Optimization of Regeneration and Transformation of *Fagonia indica*

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By

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Abstract

Fagonia indica (syn. *F. cretica*), commonly known as Dhamasa, is an important medicinal plant. It contains many important compounds especially active against breast and liver cancer. *In vitro* propagation of this plant to increase the mass is an important step to obtain large amount of these compounds. Furthermore, genetic engineering of this plant is very valuable in improving the genetic diversity as well as to increase secondary metabolites. The aim of this study is to optimize different tissue culture conditions as well as to establish a significant protocol for *Agrobacterium tumifaciens* mediated transformation of *F. indica*. Treatment of seeds and explants with 70% ethanol, 0.1% mercuric chloride, 5% sodium hypochlorite and terbinafine (1mg/ml) resulted in significant control of bacterial and fungal contamination. The seed germination efficiency is maximum on MS medium i.e. 45% as compared to ½ MS (30%), RMOP (10%) and B5 (6.11%). Out of three explants (shoot apical meristems, nodes and cotyledons) chose for *in vitro* propagation, shoot apical meristems has maximum regenerative potential. The optimized callus induction medium which gave 100% efficiency is MS medium supplemented with 1:1 of NAA:BAP (0.5 mg/l). The best results for shoot induction medium include MS supplemented with BAP (0.1mg/l) only. During optimization experiments, somatic embryos and protocorm like bodies are formed in number of combinations of calli and shoot induction media. The roots were induced on media containing BAP, NAA and IAA in different combinations. *A. tumifaciens* strain C58C1 harbouring a binary vector p35GUSint containing neomycin phosphotransferase (*nptII*) as selectable marker and β-glucuronidase (*GUS*) as a reporter gene, was used for plant transformation. The optimal concentration of Cefotaxime and Kanamycin for elimination of *A. tumifaciens* and transgenic explants was found to be 300 mg/l and 50mg/l respectively. Transient *GUS* expression of *A. tumifaciens*-mediated transformation showed that bacterial density of OD_{600} value of 0.5, co-infection time of 7 minutes, co-cultivation time of 1 day and acetosyringone concentration of 200 µM for explants proved to be best variables for successful transformation, as maximum transformation efficiency was obtained with these optimized conditions. Out of five explants i.e. stipules, leaves, shoot apical meristems, nodes and callus, the maximum transformation efficiency was observed in callus. The tissue culture and transformation conditions optimized in this study can be helpful for future research of *F. indica* genetic engineering.

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In the name of **ALMIGHTY ALLAH SUBHAN-O-TA'ALA**, the most Gracious, the most Merciful and the Giver of bountiful blessings and gifts. Peace of Allah be upon my **HAZRAT MUHAMMAD (S.A.W)**, his family and companions who is forever a torch of knowledge and guidance for the humanity.

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Declaration

I, Maryam Naeem,student of M.Phil Biochemistry/ Molecular Biology, session 2013-2015, hereby declare, that work presented in the thesis "Optimization of Regeneration and Transformation of *Fagonia indica*" is my own effort, except where others have been acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Signature of deponent _____

Dated: _____

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LIST OF ABBREVIATIONS

%	Percentage
µM	Micro molar

½ MS medium	Half strength Murashige and Skoog medium
AS	Acetosyringone
BAP	Benzyl aminopurine
CCM	Co-cultivation medium
Conc.	concentration
DIVA	Dispersal vicariance analysis
DNA	Deoxyribo nucleic acid
e.g.	For example
g	gram
GFP	Green fluorescent protein
GUS	β -Glucuronidase
HCl	Hydrochloric acid
hpt	Hygromycin phosphotransferase
i.e.	That is
IAA	Indolyl acetic acid
INT	Intron
ITS	Internal transcribed sequence

kb	Kilobase
LA	Luria-bertani agar
lacZ	Beta galactosidase
LB	Luria-broth
LFH	Laminar flow hood
Luc	Luciferase
mg	Milligram
mg/l	Milligram per liter
ml	Milliliter
MS medium	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
NOS	Nopaline synthase
NOS P	Nopaline synthase promoter
NOS T	Nopaline synthase terminator
NPTII	Neomycin phosphotransferase II
^{o}C	Degree centigrade

OCS	Octapine synthase
OD	Optical density
PGR	Plant growth regulator
pH	Power of hydrogen ions
PPT	L-phosphinothiricin
RMOP	Revised medium for organogenesis of plant
SAM	Shoot apical meristem
sp. and spp.	Species
TE	Transformation efficiency
T-DNA	Transfer DNA
Ti	Tumor inducing
TrnL	T RNA asparagine
UV	Ultra violet
Vir	Virulence
X-gluc	5-bromo-4-chloro-3-indolyl glucoronide
YEB	Yeast extract broth

INTRODUCTION

This study covered the selection and optimization of tissue culturing and transformation conditions of medicinally important plant i.e. *F. indica* commonly known as Dhamasa. The plant is used as decoction for the treatment of many diseases especially liver and breast cancers.

1.1. Family Zygophyllaceae

The Zygophyllaceae belongs to family of flowering plants. Species in this family are mostly found in tropical and warm climates, specifically in arid habitats. Most species are shrubby in morphology, but small trees and herbs are also present. This family includes a number of well-known species such as bean-caper and caltrop. It includes around 285 species in 22 genera. "Genera of *Zygophyllaceae* subfam. *Zygophylloideae*". *Germplasm Resources Information Network*. United States Department of Agriculture. Retrieved 2010-08-15. Zygophyllaceae is divided into five subfamilies Larreoideae, Morkillioideae, Seetzenioideae, Zygophylloideae and Tribuloideae. A molecular phylogeny of the family is also reported (Sheahan and Chase, 1996; Sheahan and Chase, 2000). Phylogenies of groups within the family have also been published. A phylogeny of the southern African species of Zygophyllum was published in 2008. In Pakistan, so far 8 genera and 22 species are reported in subtropical and warm temperate regions (http://www.tropicos.org/). Classification is shown in table 1.1.

1.1.1. Ethnobotany of family Zygophyllaceae

Aqueous decoction of the plants included in Zygophyllaceae is used in indigenous system of medicines for their ability to treat various digestive and blood vascular system disorders and early stage cancers (Saeed, 1969). The aerial parts, including flowers of certain species of Zygophyllaceae were investigated for glycosides linked to a glycan moieties such as flavonoids, terpenoids, triterpenoids or steroidal saponins (Atta *et al.,* 1982; Ansari *et al.,* 1987; EI-Hadidi *et al.,* 1988).

Table1.1. Classification of *Fagonia indica*

KINGDOM	Plantae
SUB-KINGDOM	Viridaeplantae
PHYLUM	Tracheophytae
SUB-PHYLUM	Euphyllophytina
INFRA-PHYLUM	Radiatopses
CLASS	Magnoliopsida
SUB-CLASS	Rosidae
SUPERORDER	Geranianae
ORDER	Zygophyllales
SUB-ORDER	Zygophyllineae
FAMILY	Zygophyllaceae
GENUS	*Fagonia*
SPECIES	*Indica*

1.2. Genus *Fagonia*

The genus *Fagonia* is a member of Zygophyllaceae, which is included in the eurosid I clade (APG II, 2003). A genus of about 45 species; confined to warm and arid regions of all continents except Australia. The genus consists of shrubs, shrublets or herbs with pointed or spinescent stipules. The colour of petals in this genus is either pink or purple, the capsule shape is obconical, more or less pubescent, loculicidal containing persisting sepals. The leaves of most species in this genus are 3-foliolate, but there are also species, which are consistently 1-foliolate (Beier, 2005). It is well perceived as the important medicinal plants containing genus among the folk medical scholars.

1.2.1. Geographical distribution of genus *Fagonia*

In a phylogenetic analysis of Zygophylloideae, based on molecular and morphological data, *Fagonia* was shown to have a well-supported position as sister to the genus Melocarpum, endemic to the northeast of Africa region (Beier *et al.*, 2003). *Fagonia* is distributed to the arid areas of the New and Old World (Figure. 1). It is found in Mexico, southwestern USA, Chile, and Peru in the New World, whereas in the Old World, it is found in south Africa, north Africa and the southern parts of Europe, including many of the Mediterranean islands. *Fagonia* is also found in the northeast Africa including Kenya, western Asia including Lebanon, Turkey, and the Arabian Peninsula east to Afghanistan and western India. A single species in South America is endemic to Chile and Peru, and the two species of southern Africa are confined to Botswana, Namibia, and South Africa, six species in Baja California and eight species in northeast of Africa region. The genus has not been reported from Madagascar so far. The disrupt distribution of *Fagonia* has been explained by several different hypotheses. Engler (1896, 1915) proposed that *Fagonia* is introduced into the new world by cargo-shipment of the seeds of *Fagonia cretica* from Iberian Peninsula. However, later Engler (1931), considered the distribution as prehistoric and not a result of anthropogenic dispersal. Johnston (1940) explained the distribution of *Fagonia* is due to widespread of early Tertiary desert flora. Axelrod (1950) initially considered the distribution as a result of a break-up of the range of a common pantropical tertiary ancestor and subsequent speciation. Later, Axelrod (1970) considered the distribution as being remnant of

the dry flora, whereas Stebbins and Day (1967) considered that the distribution was a result of a pre-tertiary migration from the Old to the New World via the Bering land bridge. Porter (1974) proposed a combination of sea-floor spreading and long-distance dispersal from east to west during the early tertiary, when the continents of the Old and New Worlds were much closer than at present.

1.2.1.2. Phylogenetic relationships with in genus *Fagonia*

There were few studies on the phylogenetic relationships of genus *Fagonia*. The North African *Fagonia* species are divided into four ‘‘natural groups’’: (1) the *F. kahirina–cretica– flamandii* group, (2) the *F. arabica–bruguieri* group, (3) the *F. glutinosa–latifolia* group, and (4) the *F. microphyl-la* group (Ozenda and Que´zel, 1957). El Hadidi (1966), divided the North African *Fagonia* species into three groups and later modified the ‘‘natural groups’’ species ‘‘with tri- or unifoliolate leaves’’ or ‘‘with simple leaves’’.

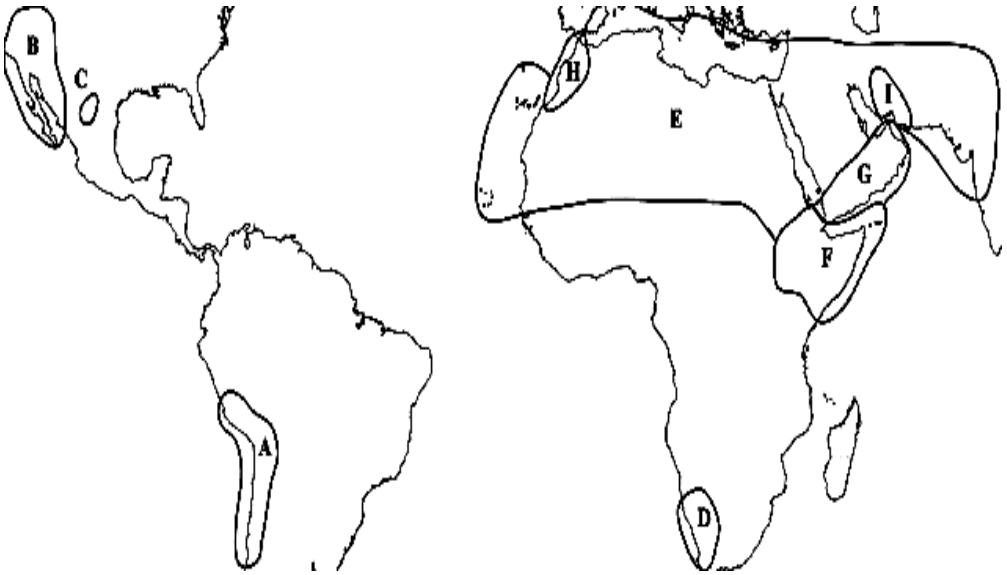


Fig 1: Distribution of *Fagonia* with in 9 particular areas; A; South America, B; North America, C; Northeastern Mexico, D; South Africa, E; Saharo-Sind, F; Horns Of Africa, G; Yemen and Oman, H; Western Morrocco, I; Southern Iran

The group with ‘‘tri- or unifoliolate leaves’’ was in turn divided into three ‘‘complexes’’: (1) *F. arabica*-complex, (2) *F. bruguieri*-complex, and (3) *F. indica*-complex. *F. bruguieri*-complex and *F. indica*-complex contained many taxa which are eliminated by Beier (2005). El Hadidi (1974) also made changes in the division of the complexes and divided them into: (1) the *F. isotricha*-complex (*F. isotricha* is now a synonym of *F. latifolia*), (2) the *F. glutinosa*-complex, (3) the *F. sinaica*-complex (*F. sinaica* is now a synonym of *F. scabra*), (4) the *F. thebaica*-complex (*F. thebaica* is now a synonym of *F. arabica*). The species of the New World *F. californica* and *F. chilens* (as *F. subaphylla*), were hypothesized to be associated with the *F. sinaica*-complex. El Hadidi (1974) also recognized that the three groups of *Fagonia* species with short stipules and trifoliolate leaves were more closely related to each other than to the group of species with long stipules and one to tri-foliolate leaves.

Phylogenetic studies done by Beier (2005) gave different distribution and phylogenetic relations among *Fagonia* spp. as compared to El Hadidi. Beier’s uses plastid trnL intron and nuclear ribosomal ITS DNA sequences of 34 species of *Fagonia* genus. The result divides the species into different clades based on the genetic and morphological resemblance (divided into 16 sub-divisions known as Clades (A-P)). There is little resolution within *Fagonia*. The genus is weakly supported, and *F. scopariais* sister to the rest of *Fagonia* form a clade (clade B). The other *Fagonia* species of the New World form a clade (clade C), and *F. acerosa*, endemic to Iran, and the widespread North African-Asian *F. Bruguieri* form a clade (clade D). Another well-supported clade comprises *F. lahovarii*, endemic to the Horn of Africa region including Yemen, *F. latistipulata*, endemic to Somalia, and *F. mollis*, restricted to Egypt, Israel/Palestine, Jordan, and northern Saudi Arabia form a clade (clade E). A clade consisting of *F. harpago*, endemic to Morocco and *F. longispina*, endemic to Morocco and Algeria form clade (clade F). The two species from southern Africa, *F. minutistipula* and *F. rangei*, form a weakly supported clade (clade G). All samples of *F. indica* and *F. paulayana*, which are widespread North African-Asian species, and *F. subinermis*, endemic to Iran, form a moderately supported clade (clade H), within which there is little

internal structure resemblance. *F. olivieri*, found in Lebanon, Syria, Iran, Iraq, and Jordan (clade P).

The result of the DIVA analysis showed 28 dispersals, and the historical distribution of the ancestor of *Fagonia* is given two alternative ancestral distributions in the analysis: North America, northeastern Mexico, and Saharo-Sind with the bordering area of western Morocco, or the same area, but including the Horn of Africa. The ancestor to all species of *Fagonia* except *F. scoparia is* also given two alternative ancestral distributions: North America, Saharo-Sind with the bordering area of Morocco, or the same area plus the Horn of Africa. Vicariance is inferred as the event that separated the ancestor of all *Fagonia* species, except *F. cretica* and *F. scoparia*, to the areas of North America and the Horn of Africa region. Considering that vicariance took place between two areas not adjoined today i.e. North America and Horn of Africa. All the NewWorld species of *Fagonia* are indicated to be a result of successive duplications (i.e., speciations within the area), with the exception of *F. chilensis* in South America, the distribution of which is conceived as a result of dispersal of the ancestor. The ancestor of all species of the Old World, except *F. cretica* endemic to the Horn of Africa, from where dispersal to Saharo-Sind or western Morocco, and southern Arabia took place. The ancestor of all species of the Old World, except *F. cretica* and *F. luntii,* had a wide distribution covering the Horn of Africa and Saharo-Sind, or the Horn of Africa and western Morocco. The ancestor of clade M, which includes: *F. gypsophila, F. indica, F. lahovarii, F. latistipulata, F. mahrana, F. mollis, F. paulayana* and *F. subinermis*, were endemic to the Horn of Africa, from where it dispersed into southern Arabia and southern Iran, as well as to Saharo-Sind. The ancestor to the species included in clade M, is endemic to Saharo-Sind or western Morocco. Subsequently, speciation within the area occurred, after which dispersal to southern Iran, southern Arabia, and southern Africa and to the Horn of Africa took place. Some of narrow endemics such as *F. hadramautica* are indicated to have evolved after relatively recent dispersals. The dispersal–vicariance analysis shows that the occurrence of *Fagonia* in South America and southern Africa is most likely a result of dispersal (Beier, 2005).

1.3. Ethnobotany of genus *Fagonia*

Plants and herbs have provided natural remedies for human ailments from time immortal. As knowledge progressed, man selected different herbs for cure of different diseases and ailments. The World Health Organization (WHO) estimated that about 80% of the world's population relies on traditional medicines for their primary health care. Most of the plants, which are used for various ailments, have not been properly investigated. About 30% of the pharmaceuticals are prepared from plants worldwide (Farnsworth and Morris, 1976). Traditionally species, present in genus *Fagonia* are used in Sind and Afghanistan for fever treatment. The plant is given as a tonic and febrifuge, and in the Peshawar Valley it is given to children as a prophylactic against small- pox. The leaves and twigs are supposed to possess cooling properties. In the Ormara hills the plant is boiled and bound upon the swellings of the neck and for scrofula. At Saruna in Jhalawan, it is boiled in water and strained, the liquid is rubbed all over the bodies of children when they got fever. In Kharan, an infusion made with hot water is used as a bath in cases of fever. The plant is considered to cure for itch in the Las Bela State and in the Levy tracts, the plant is, for this purpose pounded, mixed with milk, kept for three days and then rubbed all over the body (Kirtikar and Basu, 1975). Species of *Fagonia* have been found to contain saponins, alkaloids , terpenoids, sterols , flavonoids , coumarins , trace elements, proteins and amino acids (Saleh, Hasan and Aftab, 2011). Species of *Fagonia* i.e. *F. mollis* contains number of saponins and its derivative i.e. oleanolic acid-3-O-6'-O-methyl-β-D-glucuronopyranoside, oleanolic acid 3-O-α-L-rhamnopyranosyl (1→3)-6'-O-methyl-β-o-glucuronopyranoside and oleanolic acid 3-O-α-L-rhamnopyranosyl (1→3)-6'-O-methyl-β-o-glucuronopyranosyl28-O-β-D-glucopyranoside (Melek *et al*., 1996). The separation and characterization of ursolic acid, pinitol, nahagenin and hederagenin from other *Fagonia* species have also been reported (Rahman *et al*., 1984). The isolation of other important constituents such as docosanoate from hexane extract (Hamid *et al.,* 1989) and water soluble proteins from aqueous extract of air-dried *F. indica* has also been reported (Shaukat *et al.,* 1981). The cytotoxic effect of Kaempeferol glycosides isolated from *F. taeckholmiana* against different cells has also been reported (Ibrahim *et al.,* 2008). New erythroxane-type diterpenoids from *Fagonia* showed cancer-preventing potential (Gedara *et al*., 2003). The extracts of *Fagonia*

cretica linn are able to induce neuroprotection in rat brain model by initiating the anti-oxidant system (Rawal and Muddeshwar, 2004). Extacts from aerial parts of *F. longispina* has antibacterial and antioxidant activities (Hamidi *et al*., 2014). The anti-microbial and cytotoxic effects of *F. olivieri* extracts and fractions has also been reported (Rashid *et al.,* 2013). The alcoholic extracts of *F. schweinfurthii* has an anti-inflammatory and wound healing activity (Saleh, Hasan and Aftab, 2011). The anti-microbial activity of *F. cretica* has been investigated on different strains of bacteria (Anjum *et al.,* 2007). Adronergic effects of *F. cretica* alcoholic extracts on immunomodulation of albino rats is reported by Abirami *et al*. (1996). The anti-allergic effects of *F. bruguieri* were investigated on albino guinea pigs (Yahya *et al*., 2007).

1.4. *Fagonia indica*

Fagonia indica is a small spiny undershrub found mostly in arid, desert regions of Pakistan, India, Africa and parts of Europe, such as Balearic Islands in Spain, where it grows in rocky coastlines. It is widely distributed in Pakistan, Afghanistan, the Indian sub-continent and Egypt (Hussain *et al*., 2007).

1.4.1. Common name

Dhamasa, Dhamaya or Kandiara

1.4.2. English name

Southern Cyprus

1.4.3. Synonyms

Fagonia cretica

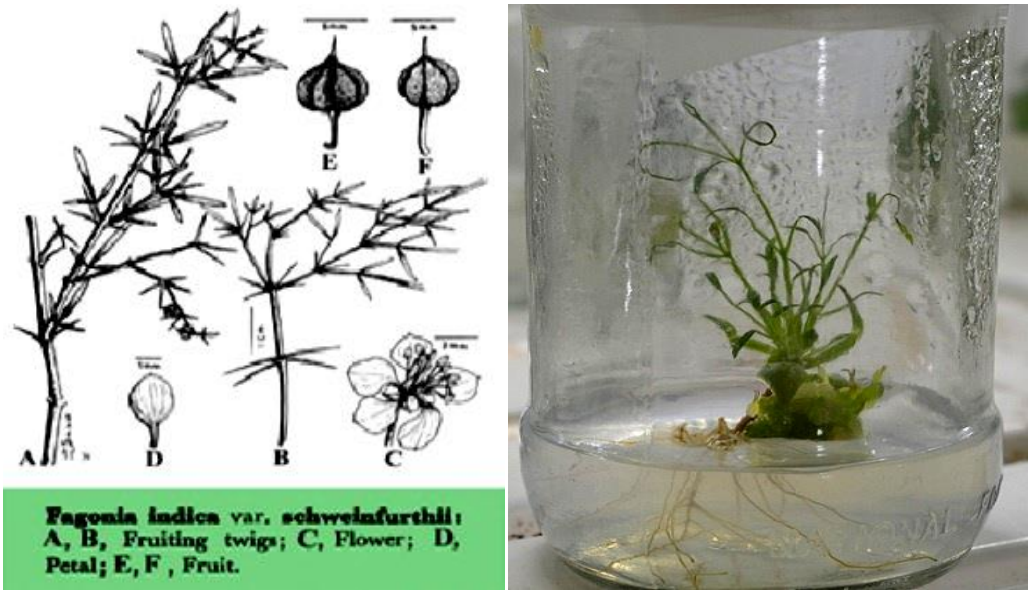
1.4.4. Controversy in naming of *Fagonia indica*

There is a confusion on whether we use the name *F. cretica* or *F. indica* for this plant because the research on this plant had been conducted under the name *F. cretica* by Saeed *et al*. (2003); Razi *et al*. (2011) and Lam *et al*. (2012). Apparently the *F. cretica* is limited to Mediterranean and Middle Eastern regions according to new revision of Beier *et al*. (2005). In Pakistan, *F. cretica* is used as synonym of

F. indica or particularly its variety *schweinfurthii* according to flora of Pakistan under the classification of university of Missouri (http://www.tropicos.org/Name/100336325?projectid=32). Our lab also published papers of *F. indica*, using its synonym *F. cretica* (Molecular Biology Lab Biochemistry dept. supervisor: Dr Bushra Mirza (HOD) Quaid-e-Azam university Islamabad) (Hussain *et al.,* 2007) (Saleem *et al.,* 2014)

1.5. Morphology

It is profuse, branched, pale green, glandular, annual or perennial shrublets up to 60 cm high. Internodes: 2.5-5.0 cm long. Leaves: opposite, uni or trifoliate, from 3 – 30 mm long, deeply striate, narrowly lanceolate, mucronate, distinctly articulate at the base. Stipules: 2 pairs of sharp slender thorns, sometimes exceeding 12 mm in length, aculeate, shorter than leaves and internodes, patent, not reflexed (Nasit, Ali .Eds. Flora of West Pakistan http://www.tropicos.org/). Leaflets: linear, acute, sessile, or with very short petioles (Beier, 2005). Stem basally woody, branches prostrate to erect, terete, striate, with up to 1-3 (-3.5) cm long internodes. Flowers: solitary in the axils, fragrant, 12mm in diameter, spathulate, obtuse. Stamen: 6 mm long. Capsule: 4×4 mm, softly hairy, pedicels 3-8 mm long, with pink to purple petals, reflexed, equal to the length of fruits. Fruit: a capsule, 3-5 mm long, 2-5 mm wide, short pubescent with persistent style. Fig 1.2. (A) shows *Fagonia indica* illustration. Seeds flat rounded and brown in colour (Bhandari, 1990; Eman, 2011).



(A) (B)

Fig 1.2. *Fagonia indica* (A) Illustration of *Fagonia indica* (B) Tissue cultured *Fagonia indica.*

1.6. Distribution of *Fagonia indica* across the world

Fagonia indica is present commonly in Pakistan, India, Iran, Aden, Eritrea, Aethopia, Sudan, Somalia, Kenya, Garcin, north and east of Africa and semi arid regions of world (http://www.tropicos.org/).

1.6.1. Distribution of *Fagonia indica* across Pakistan

C-6 Mianwali Dist, D-8 Lahore Dist: about 8 miles from Patoki on way to Lahore, F-5 Sanghar Dist: Haji Fateh Ali Goth, between Kandiari and Sanghar, G-4 Dadu Dist: 8 miles from Thana Bulla Khan on way to Karachi, Karachi Dist: 10 miles from Karachi on way to Bella, near Zoology dept., Karachi University Campus, North Nazimabad: common on sandy hillocks, Nasirabad: sandy clay soil, common, Karachi: rocky desert, G-5 Hyderabad Dist: between Tando Jam and Tando, G-6 Thar Parker Dist: near Nagar Parker plains, on way to Virawa, Botanical garden of Quaid-e-Azam university Islamabad.(http://www.tropicos.org/).

1.7. Ethnobotany/ Medical importance of *Fagonia indica*

Fagonia species were extensively studied by many workers regarding their medicinal uses, since these plants are antitumor, antioxidant, analgesic, astringent, febrifuge and prophylactic against small-pox agents. Species of *Fagonia* were also used in the indigenous system for the treatment of cancers, fever, asthma, urinary discharges, toothache, stomach troubles and kidney diseases. *F. indica* is very bitter in taste. In scientific and folk literature, it is reputed as medicinal plant with rich therapeutic potential. An aqueous decoction of plant is popular remedy for the treatment of skin lesions and boils (Hussain *et al.,* 2007). It is reputed in the indigenous system of medicine as tonic, febrifuge and prophylactic against small pox. It is well documented for the treatment of fever such as typhoid, thirst, vomiting, dysentery, asthma, urinary discharges and filtration process, liver and stomach problems (Saeed and Sabir, 2003). Boiled plant is used to induce abortion. It is also famous for its antivenom, anti-inflammatory and antipyretic activity (Satpute *et al.,* 2009). An aqueous decoction of the aerial parts of the plant is used for treatment of cancer especially for breast cancer in its early stages and for treatment of various diseases of digestive and blood vascular system (Saeed *et al.,* 1969). *F. Indica* has been shown to elevate GSH levels as well as having strong free radical scavenging properties (Rawal *et al.,* 2004). Plant extracts has shown antitumor and cytotoxic properties *in vitro (*Hussain *et al.,* 2007*).* Compounds isolate from *F. Indica* has cytotoxic activity towards erythrocytes and leucocytes (Saeed and Sabir, 2003). It has also shown anti-inflammatory properties including platelets aggregation, down regulation of COX2 and upregulation of VEGF (Rawal *et al.,* 2004). A saponin isolated from *F. Indica* has been shown to selectively induce aponecrosis in cancer cells (Waheed *et al.,* 2012). Antibacterial effects of *F. Indica* have also been reported (Gehlot and Bohra, 2000). Effects of powdered *Fagonia indica* plant and triterpenoid from its ethanolic extract showed that the saponins had highly significant decreasing effects on the amount of total leukocyte count of rabbit's blood (Asif *et al.,* 2003). The plant leaf extracts were found most effective against *Salmonella typhi* (Geholt *et al.,* 2000). The plant extract showed molluscicidal activity (Shoeb *et al.,* 1987). Furthermore, an aqueous extract of *F. indica* was able to significantly increase survival time in mice with induced tumors. Recently, it is reported that an aqueous extract of *F. Indica* can induce cell cycle

arrest and apoptosis in human breast cancer cell lines via p53-dependent and independent mechanisms with activation of DNA damage (Lam *et al.,* 2012). Analgesic and antimicrobial activity of *Fagonia indica* extracts has been reported (Sharma *et al.,* 2009). *Fagonia* species contains potent antifungal, antibacterial agents and has cytotoxic property (Zhang *et al.,* 2008; Gupta *et al.,* 2009). Alkaloids (Sharawy and Alshammari, 2009), terpenoids (Perrone *et al.,* 2007), sterols (Shoeb *et al.,*1994), flavonoids (Ibrahim *et al.,*2008), proteins and amino acids (Sharma *et al.,* 2010) were so far reported from this plant.

F. indica is rich in various types of chemical constituents such as triterpenoids, saponins, coumarins,f lavanoids and tannins(Abdel-Khaliq *et al.,* 2001). It also contains flavanoids such as quercetin, kaempferol, isorhamntin-α-3-O-rhamnoside, quercetin 3-O-β-D-glucopyranosyl-(1”-6”’)-β-D-glucopyrannoside and quercetin 3-O-β-D-galactopyranoyl-(6’-1’’’)-α-L-2’’’acetylrhamnose-(3’’’-1’’’’)-β-D-glucopyranoside. Major saponins isolated so far from this plant include 3-O-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] hederagenin 28-O-β-D-glucopyranosyl ester, 3-O-] β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] oleanolicacid, 28-O-[β-D-glucopyranosyl(1→6)-β-D- glucopyranosyl] ester, 3-O-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl], 27-hydroxy oleanolic acid, 3β-O-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] olean-12-en-27-al-28-oic acid (Ansari *et al.,* 1988; Abdel Khalik *et al.,* 2001).

1.8. Tissue culturing in *Fagonia* species

So far, few papers were reported on tissue culturing of the species included in genus *Fagonia.* Callus induction in *Tribulus terrestris*, using leaf and stem as an explants for detection of metabolites has been reported (Erhun and Sofowora, 1985). Callogenesis and somatic embryogenesis from cotyledon explants of *Fagonia indica burm* was also reported (Ebrahimi and Payan, 2013). *In vitro* Propagation through seeds of *Guaiacum sanctum* was reported by Valverde-Cerdas, Rojas-Vargas and Hine-Gómez (2008). Callogenesis of *Fagonia arabica* L for chemical analysis was also reported (Eman *et al.,* 2010). Callogenesis of *F. indica* and *Fagonia bruguieri Dc*. for phytochemical screening had also been reported (Eman andAlam, 2010).

1.8.1. Limitation of tissue culturing

The less work on *in vitro* propagation of genus *Fagonia spp*. is due to high rate of contamination, poor regeneration capacity of explants and poor germination rate of the seeds. The seeds give poor germination on artificial media with browning of cotyledons and retarded growth (Valverde-Cerdas *et al.,* 2008).Although many attempt has been made on clonal propagation of medicinal important plants in this genus but results are non-satisfactory. Uptill now, only callogenesis of few species are reported. The high rate of fungal contamination is the main hindrance in establishing the tissue culture conditions for plants included in *Fagonia* as well as their poor growth *in vitro*.

1.9. Plant tissue culturing

Plant cell and tissue culture is defined as the capability of any plant part to regenerate into complete plant, tissue or organ under sterilized and controlled conditions (Murishage and Skoog, 1974). Clonal propagation, rapid propagation, micropropagation, *in vitro* propagation (in vitro means ‘’in glass’’ or ‘’inartificial conditions’’ (Thiart, 2003)) are names, that are used as an alternative of term tissue culture. The basic principle of tissue culture technique is based on concept of totipotency i.e. every single cell with in plant has the potential to give rise a whole plant (Fowler *et al.,* 1993). It is one of the most incredible property of plants,which fascinate a number of scientists to run a number of experiments to elucidate the properties of various plants and ultimately succeeding in establishment of efficient methods of growing plant cells and tissues under sterilized environment (Zryd, 1988). Each cells of different tissues of plant has different level of differentiation and specialization, so therefore the extent of totipotency is different in each cell of a plant (Cassells and Gahan, 2006).

The first works in the field of plant tissue cultivation date back to beginning of 19th century and linked with the names of three outstanding scientists: Vochting (1878), who first reported the concept of polarity in plant cells and tissue through *in vitro* propagation, Rechinger (1893), who reported callus induction in poplar stem, candeloin and beet roots, when placed on wet filter paper and Haberlandt, also called the father of plant tissue culture, who for the first time separated and

cultured the plant cells on Knop's solution (Krikorian and Berquam., 1969). The first successful report on true plant tissue culture was given by Gautheret (1934), when he tissue cultured cambial tissues of *Acerpsedoplatanus.*

In conventional method of cultivation, many plants under certain climatic conditions produce seeds and some of them are even unable to produce seeds and flowers. They also need long time for their growth and development. The advantages of plant tissue culturing is far more as compared to conventional method of plant cultivation (Prakash and Staden., 2007). It requires less time, space and much less expenses as compared to conventional methods. It also helpful in production of plants from seeds that otherwise unable to germinate e.g. orchids and nepenthes (pitcher plant) (Fasolo and Predieri, 1988).

There are three different strategies of tissue culture that are used for the regeneration of plants which are (1) using apical meristem (shoot tips or nodes), (2) organogenesis (direct or indirect using calli) and (3) somatic embryogenesis. Shoot initiation and multiplication, shoot elongation and *in vitro* rooting from shoot to form stably growing plantlets are the three common pathways through which cells or tissues are regenerated into a complete plant. The success rate of plant tissue culture depends upon many factors. One of the important factors is source from which we take the explants i.e. age, size, type and position of the explants (Gamborg *et al.,* 1976). All plant cells have different ability to regenerate into new plant (Sasikumar*et al.,* 2009).Shoots, roots, stem, leaves, flower and callus are used as explants in plant tissue culture but shoot tips, root tips and nodal buds are most commonly used explants in tissue culture technique. The main reason for their common use is the high rate of cell division in these regions (as they contain undifferentiated meristematic tissues) and their ability to produce high level of growth regulators such as auxin and cytokinins. Young explants have more totipotency as compared to mature explants. Small size explants sometimes show less growth while using large explants enhance the chances of contamination (Staba and Seabrook, 1980).

1.9.1. Advantages

The techniques of tissue culture are practically applied on all types of plants ranging from cash crops and foods to medicinal and ornamental plants and even trees. It has increased the mass production of vegetative propagated crops (Sutherland *et al.,* 2005). It is widely used for large scale multiplication of plants in short time. Apart from research tool, it is important in the area of disease elimination, plant improvement, plant propagation and production of secondary metabolites. Using a plant tissue culture technique, single explants can be multiplied into thousands of explants in relatively shorter period of time and space, under sterilized conditions, irrespective of the season and weather on a year round basis (Akin-Idowu *et al.,* 2009). This technique also help us in the field of fundament botany, especially in the field of agriculture, horticulture, plant breeding, forestry, somatic cell hybridization, phytopathology and plant metabolites production at industrial level.

It also help us to understand the phenomena and advantages of biotransformation, forestry, genetic engineering, maintaining pathogen free plant, morphogenesis, comparison of totipotency rate among plants, differentiation rate, cell division rate among different area of the plant, cell nutrition, cell preservation, somatic hybridization, metabolism, radio biology. The production of pure haploid plants through tissue culture technique from anthers or isolated microspores and of protoplasts from higher plant cells has been used as the basic tools for genetic engineering and somatic hybridization. One of the significant developments in the field of plant tissue culture is the isolation, culture and fusion techniques which have their special importance in studies of plant improvement by cell modification and somatic hybridization.

1.9.2. Disadvantages

Beside various advantages, the tissue culture technique is facing some serious challenges. It requires keen and close attention of the researchers in order to overcome infection that may proceed through generations. The unavailability of standard protocol for each plant is another major issue in this technique because the

genotyping is different for each plant (Israeili *et al.*, 1995). In plant cell and tissue culture, the chances of soma clonal variation are ubiquitous (Côte *et al.,* 1992). According to Israeli *et al*. (1995), the somaclonal variations rate depends upon genotype, e.g. shoot tip of Musa species vary from 0-70%, however this genetic instability may be by chance, as it may also provide us novel items and foods in large quantity in less time (Vuylsteke and Swennen, 1993). The issue of biosafety and resistance in pests and weeds of pesticides and herbicides due to gene leakage from transgenic plants to wild plants is serious issue in using biotransformation technique through tissue culture technique. The destruction of biosphere and unavoidable genetic variability may cover the benefits of this technique.

1.10. Plant genetic transformation

Developments in biotechnology have revolutionized the way, to introduce any new trait or characteristic, by utilizing the technique of genetic engineering, which otherwise by conventional breeding involves sexual hybridization, which is a very lengthy procedure. Genetic transformation is the process through which the genetic makeup of an organism is altered by introducing a fragment of foreign DNA. It involves the successful introduction, integration and expression of a gene from its normal location into a cell of a tissue that does not contain it. Genetic engineering is a young branch of science. The first report of transgenic plant was reported by Bevan *et al*. (1983); Herrera-Esrrella *et al*. (1983), they use modified Ti plasmid as a vector and allow insertion of it into host plant DNA. The genetic engineering results into either stable or transient expression of incorporated DNA. In stable transformation, gene is stably integrated into chromosome so that the cell that has been transformed and all of its daughter cell contain the foreign gene, hence it is time consuming process (Hansen and Wright, 1999; Newell, 2000). In transient expression gene is not transferred to germ cells, so the next progeny will not be transformed. This technique is beneficial to study metabolic processes and development of improved transformation methods (Gheysen *et al.,* 1989).

Phytotransformation plays a very important role in increasing the dependence of mankind on crops with more yield and nutritional value. Since 1980s scientists have used different transformation techniques and have succeeded in production of large number of transgenic plants (Chen *et al.,* 1998; Gahakwa *et al.,* 2000).

Foreign genes taken from different plants and bacteria had been incorporated successfully in major crops such as rice (Hieri and Komari, 2008; Ozawa, 2009), tobacco (Barone *et al.*, 2009), bean (Rech *et al*., 2008; Sillero *et al*., 2010), maize (Reyes *et al.*, 2010; Frame *et al*., 2011), cotton (Rech *et al*., 2008), potato (Begam *et al*., 2008), sweet potato (Yanga *et al*., 2011), wheat (Ogawa *et al*., 2008; Zale *et al*., 2009), barley (Ibrahim *et al*., 2010) and spruce (Pavingerová *et al*., 2011), to get desirable characteristics such as pesticides, disease, herbicide and insecticide resistance in order to increase crop yield and decrease the expenses of pesticides.

According to Hansen and Wright (1999), there are certain principles that must be fulfilled in order to get the successful plant transformation method. These are: (i) the transformation method should be simple, genotype-independent, and easy to perform, well organised, highly reproducible and cost effective. (ii) An efficient and competent method for delivery of foreign DNA. (iii) Selectable markers for transgenes. (iv) Transgenes should be viable and recovered at a comparable rate. (v) Target cells or tissues should be competent for propagation or expression. (vi) A fixed timeframe in culture in order to evade somaclonal variation and infertility.

At present, there are two major systems that fulfil the criteria of Hansen and Wright (1999) i.e. Direct DNA delivery and *Agrobacterium*-mediated transformation method.

1.10.1. Direct DNA delivery

This technique includes biolistic (particle bombardment), silicon carbide whiskers, electroporation of tissues, injection of foreign DNA and the protoplast system. The later can be transformed using polyethylene glycol (PEG) fusion and microinjection or electroporation (Newell, 2000). The most frequently used methods for transformation are gene gun (biolistic approach) and *Agrobacterium*-mediated transformation. The biolistic method is used for both nuclear and chloroplast transformation while *Agrobacterium*-mediated is solely for nuclear based transformation and is one of the most reasonable method (Gasparis *et al.*, 2000).

1.10.2. *Agrobacterium*-mediated transformation

The genus *Agrobacterium* comprises of species that are normally present in soil microflora. Most of them are saprophytes that mainly live on decaying organic matter (Daniel *et al.,* 2011). *Agrobacterium* is a common soil born gram-negative bacterium. Virulent types of *Agrobacterium* species infect almost all types of plant species but form tumors in herbaceous dicotyledonous and woody plants and sometimes in monocotyledonous species (Pitzschke and Hirt, 2010). This genus includes many species classified on their host range and the extent of pathogenicity. Some species are avirulent such as *A. radiobacter* (Gelvin, 2003) and many others cause neoplastic disease in plants e.g. *A. rubi* causing cane gall disease, *A. rhizogenes* causing hairy root disease, *A. vitis* causes crown gall of grape and *A. tumificiens* resulting in crown gall disease (Matthysse, 2006).

Agrobacterium-mediated transformation has played a major role in the development of genetic engineering of plants and involves *A. tumificiens* and *A. rhizogenes* mediated transformation. *A. tumificiens* carry tumor inducing (Ti) plasmid, while *A. rhizogenes* carry root inducing (Ri) plasmid. *A. tumificiens* transfer its plasmid fragment called T-DNA into host cell DNA which results in the formation of crown galls in host plant. Crown galls are chimeras due to modified growth of transformed and related untransformed cells. Tissues become chimeric because T-DNA coding for the enzymes involved in synthesis of auxin and cytokinin, resulting in the growth changes. On the other hand, *A. rhizogenes* stimulates hairy root formation in dicotyledonous (Newell, 2000; Lacroix, 2006), however, root induced due to infection *of A. rhizogenes* are not chimeric and made of totally transformed cells (Schmülling*et al.*,1998).

Agrobacterium-mediated transformation is commonly used for transforming genes in almost all types of plants because of its potential to produce transgenic plants at higher frequency. Overall, this technique is relatively simple, easy to perform, efficient and cost effective as compared to other genetic transformation methods (Walden and Wingender, 1995). One or few copies of gene (even of large size) can be integrated in the host cells without undesired gene silencing or fragmentation of foreign gene (Murray *et al.,* 2004).

The efficiency of *Agrobacterium*-mediated transformation varies and depends on many factors, including plant species, *Agrobacterium* strains, medium, explants, antibiotics, co-cultivation, and selection. Although numerous other methods of transformation has been developed by other scientists but this method is preferential over others so far, for the genetic manipulation of plant cells. The reason is that uptill now no other organism across the kingdom has been discovered which has the ability to transform the genetic material of host cells of other kingdom species. Due to this property of inter-kingdom transformation, *A. tumificiens* has also the ability to transform fungi and human cells as well (Veena *et al*., 2007).

1.10.2.1. Molecular mechanism of *A.tumificiens*-mediated transformation

The mechanism of processing and transfer of T-DNA from *Agrobacterium* to the nuclear genome of the host cell has been given in a number of reviews (McCullen and Binns, 2006; Karami *et al* ., 2009; Gelvin, 2009). The crown gall disease caused by *A. tumificiens* involves two major elements i.e. transformation and tumorigenesis. Transformation is the transfer of tumor inducing DNA into nuclear genome of host plant whereas tumorigenesis is the consequential alteration in the metabolism of plant cell resulting in cell proliferation and production of essential nutritive compounds for *Agrobacterium* itself (Escobar and Dandekar, 2003).

Agrobacterium tumificiens has a large number of tumor inducing (Ti) plasmid. It ranges in size from 200 to 800 kb. Ti plasmid contains Transfer DNA (T-DNA) and many other functional parts for virulence (*vir*), conjugation(*con*) and the origin of replication (*ori*). T-DNA and *vir* genes are necessary for inducing plant tumors. *Vir* genes play a key role in delivering the T-DNA to host cell genome (Gustavo and Riva, 1998) while T-DNA has a role in the genetic manipulation of host plant cell as well as in the production of enzymes that helps in the synthesis of opine. Opine is conjugate of sugar and amino acid which act as a source of carbon and nitrogen for *Agrobacterium* (Tempe and Petit, 1982).

T-region varies in size from 10 to 30 kb while the *vir* region is about 30 kb (Suzuki *et al*., 2000). Normally, T-region accounts for less than 10% of the total Ti plasmid. In Ti plasmid there may be single or multiple T-DNA regions. Any gene located in

the T-DNA region is transferred. T-regions are defined by flanking sequences known as the T-DNA border. This border region comprises of 25 bps sequence which are homologous to each other (Gelvin, 2009). The T-DNA is transferred to the host genome from Ti plasmid through a special system known as the type IV secretion system (T4SS). This region is randomly inserted into host genome by process of recombination (Lacroix *et al*., 2006).

Activated vir G binds to other *vir* genes called vir box and induces their expression. The vir D1 and vir D2 are responsible for the generation of T-strand i.e a single-stranded T-DNA copy, by specifically identifying and excising the bottom strand , of which the right one is the start site and hence more important. After excising, virD2 remains covalently bound to the T-strand at 5' end and form a complex which is then transferred into the nucleus through nuclear target signals (NLS) along with vir E2 and virD2. T-DNA then integrates randomly into the plant nuclear genome as asingle or multiple copies (McCullens and Binns, 2006).

1.11. Marker genes

Transformed cells can be efficiently selected and screened due to the development of the number of bacterial marker genes. For the selection of transformed cells and tissues usually an antibiotic or herbicide is included in the medium, from which transformed plants are generated. For this purpose, two types of indicator genes are commonly used, namely selectable and non-selectable markers.

1.11.1. Selectable markers

The transformed cells can be selected from the mixture of both transformed and un-transformed cells by using selectable marker genes. For this purpose a variety of selectable genes are used such as neomycin phosphotransferase II (*NPTII*), that confers resistance to antibiotic Kanamycin, hygromycin phosphotransferase (*hpt)* genes shows resistance to antibiotic Hygromycin and bialaphos resistance (*bar*) genes that show resistance to L-phosphinothiricin (PPT). Out of these, the most commonly used selectable marker gene is neomycin phosphotransferase II (*NPTII*) in dicotyledonous plants (Irdani *et al.,* 1998). Enzymes encoded by *NPT II* produce specific properties normally not found in plant tissues. *NPT II* is also useful to study transport among plant organelles as it can bear amino terminal fusion and

still maintain its enzymatic activity (Teemu *et al*., 1989). *NPT II* protein doesnot cause any risk for environment and for plants as well (Fuchs *et al.,* 1993).

1.11.2. Non-selectable markers

Non-selectable markers are also identified as scorable markers or reporter genes. The reporter genes perform their function by coding for an enzymes having activity on substrates normally not found in the plant host. During plant transformation study, the most frequently used reporter gene is *GUS* (β-glucuronidase) (Newll, 2000). Example of the reporter genes used are: β-galactosidase (*Lac Z*), green fluorescent protein (*GFP*), chloramphenolacyl transferasre (*CAT),* firefly luciferase (*LUC*), octapine synthase (*OCS*), and nopaline synthase (*NOS*). *GFP* was isolated from the jelly fish *Aequoreas Victoria. GFP* is one of the most sophisticated and advanced reporter gene because it does not require the substrate to be supplied to plants rather it makes the transgenic cells visualized by excitation with light.

GUS gene was initially isolated and sequenced from *E. coli* (Jefferson *et al.,* 1987). It is also found in other bacteria namely; *Staphylococcus warnari*, *Bacillus sp.*, *Salmonella sp.*, *Enterobacter sp.*, *Thermotoga maritime* and *S. homini* (Jefferson and Mayer, 2003). There are many advantages of using *GUS* as reporter gene. It showed expression at a wide range of pH i.e. 4-8 and is thermally stable upto 50. *GUS* gene has the ability to express in all the tissues of shoot and roots and is stably inherited to offsprings (Rashid *et al.,* 1996). In order to detect the expression of *GUS* gene *in vitro* and *in vivo* various substrates are available. All these substrates have the sugar glucopyrannosiduronic acid which is attached to hydroxyl group of florogenic, chromogenic or other detectable molecule through glucosidic linkage. The most widely used substrate for the detection of *GUS* is 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). *GUS* cleaves X-Gluc substrate that results in the formation of colourless glucuronic acid and indigo blue chromogenic precipitate (Naleway, 1992).

1.12. Aims and Objectives

- To select and optimize the best tissue culturing conditions of *Fagonia indica.*
- To select and optimize the best transformation conditions of *Fagonia indica.*

MATERIALS AND METHODS

The aim of the present study was to determine the seed germination efficiency, to optimize the tissue culture conditions (calli induction, roots induction, and shoots induction) and to optimize the transient transformation as well as stable transformation of species of *Fagonia i.e. Fagonia indica* using *Agrobacterium tumifaciens* strain C58C1 containing plasmid 35SGUSTint. The details of materials used and methods have been given in the following chapter. The following research took place in Plant molecular biology laboratory, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad.

2.1. Working precautions

Sterilized conditions were maintained during seeds germination, tissue culturing and streaking of bacterial culture on Luria Agar in order to minimize the chances of contamination, cross contamination and for personal safety. For this purpose following measures were taken.

- Lab coat was used during working in the laboratory.
- All equipments and materials used in plant tissue culturing and culturing of micro-organism were sterilized by autoclaving at 121 °C for 20 minutes.
- Disinfectants such as 70% ethanol or 5% sodium hypochlorite (bleach solution) were used to swap benches and laminar flow hood (LFH).
- For surface sterilization, whole arrangement (LFH and apparatus) was exposed to ultraviolet radiations for 15-20 minutes.
- A disinfectant soap or hand sanitizer was used for washing hands before and after working with microorganisms.
- Sterilized hand gloves were used, while working in the LFH.
- All contaminated materials were autoclaved before their disposal.

2.2. Laboratory glassware and chemicals

The 9 cm petri plates were procured from Corning® USA while all the micropipette tips were purchased from Axygen Scientific USA. The microcentrifuge tubes (1.5 and 2 ml) were obtained from Eppendorf Germany, while the syringes and syringe filters (0.2µm) were purchased from Sartorious Ltd, UK. The glassware made of

borosilicate glass was used during the whole experimentation and was purchased from Pyrex®. All the glassware was washed using commercial detergent. It was later dipped for 30 minutes in 10% bleach solution and dried in a conventional oven at 200°C for 15 minutes. Flasks were plugged with fine absorbent cotton covered with aluminium foil while petri-plates were enclosed in brown paper or newspaper and autoclaved for 20 minutes at 121°C temperature keeping the pressure at 15 psi. The surgical instruments used in tissue culturing were autoclaved for 20 minutes at 121°C keeping the pressure at 15 psi.

All the chemicals used in the experimental work were of the highest grade of purity. The chemicals, reagents and Murashige and Skoog (MS) medium used in the present research work were purchased from Sigma Chemical Co., USA and were either molecular biology grade or analytical grade. Molecular biology reagents were acquired from Fermentas, Invitrogen and Sigma whereas different molecular biology kits were obtained from Promega, Qiagen and Invitrogen. Moreover, Agar and Luria Bertini (LB) medium were obtained from "DIFCO" laboratories, USA. All of the solutions were prepared using distilled water.

2.3. Control of bacterial and fungal contamination by antibiotics in plant tissue culture

Antibiotics are used to reduce or eliminate contamination of micro-organism (bacteria, fungus etc) in plant tissue culture media because the microbes have same requirements as those of plants grown *in vitro*. The sugar present in medium attracts a variety of micro-organisms which grow faster than that of cultured plant tissues in medium and ultimately kill plant cells. It is therefore, necessary to have complete aseptic conditions around media. The commonly used antibiotics are Cefotaxime which is a third-generation broad-spectrum cephalosporin antibiotic and has activity against numerous Gram-positive and Gram-negative bacteria. Streptomycin and spectinomycin, which are antimycobacterial, are also commonly used.

Sodium hypochlorite (NaOCl), also known as danchlor solution is used commonly to tackle fungal contamination. It is a potent anti-fungal agent and kills wide

variety of fungal spores. Commercial bleach is also used as substitute anti-fungal agent and is less expensive as compared to danchlor solution.

Hydrogen Peroxide (H_2O_2) solution is also used for sterilization purposes and is easy to remove from plant material as compared to NaOCl. Mercuric Chloride ($HgCl_2$) (0.1-0.5%) is commonly used for sterilization of seeds and plant materials. Surfactants such as *tween 20* and *triton X 100* are also used in sterilization of some explants which are difficult to sterilize completely.

The antibiotics are filtered sterilized because they are heat labile and add to culture media before pouring while detergents and disinfectants used were autoclaved for 20 minutes at 121°C temperature keeping the pressure at 15 psi or filtered sterilized using filtration assembly.

All the plant tissue culture work was done inside the laminar flow hood (LFH).

2.3.1. Sterilization of laminar flow hood

For tissue culturing of plants, aseptic conditions were maintained by a laminar flow hood (LFH) fitted with a High-efficiency particulate absorption (HEPA) filter providing a constant flow of air across working area. Before using, the LFH was cleaned by swabbing with 70% ethanol or bleach using autoclaved cotton. Further sterilization of LFH was done by using UV light for 15-20 minutes. Surgical instruments were autoclaved and dipped in 70 % ethanol or bleach inside the LFH. After using, the surgical instruments (forceps and scalpels) were again dipped in ethanol, re-flamed and then reused.

2.4. Seed germination

The experiments of the present research were performed using seeds of *Fagonia indica*. These seeds were purchased from Mianwali, Pakistan. The seeds were kept in sealed plastic envelopes or placed in air tight jars. These jars were stored at cool and dry place with temperature less than 20°C and relative humidity not exceeding 30%.

The surface sterilization of seeds was performed prior to germination. Seeds were washed with autoclaved distilled water to remove dirt and debris. The seeds were

divided into two group's i.e. imbibed and non-imbibed, to check the efficiency of germination of imbibed and non-imbibed seeds. Few seeds were also cold treated in order to break the dormancy. The morphology of seeds used was also different i.e. old or mature seeds which were brown in colour and young seeds which were green in colour. Both types of seeds were used for germination.

2.4.1. Surface sterilization of seeds

The seeds were dipped in different sterilizing agents, in order to optimize the best combination of sterilized agents for seeds sterilization and to check the effect of sterilizing agents on seeds germination efficiency. The sterilizing agents used are: 70% (w/v) ethanol solution, 5% Sodium hypochlorite and 0.1% Mercuric Chloride. These sterilizing agents were used with different exposure times (table 2.1). During sterilization, the seeds were continuously shaked with sterile forceps to increase the surface area of seeds with sterilizing agents, the seeds were then washed thrice with autoclaved distilled water. The seeds were dried on autoclaved filter paper. All the work was done inside laminar flow hood.

Table 2.1: Sterilizing agents used and their duration of exposure.

Sr No.	Combinations of sterilizing agents	Duration of exposure (seconds)
1	70% (w/v) ethanol	30
	sodium hypochlorite	30
	0.1% mercuric chloride	30
2	70% (w/v) ethanol	30
	sodium hypochlorite	45
	0.1% mercuric chloride	30
3	70% (w/v) ethanol	60

	sodium hypochlorite	30
	0.1% mercuric chloride	30
4	70% (w/v) ethanol	60
	sodium hypochlorite	45
	0.1% mercuric chloride	30
5	70% (w/v) ethanol	120
	sodium hypochlorite	30
	0.1% mercuric chloride	30
6	70% (w/v) ethanol	120
	sodium hypochlorite	45
	0.1% mercuric chloride	45

2.4.2. Preparation of seed germination medium

Seed germination required usually ½ Murashige and Skoog (½MS) medium (Murashige and Skoog, 1962) which was prepared by adding MS medium (4.4/2 g) and sucrose (30 g) in 1 litre de-ionized distilled water. Once the contents were mixed by stirring, the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH and solidified with 0.5% agar (gelrite or gum powder). The flask containing ½MS medium was plugged with non-absorbent cotton and wrapped with aluminium foil and finally autoclaved for 20 minutes at 121°C and 15 psi. Once the sterilization was completed by autoclaving, the media was left to cool in a laminar flow hood. Roughly 40-45 ml of the medium was dispensed in each 9 cm sterilized petri plate and allowed to solidify.

2.4.3. Seed inoculation

The sterilized dried seeds were inoculated using sterilized forcep in petri plates containing medium under aseptic conditions in LFH near the lamp. The plates were sealed with parafilm and kept in growth room for 15 days approximately at 25±2 °C under light.

2.4.4. Seed germination efficiency

In order to check the optimize media for seed germination, the seeds were germinated on four different types of media including half strength MS (½ MS) media, MS (murishage and skoog medium), B5 (Gamborg medium) and RMOP (revised medium for organogenesis of plants) in order to compare the seed germination efficiency of *Fagonia indica*. table 2.2. shows the chemical composition of ½ MS, MS, B5 and RMOP media, respectively.

Table 2.2: Chemical composition of seed germination media

Medium	Components	Conc. g/l
½ MS	MS basal salts Sucrose Agar	2.2 g 30 g 10 g
MS	MS basal salts Sucrose Agar	4.4 g 30 g 10 g
	MS B5 macronutrients B5 micronutrients	4.4 g 100 ml 10 ml

B5	B5 vitamins $MgSO_4 \times 7H_2O$ Sucrose Agar	10 ml 0.983 g 20 g 10 g

The experiment was performed in four batches with the gap of two weeks between two consecutive batches. Furthermore, in each batch 5 plates of each combination were used, each containing 7 seeds. In each combination, 21 non-imbibed and 14 imbibed seeds were inoculated. Furthermore, 2 batches contain mature seeds and 2 batches contain green seeds. The experiment is summarized in the following table 2.3.

Table 2.3: Seed germination optimization experiment.

Sr. no.	Media	Total no. of plates (A)	Total no. of seeds in each plate (B)	Total no. of seeds (A×B)	Contaminated seeds	Germinated seeds
1	½ MS	5×4	7	140	5	42
2	MS	5×4	7	140	1	63
3	B5	5×4	7	140	2	9
4	RM OP	5×4	7	140	3	14

2.5. Regeneration/ *in vitro* propagation through explants

2.5.1. Preparation of explants

A piece or part of the plant tissue taken from original whole plant body and transferred to an artificial tissue culture medium for growth or maintenance, is known as explants material or also called primary explants. The choice of tissue depends upon the ultimate goal of tissue culture project. Any piece of plant tissue can be used as explants.

Various factors of an explant tissue source influence the culture, on tissue culture media. These include:

- Physiological and ontogenetic age of organ or tissue
- Genetic variation and genetic drift
- Season in which explants tissue is obtained
- Quality of source plant
- Size of explants

During this research, three types of explants are used for subculturing/clonal propagation *in vitro* which are:

1) Stem meristematic region i.e shoot apical meristem (SAM)
2) Nodal region of stem
3) Cotyledons (small two leaves stage explants obtained from seeds *in vitro*).

The SAM and nodes were cut from wild plants of *Fagonia indica* obtained from the Botanical Garden of Quaid-i-Azam University Islamabad, therefore these explants need sterilization treatment in order to get rid of possible contaminations on culture media.

Multiple sterilization treatments were given to explants to avoid possible contaminations. The sterilizing agents used are described in table 2.4.

Table 2.4: Sterilizing agents used for explants sterilization

Sr No.	Sterilizing agent used at one time	Time of exposure (minutes)
1	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 15% Commercial bleach	10 3 10
2	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 15% commercial bleach	15 3 10
3	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 5% sodim hypochlorite	10 3 15
4	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 5% sodium hypochlorite (NaOCl) cefotaxime	15 3 15 2
5	70% ethanol 0.2% mercuric chloride ($HgCl_2$) 5% sodium hypochlorite (NaOCl)	10 2 15

	Nystatin	2
6	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 5% sodium hypochlorite (NaOCl) terbinafine (1 mg/ml)	10 2 10 2

2.5.2. Shoot apical meristems explant preparation

Apical meristems of young green plant were used for regeneration. About 1 cm long piece of shoot apical meristems were carefully removed from the seedlings using a sharp blade under LFH.

2.5.3. Nodes explant preparation

The nodes of young green plant were used for regeneration. They were cut into small pieces of 2x4 mm using blade under aseptic conditions.

2.5.4. Cotyledons explant preparation

The cotyledons were obtained by seed germination *in vitro*. Cotyledons are 10 days old.

2.6. Regeneration media

2.6.1. Calli induction media

Callus is amorphous aggregate of loose parenchyma cells which is a natural ability of plant and usually appear on wounded side of plant body. Callus contains no organised meristems, it is somewhat abnormal tissue which has the potential to produce normal roots and embryoids and in turn develop into complete plantlets.

Calli induction medium was prepared by adding MS medium (4.4 g) and sucrose (30 g) to 1 litre de-ionized distilled water. They were mixed by stirring and the pH

was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH followed by the addition of 0.5% agar. After autoclaving the media, it was left to cool in a laminar flow hood. When the temperature dropped around 50°C, different combinations of growth hormones already sterilized by using 0.2 μm syringe filter were added to the media. Roughly 30 ml of the medium was poured in each magenta jars and allowed to solidify. Finally, the magenta jars were sealed with parafilm and kept at 25±2°C in growth room until use.

2.6.1.1. Plant growth regulators

Two types of hormones are used to optimize callus protocol. They are:

- **BAP** (benzyl amino purine)
- **NAA** (naphthalene acetic acid)

The whole series of hormonal combinations is summarized in the following table 2.5.

Table 2.5: Hormonal combinations for optimization of calli induction experiment.

Sr No	NAA(50 mg/100 ml) working concentration	BAP(50 mg/100 ml) working concentration	Ratio NAA:BAP	No. of explants/combi-nation
1	0.5 mg/l	0 mg/l	1:0	100
2	0.5 mg/l	0.5 mg/l	1:1	100
3	1 mg/l	0.5 mg/l	2:1	100
4	1.5 mg/l	0.5 mg/l	3:1	100
5	2 mg/l	0.5 mg/l	4:1	100

6	2.5 mg/l	0.5 mg/l	5:1	100
7	3 mg/l	0.5 mg/l	6:1	100
8	3.5 mg/l	0.5 mg/l	7:1	100
9	4 mg/l	0.5 mg/l	8:1	100
10	4.5 mg/l	0.5 mg/l	9:1	100
11	5 mg/l	0.5 mg/l	10:1	100
Sr No	BAP(50 mg/100 ml) working concentration	NAA(50 mg/100 ml) working concentration	Ratio NAA:BAP	No. of explants/comb-ination
12	0.25 mg/l	0 mg/l	1:0	100
13	0.25 mg/l	0.25 mg/l	1:1	100
14	0.5 mg/l	0.25 mg/l	2:1	100
15	0.75 mg/l	0.25 mg/l	3:1	100
16	1 mg/l	0.25 mg/l	4:1	100
17	1.25 mg/l	0.25 mg/l	5:1	100
18	1.50 mg/l	0.25 mg/l	6:1	100
19	1.75 mg/l	0.25 mg/l	7:1	100
20	2 mg/l	0.25 mg/l	8:1	100

21	2.25 mg/l	0.25 mg/l	9:1	100
22	2.5 mg/l	0.25 mg/l	10:1	100

2.6.1.2. Explants inoculation

Sterilized shoots apical meristems (SAM), nodes and cotyledons were then inoculated on the plates supplemented with different hormonal combination. A total of 100 explants (30 nodes, 30 cotyledons and 40 shoot apical meristems) were inoculated per combination. The jars were sealed with parafilm and kept in growth room at 25±2°C under light for 10-15 days.

2.6.2. Shoot induction media

Shoot induction medium was prepared by adding MS medium (4.4 g) and sucrose (30 g) to 1 liter de-ionized distilled water. They were mixed by stirring and the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH followed by the addition of 0.5% agar. After autoclaving the media, it was left to cool in a laminar flow hood. When the temperature dropped around 50°C, different combinations of growth hormones already sterilized by using **0.2** μm syringe filter were added to the media. Roughly 30 ml of the medium was poured in each magenta jars and allowed to solidify. Finally, the magenta jars were sealed with parafilm and kept at 25±2°C in growth room until use.

2.6.2.1. Plant growth regulators

Two types of hormones are used to optimize shoot induction protocol from different explants. They are:

- **BAP** (benzyl amino purine)
- **NAA** (naphthalene acetic acid)

The whole series of hormonal combination is summarized in the following table 2.6.

Table 2.6: Hormonal Combinations for optimization of shoot induction experiment.

Sr No	BAP(50 mg/100 ml) working concentration	NAA(50 mg/100 ml) working concentration	Ratio BAP:NAA	No. of calli used
1	0.1 mg/l	-		9
2	0.2 mg/l	-		9
3	0.1 mg/l	0.1 mg/l	1:1	9
4	0.1 mg/l	0.05 mg/l	2:1	9
5	0.1 mg/l	0.075 mg/l	3:1	9
6	0.1 mg/l	0.025 mg/l	4:1	9

2.6.2.2. Explants inoculation

Calli induced through explants *in vitro* were then inoculated on the magenta jars supplemented with different hormonal combination. A total of 9 calli were inoculated per combination. The jars were sealed with parafilm and kept in growth room at 25±2°C under light for 10-15 days

2.6.3. Root Induction Medium

Root induction medium was prepared by adding MS medium (4.4 g) and sucrose (30 g) to 1 litre de-ionized distilled water. They were mixed by stirring and the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH followed by the addition of 0.5% agar. After autoclaving the media, it was left to cool in a laminar flow hood. When the temperature dropped around 50°C, different combinations of growth hormones already sterilized by using 0.2 μm syringe filter were added to the media.

Roughly 30 ml of the medium was poured in each magenta jars and allowed to solidify. Finally, the magenta jars were sealed with parafilm and kept at 25±2°C in growth room until use.

2.6.3.1. Plant growth regulators

Three types of hormones are used to optimize shoot induction protocol from different explants. They are:

- **BAP** (benzyl amino purine)
- **NAA** (naphthalene acetic acid)
- **IAA** (indole acetic acid)

The whole series of hormonal combination is summarized in the following table 2.7.

Table 2.7 Hormonal combinations for optimization of root induction experiment.

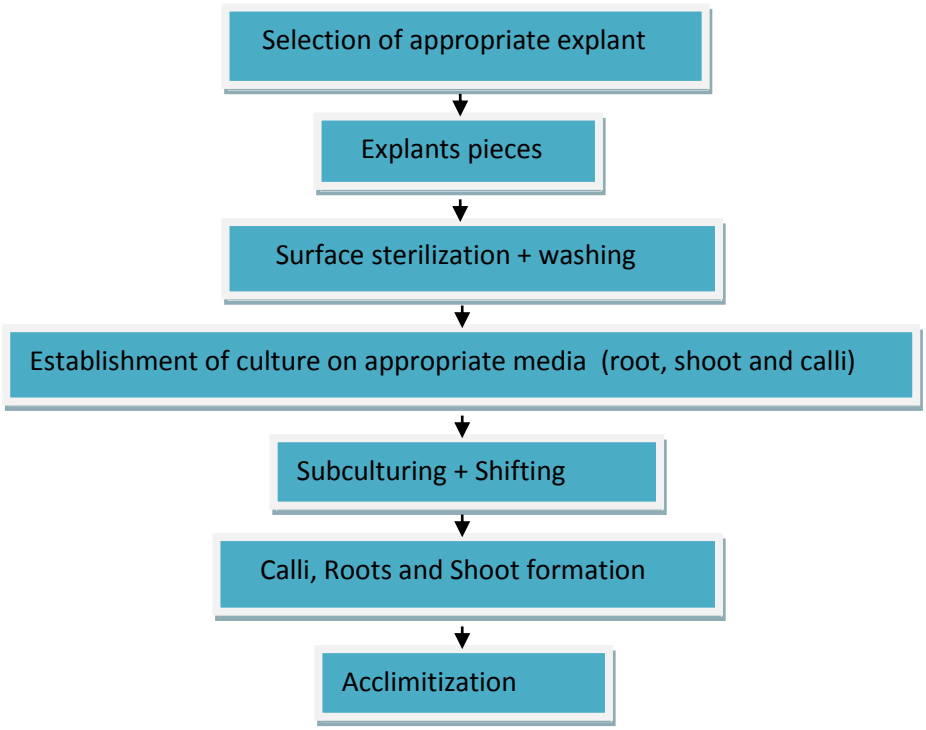
Sr. No	BAP(50 mg/100 ml) working concentration	IAA (50 mg/100 ml) working concentration	NAA(50 mg/100 ml) working concentration	Ratio	No. of calli used
1	0.1 mg/l	-	0.1 mg/l	1:1	9
2	0.1 mg/l	-	0.2 mg/l	1:2	9
3	-	-	0.1 mg/l	0:1	9
4	0.1 mg/l	0.1 mg/l	-	1:1	9
5	0.1 mg/l	0.2 mg/l	-	1:2	9

6	-	0.1 mg/l	-	0:1	9
7	-	-	-	-	9

2.6.4.1. Somatic embryogenesis

Somatic embryos first observed by Steward *et al*. (1958), which they commonly called embryoids at that time. Somatic embryos closely resemble zygotic embryos in their structure but their organization is more generally variable as compared to zygotic embryos. Somatic embryos are observed in different calli induction medium with different morphology.

2.7. Major stages of tissue culture/ clonal propagation



2.8. Optimization of transformation

Since no work has been reported on the transformation of *Fagonia indica* so far, therefore different parameters have to be optimized for stable transformation. Furthermore, transient *GUS* assay has to be performed in order to check whether the transformation is possible or not. The parameters include

- Cefotaxime concentration optimization
- Kanamycin concentration optimization
- Acetosyringone concentration optimization
- OD of bacterial culture (OD_{600})
- Co-infection period
- Co-cultivation period

2.8.1. Explant sensitivity against antibiotic Kanamycin

In order to check the sensitivity of calli and shoots generated *in vitro* of *Fagonia indica* against Kanamycin (40 mg/ml) and to select the optimal concentration of this antibiotic, we supplemented five different concentrations of filter sterilized Kanamycin to autoclaved shoot induction media under aseptic conditions in LFH. The explants (calli and shoots) were gently placed on the media having 20, 40, 60, 80 and100 mg/l Kanamycin and kept for four weeks in a growth room. The media was refreshed once during the experiment and the data was taken for number of surviving explants after four weeks. The experiment was conducted in three replicates with 20 explants in each replication.

2.8.2. Explant sensitivity against Cefotaxime

The sensitivity of explants against Cefotaxime was studied by using different concentrations of antibiotic. Filter sterilized Cefotaxime of five different concentrations i.e. 100, 300, 500, 700 and 900 mg/l were added to autoclaved shoot induction medium. The calli induced *in vitro* from SAM explants were prepared and cultured on media supplemented with the above concentrations of Cefotaxime. After four weeks the data for explant survival and shoot induction was recorded.

2.8.3. Co-cultivation medium

Co-cultivation medium (CCM) was prepared by adding acetosyringone of various concentration (100 µM, 200 µM, and 400 µM) to autoclaved MS medium containing best calli induction and shoot induction hormonal combination. It was

then poured in magenta jars under LFH and allowed to solidify followed by sealing with parafilm and kept in the growth room for further use.

2.8.4. Selection medium

Selection medium was prepared by adding MS, sucrose and agar in distilled water. After adjusting the pH, autoclaved MS medium containing best calli induction and shoot induction hormonal combination. Media was cooled to 50^{o}C in LFH. Then 50 mg/L antibiotic Kanamycin and 300mg/l of Cefotaxime were added to it for the selection of transformants. Medium poured in magenta jars was left in LFH to solidify. Magenta jars sealed with parafilm were kept in growth room until further use.

2.9. Optimization of *Agrobacterium*-mediated transformation

2.9.1. Bacterial culture preparation

A. tumifaciens (strain C58C1 harboring p35SGUSINTvector) was inoculated on Luria-Bertani (LB) and LB-agar (LA) on selection medium supplemented with 100 mg/l of Kanamycin. A single colony of bacteria was picked with the help of bacterial loop and inoculated in broth (LB having 0.01 % tryptone, 0.005 % yeast extract, 0.01 % sodium chloride) supplemented with 100 mg/l Kanamycin. These bacterial cultures were placed at 28°C in a shaker incubator (Sheldon 1575R-2) at 120 rpm overnight. After achieving bacterial growth, optical density (OD) was maintained at three different concentrations (0.3, 0.6, 0.9) by adding autoclaved LB medium. The bacterial culture having optimum OD was used for streaking on LA to acquire pure colony. Then these pure colonies were inoculated in with selection. LB was prepared by dissolving 2.5 g LB broth (Sigma) in 100 ml distilled water. In each 100 ml conical flask 50 ml LB broth was poured. pH was adjusted at 7.0 and the medium was autoclaved. LA was prepared by adding 0.5g agar in 100 ml LB. After autoclaving 100 mg/l Kanamycin was added to both LA and LB for selection in LHF. For checking the best transformation efficiency, culture at different OD_{600} was used i.e. 0.1, 0.25, 0.5, 0.75, 1.0, 1.25.

2.9.2. *A. tumifaciens* strain and plasmid construction

Agrobacterium tumifaciens strain C58C1 having p35SGUSINT was used for transformation of *Fagonia indica* (Jefferson *et al*., 1987). T-DNA of p35SGUSINT contains *NPTII* gene flanked by NOS promoter and NOS terminator and *GUS* gene with CaMC35S promoter and NOS terminator (Figure.2.1). Prior to infection, *Agrobacterium tumifaciens* strain C58C1 was grown overnight in an incubator at 28ºC with constant shaking at 120 rpm in YEB medium supplemented with 100 mg/l of selective antibiotic Kanamycin. In transformation studies, *Agrobacterium tumifaciens* strain C58CI containing p35SGUSint harbouring *GUS* gene was kindly provided by Dr. Sarah R. Grant, University of North Carolina, Chapel Hill, USA. C58CI is resistant to Kanamycin and the selectable marker for *GUS* gene is *NPTII*.

The map of the plasmid is given below:

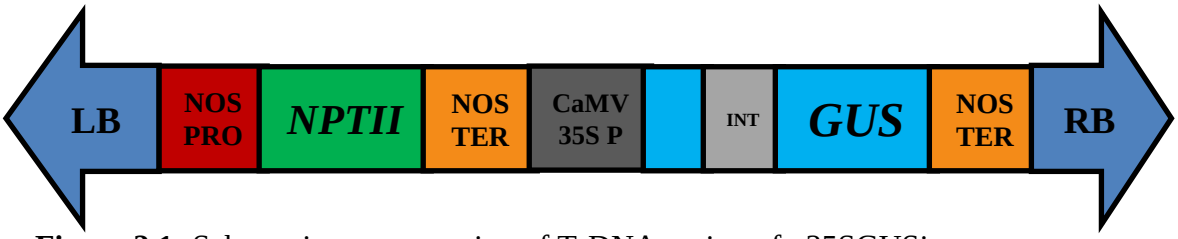


Figure 2.1: Schematic representation of T-DNA region of p35SGUSint

LB: Left border**; NOS P:** Nopaline synthase promoter**; *NPTII*:** Neomycin phosphotransferase gene**; NOS T:** Nopaline synthase terminator**; 35S P:** CaMV35S promoter**; *GUS*:** β- glucuronidase gene**; INT:** intron**; RB:** Right border

2.9.3. Transformation procedure

2.9.3.1. Maintenance of *Agrobacterium* culture

The *Agrobacterium tumifaciens*strain C58C1 containing p35SGUSint was routinely kept at 4°C on LB plates having 1 % agar supplemented with 100 mg/l of antibiotic Kanamycin. Aseptically a single cell colony of *A. tumifaciens* was picked and inoculated on LB broth followed by keeping on a shaker incubator at 28°C and 225 rpm. The *Agrobacterium* cells were collected by centrifugation and resuspended in liquid MS medium for a short time before using in the transformation experiments.

2.9.3.2. Cocultivation

A. tumifaciens C58C1 containing plasmid 35SGUSint was grown overnight in liquid LB medium (1% tryptone, 0.5 % yeast extract, and 1% sodium chloride, pH 7). Medium was supplemented with Kanamycin (100 mg/l), added to cold media, after autoclaving in LFH. After inoculation, bacterial cultures were maintained at 28^{o}C and 225 rpm in shaking incubator. After one day of pre-culturing, explants were immersed in the bacterial suspensions for 3 different co-infection periods i.e. 5, 10 and 15 minutes. Subsequently, the explants were blotted on sterilized filter paper followed by keeping on co-cultivation medium i.e. MS medium with NAA and BAP. About 100 explants (20 SAM, 20 nodes, 20 leaves, 20 stipules and 20 calli) were co-cultivated in each parameters. The magenta jars with explants were kept in growth room at 25^{o}C in dark for one, two and three days, respectively.

2.9.3.3. Selection

After the completion sof co-cultivation, the explants were washed with washing medium (WM) consisted of sterilized liquid MS medium. In order to kill bacteria, the medium was supplemented with either 300 mg/l Cefotaxime and blotted on sterilized filter paper. All the antibiotics were filter sterilized and added to the autoclaved medium. The explants were then transferred to the magenta jars containing selection medium supplemented with Kanamycin (50 mg/l). These jars were then kept at 25^{o}C, 16h of photoperiod with relative humidity not exceeding 60%.

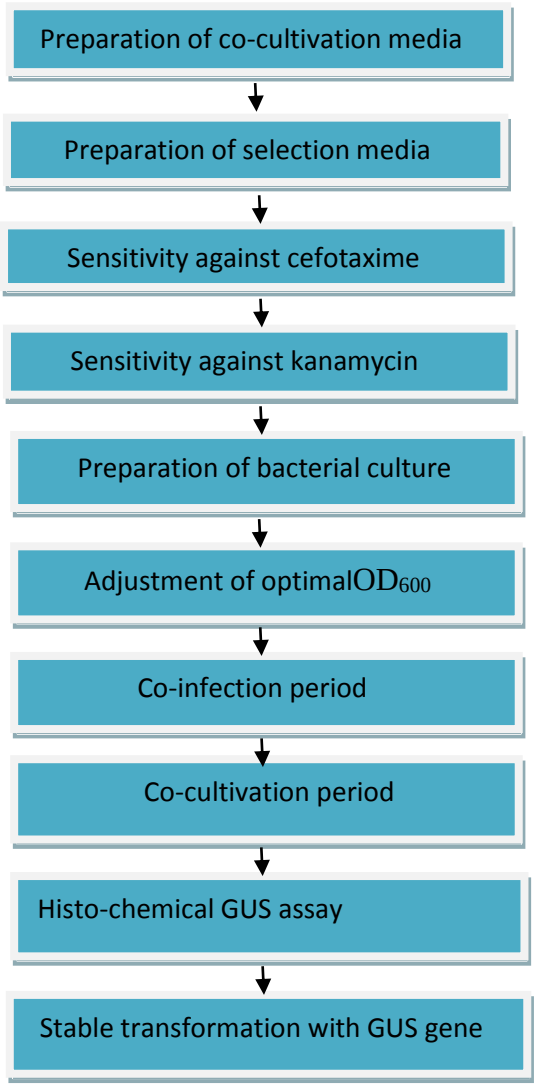
2.9.3.4. Histochemical *GUS* assay

The histochemical analysis was conducted following the protocol of Jefferson *et al*. (1987). This assay was conducted to find out β-glucuronidase (*GUS*) gene expression in *Fagonia indica* explants. The explants were immersed in 1 mM X-Gluc solution in 1.5 ml tubes and kept them in vacuum cell by keeping 200 mbar vacuums for 10 minutes to facilitate infiltration. The explants were incubated in dark at 37°C for overnight in X-Gluc solution. After 12-16 hours, *GUS* solution was replaced with 70% ethanol followed by 95% ethanol to completely remove the chlorophyll to visualize the blue color of *GUS* expression with naked eye. Composition of *GUS* solution is given in following table 2.8.

Table 2.8: Composition of *GUS* solution.

Stock Solution	Amount of stock per ml solution
X-Gluc (1 mg /20 µl Dimethyl Formamide)	20 µl
50 mM Phosphate buffer (pH 7.0)	500 µl
20% Methanol	200 µl
0.5% Triton X-100	5 µl
Distilled H_2O	275 µl

2.10. Major stages of transformation optimization



RESULTS

In the present study, seed germination, tissue culture and regeneration conditions of *Fagonia indica* were optimized. Moreover, conditions for successful transformation of *F. indica* with *Agrobacterium tumifaciens* strain C58C1 containing plasmid 35SGUSTint were also established.

3.1. Seed germination

3.1.1. Surface sterilization of seeds

The experiments of the present research were performed using seeds of *Fagonia indica*. As the seed germination of *Fagonia* species are difficult to grow *in vitro* due to poor germination rate and high contamination issues (Valverde-Cerdas *et al*., 2008), therefore different sterilization agents had a very important role in controlling the contamination and breaking the dormancy of seeds. For surface sterilization of *Fagonia indica* seeds, 3 types of sterilizing agents in different combination and exposure time were used. The sterilizing agents are 70% (w/v) ethanol, sodium hypochlorite (5%) and 0.1% mercuric chloride. Seeds exposed to these sterilizing agents resulted into 100% sterilization with no bacterial and fungal contamination. Longer duration to sodium hypochlorite and 0.1% mercuric chloride treatment resulted in the reduction of seed germination efficiency, as they are highly toxic and longer exposure to them results into invasion of these agents through seed coat into the inner soft tissues (embryo) and killing them (table 3.1).

The combination of sterilization agents i.e. 70% (w/v) ethanol for 2 minutes, sodium hypochlorite for 45 seconds and 0.1% mercuric chloride for 30 seconds results into maximum sterilization along with germination rate.

The germination efficiency obtained after above mentioned treatment was 45%.

Table 3.1: Effect of sterilizing agents on seed sterilization and germination

Sr No.	Combinations of sterilizing agents	Duration of exposure (seconds)	Germination efficiency
1	70% (w/v) ethanol sodium hypochlorite 0.1% mercuric chloride	30 30 30	2%
2	70% (w/v) ethanol sodium hypochlorite 0.1% mercuric chloride	30 45 30	5%
3	70% (w/v) ethanol sodium hypochlorite 0.1% mercuric chloride	60 30 30	5%
4	70% (w/v) ethanol sodium hypochlorite 0.1% mercuric chloride	60 45 30	15%
5	70% (w/v) ethanol sodium hypochlorite	120 45	45%

	0.1% mercuric chloride	30	
6	70% (w/v) ethanol sodium hypochlorite 0.1% mercuric chloride	120 30 30	23%

3.1.2. Seed germination medium

For the germination of *Fagonia indica* seeds, four different media were used, which were 1) Half strength MS (½ MS) medium, 2) Murashige and Skoog (MS) medium, 3) B5 medium and 4) RMOP medium. Sterilized seeds were inoculated under aseptic conditions onto the media that resulted in the formation of hypocotyls and cotyledonary leaves within a week.

The seeds were germinated on all the media combinations with low germination efficiency rate. The germination rate of non-imbibed cold treated seeds was more as compared to imbibed ones. Furthermore, it was observed that efficiency of germination of green young seeds was more as compared to brown mature seeds.

3.1.3. Germination efficiency

Seeds of *Fagonia indica* were germinated on four different types of media including 1) Half strength MS (½ MS) medium, 2) Murashige and Skoog (MS) medium, B5 medium and RMOP medium in order to compare the efficiency of seed germination. *F. indica* seeds germinated on MS were significantly dense than those germinated on ½ MS (figure 3.1). Full MS showed 45% germination efficiency as compared to ½ MS, B5 and RMOP media showing 30%, 6% and 10%, respectively (table 3.2).

Table 3.2: Seed germination efficiency of *F. indica* on four different media

Sr No.	Media	Total no. of plates (A)	Total no. of seeds in each plate (B)	Total no. of seeds (A×B)	Germinated seeds	Germination efficiency (%)
1	½ MS	5×4	7	140	42	30%
2	MS	5×4	7	140	63	45%
3	B5	5×4	7	140	9	6.11%
4	RMOP	5×4	7	140	14	10%

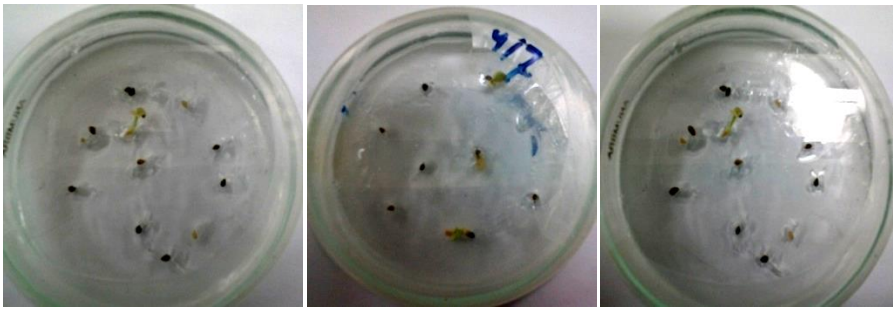


Figure 3.1: Germination of seeds on MS media.

3.2. Regeneration */in vitro* propagation through explants

3.2.1. Type of explants used

Three types of explants were used for *in vitro* propagation.

- Shoot Apical Meristems (SAM).
- Nodes.
- Cotyledons (germinated *in vitro* from seeds)

The shoot apical meristems of about 1 cm in size gave best regenerative capability on artificial media among the three as shown in figure 3.1. The nodes gave very little, while cotyledons gave no result.

The SAM has undifferentiated mass of cells that has the ability to give rise to shoot, leaves, buds and tracheids etc.

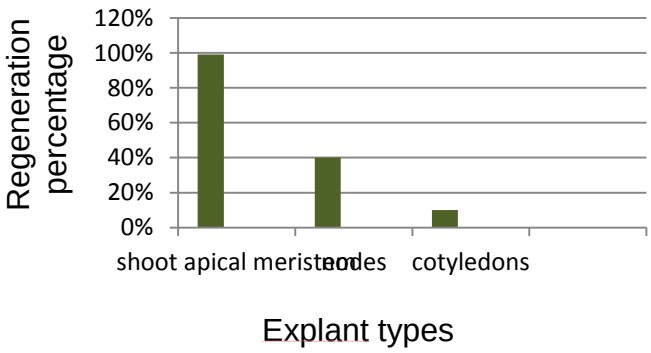


Figure 3.2: SAM has highest regenerative potential.

3.2.2. Sterilization treatment of explants

The nodes and shoot apical meristems excised from wild plant were treated with 70% ethanol, 0.1% mercuric chloride, 15% bleach and different anti-fungal agents (nystatain, terbinafine) to avoid the possible contamination and check the regeneration capacity of explants. The combination no. 6 including 70% ethanol for 10 minutes, 0.1% mercuric chloride ($HgCl_2$) for 2 minutes, 5% sodium hypochlorite (NaOCl) for 10 minutes and terbinafine (1 mg/ml) for 2 minutes gave best results, which ensured best sterilization and

maximum regeneration capability of explants i.e. 99%, while remaining of the combinations either kill the explants or contamination is not totally controlled as shown in table 3.3.

Table 3.3: Sterilizing agents efficiency and time of exposure

Sr No.	Sterilizing agent used at one time	Time of exposure (minutes)	Percentage contamination rate	Regenerative capacity
1	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 15% Commercial bleach	10 3 10	60 %	10 %
2	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 15% bleach	15 3 10	65 %	5 %
3	70% ethanol 0.1% mercuric chloride ($HgCl_2$) 5% sodium hypochlorite	10 3 15	35 %	25 %

4	70% ethanol 0.1% mercuric chloride 5% sodium hypochlorite cefotaxime	15 3 15 2	15 %	55%
5	70% ethanol 0.2% mercuric chloride ($HgCl_2$) 5% sodium hypochlorite (NaOCl) Nystatin	10 2 15 2	20 %	45 %
6	70% ethanol 0.1% mercuric chloride 5% sodium hypochlorite (NaOCl) terbinafine(1 mg/ml)	10 2 10 2	2 %	99 %

3.3. Callus induction

In order to compare regeneration efficiency of explants, different series of hormonal combinations were used, using full MS media. Callus formation was initiated within 3-4

days of explants inoculation and was not observed in all series of hormonal combination. Two series of hormonal combinations were used i.e. NAA+BAP (the concentration of BAP remains constant and the working concentration for both hormones was 0.5 mg/l) and BAP+NAA (the concentration of NAA was constant and the working solution for both of the hormones was 0.25 mg/l) as show in table 3.4.

According to experimental results hormonal combination of NAA+BAP i.e. 1:1 gave the best result as highest number of callus was obtained on this combination i.e. 100%.

The calli induced on 1:0 NAA:BAP hormonal combination was cream in color and have a soft texture. The efficiency was 56% on this combination. When these calli were shifted on 1:1 NAA:BAP combination, they started to turn green in color due to chloroplast formation, displaying the role of BAP in differentiation of plant cells. The combination 1:1 NAA:BAP gave 100% result (table 3.4) The calli induced on this combination were green in color containing chloroplast and has variety of appearance from hard to brittle.

The other series gave best result on 10:1 (BAP:NAA, 0.25 mg/l) combination with 70% efficiency. The calli produced through these combinations were green in color, brittle (figure 3.3) and induce somatic embryos after 1 month.

Table 3.4: Hormonal combinations for calli induction and percentage efficiency

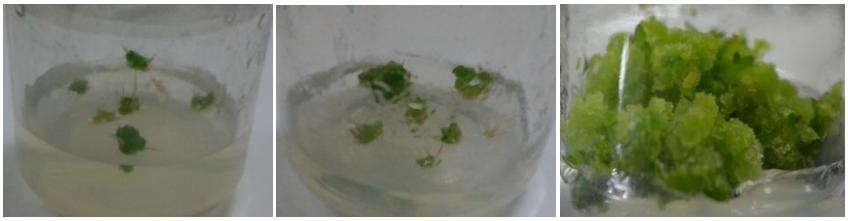
Sr No.	NAA (50 mg/100 ml) working conc.	BAP (50 mg/100 ml) working conc.	Ratio NAA:BAP	No. of explants/com-bination	Percentage efficiency
1	0.5 mg/l	0 mg/l	1:0	100	56%
2	0.5 mg/l	0.5 mg/l	1:1	100	100%
3	1 mg/l	0.5 mg/l	2:1	100	39%
4	1.5 mg/l	0.5 mg/l	3:1	100	15%

5	2 mg/l	0.5 mg/l	4:1	100	-
6	2.5 mg/l	0.5 mg/l	5:1	100	-
7	3 mg/l	0.5 mg/l	6:1	100	-
8	3.5 mg/l	0.5 mg/l	7:1	100	-
9	4 mg/l	0.5 mg/l	8:1	100	-
10	4.5 mg/l	0.5 mg/l	9:1	100	-
11	5 mg/l	0.5 mg/l	10:1	100	-
Sr No.	BAP (50 mg/100 ml) working conc.	NAA (50 mg/100 ml) working conc.	Ratio NAA:BAP	No. of explants/com-bination	Percentage efficiency
12	0.25 mg/l	0 mg/l	1:0	100	-
13	0.25 mg/l	0.25 mg/l	1:1	100	-
14	0.5 mg/l	0.25 mg/l	2:1	100	-
15	0.75 mg/l	0.25 mg/l	3:1	100	-
16	1 mg/l	0.25 mg/l	4:1	100	-

17	1.25 mg/l	0.25 mg/l	5:1	100	10%
18	1.50 mg/l	0.25 mg/l	6:1	100	15%
19	1.75 mg/l	0.25 mg/l	7:1	100	29%
20	2 mg/l	0.25 mg/l	8:1	100	35%
21	2.25 mg/l	0.25 mg/l	9:1	100	43%
22	2.5 mg/l	0.25 mg/l	10:1	100	70%

NAA:BAP (1:0) NAA:BAP (1:0) NAA:BAP (1:0)

3 DAYS OLD 7 DAYS OLD 14 DAYS OLD



NAA:BAP (1:1) NAA:BAP(1:1) NAA:BAP(1:1)

3 DAYS OLD 7 DAYS OLD 14 DAYS OLD

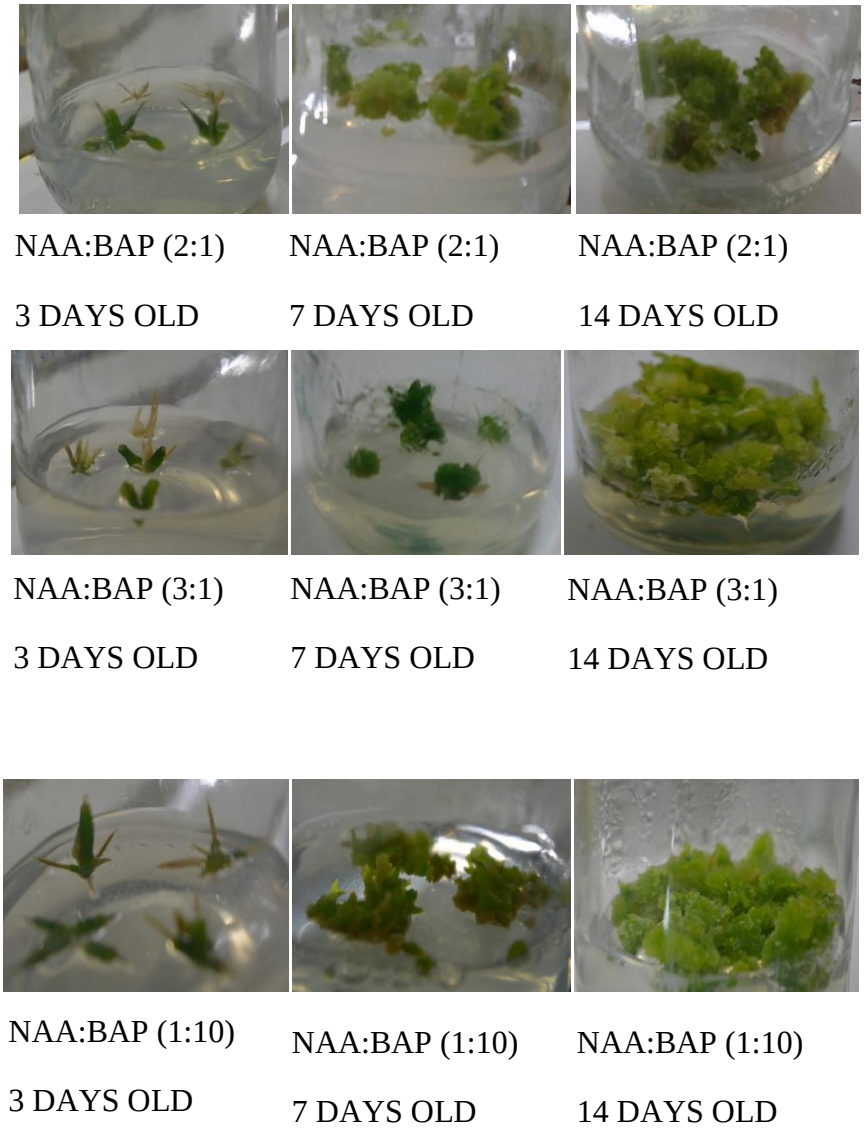


Figure 3.3: Calli formation on different hormonal combination.

After 3 weeks of callus induction, callus could be classified into five groups: whitish cream, brownish yellow, brittle green, hard green, green calli containing somatic embryos.

3.4. Shoot regeneration

Shoot regeneration takes place when cell clumps or callus were transferred on regeneration medium. The time required for *Fagonia indica* regeneration was around 3 weeks. Only green calli containing somatic embryos produce shoots, thus selected as a plant material for experiments designed to optimize shoot regeneration (figure 3.4, A).

3.4.1. Type of explants used

In order to determine best shoot regeneration, calli from different types of explants as well as different types of shoot regeneration media were studied.

For indirect organogenesis of shoot from nodes explant, nodes were regenerated onto medium containing growth regulators for calli induction. Depending upon the type of callus inducing medium, the nodes were induced into calli within 10-14 days. The calli that were induced resulted in shoot regeneration after 2-3 weeks when transferred into shoot regeneration medium supplemented with NAA and BAP hormonal combination (figure 3.4, B and C).

For indirect regeneration of shoot from SAM explant, shoot apical meristems of about 1 cm were used. The shoot explants were induced into calli within 7 days, depending upon the type of callus inducing media. The calli produced then regenerated into shoot in 2-3 weeks when transferred into shoot regeneration media containing NAA and BAP. Shoot apical meristems (SAM) explants showed 90% of regeneration efficiency (figure 3.4, D).

A B C D

Figure 3.4: Shoot formation on shoot induction medium. (A) from callus (B) and (C) from nodes (D) from shoot apical meristems.

3.4.2 Regeneration efficiency

For shoot regeneration, different concentration of BAP and NAA were used. Results showed that most suitable medium to induce shoot regeneration was MS along with hormone BAP (0.1 mg/l). By using this combination, the process of explant regeneration becomes faster, which was three weeks compared to other hormonal combination (NAA+BAP) which first induces somatic embryogenesis and later, after 3 weeks

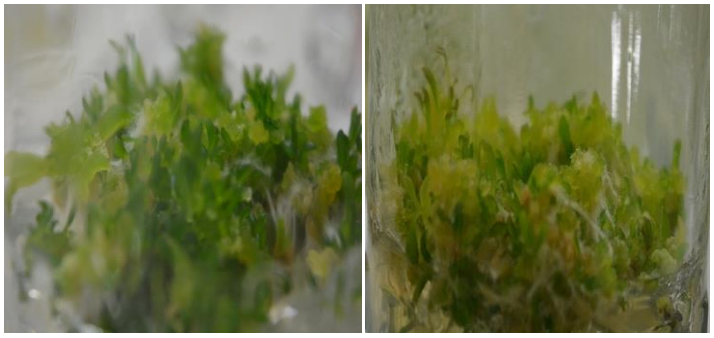
approximately formed shoots. The regenerative efficiency on BAP (0.1 mg/l) was 40% which is approx. three times more as compared to BAP:NAA combination i.e. 15% as described in table 3.5.

Table 3.5: Hormonal combination for shoot regeneration and percentage efficiency.

Sr No.	BAP (50 mg/100 ml) working conc.	NAA (50 mg/100 ml) working conc.	Ratio BAP:NAA	No. of explants used	Percentage efficiency
1	0.1 mg/l	-		30	40%
2	0.2 mg/l	-		30	-
3	0.1 mg/l	0.1 mg/l	1:1	30	15%
4	0.1 mg/l	0.05 mg/l	2:1	30	-
5	0.1 mg/l	0.075 mg/l	3:1	30	-
6	0.1 mg/l	0.025 mg/l	4:1	30	-
7	-	-	-	30	-

3.5. Somatic embryogenesis

Somatic embryos (embryoids) were also formed on different hormonal combination for callogenesis and shoot formation. On calli induction medium, 10:1 (BAP:NAA) embryoids were formed 1 month after the induction of callus (figure 3.5, A). The combination 0.05:0.1 (mg/l) of BAP:NAA for shoot regeneration also induce somatic embryos (figure 3.5, B).

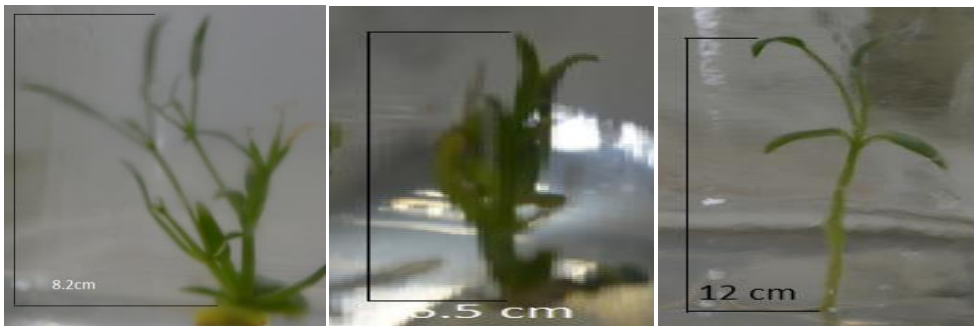


A B

Figure 3.5: Somatic embryos formation. (A) on calli induction medium (B) on shoot regeneration medium.

3.6. Shoot length

The length of shoots regenerated on shoot regeneration medium is approximately between a range of 5 to 12 cm (figure 3.6).



A B C

Figure 3.6: Different length of shoot obtained. (A) 8.2 cm, (B) 6.5 cm and (C) 12 cm.

3.7. Root regeneration from regenerated shoots and somatic embryos

Root regeneration takes place when somatic embryos or regenerated shoots were transferred on root regeneration medium. The time required for root regeneration was around 1 week. Three different types of hormones were used to develop roots i.e. NAA, IAA and BAP. The table 3.6 showed the hormonal combination and root formation efficiency. Figure 3.7 showed root formation on different hormonal combination.

Table 3.6: Hormonal combinations for root regeneration and percentage root efficiency

Sr No	BAP(50 mg/100 ml) working conc.	IAA(50 mg/100 ml) working conc.	NAA(50 mg/100 ml) working concentration	Ratio	No. of calli used	Perc-entage efficie-ncy
1	0.1 mg/l	-	0.1 mg/l	1:1	9	100%
2	0.1 mg/l	-	0.2 mg/l	1:2	9	20%
3	-	-	0.1 mg/l	0:1	9	-
4	0.1 mg/l	0.1 mg/l	-	1:1	9	30%
5	0.1 mg/l	0.2 mg/l	-	1:2	9	-
6	-	0.1mg/l	-	0:1	9	-
7	-	-	-	-	9	-

A B C

Figure 3.7: Roots formation on different hormonal combination. (A) BAP:NAA (0.1:0.1 mg/l), (B) BAP:NAA (0.1:0.2 mg/l), (C) BAP:IAA (0.1:0.1 mg/l).

3.7.1. Regeneration efficiency

For roots regeneration, BAP, NAA and IAA were used at different concentrations Results showed that most suitable medium to induce root regeneration was MS along with hormone BAP (0.1 mg/l) and NAA (0.1 mg/l). The efficiency rate was 100%. By using this combination, the process of regeneration becomes faster, which was approx. 1 week as compared to other hormonal combination (BAP+IAA), which first undergo somatic embryogenesis and later after 3 weeks approx. formed roots. The other combination IAA:BAP also formed the roots but the percentage was low i.e. 30% as shown in table 3.6.

The results obtained, while using IAA and NAA is similar, as same concentration of both induced root formation along with BAP but the efficiency is three times approx. low with IAA as compared to NAA.

3.8. Morphology comparison

The morphology of tissue cultured *Fagonia indica* is quite distinct from the wild plant. The shoots are fragile and root system is not well developed. The stipules are small and leaves are not very distinct from stipules (figure 3.8).

A B C D

Figure 3.8: Morphological comparison of wild and *in vitro* propagated plant of *Fagonia indica*. (A) complete tissue cultured plant, (B) different parts of tissue cultured plant, (C) wild plant, (D) different parts of wild plant. (C) and (D) Source: botanical garden Quaid-i-Azam University Islamabad.

3.9. A*grobacterium*-mediated transformation of *Fagonia indica*

Five types of explants were used for transformation i.e. nodes, calli, cotyledonary leaves, stipules and inter-nodal region. These explants were taken both from wild and tissue cultured plant of *Fagonia indica*. These explants were pre-cultured on pre-culturing media for one day. We used two types of hormonal combination i.e. calli induction medium BAP:NAA (10:1) and shoot induction medium BAP (0.1 mg/l) for transformation of *Fagonia indica*. Plants were co-infected with bacteria and then grown on co-cultivation media along with bacterial culture. Co-cultivation medium was also supplemented with acetosyringone to enhance transformation efficiency. After co-cultivation, plants were shifted to selection media. Table 3.7 shows chemical composition of transformation media.

Table 3.7: Composition of media used for *Agrobacterium*-mediated transformation

Media	Composition	Working concentration
MS	MS Agar Sucrose	4.4 g 8 g 30 g
Pre-culturing medium	MS media NAA+BAP	 0.25+2.5 mg/l
Co-cultivation medium	MS media+ NAA+BAP+ Acetosyringone	0.25+2.5 mg/l 0.04 g/l
Selection medium	MS media NAA+ BAP/BAP Kanamycin	 0.25+2.5/0.1 mg/l 1 mg/ml

3.9.1. Sensitivity to Kanamycin

Prior to transformation, an effective concentration of antibiotic for the selection of transformed cells was determined by culturing calli (induced from SAM) on shoot induction medium i.e 0.1 m/l BAP. Medium was supplemented with five different concentrations of Kanamycin i.e. 20, 40, 60, 80 and 100 mg/l. Explants became dry and yellow (figure 3.9) on SIM containing 40 and 60 mg/l of Kanamycin. Kanamycin at concentration of 80 and 100 mg/l caused complete necrosis of the explants after three weeks while concentration 20 mg/l has no effect on explants growth and they continue to grow and produce somatic embryos after 3 weeks. In order to finalize, which concentration of Kanamycin is best for selection, the in between concentration of 40 and 60 mg/l i.e. 50 mg/l was used. The result showed that Kanamycin at 50 mg/l concentration caused total inhibition of growth of calli on SIM (shoot induction medium). These results showed that Kanamycin is an effective selection marker. Hence this concentration was used for the selection of transformed plants.

20 mg/l 40 mg/l 60 mg/l

80 mg/l 100 mg/l

Figure 3.9: Calli inoculated on SIM supplemented with different concentrations of Kanamycin, in order to optimize selection medium for transformants.

3.9.2. Sensitivity of explants to Cefotaxime

The sensitivity of explants against cefotaxime was studied by using different concentration of antibiotic. Filter sterilized cefotaxime of five different concentration i.e. 100, 300, 500, 700, 900 mg/l were used. The best result is on 300 mg/l, which eliminate the bacteria and has no effect on plant growth, while higher amount result in necrosis of plant cells and on 300 mg/l, the contamination rate is not controlled.

3.10. Factors affecting transformation

3.10.1. Effect of optical density (OD) of *Agrobacterium* culture

Explants were co-cultured with *Agrobacterium* culture of varying optical density (OD_{600}) i.e. 0.25, 0.5, 0.75 and 1.0 and 1.25. Kanamycin concentration was kept at 50 mg/l. Highest percentage of *GUS* expression (87.5%) was observed when OD_{600} was 0.5 (table 3.8). Lowest percentage of *GUS* expression (6.25%) was observed at the highest OD value recorded i.e. 1.25 (figure 3.10).

Table 3.8: Effect of $OD_{(600)}$ of *A. tumifaciens* culture on transient *GUS* expression.

$OD_{(600)}$	Calli	internodes + nodes	Stipules	leaf	number of explants used for *GUS* staining	number of explants showing *GUS* expression	percentage of explants *GUS* expression
0.25	10	10	10	10	16	3	18.75
0.5	10	10	10	10	16	14	87.5
0.75	10	10	10	10	16	7	43.75

1.0	10	10	10	10	16	4	25
1.25	10	10	10	10	16	1	6.25

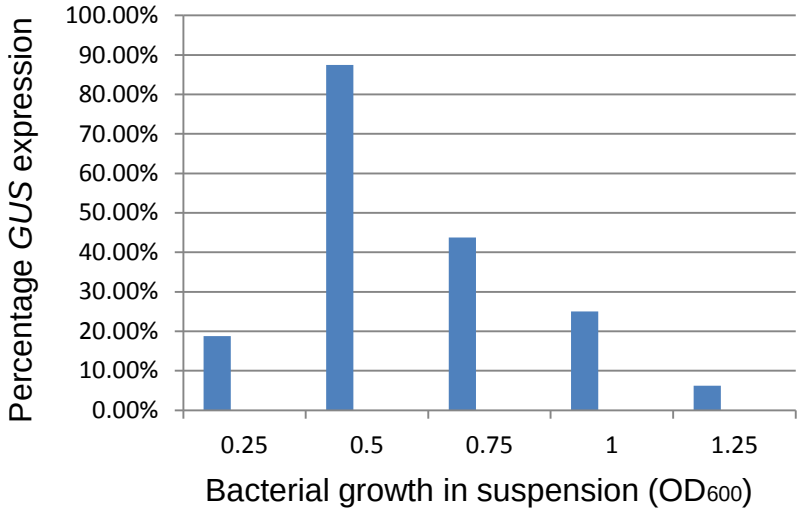


Figure 3.10: Effect of OD of bacteria on transformation expression

3.10.2. Co-cultivation time

Co-cultivation duration also affected the transformation efficiency. After infection plants were placed on co-cultivation media (SIM) for 24, 48 and 72 hours, respectively. Transformation efficiency was maximum (50%) by co-cultivating the explants for 24 hours in dark (figure 3.11). Extending the co-cultivation time up to three days increased the transient transformation frequency but also increased the contamination rate.

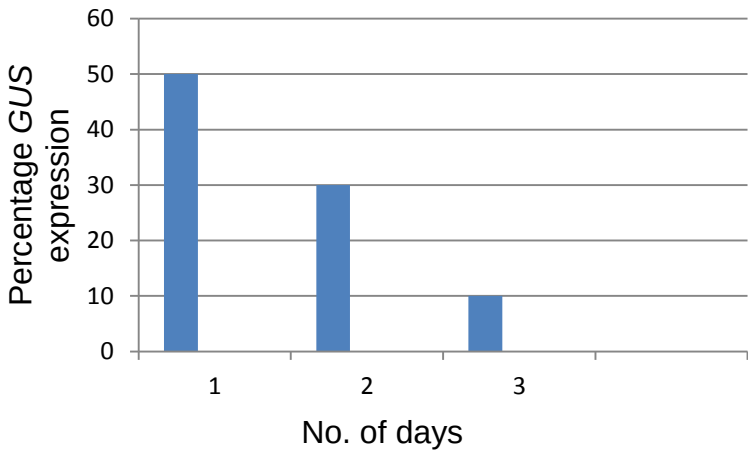


Figure 3.11: Co-cultivation effect on transformation expression

3.10.3. Age of explant

Age of the explant is a critical aspect in transformation experiment. Explants excised from young plants (both wild and tissue cultured) showed higher transient transformation rate (70%) than those excised from old plant. Our findings showed that younger explants are more susceptible to *Agrobacterium* than older explants.

3.10.4. Explant type

Explant type is also a very important factor affecting plant transformation efficiency. Five types of explants were used i.e. calli, leaf, stipules internodes and nodes. Transformation efficiency was assessed as the proportion of the blue inclusion in the putative transformed explants. In calli, the transformation efficiency was higher (70%) than that of stipules (40%), leaves (35%), internodes (30%) and nodes (18%) as shown in figure 3.12.

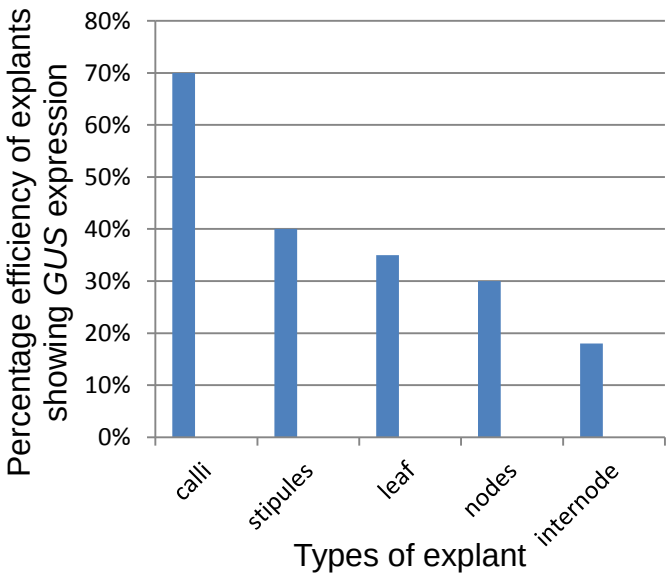


Figure 3.12: Effect of explants types on transformation expression

3.10.5. Effect of acetosyringone

Virulence inducer (acetosyringone concentration) is found noteworthy in transformation of different explant types. Above optimum AS value (200 µM), a little increase in TE was observed but it was difficult to remove the *Agrobacterium* from the explants and they eventually die as shown in figure 3.13.

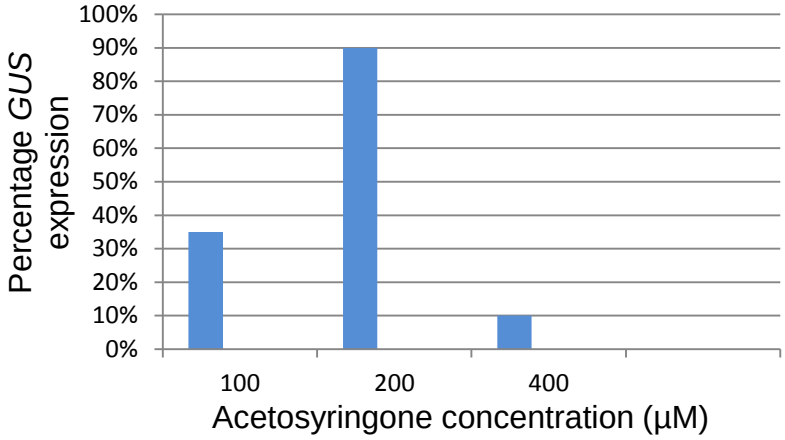


Figure 3.13: Effect of acetosyringone on transformation expression

3.11. *GUS* analysis

Histochemical *GUS* assays were used to assess transient and stable expression of the *GUS* gene. In order to optimize conditions of transformation, transient *GUS* expression was scored after 24, 48 and 72 hours of co-cultivation with *Agrobacterium tumifaciens* by counting the number of explants showing *GUS* expression. In the present study, five types of explants were used for the analysis of *GUS* expression. Stable *GUS* expression in Kanamycin-resistant putative transgenic plants was also examined. For *GUS* expression study, the tissue materials were immersed in *GUS* substrate solution for 24 hours at 37°C. Following incubation, tissues were washed with 95% ethanol, and examined. Transient *GUS* expression was observed in all types of explants which were stained blue that can be seen through microscope (figure3.14) and also with the naked eye (figure 3.15). Results indicated that 80% of the explants showed *GUS* activity after three days of co-cultivation with C58C1strain and highest expression was observed in calli due to fragile cell wall and undifferentiated cells as their cell wall is not lignified and there are easy to penetrate by foreign DNA.

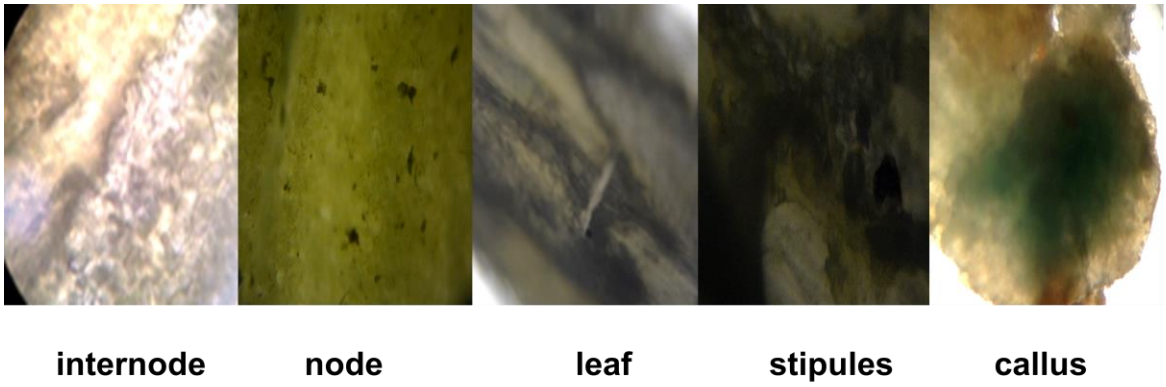


Figure 3.14 Microscopic view of *GUS* transformed explants of *Fagonia indica*.

Figure 3.15: Transient *GUS* assay. (A) shoot, (B) SAM, (C) nodes and (D) callus rsespectively.

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DISCUSSION

Fagonia indica is a small spiny undershrub found mostly in arid desert regions of Pakistan. It was extensively studied by many researchers regarding its medicinal importance and uses, since this plant is antitumor, antioxidant, analgesic, astringent, febifuge and prophylactic against small pox agents. In folk literature, it is reputed as a medicinal plant with rich therapeutic potential. It is also famous for its anti-venom, anti inflammatory and antipyretic activity (Satpute *et al*., 2009). It is a potent anti fungal, anti bacterial agent and has cytotoxic activity (Zhang *et al*., 2004; Gupta *et al*., 2009).

Establishment of tissue culture conditions was a prerequisite for this study and this was achieved by optimizing the tissue culturing conditions and transformation conditions for this plant since, there is a requirement of standard technique for tissue culturing, which in turn helps to increase production of secondary metabolites and important compounds *in vitro* and to obtain large amount of plants in short time.

Seed germination is one of the important steps in the life cycle of plant. For *in vitro* germination of seeds, proper sterilization procedure is very essential. Sterilization agents like sodium hypochlorite, tween 20 and mercuric chloride are used (Oyebanji *et al*., 2009). Sodium hypochlorite is one of the commonly used surface disinfectants. According to Talei *et al.* (2011), ethanol is strong sterilizing and highly phytotoxic chemical, therefore seed should be exposed to it for a short period of time. For efficient sterilization of seeds, ethanol is normally used prior to the treatment with other sterilizing agents like tween 20 and bleach (Oyebanji *et al*., 2009). Surface sterilizing agents such as 70% ethanol and 10% sodium hypochlorite play an important role in germination of seeds.

The choice of medium highly effects the germination of seeds due to variation in their organic and inorganic nutrients (Arditti, 1982). *Fagonia indica* seeds were inoculated on four different media i.e. MS, ½ MS, B5 and RMOP to check the best medium for their germination, as the species of Zygophyllaceae family are usually recalcitrant and have low germination efficieny *in vitro* (Valverde-Cerdas *et al*., 2008), therefore when seeds were inoculated on simple MS medium, they gave maximum germination efficiency i.e. 45%, as compared to ½ MS (30%), RMOP (10%) and B5 (6.11%). MS medium contain high amount of micro and macro nutrients and also ammonium nitrate and potassium (Murishage and Skoog, 1962). The ammonium ions enhance the growth and differentiation of seeds (Kramer and Kozlowski, 1979).

In vitro regeneration of cells, tissues or organs depends on many physical and chemical factors. These factors include temperature, light, humidity, pH conditions, phytohormones, nutrient supply, type or age of explants and genotype (Nurazah *et al*, 2009; Parveen and Shahzad, 2014). Since wild explants were used in this study, there was a need of sterilization reagents to avoid possible contaminations. Different sterilizing agents were used to control contamination rate. 70% ethanol for 10 minutes, 0.1% mercuric chloride for 2 minutes, 5% sodium hypochlorite for 10 minutes and terbinafine (1mg/ml) for 2 minutes gave best results with 100% contamination control. 70% ethanol is effective phytotoxic chemical that worked against large number of bacteria (Talei *et al*., 2011). Sodium hypochlorite releases oxygen gas as a by product that enhances oxidative respiration which in turn promoted seed germination (Vujanovic *et al*., 2000). 0.1% mercuric chloride is a good anti bacterial and anti fungal agent as described by Kishor in his book Plant Tissue Culture and Biotechnology: Emerging Trends. Terbinafine is a squalene epoxidase inhibitor and is potent anti fungal agent for a long range of dermatophytes group of fungi. Amiri *et al*. (2013) also described the importance of these sterilizing agents to control contamination rate during *in vitro* propagation of Rootstock Mariana. MS medium, supplemented with 2 different phytohormones (NAA and BAP) in different combination i.e. 20 series of hormones were used to optimize the callus induction medium for *Fagonia indica*. Three different explants i.e. shoot apical meristems (SAM), nodes, cotyledons were used. The best explants for calli induction were shoot apical meristems (SAM) as it contain undifferentiated meristematic tissues which has high totipotency. Sharma and Nautiya (1959), also described the effect of different explants on shoots regeneration through indirect organogenesis of a Laurel from Himalaya and proved that apical meristems have high regenerative potential on artificial medium. The best combination for calli induction was 1:1 ratio (working concentration 0.5 mg/l for both BAP and NAA). It gave green calli with various textures and hardness. The combination 10:1 (BAP:NAA) gave second best result, with 70% efficiency. There were many protocorm like bodies (PLBs) formed along with calli on these combination. These results are in accordance with Roy and Banerjee (2003), who studied callus induction from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. f. using different combinations of BAP and NAA.

We also investigated the organogenesis of calli to form shoots. For this, we used MS medium supplemented with NAA and BAP in different combinations. The best result was obtained with MS containing BAP (working concentration 0.05 mg/l). The result is supported by

Malik and Saxena (1991), they proved that BAP is required for induction and development of shoots while working on *Phaseolus vulgaris.* BAP has been reported to enhance the regeneration of plant (Salekjalali, 2012). Shoot regeneration is much faster while using SAM as explant as compared to nodal regions of explants.

For root regeneration, developed shoots and calli were inoculated on MS medium supplemented with three phytohormones BAP, IAA and NAA. The best result was on NAA:BAP (1:1), which gave 100% root induction.

Plant transformation is a key methodology that has allowed transfer and expression of novel genes and helped to investigate about the functions of plant genes. A number of common problems related to successful transformation of plant species include low transformation efficiency, instability of transgene expression, somaclonal variations and inability to regenerate whole plant. Plant genetic transformation plays a key role in production of transgenic organisms and has enhanced the potential uses of crops and vegetables for pharmaceutical purposes (Arntzen *et al*., 2001; Potenza, 2004).

This project was conducted to optimize transformation conditions for *Fagonia indica* using *Agrobacterium* strain C58C1 as a vector and *Gus* gene as reporter marker to check the frequency of transformation.

The explants used for transformation were SAM, leaves, stipules, nodes and calli. The best transformation efficiency is of calli. Frame *et al.* (2001) reported highest transformation efficiency for callus during transformation of maize.

The antibiotic concentration in selection medium is used to control bacterial outgrowth after co-infection and co-cultivation period. As these antibiotics normally have some negative effects on regeneration of explants, therefore Cefotaxime concentration was optimized for complete elimination of *Agrobacterium* without affecting regeneration potential of explants to induce calli and shoots. The shoot induction medium was supplemented with 100, 300, 500, 700, 900 mg/l of Cefotaxime. It was observed that calli on 700 and 900 mg/l of Cefotaxime were grown very slowly and became yellow. It might be due to by product of antibiotic that acts as growth regulator and altered the growth (Lin *et al*., 1995). The calli on 300 and 500 mg/l did not effected by antibiotic and grew normally into calli and shoots, so 300 mg/l was used as standard to eliminate bacteria. Many reports on the use of Cefotaxime concentration below 500 mg/l, showed that the *Agrobacterium* growth could effectively be eliminated by Cefotaxime (Lupan *et al*., 2010).

Tissue resistance against Kanamycin is the most common selection markers for the procurement of transgenics (Nap *et al*, 1992). Kanamycin selection is so important that even some research groups use this tool to calculate transformation frequency (Wang *et al*, 2012). Prior to transformation, we optimized an optimal concentration of Kanamycin for selection of transformed cells by culturing explants on shoot induction medium supplemented with different concentration of Kanamycin i.e 20, 40, 60, 80, 100 mg/l. Inoculated calli were turned yellow on 40 and 60 mg/l. so we concluded mean i.e 50 mg/l to be best for selection. The explants on 100 mg/l undergo necrosis and the same were found in many plant species including *Arabidopsis* (Ayre *et al*., 2003), tomato (Ying *et al*., 2008), lettuce (Ahmed *et al*.,2007). carnation (Kanwar and Kumar, 2011) and mulberry (Chitra *et al*., 2014).

There are various factors that affect transformation efficiency, one of them is optical density (OD) of bacterial culture. Efficiency of transformation was found to be highly dependent on the concentration of bacteria (OD_{600}). The optimal OD of *Agrobacterium tumificiens* for inoculation of explants was obtained by co-culturing explants with bacterial culture at OD_{600} of 0.25, 0.5, 0.75, 1.0 and 1.25. It was observed that highest *GUS* expression was observed at 0.5 OD_{600} and least expression was at 1.25 OD_{600}. Similar results were reported by Yadav *et al*. (2012) for *Cochliobolus sativus.* Bacteria in liquid inoculation medium are likely at the hypervirulent active log phase from 0.5-1.0 OD_{600} therefore, inducing maximum TE. Nanasato *et al*. (2013) also reported highest *GUS* expression for *C. sativus* at 0.5 OD_{600}.

The explants were incubated for five different durations (5, 7, 10, 12 and 15minutes). It was observed that lesser infection time led to low transformation efficiency while higher infection time led to contamination of explants with bacteria. Hence optimal infection time is to be determined for successful transformation (Khan *et al*., 2013). The optimal infection time for successful transformation was 7 minutes. The highest *GUS* expression was observed when explants were incubated on bacterial suspension for 7 minutes (approx.) similar result was reported by Lupan *et al*. (2010).

The effect of co-cultivation time on transformation efficiency was determined by co-cultivating explants infected with *Agrobacterium tumificiens* for 3 different durations i.e 1, 2 and 3 days. The best efficiency was observed, when *Fagonia indica* explants were co-cultivated for 1 day. This is contradictory to most of the related research in which maximum efficiency of *GUS* was observed at 2-3 days (Hamid *et al*., 1995; Lupan *et al*., 2010).

The co-cultivation medium was supplemented with hormones and acetosyringone was added to enhance the bacterial virulence (Godwin *et al*., 1991). Acetosyringone along with C58C1strain of *Agrobacterium tumificiens* increases the tumor production and phenolic release tenfold than without it (Holford *et al.*,1992). We use 200 µM of acetosyringone during co-infection period and in co-cultivation medium. Acetosyringone concentration used in infection and inoculation media is considered safe at up to 200 µM (Stachel *et al*., 1985). In another species of Lamiaceae (*Pogostemoncablin*), highest transformation frequency was found at 150 µM AS concentration (Paul *et al*., 2012).

Age of explants is also important in determining the transformation efficiency. Calli of 45 days old were used, which gave maximum efficiency as compared to 15 days and 70 days old calli. These results were supported by Tripathi *et al*. (2010), while doing a study on rice.

Transformation frequency was ultimately a manifestation of *GUS* expression because it is facile, authentic, relatively low priced, safe, need no particular machinery or apparatus, and is clearly visible to the naked eye (Jefferson 1987; Jefferson 1988). In the present study, plant transformation binary vector p35SGUSint containing *GUS* reporter gene, expressed under CaMV35S promoter in *Agrobacterium tumificiens* strain C58C1 was used. Moreover, p35SGUSint encloses chimeric *gusA* gene with a plant intron (*gusAint*), so it cannot be expressed in bacterium thus making it a useful tool for transformation (De Bondt *et al*., 1994). A clear blue colour was observed when putative transformants were stained with *x-gluc* reagent and was observed with the naked eye. The positive control gave no result while the frequency of blue colour was maximum in calli among the explants used for transformation.

Conclusion and future strategies

Following conclusions can be drawn from the present study:

- Sterilizing agents like sodium hypochlorite and terbinafine play an important role in surface sterilization of seeds and explants used for *in vitro* propagation.
- The seeds are recalcitrants and maximum germination efficiency was shown on MS medium.
- Factors like explant type, genotype and medium composition effects the *in vitro* regenerative potential of explants used.

- Among the explants used, shoot apical meristems has the highest regenerative potential.
- The best callus induction medium is NAA:BAP (1:1; working concentration 0.5 mg/l).
- The best shoot induction medium contains only BAP (0.1mg/L) in MS medium.
- The best root induction medium contains NAA, BAP and IAA.
- There is a formation of many protocorm like bodies (PLBs) and somatic embryos along with callus, which later turned into complete plantlets.
- *GUS* reporter expression was observed in all kind of explants type but maximum in calli.

In the light of above mentioned research, further investigation may be carried out for the stable transformation of *Fagonia indica*, as conditions for transformation had been optimized. Since plant has important medicinal role in both folk and allopathic field and no stable transformation has been reported so far, thus plant can be transformed with genes that increase the secondary metabolites, which play role in the cure of different disease. Molecular analysis can also be performed on transformed plants.

Cell suspension cultures can also be produced and *in vivo* and *in vitro* bioassays can also be carried out for relative comparison of transformed and untransformed plants.

Mphil Thesis By Maryam Naeem

by Maryam Naeem

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This study covered the selection and optimization of tissue culturing and transformation conditions of medicinally important plant i.e. *F. indica* commonly known as Dhamasa. The plant is used as decoction for the treatment of many diseases especially liver and breast cancers.

1.1. Family Zygophyllaceae

The Zygophyllaceae belongs to family of flowering plants. Species in this family are mostly found in tropical and warm climates, specifically in arid habitats. Most species are shrubby in morphology, but small trees and herbs are also present. This family includes a number of well-known species such as bean-caper and caltrop. It includes around 285 species in 22 genera. Zygophyllaceae is divided into five subfamiliesLarreoideae, Morkillioideae, Seetzenioideae and Tribuloideae. A molecular phylogeny of the family is also reported. In 2008, phylogeny of the southern African species of this family was published. In Pakistan, so far 8 genera and 22 species are reported in subtropical and warm temperate regions

Ethnobotany

Aqueous decoction of the plants included in Zygophyllaceae is used in native system of medicines for their ability to treat various digestive and blood vascular system disorders and early stage cancers The aerial parts, including flowers of certain species of Zygophyllaceae were investigated for glycosides linked to a glycan moieties such as flavonoids, terpenids, triterpenoids or steroidal saponins

1.2. Genus *Fagonia*

The genus *Fagonia* belongs to family Zygophyllaceae, which is a part of eurosid I clade. A genus of about 45 species; restricted to warm and arid regions of all the 7 continents with exception to Australia. The genus consist of shrubs, shrublets or herbs with pointed or spinescent stipules.The colour of petals in this Genus is either pink or purple, the capsule shape is obconical, loculicidal containing persisting sepals. The leaves of majority of the species present in this genus are 3-foliolate, but there are also some species, which are consistently 1-foliolate It is well perceived as the important medicinal plant containing genus among the folk medical scholars.

1.2.1.Geographical distribution of Genus *Fagonia*

Based on molecular and morphological analysis, phylogeny of Zygophylloideae showed *Fagonia* position as a sister of genus Melocarpum, endemic to the northeast of Africa region *Fagonia* is distributed to the arid areas of the New and Old World. It is found in Mexico, USA, Chile, and Peru in the New World, whereas in the Old World, it is found in south Africa, north Africa and the southern parts of Europe and most of the Mediterranean islands. *Fagonia* is also found in the northeast Africa including Kenya, western Asia including Lebanon, Turkey, and the Arabian Peninsula east to Afghanistan and western India. The genus has not been so far reported from Madagascar. The disrupt distribution of *Fagonia* has been supported by different hypotheses. suggested that *Fagonia* is introduced into the new world by cargo-shipment of the seeds of *Fagonia cretica* from Iberian Peninsula. However, later Engler suggested that, it may be prehistoric and not the aftermath of anthropogenic dispersal in 1931described the distribution of *Fagonia* is because of widespread of early Tertiary desert flora. suggested initially, that the distribution was as a result of a break-up of the range of a common pantropical tertiary ancestor but later proposed as a remaining of the dry flora. proposed, that the distribution was a result of a pre-tertiary movements from the Old to the New World through the Bering land bridgesuggested a mixture of sea-floor spreading and long-distance dispersal from east to west during the early tertiary, when the both Old and New Worlds continents were much closer to each other than at present.

1.2.1.2. Phylogenetic relationships with in genus *Fagonia*

There were few studies on the phylogenetic relationships of genus *Fagonia*. The North African *Fagonia* species are divided into four ‘‘natural groups’’: (1) the *F. kahirina–cretica–flamandii* group, (2) the *F. arabica–bruguieri* group, (3)the *F. glutinosa–latifolia* group, and (4) the *F. microphyl-la* group divided the North African *Fagonia* species into three groups and later modified the ‘‘natural groups’’ species ‘‘with tri- or unifoliolate leaves’’ or ‘‘with simple leaves’’

The ‘‘unifoliolate leaves’’ was in turn divided into three ‘‘complexes’’: (1) *F. arabica*-complex, (2) *F. bruguieri*-complex, and (3) *F. indica*-complex. El Hadidi (1974) also made changes in the division of the complexes and divided them into: (1) the *F. isotricha*-complex (*F. isotricha* is now a synonym of *F. latifolia*), (2) the *F. glutinosa*-complex, (3) the *F.*

sinaica-complex (*F. sinaica* is now a synonym of *F. scabra*), (4) the *F. thebaica*-complex (*F. thebaica* is now a synonym of *F. arabica*also reported that the three groups of *Fagonia* species with short stipules and trifoliolate leaves were more closely associated to each other as in comparison to the group of species with long stipules and one to tri-foliolate leaves.

Phylogenetic studies done gave different distribution and phylogenetic relations among *Fagonia* spp. as compared to El Hadidi. Beier's uses plastid trnL intron and nuclear ribosomal ITS DNA sequences of 34 species of *Fagonia* genus. The result divides the species into different clades based on the genetic and morphological resemblance (divided into 16 sub-divisions known as Clades (A-P)). There is small distinction among the members of *Fagonia*. The genus is weakly supported, and *F. scoparia is* sister to the rest of *Fagonia* (clade B). *Fagonia* species belonging to the New World form a clade (clade C), and *F. acerosa*, (Iran), and *F. Bruguieri* (North African-Asian) form clade (clade D). *F. lahovarii*, endemic to the Horn of Africa region including Yemen, *F. latistipulata*, endemic to Somalia, and *F. mollis*, restricted to Egypt, Israel/Palestine, Jordan, and northern Saudi Arabia form clade (clade E). *F. harpago*, endemic to Morocco, and *F. longispina*, endemic to Morocco and Algeria (clade F). The two species from southern Africa, *F. minutistipula* and *F. rangei*, form a weak clade (clade G). All samples of *F. indica* and *F. paulayana*, which are widely spread to North African-Asia and *F. subinermis*, endemic to Iran, form a moderately supported clade (clade H). *F. olivieri*, found in Lebanon, Syria, Iran, Iraq, and Jordan form clade (clade P).

The result of the DIVA analysis showed 28 dispersals, and the historical distribution showed two alternative ancestral distributions in the analysis: North America, northeastern Mexico, and Saharo-Sind with the bordering area of western Morocco, including the Horn of Africa. Vicariance is referred to the phenomena, that separated the ancestor of all *Fagonia* species, except *F. cretica* and *F. scoparia*, to the areas of North America and the Horn of Africa region. Considering that vicariance took place between two areas not adjoined today i.e. North America and Horn of Africa. All the New World species of *Fagonia* are indicated to be a result of successive duplications (i.e., speciations within the area), with the exception of *F. chilens is* in South America, the distribution of which is conceived as a result of dispersal of the ancestor. The ancestor of all species of the Old World, except *F. cretica* endemic to the Horn of Africa, from where dispersal to Saharo-Sind or western Morocco, and southern Arabia took place. The ancestor of clade M, which includes: *F. gypsophila*, *F. indica*, *F.*

lahovarii, F. latistipulata, F. mahrana, F. mollis, F. paulayana and *F. subinermis*, were endemic to the Horn of Africa, from where it dispersed into southern Arabia and southern Iran, as well as to Saharo-Sind. The ancestor to the species included in clade M, is endemic to Saharo-Sind or western Morocco. Subsequently, speciation within the area occurred, after which dispersal to southern Iran, southern Arabia, and southern Africa and to the Horn of Africa took place. Some of narrow endemics such as *F. hadramautica* are indicated to have evolved after relatively recent dispersals. The dispersal–vicariance analysis shows that the occurrence of *Fagonia* in South America and southern Africa is most likely a result of dispersal (Beier, 2005).

1.3. Ethnobotany

Plants and herbs have provided natural remedies for human ailments from time immortal. As knowledge progressed, man selected different herbs for cure of different diseases and ailments. The World Health Organization (WHO) estimated that about 80% of the world's population relies on traditional medicines for their primary health care. Most of the plants, which are used for various ailments, have not been properly investigated. About 30% of the pharmaceuticals are prepared from plants worldwide.Traditionally it is used in Sind and Afghanistan for fever treatment. The plant is given as a tonic and febrifuge, and in the Peshawar Valley it is given to children as a prophylactic against small- pox. The leaves and twigs are supposed to possess cooling properties. In the Ormara hills the plant is boiled and bound upon the swellings of the neck and for scrofula. At Saruna in Jhalawan it is boiled in water and strained, the liquid is rubbed all over the bodies of children when they get fever. In Kharan, an infusion made with hot water is used as a bath in cases of fever. The plant is considered to cure for itch in the Las Bela State and in the Levy tracts it is for that purpose pounded, mixed with milk, kept for three days and then rubbed all over the body. Species of *Fagonia* have been found to contain coumarins, alkaloids, terpenoids, sterols, saponins, flavonoids, proteins and amino acids, trace elements. Specie of *Fagonia, F. mollis* contains number of saponins and its derivative i.eoleanolic acid-3-O-6'-O-methyl-β-D-glucuronopyranoside, oleanolic acid 3-O-α-L-rhamnopyranosyl (1→3)-6'-O-methyl-β-o-glucuronopyranoside and oleanolic acid 3-O-α-L-rhamnopyranosyl (1→3)-6'-O-methyl-β-o-glucuronopyranosyl 28-O-β-D-glucopyranoside (Melek *et al*., 1996).The separation and characterization of ursolic acid, pinitol and nahagenin hederagenin from other *Fagonia* species have also been reported. The isolation of other important constituents such as

docosanoate from hexane extract and water soluble proteins from aqueous extract of air-dried *F. indica* has also been reported The cytotoxic effect of Kaempeferol glycosides isolated from *F. taeckholmiana* against different cell has also been reported (Ibrahim *et al*., 2008). The extracts of *Fagonia cretica linn* are able to induce neuroprotection in rat brain model by initiating the anti-oxidant system . Extacts from aerial parts of *F. longispina* has antibacterial and antioxidant activities .The anti-microbial and cytotoxic effect of *F. olivieri* extracts and fractions has also been reported. The alcoholic extracts of *F.schweinfurthii* has an anti-inflammatory and wound healing activity (. The anti-microbial activity of *F.cretica* has been investigated on different strains of bacteria . Adronergic effects of *F.cretica* alcoholic extracts on immunomodulation of albino rats is reported by Abirami *et al* in 1996. The anti-allergic effects of *F.bruguieri* were investigated on albino guinea .

1.4.*Fagoniaindica*

*Fagoniaindica*is a small spiny undershrub found mostly in arid, desert regions of Pakistan, India, Africa and parts of Europe, such as Balearic Islands in Spain, where it grows in rocky coastlines. It is widely distributed in Pakistan, Afghanistan, the Indian sub-continent and Egypt.

1.4.1.Common Name

Dhamasa, Dhamaya or Kandiara

1.4.2. English Name

Southern Cyprus

1.4.3. Synonyms

Fagoniacretica

1.4.4. Controversy in naming of *Fagoniaindica*

There is a confusion on whether we use the name *F. cretica*or *F. indica* for this plant because the research on this plant has been done under the name *F. cretica* by Apparently the *F. cretica* is limited to Mediterranean and Middle Eastern regions according to new revision of. In Pakistan, *F.cretica* is used as synonym of *F. indica* or particularly its variety *schweinfurthii* according to flora of Pakistan under the classification of university of Missouri(http://www.tropicos.org/Name/100336325?projectid=32). Our lab also published

papers of *F.indica*, using its synonym *F. cretica* (Molecular Biology Lab Biochemistry dept. supervisor: Dr BushraMirza (HOD) Quaid-e-Azam university Islamabad)

1.5. Morphology

It is profuse, branched, pale green, glandular, annual or perennial shrubletsup to 60 cm high, internodes 2.5-5.0 cm long; leaves opposite, uni or trifoliate, from 3 – 30 mm long, deeply striate, narrowly lanceolate, mucronate, distinctly articulate at the base; stipules 2 pairs of sharp slender thorns, sometimes exceeding 12 mm in length, aculeate, shorter than leaves and internodes, patent, not reflexed(Nasit, Ali .Eds. Flora of West Pakistan http://www.tropicos.org/). Leaflets linear, acute, sessile, or with very short petioles(Beier, 2005) stem basally woody, branches prostrate to erect, terete, striate, with up to 1-3 (-3.5) cm long internodes; Flowers *ca*. solitary in the axils, fragrant, 12mm in diameter,spathulate, obtuse;stamen *ca*. 6 mm long; capsule *ca*. 4×4 mm , softly hairy; pedicels 3-8 mm long, with pink to purple petals, reflexed, equal to the length of fruits; fruit a capsule 3-5 mm long, , 2-5 mm wide, short pubescent with persistent style; Seeds flat rounded and brown in colour (Bhandari, 1990; Eman, 2011). Fig 1.2 shows *Fagoniaindica*.

1.6. Distribution of *Fagoniaindica* across the world

Fagoniaindica is present commonly in Pakistan, India, Iran, Aden, Eritrea, Aethopia, Sudan, Somalia, Kenya, Garcin, north and east of Africa and semi arid region(http://www.tropicos.org/).

1.6.1. Distribution of *Fagoniaindica* across Pakistan

C-6 MianwaliDist, D-8 Lahore Dist; About 8 miles from Patoki on way to Lahore, F-5 SangharDist; Haji Fateh Ali Goth, between Kandiari and Sanghar, G-4 DaduDist; 8 miles from Thana Bulla Khan on way to Karachi, Karachi Dist; 10 miles from Karachi on way to Bella, near Zoology dept., Karachi University Campus, North Nazimabad common on sandy hillocks, Nasirabad, sandy clay soil, common, Karachi, rocky desert, G-5 Hyderabad Dist; between Tando Jam and Tando, G-6 Thar Parker Dist; near Nagar Parker plains, on way to Virawa, Botanical garden of Quaid-e-Azam university Islamabad.(http://www.tropicos.org/).

1.7. Ethnobotany/Medical importance

F. indicia is very bitter in taste.*Fagonia* species were extensively studied by many workers regarding their medicinal uses, since these plants are antitumor, antioxidant, analgesic, astringent, febrifuge and prophylactic against small-pox agents. Species of *Fagonia*were also used for the treatment of cancer in the indigenous system, fever, asthma, urinary discharges, toothache, stomach troubles and kidney diseases. In scientific and folk literature, it is reputed as medicinal plant with rich therapeutic potential. An aqueous decoction of plant is popular remedy for the treatment of skin lesions and boils. It is reputed in the indigenous system of medicine as tonic, febrifuge and prophylactic against small pox. It is well documented for the treatment of fever such as typhoid, thirst, vomiting, dysentery, asthma, urinary discharges and filtration process, liver and stomach problems. Boiled plant is used to induce abortion. It is also famous for itsantivenom, anti-inflammatory and antipyretic activity. An aqueous decoction of the aerial parts of the plant is used for treatment of cancer especially for breast cancer in its early stages and for treatment of various diseases of digestive and blood vascular system. *F. Indica* has been shown to elevate GSH levels as well as having strong free radical scavenging properties. Plant extract has shown antitumor and cytotoxic properties *in vitro*. Compounds isolate from *F. Indica* has cytotoxic activity towards erythrocytes and leucocytes. It has also shown anti-inflammatory properties including platelets aggregation, down regulation of COX2 and upregulation of VEGF. A saponin isolated from *F. Indica*has been shown to selectively induce aponecrosis in cancer cells (Waheed*et al*., 2012). Antibacterial effects of *F. Indica* have also been reported (Gehlot and Bohra, 2000). Effects of powdered *Fagoniaindica* plant and triterpenoid from its ethanolic extract showed that the saponins had highly significant decreasing effects on the amount of total leukocyte count of rabbit's blood .The plant leaf extracts were found most effective against Salmonella typhi.). The plant extract showed molluscicidal activity (Shoeb*et al*., 1987). New erythroxane-type diterpenoids from *Fagonia* showed cancer-preventing potential (Gedara*et al*., 2003). Furthermore, an aqueous extract of *F. indica* was able to significantly increase survival time in mice with induced tumors. Recently it is reported that an aqueous extract of *F. Indica*can induce cell cycle arrest and apoptosis in human breast cancer cell lines via p53-dependent and independent mechanisms with activation of DNA damage.Analgesic and antimicrobial activity of *Fagoniaindica* extracts has been reported (Sharma *et al*., 2009).*Fagonia* species is a potent antifungal, antibacterial agents and has cytotoxic property*F. indica*is rich in various types of chemical constituents such as

triterpenoids,saponins,coumarins,flavanoids and tannins. It also contains flavanoids such as quercetin, kaempferol, isorhamntin-α-3-O-rhamnoside, quercetin 3-O-β-D-glucopyranosyl-(1"-6"')-β-D-glucopyrannoside and quercetin 3-O-β-D-galactopyranoyl-(6'-1''')-α-L-2'''acetylrhamnose-(3'''-1'''')-β-D-glucopyranoside. Major saponins isolated so far from this plant include 3-O-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]hederagenin 28-O-β-D-glucopyranosyl ester, 3-O-]β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] oleanolicacid, 28-O-[β-D-glucopyranosyl(1→6)-β-D- glucopyranosyl] ester, 3-O-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl],27-hydroxy oleanolic acid, 3β-O-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] olean-12-en-27-al-28-oic acid.

1.8. Tissue culturing in *Fagonia* species

So far, few papers were reported on tissue culturing of species included in genus *Fagonia.* So far, few papers were published regarding to the *invitro*propagation of the species including in genus *Fagonia.* Callus induction in *Tribulusterrestris,* using leaf and stem as an explants for detection of metabolites has been reported. Callogenesis and somatic embryogenesis from cotyledon explants of *Fagoniaindicaburm* was also reported. *In vitro* Propagation through seeds of *Guaiacum sanctum* was reported. Callogenesis of *Fagoniaarabica* L for chemical analysis was also reported. Callogenesis of *F. indica* and *Fagoniabruguieri Dc.* for phytochemical screening had also been reported.

1.8.1. Limitation of tissue culturing

The less work on *invitro*propagation of genus *Fagonia spp.* is due to high rate of contamination, poor regeneration capacity of explants and poor germination rate of the seeds. The seeds give poor germination on artificial media with browning of cotyledons and retarded growth . Although many attempt has been made on clonal propagation of medicinal important plants in this genus but results are non-satisfactory. Uptill now, only callogenesis of few species are reported. The high rate of fungal contamination is the main hindrance in establishing the tissue culture conditions for plants included in *Fagonia* as well as their poor growth*in vitro*.

1.9.Plant tissue culturing

Plant cell and tissueculture is defined as the capability of any plant part to regenerate into complete plant, tissue or organ under sterilized and controlled conditions). Clonal

propagation, rapid propagation, micropropagation, *in vitro* propagation (in vitro means ''in glass'' or ''inartificial conditions'' are names, that are used as an alternative of term tissue culture. The basic principle of tissue culture technique is based on concept of totipotencyi.e every single cell with in plant has the potential to give rise a whole plant). It is one of the most incredible property of plants,which fascinate a number of scientists to run a number of experiments to elucidate the properties of various plants and ultimately succeeding in establishment of efficient methods of growing plant cells and tissues under sterilized environment). Each cells of different tissues of plant has different level of differentiation and specialization, so therefore the extent of totipotency is different in each cell of a plant

The first works in the field of plant tissue cultivation date back to beginning of 19^{th} century and linked with the names of three outstanding scientists. The first successful report on true plant tissue culture was given by when he tissue cultured cambial tissues of *Acerpsedoplatanus.*

In conventional method of cultivation, many plants under certain climatic conditions produce seeds and some of them are even unable to produce seeds and flowers. They also need long time for their growth and development. The advantages of plant tissue culturing is far more as compared to conventional method of plant cultivation. It requires less time, space and much less expenses as compared to conventional methods. It also helpful in production of plants from seeds that otherwise unable to germinate e.g orchids and nepenthes (pitcher plant)

There are three different strategies of tissue culture that are used for the regeneration of plants which are (1) using apical meristem(shoot tips or nodes), (2) organogenesis (direct or indirect using calli) and (3) somatic embryogenesis. Shoot initiation and multiplication, shoot elongation and*in vitro* rooting from shoot to form stably growing plantlets are the three common pathways through which cells or tissues are regenerated into a complete plant. The success rate of plant tissue culture depends upon many factors. One of the important factors is source from which we take the explants i.e age, size, type and position of the explants. All plant cells have different ability to regenerate into new plant .Shoots, roots, stem, leaves, flower and callus are used as explants in plant tissue culture but shoot tips, root tips and nodal buds are most commonly used explants in tissue culture technique. The main reason for their common use is the high rate of cell division in these regions (as they contain undifferentiated meristematic tissues) and their ability to produce high level of growth regulators such as auxin and cytokinins. Young explants have more totipotency as compared to mature explants.

Small size explants sometimes show less growth while using large explants enhance the chances of contamination (Staba and Seabrook, 1980).

1.9.1.Advantages

The techniques of tissue culture are practically applied on all types of plantsranging from cash crops and foods to medicinal and ornamental plants and even trees. It has increased the mass production of vegetative propagated crops (Sutherland *et al*., 2005).It is widely used for large scale multiplication of plants in short time. Apart from research tool, it is important in the area of disease elimination, plant improvement, plant propagation and production of secondary metabolites. Using a plant tissue culture technique, a single explants can be multiplied into thousands of explants in relatively shorter period of time and space under sterilized conditions, irrespective of the season and weather on a year round basis (Akin-Idowu*et al*., 2009). This technique also help us in the field of fundament botany, especially in the field of agriculture, horticulture, plant breeding, forestry, somatic cell hybridization, phytopathology and plant metabolites production at industrial level.

It also help us to understand the phenomena and advantages of biotransformation, forestry, genetic engineering, maintaining pathogen free plant, morphogenesis, comparison of totipotency rate among plants, differentiation rate, cell division rate among different area of the plant, cell nutrition, cell preservation, somatic hybridization, metabolism, radio biology. The production of pure haploid plants through tissue culture technique from anthers or isolated microspores and of protoplasts from higher plant cells has been used as thebasic tools for genetic engineering, and somatic hybridization. One of the significant developments in the field of plant tissue culture is the isolation, culture and fusion techniques which have their special importance in studies of plant improvement by cell modification and somatic hybridization.

1.9.2. Disadvantages

Beside various advantages, the tissue culture technique is facing some serious challenges. It requires keen and close attention of the researchers in order to overcome infection that may proceed through generations. The unavailability of standard protocol for each plant is another major issue in this technique because the genotyping is different for each plant. In plant cell and tissue culture, the chances of soma clonal variation are ubiquitous the somaclonal variations rate depending upon genotype, from shoot tip of Musa species, vary from 0-70%,

however this genetic instability may be by chance as it may also provide us novel items and foods in large quantity and less The issue of biosafety and resistance in pests and weeds of pesticides and herbicides due to gene leakage from transgenic plants to wild plants is serious issue in using biotransformation technique through tissue culture technique. The destruction of biosphere and unavoidable genetic variability may covered the benefits of this technique.

1.10.Plant Genetic Transformation

Developments in biotechnology have revolutionized the way to introduce any new trait or characteristic by utilizing the technique of genetic engineering, which otherwise by conventional breeding also involve sexual hybridization, which is a very lengthy procedure. Genetic transformation is the process through which the genetic makeup of an organism is altered by introducing a fragment of foreign DNA. It involves the successful introduction, integration and expression of a gene from its normal location into a cell of a tissue that does not contain it. Genetic engineering is young branch of science. The first report of transgenic plant was reported, they use modifiedTi plasmid as a vectorand allow insertion of it into host plant DNA. The genetic engineering result into either stable or transient expression of incorporated DNA. In stable transformation, gene is stably integrated into chromosome so that the cell that has been transformed and all of its daughter cell contain the foreign gene, hence it is time consuming process. In transient expression gene is not transferred to germ cells, so the next progeny will not be transformed. This technique is beneficial to study metabolic processes and development of improved transformation methods (Gheysen*et al*., 1998).

Phytotransformation plays a very important role in increasing the dependence of mankind on crops with more yield and nutritional value. Since 1980s scientists have used different transformation techniques and have succeeded in production of large number of transgenic plants (Chen *et al*., 1998; Gahakwa*et al*., 2000).Foreign genes taken from different plants and bacteria had been incorporated successfully in major crops such as to get desirable characteristics such as pesticides, disease, herbicide and insect resistance in order to increase crop yield and decrease the expenses of pesticides.

According to Hansen and Wright (1999), there are certain principles that must be fulfilled in order to get the successful plant transformation method. These are: (i) the transformation method should be simple, genotype-independent, and easy to perform, well organised, highly

reproducible and cost effective. (ii) An efficient and competent method for delivery of foreign DNA. (iii) Selectable markers for transgenes.(iv) Transgenes should be viable and recovered at a comparable rate. (v) Target cells or tissues should be competent for propagation or expression.(vi) A fixed timeframe in culture in order to evade somaclonal variation and infertility.

1.10.1.Direct DNA Delivery

This technique includes biolistic (particle bombardment), silicon carbide whiskers, electroporation of tissues, injection of foreign DNA and the protoplast system. The later can be transformed using polyethylene glycol (PEG) fusion and microinjection or electroporation. The most frequently used methods for transformation are gene gun (biolistic approach) and *Agrobacterium*-mediated transformation. The biolistic method is used for both nuclear and chloroplast transformation while *Agrobacterium*-mediated is solely for nuclear based transformation and is one of the most reasonable application.

1.10.2.*Agrobacterium*-mediatedtransformation

The genus *Agrobacterium* comprises of species that are normally present in soil microflora. Most of them are saprophytesthat mainly live on decaying organic matter. *Agrobacterium* is a common soil born gram-negative bacterium. Virulent typeof *Agrobacterium* species infect almost all types of plant species but form tumors in herbaceous dicotyledonous and woody plants and sometimes in monocotyledonous species. This genus includes many species classified on their host range and the extent of pathogenicity. Some species are avirulent such as *A. radiobacter* and many others cause neoplastic disease in plants namely *A. rubi* causing cane gall disease, *A. rhizogenes* causing hairy root disease, *A. vitis* causes crown gall of grape and *A. tumificiens* resulting in crown gall disease.

Agrobacterium-mediated transformation has played a major role in the development of genetic engineering of plants and involves *A. tumificiens* and *A. rhizogenes* mediated transformation. *A. tumificiens* carry tumor inducing (Ti) plasmid while *A. rhizogenes* carry root inducing (Ri) plasmid. *A. tumificiens* transfer its plasmid fragment called T-DNA into host cell DNA which involves in the formation of crown galls in host plant. Crown galls are chimeras due to modified growth of transformed and related untransformed cells. Tissues

become chimeric because T-DNA coding for the enzymes involved in synthesis of auxin and cytokinin, resulting in the growth changes. On the other hand, *A. rhizogenes* stimulates hairy root formation in dicotyledonous (Newell, 2000; Lacroix, 2006), however, root induced due to infection *of A. rhizogenes* are not chimeric and made of totally transformed cells (Schmüllinget *al*., 1988).

Agrobacterium-mediated transformation is commonly used for transforming genes in almost all types of plants because of its potential to produce transgenic plants at higher frequency. Overall, this technique is relatively simple, easy to perform, efficient and cost effective as compared to other genetic transformation methods. One or few copies of gene (even of large size) can be integrated in the host cells without undesired gene silencing or fragmentation of foreign gene.

The efficiency of *Agrobacterium*-mediated transformation varies and depends on many factors, including plant species, *Agrobacterium* strains, medium, explants, antibiotics, co-cultivation, and selection. Although numerous other methods of transformation has been developed by other scientists but this method is preferential over others so far, for the genetic manipulation of plant cells. The reason is that uptill now no other organism across the kingdom has been discovered which has the ability to transform the genetic material of host cells of other kingdom species. Due to this property of inter-kingdom transformation, *A. tumificiens* has also the ability to transform fungi and human cells as well

1.10.2.1 Molecular mechanism of *A.tumificiens*-mediated transformation

The mechanism of processing and transfer of T-DNA from *Agrobacterium* to the nuclear genome of the host cell has been given in a number of reviews (McCullen and Binns, 2006; Karamiet *al*., 2009; Gelvin, 2009). The crown gall disease caused by *A. tumificiens* involves two major elements i.e transformation and tumorigenesis. Transformation is the transfer of tumor inducing DNA into nuclear genome of host plant whereas tumorigenesis is the consequential alteration in the metabolism of plant cell resulting in cell proliferation and production of essential nutritive compounds for *Agrobacterium* itself

Agrobacterium tumificiens has a large number of tumor inducing (Ti) plasmid. It ranges in size from 200 to 800 kb. Ti plasmid contains Transfer DNA (T-DNA) and many other functional parts for virulence (*vir*), conjugation(*con*) and the origin of replication (*ori*). T-DNA and *vir* genes are necessary for inducing plant tumors. *Vir* genes play a key role in

delivering the T-DNA to host cell genome (Gustavo *et al*., 1998) while T-DNA has a role in the genetic manipulation of host plant cell as well as in the production of enzymes that helps in the synthesis of opine. Opine is conjugate of sugar and amino acid which act asa source of carbon and nitrogen for *Agrobacterium* (Tempe and Petit, 1982).

T-region varies in size from 10 to 30 kb while the *vir* region is about 30 kb (Suzuki *et al*., 2000). Normally, T-region accounts for less than 10% of the total Ti plasmid. In Tiplasmid there may be single or multiple T-DNA regions. Any gene located in the T-DNA region is transferred. T-regions are defined by flanking sequences known as the T-DNA border. This border region comprises of 25 bps sequence which are homologous to each other. The T-DNA is transferred to the host genome from Ti plasmid through a special system known as the type IV secretion system (T4SS). This region is randomly inserted into host genome by process of recombination

The infection of *Agrobacterium* initiates at the wounded sites of the host plant. The cells of the injured plant release some phenolic compounds called acetosyringone(AS). Acetosyringone act as a strong inducer of *Agrobacterium tumificiens*. It binds to the membranous protein called vir A and activates it through autophosphorylation which in turn activates vir G by phophorylating one of its aspartate residues).

Activated vir G binds to other *vir* genes called vir box and induces their expression. The vir D1 and vir D2 are responsible for the generation of T-strand i.e a single-stranded T-DNA copy, by specifically identifying and excising the bottom strand , of which the right one is the start site and hence more important. After excising, virD2 remains covalently bound to the T-strand at 5' end and form a complex which is then transferred into the nucleus through nuclear target signals (NLS) along withvir E2 and virD2. T-DNA then integrates randomly into the plant nuclear genome as asingle or multiple copies.

1.11. Marker genes

Transformed cells can be efficiently selected and screened due to the development of the number of bacterial marker genes. For the selection of transformed cells and tissues usually an antibiotic or herbicide is included in the medium, from which transformed plants are generated. For this purpose, two types of indicator genes are commonly used namely selectable and non-selectable markers.

1.11.1. Selectable markers

The transformed cells can be selected from the mixture of both transformed and un-transformed cells by using selectable marker genes. For this purpose a variety of selectable genes are used such as neomycin phosphotransferase II (*NPTII*), that confers resistance to antibiotic kanamycin, hygromycin phosphotransferase (*hpt)* genes shows resistance to antibiotic hygromycin and by bialaphos resistance (*bar*) genes that show resistance to L-phosphinothiricin (PPT). Out of these, the most commonly used selectable marker gene is neomycin phosphotransferase II (*NPTII*) in dicotyledonous plants (Irdani*et al*., 1998). Enzymes encoded by *NPT II*produce specific properties normally not found in plant tissues. *NPT II* is also useful to study transport among plant organelles as it can bear amino terminal fusion and still maintain its enzymatic activity.*NPT II* protein doesnot cause any risk for environment and for plants as well).

1.11.2.Non-selectable markers

Non-selectable markers are also identified as scorable markers or reporter genes. The reporter genes perform their function by coding for an enzymes having activity on substrates normally not found in the plant host. During plant transformation study, the most frequently used reporter gene is *GUS* (β-glucuronidase) (Newll, 2000). Example of these reporter genes used are: β-galactosidase (*Lac Z*), green fluorescent protein (*GFP*), chloramphenolacyltransferasre (*CAT),* firefly luciferase (*LUC*), octapine synthase (*OCS*), and nopaline synthase (*NOS*). *GFP* was isolated from the jelly fish *Aequoreas Victoria. GFP* is one of the most sophisticated and advanced reporter gene because it does not require the substrate to be supplied to plants rather it makes the transgenic cells visualized by excitation with light.

GUS gene was initially isolated and sequenced from *E. coli*.It is also found in other bacteria namely; *Staphylococcus warnari*, *Bacillus sp., Salmonella sp., Enterobacter sp., Thermotoga maritime* and *S. hominis*There are many advantages of using *GUS*as reporter gene. It showed expression at awide range of pH i.e. 4-8 and isthermally stable upto 50. *GUS* gene has the ability to express in all the tissues of shoot and roots and is stably inherited to offsprings In order to detect the expression of *GUS*gene in vitro and in vivo various substrates are available. All these substrates have the sugar glucopyrannosiduronic acid which is attached to hydroxyl group of florogenic, chromogenic or other detectable molecule through glucosidic linkage. The most widely used substrate for the detection of *GUS* is 5-bromo-4-chloro-3-

indolyl-β-D-glucuronide (X-Gluc). *GUS* cleaves X-Gluc substrate that results in the formation of colourless glucuronic acid and indigo blue chromogenic precipitate

1.8.1. Aims and Objectives

- To select and optimize the best tissue culturing conditions of *Fagoniaindica.*
- To select and optimize andthe best transformation conditions of *Fagoniaindica.*

MATERIAL AND METHODS

The aim of the present study was to determine the seed germination efficieny, to optimize the tissue culture conditions (calli induction, roots induction, and shoots induction) and to optimize the transient transformation as well as stable transformation of species of *Fagonia i.e Fagonia indica* using *Agrobacterium tumifaciens* strain C58C1 containing plasmid 35SGUSTint. The details of materials used and methods have been given in following chapter. The following research took place in Plant molecular biology laboratory, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan under the supervision of Dr Bushra Mirza.

2.1. Working Precautions

Sterilized conditions were maintained during seeds germination, tissue culturing and streaking of bacterial culture on Luria Agar in order to minimize the chances of contamination, cross contamination and for personal safety. For this purpose following measures were taken.

- Lab coat was used during working in the laboratory.
- All equipments and materials used in plant tissue culturing and culturing of micro-organism were sterilized by autoclaving at 121 °C for 20 minutes.
- Disinfectants such as 70% ethanol or 5% Sodium hypochlorite (bleach solution) were used to swap benches and laminar flow hood (LFH).
- For surface sterilization, whole arrangement (LFH and apparatus) was exposed to ultraviolet radiations for 15-20 minutes.
- A disinfectant soap or hand sanitizer was used for washing hands before and after working with microorganisms.

- ❖ Sterilized hand gloves were used, while working in the LFH.
- ❖ All contaminated materials were autoclaved before their disposal.

2.2.1. Laboratory Glassware and Chemicals

The 9 cm petri plates were procured from **Corning® USA while all the micropipette tips were purchased from Axygen Scientific USA. The microcentrifuge tubes (1.5 and 2 ml) were obtained from Eppendorf Germany while the syringes and syringe filters (0.2 μm) were purchased from Sartorious Ltd, UK.** The glassware made of borosilicate glass was used during the whole experimentation and was purchased from Pyrex®. All the glassware was washed using commercial detergent. It was later dipped for 30 minutes in 10% bleach solution and dried in a conventional oven at 200°C for 15 minutes. Flasks were plugged with fine absorbent cotton covered with aluminium foil while petri-plates were enclosed in brown paper or newspaper and autoclaved for 20 minutes at 121°C temperature keeping the pressure at 15 psi. The surgical instruments used in tissue culturing were autoclaved for 20 minutes at 121°C temperature keeping the pressure at 15 psi.

All the chemicals used in the experimental work were of the highest grade of purity. The chemicals, reagents and Murashige and Skoog (MS) medium used in the present research work were purchased from Sigma Chemical Co., USA and were either molecular biology grade or analytical grade. Molecular biology reagents were acquired from Fermentas, Invitrogen and Sigma whereas different molecular biology kits were obtained from Promega, Qiagen and Invitrogen. Moreover, Agar and Luria Bertini (LB) medium were obtained from "DIFCO" laboratories, USA. All of the solutions were prepared using distilled water.

2.3.1. Control of bacterial and fungal contamination by antibiotics in plant tissue culture

Antibiotics are used to reduce or eliminate contamination of micro-organism (bacteria, fungus etc) in plant tissue culture media because the microbes have same requirements as those of plants grown *in vitro*, the sugar present in media attracts a variety of micro-organism which grow faster than that of cultured plant tissues in medium and ultimately kill plant cells. It is therefore, necessary to have complete aseptic conditions around media. The commonly used antibiotics are cefotaxime which is a third-generation broad-spectrum cephalosporin

antibiotic and has activity against numerous Gram-positive and Gram-negative bacteria. Streptomycin and spectinomycin which are antimycobacterial, are also commonly used.

Sodium hypochlorite (NaOCl), also known as danchlor solution is used commonly to tackle fungal contamination. It is potent anti-fungal agent and kills wide variety of fungal spores. Commercial bleach is also used as substitute anti-fungal agent and is less expensive as compared to danchlor solution.

Hydrogen Peroxide (H2O2) solution is also used for sterilization purposes and is easy to remove from plant material as compared to NaOCl. Mercuric Chloride (HgCl2) (0.1-0.5%) is commonly used for sterilization of seeds and plant materials. Surfactants such as *tween 20* and *triton X 100* are also used in sterilization of some explants which are difficult to sterilize completely.

The antibiotics are filtered sterilized because they are heat labile and add to culture media before pouring while detergents and disinfectants used were autoclaved for 20 minutes at 121°C temperature keeping the pressure at 15 psi or filtered sterilized using filtration assembly.

All the plant tissue culture work was done inside the laminar flow hood (LFH).

2.4.1. Sterilization of Laminar Flow Hood

For tissue culturing of plants, aseptic conditions were maintained by a Laminar Flow Hood (LFH) fitted with a High-efficiency particulate absorption (HEPA) filter providing a constant flow of air across working area. Before using, the LFH was cleaned by swabbing with 70% ethanol or bleach using autoclaved cotton. Further sterilization of LFH was done by using UV light for 15-20 minutes. Surgical instruments were autoclaved and dipped in 70 % ethanol or bleach inside the LFH. After using, the surgical instruments (forceps and scalpels) were again dipped in ethanol, re-flamed and then reused.

2.5.1. Seed Germination

2.5.1.2. Plant Material

The experiments of the present research were performed using seeds of *Fagonia indica*. These seeds were purchased from Mianwali, Pakistan. The seeds were kept in sealed

plastic envelopes and placed in air tight jars. These jars were stored at cool and dry place with temperature less than 20°C and relative humidity not exceeding 30%.

The surface sterilization of seeds was performed prior to germination. Seeds were washed with autoclaved distilled water to remove dirt and debris. The seeds were divided into two groups i.e imbibed and non-imbibed, to check the efficiency of germination of imbibed and non-imbibed seeds. Few seeds were also cold treated in order to break the dormancy. The morphology of seeds used is also different i.e old or mature seeds which are brown in colour and young seeds which are green in colour. Both types of seeds are used for germination.

2.5.1.3. Surface Sterilization of Seeds

The seeds were dipped in 70% (w/v) ethanol solution for 2-3 minute and then in 5% Sodium hypochlorite for 45 seconds and then in 0.1% Mercuric Chloride for 30 seconds by continuous shaking with sterile forceps, the seeds were then washed thrice with autoclaved distilled water. The seeds were dried on autoclaved filter paper. All the work was done inside laminar flow hood.

2.5.1.4. Preparation of Seed Germination Medium

Seed germination required usually ½ Murashige and Skoog (1/2MS) medium (Murashige and Skoog, 1962) which was prepared by adding MS medium (4.4/2 g) and sucrose (30 g) in 1 liter de-ionized distilled water. Once the contents were mixed by stirring, the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH and solidified with 0.5% agar (gelrite or gum powder). The flask containing ½ MS medium was plugged with non absorbent cotton and wrapped with aluminium foil and finally autoclaved for 20 minutes at 121°C and 15 psi. Once the sterilization was completed by autoclaving, the media was left to cool in a laminar flow hood. Roughly 40-45 ml of the medium was dispensed in each 9 cm sterilized petri plate and allowed to solidify.

2.5.1.5. Seed Inoculation

The sterilized dried seeds were inoculated using sterilized forcep in petri plates containing medium under aseptic conditions in LFH near the lamp. The plates were sealed with parafilm and kept in growth room for 15 days approximately at 25±2 °C under light.

2.5.1.6. Seed Germination Efficiency

In order to check the optimize media for seed germination, the seeds were germinated on four different types of media including half strength MS (½ MS) media, MS, B5 and RMOP in order to compare the seed germination efficiency of *Fagonia indica*. Table 2.1. shows the chemical composition of ½ MS, MS, B5 and RMOP media, respectively

The experiment was performed in four batches with the gap of two weeks between two consecutive batches. Further, in each batch 5 plates of each combination were used, each containing 7 seeds. In each combination, 21 non-imbibed and 14 imbibed seeds were inoculated. Furthermore, 2 batches contain mature seeds and 2 batches contain green seeds

During this research, three types of explants are used for subculturing/clonal propagation *in vitro* which are:

- stem meristematic region i.e shoot apical meristem (SAM)
- Nodal region of stem
- Cotyledons (small two leaves stage explants obtained from seeds *in vitro*).

. The SAM and nodes were cut from wild plants of *Fagonia indica* obtained from the Botanical Garden of Quaid-i-Azam University Islamabad, therefore these explants need sterization treatment in order to get rid of possible contaminations on culture media.

Multiple sterilization treatments were given to explants to avoid possible contaminations. The sterilizing agents used are described in table 2.3.

2.6.2.1. Shoot Apical Meristems Explant Preparation

Apical meristems of young green plant were used for regeneration. About 1 cm long piece of shoot apical meristems were carefully removed from the seedlings using a sharp blade under LFH.

2.6.2.2. Nodes Explant Preparation

The nodes of young green plant were used for regeneration. They were cut into small pieces of 2x4 mm using blade under aseptic conditions.

2.6.2.3. Cotyledons Explant Preparation

The cotyledons were obtained by seed germination *in vitro*. Cotyledons are 10 days old.

2.6.3.1. Regeneration Media

2.6.3.1.2. Calli induction media

Callus is amorphous aggregate of loose parenchyma cells which is a natural ability of plant and usually appear on wounded side of plant body. Callus contains no organised meristems, it is somewhat abnormal tissue which has the potential to produce normal roots and embryoids and in turn develop into complete plantlets.

Calli induction medium was prepared by adding MS medium (4.4 g) and sucrose (30 g) to 1 litre de-ionized distilled water. They were mixed by stirring and the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH followed by the addition of 0.5% agar. After autoclaving the media, it was left to cool in a laminar flow hood. When the temperature dropped around 50°C, different combinations of growth hormones already sterilized by using **0.2** µm syringe filter were added to the media. Roughly 30 ml of the medium was poured in each magenta jars and allowed to solidify. Finally, the magenta jars were sealed with parafilm and kept at 25±2°C in growth room until use.

2.6.3.1.4. Explants Inoculation

Sterilized shoots apical meristems (SAM), nodes and cotyledons were then inoculated on the plates supplemented with different hormonal combination. A total of 100 explants (30 nodes, 30 cotyledons and 40 shoot apical meristems) were inoculated per combination. The jars were sealed with parafilm and kept in growth room at 25±2°C under light for 10-15 days.

Two types of hormones are used to optimize shoot induction protocol from different explants. They are:

- **BAP** (benzyl amino purine)
- **NAA** (naphthalene acetic acid)
- **2.6.3.1.7. Explants Inoculation**

- Calli induced through explants *in vitro* were then inoculated on the magenta jars supplemented with different hormonal combination. A total of 9 calli were inoculated per combination. The jars were sealed with parafilm and kept in growth room at 25±2°C under light for 10-15 days
- **2.6.3.1.8. Root Induction Medium**
- Root induction medium was prepared by adding MS medium (4.4 g) and sucrose (30 g) to 1 litre de-ionized distilled water. They were mixed by stirring and the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH followed by the addition of 0.5% agar. After autoclaving the media, it was left to cool in a laminar flow hood. When the temperature dropped around 50°C, different combinations of growth hormones already sterilized by using **0.2** µm syringe filter were added to the media. Roughly 30 ml of the medium was poured in each magenta jars and allowed to solidify. Finally, the magenta jars were sealed with parafilm and kept at 25±2°C in growth room until use.

Shoot induction medium was prepared by adding MS medium (4.4 g) and sucrose (30 g) to 1 liter de-ionized distilled water. They were mixed by stirring and the pH was adjusted at 5.8 by adding 0.1N HCl or 0.1N NaOH followed by the addition of 0.5% agar. After autoclaving the media, it was left to cool in a laminar flow hood. When the temperature dropped around 50°C, different combinations of growth hormones already sterilized by using **0.2** µm syringe filter were added to the media. Roughly 30 ml of the medium was poured in each magenta jars and allowed to solidify. Finally, the magenta jars were sealed with parafilm and kept at 25±2°C in growth room until use.

2.6.4.1. Somatic Embryogenesis

Somatic embryos commonly called embryoids at that time. Somatic embryos closely resemble zygotic embryos in their structure but their organization is more generally variable as compared to zygotic embryos. Somatic embryos are observed in different calli induction medium with different morphology

2.8.1. Optimization Of Transformation

Since no work has been reported on the transformation of *Fagonia indica* so far, therefore different parameters have to be optimized for stable transformation. Furthermore, transient *GUS* assay has to be performed in order to check whether the transformation is possible or not. The parameters include

- Cefotaxime concentration optimization
- Kanamycin concentration optimization
- Acetosyringone concentration optimization
- OD of bacterial culture (OD_{600})
- Co-infection period
- Co-cultivation period

2.8.2.1 Explant Sensitivity against Antibiotic Kanamycin

In order to check the sensitivity of calli and shoots generated *in vitro* of *Fagonia indica* against kanamycin (40 mg/ml) and to select the optimal concentration of this antibiotic, we supplemented five different concentrations of filter sterilized kanamycin to autoclaved shoot induction media under aseptic conditions in LFH. The explants (calli and shoots) were gently placed on the media having 20, 40, 60, 80 and100 mg/L kanamycin and kept for four weeks in a growth room. The media was refreshed once during the experiment and the data was taken for number of surviving explants after four weeks. The experiment was conducted in three replicates with 20 explants in each replication.

2.8.2.2. Explant Sensitivity against Cefotaxime

The sensitivity of explants against cefotaxime was studied by using different concentrations of antibiotic. Filter sterilized cefotaxime of five different concentrations i.e. 100, 300, 500, 700 and 900 mg/L were added to autoclaved shoot induction medium. The calli induced *in vitro* from SAM explants were prepared and cultured on media supplemented with the above concentrations of cefotaxime. After four weeks the data for explant survival and shoot induction was recorded.

2.8.2.3. Co-cultivation Medium

Co-cultivation medium was prepared by adding acetosyringone of various concentration (100 µM, 200 µM, and 400 µM) to autoclaved MS medium containing best calli induction and shoot induction hormonal combination. It was then poured in magenta jars under LFH and allowed to solidify followed by sealing with parafilm and kept in the growth room for further use.

2.8.2.4. Selection Medium

Selection medium was prepared by adding MS, sucrose and agar in distilled water. After adjusting the pH, autoclaved MS medium containing best calli induction and shoot induction hormonal combination. Media was cooled to 50°C in LFH. Then 50 mg/L antibiotic kanamycin and 300mg/L of cefotaxime were added to it for the selection of transformants. Medium poured in magenta jars was left in LFH to solidify. Magenta jars sealed with parafilm were kept in growth room until further use.

2.8.3.1. Optimization of *Agrobacterium* Mediated Transformation

2.8.3.1.1. Bacterial Culture Preparation

A. tumifaciens (strain C58C1 harboring p35SGUSINT vector) was inoculated on Luria-Bertani (LB) and LB-agar (LA) on selection medium supplemented with 100 mg/L of kanamycin. A single colony of bacteria was picked with the help of bacterial loop and inoculated in broth (LB having 0.01 % tryptone, 0.005 % yeast extract, 0.01 % sodium chloride) supplemented with 100 mg/L kanamycin. These bacterial cultures were placed at 28°C in a shaker incubator (Sheldon 1575R-2) at 120 rpm overnight. After achieving bacterial growth, optical density (OD) was maintained at five different concentrations (0.25, 0.5, 0.75 1.0 and 1.25) by adding autoclaved LB medium. The bacterial culture having optimum OD was used for streaking on LA to acquire pure colony. Then these pure colonies were inoculated in with selection. LB was prepared by dissolving 2.5 g LB broth (Sigma) in 100 ml distilled water. In each 100 ml conical flask 50 ml LB broth was poured. pH was adjusted at 7.0 and the medium was autoclaved. LA was prepared by adding 0.5g agar in 100 ml LB. After autoclaving 100 mg/L kanamycin was added to both LA and LB for selection in LHF. For checking the best transformation efficiency, culture at different OD was used i.e 0.1, 0.25, 0.5, 0.75,1.0, 1.25.

2.8.3.1.2. *A. tumifaciens* strain and plasmid construction

Agrobacterium tumifaciens strain C58C1 having p35SGUSINT was used for transformation of *Fagonia indica* (Jefferson *et al*., 1987). T-DNA of p35SGUSINT contains *NPTII* gene flanked by NOS promoter and NOS terminator and *GUS* gene with CaMC35S promoter and NOS terminator (Fig. 2.1). Prior to infection, *Agrobacterium tumifaciens* strain C58C1 was grown overnight in an incubator at 28ºC with constant shaking at 120 rpm in YEB medium supplemented with 100 mg/L of selective antibiotic kanamycin. In transformation studies, *Agrobacterium tumifaciens* strain C58CI containing p35SGUSint harboring *GUS* gene was kindly provided by Dr. Sarah R. Grant, University of North Carolina, Chapel Hill, USA. C58CI is resistant to kanamycin and the selectable marker for *GUS* gene is *NPTII*.

2.8.4.1. Transformation Procedure

2.8.4.1.2. Maintenance of *Agrobacterium* culture

The *Agrobacterium tumifaciens* strain C58C1 containing p35SGUSint was routinely kept at 4°C on LB plates having 1 % agar supplemented with 100 mg/L of antibiotic kanamycin. Aseptically a single cell colony of *A. tumifaciens* was picked and inoculated on LB broth followed by keeping on a shaker incubator at 28°C and 225 rpm. The *Agrobacterium* cells were collected by centrifugation and resuspended in liquid MS medium for a short time before using in the transformation experiments.

2.8.4.1.3. Cocultivation

A. tumifaciens C58C1 containing plasmid 35SGUSint was grown overnight in liquid LB medium (1% tryptone, 0.5 % yeast extract, and 1% sodium chloride, pH 7). Medium was supplemented with kanamycin (100 mg/L), added to cold media, after autoclaving in LFH. After inoculation, bacterial cultures were maintained at 28°C and 225 rpm in shaking incubator. After one day of pre-culturing, explants were immersed in the bacterial suspensions for 3 different co-infection periods i.e 5, 10 and 15 minutes. Subsequently, the explants were blotted on sterilized filter paper followed by keeping on co-cultivation medium i.e. MS medium with NAA and BAP. About 100 explants (20 SAM, 20 nodes, 20 leaves, 20 stipules and 20 calli) were cocultivated in each parameters. The magenta jars with explants were kept in growth room at 25°C in dark for one, two and three days, respectively.

2.8.4.1.4. Selection

After the completion of co-cultivation, the explants were washed with washing medium (WM) consisted of sterilized liquid MS medium. In order to kill bacteria, the medium was supplemented with either 300 mg/L cefotaxime and blotted on sterilized filter paper. All the antibiotics were filter sterilized and added to the autoclaved medium. The explants were then transferred to the magenta jars containing selection medium supplemented with kanamycin (50 mg/L). These jars were then kept at 25°C, 16h of photoperiod with relative humidity not exceeding 60%.

2.8.5.1. Histochemical *GUS* assay

The histochemical analysis was conducted following the protocol of Jefferson *et al.* (1987). This assay was conducted to find out β-glucuronidase (*GUS*) gene expression in *Fagonia indica* explants. The explants were immersed in 1 mM X-Gluc solution in 1.5 ml tubes and kept them in vacuumcell by keeping 200 mbar vacuum for 10 minutes to facilitate infiltration. The explants were incubated in dark at 37°C for overnight in X-Gluc solution. After 12-16 hours, *GUS* solution was replaced with 70% ethanol followed by 95% ethanol to completely remove the chlorophyll to visualize the blue color of *GUS* expression with naked eye. Composition of *GUS* solution is given in following Table 2.8.

RESULTS

In the present study, seed germination, tissue culture and regeneration conditions of *Fagonia indica* were optimized. Moreover conditions for successful transformation of *F. indica*with *Agrobacterium tumifaciens* strain C58C1 containing plasmid 35SGUSTint has also been established.

3.1. Seed Germination

3.1.1.Surface Sterilization of Seeds

The experiments of the present research were performed using seeds of *Fagonia indica*. As the seed germination of *Fagonia* species are difficult to grow *in vitro* due to poor germination rate and high contamination issues. Sterilization agents had a very important role in controlling the contamination and breaking the dormancy of seeds. For surface sterilization of *Fagonia indica* seeds, 3 types of sterilizing agents in different combination and exposure time

were used. The sterilizing agents are 70% (w/v) ethanol, sodium hypochlorite (5%) and 0.1% Mercuric Chloride. Seeds were exposed to these sterilizing agent results into 100% sterilization with no bacterial and fungal contamination. Longer duration to sodium hypochlorite and 0.1% Mercuric Chloride treatment resulted in the reduction of seed germination efficiency as they are highly toxic and longer exposure to them result into invasion of these agent through seed coat into the inner soft tissues (embryo)and killing them.(Table 3.1).

The combination of sterilization agent's i.e. 70% (w/v) ethanol for 2 minutes, sodium hypochlorite for 45 seconds and 0.1% Mercuric Chloride for 30 seconds result into maximum sterilization along with germination rate.

The germination efficiency obtained after above mentioned treatment was 45%.

3.1.2. Seed Germination Medium

For the germination of *Fagonia indica* seeds, four different media were used such as 1) Half strength MS medium, 2) Murashige and Skoog (MS) medium, 3) B5 medium and 4) RMOP medium. Sterilized seedswere inoculated under aseptic conditions onto the media that resulted in the formation of hypocotyls and cotyledonary leaves within a week.

The seeds were germinated on all the media combinations with low germination efficiency rate. The germination rate of non-imbibed cold treated seeds are more as compared to imbibed ones. Furthermore, it was observed that efficiency of germination of green young seeds was more as compared to brown mature seeds.

3.1.3.Germination Efficiency

Seeds of *Fagonia indica* were germinated on four different types of media including 1) Half strength MS (½ MS) medium, 2) Murashige and Skoog (MS) medium, B5 medium and RMOP medium in order to compare the efficiency of seed germination. Sterilized seeds were placed on different media without the addition of plant growth regulators (PGR) in laminar flow hood (LFH). When seeds were germinated on full MS media, there was an increase in germination efficiency. *F. indica*seeds germinated on MS were significantly more dense than those germinated on ½ MS (figure 3.1). Full MS showed 45% germination efficiency as compared to ½MS, B5 and RMOP media showing 30%, 6% and 10%, respectively (table 3.2).

The shoot apical meristems of about 1 cm in size gave best regenerative capability on artificial media among the three. The nodes gave very little, while cotyledons gave no result.

The SAM has undifferentiated mass of cells that has the ability to give rise to shoot, leaves, buds and tracheids etc.

3.2.2. Sterilization Treatment of Explants

The nodes and shoot apical meristems excised from wild plant were treated with 70% ethanol, 0.1% mercuric chloride, 15% bleach and different anti-fungal agents (nystatain, terbinafine) to avoid the possible contamination and check the regeneration capacityof explants. The combination no. 6 including 70% ethanol for 10 minutes, 0.1% Mercuric Chloride ($HgCl_2$) for 2 minutes, 5% sodium hypochlorite (NaOCl) for 10 minutes and Terbinafine (1mg/ml) for 2 minutes gave best results, which ensured best sterilization and maximum regeneration capability of explants i.e 99% while remaining of the combinations either kill the explants or contamination is not totally controlled as shown in table 3.3.

3.3. Callus Induction

In order to compare regeneration efficiency of explants, different series of hormonal combinations were used, using full MS media. Callus formation was initiated within 3-4 days of explants inoculation and was not observed in all series of hormonal combination. Two series of hormonal combinations were used i.e. NAA+BAP (the concentration of BAP remains constant and the working concentration for both hormones was 0.5mg/l) and BAP+NAA (the concentration of NAA was constant and the working solution for both of the hormones was 0.25mg/l) as show in table 3.4.

According to experimental results hormonal combination of NAA+BAP i.e 1:1 gave the best result as highest number of callus was obtained on this combination i.e 100%.

The calli induced on 1:0 NAA:BAP hormonal combination was cream in colour and have a soft texture. The efficiency is 56% on this combination. When these calli were shifted on 1:1 NAA:BAP combination, they started to turn green in colour due to chloroplast formation, displaying the role of BAP in differentiation of plant cells. The combination 1:1 NAA:BAP gave 100% result. The calli induced on this combination was green in colour containing chloroplast and has variety of appearance from hard to brittle.

The other series gave best result on 10:1 (BAP:NAA, 0.25 mg/L) combination with 70% efficiency. The calli produced through these combinations are green in colour, brittle and induce somatic embryogenesis after 1 month as shown in figure 3.2.

3.4. Shoot Regeneration

Shoot regeneration takes place when cell clumps or callus were transferred on regeneration medium. The time required for *Fagonia indica* regeneration was around 3 weeks. Only green calli containing somatic embryos produce shoots, thus selected as a plant material for experiments designed to optimize shoot regeneration (figure 3.3, A).

3.4.1 Type of Explants Used

In order to determine best shoot regeneration, calli from different types of explants as well as different types of shoot regeneration media were studied.

For indirect organogenesis of shoot from nodes explant, nodes were regenerated onto medium containing growth regulators for calli induction. Depending upon the type of callus inducing medium, the nodes induced into calli within 10-14 days. The calli that were induced resulted in shoot regeneration after 2-3 weeks when transferred into shoot regeneration medium supplemented with NAA and BAP hormonal combination (figure 3.3, B and C).

For indirect regeneration of shoot from SAM explant, shoot apical meristems of about 1 cm were used. The shoot explants induced into calli within 7 days depending upon the type of callus inducing media. The calli produced then regenerated into shoot in 2-3 weeks when transferred into shoot regeneration media containing NAA and BAP.Shoot apical meristems (SAM) explants showed 90% of regeneration efficiency (figure 3.3, D).

3.7. Root Regeneration from Regenerated Shoots and Somatic Embryos

Root regeneration takes place when somatic embryos or regenerated shoots were transferred on root regeneration medium. The time required for root regeneration was around 1 week. Three different types of hormones were used to develop roots i.e

3.7.1. Regeneration Efficiency

For roots regeneration, BAP, NAA and IAA were used at different concentrations Results showed that most suitable media to induce root regeneration was MS along with hormone BAP (0.1mg/l) and NAA (0.1mg/l). The efficieny rate is 100%. By using this combination, the process of regeneration becomes faster, which was approx. 1 week compared to other hormonal combination (BAP+IAA) which first induces somatic embryogenesis and later after 3 weeks approximately formed roots. The other combination IAA:BAP also formed the roots but the % age is low i.e 30% as shown in table 3.6.

The results obtained, while using IAA and NAA is similar, as same concentration of both induce root formation along with BAP but the efficiency is three times approx. low with IAA as compared to NAA.

3.8. Morphology Comparison

The morphology of tissue cultured *Fagonia indica* is quite distinct from the wild plant. The shoots are fragile and root system is not well developed. The stipules are small and leaves are not very distinct from stipules as shown in figure 3.7.

3.9. A*grobacterium*-Mediated Transformation of *Fagonia indica*

Five types of explants were used for transformation i.e. nodes, calli,cotyledonary leaves, stipules and intermodal region. These explants were taken both from wild and tissue cultured plant of *Fagonia indica*. These explants were pre-cultured on pre-culturing media for one day. We used two types of hormonal combination i.e calli induction medium BAP:NAA (10:1) and shoot induction medium BAP (0.1mg/L) for transformation of *Fagonia indica*. Plants were co-infected with bacteria and then grown on co-cultivation media along with bacterial culture. Co-cultivation medium was also supplemented with acetosyringone to enhance transformation efficiency. After co-cultivation, plants were shifted to selection media. Table 3.7 shows chemical composition of transformation media.

3.9.1. Sensitivity to Kanamycin

Prior to transformation, an effective concentration of antibiotic for the selection of transformed cells was determined by culturing calli (induced from SAM) on shoot induction medium i.e 0.1m/L BAP. Medium was supplemented with five different concentrations of kanamycin i.e. 20, 40, 60, 80 and 100 mg/L. Explants became dry and yellow (Figure 3.8) on SIM containing 40 and 60 mg/L of kanamycin. Kanamycin at concentration of 80 and 100

mg/L caused complete necrosis of the explants after three weeks while concentration 20mg/L has no effect on explants growth and they continue to grow and produce somatic embryos after 3 weeks. In order to finalize, which concentration of Kanamycin is best for selection, the inbetween concentration of 40 and 60 mg/L i.e 50 mg/L was used.The result showed that Kanamycin at 50 mg/L concentration caused total inhibition of growth of calli on SIM (shoot induction medium). These results showed that kanamycin is an effective selection marker. Hence this concentration was used for the selection of transformed plants.

3.9.2. Sensitivity of Explants to Cefotaxime

The sensitivity of explants against cefotaxime was studied by using different concentration of antibiotic. Filter sterilized cefotaxime of five different concentration i.e 100, 300, 500, 700, 900 mg/L were used. The best result is on 300 mg/L which eliminate the bacteria and has no effect on plant growth, while higher amount result in necrosis of plant cells and 300mg/L, the contamination rate is not controlled.

3.10. Factors Affecting Transformation

3.10.1 Effect of Optical Density (OD) of *Agrobacterium* Culture

Explants from 2-day old seedlings were co-cultured with *Agrobacterium* culture of varying optical density (OD600) i.e. 0.25, 0.5, 0.75 and 1.0 and 1.25. Kanamycin concentration was kept at 50 mg/L. Highest %age of *GUS* expression (87.5%) was observed when OD was 0.5 (Table 3.8). Lowest percentage of *GUS* expression

3.10.2. Co-cultivation Time

Co-cultivation duration also affected the transformation efficiency. After infection plants were placed on co-cultivation media (SIM) for 24, 48 and 72 hours, respectively. Transformation efficiency was maximum (50%) by co-cultivating the explants for 24 hours in dark (figure 3.10). Extending the co-cultivation time up to three days increased the transient transformation frequency but also increased the contamination rate

3.10.3. Age of Explant

Age of the explant is a critical aspect in transformation experiment. Explants excised from young plants (both wild and tissue cultured) showed higher transient transformation rate (70%) than those excised from old plant. Our findings showed that younger explants are more susceptible to *Agrobacterium* than older explants.

3.10.4. Explant Type

Explant type is also a very important factor affecting plant transformation efficiency. Fivetypes of explants were used i.e. calli, leaf, stipules internodes and nodes. Transformation efficiency was assessed as the proportion of the blue inclusion in the putative transformed explants. In calli the transformation efficiency was higher (70%) than that ofstipules(40%), leaves (35%), internodes (30%) and nodes (18%)as shown in Figure 3.11.

3.10.5. Effect of Acetosyringone

Virulence inducer (acetosyringone concentration) is found noteworthy in transformation of different explant types. Above optimum AS value (200µM), a little increase in TE was observed but it was difficult to remove the *Agrobacterium* from the explants and they eventually die.

3.11. *GUS* Analysis

Histochemical *GUS* assays were used to assess transient and stable expression of the *GUS* gene. In order to optimize conditions of transformation, transient *GUS* expression was scored after 24, 48 and 72 hours of co-cultivation with *Agrobacterium tumifaciens* by counting the number of explants showing *GUS* expression. In the present study, five types of explants were used for the analysis of *GUS* expression. Stable *GUS* expression in kanamycin-resistant putative transgenic plants was also examined. For *GUS* expression study, the tissue materials were immersed in *GUS* substrate solution for 24 hours at 37°C. Following incubation, tissues were washed with 95% ethanol, and examined. Transient *GUS* expression was observed in all types of explants which were stained blue that can be seen through microscope (figure 3.12) and also with the naked eye (Figure 3.13). Results indicated that 80% of the explants showed *GUS* activity after three days of co-cultivation with C58C1strain

DISCUSSION

Fagonia indica is a small spiny undershrub found mostly in arid desert regions of Pakistan. It was extensively studied by many researchers regarding its medicinal importance and uses, since this plant is antitumor, antioxidants, analgesic, astringent, febifuge and prophylactic against small pox agents. In folk literature, it is reputed as a medicinal plant with rich therapeutic potential. It is also famous for its anti-venom, anti inflammatory and antipyretic activity (Satpute *et al*., 2009). It is a potent anti fungal, anti bacterial agent and has cytotoxic activity (Zhang *et al*., 2004 and Gupta *et al*., 2009).

Establishment of tissue culture conditions was a prerequisite for this study and this was achieved by optimizing the tissue culturing conditions and transformation conditions for this plant since, there is a requirement of standard technique for tissue culturing, which in turn help to increase production of secondary metabolites and important compounds *in vitro* and to obtain large amount of plants in short time.

Seed germination is one of the important steps in the life cycle of plant. For *in vitro* germination of seeds, proper sterilization procedure is very essential. Sterilization agents like sodium hypochlorite, tween 20 and mercuric chloride are used (Oyebanji *et al*., 2009). Sodium hypochlorite is one of the commonly used surface disinfectants. According to Talei *et al.* (2011), ethanol is strong sterilizing and highly phytotoxic chemical, therefore seed should be exposed to it for a short period of time. For efficient sterilization of seeds, ethanol is normally used prior to the treatment with other sterilizing agents like tween 20 and bleach (Oyebanji *et al*., 2009). Surface sterilizing agents such as 70% ethanol and 10% sodium hypochlorite play an important role in germination of seeds.

The choice of medium highly effect germination of seeds due to variation in their organic and inorganic nutrients (Arditti, 1982). *Fagonia indica* seeds were inoculated on four different media i.e MS, ½ MS, B5 and RMOP to check the best medium for their germination, as the species of this family is usually recalcitrant and have low germination efficieny *in vitro* (Valverde-Cerdas *et al*., 2008), therefore when seeds were inoculated on simple MS medium, they gave maximum germination efficiency i.e 45%, as compared to ½ MS (30%), RMOP (10%) and B5 (6.11%). MS medium contain high amount of micro and macro nutrients and also ammonium nitrate (NH4)(NO3) and potassium (Murishage and Skoog, 1962). The ammonium ions enhances the growth and differentiation of seeds (Kramer and Kozlowski, 1979).

In vitro regeneration of cells, tissues or organs depends on many physical and chemical factors. These factors include temperature, light, humidity, pH conditions, phytohormones, nutrient supply, type or age of explants and genotype (Nurazah *et al*, 2009; Parveen and Shahzad, 2014). MS medium, supplemented with 2 different phytohormones (NAA and BAP) in different combination i.e 20 series of hormones were used to optimize the callus induction medium for *Fagonia indica*. Three different explants i.e shoot apical meristems (SAM), nodes, cotyledons were used. The best explants for calli induction were shoot apical meristems (SAM) as it contain undifferentiated meristematic tissues which has high totipotency. Gunjan Sharma and Anant Ram Nautiya, also described the effect of different explants on shoots regeneration through indirect organogenesis of a Laurel from Himalaya and proved that apical meristems has high regenerative potentials on artificial media in 1959. The best combination for calli induction was 1:1 ratio (working concentration 0.5 mg/L for both BAP and NAA). It gave green calli with various textures and hardnous. The combination 10:1 (BAP:NAA) gave second best result, with 70% efficiency. There were many protocorm like bodies (PLBs) formed along with calli on these combination. These results are in accordance with Roy and Banerjee (2003), who study callus induction from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. f. using different combinations of BAP and NAA. Different sterilizing agents combination were used to control contamination rate. 70% ethanol for 10 minutes, 0.1% mercuric chloride for 2 minutes, 5% sodium hypochlorite for 10 minutes and terbinafine (1mg/ml) for 2 minutes gave best results with 100% contamination control. 70% ethanol is effective phytotoxic chemical that worked against large number of bacteria. (Talei *et al.,* 2011). Sodium hypochlorite releases oxygen gas as a by product that enhances oxidative respiration which in turn promoted seed germination (Vujanovic *et al*., 2000). 0.1% mercuric chloride is a good anti bacterial and anti fungal agent as described by P.B. Kavi Kishor in his book Plant Tissue Culture and Biotechnology: Emerging Trends. Terbinafine is a squalene epoxidase inhibitor and is potent anti fungal agent for a long range of dermatophytes group of fungi. Amiri *et al*. (2013) also described the importance of these sterilizing agents to control contamination rate during *in vitro* propagation of Rootstock Mariana.

We also investigated the organogenesis of calli to form shoots. For this, we used MS medium supplemented with NAA and BAP in different combinations. The best result obtained with MS containing BAP (working concentration 0.1mg/L). The result is supported by K.A Malik and P.K. Saxena (1991), which proved that BAP is required for induction and development of

shoots while working on *Phaseolus vulgaris*. BAP has been reported to enhance the regeneration of plant (Salekjalali, 2012). Shoot regeneration is much faster while using SAM as explant as compared to nodal regions of explants.

For root regeneration, developed shoots and calli were inoculated on MS medium supplemented with three phytohormones BAP, IAA and NAA. The best result was on NAA:BAP (1:1), which gave 100% root induction.

Plant transformation is a key methodology that has allowed transfer and expression of novel genes and helped to investigate about the functions of plant genes. A number of common problems related to successful transformation of plant species include low transformation efficiency, instability of transgene expression, somaclonal variations and inability to regenerate whole plant. Plant genetic transformation plays a key role in production of transgenic organisms and has enhanced the potential uses of crops and vegetables for pharmaceutical purposes (Arntzen *et al*., 1998; Potenza, 2004).

This project was conducted to optimize transformation conditions for *Fagonia indica* using *Agrobacterium* strain C58C1 as a vector and *Gus* gene as reporter marker to check the frequency of transformation.

The explants used for transformation were SAM, leaves, stipules, nodes and calli. The best transformation efficiency is of calli. Frame *et al.* (1999) reported highest Transformation efficiency for callus during transformation of maize.

The antibiotic concentration in selection medium is used to control bacterial outgrowth after co-infection and co-cultivation period. As these antibiotics normally have some negative effects on regeneration of explants, therefore cefotaxime concentration was optimized for complete elimination of *Agrobacterium* without affecting regeneration potential of explants to induce calli and shoots. The shoot induction medium was supplemented with 100, 300, 500, 700, 900 mg/L of cefotaxime. It was observed that calli on 700 and 900mg/L of cefotaxime were grow very slowly and became yellow. It might be due to byproduct of antibiotic that act as growth regulator and alter the growth (Lin *et al*., 1995). The calli on 300 and 500 mg/L did not effected by antibiotic and grow normally into calli and shoots, so 300 mg/L was used as standard to eliminate bacteria. Many reports on the use of cefotaxime concentration below 500 mg/L, showed that the *Agrobacterium* growth could effectively be eliminated by cefotaxime (Lupan *et al*., 2010).

Tissue resistant against kanamycin is the most common selection markers for the procurement of transgenics (Nap *et al*, 1992). Kanamycin selection is so important that even some research groups use this tool to calculate transformation frequency (Wang *et al*, 2012). Prior to transformation, we optimized an optimal concentration of Kanamycin for selection of transformed cells by culturing explants on shoot induction medium supplemented with different concentration of Kanamycin i.e 20, 40, 60, 80, 100 mg/L. Calli inoculated were turned yellow on 40 and 60 mg/L. so we concluded mean i.e 50 mg/L to be best for selection. The explants on 100 mg/L undergo necrosis and the same were found in many plant species including arabadopsis (Ayre *et al*., 2003), tomato (Ying *et al*., 2008), (Ahmed *et al*.,2010). carnation (Kanwar and Kumar 2011) and mulberry (Chitra *et al*., 2014).

There are various factors that affect transformation efficiency, one of them is optical density (OD) of bacterial culture. Efficiency of transformation was found to be highly dependent on the concentration of bacteria (OD_{600}). The optimal OD of *Agrobacterium tumificiens* for inoculation of explants was obtained by co-culturing explants with bacterial culture at OD_{600} of 0.25, 0.5, 0.75, 1.0 and 1.25. It was observed that highest *GUS* expression was observed at 0.5 OD_{600} and least expression was at 1.25 OD_{600}. Similar results were reported by Miao *et al.* (2009) for *Cochliobolus sativus*. Bacteria in liquid inoculation medium are likely at the hypervirulent active log phase from 0.5-1.0 OD_{600} so therefore, inducing maximum TE (Yadav *et al*., 2012). Nanasato *et al*. (2013) also reported highest *GUS* expression for *C. sativus* at 0.5 OD_{600}.

The explants were incubated for five different durations (5, 7, 10, 12 and 15minutes). It was observed that lesser infection time led to low transformation efficiency while higher infection time led to contamination of explants with bacteria. Hence optimal infection time is to be determined for successful transformation (Khan *et al*., 2013). The optimal infection time for successful transformation is 7 minutes. The highest *GUS* expression was observed when explants were incubated on bacterial suspension for 7 minutes approx. similar result was reported by lupan *et al*. (2010).

The effect of co-cultivation time on transformation efficiency was determined by co-cultivating explants infected with *Agrobacterium tumificiens* for 3 different durations i.e 1, 2 and 3 days. The best efficiency was observed, when *Fagonia indica* explants were co-cultivated for 1 day. This is contradictory to most of the related research in which maximum efficiency of *GUS* was observed at 2-3 days (Hamid *et al*., 1996; Lupan *et al*., 2010).

The cocultivation medium was supplemented with hormones and acetosyringone was added to enhance the bacterial virulence (Godwin *et al*., 1991). Acetosyringone along with C58C1strain of *Agrobacterium tumificiens* increase the tumor production and phenolic release tenfold than without it (P. Holford *et al.,*1992). We use 200 µM of Acetosyringone during co-infection period and in cocultivation medium. Acetosyringone concentration used in infection and inoculation media is considered safe at up to 200 µM (Stachel *et al*., 1985). In another species of Lamiaceae (*Pogostemoncablin*), highest transformation frequency was found at 150 µM AS concentration (Paul *et al*., 2012).

Age of explants is also important in determining the transformation efficiency. A calli of 45 days old is used, which gave maximum efficiency as compared to 15 days and 70 days old calli. These result were supported by R.M. Tripathi *et al*. (2010) while doing a study on rice.

Transformation frequency was ultimately a manifestation of *GUS* expression because it is facile, authentic, relatively low priced, safe, need no particular machinery or apparatus, and is clearly visible to the naked eye (Jefferson 1987; Jefferson 1988). In the present study, plant transformation binary vector p35SGUSint containing *GUS* reporter gene expressed under CaMV35S promoter in *Agrobacterium tumificiens* strain C58C1. Moreover, p35SGUSint encloses chimeric *gusA* gene with a plant intron (*gusAint*), so it cannot be expressed in bacterium thus making it a useful tool for transformation (De Bondt *et al*., 1994). A clear blue colour was observed when putative transformants were stained with *x-gluc* reagent and was observed with the naked eye. The positive control gave no result while the frequency of blue colour is maximum in calli among the explants used for transformation. This result is contradictory to most research work in which cotyledons show maximum transformation efficiency (Ahmed *et al*., 2007; Lupan *et al*., 2010).

Conclusion and future strategies

Following conclusions were drawn from the present study:

- Sterilizing agents like sodium hypochlorite and terbinafine play important role in surface sterilization of seeds and explants used for *in vitro* propagation.
- The seeds are recalcitrants and maximum germination efficiency was shown on MS medium.
- Factors like explants type, genotype and medium composition effect the *in vitro* regenerative potential of explants used.

- Among the explants used shoot apical meristems has the highest regenerative potential.
- The best callus induction medium is NAA:BAP (1:1; working concentration 0.5mg/L).
- The best shoot induction medium contain only BAP (0.1mg/L) in MS medium.
- The best root induction medium contains NAA, BAP and IAA.
- There are formation of many protocorm like bodies (PLBs) and somatic embryos along with callus, which later turned into complete plantlets.
- *GUS* reporter expression was observed in all kind of explants type but maximum in calli.

In the light of above mentioned research, further investigation may be carried out for the stable transformation of *Fagonia indica*, as conditions for transformation had been optimized. Since plant has important medicinal role in both folk and allopathy field and no stable transformation has been reported so far, thus plant can be transformed with genes that increase the secondary metabolites, which play role in the cure of different disease. Molecular analysis can also be performed on transformed plants.

Cell suspension cultures can also be produced and *in vivo* and *in vitro* bioassays can also be carried out for relative comparison of transformed and untransformed plants.

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