Studies on tissue culture and *Agrobacterium*-mediated genetic transformation in safflower (*Carthamus tinctorius* L.)

Thesis submitted to the University of Hyderabad for

the award of the degree of Doctor of Philosophy in Plant Sciences

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Certificate

This is to certify that I, **Sri Shilpa Katragadda** have carried out the research work embodied in the present thesis entitled "**Studies on tissue culture and *Agrobacterium*-mediated genetic transformation in safflower (*Carthamus tinctorius* L.)"** and submitted for the degree of **Doctor of Philosophy** was accomplished for the full period prescribed under Ph.D ordinances of the University, under the supervision of Dr. M. Sujatha at the Directorate of Oilseeds Research, Rajendranagar, Hyderabad and in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma of any university.

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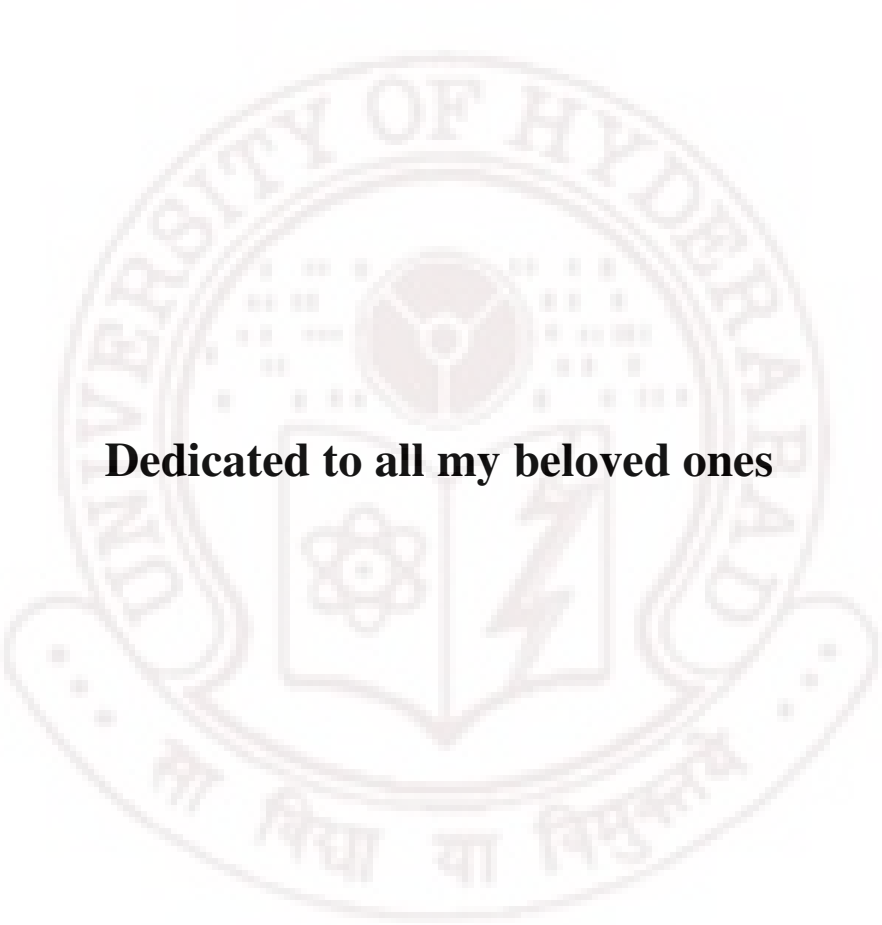

Dedicated to all my beloved ones

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Sri Shilpa Katragadda

Abbreviations

µg	: Microgram
µl	: Microlitre
µM	: Micromolar
µm	: Micro meter/micron
2,4,5-Cl_3POP	: 2,4,5-Trichlorophenoxypropionic acid
2,4-D	: 2,4-Dichlorophenoxyacetic acid
2iP	: N^6-[2-Isopentenyl]adenine/6-γ-γ-(Dimethylallylamino)-purine
$AgNO_3$	: Silver nitrate
B5	: Gamborg B5 medium
BAP	: 6-Benzylaminopurine/N6-Benzyladenine
bp	: Basepairs
BSA	: Bovine serum albumin
cDNA	: Complementary DNA
CH	: Casein hydrolysate
CRD	: Completely randomized design
CTAB	: Cetyltrimethylammonium bromide/N-Cetyl-N,N,N,-trimethylammonium bromide
CV	: Coefficient of variation
DMF	: N,N-Dimethylformamide
DMRT	: Duncan's multiple range test
DMSO	: Dimethyl sulfoxide/Methyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxyribonucleotide triphosphates
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
FAA	: Formalin acetic acid alcohol
GA_3	: Gibberellic acid 3/Gibberellin A_3
hr	: Hour
IAA	: Indole-3-acetic acid/3-Indoleacetic acid
IBA	: Indole-3-butyric acid/4-(3-Indolyl)butanoic acid
kan	: Kanamycin monosulphate monohydrate
KN	: 6-Furfurylaminopurine/N6-Furfuryladenine
l	: Litre
LB	: Luria Bertani
LS	: Linsmaier and Skoog medium
M	: Molar
mg	: Milligram

min	: Minutes
mM	: Milli molar
MS	: Murashige and Skoog medium
MSG	: Murashige and Skoog Germination medium
NAA	: 1-Naphthaleneacetic acid/α-Naphthaleneacetic acid
ºC	: Degree centigrade
OD	: Optical density
PCR	: Polymerase chain reaction
PGA	: Phloroglucinol/1,3,5-Trihydroxybenzene
RNA	: Ribonucleic acid
RT PCR	: Reverse transcriptase PCR
rpm	: Revolutions per minute
SDS	: Sodium dodecyl sulfate/Dodecyl sodium sulfate
sec	: Seconds
$T_{10}E_1$	: Tris-EDTA
TBA	: Tertiary Butyl alcohol/2-Methyl-2-propanol
TDZ	: Thidiazuron/N-Phenyl-N'-1,2,3-thiadiazol-5-ylurea
Thiamine-HCl	: Thiamine hydrochloride
TIBA	: 2,3,5-Triiodobenzoic acid
T_m	: Melting temperature
X-GlcA	: 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YEP	: Yeast extract peptone

Contents

Chapter 1
Introduction

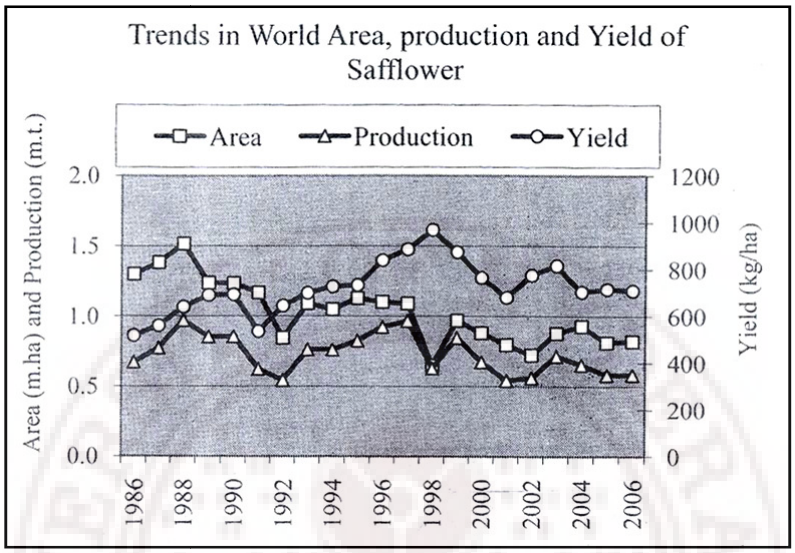
Safflower (*Carthamus tinctorius* L.) is one of the most important oilseed crops of the semi-arid regions belonging to the family Asteraceae (Compositae). The genus *Carthamus* is composed of about 25 species that are indigenous to the Mediterranean region and distributed from Spain to North America, West Asia, India. Safflower is one of the world's oldest crops, and the seeds have been found in Egyptian tombs over 4,000 years old and its use was recorded in China approximately 2200 years ago. Based on the closely related wild species, safflower was believed to have originated in an area bound by the Eastern Mediterranean and Persian Gulf, encompassing Southern parts of former USSR, Western Iran, Iraq, Syria, Southern Turkey, Jordan and Israel (Knowles, 1969). Presently, safflower is cultivated on a commercial scale in India, USA, Mexico, Ethiopia, Australia, China, Argentina and Russia and to a limited extent in Pakistan, Italy, Spain, Portugal and Iran. The cultivated safflower (*C. tinctorius* L.) was believed to have originated from *C. lanatus* and *C. oxyacantha* (Weiss, 1971; Knowles and Schank, 1964).

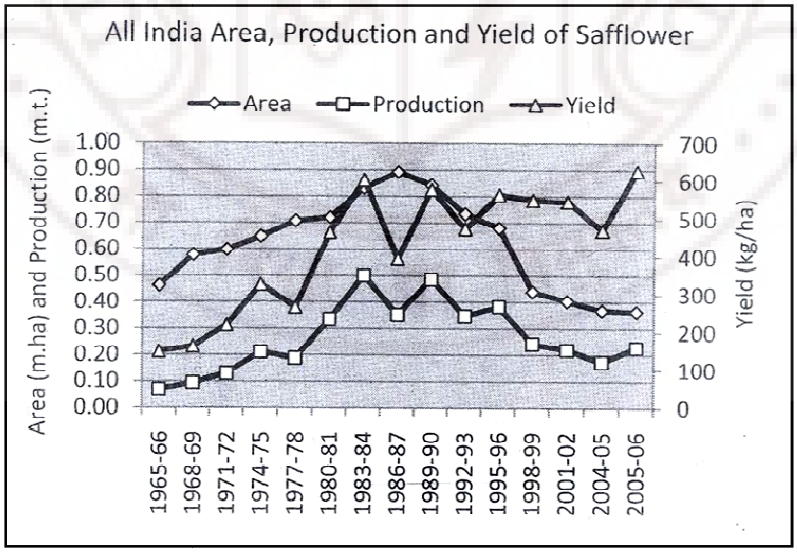
India is the largest safflower producing country in the world and ranks first in the world with an area of 420 thousand hectares accounting for 51.1% of total area in the world (822 thousand hectares) and with a productivity of 230 thousand metric tonnes accounting to 39.4% of the total production in the world (583 thousand metric tonnes) in 2006 (Figure 1.1). In India, Maharashtra is leading in terms of acreage and production followed by Karnataka, Andhra Pradesh and Orissa (2005-06).

1.1 Economic importance

Safflower is a multipurpose crop grown for its foliage, flowers and seeds (oil) which have diversified uses. A tea made from safflower foliage is used to prevent abortions and infertility in

Figure 1.1 Trends in area, production and yield of safflower in World and India





Source: FAO database

women. Young leaves are high in vitamin A, iron, phosphorous and calcium are used as leafy vegetable and salads. Safflower dye extracted from its glittering florets is used as natural colouring agent for food (cakes, biscuits, ice creams, etc.), medicinal formulations (tablets), cosmetics (lipsticks) and fabrics (yarn). In China, safflower is grown extensively for its flowers which are used in medicines for treating many illnesses like hypertension, spasm of blood vessels, chronic bronchitis, etc. (Dajue and Zhou, 1993).

Safflower oil is used for industrial and edible purposes. The oil is used in the manufacture of soaps, paints, varnishes, etc. The oil is used as cooking oil, salad oil and margarine. The oil is highly desirable for human nutrition because of its highest degree of polyunsaturation (75.0% PUFA) and elevated levels of ∞-tocopherol (Furuya et al., 1987). The PUFA lowers blood cholesterol which is advantageous in health point of view. There is a considerable health food market for safflower oil, especially in North America, Germany and Japan. Furthermore, safflower oil is stable and its consistency does not change at low temperatures, making it suitable for use in chilled foods (stable up to -12 ºC). Apart from all these uses, the forage is also used as animal meal or stored as hay or silage. The stalks are cellulose rich and can be used in paper and pulp industries. The soot obtained from charred safflower plants is used to make eye liner in Egyptian cosmetics (Ramanjaneyulu, 2003).

1.2 Crop features

Safflower is basically a self-pollinated crop with some degree (5-10%) of out crossing. It is grown as a *rabi* crop during winter season. The crop's productivity depends on irrigation at early elongation or at flowering stage. However, the crop's productivity is limited by low yield and vulnerability to abiotic and biotic stresses. Safflower is a rain-fed crop but, in general suffers

severely due to moisture stress. The crop can withstand salt stress and can be grown on salt-affected soils as it can tolerate salinity in irrigation water up to 4-8 ds/m (Anjani et al., 2005).

The crop is affected by biotic stresses in the form of insect pests and diseases which reduce the yield and productivity to a large extent (Singh and Prasad, 2005). The major diseases affecting the crop include *Alternaria* leaf blight, *Fusarium* wilt, *Phytophthora* root rot, *Ramularia* leaf spot causing yield losses to the tune of 25-100% depending on the crop age and the level of incidence. The crop is attacked by insect pests like aphids (Lygus bug, Rutherglen bug), boll worms (*Heliothis*), flies (*Acanthiophilus helianthi*), etc. resulting in moderate to heavy losses in yield and production. Although sources of resistance to the major biotic stresses are available in the primary and secondary gene pools, introgression from wild allies is constrained by the existence of sexual crossability barriers and ploidy differences. The studies regarding the abiotic stresses and genetic control of tolerance to them in safflower are largely lacking. Genetic improvement studies were mostly confined to development of high yielding varieties and hybrids. Hybrids based on genetic and cytoplasmic male sterility system were developed. DSH-129 (DOR), MKH-11 (MAHYCO), MRSA-521 (MAHYCO), NARI-NH-1 (NARI), NARI-H-15 (NARI) are the safflower hybrids released with increased oil content and moderate resistance to wilt, leaf blight and aphids. However, plant-breeding programs aimed at development of improved varieties with agronomically desirable attributes, such as, resistance to biotic and abiotic stresses, oil quality, viable pollination control systems are rather limited. Hence, it is necessary to exploit *in vitro* techniques for genetic improvement of safflower for various desirable attributes. Genetic engineering may serve as an efficient tool in broadening the genetic base for improvement of the agronomic characters. For genetic modification, a reliable regeneration and transformation system must be in place.

1.3 Tissue culture studies

Tissue culture studies in safflower include callus-mediated regeneration (Rani et al., 1996; Rani and Rao, 1998; Radhika et al., 2006; Sujatha and Dinesh Kumar, 2007), direct organogenesis (George and Rao, 1982; Tejovathi and Anwar, 1987; Tejovathi and Anwar, 1993; Sujatha and Suganya, 1996; Tejovathi and Das, 1997; Nikam and Shitole, 1999; Mandal and Gupta, 2001; Radhika et al., 2006; Sujatha and Dinesh Kumar, 2007) and somatic embryogenesis (Mandal et al., 1995; Mandal et al., 2001; Mandal and Gupta, 2003; Walia et al., 2007; Vijaya Kumar et al., 2008a).

Among the basal media, Murashige and Skoog salt medium proved to be superior for morphogenic response (Prasad et al., 1991; Chatterji and Singh, 1993). Most of the studies reported for shoot regeneration used media supplemented with growth regulators 6-Benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA). Anthers of safflower were also cultured *in vitro* and haploids were recovered successfully (Prasad et al., 1991). *In vitro* capitula induction was an interesting aspect in safflower tissue cultures (Tejovathi and Anwar, 1984; Radhika et al., 2006). Cell cultures of safflower have been established for production of tocopherol (Wang et al., 1999), antioxidant kinobeon (Wakayama et al., 1994) and pigments (Hanagata and Karube, 1994; Gao et al., 2000). Callus and cell culture studies for selection of resistant *Fusarium oxysporum* f. sp *carthami* cell lines (Suganya et al., 1997) and for tolerance to sodium chloride (Nikam and Shitole, 1997) were also reported. Despite the research expanded over the past three decades, rooting of regenerated shoots and post acclimatization survival remained a problem in safflower tissue culture. Improvement in rooting was attempted (Tejovathi and Anwar, 1984; Nikam and Shitole, 1999; Orlikowska and Dyer, 1993; Baker and

Dyer, 1996) but the problem of rooting still persists. The major constraints in safflower regeneration are-

- Low frequency of shoot regeneration
- Sensitivity of regenerated shoots to media water content
- High relative humidity in culture vessel
- Differential rooting responses among cultivars
- Efficient *in vitro* plant regeneration systems applicable for a wide range of genotypes is still lacking

Hence, there is a need to develop a suitable protocol for improving regeneration in safflower.

1.4 Genetic transformation

Genetic transformation plays a major role in modern biotechnology and agricultural sciences. The basic principle of transformation is to alter the genetic makeup of the system by integrating foreign DNA. The challenges being faced by the conventional breeding methods can be circumvented to a certain extent by genetic engineering. The development of novel technology for gene transfer has opened new dimensions in plant improvement programs (James, 2008). Although, several reports on successful transformation of crop plants are available much remains to be solved in crops like safflower. Genetic transformation in plants can be carried out by direct gene transfer or vector-mediated gene transfer methods. However, in safflower, genetic engineering studies so far are confined to vector-mediated (*Agrobacterium*-mediated) transformation.

Genetic transformation experiments in safflower have been developed for the American (Centennial) and Indian (A-1, A-300) cultivars using *Agrobacterium*-mediated transformation

method. Transformation through callus-mediated regeneration (Ying et al., 1992; Orlikowska et al., 1995; Rao and Rohini, 1999) and embryo transformation through *in planta* (Rohini and Rao, 2000) have been reported. Ying et al. (1992), Rao and Rohini (1999) used seedling explants and obtained transformed shoots on MS medium supplemented with BAP and NAA while in the studies of Orlikowska et al. (1995) the seedling explants showed shoot induction on medium supplemented with Thidiazuron (TDZ) and NAA. To overcome the difficulties in tissue culture based transformation, Rohini and Rao (2000) adopted *in planta* method of transformation. In all these studies, the gene constructs harboured *npt* II as plant selection marker and *Uid* A as reporter gene. The introduced transgene was initially confirmed by GUS histochemical assay. Further, confirmation by molecular studies was done through PCR analysis for the genes of interest and the vector genes (*npt* II, *Uid* A, *vir*C), Southern analysis, non-denaturing PAGE for neomycin phosphotransferase activity, Western blot and Dot blot (Ying et al., 1992; Orlikowska et al., 1995; Rao and Rohini, 1999; Rohini and Rao, 2000). Most of the studies were confined to the characterization of primary transformants. In the recent past, safflower has received great importance as a model plant in biofarming. Safflower is being used as a production vehicle for various pharmaceuticals, neutraceuticals and cosmaceuticals such as human insulin, apolipoprotein A1, growth hormones, carp growth hormones n-6 γ-linolenic acid (GLA), n-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Reviewed by Sujatha, 2007). Arcardia Biosciences has successfully demonstrated 65.0% GLA in the oil from genetically transformed safflower seeds. However, the major constraints in genetic transformation studies of safflower are –

- Lack of genotype-dependent regeneration system
- Low frequency of transformation

- Growth retardation of shoots following long time exposure to selection
- Poor rooting and low survival following acclimatization of selected shoots

Against the above background the present investigation has been undertaken with the following objectives—

- To optimize parameters for establishing a reproducible regeneration protocol in safflower
- To establish a highly efficient transformation protocol using *Agrobacterium*-mediated transformation method
- To confirm the putative transformants for integration of the introduced gene through molecular analysis
- Transformation of safflower with an agronomically desirable gene (*atp 9*) and confirmation of integration of the gene by GUS assay and PCR studies

Chapter 2
Review of literature

Safflower is a versatile crop with multiple uses. It is grown for edible oil (linoleic rich types), industrial oil (oleic rich types), birdseed and animal meal. The dye obtained from safflower florets has high medicinal value. India is the leading producer of safflower with 51.1% in area and 39.4% in seed production. Safflower oil has high linoleic acid content (75.0%) and is known to reduce blood cholesterol level, demands a premium price among edible oils and is competitive from health viewpoint with canola and olive oil. Safflower is now being used as a model plant in molecular farming for production of human insulin, apolipoprotein A1, growth hormones, etc. With the gaining importance of the crop, it is necessary to develop *in vitro* techniques for genetic enhancement of the crop for agronomically important traits, which are difficult to be introgressed through conventional breeding.

In vitro regeneration of Indian, American and Turkish cultivars of safflower was attempted. In most of the studies, shoot regeneration was from immature embryos and seedling explants (mostly cotyledonary leaf). It was observed that juvenile tissues were more amenable for *in vitro* manipulations when compared to mature tissues. However, an efficient *in vitro* plant regeneration system applicable for a wide range of genotypes/cultivars is still lacking. The sensitivity of the regenerated shoots to media water content, humidity in culture vessels and differential rooting response are the major constraints in safflower regeneration experiments.

Genetic engineering is a non-conventional tool for gene transfer and had yielded promising results in genetic enhancement of some agriculturally important crop plants (James, 2008). The prerequisites for a reproducible transformation system are an efficient tissue culture system, a method of delivering plant expressible DNA into totipotent cells of the culture system and the ability to identify and select regenerated tissues that have been stably transformed with introduced DNA (Hewezi et al., 2002). The techniques followed commonly to introduce genes

into plant cells are vector-mediated/indirect gene transfer (*Agrobacterium*-mediated transfer, viral vetor-mediated transfer) and vectorless/direct gene transfer (chemical-mediated, microinjection, electroporation, particle bombardment, lipofection, etc.). Of all the methods available, *Agrobacterium*-mediated transformation method has been widely used due to the following advantages over other transformation methods—

- Ability to transfer large DNA fragments
- Integration of single or few copies of the transgene leading to fewer problems with transgene co-suppression and instability
- Ability to transfer any foreign DNA placed between the T-DNA irrespective of its source
- Transformation efficiency is relatively high as compared to other methods

2.1 Tissue culture studies

The term 'plant tissue culture' is commonly used for *in vitro* and aseptic cultivation of any plant part on a nutrient medium (Dixon, 1985). With the recent developments in molecular biology and genetic engineering, tissue culture has gained importance for gene transfer into plants. Phytohormones play an important role in establishment of callus culture, morphogenesis and regeneration of whole plants. It was observed that the balance in the levels of cytokinin and auxin determines specific morphogenetic pattern, a relatively low auxin and high cytokinin produces shoots, whereas high auxin and low cytokinin level stimulates rooting and almost equal proportions of each component results in undifferentiated callus (Skoog and Miller, 1957).

In vitro plant regeneration is governed by many factors. The callus induction, differentiation and maintenance of the material *in vitro* are determined by physical and chemical

components of the nutrient medium. The first major completely defined medium for *in vitro* plant regeneration was developed by Murashige and Skoog (1962). Of the growth substances, auxins have been proved to be an essential supplement for establishing tissue culture (Gautheret, 1966). Cytokinins control cell division and cell growth by exerting influence on specific proteins and nucleic acid metabolism. In addition to growth hormones, the physical environment plays an important role in organogenesis, the major factors being the physical state of the medium, light, temperature, humidity, age of the explants and so on (Murashige, 1974). The studies on various aspects of tissue culture in safflower are reviewed below.

George and Rao (1982) have reported callus formation and differentiation of 2 to 4 shoot buds per explant on medium with MS salts+0.2-2.0 mg/l BAP+0.5 mg/l NAA in about 40% hypocotyl cultures of NP-9 black and Th-10 black. They also observed that cotyledons were more responsive than hypocotyl tissues on MS+BAP+NAA medium. The shoot buds developed on BAP+NAA medium were very small and most of them failed to develop into normal plantlets. In the variety partial hull black, good callus growth occurred, but shoot differentiation was observed only in 10% of the cotyledon and hypocotyl explants. Rooting of shoot buds was obtained in about 10% of cultures on hormone free-MS basal medium. The MS medium containing higher concentrations of sucrose was found to be effective in root induction. About 50% of the cultures of NP-9 black and Th-10 black produced normal roots when sucrose concentration was increased from 3 to 6, 7 or 8% in the medium. Under continuous illumination, many of the rooted plantlets as well as the rootless micro-shoots produced flowers.

Goyal and Pillai (1983) reported profuse callusing from meristems (shoot apex) of safflower on MS medium supplemented with 0.04-0.08 mg/l KN with 1.5 and 3.0 mg/l NAA.

Negatively geotropic roots were formed in large numbers on MS medium with 0.04 mg/l KN+3.0 mg/l NAA.

Tejovathi and Anwar (1984) reported *in vitro* capitula (head inflorescence) induction in two varieties (A-1, Manjira) of safflower from the inner surface of the cotyledons on MS medium with 0.5 mg/l BAP or KN and 0.1 mg/l NAA. Complete blooming of florets in a capitulum was observed within 55-90 days after culture initiation. The pollen fertility of *in vitro* produced flowers ranged from 90.0-95.0%. Embryo development was normal and few seeds were also recovered.

Tejovathi and Anwar (1986) cultured cotyledons of 2-day-old seedlings of two safflower varieties A-1 and Manjira, devoid of meristem, shoot and root primordia on MS medium supplemented with 0.5 mg/l BAP, 0.1 mg/l NAA and 3% sucrose. Multiple shoots obtained from these cultures were transferred to MS medium with various concentrations of NAA (or) IAA and BAP (or) KN (or) TIBA. The shoots were rooted on MS medium supplemented with 2,4,5-trichlorophenoxy propionic acid (2,4,5-Cl_3POP).

Anwar et al. (1989) cultured cotyledonary explants on seven different media (LS, MS, B5, Blaydes, Chaleffs, Wood and Hsienmiaos). Callus initiation was observed within 7-10 days of inoculation and the frequency of callusing was maximum (91.1%) on MS medium followed by Chaleff's and B5 media. Poor callusing was observed on Wood's medium. The ratio of dry weight, fresh weight was highest (38.9%) on MS medium followed by B5 (34.1%) whereas, it varied from 10.2-19.8% on other media. In seven different explants tested (cotyledons, root, apical bud, hypocotyl, stem, leaf and seedling base), the frequency of callus induction was maximum with cotyledonary explants (90-95%) followed by root (85-90%), apical bud (80-90%), hypocotyl (80-90%), stem (60-68%) and leaf (55-60%). Minimum callusing (30-40%) was

noticed in seedling base explant.

Prasad et al. (1990) studied influence of culture medium, genotype and cold pre-treatment on anther culture response in safflower and reported that MS medium was the most responsive for callus induction among the five different basal media (N6, B5, Chaleff's, LS and MS) tested. The MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l NAA and 2% sucrose was selected to assess the genotypic differences in the induction of haploid calli in 10 genotypes of safflower including the local varieties. The most responsive genotype was Manjira, with 48.6% callus induction. The MS medium supplemented with varying concentrations of BAP and NAA with 2% sucrose showed relatively less callusing, but the frequency of shoot bud initiation increased.

Padmaja et al. (1990) found that among several media tested, MS medium supplemented with 2.0 mg/l 2,4-D was effective in callusing. The ratio of fresh weight, dry weight was highest (38.9%) on MS medium with 2.0 mg/l 2,4-D followed by B5 medium (34.1%) whereas in others it varied from 10.2 to 19.8% suggesting the superiority of MS medium over other media. When different explants were tested for their callusing efficiency, the frequency of callusing was maximum with cotyledonary explants (90.0-95.0%) followed by root (85.0-90.0%), apical bud (80.0-90.0%), stem (60.0-68.0%), leaf (55.0-60.0%) and shoot base (30.0-40.0%) explants. The callus cultures initiated from cotyledons exhibited morphogenesis (rosettly arranged leaf-like structures, shoot buds, root initiation and embryoid formation) on MS medium supplemented with 0.5-6.0 mg/l BAP in conjunction with 0.1-2.0 mg/l NAA. In general, MS medium supplemented with 0.5 mg/l BAP+0.1 mg/l NAA showed increased frequency (87.0%) of morphogenic response.

Prasad and Anwar (1991) studied the influence of culture medium on induction of androgenic callus from cultured anthers. Of five different basal media, MS medium was the most

responsive. Fourteen different genotypes including four F_1 hybrids and two *in vitro* regenerated anther donor plants were tested for genotypic differences. The most responsive genotype was cv. Manjira, where 48.6% of anthers initiated callus formation. Plant growth factors such as NAA, 2,4-D and IAA (auxins), BAP and KN (cytokinins) and gibberellin used singly and in combination revealed that a low cytokinin to auxin ratio stimulated shoot induction.

Prasad et al. (1991) obtained shoot regeneration from cultured anthers with uninucleate microspores on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA and rhizogenesis on half-strength MS medium supplemented with 0.1 mg/l NAA and low level of sucrose (1%).

Reddy and Devi (1991) cultured cotyledon explants on MS medium supplemented with 3% sucrose and varying levels of 2,4-D (1.0, 2.0 and 5.0 mg/l) for 2-3 weeks. Frequency of callus induction was high on medium with 1.0 mg/l 2,4-D in Manjira and HUS-305 genotypes. Shoot differentiation was achieved with varying frequencies from cotyledon-derived callus on MS medium containing BAP. Shoots were rooted on hormone-free MS medium. These studies suggested that shoot morphogenesis could be induced from cotyledonary callus culture.

Singh (1991) carried out morphogenetic studies in callus and organ cultures of cultivated (*C. tinctorius* L. cv. CO-1) and wild safflower (*C. oxyacantha*) and observed that leaf and cotyledonary wings were more potent and showed better callus induction potential than the root and shoot explants. The concentration of cytokinin appeared to be a critical hormonal supplement during regeneration phase. A comparatively higher concentration of cytokinin (1.0-2.5 mg/l BAP) and a lower level of auxin (0.25-1.0 mg/l NAA) was found optimal for callus induction. Callus proliferation rate and greening was increased with addition of adenine sulphate (5.0-10.0 mg/l). Morphogenetic response through organogenesis was observed after 5 weeks

when cultures were placed on medium containing enhanced levels of BAP (8.0-10.0 mg/l). Shoot buds were regenerated from leaf and cotyledonary derived callus. Rooting and further establishment of regenerated plantlets was successfully achieved on half-strength MS medium devoid of hormones. Shoot apices of wild safflower *C. oxyacantha* proliferated on MS medium with NAA (0.5 mg/l)+GA_3 (20.0 mg/l)+ascorbic acid (5.0 mg/l).

Among the five genotypes used (Manjira, A-1, Sagarmuthyalu, CO-1 and S-4), Manjira exhibited superior response for callus induction, shoot regeneration and subsequently the plantlet regeneration suggesting the role of explant type and the genotype in safflower tissue culture (Anwar et al., 1993).

Zhanming and Biwen (1993) reported profuse callus growth of different seedling explants of safflower cultured on MS basal medium supplemented with 2,4-D (0.25 mg/l) singly or in combination with BAP (0.5 mg/l). Adventitious bud differentiation was induced on medium supplemented with BAP (0.5-5.0 mg/l)+NAA (0.1-0.5 mg/l) from the young leaf and shoot apex. The hormonal combination of 2.0 mg/l NAA with 0.05 mg/l KN was most suitable for adventitious root differentiation of the explants. There was correlation between the adventitious root and bud differentiation and the source of explants.

Tejovathi and Anwar (1993) observed that low concentrations of BAP and NAA induced 10-12 shoot buds per explants from seedling cotyledons of safflower cultivars, A-1 and Manjira. Although A-1 showed a wider plasticity of growth regulator requirement, it showed a slightly lesser degree of shoot bud regeneration than Manjira. Conventionally used auxins like NAA, IAA, IBA failed to induce roots in the *in vitro* formed shoots, whereas, 2,4,5-Cl_3POP at 1.0 and 2.0 mg/l induced rooting of the shoots at a high frequency, which facilitated the recovery of plantlets.

Huetteman and Preece (1993) reported that low concentrations of TDZ (0.2 mg/l) could induce greater axillary proliferation than many other cytokinins, however, although at times it may inhibit shoot elongation. In some cases, it is necessary to transfer shoots to an elongation medium containing a lower level of TDZ and/or a less active cytokinin. At concentrations higher than 0.2 mg/l, TDZ can stimulate the formation of callus, adventitious shoots or somatic embryos. Subsequent rooting of micro-shoots was unaffected or slightly inhibited by prior exposure to TDZ.

Orlikowska and Dyer (1993) reported direct shoot regeneration from primary seedling explants and immature embryos of the American safflower cv. Centennial. Direct shoot regeneration from primary explants was obtained on MS medium supplemented with 0.5 mg/l BAP or 0.1 mg/l TDZ and 0.1 mg/l NAA. Shoot regeneration from immature embryos was observed on MS medium containing TDZ and NAA. The shoots were healthy and elongated on MS supplemented with 1.0 or 2.0 mg/l 2iP and rooted on half-strength MS with 1 mg/l NAA.

Chatterji and Singh (1993) studied the induction of morphogenetic calli from leaf explants of an elite safflower cultivar, CO-1. Leaf explants from three-week-old *in vitro* grown seedlings exhibited callusing potential on MS medium with NAA (1.0-1.5 mg/l) and BAP (0.5-1.0 mg/l). Shoot bud primordia surfaced when the level of BAP was raised to 5.0 mg/l in combination with 0.25 mg/l NAA after four weeks of subculturing. For leafy shoot development, shoot buds were transferred to half-strength basal MS with adenine sulphate (20 mg/l). The leafy shoots failed to produce roots on hormone-free medium.

Udhyakumar and Ramaswamy (1996) studied the *in vitro* response of three different explants (leaf, flower bud and root) of two safflower varieties (CO-1 and JSI-7) for callus induction on MS basal medium supplemented with different levels of 2,4-D, NAA and BAP. The

selected varieties showed callus induction on the five media combinations tested. The leaf explants of both varieties recorded a maximum of 87.5% callus induction. The best media combinations for callus induction in leaf, flower bud and root explants of CO-1 and JSI-7 were found to be MS+2,4-D (2.0 mg/l) and MS+BAP (2.0 mg/l)+NAA (0.1 mg/l), respectively.

Baker and Dyer (1996) reported that 76% of shoots directly regenerated from primary explants rooted after 7 days exposure to 10.0 mg/l BAP. Auxin source, concentration or exposure time did not greatly affect root formation or morphology, but strongly affected callus production. The use of NAA (5.0 mg/l) or IBA (5.0 or 10.0 mg/l) did not promote callus formation and shoots produced a single taproot with good branching that arose from shoot base. A seven-day exposure to 10 mg/l IBA in root induction media, followed by incubation in media containing 15 g/l sucrose and 1.0 g/l activated charcoal for 21 days was found optimal for rhizogenesis.

Rani et al. (1996) reported that cotyledonary leaf was efficient for callus induction, growth and differentiation in safflower genotypes (MS-105 and Manjira). The MS medium supplemented with 5.0 mg/l NAA and 0.25 mg/l BAP induced maximum callus (65.4%) irrespective of genotypes and explants. MS medium supplemented with 2.0 mg/l NAA was significantly superior to other hormonal combinations in terms of differentiation. They found maximum frequency of direct rhizogenesis and caulogenesis from both cotyledonary leaf and hypocotyl explants on MS medium supplemented with 5.0 mg/l NAA and 0.1 mg/l NAA+0.25 mg/l BAP, respectively.

Sujatha and Suganya (1996) assessed the efficacy of different seedling explants of safflower for direct and callus-mediated shoot regeneration. All the four types of explants (root, hypocotyl, cotyledon and leaf explants) cultured on MS medium supplemented with 1.0 mg/l BAP and NAA regenerated shoots. However, the frequency of direct regeneration as well as

callus-mediated shoot regeneration and number of shoots per responding explant were significantly high with leaf when compared to other explants. Regenerated shoots elongated on MS medium fortified with 0.2 mg/l BAP. Well-developed shoots rooted with a frequency of 32% on half-strength MS medium supplemented with 0.5 mg/l NAA.

Suganya et al. (1997) obtained and maintained callus on MS medium supplemented with 1.0 mg/l each of BAP and NAA, while shoot regeneration was achieved on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA.

Rani and Rao (1998) studied callus differentiation in tissue cultures of safflower. Callus was induced from cotyledonary leaf and hypocotyl explants of two safflower genotypes, MS-105A line and Manjira on MS medium supplemented with 0.1, 0.5, 1.0, 2.0 and 5.0 mg/l of NAA in combination with 0.25 mg/l BAP and KN. MS medium supplemented with 2.0 mg/l BAP gave maximum shoot differentiation from five-week-old primary calli. Rhizogenesis of *in vitro* differentiated shoots was achieved on growth regulator-free MS medium, however, MS medium supplemented with 2.0 mg/l NAA was found good for rhizogenesis.

Nikam and Shitole (1999) reported callus induction and *in vitro* plantlet regeneration systems for safflower cv. Bhima using root, hypocotyl, cotyledon and leaf explants. Supplementation of the medium with an auxin : cytokinin ratio > 1 enhanced the growth rate of callus cultures, however for 2,4-D the ratio was < 1.34. The growth regulators, IAA, NAA, BAP and KN alone or in combinations were found effective for callus induction and regeneration in all the explants. The calli could be maintained for over 32 months. BAP (1.0 mg/l) combined with casein hydrolysate (CH, 10 mg/l) yielded the highest rate of shoot production on hypocotyl (3-6) and cotyledon (5-7) explants and cotyledonary derived callus (4-8). More shoots were produced on explants cut from the most basal region of cotyledons from 5 to 7-day-old seedlings

than from older seedlings or more distal cut sites. Apolar placement of explants inhibited shoot regeneration. The shoot regeneration potential remained upto 7 months in calli developed on NAA+BAP. Of three media tested, MS was superior to Mitchell and Gildow and Gamborg medium. Rooting of shoots was not efficient, and 42% of the shoots developed roots on MS medium containing sucrose (7-8%)+IAA (0.5-1.0 mg/l). Capitulum induction was observed in callus-mediated shoots on cotyledons as well as from shoots on rooting medium with sucrose (1-9%), IAA, NAA and IBA (0.1-5.0 mg/l). Well-developed plantlets were transferred to the field with a 34% success rate.

In studies of Mandal and Gupta (2001) adventitious shoot buds were induced from adaxial surface of cotyledons of eight safflower cultivars on MS medium supplemented with BAP. The variety S144 showed maximum shoot organogenesis (54.4%) with 2.0 mg/l BAP. Highest number of normal shoots was obtained with lower concentration of BAP (0.5 mg/l). Root induction was best on medium supplemented with NAA followed by IAA and IBA. Rooted plantlets that were transferred to soil appeared morphologically normal.

Mandal et al. (2001) studied the factors affecting somatic embryogenesis from cotyledonary explants of eight safflower cultivars. Among all the tested genotypes, Girna showed best response. Incorporation of silver nitrate in the medium increased the embryogenic frequency and also the number of embryos per responding explant with no effect on germination.

Mandal and Gupta (2003) investigated the influence of auxin type and concentration on somatic embryogenesis from cotyledonary explants of safflower. High frequency of somatic embryos was obtained at 2.0 mg/l NAA. Maximum number of somatic embryos at the cotyledonary stage was obtained with 1.0 mg/l NAA+0.5 mg/l BAP.

Radhika et al. (2006) reported adventitious shoot regeneration from different explants of safflower. MS medium supplemented with TDZ (0.5-5.0 mg/l) and NAA (0.1-0.5 mg/l) combinations showed high frequency of shoot regeneration. Shoot elongation was achieved on medium with 0.5 mg/l KN. Rooting was obtained on half-strength MS medium with 0.5 mg/l NAA. The study for the first time demonstrated the amenability of roots for *in vitro* regeneration.

Walia et al. (2007) reported proliferation and shoot differentiation from safflower endosperm. Embryo differentiated only from the calli developed on medium supplemented with BAP, KN or TDZ with the last eliciting maximum embryogenic response. Somatic embryos were induced on medium supplemented with 100 mg/l adenine sulphate. Embryos matured and developed into shoots on MS basal medium supplemented with 0.2 mg/l gibberellic acid. Rooting was tested on half-strength MS+0.2 mg/l NAA, but even embryo-derived shoots failed to root and the shoots turned brown.

In vitro bud regeneration of cultivated safflower and wild *Carthamus* spp from leaf explants and axillary buds were carried out by Sujatha and Dinesh Kumar (2007). Among eleven *Carthamus* spp, *C. tinctorius* and *C. arborescens* showed highly prolific adventitious shoot regeneration on MS medium supplemented with TDZ+NAA. Shoot elongation was achieved on MS medium with 1.0 mg/l KN or 0.5 mg/l BAP. Elongated shoots were rooted on MS medium with 1.0 mg/l each of IBA and phloroglucinol (PGA).

Basalma et al. (2008) reported adventitious shoot regeneration from safflower cv, Dincer (Turkish cultivar) using cotyledonary leaf explants. Highest shoot regeneration was obtained on MS medium with 0.5 mg/l TDZ and 0.25 mg/l IBA. Shoot regeneration from cotyledonary nodes and meristem tips was obtained on MS medium supplemented with various concentrations of BAP alone or in combination with NAA.

Vijaya Kumar et al. (2008a) reported cyclic somatic embryogenesis and efficient plant regeneration from cotyledon and leaf explants of safflower cv. NARI-6. High frequency of embryogenic callus was obtained from cotyledon explants on MSG (Murashige and Skoog Germination) basal medium supplemented with TDZ, 2-isopentenyl-adenine (2iP) and IBA. Embryo germination was achieved in 1/4 MSG with 1.5% sucrose, 0.2 mg/l GA_3, 250 mg/l glutamine, 100 mg/l arginine and 100 mg/l proline. Frequency of rooting was maximum on medium with putrescine (1.5 mg/l) in this genotype.

Thus, in most of the tissue culture studies of safflower MS medium supplemented with BAP and NAA at varying concentrations was used successfully for stimulating different organogenic responses from seedling tissues. For somatic embryogenesis, medium with 2,4-D, IAA and NAA was found favourable.

2.2 Transformation studies

Ying et al. (1992) have studied *Agrobacterium tumefaciens*-mediated transformation of safflower cv. Centennial. Efficient callus formation and shoot regeneration were obtained from cotyledon, stem and leaf explants on MS medium supplemented with 1.0 mg/l each of BAP and NAA. They further reported that substitution of 2,4-D for NAA did not improve efficiency of callus formation. Callus growth was visible on over 80.0% of explants after fourteen days. Shoot buds regenerated from 26.0% of leaf-derived calli on callus induction medium. However, attempts to induce roots in the regenerated shoots were not successful. Explants of Centennial subjected to transformation with *A. tumefaciens* containing *npt* II and *gus* genes produced kanamycin-resistant calli from which buds were regenerated. Transformation and stable integration of transgene was confirmed by GUS assay and DNA hybridization.

Factors (such as bacterial strain, acetosyringone, co-cultivation period) influencing *A. tumefaciens*-mediated transformation and regeneration of safflower cultivar 'Centennial' were examined by Orlikowska et al. (1995). Shoots were formed on medium with MS salts and B5 vitamins supplemented with TDZ (0.01 mg/l)+NAA (0.1 mg/l)+$AgNO_3$ (2.5 mg/l)+carbenicillin (500 mg/l). Shoot elongation was optimal on MS medium with KN (1.0 mg/l)+NAA (0.05 mg/l)+$AgNO_3$ (2.5 mg/l)+carbenicillin (500 mg/l). Rooting was induced on half-strength MS medium supplemented with NAA (1.0 mg/l)+$AgNO_3$ (2.5 mg/l)+vitamin B_2 (1 mg/l) and incubated in dark for 5 days and then transferred to light. The *A. tumefaciens* strain EHA 105 with p35SGUSInt was more infective than LBA 4404 with pBI121 which was determined in terms of β-glucuronidase activity. Regeneration efficiency decreased in transformed shoots when compared to untransformed explants (controls) on co-cultivation with EHA 105 and the decrease aggravated with acetosyringone addition. However, regeneration declined with increase in acetosyringone concentration from explants co-cultivated with LBA 4404. The presence of the transgene was confirmed in the putative transformants by PCR and Southern hybridization assay.

Rao and Rohini (1999) studied gene transfer into two Indian cultivars (A-1 and A-300) of safflower using *A. tumefaciens*-mediated transformation method. The binary vector pKIWI 105 lacking bacterial ribosome binding site and harbouring *Uid* A gene was used. Shoot induction was obtained on MS medium supplemented with 0.1-5.0 mg/l BAP and 0.01-1.0 mg/l NAA. Putative transformants were confirmed for the presence of the introduced gene by GUS histochemical assay and western blot analysis for *npt* II expression assay. Transgene integration was examined by PCR and dot blot hybridization. Extended periods of callus-mediated regeneration led to hyperhydricity and vitrification of the shoots. The axillary shoots of A-1 did

not root on medium supplemented with NAA or IBA while the shoots of A-300 formed roots only on medium with 0.1 mg/l NAA.

Rohini and Rao (2000) reported *in planta* transformation using embryos bypassing tissue culture regeneration in safflower. Embryo axes of germinating seeds were used for *A. tumefaciens*-mediated transformation. After 24 hours of co-cultivation, explants were decontaminated with cefotaxime and allowed to germinate and transferred to green house. Putative transformants were confirmed by GUS histochemical assay, PCR amplification of *Uid* A and *npt* II marker genes and Southern analysis. The frequency of transformation was 5.3% in A-1 and 1.3% in A-300.

2.3 Transformation with *atp 9* gene

In a highly self-pollinating crop like safflower, the development of male sterility lines is of great importance in hybrid seed production. The conventional hybrid seed production is based on genetic male sterility (GMS) and only recently a cytoplasmic male sterility (CMS) based hybrid (MRSA-521) bred from private sector (MAHYCO) has been released. The GMS based hybrid breeding system is constrained by the difficulties in rouging of the offtypes due to the spiny nature of the crop, and identification of offtypes only at the flowering stage and thus, is highly uneconomical. The limitations with the conventional hybrid breeding programme has opened doors for the exploitation of the transgenic technology for the production of male sterile lines. Male sterility may be due to the absence/malformation of male organs (stamens), failure in normal development of microsporogenous tissue (anther), abnormal/deformed/inviable pollen, etc. Genetic engineering can be used for the production of male sterile plants by manipulating tapetum/stomium that hinders anther development, destruction of the connective or stomium

leading to failure of pollen release, altering gene expression of male reproductive organ (temporal/spatial regulation, etc.). It has been demonstrated that impairment in the mitochondrial function especially in the tapetal cell layer of anther (requires high energy during pollen formation and development) leads to male sterility. RNA editing (C-U), a post-transcriptional process that occurs in plants had been advantageous in production of male sterile lines. Targeted expression (tapetum) of the unedited mitochondrial genes led to the formation of abnormal pollen leading to male sterility.

Mariani et al. (1990), reported the formation of male sterile transgenics in tobacco and rapeseed by using barnase-barstar system, where a ribonuclease gene from *Bacillus amyloliquifaciens* (barnase) was cloned using a tapetum specific promoter TA 29. The expression of the transgene lead to abalation of tapetal cell layer resulting in failure of pollen development.

Hernould et al. (1993) reported that mitochondrial dysfunction leads to cytoplasmic male sterility in plants. The use of unedited *atp 9* of wheat ATP synthase subunit 9 (*atp 9*) fused to the coding sequence of a yeast *cox IV* transit peptide resulted in the production of transgenic plants with fertile, semifertile and male sterile phenotype. Pollen fertility ranged from 31-75% in fertile plants, 10-20% in semifertile and < 2% in male sterile plants.

Hernould et al. (1998) reported that transformation of tobacco with an unedited copy of the mitochondrial *atp 9* gene sequence fused to the yeast *cox IV* targeting presequence showed several anther abnormalities leading to pollen abortion. Cytological studies revealed that the tapetal development was impaired. The mitochondria of tapetal cells showed degeneration signs which was correlated with the presence of the transcript and translated product of the unedited *atp 9* leading to male sterility.

Gomez-Casati et al. (2002) reported production of transgenic male sterile plants of *Arabidopsis* using the unedited *atp 9* (*uatp 9*) under the control of three different promoters- CaMV 35S, apetala 3 and A 9.

At the Directorate of Oilseeds Research, a project for the production of male sterile lines using unedited genes (*orfH 522*, *nad 3* and *atp 9*) is under progress. The study uses TA 29 promoter (tapetum specific), for expression of the unedited gene only in the tapetal cell layers, with a *cox IV* transpeptidase gene (for translocation of the peptide into mitochondria) and a *nos* terminator.

2.4 Conclusion

The studies on regeneration and transformation from different explants and genotypes of safflower are tabulated in Table 2.1.

Table 2.1 Tissue culture and transformation studies in safflower

Explant	Type of morphogenetic response	Medium (mg/l)	Reference
Tissue culture			
Hypocotyl, cotyledons	Multiple shoots	MS+0.2-2.0 BAP+0.5 NAA	George and Rao, 1982
Cotyledons	Induction of capitula	MS+0.5 BAP+0.1 NAA MS+0.5 KN+0.1 NAA	Tejovathi and Anwar, 1984
Leaf	Oil accumulation	MS+1.0 BAP+0.25 NAA+5% sucrose+1 g/l CH+10% coconut water	Singh and Chatterji, 1991
Primary seedling explants	Shoot regeneration from leaf	MS+8.0 BAP+0.5 NAA+5.0 adenine sulphate	Singh, 1991
Shoot apices of *C. oxyacantha*	Shoot proliferation	MS+0.5 NAA+20.0 GA_3+5.0 ascorbic acid	
Anther	Shoot regeneration	MS+2.0 BAP+0.5NAA	Prasad et al., 1991
Cotyledons	*In vitro* rooting	MS+1.0-2.0 2,4,5-Cl_3POP	Tejovathi and Anwar, 1993
Leaf	Shoot buds	MS+5.0 BAP+0.25 NAA	Chatterji and Singh, 1993
Primary seedling explants	Adventitious shoot regeneration	MS+0.5 BAP+0.1 NAA MS+0.1 TDZ+0.1 NAA	Orlikowska and Dyer, 1993
Immature embryos	Shoot regeneration	MS+0.01 TDZ+0.1-10.0 NAA	
Cotyledons	Somatic embryos	MS+0.5 BAP+1.0-2.0 NAA	Mandal et al., 1995
Primary seedling explants	Adventitious shoots	MS+0.5-5.0 BAP+0.1-0.5 NAA	Zhanming and Biwen, 1993
Cotyledons, hypocotyl	*In vitro* rooting	MS+10.0 IBA for 7days followed by MS+1.5% sucrose+1 g/l activated charcoal for 21 days	Baker and Dyer, 1996
Hypocotyl, cotyledons	Shoot regeneration	MS+0.25 BAP+0.1 NAA	Rani et al., 1996
Primary seedling explants	Shoot regeneration	MS+1.0 BAP+1.0 NAA	Sujatha and Suganya, 1996

Primary seedling explants	Selection of calli and shoots resistant to *Fusarium oxysporum*	MS+1.0 BAP+1.0 NAA for callus MS+1.0 BAP+0.1 NAA for shoots	Suganya et al., 1997
Cotyledons	Selection of calli resistant to sodium chloride	MS+0.5 BAP+1.5 NAA	Nikam and Shitole, 1997
Primary seedling explants	Direct shoot regeneration	MS+1.0 BAP+10.0 CH	Nikam and Shitole, 1999
Cotyledons	Adventitious shoots	MS+0.5-2.0 BAP	Mandal and Gupta, 2001
Cotyledons	Somatic embryos	MS+0.5 BAP+1.0 NAA	Mandal and Gupta, 2003
Cotyledonary node, stem node	Shoot buds	MS+B5 vitamins+4.5 BAP+1.5 KN	Vijaya Kumar and Kumari, 2005
Primary seedling explants including roots	Shoot regeneration	MS+0.5-5.0 TDZ+0.1-0.5 NAA	Radhika et al., 2006
Endosperm	Somatic embryos	MS+0.5-2.0 BAP/0.5-2.0 KN/0.2-0.5 TDZ	Walia et al., 2007
Leaf	Shoot regeneration	MS+0.2-1.0 TDZ+0.2-1.0NAA MS+1.0 BAP+0.5-1.0 NAA	Sujatha and Dinesh Kumar, 2007
Axillary bud	Shoot regeneration	MS+0.5-1.0 BAP+0.5-2.0 KN	
Cotyledons, leaf	Somatic embryogenesis	MS+6.0 TDZ+1.5 2iP+2.5 IBA	Vijaya Kumar et al., 2008a
Cotyledonary leaf	Adventitious shoots	MS+0.5TDZ+0.25IBA	Basalma et al., 2008
Transformation			
Primary seedling explants	Transformed shoots	MS+1.0 BAP+1.0 NAA	Ying et al., 1992
Primary seedling explants	Transformed shoots	MS salts+B5 vitamins+0.01 TDZ+0.1 NAA+2.5 $AgNO_3$+500 carbenicilin	Orlikowska et al., 1995
Primary seedling explants	Transformed shoots	MS+1.0 BAP+0.1 NAA	Rao and Rohini, 1999
Embryos	*In planta* transformed shoots	-	Rohini and Rao, 2000

Chapter 3
Materials and methods

The present investigations were carried out at the Genetics and Biotechnology laboratory, Directorate of Oilseeds Research, Rajendranagar, Hyderabad with the objectives of development of reliable and reproducible protocols of tissue culture regeneration and genetic transformation in safflower (*C. tinctorius* L.). The work mainly involved the study of the various combinations and concentrations of plant growth regulators influencing regeneration and optimization of various parameters that enhance transformation frequency.

3.1 Plant material

The seed material of safflower cv. HUS-305 used in the present study, was obtained from the Directorate of Oilseeds Research (DOR), Hyderabad. Some experiments were carried out with other genotypes like Manjira, Sharda, A-1, A-2, A-300, NARI- 6, JSF-1 and JLSF-414.

3.2 Equipments/Instruments

Equipment	Company/Suppliers
Autoclave	Sanyo, Lab-tech
Bio-imaging system	Syngene
Centrifuges	Heraeus, Sorvall
Electrophoresis units	Biorad, Consort
Freezer (-20 ºC)	Sanyo
Hot air oven	JSR
Incubators	JSR
Laminar air flows	Klenzaids, Klenz Flo
Micro oven	Kenstar, LG, Bajaj
Microscopes	Leitz/Leica
Milli Q unit	Millipore
pH meter	Eutech
Pipetman	Eppendorf
Water bath or Incubator	Julabo
Spectrophotometer	Genway
UTL freezer (-80 ºC)	Thermo Electron Corporation
Vacuum pump	Millipore
Vortex mixer	Genei
Water bath	Haake

3.3 Chemicals and consumables

The chemicals used in the present study were procured from Amersham Biosciences, Bangalore Genei, Duchefa, Genetix, Himedia Chemicals, InVitrogen, Life Technologies, Fermentas, Promega Life Science, Sigma, SD Fine Chemicals, USB, Qiagen, Qualigens fine chemicals. Consumables like glassware, plastic ware, scalpel blades, forceps, scalpels, filter papers, pipette tips, eppendorf tubes, etc., were obtained from Axygen, Borosil, Tarsons, etc. Other standard solutions, buffers and reagents were prepared according to the procedures given by Sambrook and Russel (2001).

3.4 Plasmid DNA vectors and gene constructs

The plasmid DNA vectors used for transformation in the present study were developed at the Directorate of Oilseeds Research and maintained in *E. coli* (Kalyani et al., 2007; Yamini, 2007). The gene constructs used in the present study were pCAMBIA 1391Z with CaMV 35S promoter for *gusA* reporter gene and pCAMBIA 1305.2 TCAN harbouring the unedited *atp 9* gene. The construct pCAMBIA 1305.2 TCAN was mobilized into *Agrobacterium tumefaciens* strain, LBA 4404 and used for transformation while the pCAMBIA 1391Z available in *Agrobacterium* was directly used (Kalyani et al., 2007). The *Agrobacterium* vectors were obtained from CAMBIA (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia).

3.4.1 pCAMBIA 1391Z+CaMV 35S promoter (CAMBIA, Australia)

pCAMBIA1391Z is a binary vector with hygromycin resistance gene (*hpt*) for selection in plants and kanamycin resistance gene for selection in bacteria (Figure 3.1). The vector has pUC9 polylinker and *gusA* reporter gene. The truncated *lacZα* is functional for blue/white screening of

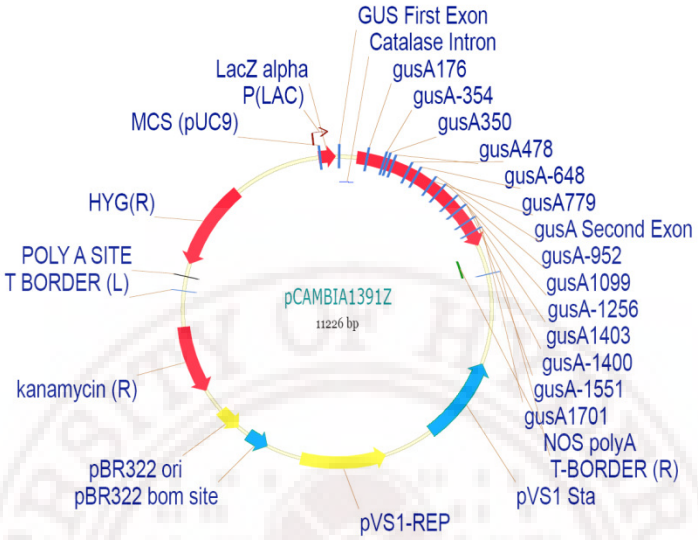


Figure 3.1 pCAMBIA 1391Z vector map

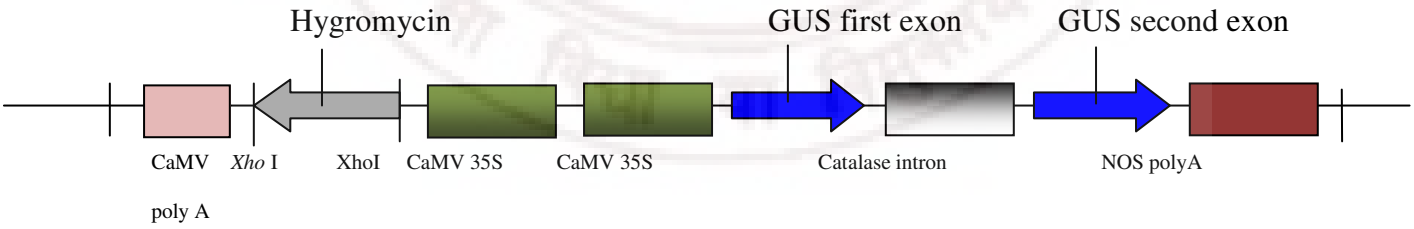


Figure 3.2 pCAMBIA 1391Z+CaMV 35S promoter vector

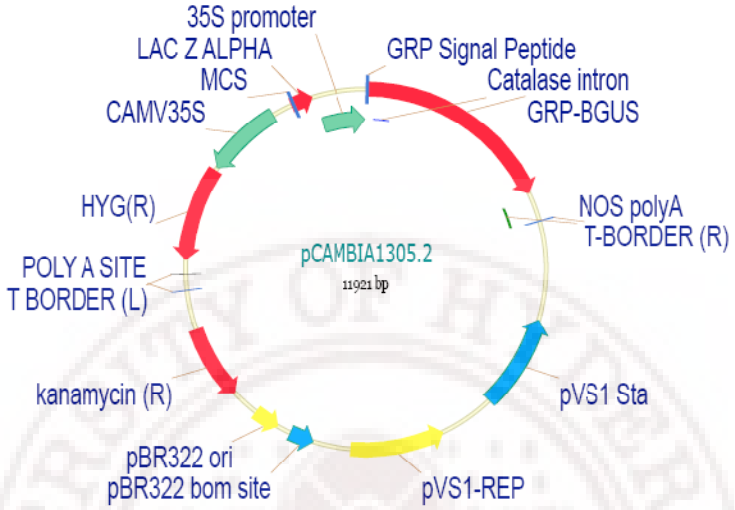


Figure 3.3 pCAMBIA 1305.2 vector map

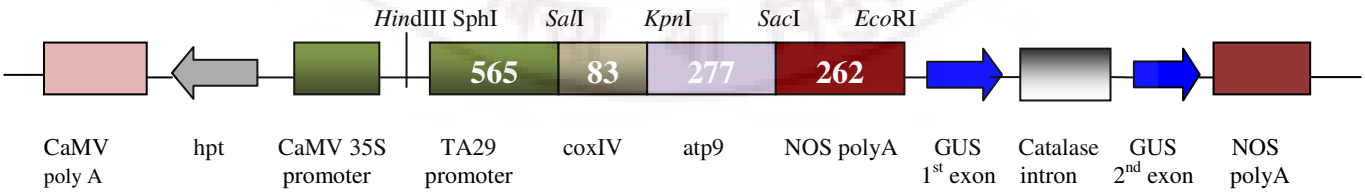


Figure 3.4 pCAMBIA 1305.2 TCAN vector

clones. However, the vector is promoterless version of *gusA*, and hence, CaMV 35S promoter was cloned upstream to the *gusA* reporter gene. The vector plasmid pRT100 upon restriction with *Hin*d III and *Nco* I releases the CaMV 35S promoter (~ 440 bp) which was cloned into pCAMBIA1391Z to obtain pCAMBIA 1391Z+CaMV 35S promoter resulting in a construct with promoter for *gusA* as depicted in Figure 3.2 (Kalyani et al., 2007). Thus, the construct has *gusA* with a catalase intron, for specific expression in eukaryotes. As the reporter gene is now functional, it was used for simple and sensitive analysis of gene function based on its presence in the regenerated plants by GUS histochemical assay.

3.4.2 pCAMBIA 1305.2 TCAN (CAMBIA, Australia)

pCAMBIA 1305.2 is a 11921 bp binary vector with hygromycin resistance gene (*hpt*) for selection in plants and kanamycin resistance gene for selection in bacteria (Figure 3.3). The *atp 9* gene cassette as TCAN has been cloned into pCAMBIA 1305.2 (Yamini, 2007). The vector TCAN has: T-TA 29 (tapetum specific promoter), C- *coxIV* transit peptide, A- unedited *atp 9* gene, N-*nos* terminator. The vector has the fully functional *gusA* reporter gene for analysis of the regenerated plants by GUS histochemical assay (Figure 3.4).

3.5 Bacterial strains

The bacterial strain DH5α of *Escherichia coli* was used to maintain the plasmid while *Agrobacterium tumefaciens* strain LBA 4404 was used for harbouring the plasmid for *Agrobacterium*-mediated transformation study.

3.6 Storage of bacterial cultures

The bacterial cultures of *E. coli* and *A. tumefaciens* were stored as glycerol stocks. To 0.85 ml of

the bacterial culture, 0.15 ml of sterile glycerol (sterilized by autoclaving for 15 min at 121 ºC) was added. The mix was vortexed to ensure even dispersion of glycerol. The culture was transferred to storage tube and tightly plugged. The tubes were frozen in liquid nitrogen and then transferred to -70 ºC for longterm storage. The stored bacteria were revived by scraping the frozen surface of the culture with a sterile needle and then immediately streaked onto the surface of the agar plate containing LB medium with appropriate antibiotics. The frozen cultures were returned to -70 ºC.

3.7 Growth conditions

- **Growth conditions for micro-organisms**
- **Plant growth conditions**

3.7.1 Growth conditions for micro-organisms

The *E. coli* strains were incubated and cultured either in Luria Bertani (LB) broth with continuous shaking at 200 rpm or in Luria Bertani (LB) agar solidified medium at 37 ºC. The *Agrobacterium* strain was grown in liquid Yeast Extract Peptone (YEP) medium with continuous shaking at 180 rpm or in the solid YEP medium at 28 ºC. These bacterial strains were cultured and selected with appropriate selection markers.

3.7.2 Plant growth conditions

The culture plates with the medium and explants were incubated in growth room at temperature of 27±1 ºC under 16/8 hr photoperiod provided by cool fluorescent lights at an intensity of 30 $\mu mol\ m^{-2}\ s^{-1}$.

3.8 Restriction enzymes, Markers

The restriction enzymes *Hin*d III, *Eco*R I, *Xho* I, etc. and their corresponding buffers for restriction analysis used in the present investigation were obtained from Promega and Genei. The DNA markers like lambda DNA, lambda *Hin*d III, *Eco*R I double digest were obtained from Genei (Bangalore, India). Restriction analysis was done to confirm the developed clones. DNA digestion was carried out in a reaction volume of 50 µl with 1 µg of plsmid DNA, 5-10 Units of the appropriate restriction enzyme, 1X final concentration of appropriate reaction buffer, bovine serum albumin (BSA) to a final concentration of 100 µg/ml and the final volume was made up with sterile water and incubated at 37 ºC for 30-60 min. The digested products were run on 1.0% agarose gel to check for restriction digestion.

3.9 Media preparation

- **Bacteriological media**
- **Tissue culture media**

3.9.1 Bacteriological media

The *E.coli* strains were cultured in Luria Bertani (LB) medium obtained readily in the form of powder for making broth or agar (Table 3.1). As there is a tendency for *Agrobacterium* to form clumps in LB due to the presence of Mg^{+2} ions, yeast extract peptone (YEP) medium was used (Table 3.2). The media were sterilized by autoclaving at 121 ºC for 15 min. These were used as broth for growing liquid culture or as agar plates (1.5%) for streaking and maintaining bacteria.

3.9.2 Tissue culture media

The Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as basal medium in the present study. The basal salt medium (Table 3.3) was obtained in ready-made

Table 3.1 Composition of LB medium

Components	g/l
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
Sodium chloride	5.0
pH 7.0, Agar 1.5%	

Table 3.2 Composition of YEP medium

Components	g/l
Bacto yeast extract	5.0
Peptone	10.0
Sucrose	1.0
$MgSO_4.7H_2O$	0.5
pH 7.0-7.2, Agar 1.5%	

Table 3.3 MS media composition

Ingredients	milligrams/litre
Potassium nitrate	1900.0
Ammonium nitrate	1650.0
Magnesium sulphate	180.7
Potassium phosphate monobasic	170.0
Manganese sulphate.H_2O	16.9
Boric acid	6.2
Potassium iodide	0.8
Molybdic acid (sodium salt).$2H_2O$	0.3
Zinc sulphate.$7H_2O$	8.6
Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Ferrous sulphate.$7H_2O$	27.8
Na_2EDTA	37.3
myo-Inositol	100.0
Thiamine-hydrochloride	0.1
Pyridoxine-hydrochloride	0.5
Nicotinic acid (Free acid)	0.5
Glycine (Free base)	2.0
Sucrose	30000
Total gm/litre	34.1

powder form (Himedia, Bangalore) with MS salts, vitamins, sucrose (30 g/l) but devoid of calcium chloride and agar. One medium vial (PT010) was used to make one litre of medium. One vial (34.08 g of dehydrated medium) was suspended in 600 ml distilled water to which calcium chloride (0.4 g) and heat stable components (plant growth regulators, other growth promoting substances) were added and the pH was adjusted to 5.7-5.8 using 1N HCl or 1N NaOH. The solution was made to 1,000 ml with distilled water, to which the gelling agent, agar (0.8%) was added and sterilized by autoclaving at 121 ºC for 15 min. The medium was allowed to cool to about 45 ºC and then the filter sterilized heat labile supplements ($AgNO_3$, antibiotics) were added if, necessary and then the desired amount of medium was dispensed aseptically into sterile culture vessels.

3.9.2.1 Stocks

The stock solutions of hormones, antibiotics, calcium chloride, acetosyringone were prepared and working concentrations were taken from the stocks.

The volume of stock solution required was calculated using the formula-

$$\text{Volume of stock solution required} = \frac{\text{Concentration required} \times \text{Volume of the medium}}{\text{Concentration of the stock solution}}$$

3.9.2.2 Plant growth regulators

The plant growth regulators TDZ, KN, BAP, 2,4-D, NAA, IAA, IBA and PGA were prepared as 1 mg/ml stocks. The required amount of hormone powder was taken into autoclaved glass bottle/vial and then dissolved in 2-3 drops of appropriate solvent (Table 3.4) and then made to the final volume with sterile water and stored at 4 ºC. Heat stable hormones were added prior to autoclaving while, heat labile compounds were filter-sterilized and added to the cooled autoclaved medium. The sterilization method of the used hormones and their corresponding solvents are tabulated in Table 3.4.

Table 3.4 Preparation of stock solution of plant growth regulators

Plant growth regulator		Solvent	Sterilization
2,4,5-Cl_3POP	2,4,5-Trichlorophenoxypropionic acid	Water	Filtration
2,4-D	2,4-Dichlorophenoxyacetic acid	1NaOH/ethanol	Autoclavable
2iP	N^6-[2-Isopentenyl]adenine (6-γ-γ-(Dimethylallylamino)-Purine)		
BAP	6-Benzylaminopurine (N^6-Benzyladenine)	1N NaOH	Autoclavable/ Filtration
IAA	Indole-3-acetic acid (3-Indoleacetic acid)	1NaOH/ethanol	Autoclavable/ Filtration
IBA	Indole-3-butyric acid (4-(3-Indolyl)butanoic acid)	1NaOH/ethanol	Autoclavable/ Filtration
KN	6-Furfurylaminopurine (N^6-Furfuryladenine)	1N NaOH	Autoclavable/ Filtration
NAA	1-Naphthaleneacetic acid (α-Naphthaleneacetic acid)	Water/ ethanol	Autoclavable
PGA	Phloroglucinol (1,3,5-Trihydroxybenzene)	Water	Autoclavable
TDZ	Thidiazuron (N-Phenyl-N'-1,2,3-thiadiazol-5-ylurea)	Water/DMSO	Filtration

Table 3.5 Preparation of antibiotics

Antibiotics	Stock concentration (mg/ml)	Solvent
Cefotaxime Sodium	250	Water
Hygromycin B	20	Water
Kanamycin monosulphate monohydrate	50	Water
Rifampicin	25	DMSO
Streptomycin sulphate	25	Water

3.9.2.3 Silver nitrate

Silver nitrate stock was made as 1 mg/ml with sterile milliQ water and stored at 4 ºC in amber coloured bottle. Silver nitrate is heat labile and hence filter sterilized stock was added to the autoclaved medium under sterile conditions.

3.9.2.4 Antibiotics

The antibiotics (bacterial growth controlling agents) like cefotaxime, kanamycin, streptomycin, rifampicin and hygromycin stocks (Table 3.5) were prepared by dissolving the calculated amount according to the stock concentrations, filter sterilized and stored at 4 ºC.

3.9.2.5 Acetosyringone

Acetosyringone (3',5'-dimethoxy-4'-hydroxy acetophenone) is a potent *vir*-gene enhancer. It is prepared by dissolving in ethanol to the required stock concentration (100 mM), filter sterilized and stored at 4 ºC.

3.9.2.6 Calcium chloride

A quantity of 4.4 g of calcium chloride ($CaCl_2.2H_2O$) was weighed and dissolved in about 50 ml of distilled water and the final volume was made to 100 ml with distilled water. From this, 10 ml solution was added to one litre of medium to make a final concentration of 0.44 g/l.

3.9.2.7 Pectinase

The solution of pectinase (Sigma) was freshly aliquoted and filter sterilized. The required concentration of the sterilized enzyme was added to the medium to make 0.1, 0.5, 1.0 and 2.0% solution.

3.10 Sterilization

The media, beakers, culture vessels (conical flasks, test tubes, petriplates), surgical instruments (forceps, scalpels, syringes), miscellaneous items (filter papers, blotting papers, distilled water),

plastic ware (pipette tips, PCR tubes, eppendorf tubes) were sterilized by autoclaving at 121 ºC for 15 min. Instruments such as forceps, scalpels, etc. used for inoculation were sterilized by dipping in alcohol and flaming before and while using in the laminar air flow. Seeds were surface sterilized by using 0.1% mercuric chloride. Heat labile hormones, antibiotics, etc. were filter sterilized (pore size 0.2 µm) and used.

3.10.1 Seed sterilization

A preliminary study was done to standardize the concentration of mercuric chloride ($HgCl_2$) for surface sterilization of seeds. Concentrations of mercuric chloride used for surface sterilization were 0.05%, 0.1% and 0.2%. In each concentration, the seeds were treated for 8, 10, 12, 15 and 20 min. Seeds treated with sterile distilled water were used as controls. Excess mercuric chloride was removed and traces were washed off by rinsing the seeds four times with sterile distilled water. In the fourth wash, Bavistin (a fungicide) at 1 mg/ml was added to the sterile water. The seeds were then transferred to autoclaved petriplates lined with moistened paper. These petriplates were moistened everyday with sterile distilled water. The germination percentage was recorded after 8 days for deciding the optimum concentration of $HgCl_2$ and the time of treatment. As maximum percentage of germination without any contamination or browning was recorded with the treatment of seeds with 0.1%, $HgCl_2$ for 15 min the same concentration and time were used for surface sterilization of seeds.

Thus, for seed sterilization healthy seeds were selected and washed thoroughly under running tap water for about 1-2 hours with 2-3 drops of labolene (detergent), then treated with 0.1% $HgCl_2$ for 15 min. The solution was discarded and washed three times with sterile water for 5 min each and final wash was carried out for 8 min with Bavistin treated sterile water. After complete removal of the water the seeds were dried on sterile filter paper and inoculated onto half-strength MS media.

3.11 Tissue culture

The regeneration studies were carried out on MS medium supplemented with different hormones. The various parameters tested for regeneration are listed in Table 3.6. The seedling explants (root, hypocotyl, cotyledonary leaf and primary leaf) of 6 to 8-day-old (unless mentioned) were cut into 0.5 cm size under the laminar air flow and transferred to MS agar medium supplemented with different concentrations and combinations of hormones (Table 3.7). The regenerated shoots were subjected to multiplication and elongation on the respective media (Table 3.6). All the cultures were maintained at 27 ± 1 ºC under a 16/8 hr light/dark photoperiod with light at an intensity of 30 µmol m^{-2} s^{-1}. The elongated shoots were subjected to rooting on MS medium supplemented with different auxins (Table 3.6). Subsequently, the rooted shoots were acclimatized in the culture room, transferred to soil and grown in green house for some time (7-10 days) and finally to field conditions. The parameters standardized for optimal regeneration are presented in Table 3.8.

3.12 *Agrobacterium*-mediated transformation

The *Agrobacterium* clone LBA 4404 harbouring the desired gene construct was grown at 28 ºC overnight on YEP containing the appropriate antibiotics (25 mg/l streptomycin, 50 mg/l kanamycin and 25 mg/l rifampicin) and used for co-cultivation. The seedling explants (hypocotyl, root, cotyledonary leaf and primary leaf) from 6 to 8-day-old seedlings were cut into 0.5 cm segments under the laminar air flow into sterile tubes containing *Agrobacterium* culture diluted to a final concentration of 0.5 OD with liquid MS medium. The tubes were kept for 10 min under agitation (180 rpm) and subjected to vacuum infiltration for 30 min. The explants were dried on sterile blotting paper and transferred to MS agar medium supplemented with 0.2

Table 3.6 Tissue culture parameters tested

Parameter	Variables tested
Media	MS+TDZ (0.2-0.5 mg/l)+NAA (0.2-0.5 mg/l)
	MS+TDZ (0.5-1.0 mg/l)+IBA (0.1-0.5 mg/l)
	MS+TDZ (0.5-1.0 mg/l)+IAA (0.1-0.5 mg/l)
	MS+BAP (0.5-2.0 mg/l)+NAA (0.5-2.0 mg/l)
	MS+KN (0.0-1.0 mg/l)+2,4-D (0.2-1.0 mg/l)
Genotype	A-1, Bhima, CO-1, HUS-305, JSF-1, Manjira, Tara
Seedling age	8, 10, 12, 14, 16, 20 days
Shoot multiplication	MS+BAP (0.2 , 0.5 mg/l)
	MS+BAP (0.2, 0.5 mg/l)+thiamine-HCl 4.0 mg/l
Shoot elongation	MS+BAP (0.2, 0.5 mg/l)+KN (0.5, 1.0 mg/l)+/-$AgNO_3$ (1.0 mg/l)
	MS+KN (0.5, 1.0 mg/l)
	MS+KN (0.5, 1.0 mg/l)+2iP (1.0 mg/l)+/-$AgNO_3$ (1.0 mg/l)
Rooting	1/2 MS+NAA (0.5, 1.0 mg/l)
	1/2 MS+IBA (0.5, 1.0 mg/l)+PGA (0.5, 1.0 mg/l)
	1/2 MS+2,4,5-Cl_3POP (1.0, 2.0 mg/l)
	Pulse treatment with IBA, NAA

Table 3.7 Combinations and concentrations of growth regulators incorporated in MS medium

Combinations	Growth regulator	Concentrations (mg/l)				
1	TDZ	0.2	0.5	1.0	2.0	5.0
	NAA	0.0	0.1	0.2	0.5	1.0
2	TDZ	0.5	1.0	-	-	-
	IBA	0.1	0.2	0.5	-	-
3	TDZ	0.5	1.0	-	-	-
	IAA	0.1	0.2	0.5	-	-
4	BAP	0.5	1.0	2.0	-	-
	NAA	0.5	1.0	2.0	-	-
5	KN	0.0	0.1	0.5	1.0	-
	2,4-D	0.2	0.5	1.0	-	-

Table 3.8 Sequential media used for regeneration and transformation study

Step	Basal medium	Agar (g/l)	Growth regulator (mg/l)	Others (mg/l)
Seed germination	MS salts and vitamins	8	-	-
Co-culltivation	MS salts and vitamins	8	0.2 TDZ +0.2 NAA	-
Shoot initiation	MS salts and vitamins	8	0.2 TDZ +0.2 NAA	-
Shoot multiplication	MS salts and vitamins	8	0.5 BAP	4.0 thiamine-HCl +1.0 $AgNO_3$
Shoot elongation	MS salts and vitamins	8	0.5 KN +1.0 2iP	1.0 $AgNO_3$
Rooting	Half-strength MS salts and vitamins	7	1.0 NAA/1.0 mg/l IBA+PGA	-

Table 3.9 Different parameters assessed for enhancing the transformation efficiency

Parameter	Variables tested
Genotype	A-1, HUS-305, A-2, NARI-6, JSF-1, Sharda, JLSF- 414
Seedling age	8, 12, 16, 20 days
Co-cultivation period	2, 4 , 6 days
Bacterial culture density	0.1, 0.2, 0.5 , 1.0 OD
Enzyme treatment (pectinase)	0.1%, 0.5%, 1.0%, 2.0% (v/v)
Potent *vir* gene enhancer (acetosyringone)	50, 150, 200 µM
Explant type	Root, hypocotyl, cotyledonary leaf, primary leaf
Explant injury / wounding response	Mechanical wounding

mg/l TDZ and 0.2 mg/l NAA without any selection pressure and co-cultivated in dark at 28 ºC for 2 days. After 48 hours of co-cultivation, the explants were cultured on the regeneration medium, MS medium supplemented with TDZ and NAA with 250 mg/l cefotaxime and 10 mg/l hygromycin. After 15 days of incubation, the responding explants were transferred to MS medium supplemented with 0.5 mg/l BAP, 250 mg/l cefotaxime and 15 mg/l hygromycin. The proliferated hygromycin resistant shoots were subsequently transferred to 0.2-0.5 mg/l KN. The elongated shoots were subjected to rooting on half-strength MS medium supplemented with 1 mg/l NAA/IBA+PGA, 250 mg/l cefotaxime and 5 mg/l hygromycin. The transformation parameters studied are presented in the Table 3.9.

3.13 Isolation of plasmid DNA

The plasmid DNA was isolated by alkali hydrolysis method (Bimboim and Doly, 1979). For plasmid mini-preparation, *E. coli* cells containing the desired gene construct were inoculated in 10 ml of LB medium with the appropriate antibiotic (kan 50 mg/l) and grown overnight by shaking at 200 rpm at 37 ºC. About 5 ml of overnight grown culture was pelleted out at 12,000 rpm for 1 min. Traces of the medium were also removed and to the pellet 250 µl ice-cold STET (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA pH 8.0, 5% Triton X-100) was added, vortexed thoroughly and repelleted at 12,000 rpm for 1 min. The supernatant was decanted and the pellet was resuspended in 200 µl of ice-cold solution-I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) by vortexing. To this 200 µl of freshly prepared solution-II (200 mM NaOH, 1% (w/v) SDS) was added, mixed gently by inverting and kept on ice for 5 min. To this lysate, 150 µl of ice-cold solution-III (3M potassium acetate, pH 4.8) was added and mixed gently and placed on ice for 5 min. The supernatant was collected by centrifugation at 12,000

rpm for 15 min at 4 ºC. To the supernatant, equal volumes of phenol:chloroform:isoamylalcohol (25:24:1) mixture was added, mixed gently and centrifuged at 12,000 rpm for 15 min at 4 ºC. To the supernatant, equal volumes of chloroform:isoamylalcohol (24:1) was added mixed by inverting and centrifuged as above. The DNA was precipitated by adding two volumes of pre-chilled absolute ethanol and kept at -20 ºC for 15-30 min. Finally, the DNA was pelleted by centrifugation at 12,000 rpm for 10 min. The pellet was washed twice with 70% (v/v) ethanol, air dried and resuspended in 20-25 µl of $T_{10}E_1$ buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA 8.0) and stored at -20 ºC.

3.14 Extraction of plant genomic DNA

The plant genomic DNA was isolated using the modified CTAB procedure (Doyle and Doyle, 1990). The leaf material was ground to a fine powder in liquid nitrogen using pre-chilled mortar and pestle. The powder was suspended in pre-warmed extraction buffer (2% (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.2% (v/v) β-mercaptoethanol added before DNA extraction) and incubated at 60 ºC for 30-60 min in a water bath with occasional mixing by gentle swirling. The mixture was centrifuged at 8,000 rpm for 10 min. To the supernatant, equal volumes of chloroform:isoamylalcohol (24:1) solution was added, mixed gently and centrifuged at 8,000 rpm for 10 min and the extraction was repeated once again. The final aqueous phase was mixed with 2/3 volume of ice-cold iso-propanol and incubated at -20 ºC for 30-60 min. The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded carefully. The pellet was rinsed with 70% (v/v) ethanol twice, air-dried and resuspended in $T_{10}E_1$ buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA 8.0).

3.14.1 Purification of DNA

To the DNA sample, 5 µl of RNaseA (10 mg/l) was added and incubated for 1 hr at 37 ºC. To this, equal volumes of chloroform:isoamylalcohol (24:1) solution was added, mixed gently and the suspension was centrifuged at 12,000 rpm for 10 min. The aqueous phase was mixed with 2/3 volume of ice-cold iso-propanol and incubated at -20 ºC for 30-60 min. The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded carefully. The pellet was rinsed with 70% (v/v) ethanol twice, air-dried and re-suspended in $T_{10}E_1$ buffer and stored at -20 ºC.

3.15 Preparation of *E. coli* competent cells and transformation

A single colony of *E. coli* (DH5α) culture was inoculated into 5 ml of LB broth and incubated overnight with constant shaking at 37 ºC. Two ml of overnight culture was added to 50 ml of LB medium (without any antibiotics) and allowed to grow with vigorous shaking at 37 ºC until the culture grew to an OD_{600} of 0.5. The cells were chilled on ice and the cell suspension was subjected to centrifugation at 4,000 rpm for 5 min at 4 ºC. The supernatant was discarded and the pellet was suspended in 40 ml of ice-cold 100 mM $CaCl_2$ and incubated on ice for 20 min and centrifuged again at 4,000 rpm for 5 min. The pellet was finally resuspended in 3 ml ice-cold 100 mM $CaCl_2$, 15% (v/v) sterile glycerol, mixed and stored at -70 ºC in aliquots of 0.2 ml of competent cells.

For transformation, one microlitre of plasmid DNA (10-50 ng/µl) was added to the competent cells, carefully mixed and incubated on ice for 30 min. The cells were subjected to heat shock at 42 ºC for 90 seconds and then immediately kept on ice for 1 minute. LB medium (0.8 ml) was added to the treated cells and further incubated by shaking at 200 rpm at 37 ºC.

Aliquots (100-200 µl) of the transformed cells were spread on selective plates (LB agar+Kan 50 mg/l) and incubated at 37 ºC overnight.

3.16 Preparation of *Agrobacterium* competent cells and transformation

A single colony of *Agrobacterium* strain LBA 4404 was grown in 5 ml of YEP broth overnight at 28 ºC with constant shaking at 180 rpm. Two ml of the overnight culture was added to 50 ml YEP broth and incubated at the same conditions with vigorous shaking until the culture grew to an OD_{600} of 0.5-1.0. The culture was chilled on ice and then centrifuged at 3,000 g for 5 min at 4 ºC. The supernatant was discarded and the cell pellet was resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ solution and dispensed as 0.1 ml aliquots into pre-chilled eppendorf tubes and stored at -80 ºC for future use.

Transformation of *Agrobacterium* with the recombinant construct pCAMBIA 1305.2 TCAN was carried out following the freeze thaw method by mixing 1 µg of plasmid DNA with the *Agrobacterium tumefaciens* (LBA 4404) competent cells and immediate freezing in liquid nitrogen. The cells were thawed by incubating the eppendorf tube at 37 ºC for 5 min to which 1 ml of YEP medium was added and incubated at 28 ºC for 2-4 hr with gentle shaking. This period allows the bacteria to express the antibiotic resistance genes. Later, the tubes were centrifuged for 30 sec and the supernatant was discarded. The cell pellet was re-suspended in 0.1 ml YEP medium and the cells were spread on YEP agar plate supplemented with 25 mg/l streptomycin, 50 mg/l kan and 25 mg/l rifampicin and incubated at 28 ºC. The transformed colonies that appeared after 2-3 days were subjected to colony PCR and also confirmed by restriction digestion and PCR amplification of the purified recombinant plasmid.

3.17 Extraction of total RNA

RNA was extracted from leaves obtained from the transformed shoots and untransformed shoots (control) using RNA isolation kit (RNAqueous, Ambion) according to the manufacturer's instruction. The leaf material was ground to fine powder in liquid nitrogen using pre-chilled mortar and pestle. To the lysate equal volume of 64% ethanol was added and mixed gently. The lysate mixture was passed through a filter cartridge attached to a collection tube and centrifuged at 10,000 g for 30 sec, the flow-through was discarded and the collection tube was replaced. To this, 700 µl wash solution I was added and centrifuged at 10,000 g for 30 sec and the flow-through was discarded and the above step was repeated twice with 500 µl of wash solution #2/3. The filter cartridge was placed into a fresh collection tube and 40 µl of pre-heated elution solution was pipetted on to the center of the filter. The eluate was recovered by centrifugation at 10,000 g for 30 sec. To elute the traces of RNA left out, 50 µl of second aliquot of hot elution solution was pipetted to the center of the filter and re-spinned at 10,000 g for 30 sec. The RNA obtained was stored at -70 ºC.

3.18 Confirmation of the putative transgenics

The transgenic shoots obtained were initially confirmed for the introduced reporter gene by GUS histochemical assay and later by the PCR analysis. The transformed shoots were checked for the transcription of the gene by RT-PCR analysis. Southern blot analysis was carried out to confirm the stable integration of the foreign DNA.

3.18.1 GUS assay

GUS histochemical assay was performed to confirm the putative transformants (Jefferson, 1987). X-GlcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) is a substrate for β-D-glucuronidase

(GUS) encoded by the *gusA* gene. The substrate is used as a qualitative histochemical marker of specific GUS expression in cells and tissues. X-GlcA is cleaved by GUS at the β1 glucuronic bond between glucuronic acid and the 5-bromo-4-chloro-3-indolyl part of X-GlcA via hydrolysis. This enzymatic cleavage of X-GlcA results in the precipitation of a water insoluble blue dichloro-dibromo-indigo precipitate. This reaction was used to confirm the presence of the transgene. X-GlcA solution was prepared by dissolving 5 mg of X-GlcA cyclohexylammonium salt in 50 µl dimethyl formamide (DMF) and 10 ml of autoclaved 0.05 M phosphate buffer solution at pH 7.0 (Sambrook and Russel, 2001). To this, 30 µl of Triton X 100 was added. Regenerated shoots obtained from the transformation experiments were immersed in GUS staining solution and incubated overnight in dark at 37 ºC. Before analysis, chlorophyll was bleached by extraction in ethanol. Untransformed explants (control) which were cultured under identical conditions served as control.

3.18.2 PCR analysis

For the amplification of the desired DNA fragments, PCR analysis was done with appropriate gene-specific primers and conditions. A reaction volume of 20 µl solution was prepared in sterile 0.2 ml thin-wall PCR tubes with 25 þM each of forward and reverse primers, 50-100 ng of genomic DNA or 5 ng/µl plasmid DNA or bacterial colony (colony PCR) or PCR product or 2-5 µl of first strand cDNA as a template, 100 µM of each dNTP, 1Unit of *Taq* DNA polymerase, 1X concentration of *Taq* DNA polymerase buffer (10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin) and milliQ water to make up the total volume. Each PCR aliquot was mixed and PCR reaction was carried out in Eppendorf Master Cycler. The amplified products were run on 1% agarose gel to check for amplification.

Table 3.10 PCR reaction mix for 20 µl reaction

Components	**Concentration**	**Mix (in 20 µl)**									
		FC	***gus* (µl)**	**FC**	***hpt* (µl)**	**FC**	***Vir* D2 (µl)**	**FC**	***actin* (µl)**	**FC**	***atp 9* (µl)**
Sterile milliQ water	-	-	13.8	-	13.8	-	13.6	-	11.0	-	10.9
Buffer A	10X	1X	2.0	1X	2.0	1X	2.0	1X	2.0	1X	2.0
dNTPs	10 mM	150 µM each dNTP	1.2	150 µM each dNTP	1.2	150 µM each dNTP	1.2	100 µM each dNTP	0.8	100 µM each dNTP	0.8
Forward primer	25 þM for *gus*, *hpt* and v*ir* D2, 2þM for *actin* and *atp 9*	0.38 µM	0.3	0.5 µM	0.4	0.5 µM	0.4	0.2 µM	2.0	0.2 µM	2.0
Reverse primer	25 þM for *gus*, *hpt* and v*ir* D2, 2þM for *actin* and *atp 9*	0.38 µM	0.3	0.5 µM	0.4	0.5 µM	0.4	0.2 µM	2.0	0.2 µM	2.0
Taq DNA polymerase	3 U/µl	1.2 U	0.4	0.48 U	0.2	1.2 U	0.4	0.6 U	0.2	0.9 U	0.3
DNA (ng)	-	50-100 ng	2.0	50-100 ng	2.0	50-100 ng	2.0	50-100 ng	2.0	50-100 ng	2.0

FC- Final concentration

Plasmid DNA was used at a concentration of 5-10 ng

DNA from transformed and non-transformed (control) plants was extracted by CTAB method and used for PCR analysis (Doyle and Doyle, 1990). The PCR was done specifically to amplify the *Uid* A and *hpt* genes. To overrule the possibility of *Agrobacterium* contamination, PCR was performed to check for amplification of the bacterial virulence gene (*vir* D2). PCR was also done for the internal control (*actin*) gene. The PCR mix (Table 3.10) and conditions for the five gene amplifications are presented in Table 3.11. The primer sequences and the fragment sizes are given in Table 3.12. As a positive control, the corresponding plasmid DNA was used as a template. The negative control and plant control were set with no DNA and untransformed plant DNA, respectively. The amplified products were mixed with 1X loading dye, electrophoresed using horizontal electrophoresis unit on 1% agarose gel in 1X TAE buffer (0.5 µg/ml ethidium bromide). The amplified products were visualized and documented in gel documentation unit (Syngene). PCR analysis of transformants harbouring the unedited *atp 9* gene was carried out with TA 29 forward and *nos* reverse primers to amplify the entire gene cassette. The primer sequences and the amplicon sizes are given in Table 3.12.

3.18.2.1 Primers used

The constructs pCAMBIA 1391Z+CaMV 35S in *E. coli* which were already developed in DOR were used in the present study. The gene cassette from *E. coli* was cloned into *A. tumefaciens* and used for transformation studies. The primers used for confirming the obtained clones and the transgenic plants are as listed in Table 3.12.

The melting temperature (T_m) of the primers was calculated by using the formula-

$$T_m = 2\,(A+T)+4\,(G+C)\ ^{o}C$$

Where A, T, G and C represent the number of corresponding nucleotides in the primers.

Table 3.11 PCR conditions

A. PCR profile for *gus* gene amplification

Step	Temperature ºC	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	30
Annealing	64	30 sec	
Extension	72	1.2 min	
Final extension	72	5 min	1
Hold	4	∞	--

B. PCR profile for *hpt* and *vir* D2 gene amplification

Step	Temperature ºC	Time	Cycles
Initial denaturation	94	3-5 min	1
Denaturation	94	30 sec	30
Annealing	60	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	1
Hold	4	∞	--

C. PCR profile for *actin* gene amplification

Step	Temperature ºC	Time	Cycles
Initial denaturation	94	3-5 min	1
Denaturation	92	30 sec	35
Annealing	55	30 sec	
Extension	72	30 sec	
Final extension	72	7 min	1
Hold	4	∞	--

D. PCR profile for *atp 9* gene amplification

Step	Temperature ºC	Time	Cycles
Initial denaturation	92	2 min	1
Denaturation	92	30 sec	35
Annealing	58	30 sec	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	∞	--

Table 3.12 Primers used for amplification of different genes

S.No.	Primer		Primer length (bp)	T_m (ºC)	Sequence	Amplicon size (bp)
1	*gus*	F	21	66	5' ggt ggg aaa gcg cgt tac aag 3'	1200
		R	21	68	5' ggt tac gcg ttg ctt ccg cca 3'	
2	*hpt*	F	21	64	5' cac aat ccc act atc ctt cgc 3'	520
		R	21	66	5' gca gtt cgg ttt cag gca ggt 3'	
3	*vir* D2	F	24	68	5' tca agt aat cat tcg cat tgt gcc 3'	487
		R	21	64	5' gcc gtg acg aag tga aat ctc 3'	
4	*actin*	F	20	56	5' ctt gac gga aag agg tta tt 3'	450
		R	20	60	5' gat cct cca atc cag aca ct 3'	
5	TA 29	F	33	65	5' tac atg cat gca tct agc taa gta taa ctg gat 3'	1184
6	*nos*	R	30	67	5' gga att ctc ccg atc tag taa cat aga tga 3'	

F-Forward, R-Reverse, T_m-Melting temperature

The annealing temperature (T_A) was calculated as-

$$T_A = T_m - 5\ ^{\circ}C$$

The primers were designed in such a way that they do not complement in their secondary structure. The T_m of forward and reverse primers was approximately the same.

3.18.3 RT-PCR

The first strand cDNA synthesis was carried out with random hexamer primers by using RT-PCR Kit (Genei, Bangalore) according to the manufacturer's protocol. In a sterile RNase-free tube, 1-5 µl (10-50 ng) of RNA was taken and the volume was made to 9 µl with sterile RNase free water. To this, 1 µl of random primer was added and placed at 65 ºC for 10 min and then transferred to room temperature for 2 min. The vial was spinned and the reagent mix (1 µl RNase inhibitor, 1 µl 0.1 M DTT, 4 µl RT buffer (5X), 2 µl of 30 mM dNTP mix, 0.5 µl reverse transcriptase, 1 µl of sterile RNase free water) was added. The solution was mixed well and incubated at 42 ºC for 1 hr and the reaction was terminated by heat inactivation at 70 ºC for 10 min. The cDNA obtained was immediately placed on ice. RT-PCR analysis was performed with the cDNA by using *gus* and *hpt* forward and reverse primers under similar conditions as PCR (Table 3.10, 3.11 A, B).

3.18.4 Southern blot analysis

Genomic DNA (2-8 µg) was digested with *Xho* I (8 Units/µg DNA) in appropriate buffer at 37 ºC overnight to release a 1094 bp fragment of the *hpt* gene. The digested DNA was electrophoresed on 0.8% agarose gel for 12-15 hr at 30 V. The gel was then soaked in depurination solution (0.2 N HCl) for 15 min and then subjected to denaturation by soaking in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min. Following this, the gel was

neutralized with neutralizing buffer (1 M Tris-HCl pH 7.5, 1.5 M NaCl) for 30 min. The DNA was transferred to nylon membrane using vacuum blotting apparatus (Hoefer scientific, USA). A window was cut using polythene sheets to exactly fit the gel area of transfer. One sheet of Whatman filter paper No. 3 was cut 1 cm excess of length and breadth. A sheet of Hybond membrane N+ blotting membrane (GE healthcare, Inc.,UK) was cut to the same size as the Whatman filter paper and was marked by cutting at the left top corner to mark the starting of the gel and was soaked in sterile distilled water. The Whatman filter paper and the window were adjusted on the blotting apparatus so that equal part of the filter paper is covered by the window and 10 ml of water was flooded over the filter paper to get it fixed on the stainless steel mesh. The nylon membrane was soaked on sterile distilled water and placed exactly over the Whatman sheet. The window was replaced and the safety clamps were fixed. The gel was carefully placed over the window with the help of a clean X-ray film, taking care that all parts of the window are covered by the gel and that all the parts of the gel carrying DNA is covered within the window. Care was taken to avoid trapping of air-bubbles beneath the membrane. The vacuum pump was connected through a liquid trap and was operated at 35 KPa. A slight depression on the gel could be felt within minutes of starting the vacuum. The alkali transfer solution (0.4 N NaOH) was poured slowly over the gel to cover the gel completely and the transfer was carried out for 25 minutes. The DNA transferred onto the membrane was UV-crosslinked, pre-hybridized at 65 ºC for 6-8 hr in phosphate buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% (w/v) SDS, 1 mM EDTA, pH 7.0 and 1% BSA) and hybridized for 16 hr with p^{32} (CTP) labelled probe (Amersham, Ready-to-go DNA labeling beads). After hybridization, the membrane was washed twice with wash solution I (2X SSC, 0.1% SDS) at 65 ºC for 10 min, followed by wash solution

II (1X SSC, 0.1% SDS) at 65 ºC for 15 min and wash solution III (0.1X SSC, 0.1% SDS) at 65 ºC for 15 min. The membrane was blotted on a Whatman filter paper No. 3 to remove excess of wash buffer and wrapped with saran wrap. The membrane was then placed in X-ray cassette. An X-ray film of appropriate size, marked by cutting at the left top edge was placed and the cassette was closed and kept in -80 °C. After 24 hours, the cassette was taken out, allowed to thaw and the X-ray film was developed.

3.19 Statistical analysis

The tissue culture and transformation experiments were laid out as completely randomized design (CRD) with three replications. Each replication consisted of 10-20 plates with seven explants per plate. Each experiment was repeated at least three times. Data sets with percentages were transformed into arcsine angular transformation values prior to statistical analysis. The data were subjected to statistical analysis using single factor completely randomized block design in order to study the effect of different treatments on regeneration and genetic transformation of safflower. Duncan's Multiple Range Test (DMRT) was carried out for comparison of means at significance (p) level 0.05%. Coefficient of variation (CV) and grand mean are indicated. Analysis of variance and mean separation by DMRT was computed by using Statistical Analysis System (SAS version 9.1).

Chapter 4
Tissue culture

4.1 Results

Tissue culture studies were undertaken in safflower to determine the effect of different hormonal concentrations and combinations for callus induction and shoot regeneration, variation among genotypes, explants and seedling age for shoot regeneration. The genotype used for all the studies was HUS-305 unless otherwise mentioned.

4.1.1 Studies on callogenesis and shoot induction on different media

The response of different explants (root, hypocotyl, cotyledonary leaf and primary leaf) for callusing and shoot induction of cultivar HUS-305 was assessed on MS medium supplemented with various concentrations and combinations of cytokinins (TDZ, BAP, KN) and auxins (NAA, IBA, IAA, 2,4-D).

Segments of root, hypocotyl, cotyledonary leaf and primary leaf were excised aseptically from 8-day-old seedlings of safflower grown on half-strength MS medium. The variation in response of different explants on MS medium supplemented with different concentrations and combinations of hormones were recorded. All the cultured explants showed initial expansion followed by swelling and callus initiation from the cut end, which were directly in contact with the medium surface. The callus observed on different growth regulators varied in colour and texture. The callus obtained on MS medium supplemented with TDZ and NAA was green, with smooth round nodular structures. Increasing concentrations of TDZ showed hyperhydricity. TDZ in combination with IBA and IAA showed light yellow to cream coloured flaky callus. When cultured on BAP and NAA combination, the callus was white to light green in colour, loose and friable. The explants cultured on KN+2,4-D showed only swelling and enlargement at the cut

ends without further development. Media containing TDZ in combination with NAA, IBA, IAA showed morphogenic response shoot formation from all explants investigated but KN in combination with 2,4-D failed to respond.

The results obtained by culturing different explants on MS medium supplemented with various hormonal combinations and concentrations that led to callogenesis and shoot induction are presented in detail as follows-

- Callusing and shoot induction on MS medium supplemented with TDZ and NAA
- Callogenesis and shoot induction on MS medium supplemented with TDZ and IBA
- Effect of TDZ and IAA on shoot regeneration
- Effect of MS medium supplemented with BAP and NAA on shoot regeneration
- Effect of KN and 2,4-D on shoot regeneration

4.1.1 Callusing and shoot induction on MS medium supplemented with TDZ and NAA

Callusing and shoot induction on MS medium supplemented with 25 combinations and concentrations of TDZ (0.2, 0.5, 1.0, 2.0, 5.0 mg/l) and NAA (0, 0.1, 0.2, 0.5, 1.0 mg/l) from different explants (root, hypocotyl, cotyledonary leaf and primary leaf) derived from seedlings of different ages (10-20 days) were studied (Table 4.1). Regardless of media and explants, callus-mediated shoot regeneration (Figure 4.1 A-C) was observed. Callus initiation was observed in all the explants from the cut surfaces which were in direct contact with the media. The callus obtained was greenish in colour with smooth texture, from which green shoot-like structures emerged. All the tested combinations promoted shoot regeneration from the cultured explants but with varied frequencies (Table 4.1).

Table 4.1 Frequency of shoot regeneration from different explants of safflower on MS medium supplemented with different concentrations of TDZ and NAA

Hormone concentration (mg/l)		Explants			
TDZ	**NAA**	**R**	**H**	**CL**	**PL**
0.2	0.0	23.1	21.6	19.9	25.9
0.2	0.1	32.4	26.7	17.6	36.9
0.2	0.2	61.0	65.1	20.6	28.5
0.2	0.5	67.1	68.3	50.0	**82.5**
0.2	1.0	50.0	37.6	33.3	38.6
0.5	0.0	57.3	26.1	27.0	25.9
0.5	0.1	31.0	63.0	52.3	41.0
0.5	0.2	75.1	67.1	58.7	37.6
0.5	0.5	**92.0**	71.1	47.7	56.6
0.5	1.0	88.6	68.0	17.6	51.8
1.0	0.0	24.4	28.2	19.0	39.3
1.0	0.1	59.9	48.6	30.9	41.6
1.0	0.2	58.6	57.3	43.8	64.5
1.0	0.5	47.6	**93.0**	52.5	22.2
1.0	1.0	53.8	51.3	61.0	38.0
2.0	0.0	38.6	58.0	56.7	24.9
2.0	0.1	48.9	82.5	**68.1**	35.8
2.0	0.2	72.1	81.8	55.6	70.8
2.0	0.5	60.3	88.1	50.0	45.6
2.0	1.0	61.0	84.2	32.8	17.6
5.0	0.0	29.1	10.8	11.0	30.8
5.0	0.1	62.0	62.9	20.0	73.1
5.0	0.2	65.6	52.1	14.8	59.6
5.0	0.5	58.7	48.7	21.9	63.1
5.0	1.0	48.2	75.0	11.4	32.9
Grand mean		55.0	57.4	36.7	43.3

R - Root, H - Hypocotyl, CL - Cotyledonary leaf, PL - Primary leaf. Explants were obtained from 10 to 20-day-old seedlings. Data was scored 3 weeks after culture initiation

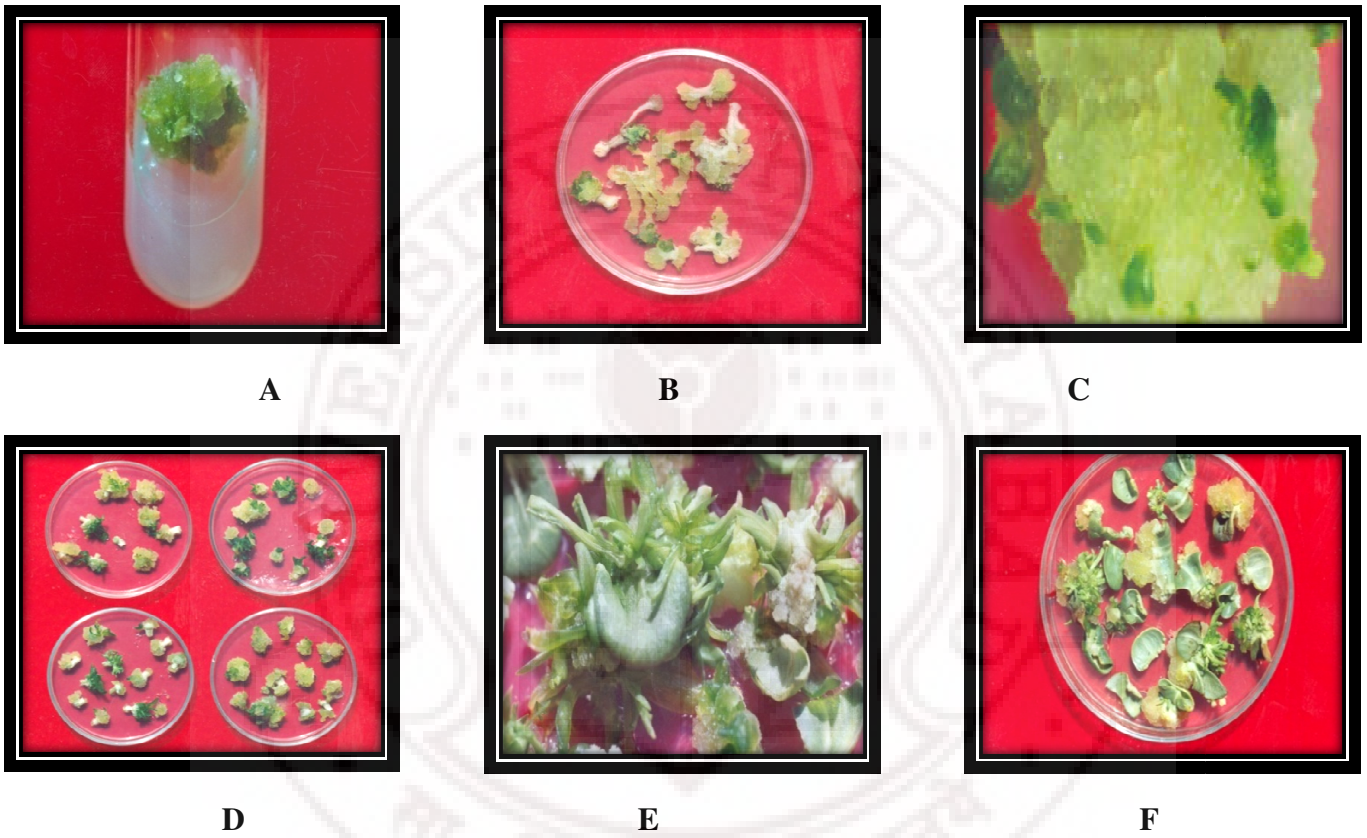


Figure 4.1 Callusing and shoot induction from seedling explants on MS medium supplemented with different concentrations of TDZ and NAA A. Formation of green callus from root explants, **B**. Creamy callus with leafy shoots from root explants, **C**. Green shoot-like structures from hypocotyl derived callus, **D**. Shoot regeneration from hypocotyl explants, **E**. Prolific shoot regeneration from cut margins of cotyledonary leaf explant, **F**. Callusing and shoot induction from cotyledonary leaf explant

Shoot induction frequency ranged from 10.8 to 93.0%. Differential response was observed with different explants and shoot induction was maximum from hypocotyl explants (93%). In case of roots the shoot regeneration frequency varied from 23.1 to 92.0% with an average frequency of 55.0%. In hypocotyl explants, the shoot regeneration frequency varied from 10.8 to 93.0% with an average of 57.4%. In leaf explants the frequency of regeneration was lower as compared to the root and hypocotyl explants and varied from 11.0 to 68.1% with an average of 36.7% in cotyledonary leaf and from 17.6 to 82.5% with an average of 43.3% in primary leaf. Averaged over all the media, hypocotyl explants showed maximum shoot regeneration.

Medium fortified with 0.5 mg/l and 1.0 mg/l TDZ, with varied concentration of NAA showed maximum frequency of shoot regeneration from root (Figure 4.1 A, B) and hypocotyl (Figure 4.1 D) explants. However, no definite trend was observed with regard to the growth regulator combinations on shoot regeneration from cotyledonary leaf and primary leaf tissues. Among the NAA concentrations tried (0 to 1.0 mg/l), 0.5 mg/l proved to be optimal but the concentration of TDZ required in combination varied with the explant type. Frequency of regeneration was maximum from root (92.0%), hypocotyl (93.0%) and primary leaf (82.5%) when 0.5 mg/l NAA was used in conjunction with 0.5, 1.0 and 0.2 mg/l TDZ, respectively. However, for cotyledonary leaf explants (Figure 4.1 E, F), a high ratio (20:1) of TDZ (2.0 mg/l) to NAA (0.1 mg/l) was found to give maximum shoot regeneration (68.1%).

Overall it was observed that shoot induction was obtained on a broad range of TDZ and NAA concentrations. Shoot induction was maximum in hypocotyl tissues followed by root explants. Media with increasing concentrations of TDZ resulted in vitrified and watery shoots from all the explants.

4.1.1.2 Callogenesis and shoot induction on MS medium supplemented with TDZ and IBA

Six combinations and concentrations of TDZ (0.5, 1.0 mg/l) and IBA (0.1, 0.2, 0.5 mg/l) incorporated in MS medium were tested for callogenesis (Table 4.2) and shoot induction (data not presented) from root, hypocotyl, cotyledonary leaf and primary leaf explants of 8-day-old seedlings. On medium supplemented with TDZ and IBA, light yellow to cream coloured flaky callus was observed (Figure 4.2). Shoot induction was observed in few calli but was mostly vitrified (Figure 4.2 A). Averaged over explants maximum frequency of callusing was observed from primary leaf followed by root, cotyledonary leaf and hypocotyl explants. Among the hormonal concentrations tried, 1.0 mg/l TDZ was found to be superior over 0.5 mg/l in terms of frequency of callusing. Statistical analysis of the data revealed that the concentrations of TDZ and IBA had significant influence on frequency of callusing.

Medium supplemented with 1.0 mg/l TDZ+0.5 mg/l IBA was found superior in producing maximum frequency of callusing from root (66.0%) and hypocotyl (40.1%) explants while medium supplemented with 1.0 mg/l TDZ+0.2 mg/l IBA stimulated maximum frequency of callusing from cotyledonary leaf (52.3%) and primary leaf (74.0%) explants, respectively. Further, the varied concentrations of hormones showed variation in callusing among different explants. On medium incorporated with 0.5 mg/l TDZ, the frequency of callusing decreased with increase in IBA concentration from 0.1 mg/l (22.8% and 37.6%) to 0.2 mg/l (4.4% and 13.3%) and increased at 0.5 mg/l (35.6% and 23.4%) in root and cotyledonary leaf, respectively. On the contrary, in hypocotyl explants, the frequency of callusing increased with increase in IBA concentration from 0.1 mg/l (6.7%) to 0.2 mg/l (27.0%) and decreased at 0.5 mg/l (14.5%). However, in primary leaf the frequency of callusing gradually increased with increase in IBA concentration.

Table 4.2 Frequency of callusing (%) from seedling explants of 8-day-old seedlings on MS medium with TDZ and IBA

Concentration of hormone (mg/l)		Explant			
TDZ	**IBA**	**R**	**H**	**CL**	**PL**
0.5	0.1	22.8^{e}	6.7^{f}	37.6^{d}	11.2^{f}
0.5	0.2	4.4^{f}	27.0^{c}	13.3^{f}	25.5^{e}
0.5	0.5	35.6^{d}	14.5^{e}	23.4^{c}	27.8^{d}
1.0	0.1	50.2^{c}	17.6^{d}	46.0^{b}	**74.0^{a}**
1.0	0.2	52.8^{b}	37.6^{b}	**52.3^{a}**	45.0^{c}
1.0	0.5	**66.0^{a}**	**40.1^{a}**	38.9^{c}	50.0^{b}
Grand mean		38.6	24.0	35.3	39.0

R - Root, H - Hypocotyl, CL - Cotyledonary leaf, PL - Primary leaf. The percentage values were converted to arcsin angular transformed values before statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Data scored after 15 days of culture initiation. Experimental design was simple RBD with three replications and about 50 explants per replication

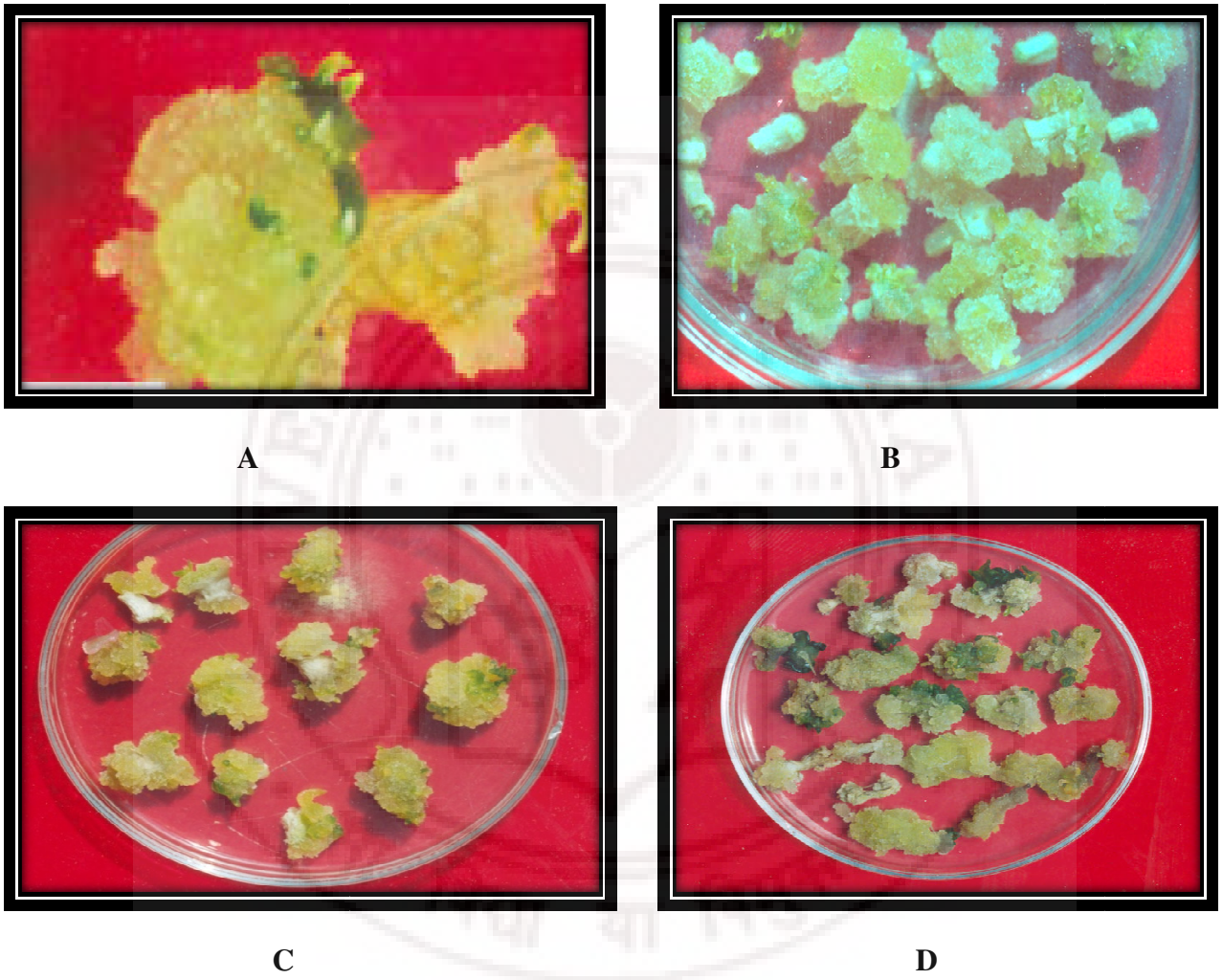


Figure 4.2 Callusing and shoot induction from seedling explants of safflower on MS medium supplemented with TDZ and IBA **A**. Leafy shoots emerging from hypocotyl derived callus, **B, C**. Yellowish flaky calli from hypocotyl with some explants showing regeneration, **D**. Callusing with shoot differentiation from root explants

On media fortified with 1.0 mg/l TDZ, the frequency of callusing increased with increase in concentration of IBA in root and hypocotyl, while in cotyledonary leaf there was increase in frequency of callusing with increase in concentrations from 0.1 mg/l (46.0%) to 0.2 mg/l (52.3%) and decreased at 0.5 mg/l (38.9%). However, in primary leaf a reverse trend that is decrease in frequency of callusing with increase in IBA concentration from 0.1 mg/l (74.0%) to 0.2 mg/l (45.0%) and increase at 0.5 mg/l (50.0%) was observed.

Overall, it was observed that TDZ in combination with IBA promoted callogenesis (with low shoot regeneration) from different explants.

4.1.1.3 Effect of TDZ and IAA on shoot regeneration

The effect of six combinations and concentrations of TDZ (0.5, 1.0 mg/l) and IAA (0.1, 0.2, 0.5 mg/l) was assessed for shoot regeneration from root, hypocotyl, cotyledonary leaf and primary leaf explants of 8-day-old seedlings (Table 4.3). The callus obtained on medium supplemented with TDZ+IAA was yellowish, flaky and was similar to that observed on medium supplemented with TDZ and IBA (Figure 4.3 A, B). Shoot regeneration was observed from all the explants and on all the combinations tested. Shoot regeneration was associated with moderate to heavy vitrification. The frequency of shoot regeneration varied with the type of explant. Regardless of the media, maximum frequency of shoot regeneration was observed from primary leaf followed by root, hypocotyl and cotyledonary leaf explants. With regard to the hormonal concentrations, 0.5 mg/l TDZ in combination with either 0.2 mg/l or 0.1 mg/l IAA was found superior in producing maximum frequency of shoot regeneration from root (57.9%) and primary leaf (66.2%), respectively. However, high concentration of TDZ (1.0 mg/l) in combination with 0.5 mg/l IAA was found to induce maximum frequency of shoot regeneration from hypocotyl (38.1%) and cotyledonary leaf (32.0%) explants.

Table 4.3 Shoot regeneration frequency from different explants of cv. HUS-305 on MS medium supplemented with TDZ+IAA

Concentration of hormone (mg/l)		Explant			
TDZ	**IAA**	**R**	**H**	**CL**	**PL**
0.5	0.1	43.2^{c}	19.8^{f}	30.3^{b}	**66.2^{a}**
0.5	0.2	**57.9^{a}**	37.6^{b}	16.0^{f}	21.8^{f}
0.5	0.5	14.7^{f}	22.8^{d}	25.0^{d}	26.1^{e}
1.0	0.1	48.1^{b}	30.0^{c}	21.5^{e}	33.6^{d}
1.0	0.2	40.0^{d}	22.7^{e}	29.1^{c}	45.3^{c}
1.0	0.5	28.9^{e}	**38.1^{a}**	**32.0^{a}**	60.0^{b}
Grand mean		38.8	28.5	25.6	42.1

R - Root, H - Hypocotyl, CL - Cotyledonary leaf, PL - Primary leaf. The percentage values were converted to arcsin angular transformed values before statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Data scored after 15 days of culture initiation. Experimental design was simple RBD with three replications and about 70 explants per replication

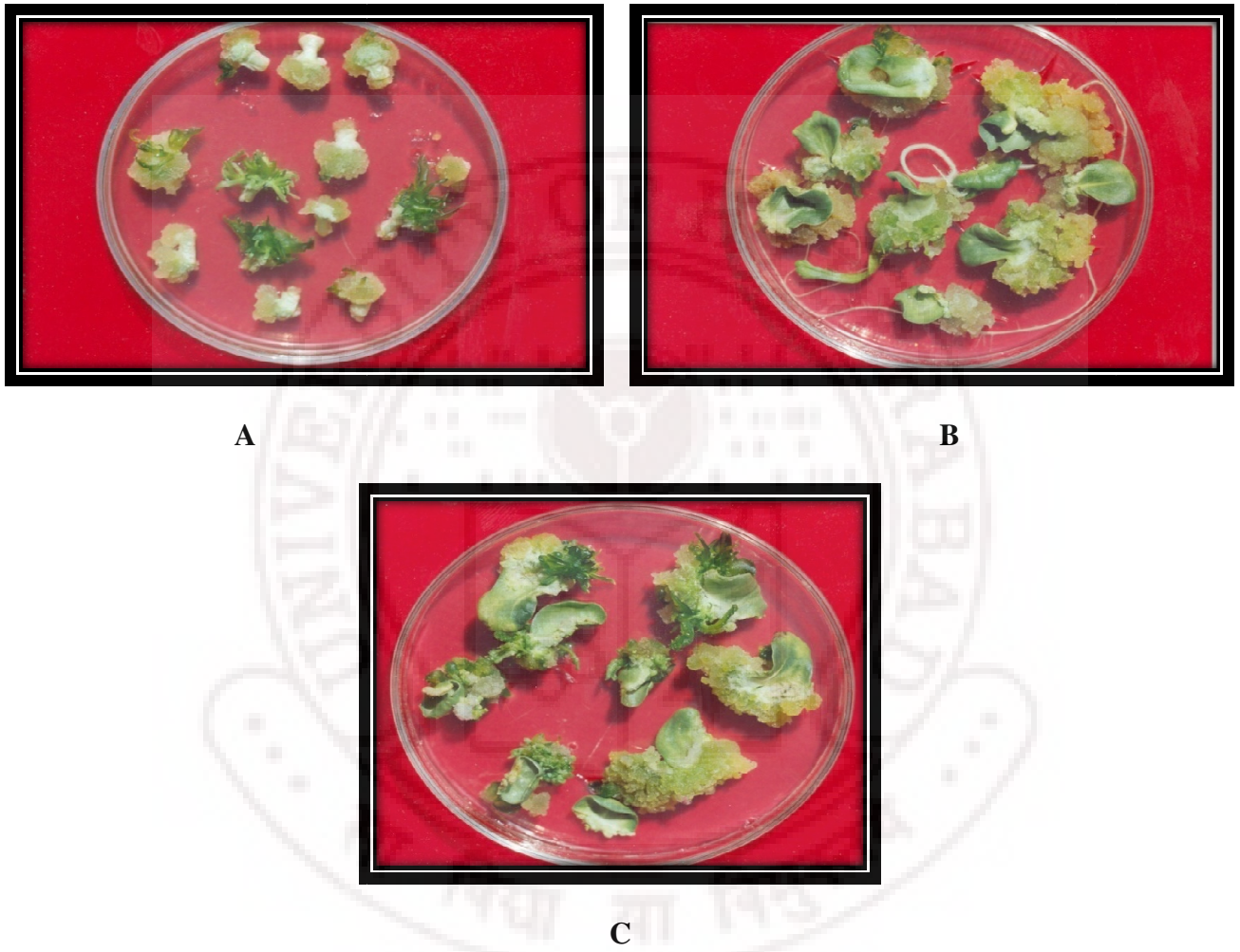


Figure 4.3 Organogenesis from seedling explants on MS medium supplemented with TDZ and IAA A. Shoot proliferation from hypocotyl explants, **B**. Callus and root formation observed from cotyledonary leaf, **C**. Callusing and shoot induction from cut margins of cotyledonary leaf

On medium supplemented with 0.5 mg/l TDZ, the frequency of shoot regeneration increased with increase in IAA from 0.1 mg/l (43.2% and 19.8%) to 0.2 mg/l (57.9% and 37.6%) and decreased at 0.5 mg/l (14.7% and 22.8%) in root and hypocotyl explants, respectively. On the contrary, in primary leaf explants a decrease in frequency of shoot regeneration with increase in IAA concentration from 0.1 mg/l (66.2%) to 0.2 mg/l (21.8%) followed by an increase at 0.5 mg/l (26.1%) was observed. However, there was no definite trend with regard to hormonal combinations and concentrations on cotyledonary leaf (Figure 4.3 C).

On medium supplemented with 1.0 mg/l TDZ, root explants showed a decrease in frequency of shoot regeneration with increase in IAA concentration while in hypocotyl explants, the frequency of shoot regeneration decreased up to 0.2 mg/l IAA and increased henceforth. In case of cotyledonary leaf and primary leaf explants the frequency of shoot regeneration increased with increasing concentration of IAA. Cotyledonary leaf explants showed rooting in most of the combinations tested (Figure 4.3 B). Statistical analysis of the data revealed that different concentration of TDZ and IAA had significant influence on shoot regeneration.

Over all the treatments, it was observed that TDZ in combination with IAA was capable of shoot induction from different explants with primary leaf and root explants showing better response as compared to hypocotyl and cotyledonary leaf explants.

4.1.1.4 Effect of MS medium supplemented with BAP and NAA on shoot regeneration

The effect of MS medium supplemented with six different combinations and concentrations of BAP (0.5 mg/l)+NAA (0.5, 1.0 and 2.0 mg/l), BAP (1.0 mg/l)+NAA (0.5 and 1.0, mg/l) and BAP (2.0 mg/l) + NAA (1.0 mg/l) for shoot regeneration from hypocotyl, cotyledonary leaf and

Table 4.4 Shoot regeneration from seedling explants on MS medium supplemented with BAP+NAA

Concentration of hormone (mg/l)		Explant		
BAP	**NAA**	**H**	**CL**	**PL**
0.5	0.5	NT	18.8^{d}	42.0^{d}
0.5	1.0	**46.7^{a}**	**33.3^{a}**	21.8^{e}
0.5	2.0	29.2^{c}	NT	21.8^{e}
1.0	0.5	27.8^{d}	22.6^{c}	**62.1^{a}**
1.0	1.0	24.0^{e}	24.0^{b}	46.6^{c}
2.0	1.0	31.5^{b}	17.2^{e}	48.5^{b}
Grand mean		31.8	23.2	40.5

H - Hypocotyl, CL - Cotyledonary leaf, PL - Primary leaf. The percentage values were converted to arcsin angular transformed values before statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Data scored after 15 days of culture initiation. Experimental design was simple RBD with three replications and about 70 explants per replication

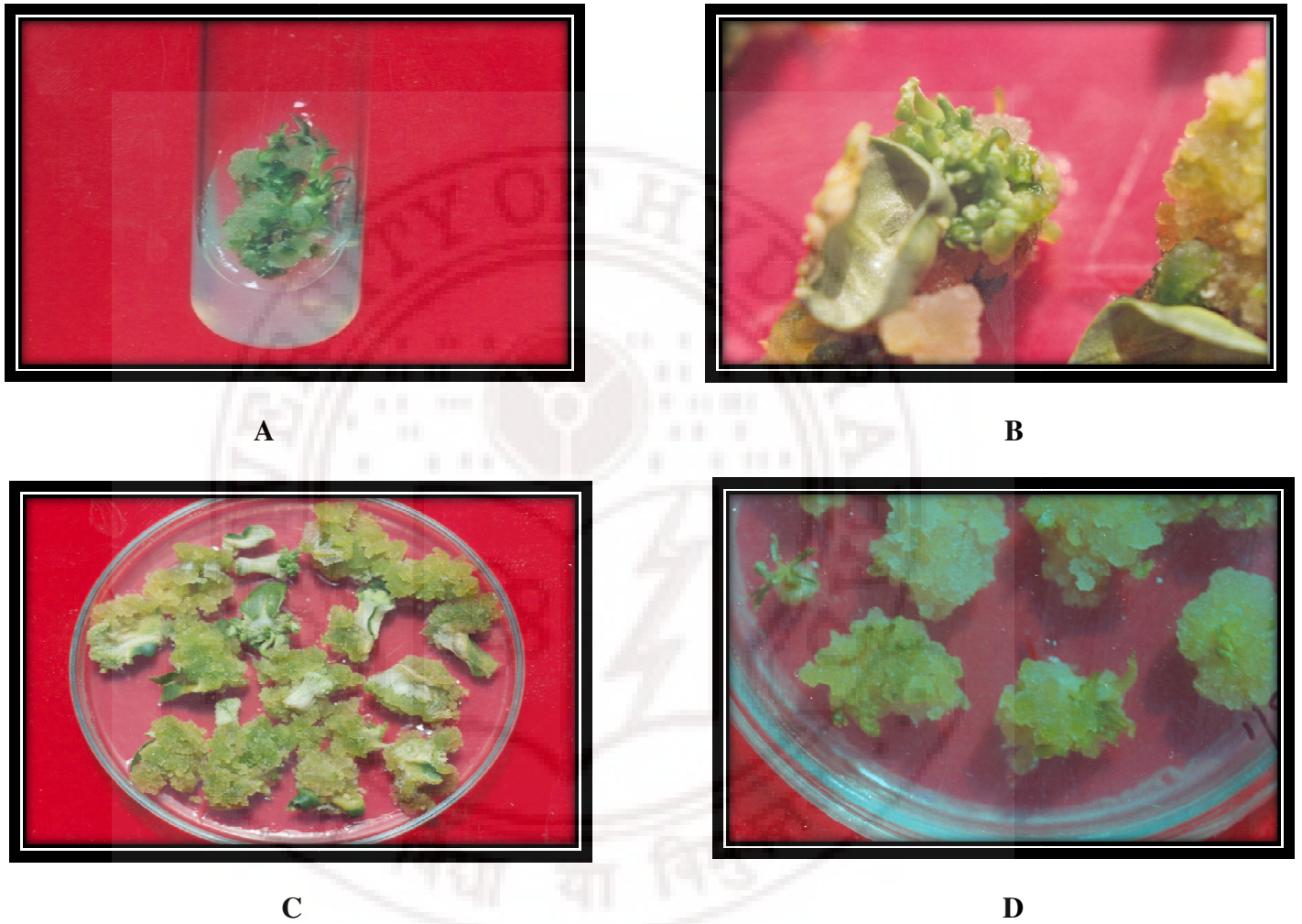


Figure 4.4 Shoot regeneration from seedling explants on MS medium supplemented with BAP and NAA A. Shoot emergence from calli of primary leaf ,**B**. Multiple shoots originating from cotyledonary leaf, **C**. Light green coloured calli from cotyledonary leaf, **D**. Embryo-like structures from hypocotyl derived calli

primary leaf of 8-day-old seedlings was studied. On medium supplemented with different concentrations and combinations of BAP and NAA, white to light green coloured loose, friable, embryogenic callus was observed. Shoot regeneration was observed from all the explants, however, significant variation was observed in terms of the frequency of shoot regeneration. With regard to the three explants, regeneration was maximum from primary leaf followed by hypocotyl and cotyledonary leaf tissues (Figure 4.4). Among the hormonal combinations and concentrations tried, 0.5 mg/l BAP+1.0 mg/l NAA was found to be significantly superior in terms of induction of maximum frequency of shoots from hypocotyl (46.7%) and cotyledonary leaf (33.3%) tissues while a higher cytokinin to auxin ratio (1.0 mg/l BAP+0.5 mg/l NAA) favoured maximum frequency of shoot regeneration from primary leaf (62.1%). The shoot quality varied with the growth regulator concentrations and shoots tended to become hyperhydric with increase in concentration of BAP. Statistical analysis of the data revealed (Table 4.4) significant influence of the various concentrations and combinations of BAP and NAA on shoot regeneration.

Overall, it was observed that medium fortified with BAP and NAA promoted shoot regeneration from seedling explants with primary leaf possessing higher regenerative ability when compared with cotyledonary leaf and hypocotyl segments.

4.1.1.5 Effect of KN and 2,4-D on shoot regeneration

In this experiment, MS medium supplemented with 12 combinations and concentrations of KN (0, 0.1, 0.5, 1.0 mg/l) and 2,4-D (0.2, 0.5, 1.0 mg/l) were assessed for induction of morphogenic response from root, hypocotyl, cotyledonary leaf and primary leaf explants of 8-day-old seedlings. All the explants failed to respond on the media tried. On all the media, the explants

bulged, expanded and remained without any dedifferentiation and differentiation. On media with varying concentrations of KN and high 2,4-D (1.0 mg/l) slight callusing was observed after a long time. Thus, the growth regulator combinations involving KN and 2,4-D failed to evoke morphogenic stimulus in seedling tissues of safflower.

4.1.2 Genotypic variations for callus and shoot regeneration

In this experiment, the efficacy of different genotypes (A-1, Bhima, CO-1, HUS-305, JSF-1, Manjira and Tara) was tested on SV1 (MS+1.0 mg/l TDZ+0.2 mg/l NAA) and SV2 (MS+0.5 mg/l TDZ+0.2 mg/l NAA) media using seedling explants (root, hypocotyl and cotyledonary leaf) from 8 to 12-day-old seedlings for callus induction and shoot regeneration (Table 4.5). The seedling explants of all the genotypes showed callus induction and shoot regeneration but the frequency varied with the genotype.

The callusing frequency from different genotypes ranged from 82.0 to 98.0% (data not presented). Regardless of the genotype, root explants showed maximum frequency of callusing followed by hypocotyl and cotyledonary leaf explants. Response of the genotypes for callusing varied with the media. On SV1 media, the frequency of callusing averaged over explants was maximum with genotype HUS-305 followed by Tara, Manjira, Bhima, CO-1, JSF-1 and A-1 while on SV2 media, the frequency of callusing was maximum with genotype HUS-305 followed by Tara, Manjira, A-1, JSF-1, CO-1 and Bhima.

The tested genotypes differentiated shoots on both the media from different explants but shoot regeneration frequency varied with genotype and explant. Averaged over treatments, maximum frequency of shoot regeneration was recorded in HUS-305 followed by Bhima, A-1, (Figure 4.5 A, B) JSF-1, Tara, Manjira and CO-1. However, the frequency of shoot regeneration

Table 4.5 Frequency of shoot regeneration from seedling explants of different genotypes on SV1 and SV2 media

Genotype	Shoot regeneration frequency (%)					
	SV1			SV2		
	R	H	CL	R	H	CL
A-1	45.0^{c}	NT	21.5^{c}	37.1^{c}	40.5^{b}	20.5^{bc}
Bhima	51.4^{b}	33.9^{c}	34.4^{b}	46.4^{b}	32.4^{d}	26.4^{b}
CO-1	0.0^{f}	11.3^{f}	6.3^{d}	11.6^{f}	NT	5.5^{d}
HUS-305	**78.9^{a}**	**70.0^{a}**	**43.1^{a}**	**68.8^{a}**	**51.8^{a}**	**36.7^{a}**
JSF-1	46.4^{c}	36.6^{b}	18.7^{c}	33.1^{c}	35.9^{b}	26.2^{b}
Manjira	21.8^{e}	31.1^{d}	19.9^{c}	19.0^{e}	21.9^{f}	16.3^{c}
Tara	41.5^{d}	28.6^{e}	17.2^{c}	26.1^{d}	25.0^{e}	14.7^{c}
Grand mean	40.7	35.2	23.0	34.6	34.6	20.9

R - Root, H - Hypocotyl, CL - Cotyledonary leaf. SV1 = MS+1.0 mg/l TDZ+0.2 mg/l NAA, SV2 = MS+0.5 mg/l TDZ+0.2 mg/l NAA. The percentage values were converted to arcsin angular transformed values before statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Data scored after 15 days of culture initiation. Experimental design was simple RBD with three replications and about 70 explants per replication

A **B** **C**

Figure 4.5 Shoot regeneration from different genotypes observed after 10 days of transfer to 0.5 mg/l BAP from SV2 A. Hypocotyl explant of A-1, **B**. Root explant of Bhima, **C**. Hypocotyl explant of HUS-305

from different genotypes on a particular media varied with regard to the explant type. On SV1 media, maximum frequency of shoot regeneration was observed from root explants of the genotypes, HUS-305 (78.9%), Bhima (51.4%), JSF-1 (46.4%), A-1 (45.0%), Tara (41.5%) and hypocotyl of Manjira (31.1%), CO-1 (11.3%) while on SV2 media, the root explants of HUS-305 (68.8%), Bhima (46.4%), Tara (26.1%), CO-1 (11.6%), and hypocotyl of A-1 (40.5%), JSF-1 (35.9%), Manjira (21.9%) showed maximum frequency of shoot regeneration. Shoot regeneration from different explants of the genotypes tested was higher on SV1 media than SV2 media. The root explants of CO-1 failed to respond on SV1 media. The frequency of shoot regeneration from cotyledonary leaf varied from 6.3% (CO-1) to 43.1% (HUS-305) and 5.5% (CO-1) to 36.7% (HUS-305) on SV1 and SV2, respectively. Cotyledonary leaf explants of all the genotypes showed lower shoot regeneration frequency compared to other explants. Among the genotypes, the shoot quality varied with the explant and the media . The genotypes JSF-1 and Bhima produced shoots that showed heavy vitrification, Manjira and Tara showed moderate vitrification while HUS-305, A-1 and CO-1 showed less vitrification on the tested media. Figure 4.5 represents the shoots from genotypes A-1, Bhima and HUS-305 after 10 days of culture on shoot multiplication medium supplemented with 0.5 mg/l BAP.

Averaged over all explants and treatments, HUS-305 was found to be superior in terms of the frequency of callusing and shoot regeneration among the tested genotypes. The frequency values when converted to angular transformed values and subjected to statistical analysis revealed that the effect of genotype on callusing was non-significant while it was significant on shoot regeneration.

4.1.3 Effect of seedling age on shoot regeneration

The influence of seedling age (8, 10, 12, 14, 16 and 20 days) on shoot regeneration from seedling explants (root, hypocotyl and cotyledonary leaf) on SV1 (MS+1.0 mg/l TDZ+0.2 mg/l NAA) and SV2 (MS+0.5 mg/l TDZ+0.2 mg/l NAA) media was studied. There was a drastic decline in frequency of shoot regeneration with increase in seedling age. The frequency of shoot regeneration ranged from 2.0 to 73.2%. Shoot regeneration was maximum in all the explants derived from 8-day-old seedlings. Statistical analysis revealed significant influence of seedling age on shoot regeneration (Table 4.6).

The explants derived from seedlings of different ages showed variation in the frequency of shoot regeneration with respect to explant and media. Frequency of shoot regeneration from all the explants was significantly higher on SV1 when compared to SV2 with 8-day-old seedlings. In case of seedlings derived from 10 to 20-day-old seedlings the frequency of shoot regeneration from hypocotyl and cotyledonary leaf was higher on SV1 than SV2. Conversely, in root explants shoot regeneration frequency was either on par (14, 16 and 20-day-old seedlings) or higher (10 and 12-day-old seedlings) on SV2 when compared to SV1.

Regardless of media and explants, the shoot regeneration frequency was maximum in 8-day-old seedlings and declined with increase in seedling age. Shoot regeneration frequency varied from 34.9 to 73.2% in 8-day-old seedlings followed by 25.1 to 43.4% in 10-day-old seedlings, 12.1 to 35.7% in 12-day-old seedlings, 3.6 to 34.9% in 14-day-old seedlings, 2.0 to 11.6% in 16-day-old seedlings. The root and hypocotyl explants of 20-day-old seedlings showed low shoot regeneration frequency (3.5%) while cotyledonary leaf failed to produce shoots. Medium supplemented with higher concentration of TDZ (SV1) showed better shoot regeneration when compared with lower concentration of TDZ (SV2) but varied with the explant

Table 4.6 Frequency of shoot regeneration from explants of seedlings of different ages on SV1 and SV2 media

Age of seedling (days)	Frequency of shoot regeneration (%)					
	SV1			SV2		
	R	H	CL	R	H	CL
8	**73.2^{a}**	**52.5^{a}**	**50.0^{a}**	**47.9^{a}**	**38.8^{a}**	**34.9^{a}**
10	31.8^{b}	37.1^{b}	28.4^{b}	43.4^{b}	34.7^{b}	25.1^{b}
12	23.4^{c}	35.7^{bc}	13.0^{c}	27.8^{c}	25.3^{c}	12.1^{c}
14	13.6^{d}	34.9^{c}	7.5^{d}	12.0^{d}	23.1^{d}	3.6^{d}
16	9.1^{e}	11.6^{d}	3.2^{e}	7.2^{e}	9.0^{e}	2.0^{e}
20	3.6^{f}	3.2^{e}	0.0^{f}	3.3^{f}	3.1^{f}	0.0^{f}
Grand mean	25.8	29.2	17.0	23.6	25.0	13.0

R - Root, H - Hypocotyl, CL - Cotyledonary leaf. SV1 = MS+1.0 mg/l TDZ+0.2 mg/l NAA, SV2 = MS+0.5 mg/l TDZ+0.2 mg/l NAA. The percentage values were converted to arcsin angular transformed values before statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Data scored after 15 days of culture initiation. Experimental design was simple RBD with three replications and about 70 explants per replication

type. With regard to explants, the root explants of 8-day-old (73.2%) and hypocotyl explants of 10 (37.1%), 12 (35.7%), 14 (34.9%) and 16 (11.6%) day-old-seedlings showed maximum frequency of shoot regeneration on SV1 while the root explants of 8 days (47.9%), 10 days (43.4%), 12 days (27.8%) and hypocotyl of 14 days (23.1%), 16 days (9.0%) old seedlings showed maximum frequency of shoot regeneration on SV2. The frequency of shoot regeneration from cotyledonary leaf varied from 3.2% (16-day-old) to 50.0% (8-day-old) and 2.0% (16-day-old) to 34.9% (8-day-old) on SV1 and SV2 media, respectively.

Overall, a clear decrease in the shoot regeneration frequency was recorded with increase in seedling age irrespective of media and explants. The experiment revealed that explants of younger seedlings responded better than those from older seedlings. The 8-day-old seedling explants were found to be best for shoot regeneration regardless of the media.

4.1.4 Shoot multiplication and elongation

The shoot clusters obtained on MS media with different combinations and concentrations of cytokinins and auxins (TDZ+NAA, TDZ+IBA, TDZ+IAA and BAP+NAA) were transferred on to medium supplemented with the cytokinins BAP, KN, 2iP singly or in combination for shoot multiplication and elongation (Figure 4.6). Shoot multiplication was assessed on medium supplemented with BAP (0.2, 0.5 mg/l) with or without thiamine-HCl (4.0 mg/l)+$AgNO_3$ (1.0 mg/l). Shoot elongation was carried on medium supplemented KN (0.5 and 1.0 mg/l) singly or in combination with BAP (0.2 mg/l) and 2iP (1.0 mg/l)+$AgNO_3$ (1.0 mg/l), respectively.

On MS medium fortified with 0.2 mg/l BAP, the shoots remained stunted and failed to multiply. Shoot multiplication was observed on MS+0.5 mg/l BAP but the shoots appeared vitrified to some extent. Medium supplemented with 0.2 or 0.5 mg/l BAP+4.0 mg/l thiamine-HCl+1.0 mg/l $AgNO_3$ promoted good shoot multiplication and shoots were healthy with no

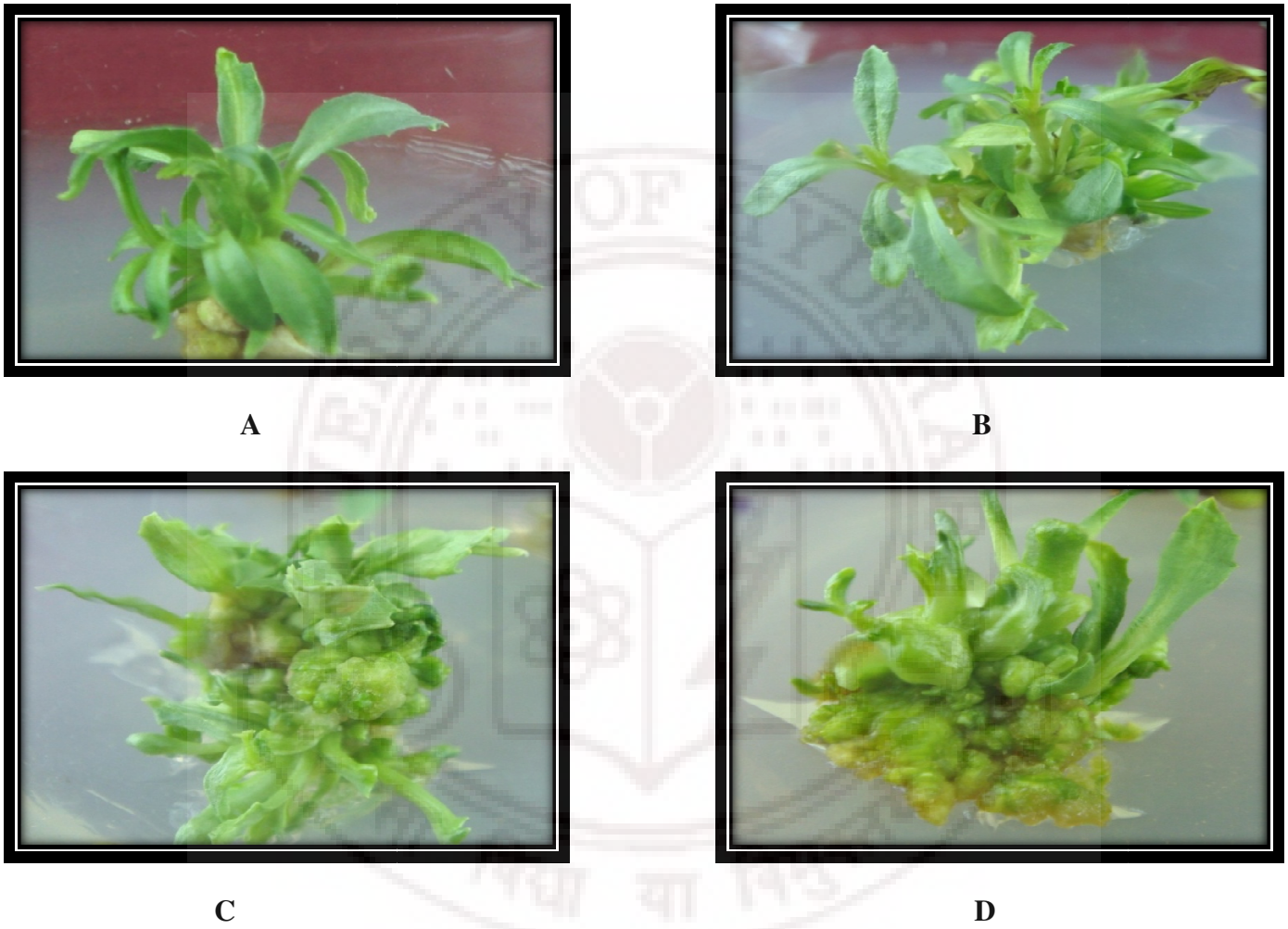


Figure 4.6 Shoot multiplication A. Well developed shoots after 12 days on medium supplemented with 0.5 mg/l BAP+1.0 mg/l $AgNO_3$, **B**. Shoot multiplication on medium supplemented with 0.5 mg/l BAP+4.0 mg/l thiamine-HCl, **C, D**. Multiple shoot buds on medium supplemented with 0.5 mg/l BAP

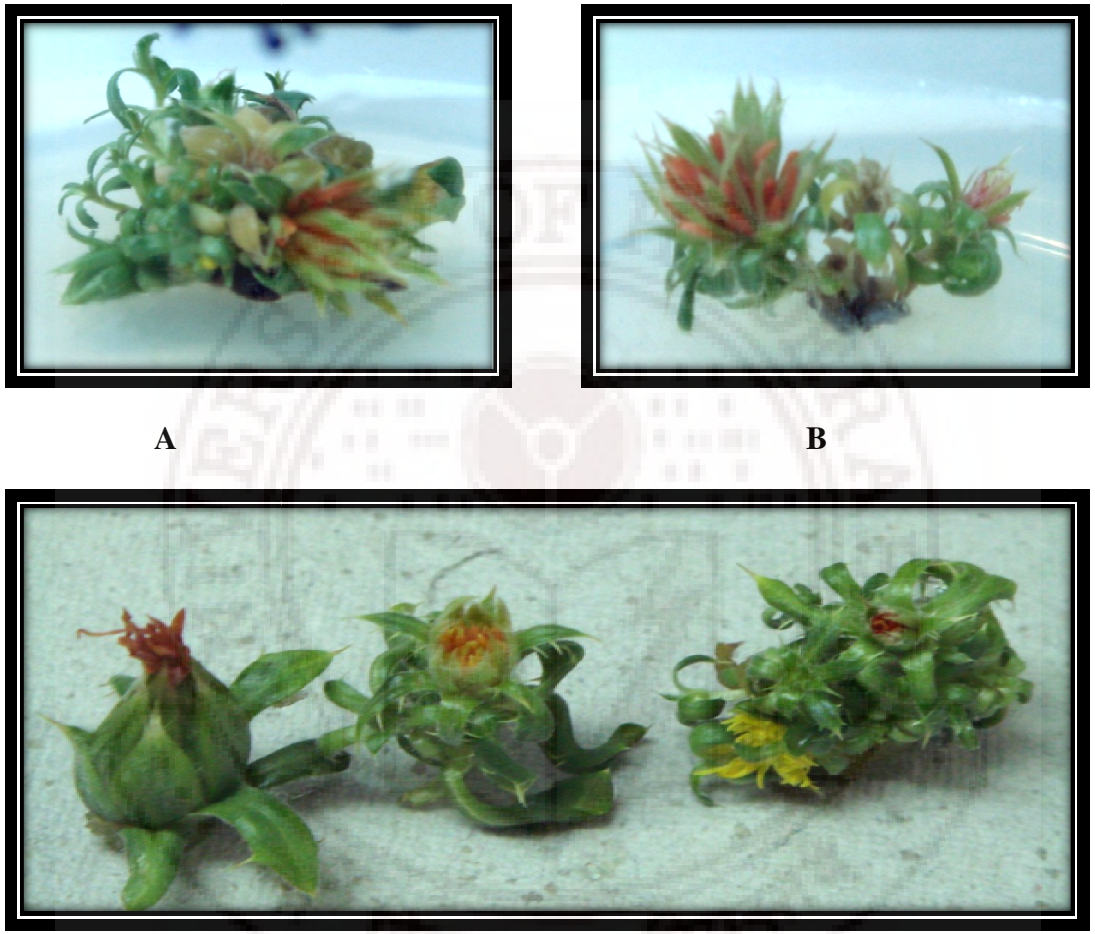
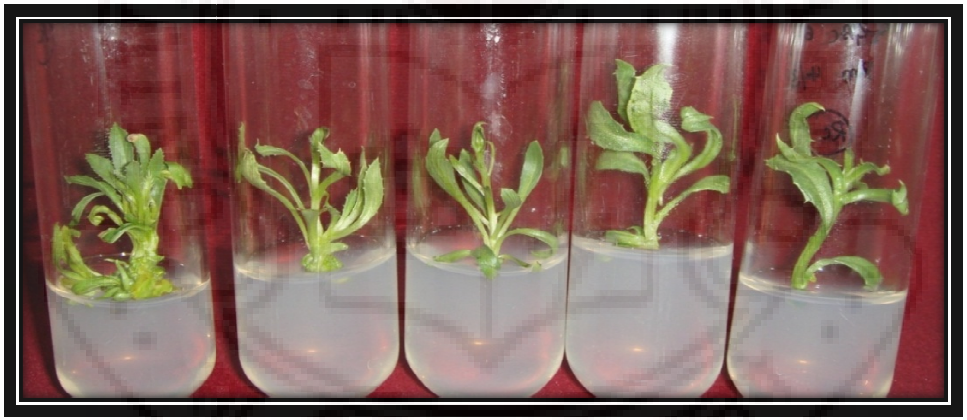


Figure 4.7 *In vitro* flowering on medium fortified with KN A, B. Shoot clusters showing *in vitro* flowering after 15 days of culture on medium supplemented with 0.5 mg/l KN, **C, D**. Unopened capitula observed after 20 days on medium supplemented with 0.2 mg/l KN

A **B**



C

Figure 4.8 Shoot elongation on MS medium supplemented with different combinations of cytokinins A. Elongated shoots after 12 days on medium supplemented with 0.2 mg/l BAP+0.5 mg/l KN, **B**. Elongated shoot with distinct nodes after 10 days on medium supplemented with 0.5 mg/l KN, **C**. Shoot elongation observed after 7 days on MS medium fortified with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$

symptoms of hyperhydricity. Shoots cultured on MS medium supplemented with KN singly or in combination with BAP showed elongation but on exposure to KN for longer periods the growth ceased. On medium supplemented with KN, some shoots produced flowers *in vitro*, but the size was small and the number of florets were reduced compared to the flowers obtained from field grown plants (Figure 4.7). The shoots with flowers ceased to grow further. The medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ promoted good elongation of shoots with well developed leaves (Figure 4.8 C). The elongated shoots after attaining a size of 4-5 cm with distinct nodes and internodes (Figure 4.8 A, B) were transferred to rooting medium for root induction.

Finally, it was observed that medium supplemented with 0.2, 0.5 mg/l BAP+4.0 mg/l thiamine-HCl+1.0 mg/l $AgNO_3$ and 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ were best for shoot multiplication and elongation, respectively.

4.1.5 Rooting

The elongated shoots with 2-3 distinct nodes were transferred on to media with various combinations and concentrations of auxins for rooting. The rooting media tested included half-strength MS media supplemented with NAA (0.5, 1.0 mg/l); IBA (0.5, 1.0 mg/l)+PGA (0.5, 1.0 mg/l); full strength MS media supplemented with 2,4,5-TCl_3POP (1.0, 2.0 mg/l); 7 days incubation on half-strength MS medium fortified with IBA (10 mg/l) and subsequent transfer to hormone free half-strength MS media; pulse treatment with NAA (250 mg/l) or IBA (100 mg/l) for 1, 5, 10 seconds followed by transfer to hormone-free half-strength MS media. Root formation was observed on half-strength MS media fortified with either 1.0 mg/l NAA (70.0%)

A **B** **C**

Figure 4.9 *In vitro* rooting of regenerated shoots A. Root formation on half-strength MS media supplemented with 1.0 mg/l NAA, **B**. Induction of roots on half-strength MS media supplemented with 1.0 mg/l IBA+1.0 mg/l PGA, **C**. Tuft of roots produced with IBA treatment

or 1.0 mg/l IBA+1.0 mg/l PGA. The roots formed on medium supplemented with NAA (Figure 4.9 A) were healthy while those on medium with IBA+PGA were fragile (Figure 4.9 B). Despite the variations in the quality of the induced roots, the rooted shoots from both the combinations sustained acclimatization and survived in the green house. The shoots failed to develop roots on MS media supplemented with 2,4,5-TCl_3POP. A tuft of roots (Figure 4.9 C) were developed on half-strength MS medium supplemented with 10 mg/l IBA for 7 days and later to hormone-free half-strength MS media but the shoots failed to survive following acclimatization. Pulse treatment with NAA and IBA showed browning of the shoot bases. Among all the treatments, tried optimal rooting was obtained on half-strength MS medium supplemented with 1.0 mg/l NAA.

The rooted shoots were acclimatized by transferring them to small pots containing sterile vermiculite and were covered with polythene bag for 4 to 5 days to maintain high humidity. After 5 days, the tips of the polythene bag were cut for ensuring gaseous exchange. The polythene cover was removed after a week and plantlets were allowed to grow in the *in vitro* conditions for 3 to 4 days. The shoots that acclimatized successfully were transferred to soil and maintained under controlled conditions of light and temperature for about one week and later transferred to field conditions. The plants obtained *in vitro* were morphologically similar to the seed raised plants. However, the stems were weak and the leaves were small when compared with the seed raised safflower plants.

4.1.6 Conclusion

The tissue culture regeneration studies were carried out with various hormonal concentrations and combinations, different genotypes, explants, seedling age and the best parameters for

achieving maximum regeneration were established. The study revealed that TDZ in combination with NAA resulted in high shoot regeneration frequency. Among the different genotypes, the cv. HUS-305 proved to be superior with higher frequency of shoot regeneration from all the seedling explants. Among the explants, primary leaf was found to be highly regenerative followed by hypocotyl and root. Explants derived from young seedlings (8-day-old) possessed better regenerative potential when compared to older seedlings (10 to 20-day-old). The schematic representation of the standardized protocol for shoot regeneration is presented in Figure 4.10.

Figure 4.10 Schematic representation of regeneration protocol optimized for seedling explants of safflower

Explants 8-day-old seedling explants

↓ 15 days

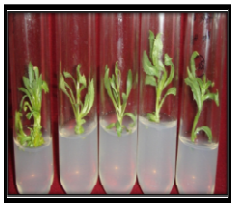
Shoot induction MS+TDZ (0.2-1.0 mg/l)+NAA (0.1-0.2 mg/l)

↓ 15 days

Shoot multiplication MS+BAP (0.5 mg/l)+thiamine-HCl (4.0 mg/l)+$AgNO_3$ (1.0 mg/l)

↓ 15 days

Shoot elongation MS+KN (0.5 mg/l)+2iP (1.0 mg/l)+$AgNO_3$ (1.0 mg/l)



↓ 15 days

Rooting ½ MS+NAA (1.0 mg/l) or IBA+PGA (1.0 mg/l)

4.2 Discussion

Safflower is an important oilseed crop with high medicinal value. The crop has multiple pharmaceutical uses; the oil is used for edible and industrial purposes, the dye obtained from the florets is used as natural food colourant and has medicinal value, the seed is used as bird feed and animal meal, etc. The crop's productivity is limited by low yield and vulnerability to a number of biotic and abiotic stresses. Although resistance to major stresses is available in the primary and secondary gene pools, introgression is limited by sexual crossability barriers and ploidy differences. Therefore, there is a need for development of *in vitro* techniques for genetic improvement of the crop for agronomically important traits. For genetic modification of a crop, an efficient and reliable *in vitro* regeneration system is a prerequisite.

Studies on regeneration in safflower demonstrated the influence of a number of variables, such as, growth regulators, genotype, explant age and explant type (George and Rao, 1982; Tejovathi and Anwar, 1984; Chatterji and Singh, 1993; Orlikowska and Dyer, 1993; Mandal et al., 1995; Baker and Dyer, 1996; Nikam and Shitole, 1999; Radhika et al., 2006) on the morphogenic potential. These studies revealed the amenability of safflower tissues for direct as well as callus-mediated shoot regeneration. Shoot regeneration via embryogenesis (Mandal et al., 1995, 2001; Mandal and Gupta, 2003; Vijaya Kumar et al., 2008a) and organogenesis (Rani et al., 1996; Mandal and Gupta, 2001) were also reported. *In vitro* regeneration systems have been established for Indian (George and Rao, 1982; Tejovathi and Anwar, 1987; Mandal and Gupta, 2001; Radhika et al., 2006; Sujatha and Dinesh Kumar, 2007; Vijaya Kumar et al., 2008a), American (Orlikowska and Dyer, 1993) and Turkish genotypes (Basalma et al., 2008). Protocols were optimized for the production of haploid plants (Prasad et al., 1991) and capitulum induction

in vitro (Tejovathi and Anwar, 1984; Seeta et al., 1999). In most of the *in vitro* plant regeneration studies the development of whole plantlets is constrained by low shoot regeneration frequency and poor rooting ability of the regenerated shoots. Furthermore, the regeneration frequency was highly genotype specific. The aim of the present study was to determine the effect of various combinations and concentrations of different cytokinins and auxins for whole plantlet development in the Indian safflower cultivar HUS-305. It was also aimed to assess genotypic differences among the released cultivars and also to improve the frequency of rooting of *in vitro* regenerants by using various auxin treatments.

4.2.1 Morphogenesis on different media

In the present investigation, the organogenic differentiation of seedling explants of safflower cv. HUS-305 was assessed on a wide range of growth regulator combinations and concentrations (TDZ+NAA-25, TDZ+IBA-6, TDZ+IAA-6, BAP+NAA-6 and 2,4-D+KN-12). In addition, the effect of some critical variables such as, genotype and explant age on the frequency of shoot regeneration was determined. Shoot multiplication and elongation was attempted on MS medium fortified with different combinations and concentrations of BAP, KN and 2iP. Rooting of the *in vitro* shoots was tested on medium supplemented with varied concentrations of NAA, IBA, 2,4,5-TCl_3POP and IBA+phloroglucinol.

Earlier studies on shoot regeneration of safflower reported the superiority of MS medium over other basal media, such as, LS, B_5, Blaydes, Chaleffs, Woods and Hsienmiaos (Anwar et al., 1989; Padmaja et al., 1990; Prasad et al., 1991; Singh, 1991). Hence, in the present study MS salt medium was used as the basal medium.

The effect of TDZ on shoot induction from different seedling explants of safflower was examined as this highly potent cytokinin-like substance facilitated efficient shoot formation in many recalcitrant woody species (Huetteman and Preece, 1993). In herbaceous annual like safflower as well, TDZ proved to be superior over BAP (Orlikowska and Dyer, 1993; Radhika et al., 2006, Basalma et al., 2008). Therefore, the effect of TDZ in combination with NAA, IBA and IAA on shoot regeneration from seedling explants of safflower was studied.

In the study of Orlikowska and Dyer (1993), seedling explants (except roots) and immature embryos of American safflower cultivars, Centennial and Montola produced leafy structures on medium supplemented with TDZ and NAA. However, in this study very low concentrations of TDZ (0.001, 0.01, 0.1 and 1.0 mg/l) were used unlike in the present study where a wider concentration range involving 25 combinations of TDZ (0.2, 0.5, 1.0, 2.0 and 5.0 mg/l) and NAA (0, 0.1, 0.2, 0.5 and 1.0 mg/l) was used. In the present investigation, the amenability of different seedling explants (root, hypocotyl, cotyledonary leaf and primary leaf) for shoot differentiation on most of the tested combinations and concentrations of TDZ and NAA in addition to those reported by Radhika et al. (2006) and succeeded in production of better quality shoots. Initially green callus was observed from the cut ends that were in direct contact with the medium from which greenish shoot-like structures emerged. Callus-mediated shoot regeneration was also observed in the studies of Radhika et al. (2006). Regardless of the explant type, shoot regeneration was observed in almost all the combinations tested with frequencies ranging from 10.8 to 93.0%. Healthy shoots were observed on medium with lower concentrations of TDZ while increasing concentrations of TDZ showed an increase in the hyperhydric shoots and was in agreement with the studies of Radhika et al. (2006).

The morphogenetic response varied with the explant type. It was observed that primary leaf was more amenable to *in vitro* regeneration followed by root and hypocotyl explants. In safflower, most of the previous studies used primary seedling explants (hypocotyl, cotyledon, leaf and root) for *in vitro* shoot regeneration and the frequency of shoot regeneration reported varied from 2.1 to 98.5% (George and Rao, 1982; Zhanming and Biwen, 1993; Tejovathi and Anwar, 1993; Sujatha and Suganya, 1996; Suganya et al., 1997; Mandal and Gupta, 2001, 2003; Radhika et al., 2006; Basalma et al., 2008). In the studies of Singh (1991), Vijaya Kumar and Kumari (2005) and Sujatha and Dinesh Kumar (2007), meristematic regions (axillary bud, shoot apex and cotyledonary node) were used. Anthers were also cultured as a prelude for doubled haploid production and the maximum frequency of shoot regeneration was 30.8% (Prasad et al., 1991). In our study, cotyledonary leaf explant exhibited low shoot regeneration capacity compared to other explants. The low regeneration potential of the cotyledonary explant was reported in safflower (Nikam and Shitole, 1999; Radhika et al., 2006) and in other plant species (Angelini and Allavena, 1989). The availability of primary leaf is not continuous and hence, most of the experiments in the present investigation were carried out with root and hypocotyl explants. Root derived callus of Manjira (Sujatha and Suganya, 1996) and American cultivars (Orlikowska and Dyer, 1993) failed to differentiate shoots but the studies of Radhika et al. (2006) and also the present investigation demonstrated the amenability of root tissues for morphogenesis. In the present investigation, polarity differences in the explants were not observed in contrary to the study of Nikam and Shitole (1999) where apolar placement of all hypocotyl explants except from 2 cm apical region of hypocotyl failed to differentiate shoots.

With regard to TDZ+NAA combination, the most effective medium for shoot induction with minimum hyperhydricity was 0.2-1.0 mg/l TDZ and 0.2 mg/l NAA with hypocotyl and root explants showing maximum response.

The effect of TDZ in combination with IBA was studied based on the report of 33.33% frequency of shoot regeneration from Turkish safflower cultivar, Dincer (Basalma et al., 2008). However, in the present study, TDZ+IBA combination promoted only callogenesis in the tested combinations from all the explants. In combination with high concentration of TDZ, very low frequency of shoot regeneration was recorded but the shoots were hyperhydric. Differences in morphogenic response on BAP+IBA could be attributed to the genotypic differences among the Turkish and Indian safflower cultivars.

The effect of TDZ in combination with IAA on *in vitro* regeneration in safflower was also studied. On medium with TDZ and IAA the seedling explants initially produced callus with varied frequencies. Likewise, in the study of Nikam and Shitole (1999) callus induction was reported in cultivar Bhima on medium supplemented with IAA alone or in combination with cytokinins. In the present study, shoot induction was observed but with varied frequencies (14.7-66.2%) after 15 days of incubation on medium supplemented with TDZ and IAA. The results observed in the study were parallel to the previous observations (George and Rao, 1982; Tejovathi and Anwar, 1987) that combinations of IAA or NAA with BAP induced shoot buds. The shoots induced on TDZ+IAA supplemented media appeared vitrified compared to those obtained on medium supplemented with TDZ+NAA. Cotyledonary leaf explants showed rhizogenesis in most of the TDZ+IAA combinations and similar response was noticed in the studies of Nikam and Shitole (1999).

In the study of the effectiveness of TDZ in combination with different auxins (NAA, IBA and IAA) for shoot regeneration, the hormonal combination with NAA was found to be superior over other combinations with a maximum frequency of shoot regeneration of 93.0%. The regeneration rates obtained are unusually high which can be easily be converted to higher transformation frequencies. The most effective medium for shoot induction with minimum hyperhydricity was found to be 0.2-1.0 mg/l TDZ and 0.2 mg/l NAA.

In addition to the study of the effect of TDZ in combination with auxins (NAA, IBA, IAA) the influence of BAP+NAA was also studied as most of the investigations used this combination for tissue culture experiments in safflower (Tejovathi and Anwar, 1984; Padmaja et al., 1990; Singh, 1991; Zhanming and Biwen, 1993; Orlikowska and Dyer, 1993; Chatterji and Singh, 1993; Tejovathi and Anwar, 1993; Mandal et al., 1995; Sujatha and Suganya, 1996; Rani et al., 1996; Suganya et al., 1997; Rani and Rao, 1998; Nikam and Shitole, 1999; Rao and Rohini, 1999; Mandal and Gupta, 2001, 2003; Mandal et al., 2001; Radhika et al., 2006; Walia et al., 2007). Medium supplemented with BAP+NAA promoted white to green loose embryogenic calli from the cut regions which were in direct contact with the medium surface. Similar response was reported earlier (Mandal et al., 1995; Sujatha and Suganya, 1996; Mandal et al., 2001, 2003; Walia et al., 2007). Shoot regeneration was observed from all the explants in all the combinations of BAP and NAA tried but the frequencies varied with the explants tissue. Shoot regeneration varied from 33.3% in cotyledonary leaf to 62.1% in primary leaf. Shoot regeneration was maximum in primary leaf followed by hypocotyls (46.7%) and cotyledonary leaf, which was in accordance to the study of Sujatha and Suganya (1996) wherein primary leaf (48.9%) produced maximum shoot regeneration followed by hypocotyl (30.7%) and cotyledon (25.3%) explants. Increasing concentrations of BAP showed an increase in hyperhydricity.

Studies of Orlikowska and Dyer (1993), Sujatha and Suganya (1996) and Mandal and Gupta (2001) reported that higher concentrations of BAP showed an increase in the frequency of hyperhydric shoots. Overall, it was observed that BAP at a concentration of 0.5 mg/l produced maximum frequency of shoots (Tejovathi and Anwar, 1993; Mandal et al., 1995, 2001; Mandal and Gupta, 2001, 2003; Radhika et al., 2006). It was observed that medium supplemented with NAA in combination with TDZ was more effective in shoot regeneration than with BAP which could be due to the fact that TDZ is a more potent cytokinin (Huetteman and Preece, 1993).

In the present investigation, the effect of various combinations and concentrations of KN and 2,4-D was also tested. The explants failed to elicit an organogenic response on media supplemented with KN in combination with 2,4-D. Similarly, shoot differentiation failed to occur on medium supplemented with KN+2,4-D in safflower (Radhika et al., 2006) and sunflower (Sujatha and Prabakaran, 2001).

In the present investigation, the frequency of shoot regeneration was 93.0% which indeed is very high compared to the previous studies. George and Rao (1982) reported an average of 8-10 shoot buds from cotyledons in 65.0-70.0% of the cultures. The studies of Ying et al., (1992) reported shoot bud regeneration from 26.0% of leaf-derived calli. Direct shoot regeneration of 48.9% was reported from leaf explants (Sujatha and Suganya, 1996). Nikam and Shitole (1999) reported a maximum of 3-6 shoots from hypocotyl, 5-7 from cotyledon explants and 4-8 from cotyledonary-derived callus. Mandal et al. (1995) reported maximum somatic embryogenesis of 54.0% with a mean number of 14.7±4.1 somatic embryos per responding explant. Shoot regeneration frequency in the studies of Basalma et al. (2008) was 33.3% with 6.5 shoots/explant. Thus, the frequency of shoot regeneration of 93.0% obtained in the present study

was very high compared to all the previous reports and the protocol can easily be converted to high transformation frequency. Furthermore, the number of shoots per responding explant were innumerable (countless) which continued to proliferate on subculture medium as well. TDZ is known to have carryover effect (Huetteman and Preece, 1993) which probably could have facilitated multiple shoots with continous proliferation ability. The study revealed that maximum frequency of shoot regeneration from different seedling explants of safflower cv. HUS-305 can be obtained on medium supplemented with 0.2-1.0 mg/l TDZ and 0.2 mg/l NAA.

4.2.2 Genotypic differences

In the present investigation, genotypic variations for shoot regeneration were assessed which indicated strong influence of the genotype in evoking a morphogenic response. Earlier studies in safflower also reported the effect of genotype on shoot regeneration (George and Rao, 1982; Prasad et al., 1991; Ying et al., 1992; Tejovathi and Anwar, 1993; Rani et al., 1996; Suganya et al., 1997; Radhika et al., 2006). In the present callusing at a frequency of 82.0-98.0% and shoot regeneration at a frequency of 5.5-78.9% was recorded among the genotypes tested on medium with 0.5 and 1.0 mg/l TDZ and 0.2 mg/l NAA. Among the seven genotypes (A-1, Bhima, CO-1, HUS-305, JSF-1, Manjira and Tara) tested maximum frequency of shoot regeneration shoot regeneration was recorded in HUS-305 (58.2%) followed by Bhima (37.5%), A-1 (32.9%), JSF-1 (32.8%), Tara (25.5%), Manjira (21.6%) and CO-1 (8.7%) irrespective of explant and media. In the study of Seeta (1991) on five safflower varieties (Manjira, A-1, Sagaramuthyalu, CO-1 and S-4) Manjira gave better response in terms of callus induction (69.0-96.0%). In the experiments of Suganya et al. (1997) on five different safflower genotypes (Tara, Manjira, A-1,

86-36-93A and 237550) callusing frequency ranged from 77.3 to 94.9% with Manjira showing maximum frequency of callusing. The studies of Ying et al. (1992) and Rani et al. (1996) also reported variation among genotypes in terms of the frequency of callusing.

Genotypic differences existed with regard to the frequency of shoot regeneration as well. George and Rao (1982) reported maximum shoot regeneration frequency of 60.0% and 70.0% from NP-9 black and Th-10 black, respectively. The studies of Prasad et al. (1991) reported maximum frequency of shoot regeneration in Manjira (30.8%) followed by Tara (25.9%), A-1 (21.6%) and S-4 (18.8%) from cultured anthers. Ying et al. (1992) reported that of the ten varieties (not mentioned) tested, frequency of shoot bud induction was highest in the cv. Centennial. The study of Tejovathi and Anwar (1993) revealed that the genotype A-1 (20.0-68.0%) exhibited a wider plasticity for growth regulator requirements, but it showed a slight lesser degree of shoot bud regeneration than cv. Manjira (20.0-87.0%). Furthermore, the experiments of Suganya et al. (1997) on five safflower genotypes (Tara, Manjira, A-1, 86-36-93A and 237550) revealed a shoot regeneration frequency of 2.8-14.6% with Tara (14.6%) showing maximum frequency of shoot regeneration. In the experiment of Mandal and Gupta (2001) wherein eight cultivars (JSI-46, Bhima, A-300, S-144, Saradha, A-1, Tara and Girna) were tested for shoot organogenesis, S-144, Saradha and Tara were identified as highly organogenic with more than 45.0% of the explants responding while A-1 was found to be recalcitrant to regeneration. Further, Mandal and Gupta (2001) reported that the number of shoots produced per explant also varied significantly from 5.0-11.1 and the highest number of shoots was obtained from the cv. Bhima. The differential response of the spineless variety CO-1 to *in vitro* regeneration has been demonstrated by Singh and Chatterjee (1991). Mandal et al.

(2001) reported the profound effect of genotype on somatic embryogenesis wherein out of the eight genotypes (JLSF-1, APR-3, A-1, Bhima, Girna, Tara, S-144 and A-300) studied, highest embryogenic frequency was observed in Girna (51.7%) followed by APR-3 (50.0%), S-144 (41.7%), JLSF-1 (40.0%), Bhima (25.0%), Tara (20.7%) and A-1 (16.7%) while A-300 failed to exhibit embryogenic response. All these studies suggest that there is a marked influence of genotype in safflower regeneration. The studies of Khehra and Mathias (1992), Chengalrayan et al. (1998) and Tang et al. (2003) substantiate the fact that genotype plays a major role in eliciting morphogenic response in oilseed crops. The significant effect of genotype may be related to the effect of genes, sex of the plant, maternal influence, etc. (George, 1993). In close agreement, to the previous studies, the present investigation also revealed that the regeneration potential of safflower depends on the genetic makeup of the donor explant. However, the present investigation proved superior to those reported earlier as the effect of the genotype on shoot regeneration was minimized with the use of TDZ+NAA combination. Finally, among all the tested genotypes, HUS-305 was found to be superior in terms of callusing (98%) and shoot regeneration (78.9%).

4.2.3 Explant age

The experiment on the effect of seedling age on shoot regeneration indicated that the frequency of shoot regeneration varies with the age of the donor explant. Regardless of the explant type, maximum frequency of shoot regeneration was recorded from 8-day-old seedlings (73.2%) with a constant reduction in regeneration frequency with increase in age of the donor explant. Use of 2 to 3-day-old seedlings resulted in shoot bud frequency of 18.0-75.0% (Padmaja et al., 1990).

Mandal and Gupta (2001) reported a maximum frequency of shoot regeneration of 54.4% with 10.2 shoots/explant when 5 to 6-day-old seedlings were used. Regeneration studies with 7-day-old seedling explants reported shoot regeneration with frequencies ranging from 14.6-70.0% (George and Rao, 1982; Zhanming and Biwen, 1993; Anwar et al., 1993; Suganya et al., 1997). In the experiments of Orlikowska and Dyer (1993), cotyledons of 3 to 7-day-old seedlings produced leafy structures, while those from older seedlings (10 to 20-day-old seedlings) failed to respond. The study of Nikam and Shitole (1999) reported that explants from 4 to 7-day-old seedlings were more regenerative than younger (2 to 3-day-old) and older (8 to 15-day-old) seedlings. With regard to somatic embryogenesis, Mandal et al. (2001) reported that 10-day-old cotyledons were more responsive than those derived from 5 or 15-day-old seedlings. Significant differences were also observed among the different segments of cotyledons of the same age and whole cotyledons were more responsive than segmented cotyledons. The influence of explant age on shoot regeneration was also reported in other plant species like sunflower (Paterson and Everett, 1985), bean, pea (Angelini and Allavena, 1989) and blume (Kai et al., 2008). The effect could be due to differences in endogenous hormone levels which change with age of the tissue. Alternatively, older tissues might produce some inhibitory factors like loss of promoting factors (Mroginski and Kartha, 1984), decrease in amount of DNA per nucleus (Hasemann and Schroder, 1982) or fragmentation of DNA (Halperin, 1986).

Finally, the study revealed age-dependent response for *in vitro* regeneration of safflower and that younger tissues were more responsive compared to the older seedlings. Maximum shoot regeneration was recorded from root and hypocotyl explants of 8-day-old seedlings of HUS-305.

4.2.4 Shoot development and rooting

Shoot multiplication and elongation was carried out on various combinations and concentrations of auxins and cytokinins. Shoot multiplication was attempted on medium supplemented with 0.2 and 0.5 mg/l BAP with or without 4.0 mg/l thiamine-HCl+1.0 mg/l $AgNO_3$. On medium fortified with 0.5 mg/l BAP, vitrification of shoots was observed. Our observations were in agreement with the previous studies where high concentrations of BAP resulted in an increase of hyperhydric shoots (Mandal and Gupta, 2001). A similar increase in frequency of hyperhydric shoots with increasing concentration of BAP was reported in other crops (Ziv, 1991). However, on medium with lower concentration of BAP (0.2 mg/l) for a longer time the shoots failed to multiply. Studies of Vijaya Kumar et al. (2008a, 2008b) showed positive effect of thiamine-HCl on shoot regeneration in safflower. Our study also proved that addition of 4.0 mg/l thiamine-HCl to medium along with 0.5 mg/l BAP promoted the growth of well-developed shoots. Furthermore, addition of 1.0 mg/l $AgNO_3$ facilitated the production of healthy shoots. Silver nitrate when added at a concentration of 2.5 mg/l to the elongation medium prevented hyperhydricity and improved shoot health in regenerated safflower shoots (Orlikowska and Dyer, 1993). With regard to embryogenesis, Mandal et al. (2001) reported that embryogenic frequency as well as number of embryos per responding explants was found to increase with an increase in $AgNO_3$ concentration (0-75 µM) and a maximum of 83.3% embryogenic response with 22.1 somatic embryos per responding explant were obtained at 50 µM concentration. The influence of $AgNO_3$ in the production of well-developed shoots was also demonstrated in *Brassica* (Chi et al., 1990) and sunflower (Mayor et al., 2003).

Shoot elongation was tested on medium supplemented with 0.2 mg/l BAP+0.5 mg/l KN and 1.0 mg/l KN. However, on long period of exposure the shoots remained stunted. In addition, some shoots showed precocious flowering and such shoots failed to develop further. The formation of flowers *in vitro* has been reported in safflower previously on medium supplemented with BAP or KN singly and in combination (George and Rao, 1982; Tejovathi and Anwar, 1984; Anwar et al., 1989; Orlikowska and Dyer, 1993; Chatterji and Singh, 1993; Seeta et al., 1999; Nikam and Shitole, 1999; Radhika et al., 2006). In the present study, well-elongated shoots were obtained on medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$. The effect of 2iP in the production of well-elongated healthy shoots in safflower (Orlikowska and Dyer, 1993) and plum (Ambrozic et al., 1991) was reported.

The previous reports suggest that rooting of *in vitro* regenerated shoots of safflower is a continuous obstacle in obtaining whole plantlets. The rooting of shoots in safflower was attempted on hormone-free MS medium (George and Rao, 1982; Singh, 1991; Anwar et al., 1993; Chatterji and Singh, 1993), full-strength MS or half-strength MS supplemented with NAA (George and Rao, 1982; Goyal and Pillai, 1983; Prasad et al., 1991; Orlikowska and Dyer, 1993; Mandal et al., 1995; Sujatha and Suganya, 1996; Baker and Dyer, 1996; Rani et al., 1996; Suganya et al., 1997; Rani and Rao, 1998; Nikam and Shitole, 1999; Mandal and Gupta, 2001; Radhika et al., 2006; Vijaya Kumar et al., 2008a), MS medium supplemented with varying concentrations of sucrose (George and Rao, 1982; Prasad et al., 1991; Radhika et al., 2006), full-strength MS or half-strength MS fortified with IBA, PGA, Putrescine (Orlikowska and Dyer, 1993; Sujatha and Dinesh Kumar, 2007; Vijaya Kumar et al., 2008a), MS medium incorporated with 2,4,5TCl_3POP (Anwar et al., 1989; Tejovathi and Anwar, 1993; Orlikowska and Dyer,

1993; Chatterji and Singh, 1993) and MS medium incorporated with safflower embryo extracts (Tejovathi and Anwar, 1993).

In the present investigation, rooting of elongated safflower shoots was tested on half-strength MS media supplemented with NAA (0.5, 1.0 mg/l); IBA (0.5, 1.0 mg/l)+PGA (0.5, 1.0 mg/l); full strength MS media supplemented with 2,4,5-TCl_3POP (1.0, 2.0 mg/l); 7 days incubation on half-strength MS medium fortified with IBA (10 mg/l) and subsequent transfer to hormone free half-strength MS media; pulse treatment with NAA (250 mg/l) or IBA (100 mg/l) for 1, 5, 10 seconds followed by transfer to hormone-free half-strength MS media. Of all the treatments tried, roots developed on half-strength MS medium supplemented with 1.0 mg/l NAA with a frequency of 70.0% and survived following acclimatization. Similarly, Orlikowska and Dyer (1993), Mandal and Gupta (2001), Sujatha and Dinesh Kumar (2007) reported rooting of regenerated shoots of safflower on half-strength MS medium supplemented with 1.0 mg/l NAA with a frequency of 31.0, 72.0 and 36.0%, respectively. The studies of Sujatha and Suganya (1996) reported rooting of safflower shoots on half-strength MS medium supplemented with 0.5 mg/l NAA with a frequency of 32%. Rani and Rao (1998) reported rhizogenesis on MS medium supplemented with 2 mg/l NAA with a frequency of 43.5%. The differences in the frequency of root induction may be attributed to the differences in endogenous hormone levels among the genotypes. Shoots cultured on medium with IBA and PGA produced roots but were fragile compared to those obtained on medium supplemented with NAA. Sujatha and Dinesh Kumar (2007) reported rooting on half-strength MS medium supplemented with 1.0 mg/l IBA and PGA with a frequency of 58.0% and 85.7% from *C. tinctorius* and *C. arborescens*, respectively. In the present study, incorporation of 2,4,5-TCl_3POP failed to promote rooting unlike in the study of

Tejovathi and Anwar (1993) where it induced rooting at a frequency of 10.0-75.0%. Orlikowska and Dyer (1993) reported that even though rooting on NAA medium was slightly less than 2,4,5-TCl_3POP, roots were healthy and plants survived on transfer to soil. Well-developed roots were obtained on MS medium with 10.0 mg/l IBA incubated for 7 days followed by transfer to half-strength MS medium (Baker and Dyer, 1996), but the shoots failed to survive on acclimatization. In the rooting experiments of Baker and Dyer (1996), 76.0% of shoots rooted after a 7-day exposure to 10 mg/l IBA and stated that additional research was required to improve the survival of rooted shoots when transferred to soil. The dipping of shoots in hormone solution (NAA/IBA) for short period induced roots in some plant species (Economou and Read, 1981; Lane and Dougald, 1982; Fiorino and Leva, 1983). However, in the present study pulse treatment failed to produce roots and resulted in shoot browning from the base, which may be due to the differences in the plant species and in the differences in the endogenous hormone levels.

Thus, it was observed that the shoot multiplication and elongation were better on medium with 0.5 mg/l BAP+4.0 mg/l thiamine-HCl+1.0 mg/l $AgNO_3$ and 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$, respectively while rooting was better on half-strength MS medium supplemented with 1.0 mg/l NAA. The rooted shoots survived acclimatization and remained healthy on transfer to soil.

4.2.5 Conclusion

The present investigation revealed that the *in vitro* regeneration potential in safflower is dependent on the growth regulators supplemented in the media, explant age, explant type and genotype. The protocol followed in the present study showed maximum shoot regeneration

frequency of 93.0%, which is very high compared to all the previous studies on safflower regeneration. With regard to explant, seedling explants viz., root and hypocotyl were found highly regenerative compared to cotyledonary leaf and primary leaf. Furthermore, frequency of shoot regeneration was influenced by age of the seedling and juvenile tissues were found to be highly responsive for *in vitro* regeneration. Genotype had a profound effect on shoot regeneration and the cultivar HUS-305 showed maximum shoot regeneration among the tested genotypes. It was observed that MS medium supplemented with 0.2-1.0 mg/l TDZ+0.2 mg/l NAA showed maximum frequency of healthy shoots. Shoot development and elongation was better on medium with 0.5 mg/l BAP+4.0 mg/l thiamine-HCl+1.0 mg/l $AgNO_3$ and 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$, respectively. The shoots produced roots of good quality on half-strength MS medium fortified with 1.0 mg/l NAA and remained healthy on transfer to soil (after acclimatization). With the standardized protocol shoot regeneration is achieved within 15 days and complete plant was obtained within 2 months of culture initiation.

4.3 Summary

In the present investigation on tissue culture responses of seedling explants of safflower, it was observed that the seedling explants of safflower were amenable for *in vitro* regeneration. The morphogenic potential was influenced significantly by various combinations and concentrations of growth regulators, genotype of donor explant, explant type and age. The current study revealed that the frequency of shoot induction of the seedling explants (root, hypocotyl, cotyledonary leaf and primary leaf) was maximum on MS medium supplemented with TDZ and NAA, and the shoot quality varied with the concentration of the hormones. Higher concentration of TDZ led to the formation of vitrified shoots. The optimal concentrations of TDZ and NAA for maximum shoot induction with minimum hyperhydricity were 0.2-1.0 mg/l and 0.2 mg/l, respectively. The MS medium fortified with TDZ+IBA, TDZ+IAA and BAP+NAA promoted shoot regeneration but the frequency of shoot regeneration was less and/or the shoots appeared watery and vitrified. On medium supplemented with 2,4-D+KN the explants failed to respond.

Among the seven genotypes tested, HUS-305 showed maximum frequency of shoot regeneration. With regard to the explant type, primary leaf was found to be highly responsive for shoot regeneration followed by root and hypocotyl explants, but due to the limitations of availability of primary leaf tissue in 8-day-old-seedlings, the present study was confined to optimization with root and hypocotyl explants. The age of donor tissue also played a major role and juvenile tissues (8-day-old) were found to be highly efficient for *in vitro* shoot regeneration as compared to explants obtained from older seedlings.

Medium supplemented with BAP (0.5 mg/l) in combination with thiamine-HCl (4.0 mg/l) and $AgNO_3$ (1.0 mg/l) favoured maximum shoot proliferation with low or nil hyperhydricity.

Shoot elongation was good on MS medium fortified with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$. Frequency of rooting was maximum on half-strength MS medium supplemented with 1.0 mg/l NAA. The rooted shoots survived following acclimatization and remained healthy on transfer to soil.

Chapter 5

Agrobacterium-mediated genetic transformation

5.1 Results

The tissue culture protocol being optimized served as a prelude for genetic transformation experiments in safflower. For genetic transformation of safflower, the *Agrobacterium*-mediated transformation has been adopted. Among the different genotypes of safflower, HUS-305 a wilt resistant genotype was selected for transformation studies as the genotype showed consistent overall response for shoot regeneration on the media tested. The gene construct used was pCAMBIA 1391Z with CaMV 35S promoter upstream to the *gus* reporter gene (already developed at DOR). The gene construct has hygromycin (*hpt* gene) as plant selection marker and kanamycin resistant gene for selection in bacteria. The *Agrobacterium tumefaciens* strain, LBA 4404 was used as the plant transformation vector. Genetic transformation experiments were carried out to evaluate the efficacy of different parameters that enhance the transformation efficiency. The variables tested were the genotype, seedling age, co-cultivation period, bacterial titer, enzymatic pre-treatment of target tissues, use of compounds that induce *vir*-gene enhancer (acetosyringone), explant type and explant injury. Transformation efficiency was recorded in terms of the GUS positive explants in GUS histochemical assay and the number of shoots obtained after three cycles of selection. Molecular characterization of the transformants was done by PCR, RT-PCR and Southern analysis for confirmation of the presence of the introduced gene.

The root and hypocotyl explants of genotype, HUS-305 were used for the transformation experiments unless mentioned. From the tissue culture studies it was concluded that MS medium supplemented with 0.2-1.0 mg/l TDZ in combination with 0.2 mg/l NAA was optimal for obtaining maximum shoot regeneration. However, lower concentrations of TDZ (0.2 mg/l) favoured the formation of healthy and better quality shoots with less hyperhydricity and hence, in the transformation studies MS medium supplemented with 0.2 mg/l TDZ+0.2 mg/l NAA

(SV17) was used for the first cycle of selection followed by 0.5 mg/l BAP for the second cycle of selection and 0.2 mg/l BAP+0.5 mg/l KN for the third cycle of selection with a selection regime of 10-15-15 mg/l hygromycin for a period of 15 days each. After the release of selection pressure, the putative transformed shoots were transferred to MS medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$. To keep check on the excess *Agrobacterium* growth, cefotaxime at a concentration of 250 mg/l was used.

5.1.1 *Agrobacterium* elimination

Prior to *Agrobacterium*-mediated transformation experiments, the lethal effect of cefotaxime, (the widely used antibiotic for *Agrobacterium* elimination after co-cultivation) was determined by incubating 8-day-old seedling explants on MS medium with cefotaxime concentrations ranging from 100 - 500 mg/l (100, 150, 200, 250, 400 and 500 mg/l). The cultured explants showed no obvious difference on medium with 125-250 mg/l cefotaxime and control (no cefotaxime). At a concentration of 400 mg/l cefotaxime, the explants showed 50.0% browning and at a concentration of 500 mg/l, 95.0% of explants showed browning (data not presented). Hence, cefotaxime at 250 mg/l was incorporated in the media to check *Agrobacterium* growth after co-cultivation and at this concentration, the selected shoots appeared healthy as well.

5.1.2 Sensitivity to hygromycin

The gene construct used in the present study harboured *hpt* gene as plant selection marker for selection on hygromycin. Hygromycin belongs to the family of aminoglycoside antibiotics and is inactivated with hygromycin phosphotransferase. Determination of the optimum dose of the antibiotic is important for the selection of transformed plants. The effect of different

Table 5.1 Sensitivity of root and hypocotyl explants of 8 to 10-day-old seedlings to hygromycin

Concentration of hygromycin (mg/l)	Frequency of browning (%) at different days of culture											
	Root						Hypocotyl					
	2	4	7	10	13	16	2	4	7	10	13	16
0	0.0^{j}	0.0^{i}	0.0^{j}	0.0^{i}	0.0^{h}	0.0^{d}	0.0^{j}	0.0^{j}	0.0^{j}	0.0^{i}	0.0^{i}	0.0^{e}
5	20.5^{i}	28.1^{h}	30.3^{i}	36.4^{h}	44.3^{g}	57.2^{c}	11.6^{i}	15.3^{i}	23.4^{i}	28.3^{h}	38.4^{h}	44.5^{d}
10	26.4^{h}	36.7^{g}	46.2^{h}	53.2^{g}	73.5^{f}	73.5^{b}	14.9^{h}	22.1^{h}	25.9^{h}	44.3^{g}	55.6^{g}	62.3^{c}
20	27.6^{g}	36.7^{g}	52.6^{g}	78.6^{f}	82.4^{e}	**100.0^{a}**	21.2^{g}	25.0^{g}	48.1^{g}	62.3^{f}	77.5^{f}	94.0^{b}
30	36.3^{f}	54.6^{f}	74.3^{f}	81.4^{e}	96.2^{d}	100.0^{a}	25.5^{f}	57.2^{f}	71.2^{f}	93.1^{e}	93.2^{e}	**100.0^{a}**
40	54.2^{e}	70.0^{e}	82.3^{e}	95.0^{c}	98.3^{c}	100.0^{a}	38.3^{e}	69.4^{e}	83.1^{e}	94.2^{d}	95.5^{c}	100.0^{a}
50	56.3^{d}	69.2^{d}	85.5^{d}	94.2^{d}	99.7^{b}	100.0^{a}	57.0^{d}	71.3^{d}	85.3^{d}	95.3^{c}	95.3^{d}	100.0^{a}
60	69.2^{c}	77.8^{c}	91.6^{c}	98.3^{b}	**100.0^{a}**	100.0^{a}	62.3^{c}	78.1^{c}	89.4^{c}	97.0^{b}	97.8^{b}	100.0^{a}
80	74.1^{b}	81.2^{b}	94.3^{b}	**100.0^{a}**	100.0^{a}	100.0^{a}	71.3^{b}	82.0^{b}	95.5^{b}	**100.0^{a}**	**100.0^{a}**	100.0^{a}
100	**91.3^{a}**	**91.3^{a}**	**100.0^{a}**	100.0^{a}	100.0^{a}	100.0^{a}	**84.2^{a}**	**91.3^{a}**	**100.0^{a}**	100.0^{a}	100.0^{a}	100.0^{a}
Grand mean	45.6	54.6	65.7	73.7	79.4	83.1	38.6	51.2	62.2	71.4	75.3	80.1

Hygromycin sensitivity of untransformed root and hypocotyl explants on MS medium recorded after 2, 4, 7, 10, 13 and 16 days of culture initiation. The percentage values were converted to arcsin angular transformed values before statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

Figure 5.1 A Kill curve for hypocotyl explants on hygromycin

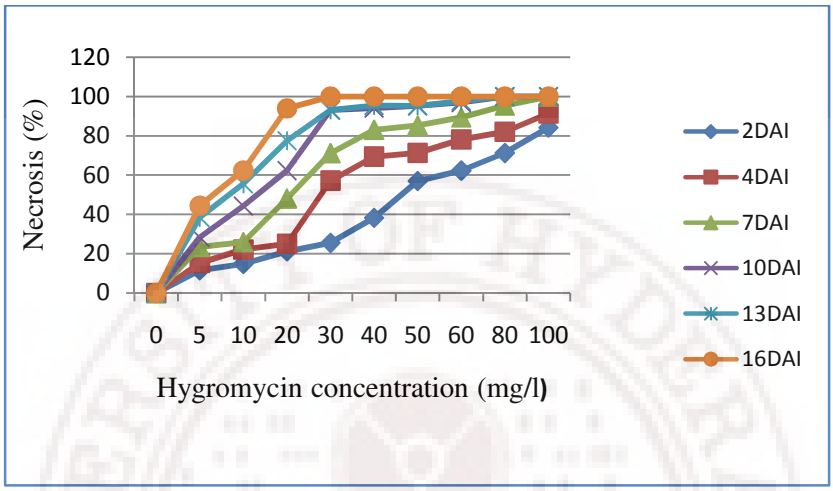
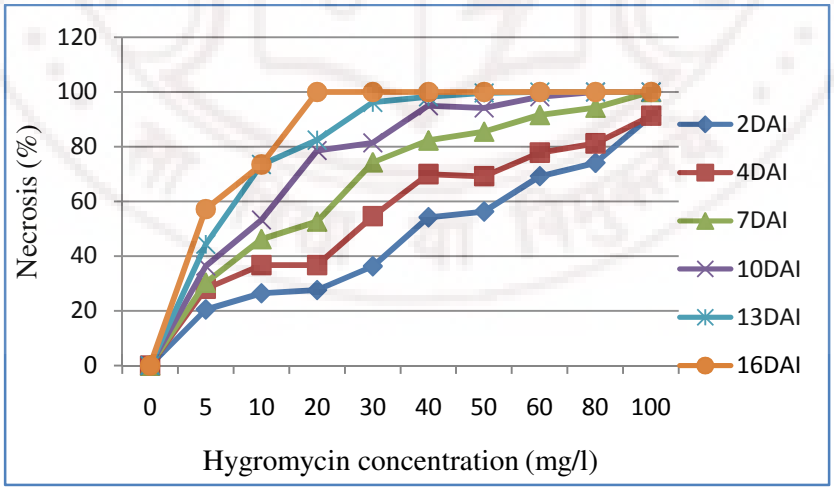


Figure 5.1 B Kill curve for root explants on hygromycin



concentrations of hygromycin (5, 10, 20, 30, 40, 50, 60, 80 and 100 mg/l) on survival of root and hypocotyl explants of 8 to 10-day-old seedlings cultured on MS media was determined. The number of explants showing browning and necrosis on different concentrations of hygromycin was recorded after 2, 4, 7, 10, 13 and 16 days of culture initiation (Table 5.1, Figure 5.1 A, 5.1 B). The frequency values when converted to angular transformed values and subjected to statistical analysis revealed significant effect of different concentrations of hygromycin on the survival of the explants. With an increase in hygromycin concentration there was a drastic decline in the survival frequency of both the explants. Likewise, with increase in hygromycin concentration the time taken for explant browning was reduced.

The explants cultured on control media (without hygromycin) showed 100.0% survival. The hygromycin concentration of 5 mg/l showed 44.3% and 38.4% browning of root and hypocotyl explants, respectively after 13 days of culture initiation. The LD_{50} for root explants (53.2%) and hypocotyls (55.6%) was obtained with hygromycin at 10 mg/l after 10 days and 13 days of culture, respectively. At a concentration of 20 mg/l, root explants showed 100.0% browning (LD_{100}) while 94.0% of the hypocotyls showed browning after 16 days of culture. The hygromycin concentration of 30 mg/l showed browning of more than 50.0% in both the explants within 4 days of exposure and about 100.0% necrosis by the end of 16 days. The hygromycin concentration of 40 mg/l showed about 80.0% browning from both the explants within 7 days of exposure. Higher concentrations of hygromycin showed browning of the explants within 2 days of culture.

From this study, the optimum dose of hygromycin was determined as 10 mg/l for both root and hypocotyl explants, as 50.0% survival was observed even after 13 days on hygromycin. A concentration of 20 mg/l hygromycin was considered as LD_{100} for both the explants.

5.1.2.1 Antibiotic Selection regime

To establish a selection regime for hygromycin, 8-day-old seedling explants were subjected to transformation with bacterial culture of 0.5 OD and co-cultivated for 2 days under dark and subjected to selection pressure of 10-15-20 mg/l on hygromycin pressure for 12 to 15 days on each concentration of hygromycin. Untransformed explants were kept as regeneration control on selection free (no hygromycin) media and as transformation control on selection (with hygromycin) media. After the first cycle of selection, the explants in the regeneration control plates showed callusing and shoot initiation while those on the transformation control plates showed slight browning without any differentiation (Figure 5.2). After the second cycle of selection, the explants on transformation control plates showed complete browning and necrosis. The GUS histochemical assay was performed after each cycle of selection and the frequency was calculated based on the GUS positive shoots among the surviving shoot cultures. To detect the presence of endogenous GUS-like activity in the cultured tissues, the uninfected tissues were also subjected to GUS histochemical assay (Figure 5.3). GUS anaysis showed no staining of uninfected tissues while explants subjected to transformation and selection were positive and showed blue colouration. The frequency of GUS positive shoots (number of GUS positive shoots over the total number of shoots incubated in X-gluc) obtained after the first cycle of selection was 89.9% while it was 98.5% after second and third cycles of selection. The frequency of shoot regeneration as well as the number of shoots per surviving explant decreased with each cycle of selection. To decrease the selection pressure on the putative transformants the concentration of hygromycin in the third cycle of selection was decreased to 15 mg/l and a selection regime of 10-15-15 mg/l was followed. The frequency of putative transformants on selection regime of 10-15-15 mg/l hygromycin showed no significant difference from that of 10-15-20 mg/l as evident from GUS analysis.

A B

Figure 5.2 Regeneration control vs transformation control after 7 days of culture of hypocotyls from 8-day-old seedlings on MS medium supplemented with 0.5 mg/l BAP A. Untransformed explants on medium without selection, **B**. Untransformed explants on medium with selection agent (15 mg/l hygromycin)

A B C

Figure 5.3 GUS histochemical assay of explants after 10 days of second cycle of selection (hygromycin 15 mg/l) A. Shoots from regeneration control showing no blue colouration, **B**. Transformation control explants showing no blue colouration, **C**. Putative transformed shoots showing blue colouration

Finally, to maintain a balance between the elimination of untransformed and transformed tissues a stringent selection regime of 3 cycles with hygromycin 10-15-15 mg/l was adopted for all transformation studies.

5.1.3 Factors influencing transformation

5.1.3.1 Genotype

For identification of the best genotype suitable for transformation, the seedling explants (root, hypocotyl) of seven safflower genotypes (A-1, A-2, HUS-305, JLSF-414, JSF-1, NARI-6 and Sharda) were subjected to transformation. The 8 to 10-day-old seedling explants were subjected to transformation with bacterial culture of 0.5 OD and co-cultivated for 2 days under dark conditions and transferred to selection media. The GUS frequency in putative transformants recovered after the final cycle of selection ranged from 35.0 to 100.0% in different genotypes (Figure 5.4). The frequency of GUS positive shoots from some genotypes was high but the regeneration frequency of the putative transformants remained a check point. The genotype HUS-305 was found to be superior among all the tested genotypes in terms of high transformation efficiency as evident from GUS staining. The frequency of the hygromycin resistant calli (after first cycle of selection) and putative transformed shoots obtained following the second and third cycles of selection from both the explants were tabulated (Table 5.2). The frequency of hygromycin resistant calli after the first cycle of selection and the putative transformed shoots after the second and third cycles of selection was subjected to statistical analysis which revealed that the genotype plays a significant role in transformation.

Table 5.2 Frequency of callusing and shoot regeneration from transformed explants of different genotypes

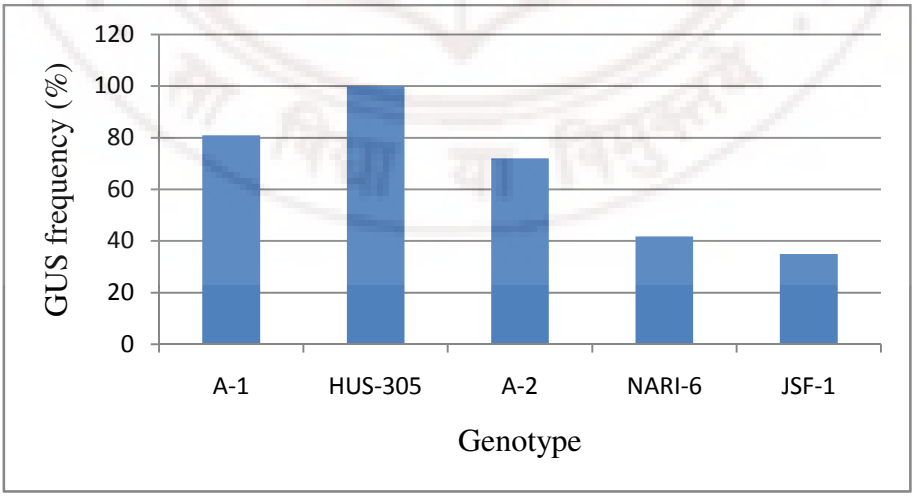
Genotype	Selection I*		Selection II**		Selection III**	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
A-1	40.1^{c}	32.3^{c}	22.1^{c}	24.4^{c}	9.5^{d}	10.9^{c}
A-2	31.1^{d}	34.6^{d}	21.1^{c}	24.9^{c}	24.9^{c}	11.2^{c}
HUS-305	**70.4^{a}**	**70.7^{a}**	**60.3^{a}**	**59.6^{a}**	**51.4^{a}**	**47.6^{a}**
JLSF-414	28.9^{e}	28.9^{e}	17.0^{d}	10.9^{f}	9.5^{d}	5.6^{d}
JSF-1	38.5^{c}	32.4^{c}	17.8^{d}	21.5^{d}	11.0^{d}	13.1^{c}
NARI-6	52.5^{b}	47.1^{b}	29.7^{b}	33.4^{b}	15.9^{b}	19.2^{b}
Sharda	27.5^{e}	23.5^{e}	13.6^{e}	16.6^{e}	6.1^{e}	4.0^{d}
Grand mean	41.3	38.5	25.9	27.3	18.3	15.9
CV (%)	1.9	2.2	2.2	3.1	4.5	9.2

Transformation was carried out with bacterial titer of 0.5 OD_{600} and co-cultivation for 2 days. Data was recorded after 15 days of incubation on each cycle of selection. The percentage values were converted to arcsin angular transformed values prior to statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

* Data represents frequency of callusing following first selection

** Data represents frequency of shoot regeneration after the second and third cycles of selection

Figure 5.4 GUS assay of different genotypes



Assessment of the *Agrobacterium*-mediated transformation efficiency of different genotypes on the basis of GUS positive shoots. Data represents average frequency of GUS expression in the root and hypocotyl explants

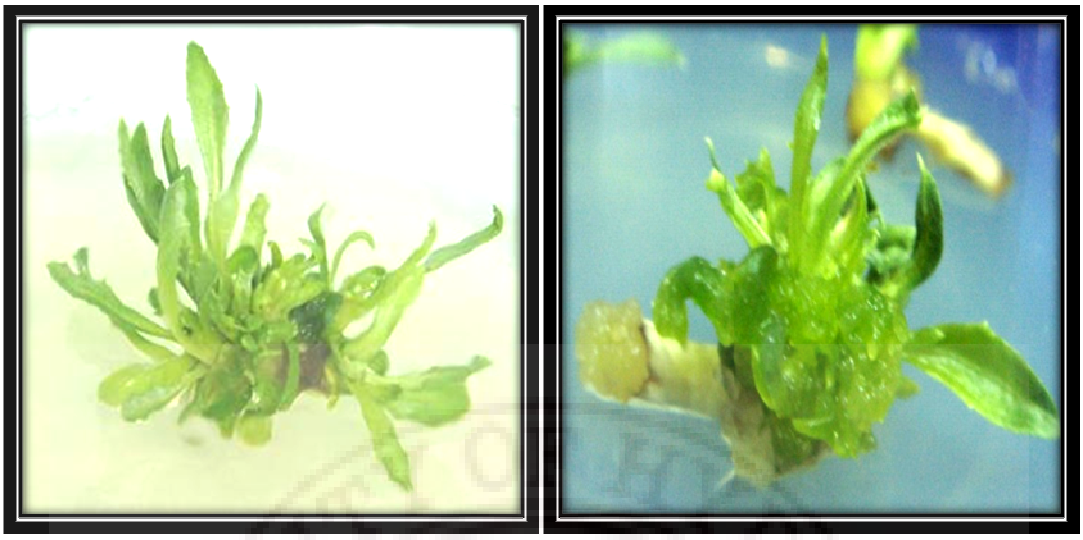


Figure 5.5 Selection of transformed shoots from hypocotyl explants on MS medium supplemented with 0.5 mg/l BAP and 15 mg/l hygromycin (second selection) A. Putative transformed shoots from HUS-305, **B.** Putative transformed shoots from NARI-6 showing vitrification

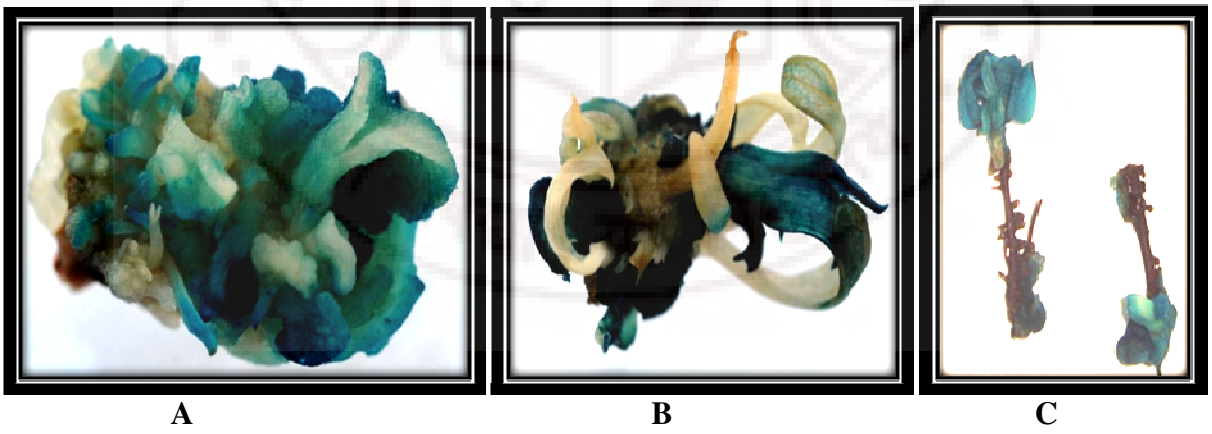


Figure 5.6 Transformed shoots showing blue colouration following GUS staining after second selection A. Transformed shoots obtained from hypocotyl explants of NARI-6 showing uniform blue colouration, **B.** Transformed shoots obtained from hypocotyl explants of HUS-305 showing some unstained sectors, **C.** The shoots obtained from root explants of JSF-1 showing blue colouration

After the first cycle of selection, the two explants from all the genotypes showed callus formation from the cut ends, but with varying frequencies (Table 5.2). Shoot induction and proliferation of putative transformants was observed on the second and third cycles of selection (Figure 5.5). After the third cycle of selection, the genotype HUS-305 showed maximum frequency of putative transformed shoots from both the explants (51.4% from root, 47.6% from hypocotyl) followed by NARI-6 and A-2 with an average frequency of 18.0%. The genotypes JSF-1 and A-1 had an average frequency of 12.0% and 10.0% of the putative transformants while the genotypes Sharda and JLSF-414 had poor transformation and the frequency was less than 10.0% after the third cycle of selection. The transformed shoots recovered from JSF-1 and NARI-6 showed moderate to heavy vitrification. GUS frequency was maximum in HUS-305 (100.0%) followed by A-1 and A-2 with 81.0% and 72.0%, respectively (Figure 5.6). The GUS frequency in the genotypes NARI-6 and JSF-1 was less than 45.0% while the transformants obtained from genotypes JLSF-414 and Sharda were not tested (less number of transformants were obtained).

Overall, it was observed that the genotype had a significant influence on transformation. Among all the genotypes, HUS-305 proved to be superior in terms of its high regeneration ability and maximum frequency of GUS positive shoots from both the explants even after three cycles of selection. The other genotypes being tested responded well initially but failed to produce healthy shoots after final selection.

5.1.3.2 Seedling age

The influence of seedling age on transformation was assessed by subjecting root and hypocotyl explants from seedlings of different ages (8, 12, 16 and 20 days) to transformation with bacterial

Table 5.3 Frequency of callusing and shoot regeneration from transformed explants of seedlings of different ages

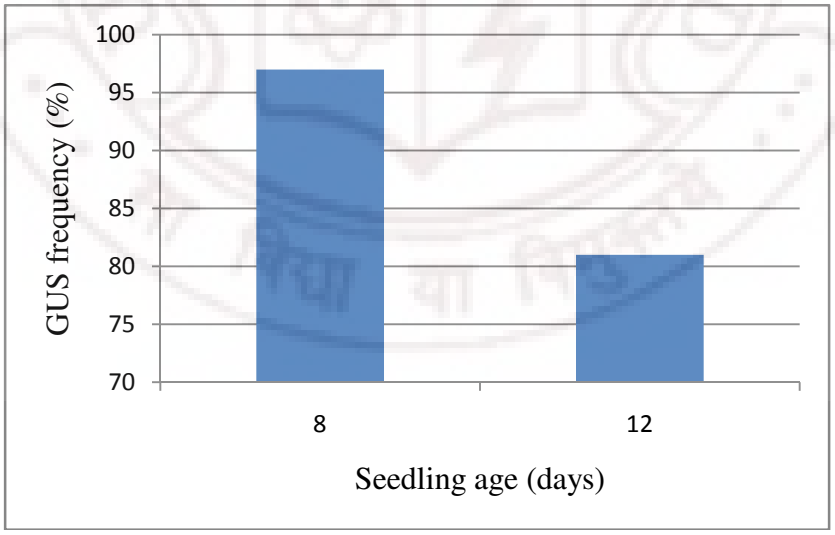
Seedling age (days)	Selection I*		Selection II**		Selection III**	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
8	**65.8[a]**	**68.3[a]**	**55.8[a]**	**60.6[a]**	**50.9[a]**	**53.0[a]**
12	27.3[b]	22.8[b]	17.6[b]	20.5[b]	7.3[b]	9.6[b]
16	13.7[c]	14.5[c]	7.4[c]	11.1[c]	5.9[b]	6.0[c]
20	5.3[d]	8.0[d]	4.6[d]	4.1[d]	1.6[c]	1.6[d]
Grand mean	28.0	28.4	21.3	24.1	16.4	17.5
CV (%)	7.1	7.8	3.8	6.1	14.1	4.2

Transformation was carried out with bacterial titer of 0.5 OD_{600} and co-cultivation for 2 days. Data was recorded after 15 days of incubation on each cycle of selection. The percentage values were converted to arcsin angular transformed values prior to statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

* Data represents frequency of callusing following the first cycle of selection

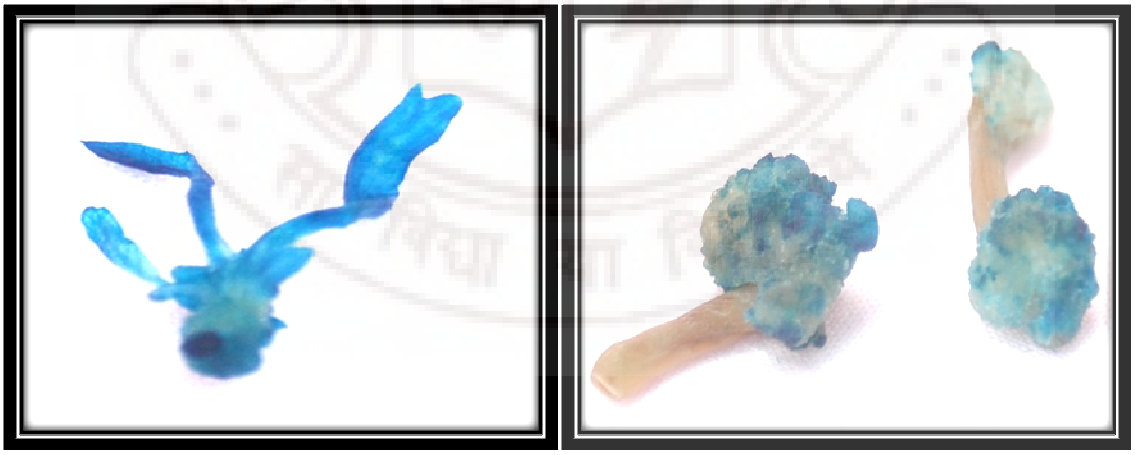
** Data represents frequency of shoot regeneration after the second and third cycles of selection

Figure 5.7 GUS assay of seedling explants of different ages



Assessment of the *Agrobacterium*-mediated transformation efficiency from seedlings of different ages on the basis of GUS positive shoots. Data represents average frequency of GUS positive shoots from both the explants

Figure 5.8 Putative transformants- Putative transformed shoots emerging from hypocotyl explant of 8-day-old seedlings on 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ after 10 days of release of selection pressure following the third cycle of selection



A B

Figure 5.9 GUS staining of putative transformed shoots from hypocotyls of seedlings of different ages A. The shoots of 8-day-old seedling explant showing uniform blue colouration after third selection cycle, **B.** The calli and emerging shoots showing blue colouration with central brown unstained portions from 12-day-old seedling explants after the second selection cycle

culture of 0.5 OD and co-cultivation for 2 days under dark conditions and subsequent transfer to selection media. The frequency of primary transformants after the three cycles of selection was recorded (Table 5.3). Statistical analysis revealed significant influence of seedling age on transformation. GUS histochemical assay showed GUS frequency of above 80.0% from seedlings of different ages 8, 12 days) after the final cycle of selection (Figure 5.7).

Regarless of the explant type and selection cycle, frequency of transformation declined with increase in seedling age. On the first cycle of selection, only callus formation from the tips of the explants was observed. The 8-day-old seedling explants showed maximum frequency (65.8% from root and 68.3% from hypocotyl) of hygromycin resistant calli. After the second cycle of selection, putative transformed shoots were observed from the calli but the central portion of the explants showed slight browning. The 8-day-old seedling explants showed maximum frequency (55.0-60.0%) of putative transformed shoots while 20-day-old seedling explants showed the least frequency (4.0%). The frequency of transformed shoots from 8-day-old seedlings (50.9% from root and 53.0% from hypocotyl) after the third cycle of selection was comparable to that from the second cycle of selection (55.8% from root and 60.6% from hypocotyl) while a two-fold decrease in the frequency of transformed shoots was observed in explants derived from 12, 16 and 20-day-old seedlings. GUS histochemical assay of the putative transformed shoots obtained from 8-day-old seedling explants after three cycles of selection showed uniform blue colouration (Figure 5.9). However, some sections of the callus obtained from 12-day-old seedling explants showed unstained regions when subjected to GUS histochemical assay after the second cycle of selection. The frequency of GUS positive shoots after the third cycle of selection from 8-day-old seedlings and 12-day-old seedlings was 97.0%

and 81.0%, respectively. The GUS assay of putative transformants from 16 and 20-day-old seedlings was not tested.

Overall, the study revealed that the rate of transformation and seedling age are inversely proportional to each other. The explants obtained from 8-day-old seedlings (Figure 5.8) were found to be best for *Agrobacterium*-mediated transformation of safflower.

5.1.3.3 Period of co-cultivation

The influence of the period of co-cultivation on transformation was studied by subjecting seedling explants (root, hypocotyl) of cv. HUS-305 for transformation with 2, 4 and 6 days of co-cultivation (Table 5.4). Transformation of 8-day-old seedling explants was carried out with a bacterial concentration of 0.5 OD and co-cultivated for different time intervals under dark conditions and transferred to selection media. The increase in the period of co-cultivation had a negative effect on transformation. With increase in co-cultivation period, the bacterial contamination increased causing severe damage to the explants leading to decrease in the frequency of transformation. However, stable GUS frequency of 100.0% was observed from the shoots obtained after third cycle of selection with co-cultivation of explants for 2, 4 and 6 days.

The increase in the number of days for co-cultivation showed a clear decline in the frequency of putative transformants which was mainly due to the excessive bacterial growth observed in explants incubated for more than 2 days. On the first cycle of selection, callusing was observed from the cut region of the explants that were subjected to transformation while untransformed tissues showed browning on selection media (transformation control) and shoot induction on selection free media (regeneration control). After the second cycle of selection, the frequency of selected shoots on hygromycin was maximum from both root (53.9%) and

Table 5.4 Frequency of callusing and shoot regeneration from transformed explants subjected to different periods of co-cultivation

Co-cultivation period (days)	Selection I		Selection II		Selection III	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
2	**68.4[a]**	**68.4[a]**	**53.9[a]**	**57.3[a]**	**48.1[a]**	**47.4[a]**
4	47.9[b]	46.9[b]	27.3[b]	32.6[b]	16.6[b]	17.0[b]
6	41.7[c]	40.3[c]	25.6[b]	25.5[c]	12.0[c]	13.0[c]
Grand mean	52.6	51.9	35.6	38.5	25.6	25.8
CV (%)	1.6	1.8	1.7	2.1	0.6	3.7

Transformation was carried out with co-cultivation periods of 2, 4 and 6 days with bacterial titer of 0.5 OD_{600}. Data was recorded after 15 days of incubation on each selection cycle. The percentage values were converted to arcsin angular transformed values prior to statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

* Data represents frequency of callusing following the first cycle of selection

** Data represents frequency of shoot regeneration after the second and third cycles of selection

Figure 5.10 Primary transformants obtained after 6 days of culture on MS medium supplemented with 0.2 mg/l BAP+0.5 mg/l KN+15 mg/l hygromycin (third cycle of selection) A. Transformed shoots obtained with co-cultivation for 2 days, **B**. Transformed shoots obtained with co-cultivation of the explants for 4 days showing vitrification

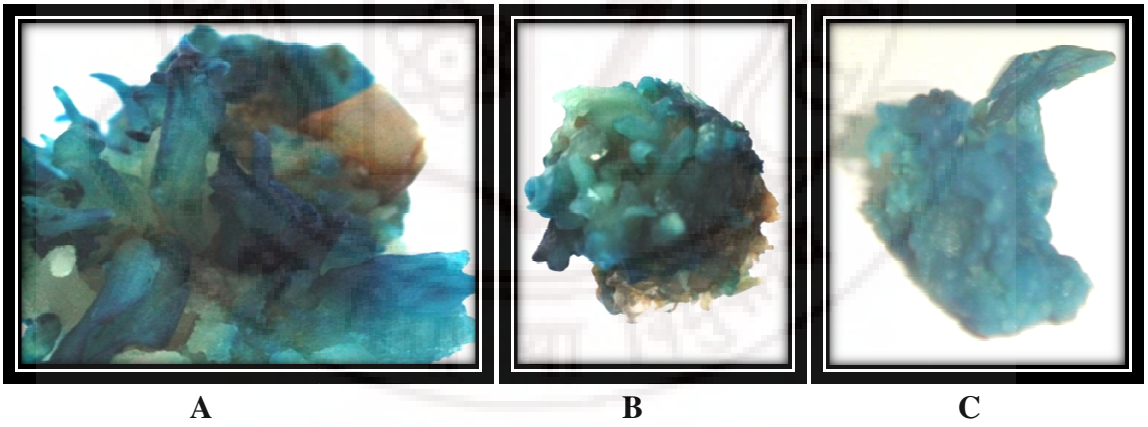


Figure 5.11 Transformed shoots showing uniform blue colouration from 8-day-old hypocotyl explants co-cultivated for A. 2 days, **B**. 4 days, **C**. 6 days. The shoots were assayed for GUS reaction after 5 days of incubation on second selection cycle

hypocotyl (57.3%) explants co-cultivated for 2 days. Even after the final selection, the average frequency of putative transformed shoots (Figure 5.10) over the explants was maximum from explants co-cultivated for 2 days (48.0%) followed by 4 days (17.0%). The frequency of putative transformants recovered from explants co-cultivated for 6 days was only 12.5%. The GUS assay studies showed uniform blue coloration (Figure 5.11) of the transformed tissues from explants co-cultivated for different time periods. Statistical analysis of the frequency of primary transformants revealed significant influence of the co-cultivation period on transformation.

Thus, the study revealed that the length of co-cultivation period influenced transformation in terms of the frequency of putative transformants although the frequency of GUS positive shoots remained the same. The increase in duration of co-cultivation resulted in a decline of the frequency of transformed shoots due to overgrowth of the bacteria. Thus, a co-cultivation period of 2 days (dark) was found to be ideal.

5.1.3.4 Bacterial cell density

The influence of bacterial cell density (OD_{600} 0.1, 0.2, 0.5, 1.0) on transformation was examined by subjecting 8-day-old seedling explants (root, hypocotyl) to transformation with a co-cultivation period of 2 days (Table 5.5). To test the effect of the bacterial cell densities, serial dilutions of the overnight grown bacterial culture in liquid MS medium was used. With an increase in the bacterial cell titer, the frequency of transformed shoots increased up to 0.5 OD and declined thereafter with further increase in the bacterial concentration. The decrease in the frequency of transformed shoots at high bacterial cell density was attributed to the excessive bacterial growth. The GUS histochemical study (after final selection) revealed an increase in the GUS frequency with increase in the culture density (Figure 5.12).

Table 5.5 Frequency of callusing and shoot regeneration from transformed explants of different bacterial cell densities

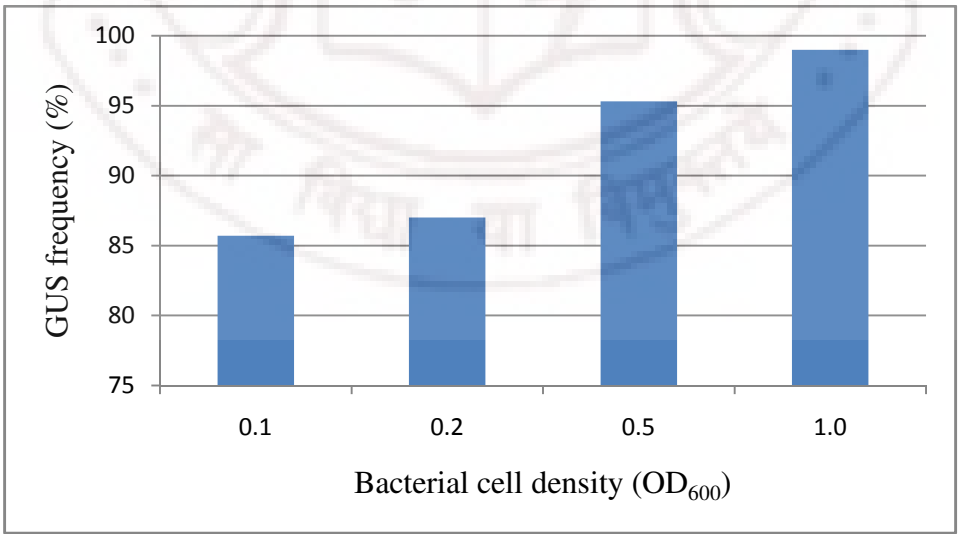
Optical density (OD_{600})	Selection I		Selection II		Selection III	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
0.1	25.9^{c}	27.5^{c}	15.7^{c}	18.7^{b}	9.2^{c}	14.1^{b}
0.2	35.6^{b}	41.7^{b}	27.6^{b}	22.5^{b}	16.2^{b}	10.9^{b}
0.5	**71.5^{a}**	**64.1^{a}**	**51.8^{a}**	**52.1^{a}**	**46.4^{a}**	**41.9^{a}**
1.0	21.2^{c}	26.1^{c}	16.7^{c}	16.0^{b}	8.3^{c}	4.3^{c}
Grand mean	38.5	39.8	27.9	27.3	20.0	17.8
CV (%)	4.8	2.6	1.7	10.4	8.1	6.7

Transformation with bacterial cell densities of 0.1, 0.2, 0.5 and 1.0 OD_{600} with co-cultivation period of 2 days. Data was recorded after 15 days of incubation on each cycle of selection. The percentage values were converted to arcsin angular transformed values prior to statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

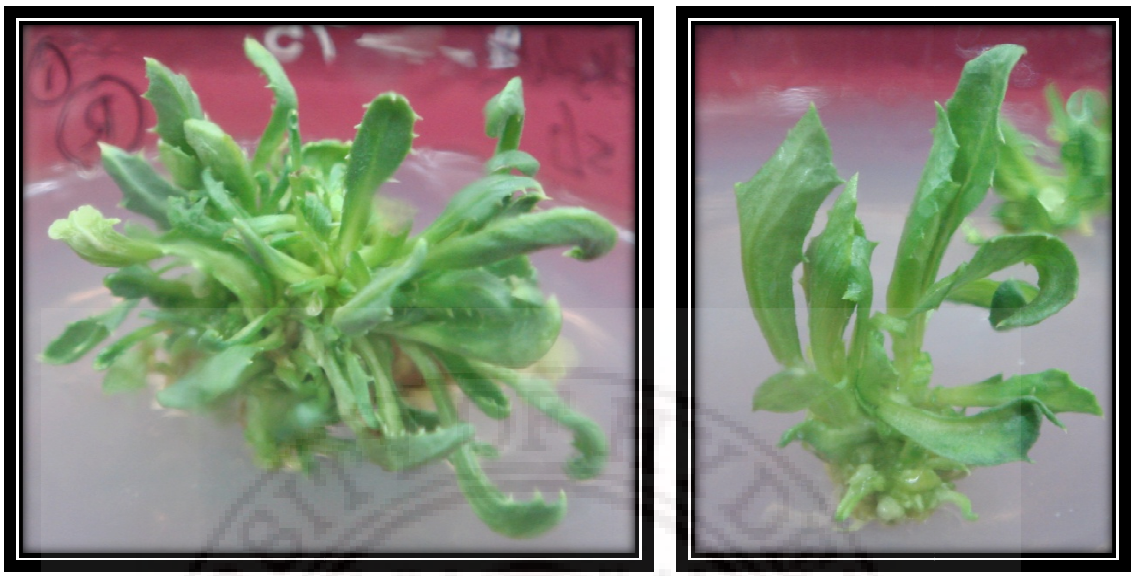
* Data represents frequency of callusing following the first cycle of selection

** Data represents frequency of shoot regeneration after the second and third cycles of selection

Figure 5.12 GUS assay with different bacterial cell densities

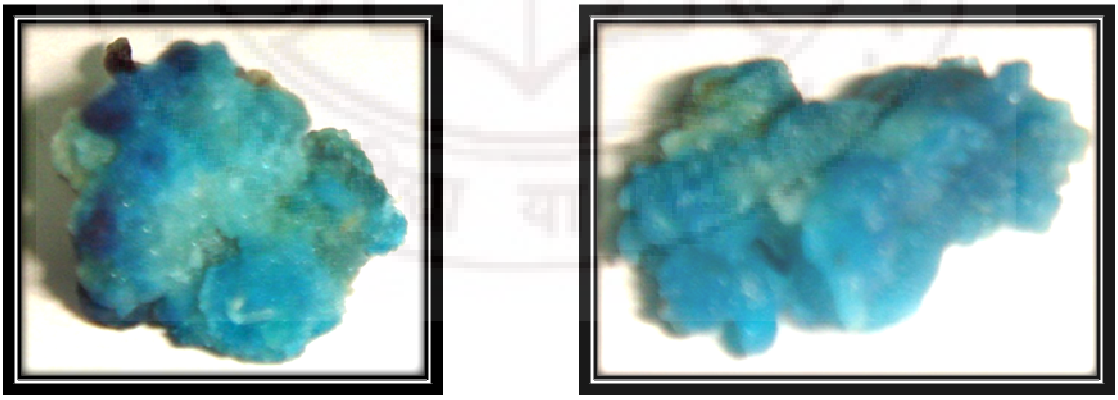


Assessment of the *Agrobacterium*-mediated transformation efficiency of seedling explants with different bacterial cell densities on the basis of GUS positive shoots. Data represents average frequency of GUS positive shoots (%) from both the explants



A **B**

Figure 5.13 Primary transformed shoots obtained after 15 days of culture on 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ (after release of the third cycle of selection) A. Transformed shoots obtained from hypocotyl explants co-cultivated with a bacterial titer of 0.5 OD, **B**. Transformed shoots obtained from hypocotyl explants co-cultivated with a bacterial titer of 1.0 OD



A **B**

Figure 5.14 Transformed shoots showing uniform blue colouration from 8-day-old root explants co-cultivated with bacterial titer of A. 0.1 OD, **B**. 0.5 OD. The shoots were assayed after 12 days on the second cycle of selection

Irrespective of the bacterial cell density the explants after the first cycle of selection showed callusing from the cut ends. Maximum frequency of hygromycin resistant calli was obtained from root (71.5%) and hypocotyl (64.1%) explants transformed with a bacterial culture density of 0.5 OD. Following the second cycle of selection, the average frequency of surviving shoots from explants transformed with bacterial cell densities of 0.1, 0.2, 0.5 and 1.0 OD was 17.0%, 25.0%, 52.0% and 16.0%, respectively. After the third cycle of selection there was a decrease in the frequency of transformed shoots and the maximum frequency of transformed shoots were recorded from explants (root 46.4% and hypocotyl 41.9%) transformed with bacterial culture of 0.5 OD (Figure 5.13 A). The frequency of putative transformants from explants transformed with bacterial culture densities of 0.1, 0.2 and 1.0 OD (Figure 5.13 B) was less than 20.0% (irrespective of explant type). The GUS assay after final selection showed brilliant blue (Figure 5.14) colouration in the shoots transformed with different bacterial cell densities but with varied frequency. Maximum GUS frequency (99.0%) was observed in shoots recovered from transformation with bacterial cell culture density of 1.0 OD. The frequency of GUS stained explants from putative transformed shoots obtained with co-cultivation at bacterial cell densities of 0.1, 0.2 and 0.5 OD was 85.7%, 87.0% and 95.3%, respectively. The frequency of GUS positive shoots was high with bacterial culture of 1.0 OD but the frequency of transformed shoots following three cycles of selections was less due to excessive bacterial growth and hence, a bacterial titer of 0.5 OD was found to be optimum.

The study of the effect of bacterial cell densities on *Agrobacterium*-mediated transformation of the seedlings explants revealed that the frequency of transformed shoots increased with the increase in the bacterial titer and reached to a peak at 0.5 OD and declined

thereafter. It was observed that the bacterial cell density has significant influence on enhancing transformation efficiency and that 0.5 OD was found to be significantly superior.

5.1.3.5 Enzymatic pretreatment

The effect of the macerating enzyme (pectinase) on the transformation efficiency was examined. The cell wall digesting enzyme pectinase was used to enhance the penetration zone of the vector in the target tissues. Pectinase was used at concentrations of 0.1, 0.5, 1.0 and 2.0% prior to vacuum infiltration. The effect of pectinase pretreatment on subsequent survival of the transformants was assessed following three cycles of selection (data not presented). Pectinase had a negative effect at all the concentrations tested when compared to the untreated explants. During the first cycle of selection, pectinase effect was non-significant and the survival rate of the calli recorded from infected explants was comparable to that of the untreated explants. However, a drastic decline in shoot induction was observed following the second and third cycles of selection. The explants showed heavy necrosis, contamination and failed to develop further at all the concentrations studied.

Finally, it was observed that the use of pectinase for enhancing transformation frequency in safflower tissues is not ideal as the treatment resulted in excessive bacterial growth.

5.1.3.6 Acetosyringone treatment

The influence of the virulence gene inducer on transformation was tested by co-cultivating the explants (root, hypocotyl) in the presence of different concentrations (50, 150, 200 µM) of a potent *vir* gene enhancer acetosyringone (Table 5.6). Acetosyingone was added to the bacterial

suspension liquid MS medium containing the explants before subjecting the explants to vacuum infiltration. The explants were subjected to transformation with bacterial culture of 0.5 OD and co-cultivation for 2 days (dark) followed by three cycles of selection on hygromycin. Acetosyringone was found to have significant influence on transformation. The frequency of transformed shoots increased with an increase in acetosyringone concentration up to 150 µM and declined drastically at a concentration of 200 µM. It was observed that acetosyringone treatment at a concentration of 150 µM was found to be significantly superior regardless of explant type. The effect of acetosyringone on transformation efficiency varied with the explant type and the selection cycle. Differences among different concentrations of acetosyringone on transformation from hypocotyl explants was evident from the first cycle of selection itself. However, in case of root explants, differences among acetosyringone treatments (excluding 200 µM) were not significant during the first cycle of selection. Following the second cycle of selection the average frequency of putative transformed shoots from both explants treated with 150 µM acetosyringone was maximum (57.0%) followed by 50 µM treatment (53.5%) and control explants (53.0%). However, the explants treated with 200 µM acetosyringone showed less frquency (35.0%) of putative transformed shoots. After the final cycle of selection, the frequency of putative transformants averaged over explants was maximum in explants treated with 150 µM acetosyringone (50.0%) followed by 50 µM (47.0%), untreated (44.0%) and 200 µM treated(26.0%) explants. It was observed that the acetosyringone concentration beyond 150 µM promoted excessive bacterial growth. The GUS frequency of the putative transformants from untreated and treated explants was 93.0% (Figure 5.15). Though GUS frequency was high and

Table 5.6 Frequency of callusing and shoot regeneration from transformed explants of different concentrations of acetosyringone

Concentration of acetosyringone (µM)	Selection I		Selection II		Selection III	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
0	67.3[a]	67.5[a]	54.4[b]	52.1[b]	46.4[b]	42.9[c]
50	70.2[a]	68.6[ab]	55.3[ab]	52.3[b]	48.3[ab]	46.6[b]
150	**73.2[a]**	**72.0[a]**	**60.4[a]**	**54.6[a]**	**51.3[a]**	**48.8[a]**
200	53.3[b]	46.7[c]	38.9[c]	31.1[c]	27.0[c]	25.3[d]
Grand mean	66.0	63.7	52.2	47.5	43.2	40.9
CV (%)	4.9	2.4	3.5	1.4	3.2	1.3

Transformation was carried out with bacterial titer of 0.5 OD_{600} and co-cultivation for 2 days. Data was recorded after 15 days of incubation on each cycle of selection. The percentage values were converted to arcsin angular transformed values prior to statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

* Data represents frequency of callusing following the first cycle of selection

** Data represents frequency of shoot regeneration after the second and third cycles of selection

A **B** **C**

Figure 5.15 GUS staining of putative transformants recovered after selection following transformation in the presence of acetosyringone **A**. Calli of hypocotyl explants after first selection of transformation with 50 µM acetosyringone **B**. Calli of hypocotyl explants after first selection of transformation with 150 µM acetosyringone **C**. Blue coloured shoots observed after 12 days of second selection from roots transformed with 200 µM acetosyingone

similar for untreated and treated explants, the regeneration frequency of the putative transformants with 150 µM acetosyringone treated explants was comparatively high.

The experiment thus revealed the significant influence of acetosyringone on the production of putative transformed shoots. The acetosyringone concentration of 150 µM was found to be superior in terms of high frequency of regeneration of primary transformants. The increase in acetosyringone beyond 150 µM resulted in excessive bacterial growth.

5.1.3.7 Explant

Explant type is also one of the important factors affecting *Agrobacterium*-mediated transformation. Root, hypocotyl, cotyledonary leaf and primary leaf of 8-day-old seedlings were subjected to transformation with bacterial culture of 0.5 OD and co-cultivation period of 2 days to identify the explants that produce maximum number of transformed shoots (Table 5.7).

After the first cycle of selection it was observed that root and primary leaf explants produced maximum frequency of hygromycin resistant calli but after the third cycle of selection the frequency of primary transformants was maximum from root and hypocotyl explants followed by primary leaf. The transformation efficiency from cotyledonary leaf remained low on all the cycles of selection. After the first cycle of selection, maximum frequency of hygromycin resistant calli were observed in root (72.1%) and primary leaf (69.4%) explants while it was significantly low in hypocotyl (64.2%) followed by cotyledonary leaf explants (37.1%). After the second cycle of selection, a significant difference in frequency of transformed shoots was observed among explants. Root explants (58.9%) recorded maximum frequency of transformed

Table 5.7 Frequency of callusing and shoot regeneration from different seedling explants subjected to transformation

Explant	Seletion I	Selection II	Selection III
Root	**72.1[a]**	**58.9[a]**	49.0[a]
Hypocotyl	64.2[b]	54.4[ab]	**50.9[a]**
Cotyledonary leaf	37.1[c]	21.5[c]	14.3[c]
Primary leaf	69.4[a]	46.6[b]	30.3[b]
Grand mean	60.7	45.3	36.1
CV (%)	2.2	5.7	3.9

Transformation was carried out with bacterial titer of 0.5 OD_{600} and co-cultivation for 2 days. Data was recorded after 15 days of incubation on each cycle of selection. The percentage values were converted to arcsin angular transformed values prior to statistical analysis. Means in a column followed by same letters are not significantly different according to DMRT at p=0.05. Experimental design: Single factor completely randomized design with three replications

* Data represents frequency of callusing following the first cycle of selection

** Data represents frequency of shoot regeneration after the second and third cycles of selection

A **B**

Figure 5.16 Putative transformed shoots obtained from different explants of 8-day-old seedlings of HUS-305 after the third cycle of selection. Observations were taken after 4 days of culture on MS medium supplemented with 0.5 mg/l KN **A**. Transformed shoots from hypocotyl explants **B**. Transformed shoots from root explants

shoots followed by hypocotyl (54.4%), primary leaf (46.6%) and cotyledonary leaf (21.5%) explants. Following the third cycle of selection the frequency of putative transformants was maximum from hypocotyl (50.9%) and root (49.0%) explants followed by primary leaf (30.3%) and cotyledonary leaf (14.3%). Figure 5.16 represents putative transformed shoots after the third cycle of selection. The GUS assay showed uniform blue colouration (Figure not shown) in the shoots obtained from root and hypocotyl explants. The GUS staining of shoots obtained from cotyledonary leaf showed some untransformed sections (after third cycle of selection) while GUS staining of putative transformants from primary leaf were not tested.

The study revealed that the explant type plays a major role in transformation and that root and hypocotyl explants were significantly superior over other explants tested.

5.1.3.8 Explant injury

Manual wounding (small incisions) of the explants (root, hypocotyl) was carried out to study the influence of explant injury on transformation efficiency. Explant injury resulted in overgrowth of the bacterium and the explants showed heavy necrosis. Antibiotic treatment failed to cure the explants. The study revealed that wounding of explants has a detrimental effect on transformation.

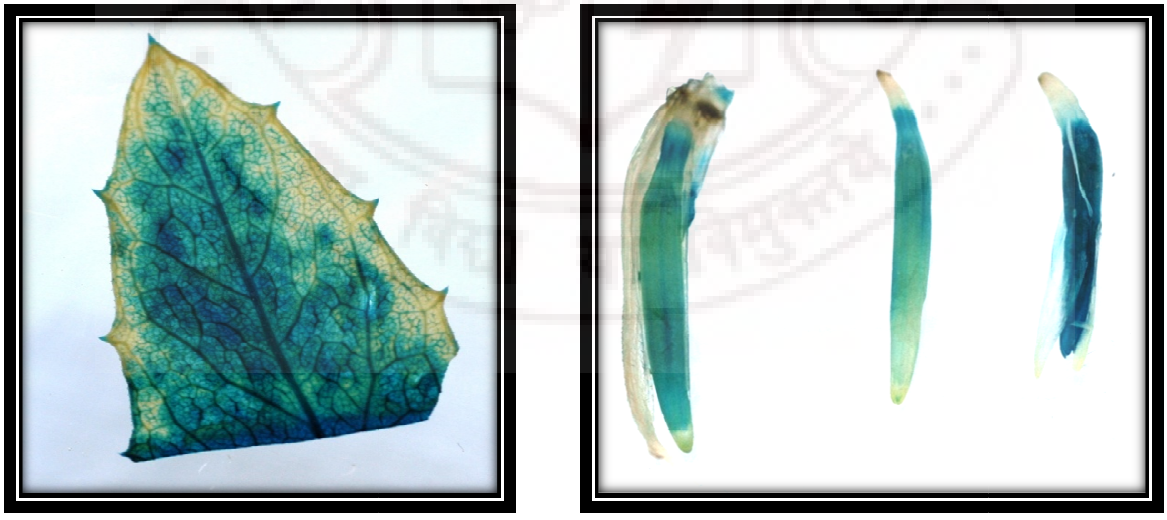
5.1.4 Elongation and rooting of transformed shoots

The primary transformants obtained after the third cycle of selection from all the above experiments were transferred to elongation medium, viz., MS + 0.5 mg/l KN + 1.0 mg/l 2iP + 1.0 mg/l $AgNO_3$ and / or 1.0 mg/l KN + 1.0 mg/l $AgNO_3$ (Figure 5.17). A total of 469 putative

Figure 5.17 Elongation- Elongated putative transformed shoots on 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ after three cycles of selection

Figure 5.18 Rooting- Rooted transformed shoots on half-strength MS medium supplemented with 1.0 mg/l NAA

Figure 5.19 Acclimatization and establishment of transformed shoots



A **B**

Figure 5.20 GUS histochemical assay of T_0 plants A. The blue colouration of leaf segment, **B**. Entire floret showing uniform blue colouration

transformed shoots that showed elongation were transferrered to rooting medium on to half-strength MS+1.0 mg/l NAA (Figure 5.18). The frequency of rooting of the primary transformants was less compared to that of the regenerated shoots. About 84 shoots showed rooting, however some rooted shoots failed to survive on acclimatization. The shoots that survived on sterile vermiculite were transferred to soil in pots and left in the greenhouse (Figure 5.19). Of the 13 plants that were transferred to green house, only 7 were able to survive. The GUS histochemical assay study of the leaf and floral parts of the sexually mature plants showed intense blue colouration confirming the presence of the transgene (Figure 5.20).

5.1.5 Molecular analysis

The putative transformants obtained were confirmed for the presence of the introduced gene by PCR, RT-PCR and Southern analysis.

5.1.5.1 PCR

The putative transformants obtained after the three cycles of selection were subjected to PCR with *Uid* A and *hpt* gene specific primers. PCR was also carried to check the amplification of the house keeping gene, *actin*. PCR was done to check the persistence of the residual *Agrobacterium* by amplifying the *vir* D2 region. The PCR amplification of 24 transformants with the *Uid* A and *hpt* gene specific primers showed DNA fragments of the expected size of 1200 bp and 520 bp, respectively confirming the presence of the introduced DNA in the transformed shoots (Figures 5.21, 5.22). Both *Uid* A and *hpt* DNA fragments were not detected in the control plants. With regard to *vir* D2 amplification, amplified product of 487 bp was observed only in the positive control while no band was detected in the untransformed and transformed shoots which indicates

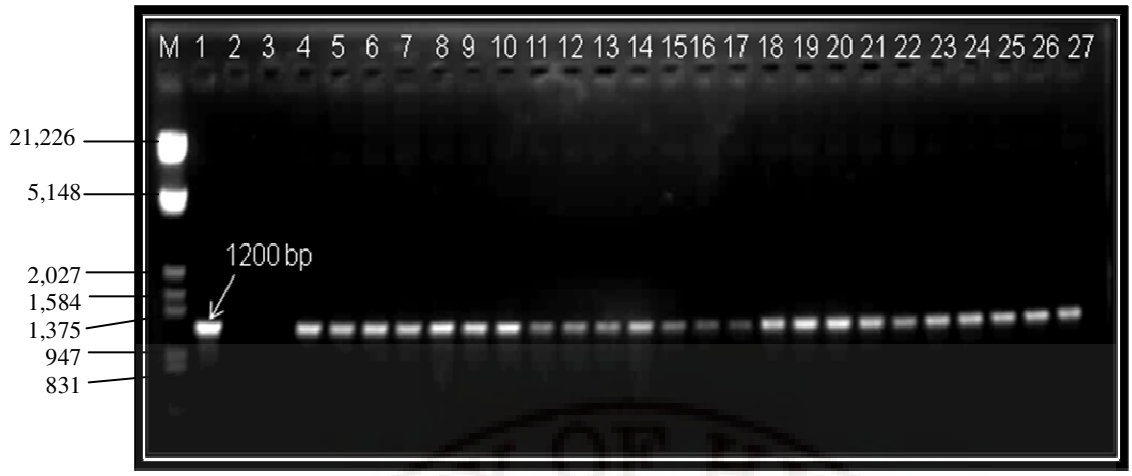


Figure 5.21 PCR analysis of putative transformants showing amplification of 1200 bp fragment of the *Uid* A gene Lane M: λ DNA double digest with *Eco*R I/*Hin*d III, lane 1: Positive control (plasmid DNA pCAMBIA 1391Z+CaMV 35S), lane 2: Negative (no DNA) control, lane 3: Plant (untransformed safflower) control, lanes 4-27: DNA from putative transformants

Figure 5.22 PCR analysis of putative transformants showing amplification of 520 bp fragment of the *hpt* gene Lane M: λ DNA double digest with *Eco*R I/*Hin*d III, lane 1: Positive control (plasmid DNA pCAMBIA 1391Z+CaMV 35S), lane 2: Negative (no DNA) control, lane 3: Plant (untransformed safflower) control, lanes 4-27: DNA from putative transformants

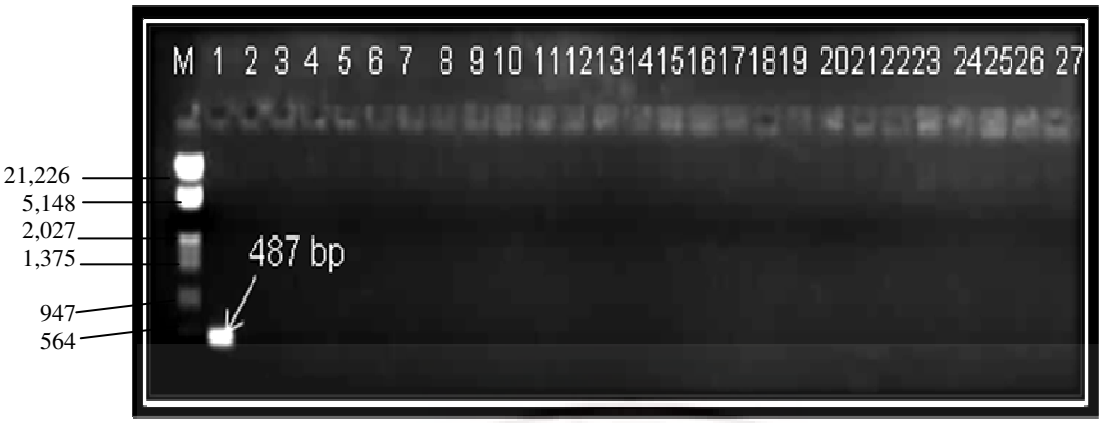


Figure 5.23 PCR analysis of putative transformants showing amplification of 487 bp fragment of the *vir* D_2 gene Lane M: λ DNA double digest with *ECo*R I/*Hin*d III, lane 1: Positive control (plasmid DNA pCAMBIA 1391Z+CaMV 35S), lane 2: Negative (no DNA) control, lane 3: Plant (untransformed safflower) control, lanes 4-27: DNA from putative transformants

Figure 5.24 PCR analysis of putative transformants showing amplification of 450 bp fragment of the *actin* gene Lane M: λ DNA double digest with *EcoR* I/*Hin*d III, lane 1: Positive control (plasmid DNA pCAMBIA 1391Z+CaMV 35S), lane 2: Negative (no DNA) control, lane 3: Plant (untransformed safflower) control, lanes 4-27: DNA from putative transformants

Figure 5.25 RT-PCR of the cDNA showing amplification of *Uid* A and *hpt* gene transcripts Lane M: λ DNA double digest with *ECo*R I/*Hin*d III, lane 1: DNA from pCAMBIA 1391Z+CaMV 35S showing amplification of 1200 bp fragment of *Uid* A, lanes 2 and 10: Negative (no DNA) control, lanes 3 and 11: cDNA from untransformed plant (control), lanes 4-8: cDNA from putative transformants showing amplification of 1200 bp fragment of *Uid* A, lane 9: DNA from pCAMBIA 1391Z+CaMV 35S showing amplification of 520 bp fragment of *hpt*, lanes 12-16: cDNA from putative transformants showing amplification of 520 bp fragment of *hpt* gene

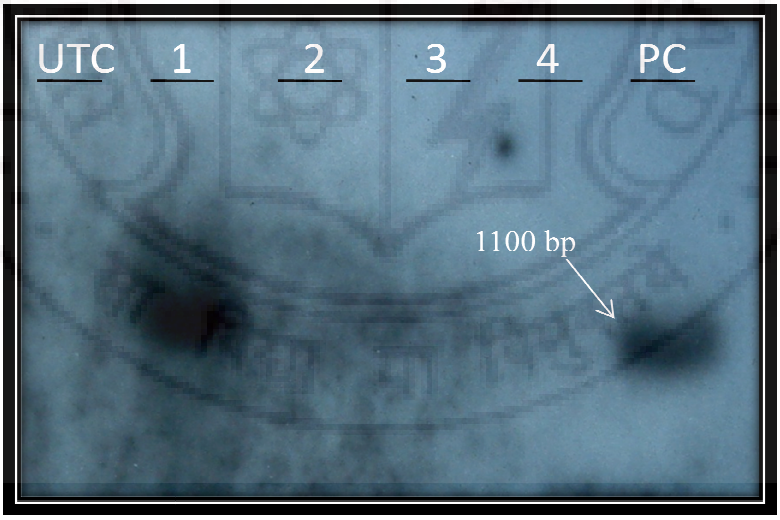


Figure 5.26 Southern blot analysis of PCR and RT-PCR positive plants using a *hpt* amplified fragment as the probe Lane UTC: DNA from untransformed plant (control) digested with *Xho* I, lanes 1-4: DNA (8, 6, 4, 2 µg) from RT-PCR positive plants digested with *Xho* I, lane PC: DNA from pCAMBIA 1391Z+CaMV 35S digested with *Xho* I.

the absence of *Agrobacterium* contamination (Figure 5.23). PCR analysis for the amplification of the internal control gene, *actin* revealed the presence of band of specified size (450 bp) in control (untransformed) and transformed plants (Figure 5.24).

5.1.5.2 RT-PCR

About five rooted transformants that established well in the soil were subjected to RT-PCR analysis. The RT-PCR analysis of these transformants with *Uid* A and *hpt* gene sequences showed the expected band size of 1200 bp and 520 bp, respectively in all the five plants tested (Figure 5.25). Amplification was not observed in the control (untransformed) plants for both *Uid* A and *hpt* genes.

5.1.5.3 Southern analysis

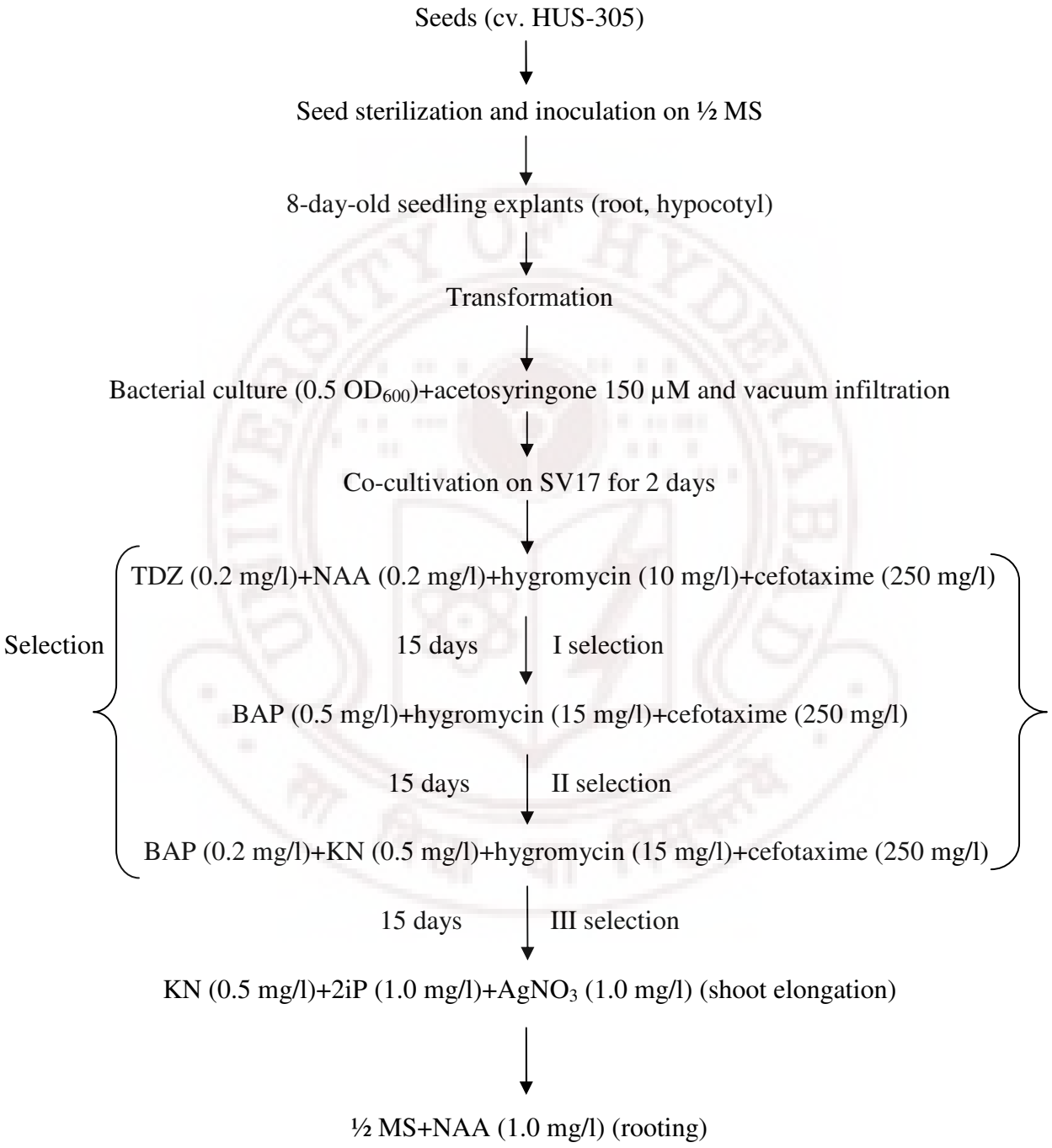
Southern hybridization was done to check for the stable integration of the transgene. The genomic DNA isolated from the transformed plants was purified and digested with *Xho* I that released the *hpt* gene fragment. Hybridization with a P^{32} labelled radioactive probe of *hpt* showed an expected 1.1 kb band indicating the presence of the selectable marker gene in the transformed plant. Hybridization signals were not detected in the digested DNA of the control plants. Out of the four putative transformants that were positive for PCR and RT-PCR, only one plant showed a thick band of the expected size (Figure 5.26). However, the concentrations of the transformed plant DNA used varied as 8, 6, 4 and 2 µg.

5.1.6 Conclusion

In the present study, different variables such as, genotype, seedling age, co-cultivation period, bacterial titer, enzymatic pre-treatment of target tissues, acetosyringone (*vir*-gene enhancers),

explant type and explant injury, the ideal parameters for obtaining maximum transformation frequency were established. Based on these investigations, it was concluded that the genotype HUS-305 is more responsive for transformation as compared to the other genotypes. Among the different explants, root and hypocotyl were more efficient for transformation. The explants of 8-day-old seedlings were more amenable for transformation compared to those derived from older seedlings. The bacterial concentration of 0.5 OD_{600}, incubation with 150 µM acetosyringone and co-cultivation period of 2 days were found to be optimum for efficient transformation. A selection regime of 10-15-15 mg/l hygromycin was found to be ideal for selection of putative transformants with nil or low frequency of escapes. The schematic representation of the standardized transformation protocol is presented in Figure 5.27.

Figure 5.27 Schematic representation of transformation protocol



5.2 Discussion

Safflower is affected by biotic stresses in the form of insect pests and diseases which reduce the yield and productivity of the crop to a large extent. Although sources of resistance to the major biotic stresses are available in the primary and secondary gene pools, introgression from wild allies is constrained by the existence of sexual crossability barriers and ploidy differences. Genetic improvement through exploitation of heterosis is hindered by the lack of availability of a viable pollination control system. Hence, it is necessary to exploit *in vitro* techniques for genetic improvement of safflower for various desirable traits. The previous reports in safflower transformation with American cultivar Centennial (Ying et al., 1992; Orlikowska et al., 1995) and Indian cultivars A-1 and A-300 (Rao and Rohini, 1999) via *Agrobacterium*-mediated transformation were limited by low frequency of putative transformants and difficulty in recovery of whole plants. Even the *in planta* method adopted by Rohini and Rao (2000) resulted in transformation frequency of 5.3% and 1.3% in A-1 and A-300, respectively. In the present study, attempts were made to enhance the frequency of transformation by optimizing different variables which are hitherto reported to improve the tissue transformability in several other crop plants including safflower.

5.2.1 *Agrobacterium* elimination

In *Agrobacterium*-mediated transformation studies, it is important to determine the optimum dose of the antibiotic (bacteriostat) to control bacterial overgrowth without inhibiting shoot regeneration. In the present study, cefotaxime at a concentration of 250 mg/l was found to be optimal for controlling bacterial overgrowth following co-cultivation. Cefotaxime at a

concentration above 250 mg/l showed more than 50.0% browning of the explants. In safflower transformation, the bacteriostat (cefotaxime/carbenicillin) was used at concentration of 250 mg/l (Rao and Rohini, 1999) and 500 mg/l (Rohini and Rao, 2000; Ying et al., 1992). A cefotaxime concentration of 250 mg/l was used for the *Agrobacterium*-mediated transformation studies in sunflower (Sawahel and Hagran, 2006) and tea (Jeyaramraja and Nithya, 2005). Mihalijevic et al. (2001) reported that cefotaxime was less toxic to the tissues and suitable for *Agrobacterium* elimination. It was also believed that cefotaxime is converted by cell metabolism to an unknown compound with growth regulator activity (Borrelli et al., 1992; Mathias and Mukasa, 1987; Nakano and Mii, 1993; Yu and Wei, 2008).

5.2.2 Sensitivity to hygromycin

For identification of the transformants from the nontransformants a selectable marker is used. The construct pCAMBIA 1391Z+35S harbours *hpt* gene for selection of transformants on hygromycin. Earlier experiments in safflower were with construct harbouring *npt II* gene for selection on kanamycin. The sensitivity of explanted tissues varies with the antibiotic type and concentration. In general, concentrations of kanamycin required for any plant species is higher than those used for hygromycin. In safflower, kanamycin was used at concentrations of 50 mg/l and 100 mg/l in safflower by Ying et al. (1992) and Rao and Rohini (1999), respectively. The concentrations of kanamycin and geneticin used by Orlikowska et al. (1995) for safflower transformation were not clear and ranged from 12.5 to 60 mg/l. In experiments of Rao and Rohini (1999), the selection agent (kanamycin) was added to the medium after one week of co-cultivation. In the study of Ying et al. (1992), the transformation efficiency of safflower recorded

in terms of GUS activity varied with the selection agent wherein calli selected on gentimycin showed uniform GUS activity while those from kanamycin showed chimaerics.

As all the earlier experiments were with selection on kanamycin, the current experiments were carried out to determine the optimum dose of hygromycin on the survival of the seedling explants (root and hypocotyl). In the present investigation the survival of the explants was tested on various concentrations of hygromycin (5, 10, 20, 30, 40, 50, 60, 80 and 100 mg/l) and the explant browning was recorded periodically (2, 4, 7, 10, 13 and 16 days after culture initiation).With increase in hygromycin concentration there was drastic decline in the survival frequency of both the explants. Furthermore, with increase in hygromycin concentration the time taken for explant browning decreased. Hartman (1991) suggested that hygromycin is a good antibiotic for use as a selectable marker. Schrott (1995), Catlin (1990) also reported that the tissues of many plant species showed higher sensitivity to hygromycin than kanamycin and other antibiotics. Low concentration (2.0 mg/l) of hygromycin was highly toxic to embryo cultures of *Picea* (Mihaljevic et al., 2001). Conversely, in the present study a LD_{50} of 10 mg/l hygromycin was recorded. The ability of the tissues to survive at such high concentrations may be attributed to the differences in the plant species.

With regard to the selection cycles, the *Agrobacterium*-mediated studies in safflower employed three cycles of selection with a fixed concentration of the selection agent (Ying et al., 1992; Orlikowska et al., 1995 and Rao and Rohini, 1999). In the present investigation three cycles of selection were used but with a selection regime of 10-15-15 mg/l hygromycin not only to prevent escapes but also to recover a higher frequency of transformants. The concentrations of

10-15 mg/l hygromycin were used by Zhao et al. (2007) in *Agrobacterium*-mediated transformation of cotton.

Finally, in the present study a stringent selection regime of 10-15-15 mg/l hygromycin was adopted for all the transformation experiments.

5.2.3 Genotype

To assess the influence of genotype on transformation, seedling explants of seven different genotypes viz., A-1, A-2, HUS-305, JLSF-414, JSF-1, NARI-6 and Sharda were tested. The study revealed significant differences among the different genotypes in terms of the transformation frequency. Among the tested genotypes, the cv HUS-305 was found to be superior in terms of its high regenerative ability and maximum frequency of GUS positive shoots after three cycles of selection. Earlier studies on transformation in safflower were carried out with either a single genotype-Centennial (Ying et al., 1992; Orlikowska et al., 1995) or two (A-1, A-300) genotypes (Rao and Rohini, 1999; Rohini and Rao, 2000). Hence, differences in transformation due to genotype were not evident from the previous studies. However, Rohini and Rao (2000) reported the differences in the frequency of transformation among the two genotypes A-1 and A-300 viz., A-1 (5.3%) was more efficient than A-300 (1.3%) in the *in planta* method of transformation. The significant role of genotype in the determination of transformation efficiency was also reported in sunflower (Gurel and Kazan, 1999), Broccoli (Jong and Botella, 2002), *Capsicum* (Sanatombi and Sharma, 2008), citrus (Gutierrez et al., 1997) and soybean (Sheng et al., 2008). The differences in transformation efficiency among different genotypes may be

attributed to the differences in regeneration potential of the genotypes. The previous studies (Radhika et al., 2006; Neetika et al., 2005) and also the present study on tissue culture in safflower indicated high frequency of shoot regeneration in HUS-305. The high regenerative potential indicates the presence of maximum number of totipotent cells that are more amenable to transformation leading to high frequency of transformation in HUS-305.

5.2.4 Seedling age

The influence of seedling age of the donor explant on transformation was assessed and the study demonstrated that seedling age and transformation efficiency were inversely proportional. Among the different seedling ages tested, 8-day-old seedlings were found to be highly responsive/amenable for *Agrobacterium*-mediated transformation. Further, the percentage of GUS positive shoots decreased with the increase in the seedling age. In the previous studies of safflower, explants from 3 to 6-day-old seedlings were used (Orlikowska et al., 1995), but the studies were limited to the use of cotyledonary leaf explants unlike in the present study wherein hypocotyl and root explants were used. In the safflower transformation experiments of Ying et al. (1992), the use of 3-4 week old seedling explants (hypocotyl, leaf) showed callusing and the frequency of shoot bud induction was only 17-25%. However, in the present investigation the use of 8-day-old seedling explants showed a two fold increase in the frequency of putative transformants compared to the study of Ying et al. (1992) clearly suggesting the influence of seedling age in safflower transformation. In most of the plant transformation studies, the age of explants was found to be crucial and transformation efficiency was relatively high in explants derived from younger seedlings when compared to those from older seedlings (Bond and Roose,

1998; Jong and Botella, 2002; Jeyaramraja and Nithya, 2005; Sridevy et al., 2008). Studies of Radhika et al. (2006) and the present investigation on tissue culture studies on safflower demonstrated higher regenerative ability of juvenile tissues compared to the older tissues. High transformation efficiency of the younger tissues could be due to the fact that many cells divide actively in the younger seedlings and hence, are susceptible to TDNA integration to a greater extent compared to the older tissues. The differences in the transformation efficiency among younger and older seedlings could also be attributed to the differences in the cell wall composition that may influence the bacterial binding (Bond and Roose, 1998).

5.2.5 Period of co-cultivation

In the present investigation, the duration of co-cultivation showed a clear influence on transformation. There was a decline (three fold) in the frequency of putative transformants with an increase in the co-cultivation period from 2 to 6 days but the frequency of GUS positive shoots remained the same. The previous reports in safflower transformation employed a co-cultivation period of 2-3 days (Orlikowska et al., 1995; Ying et al., 1992; Rao and Rohini, 1999). Orlikowska et al. (1995) reported that in safflower a specific time interval after transformation is necessary in order to exploit regeneration potential. Influence of co-cultivation period on *Agrobacterium*-mediated transformation has been reported in many plant species (Cervera et al., 1998; Uranbey et al., 2005; Wang et al., 2009). An increase in the co-cultivation duration resulted in excess bacterial overgrowth leading to tissue necrosis. Similar problem of excessive growth of *Agrobacteria* with an increase in co-cultivation period has been reported (Cervera et al., 1998; Saima and Bushra, 2004; Wang and Zu, 2007; Wang et al., 2009; Srivastava et al., 2009). In the present study, a co-cultivation period of 2 days (in dark) was found to be optimum

for obtaining maximum frequency of transformed shoots. A co-cultivation period of 2 days was adopted in other plant species like *Antirrhinum majus* (Holford et al., 1992), *Glycine max* (Li et al., 2004), *Nicotiana tabacum* (Uranbey et al., 2005) and *Vigna radiata* (Saima and Bushra, 2004). Belarmino and Mii (2000) reported that in orchid transformation, extending the co-cultivation period showed no increase in the GUS expression but caused tissue necrosis as observed in the present study on safflower.

5.2.6 Bacterial cell density

A bacterial cell density of 0.1 to 2.0 OD has been reported for transformation of different crop plants (Mohamed et al., 2004; Wang et al., 2009). Bacterial cell densities varies with plant species, explanted tissue, duration of co-cultivation and mode of regeneration. In previous studies on safflower, a bacterial cell dilution of 1:10 (Orlikowska et al., 1995) and 0.2 OD (Ying et al., 1992) was employed for *Agrobacterium*-medited transformation. However, in case of sunflower, higher bacterial cell density (2.0 OD) was used as most of the studies used cotyledons from mature seeds as target tissues for transformation. In the present study, the effect of different bacterial densities (0.1, 0.2, 0.5 and 1.0 OD_{600}) on regeneration and GUS histochemical activity was assessed which revealed that the frequency of putative transformed shoots increased with increase in bacterial cell density up to 0.5 OD and declined drastically thereafter. However, the frequency of GUS positive shoots increased gradually with increase in the bacterial cell density. The decrease in the frequency of putative transformants at high bacterial concentrations may be ascribed to the increase in the bacterial contamination resulting in tissue necrosis. Such decline in transformation efficieny with increase in bacterial cell density has been reported in safflower (Orlikowska et al., 1995), ramie (Wang et al., 2009) and black gram (Saini and Jaiwal, 2007).

With regard to the GUS activity, the results of the present study that GUS activity increased as the bacterial concentration increased were in agreement with those reported in sunflower (Mohamed et al., 2004). The present investigation thus, revealed that a bacterial cell density of 0.5 OD is optimal for achieving maximum transformation efficiency with controllable levels of bacterial contamination. Similar bacterial cell density (0.5 OD) was found to be ideal in the transformation of rice (Kumar et al., 2005) and ramie (Wang et al., 2009).

5.2.7 Enzymatic pretreatment

For efficient transformation, the penetration of the vector into the target tissues is of great importance. Therefore, the cell wall digesting enzyme pectinase was used to enhance the transformation frequency in sunflower (Alibert et al., 1999). In the present study use of pectinase showed negative effect on safflower transformation leading to heavy bacterial contamination resulting in severe necrosis of the tissues. The results are in agreement with those of Weber et al. (2003) and Sujatha and Sailaja (2005) who observed detrimental effect of pectinase in transformation of sunflower and castor, respectively.

5.2.8 Acetosyringone

Acetosyringone is a phenolic compound that attracts *Agrobacterium* and enhances the activity of virulence genes resulting in an increase of transformation efficiency (Stachel et al., 1985). In the present study, acetosyringone was used at concentrations of 50, 150, 200 µM to assess its effect on enhancing the transformation efficieny in safflower. In the previous safflower transformation studies, acetosyringone was used at a concentration of 100 µM in the induction medium after

Agrobacterium-mediated transformation (Orlikowska et al., 1995) and a high concentration (100 mM) was used in the bacterial culture medium for *in planta* transformation (Rohini and Rao, 2000). The use of wounded tobacco leaf extract for *vir* gene induction in *in planta* safflower transformation (Rohini and Rao, 2000) improved transformation efficiency by reducing the symptoms of hypersensitivity and increasing the number of embryos expressing GUS. In the present investigation, the frequency of transformed shoots increased with increase in the concentration of acetosyringone up to 150 µM and showed a drastic decline at 200 µM due to excessive bacterial contamination. Orlikowska et al. (1995) reported uniform decline in the regeneration with increasing acetosyringone on transformation of American safflower cultivar, Centennial. These differences in the promotive effect of acetosyringone could be due to genotypic variations. Acetosyringone at a concentration of 200 µM was found to decrease transformation in ramie (Wang et al., 2009). Transformation studies in most plant species indicated that acetosyrigone at an appropriate concentration enhances the efficiency of transformation (Mohamed, 2004; Agnieszka et al., 2004; Sunil Kumar and Keerthi, 2001; Srivastava et al., 2009). With regard to the GUS staining there were no significant differences in terms of the frequency of GUS positive shoots at different concentrations of acetosyrigone. Similarly, the study of Rohini and Rao (2000) on *in planta* safflower transformation the use of *Agrobacterium* treated with acetosyringone (100 mM) did not have any effect on the transformation efficiency. Likewise, frequency of GUS positive shoots in groundnut was not influenced by different concentrations of acetosyringone (Ming et al., 1996). However, Orlikowska et al. (1995) reported that in safflower cv Centennial, addition of acetosyringone

(25-100 µM) increased the number of blue sectors in explants co-cultivated with EHA 105 in a dose dependent manner but did not give consistent effects in explants co-cultivated with LBA 4404.

The present study revealed that acetosyringone treatment at a concentration of 150 µM could enhance the frequency of transformed shoots.

5.2.9 Explant type

Explant type is a very important factor affecting plant transformation. In the present study, the response of four types of seedling explants viz., root, hypocotyl, cotyledonary leaf and primary leaf for *Agrobacterium*-mediated transformation was studied. The study revealed significant differences in the transformation ability among the explants. It was observed that root and hypocotyl explants were highly efficient for transformation followed by primary leaf and cotyledonary leaf. In safflower transformation studies, cotyledons, leaves, epicotyls, embryo axis were used for transformation but the differences in the transformability of the explants was not elucidated or determined (Ying et al., 1992; Orlikowska et al., 1995; Rao and Rohini, 1999; Rohini and Rao, 2000). Mukhopadhyay et al. (1992) reported that in oilseed *Brassica campestris*, the hypocotyl explants were more amenable to transformation compared to cotyledons. Similarly, differences in transformation with regard to the explant type have been reported in other plant species like tomato (Oktem et al., 1999), bean (Saima and Bushra, 2004), oak (Rube and Ricardo, 2007) and *Capsicum* (Sanatombi and Sharma, 2008). The variation in the transformation efficiencies of different explants may be related to the differences in the regenerative potential of the explant. In the present investigation on tissue culture studies, it was

observed that root and hypocotyl explants were more amenable for *in vitro* regeneration when compared to cotyledonary leaf. Similar differences with regard to the frequency of transformation from different explants has been observed.

The study revealed significant influence of explant type on transformation and that root and hypocotyl explants were more amenable for transformation compared to leaf explants.

5.2.10 Explant injury

Wounding of the explant prior to co-cultivation enhanced the formation of transformed shoots in cereals (Potrykus, 1990) and grasspea (Barik et al., 2005). Conversely, in the present study manual wounding of the explants failed to promote the recovery of transformants due to heavy bacterial contamination. In the previous experiments on genetic transformation in safflower, explants were not subjected to any kind of mechanical injury (Ying et al., 1992; Orlikowska et al., 1995) with the exception of the studies of Rao and Rohini (1999) and Rohini and Rao (2000) wherein the cotyledonary nodes of seedling segments and embryo axis were pricked with fine needle. Tissues harbouring meristematic cells are deep seated and are in active state of division and such cells respond to mechanical injury. Likewise, in meristem-based transformation of castor, wounding of explants enhanced the overall frequency of transformation (Sujatha and Sailaja, 2005). Wounding could have enhanced the sites of bacterial penetration into the tissues leading to uncontrollable bacterial overgrowth and tissue necrosis. McGranahan et al. (1998) and Ducrocq et al. (1994) also reported that wounding was not essential for efficient transformation in walnut and *Datura*, respectively. The present study revealed a negative effect of explant injury on transformation in safflower.

5.2.11 Elongation and Rooting

The putative transformed shoots obtained after the third cycle of selection were elongated on MS medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ and/or 1.0 mg/l KN+1.0 mg/l $AgNO_3$ which were found to be best for elongation of regenerated plants from the tissue culture experiments that were carried out as part of the current investigation. The frequency of transformed shoots that showed elongation was comparatively less than the normal tissue culture raised shoots. This may be due to the interference of several agents, processes and compounds during transformation and selection. Studies of Ying et al. (1992) also showed that the frequency of shoot regeneration following transformation (15.0%) was much lower when compared to the regeneration (26.0%) experiments.

Further, it has been observed that the frequency of rooting of the transformed shoots was less compared to that of untransformed shoots which could be due to adverse effect of T-DNA integration, hypersensitive response or inhibitory effect of kanamycin. In the study of Ying et al. (1992) putative transformed shoots failed to produce roots unlike in the present study where rooting was achieved on half-strength MS+1.0 mg/l NAA. The rooted shoots were successfully acclimatized and maintained under high humidity and transferred to soil.

The GUS histochemical assay of the leaf and florets of the T_0 plant also showed blue colouration confirming the presence of the transgene. In the previous investigations on safflower transformation, GUS histochemical assay was confined to calli and regenerating shoots (Ying et al., 1992; Rao and Rohini, 1999) with the exception of the study of Rohini and Rao (2000) wherein leaves of the green house established plants were subjected to GUS histochemical analysis. Further, the blue colouration was confined to sectors (Ying et al., 1992 and Rao and Rohini, 1999). However, in the present investigation uniform blue colouration of whole plants

was observed even when parts of sexually mature plants were subjected to GUS histochemical assay.

5.2.12 Molecular analysis

5.2.12.1 PCR

PCR analysis of the putative transformants with *Uid* A and *hpt* gene specific primers resulted in amplicons of the expected sizes of 1200 bp and 520 bp, respectively. In the present investigation 100.0% of the putative transformants (24/24) showed the amplified product when amplified with selectable marker (*hpt*) and reporter (*Uid* A) gene specific primers. In the study of Orlikowska et al. (1995), the PCR analysis showed amplification of 66.0% (4/6) for marker gene (*npt* II) and 50.0% (3/6) for the reporter gene (*Uid* A). In the *in planta* transformation studies of Rohini and Rao (2000), PCR amplicons for independent gene sequences were obtained at a frequency of 48.0% and 33.0% for *Uid* A and *npt II*, respectively in A-1 and 30.0% and 50.0% in A-300. However, when the plants were tested for the presence of both the genes, only 20.0% (A-300) to 30.0% (A-1) of the putative transformants showed the presence of the two genes. Conversely, in the present investigation, 100.0% of the putative transformants showed amplicons for the presence of the two genes indicating that the protocol being developed results in very low frequency of escapes which could be due to the high proliferative ability of selected shoots, stringency of the selection, the number of selection cycles and the use of hygromycin as plant selection marker. PCR study was also carried out with the v*ir* D2 gene specific primers to rule out the presence of bacterial contamination. The *vir* D2 gene amplification was observed only from the bacterial DNA confirming the absence of residual bacteria. Similar results were recorded with *vir* C specific primers in the transformation of safflower (Rao and Rohini, 1999;

Rohini and Rao, 2000). The amplification of the house keeping gene *actin* showed a band of expected size (450 bp) in untransformed safflower and putative transformants but not in the plasmid DNA. Finally, the PCR study revealed the presence of the transgene in the putative transformants.

5.2.12.2 RT-PCR

It is not only essential to determine the presence of the transgene but it is also important to check the expression of the introduced gene. Transcript level testing was done by using RT PCR analysis to check the presence of the transcript in the putative transformants. Rao and Rohini (1999) carried out Western blotting for the detection of β-glucuronidase protein in the putative transformants using an anti β-glucuronidase antibody and reported the presence of GUS protein in all the transformed calli of A-300 and multiple shoots of A-1. In the present investigation, RT-PCR analysis of five rooted transformants with *Uid* A and *hpt* specific primers showed the bands of expected sizes 1200 bp and 520 bp for *Uid* A and *hpt* genes, respectively in all the five primary transformants (100.0%) confirming the active transcription of the transgenes in all the tested primary transformants thus, confirming the efficieny of the protocol being employed.

5.2.12.3 Southern analysis

Southern analysis of the four putative transformed shoots (one plant died after transfer to green house) that were positive in PCR and RT PCR studies was carried by using 8, 6, 4 and 2 µg of DNA for plants 1, 2, 3 and 4, respectively. The studies of Southern analysis in safflower transformation for the marker (*npt* II) and reporter (*Uid* A) gene used 1.5-10 µg of DNA (Ying et al. 1992; Orlikowska et al., 1995; Rohini and Rao, 2000). In the present study, Southern hybridization was done for the *hpt* gene by restriction of the DNA with restriction enzyme *Xho* I

which cuts on either side of the gene releasing the *hpt* fragment of 1094 bp intact. Out of the four transformants analyzed one plant showed positive signal at 1.1 kb confirming the presence of the transgene. The lack of signal in the other three transformants could probably be due to the differences in the DNA concentrations used. As restriction with *Xho* I does not idicate the copy number, the analysis confirms only the stable integration of the transgene. Ying et al. (1992) failed to carry out Southern analysis of the putative transformants of safflower due to the difficulties in obtaining sufficient amount of DNA from the transformants, but 100.0% of the calli showed positive signal for the marker gene (*npt* II). The main aim of the present study was to optimize various parameters for efficient transformation which was proved by the stable GUS expression. Further PCR, RT-PCR and Southern analysis confirmed the stable integration of the introduced gene.

5.2.13 Conclusion

The present investigation revealed that the efficiency of *Agrobacterium*-mediated transformation in safflower is dependent on selection, genotype, seedling age, period of co-cultivation, bacterial cell density, acetoyringone and explants type. The protocol followed in the present study showed a maximum frequency of 51.0% of putative transformants after final cycle of selection with hypocotyl explants of 8-day-old seedlings of cv. HUS-305, co-cultivated with bacterial cell density of 0.5 OD_{600} and a co-cultivation period of 2 days followed by selection. MS medium supplemented with 0.2 mg/l TDZ+0.2 mg/l NAA (SV17) was used for the first cycle of selection followed by 0.5 mg/l BAP for the second cycle of selection and 0.2 mg/l BAP+0.5 mg/l KN for the third cycle of selection with a selection regime of 10-15-15 mg/l hygromycin for a period of

15 days each. After the release of selection pressure, the putative transformed shoots were transferred to MS medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$. Thus, the transformation frequency in the present study is very high (51.0%) as compared to 23-34% (Rao and Rohini, 1999), 1.3-5.3% (Rohini and Rao, 2000) and 15.0% (Ying et al., 1992). With the optimized transformation protocol putative transformants are obtained within 8-10 weeks of culture initiation.

5.3 Summary

The present investigation was undertaken to optimize various parameters for enhancing the frequency of transformation in safflower. Prior to the transformation study, the sensitivity of the explants to the antibiotics, cefotaxime (bacterial control) and hygromycin (plant selection marker) were studied and a selection regime for hygromycin was determined. The various parameters studied included genotype, seedling age, period of co-cultivation, bacterial cell density, enzymatic pretreatment, acetosyringone treatment, explant type and explant injury.

For controlling bacterial growth after transformation a concentration of 250 mg/l cefotaxime was found to be ideal. The selection regime optimized was 10-15-15 mg/l hygromycin. The study revealed that the genotype had significant influence on transformation and that among the studied genotypes, the cv. HUS-305 showed maximum transformation efficiency. With regard to seedling age, the younger tissues were more responsive compared to the older tissues even after transformation. The period of co-cultivation and bacterial cell density also influenced the transformation. The co-cultivation period of 2 days with a bacterial titre of 0.5 OD was found to be optimal. Enzymatic pretreatment and explant injury had negative influence on transformation in safflower due to uncontrollable bacterial growth. The use of the *vir*-gene enhancer, acetosyringone had a positive effect up to 150 µM and a negative effect thereafter. With regard to the explant type, root and hypocotyl were more amenable than cotyledonary leaf and primary leaf explants. The transformation efficiency was checked by GUS histochemical assay. The presence of the introduced gene and stable integration of the *hpt* and *Uid* A genes were confirmed through PCR, RT-PCR and Southern analysis of the primary transformants. In the present investigation the transformation frequency was increased to 50% which is very high compared to the previous studies.

Chapter 6

Transformation with unedited *atp 9* gene

6.1 Results

At the Directorate of Oilseeds Research, gene constructs for the development of male sterile lines in safflower have been developed by using *orf H522* (Narasimha et al., 2009) from sunflower and unedited mitochondrial genes, *nad 3* and *atp 9* (Yamini, 2007). In the present study, the *atp 9* gene was used for safflower transformation. The gene construct has TA 29-a tapetum specific promoter followed by *cox IV* transit peptide for mitochondrial targeting of the gene product, the unedited *atp 9* gene with a *nos* terminator. The plasmid vector used was pCAMBIA 1305.2 which has hygromycin as plant selection marker and the *Uid* A gene for GUS reporter activity. In the present study, the gene construct was mobilized into *Agrobacterium* strain LBA 4404 and used for transformation.

6.1.1 Mobilization of the developed construct into *Agrobacterium*

The gene construct pCAMBIA 1305.2 TCAN (T-TA29 promoter, C-coxIV transit peptide, A-unedited *atp 9* gene, N-nos terminator), already developed at the Directorate of Oilseeds Research (Yamini, 2007) was mobilized into *Agrobacterium* strain LBA 4404. After 2 days of incubation on YEP medium (with antibiotics), 12 colonies were observed. The transformed colonies were confirmed for the presence of the gene construct by colony PCR, PCR of plasmid DNA and restriction digestion analysis.

6.1.1.1 Colony PCR

Colony PCR was done for preliminary confirmation of the clones. Instead of plasmid DNA, the bacterial cells were used in the PCR reaction mix and the PCR was set as per details provided in section 3.18.2. The primer set used was TA 29 forward and *nos* reverse to check for the presence of the entire fragment. Out of the 12 clones, 9 clones showed the amplified product of the desired size (1187 bp) of TCAN fragment (Figure 6.1).

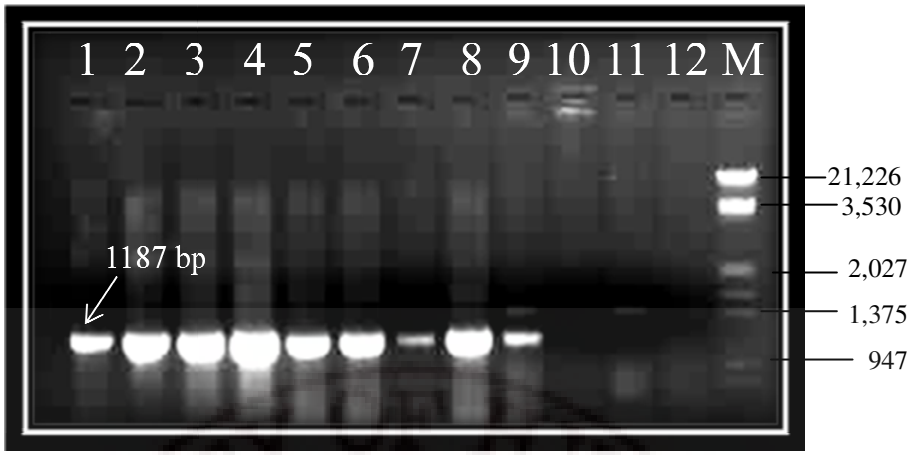


Figure 6.1 Colony PCR for confirmation of the presence of *uatp 9* by using the TA29 forward and *nos* reverse primers Lanes 1-12: Colonies obtained after transformation of the pCAMBIA 1305.2 TCAN into *Agrobacterium*, lane M: λ DNA double digest with *Eco*R I/*Hin*d III

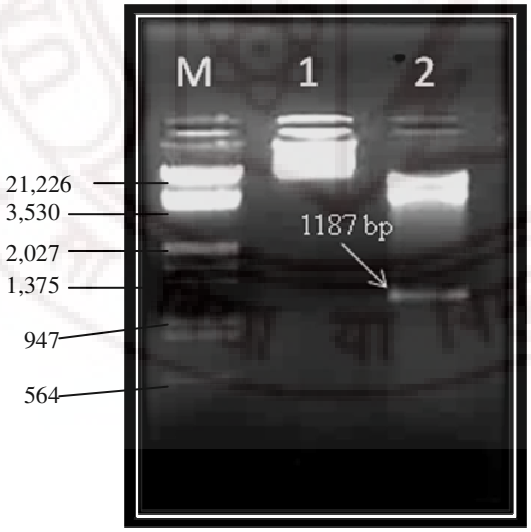


Figure 6.2 Confirmation of LBA 4404 pCAMBIA 1305.2 TCAN in *Agrobacterium* by restriction with *Hin*d III and *Eco*R I Lane M: λ DNA double digest with *Eco*R I/*Hin*d III, lane 1: Unrestricted plasmid DNA, lane 2: Restricted plasmid DNA

6.1.1.2 Restriction analysis

The plasmid DNA from the obtained clones upon restriction digestion with *Hin*d III and *Eco*R I showed the release of the insert (1187 bp) confirming the presence of the gene construct (Figure 6.2).

6.1.1.3 PCR analysis

PCR analysis of the plasmid DNA with TA 29 forward and *nos* reverse primers showed the fragment of desired size (1187 bp) confirming the presence of the insert in the bacterial colonies obtained (Figure not presented).

6.1.2 *Agrobacterium*–mediated transformation with unedited *atp 9* gene

With the developed gene construct harbouring the unedited version of *atp 9* (for induction of male sterility) and the optimized transformation protocol, transformation experiments were carried out. The 8-day-old seedling explants (root and hypocotyl) were subjected to vacuum infiltration with the bacterial (LBA 4404: pCAMBIA 1305.2 TCAN) concentration of 0.5 OD in the presence of 150 µM acetosyringone and cocultivated in dark for 2 days and transferred to selection media.

The first cycle of selection was carried on SV17 (MS+0.2 mg/l TDZ+0.2 mg/l NAA), followed by the second cycle of selection on 0.5 mg/l BAP and the final selection on media with 0.2 mg/l BAP+0.5 mg/l KN. At all the steps, cefotaxime at a concentration of 250 mg/l was used to check bacterial contamination. The selection regime followed was 10-15-15 mg/l hygromycin for both root and hypocotyl explants for the first, second and third cycles of selection, respectively. After first cycle of selection calli formation was observed from the cut ends. Following the second cycle of selection, shoot formation was observed (Table 6.1). The putative transformants obtained after the third cycle of selection are represented in Figure 6.3 A. The

Table 6.1 Transformation using *uatp 9* gene construct

Explant	Total number of explants	Selection I *	Selection II **	Selection III **
Root	1352	963	743	683
Hypocotyl	1502	1021	862	598

Data represents the number of putative transformants from 8-day-old seedling explants transformed with bacterial cell density of 0.5 OD_{600} with co-cultivation period of 2 days. Data was recorded after 15 days on each selection. The gene construct used was pCAMBIA 1305.2 TCAN

* Data represents number of explants callusing following the first cycle of selection

** Data represents number of explants with shoot regeneration after the second and the third cycles of selection

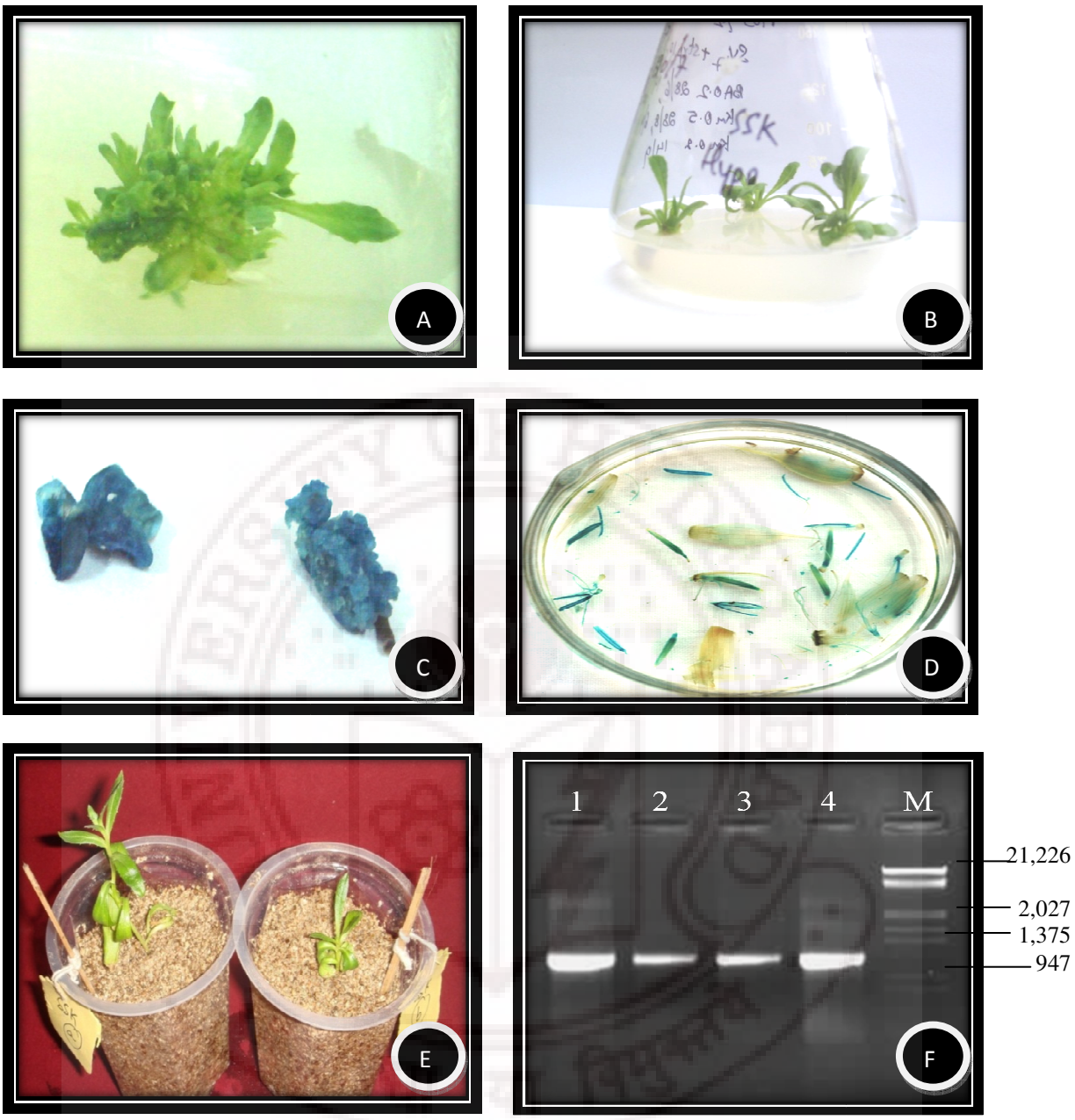


Figure 6.3 Transformation with pCAMBIA 1305.2 TCAN (*uatp 9*) construct. A. Putative transformed shoots after the final cycle of selection, **B**. Transformants on elongation medium, 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$, **C**. Transformed shoots showing blue colouration after the second cycle of selection following the GUS assay, **D**. The floral organs of the transformed shoots showing blue colour after GUS staining, **E**. The primary transformed shoot in the soil, **F**. PCR analysis of the putative transformants for the presence of transgene *atp 9*: Lanes 1-4: Putative transformants; lane M: λ DNA double digest with *Eco*R I/*Hin*d III

shoots were elongated on MS medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l $AgNO_3$ (Figure 6.3 B). The shoots rooted on half-strength MS medium supplemented with 1.0 mg/l NAA, but with low frequency compared to the untransformed shoots. The rooted shoots were acclimatized and transferred to soil (Figure 6.3 E). The primary transformants were confirmed by GUS histochemical assay and PCR analysis.

6.1.3 Confirmation of the putative transformants

6.1.3.1 GUS histochemical assay

The presence of the transgene in the putative transformants was confirmed by the blue colouration observed after GUS staining of the obtained shoots. GUS histochemical assay of shoots recovered after the third cycle of selection showed GUS positive shoots with a frequency of 98.0% (Figure 6.3 C, D) confirming the presence of the transgene.

6.1.3.2 PCR analysis

PCR analysis of four primary transformants with TA 29 forward primer and *nos* reverse primer showed the amplicon size of 1187 bp (Figure 6.3 F) in all the four plants confirming stable integration of the transgene. Further characterization of the transformants is underway.

6.2 Discussion

Safflower is predominantly self-pollinated crop. For exploitation of heterosis and hybrid seed production development of male sterile lines assumes importance. At the Directorate of Oilseeds Research, gene constructs for induction of male sterility in safflower have been developed. These constructs include the *orf H522* gene from sunflower which is known to cause male sterility in the sterile *Pet 1* cytoplasm and unedited mitochondrial genes from safflower viz., *nad 3* and *atp* 9. Genetic transformation of tobacco with *orf H522* gene resulted in male sterility (Narasimha et al., 2009). The constructs were under TA 29 (tapetum specific promoter) for tissue specific expression of the genes. For targeting the polypeptide to mitochondria, a *cox IV* transit peptide was used. The construct used *nos* as the terminator sequence. In the present study, the gene construct harbouring *atp 9* (unedited) was used for induction of male sterility in safflower. The study was confined to the GUS histochemical assay and PCR confirmation of the primary transformants. Further characterization of the putative transformants is under progress.

Most of the studies for induction of male sterility were aimed at the disruption of the mitochondrial function which reduces the ATP production that severely affects pollen fertility. In the present study, unedited *atp 9* gene (from safflower) was intended to express in the tapetal cell layer to induce male sterility. The gene is driven by the T A29 promoter (tapetum specific promoter), which was fused to the *cox IV* transit peptide (from yeast) to target the resultant peptide into mitochondria. Similar approach for the production of male sterile lines had been adopted in tobacco (Hernould et al., 1998) and *Arabidopsis* (Gomez-Casati et al., 2002). RNA editing in *atp 9* gene has been reported in several plant species such as wheat (Begu et al., 1990), Petunia (Wintz and Hanson, 1991) and tobacco (Hernould et al., 1993). The previous study (Yamini et al., 2008) reported that safflower *atp 9* trancript undergoes editing at 12 sites, 10

complete and 2 partial edit sites resulting in shortening of the *atp 9* polypeptide by 12 amino acids due to the stop codon created by editing at nucleotide position 223. Similar observation were reported in *Oenothera* (Schuster and Brennicke, 1990), sorghum (Salazar et al., 1991) and *Petunia* (Wintz and Hanson, 1991).

This is the first attempt at transformation of safflower using an agronomically desirable gene. The previous studies were confined to genetic transformation of safflower using constructs harbouring only the selectable and reporter marker genes (*Uid* A, *npt* II). Use of the optimized protocol for transformation resulted in the development of transgenics through deployment of the unedited *atp 9* gene. All the putative transformants were confirmed to harbour the introduced gene through PCR analysis. Further studies on characterization of the transgenics are under progress.

6.3 Summary

The present study was aimed to transform safflower with an agronomically desirable trait, that induces male sterility (unedited *atp 9*). The basis of the study is the expression of unedited version of the important mitochondrial gene (*atp 9*) under tapetum specific promotor, targeting the resultant polypeptide to the mitochondria that impairs the mitochondrial functionality leading to the male sterility.

With the optimized transformation parameters the *Agrobacterium*-mediated transformation was carried out with the gene construct pCAMBIA 1305.2 TCAN (T-TA 29 taptum specific promoter, C-*cox IV* transit peptide, A-*atp 9* unedited version, N-*nos* terminator). The putative transformants obtained were confirmed by GUS histochemical assay and PCR analysis. The GUS assay showed GUS positive shoots with a frequency of 98.0%. The PCR study (TA 29 forward and *nos* reverse primers) showed the amplicon of expected size (1187 bp) confirming the presence of the transgene. The further characterization of the tranformants (RT-PCR Southern analysis, pollen study, progeny analysis) is an ongoing project at the Directorate of Oilseeds Research.

Chapter 7
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