To Study Callus Induction from Explants of *Nicotiana tabacum*

Project report submitted to Central University of Punjab

For the award of

Master of Science

In

Life Sciences with Specialization in Plant Sciences

By
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Supervisor

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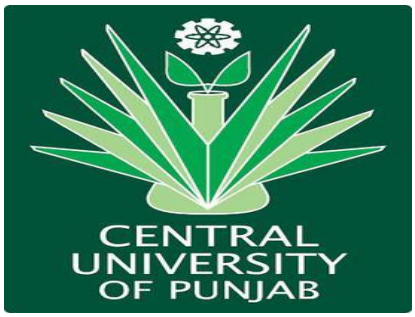

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May, 2018

CERTIFICATE

I declare that the dissertation entitled **"To study callus induction from explants of *Nicotiana tabacum*"** has been prepared by me under the guidance of Dr. Vinay Kumar, Assistant Professor, Department of Plant Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda for the award of degree of MSc. in Life Sciences with Specialization in Plant Sciences. No part of this dissertation has formed the basic for the award of any degree or fellowship previously.

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ABSTRACT

To study callus induction from explants of *Nicotiana tabacum*.

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Due to a great importance of callus most of the work has been carried out on regeneration aspect of callus including somatic embryogenesis, which is very important aspect of plant tissue culture and crop improvement. A large number of plants have been regenerated in plant tissue facility using various explants in phytohormone supplemented MS media. In present proposal, three selected phytohormone (NAA, BAP and 2,4-D) in different concentration combinations have been used for callus induction using leaf and seedling as explants in tobacco. It was found that best combination for induction of callus is BAP and NAA as compared to BAP and 2,4-D. Color, texture and size of callus were assessed after 25 days of culture. Rate of growth was found more in the culture media containing BAP and NAA combination. It was observed that color of callus was greenish in culture media having BAP and NAA and yellowish brown in the culture medium containing BAP and 2,4-D. In conclusion, this work help to establish that both seedling and leaf explants can be used for callus formation with appropriate supply of phytohormones.

Reetu Dr. Vinay Kumar

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Murashige and Skoog	MS
2	Callus inducing media	CIM
3	Milliliter	ML
4	Liter	L
5	6- benzylaminopurine	BAP
6	1-naphthaleneacetic acid	NAA
7	2,4-dichlorophenoxyacetic acid	2,4-D
8	Wound induced dedifferentiation	WIND1
9	Degree Celsius	˚C
10	Somatic embryo	SE
11	Zygotic embryo	ZE
12	Random amplified polymorphic DNA	RAPD
13	Centimeter	cm.
14	Polymerases chain reaction	PCR
15	Plant growth regulators	PGRs
16	Indole-3-acetic acid	IAA
17	Ultraviolet radiation	UV
18	Milligram	mg

Chapter 1

Introduction

Callus is unorganized cell mass having highly vacuolated and differentiated cells. Callus can be induced naturally as well in artificial environmental conditions. In plant species, callus generates naturally in stress conditions like during pathogen infections or wounding. Callus are induced in laboratory under aseptic conditions when cells or tissues grown on agar- gelled medium containing proper nutrients, PGRs etc. Plant growth regulators help in callus induction under *in-vitro* conditions. Cytokinins and auxins are major PGRs help in callus induction. The developmental fates of callus culture cells are determined by balance of cytokinins and auxins plant growth regulators (Gill *et. al.* 1992). Under the influence of plant growth regulators cells of callus become committed to generate whole plant. Joint actions of both hormones regulate the cell division. Auxins affect DNA replication process and cytokinins control events leading to mitosis and cytokinesis (Gasper *et. al.* 1996). A piece or part of plant is used for callus induction called explants. Cells, tissue or any organ of plant may act as explants. Portions of roots, leaves, shoots, buds, flowers are taken as explants. Success of work in plant tissue culture depends on choice of correct explants (George *et. al.* 2008). It is very difficult to culture hard parts of plants such as bark. Soft and meristematic tissues, buds, leaves and stems are easy to culture. Callus is formed from explants due to change in metabolism and appearance of cells. There are different types of callus on the basis of morphological appearance. Callus may be compact or friable. Compact callus is hard callus and cells are less separable but friable callus being soft, cells are separable. Sometimes callus may generates organs and on the basis of organ regeneration callus may be shooty, embryonic and rooty.

A lot of plants species had been studied for callus development. *Arobidopsis thaliana* and *Nicotiana tabacum* are considered as model plants mostly used in plant tissue culture for various studies. It is very difficult to culture woody plants such as bamboo plant.

Nicotiana tabacum being a model plant, most commonly used for Agrobacterium-mediated transformation. *Agrobacterium tumifaciens* (gram-negative bacterium) act as a tool to make transgenic plant. Scientists have considered this bacterium as a natural genetic engineer (Gelvin *et. al.* 2003). We generally take callus of leaf explants of tobacco for Agrobacterium- mediated transformation. It takes a lot of time to grow plant for preparation of mature leaf explants. In this work we check the callus induction from seedling as well as leaves explants. Callus induction from seedling explants will decrease wait time for transformation mediated experiments.

Different types and different concentrations of plant growth regulators influence the morphology of callus. As it is already known that only auxins and cytokinins plant growth regulators used for callus induction. In general, equal concentration of auxin and cytokinin leads to formation of callus. It was found that culture media having more concentration of cytokinin lead to development of shoot. If the concentration of auxin is more as compare to cytokinin in callus culture leads to development of root in callus. NAA and 2,4-D both are synthetic auxins but influence the callus morphology . NAA is mostly used for organogenesis while 2,4-D is used for callus formation and suppresses organogenesis.

The present study was designed to induce callus from seedling and leaves explants of *Nicotiana tabacum.* Five different types of culture medium were prepared for callus induction from seedling and leaf explants. Analysis of morphological characterstic of callus was done to see the effect of different hormonal concentrations and combinations. The color, texture and size of callus was assessed after 25 days of inoculation of explants in culture medium.

Hypothesis

To induce the callus from various explants of *Nicotiana tabacum* by different combinations of hormonal treatments.

Chapter 2

Review of Literature

Plant tissue culture technique is most widely used for multiplication of plants. It is a science of growing tissues or cells in a artificial nutrient media in sterile conditions. Haberlandt formulated the principle of cell culture and gave totipotency concept. The plant tissue culture technique is based on the totipotency concept. *In-vitro* culture of plant tissues or cells need generally two basic requirements. First, a proper nutrient medium for growth and development. There are different nutrient medium but MS media is most widely used in tissue culture as it provide all inorganic nutrients, vitamins and carbon sources for growth and development. Second requirement is aseptic conditions, plant tissues and cells and organs should be free form any microbial contaminations. So before inoculation explants should be sterilized with ethanol, detergents etc.

2.1 Callus

Plant cells are highly plastic for cell differentiation. Under stress conditions, plant cells are being able to induce cell masses. The unorganized masses of cells refer as tumors or callus. Stress conditions may be pathogen infections or wounding etc. that are able to generate callus naturally. Callus has differentiated and highly vacuolated cells (Ikeuchi *et. al.* 2013). The “callus” term originates from callum (latin word) which mean hard. In term of medicine, “callum” means thickening of dermal tissue. Callus cells are highly totipotent i.e. a single cell can regenerate a whole plant. Callus is exploited as a source of *in-vitro* tissue and organ regeneration. Callus is also induced under artificial environments by providing nutrient medium, plant growth regulators and aseptic conditions. Many physical factors like Ph, temperature, light and photoperiods effect the callus culture. Light plays very important role in the induction of callus. Callus generates either the presence or absence of light. In some plants like wheat, maize callus induction require dark or absence of light. Callus formation in some plants like chili, tomato need light (Siddique *et. al.* 2015). Callus induction of tobacco requires light of 16/8 hours light or dark photoperiods (Yanjie *et. al.* 2004). In tobacco plant, callus induction and plant regeneration calli develop in light rather than dark.

It has been observed that low impulse of electric current also affect the callus culture. Rathore *et. al.* 1985 found that 1-2 microamps electric current stimulate shoot differentiation in tobacco callus culture. It has been observed that electric current

increases the growth of callus. 1 microampere current passed through the culture medium containing callus of tobacco lead to increase in growth of callus.

There are different types of callus. Callus may be friable or compact (Ikeuchi *et. al.* 2013). Friable callus is soft and easily separable. Compact callus is hard callus and not easily separable. Callus may be embryonic, shooty or rooty on the basis of organ regeneration.

There are different ways to induce callus. The following protocols of callus induction are described below:

- Under natural conditions, callus is formed by wounding and infection of pathogen in plants. *Agrobacterium tumefaciens* is a pathogen cause crown gall disease by infecting plant and make a tumor like mass of cells. It was found that wound induced callus accumulate pathogen- related gene and phytoalexins that helps in defense processes. Wound-induces calli are highly pluripotent and used in horticulture.
- Callus may be induced *in-vitro* under artificial environmental conditions. Plant parts, tissues, cells are placed in the nutrient medium containing PGRs under aceptic conditions lead to formations of callus. Plant growth regulators help in the induction of callus. Auxins and cytokinins are plant growth regulators generally used for the formation of callus. Differentiation and dedifferentiation states depend upon balance between auxin and cytokinin hormone. Other hormone such as abcisic acid or brassinosteroids may substitute cytokinins or auxins in callus formation in some species *(Ikeuchi et. al*. 2013). Balance between auxin and cytokinin helps in the formation of callus.
- In tobacco, callus formation is induced in phytohormones free medium with the help of *AtWIND1* (*Iwase et. al.* 2013). AP2/ERF transcription factor WIND1 (WOUND INDUCED DEDIFFERENTIATION 1) also called RAP2,4 induces cell differentiation in *Arabidopsis thaliana*. Iwase et. al. introduced *35S:AtWIND1* into *Nicotiana tabacum* SR 1 and found that WIND1 helps in callus induction as well as establishment of callus cell lines.

Plant growth regulators effect the morphology of callus. Different plant growth regulators and different concentrations of plant growth regulators are main factors that affect the

callus morphology. In general, equal concentration of cytokinin and auxin hormone leads to formation of callus. If the concentration of auxin is kept high as compare to cytokinin in the culture medium will lead to root formation. Culture medium containing high amount of cytokinin as compare to auxin will lead to shoot formation. It has been found that NAA (auxin) is mostly used in organogenesis and 2,4-D is most effective for callus induction without any differentiation.

It had been observed that when foliar explants of N. *tabacum* were cultured in basal medium containing benzyladanine lead to formation of bud. Tanguy et. al. cultured foliar explants in basal media containing different combinations of benyladanine and 2,4-D. Level of hydroxycin-namoyl putrescines and amines were higher in callus culture containing 2,4-D alone instead of callus culture containing both 2,4-D and benzyladanine. Growth rate of callus was found to higher in the medium containing 2,4-D and benzyladanine (Tanguy *et. al.* 1988). These cultures are to study relationship cell devisions, amines and hydroxycin-namoyl putrescines.

Callus culture is most important for the plant regeneration, clonal propagation and genetic manipulation in plants. Transgenic plant can be prepared by inserting gene into callus. The gene of interest expresses itself after regenerating into whole plant. There are several method to insert a gene into callus i.e. *Agrobactrium tumefaciens*-mediated transformation, biolistic bombardment etc.

It was observed that repeated subculturing of tabacco callus give rise to somaclonal variations (Ghartaval *et. al.* 2010). Ghartaval et. al. done repeated subculturing of tobacco callus in a medium containing 0.5 mg/l kinetin and 4 mg/l 2,4-D resulting somaclonal variations. Long–term culture of callus leads to somaclonal variations. These variations are detected by RAPD using PCR. The result was indentified using 20 random RAPD primers and polymorphism of amplified DNA bands was seen in the electrophoresis pattern of three (OPC-09, OPA -10, OPR-12) random primers.The polymorphism indicates the somaclonal variation due to repeated subculturing.

Ce^{3+} and La^{3+} promote cell growth and plant growth. Recently it was found that $LaCl_3$ and $CeCl_3$ exert some effect on callus. Biological effects of these elements on callus induction are unclear. Low concentration (< 15 mg/l) of Ce^{3+} (rare element) increases the callus induction of *Nicotiana tabacum* (Song *et. al.* 2018). La^{3+} at concentration

more than 5mg/L inhibits the callus induction in tobacco. Ce^{3+} may help in improving enzyme activity resulting increase in callus.

Callus cells may form somatic embryo by changing the concentrations of plant growth regulators. Somatic embryogenesis is a process of formation of embryo from somatic cells. Sometimes direct SE can be formed in the culture medium. It has been found that in a medium containing 0.1 mg/L NAA and 1 mg/L BAP. It has been demonstrated that thidiazurone substituted the BAP-NAA combination for direct somatic embryogenesis in *Nicotiana tabacum*. Embryonic stages during the development of somatic embryo are similar as zygotic embryo.

Broman et. at. (1988) studied the callus of *N. tabacum* using explants pretreated with abscisic acid and found the improvement in cold damage. This study confirmed that abscisic acid make resistance to cold stress. Cells of abscisic acid treated tissue could be able to survive under subzero temperature. These cells were able to regenerate callus.

2.2 *Nicotiana tabacum*- a model plant

Nicotiana tabacum is a model plant for plant sciences, plant tissue culture and genetic engineering. The genus *Nicotiana* contains 76 species. It is a herbaceous, annually-grown plant and native of America. Tobacco is eudicot plant because of having two cotyledons; leaves have web-like veins and main tap root system. The species of genus *Nicotiana* grow in subtropical and tropical area. *Nicotiana tabacum* belongs to family *Solanaceae* and commercial non-food crop cultivated in nearly 120 countries. *Nicotiana tabacum* (2n=48) is a natural allotetraploid having 4.5 Gb genome size. It was formed approx. 6 million years ago by the interspecific hybridization between two diploid species i.e*. N. sylvestris* (maternal S genome donor, 2n=24) and *Nicotiana tomentosiformis* (paternal T genome donor, 2n=24) (Ganapathi *et. al.* 2004*). In-vitro* studies with *Nicotiana tabacum* tissue culture have provided idea of control of growth, development and differentiation. First time, transgenic plants were developed in tobacco plant. This plant is being also used for the production of antibodies, chemicals for medicine and recombinant proteins (Ganapati *et. al.* 2004). For example, 360 million doses of anthrax can be produced in one acre of tobacco (Pathi *et. al.* 2013). Being a

model plant *Nicotiana tabacum* provides a way for improvement of other crop plants. This plant has been used in many research works for studying physiology, morphogenesis and metabolism. The concept of totipotency was first time explained with tobacco plant by regenerating whole plant from single cell. A majority of discoveries in tissue culture, molecular biology and plant cell culture have been originated from experiments with this plant. This plant is used to study biological processes. First time standardization of growth medium for callus induction of *Nicotiana tabacum* was done by Murashige and skoog in 1962 using IAA and kinetin.

Because of following reasons*, Nicotiana tabacum* is considered as a model in plant tissue culture and genetic engineering:

- It is small plant and very easy to grow. The optimum temperature required for growth can be provided in laboratory. This plant easily grows in nutrient media and soil without any difficulty.
- It is very easy to culture any part of *Nicotiana tabacum* under aseptic conditions. This is possible because of the vegetative nature of the plant. The optimum condition for culture can be provided under laboratory conditions.
- This plant produces a large number of seeds and it is very easy to cross for experiment regarding improvement. Thousands of seeds are produces per cross and shows a large phenotypic diversity.
- Tobacco plants take only 3 months of time from seed to next generation seeds. One plant of tobacco generates million seeds. Seeds of tobacco plants are very small.

Tobacco is short- lived perennial plant. In tobacco plant, more than 2500 compounds have been found. Major discoveries in the field of plant tissue culture have been possible from experiment on tobacco plant. Optimization of Murashige and Skoog nutrient media was done working on tobacco tissue culture. Many recombinant antibodies have been made from transgenic tobacco (Ganapathi *et. al.* 2004). Many secondary metabolites such as nicotine, aliphatic alkenes, scopoletin, cycloartinol etc. have been observed in tobacco cell cultures. Tobacco is used as a plant factory for the production of vaccines, proteins, antibodies and enzymes (Ganapathi *et. al.* 2004). Tabata *et. al.* (1971) has observed that amount of nicotine was found to be increased in

the presence of kinetin in culture medium. Auxins like IAA have shown the inhibitory effect on nicotine production. Auxins inhibit the nicotine production in the presence of kinetin hormone. Nicotine production increases in tobacco callus culture due to ultraviolet radiations. Low intensity UV-A radiation showed a positive effect nicotine production in callus culture of pith tissue of *N. tabacum* (Kartusch *et. al.* 1990). Many proteins are being able to express in tobacco culture. Gurusamy *et. al.* 2017 observed that tobacco hairy root culture was able to express recombinant erythropoietin of human. It is favorable to produce recombinant proteins in tobacco because of maintaining genetic stability, fast reproduction and growth rate.

Objectives

Observing the importance of callus in *N. tabacum*, following objectives were taken for study:

1 *In-vitro* regeneration of tobacco plants from seeds under aseptic conditions.

2 Callus induction from explant(s) of *Nicotiana tabacum*

Chapter 3

Materials and Methods

3.1 Plant materials

Seeds of wild variety *Nicotiana tabacum* were taken for this work. Seeds were taken in 2ml micro centrifuge tube. The seeds were obtained from Institute of Himalayan Bioresource Technology, Palampur (HP). This Project work was done in Central university of Punjab, Bathinda.

3.2 Preparation of Murashige and Skoog media for seeds germination

Murashige and Skoog is a synthetic nutrient media and support growth of most of the plant cells in culture media. This growth medium contains the entire essential inorganic and organic compound necessary for growth and development.

Table 1: Composition of different constituents in preparation of MS media

Constituents	**Amount**
Murashige and Skoog basal salt mixture	4.2 gm/liter
Murashige and Skoog vitamin solution, 1000 x	1 ml/liter
Sucrose	30 gm/liter
PH	5.8
Agar	8 gm/liter

As per the above concentration, Constituents were taken. Media was adjusted at pH 5.8 before autoclaving . PH was adjusted with the help of 1N NaOH and 1 N HCl. After pH set, agar was added. Media was sterilized by heating in autoclave at 15 p.s.i pressure and 121˚C temperature for 20 min. Nearly 30-35 ml media poured in each petri plate under axenic condition in laminar air flow cabinet.

3.3 Seeds sterilization of *Nicotiana tabacum*

Sterilization is a process to kill microbes. Sterilization is the necessary step to prevent the contamination. Aseptic conditions are required for the sterilization process. Seeds of *Nicotiana tabacum* were sterilized before inoculation in the petri plate having MS media. The whole sterilization process was done in the laminar air flow cabinet under aseptic conditions.

Following steps take place in the sterilization of seeds:

1 Seeds of *Nicotiana tabacum* were taken in the 2 ml microcentrifuge tube.

2 Wash with 1.5 ml 10% TWEEN-20 for the duration of 2-3 min.

3 After washing with detergent, seeds were sterilized with 70% ethanol for 30 sec.

4 Next step is washing with 0.001% (w/v) mercuric chloride for the duration of 5 min.

5 After washing with mercuric chloride, rinse with autoclaved water for 5-6 times.

Repeated washing with autoclave water ensure the removal of detergent. The presence of detergents may create difficulties in the germination of seeds.

3.4 Inoculation and germination of *Nicotiana tabacum* seeds

Seeds of *Nicotiana tabacum* were inoculated in the petri plates containing MS basal media. Inoculation is a process of transfer explants to a media. The inoculation process was done in the laminar air flow cabinet under aseptic conditions. Before working, laminar air flow cabinet was sterilized by turning on UV light for 15 min. The surface of cabinet was sterilized 70% ethanol to prevent the chances of contamination. With the help of pipette, seeds were taken and inoculated in the MS media. Petri plates were sealed with parafilm and kept in the tissue culture racks having proper environment for growth. Seeds started to germinate after 5 to 6 days. After 25-days, plantlets were transferred the conical flasks having MS media for further growth.

3.5 Callus induction from seedling explants of *Nicotiana tabacum*

a) Callus induction in callus inducing media

4-5 days old Seedling of tobacco grown in MS media were taken and inoculated in callus inducing media. Callus inducing media was prepared by adding NAA and BAP in MS media. Plant growth regulators are not added directly into media. Stock solutions of plant growth regulators are used for preparation of callus inducing media. Stock solution of BAP, NAA and 2,4-D hormones were prepared according to compositions given in table 2,3 and 4 respectively. PGRs were first dissolved in 1 N NaOH (2,4-D in EtOH) then dilute the mixture with autoclave water.

Table 2: Composition of constituents in stock solution of 6-benzylaminopurine (BAP)

Compounds	Composition
BAP	5 mg
1 N NaOH	2 ml
Autoclave water	3 ml
Total volume	5 ml

Table 3: Composition of constituents in stock solution of 1-napthaleneacetic Acid (NAA)

Compounds	Composition
NAA	5 mg
1 N NaOH	2ml
Autoclave Water	3ml
Total volume	5ml

Table 4: Composition of constituents in stock solution of 2,4-dichlorophenoxyacetic Acid (2,4-D)

Compounds	Composition
2,4-D	5 mg
1 N Ethanol	2ml
Autoclave Water	3ml
Total volume	5ml

Callus inducing media contained the constituents MS media , 0.2 mg/L BAP and 2.0 mg/L NAA. Callus inducing media was poured in petri plates. Seedlings explants of 4-5 days old were inoculated in the petri plates containing CIM with the help of sterilized forceps in laminar air flow cabinet. Petri plates were sealed with the parafilm. The plates

containing explants were kept in tissue culture rack at 25-28℃ temperature and 16/8 light or dark photoperiod.

b) Callus induction from seedling explants in different callus inducing media having different hormonal combinations

Five type of diffferent callus inducing media were prepared containing different combinations of auxins and cytokinins and different hormonal concentrations to see effect on callus induction and morphology. All five types of CIM like MS -1, MS-2, MS-3, MS-4 and MS-5 were prepared according to composition of constituents given the table 5. All were prepared under aseptic conditions. 4-5 days old seedling were inoculated in petri plates containing callus inducing media with the help of forceps and kept under aseptic conditions.

Table 5: Different concentration of auxins and cytokinins for callus formation from seedlings and leaf explants

Serial No.	Callus inducing Media	Composition of hormones in Media
1	MS-1	MS + 0.2 mg/L BAP + 1.0 mg/L NAA
2	MS-2	MS + 0.2 mg/L BAP + 2.0 mg/L NAA
3	MS-3	MS + 0.2 mg/L BAP + 3.0 mg/L NAA
4	MS-4	MS + 0.2 mg/L BAP + 2.0 mg/L 2,4-D
5	MS-5	MS + 0.0 mg/L BAP + 2.0 mg/L 2,4-D

3.6 Callus induction from leaf explants of *Nicotiana tabacum*

a) Callus induction from leaf explants in callus inducing media

Leaves of 6-7 weeks old plants of *N. tabacum* grown under aseptic conditions were taken as explants and cut into small pieces with the help of sterilized blade. As the plants were grown under aseptic conditions so leaf sterilization process was not done. Callus inducing media containing MS, 0.2 mg/L BAP and 2.0 mg/L NAA was prepared and poured in the petri plates in laminar air flow cabinet. Leaves pieces were inoculated

in callus inducing media. Culture media having leaf explants were kept in tissue culture racks with 27-28℃ temperature and 16/8 hours light or dark photoperiod.

b) Callus induction from leaf explants in different callus inducing media having different hormonal combinations

Leaves of 6-7 weeks old tobacco plants grown under aseptic conditions were taken as explants. Leaves were cut in small pieces with sterilized blade and inoculated in different culture media having composition according to given in table no. 5. After inoculation, petri plates sealed with parafilm and kept in appropriate conditions.

3.7 Callus Imaging

Image or picture was capture with digital camera. Images of germinating seeds, seedling, plantlets, and callus were taken at different days. Images of callus of seedling explants were captured at 0, 5, 10, 15, 20, 25 days. Images of callus using leaf explants were captured at 0, 10 and 25 days.

Chapter 4

Results

4.1 *In- vitro* seeds germination of *Nicotiana tabacum* in aseptic conditions

Seeds of wild variety of *Nicotiana tabacum* were taken and sterilized to prevent bacterial and fungal contamination. The whole sterilization process was done in laminar air flow cabinet. 180 seeds were inoculated in petri plates containing MS media. After inoculation, petri plates were sealed with the help of papafilm. The petri plates were kept in tissue culture racks. Temperature and other conditions required for germination were provided in laboratory. It was observed that seeds started to germinate after 5-6 days.

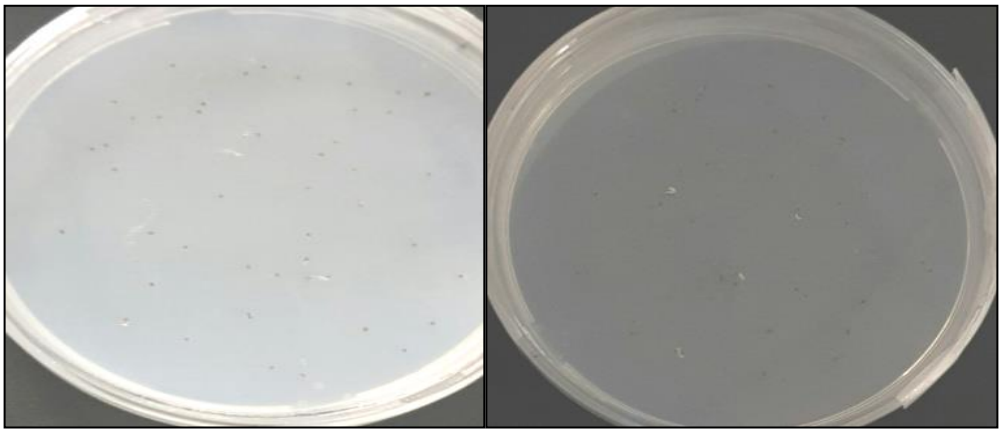

Figure 1: Seeds inoculated in MS media **Figure 2:** Picture depicting seeds germination at 5 days

Figure 3: Picture of 10 days old seedlings germinating in MS Media

180 seeds of wild variety of *Nicotiana tabacum* were sprinkled in MS media and it was found that 123 seeds were germinated. Seed germination efficiency was found to be 68.33%. A magnified image of 10 days old seedling can be observed in figure 3.

Seed germination efficiency= Germinated seeds/ seed sprinkled × 100

Table 6: Seeds germination efficiency

Seeds sprinkled	Germinated seeds	Germination efficiency
180	123	68.33%

4.2 Regeneration of plants under aseptic conditions for explants preparation

Nicotiana tabacum plants were generated under aseptic environment for explants preparation. After 25 days, plantlets were transferred to conical flask containing MS media for further growth.. MS media was prepared and poured in conical flasks. Nearly 100 ml MS media were poured in each conical flask. 25 days old plantlets were transferred with the help of sterilized forceps. Leaves of 6-7 weeks old plants were taken as explants for callus induction.

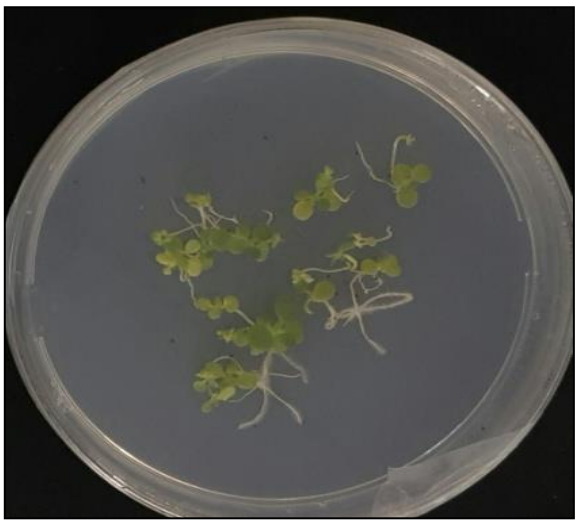

Figure 4: Picture of 15 days old seedlings in MS

Figure 5: Plantlets (25 days old) in MS media

Figure 6: Plants (6 weeks old) of *N. tabacum* germinating in MS media.

4.3 Callus induction from seedling explants of *N. tabacum* in callus inducing media

4-5 days old seedlings were transferred to MS media containing plant growth regulators. Callus inducing media contains MS media, 0.2 mg/L BAP and 2.0 mg/L NAA. BAP is 6-benzylaminopurine (synthetic cytokinin) and NAA (1-naphthaleneacetic acid) is a synthetic auxin. These PGRs help in callus induction. CIM was prepared and poured in petri plates. Seedlings explants were inoculated in CIM with the help of sterilized forceps under aseptic conditions. After inoculation, the petri plates were kept at temperature 27-28℃ and 16/8 hours light or dark photoperiods. Callus started to form after 3 days of inoculation. Image of callus were taken with the help of digital camera at 5, 10, 15, 20, 25 days.

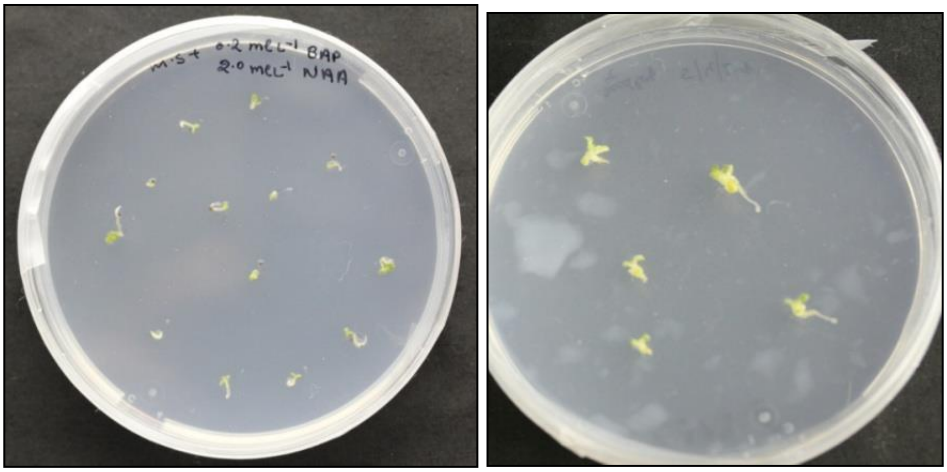

Figure 7: Seedling explants (4-5 days) inoculated in CIM **Figure 8:** Picture of callus (5 days) in callus inducing media

Callus was induced at this hormonal concentration from leaf explants of two cultivars i.e. SPTG-172 and K-399 of *N.tabacum* (Ali et. al. 2007).

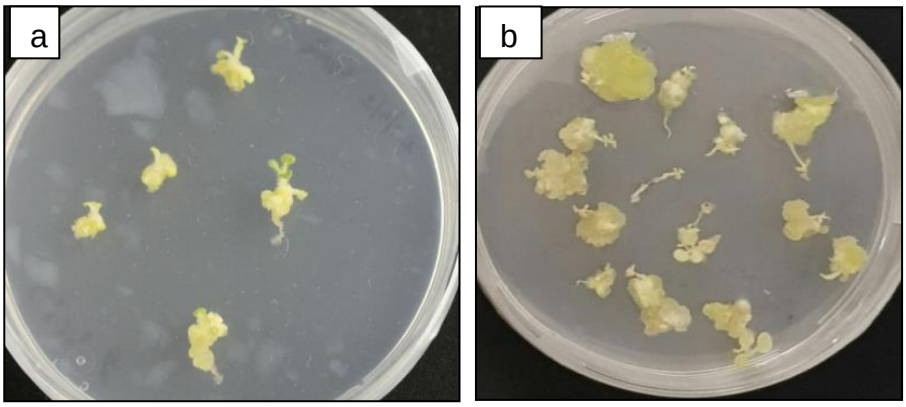

Figure 9: Picture depicting 10 days (a) and 15 days (b) old callus from seedling explants in callus inducing media

Picture of callus from seedling explants in culture media indicating that greenish color callus, very soft and fleshy. Callus grows gradually in this culture media and growth rate is very good.

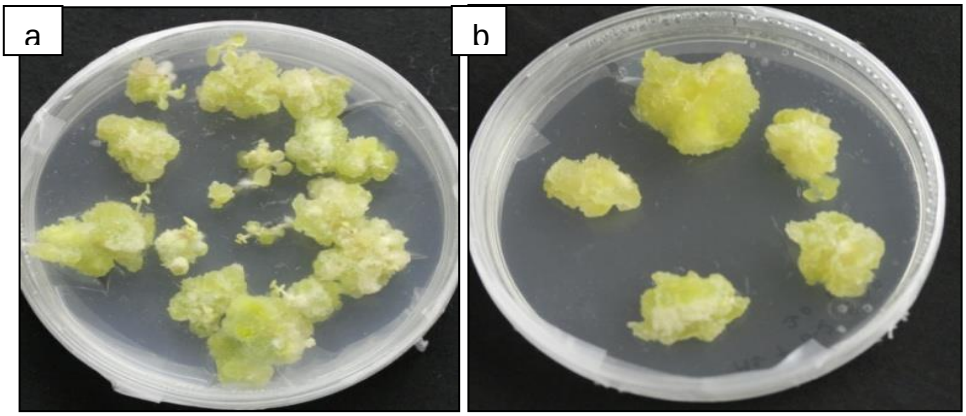

Figure 10: Picture depicting 20 days (a) and 25 (b) days old callus using seedling explants in callus inducing media

4.4 Callus induction from leaf explants of *Nicotiana tabacum* in callus inducing media

Leaves from 6-7 weeks old plants grown in aseptic conditions were taken as explants. Leaves were cut into 2- 3 cm in size with the help of sterilized blades. Leaves pieces were inoculated into petri plate containing callus inducing media. The inoculation of explants was done under aseptic conditions in laminar air flow cabinet. Leaves pieces were transferred to petri plates containing MS media with 0.2 mg/L BAP and 2.0 mg/L NAA hormonal concentration. The culture media were kept at 27-28℃ temperature and 16-8 hours light/dark period.

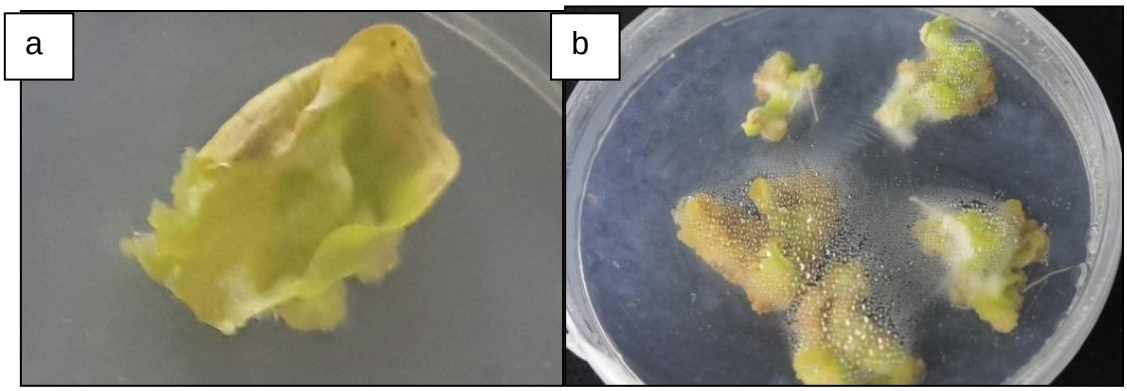

Figure 11: Picture of 10 days (a) and 25 days (b) old green callus using leaf explants in callus inducing media

It was observed that Callus started to form after 3-4 days of inoculation. A green color callus formed at this concentration. Magnified image of 25 days old calli as shown above indicating that roots started to form in callus at this hormonal concentration. Callus was greenish in color. Observational analysis showing callus was rough and rooty.

4.5 Callus induction from seedling explants in different callus inducing media containing different hormonal combinations

4-5 days old seedlings were inoculated to MS media containing different hormonal concentrations. Different callus inducing media like MS-1, MS-2, MS-3, MS-4, and MS-5 and were prepared according to composition given in table 5. After inoculation the cultures were kept at 27-28℃ temperature in tissue culture racks. Callus started to form after 3 days after inoculation of seedling in all callus inducing media.

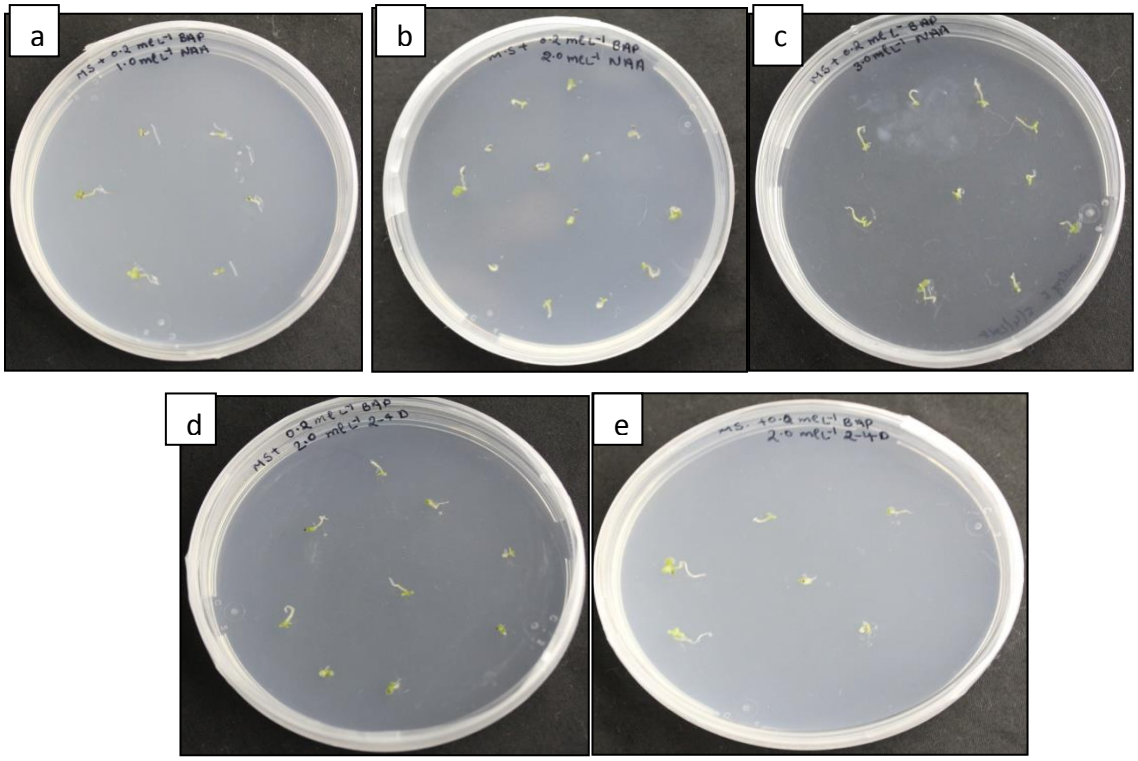

Figure 12: Seeding explants inoculated with media supplemented with different hormonal combinations (a) MS-1; media with 0.2 mg/L BAP and 1.0 mg/L NAA (b) MS-2 media with 0.2 mg/L BAP and 2.0 mg/L NAA (c) MS-3 media with 0.2 mg/L BAP and 3.0 mg/L NAA (d) MS-4 media with 0.2 mg/L BAP and 2.0 mg/L 2,4-D (e) MS-5 media with 0.0 mg/L BAP and 2.0 mg/L 2,4-D

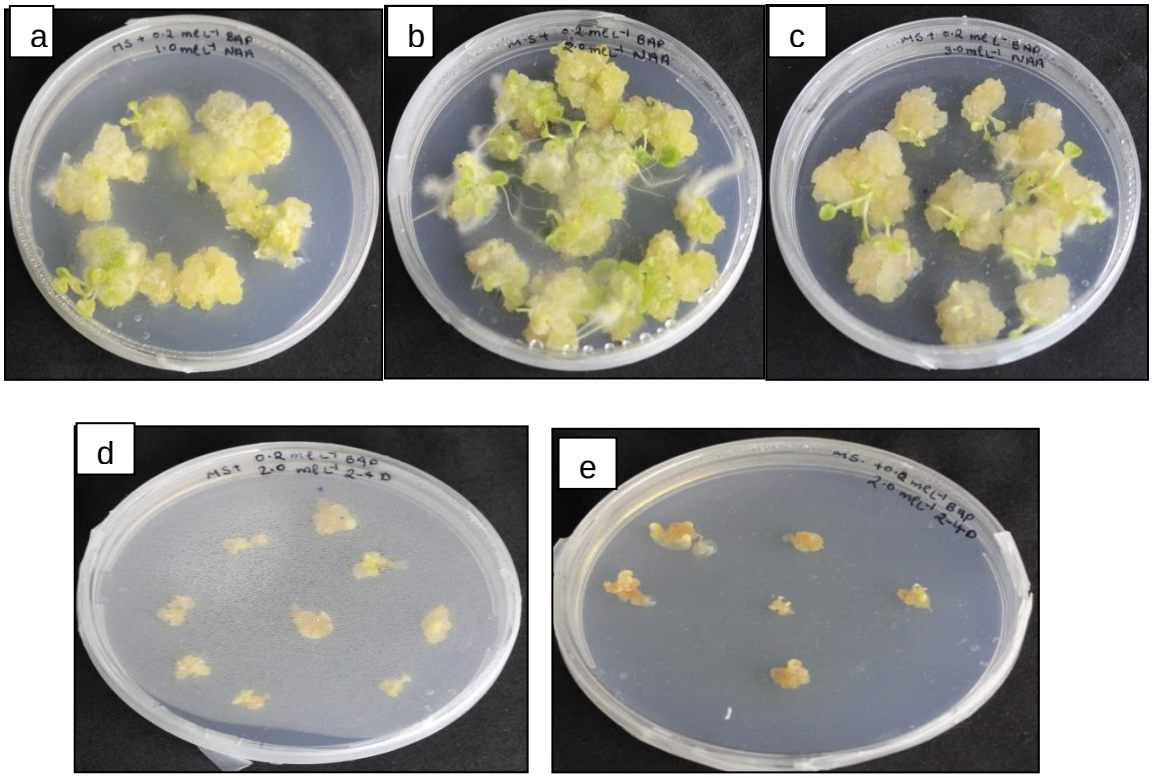

Figure 13: Picture of 25 days old callus from seedling explants in media supplemented with different hormonal combinations (a) MS-1; media with 0.2 mg/L BAP and 1.0 mg/L NAA (b) MS-2 media with 0.2 mg/L BAP and 2.0 mg/L NAA (c) MS-3 media with 0.2 mg/L BAP and 3.0 mg/L NAA (d) MS-4 media with 0.2 mg/L BAP and 2.0 mg/L 2,4-D (e) MS-5 media with 0.0 mg/L BAP and 2.0 mg/L 2,4-D

Callus started to grow with different rate in different media. It was found that growth rate of culture was more in the culture media containing combinations of BAP and NAA hormone as compare to combinations of BAP and 2,4-D. Yellowish brown Color of callus was obtained in culture media containing BAP and 2,4-D. Observational analysis of callus at different hormonal concentration at 25 days has been listed in table 7 .

Table 7: Effect of auxins and cytokinins on callus induction from seedling explants of *N. tabacum*

Sr. No.	Callus Inducing Media	Size of Callus after 25 days	Colour of Callus	Texture of Callus
1	MS-1	1.7- 2.0 cm.	Green	Friable
2	MS-2	2.0- 2.6 cm.	Green	Friable
3	MS-3	2.0- 2.8 cm.	Green	Friable
4	MS-4	0.8- 1.2 cm.	Yellowish brown	Friable
5	MS-5	0.5-0.8 cm.	Yellowish brown	Friable

4.6 Callus induction from leaf explants at different callus inducing media having different hormonal combinations

Leaves of 6-7 weeks of *N. tabacum* grown in aseptic conditions were taken as explants. Leaves were cut in small pieces and inoculated in different CIM having different hormonal combination of auxin and cytokinin such as MS-1, MS-2, MS-3, MS-4, and MS-5 having composition listed in table no. 5. All culture were kept in proper light and 27-28℃ temperature. Callus started to form after 3-4 days of inoculation.

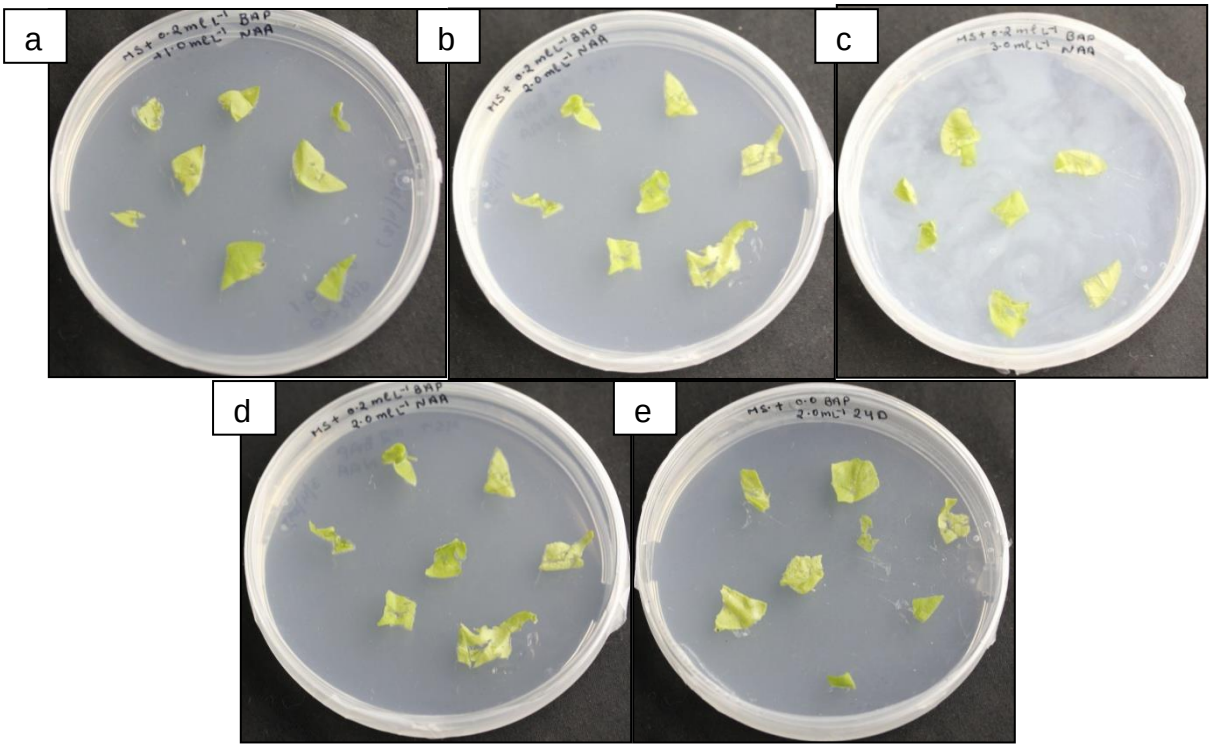

Figure 14: Leaf explants inoculated with media supplemented with different hormonal combinations (a) MS-1; media with 0.2 mg/L BAP and 1.0 mg/L NAA (b) MS-2 media with 0.2 mg/L BAP and 2.0 mg/L NAA (c) MS-3 media with 0.2 mg/L BAP and 3.0 mg/L NAA (d) MS-4 media with 0.2 mg/L BAP and 2.0 mg/L 2,4-D (e) MS-5 media with 0.0 mg/L BAP and 2.0 mg/L 2,4-D

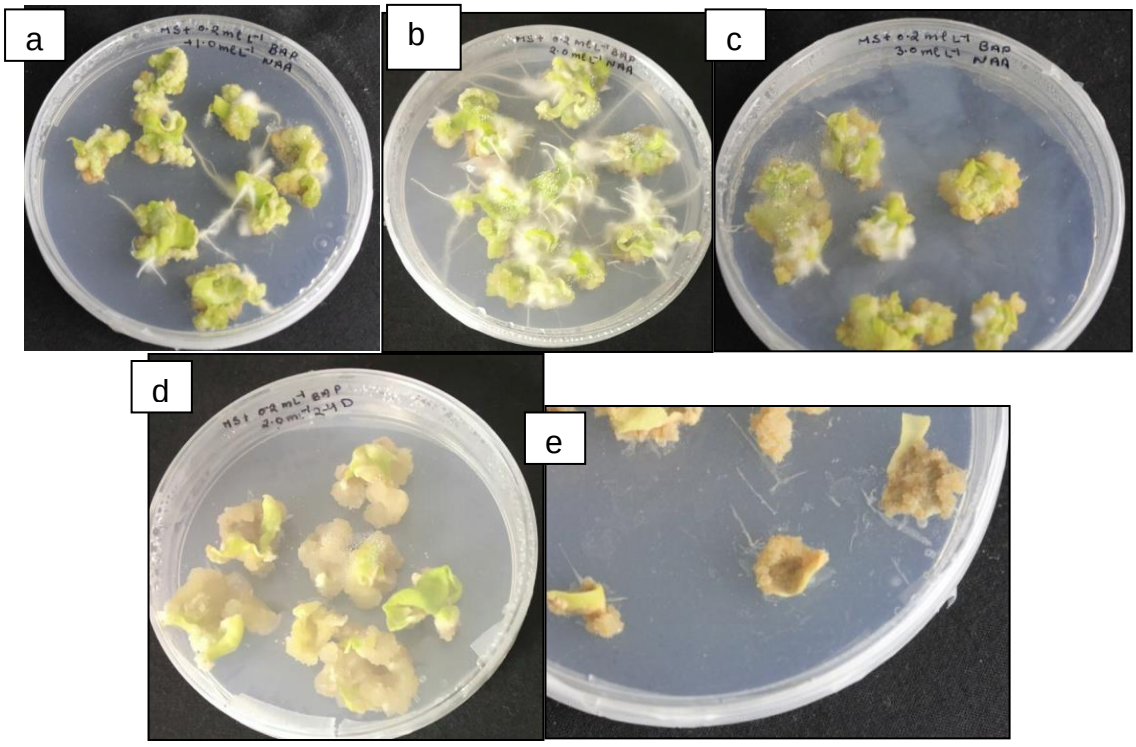

Figure 15: Picture of 25 days old callus from leaf explants in media supplemented with different hormonal combinations (a) MS-1; media with 0.2 mg/L BAP and 1.0 mg/L NAA (b) MS-2 media with 0.2 mg/L BAP and 2.0 mg/L NAA (c) MS-3 media with 0.2 mg/L BAP and 3.0 mg/L NAA (d) MS-4 media with 0.2 mg/L BAP and 2.0 mg/L 2,4-D (e) MS-5 media with 0.0 mg/L BAP and 2.0 mg/L 2,4-D

It was observed that yellowish brown color of callus in culture media containing BAP and 2,4-D combination and green color callus in the culture media containing BAP and NAA. Rooty callus was obtained in BAP and NAA containing culture media.

Table 8: Effect of different auxin and cytokinin combinations on callus induction of leaf explants of *N. tabacum*

Sr. No.	Callus inducing media	Color of callus	Texture of Callus
1	MS-1	Greenish	Rough, rooty
2	MS-2	Greenish	Rough, rooty
3	MS-3	Greenish	Rough, rooty
4	MS-4	Yellowish – brown	Friable
5	MS-5	Yellowish-brown	Friable

Discussions

The current work was conducted for callus induction and optimization the media for callus induction from explants of *N. tabacum.* The work was done under aseptic conditions and these conditions were maintained throughout the work. Seeds were sterilized and germinated in simple MS media having no plant growth regulators. In MS media seeds germinated after 5-6 days. Quality and properties and rate of growth of callus depend on the growth regulators, growth media etc. It was observed that callus induction takes 3 and 3-4 days from seedling and leaf explants in Callus inducing media respectively. It was found from this study of callus induction from explants of tobacco plant, low concentration of cytokinin and high concentration of auxins was superior for callogenesis. Ali *et. al.* 2007 observed the callus induction from leaf explants in 0.2 mg/L BAP and 2.0/3.0 mg/L NAA. In the present study, callus induction from 3-4 days seedling gave better result at this hormonal concentration. Callus induction from seedling save a lot time. In this work five different hormonal concentration were taken to see the response of different hormone on callus. It was found that green and large size callus obtained from seedling in culture medium containing BAP and NAA hormone combinations. So the growth rate was found more in the culture medium having BAP and NAA. MS-5 media was not found suitable for callus induction. In case of callus induction from leaf explants, callus was rough and rooty in culture medium containing BAP and NAA. No root formed in culture medium containing NAA and 2,4-D . A yellowish brown and without root callus was formed in MS-4. This indicates that different hormones show different effect on the callus properties and quality. If we compare MS-2 and MS-4 callus inducing medium, both media having same concentration of auxin but one having NAA and other having 2,4-D. The morphological charactersties of callus were found different in MS-2 and MS-4 culture media indicating different hormone having same concentration have different effect.

Chapter 5

Conclusions

Seeds of *N. tabacum* germinated in simple Murashige and Skoog media and found 68.33 % seed germination efficiency. Seed germinated after 5-6 days in MS media. Callus induction from seedling takes only 3 days in culture media containing MS, 0.2 mg/L BAP and 2.0 mg/L NAA. Callus was found to greenish and soft. Callus induction from leaf explants take 3-4 days in CIM containing MS, 0.2 mg/L BAP and 2.0 mg/L NAA. It was observed the callus was rooty and greenish in color. Five different types of media containing different BAP, NAA and 2,4-D hormonal concentration was used for callus induction from seedling and leaves explants of *Nicotiana tabacum.* The result of different hormonal concentration showed that combination of BAP and NAA was better than BAP and 2,4-D for callus induction from seedling. The size and growth rate of callus of seedling explants was found more in MS media supplemented with BAP and NAA as compare to BAP and 2,4-D. When only 2,4-D was used only a small mass of cell was obtained after 25 days. So MS-5 media was not found suitable media for callus induction. In this study it was observed that MS medium supplemented with 0.2mg/L BAP and 2.0 or 0.3 mg/L NAA gave large size callus from seedling explants. The color of callus was yellowish brown in culture medium containing BAP and 2,4-D. Green color callus was obtained in culture medium containing BAP and NAA. Callus of leaf explants was rooty and rough in culture media containing NAA and BAP but in medium containing NAA and 2,4-D, callus was yellowish brown and without any root. Callus induction from seeding will be beneficial for Agrobacterium-mediated transformation experiments to decreases the time wait. As most of these experiments are done on the callus of mature leaf explants. So callus induction from seedling explants save a lot of time.

References

Ali, G., Hadi, F., Ali, Z., Tariq, M., & Khan, M. A. (2007). Callus induction and in vitro complete plant regeneration of different cultivars of tobacco (Nicotiana tabacum L.) on media of different hormonal concentrations. *Biotechnology*, *6*(4), 561-566.

Ganapathi, T. R., Suprasanna, P., Rao, P. S., & Bapat, V. A. (2004). Tobacco (Nicotiana tabacum L.)-A model system for tissue culture interventions and genetic engineering.

Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant callus: mechanisms of induction and repression. *The Plant Cell*, *25*(9), 3159-3173.

Iwase, A., Mitsuda, N., Ikeuchi, M., Ohnuma, M., Koizuka, C., Kawamoto, K., ... & Sugimoto, K. (2013). Arabidopsis WIND1 induces callus formation in rapeseed, tomato, and tobacco. *Plant signaling & behavior*, *8*(12), e27432.

Husin, M. A. G., Hasan, M., & Taha, R. M. (2005). Callus Induction from Tobacco (Nicotiana tabacum) Leaf Explants for the Production of Quinone. *Asia-Pacific Journal of Chemical Engine ering*, *13*(5-6), 563-572.

Geethalakshmi, S., Hemalatha, B., & Saranya, N. (2016). Optimization of Media Formulations for Callus Induction, Shoot Regeneration and Root Induction in Nicotiana benthamiana.

Rahman, M. A., Alam, M. A., Hossain, M. R., Hossain, A., & Afroz, R. (2010). In vitro regeneration of popular tobacco varieties of Bangladesh from leaf disc. *Bangladesh Journal of Agricultural Research*, *35*(1), 125-134.

Neelu Joshi (2003). In vitro growth and shoot multiplication in Nicotiana tabacum L. – influence of gelling agent and carbon sources. *International Journal of Plant Developmental Biology*

Pathi, K. M., Tula, S., & Tuteja, N. (2013). High frequency regeneration via direct somatic embryogenesis and efficient Agrobacterium-mediated genetic transformation of tobacco. *Plant signaling & behavior*, *8*(6), e24354.

SEDGHI, G. N., BAKHSHI, K. G., & Karimi, F. (2011). Evaluation of somaclonal variation during repetitious subcultures of tobacco (Nicotiana tabacum L.) callus.

Siddique, A. B., & Islam, S. M. (2015). Effect of light and dark on callus induction and regeneration in tobacco (Nicotiana tabacum L.). *Bangladesh J Bot*, *44*(4), 643-651.

Song, G., Zhang, P., Shi, G., Wang, H., & Ma, H. (2018). Effects of CeCl 3 and LaCl 3 on callus and root induction and the physical response of tobacco tissue culture. *Journal of Rare Earths*.

Gaspar, T., Kevers, C., Penel, C., Greppin, H., Reid, D. M., & Thorpe, T. A. (1996). Plant hormones and plant growth regulators in plant tissue culture. *In Vitro Cellular & Developmental Biology-Plant*, *32*(4), 272-289.

Rathore, K. S., & Goldsworthy, A. (1985). Electrical control of growth in plant tissue cultures. *Nature Biotechnology*, *3*(3), 253.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.

Martin-Tanguy, J., Martin, C., Paynot, M., & Rossin, N. (1988). Effect of hormone treatment on growth bud formation and free amine and hydroxycinnamoyl putrescine levels in leaf explant of Nicotiana tabacum cultivated in vitro. *Plant physiology*, *88*(3), 600-604.

Tabata, M., Yamamoto, H., Hiraoka, N., Marumoto, Y., & Konoshima, M. (1971). Regulation of nicotine production in tobacco tissue culture by plant growth regulators. *Phytochemistry*, *10*(4), 723-729.

Kumar, V., & Maherchandani, N. (1988). Differentiation in callus cultures of a tobacco (Nicotiana tabacum cv. White Burley) variant: some biochemical aspects. *Plant cell, tissue and organ culture*, *14*(3), 177-185.

BORNMAN, C. H., & JANSSON, E. (1980). Nicotiana tabacum callus studies. X. ABA increases resistance to cold damage. *Physiologia Plantarum*, *48*(4), 491-493.

Gurusamy, P. D., Schäfer, H., Ramamoorthy, S., & Wink, M. (2017). Biologically active recombinant human erythropoietin expressed in hairy root cultures and regenerated plantlets of Nicotiana tabacum L. *PloS one*, *12*(8), e0182367.

Thorpe, T. A. (2007). History of plant tissue culture. *Molecular biotechnology*, *37*(2), 169-180.

George, E. F., Hall, M. A., & De Klerk, G. J. (2008). Plant tissue culture procedure-background. In *Plant propagation by tissue culture* (pp. 1-28). Springer, Dordrecht.

Gelvin, S. B. (2003). Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and molecular biology reviews*, *67*(1), 16-37.

KARTusCH, R. O. B. E. R. T., & Mittendorfer, B. (1990). Ultraviolet radiation increases nicotine production in Nicotiana callus cultures. *Journal of plant physiology*, *136*(1), 110-114.