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Genetic Transformation & Gus Gene Expression in *Piper longum*

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Abstract: ***Piper longum is a medicinal plant of great importance. It has antioxidant, anticancerous & antibacterial activity.Genetic transformation of Piper longum can improve its existing properties for the betterment of mankind.Gus gene expression was observed in in vitro grown callus tissue of Piper longum when it was transformed using Agrobacterium tumefaciens Agrobacterium strain containing plasmid pBECK2000 carrying GUS gene and kanamycin gene is used. The Agrobacterium strain at concentration 600nm of 0.538 OD and callus of Piper longum was Co-cultivated at 28°C for 2-3 days incubation, at different acetosyringone concentration (50-200µM) infection time varies from 5-10 min . Genomic DNA was isolated from the infected callus and Molecular analysis for Gus gene expression in plant was done by Polymerase chain reaction which shows stable transformation. In this paper Piper longum is transformed to raise transgenic plants of Piper longum with improved medicinal properties.***

Keywords: Agrobacterium tumefaciens , Acetosyringone, GUS gene, Kanamycin, Piper longum, Plasmid pBECK2000

1. Introduction

Piper longum is a medicinal plant ,it is found almost all over india.It is slender aromatic climber with perennial woody stems.Common name of *Piper longum* is Pippali, Indian long pepper &Pipal .*Piper longum* is widely used in ayurvedic and unani systems of medicine particularly in diseases of respiratory tract eg-cough,bronchitis,asthma,etc.The herb has nerve depressant and antagonistic effects on electro-shock and chemo-shock seizures.

The oil extracts of *Piper longum* has antibacterial properties.It can also be used to treat stomachaches,diseases of spleen and tumors.It is good for gonorrhea,paralysis of tongue advised in diarrhea,cholera,chronic malaria,viral hepatitis.It is used as a sedative in insomnia & epilepsy. It helps to cure leprosy. *Argrobacterium tumefaceins* naturally infects dicotyledons. *Piper longum* was successfully transformed by *Argrobacterium tumifaciens* strain LBA4404 harboring plasmid pBECK 2000 containing vectors carrying GUS gene and Kanamycin gene.

Argrobacterium tumifaciens is a soil bacterium that can genetically transform plant cells with a segment of DNA known as T-DNA.*Piper longum* is transformed to obtain transgenic plants with disease resistant variety , high nutrient content and improved medicinal properties.

2. Materials & Methods

(A) *Piper longum*
(B) *Agrobacterium tumefaciens* strain with the desired gene construct

Screening of Agrobacterium was done on LB media
Composition of LB media
Tryptone- 2.5 gm, Yeast extract- 1.25 gm, NaCl-1.25gm,Agar-37.5gm was dissolved in 250 ml of distilled water pH - 7±0.2.
MS media was prepared for the inoculation of explants of *Piper longum.*

Composition of MS media
Macronutrients-Ammonium nitrate- 6.6 gm,Potassium nitrate-7.6gm,Calcium Chloride-1.76gm, Magnesium Sulphate- 1.48gm, Potassium dihydrogen-0.68gm was dissolved in 250ml of distilled water. Micronutrients-Potassium Iodide – 0.083 gm, Boric acid – 0.62 gm, Manganese Sulphate-2.23gm,Zinc Sulphate – 0.86 gm, Sodium Molybdate- 0.025gm,Copper Sulphate-0.002gm,Cobalt Chloride- 0.002 gm was dissolved in 100ml of distilled water. Iron Source- Sodium EDTA – 0.373gm, Ferous Sulphate – 0.278 gm was dissolved in 100ml of distilled water. Organic Supplements- Ionositol-1gm, Nicotinic acid- 0.005gm,Pyridoxin-HCl– 0.005gm,Thiamine HCl- 0.001 gm, Glycine – 0.02 gm was dissolved in 100ml of distilled water.

Preparation of MS basal media for 1 litre
500 ml ofDW was taken in a conical flask then 62.5 ml macronutrients was added then 1ml of micronutrients were added after that10ml of iron source & 10 ml of organic supplement was added 30gm of sucrose was added then volume was maintained upto 1l then pH was maintained at 5.7 then 8gm agar-agar was added& media was distributed in two parts and 2 different concentration of three growth regulators were used: For the 1st 500ml of MS media-IAA(Indole acetic acid)- 1mg/l, BAP(Benzyl amino purine)-2 mg/l, KIN(Kinetin)-1mg/lFor the 2nd 500ml of MS media-IAA(Indole acetic acid)- 2mg/l, BAP(Benzyl amino purine)2mg/l,KIN(Kinetin)-1mg/l

1. Sterilization of the explant of *Piper longum*
At first the explant i.e. leaves and stems of *Piper longum* were washed with tap water 3-4 times. Then the explants were washed with 0.5% of tween-20 for 10-15 min. Then they were washed with tap water to remove the detergent completely.Then it was washed with 0.5% of Bavastin for 10 min.Then again explants were washed with tap water.Then the explants were washed with distilled water. The explants were dipped in 0.1% $Hgcl_2$ solution for 1 min. inside LAF. Finally the explants were washed with autoclaved distilled water for 5-7 times and then inoculated to the hormonal MS media,with three types of hormones:

IAA,BAP,Kinetin.
Callus induction was allowed to continue for 3-4 weeks.

2. Transformation of *Piper longum*

At first stock of kanamycin 100mg/ml and Rifamycin100mg/ml was prepared. Then 100 ml LB broth was prepared and autoclaved. After that both the antibiotics and 1 ml of agrobacterium was dispensed in this media.Then It was left for 24 hrs. At 120-130 r.p.m. at 28 °c.After incubation growth of Agrobacterium was seen in the media. Then LB agar was prepared and both the antibiotics were added to it. Then with the help of sterile inoculating loop bacteria was taken and streaking was done on the LB agar plate.After sufficient growth the colonies were observed on the petriplates.

a) **Primary Culture**
After sufficient growth of the colonies LB broth was prepared and Kanamycin and Rifamycin were added to it.This broth containing antibiotics was divided into small conical flask.Single isolated colony of Agrobacterium was taken from petriplate and was inoculated in the broth of flask and left at 28°C at 130 r.p.m. for 1 day in incubator shaker.This culture is called starter culture.

b) **Secondary culture**
After sufficient growth of starter culture O.D. was taken at 600 nm.the O.D. should be between 0.5-0.6

c) **Cocultivation of *Piper longum* callus with Agrobacterium.**
Hormonal media containing growth regulators IAA,BAP,and kinetin for *Piper longum* was prepared. Acetosyringone with different concentration was added, it is then dispensed in sterile petriplates .Callus of *Piper longum* was cut into small pieces in sterile condition and then dipped in MS liquid broth containing Agrobacterium. Callus were taken out from the broth and dried on blotting paper and transfered to MS regenerated media these plates were kept in dark for 2-4 days at 28°c this is cocultivation.

d) **Callus washing**
After incubation there was appearance of thin film of Agrobacterium around callus. Then callus was transfered to the washing media containing cefotaxime .Then they were blot dried and transfered to the regeneration MS hormonal media.

3. Genomic DNA extraction from the callus of *Piper longum*

Prechilled motar and pistle were taken and callus of *Piper longum* was crushed then 2ml EDTA,2ml tris buffer and 1ml SDS was addedThen 2ml mixture of chloroform and amyl alcohol in ratio of 5:1was added**.**The required mixture was taken in equal amount in 3 eppendorfs.The level was doubled by adding mixture of Phenol,chloroform and isoamyl alcohol in the ratio 25:24:1,Then the mixture was centrifuged at 10,000 rpm for 10min at 4°c.Supernatent was transfered to fresh eppendorf,then isopropanol was added.Centrifugation was done at 15000rpm for 10 min.70% of ethanol was added to wash the pellet.Then centrifugation was done at 15000rpm for 10 min.Then the pellet was dried and dissolved inTE buffer,gel electrophoresis was done to check DNA on 0.8% agrose gel.

4. Molecular analysis is done by Polymerase Chain Reaction to confirm stable confirmation

3. Results & Discussion

Agrobacterium tumifaciens strain containing plasmid pBECK2000 at concentration 0.538 OD at600nm for 3 days of co-cultivation in dark with 100 µM acetosyringone concentration and with infection time of 7 min showed stable transformation which was confirmed by performing PCR further agrose gel electrophoresis showed band of transformed DNA .PCR was performed with vector specific primer:- vector specific primer UID A/GUS-FP was used as a forward primerandUIDA/GUS-RP as a reverse primer to check the plant transformation. Analysis of Amplified Product was done by agrose gel electrophoresis

Table 3: Effect of Acetosyringone concentration

Acetosyringone concentration (µM)	*Infection in Callus*
50	++
100	++++
200	Over growth of bacteria

Table 4: Effect of time duration during infection

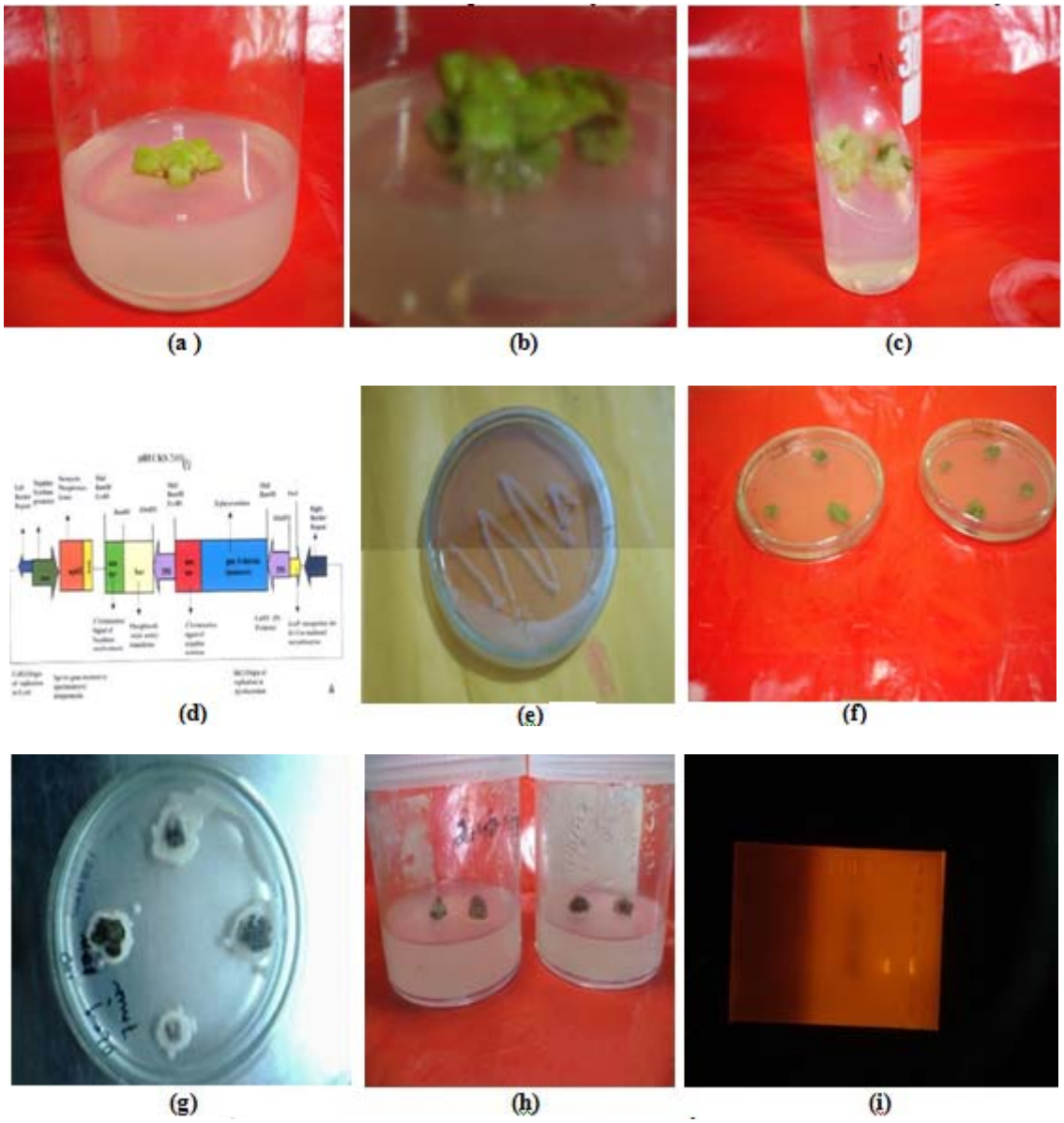
Time(min)	Infection in Callus
5	++
7	++++
10	Over growth of bacteria

Table 5: Effect of co-cultivation period

Co-cultivation period(days)	No. of callus inoculated	No. of infected calli
01	7	3
03	4	4
05	5	Over growth of bacteria

Table 6: Effect of optical density at 600nm on transformation efficiency

Parameters studied optical density at 600 nm	No. of callus inoculated	No. of infected calli (Transformed Calli)
0.398	7	3
0.538	4	4
1.00	5	Over growth of bacteria around calli



(a) explant during 1st week of inoculation (b) explants during 2nd week of inoculation (c) Induction of callus from leaf of *P. longum*(d) Structure of plasmid pBECK2000 used for transformation of *Piper longum* (e) transformed colonies of Agrobacterium on LB agar plate(f) Callus before 2-3 days incubation in dark (g) Callus after incubation(h)Transformed Callus in Regeneration media (i) Analysis of amplified product by Agrose gel electrophoresis

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