An effective protocol for optimization of in-vitro micropropagation and genetic transformation of *Furcraea foetida*

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Abstract

Furcraea foetida (Mauritius hemp) belongs to the family Agavaceae, is cultivated for its ornamental, fiber and medicinal value and can survive in xerophytic conditions. Despite its importance, Agavaceae family has not been genetically exploited. In the presented study, a rapid and efficient protocol for micropropagation and genetic transformation of *Furcraea foetida* has been established. For this purpose, several sets of experiments were conducted. *F. foetida* displayed best shooting and multiplication at Murashige and Skoog (MS) medium containing 6-Benzylaminopurine (BAP) 1.5 mg /L. Successful rooting was induced on MS medium without any plant growth regulator (PGR). The best transformation efficiency was achieved when two weeks old, regenerated shoots were infected with *Agrobacterium tumefaciens* strain LBA 4404 and co-cultivated for two days on MS medium prepared with BAP 1.5 mg/L and 250 mM acetosyringone. The transformed explants were developed on medium enriched with BAP 1.5 mg/L, cefotaxime 500 mg/L and kanamycin 150 mg/L. The transient GUS histo-chemical assay was carried out in order to find out the transformation. The molecular analysis of transformed shoots was done using PCR which revealed the integration of foreign gene into *F. foetida* genome. The objective of this study is to exploit *Furcraea* as bioreactor for molecular farming to produce recombinant pharmaceuticals.

Introduction

Furcraea Foetida is an evergreen perennial shrub and belongs to the family Agavaceae. It has sword-shape leaves, with 1-1.8 m length and 10–15 cm breadth (Huxley, 1992). It is used as an ornamental in landscaping of various gardens and lawns. It is cultivated in tropical and subtropical regions for its leaves, which are traditionally used to produce a natural fiber. The leaves of *F. foetida* has various medicinal uses such as anti-inflammatory effects to aid in bronchitis wound healing, hepatitis, stomach ache and problems of uterus (Van Ande *et al.*, 2007). Besides fiber source, the plant is also being used as folk medicine for the treatment of febrifuge, back pain, cold and syphilis (DePhillips *et al.*, 2004). The roots of *F. foetida* are used as blood purifying tonics in folk medicine. They also contain large amount of saponins and used as soap in cosmetic industry.

One of the problems with agave gardening is its long life span, as it is noted that *F. foetida* takes 15–20 years to mature. During this lengthy period, the plant could be susceptible to diseases and pests (Benitez *et al.*, 2007). Conventional long propagation of Agave resulted in reduced genetic variability which might make it susceptible to new and destructive pathogens (Gil *et al.*, 2007). The long growth cycle made it difficult to bring about genetic changes employing the conventional breeding methods in agave species. Tissue culture techniques can be absolute to regenerate plants in big number at a shorter period. Few articles reported the micropropagation of some species of Agavaceae family (Robert *et al.*, 2006; Patel, 2009 and Maria *et al.*, 2007). So far, there is no report on the micropropagation of *F. foetida*.

There are various techniques which can be exploited to improve the quality and genetic traits of any plant such as improved maturity period, carbohydrate content and disease resistance (Valenzuela *et al.*, 2006).

Genetic transformation is an efficient tool to improve any variety to get desirable characters without considering any specie barrier.

The use of *Agrobacterium* for the delivery of DNA; to the host cell possess several advantages over other mentioned methods. *Agrobacterium* has been used for efficient transformation of monocotyledonous plants (Ishida et al. 1996; Tingay *et al.* 1997; Hiei et al. 1994). The transgenic technology might be used as bioreactors to produce recombinant pharmaceutical proteins. Genetically engineered plants, performing as bioreactors, can competently produce recombinant proteins in bigger quantities than those produced using other living systems. To set up a thriving study of experimental plant genetic engineering, it is significant to build up systems for the revival of whole plants in big numbers from prime explants. This is necessary to do while optimizing the method for introducing foreign genes into the desired species.

The present study is the very first report to develop an *in-vitro* micropropagation system for *Furcraea foetida* and the establishment of a successful *Agrobacterium* mediated protocol for genetic transformation to exploit the plant as bioreactor for molecular farming.

Materials And Method

PLANT MATERIAL: The plant material of *Furcraea foetida* was gathered from the green house of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. It was identified by Dr. Saifullah Khan.

STERILIZATION:

Freshly grown shoot tips were selected as an explants source. Shoot tips were washed in running tap water for 10 minutes to remove the dust and other pollutants. They were then surface sterilized by 0.5% of commercially available sodium hypochlorite marketed as Robin Bleach. Three drops of Tween-20 were also added to decrease the surface tension and placed on a gyratory shaker for 20 minutes with continuous gentle stirring. This was followed by rinsing the explants in sterile distilled water 3 times for 5 minutes interval to remove traces of NaOCl. The meristematic shoots were cut into approximately 2 cm long segments and used as explants for shoot initiation under *in-vitro* conditions.

SHOOT INDUCTION:

Shoot induction and multiplication were evaluated using Murashige and Skoog (1962) salts fortified with various concentration levels of BAP, kinetin and zeatin (Table 1). 3% sugar was added as the sole carbon source and 2.5 gm/L of phytagel as a solidifying agent. The pH of the medium was adjusted to 5.75.

The sterilization was achieved by using autoclave with 15 psi. pressure for 15 minutes. Data were recorded twice a month for number of shoots and shoot length for eight weeks and statistically analyzed for selection of most appropriate PGR.

ROOT INDUCTION:

The *in-vitro* rooting in *Furcraea* was studied using MS medium with various levels of two auxins Indole-3-acetic acid (IAA), and Indole-3-butyric acid (IBA). Control was MS medium, without any PGR. 2.5% of sugar and 2.5 gm/L of phytigel as a solidifying agent was used in all the media formulations for root induction. Sterilization was done in an autoclave, with a pH level 5.6-5.7 prior to autoclaving. Data were recorded each week for four weeks and statistically analyzed.

AGROINFECTION:

Bacterial strains and plasmids:

For genetic transformation, *Agrobacterium tumefaciens* strain LBA 4404 hauling the binary plasmid pBI121 was used (Bevan, 1984). The strain LBA 4404 was selected because it has been reported to use for monocotyledonous plants transformation efficiently (Rachmawati *et al.*, 2004; Hei *et al.*, 1994)

Susceptibility of explants to kanamycin:

Dose-response test was carried out on leaf section of *F. foetida* in nutrients containing diverse concentrations of kanamycin as 0, 25, 50, 100, 125, 150, 175 and 200 mg/L.

Concentration of acetosyringone:

The concentrations of acetosyringone were selected on previous reports of transformation of monocotyledonous plants (Dong and Qu, 2005). Leaf segments were used as target tissues for transformation assays.

Co-cultivation Period:

The strain of *A. tumefaciens*, LBA 4404 was cultivated overnight at 28° C until an optical density of 1 at OD 600 was obtained in 25 ml of Luria-Bertani medium (LB) containing 50 mg/L kanamycin and 50mg/L Rifampicin. Cells were concentrated by centrifugation at 5000 rpm for 10 minutes and resuspended in 25 ml MS liquid medium and acetosyringone was added at various concentrations. Explants were cut with a sterile scalpel in petri dishes avoiding dehydration during the cutting process by placing them in MS medium. A number of 150 explants were obtained and transferred to the *Agrobacterium* solution, where they remained for 30 minutes with gentle shaking. The explants were then transferred to sterile filter paper to eliminate the excess liquid MS medium and placed in petri dishes containing MS without antibiotics, supplemented with 1.5 mg/L BAP, and various levels of acetosyringone. The explants were co-cultivated at 28 °C for 24, 48, and 72 hrs in the dark, and finally washed with sterile distilled water containing 500 mg/L cefotaxime. The explants were transferred to MS medium supplemented with 1.5 gm/L BAP, 125 mg/L kanamycin and 500mg/L cefotaxime. The cefotaxime was used to eliminate the agrobacteria.

The regenerated explants were transferred to fresh MS medium supplemented with 1.5 mg/L BAP and 125mg/L Kanamycin for every 6 weeks.

Histochemical GUS assay:

Histochemical GUS assay was carried out on regenerated plants produced by co-cultivation with *Agrobacterium* after four months. Similar procedure was done on plants incubated for one week after the co-cultivation. Untransformed tissues were used as negative controls. The procedure is similar as used by Jafferson (1987), with some modifications.

Transgene detection by Polymerase Chain Reaction (PCR):

Genomic DNA from transformed *F. foetida* was extracted based on an adapted method reported by Doyle and Doyle (1990). Transformation analysis was carried out by the PCR employing specific oligonucleotides for the reporter GUS gene and selection marker nptII.

The primer sequence for GUS was used: Forward 5'CAACGTCTGCTATCAGCGCGAAGT 3' and Reverse 5'TATCCGGTTCGTTGGCAATACTCC 3'. Thermocycler conditions: 5 minutes at 94°C, followed by 35 cycles of 30 sec. at 94°C, 30 sec. at 55°C and 90 sec. at 72°C, with a final extension of 5 minutes at 72°C, approximately 1000 b.p. fragment was amplified.

The oligonucleotides for nptII forward 5'GAGGCTATTCGGCTATGACTG 3' and reverse nptII 5'TAGAAGGCGATGCGCTGCGA 3' were used as follows: initial denaturation of 4 minutes at 95°C, followed by 35 cycles of 30 sec. at 95°C, 1 minute at 59°C and 1 minute at 72°C, with a final extension of 5 minutes at 72°C amplifying a fragment of approx. 700 b.p. In both cases, the reaction was carried out with 100 ng of the template DNA. The amplification product was detected by electrophoresis in a 1.2 % agarose gel and visualized under UVITECH gel documentation system.

Results And Discussion

Effect of PGRs on shoot induction and multiplication

In this study, we have developed an efficient protocol for micropropagation and genetic transformation of *F. foetida*. For this, several experiments were conducted. In first such experiment, shoot multiplication medium was optimized. Cytokinin influences various aspects of the plant development and its physiology (Haberer et al., 2002). For the micropropagation of *Furcraea*, cytokinins (BAP, kinetin and zeatin) were used in various concentrations without auxin. In this investigation, 95% (data not shown) of the *in-vitro* induced shoot tips were viable and axillary bud formation was evident after four and six weeks of culture respectively (Fig. 1a; Fig. 1b). This response is consistent with various reports for *in-vitro* cultures of other *Agavaceae* members reported previously like *A. sisalana* (Das, 1992) and *A. parrasana* (Santacruz et al., 1999). Our result shows that T3 medium containing BAP 1 mg/L, yielded highest number of shoots i.e., 13.4 ± 0.24 shoots per replication where as the length of shoot was 2.64 ± 0.09 cm (**Table. 1**; Fig. 4a). As BAP concentration increased up to 2mg/L, the decline in number of shoots was observed and also no significant effect on shoot length. Kinetin showed maximum number of shoots (11.6) with shoot length of 2.86 cm at the concentration of 2mg/L (Fig. 4b). Zeatin exhibited similar

response as kinetin, when used at concentration of 2 mg/L (**Table. 1**). The use of BAP was preferred for mass multiplication of *F. foetida* as it is economical than zeatin and also gave good results at lower concentration than Zeatin because higher concentration of growth regulators are prone to somaclonal variation in micropropagated plants. According to a classical report of Skoog and Miller (1957), the balanced ratio of auxins and cytokinin leads towards the dedifferentiation and formation of callus; high ratios of cytokinin to auxin promote the shoot induction while high auxin promotes root development. The use of cytokinin/auxin combination did not yield remarkable response (Data not shown). Although, there are reports for micropropagation of Agave that validate the requirement for moderate to low concentrations of auxin, with a cytokinin for effective induction of organogenesis and shoot development (Powers and Backhaus, 1989; Binh et al., 1987; Robert et al., 1996; Rodriguez et al. 1996 b).

Effect of PGRs on root development

To study the effect of auxins on the root development, various levels of IBA and IAA were used. Experimental data showed that there is no significance difference among all the treatments applied including control (without auxin). Almost 100% of the shoots developed (≥ 3 cm lengths) exhibit rooting after 15–20 days of culture in medium supplemented with IBA and IAA (Fig. 1c), and also in medium without auxins. The average number of roots observed on all the treatments was 6–7 (Fig. 5a), with average root length of 6 cm (**Table. 2**; Fig. 5b). For root induction, IBA (7.4) proved to be more effective than the IAA (7.38) and control (6.9).

All the treatments tested quantified high percentages of rooted shoots. However, the most striking difference between them was the shortest time required for rooting the shoots cultured in the presence of auxin, compared to the time required for those grown in the absence of them. The results are in accordance to the previous reports (Maria et al., 2006; Villalobos et al., 1991; Yépez et al., 2004), as the axillary shoots were rooted in medium without auxin, although auxins stimulate faster root development if the medium contains IBA. Similar results reported by Santacruz et al. (1999), for root induction of *A. parrasana* without auxin or supplemented with various levels of IBA, lacking significant differences between treatments. Furthermore, Hazra et al. (2002) reported rooting of shoots of *A. sisalana* in medium supplemented with IAA. In other Agavaceas species such as *A. arizonica* (Powers and Backhaus, 1989), *A. cantala*, *A. furcroydes* (Binh et al., 1990), *A. sisalana* (Binh et al., 1990; Das, 1992), the basal medium was sufficient to stimulate the formation and development of roots. On the basis of present study, it can be concluded that endogenous auxin is enough for the development of roots in micropropagated shoots and there is no need of exogenous auxin to induce the rooting in *F. foetida*.

Optimization of *Agrobacterium tumefaciens* transformation

Prior to initiate stable transformation procedure, it is important to examine some preliminary attributes of target plant to optimize the genetic transformation protocol. These examinations help to optimize various factors which have great impact on a successful stable transformation. In present work, we have studied effect of age of explant, co-cultivation time and concentration of acetosyringone.

Minimum inhibitory concentration of kanamycin

At first, the lethal effect of kanamycin on explants, used as a selection marker was checked. The presence of kanamycin is considered lethal for plants even at very low concentration. Various levels of kanamycin were used to detect the dose response effect on *F. foetida* using media containing various concentrations of kanamycin (0, 25, 50, 75, 100, 125 mg/L). The effect of various concentrations of kanamycin on regeneration response is described in **Figure. 8**. In this investigation, it was observed that the plant showed a remarkable resistance against kanamycin. Approximately 60% plants shows viability at 75 mg/L kanamycin for 10 weeks (**Figure. 8**). 125 mg/L of kanamycin was observed to be effective to select the transformed *F. foetida* explants and caused complete necrosis of the untransformed explants within three weeks, and thus was successfully used for selection of transformants. This finding is contrary to the previous report on same genus *A. salmiana* () that shows susceptibility at 25 mg/L. Hence, kanamycin at a concentration of 125 mg/l was used for selection of transformants. In an earlier report, Phogat et al. (1999) selected transformed calli of *Vigna radiata* on 100 mg/L of kanamycin concentration.

Influence of acetosyringone on transformation efficiency

As *F. foetida* is not dicotyledonous, therefore, in this study acetosyringone is used in the co-cultivation medium with *Agrobacterium*. Acetosyringone, a phenolic compound which is produced during wounding is known to stimulate the transformation of dicotyledonous plants by attracting the *Agrobacterium* (Stachel et al., 1985). The study shows that the acetosyringone concentration and co cultivation time have significant impact on transformation efficiency. In control plants without acetosyringone there was no transformation event observed. The level of these phenolics is insufficient in monocots to serve as an inducer of the *vir* genes (Smith and Hood, 1995).

Acetosyringone is required for the activation of the *Agrobacterium* virulence machinery (Stachel et al., 1985; Hiei et al., 1994). Explants were transferred onto solid MS medium (co-culture medium) containing different acetosyringone concentrations 0.0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM) for two days. The results in **Fig. 7** indicated that the addition of acetosyringone at 250 mM was the best treatment giving the highest number of transformed explants. On the other hand, the further increase in acetosyringone lower the number of transformants. In the case of the control treatment where the acetosyringone was not added to medium, no transformation event was observed. A positive effect of phenolic compounds such as acetosyringone on *Agrobacterium*-mediated transformation has also been demonstrated in several other studies in a wide range of species, i.e. maize, (Shen et al., 1993), *Antirrhinum majus*, (Godwin et al., 1991), *Picea abies* and *Pinus taeda*, (Wenck et al., 1999) and white pine, (Levee et al., 1999). The efficiency did increase three fold when it was included in the co-cultivation medium. Hiei et al., (1994); Ishida et al. (1996); Wenck et al. (1999) found that high levels of 100 µM acetosyringone or greater used in some other systems were not beneficial.

In this study, the presence of higher acetosyringone concentration (250 mM) in co-cultivation medium resulted in the highest transient expression. However 300 mM acetosyringone concentrations in co-

cultivation medium or lower acetosyringone concentration i.e., 50 mM did not have effect on *Agrobacterium* transformation. These observations indicated that the amount of acetosyringone treatment is a critical factor for *Agrobacterium* transformation of *Furcraea*. The lower acetosyringone concentration was probably not enough to induce *Agrobacterium* vir genes efficiently and higher acetosyringone may have toxic effects on plant cells or are inhibitory to *Agrobacterium*. De Clercq et al., (2002) investigated and found an increasing number of uid A expressing zones with increasing acetosyringone concentration and recorded a maximum of 0.2 mM acetosyringone. When 2 mM acetosyringone was used, explants showed necrotic zones. More than 10% of the explants did not survive under such co-culture conditions.

Influence of co-cultivation time and age of explant on transformation efficiency

Co-cultivation time also has great effect on transformation percentage. The co-cultivation time of 48 hours provide best results as shown in Fig. 6, while 24 hour co-cultivation time showed very low transformation events. When the time duration was increased up to 72 hours, it resulted in overgrowth of bacteria leading to the low plant regeneration percentage and necrosis. Besides the co-cultivation time and acetosyringone levels, age of explants also has strong impact on transformation efficiency. In this study, two weeks old plantlets developed from somatic embryos were proved best to get optimum transformation. GUS histochemical assay showed the transient transformation events. It was observed that transformation percentage was almost 45% when 14 days old plantlets were co-cultivated for 48 hours. As the age of the plant increased, the ability of transformation decreased as shown in **Fig. 9**. The length of co-cultivation has significant positive effect on efficiency of transformation in spinach as explained by Cervera et al. and Cao et al. [17, 18]. By increasing co-cultivation duration, number of GUS spot per plant and percent of explants with blue spot were increased (Fig. 3). This finding is similar to Shuangxia *et al.* found that shoot co-cultivation duration of 48 hours was optimal for developing a highly efficient method of transforming embryogenic callus [19]. The age of explant before transformation also has positive impact on regeneration potential of transformed events. In this study we observed that better regeneration potential of transformed shoots were observed when 2 weeks old explants were used for co cultivation(Fig. 2)

Expression analysis using GUS histochemical assay

The expression of putative transgenic plants and non transformed controls were analyzed by using GUS assay to check the plants after co-cultivation as well as eight months after transformation. The plants which showed blue spots considered GUS-positive (Fig. 3). The control plants did not yield any positive GUS symbol.

Molecular analysis of transformed *Furcraea foetida*

The plants that were GUS positive, yielded signals specific for *nptII*. Non-transformed controls did not show *nptII* specific signals as using molecular analysis by PCR. Genomic DNA was amplified with two specific primers pairs: primers specific for the selection marker *nptII* and primers specific for GUS. The

presence of PCR-products for *nptII* as well as GUS indicated that these plants were transformed. Control plants did not give any band.

The *nptII* gene specific primer pair gave a 740 b.p. bands in the DNA extracted from all the four GUS positive putative transformed *Furcraea* plant including the plasmid DNA which used as positive control (Fig. 10a). There was no amplified product observed in control non transformed plant DNA.

The UDI A gene specific primer set also amplified a product of 1kb in all four transformed plants and positive control plasmid DNA. There was no amplification from untransformed control plant observed (Fig. 10b).

Table 1
Effect of Cytokinin on Shoot induction and multiplication in *Furcraea*

PGR	Conc.	Code	No. of Shoots	Shoot Length (cm)
Control	0	T1	2.8 ± 0.37	1.8 ± 0.12
BAP	0.5	T2	5.4 ± 0.40	2.3 ± 0.25
	1	T3	13.4 ± 0.24	2.64 ± 0.09
	1.5	T4	12 ± 0.31	3 ± 0.03
	2	T5	10.8 ± 0.37	3 ± 0.15
KINETIN	0.5	T6	6 ± 0.31	2.18 ± 0.09
	1	T7	7.8 ± 0.37	2.16 ± 0.10
	1.5	T8	10.4 ± 0.24	2.4 ± 0.24
	2	T9	11.6 ± 0.24	2.86 ± 0.09
ZEATIN	0.5	T10	5.4 ± 0.40	2.14 ± 0.09
	1	T11	8.8 ± 0.37	2.22 ± 0.10
	1.5	T12	10.2 ± 0.20	2.44 ± 0.06
	2	T13	12 ± 0.31	2.94 ± 0.11
Values are Means ± SD				

Table 2
Effect of Auxin on root induction in Furcraea

Auxin	Conc.	Code	No. of Roots	Root Length (cm)
IAA	0	R1	6.9 ± 0.30	6.2 ± 0.18
	0.1	R2	6.86 ± 0.08	6.06 ± 0.03
	0.25	R3	7.16 ± 0.19	6.4 ± 0.17
	0.5	R4	7.38 ± 0.21	6.8 ± 0.23
IBA	0.1	R5	6.88 ± 0.25	6.1 ± 0.30
	0.25	R6	7.2 ± 0.34	6.36 ± 0.32
	0.5	R7	7.42 ± 0.17	6.7 ± 0.18

Abbreviations

IAA: Indole-3-Acetic Acid **IBA:** Indole butyric acid **BAP:** 6-Benzylaminopurine **PGR:** Plant Growth Regulators **GUS:** β -glucuronidase

Declarations

ETHICS APPROVAL: As there was no animal model or environmental hazardous chemicals were used in this study; so it was not liable to get ethical approval.

CONSENT FOR PUBLICATION: Not Applicable

AVAILABILITY OF DATA AND MATERIALS: Not Applicable

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Sheeba Naz (Research work including in-vito propagation, initiation, multiplication, rooting of Furcraea)

Naheed Kauser (Research Work Agrobacterium Transformation and regeneration in Furcraea; write up)

Hammad Afzal Kayani (Statistics, write-up)

Bushra Ahmed Saeed (Graphs, observations, formulations, editing)

Saifullah Khan (Supervisor, Idea generation, working in his lab)

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Figures

Figure 1

various stages of micropropagation of *Furcraea foetida*. **a** Initiation ; **b** Shoot multiplication; **c** Rooting

Figure 2

Regeneration stages of *Furcraea foetida* after co-cultivation with *Agrobacterium tumefaciens* on medium with 125mg/lit Kanamycin

Figure 3

GUS histochemical assay; Blue spots showing the transformation events after 3 days of co-cultivation

Figure 4

a: Assessment of effect of various levels of cytokinins on the development of average number of shoots.

b: Effect of various levels of cytokinins on the length of shoots. Mean values with ($\pm$) standard Error

Figure 5

a: Assessment of effect of various levels of Auxins on the development of average number of roots.

b: Effect of various levels of Auxins on the length of roots. Mean values with ($\pm$) standard Error

Figure 6

Impact of co-cultivation time on transformation

Figure 7

Effect of Ascetosyrene conc. on transformation

Effect of Kanamycin Concentration on plant

Figure 8

Effect of Kanamycin conc. on transformation

Figure 9

Impact of age of explant on transformation

Figure 10

PCR amplification of 700 bp nptII gene (**a**) and 1000 bp GUS (**b**); L is 100bp ladder, lane 1-4 transformed Plants, lane 5 untransformed control and lane 6 plasmid DNA.