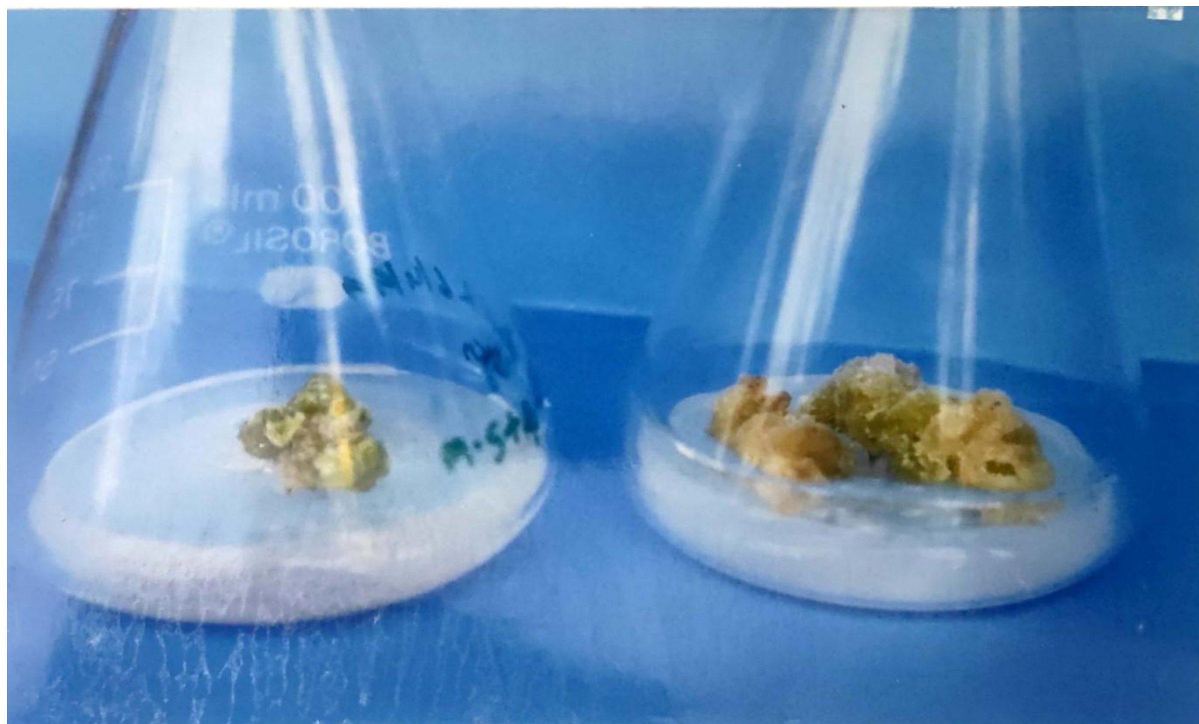

Figure C : Showing fragile nodular and green callus developed on MS medium supplemented with 2, 4-D (4.0 mg/l) + Kn(1.0 mgL-1)

Growth of the callus was studied in terms of fresh and dry matter weight after 30 days of inoculation of the callus on MS medium supplemented with different concentrations of auxins and cytokinins.

Effect of Auxins

The effect of NAA, IAA, IPA, IBA, and 2, 4, and 5-T in the concentrations of 0.5 mgL^{-1}, 1.0 mgL^{-1}, and 2.0 mgL^{-1} was studied on callus growth.

Effect of NAA

In basal MS medium supplemented with 5 mg/l NAA, fresh weight of the callus observed was 324 mg and the dry weight was 34 mg after 30 days. Basal medium supplemented with 1.0 mg/l NAA showed 467 mg of fresh weight and dry weight 48 mg of dry weight after 30 days. While in 2.0 mg/l NAA supplemented media, fresh weight of the callus was 593 mg and dry weight was52 mg

The callus was fragile, slow to grow, nodular, and light brown.

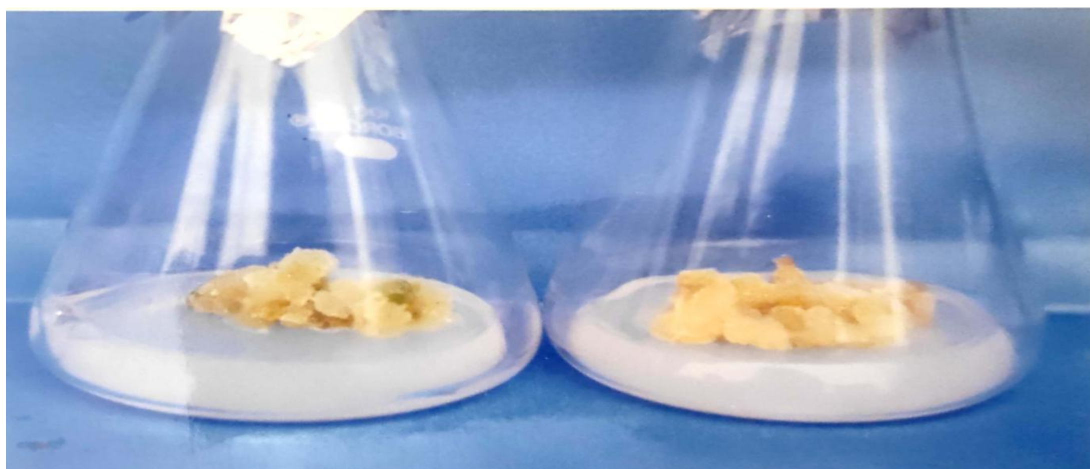

Figure D: Showing Fragile nodular and yellowish white callus developed on MS medium supplemented with 2.0 mg/l NAA.

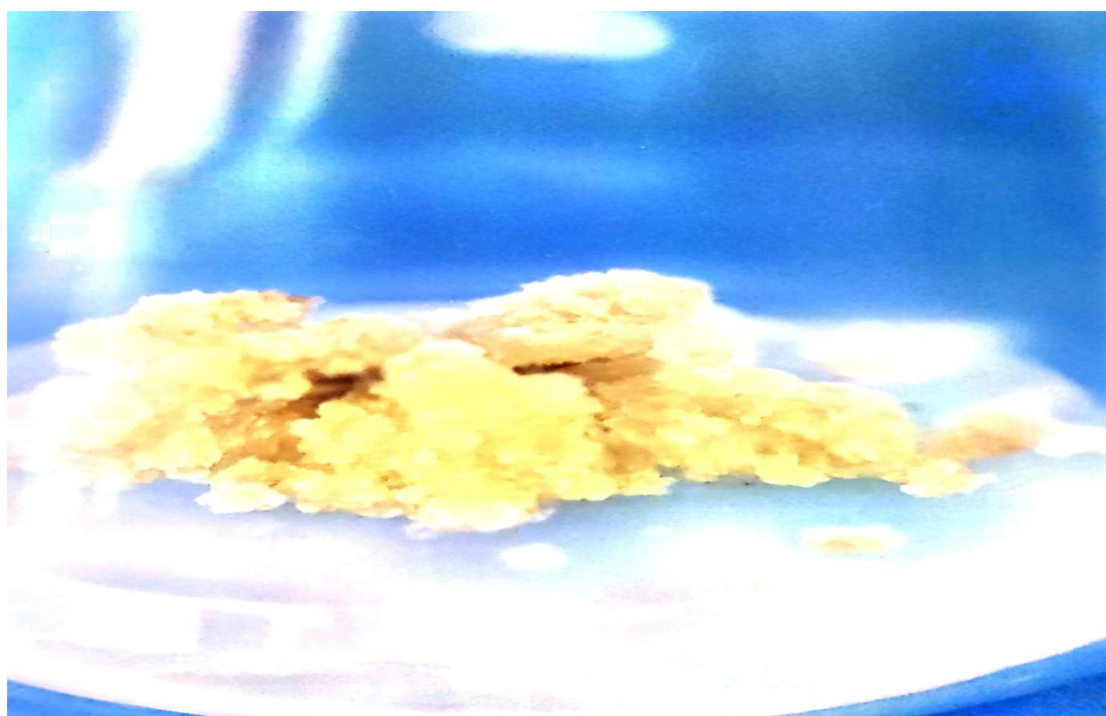

Figure E: Showing Fragile nodular and yellowish white callus developed on MS medium supplemented with 2.0 mg/l NAA.

Effect of IBA

In basal MS medium supplemented with 5 mg/l IBA, fresh weight of the callus observed was 462 mg, and the dry weight was 42 mg after 30 days. While in 1.0 mg/l IBA supplemented media, fresh weight of the callus was noted as 604 mg and dry weight was 78 mg. The callus was fragile, nodular, and yellowish, and after some time color changed to light brown. While in 2.0 mg/l IBA supplemented media, fresh weight of the callus was noted was 947 mg and dry weight was138 mg.

Effect of IPA

In basal medium supplemented with 0.5 mg/l IPA, the fresh weight of the callus observed was 368 mg and the dry weight was 31 mg after 30 days, and with 1 mg/l IPA, 416 mg of fresh weight and 38 mg of dry weight.
The callus developed was compact and yellowish-white in color. While in 2.0 mg/l IPA supplemented media, fresh weight of the callus was noted as 464 mg and the dry weight was 45 mg.

Effect of 2, 4, 5-T

In Basal medium supplemented with 5 mg/l 2, 4, 5-T, the fresh weight of the callus observed was 1130 mg, and the dry weight was 123 mg after 30 days. BM supplemented with 1 mg/l, 2, 4, 5-T showed 1262 mg of fresh weight and 136 mg of callus noted was 2310 mg? and dry weight as 196 mg after 30 days at 2.0 mg/l 2, 4, 5-T supplemented media. Callus growth was rapid, fragile, nodular, and yellowish white callus was observed and changed into the green after some time.
Table 2: Response of explant of E. Foliata on different concentrations of auxins for callus growth, nature of callus

S.No. M.S. Medium supplemented Nature of callus Growth of callus
With auxins (mgL^{-1})

S.No.	MS Medium supplemented with auxins (mgl^{-1})	Nature of Callus	Growth of Callus
1	MS + 0.5 NAA	Fragile	+++
2	MS + 1.0 NAA	Fragile	++
3	MS + 2.0 NAA	Fragile	+++
4	MS + 0.5 IAA	Compact	++

5	MS + 1.0 IAA	Compact	++
6	MS + 2.0 IAA	Compact	+++
7	MS + 0.5 IBA	Fragile	++
8	MS + 1.0 IBA	Fragile	+++
9	MS + 2.0 IBA	Fragile	+++
10	MS + 0.5 IPA	Fragile	++
11	MS + 1.0 IPA	Fragile	++
12	MS + 2.0 IPA	Fragile	++
13	MS + 0.5 2, 4,	Fragile	++
14	MS + 1.0 2, 4, 5-T	Fragile	++
15	MS + 2.0 2, 4, 5-T	Fragile	+++
16	MS + 2.0 2, 4-D + 0.5 Kn	Fragile	+++

+Slow ++ Good +++Very Good

Table 3: Growth of callus in terms of fresh and dry matter weights under different concentrations of auxins after 30 days.

S.No. MS Medium Supplement Fresh Weight Dry (Weight)
With Auxins (mgL^{-1}) (mg) (mg)

S.No.	MS Medium supplemented with auxins (mgl^{-1})	Fresh weight (mg)	Dry weight (mg)
1	MS + 0.5 NAA	324	34
2	MS + 1.0 NAA	467	48
3	MS + 2.0 NAA	593	52
4	MS + 0.5 IBA	465	42
5	MS + 1.0 IBA	604	76
6	MS + 2.0 IBA	947	138
7	MS + 0.5 IPA	368	31
8	MS + 1.0 IPA	416	38
9	MS + 2.0 IPA	464	45
10	MS + 0.5 2, 4, 5-T	1130	123
11	MS + 1.0 2, 4, 5-T	1262	136
12	MS + 2.0 2, 4, 5-T	2310	196

Table 4: Response of explant of E. foliata on different concentrations of cytokinins for callus growth and nature of callus.

S.No. M.S. Medium Supplemented Growth of callus Growth of Nature
With auxins (mgL^{-1})

S.No.	MS Medium supplementedwith auxins (mgl^{-1})	Nature of Callus	Growth of Callus
1	MS + 0.5 Kn	Fragile	++
2	MS + 1.0 Kn	Fragile	+++
3	MS + 2.0 Ka	Compact	+++
4	MS + 0.5 BAP	Fragile	++
5	MS + 1.0 BAP	Fragile	+++
6	MS + 2.0 BAP	Fragile	+++

+ = Poor
++ = Good
+++ = Very Good

Table 5: Growth of callus in terms of fresh and dry matter weights under different cytokinins concentrations after 30 days.

S.No. M.S. Medium Supplemented Growth of callus Growth of Nature
With auxins (mgL^{-1})

S.No.	MS Medium supplemented with auxins (mgl^{-1})	Fresh weight (mg)	Dry weight (mg)
1	MS + 0.5 Kn	1238	82
2	MS + 1.0 Kn	3532	215
3	MS + 2.0 Kn	3158	164
4	MS + 0.5 BAP	683	38
5	MS + 1.0 BAP	2185	196
6	MS + 2.0 BAP	3317	234

Effect of Cytokinin

The effect of Kn and BAP in the concentration of 0.5 mgL^{-1}; 1.0 mgL^{-1} and 2.0 mgL^{-1} was studied in callus growth. The best growth was observed on the medium supplemented with 2.0 mgL^{-1} BAP followed by 1.0 mgL^{-1} Kn and 2.0 mgL^{-1}?

Effect of Kn

In basal medium supplemented with 0.5 mgL^{-1} Kn, the fresh weight of the callus observed was 1238 mg and the dry weight was 82 mg after 30 days. BM supplemented with 1.0 mgL^{-1} Kn showed 3532 mg of fresh weight and 215 mg of dry weight after 30 days. While in 2.0 mgL^{-1} Kn supplemented media, fresh weight of the callus was noted as 3158 mg and dry weight as 164 mg after 30 days.
Figure E: Fragile nodular and green callus developed on MS medium supplemented with 4.0mg/l 2, 4 D along with 1.0 Kn.

Effect of BAP

In BM supplemented with 0.5 mgL^{-1} BAP, the fresh weight of the callus observed was 683 mg and the dry weight was 38 mg after 30 days. BM supplemented with 1.0 mgL^{-1} BAP showed 2185 mg of fresh weight and 196 mg of dry weight after 30 days. While in 2.0 mgL^{-1} BAP supplemented media fresh weight of the callus was noted as 3317 mg and the dry weight as 234 mg after 30 days.
Light brown, fragile, and nodular callus was observed, and the growth of the callus was very fast.

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