Optimization of explants for *Agrobacterium* mediated gene transfer in *Eleusine coracana* (PES-400)

Thesis

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By

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Pantnagar
August, 2017

CERTIFICATE

This is to certify that the thesis entitled **"Optimization of explants for *Agrobacterium* mediated gene transfer in *Eleusine coracana* (PES-400)"** submitted in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture,** with major in **Molecular Biology and Biotechnology** of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by **Ms. Masochon Raingam, Id. No. 48102** under my supervision and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been acknowledged.

Pantnagar
August, 2017

(Pushpa Lohani)
Chairperson
Advisory committee

CERTIFICATE

We, the undersigned, members of the Advisory Committee of **Ms. Masochon Raingam, Id. No. 48102**, a candidate for the degree of **Master of Science in Agriculture,** with major in **Molecular Biology and Biotechnology** agree that the thesis entitled **"Optimization of explants for *Agrobacterium* mediated gene transfer in *Eleusine coracana* (PES-400)"** may be submitted in partial fulfillment of the requirements for the degree.

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LIST OF ABBREVIATIONS

%	Percent
˚C	Degree Celsius
2, 4-D	2, 4-Dichlorophenoxy acetic acid
BAP	Benzyl amino purine
DMSO	Dimethyle Sulphoxide
et al.	Etalia / And other people
g	Gram
HCl	Hydrochloride acid
HgCl2	Mercuric chloride
hpt II	Hygromycin phosphotransferase II
mg	Milligram
ml	Milliliter
MS	Murashige and Skoog's media
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
npt II	Neomycin phosphotransferase II
T DNA	Transfer DNA
Ti plasmid	Tumor-Inducing Plasmid
UV	Ultra Violet
viz.	Videlicet (namely)
Vir	Virulence
YEM	Yeast Extract Mannitol

Introduction

Chapter 1 INTRODUCTION

Finger millet (*Eleusine coracana*), also known as African millet belongs to the family Poaceae. It is the primary food source for millions of people living in tropical dry land regions. It is extensively grown as cereal crop in the semi arid tropical regions of Africa and Asia. Finger millet is an annual cereal crop growing upto a height of 170 cm. It gets its name "Finger millet" from the arrangement of its panicle, 4-19 finger-like spikes resembling a fist when matured. It is known as *ragi* and *mandua* in India. It is a fast growing cereal crop that reaches maturity within 3 to 6 months and occasionally in only 45 days.

Finger millet posses many desirable properties in terms of adaptability and nutrition.

It is an agronomically sustainable crop adapted to a wide range of soil conditions and high altitudes. It can maintain optimum yields even in marginal lands, withstand drought and saline conditions, requires little irrigation and other inputs like fertilizers. Finger millet outperforms pearl millet and sorghum in drier condition. It is the fourth millet in terms of worldwide production after sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*) and foxtail millet (*Setaria italica*) **(Upadhyaya *et al.*, 2007).** It is estimated that nearly 10% of the world's 30 million tons of millet produced is finger millet **(Dida *et al.*, 2008).** Millets are proposed to provide alternative climate-smart crops because of their ability to adapt to harsh environmental condition better than the current major crops of the world. It is considered as a helpful famine crop by FAO as it can be stored easily for lean years. Finger millet is considered to be an ideal crop for food and nutritional security because of its high nutritive and satiety value, medicinal properties, excellent grain storage properties and a good resistance to biotic and abiotic stress. The nutritive value of finger millet grain is superior to rice and comparable to wheat. Finger millet has the highest calcium content among all cereals.It is a rich source of iron, dietary fiber, phytates, protein, minerals, phenolics, carbohydrates, vitamins like thiamine, riboflavin and essential amino acids like methionine, isoleucine, leucine and phenylalanine which is lacking in the diets of millions of the poor people **(Devi *et al.*, 2014; Kumar *et al.*, 2016).**

Inspite of its tremendous potential, this crop is under-explored and their yield is very low as they are mostly cultivated in areas with poor moisture and fertility. The abiotic and biotic stresses are the two major limitations in cereal production faced by farmers and scientists, amounting to a yield loss of 30%-60% per year all over the world **(Dhlamini *et al.,* 2005).** Biotic stresses such as insect pests and diseases are a cause for substantial yield losses to diverse types of millets. However, abiotic stresses are the major contributor to losses every year **(Tadele, 2016).** It limits the crop plant from expressing their full genetic potential there by reducing their agricultural productivity. The importances of heritable alteration made possible by recombinant DNA technologies to produce a genotype that can tolerate stressful conditions are crucial to this success. Recombinant DNA technology is a potential tool to introduce genetic elements directly into a cell's genome. It move genes of interest across sexually incompatible species and thereby finds a solution to problems that defy conventional breeding and selection approaches. It is a constraint in breeding that it does not allow insertion of exogenous genes from unrelated species. Genetic transformation using direct or indirect methods offers an effective means to integrate the beneficial genes from wild relatives into crop plants, from one species to another and across sexually incompatible species for the production of genetically altered plant. This techniques has been widely used for studying genetically controlled traits; gene function, gene discovery and varietal improvement. In the plant biotechnology, transformation is defined as the process of DNA introduction into a plant cell, leading to a permanent change in the genetic makeup of the target cell and its derivatives.

Methods of gene transfer into plant cells include *Agrobacterium*-mediated transformation, direct gene transfer into protoplasts (polyethylene glycol, Electroporation), and particle (microprojectile) bombardment. In monocots, particle bombardment and *Agrobacterium*-mediated transformation are commonly used method for genetic manipulation. The Particle bombardment method of gene transfer is a robust, relatively efficient and widely used method. The drawback of Particle bombardment method is that the DNA integration sites are often very complex. *Agrobacterium*-mediated transformation has many advantageous features, these includes reduction in transgene copy number, stable integration with fewer rearrangements of long molecules of DNA with defined ends and ability to generate

lines free from selectable marker genes and other advantages include high quality and fertility of resultant transgenic plants. Above all *Agrobacterium*-mediated transformation is inexpensive and convenient as compared to other methods **(Komari *et al.*, 1998).**

Agrobacterium tumefaciens is a gram negative soil bacterium which naturally infects the wound sites in dicotyledonous plants and induces crown gall diseases. It was generally believed that monocotyledonous plants were non transformable by *Agrobacterium* as they were outside the natural host until **Hiei *et al.*** in 1994 reported an efficient generation of transgenic rice from mature embryo derived callus. After two years **Ishida *et al.*** also reported successful transformation in maize by co-cultivating immature embryo and *A. tumefaciens.* The recalcitrance of monocots to *in vitro* regeneration and lack of active cell divisions at the wound site were the key reason for the failure. For these reasons, the use of actively dividing cells and tissue culture technology for obtaining actively dividing cells or about to divide and regenerate are critical for successful transformation in cereals. Further, the identification of inducers like acetosyringone has made possible the induction of virulence genes by artificially adding the chemicals. The target material for *Agrobacterium* transformation can be embryonic culture, immature embryo, mature seeds, derived, calluses, shoot apices, excised leaf blades, root cotyledons, stem segments, callus suspension culture and meristems. The success of transformation is highly dependent on *in vitro* regeneration. The most important factor to be considered while choosing the target explants is their susceptibility to *Agrobacterium* and their morphogenetic potential as it may differ even within a plant depending on the types of tissues, organs and cell types. Transformation is a complex process involving many critically important factors like the types, age and genotype of the tissues infected, culture media, the strains and type of vector, the selection markers and selective agents, temperature and pH. *Agrobacterium*-mediated gene transfer is considered as an efficient and indirect method of gene transfer into plant genomes through the interaction between bacterial and host plant. However, it is still difficult to achieve high reproducibility and consistency of transformation events since it is a multi factor, complex interaction process. It is difficult to analyze which factor contributes the most to the transformation efficiency**. Jha *et al.* (2011)** suggested that a step by step optimization of such factors will be of considerable importance for

the establishment of successful transformation systems in monocotyledonous crops. Millets have received little attention in comparison to other cereal crops such as rice, maize, wheat etc. and thus remain largely unimproved. Till date, there are limited numbers of reports on *Agrobacterium* mediated transformation in finger millet.

For genetic improvement of finger millet, development of efficient protocol for transformation is necessary. In monocots, particle bombardment and *Agrobacterium*-mediated transformation are commonly used method for genetic manipulation. Considering the many advantageous features, cost and convenience, *Agrobacterium*-mediated transformation method is a method of choice in this present investigation.

This study is taken up with the objective of establishing an efficient method and to obtain further information on finger millet tissue culture, genetic transformation and finds out the best explants that can be used for genetic transformation. The difference in the competence of *Agrobacterium* to infect a specific tissue, organs, genotype or species remains a major drawback of this method for the introduction of the novel gene in cereal crops. Moreover, a huge variation in callus induction and regenerative capability was observed within the genotypes of same species. Most of the researchers use embryonic callus as target explants for transformation studies in millets. Even though there are reports where the use of immature embryo is favoured over somatic embryos **(Bartlett *et al.*, 2008)** and others explants as an ideal target for cereals, it comes with many disadvantages. Collection and maintainance of immature embryo is a tedious task. There are other reports where leaf segments, immature embryo, pollen grain were directly used as a target explants **(Wüthrich and Tadele, 2012).** In order to enhance the transformation and understand the complexity, there is a need to identify the best explants for *Agrobacterium* infection, callus induction and regeneration, acetosyringone concentration, inoculation, co-culture, co-cultivation condition, bacterial strain, cell density selection, temperature, light, pH of finger millet. Taking note of all the important factors, an attempt was made to optimize the explants for *Agrobacterium* mediated gene transfer in finger millet (PES 400). The culture method developed can be used for further studies in transformation of finger millet.

The response of coleoptile, mesocotyl and seeds were evaluated on MS agar nutrient media with different concentration and combination of plant growth regulators 2, 4-D, NAA, kinetin, and BAP on callus production and somatic embryogenesis were

studied. The sensitivity of different explants to different antibiotic that will be used for selection of transformed explants and killing excess *Agrobacterium* after infection and co-cultivation was also studied. The effect of infection and co-cultivation time was also evaluated.

Finger millet is chosen as a model system for this present investigation because it is one of the most nutritious and hardy crop that can survive at extreme climatic and soil conditions. Many genes involved in adaptation to biotic and abiotic stress can be fished out to use it for further improvement and development of superior crop variety. Our lab has been working on *Myb* gene, which is known to play a significant role in the regulation of drought response. In order to understand the gene function, the cloned *Myb* gene needs to be transferred to a drought sensitive finger millet variety PES 400.Phenotypical and biochemical analysis can then be carried out in the transformed finger millet plant at phenotypical and biochemical level while trancriptome analysis of the gene is performed to find out which gene is suppressed or upregulated. The standardization of a protocol is required for successful transformation through *Agrobacterium* for expression analysis of *Myb* gene and to study its role in abiotic stress.

In view of above, the present investigation has been planned with the following major objectives:-

1. Optimization of different combination of hormones for callus induction in *Eleusine coracana.*
2. *Agrobacterium* mediated gene transfer.
    i) Optimization of antibiotic concentration.

       Hygromycin and kanamycin; Selection of transformed callus and other explants.

       Cefotaxime: To remove excess *Agrobacterium* after infection

    ii) Optimization of acetosyringone concentration for bacterial infection of callus and other explants.

    iii) Optimization of infection time for different explants.

    iv) Optimization of co-cultivation time.
3. Selection of transformed explants using selectable marker.

Review of Literature

Chapter 2 REVIEW OF LITERATURE

This chapter gives a detailed account of literature and research studies which are directly or indirectly related with the present investigation. The collected reviews were organized under the following headings and subheadings.

2.1 A Brief description of Finger Millet

Finger millet is one of the minor cereal crop extensively cultivated in the semi-arid regions of Asia and Africa. It is the primary food source for millions of people living in tropical regions of Africa and Asia **(Devi *et al.*, 2014)**. Finger millet gets its name from the arrangement of its panicle, 4-19 finger-like spikes arranged serially, resembling a fist when matured**.** It is known by different name in different countries. In India, it is known as *Ragi, Koddo* in Nepal, *Finger millet*; *African millet; Koracan* in English, *Rapoka, zviyo, niera, mazhovole, rukweza, uphoka, poha* in Zimbabwe, *kambale, lupoke, mawele, majolothi, awale, bule* in Zambia, *Petit millet*, *eleusine cultivee, Bulo* in Uganda, *Hawere, lipoke, usanje, khawke, malesi, mulirubi, lupodo, mawe* in Malawi; *Limbi, mugumbi* in Kenya, *Dapussa, Tokusa, Bavankiya* in Ethiopia*; petit mil, eleusine cultivee, coracan, koracan* in France **(Singh and Raghuvanshi, 2012).**

2.1.1 Taxonomy and Morphology

The botanical name of finger millet is *Eleusine coracana* (L.) Gaertn. The subspecies *coracana* belongs to the family Poaceae. Finger millet is a fast growing tufted annual cereal crop growing up to a height of 170 cm **(FAO. 2012).**It reaches maturity within 3 to 6 months and occasionally in only 45 days **(Dida *et al.*, 2006).** All the florets except the terminal ones are perfect flower.Finger millet grain is reddish brown in colour, oblong to round and oval shape.

2.1.2 Origin and Distribution

Finger millet originates in Africa. It is native to the Ethiopian and Ugandan highlands. The archaeological record of finger millet from Ethiopia dates back to about the 5000 years ago. It later spreads to Asia around 3000 years ago **(Hilu *et al.*, 1979; Andrea *et al.*, 1999)**. The finger millet are mainly cultivated in Southern and Eastern African countries; Sudan, Tanzania, Malawi, Uganda, Kenya, Zaire, Zimbabwe,

Zambia, and Mozambique and Southern Asia mainly in India and Nepal **(National Research Council, 1996).** India is the largest producer of finger millet.In India, production area of finger millet stands sixth after wheat, rice, maize, sorghum and bajra. Karnataka, the major producer contributes up to 58% of global finger millet production.It is estimated that nearly 10% of the world's 30 million tons of millet produced is finger millet **(Dida *et al.*, 2008).** Finger millet is the fourth millet in terms of worldwide production after sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*) and foxtail millet (*Setaria italica*) **(Upadhyaya *et al.*, 2007).**

2.1.3 Climatic requirements

Finger millet is adapted to a wide range of soil conditions. Despite the fact that finger millet prefers a fertile, well-drained sandy to sandy loam soils with a pH ranging from 5 to 7 **(Dida *et al.*, 2006),** 5.0–8.2 **(Duke 1978, 1979)**, it can also grow well in lateritic or black heavy vertisols. Finger millet has some tolerance to alkaline and moderately saline soils **(Dida *et al.*, 2006).** It can grow well in high altitudes. Finger millet is commonly found at altitudes between 1000 and 2000 in eastern and southern Africa, and up to 2500-3000 meters in the Himalayas.It grows best at an average temperature of 23°C but can withstand cooler and hotter conditions **(FAO, 2012).** An annual rainfall ranging from 500 to 1000 mm is suitable for their growth, provided it is well distributed across the growing season **(Dida *et al.*, 2006)**. Finger millet was also reported to grow best in an environment with medium rainfall (29–429 cm) and yearly temperature range of 11 to 27°C **(Duke, 1978, 1979)**.Finger millet performs better than pearl millet and sorghum in drier condition**(de Wet, 2006).**

2.2 Finger millet for food and nutritional security

The problem of food insecurity and malnutrition is an impediment in development. Nutritional well being is indispensable for good health, development and maximization of human genetic potential **(Singh and Raghuvanshi, 2012).**Finger millet posses many desirable properties in terms of adaptability and nutrition. Millets might provide alternative climate-smart crops, as their adaptations to challenging environment are better than the current major crops of the world. Inspite of its tremendous potential, this crop is under-explored. Finger millet is considered to be an ideal crop for food and nutritional security because of its high nutritive and satiety

value, medicinal properties, excellent grain storage properties and a good resistance to biotic and abiotic stress. Biotic stresses such as insect pests and diseases are a cause for substantial yield losses to diverse types of millets. However, abiotic stresses are the biggest contributor to losses every year **(Tadele, 2016).** Finger millet is one of the most nutritious among all major cereals. It considered as Potential "super cereal" by United States National Academies **(Kumar *et al.*, 2016)** and a "famine crop" by **FAO (2012)** for all the right reasons.

2.3 Health Benefits of finger millet

The nutritive value of finger millet grain is superior to rice and equivalent to wheat. It is rich in calcium (0.34%), dietary fiber (18%), phytates (0.48%), protein (6%–13%) minerals (2.5%–3.5%), and phenolics (0.3%–3%), 65–75% carbohydrates. Finger millet has the highest calcium content among all cereals.It is a rich source of thiamine, riboflavin, iron, methionine, isoleucine, leucine, phenylalanine and other essential amino acids which is lacking in the diets of millions of the poor people. It also contain phytates (0.48%), polyphenols (0.2–3.0%), tannins (0.61%), trypsin inhibitory factors, and dietary fiber which were once considered as "anti nutrients" due to their metal chelating and enzyme: inhibition activities are now termed as neutraceuticals. Health beneficial effects of finger millet includes anti-diabetic, antitumerogenic, antioxidant, anti-diarrheal, antiulcer, anti-inflammatory, atherosclerogenic effects and antimicrobial properties. Finger millet based diet was known to reduce the glycemic index in diabetic animals. These anti-diabetic properties of finger millet were attributed to its high polyphenols and dietary fibre contents. Phenolics controls the postprandial blood glucose levels by partially inhibiting amylase and α-glucosidase during enzymatic hydrolysis of complex carbohydrates and delaying glucose absorption while dietary fiber slows down gastric emptying or form un-absorbable complexes with available carbohydrates in the gut lumen **(Devi *et al.*, 2014)**.Antioxidant capacity of finger millet is also attributed to the high total phenolic content as well as flavonoids such as catechin, gallocatechin, epicatechin, procyanidin dimmer, levels of enzymatic (catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase) and non-enzymatic antioxidants like glutathione, vitamin E and C**(Kumar *et al.*, 2016; Singh and Raghuvanshi, 2012).**

2.4 Adaptation of finger millet to drought stress

Drought is one of the major constraints in crop production worldwide. Global warming, occurrences of drought and abnormal weather events have lately been observed all over the world **(Nakashima *et al.*, 2014).** Shrinking agricultural land and increasing population has posed a serious threat to food security. Genetic engineering of drought tolerant crops will be a key in the challenge of feeding a growing population.

Millets are resilient to the extreme climatic and soil conditions.It is a hardy crop extensively cultivated in the semi-arid regions of Asia and Africa, where many crops fail to grow.It is relatively easy to grow finger millet under stressful condition, without impeding the net productivity **(Puranik *et al.,* 2011).** Both abiotic and biotic stresses caused considerable yield losses to diverse types of millets. However, abiotic stresses like drought and salinity are the biggest contributor to losses every year. In general, millets perform better than cereals such as wheat and rice in semi-arid environments, these challenging climatic and soil conditions are by no means an optimum environment for millet cultivation. Antioxidant capacity of finger millet which reduce the damaging effects of high production of ROS Reactive oxygen species caused by stresses such as drought and salinity is one of its attribute for adaptation to abiotic stress. A transcriptome-wide study of finger millet plants exposed to drought obtained 2824 genes that were differentially expressed under these conditions. There is a need to identify the genotypes having greater adaptation to the adverse environment so that higher yield could be obtained **(Tadele, 2016).** Transcriptome sequencing of millets after exposure to moisture-deficit condition provides information on genes differentially regulated under exposure to abiotic stresses particularly to drought. Those genes which are responsible for their adaptability to a wide range of environment can be used for biotechnological manipulation to improve drought tolerance and productivity, through their over expression or silencing.

2.5 Finger Millet Tissue Culture

Among biotechnological techniques, plant tissue culture techniques have enormous potential and wide application for crop improvement. High frequency plant regeneration from tissue culture is pre-requisite for successful genetic transformation

and selection of the transgenic plants. The success of transformation especially cereal crops are highly dependent on in vitro regeneration. Most of the cereal crops use actively dividing cells as a target for *Agrobacterium* infection.The regeneration of transformed explants through organogenesis or somatic embryogenesis is an integral part of genetic transformation. The first regeneration studies in millets were performed in the 1970s for proso-, finger-, pearl- and kodo- millets by **Rangan (1976**). He was the first to report callus formation and plantlet regeneration from mesocotyl explants of finger millet. Regeneration of plant was achieved either by somatic embryogenesis or organogenesis. Somatic embryos appeared to be superior for efficient transformation and recovery of transgenic plants **(Veluthambi *et al.,* 2003)**. Direct development of somatic embryos is rare in finger millet as compared to indirect somatic embryogenesis through callus. The capability of regeneration is influenced by various factors like explants, plant growth regulators (PGRs), media and environmental factors such as pH, temperature and light **(Plaza-Wüthrich and Tadele, 2012).**

2.5. 1 Plant growth regulators and explants for callus induction

The transformation of plant is possible because of their totipotency. Plant hormones play an important role in growth and differentiation of cultured cells and tissues. There are many classes of plant growth regulators used in culture media. These include: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Several forms of auxin include 2, 4 dichlorophenoxyacetic acid (2, 4-D), Naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), dicamba and picloram. 2, 4-dichlorophenoxy acetic acid (2, 4-D) is the most popular auxin for culturing finger millets. Cytokinins are known to stimulate cell division, induce shoot and axillary shoot proliferation, cell enlargement in young leaves, tissue differentiation, flowering, fruiting, and delay of aging in leaves. As many as 100 synthetic and natural cytokinins were known.Some popular forms of cytokinins are; Kinetin, thidiazuron (TDZ), zeatin, 6-benzylaminopurinen (BAP). Auxins and cytokinins are the two most widely used plant growth regulators in plant regeneration. A number of factors are considered while choosing plant growth regulators for *in vitro* regeneration studies; the types of phytohormones, their concentrations and combinations. They are used either in combination or alone as their concentration in the medium determines the type of tissue or organ to be generated. The intermediate ratio induces callus formation. Somatic embryo was also obtained when auxin or cytokine alone was used. High auxin to

cytokinin ratio induce development of root while low ratio ratio promotes shoot development **(Kaur and Kothari, 2004).**The concentration of the auxin has to be decreased for long-term maintenance of the callus to reduce chromosomal abnormalities and poor regeneration **(Kumar *et al.*, 2001; Plaza-Wüthrich and Tadele, 2012).** In finger millet embryonic callus developed shoot at low Auxin to cytokinin ratio **(Girgi *et al.*, 2002).** Cytokinin alone was also observed to be successful for regeneration(**Sankhla *et al.*, 1992; Latha *et al.*, 2005;Ceasar and Ignacimuthu, 2008; Yemets *et al.*, 2003; Nethra *et al.*, 2009**).There are many studies which reported that none of the plant growrth regulators were necessary for shoot regeneration in finger millet **(Eapen and George, 1990; Mehta and Nag, 2012).** About half of millet regeneration studies applied plant growth regulators together to initiate shoot and root simultaneously while other remaining studies allowed shoot to develop first followed by rooting **(Plaza-Wüthrich and Tadele, 2012). Mehta and Nag (2012)** observed direct plant regeneration when *in vitro* grown mesocotyl explants were cultured on Murashige and skoog media supplemented with NAA(0.5-5.0 mg/l).They found that the frequency of embryogenic callus production was 100% whenever 2, 4-D was added in the the medium whereas direct plantlet formation from mesocotyl explants was observed in the medium devoid of 2, 4-D. Green cell masses were observed on surface of seed and mesocotyl calli when implanted on 2, 4-D (2.00-5 mgL-1) supplemented medium. The Green nodulated seed-calli and mesocotyl callus developed into a whole plant when it was transferred to auxin-free medium or medium devoid of growth regulator.

The identification of the most suitable explants is extremely important for increasing the competence of plant regeneration. Mature seeds, leaf, shoot tips, shoot apical meristem, mesocotyl, root, immature inflorescence were used as explants for regeneration studies in fingermillet. The callus formation and plantlet regeneration of millet from mesocotyl explants was first reported in 1976 by Rangan. **Mohanty *et al.*, 1985** used root of finger millet as explants, callus induction rate was found out to be more than 90%. The Shoot Apical Meristem which contains the zone of actively dividing cells was demonstrated to be suitable explant by **Eapen and George (1990)**. The mesocotyl part was also used as an explants by many researchers for induction of callus and somatic embryogenesis in finger millet **(Rangan, 1976; Mohanty *et al.*, 1985; Eapen and George, 1990, Mehta and Nag, 2012)**. Mature seeds and embryos were also widely used for induction of callus and somatic embryogenesis **(Sivadas *et***

***al.*, 1990, Poddar *et al.*, 1997, Gupta *et al.*, 2001, Kothari *et al.*, 2004, Dosad and Chawla, 2015)**. Leaves were commonly used as explants for callus induction and regeneration in dicots but this was not true for monocots. The vegetative part of the monocots did not proliferate readily. There is no report of successful regeneration from leaf explants in cereals **(Saalbach and Koblitz, 1978)**. A huge variation in callus induction and regenerative capability was observed within the genotypes of same species. **Dosad and Chawla (2015)** performed an in vitro plant regeneration from mature seeds of two finger millet variety PR 202 and PES 400 through somatic embryogenesis observed that the response for embryogenic callus development for PR 202 ranged from 29.5% to 78.2 % and 10 to 35.7 % for PES 400. **Ducan *et al* (1985)** made an interesting observation that the response of well nourished explant from well nourished plants and malnourished plants were different**.** The explants and plant growth regulators used for *in vitro* regeneration studies of millet are shown in Table 2.1.

Table 2.1: Explants and growth regulators used for callus induction in millets

Explant	Growth regulator	References
Mature seed	2, 4-D alone or with KIN	Sivadas *et al.* (1990), Poddar *et al.* (1997), Gupta *et al.* (2001), Kothari *et al.* (2004)
Immature embryo	2, 4-D alone or with KIN	Vishnoi and Kothari, 1995
Mature seed	2, 4-D alone or with BAP, KIN	Dosad and Chawla(2015)
Shoot tip	2, 4-D alone or with BAP, KIN	Anjaneyulu *et al.*(2011)
Shoot tip	thidiazuran and kinetin	Ceasar and Ignacimuthu (2008)
Mature embryo and epicotyl	2, 4-D	Patil *et al.*, 2009
Leaf and root explant and mature seed	2, 4-D or 3, 6-D or dicamba; ABA, BA; KIN	Bekele *et al.* (1995) and Mekbib *et al.* (1997)
Seeds and Mesocotyl	2, 4-D, NAA, IAA, IBA alone or in combination with kinetin and BAP	Mehta and Nag (2012)
immature inflorescence, mature seed	2, 4-D in combination with zeatin or coconut water or picloram + kinetin	George and Eapen, 1989, 1990
Shoot Apical Meristem	Picloram and kinetin	Eapen and George, 1989
Shoot Apical Meristem	Picloram, BA, dicamba	Pius *et al.*, 1994
Shoot Apical Meristem	Picloram and kinetin	Satish *et al.*, 2001
Shoot Apical meristem	2, 4D alone or in combination with Kinetin	Kumar *et al.*, 2001

2.6 Methods of transformation

Transformation is a powerful research tools for studying the function of genetically controlled traits, gene discovery and varietal improvement. Genetic transformation use direct or indirect methods to integrate valuable genes from wild relatives into crop plants, from one species to another and across sexually incompatible species for the production of genetically altered plant.

Transformation in plants consists of three main steps; target gene, plant tissue to be targeted and transformation methods. There are many methods for transformation of gene into plant cells viz; Physical methods such as microinjection and biolistics transformation, Chemical methods involving DNA transfer by calcium phosphate method, Liposome mediated transfer, Transfer of DNA by use of polyethene glycol and Electrical methods like Electroporation. All these methods have their own advantage and disadvantages. Agrobacterium mediated gene transfer and microprojectile bombardment are the most commonly used methods for transformation of gene in plants owing to their ease and cost of transformation. Microprojectile bombardment is a robust and efficient method of gene transfer. However, *Agrobacterium* –mediated gene transfer is considered to be more advantageous for many reasons. *Agrobacterium* - mediated transformation is simple compared to other methods. The DNA integration site of particle bombardment is complex and random while gene delivery via *Agrobacterium* is site specific and stable. It can delivers a single copy of DNA fragments with defined ends and fewer rearrangements there by reducing the problem of transgene silencing **(Komari and Kubo, 1999)**.

2.7 *Agrobacterium* Mediated Gene Transfer

Agrobacterium tumefaciens is a Gram-negative soil-borne, rod-shaped bacterium commonly associated with the roots of plants **(Kado and Hooykaas, 1991).** *Agrobacterium* mediated gene transfer is an effective method for transferring gene to plants. The transfer of gene from *Agrobacterium tumefaciens* to plant cells involves the following five steps:

(1) Bacterial colonization and attachment:

(2) Induction of bacterial virulence system,

(3) Generation of T-DNA transfer complex

(4) Transfer of T -DNA

(5) T-DNA integration into plant genome.

The efficiency of *Agrobacterium* transformation is influenced by many factors such as bacterial strain, cell density, antibiotics, plant species and genotype, explants(organs, cell and tissue types), explants wounding, light and temperature **(Karami, 2008).**The cereals were considered to be resistant to *Agrobacterium* infection for a very long time before the main factors for successful agrobacterial transformation was identified. One of which is; *vir* gene expression activation by phenolic signal **(Sood *et al.*, 2011).** Now it is possible to induce the expression of *vir* genes by adding artificial phenolic compounds like acetosyringone. The *vir* gene encodes Vir proteins which mediates the excision of T-DNA from the Ti plasmid and export o the T-DNA piece from the bacteria into host chromosome.

2.8 Host range of *Agrobacterium*

Agrobacterium has broad host range. It can transfer DNA to angiosperms (dicot and monocot), gymnosperms and fungi (yeasts, ascomycetes and basidiomycetes). It was also reported to transfer DNA to human cells **(Gelvin, 2003)**. Till date, the genetic mechanism of host range remains obscure. It was reported that the major genetic determinant of host range was the Ti plasmid rather than chromosomal genes. The Ti plasmid has several virulence (*vir*) genes;*vir* C **(Yanofsky and Nester, 1986)**, *vir* F **(Regensburg-Tuink and Hooykaas, 1993)**, and *vir* H **(Jarchow *et al.*, 1991)** which were reported to be involved in determining the range of plant species. The host range determination is a complex process. It is controlled by multiple genetic factors of the bacterium and the host plant and interaction of particular Ti plasmids with certain bacterial chromosomal backgrounds **(Gelvin, 2003)**. For example, **Hood *et al.* (1987)** observed that Ti plasmid pTiBo542 in its natural host strain *A. tumefaciens* Bo542 cannot transform leguminous plant species but it became virulent when it is placed in another strain *A. tumefaciens* C58 chromosomal background. It was thought that monocotyledons cannot be transformed but **Potrykus (1990)** reported that cereals (monocotyledons) are difficult to transform because they do not show wound response.The specific attachment of *A. tumefaciens* to the cells of bamboo and other

monocotyledons were observed by **Douglas *et al.* (1985)** but the induction was limited due to lack of production of the compounds such as acetosyringone that induce the virulence genes involved in the transfer of T-DNA **(Usami *et al.*, 1987).**

2.9 Transfer DNA (T-DNA)

Ti (tumor inducing) plasmid is a large plasmid with the size range of 200 to 800 kbp **(Gelvin, 2003)**. It contains 35-kb virulence (*vir*) region consisting of seven loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*) and T-region (transferred DNA-region).T-regions on native Ti regions represent less than 10% of the Ti plasmid. It is approximately 15 to 30 kbp in size depending on the strain **(Zambryski *et al.* 1980**). T-regions represent less than 10% of the Ti plasmid. Ti plasmids can have one or multiple T-regions as reported by (**Gelvin, 2003)**. The T-regions of octopine and nopaline type plasmids are defined by T-DNA right and left border sequences of 25 base pairs in length. The border sequences are highly homologous (Figure 2.1). It delimits and flanks the T-region in a directly repeated orientation (**Suzuki *et al.*, 2000).** The T-DNA borders are the target of the VirD1/VirD2 border-specific endonucleases. The excised single strand T-DNA is then transferred from the bacterial cell to the host plant by the Vir proteins. Deletion of the right border reduces virulence, whereas the left border does not. This suggested that T-DNA transfer is directed from the right to left border **(Howard *et al.*, 1992).** The importance of right border for *Agrobacterium* pathogenecity over the left border is further supported by other findings. **Jasper *et al.*** in the year 1994 reported that the VirD2 protein alone can bind the single stranded right border sequence (5`end) and cleave a single-stranded T-DNA. The VirD2 protein remained on the 5'end (right border) of the resulting single stranded. T-DNA of octopine-type Ti plasmid has an overdrive sequence near the right border, but not left border, which may augment the functional polarity of right border and left border **(Gelvin, 2003)**. Further **Peralta *et al.* (1986)** found that the overdrive sequence, a 15-bp element with a highly conserved 6-bp core is present near the right side borders of the T-DNA in three different Ti plasmids.The Overdrive sequences is shown to enhance the efficiency transmission of T-strands to plants (**Peralta *et al.*, 1986 Binns and Thomashow, 1988**).

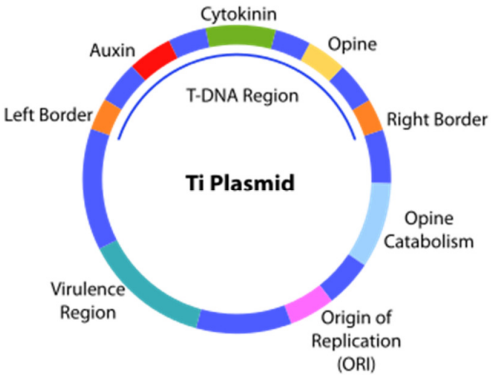


Figure 2.1: Structure of Ti plasmid (Image source: Wikipedia)

Agrobacterium transfers the T-DNA from the Ti plasmid to the plant cell which upon expression of its gene causes crown gall disease in the host plant.The tumor induction is initiated by bacterial recognition of monosaccharides and phenolic compounds secreted by the plant wound site.The transfer DNA has the genes for opine synthesis and tumor inducing factors. Stable integration of the T-DNA into the host plant chromosomal DNA and its expression causes neoplastic growth of the tumors. The tumor is the results from unregulated accumulation of the plant hormones auxin and cytokinin encoded by the T-DNA genes. These genes also direct the synthesis of unusual compounds called opines for bacterial consumption. Opine is the condensation product of an amino acid with a keto acid or sugar. It serves as a major carbon and nitrogen source for *Agrobacterium* growth (**Binns and Thomashow, 1998; Yildiz *et al.*, 2016).** *Agrobacterium* classification is usually based on the disease symptomology (type of opine produced) and host range. Most common *Agrobacterium* strains produce either octopine or nopaline form of opines **(Hooykaas and Beijersbergen, 1994),** a derivatives of arginine. The transfer of T-DNA is mediated by products encoded by the 30-40 kb *vir* region of the Ti plasmid. The *vir* region of Ti plasmid is an operon consisting of at least 6 essential operons;*vir A, vir B, vir C, vir D, vir E, virG* and two non essential; *virF* and *virH.* The *virA* and *virG* are expressed constitutively. They code for a two-component (VirA-VirG) system which activates the transcription of the other *vir* genes **(De la Riva *et al.*, 1998).** The *vir* genes do not enter the plant cell but it must be expressed in the bacterium for successful transfer of the T-DNA. Under normal bacterial growth condition the *vir* genes are not expressed.

2.10 Induction of *vir* genes

The expression of *vir* gene is induced by plant signal molecules that are mostly phenolic compounds. These compounds are synthesized in wounded plant tissues. Acetosyringone is the first inducer to be characterized. Other phenolics that can induce *vir* genes include vanillin, sinapinic acid, and p-hydroxybenzoic acid **(Binns and Thomashow, 1998)**. Experiments by **Ashby *et al.* (1987)** and **Parke *et al.* (1987)** indicates that *Agrobacterium tumefaciens* is chemotactic towards phenolic compounds. The three genetic loci that are influential in attachment (chemotaxis) to the plant cells are *chvA*, *chvB* and *pscA*(exoC), respectively. They are encoded by the chromosome of *Agrobacterium* and are constitutively expressed. The receptor protein coded by *virA* gene detects the signal molecules which are generally phenolic compounds such as acetosyringone. The phenolics and certain class of monosaccharides which acts sinergistically with phenolic compound leaks out of damaged plant cells at acidic pH (5.0 to 5.8).Activated VirA consequently stimulates the cytoplasmic DNA binding protein virG by transferring its phosphate to a conserved aspartate residue. The phosphorylated virG act as a transcription factor that regulates the expression of *vir* genes. virC, a helicase enables the T-DNA to separate from the borders, while *virD* gene recognize the T-DNA border sequences and nick (endonuclease activity) the bottom strand at each border; *virB* and *virE* genes facilitate the movement of the T-DNA from the bacterium to the plant cell **(Zambryski1; 1992; de la Riva *et al*., 1998).** The *vir G* gene is reported to contribute in "hypervirulence" of particular strains by many researchers. Increase in the expression of *vir* gene was observed when the copy number of nopaline-type *virG* is increased **(Rogowsky *et al.*, 1987)**. Under normal condition, induction of *vir* gene is very scanty at neutral or alkaline pH. **Liu *et al.* (1993)** reported that presence of multiple copies of *virG* altered the pH response profile for *vir* gene induction. They observed substantial degree of *vir* gene induction in rich medium with pH 8.5.

2.11 Factors influencing transformation in millet

Most of the millets transformation used microprojectile bombardment and *Agrobacterium*-mediated transformation method. Despite the drawbacks of microprojectile bombardment method, it is used more often than the *Agrobacterium*-mediated transformation in millets **(Plaza-Wüthrich and Tadele, 2012).** There was no

prior report on *Agrobacterium*-mediated transformation until **Sharma *et al.*** in 2011 reported successful transformation in finger millet PR-202.The genetic engineering of monocots were initially difficult due to their recalcitrance to *in vitro* regeneration and their resistance to *Agrobacterium*-mediated infection due to lack of wound response which act as an inducer for the virulence genes. Nowadays, there are many reports available for the in vitro culture of millets **(Kothari *et al.*, 2005)** and the inducers for virulence genes has been identified. Induction of virulence genes is possible by artificially added chemicals like acetosyringone. However, there is a huge variation in callus induction, regenerative capability and competence of *Agrobacterium* to infect certain type and part of explants within the genotypes of same species. Development of efficient protocols for in vitro regeneration system is an important prerequisite for successful transformation and recovery of transgenic millet crops **(Ceasar and Ignacimuthu, 2009)**. *Agrobacterium*-mediated transformation is a multi factor, complex interaction process. It is very difficult to analyze which factor contributes the most to the transformation efficiency. A step by step optimization of such factors like screening of the most responsive genotype and explant, *Agrobacterium* strain, binary vector, selectable marker gene and promoter, inoculation and co-culture conditions, and tissue culture medium, plant growth regulators, culture condition and their interaction is essential for establishment of successful transformation systems in monocotyledonous crops **(Jha *et al.*, 2011).**

2.11.1 Target material for transformation

While choosing the target material for *Agrobacterium* mediated transformation, the ability of the explants to regenerate should be considered since totipotency is one of the most important factor that determines the transformation system in most plants. It is is indispensible for successful genetic transformation of plants. Depending on the type of host cell and objective of experiment, a variety of explants target material of *Agrobacterium* can be used for transformation; embryonic culture, immature embryo, mature seeds derived callus, shoot apices, excised leaf blades, root cotyledons, stem segments, callus suspension culture and meristems **(Karami, 2008)**. The susceptibility to *Agrobacterium* transformation may differ within a plant depending on the types of tissues, organs and cell types. Millet transformation commonly use embryogenic callus as target material for *Agrobacterium*. However, explants such as leaf segments,

immature embryo, pollen grains and shoot tips were also used directly as explants target for *Agrobacterium* mediated transformation **(Girgi *et al.*, 2002, 2006**, **Goldman *et al.*, 2003; Latha *et al.*, 2006; Gupta *et al.*, 2001; Latha *et al.*, 2005; Yemets *et al.*, 2008; Sharma *et al.* 2011; Liu *et al.*, 2005;Qin *et al.*, 2008;Wang *et al.*, 2011**). These difference in the competence of *Agrobacterium* to infect certain type and part of explants remains to be the major problem in using this method of transformation routinely for transferring gene of interest into cereal crops **(Shrawat and Lörz, 2006)**. So, screening of the most responsive genotype and explant, *Agrobacterium* strain, binary vector, selectable marker gene and promoter, inoculation and co-culture conditions, and tissue culture medium, plant growth regulators, culture condition and their interaction is essential for successful transformation. Majority of *Agrobacterium* mediated gene transfer in cereals use actively dividing tissues owing to their excellent morphogenetic potential **(Karami, 2008)**. Somatic embryos is preferred over organogenesis for genetic transformation because it originates from a single cell. The regenerated plants from somatic embryos were transformed uniformly with higher transformation frequencies and without chimeric variations **(Wilkins *et al.*, 2004).** The embryo in which differentiation of shoot apical meristem has started and the mesocotyl part of the maize has been reported to show competence for transformation **(Schlappi and Hohn, 1992; Ritchie et al, 1993)**. Immature zygotic embryo has been widely used as an explants for development of embryogenic callus lines, cell suspensions and protoplasts for transformation of many important cereal crops.However, the problem arose due to their lack of competence in certain elite lines and their regenerability was also reduced after a few months of in vitro culture. Above that obtaining immature embryo and their maintenance takes lots of time and effort since its availability is restricted to the growing season. Scientist has been working on development of efficient and genotype independent regeneration system that can be maintained *in vitro* for long time. With their efforts now there are many reports on successful manipulation of shoot apical meristems in maize, oat, sorghum, millet, wheat, and barley. These reports indicate shoot apical meristems as a suitable explants for transformation of certain cereal crops (**Sticklen and Oraby, 2005**).

2.11.2 Bacterial strain, vector and selectable used in millet transformation

The use of appropriate bacterial strains, binary vectors, reporter and marker genes, promoters and molecular biology techniques are necessary for successful

transformation and transformants selection **(Riva *et al.*, 1998)**. The *Agrobacterium* strain GV3101 (C58 derivative) having rifampicin resistance gene on the chromosome is frequently used to transform many binary vectors, e.g., pBI121, pGPTV, pCB301, pCAMBIA, and pGreen, into *Arabidopsis*. *Agrobacterium* strain LBA4404 is commonly used for tobacco transformation but it was found to be less efficient for *Arabidopsis* (Narusaka *et al.*, 2012). For cereals transformation hypervirulent *Agrobacterium* strains such as EHA101 and EHA105 in maize, AGL-0 and AGL-1 in barley and wheat as well as hypervirulent derivatives of LBA4404 in maize, barley and wheat are used (**Hensel *et al.*, 2009**). The *LBA4404, EHA101* and derivatives of *EHA101* namely *EHA105, AGL0* and *AGL1* are the most widely used *Agrobacterium* strains for millet transformations **(Plaza-Wüthrich and Tadele, 2012)**. Promoters frequently used are CaMV 35S, Actin 1 promoter isolated from rice Ubiquitin1 promoter from maize and termination sequence like *nos* to signal the termination of gene expression. Most of the finger millet transformation use binary vector pCAMBIA.The binary vector pCAMBIA 1301 has *nptII* gene as bacterial selection marker, *hptII* gene as plant selectable and intron-containing β-glucuronidase (GUS) as a reporter gene; both are driven by the CaMV35S promoter **(Babu *et al.*, 2012)**.

Sharma *et al.* (2011) used EHA 105 harbouring pBI121 for transformation.The plasmid pBI121 contains β-glucuronidase (uidA) as a reporter gene, and neomycin phosphotransferase II *(npt II*) as a selective marker gene, both genes are driven by the CaMV 35S promoter. Using *Agrobacterium* strain LBA4404 harboring pCAMBIA1301 vector and hygromycin as selection marker and embryogenic callus as target material, two genotypes of finger millet were transformed by **Ceasar and Ignacimuthu (2011). Liu *et al.* (2005)** developed a protocol for *Agrobacterium*-mediated transformation in foxtail millet by using LBA4404 strain harboring a binary vector pBI121 for infecting inflorescence-derived calli. 50 mg/L Kanamycin was used for selecting the transformed plants. They obtain a transformation efficiency of 5-9%. Further confirmation was done by Southern hybridization.

Selectable markers protect the organism from a selective agent that would normally kill the cell or prevent its growth. Different selectable marker genes are used for selection of transformed explants. They are introduced together with the transgene or gene of interest so that they are expressed along with it. The selectable markers are

usually antibiotics such as kanamycin and hygromycin or herbicides such as glyphosate and PPT. Antibiotics are exclusively used as a selectable marker in bacteria while in plants, antibiotics that kill the chloroplast and mitochondria are used. Hygromycin is an amino glycoside antibiotic that can kill fungi, bacteria and higher eukaryotic cells. It is produced by *Streptomyces hygroscopicus*. It inhibits the protein synthesis by interfering with spontaneous reverse translocation of tRNAs and mRNA on the ribosome. Hygromycin stabilizes the tRNA-ribosomal acceptor site, causing mistranslation at 70S ribosome (**Cabanas *et al.*, 1978; Gonzales *et al.*, 1978; Borovinskaya *et al.*, 2008**). Hygromycin is widely used as a plant selectable marker in research. The cells that express *hpt* gene are resistant to hygromycin. The hygromycin phosphotransferase gene is originally derived from the *Escherichia coli* bacteria. It codes for the enzyme hygromycin phosphotransferase which detoxifies the aminocyclitol antibiotic hygromycin B. Many plants have been transformed with the *hpt* gene. Hygromycin B was found out to be very effective in the selection of a wide range of plants, including monocotyledonous and transformed mammalian cells. Most of the plants especially cereals exhibit higher sensitivity to hygromycin B than to kanamycin. EPSP synthase is an enzyme involved in aromatic amino acids biosynthesis in bacteria and plants. In absence of EPSP synthase, accumulation of shikimate, inhibition of aromatic amino acids and secondary metabolites synthesis leads to cell death. 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase is isolated from *Salmonella typhimurium* conferring resistance to the herbicide glyphosate is used as a selectable marker for glyphosate resistant plant. Phosphinothricin N-acetyltransferase (PAT) Or PPT selection system contains genes coding either for bialaphos resistant genes *bar* isolated from Streptomyces hygroscopicus. PPT acetyltransferase *(pat)* isolated from the bacterium *Streptomyces viridochromogenes,* or glufosinate (an ammonium salt of PPT) have been used as a plant selectable marker. The PPT (ammonium glufosinate) is analogous to glutamate which is a substrate of glutamate synthetase (GS). Glutamate is a competitive inhibitor of glutamate synthetase.GS catalyzes the conversion of glutamate to glutamine which removes the toxic ammonia from the cell. Inhibition of GS results in ammonia accumulation leading to chloroplast disruption and eventually photosynthesis inhibition **(Lindsey, 1992; Sundar and Sakthivel, 2008; Ji *et al.*, 2013).**

2.11.3 Effect of temperature

Temperature is an important factor that influence the transformation efficiency of *Agrobacterium* mediated gene transfer. The importance of temperature has been demonstrated by **Braun** in the year 1947 and **Brown** in the year 1942. They observed that during bacterial infection the formation of tumor was inhibited at 28˚C and above. Also, the size of the crown gall tumor increases with decrease in temperature. In monocots most of the co-cultivation was carried out at 24-25˚C **(Sharma *et al*, 2011)**. The report for optimal temperature for co-cultivation time varies in between crops and even in between genotype of the same species. For example, when maize immature embryo is used as a target material for transformation, higher frequency of transformation was obtained when co-cultivation is carried out at 20 ˚ C and sub culturing at 28℃ **(Frame *et al.*, 2002)**. In finger millet, *Agrobacterium* mediated gene transfer co-cultivation was carried out at 22˚ C (**Sharma *et al.*, 2011**), 25°C **(Jayasudha *et al.,* 2014; Hema *et al.,* 2014)** and 26 ℃ **(Ceasar and Ignacimuthu, 2011, Ignacimuthu and Ceasar, 2012)**. **Dillen *et al.* (1997)** reported that the optimum temperature for co-cultivation is 22℃ irrespective of the type of helper plasmid. They observed that the transient expression level of *uid*A was lower at 25 ℃ than in 22℃. While very low level of expression and no expression was observed at 27 ℃ and 29 ℃. **Sharma *et al.* (2011)** observed the highest transformation frequency at 22 ℃ in finger millet. The frequency of transformation and number of plantlets was reduced when the temperature increased beyond 24 ℃ and no transformation at 28℃. This result indicates that the temperature which is optimum for their growth does not necessarily coincide with their ability to transfer gene. The reason behind their inability to transform may be because the temperature is not favorable for expression of *virG* and *virD* genes. **Alt-Mörbe *et al.* (1989)** studied the effect of temperature, sucrose, acetosyringone and pH on *virG* and *virD* genes expression. They reported that temperature below 28°C is required for the expression *virG* gene coding for VirG protein which along with VirA activates the transcription of the other *vir* genes. The induction of *vir* genes; virD2 **(Alt-Mörbe *et al.*, 1988)**, virG and virB **(Fullner and Nester, 1996; Melchers *et al.*, 1989)** were high at 20-25 degree Celsius. Most of the *Agrobacterium*-based transformation protocols carry out infection of explants at 28 ℃ or lower. There are many reports where a brief heat shock or cold shock by centrifugation is given just before infection

of the explants by *Agrobacterium* to enhance the efficiency of transformation **(Khanna *et al.,* 2004; Cao *et al.,* 2006; Hiei *et al.,* 2006)**. As reviewed by **Karami (2008)**, the optimal temperature for T-DNA delivery and optimal temperature for stable transformation may not be same for a given species or explants. He further suggested that the optimal temperature for both delivery and stable transformation should be evaluated with each specific explants and *Agrobacterium* strain used for infection. Most of the regeneration of millets was carried out at 24±2°C **(Jayasudha *et al.*, 2014; Ceasar and Ignacimuthu 2011, Ignacimuthu and Ceasar, 2012).**

2.11.4 Effect of Infection and Co-Cultivation condition

Acetosyringone and cell extracts from dicots were added in infection and co-cultivation media to improve *Agrobacterium* mediated gene transfer in cereals **(Plaza-Wüthrich and Tadele, 2012).** Different studies have shown that acetosyringone below 100µM could not induce bacterial virulence, while high concentrations of acetosyringone as high as 500µM is harmful for the bacteria and explant growth (**De Clercq *et al.*, 2002**). **Ahmadpour *et al.* (2015)** obtained highest transformation at 200 µM acetosyringone in wheat immature embryo. Transformation in finger millet mostly used acetosyringone concentration in between 100-200.

Optimal co-cultivation period is crucial for enhancing the transformation efficiency. The co-cultivation time beyond the optimal causes overgrowth of *Agrobacterium* and below drastically reduce the transformation efficiency as the duration is too short for the *Agrobacterium* to insert the T-DNA into the target cell.Normally co-cultivation was done for 2–5 days in *Agrobacterium*-mediated gene transfer at different temperature **(Somleva *et al*, 2002). Lee *et al.* (2006)** performed an experiment on orchardgrass callus to study the effect of co-cultivation.The callus was co-cultivated with *Agrobacterium* for 1–7 days. They found 3 days co-cultivation to be optimal. 3 days of co-cultivation was reported to be optimal for finger millet by **Ceasar and Ignacimuthu (2011)** and peal millet by **Jha *et al.* (2011)**.

2.11.5 Control of *Agrobacterium*

For elimination of *Agrobacterium* after co-cultivation, one or more types of antibiotic is added to the culture medium. The presence of bacteria can interfere with the identification of transformed genes, growth and development or may kill the

transformed explants. Overgrowth of *Agrobacterium* reduce oxygen supply and interfere with the respiration process of the tissues **(Matzk *et al.*, 1996).** Elimination of *Agrobacterium* is important for successful recovery of transgenic cells and tissues **(Silva and Fukai, 2001;Karami, 2008)**. Antibiotics such as carbinenicillin, timentin, ticarcillium, vancomycin and cefotaxime have been used to inhibit the growth of *Agrobacterium* cells after co-cultivation **(Jones *et al.*, 2015).** Carbenicillin and cefotaxime are the two commonly used antibiotics for suppression of *Agrobacterium* cells **(Mathias and Boyd 1986; Karami, 2008)**. Cefotaxime is reported to show higher regeneration rate compared to clavamox and carbenillin. The studies on the influence of antibiotics on callus or explants morphogenesis and plant regeneration reported some antibiotics are harmful for the explants while others are reported to be beneficial. Cefotaxime is a broad spectrum cephalosporin antibiotic with low toxicity to eukaryotic cells. It is effective against gram-negative and Gram-positive bacteria at low doses **(Mathias and Boyd, 1986).** Cefotaxime is one of the most commonly used antibiotics **(Nauerby *et al.*, 1997; Karami, 2008). Mathias and Boyd (1986)** for the first time reported the effect of cefotaxime and carbenicillin. They observed that these antibiotics promote callus growth and enhance the organogenesis (shoot regeneration) in wheat. The following year **Mathias and Mukasa** observed a similar result in barley and again in cultured durum wheat by **Borrelli *et al.* (1992)** and in pearl millet **(Pius *et al.*, 1993).** Determining optimal optical density of *Agrobacterium* for inoculation is important as explants were wholly colonized by *Agrobacterium* during co- cultivation, selection and regeneration stages at high O.D **(Jha *et al.*, 2011).**

2.12 *Agrobacterium* mediated Transformation in millet

Sharma *et al.* (2010) develop a protocol for *Agrobacterium*-mediated transformation finger millet variety PR- 202. Seed derived green nodular calli were used as target for *Agrobacterium tumefaciens*, LBA 4404 harboring plasmid pBI-121 and EHA-105 harboring plasmids pCNL-56 and p35S-GUS-INT possessing *nptII* gene under 35SCaMV promoter. The highest transformation frequency of 44.4% was observed in a calli infected (25 minutes), co-cultivated (2days) and incubated at 22℃ for 15 days followed by final incubation at 26 ± 1˚C. They performed pretreatment of calli with anti-necrotic mix and surfactant (Tween-20).

was used during infection to enhance the survival of GUS positive calli. They observed that addition of higher level of CuSO4 in the regeneration medium significantly increase the recovery of transformed plants.

Jha *et al.* (2011) used super virulent Agrobacterium EHA105 harboring pCAMBIA 1301 to investigate and optimize T-DNA delivery into shoot apices of pearl millet. Highest transformation frequency of 5.79% was observed when the shoot apex explants were infected with *Agrobacterium* of 1.2 O.D at 600 nm for 30 minutes under a negative pressure of 0.5 $\times 10-5$ Pa and co-cultivated in co-cultivation media containing 400 µMole acetosyringone for 3 days at 25℃ in 16 h light.

Ceasar and Ignacimuthu (2011) the *Agrobacterium* strain LBA4404 harboring binary vector pCAMBIA1301 was used to infect two finger millet genotypes, GPU 45 and CO 14. The optimal conditions for the *Agrobacterium* mediated transformation was observed when callus obtained on 16th day after callus induction was inoculated for 30 minutes with *Agrobacterial* inoculm and 3 days of co-cultivation on filter paper placed on medium supplemented with 100 µM acetosyringone. Both finger millet genotypes were transformed by *Agrobacterium*. A frequency of 19% transient expression with 3.8% stable transformation was achieved in genotype GPU 45 using optimal conditions.

Ignacimuthu and Ceasar (2012) develop a transgenic finger millet plants conferring resistance to leaf blast disease by introducing a rice chitinase (*chi11*) gene under the control of maize ubiquitin promoter into finger millet through *Agrobacterium*-mediated transformation. They used *Agrobacterium* strain LBA4404 harboring *pHyg-Chi.11*. They obtained a transient expression frequency of 16.3% to 19.3% and stable expression frequency of 3.5% to 3.9% using the protocol developed by them in 2011. Integration of 3.1 kb of chitinase gene was confirmed by southern blot analysis.

Babu *et al.* (2012) infected embryogenic finger millet callus with *Agrobacterium* strain LBA 4404 harboring binary vector pCAMBIA1301 carrying PDH45 gene to develop transgenics for salt tolerance. Then calli explants were soaked in bacterial suspension supplemented with 100 µM acetosyringone for 3-4 minutes, co cultivated for 2 days and washed with 200mg/l cefotaxime. The co cultivated callus

were transferred to regeneration media containing 50 mg/l cefotaxime and 30mg/l hydromycin for three week in the dark.The surviving callus were regenerated in half strength MS media containing 0.5 mg.L-1BA, 3.0 mg.L-1 2, 4-D, and 30mg/l hygromycin, 40µg.mL-1 of cefotaxime incubated under light. Around one percent transformed calli survived on regeneration media containing hygromycin.

Hema *et al*. (2014) successfully transferred *mtlD* gene in fingermillet by using 45 day old embryonic callus as target explants for *Agrobacterium* strain EHA105 harboring pCAMBIA 1380 carrying *mtlD* gene.MS media with 3.0 mg/L auxin (2, 4-D) and 0.5 mg/L benzyl adenine (BA) was used for callus induction.Infection was carried out for 10 minutes in liquid co-cultivation medium supplemented with 100mM acetosyringone and bacterial suspension of 0.1 OD at 600 nm.Co cultivation was done for 48 hours in dark at 25 C.Sterile water with 300 mg/L cefotaxime is used for washing(4-5 times).Selection for transformed callus was carried out in antibiotic selection medium (MS+0.5 mg/L BA+3 mg/L 2, 4-D+ 30 mg/L hygromycin and 300 mg/L cefotaxime. Regeneration of transformed calli was carried out in presence of hygromycin with 0.5mg/l BAP for shooting and 0.5 BAP and 0.1mg/l NAA for 2 weeks.

Jalaja *et al.* (2016) used pearl millet's immature inflorescence derived embryogenic calli for transformation. Callus induction obtained by inoculating in MS media containing 2mg/l 2, 4-D, 3% sucrose and 2% agar, incubated in dark at 25±2℃. *A. tumefaciens* strains (GV2600), harboring vector pCAMBIA2300 with osmotin and chitinase and pCAMBIA 1300 containing *bar gene* as plant selection marker was used for transformation. The cell density of bacterial culture (O.D - 0.6), acetosyringone concentration of 100 µM, 15 min infection time, co-cultivation in dark for 2-days, followed by regeneration of callus after 15 days in regeneration media (MS Basal + Kinetin 2mg/l + NAA 0.2mg/l) containing 5mg/1 phosphinothricin. The percentage frequency of survival of explants was 5%.

Materials and Methods

Chapter 3 MATERIALS AND METHODS

This chapter covers the experimental details about the materials, procedures and techniques used for optimization of explants for *Agrobacterium*-mediated gene transfer in Finger millet PES-400.The experiments were carried out in teaching laboratory, Central Laboratory and Plant Tissue Culture Laboratory in the Department of Molecular Biology and Genetic Engineering, G.B Pant University of Agriculture and Technology,Pantnagar-263145 in the year 2017.Material and Methods for the present investigation are divided into following heads and sub-heads;

3.1 Equipments and Apparatus

Plastic wares used for the experiment were procured from Tarson,India .Glass wares were purchased from Borosil, India

Equipments and apparatus used in the present study were as follows:

(1)	Glasswares	
a)	Conical flasks	: 100 ml, 250 ml, 500 ml capacity
b)	Beakers	: 100-1000 ml capacity
c)	Measuring cylinders	: 10-1000 ml capacity
d)	Pipettes	: 0.1-1 ml capacity
e)	Petri plates	: 00x50 mm and 150x75 mm diameter
f)	Reagent bottles	: 100 and 500 ml capacity
g)	Jam bottles	: 500ml capacity
2)	Electronic balance	: 0.001-310 gm, Shimadzu
3)	Autoclave	: Labtech,korea
4)	Laminar air flow cabinet	: Lab Companion,BC-11,Korea
5)	pH meter	: EUTECH,Singapore
6)	Refrigerator	: LG

7) Microwave Oven : LG (GRILL), India

8) Centrifuge : Eppendorf, Germany

9) Camera : Nikon

10) Deep F reezer (-20) : Vestfrost, BSF -345,Denmark

11) Micropipette :Eppendorf

10) Other accessories : Forceps, scissors, spatulas, scalpel,Spirit lamp, aluminum foil, sterile blade and plastic measuring cylinders (50-1000 ml capacity),wash bottle, cotton, rubbers, blotting papers,and tissue paper.

3.2 Chemicals and Phytohormones

The Chemicals used were Plant tissue culture grade, molecular and analytical grade. Most of the chemicals and phytohormones were procured from Himedia. The chemicals (Acetosyringone, Agar, Cefotaxime, DMSO, Ethanol, Glycerol, Hg (Mercuric Chloride), Hygromycin, HCl (hydrochloric acid), kanamycin, MS media, NaOH(Sodium Hydroxide) pellet, Rifampicin, Sucrose, Tween-20, YEM Agar, YEM Broth and phytohormones (2,4 D; BAP, NAA and Kinetin) were purchased from Himedia.

3.3 Stock solutions and Storage condition

The stock solutions for antibiotics, phytohormones, phenolics to be used for the experiment were prepared by dissolving it in their respective solvents. A stock solution is a concentrated solution that will be diluted into a working concentration.

Table 3 .1 : Plant growth regulators and acetosyringone stock solution

S.No.	Chemicals/Hormones (Solvent)	Concentration (mg/ml)	Storage
1.	2,4 D(Ethanol)	1 mg/ml	4℃
2.	NAA	1 mg/ml	4℃
3.	BAP	1 mg/ml	4℃
4.	Kinetin	1 mg/ml	4℃
5	Acetosyringone(DMSO)	1Molar	-20℃

Table 3. 2 : Antibiotic stock solution

S.No.	Antibiotics	Stock Concentration(mg/ml)	Storage condition
1	Cefotaxime	100	-20℃
2	Hygromycin	50	4℃
3	Kanamycin	50	4℃
4	Rifampicin	3250	-20(dark)℃

3.4 Plant material

Eleusine coracana (L.) Gaertn

Common Name : Finger millet

Vernacular Name : Ragi

Variety :PES-400

The finger millet seeds of PES-400 were provided by the Department of Molecular Biology and Geneting Engineering, CBSH,G.B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand .

3.5 Agrobacterium strain and plasmid vector :

Agrobacterium strain harboring binary vector were provided by NRCPB,Pusa Campus, New Delhi- 110012 were used for standardizing essential basic condition such as acetosyringone concentration, infection time and co-cultivation time for transformation

1.LB 4404 harboring vector pCAMBIA 1301

2.EHA105 harboring vector pBI121.

Table 3.3: Bacterial strain used for transformation

Strain	Characteristics	Antibiotic resistance
Agrobacterium tumefaciens-EHA 105	Cured strain of A281 .Harbours pTiB0542 plasmid	Rifampicin
Agrobacterium tumefaciens-LBA 4404	Cured strain. Harbours pAL4404	Rifampicin

Table 3. 4 : Plasmid Vector used for transformation (Appendix III)

Vector	Bacterial selectable marker	Plant selectable marker
pBI121	Kanamycin	Kanamycin
pCAMBIA1301	kanamycin	Hygromycin

3 .6 Sterilization of plastic wares and glasswares

Jam bottles, conical flask and glasswares were washed with detergent and kept in oven for 2-3 hrs. Petriplates, forceps and scalpels were autoclaved at 121 degree Celsius for 15 minutes. Plastic wares were washed thoroughly and kept for drying.

3.7 Procurement of explants for *Agrobacterium* mediated transformation

Explants for callus induction : Seed, coleoptile, mesocotyl

Explants for *Agrobacterium* infection : Seeds, coleoptile, mesocotyl, callus/ embryonic callus.

3.7.1 Maintenance of aseptic condition

Inoculation of explants and all the aseptic manipulation were carried out in a vertical laminar flow chamber. The laminar was wiped with 70% ethanol. All the materials and media except the explants and *Agrobacterium* were kept inside the laminar and exposed to UV light for 15 minutes. The *Agrobacterium* used for transformation and contaminated cultures were disposed only after autoclaving.

3.7.2 Preparation of Callus induction media and Basal media

Murashige and Skoog (MS) is a plant growth medium formulated by Murashige and Skoog in 1962 .It provides all essential macroelements and microelements. Basal media contains all the compounds needed for the plant growth and development. The plant cells used the nutrients in the media as a building block or organic molecule synthesis and as a catalyst in enzymetic reactions. It includes certain compound that can only be made by an intact plant. The plant tissue culture growth medium consisted of 95% water, macroelements and microelements, vitamins, amino acids and sugars. MS media used in the study contains macroelements in the form of Ammonium nitrate, Potassium nitrate, Magnesium sulphate heptahydrate, Potassium dihydrogen phosphate as a source of phosphate, calcium chloride dehydrate. The microelements includes

Boron, Manganese, Molybdenum, Copper, Iron and Zinc provided in the form of Boric acid,Cobalt chloride hexahydrate, Copper sulphate pentahydrate, Manganese sulphate monohydrate,Ferrous sulphate heptahydrate, Zinc sulphate heptahydrate which plays a vital role in plant metabolism. Vitamins;Thiamine, nicotinic acid, pyridoxine act as enzymatic cofactors for essential metabolic function. Glycine serves as an amino acid source. Succrose is used as a carbohydrate source as most plant culture is unable to carry out photosynthesis effectively **(Murashige and Skoog, 1962).** The media with different hormone and hormone concentration were maintained at pH5.8, the most recommended pH for in vitro studies in plants **(Sharma *et al*., 2011).**

Solidifying Agent for Callus induction media : 0.8% Agar (plant tissue culture grade)

Table 3.5: Composition of Murashige and Skoog (MS) medium

CONSTITUENTS	**Quantity (mg L-1)**
Macro nutrients	
Ammonium nitrate	1650
Potassium nitrate	1900
Magnesium sulphate heptahydrate	370
Potassium dihydrogen phosphate	170
Calcium chloride dehydrate	440
Micro nutrients	
Boric acid	6.2
Manganese sulphate monohydrate	22.3
Zinc sulphate heptahydrate	8.6
Sodium molybdate dehydrate	0.25
Copper sulphate pentahydrate	0.025
Cobalt chloride hexahydrate	0.025
Potassium iodide	0.83
Iron source	
Ferrous sulphate heptahydrate	27.8
Na2EDTA	37.3
MS organics	
Glycine	2.0
Thiamine-HCl	0.1
Pyridoxine-HCl	0.5
Nicotinic acid	0.5
Carbon source	
Sucrose	30000

Callus Induction media

For callus induction different concentration and combinations of various growth regulators were added to MS media with 3% succrose, 0.8 % Agar. The composition and combination of hormone used for callus induction from matured seed, mesocotyl and coleoptile are listed below;

Table 3.6: concentrations and combinations of hormone used for callus induction

S. No	Hormone concentration (mg/l)	Stock:1mg/ml
		Working (for 500 ml)
1	-	
2	3(2,4 D)+1.5 NAA	1500/750
3	2.5(2,4 D)+2.5 NAA	1200/1200
4	4(2,4 D)	2000
5	3(2,4 D)	1500
6	0.25(2,4 D)	125
7	2.5(2,4 D)+0.5Kn	1200/250
8	4(2,4 D)+0.5 BAP	2000/250
9	3(2,4 D)+0.25 BAP	1500/125
10	3(2,4 D)+1 BAP	1500/500
11	1(2,4 D)+1 NAA	500/500
12	2NAA	1000
13	1.5 NAA	750
14	1 NAA	500
15	0.5BAP	250
16	1BAP	500

Procedure

1. The MS media powder for 1 litre was dissolved in 600 ml of water. Stirred with glass rod till it is completely dissolved in water.
2. Dissolved 30 g sucrose .Make the volume up to 1 litre
4. Different hormone concentrations and combinations were added as given in Table 3.1
5. The pH of the media was maintained at 5.8 with NaOH or HCl prior to autoclaving
6. 0.8 % agar was added to the callus induction media and media was melted in microwave oven.

7. Poured in jam bottles labelled with different hormone concentration as in the table (approximately 25 ml per jam bottle)
8. The jam bottles were capped loosely and kept for autoclaving
9. Autoclaving of the media was carried out at 121 C at 15psi for 15 minutes
10. Removed the jam bottles containing media from the autoclave
11. When the media has cooled down, the jam bottles were capped tightly.

3.7 .3 Sterilization of seeds

The husk of mature finger millet seeds were removed and washed with tap water 4-5 times. The seeds were soaked in water for 3-4 hours. The seeds were washed with distilled water. Two to three drops of Tween-20 was added and swirled for around 6 minutes. The seeds were washed again four to five times with double distilled water. The final sterilization of seeds with 0.1% HgCl2 was carried out in a laminar airflow .Swirled the petriplate for one minute and rinsed thoroughly with sterile double distilled water. The seeds were blotted dry with sterile tissue paper.

3.7.4 Germination of seeds

To get coleoptiles, mesocotyl, root and leaf explants, seedlings were grown *in vitro* in MS basal media. In a laminar air flow, 15-20 sterilized seeds were inoculated per jam bottle containing a basal media with the help of sterilized forceps. The inoculated bottles were kept in the dark for germination at 25˚ C in a culture room .The bottles were monitored every day. The contaminated jam bottles were removed immediately.

3.7.5 Inoculation of explants in callus induction media

Sterilized seeds, 6 days old coleoptiles and 15-20 days old mesocotyl explants were used for callus induction .The explants were transferred to the callus induction media, 5-10 explants per jam bottle for mesocotyl and coleoptile, 15-20 for seeds. Some bottles were incubated under photoperiod of 16 hour light and 8 hour dark while others were kept in the dark at 25˚C.

3.8 Proliferation of callus

For proliferation of induced calluses, the calluses were transferred to a proliferation media. The proliferation media contains the same concentrations and

combinations of hormone as in callus induction media. After every 20-25 days the callus was transferred to a fresh media. All the jam bottles were kept in the dark for proliferation

3.9 Antibiotic sensitivity test

The effects of different concentrations of antibiotics for each explant were tested. Triplicates were made for each antibiotic concentration by adding the desired antibiotic concentration in callus induction media. Growth was observed for 2 weeks and then sensitivity of the explants to antibiotics was scored based on the degree of browning/necrosis and callus induction rate.

The toxicity of different levels of the antibiotic to non-transformed finger millet seeds, mesocotyl, coleoptiles and callus were tested to identify the concentration levels of antibiotics that will be best for killing excess *Agrobacterium* without affecting the explants and for selecting transformed from non-transformed plants and callus tissues. The antibiotics were added in callus induction media (MS Media with 3% sucrose, 0.8%agar, 3mg/l 2,4 D and 1.5 NAA) The cultures were maintained in triplicates and incubated in the dark at 25° C. Antibiotics for which sensitivity test was performed were kanamycin, cefotaxime and hygromycin.

3.9.1 Kanamycin sensitivity test

To test the sensitivity of finger millet explants to kanamycin, concentration ranging from 0-400 mg/l (Table 3.2) were added in callus induction media. All the explants were inoculated and kept in dark at 25℃. The readings were taken after fifteen days.

Table 3.7: Kanamycin concentration used for toxicity test

S.no	Kanamycin concentration(mg/l)	Stock 50mg/ml
		Working concentration for 60 ml media (µL)
1	0	0
2	50	60
4	100	120
5	200	240
6	300	360
7	400	480

3.9.2 Cefotaxime sensitivity test

Cefotaxime concentration ranging from 0-500mg/l (Table 3.3) was added to the callus induction media to see the response of finger millet to cefotaxime. The explants were inoculated in the media and incubated in the dark at 25°C. Readings were recorded after 15 days of explants inoculation

Table 3.8: Cefotaxime concentration used for toxicity test

S.No.	Cefotaxime concentration(mg/l)	Stock 100mg/ml
		Working concentration for 60 ml media (µL)
1	0	0
2	200	120
4	250	150
5	300	180
6	350	210
7	400	240
8	450	270
9	500	300

3.9.3 Hygromycin sensitivity test

Most of the plants especially cereals are reported to exhibit higher sensitivity to hygromycin B than to kanamycin. In order to test the lethal concentration of antibiotic different concentration of hygromycin B was added in a callus induction media (Table 3.4). The seeds, coleoptiles, mesocotyl and callus were inoculated in the media. The final average reading of the callus induction, induction rate and color of explants were recorded after 7days, 15 days and 20 days

Table 3.9: Hygromycin concentration used for toxicity test

S. no	Hygromycin concentration (mg/l)	Stock 50mg/ml
		Working concentration for 60 ml media(µL)
1	0	0
2	15	18
4	20	24
5	25	30
6	30	36

3.10 *Agrobacterium* mediated gene transfer

The optimized concentration of media with plant growth regulators for callus induction and antibiotics were used for further optimization of acetosyringone, infection time and co-cultivation period

3.10.1 Growth and maintenance of bacterial culture

The optimal growth of *A. tumefaciens* is at 28°C. The bacterial cell experience heat shock at temperatures above 30°C which might cause errors in cell division. Depending on the type of bacterial culture media, aeration and culture condition, doubling time range from 2.5–4h **(Morton and Fuqua, 2012).** Yeast Extract Mannitol (YEM) and Yeast Extract Peptone (YEP) were commonly used for growing *A. tumefaciens.* Here YEM was used as a culture medium supplemented with 20 mcg/l rifampicin and 50 mcg/ml kanamycin was used for growing the bacteria .YEM is a rich medium that supports the growth of *A. tumefaciens* very well. LB can also be used but it has been demonstrated to cause irregular cell division. YEM medium shortens the doubling time of *A. tumefaciens* and can support higher cell densities **(Morton and Fuqua, 2012).**

Table 3.10 : Composition of YEM broth

Ingredients	Gms / Litre
Yeast extract	1.000
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100
Final pH (at 25°C) 7.0±0.2	

Table 3.11 : Composition of YEM agar

Ingredients	Gms / Litre
Yeast extract	1.000
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100
Agar	15.000
Final pH (at 25°C) 7.0±0.2	

YEM agar and broth preparation

1. In 20 ml double distilled water, 0.67 g YEM AGAR (for plate) and 0.236g YEM Broth (for broth culture) was added.
2. Swirled the conical flask and put a cotton plug.
3. Wrapped the cotton plug with a paper making sure that it is fully covered till the neck of the conical flask.
4. Autoclave for 15 minutes at 121psi.
5. Switched on the laminar air flow chamber and the blower. The laminar bench wiped with 70% ethanol. Kept all the requirements inside and switched on the UV for 15 minutes
6. The antibiotics were added as shown in the table 3.5 in YEM broth and YEM agar when the media has cooled down to around 40˚ C. Swirled conical flask for proper mixing and distribution of the antibiotics
7. Pour in sterile petri plates in laminar air flow. Wrap the plates and broth with aluminium foil and store at 4˚C.

Table 3.12: Antibiotic concentration for growing *Agrobacterium*

Antibiotics	Stock solution	Working solution	For 20 ml YEM broth/agar
1.Kanamycin	50 µg /ml	50 µg/l	4 µL
2.Rifampicin	3250 µg/ml	20 µg/l	1.23 µL

Bacterial culture

The bacteria culture was streaked into YEM agar plates. The culture were kept in incubator under dark condition at 28°C in the dark for 2 days. A single colony of bacteria was picked up from the plate and cultured in a broth in an orbital shaker (110 rpm) at 28°C in the dark .

O.D of bacteria was measured with spectrophotometer .Optical density of 0.7-0.9 was obtained after incubation for 42 hours.

3.10.2 *Agrobacterium* infection

Infection media

Infection media consist of half strength of MS media with 3% sucrose at pH 5.8 and different acetosyringone concentration. Different concentration of acetosyringone was added (Table 3.6) after the media has cooled down to around 40 degree Celsius.

Table 3.13: Concentration of acetosyringone added in infection media.

S.no	Concentration	STOCK:1M in DMSO
		Working solution for 20 ml of infection media
1	100 µM	2µL
2	150 µM	3 µL
3	200 µM	4 µL
4	300 µM	6 µL

Procedure

After the bacterial culture is grown up to an optical density of 0 .8 at OD600. Transferred the bacterial broth culture to an oakridge tube. This is carried out in a laminar air flow chamber.

1. Centrifuged at 10,000 rpm at 4°C for 10 minutes.
2. The pellets were re-suspended in half strength of MS media with different concentration of acetosyringone.
3. The explants were inoculated in the media with bacterial suspension for 15, 25, 30, 40 minutes each.
4. The explants were blotted dry with the Whatman filter paper.
5. Ten to fifteen explants were inoculated per Petri plates (co-cultivation media) .

Co-cultivation media: In order to enhance the transformation efficiency, acetosyringone of different concentrations (100 µM, 150 µM, 200 µM) were added to MS media with 3% sucrose, 0.8% Agar, 3 mg/L 24D and 1.5 mg/L NAA.

Table 3.14 :Concentration of acetosyringone added in co-cultivation media

S. No	Concentration (µM)	Stock:1 M
		(FOR 500ml)
1	100	50
2	150	75
3	200	100
4	300	150

3.10.3 Co-cultivation

In order to find out the optimum co-cultivation period, the inoculated petri plates in co-cultivation media were kept for co-cultivation under dark condition for 1, 2 and 3 days at 22˚C.

3.10.4 Elimination of *Agrobacterium*

After co-cultivation, the explants were washed with wash media (Sterile water with 300mg/ L cefotaxime) three to four times to kill *Agrobacterium* on the surface of explants. The explants were blotted dry with whatman filter paper and then transferred to a selection media.

3.10.5 Selection of transformed explants

For selection of transformed explants, the inoculated explants on a selection media (MS media, 0.8% Agar, 3% succrose, 3mg/l (2,4 D)+1 mg/l NAA, 25mg/l Hygromycin and 300 mg/l cefotaxime). The inoculated petri plates were kept in the dark at 25℃. The readings were recorded after 15 days of incubation.

Results and Discussion

Chapter 4 RESULTS AND DISCUSSION

The present study was undertaken with an objective to optimize the explants for *Agrobacterium* mediated gene transfer in finger millet PES 400. The explants used for optimization studies were; seeds, coleoptile, mesocotyl and callus. The type of explant used as a target for *Agrobacterium* mediated transformation must be competent and suitable for regeneration for successful recovery of whole transgenic plant. Various parameters known to influence the transformation efficiency were optimized using different concentration of plant growth regulators, antibiotics, acetosyringone, infection time, co-cultivation period and selection.

4.1 Optimization of different combination of hormones for callus induction

Different concentrations and combinations of hormones were used to find out which gives the best result. Sterilized viable seeds, coleoptile and mesocotyl were inoculated in the jam bottles containing callus induction media.MS media devoid of hormone was used as a control. Some of the inoculated jam bottles were kept in culture room at 25 ℃±1 with photoperiod of 16 hour light and 8 hours dark while others were kept in the dark.

Callus initiation was observed after 8 days of inoculation. All the three explants; seeds, mesocotyl, coleoptile were capable of producing callus in presence of 2, 4-D, NAA alone or in combination with Kinetin and BAP. The auxins 2, 4-D and NAA were essential for callus formation while cytokines alone did not show any callus induction. Callus induction frequencies varied with the type of plant growth regulator used, their concentrations and combinations.

Incubation of explants in dark showed better response than those kept in 16 hour light and 8 hours dark. This phenomenon has been reported by **Mohamed *et al.* (1992)**, **Padua *et al.* (1998)** and **Compton (1999)**. They suggested that incubation in the dark improved the morphogenetic capacity of the callus or explants by preserving endogenous light sensitive hormone or by preventing accumulation of phenolic compounds.

A good callus induction was observed in most of the hormone concentrations and combinations used in this present investigation (Table 4.1).100% callus induction was observed when seeds were inoculated in MS media supplemented with 3mg/l (2, 4 D)+1.5mg/l NAA, 1 mg/l (2, 4 D)+ 1mg/l NAA, 2.5 mg/l (2, 4D)+ 2.5 mg/l NAA (Figure 4.6), 0.25mg/l (2, 4 D), 2mg/l NAA, 3 mg/l (2, 4 D) (Figure 4.6) and 4 mg/l (2, 4 D). High callus induction frequency of 99.5%, 99 % and 98% was also observed in 2mg/l NAA, 1.5mg/l NAA and 2.5 mg/l (2, 4 D)+0.5mg/l Kinetin respectively. The size of the callus was biggest in MS media supplemented with 2 mg/l NAA (Figure 4.2)

The best response for coleoptiles were found in hormone concentration and combination of 3 mg/l (2, 4 D)+1.5mg/l NAA (Figure 4.4), 1mg/l (2, 4 D)+1mg/l NAA, 2.5 mg/l (2, 4D)+2.5 mg/l NAA, 2.5 mg/l (2, 4D)+0.5 mg/l Kinetin, 0.25 mg/l (2, 4 D), 2 mg/l NAA, 3 mg/l (2, 4 D) with 83.3%, 80%, 75%, 74.6%, 73.1%, 70%, 69.6% respectively. For mesocotyl best response was found in 3 mg/l (2, 4 D) (Figure 4.3), 1 mg/l (2, 4 D)+1 mg/l NAA, 3mg/l (2, 4 D)+1.5 mg/l NAA (Figure 4.4), 0.25 mg/l (2, 4 D), 2 mg/l NAA with 90%, 79%, 77%, 75%, 69.6% respectively. This observation contrast with the observation by **Dosad and Chawla (2015)** where they reported that the use of 2, 4 D alone showed poor callusing in finger millet PES 400 (Table 4.1).Greenish callus was observed when 3mg/l (2, 4 D) was used in combination with 0.25mg/l BAP (Figure 4.5).

After 30-40 days of inoculation, the calluses were transferred to a fresh media. Subsequent sub-culturing was done after every 25 days of culture. Somatic embryos are preferred for genetic transformation because somatic embryos usually originate from a single cell. It is a suitable target for genetic manipulation. The regenerated plants from somatic embryos are transformed uniformly with higher transformation frequencies and without chimeric variations **(Wilkins *et al*., 2004).** Embryogenic nodular callus was obtained after 45 days of incubation in a callus induction media containing 1.5 mg/l NAA (Figure 4.13), 3 mg/l (2, 4 D)+1.5 mg/l NAA (Figure 4.14), 2.5 mg/l (2, 4D)+2.5 mg/l NAA, 4 mg/l (2, 4 D) (Figure 4.15), 3 mg/l (2, 4 D), 2.5 mg/l (2, 4 D)+0.5 mg/l Kinetin, 1 mg/l (2, 4 D)+1 mg/l NAA (Figure 4.16), 2 mg/l NAA and 1 mg/l NAA.

In control (MS media devoid of hormone), mesocotyl regenerated into whole plant with excellent rooting after 25 days of inoculation (Figure 4.7). This observation was in concurrence with the report by **Mehta and Nag (2012)**. Mesocotyl derived calluses were more compact than the seed derived calluses.

Table 4.1 Callus Induction Frequency (%) at different hormone concentrations and combinations (S.C.IM: Seed callus induction media, C.C.IM: Coleoptile callus induction media, M.C.IM: Mesocotyl callus induction media, N.R: No Response)

S. No.	Hormone (mg/l)	Callus induction frequency (%)			Color	Embryonic
		S.C.I M	C.C.IM	M.C.I M		
1	3 (2, 4 D)+1.5 NAA	100±0	83.3±0.96	77±1.2	White	Yes
2	2.5 (2, 4D)+2.5 NAA	100±0	75±0.57	71.4±0.6	White	Yes
3	4 (2, 4 D)	100±0	63±2.6	60±1	White	Yes
4	3 (2, 4 D)	100±0	69.6±2.49	90±0.14	White	Yes
5	0.25 (2, 4 D)	100±0	73.1±0.85	75±2	White	No
6	2.5 (2, 4 D) +0.5Kinetin	98±2.6	74.6±3	62.5±1.5	Cream	Yes
7	4 (2, 4 D)+0.5 BAP	96±5.2	56±1.05	48±1.7	Brownish	No
8	3 (2, 4 D)+0.25 BAP	100±0	68.7±1.12	50±2.6	Greenish	No
9	3 (2, 4 D)+1 BAP	99±1	47±1.73	41±1.05	Brownish	No
10	1 (2, 4 D)+1 NAA	100±0	80±2	79±2	White	Yes
11	2 NAA	99.5±0.5	70±1.11	69.6±2.06	White	Yes
12	1.5NAA	99±2	55±2	50±2.64	White	Yes
13	1 NAA	80±2.64	50±1.99	53.9±1.1	White	Yes
14	0.5BAP	N.R	N.R	N.R	N.R	N.R
15	1BAP	N.R	N.R	N.R	N.R	N.R
16	1Kinetin	N.R	N.R	N.R	N.R	N.R
17	Basal media	N.R	N.R	N.R	N.R	N.R

$$\text{Callus Induction} = \frac{\text{Total number of explants inoculated}}{\text{Total number of callus induced}} \times 100$$

The best response and maximum number of embryogenic callus was obtained when 2, 4 D and NAA is used alone or in combination. Seeds inoculated in 1 mg/l, 1.5 mg/l and 2 mg/l NAA develop shoot, root as well as callus in the mesocotyl region. When NAA is used in combination with 2, 4 D, seed explant develops a callus with shoot. The best hormone concentration for all the explants (seed, coleoptile, mesocotyl) used in this study was obtained at 3mg/l 2, 4 D +1.5 mg/l NAA and 1mg/l 2, 4D + 1mg/l NAA but for seed 2NAA is the best. This result is similar to the observation by **Kothari *et al.* (2004)** and **Yemets *et al.* (2013)** where they have suggested the use of NAA for better regeneration. The phytohormone NAA is not commonly used for callus induction in cereals. However, it is beneficial for regeneration (**Yemets *et al.*, 2013).** The response of explants to different concentration and combination is shown in Table 4.1.

4.2 Proliferation

The calluses were transferred to proliferation media (same as the callus induction media) for proliferation of callus. The best proliferation of callus was observed in 2mg/l NAA (Figure 4.9), 1.5mg/l NAA (Figure 4.10), 1mg/l NAA (Figure 4.11), 1 mg/l NAA+1mg/l 2,4 D. Embryonic tissues were observed after 20 days of inoculation in the proliferation media. Excellent proliferation and induction of callus as well as root formation was observed when the mesocotyl and callus were inoculated in MS media supplemented with 1.5mg/l NAA (Figure 4.12).

4.3 Antibiotic Sensitivity Test

Sensitivity of finger millet explants to three antibiotics namely hygromycin, kanamycin and cefotaxime was tested by exposing the explants to different concentration of antibiotics. The *Agrobacterium* which was used for the present study; LB 4404 harboring binary vector pCAMBIA 1301 and EHA105 harboring binary vector pBI121 has a selectable marker hygromycin and kanamycin resistance gene present in the T-DNA region of binary vector.The proper choice of selectable marker is required for identification of right transformants **(Plaza-Wüthrich and Tadele, 2012)**. Hygromycin and kanamycin antibiotics are commonly used as a plant selectable marker while cefotaxime, a broad spectrum cephalosporin antibiotic is one of the most common antibiotic **(Nauerby *et al.*, 1997; Karami, 2008)** used for elimination of *Agrobacterium* after co-cultivation for successful recovery of transgenic cells and tissues **(Silva and Fukai, 2001; Karami, 2008)**.

Figure 4.1: Callus induction from mature embryos on MS media supplemented with 3mg/l 2,4 D (15 days old)

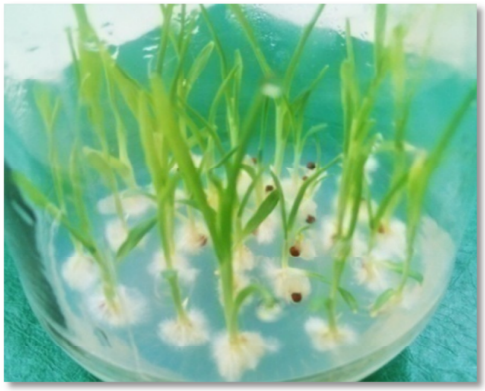


Figure 4.2:Callus induction from mature embryos on MS media supplemented with 2mg/l NAA (20 days old)

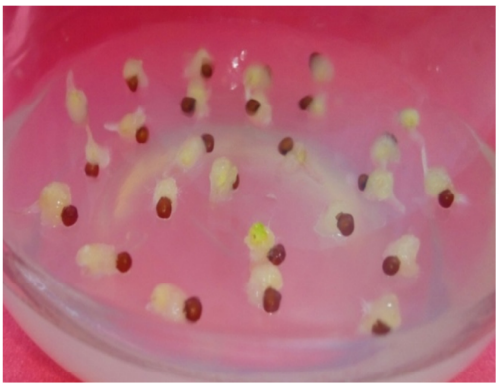


Figure 4.3:Callus induction from mature embryo on MS media supplemented with 2.5mg/l 2,4 D and 2.5mg/ l NA A

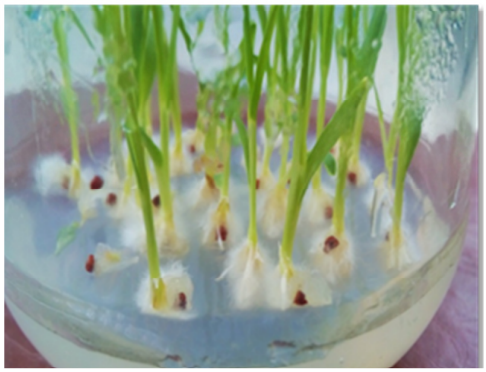


Figure 4.4 :Callus induction from mature embryo on MS media supplemented with 1.5mg/l NAA

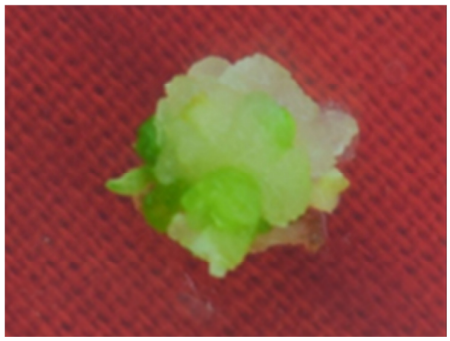


Figure 4.5: Green callus from mature embryo in MS media supplemented with 3mg/l 2, 4 D and 1.5 mg/l BAP

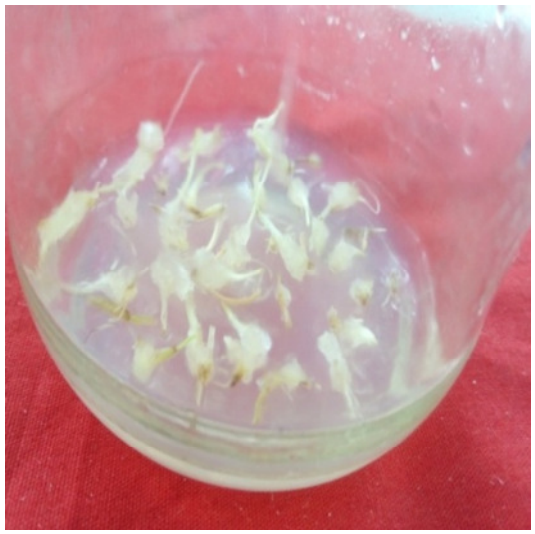


Figure 4.6: Callus induction from mesocotyl on MS media supplemented with 3mg/l 2,4 D

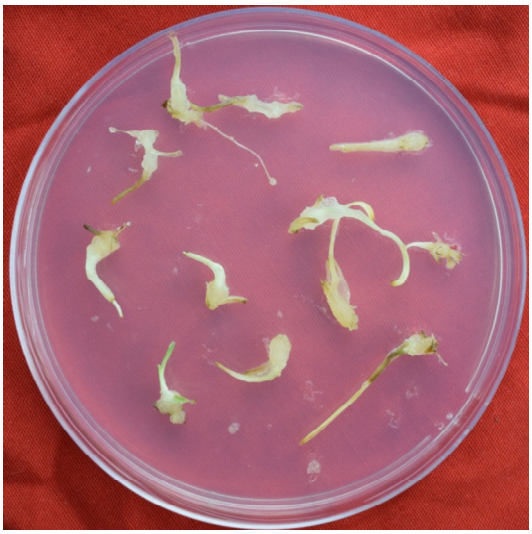


Figure 4.7: Callus induction from mesocotyl and coleoptile on MS media supplemented with 3mg/l 2,4 D and 1.5 mg/l NAA

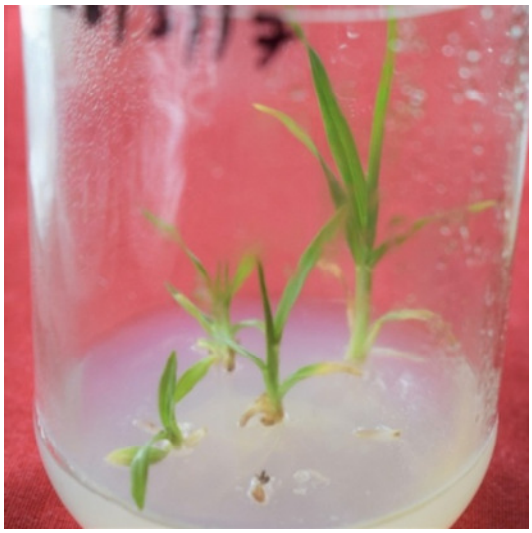


Figure 4.8: Mesocotyl inoculated in basal media regenerated into whole plant with excellent rooting

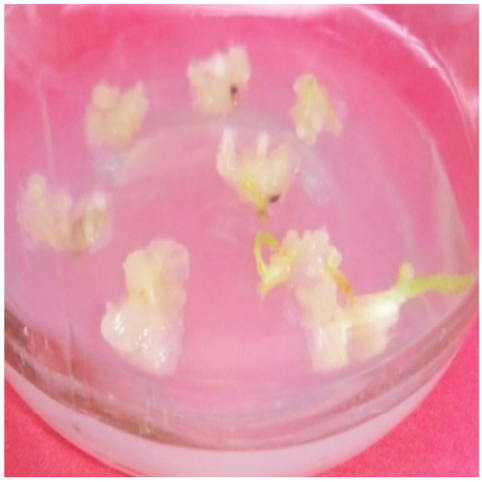


Figure 4.9:Proliferation of callus in MS media supplemented with 2mg/l NAA

Figure 4.10:Proliferation of callus in MS media supplemented with 1.5mg/l NAA

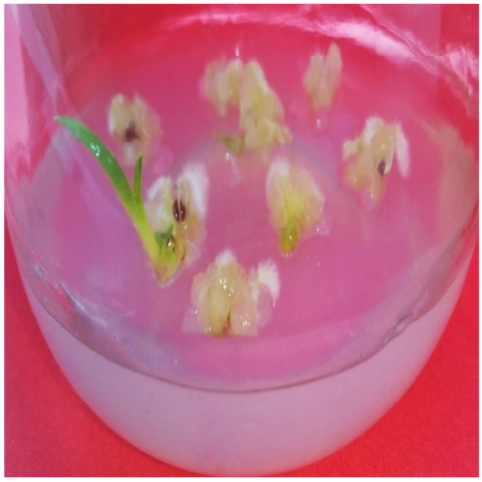


Figure 4.11:Proliferation of callus in MS media supplemented with 1mg/l NAA

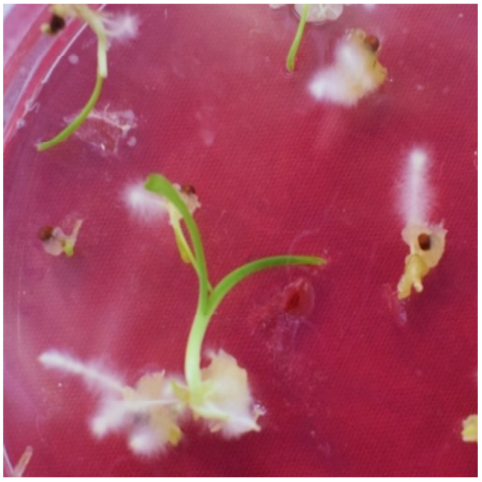


Figure4. 12: Formation of root from callus and mesocotyl in 1mg/l NA A

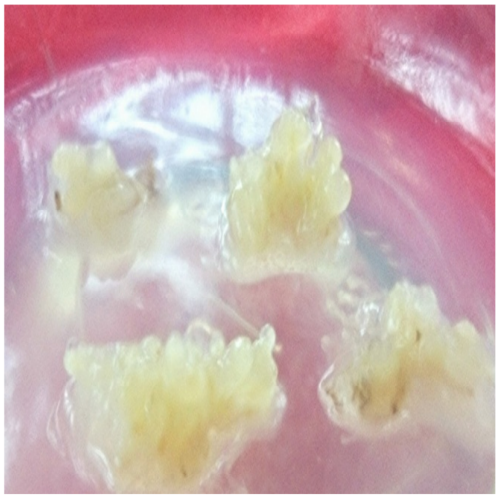


Figure 4.13: Embryonic callus in MS media supplemented with 1.5mg /l NAA

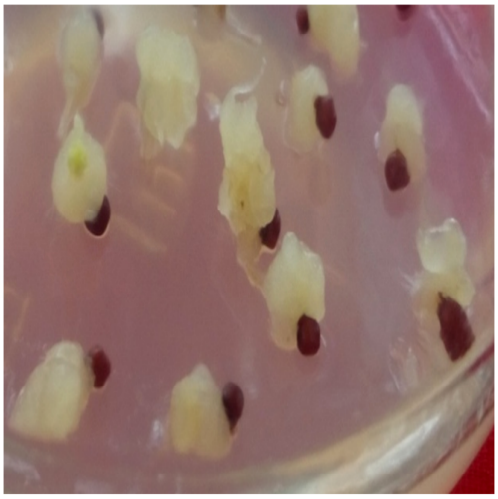


Figure 4.14: Embryonic callus in MS media supplemented with 3mg/l 2,4 D and 1.5 mg/l NAA

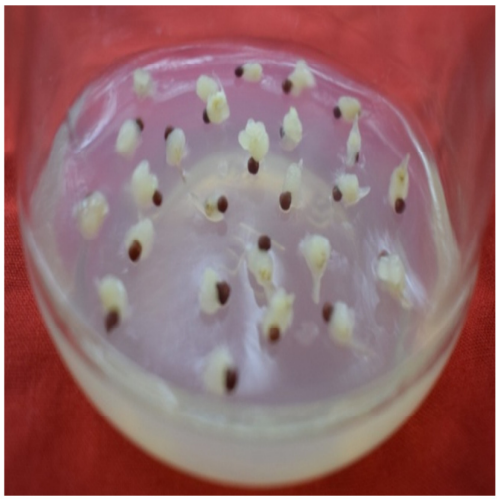


Figure 4.15: Embryonic callus in MS media supplemented with 4 mg /l 2,4D

Figure 4.16 :Embryonic callus obtained from 1mg/l 2,4 D and 1mg/l NAA

4.3.1 Sensitivity test for hygromycin

The binary vector pCAMBIA 1301 which was used for the investigation carries *hpt* gene in its T-DNA region which gives resistance to antibiotic hygromycin **(Waldron *et al*., 1985).** The ribosome of plant's mitochondria and chloroplast are similar to those ribosome found in bacteria. Like bacteria, these organelles are also susceptible to aminoglycoside antibiotics. Therefore, in presence of hygromycin B, transformed tissue with have *hpt* genes are expected to survive while those non-transformed explants will show a chlorosis due to lack of chlorophyll synthesis and inhibition of growth **(Benveniste and Davies, 1973; Brasileiro, 1998).**

The sensitivity of finger millet PES 400 explants to antibiotic hygromycin was tested to find out the optimal selection conditions for recovery of transformed explants in *Agrobacterium* mediated transformation. The explants were inoculated in a callus induction media supplemented with different concentration of hygromycin. Callus induction was observed in explants that were inoculated in a callus induction media without hygromycin (Figure 4.17 A). Browning of the explants was visible after seven days of inoculation in a selection media containing 20mg/l hygromycin (Table 4.2).The frequency and intensity of browning increased with the increase in concentration of hygromycin (Table 4.3).Callus induction from coleoptiles and mesocotyl was inhibited in presence of 15mg/l and above hygromycin concentration. The callus turned brown and stops proliferating. However, callus induction was observed in seed explants irrespective of the hygromycin concentration (Figure 4.18).

After 15 days of incubation, intense browning of coleoptiles, mesocotyl and callus explants was observed due to cell death at concentration above above 20 mg/l (Figure 4.19). The callus formed from the seed explants turns yellow as it comes in direct contact with the media (Figure 4.17 B- E). On the twentieth day after inoculation, 100% of the coleoptile, mesocotyl and callus explants showed intense browning in all the selection media supplemented with 15mg/l -30 mg/l hygromycin concentration. (Figure 4.13).However, some callus that was induced from seeds appeared white in color at 15mg/l and 20mg/l hygromycin. Based on this observation, 25 mg/l and above hygromycin concentration is an ideal concentration for selection of transformed tissues and elimination of non-transformed tissues. The same concentration (25 mg/l) was recommended by **Ceaser and Ignacimuthu (2011)** for selection of transformed tissues for two finger millet variety GPU 45 and CO 14.

Table 4.2: Hygromycin sensitivity test (SD: seed, COL: Coleoptile, MS: mesocotyl, CL: callus)

Hygromycin (mg / l)	Percentage of browning											
	Seventh day				Fifteenth day				Twentieth day			
	SD	COL	MS	CL	SD	COL	MS	CL	SD	COL	MS	CL
0	0	2	3	0	0	2	3	0	0	2	3	0
15	0	0	15	30	24	69	75	60	40	100	100	100
20	0	38	31	50	40	90	100	100	60	100	100	100
25	0	33	60	71.4	60	100	100	100	90	100	100	100
30	0	40	75	80	44.1	100	100	100	90	100	100	100

Table 4.3: Intensity of explants browning in presence of hygromycin [+ (yellow), ++ (light brown), +++ (dark brown), ++++ (intense brown), +++++ (black)]

Hygromycin concentration (mg/l)	Intensity of browning											
	Seventh day				Fifteenth day				Twentieth day			
	SD	COL	MS	CAL	SD	COL	MS	CAL	SD	COL	MS	CL
0	-	+	+	-	-	-	-	-	-	-	-	-
15	-	-	+	+	+	++	+++	++	++	++	+++	+++
20	-	+	+	++	+	+++	++++	++	++	+++	++++	+++
25	-	++	++	++	++	+++	++++	+++	+++	+++	++++	++++
30	-	++	++	++	++	+++	++++	+++	+++	++++	+++++	++++

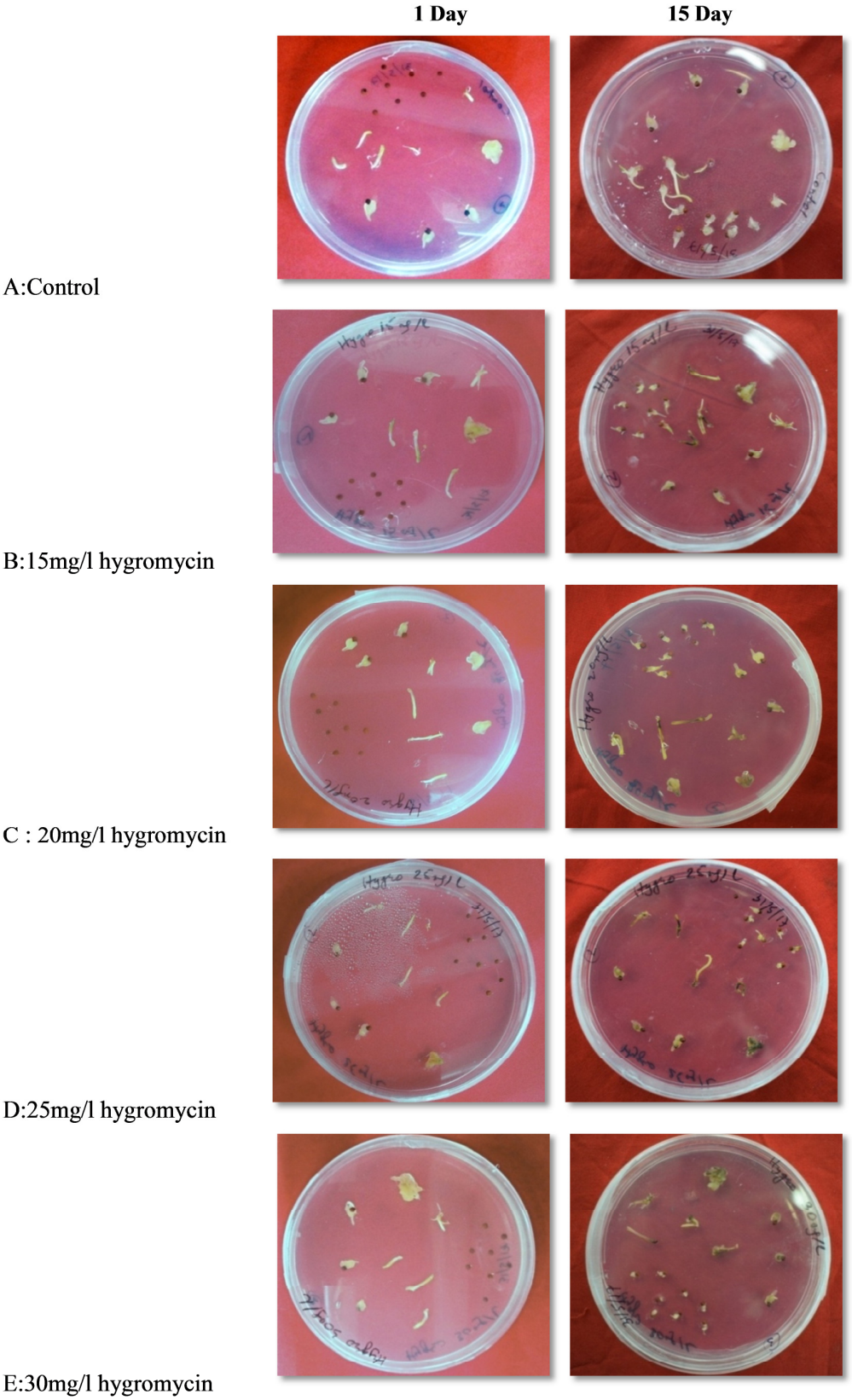


Figure 4.17:Sensitivity test for hygromycin

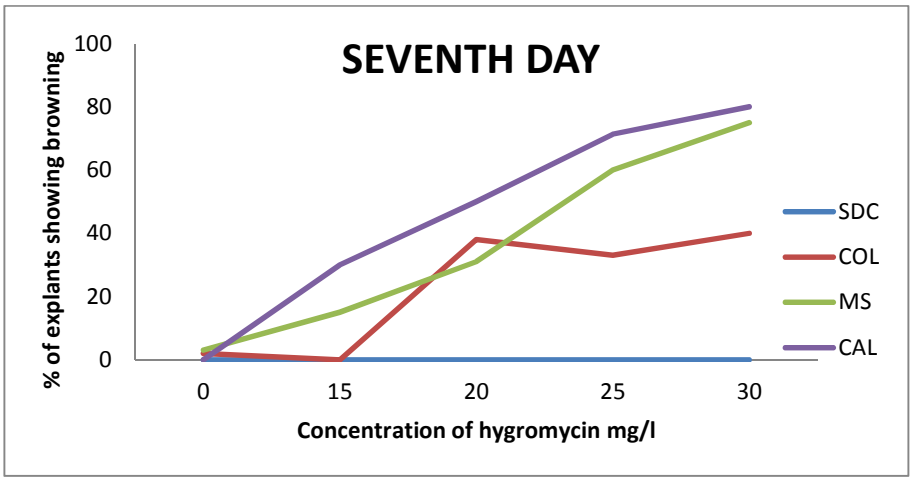


Fig 4.18: Percentage of explants showing browning after 7 days of inoculation in Callus induction media containing different concentration hygromycin.

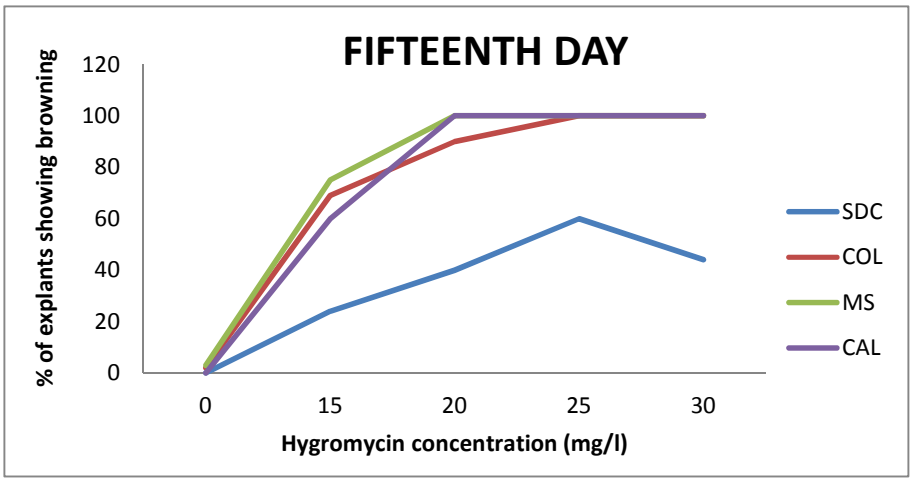


Fig 4.19: Percentage of explants showing browning after 15 days of inoculation in Callus induction

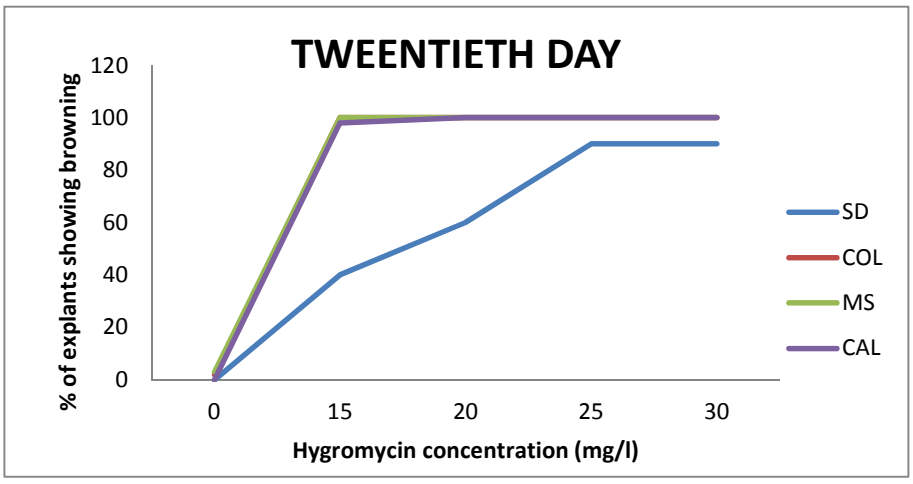


Fig 4.20: Percentage of explants showing browning after 20 days of inoculation in Callus induction media containing different concentration hygromycin.

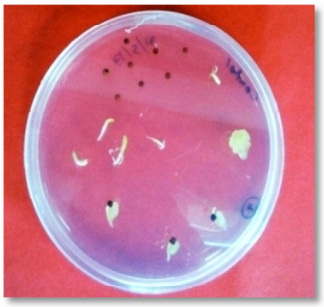
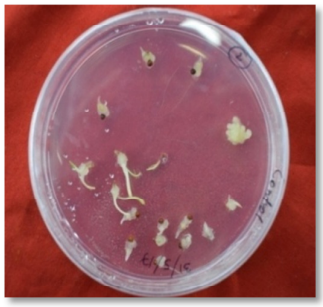
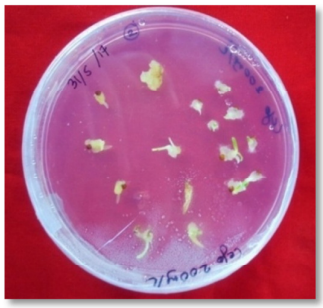
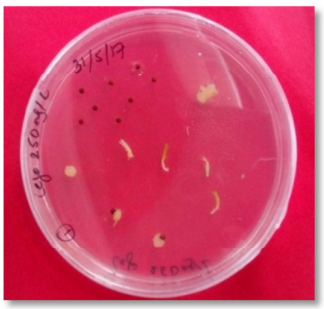
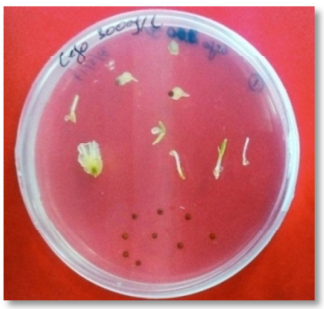
4.3.2 Sensitivity test for cefotaxime

Cefotaxime concentrations ranging from 200mg/l to 500 mg/l was added in the callus induction media to study its effect in plant cells. The observation was recorded after 15 days of inoculation in the dark at 25℃ (Table 4.4). Excellent callus induction of all the explants was observed. Initiation of embryonic callus from the callus was also observed. It indicates that cefotaxime does not have any harmful effects to finger millet explants. In fact, it enhanced the rate of callus induction and somatic embryogenesis (Table 4.4). Similar observation has been reported in wheat **(Mathias and Boyd, 1986)**, barley **(Mathias and Mukasa, 1987)** and pearl millet **(Pius *et al*., 1993)**.

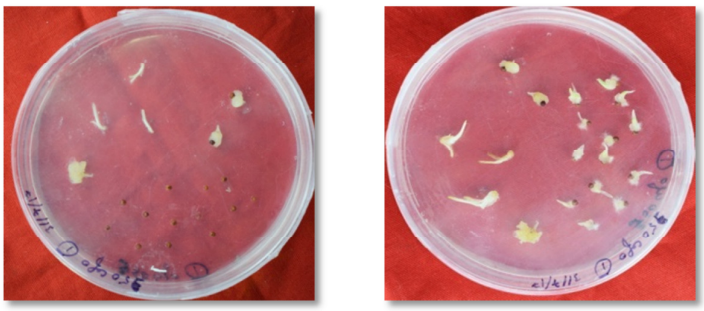
Yepes and Aldwinekle (1994) observed that cefotaxime enhanced regeneration and shoot development at a dose of 250 mg / l in apple. Similar to their observation, a much faster and better shoot development was observed at 200mg/l, 250mg/l and 300 mg/l cefotaxime concentration (Figure 4.22). So, any concentration at this range (100-500) can be used for elimination of *Agrobacterium* without causing any harm to the explants. Based on this observation, presence of cefotaxime concentration at 200mg/l and 250mg/l will be beneficial not only for the elimination of *Agrobacterium* but also for the regeneration process of the transformed explants.

Table 4.4: Response of explants to cefotaxime (C. I: Callus Induction, S.I: shoot initiation)

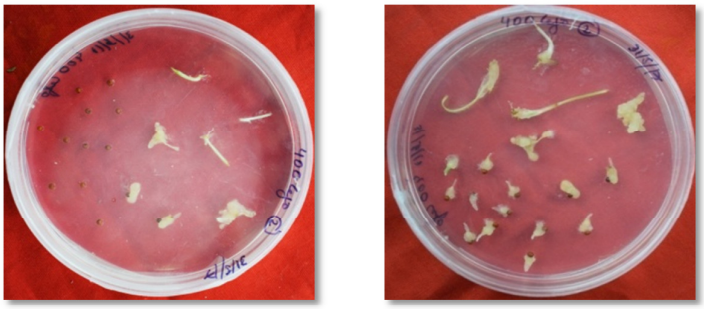
Cefotaxime concentration mg/ml	Callus induction (15 days after inoculation) Degree of induction /% of survival				C.I	S.I	Browning
	Seed	Coleoptile	Mesocotyl	Callus			
0	100	92	93	100	+++++	++	No
200	100	91	90	100	+++++	+++++	No
250	100	94	92	100	+++++	+++++	No
300	100	91	90	100	+++++	+++++	No
350	100	93	89	100	+++++	+++	No
450	100	92	90	100	+++++	+++	No
500	100	93	90	100	+++++	+++	No

	1 day	15 days
Control		
200mg/l cefotaxime		
250 mg/l cefotaxime		
300mg/l cefotaxime		

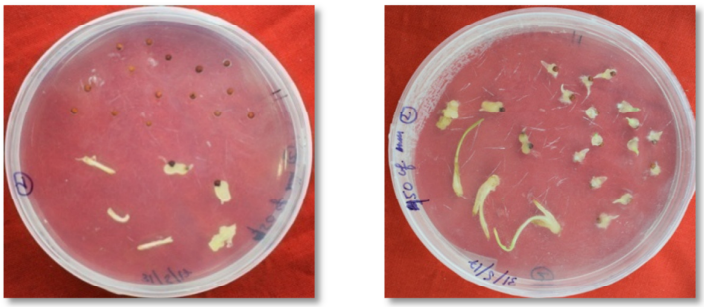
350mg/l cefotaxime



400mg/l cefotaxime



450mg/l cefotaxime



500mg/l cefotaxime

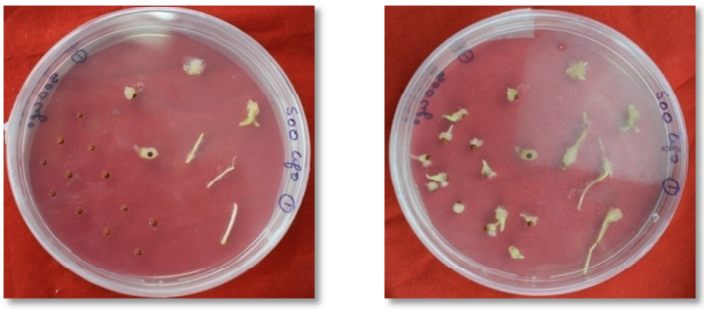


Figure 4.21:Explants after 15 days inoculation on callus induction media containing cefotaxime

A : 200mg/l

B : Control

C : 250mg/l

Figure 4.22: Formation of shoot from callus in callus induction media containing cefotaxime

4.3.3 Kanamycin toxicity test

Seeds, callus, 7 day old coleoptiles and 25 days old mesocotyl explants procured from seedlings grown in 0.8% agar with 3% sucrose was inoculated in a callus induction media supplemented with different kanamycin concentration ranging from 100-400 mg/l. **Sharma *et al*. (2010)** used nodular callus obtained from the seeds of *Eleusine coracana* PR-202 for studying the factors that influence *Agrobacterium* - mediated genetic transformation. After co-cultivation, they incubated the explants on modified maintenance medium without antibiotics without antibiotics for 10-15 days. For selection of transformed callus, they exposed the callus to increasing concentration of kanamycin (10 mg/l, 25 mg/l, 50 mg/l) with selection cycle of 15 days.

However, no browning of finger millet explants was observed in presence of kanamycin even at a very high concentration (400 mg/l) (Figure 4.24, Table 4.5) which indicates that finger millet (PES-400) is resistant to kanamycin. The percentage of seeds callus induction was almost perfect and the callus survival percentage was 100 (Figure 4.23).This result coincides with the result of **Jalaja *et al.* (2016)**. They reported that pearl millet callus was insensitive to kanamycin as it can survive even upto 300mg/l. However, there are reports by **Liu *et al. (*2007)** in foxtail millet and **Sharma *et al*. (2011)** in finger millet where kanamycin (50mg/l) is used for selecting transformed callus explants. Though other methods (Gus assay, PCR and southern hybridization) were used for analyzing transient and stable transformation

Table 4.5: Percentage of callus induction in callus induction media supplemented with different concentration of kanamycin

Kanamycin concentration mg/ml	Callus induction (15 days after inoculation). Degree of induction /% of survival				Browning
	Seed	Coleoptile	Mesocotyl	Callus survival %	
0	100	75	50	100	No
100	100	73	52	100	No
200	100	74	49	100	No
300	98	76	50	100	No
400	100	75	51	100	No

Mesocotyl explant showed poor callus induction rate even in control. This might be due to the quality of the explants since the mesocotyl explants is taken from overpopulated seedlings grown in 0.8% agar and sucrose. This observation may indicate that explants taken from a nutrient deficient has adverse effect on callus induction. **Ducan *et al*.** in **1985** observed a difference in the callus induction frequency of explants from nourished plants and malnourished plants.

Table 4.6: Degree of callus induction in callus induction media supplemented with different concentration of kanamycin. [++ (poor), +++ (moderate), ++++ (good), +++++ (excellent)]

Kanamycin mg/ml	Callus induction.Degree of induction/% of explants induced (15 days after inoculation)			Browning
	Seed	Coleoptile	Mesocotyl	
0	++++	++++	++	No
100	++++	++++	++	No
200	++++	++++	++	No
300	++++	++++	++	No
400	++++	++++	++	No

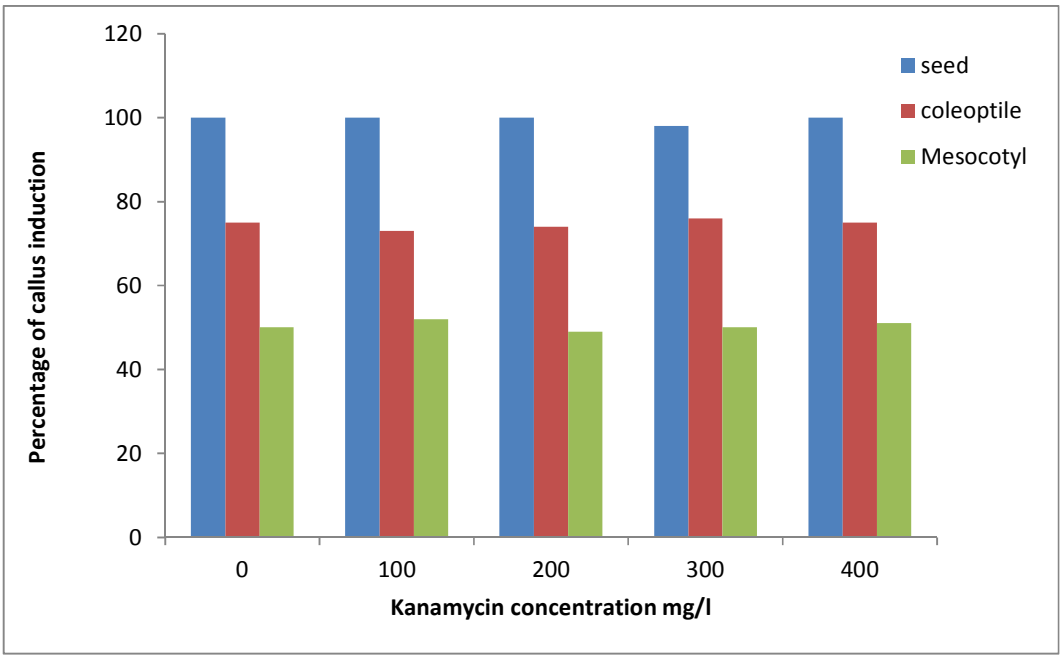
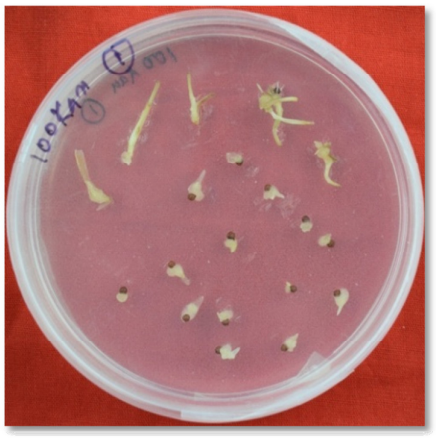


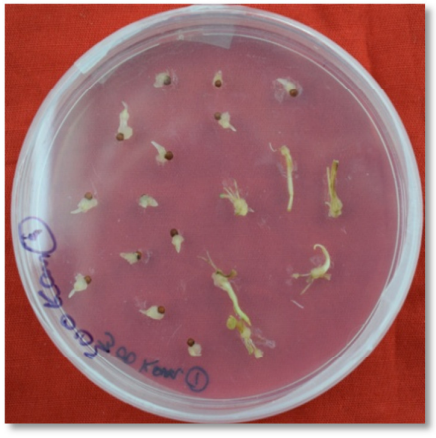
Figure 4.23 Effect of kanamycin on callus induction



100mg/l kanamycin

200mg/l kanamycin

Control



300mg/l kanamycin

400mg/l kanamycin

Figure 4.24: Explants after 15 days inoculation in callus induction media containing kanamycin

Binary vector pBI121 or any other vector which has only kanamycin antibiotic resistance gene (*npt II* gene) as a plant selectable marker will not be suitable if no other selectable marker or reporter gene or other gene of interest which confers new traits to the tissue is present in the T- D NA region.

4.4 *Agrobacterium* mediated gene transfer

The efficiency of agrobacterium transformation is influenced by many factors such as bacterial strain, cell density, antibiotics, inoculation time, co-cultivation time, acetosyringone concentration, plant species and genotype, explants (organs, cell and tissue types), light and temperature **(Karami, 2008).** The optimized hormone concentration for callus induction (3mg/l 2, 4 D+1.5 NAA), cefotaxime concentration (300mg/l) for elimination of *Agrobacterium* after co-cultivation and 25mg/l hygromycin for selection were used for optimizing acetosyringone concentration, infection time and co-cultivation time using LBA 4404 harboring pCAMBIA vector having hygromycin as a plant selectable marker. The co-cultivation and selection was carried out at 22˚C. The *Agrobacterium* strain EHA105 harboring pBI121 which has kanamycin as bacteria and plant selectable marker was not used for the present study as finger millet was found to be resistant to kanamycin. Finger millet in normal condition cannot survive in presence of hygromycin. Those explants which survived on selection media containing hygromycin were putative transformed explants

Growth of *Agrobacterium*

OD of 0.7-0.9 at 600 nm was obtained after 42 hrs of incubation in the dark at 28°C which is the optimum temperature for the growth of *Agrobacterium*. The bacterial cell experience heat shock at temperatures above 30°C which might cause errors in cell division. Depending on the type of bacterial culture media, aeration and, culture condition, doubling time range from 2.5–4h **(Morton and Fuqua, 2012).**

4.4.1 Effects of direct selection on explants

After co-cultivation, the explants were washed with 300 mg/l cefotaxime to remove excess *Agrobacterium*. The explants were then transferred to a selection media. There are two methods for selection of transformed tissues; direct and indirect method of selection. In Direct method, the explants were directly transferred to a selection media supplemented with selectable marker like hygromycin right after co-cultivation whereas Indirect method of selection allows the explants to grow for some days in callus induction media or proliferation media before exposing them to antibiotics.

Direct method of selection was used in this investigation. The explants were subjected to direction selection by transferring all the explants into a selection right after co-cultivation. The readings were recorded after 15 days of incubation in the dark at 22℃. The coleoptile and mesocotyl explants showed very poor callus induction. This condition has been observed by **Jha *et al.* (2011)** in *Pennisetum glaucum* when shoot apex used as target explants were cultured on a selection media right after co-cultivation. They suggested that the shoots must have suffered a combined stress of *Agrobacterium* and selection.To reduce this problem, they transferred the explants to recovery medium without hygromycin for 7days (indirect selection). **Sharma *et al.* (2011**) also incubated the finger millet callus on modified maintenance medium without antibiotics for 10-15 days after co-cultivation before transferring it into selection media.The callus initiation would have been better if explants were allowed to undergo a recovery phase after co-cultivation specially if the coleoptiles and mesocotyl are used as initial explants target for *Agrobacterium* transformation.

However, a study by **Raja *et al.* (2010)** in wheat reported the advantage of direct selection in wheat calli. They performed both direct and indirect method of selection to study the effect on transformation efficiency. When these two methods were compared, they found out that direct method of selection shows higher number of resistant callus. Till date, there is no report in finger millet where comparative study was conducted on direct and indirect method of selection. Further research can be done in this method of selection using different target explants and culture conditions.

4.4.3 Effect of infection time, co-cultivation time and acetosyringone concentration

Two controls were maintained for comparison of transformed and non transformed explants; Control 1: Explants inoculated in callus induction media without hygromycin and *Agrobacterium* infection. All the explants in control 1 showed good callus induction and proliferation (Figure 4.25A). ii) Control 2: Explants inoculated in callus induction media with hygromycin but without *Agrobacterium* infection (Figure 4.25B). iii) Control 3: Infection in absence of acetosyringone (Figure 4.25C and 4.25J). All the explants in control 2 and control 3turned brown after a week due to cell death.

Highest transformation frequency was observed in 30 minutes infection time while the lowest was observed in 15 minutes infection time irrespective of the acetosyringone concentration and co-cultivation time. The explants survival rate

increased from 25 minutes to 30 minutes infection time.15 minutes infection time was not enough for T-DNA transfer. The mesocotyl and coleoptiles survival rate was 0% at 15 minutes infection (Figure 4.25 Q and R). Overgrowth of *Agrobacterium* was observed in callus explant infected for 40 minutes and co-cultivated for 3 days but no *Agrobacterium* overgrowth was observed in mesocotyl and coleoptile explants co-cultivated for 3 days (Figure 4.26).

Transformation efficiency was drastically reduced due to overgrowth of *Agrobacterium*.Prolonged infection time adversely affect the explants because of *Agrobacterium* overgrowth **(Jha *et al*., 2011)**. The susceptibility of the callus for *Agrobacterium* colonization might be because of their uneven surface due to active cell division. It must have trapped a number of bacterial cells making it easier and more susceptible to be colonized. The infection time of 30 minutes was recommended by **Lee *et al*. (2006)** for orchard grass, **Jha *et al*. (2011)** for pearl millet, **Ceasar and Ignacimuthu (2011)** for finger millet, **Zhao *et al.* (2011)** for Chinese upland rice.

Addition of acetosyringone is essential for the transformation in finger millet. The explants infected with *Agrobacterium* in absence of acetosyringone do not produce any positive response irrespective of infection time and co-cultivation time (Figure 4.25C). Monocots did not synthesize phenolic compounds that induce the *vir* gene expression. Addition of phenolic compounds like acetosyringone during plant and bacterial interaction is necessary to stimulate the gene transfer **(Stachel *et al*., 1985)**. To enhance transformation efficiency acetosyringone was added in the infection and co-cultivation media at different concentration; 100 µM, 150 µM and 200 µM acetosyringone. The highest transformation efficiency was obtained in presence of 200 µM acetosyringone concentration. Increasing the concentration of acetosyringone from 100 to 200µM considerably improved the transformation frequency (Table 4.7-4.9). **Sharma *et al*. (2011)** and **Ahmadpour *et al*. (2015**) reported 200 µM acetosyringone to be the optimum concentration for finger millet and wheat respectively. While **Ignacimuthu and Ceasar (2012)** found 100 µM acetosyringone to be the most favorable concentration for *Agrobacterium* mediated transformation. The optimum concentration of acetosyringone varies as per the need of individual plant species **(Hiei *et al*., 1994, 1997).** Many researchers reported 400 µM acetosyringone to be the optimum concentration for wheat **(Lin and Zhang, 2005; Terada and Shimamoto, 2004; Raja *et al*., 2010**), pearl millet

(Jha *et al.* 2011, Ramadevi 2013). The variation in the requirement of acetosyringone for successful transformation may be due to differences in the infection time and co-cultivation duration and competence of target tissues **(Shrawat *et al.*, 2007).** Different studies have shown that acetosyringone below 100µM could not induce bacterial virulence, while high concentrations of acetosyrigone as high as 500µM is harmful for the bacteria and explant growth **(De Clercq *et al.*, 2002).**

Transformation Frequency (%)

$$= \frac{\text{Total number of explants surviving in selection media}}{\text{Total number of explants infected}} \times 100$$

Table 4.7: Effect of 100 µM acetosyringone, infection time and Co-cultivation time on transformation frequency (E.I: Explant inoculated, E.S: Explant survived, T.F: Transformation frequency, N.A: Not Applicable, O.A: Overgrowth of *Agrobacterium*)

Infection time Minutes	Co-cultivation time (1D:1 day, 2D:2 days, 3D:3 DAYS)												
	100 µM	SEED			COLEOPTILE			MESOCOTYL			CALLUS		
		1D	2D	3D	1D	2D	3D	1D	2D	3D	1D	2D	3D
15	E.I	35	35	35	32	30	36	34	42	38	36	39	45
	E.S	NA	NA	NA	0	0	0	0	0	0	0	0	1
	T.F%	NA	NA	NA	0	0	0	0	0	0	0	0	2.2
25	E.I	35	35	35	49	46	50	34	38	40	42	50	54
	E.S	NA	NA	NA	0	0	2	0	1	2	1	3	4
	T.F%	NA	NA	NA	0	0	4	0	2.6	5	2.3	6	7.4
30	E.I	35	35	35	46	44	48	36	33	34	50	48	51
	E.S	NA	NA	NA	0	0	2	0	1	2	2	3	5
	T.F%	NA	NA	NA	0	0	4.1	0	3	5.8	4	6.2	9.8
40	E.I	35	35	35	44	52	42	36	44	42	48	45	57
	E.S	NA	NA	NA	0	0	1	1	0	2	0	1	OA
	T.F%	NA	NA	NA	0	0	2.3	2.7	0	4.7	0	2.2	NA

Table 4.8: Effect of 150 µM acetosyringone, infection time and Co-cultivation time on transformation frequency (E.I: Explant inoculated, E.S: Explant survived, T.F: Transformation frequency, N.A: Not Applicable, O.A: Overgrowth of *Agrobacterium*)

Minutes	150 µM	SEED			COLEOPTILE			MESOCOTYL			CALLUS		
		1D	2D	3D	1D	2D	3D	1D	2D	3D	1D	2D	3D
15	E.I	35	35	35	38	40	42	30	32	32	42	48	38
	E.S	NA	NA	NA	0	0	0	0	0	0	1	2	2
	T.F%	NA	NA	NA	0	0	0	0	0	0	2.3	4.1	5.2
25	E.I	35	35	35	40	48	45	42	32	38	36	42	39
	E.S	NA	NA	NA	0	1	0	1	0	2	0	2	3
	T.F%	NA	NA	NA	0	2	0	2.3	0	5.2	0	4.7	7.6
30	E.I	35	35	35	42	46	44	40	42	32	43	38	48
	E.S	NA	NA	NA	0	1	2	0	1	2	2	3	5
	T.F%	NA	NA	NA	0	2.1	4.5	0	2.3	6.2	4.6	7.8	10.4
40	E.I	35	35	35	40	44	47	42	35	38	39	48	42
	E.S	NA	NA	NA	0	0	1	1	1	0	1	0	OA
	T.F%	NA	NA	NA	0	0	2.1	2.3	2.8	0	2.5	0	NA

Table 4.9 Effect of 200 µM acetosyringone, infection time and Co-cultivation time on transformation frequency (E.I: Explant inoculated, E.S: Explant survived, T.F: Transformation frequency, N.A: Not Applicable, O.A: Overgrowth of *Agrobacterium*)

Minutes	200 µM	SEED			COLEOPTILE			MESOCOTYL			CALLUS		
		1D	2D	3D	1D	2D	3D	1D	2D	3D	1D	2D	3D
15	E.I	35	35	35	40	42	50	34	40	38	36	33	39
	C.S	NA	NA	NA	0	0	0	0	0	0	0	1	2
	T.F%	NA	NA	NA	0	0	0	0	0	0	0	3	5.1
25	E.I	35	35	35	34	38	36	28	35	36	51	48	60
	C.S	NA	NA	NA	0	2	2	0	1	3	2	4	6
	T.F%	NA	NA	NA	0	5.2	5.5	0	2.8	8.3	3.9	8.3	10
30	E.I	35	35	35	44	48	45	40	34	31	45	51	46
	C.S	NA	NA	NA	1	2	3	1	2	2	2	6	7
	T.F%	NA	NA	NA	2.2	4.1	6.6	2.5	5.8	6.4	4.4	11.7	15.2
40	E.I	35	35	35	40	46	50	44	44	42	51	53	54
	C.S	NA	NA	NA	0	2	1	1	0	0	2	2	OA
	T.F%	NA	NA	NA	0	4.3	2	2.2	0	0	3.9	3.7	NA

The co-cultivation time is an essential factor influencing the competence of *Agrobacterium*-mediated gene transfer. The co-cultivation time beyond the optimal causes overgrowth of *Agrobacterium* and below drastically reduce the transformation efficiency as the duration is too short for the *Agrobacterium* to insert the T-DNA into the target cell. The extension of co-cultivation period from 1 day to 3 days increased the transformation frequency drastically (Table 4.7-4.9). Approximately 3 % increase in the transformation efficiency in callus explant was observed when co-cultivation period was extended from 2 days to 3 days. The co-cultivation for 3 days shows the highest transformation frequency while the lowest was observed in 1 day co-cultivation irrespective of the acetosyringone concentration and infection time. This result is in accordance with the observation by **Ceasar and Ignacimuthu (2011)** for finger millet**, Jha *et al.* (2011)** for pearl millet**, Wu *et al.* (2003)** and **Shrawat *et al.* (2007)** for barley callus where they reported 3 days to be an optimum co-cultivation period. Necrosis and cell death after *Agrobacterium* infection was the major limiting factor as it reduces the transformation efficiency drastically **(Dan, 2008).** The benefit of giving pre–treatment of target tissue with Anti necrotic solution has been demonstrated by **Sharma *et al.* (2011)** and **Ceasar and Ignacimuthu (2011)** in finger millet.

Among the four explants; seeds, mesocotyl, coleoptiles and callus, transformation frequency was highest in callus with the transformation efficiency of 15.2% while not much variation was observed in between coleoptiles (6.6%) and mesocotyl (6.4%) transformation efficiency (Table 4.9). Callus transformation frequency was higher than the coleoptiles and the mesocotyl irrespective of co-cultivation period, infection time and acetosyringone concentration. Seeds of finger millet were not a suitable target for *Agrobacterium* medium transformation. First of all, the seed's hard outer covering served as a barrier for infection. In order to soften the outer covering, the seeds were pre-cultured for 12 hours in a callus induction before infection. Co-cultivation of 3 days was enough for the seeds to start germination which provided an access for the *Agrobacterium* to come in contact with the living cells of the seeds. However, selection of transformed seed is difficult. After 15 days of inoculation in the selection media, all the other explants except the seeds shows drastic difference between the non-transformed and putative transformed explants as shown in Figure 4.25 and Figure 4.27. More than 20 days of incubation in the selection media will be

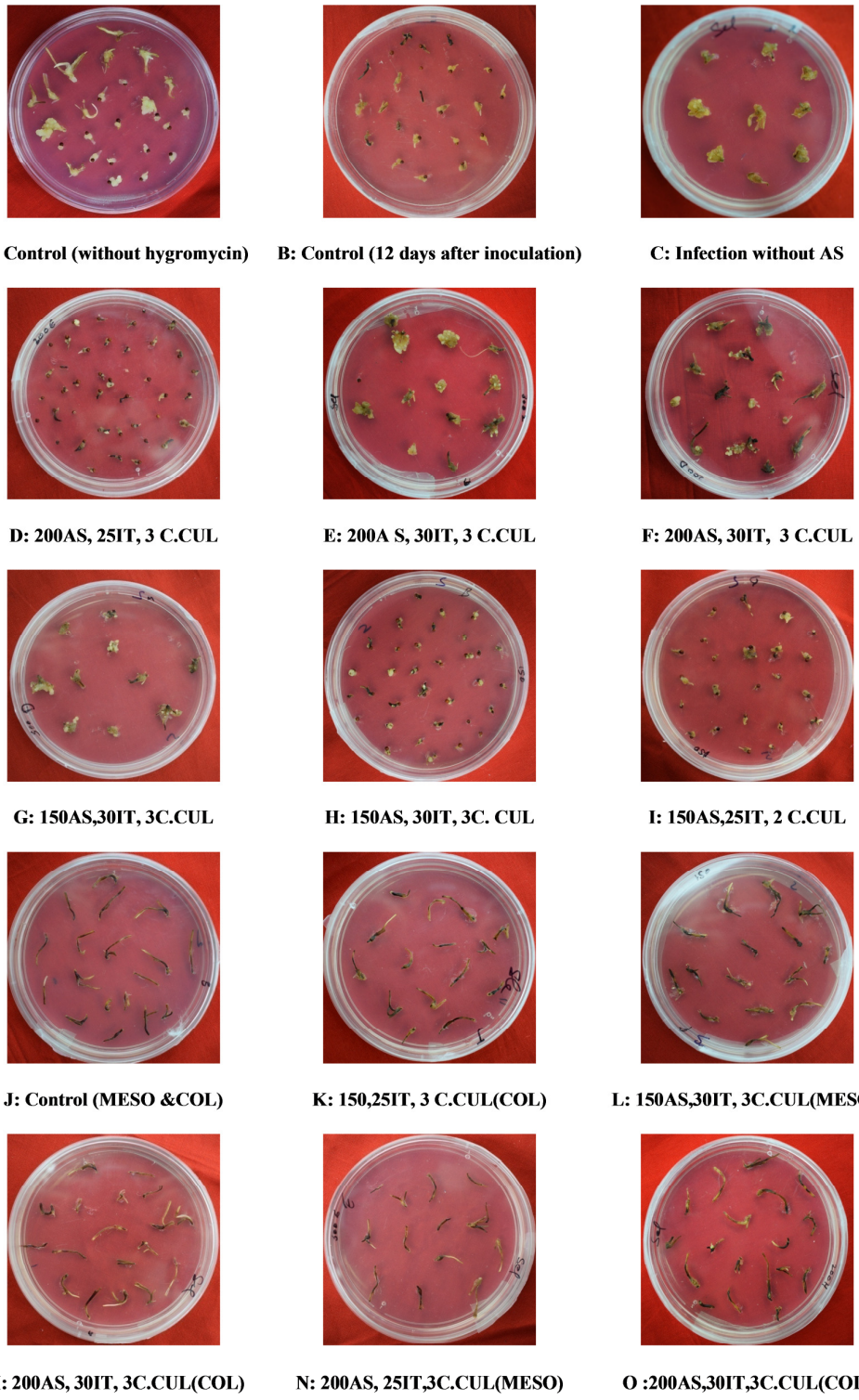


Figure 4.25: Explants after 15 days of inoculation in the selection media [AS:Acetosyringone, IT:Infection time (min.), C.CUL:Cocultivation time (Days), MESO:Mesocotyl ,COL: Coleoptile]

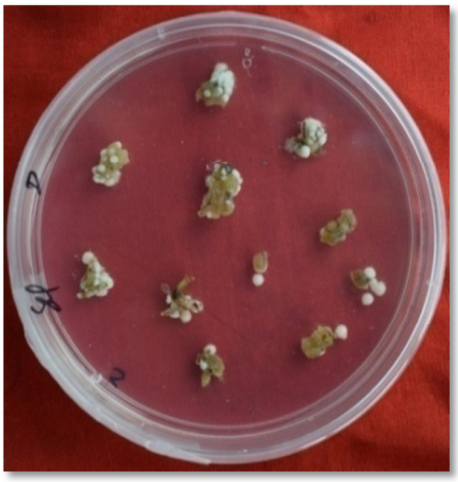
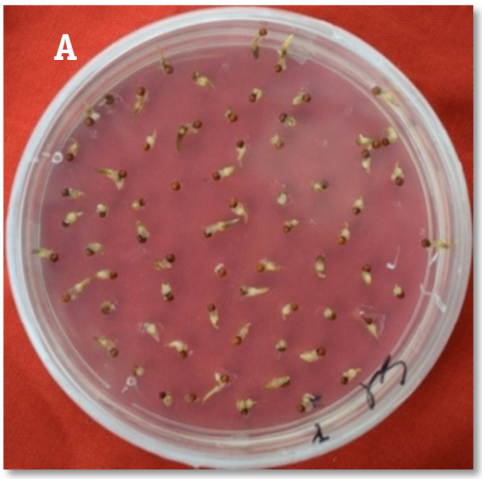


Figure 4.26: Overgrowth of *Agrobacterium* at 40 minutes infection time and 3 days co-cultivation



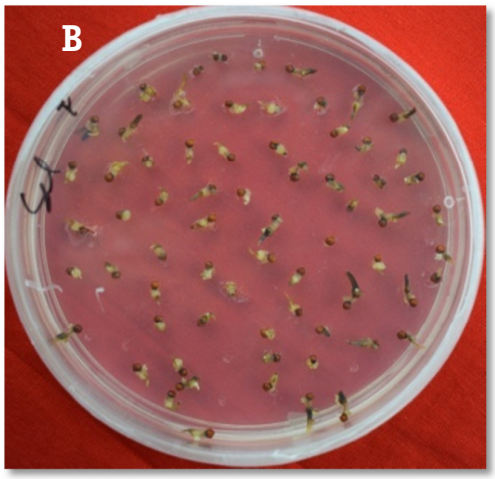


Figure 4.27: Seed explants after 15 days (A) and 20 days (B) of inoculation in the selection media

needed for selection if seeds are used as target explants. Selection on the fifteenth day is likely to give false positive result. Coleoptiles and mesocotyl explants were found to be more susceptible to tissue injury than callus, browning at the wounded site was observed. Some of the mesocotyl and coleoptiles explants must have died even before infection by *Agrobacterium*.

The present investigation entitled "Optimization of explants for *Agrobacterium* mediated gene transfer in *Eleusine coracana* (PES-400)" was focused on identification of the most suitable explants for *Agrobacterium* mediated transformation by finding out the optimum condition for various processes involved in *Agrobacterium* mediated transformation. Callus was the best target explant for *Agrobacterium.* Infection time of 30 minutes, acetosyringone concentration of 200µM and co-cultivation for three days were found to be optimum condition for *Agrobacterium* mediated transformation.

Summary and Conclusions

Chapter 5 SUMMARY AND CONCLUSION

Finger millet (*Eleusine coracana*) is a minor cereal crop belonging to the family Poaceae. It is the primary food source for millions of people living in tropical regions of Africa and Asia. Finger millet posses many desirable properties in terms of adaptability and nutrition. It is considered to be an ideal crop for food and nutritional security because of its high nutritive and satiety value, medicinal properties, excellent grain storage properties and a good resistance to biotic and abiotic stress. It is an agronomically sustainable crop adapted to a wide range of soil conditions and high altitudes. It can maintain optimum yields even in marginal lands, withstand drought and saline conditions, requires little irrigation and other inputs like fertilizers. Many genes involved in adaptation to biotic and abiotic stress can be fished out to use it for further improvement and development of superior crop variety.

Despite its beneficial effects and properties in terms of nutrition and adaptation, the yield is extremely low as they are mostly cultivated in marginal areas with poor moisture and fertility condition**.** The abiotic and biotic stresses are the two major limitations in cereal production faced by farmers and scientists, amounting to a yield loss of 30%-60% per year all over the world. Among this two, abiotic stresses are the biggest contributor to losses every year. It limits the crop plant from expressing their full genetic potential there by reducing their agricultural productivity. Millets have received little attention in comparison to other cereal crops such as rice, maize, wheat etc. and thus remain largely unimproved.

For genetic improvement of this important crop, development of efficient protocol for transformation is necessary. In monocots, particle bombardment and *Agrobacterium*-mediated transformation are commonly used method for genetic manipulation. Considering the many advantageous features, cost and convenience, *Agrobacterium*-mediated transformation method is a method of choice in this present investigation.

The present investigation entitled “Optimization of explants for *Agrobacterium* mediated gene transfer in *Eleusine coracana* (PES-400) was undertaken with the

objectives to optimize the hormone concentrations and combinations for callus induction in *Eleusine coracana, Agrobacterium* mediated gene transfer, optimization of antibiotic concentration (Hygromycin and Kanamycin; Selection of transformed callus and other explants, Cefotaxime: to remove excess *Agrobacterium* after infection, optimization of acetosyringone concentration for bacterial infection of explants, optimization of infection time for different explants, optimization of co-cultivation time and selection of transformed explants using selectable marker.

Here, we attempted to find out the best target material (explants) for *Agrobacterium*-mediated transformation using various parameters. The investigation was carried out in Teaching laboratory, Central laboratory and plant tissue culture laboratory in the Department of Molecular Biology and Genetic Engineering, G.B Pant University of Agriculture and Technology, Pantnagar in the year 2016-17.

The results obtained have been summarized below:

For the optimization of callus induction, Different concentrations and combinations of phytohormones 2, 4 D, NAA, Kinetin and BAP were used.MS media was used as a basal media with 3% sucrose solidified in 0.8 %agar, pH was maintained at 5.8.

100% callus induction was observed when seeds were inoculated in MS media supplemented with 3mg/l (2, 4 D)+1.5mg/l NAA, 1 mg/l (2, 4 D)+ 1mg/l NAA, 2.5 mg/l(2, 4D)+ mg/l 2.5 mg/l NAA(Figure 4.2), 0.25mg/l(2, 4 D), 2mg/l NAA, 3 mg/l(2, 4 D) and 4 mg/l(2, 4 D). 2 mg/l NAA, 1.5mg/l NAA and 2.5 mg/l (2, 4 D)+0.5mg/l Kinetin also showed a very high callus induction frequency of 99.5%, 99% and 98% respectively. The size of the callus was biggest in MS media supplemented with 2 mg/l NAA

The best response for Shoot tips were found in hormone concentration and combination of 3(2, 4 D)+1.5mg/l NAA, 1mg/l (2, 4 D)+1mg/l NAA, 2.5mg/l(2, 4D)+2.5mg/l NAA, 2.5mg/l(2, 4D)+0.5mg/l Kinetin, 0.25mg/l (2, 4 D), 2mg/l NAA, 3mg/l(2, 4 D) with 83.3%, 80%, 75%, 74.6%, 73.1%, 70%, 69.6% respectively.

For mesocotyl, best response was found in 3mg/l(2, 4 D), 1mg/l(2, 4 D)+1mg/l NAA, 3mg/l(2, 4 D)+1.5mg/l NAA, 0.25mg/l(2, 4 D), 2mg/l NAA with 90%, 79%, 77%, 75%, 69.6% respectively. The mature seeds show good callus induction in all the callus induction media.

Embryogenic callus formation was observed in MS media supplemented with 3(2, 4 D)+1.5 NAA, 2.5mg/l(2, 4D)+2.5mg/l NAA, 4mg/l (2, 4 D), 3mg/l (2, 4 D), 2.5mg/l(2, 4 D)+0.5mg/l Kinetin, 1mg/l(2, 4 D)+1mg/l NAA, 2mg/l NAA, 1.5mg/l NAA, 1mg/l NAA.

Mean callus induction rate for all explants was highest in 3mg/l (2, 4 D)+1.5mg/l NAA. So, this hormone concentration was used for optimizing other factors influencing *Agrobacterium* mediated transformation.

The calluses obtained were transferred to a callus proliferation media. Best proliferation was observed in MS Media supplemented with 1mg/l NAA, 1.5mg /l NAA, 2mg /l NAA, 1mg/l 2, 4 D + 1mg/l NAA.

Antibiotic sensitivity for cefotaxime was performed to optimize the concentration which will effectively eliminate the *Agrobacterium* without causing any harm to the explants after co-cultivation and selection. Sensitivity test for hygromycin and kanamycin was done to optimize their concentration (lethal dose) to use it for selection of transformed explants after infection and co-cultivation with the *Agrobacterium.* Explants were inoculated in callus induction media containing different concentration of antibiotics.The readings were recorded after 15 days incubation in dark at 25˚C.

Hygromycin sensitivity test: Finger millet explants were exposed to hygromycin (15mg/l-30mg/l) for 20 days. The coleoptiles and mesocotyl explants were the most sensitive, browning was observed on the seventh day followed by callus. Callus induction was observed in seeds but the callus turns brown and began to shrink. 100% of all explants turned brown on fifteenth day after exposure to hygromycin at 20mg/l except seeds. Hygromycin above 20 mg/l was found to be lethal for the explants.

Cefotaxime sensitivity test: Cefotaxime concentration ranging from 200-500 mg/l were added to the callus induction media. Callus induction and proliferation of all the explants were observed.This observation indicates that cefotaxime is not toxic for finger millet explants. Cefotaxime enhance the formation of shoot at the concentration of 200 and 250mg/l.

Kanamycin sensitivity test: Sensitivity test was performed for kanamycin concentration ranging from 100mg/l to 400mg/l Finger millet explants were found to be

resistant to kanamycin as the explants callus induction and survival was observed even in presence of 400 mg/l kanamycin concentration.

Agrobacterium strain LBA 4404 harboring plasmid vector pCAMBIA 1301was used for infecting the explants. Selection was carried out in presence of 300mg/l cefotaxime and plant selectable marker, hygromycin at 25 mg/l.

Acetosyringone concentration of 100 µM, 150 µM and 200 µM were added in the infection media and co-cultivation media. Efficiency of transformation increases with increase in acetosyringone concentration. The highest transformation efficiency was observed at 200 µM acetosyringone.

Infection time for 15, 20, 30, 40 minutes was performed. 15 minutes of infection shows the lowest transformation frequency while overgrowth of *Agrobacterium* was observed in 40 minutes. 30 minutes of infection was found to be optimal.

Co-cultivation; Explants were co-cultivated for 1, 2 and 3 days in a co-cultivation media. The highest transformation efficiency was observed in those explants co-cultivated for three days.

Among the four explants; seeds, mesocotyl, coleoptiles and callus transformation frequency were highest in callus with the transformation frequency of 15.2% while not much variation was observed in between coleoptiles (6.6%) and mesocotyl (6.4%) transformation frequency. The transformation frequency for seed was not recorded as selection was not possible in 15-20 days.

Conclusion

On the basis of the result from this study, it was concluded that callus was the best target explants for *Agrobacterium* mediated gene transfer in *Eleusine coracana* PES 400. Infection time of 30 minutes, acetosyringone concentration of 200µM and co-cultivation for three days were found to be the optimum condition for *Agrobacterium* mediated transformation at 22℃.

The most competent target explant for *Agrobacterium*-mediated transformation of finger millet PES 400 was successfully identified in this investigation. The standardized protocol can be used for improvement of this crop and for further studies of *Agrobacterium*-mediated transformation in finger millet.

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Appendices

APPENDIX I

CLASSIFICATION OF FINGER MILLET

SCIENTIFIC CLASSIFICATION

Kingdom	Plantae
(Unranked)	Angiosperm
(Unranked)	Monocots
(Unranked)	Commenelinids
Order	Poales
Family	Poaceae
Subfamily	Chloridoideae
Genus	*Eleusine*
Species	*Eleusine corana*

APPENDIX II

CLASSIFICATION OF *AGROBACTERIUM TUMIFACIENS*

SCIENTIFIC CLASSIFICATION

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhizobiales
Family	Rhizobiaceae
Genus	*Agrobacterium*
Species	*A. tumefaciens*

APPENDIX III

BINARY EXPRESSION VECTOR USED FOR THE STUDY

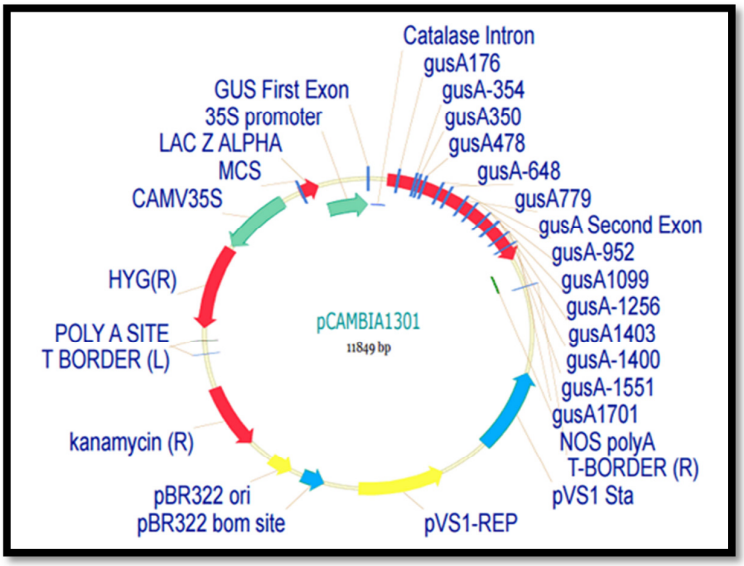

FIGURE A: Binary vector pCAMBIA1301
(Image source: Marker Gene Technologies, Inc)

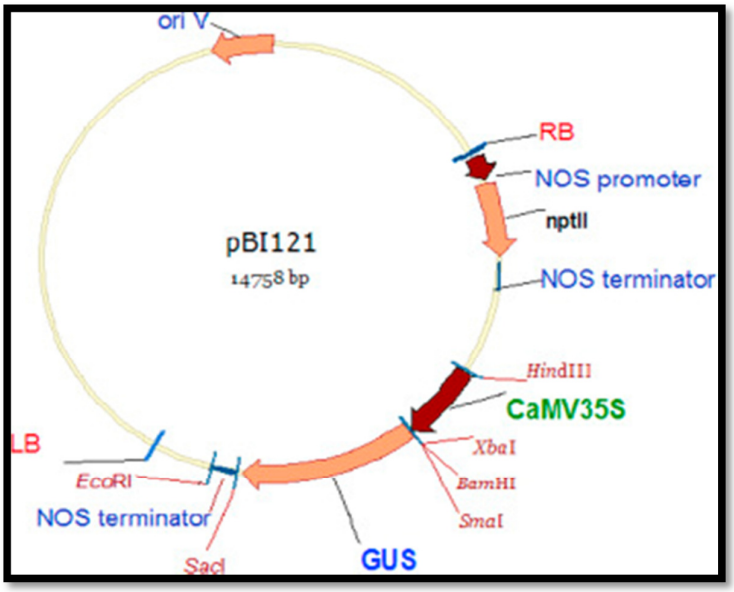

FIGURE B: Binary vector pBI121
(Image source: Rahnama *et al*., 2016)

APPENDIX IV

TABLE : THE TOP 20 MILLET-PRODUCING COUNTRIES IN THE WORLD IN 2013 (SOURCE: TADELE, 2016).

Country	Traditional millets[a]	Tef	Fonio (Acha)	Total
Production (ton)				
India	10,910,000			10,910,000
Ethiopia	848,956[b]	4,418,642		5,267,598
Nigeria	5,000,000		90,000	5,090,000
Niger	2,995,000		6,000	3,001,000
China	1,746,000			1,746,000
Mali	1,152,331		22,090	1,174,421
Burkina Faso	1,078,570		19,887	1,098,457
Sudan (former)	1,090,000			1,090,000
Guinea	215,000		429,000	644,000
Chad	582,000			582,000
Senegal	572,155		1,030	573,185
Russia	418,844			418,844
USA	418,145			418,145
Tanzania	322,731			322,731
Pakistan	310,000			310,000
Nepal	305,588			305,588
Uganda	228,000			228,000
Myanmar	185,000			185,000
Ghana	155,131			155,131
Cameroon	97,000			97,000
others	1,233,696		19,000	1,252,696
Total production	29,864,147	4,418,642[c]	587,007	34,869,796
Total area (ha)	33,118,792	3,016,521	554,451	36,689,764
Yield (ton ha^{-1})[d]	0.90	1.47	1.06	0.95

a Traditional millets include finger millet, foxtail millet, Indian barnyard millet, Japanese barnyard millet, kodo millet, little millet, pearl millet and proso millet.

b Only for finger millet.

c Only for Ethiopia.

d Average global yield except for tef where it is the national average yield for Ethiopia.

APPENDIX V

TABLE: DESCRIPTION AND BENEFITS OF MILLETS (SOURCE: TADELE, 2016).

Common name	Traditional millets			Other millets	
	Little millet	**Proso millet**	**Finger millet**	**Tef**	**Fonio**
Other names		Common millet	Ragi, African millet	Teff, lovegrass	Acha
Botanical names	*Panicum sumatrense*	*Panicum miliaceum*	*Eleusine coracana*	*Eragrostis tef*	*Digitaria exilis, D. Iburua*
Subfamily	Panicoideae	Panicoideae	Chloridoideae	Chloridoideae	Panicoideae
Tribe	Paniceae	Paniceae	Eragrostideae	Eragrostideae	Paniceae
Distribution					
Ploidy level	Tetraploid	Tetraploid	Tetraploid	Tetraploid	Diploid or hexaploid
Chromosome number	$2n = 4x = 36$	$2n = 4x = 36$	$2n = 4x = 36$	$2n = 4x = 40$	$2n = 2x = 30$ or $2n = 6x = 54$
Purpose	Food	Food, feed	Food, feed	Food, feed	Food, feed
Agronomic benefits	Abiotic stress tolerance	Drought tolerance, early maturity	Drought and salt tolerance	Water-logging tolerance, storage pest tolerance	Drought tolerance
Nutritional or	High in phytochemicals, fibre	Rich in amino acids	Rich in calcium, methionine and tryptophan	Rich in protein	Rich in amino acids
Health benefits	Anti-diabetic	Anti-cancer	Low glycaemic index, anti-oxidant	No gluten	

APPENDIX VI

TABLE: SUMMARY OF TRANSFORMATION STUDIES FOR ECONOMICALLY IMPORTANT MILLETS REGARDING EXPLANTS, METHOD AND PURPOSE OF TRANSFORMATION (SOURCE: PLAZA-WÜTHRICH AND TADELE, 2012).

Millet type (species)	Initial explant	Transformed explant	Method	Promoter	Purpose	Reference
Pearl millet (*Pennisetum glaucum*)	Immature embryo; pollen grain and shoot tip	Immature embryos; embryogenic cell suspension; embryogenic callus; pollen grain and shoot-tip clump	MB	Enhanced CaMV 35S, ZmAdh1, ZmUbi, CaMV 35S, OsAct, ZmMADS2	T	Taylor and Vasil (1991), Taylor et al. (1993), Dong et al. (1999), Devi and Stricklen (2002) and Schreiber and Dresselhaus (2003)
	Shoot tip; immature embryo; mature embryo and immature inflorescence	Embryogenic cell suspension, embryogenic callus, mature embryo	MB	CaMV 35S, ZmAdh1, Emu, ZmUbi, OsAct, double CaMV 35S, pin2	S	Lambe et al. (1995, 2000), Girgi et al. (2002, 2006), Goldman et al. (2003), O'Kennedy et al. (2004, 2011a, 2011b) and Latha et al. (2006)
Finger millet (*Eleucine coracana*)	Mature seed and shoot tip	Embryogenic callus	MB	ZmUbi, CaMV 35S, OsAct, RbcS, ppcA-L-Ft	S	Gupta et al. (2001), Latha et al. (2005) and Yemets et al. (2008)
	Mature seed	Green nodular callus	A (EHA105)	CaMV 35S	S	Sharma et al. (2011)
Barnyard millet (*Echinochloa crusgalli*)	Mature seed and leaf segment	Embryogenic callus and leaf segment	MB	ZmUbi, CaMV 35S, OsAct, RbcS, ppcA-L-Ft	S	Gupta et al. (2001)
	Cell line	Protoplasts	E	CaMV 35S	T	Hauptmann et al. (1987, 1988)
Guinea grass (*Panicum maximum*)	Immature embryo	Embryogenic cell suspension and embryogenic callus	MB	ZmAdh1, ZmUbi	T	Taylor et al. (1993)
Foxtail millet (*Setaria italica*)	Immature inflorescence	Embryogenic callus	A (LBA4404; EHA105)	Zm13, PF128	S	Liu et al. (2005), Qin et al. (2008) and Wang et al. (2011)

A: *Agrobacterium* transformation;
E: Electroporation
MB: Microprojectile bombardment
CaMV 35S: Cauliflower Mosaic Virus 35S
OsAct: rice actin ;
ZmAdh1: maize alcohol dehydrogenase 1
Emu: engineered based on truncated Adh1
pin2: potato proteinase inhibitor IIk (wound inducible)
ppcA-L-Ft: Flaveria trinervia phosphenolpyruvate carboxylase
RbcS: rice small subunit of ribulose 1, 5-biphosphate carboxylase
ZmMADS2: maize MADS-box gene 2 (pollen specific)
ZmUbi: maize ubiquitin.
S: Stable transformation of plants
T: Transient expression.

VITA

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ABSTRACT

Finger millet is the primary food source for millions of people living in tropical dry lands.It posses many desirable properties in terms of adaptability and nutrition. Finger millet can become a hub for fishing out vital genes involved in adaptation to biotic and abiotic stress which can be used for improvement and development of superior crop varieties. For genetic improvement of this important crop, development of efficient protocol for transformation is necessary. In this study, an effort was made to optimize the explants for *Agrobacterium* mediated gene transfer. Several experimental factors influencing the transformation efficiency were evaluated for four explants; seed, coleoptile, mesocotyl and callus. Among different hormone concentrations and combinations studied, the best callus induction rate for all the explants was observed in 3(2,4 D)+1.5 NAA while the size of the callus was biggest in 2 mg/l NAA .The best proliferation was observed in 1mg/l NAA, 1.5mg /l NAA, 2mg/l NAA and 1mg/l 2,4 D + 1mg/l NAA. Antibiotic sensitivity test for kanamycin showed callus induction and survival in presence of 400 mg/l kanamycin. Hygromycin above 20 mg/l was found to be lethal for finger millet explants. A good Callus induction of explants was observed in presence of 200mg/l-500mg/l cefotaxime. Cefotaxime enhanced the formation of shoot at the concentration of 200 and 250mg/l. *Agrobacterium* strain LBA4404 harboring pCAMBIA1301 and optimized callus induction media(3mg/l 2,4 D+1.5 mg/l NAA) and antibiotics (300mg/l cefotaxime and 25 mg/l hygromycin for selection) and were used for further optimization of acetosyringone concentration, infection time, co-cultivation time. Infection time of 30 minutes, acetosyringone concentration of 200µM and co-cultivation for three days were found to be optimum for *Agrobacterium* mediated transformation. Therefore, it can be concluded that callus is the best target explants for *Agrobacterium* transformation with the efficiency of 15.2 %.

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			विभाग	:	आणविक जीव विज्ञान एवं अनुवांशिक अभियांत्रिकी
मुख्य	:	आणविक जीव विज्ञान एवं जैव प्रौद्योगिकी			
शोधग्रंथ शीर्षक	:	इलेसुन कोराकाना (पी ई एस 400) में एग्रोबैक्टीरियम मध्यस्था जीन स्थनांतरण के लिये इक्सप्लेन्टस का अनुकूलन			
परामर्शदाता	:	डॉ0. पुष्पा लोहनी			

रागी उषणकटिबंधीय शुष्क भूमि में रहने वाले लोगें के लिए प्राथमिक खद्य स्रोत है। यह अनुकूलन क्षमता और पाषण में अनेक वांछनीय गुण प्रात्प हौ। रागी जैविक और अजैविक तनाव अनुकूलन में महत्त्वपूर्ण जीनों को प्राप्त कर के फसलों का सुधर और विकास करने में योगदान कर सकता है। आनुवंशिक सुधर इस महत्त्वपूर्ण फसल के परिवर्तन कार्य कुशल प्रोटोकॉल जरूरी है। इस अध्ययन में, एग्रोबैक्टीरियम मध्यस्था जीन स्थनांतरण इक्सप्लेन्टस को अनुकूलन करना प्रयास किय गया है। कई प्रायौगिक कारकों जो परिवर्तन कार्य कुशल को प्रभावित है उसको चार इक्सप्लेन्टस के लिए मूल्यांकन किय गया – बीज, कोलियपटिलइ, मेसोकोटिलइ और घटटा। विभिन्न हार्मोनों के बीच में सांदता और संयोजन अध्ययन करके सबसे अच्छा घटटा अधिषापन सब इक्सप्लेन्टस में 3(2,4 D)+1.5 NAA में देखे गए और घटटा की आकार सबसे बडी 2 mg/l NAA में देखे गए। सबसे अच्छा प्रसार 1mg/l NAA, 1.5mg /l NAA, 2mg/l NAA and 1mg/l 2,4 D + 1mg/l NAA में देखी।एंटीबायोटिक संवेदनशीलता जॉच केनामाइसिन के लिए घटटा अधिषापन और उतरजीविता 400 mg/l केनामाइसिन कि उपस्थिति में देखे गए । हाइग्रोमइसिन 20 mg/l से उपर रागी इक्सप्लेन्टस के लिए घातक देखे गए। अच्छा घटटा अधिषापन इक्सप्लेन्टस 200mg/l-500mg/l सिफोटैक्सीमइ उपस्थिति में देखे गया।सिफोटैक्सीमइ 200 और 250mg/l मात्रा में शूट कि गठन को बढ़ाति है। LBA4404एग्रोबैक्टीरियम स्टैन pCAMBIA1301शरण और अनुकूलित घटटा अधिषापन मीडिया (3mg/l 2,4 D+1.5 mg/l NAA) और एंटीबायोटिक (300mg/l सिफोटैक्सीमइ और 25 mg/l मात्रा हाइग्रोमइसिन चयन के लिए) उपयोग किया गया। एग्रोबैक्टीरियम मध्यस्थता परिवर्तन के लिए 30 मिन संक्रमण कि समय, 200µM ऐसटोसारिनगॉन कान्सन्टैशन और तीन दिन कि को कल्टवेशन अनुकूलतम देखे गया। इसलिये यह निष्कर्ष निकाला कि घटटा सब से अच्छा इक्सप्लेन्टस एग्रोबैक्टीरियम परिवर्तन के लिए 15.2 % के कार्यक्षमता के साथ

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