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STANDARDIZATION OF A PROTOCOL FOR *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION OF *P. kurroa*

BY

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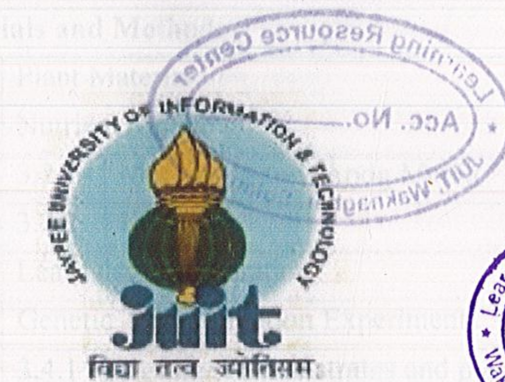

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY-WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled, **"Standardization of a protocol for *agrobacterium* mediated genetic transformation of *P.kurroa*"** submitted by **"Gaurav Gupta (071565) and Tarun Thakur (071571)"** in partial fulfillment for the award of degree of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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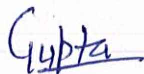
Needless to say, errors and omissions are ours.



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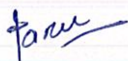

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LIST OF ABBREVIATIONS

Abbreviations	Expanded Form
MS	Murashige Skoog
BAP	Benzyl Amino Purine
IBA	Indole Butyric Acid
L	Litre
Mg	Milli gram
NAA	Napthyl Acetic Acid
LB	Luria Bertani
µl	Micro litre
µM	Micro molar
2,4-D	2, 4 – Dinitrophenol
RM	Regeneration Media

SUMMARY

In this investigation an attempt was made to standardize the protocol for *Agrobacterium* mediated genetic transformation of *Picrorhiza kurroa* Royal ex Benth using GUS gene. The method comprises of preculturing, cocultivating the explant with *Agrobacterium* and then selection of the transformed cells or tissue. Also an attempt was made for high frequency regeneration using leaf discs as explants using MS medium supplemented with different combinations and concentrations of the growth hormones. The best response of explants cultured was obtained in MS medium supplemented with 1mg/l BAP and 1mg/l IBA out of different media combinations tried so far. Leaf discs expressed the morphogenetic response in other hormonal combinations tested. *Agrobacterium* cells were selected on selective medium and used in subsequent experiments. We also investigated the key factors effecting genetic transformation by *Agrobacterium* in an attempt to establish an efficient genetic transformation system using leaf discs and root segments as explants. The transformed plant cell/tissue could survive on the selective medium containing cefotaxime (500 mg/l) and hygromycine (50 mg/l). However more efforts are required to define the protocol in order to achieve high frequency transformation and regeneration.

CHAPTER 1

INTRODUCTION

P. kurroa Royal ex Benth (Family: Scrophulariaceae) is a perennial herb, also known as kutki or karu mainly found in the North-Western Himalayan regions of India at altitudes of 3000 - 4300 m. *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting. The active constituents are obtained from the roots and rhizomes. *P. kurroa* is a rich source of hepatoprotective picrosides ; picroside-I and picroside-II and other metabolites like picroside-III, picroside-IV, apocynin, androsin, catechol, kutkoside, etc (Weinges et al., 1972; Stuppner and Wagner, 1989).

The medicinal importance of *P. kurroa* is due to its pharmacological properties like hepatoprotective (Chander et al., 1992), antioxidant (particularly in liver) (Ansari et al., 1988), antiallergic and antiasthamatic (Dorch et al., 1991), anticancerous activity particularly in liver (Joy et al., 2000) and immunomodulatory (Gupta et al., 2006). A commercial formulation named as Picroliv prepared from *P. kurroa* extracts containing picroside 1 and kutkoside was launched as a hepatoprotective drug after clinical testing (Ansari et al., 1991). Picroliv has also been shown to have immunostimulating effect in hamsters and helping to prevent infections (Puri et al., 1992; Gupta et al., 2006).

In the International Year of Mountains (2002), *Picrorhiza kurroa* was listed as an 'endangered' herb due to reckless collection from its natural habitat.

Agrobacterium system was historically the first successful plant transformation system, making the breakthrough in the plant genetic engineering. This breakthrough came by characterizing and exploiting the plasmids carried by *Agrobacterium* species. These provide natural gene transfer, gene expression and selection system.

The various approaches for integrative transformation, *Agrobacterium* mediated technique is most widely used. Gene transfer to plants via *Agrobacterium tumefaciens* is an efficient way to introduce desirable traits into crop plants. This transformation process depends on the combined action of bacterial and plant genotypes.

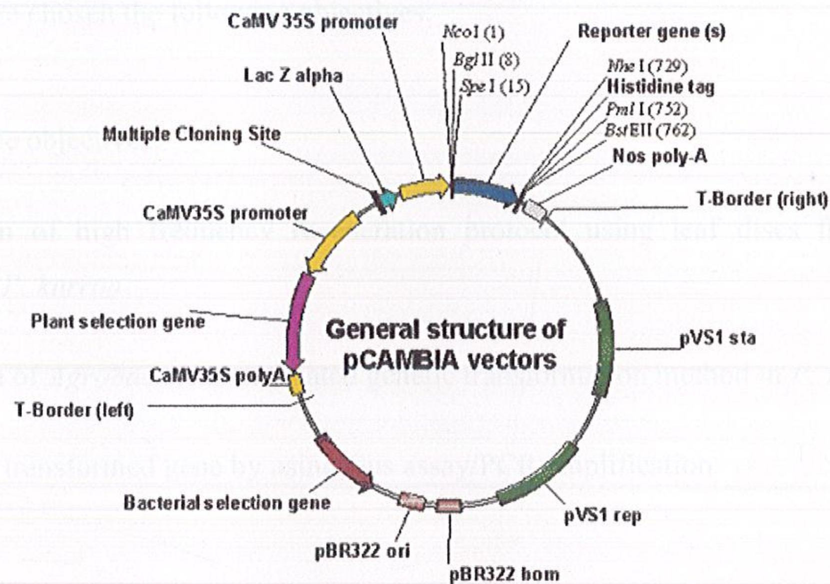


Fig1. pCambia Plasmid

Plant-based molecules are continuously gaining widespread acceptance due to their effective therapeutic properties (Dubey et al. 2004). A wide range of such plant-derived molecules of pharmaceutical interest accumulates in plant roots as secondary metabolites, which necessitates uprooting and killing of the whole plant for harnessing the compound. Indiscriminate exploitation of the natural resources through unregulated uprooting coupled with the lack of attention towards strategic cultivation practices have led to the endangered or threatened conditions of several high-altitude important medicinal plant species (Kumar et al. 1997). Realizing the threat of extinction of such endangered medicinal plant species attention has already been focused towards developing production alternatives of root-derived phytomolecules in order to meet the growing demand of pharmaceutical industries.

Various attempts have been made in recent past to increase the picroside content by conventional methods with a little success. Biotechnological interventions like genetic engineering and metabolic engineering have come to rescue the problems due to conventional methods and can only be applied if there is a stable transformation system is present for *P. kurroa*. So we have chosen the following objectives:

Here we have three objectives:

1. Standardization of high frequency regeneration protocol using leaf discs from *in vitro* grown shoots of *P. kurroa*.

2. Standardization of *Agrobacterium* mediated genetic transformation method in *P. Kurroa*

3. To confirm the transformed gene by using Gus assay/PCR amplification.

CHAPTER 2

REVIEW OF LITERATURE

The stable transformation of foreign gene(s) into plants represents one of the most significant developments in the field of plant genetic engineering. Genetic engineering method complements plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into crops and by shortening the time required for production of new varieties and hybrids. It is most suitable if a well established crop variety has to be improved with respect to one or two characters without disturbing the general genetic background. P.kurroa is one of the high-altitude important medicinal plant species (Kumar et al. 1997). Some of the work done in this plant and in other medicinal plant species with regard to genetic transformation have been reviewed here.

Chandra *et al.* (2007) developed a protocol for induction and establishment of *Agrobacterium rhizogenes* - mediated hairy root cultures of *Picrorhiza kurroa* through optimization of the explant type . The infection of leaf explants with the LBA9402 strain resulted in the emergence of hairy roots at 66.7% relative transformation frequency. This report was the first successful establishment, maintenance, growth and selection of superior hairy root clone of *Picrorhiza kurroa* with desired phyto-molecule production potential, which can serve as an effective substitute to its roots and thereby prevent the indiscriminate up-rooting and exploitation of this commercially important, endangered medicinal plant.

Vasudevan et al. (2008) developed efficient transformation protocol for cucumber cv. Poinsett 76 using *Agrobacterium* strain EHA 105.The infected plants were co-cultivated for 2 days in MS medium with BA as the growth hormone. The transformed shoots were elongated in MS medium containing BA (1.0 mg/l), Cefotaxime (300 mg/l), PPT (2.0 mg/l) along with GA3 (0.5 mg/l). The rooting of elongated shoots was achieved in MS medium with BA (1.0 mg/l),

Cefotaxime (300 mg/l), PPT (2.0 mg/l) and IBA (0.6mg/l). The molecular confirmation of transformed shoots revealed the foreign gene integration into cucumber genome.

Sheeba *et al.* (2010) introduced foreign genes into plant tissues via *Agrobacterium tumefaciens* based vectors. The transformation of *Physalis minima* was carried out using the *Agrobacterium* strain LBA 4404 pB19. 250 mg/l cefotaxime and 75 mg/l kanamycin were used for the best growth of *Physalis minima.*

He H, Xu HH, (2001) established an effective system for the *Agrobacterium* – mediated genetic transformation of *M.officinalis.* They used nodular stem segments as their explants and the *Agrobacterium* strain EHA101 was used along with the plasmid pGA482GG. MT basal medium with BA 1 mg/l was effective to inducing the direct shoot formation, and the frequency of shoot formation was 97.8%. The optimal rooting medium for regenerating shoots was MT basal medium supplemented with 0.2 to 0.5 mg.L-1 NAA, and a root induction rate over 80.0% was observed. The selection pressure for kanamycin was 50mg/l. Cefotaxime was used as antibiotics, and the concentration was 300 mg/l. After 1.5 months, 14.8% resistant shoots were emerged from the explants. Histochemical GUS assay showed that 22.2% of the resistant plants were GUS-positive.

Ya-ping Yan and Zhe-zhi Wang (2006) developed a high-frequency and simple procedure for *Agrobacterium tumefaciens* - mediated genetic transformation of the medicinal plant *Salvia miltiorrhiza.* Leaf discs were pre-cultured on MS medium supplemented with 6.6 µmol/l BAP and 0.5 µmol/l NAA for one day, then co-cultured with *A. tumefaciens* strain EHA105 harboring the plasmid pCAMBIA 2301 for three days on the same medium. Regenerated buds were obtained on selection medium (co-culture medium supplemented with 60 mg/l kanamycin and 200 mg/l cefotaxime) after two cycles' culture of 10 days each and then transferred to fresh MS medium with 60 mg/l kanamycin for rooting. Fifteen days later, the rooted plantlets were obtained and then successfully transplanted to soil. The transgenic nature of the regenerated plants was confirmed by PCR, Southern hybridization analysis and GUS histochemical assay.

Antonella Furini, Csaba Koncz, Francesco Salamini, and Dorothea Bartels (1994) conducted investigations for the *Agrobacterium tumefaciens*- mediated transformation of the desiccation-tolerant plant *Craterostigma plantagineum.* Leaf explants were inoculated with A.tumefaciens strain GV3101 carrying the gene for kanamycin- or hygromycin-resistance and the β-glucuronidase reporter gene. Parameters which affected the transformation efficiency were the age of the explant, the degree of wounding and the presence of an antioxidant in the medium. Under optimal conditions, calli originated in more than 80% of leaf explants. Transformed plants were obtained from more than 50% of the cultured calli during regeneration in the presence of a suitable antibiotic.

H. Sood and R.S. Chauhan (2009) established callus cultures from different explants such as leaf discs, nodal and root segments of *P.kurroa.* The regeneration hold a great promise in the production of metabolites in cell cultures. Callus induction was highest (70%) in root segments followed by leaf discs (56.3%) and nodal segments (38.3%) on MS medium supplemented with 2,4-D (2 mg/l) + IBA (0.5 mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v). The callus cultures derived from different explants were differentiated into multiple shoots on MS medium containing different concentrations and combinations of BA, KN and IBA. Regeneration was highest in the calli derived from root segments and leaf discs on MS + BA (2 mg/l) + KN (3 mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) with 76.7 and 72.2% calli forming shoot primordia, respectively. Most of the nodal segment derived calli got differentiated into roots rather shoots. Comparative callusing and shoot regeneration from different explants revealed that root segments are the best explant for *in vitro* studies in *P. kurroa.*

CHAPTER 3

MATERIALS & METHODS

3.1 PLANT MATERIAL

P.kurroa was grown *in vitro* on MS (Murashige and Skoog,1962) medium kinetin (1mg/l) + IBA (2mg/l) in plant growth chamber at 26±2 °C, on 16hrs light / 8hrs dark cycle.

3.2 NUTRIENT MEDIA

3.2.1 MURASHIGE & SKOOG MEDIA

The basal MS media (Murashige and Skoog 1962) with 3% sucrose solidified with 0.8% agar-agar was used to culture plant cells/tissues in vitro. The pH of media was adjusted between 5.6 and 5.7 before autoclaving. The media was supplemented with various growth regulators depending upon the experiment before autoclaving at 15lb/inch^2.

3.2.2 LB BROTH

The LB media (Luria broth, 1951) was used for the maintenance and preparation of fresh culture of *Agrobacterium*. Luria Broth – 25g/1000ml

3.3 LEAF DISC REGENERATION

The MS basal media supplemented with different combinations and concentrations of plant growth regulators (Table 1&2) were used to standardize the media for leaf disc regeneration.

BAP (mg/l) / IBA (mg/l)	1.0	2.0	3.0
0.1	1.0, 0.1 (RM1)	2.0, 0.1 (RM2)	3.0, 0.1 (RM3)
0.5	1.0, 0.5 (RM4)	2.0, 0.5 (RM5)	3.0, 0.5 (RM6)
1.0	1.0, 1.0 (RM7)	2.0, 1.0 (RM8)	3.0, 1.0 (RM9)

Table 1

Different concentrations and combinations of growth regulators IBA and BAP for leaf disc regeneration of *P. kurroa*

NAA (mg/l) / IBA (mg/l	1.0	2.0	3.0
0.1	1.0, 0.1 (RM 10)	2.0, 0.1 (RM 11)	3.0, 0.1 (RM 12)

Table 2

Different combinations of IBA and BAP for leaf disc regeneration of *P. kurroa*

3.4 GENETIC TRANSFORMATION EXPERIMENTS

3.4.1 *AGROBACTERIUM* STRAINS AND PLASMID

Disarmed *Agrobacterium* strain (GV3101) containing a reporter/marker β-glucoronidase (GUS) gene in binary vector system (pCambia 1301) along with a kanamycin resistant gene for selection in both bacteria and plant was used for co-cultivation experiment to transfer GUS gene in *P.kurroa*..

3.4.2 MAINTAINENCE OF *AGROBACTERIUM* STRAIN

Agrobacterium strain (GV3101) was maintained by sub culturing the bacterial colonies on the LB media containing 50mg/l kanamycin and 20mg/l rifampicine. After the proper growth of the *Agrobacterium*, the plates were kept at low temperature.

3.4.3 PREPARATION OF FRESH *AGROBACTERIUM* CULTURE

Overnight fresh cultures of *Agrobacterium* strains were prepared by inoculating a small amount of colony into the 10ml liquid LB medium (Luria Broth, 1951) containing 50mg/l kanamycin and 20mg/l rifampicine. 150 µl acetosyringone (100 µM) was added to the same media. These cultures were kept overnight at 28 °C in orbital shaking incubator for growth.
From this culture 5ml of inoculum was added to 100 ml LB medium containing 50mg/l kanamycin, 20mg/l rifampicine and 150 µl acetosyringone (100 µM). This culture was kept for 48hrs at 28 °C in orbital shaking incubator for growth.

3.4.4 PRE-CULTURE OF EXPLANTS

Three types of explants used were root segments, leaf discs and inter-nodal segments. Pre-culture involves culturing the explants in suitable media for some time so as to rejuvenate the cells. The explants were cut into small pieces and were cultured in MS media with growth regulators IBA (0.5mg/l) and 2,4-D (2mg/l) and were grown for 48 hour`s.

3.4.5 CO-CULTIVATION OF EXPLANTS WITH *AGROBACTERIUM*

Co-cultivation is the procedure of growing explants along with *Agrobacterium* cells containing appropriate gene construct during which acetosyringone released from plant cells induces the vir genes which bring about transformation. The fresh culture of *Agrobacterium* was centrifuged for 10 minutes and supernatant was discarded to get a pellet. This pellet was resuspended in MS liquid medium. This bacterial suspension and precultured explants were used for co-cultivation experiment. The explants were immersed into bacterial suspension for 10-15 minutes then these were blotted dry on sterile filter paper and inoculated on growth medium (MS + 100 μM acetosyringone + IBA (0.5mg/l) and 2,4-D (2mg/l)) for co-cultivation. After inoculation these were kept in culture room for varying interval of time viz. 48, 72 and 96 hours to standardize the time of co-cultivation.

3.4.6 SELECTION OF TRANSFORMED CELLS/TISSUES

Selective regeneration medium was prepared by adding cefotaxime (500mg/l) and hygromycine (50mg/l) through filter sterilization to the selective medium (MS + IBA (0.5mg/l) and 2,4-D (2mg/l)). The antibiotic cefotaxime is a broad spectrum antibiotic which is used to check for further growth of *Agrobacterium* which is not desirable at this stage. The antibiotic hygromycine is a plant selection antibiotic whose marker is present in the binary vector present in *Agrobacterium* which is used to check for the non-transformed plant cells. After co-cultivation of the explants for varying interval of time periods (48, 72 and 96 hrs) explants were transferred to the selective medium. All the cultures were kept in culture room at 26° C for further growth and differentiation. Selection involves growing co-cultivated explants into media having the antibiotic (hygromycine) whose resistance gene has been transferred through *Agrobacterium* into the explants.

CHAPTER 4

RESULTS & DISCUSSION

4.1 REGENERATION

Twelve different regeneration media which differ with respect to concentration of BAP, IBA and NAA were tried to achieve regeneration from leaf explants. The leaf explants responded to only three media namely RM4, RM7 and RM12. The regeneration response was observed in RM4 and RM7 (Table 1) after 40 days. Where as in case of RM12 the regeneration started after 30 days . However the frequency of regeneration was quite low in all the media and was not recorded. The explant turned brown and ultimately died in all other regeneration media tried. One of the reasons for poor response of the leaf explants seems to be the delicate nature of the explants. We have used the explants from *in vitro* grown cultures of *P.kurroa* which were being maintained in culture for 3-4 years. The most of leaves were very small and translucent which could be the reason for the poor survival of explants in media. We are trying to establish fresh cultures of *P.kurroa* and experiments are being designed to use explants from the plants being grown in green house. Moreover more media combination consisting of different growth regulators need to be tested to develop high frequency regeneration protocol.

Fig2. *In vitro* culture of *P.kurroa*

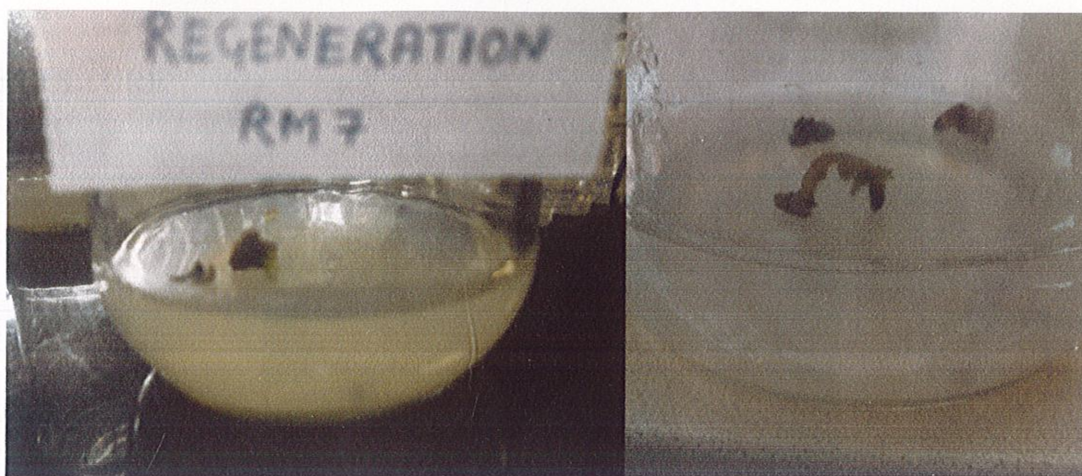


Fig3. Regeneration occurring in RM7 and RM12

4.2 TRANSFORMATION

Owing to poor response of leaf explants in the different MS media tried so far, we decided to use two more explants i.e. root segments an inter-nodal segments for transformation experiments. Out of this none of the explants showed positive response to the infection of *Agrobacterium tumiefaciens.* The necrosis of leaf explant was observed within 24 hours and died within 48-72 hours where as nodal explants survived longer. The survival of root explants was better than the leaf and internodal explants. However no callusing on regeneration was observed on these explants up to 9 days. The internodal and root explants swelled a bit on the selection media after co-cultivation. The main reason for the poor response of *P.kurroa* explant to the genetic transformation experiment seems to be the delicate nature of explants used so far. Due to the poor survival of explants after co-cultivation the other parameters/variables for transformation experiments could not be evaluated.

4.2.1 PRECULTURE

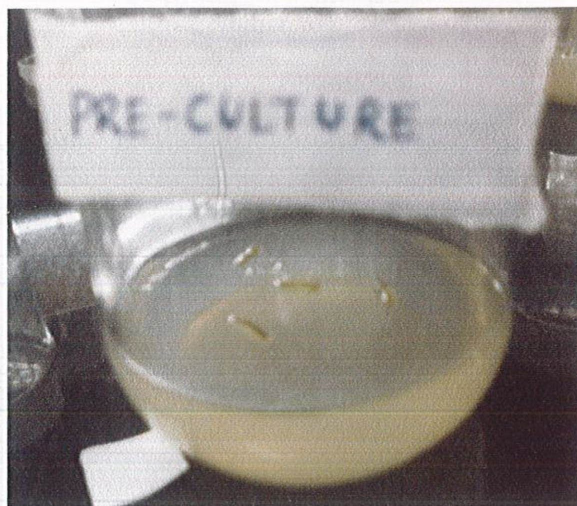
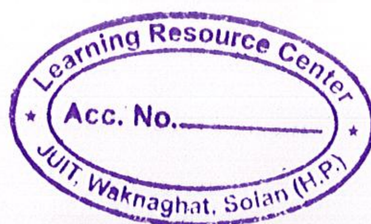


Fig4. Pre-culture after 48 hours

4.2.2 CO – CULTIVATION (48 hrs and 72 hrs)

Fig5. Explants kept for co-cultivation for 48 and 72 hours

4.2.3 PLANT SELECTION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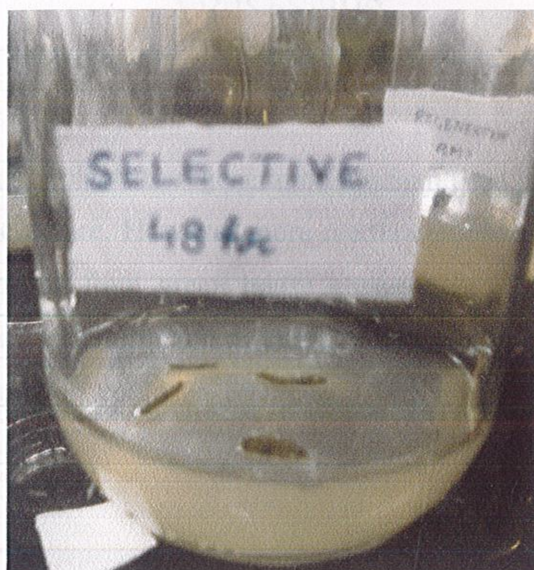

Fig6. Slight swelling was observed in the cultured explants indicating the initiation of callusing.

The level of swelling in the explants with the various co-cultivation time periods was observed to be as follows:

Co-cultivation time (hrs)	Level of swelling observed
48	+
72	++

Chapter 5

CONCLUSION

The response of leaf explants to regeneration medium tried in this investigation was poor partly due to their delicate nature. However more media combination and explants need to be tested. Even the leaf explants from green house grown plants can be tried in regeneration experiments. The success of transformation experiment also depends upon the nature of explants and we hope the transformation could be established in this plant once the issue of explants survival is resolved.

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Appendix-I

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