Silencing of cellulose synthase gene(s) of *Phytophthora infestans* for conferring late blight resistance in Indian potato (*Solanum tuberosum* L.) cultivar(s)

A thesis submitted for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

Submitted by

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Under the guidance of

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May 2024

CERTIFICATE

Certified that the thesis entitled "**Silencing of cellulose synthase gene(s) of *Phytophthora infestans* for conferring late blight resistance in Indian potato (*Solanum tuberosum* L.) cultivar(s)**" submitted by **Ms. Shivani Guleria**, Reg. no. **901700005** in the partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a record of candidate's own independent and original research work carried out by her under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

Prof. Anil Kumar
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DECLARATION

I, hereby declare that the work presented in the thesis entitled **"Silencing of cellulose synthase gene(s) of *Phytophthora infestans* for conferring late blight resistance in Indian potato (*Solanum tuberosum* L.) cultivar(s)"** in the partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala, Punjab is an authentic record of my work carried out from July 2017 to November 2023, under the supervision and guidance of **Prof. Anil Kumar**, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab. This report has not been submitted for the award of any degree or certificated in this or any other university.

Shivani Guleria

Place: Patiala, Punjab

Date: 03/05/24

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"I thank Almighty, for always allowing me to evolve and for constant blessings"

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ABBREVIATIONS

ANOVA	Analysis of Variance
BA	6-Benzyl adenine
CaMV	Cauliflower Mosaic virus
cDNA	Complementary deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
$CaCl_2$	Calcium Chloride
cm	Centimetre
DEPEC	Diethyl pyrocarbonate
DNSA	3,5-Dinitrosalicylic acid
2, 4-D	2,4-Dichlorophenoxyacetic acid
dNTPs	Deoxynucleotide triphosphate
dpi	Days post inoculation
DTT	Dithiothreitol
DI	Disease incidence
DS	Disease severity
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
g	Gram
GA_3	Gibberellin A3
HCL	Hydrochloric acid
H_2SO_4	Sulphuric acid
h	Hour
ISSR	Inter Simple Sequence Repeat
ITS	Internal transcribed spacer
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KI	Potassium Iodide
LB	Luria Broth
LA	Luria Agar
L	Litre
$MgCl_2$	Magnesium Chloride
MS	Murashige and Skoog
min	Minutes
mg	Milligram
M	Molar
mM	Millimolar
NAA	1-Naphthaleneacetic acid
N	Normal
OD	Optical density
PMSF	Phenylmethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
psi	Pounds per square inch
ppm	Parts per million
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA

SDS	Sodium Dodecyl Sulfate
s	second
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TDZ	Thidiazuron
U	Unit
µg	Microgram
µL	Microlitre
µM	Micromolar
v/v	Volume by volume
w/v	Weight by volume
YEP	Yeast Extract Peptone
YMB	Yeast Mannitol Broth
°C	Degree Celsius

ABSTRACT

Late blight is the most devastating disease of potatoes, which is caused by an oomycete *Phytophthora infestans.* The disease is known to cause significant crop losses each year. Many efforts have been made for the effective management of the late blight but the rapid development of new strains of *P. infestans* is the major constraint. The applicability of host-induced gene silencing (HIGS) in many plants against their pathogen has proven an effective approach and has driven the attention of many researchers. Therefore, in the present study, HIGS approach was adopted to silence the cellulose synthase (*CesA*) gene(s) of *P. infestans* to achieve late blight resistance in selected potato cultivars.

In the present study, two important potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' were selected based on their resistance to late blight disease. The evaluation assay of four Indian potato cultivars (Kufri Pukhraj', 'Kufri Chipsona 1', 'Kufri Jyoti' and 'Kufri Surya') with the sporal suspension of *P. infestans* revealed that the cvs. 'Kufri Pukhraj' (93.75% DI; 85.41% DS) is susceptible, 'Kufri Chipsona 1' (75% DI; 59.83% DS), 'Kufri Jyoti' (68.75% DI; 56.25% DS) is moderately resistance and 'Kufri Surya' (18.75% DI; 3.46% DS) is resistant to late blight. Therefore, the two cultivars 'Kufri Pukhraj' and 'Kufri Chipsona 1' were selected for transformation with the *Agrobacterium* harbouring hp-RNAi constructs specific to the *CesA* genes of *P. infestans*.

The process of *Agrobacterium*-mediated genetic transformation requires the optimisation of regeneration protocol as difficulties are faced in shoot organogenesis on selection-cum regeneration medium after co-cultivation. Therefore, in this study the effect of β-lactam antibiotics (used for elimination of *Agrobacterium*) and sucrose was investigated to improve shoot organogenesis. The effect of β-lactam antibiotics viz. cefotaxime, carbenicillin, and cephalexin was studied on shoot regeneration of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. Leaf and internodal explants from 21-day-old micro shoots were inoculated on MS2 medium (basal MS +10 μM $AgNO_3$+10 μM BA+15 μM GA_3) further supplemented with different concentrations (0-500 mg L^{-1}) of β-lactam antibiotics (cefotaxime, carbenicillin, and cephalexin). These antibiotics promoted shoot organogenesis in both cultivars at lower concentrations, except cephalexin. Cefotaxime (100 mg L^{-1}) induced shoot regeneration in maximum explants of cvs. 'Kufri Pukhraj' (internode 85.41%; leaf 65.27%) and 'Kufri Chipsona1' (75% internodal and 62.5%). Carbenicillin improved shoot regeneration at lower concentrations (< 300 mg L^{-1}), whereas cephalexin completely inhibited shoot regeneration in cv. 'Kufri Chipsona 1' and improved in cv.

‘Kufri Pukhraj’ upto 100 mg L^{-1}. The regeneration efficiency of internodal explants was significantly higher than that of leaf explants.

In addition, the effect of sucrose concentration (30-150 mM) was investigated on shoot organogenesis. It was observed that sucrose at 60–90 mM induced higher shoot organogenesis and number of shoots per explant, whereas lower and higher concentrations inhibited the same. Maximum shoot organogenesis was achieved from internodal explants of cv. ‘Kufri Pukhraj’ (84.44%) followed by ‘Kufri Chipsona 1’ (65.55%) on MS3 medium (basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3+100 mg L^{-1}) containing 60 mM sucrose. Sucrose was also observed to affects the activity and expression of sucrose metabolising (acid invertase and sucrose synthase) and starch metabolising enzymes (α-amylase and β-amylase).

The whole concept of silencing the *CesA* gene of *P. infestans* is based upon the differences in sequences at the N-terminal end of Pi*CesA* gene (*P. infestans*) and St*CesA* gene (potato). Strategically the ~200 bp non-homologous fragments of Pi*CesA3* and Pi*CesA2* gene to that of St*CesA* genes were selected and amplified in sense and antisense orientation. Two hp-RNAi constructs were developed targeting the Pi*CesA3* and Pi*CesA2* genes of *P. infestans*. Cloning of the ~200 bp selected fragments of Pi*CesA3* and Pi*CesA2* gene in sense and antisense orientation along the intron was performed in the hp-RNAi plasmid. The subsequent cloning of both the fragments was confirmed by amplification and restriction digestion of the ~200 bp cloned fragment with specific primers and restriction enzymes respectively. The developed RNAi constructs (Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II) were then used to perform the genetic transformation of potato cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’.

The genetic transformation of the potato cultivars through *Agrobacterium* needs optimisation of parameters affecting genetic transformation. The highest transient GUS efficiency was recorded with explants precultured for two days and infected with a bacterial density of *Agrobacterium* (OD_{590}≈0.6) for 15 min and co-cultivated (48 h) on MS4 medium (basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3 +100 µM acetosyringone) and screened on MS5 medium (basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3+100 mg L^{-1} cefotaxime+100 mg L^{-1} kanamycin). This optimised protocol was used to transform the explants of cv. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’. The putatively regenerated transgenic lines were multiplied and maintained on MS1 medium (basal MS +10 µM $AgNO_3$) for further analysis. The PCR analysis of regenerated shoots confirmed the presence of respective sense and antisense fragments, *nptII* gene, and absence of *16s rRNA* gene.

The transgenic lines were challenged with *P. infestans* to evaluate the level of resistance to late blight disease. A significantly lower disease incidence and severity were recorded in transgenic lines as compared to the control plants. The whole plant assay revealed that in transgenic lines expressing Pi*CesA3*-RNAi construct I, lower disease progression was recorded in line 4 (50% DI; 38.88% DS) of cv. 'Kufri Pukhraj' and lines 3 (37.5% DI; 37.49% DS) and line 5 (43.75% DI; 40.97% DS) of cv. 'Kufri Chipsona 1' at 10 dpi. However, in transgenic lines expressing Pi*CesA2*-RNAi construct II, minimum disease progression was recorded in line 4 (43.75% DI; 30.5% DS) of cv. 'Kufri Pukhraj' and line 1 (43.75% DI; 38.38% DS) and line 7(43.75% DI; 37.49% DS) of cv. 'Kufri Chipsona 1' at 10 dpi. The transgenic lines developed using Pi*CesA2*-RNAi construct II showed higher late blight resistance than those developed using Pi*CesA3*-RNAi construct I. Among cultivars, the transgenic lines of cv. 'Kufri Chipsona 1' showed higher late blight resistance.

Moreover, the activity of four antioxidant enzymes namely superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase, was also studied in inoculated and uninoculated control and transgenic plants. Significant differences in the activities of various enzymes were observed between the inoculated control and transgenic plants. In transgenic lines expressing Pi*CesA3*-RNAi construct I, the maximum activity of superoxide dismutase (12.67 U mg^{-1} protein), catalase (42.86 U mg^{-1} protein), peroxidase (85.39 U mg^{-1} protein), and ascorbate peroxidase (128.17 U mg^{-1} protein) was recorded in 'line 4' of cv. 'Kufri Pukhraj', while in cv. 'Kufri Chipsona 1' the 'line 3' showed the maximum activity of superoxide dismutase (15.96 U mg^{-1} protein), catalase (56.95 U mg^{-1} protein), peroxidase (95.33 U mg^{-1} protein), and ascorbate peroxidase (132.77 U mg^{-1} protein).

In transgenic lines expressing Pi*CesA2*-RNAi construct II, the maximum activity of superoxide dismutase (14.52 U mg^{-1} protein), catalase (49.45 U mg^{-1} protein), peroxidase (89.14 U mg^{-1} protein), and ascorbate peroxidase (125.64 U mg^{-1} protein), was recorded in 'line 4' of cv. 'Kufri Pukhraj' while in cv. 'Kufri Chipsona1' the 'line 7' showed the maximum activity of superoxide dismutase (14.11 U mg^{-1} protein), catalase (64.02 U mg^{-1} protein), peroxidase (98.56 U mg^{-1} protein), and ascorbate peroxidase (134.49 U mg^{-1} protein).

Chapter 1

Introduction

Potato (*Solanum tuberosum* L.) belongs to the genus *Solanum* and family *Solanaceae*. It is a tuber-bearing crop, first domesticated in South America around 8000 years ago (Singh et al. 2022; Reddy et al. 2018). Now potatoes are grown in more than 100 countries across the world. It is the third most important food crop with high nutritional and economic value (Zhang et al. 2017). It has been strongly recommended by the Food and Agriculture Organization (FAO) of the United Nations as a food security crop and for alleviating poverty (Thiele et al. 2010; Devaux et al. 2014). According to the FAO, 376 million tonnes of potatoes were produced globally in the year of 2021. China and India are the leading potato producing countries with an overall production of 94 million tonnes and 54 million tonnes respectively (FAOSTAT 2023). Therefore, for ensuring food security of the growing population sustainable potato production is a worldwide concern. Potato is a vegetatively propagated crop susceptible to various bacterial, fungal, protistan, viral, nematode pathogen and pests (Birch et al. 2012). The global yield loss due to various diseases is estimated to range from 17.2 % (8.1–21.0 %) to 100 % in severe epidemics (Savary et al. 2019).

One of the major challenges that potato production faces is the late blight disease, causing an annual economic loss of 6 billion dollars (Griffin et al. 2012; Arora et al. 2014; Derevnina et al. 2016). Late blight is the most devastating disease of potatoes caused by *Phytophthora infestans* (Mont.) de Bary, an oomycete belonging to the kingdom Stramenopiles. The pathogen is responsible for the Great Irish potato famine that happened in the year 1845. This had a tremendous impact, resulting in the death and migration of millions of people. Since then, the pathogen has spread far and wide due to the transportation of potatoes and now exists in all potato growing areas across the globe. The yield losses due to late blight can vary from 50-100% (Rasheed and Khan 2008; Ahmed et al. 2015; Guenthner et al. 2001).

Currently, the most effective strategy to control late blight is the use of fungicides on a regular and repeated basis (Rahman et al. 2008; Tsedaley et al. 2014). Contact fungicides such as chlorothalonil (Kavach), propineb (Antracol), mancozeb (Indofil M-45), as well as systemic-protectant fungicides such as Ridomil Gold (mancozeb+mefanoxam), Curzate M-8 (mancozeb+cymoxanil), Sectin (mancozeb+fenamidon), Revus (mandipropamid 250SC), Equation pro (cymoxanil+famoxadone) are mainly used (PAU 2023). This strategy, however, is costly, harmful to the environment, prevents organic farming, and results in the emergence of new fungicide-resistant strains due to sustained selection pressure (Majeed et al. 2017). Many potato breeding programs have been initiated to introduce resistance genes from wild relatives into cultivated potatoes. These genes recognise the cytoplasmic effector proteins of *P. infestans,* which help in manipulating the host cell structure, function and facilitate infection (Birch et al. 2008; Hein et al. 2009; Vleeshouwers et al. 2011). To date, more than 70 resistance genes (*R*-genes) specific to *P. infestans* (*Rpi*) have been identified from *Solanum* species. Some of the durable resistance genes reported till date are *R4*, *R8, R9a* from *S. demissum*, *Rpi-blb1*/*RB, Rpi-blb2*, *Rpi-blb3* from *S. bulbocastum*, *Rpi edn2* from *S. edinense*, *Rpi-vnt1.1* from *S. phureja, Rpi-rzc1* from *S. ruiz-ceballosii, Rpi-stol* from *S. stoloniferum, Rpi-vnt1.3* from *S. venturii, Rpi-amr3, Rpi-amr1-2273* from *S. americanum*, and *Rpi-amr1-3409* from *S. nigrescenss* (Paluchowska et al. 2022). Furthermore, in some of the resistant cultivars of *S. tuberosum* such as PB-06, S-60, Sarpo Mira, Jacqueline Lee, and Missaukee presence of the *R8* gene has been reported (Ivanov et al. 2021). Resistance cultivars with effective *R*-genes obtained through breeding are among the most effective, environment-friendly, and cost-effective methods of controlling late blight. However, the differences in ploidy levels of wild relative and cultivated potato cultivars lead to sexual incompatibility and inbreeding depressions. Moreover, the breeding process is long and requires intensive efforts to screen large progenies (Barrell et al. 2013; Dong and Zhou 2022). Further, field studies revealed that the resistance provided by the *R*-gene is race-specific, which is frequently broken by rapidly evolving strains of *P. infestans* (Paluchowska et al. 2022). These issues can be addressed effectively using various biotechnological approaches adopting novel strategies. Many studies have been carried out to clone and express the pathogenesis-related (PR) genes such as osmotin (Li et al. 1999), catalase (Yu et al. 1999), oxalate oxidase (Ghosh et al. 2016), glucose oxidase (Wu et al. 1995), thaumatin-like proteins (Ali et al. 2018), and other important genes like small G protein (Tian et al. 2023), barnase cytotoxic protein (Strittmatter et al. 1995), and transcription factors (Lawerence et al. 2019) to achieve stress tolerance. However, the lack of field studies due to government restrictions created a significant gap in the applicability of the transgenic lines generated by the aforementioned studies. Additionally, more than 50 resistance genes to *P. infestans* have been cloned such as *R1* (Ballvora et al. 2002), *R2* (Lokossou et al. 2009), *R3a* (Huang et al. 2005), *R3b* (Li et al. 2011), *R8* (Vossen et al. 2016), *R9a* (Jo 2013), *Rpi-abpt* (Lokossou et al. 2009), *Rpi-amr1*(Witek et al. 2021), *Rpi-amr3* (Witek et al. 2016), *Rpi-ber1*(Monino-Lopez et al. 2021), *Rpi-blb1*, *Rpi-blb2* (Van der Vossen et al. 2003), *Rpi-blb3* (Lokossou et al. 2009), *Rpi-bt* (Oosumi et al. 2009), *Rpi-chc1*(Monino-Lopez et al. 2021), *Rpi-edn1*(Champouret 2010), *Rpi-edn2* (De Vetten et al. 2014) *Rpi-hcb1*(Aguilera-Galvez et al. 2020), *Rpi-hjt1*(Champouret 2010), *Rpi-mcd1*(Lokossou 2010), *Rpi-mcq*, *Rpi-nrs1* (Jones et al. 2009), *Rpi-pta1*(Vleeshouwers et al. 2008), *Rpi-snk1*(Champouret 2010), *Rpi-sto1*(Vleeshouwers et al. 2008), *Rpi-sto2* (Champouret 2010), *Rpi-tar1* (Monino-Lopez et al. 2021), *Rpi-vnt1*(Foster et al. 2009), *ph-3* (Zhang et al. 2014), but as mentioned earlier the resistance achieved through *R*-gene is race specific and easily broken by new evolving strains of *P. infestans* (Paluchowska et al. 2022). The rapid adaptability of *P. infestans* to control strategies (fungicides and *R*-genes) can be explained due to the efficient and mixed reproduction cycles as well as the large number of effector genes and transposable elements in the genome (Wu et al. 2020; Rietman et al. 2012). Therefore, the development of novel control measures considering its rapid evolution in mind is an urgent requirement.

Nowadays, Host Induced Gene Silencing (HIGS), an application of RNA interference (RNAi) is widely used to develop transgenic plants expressing small interfering RNAs (siRNAs) (Nunes and Dean 2012; Mahto et al. 2020; Singh et al. 2020). These siRNAs are capable of targeting pathogen specific genes. The process of HIGS is usually initiated by the expression and processing of hairpin RNAs (hpRNAs) into siRNAs by Dicer/Dicer-like (DCL) (RNase III) enzyme (Zamore et al. 2000; Haley and Zamore et al. 2004). The siRNAs are incorporated into the RNA-induced silencing complex (RISC) and unbound by RISC; the passenger strand (sense) is degraded while the guide strand (antisense) remains bound to RISC (Akbar et al. 2022). The mature form of RISC, which includes a guide strand and an argonaute (Ago) protein, inhibits translation by targeting complementary mRNA sequences (Martinez et al. 2002; Kaur et al. 2021). This method has the potential to be used in the control of many diseases (Cheng et al. 2015; Mamta et al. 2016; Forster and Shuai 2020; Adeyinka et al. 2023) including late blight disease (Rosa et al. 2018; Kuo and Falk 2020). A few studies targeting to express specific siRNAs have been carried out which has resulted in providing moderate resistance in potato cultivars against late blight disease (Eschen-Lippold et al. 2012; Sanju et al. 2015; Jahan et al. 2015).

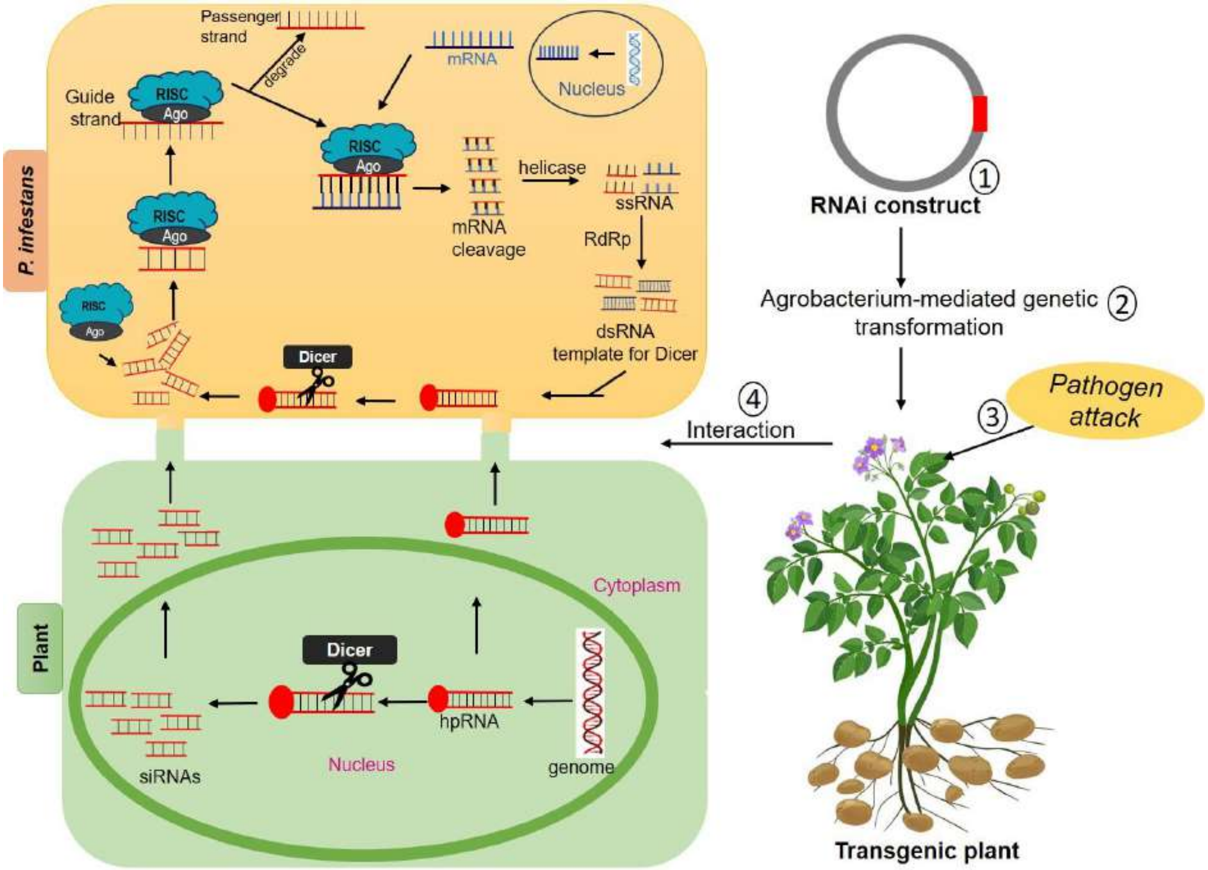


Fig. 1.1 Different steps involved in Host mediated gene silencing (HIGS) process

The applicability of the HIGS is based on the identification of target genes that are important for pathogen growth, development, and pathogenicity. In *P. infestans*, the cell wall is an important factor contributing to growth and establishing infection in the host plant. The infection cycle of *P. infestans* is initiated either by sexual reproduction, which involves A1 and A2 mating types, and their fertilization to form oospores, or by asexual reproduction which requires only one mating type. In both cases, sporangia form, which either germinate directly on the host surface and produce germ tubes or indirectly through the release of zoospore that lacks cell wall. Zoospores synthesize a cell wall, at the stage of cyst formation. Further, the cell wall produces a germ tube and forms an appressorium to invade the host tissue (Kots et al. 2017). After successful invasion, a spherical primary infection vesicle forms, from which hyphae grow intercellularly, and extend to form haustoria to extract nutrients and facilitate efficient delivery of defense and pathogenicity-related molecules (Grenville-Briggs et al. 2008; Nowicki et al. 2012; Botero et al. 2018). The formation of the cell wall at the cyst stage is very

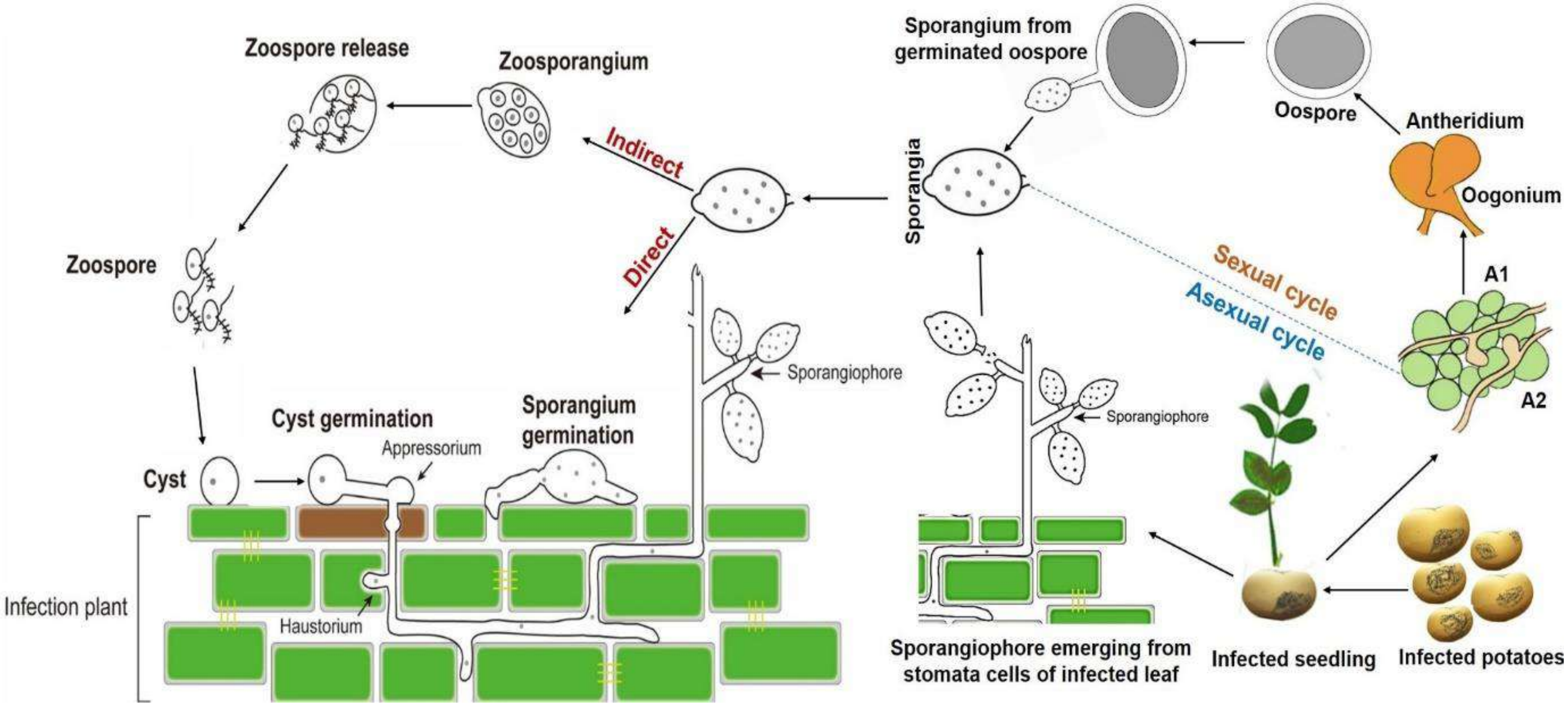


Fig. 1.2 Disease cycle of *P. infestans* on the host (adapted from Situ et al. 2020)

important for subsequent germ tube and appressorium formation (Grenville-Briggs et al. 2008; Majeed et al. 2017). The cell wall of *P. infestans* is mostly made up of cellulose, β (1-3), β (1-6) D-glucans, and very little amount of chitin (Bartnicki-Garcia 1968). Cellulose forms a microfibril structure and contributes to scaffolding and β (1→3) and β (1→6) glucans, form an amorphous matrix that is laid over and interacts with the inner microfibril layer of cellulose (Bulone et al. 1992; Helbert et al. 1997). Cellulose is a major component of the *P. infestans* cell wall and is made up of a homopolymer chain of β (1→4) glucose units. It is synthesized by plasma membrane-localized cellulose synthase (CS) complex. The complex is a rosette structure of several distinct *CesA* proteins, synthesizing an individual β-(1→4) linked glucan chain from UDP-glucose (Li et al. 2014). Further, the extrusion of multiple glucan chains from the CS complex occurs followed by the organization of glucan chains into cellulose microfibril through hydrogen bonding. The mechanism of cellulose synthesis is poorly understood in oomycetes (Fugelstad et al. 2009; Kuang et al. 2016). It has been reported that *CesA* protein contains several transmembrane domains at the N and C-terminal end along with a catalytic domain containing a conserved set of motifs (D1, D2, D3, and QXXRW) (Grenville-Briggs et al. 2008; Saxena and Brown 2000).

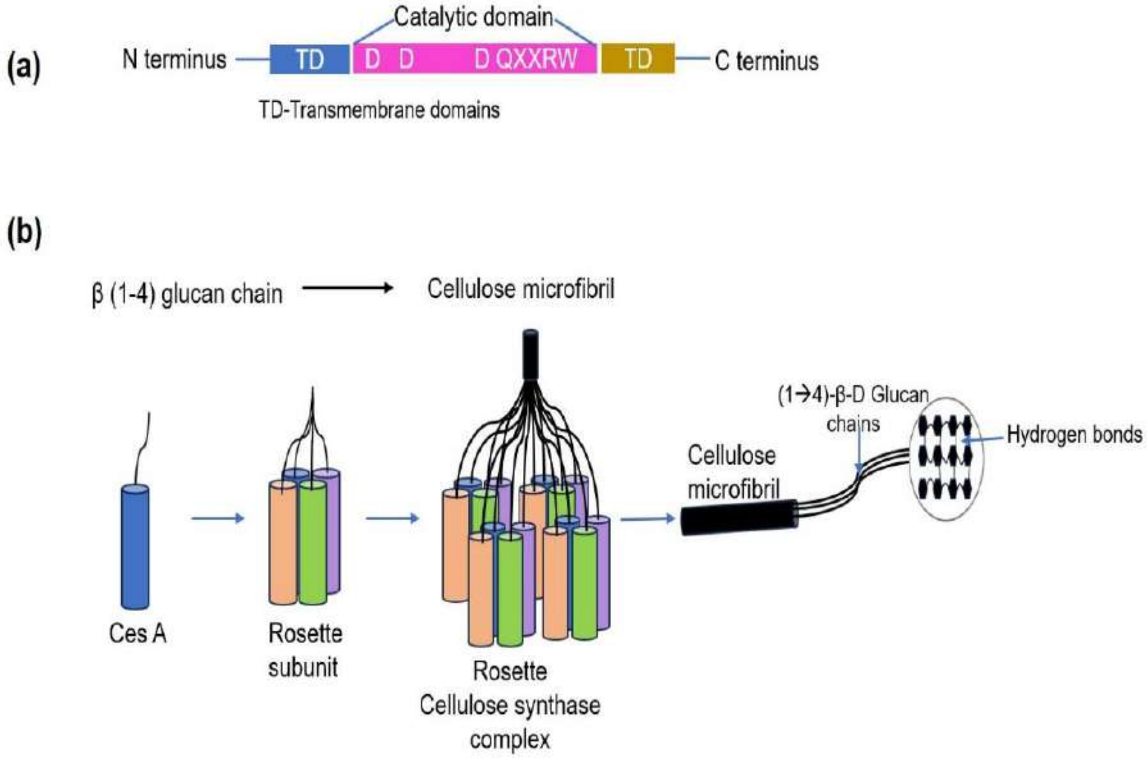


Fig. 1.3 The picture represents **(a)** conserved domains of the cellulose synthase gene and **(b)** the association to form a cellulose synthase complex for the synthesis of cellulose microfibril.

The transmembrane domain forms a channel that allows the extrusion of the newly synthesized glucan chain, the D1, D2 motif binds the sugar donor (UDP-glucose), and the D3, QXXRW motif forms a putative accepter binding region (Saxena and Brown 2000). In *P. infestans* four cellulose synthase (*CesA*) genes such as *CesA1*, *CesA2*, *CesA3,* and *CesA4* have been identified (Grenville-Briggs et al. 2008). It has been reported that the expression of *CesA* genes gets upregulated during cyst germination and appressorium formation and downregulated when the infection progresses to necrotrophic stage (Grenville-Briggs et al. 2008). Moreover, it has been reported that the C-terminal end of the *CesA* genes in *P. infestans* have higher homology to *CesA* genes in plants and bacteria. However, the N-terminal end of *P. infestans CesA* genes are divergent, and have lower homology to plant *CesA* gene. Furthermore, it has been reported that the N-terminal end of *P. infestans CesA1*, *CesA2,* and *CesA4* have structural similarities to the Pleckstrin homology (PH) domain which is a novel because no other *CesA* genes have been reported to have PH domain. Additionally, in plants *CesA* genes have a zinc finger domain which is absent in *P. infestans CesA* genes (Grenville-Briggs et al. 2008). Comparisons of *Phytophthora CesA* genes to previously published *CesA* genes revealed that they form a distinct clade, representing a novel class of cellulose synthases (Grenville-Briggs et al. 2008).

Therefore, strategically in the present study, the *CesA* genes of *P. infestans* involved in cellulose synthesis were targeted without affecting the cellulose synthesis of potatoes using HIGS. This will involve the development of a hp-RNAi constructs(s) targeting *CesA* genes of *P. infestans* followed by mobilization of the hp-RNAi construct into selected potato cultivars through *Agrobacterium*-mediated genetic transformation.

In plants, genes can be transferred using a variety of methods, including particle bombardment, direct DNA uptake, and an *Agrobacterium*-mediated gene delivery system (Chakravarty et al. 2007). However, *Agrobacterium*-mediated genetic transformation is well suited for such trait-specific modifications of dicot plants such as potatoes (Kaur et al. 2020). For undertaking *Agrobacterium*-mediated genetic transformation, the development of a high-frequency regeneration protocol (through shoot organogenesis and/or somatic embryogenesis) is a pre-requisite (Aggarwal et al. 2011; Kaur et al. 2017). Although there are many reports on the shoot regeneration of potatoes (Abd Elaleem et al. 2009; Ghosh et al. 2014; Kumlay and Ercisli 2015; Ijaz et al. 2016; Abbasi et al. 2016; Ibrahim et al. 2017; Kaur et al. 2017), yet there is a need for the improvement of shoot regeneration protocol. Further, all genetic transformation protocols employ the use of various antibiotics for the elimination of *Agrobacterium* following co-cultivation (Aggarwal et al. 2011; Kaur et al. 2020; Wen et al. 2022; Cordeiro et al. 2023). These antibiotics are reported to influence the regeneration potential of explants due to their growth regulatory activities. The stimulating effects of some of the antibiotics on shoot organogenesis have been reported (Ding et al. 2006; Bosela 2009; Grzebelus and Skop 2014; Meng et al. 2014; Tambarussi et al. 2015; Stanisic et al. 2018). Further, the inhibitory effect of some of the antibiotics on shoot organogenesis has also been reported in other plant species (da Silva Mendes et al. 2009; Qin et al. 2011). So, it is very important to investigate the effect of these antibiotics on regeneration (shoot organogenesis and/or somatic embryogenesis). A close perusal of the literature did not reveal any report investigating the effect of antibiotics on shoot organogenesis in potatoes.

In addition, sucrose is another important factor, serving as an energy source for energy-demanding processes including shoot organogenesis, rooting, and somatic embryogenesis (Kumar et al. 1991, 2002; Yaseen et al. 2013). It has also been reported that it maintains osmotic potential, which is known to influence cell division (Cosic et al. 2021). The role of sucrose as a signaling molecule has also been very well reported (Kumar et al. 1999; Leon and Sheen 2003; Gibson 2004; Skylar et al. 2011). Sucrose is known to affect the pathways activated by phytohormones by changing the activity and expression level of various important genes (Kushwah and Laxmi 2017; Sakr et al. 2018). Therefore, attempts were made to improve the shoot regeneration protocol to be adopted for the genetic transformation of the selected potato cultivar (s).

Taking all of this into consideration, the following objectives were designed for the present study

1.1 Objectives

1. Development of an efficient regeneration system using various explants of selected cultivar(s) of potato
2. Development of RNAi construct for silencing of cellulose synthase gene in *P. infestans*
3. *Agrobacterium* mediated genetic transformation of potato cultivar(s) using RNAi cassette (s) and their characterization

Chapter 2

Review of Literature

Potato is the most valuable edible crop, with over 5,000 varieties grown in different parts of the world. It is one of the most important cash crops for farmers due to its higher yield and nutritional value (Koch et al. 2020). The Food and Agriculture Organization (FAO) estimated that by 2050, 9.7 billion people will require 70% more food, likely to be 100 % more in developing countries. Potato has the potential to address issues of global food security. Efforts are also being made to improve nutritional value (Benmoussa et al. 2004; Mckibben et al. 2006; Diretto et al. 2006; Hemavathi et al. 2009; Bagri et al. 2018) and productivity of the potato crop (Wang et al. 2019; Doboch et al. 2022; Nyiraneza et al. 2021). However, the major threat to these efforts is late blight disease, which causes significant crop loss each year. The yield and management losses are estimated to be about 3-10 billion USD (Haverkort et al. 2009; Dong and Zhou 2022). Over the past years, scientists have made significant advances in understanding the molecular aspects of the pathogenicity of *P. infestans* (causal organism of late blight) (Whisson et al. 2016; Chepsergon et al. 2020; He et al. 2020) and also the development of disease management strategies (Lal et al. 2018; Tadesse et al. 2021; Dong and Zhou 2022). However, the rapid evolution and emergence of new aggressive strains of *P. infestans* pose a significant challenge to developed the control strategies for late blight (Yoshida et al. 2013; Shen et al. 2021). For feeding the growing population, there is an increased demand for food, which can be addressed by undertaking an effective potato crop improvement programme across the globe. This chapter focuses on reviewing various disease management and control strategies for late blight disease.

2.1 Introgression of late blight resistance through traditional breeding

Since the Irish Famine, the development of late blight resistance has remained a primary objective in potato breeding. It was discovered that certain wild relatives of potatoes were showing resistance to late blight, which includes both the types: horizontal (non-race specific) and vertical (race-specific), contributed by resistance (*R*) genes (Leonards-Schipper et al. 1992; Lozoya-Saldana 2011). Moreover, it is well documented that the wild potato species are valuable genetic resources contributing to genes conferring late blight resistance (Paluchowska et al. 2022). Late blight resistance was first found in *S. edinense*, a natural hybrid of *S. demissum* × *S. tuberosum* (Salaman et al. 1941). Further, the first study came from *S. demissum* (Mexican wild species, hexaploid), where 11 *R*-genes, designated *R1* to *R11*, were identified and subsequently introduced into *S. tuberosum* (Black 1951; Black et al. 1953; Malcolmson and Black 1966). Potato cultivars carrying *S. demissum* derived resistance genes, including

Pentland Dell (*R1*, *R2*, and *R3*), Pentland Ace (*R3*), and Epoka (*R4*), have been widely cultivated in Europe (Malcolmson 1969; Rudkiewicz 1985). However, it has been reported that the pathogen overcomes the resistance contributed by these genes due to the development of new strains resulting from sexual reproduction (Wastie 1991). Numerous other *R*-genes have been identified in wild relatives of potato (Paluchowska et al. 2022). However, their subsequent introduction into new potato cultivars necessitates knowledge, expertise and a time of approximately 10-15 years (Bradshaw 2017; Lenaerts et al. 2019). Additionally, several generations of backcrossing (BC) are required to eliminate undesirable traits without losing resistance. Further, crossing is time-consuming, particularly when species have different ploidy levels. For instance, it took more than 45 years to introduce a *Rpi-blb2* gene from *S. bulbocastanum* to Bionica and Toluca potato cultivars (Haverkort et al. 2016).

2.2 Fungicide-based late blight management

The use of fungicides is the oldest method of managing the late blight disease. During the 19th century copper-containing bordeaux mixture was used (Turner 2005; Thind et al. 2013). After this, the use of sulphur-based fungicides that were more effective and less phytotoxic than copper-based fungicides became widespread (Shotton 1967; Jarvis et al. 1967). The use of contact fungicides and new systemic fungicides to prevent late blight disease was practised as a management strategy (Olanya et al. 2001). Contact fungicides remain on the surface of the plant and prevent spores from germinating and entering the plant (Lal et al. 2018). However, systemic fungicides are absorbed into the plant (Dias 2012; Lal et al. 2018) and prevent the spread of infection and the formation of lesions within plants. In 1990, a very effective systemic fungicide metalaxyl was adopted to control late blight, which is now widely used throughout the world. However, within three years of its introduction, metalaxyl-resistant *P. infestans* isolates were found in field-grown potatoes (Gisi and Cohen 1996; Hu et al. 2008; Gisi and Sierotzki 2008; Perez et al. 2009; Gray et al. 2020; Gonzalez-Tobon et al. 2022). It has been reported that the sustained application of a fungicide increases selection pressure on *P. infestans*, which may lead to rapid adaptation and acquisition of resistance (Ivanov et al. 2021). Furthermore, it has been reported that the use of fungicide mixtures containing two or more fungicides provides effective control (Thind et al. 2013) and also prevents the development of resistance in *P. infestans*. Numerous fungicides have been developed and are commercially available, but none of these can effectively control *P. infestans* epidemics when conditions are highly favourable for the pathogen (Koller 1998; Mizubuti and Fry 2006). It has been reported that the schedule of fungicide spray based on a late blight forecasting model is essential (Lal et al. 2018). In addition, the removal of numerous fungicides from the EU Pesticide Directive, as well as human health and environmental concerns, encourages the development of alternative control strategies (Fry 2007).

2.3 Biological control of late blight

Biological control can be defined as the use of beneficial microorganisms to control the *P. infestans* infection. Microorganisms have significant positive effects on plant growth, suppress spread of pathogenic microorganisms, and also improves nutrient content (Meena et al. 2017; Singh et al. 2022; Koza et al. 2022; Bonaterra et al. 2022). It is an alternative to fungicides for controlling late blight and warrants further study. Until now, most of the research has centred on the bacteria *Bacillus subtilis, Pseudomonas fluorescens,* and the fungi *Trichoderma harzianum* and *Trichoderma viride,* which have delivered encouraging results (Haveri et al. 2018; Kumbar et al. 2019; Monjil et al. 2021). Haveri et al. (2018) reported that the application of *B. subtilis* and *P. fluorescens* in the soil reduced disease severity and number of blighted tubers in potato cv. ‘Kufri Jyoti’. Kumbar et al. (2019) investigated the growth inhibition activity of four *B. subtilis* isolates MTCC-2422, KU936341, KU936344, and KU936345 against *P. infestans* in cv. ‘Kufri Jyoti’. These *B. subtilis* isolates reduced the disease incidence and improved the vegetative parameters like sprouting, leaf number, plant height, fresh and dry weight.

In addition, fungi such as *T. harzianum* and *T. viride* have been reported to inhibit *P. infestans* growth and progression within plants. Hossain et al. (2014) reported that potato seed tubers treated with a formulation containing *Trichoderma* reduced the severity and incidence of late blight. Fatima et al. (2015) reported that the use of *T. harzianum* inhibited *P. infestans* mycelial growth in potato cvs. ‘Spounta’, ‘Bartina’, and ‘Condor’. In addition, Purwantisari et al. (2018) found that sporal suspension of *T. viride* induced systemic resistance against *P. infestans* in potatoes. Monjil et al. (2021) also reported that the *T. harzianum* treated potato cvs. ‘Diamant’ and ‘Challisha’ are less susceptible to *P. infestans* infection. Moreover, the increase in physical parameters such as number of tubers, fresh weight, and plant height was also documented. The reduction in *P. infestans* mycelial growth and increase in plant height and number of stems was observed when potato tubers of cvs. ‘Diamant’ and ‘Challisha’, treated with *T. harzianum* was used as seed (Khatun et al. 2021). Further, Haveri et al. (2018) reported that the addition of *T. harzianum and T viride* to the soil reduced the disease severity and decreased the number of blighted tubers in cv. 'Kufri Jyoti'.

2.4 Introgression of different genes in potato cultivars through genetic engineering

Genetic engineering has emerged as an effective tool for the improvement of potato productivity, nutritional quality, disease resistance, etc. (Bagri et al. 2018; Van Esse et al. 2020; Kavhiza et al. 2022). Cis-genesis and trans-genesis are the strategies of genetic engineering that involve the introduction of a gene (s) from sexually compatible and non-compatible species (Del Mar Martinez-Prada et al. 2021). The precision of genetic engineering has enabled rapid and significant change in crop improvement through the identification of a diverse set of novel genes. The technology overcomes the limitation of the long breeding cycle. Various genes such as resistance genes, pathogenesis-related genes, antioxidant enzyme gene(s) products, antimicrobial protein, small G protein, and transcriptional protein have been cloned in suitable binary vectors and introduced into susceptible potato cultivars to confer late blight resistance (Liu et al. 1994; Yu et al. 1999; Moon et al. 2009; Vossen et al. 2016; Tian et al. 2023).

2.4.1 Resistance genes

The direct transfer of *R*-genes from wild relatives to cultivated potato enhances the resistance of potato against rapidly evolving strains of *P. infestans*. Numerous genes for late blight resistance have been mapped and cloned (Paluchowska et al. 2022). The first study of cloning of resistance genes for late blight was reported by Ballvora et al. (2002). In this study, the *R1* gene was cloned and introduced into potato cv. 'Desiree' and the resulting transgenic lines exhibited hypersensitive response (HR) to *P. infestans* race 4. However, the transgenic lines of cv. 'Desiree' containing the *R3a* gene were resistant to *P. infestans* isolates IPO-0 and 89148-9, but susceptible to isolate H30P04 (Huang et al. 2005). Song et al. (2003) stated that the wild species of potato *S. bulbocastanum* exhibit a strong resistance to all identified races of *P. infestans*. In this study, the resistance gene *RB* from *S. bulbocastanum* is introduced into the potato cv. 'Katahdin' and transgenic lines exhibited resistance to *P. infestans* isolates US930287, US940480, MSU-96, US940501, US980008 and 126C18. Van der Vossen et al. (2003) demonstrated that the transgenic lines of cv. 'Impala' containing *Rpi-blb1* gene are resistant to *P. infestans* isolate IP0655-2A. After one year, Van der Vossen et al. (2005) cloned and introduced the *Rpi-blb2* gene into potato cvs. 'Impala' and 'Kondor' found that the transgenic lines are resistant to *P. infestans* isolates IP0655-2A and IP082001. Resistance genes such as *Rpi-pta1* and *Rpi-sto1* were introduced in potato cv. ‘Desiree’ exhibited resistance to *P. infestans* isolate 90128 (Vleeshouwers et al. 2008). In addition, the *Rpi-vnt1.1* gene was cloned and introduced in potato cv. ‘Desiree’, conferred resistance to transgenic lines against 11 isolates of *P. infestans* but susceptiblity to EC1 (Foster et al. 2009). Lokossou et al. (2009) reported that the three genes *Rpi-abpt*, *R2*, and *Rpi-blb3* introduced in transgenic lines of potato cv. ‘Desiree’ exhibited HR response to *P. infestans* isolates IPO-0 and 90128. Moreover, the transgenic lines of potato cv. ‘Lenape’ and ‘Atlantic’ containing *Rpi-bt1* gene showed reduced lesion area on inoculation of US-8 and MD-02-pet-1A2 strains (Oosumi et al. (2009). Li et al. (2011) reported that the *R3b* resistance gene cloned in potato cv. ‘Desiree’ confers resistance to *P. infestans* isolate 89148-9 but not to IPO-0. The introduction of the *R9a* gene in cv. ‘Desiree’ confers resistance to *P. infestans* isolates 89148-09 and IPO-C (Jo 2013). In field trials, Vossen et al. (2016) found that the *R8* gene introduced in potato cv. ‘Desiree’ exhibited broad-spectrum resistance to races 1, 2, 3, 4, 5, 6, 7, 10, 11 of *P. infestans* isolate IPO-C. Witek et al. (2016) reported that the introduction of the *Rpi-amr3i* gene from non-tuber bearing *S. americanum* into the potato cv. ‘Solyntal’ showed resistance to isolates 06_3928A, 88069, and EC3527 of *P. infestans*. However, the lines lacking functional *Rpi-amr3a* gene exhibited expanded necrotic lesions upon infection. Aguilera-Galvez et al. (2020) introduced the resistance genes *Rpi-mcq1* and *Rpi-blb3* into the potato cv. ‘Desiree’, reported that the transgenic lines exhibited distinct resistance to 18 diverse isolates of *P. infestans*. Witek et al. (2021) cloned the *Rpi-amr1* gene in potato cv. ‘Maris piper’ and reported the resistance in transgenic lines against 88069 isolate of *P. infestans*. Monino-Lopez et al. (2021) cloned and introduced the *Rpi-chc1.1* gene in potato cv. ‘Desiree’ and achieved resistance to *P. infestans* isolate 90128.

Numerous attempts have been made to clone the resistance gene in potato cultivars. However, all of these investigations have revealed only race-specific resistance, and further rapid development of new strains are still posing threats.

2.4.2 Pathogenesis related (PR) genes

The *PR* genes are expressed as PR proteins during pathogenic attack (biotic stress) and abiotic stress conditions in plants. Due to their pathogen specific action, PR-proteins are likely to play a significant role in the development of disease resistance (Wanderley-Nogueira 2012). By overexpressing PR proteins, scientists have attempted to impart late blight resistance in potato cultivars. Here are the few studies conducted to achieve resistance to late blight in potato plants.

Osmotin (PR-5 family), a 24 KDa protein, is one of the most exploited PR proteins to develop stress tolerant transgenic plants. Overexpression of osmotin in *S. tuberosum* (FL1607 line) has been shown to delay the symptoms following infection of isolate 175A of *P. infestans* (Liu et al. 1994), whereas expression of pA13-osmotin like protein in *S. commersonii* has been shown to reduce the sporulation of *P. infestans* isolate 184 in transgenic plants (Zhu et al. 1995). According to reports, osmotin contributes significantly increased resistance to *P. infestans* by causing spore lysis, decreasing spore viability, and inhibiting spore germination (Manghwar and Hussain 2021; Bashir et al. 2020).

Thaumatin-like proteins (TLPs), another important PR protein (PR-5 family), are believed to inhibit the growth of microorganisms and are overexpressed in response to pathogens and their elicitors. Aacharya et al. (2012) introduced *Camellia sinensis* (Cs) *TLP* gene into potato cv. 'Kufri Giriraj' which resulted in delayed symptoms of late blight against *P. infestans* race 1, 2, 3, 4, 5, 7, 8, 9, 10, and 11 in transgenic lines. In addition, Ali et al. (2018) reported that the introduction of *Arabidopsis* thaumatin-like protein 1 (ATLP1) in potato cv. 'Desiree; significantly decreased lesion size, sporulation capacity, and infection efficiency of *P. infestans* strain US-8 in transgenic lines.

Defensins (PR-12 family) are small cysteine-rich peptides (approx.15 kDa) with antimicrobial activity by creating pores in the cell membrane, resulting in membrane disruption and, eventually, cell death. Although many defensins possess antimicrobial properties (Thevissen et al. 2007), but some lack such antifungal and antibacterial action (Liu et al. 2006). In potato cv. 'Russet Ranger', Gao et al. (2000) expressed an *alfAFP* (alfalfa antifungal peptide) gene that resulted in strong resistance in transgenic lines against *Fusarium culmorum, Alternaria solani* and *Verticillium dahliae*, but those lines were susceptible to *P. infestans*. This uncovered the pathogen specific role of defensin. Nonetheless, Portieles et al. (2010) reported that the transgenic lines of cv. 'Desiree' over-expressing NmDef02 gene (*Nicotiana megalosiphon* defensins) exhibits strong resistance to *P. infestans and A. solani* under greenhouse conditions. Oxalate oxidase (PR-15 family) is an important PR protein that catalyses the oxidation of oxalate to CO_2 and H_2O_2 (Opaleye et al. 2006). Schneider et al. (2002) overexpressed the oxalate oxidase in potato cv. ‘Bintje’, which resulted in a decrease in lesion number and sporangia formed per lesion by *P. infestans* isolate 94-28. In addition, Ghosh et al. (2016) reported that the transgenic lines of potato cv. ‘Chipsona 3’ overexpressing the Osoxo4 gene (oxalate oxidase 4) showed increased resistance in transgenic plants.

In addition, it was reported that NtPRp27, a member of the PR-17 family was highly accumulated in *Nicotiana tabacum* as a result of Tobacco mosaic virus infection and additional stresses (Okushima et al. 2000). Therefore, overexpression of the StPRp27 in potato cv. ‘E3’ resulted in a decreased lesion growth rate on inoculation with *P. infestans* isolates 99189 and PY23 (Shi et al. 2012).

2.4.3 Antioxidant enzymes

The transgenic potato plants expressing gene products that function by releasing elicitors that regulating plant defenses is highly anticipated. The elicitor consists of ethylene (C_2H_4), salicylic acid (SA), and hydrogen peroxides (H_2O_2). Wu et al. (1995) reported that the expression of glucose oxidase gene isolated from *Aspergillus niger* in potato cv. ‘Russet Burbank’ causes transgenic lines to produce more H_2O_2. The increased level of H_2O_2 in transgenic lines mediates strong resistance against *Erwinia carotovora* and *P. infestans* race 0. In addition, the overexpression of catalase in the transgenic potato line FL1607 improved tolerance to *P. infestans* isolate 15 (Yu et al. 1999).

2.4.4 Small G protein

Small G proteins are important for signal transduction, stomatal closure induction, and defense responses. Zhang et al. (2014) demonstrated that the expression of an *Arabidopsis* Rop1 protein in potato cv. ‘Shepody’ resulted in H_2O_2 accumulation, smaller lesions, and reduced growth of *P. infestans* on transgenic lines. Tian et al. (2023) reported that the expression of the *StRab5b* gene in potato cv. ‘Desiree’, also resulted in the accumulation of H_2O_2 in infected leaves followed by upregulation of ROS-scavenging enzymes. Thus, inhibiting the growth of *P. infestans* and reducing the severity of leaf lesions.

2.4.5 Transcription factor

Lee et al. (2007) reported that ethylene-responsive element binding proteins (StEREBP1) of *S. tuberosum* are a transcription factor that induces the transcription of multiple stress response genes. Transgenic lines of potato cv. 'Superior' overexpressing the StEREBP1 gene showed resistance to *P. infestans* strain KACC40718 (Moon et al. 2009). This suggested that resistance was possibly induced due to the upregulation of expression of stress responsive genes encoding PR proteins (Moon et al. 2009). The Q-type C2H2 zinc finger proteins (ZFPs) are another important family in plant stress response. It can bind to two zinc molecules, enabling precise interactions with specific DNA, RNA motifs to regulate particular genes (Kielbowicz-Matuk 2012). Overexpression of the *S. tuberosum* ZFP2 protein increases resistance to *P. infestans* US11 race in potato cv. Kennebec (Lawerence et al. 2019). Moreover, Ha et al. (2017) found that the highly conserved, Zn-containing protease domain encoded by the NMMP1 gene exhibits resistance to bacterial and fungal pathogens. In potato cv. 'Daeji' overexpression of the NMMP1 gene resulted in resistance to isolate 88069 of *P. infestans*.

2.4.6 Engineered antimicrobial proteins

Antimicrobial genes encode short peptides (>50 aminoacids), having broad-spectrum antimicrobial activity. Ali and Reddy (2000) reported that pep6, pep7, pep11, and pep20 antimicrobial peptides inhibited *P. infestans* US8 strain and *Alternaia solani* growth on transgenic lines of cv. 'Desiree'. Osusky et al. (2000) expressed MsrA1 peptide (modified cecropin-melittin-chimera) in potato cv. 'Desiree' and 'Russet Burbank' to achieve resistance to *P. cactorum*, *Fusarium solani,* and *Erwinia carotovora*. After 4 years, Osusky et al. (2004) successfully expressed another antimicrobial peptide (modified temporin A) in potato cv. 'Desiree', to get resistance against *P. infestans* and *P. erythroreptica*.

2.4.7 Cytotoxic compounds

Researchers also employed cytotoxic compounds to induce rapid cell death in susceptible host plant tissues infected with *P. infestans*. Limiting cytotoxic activity to infection sites was essential for the success of this concept. Strittmatter et al. (1995) achieved this by expressing the cytotoxic molecule barnase (encodes RNAase) under the control of a promoter perp1-1 that is only responsive to pathogen attack or by inhibiting its cytotoxic effect in all non-infected tissues. However, barnase confers male sterility in plants; therefore, the barnase-inhibiting barstar gene was used to counteract its impact. The expression of fusion product Barnase-barstar gene product (barnase under the control of prp1-1) exhibited reduced sporangia formation of *P. infestans* races 1-11 in potato cv. 'Bintje'. The absence of field trials severely limited the use of the transgenic lines generated in above mentioned studies.

In light of the pathogen's rapid evolution, effective strategies for controlling late blight disease must be developed in a very short time frame. In addition, the ability to transfer genes from sources other than plants to edible crops raised public concerns and uncertainties regarding the use of genetically modified crops on health, agriculture and the environmental issues (Wolfenbarger and Phifer 2000; Mullins et al. 2006; Dona and Arvanitoyannis 2009; Bawa and Anilakumar 2012). This has also narrowed down the search area for a necessary gene. Concerns also include the spread of transgenes to other cultivar and wild relatives, which erodes genetic diversity and disrupts ecology. Therefore, new crop improvement strategies and safe methods that are acceptable to the general public must be developed.

2.4.8 RNA interference

RNA interference (RNAi) and its application have sparked the interest of biotechnologists working in different fields across the globe. The absence of transgenic proteins in RNAi-transgenic plants makes this method preferable to conventional transgenic methods (Rodrigues and Petrick 2020; Rajam 2020). In plants, Napoli et al. (1990) first studied RNAi as a co-suppression when they attempted to overexpress a gene called chalcone synthase (*chsA*) in the *Petunia* plant. Fire et al. (1998) coined the term RNAi when they studied the mechanism of RNAi in a nematode *Caenorhabditis elegans*. Dougherty et al. (1994) discovered that the plant has an RNAi-based defence mechanism by infecting transgenic *Nicotiana tabacum* with the Tobacco Etch Potyvirus (TEV) and conferred resistance through complementary viral RNA degradation. Since then, the technique has been used to develop transgenic crops that express siRNA (small interfering RNA) to gain resistance against fungus (Nowara et al. 2010; Koch et al. 2013; Ghag et al. 2014; Singh et al. 2020; Dou et al. 2020), viruses (Pooggin et al. 2003; Bonfim et al. 2007; Shimizu et al. 2011; Patil et al. 2011), nematode (Fairbairn et al. 2007; Iqbal et al. 2020) and insect pests (Mao et al. 2007; Kumar et al. 2012; Hajeri et al. 2014).

As reported, the host-induced immunity against viruses (Dougherty et al. 1994; Ratcliff et al. (1997) was extended to host-induced gene silencing (HIGS) method by developing disease resistant transgenic plants expressing hp-RNA or dsRNA specific to pathogen gene. HIGS is an RNAi-based mechanism that relies on the identification of genes required for pathogen growth, development, and infection establishment (Jahan et al. 2015; Mamta et al. 2016). It involves the introduction of hairpin RNA (hpRNAs) construct into the plant genome via *Agrobacterium* or other gene transfer methods (Mamta et al. 2016; Rajam 2020).

The success of HIGS against *P. infestans* is highly dependent on the gene that is targeted. Various studies targeting the genes involved in the infection process of *P. infestans* are discussed below

2.4.8.1 Growth-related genes

The genes involved in the infection process of *P. infestans* such as pectinesterase *(PiPEC),* G-protein β-subunit *(PiGPB1)*, glyceraldehyde 3-phosphate dehydrogenase (*PiGAPDH)* and cellulose synthase A2 *(PiCESA2)* were targeted. The RNAi constructs of these genes were introduced in cv. 'Desiree'. It was recorded that the transgenic lines expressing siRNAs for silencing of *PiGPB1* and *PiGAPDH* resulted in restricting disease progression on inoculation with *P. infestans* isolates CY29 and 88069, while the silencing of *PiPEC*, and *PiCESA2* could not reach the resistance level of *PiGPB1* and *PiGAPDH* (Jahan et al. 2015).

2.4.8.2 Effector proteins

The role of effector proteins in the process of infection is well documented in the literature, mainly secreted by haustorium and responsible for *P. infestans* virulence. Studies have been carried out to silence the *avr3a* gene of *P. infestans* for late blight resistance. Sanju et al. (2015) introduced a hairpin construct in potato cvs. 'Kufri Khyati' and 'Kufri Pukhraj' expressing siRNAs specifically targeting the *avr3a* gene of *P. infestans.* The transgenic lines challenged with *P. infestans* isolate HP10-45 showed a reduction in pathogen load and low disease progression. A similar study was also performed by Sanju et al. (2016), by targeting the *avr3a* gene through the expression of small interfering RNA (siRNA) and artificial microRNA (amiRNA) in potato cvs. 'Kufri Khyati' and 'Kufri Pukhraj'. The transgenic lines showed a small lesion area and spore production when challenged with *P. infestans* isolate HP10-45. Thakur et al. (2015) developed five gene constructs to specifically target the regions of the *avr3a* gene through amiRNA, and introduced in potato cvs. 'Kufri Pukhraj' and 'Kufri Khyati'. The transgenic lines challenged with *P. infestans* isolate Kalyani 08-1, HP10-45, and HP10-22 resulted in moderate resistance to *P. infestans*.

2.4.8.3 Susceptibility gene

Susceptibility (S) genes present in the host help the pathogen to successfully establish the disease in the host plant. Therefore, their inactivation could also result in resistance against pathogens (Garcia-Ruiz et al. 2021). Eschen-Lippold et al. (2012) achieved late blight resistance in cv. ‘Desiree’ by impairment of plasma membrane-localised S gene SYNTAXIN-RELATED1 (*StSYR1*). The protein helps transport of vesicles to selectively fuse with the membrane of the target compartment so that the molecules can be delivered. In this study, decreased susceptibility of transgenic lines to *P. infestans* isolate CRA208m2 was recorded. However, loss of *S* gene function in *StSYR1*-RNAi lines led to growth and development defects, chlorosis and necrosis. Moreover, a decrease in tuber weight, number, and overall yield was also recorded.

Table 2.1 Strategies adopted in various studies using gene silencing to control late blight

Potato cultivar	**Target gene**	***P. infestans* isolate**	**Response of transgenic lines after challenging with *P. infestans***	**References**
Desiree	SYNTAXIN-RELATED1 (*StSYR1*) (S-gene)	CRA208m2	Decreased susceptibility	Eschen-Lippold et al. (2012)
Desiree	*PiGPB1, PiCESA2, PiPEC,* and *PiGAPDH*	CY29, 88069	Higher restricted disease progression was achieved with silencing of GPB1 and GAPDH genes than PEC, and CESA2	Jahan et al. 2015
Kufri Khyati, Kufri Pukhraj	Effector protein (*avr 3a*)	HP10-45	Low disease progression and reduced pathogen load	Sanju et al. 2015
Kufri Khyati, Kufri Pukhraj	Effector protein (*avr 3a*)	Kalyani 08-1, HP10-45 and HP10-22	Moderate resistance	Thakur et al. 2015
Kufri Khyati, Kufri Pukhraj	Effector protein (*avr 3a*)	HP10-45	Moderate resistance and less disease severity	Sanju et al. 2016
Desiree	*StPMR4, StCESA3, StDMR6, StDMR1, StDND1* and *StSR4 (S-gene)*	Pic99189	Decreased susceptibility	Sun et al. 2016
Desiree	*StDeSI2*(S-gene)	88069 and EC-1	Increased susceptibility to late blight	Jiang et al. 2023

Sun et al. (2016) reported reduced susceptibility of transgenic lines of cv. 'Desiree' to isolate Pic99189 (race 1.2.5.7.10.11) of *P. infestans* by silencing *S* genes (*StPMR4, StCESA3*, *StDMR6, StDMR1*, *StDMR6*, *StDND1,* and *StSR4*). However, changes in leaf colour necrosis and reduced growth were also recorded in transgenic lines. In contrast, a study conducted by Jiang et al. (2023) reported that it is not like the silencing of S-gene can always reduce the susceptibility to late blight; in some cases, silencing of S-gene can also lead to increased susceptibility to disease. The study reported the increased colonisation of *P. infestans* isolates 88069 and EC-1 in desumoylating isopeptidase (*StDeSI2)* silenced transgenic lines than control plants.

2.5 Genetic transformation of potato cultivars for regeneration of transgenic lines

The advances in plant biotechnology tools, including gene identification and cloning, provide a solid foundation for gene transfer in plants (Giri et al. 2004; Anjanappa and Gruissem 2021). There are numerous methods for transferring genes in plants, including particle bombardment, direct DNA uptake, and by using *Agrobacterium* (Chakravarty et al. 2007; Narusaka et al. 2012; Ozyigit et al. 2020). *Agrobacterium*-mediated gene transfer is suitable for trait-specific modification of dicot plants such as potatoes (Gelvin 2003; Ziemienowicz 2014; Hwang et al. 2017). The most challenging step in *Agrobacterium*-mediated gene transfer would be the regeneration of whole plants from transformed (Aggarwal et al. 2011; Aggarwal et al. 2011; Kaur et al. 2020). This can be accomplished via direct and indirect shoot organogenesis and/or somatic embryogenesis, however it is highly dependent on genotypes; therefore, it is essential to develop efficient and reproducible protocols for callus and shoot regeneration in each potato cultivar.

2.5.1 Optimisation of callus induction and shoot regeneration protocol

The incorporation of auxins and cytokinin into a nutrient medium provides a foundation for callus induction and shoot regeneration (Bisht et al. 2023). Skoog and Miller (1957) reported that an adequate concentration of cytokinin and auxin alone or together has a substantial effect on the dedifferentiation and redifferentiation process. The first report of shoot organogenesis in potato came from tuber discs cultured on MS medium containing BA (Lam 1975). Numerous studies on the regeneration of shoots utilise various concentrations and combinations of cytokinin (BA, kinetin, TDZ, Zeatin) with auxins (NAA, 2,4-D, IBA, IAA) and GA_3. Moreover, shoot regeneration in potato is reported as a one stage (Hussain et al. 2005; Torabi

Table 2.2 Shoot regeneration in different cultivars of potato using different explants

Potato cultivar	Explant	Medium composition	Response	References
Superior	Tuber disc	Basal medium+0.4 ppm BA	Shoot formation with no roots	Lam1975
Superior	Tuber disc	Basal medium +0.2 ppm NAA	Shoot regeneration	Lam 1977
Superior	Tuber disc	Basal medium+0.03 mg L^{-1} NAA or 0.3, 1.0, 3.0 mg L^{-1}	Shoot regeneration	Jarret et al. 1980
Cara and A25/19	Shoot apices	Step 1: ½ MS medium + 3.2 mg L^{-1} IAA+ 1.0 mg L^{-1} Kinetin+ 0.5 mg L^{-1} 2,4-D Step 2: ½ MS medium+0.5 mg L^{-1} 2,4-D+ 1.0 mg L^{-1} Zeatin Step 3: ½ MS medium	Callus formation and shoot regeneration	Ahloowalia 1982
Bintje	Protoplast	MS medium+ 1 mg L^{-1} Zeatin +0.01 mg L^{-1} GA_3+ 0.01 mg L^{-1}NAA	Adventitious shoot regeneration	Bokelmann and Roest 1982
Bintje	Leaf	Step 1: MS medium+ 2.28 μM Zeatin Step 2: MS medium+ 5.78 μM GA_3 + 2.28 μM Zeatin	Shoot regeneration	Esna-Ashari and Villiers (1998)
Desiree and Igor	Internode	MS medium +0.2 mg L^{-1} NAA+2.5 mg L^{-1} Zeatin + 0.02 mg L^{-1} GA_3	Shoot regeneration	Zel and Medved (1999)
Cardinal	Leaf and Internode	MS medium+ 2 mg L^{-1} BA +2.5 mg L^{-1} NAA	Callus formation and shoot regeneration	Yasmin et al. 2003
Desiree and Maris piper), *S. commersonii* and *S. acaule*	Internode and tuber disc	MS medium +5 mg L^{-1} Zeatin+2 mg L^{-1} IAA	Shoot regeneration	Anjum and Ali 2004
Cardinal, Altamash, Diaman	Shoot tips, leaf discs, nodes, and internodes	MS medium with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} IAA	Shoot regeneration	Hussain et al. 2005

Desiree	Leaf, internode	MS medium +5 mg L^{-1} GA_3 + 2.5 mg L^{-1} BA	Shoot regeneration	Torabi et al. 2008
Diamant	Tuber disc	Step 1: MS medium+ 3.0 mg L^{-1} 2,4-D or MS medium+2.0 mg L^{-1} BA +2.0 mg L^{-1} 2,4-D Step 2: MS medium+ 5.0 mg L^{-1} TDZ Step 3: ½ MS +0.5 mg L^{-1} IBA	Callus formation and shoot regeneration	Abd Elaleem et al. 2009
Almere	Tuber disc	Step 1: MS medium + 2.0-5.0 mg L^{-1} 2,4-D Step 2: MS medium+0.5 mg L^{-1} TDZ Step 3: MS medium+1.0 mg L^{-1} IBA	Callus formation and shoot regeneration	Khalafalla et al. 2010
Asterix	Internode	MS medium +5 mg L^{-1} Zeatin	Direct shoot regeneration	Molla et al. 2011
Daraga, Sponta, Diamont, Cillan, Burun	Leaf and Internode	Step 1: Ms medium + 2 mg L^{-1} 2,4-D +0.8 mg L^{-1} Zeatin Step 2: MS medium+ 2 mg L^{-1} GA_3 + 0.8 mg L^{-1} Zeatin	Callus formation and differentiation into plantlets	Al-Sulaiman 2011
Atlantic	Leaf and Internode	WPM medium+ 5.0 mg L^{-1} Zeatin +1.0 mg L^{-1} NAA	Shoot regeneration	Rezende et al. 2013
Kufri Chipsona 1, Kufri Jyoti, Chipsona 3, Kufri Chipsona 3, Kufri Jyoti	Leaf	Step 1: MS medium+ 1.0 mg L^{-1} NAA+ 1.0 mg L^{-1} BA+ +100 mg L^{-1} PVP +1 mg L^{-1} calcium D-pantothenate Step2: MS medium+ 1.0 mg L^{-1} TDZ +3.0 mg L^{-1} BA+ 1.0 mg L^{-1} GA_3 +100 mg L^{-1} PVP +1 mg L^{-1} calcium D-pantothenate	Callus formation and adventitious shoot regeneration	Ghosh et al. 2014
Pasinler, Granola, Casper	Leaf and Internode	Step 1: MS medium +2.0 mg L^{-1} NAA+3.0 mg L^{-1} BA Step 2: MS medium+ 0.25 mg L^{-1} GA_3 +2.0 mg L^{-1} BA Step 3: MS medium+ 1 mg L^{-1} NAA or MS medium+ 1 mg L^{-1} IBA +0.1 mg L^{-1} GA_3	Callus formation, shoot proliferation	Kumlay and Ercisli 2015
Alpha	Tuber disc	Step 1: MS medium+ 2 mg L^{-1} 2,4-D Step 2: MS medium+ 5.0 mg L^{-1} BA or MS medium+ 1.0 mg L^{-1} IBA +5.0 mg L^{-1} BA	Callus formation and shoot regeneration	Abdelaleem 2015

Kuroda, FD-51-5	Tuber disc	Step 1: MS medium+ 4.5 mg L^{-1} 2,4-D Step 2: MS medium + 4.75 mg L^{-1} BA Step 3: ½ MS medium with no hormone	Callus formation, shoot proliferation	Ijaz et al. 2016
Desiree	Leaf and internode	MS medium+ 0.1 mg L^{-1} GA$_3$ +0.01 mg L^{-1} NAA+ 1 mg L^{-1} Zeatin	Callus formation and shoot regeneration	Abbasi et al. 2016
Kufri Pukhraj, Kufri Chipsona 1, Kufri Chipsona 2, Kufri Surya, Kufri Jyoti, Kufri Chandramukhi, Desiree, Kufri Khyati	Leaf and Internode	Basal MS+ 10µM AgNO$_3$ + 10µM BA+ 15µM GA$_3$	Shoot regeneration	Kaur et al. 2017
Kufri Jyoti	Leaf and Internodes	MS medium+ 1 mg L^{-1} GA$_3$ +3 mg L^{-1} BAP	Shoot regeneration	Rawat et al. 2017
Lizita, Arnova and Safari	Internode	Step 1: MS medium+ 1.0 mg L^{-1} BA +3.0 mg L^{-1} NAA Step 2: MS medium+1.5 mg L^{-1} BA +0.2 mg L^{-1} NAA	Callus formation and shoot regeneration	Ibrahim et al. 2017
Sayaka, Konafubuki, Sakurafubuki, and Norin-1.	Internodes	MS medium+2.63 mg L^{-1} Zeatin +0.27 mg L^{-1} IAA	Maximum regeneration efficiency	Ohnuma et al. 2020
Kufri Chipsona 1, Kufri Pukhraj	Leaf and Internode	MS 2 medium+ 100 mg L^{-1} cefotaxime	Maximum shoot regeneration	Guleria and Kumar 2021
Astanalyk, Monument Kunaev, Tokhtar, and Aksor	Leaf and internode	MS medium+1 mg L^{-1} zeatin+7.0 mg L^{-1} GA$_3$ +0.1 mg L^{-1} IAA	Maximum shoot regeneration	Abeuova et al. 2020
S. tuberosum	Internode	MS medium+4 mg L^{-1} AgNO$_3$ +2.25 mg L^{-1} BA	Shoot regeneration	Adly et al. 2022
Kufri Chipsona 1, Kufri Pukhraj	Leaf and Internode	MS 2 medium+ 100 mg L^{-1} cefotaxime+60 mM sucrose	Maximum shoot regeneration	Guleria and Kumar 2022
Blue Danube, Salad Blue, Violet Negretin, and Violet Queen	Tuber disc	MS medium+ 5 mg L^{-1} NAA+1mg L^{-1} TDZ +1 mg L^{-1} GA$_3$	Maximum shoot regeneration	Nagy et al. 2023

et al. 2008; Rezende et al. 2013; Campos et al. 2016), two stage (Al-Sulaiman 2011; Ghosh et al. 2014; Abd Elaleem 2015; Ibrahim et al. 2017) and three stage (Ahloowalia 1982; Khalafalla et al. 2010; Kumlay and Ercisli 2015; Ijaz et al. 2016; Bisht et al. 2021) process in which the presence of auxin is essential in the first stage of callus induction, from which shoots can be induced in stage 2 (cytokinin rich medium) and stage 3 (for root induction) (Webb et al. 1983; Adelberg and Naylor-Adelberg 2012), which is a very complicated and laborious process. However, researchers have also reported that a one-step medium eliminates the need for a two or three-step process (Kaur et al. 2017; Ohnuma et al. 2020; Guleria and Kumar 2021; Abeuova et al. 2020). In addition to auxin and cytokinin, it has been reported that the addition of $AgNO_3$ (Kaur et al. 2017; Adly et al. 2022), calcium pantothenate (Ghosh et al. 2014), sugars (Srivastava et al. 2017; Guleria and Kumar 2022), and antibiotics (Guleria and Kumar 2021) to auxin-cytokinin rich medium affects the process of shoot organogenesis.

Additionally, sugars are an important component of the regeneration medium. In general, tissues in culture have a heterotrophic mode of nutrition and require an exogenous energy source (Sumaryono et al. 2012; Tauzin and Giardina 2014). Many sugars such as glucose, fructose, maltose, sorbitol, and sucrose have been evaluated, and in the vast majority of instances (> 99%), sucrose has been found beneficial (Calamar and de Klerk 2002; Sridhar and Naidu 2011; Reddy et al. 2019). Sucrose is a disaccharide composed of glucose and fructose units, and it is the only sugar in plants that can be translocated (Lemoine et al. 2013). Reportedly, sucrose maintains osmotic potential, which is also known to affect cell division (Cosic et al. 2021). Cell division in plants is also affected by the energy level, and sucrose is likely to act as a regulator of the cell cycle (Riou-Khamlichi et al. 2000). Furthermore, it provides energy for energy-intensive processes such as shoot organogenesis, rooting, and somatic embryogenesis (Kumar et al. 1999, 2002; Yaseen et al. 2013). Sucrose is also a precursor for the synthesis of essential molecules like amino acids, nucleotides, and structural carbohydrates (Stein and Granot 2019). Notably, it is well established that sucrose acts as a signalling molecule affecting metabolic and developmental processes in plants (Kumar et al. 1999; Leon and Sheen 2003; Gibson 2004; Skylar et al. 2011). Sucrose may influence phytohormone-activated pathways by altering the activity and expression levels of numerous genes (Kushwah and Laxmi 2017; Sakr et al. 2018). Many studies have reported that sucrose affects the expression of many genes like *rolC* promoter, *CycD* cyclins gene, *Bzip* transcription factors, *MyB75*/*PAP15* gene, anthocyanin biosynthesis enzymes, patatin promoter gene, *ATB2/bZIP11* transcription factor and *ZmEREB56* transcription factor (Yokoyama et al. 1994; Riou-Khamlichi et al. 2000; Wiese et al. 2004; Teng et al. 2005; Solfanelli et al. 2006; Naumkina et al. 2007; Ramon et al. 2008; Hummel et al. 2009; Huang and Liu 2002; Cosic et al. 2021).

Antibiotics are other group of molecules that influence shoot regeneration. It has been reported that when optimised shoot regeneration protocols are used for *Agrobacterium*-mediated gene transfer, difficulties in shoot regeneration arise when antibiotics are added to the medium to eliminate *Agrobacterium* (Wiebke et al. 2006; Aggarwal et al. 2011; Ahmad et al. 2012; Tran and Sanan-Mishra 2015). The reduction in regeneration efficiency by 5-100 fold on medium supplemented with antibiotic after co-cultivation with *Agrobacterium* has been reported (Visser 1991; Kaur et al. 2020; Wen et al. 2022). Antibiotics are essential for the removal of *Agrobacterium* after co-cultivation (Aggarwal et al. 2010; Gerszberg and Grzegorczyk-Karolak 2019; Kaur et al. 2020), and without their use, it is impossible to eliminate *Agrobacterium* and achieve shoot regeneration from the transformed tissue. Antibiotics have been reported to affect morphogenetic processes such as shoot organogenesis, somatic embryogenesis, rooting, and callus induction (Yu et al. 2001; Bosela 2009; Stanisic et al. 2018; Ding et al. 2006; Grzebelus and Skop 2014; Meng et al. 2014). Moreover, it has been reported that the effect of antibiotics varies in concentrations, genotypes, cultivars, and explants. So, it is very important to investigate the effect of the antibiotics and sucrose on regeneration (shoot organogenesis and somatic embryogenesis) in target genotype(s) or cultivar(s) to develop an effective protocol to be adopted for *Agrobacterium*-mediated genetic transformations and subsequent shoot regeneration.

2.5.2 Optimisation of *Agrobacterium*-mediated genetic transfer protocol

Agrobacterium-mediated gene transfer method involves the transfer of the T-DNA region containing the gene of interest to the plant genome. In potato, the genetic transformation was first reported in the year 1983 using a wild strain of *A. tumefaciens* (Ooms et al. 1983). In no time, attempts were made to undertake trait-specific manipulation of different cultivars throughout the world (Stiekma et al. 1988; Visser et al. 1989; Jongedijk et al. 1992; Synder and Belknap 1993; Dale and Hampson 1995; Conner et al. 1992; Chakravarty and Wang-Pruski 2010; Sawahel 2002; Felcher et al. 2003; Banerjee et al. 2006; Khatun et al. 2012; Fatahillah et al. 2016; Farhanah et al. 2017). The genetic transformation methods involve the use of an

Table 2.3 *Agrobacterium*-mediated gene transfer protocols optimised in different potato cultivars

Potato cultivar	**Explant**	***A. tumefaciens* strains**	**Medium**			**Response**	**References**
			Preculture	**Co-cultivation**	**Regeneration-cum-selection medium**		
Maris Bard, Maris piper, Desiree, King Edward, Pentland Crown, Record	Stems	Wild strain T37, LBA4060, LBA1501	No preculture	MS medium	MS medium +200 µg mL^{-1} carbenicillin	Shoot formation from gall tumour	Ooms et al. 1983
Tetraploid line, Russet Burbank, Diploid line	Leaf and stems	NR	No preculture	Liquid MS medium	Step 1: MS medium+0.5 mg L^{-1} BA + 2 mg L^{-1} 2,4-D +15 mg L^{-1} glutamine +200 mg L^{-1} kanamycin+500 mg L^{-1} carbenicillin Step 2: MS medium+ 0.5 mg L^{-1} BA+15 mg L^{-1} glutamine+200 mg L^{-1} kanamycin+500 mg L^{-1} carbenicillin	Transformed callus formation and shoot regeneration	An et al. 1986
Tetraploid potato line NDD-277-2	Leaf	LBA4404	TM-2 medium+5.6 µM NAA+2 µM BA	TM-2 medium	Step 1: TM-2 medium+250 mg L^{-1} cefotaxime Step 2: MS medium+217 µM adenine sulphate+1 µM NAA+ 2 µM BA+50	Development of transformed callus and shoot formation	Shahin and Simpson 1986

					mg L^{-1} casein hydrolysate+250 mg L^{-1} cefotaxime+ 50 mg L^{-1} kanamycin+		
Pentland Dell, Maris Piper, Desiree, Golden Wonder, Maris Bard	Tuber discs	LBA4404	No preculture	MS medium+ 3 µM IAA asparatic acid + 5 µM zeatin	MS medium+ 3 µM IAA asparatic acid +5 µM zeatin +200 µg mL^{-1} carbenicillin +100 µg mL^{-1} kanamycin	Transformed shoots were developed only from cvs. Desiree and Pentland	Sheerman and Beaven 1988
Bintje, Desiree	Tuber discs	LBA4404	No preculture	MS+0.01 mg L^{-1} NAA+1 mg L^{-1} zeatin	Step1: MS medium+0.01 mg L^{-1} NAA+1mg L^{-1} zeatin+ 50-100 mg L^{-1} kanamycin+ 200 mg L^{-1} cefotaxime Step 2: MS medium+0.25 mg L^{-1} BAP+0.1 mg L^{-1} GA_3 +50-100 mg L^{-1} kanamycin+ 200mg L^{-1} cefotaxime	Development of transformed shoots	Stiekema et al. 1988
Russet Burbank Bintje, Desiree, Berolina	Leaf	C58C1	No preculture	MS medium+ 30 g L^{-1} sucrose+ 0.5 g L^{-1} MES+ 20 g L^{-1} mannitol)	MS medium devoid of sucrose +200 mg L^{-1} glutamine+20 g L^{-1} glucose+1 mg L^{-1} trans-zeatin+0.1 mg L^{-1} NAA+40 mg L^{-1} adenine sulphate +1g L^{-1} carbenicillin or 0.5g L^{-1} cefotaxime+50-100 mg L^{-1} kanamycin	Development of transformed shoots from callus	De Block 1988

Desiree	Leaf	A136, LBA4404	UM medium+0.25mg L^{-1} kinetin+5 mg L^{-1} 2,4-D	UM medium+0.25 mg L^{-1} kinetin+5 mg L^{-1} 2,4-D	MS medium+1 mg L^{-1} BA+1 mg L^{-1} IAA+10 mg L^{-1} GA_3+200 mg L^{-1} cefotaxime and vancomycin+100 mg L^{-1} kanamycin	Adventitious shoot regeneration from transformed tissues	Tavazza et al. 1989
FL1607, Desiree, Russet Burbank, Superior	Leaf	LBA4404	Basal medium+10 mg L^{-1} GA_3+200 µg L^{-1} NAA +2.24 mg L^{-1} BA	Basal medium+10mg L $^{-1}$ GA3 + 200 µg L^{-1} NAA+2.24 mg L^{-1} BA	Basal medium+10 mg L^{-1} GA_3+200 µg L^{-1} NAA+2.24 mg L^{-1} BA+ 500 mg L^{-1} carbenicillin+ 50 mg L^{-1} kanamycin	Maximum transformed shoots were achieved from FL1607 followed by Desiree, Superior and none in Russet Burbank	Wenzler et al. 1989
Homozygous diploid 79.7322 and 86.040	Leaf and stems	LBA4404	Liquid MS+10 mg L^{-1} NAA+10 mg L^{-1} BA	MS+2.25 mg L^{-1} BA + 0.175 mg L^{-1} IAA	Step 1: MS+2.25 mg L^{-1} BA+0.175 mg L^{-1} IAA+200 mg L^{-1} cefotaxime+50 mg L^{-1} kanamycin Step2: MS+2.25 mg L^{-1} BA+5 mg L^{-1} GA3+200 mg L^{-1} cefotaxime+50 mg L^{-1} kanamycin	Development of transformed shoots from the callus	Visser et al. 1989
Lemhi Russet, Russet Burbank	Tuber disc	PC2760	No preculture	MS medium	MS medium+0.03 mg L^{-1} NAA+1 mg L^{-1} BA+0.5 mg L^{-1} zeatin+200 mg L^{-1} cefotaxime+50 mg L^{-1} kanamycin	Transformed shoots were developed	Ishida et al. 1989

Russet Burbank	Stems	NR	No preculture	1/10 MS medium	Step 1: MS medium+3.0 mg L^{-1} BA+0.01 mg L^{-1} NAA+100 mg L^{-1} kanamycin+ 500 mg L^{-1} carbenicillin Step 2: MS medium+0.3 mg L^{-1} GA$_3$+ 100 mg L^{-1} kanamycin+500 mg L^{-1} carbenicillin	Transformed shoots were regenerated	Newell et al. 1991
Ilam Hardy, Iwa and Rua	leaf	LBA4404	No preculture	MS medium+40 mg L^{-1} ascorbic acid+500 mg L^{-1} casein hydrolysate+0.2mg L^{-1} NAA+2 mg L^{-1} BA	MS medium+40 mg L^{-1} ascorbic acid+500 mg L^{-1} casein hydrolysate+0.2 mg L^{-1} NAA+2 mg L^{-1} BA+250 mg L^{-1} kanamycin+250 mg L^{-1} cefotaxime	Transformed shoots were developed	Conner et al. 1992
S. verrucosum, *S. hjertingii*, *S. papita*, *S. stoloniferum*, *S. demissum*	Tuber disc	C58	No preculture	MS medium+ 1.8 mg mL^{-1} zeatin riboside+0.9 mg mL^{-1} IAA	MS medium+ 1.8 mg mL^{-1} zeatin riboside+0.9 mg mL^{-1} IAA+ 150 mg mL^{-1} kanamycin+250 mg mL^{-1} cefotaxime	Development of transformed shoots	Kumar et al. 1995
Desiree, Pentland Squire	Leaf and stem	LBA4404	MS medium+ 10 mg L^{-1} NAA+ 10 mg L^{-1} ZR	MS medium+0.2 mg L^{-1} NAA+0.02 mg L^{-1} GA$_3$+ 2 mg L^{-1} zeatin riboside	MS medium+0.2 mg L^{-1} NAA+0.02 mg L^{-1} GA$_3$+ 2 mg L^{-1} zeatin riboside+250 mg L^{-1} cefotaxime+50-100 mg L^{-1} kanamycin	Transformed shoots were developed	Kumar 1995

Desiree, Bintje, Kaptah Vandel	Internodes	C58C51	No preculture	MS medium+0.8 mg L^{-1} ZR+ 2 mg L^{-1} 2,4-D	Step1: MS medium+0.8 mg L^{-1} ZR+ 2 mg L^{-1} 2,4-D+125 mg L^{-1} kanamycin+300 mg L^{-1} cefotaxime Step2: MS medium+ 0.8 mg L^{-1} zeatin riboside+ 2 mg L^{-1} GA$_3$+125 mg L^{-1} kanamycin+300 mg L^{-1} cefotaxime	Transformed shoots with no abnormalities	Beaujean et al. 1998
Diacol Capiro and Parda Pastusa	Leaf	LBA4404	Liquid MS medium	MS medium +0.05 % casein hydrolysate, 1 mg L^{-1} GA$_3$, 40 mg L^{-1} ascorbic acid+3 mg L^{-1} zeatin riboside +1 mg L^{-1} IAA	MS medium +0.05 % casein hydrolysate, 1 mg L^{-1} GA$_3$, 40 mg L^{-1} ascorbic acid+3 mg L^{-1} zeatin riboside +1 mg L^{-1} IAA+100-150 mg L^{-1} kanamycin + 500 mg L^{-1} carbenicillin	Shoots were regenerated from transformed callus	Trujillo et al. 2001
E-potato 3, Gannongshu 2	Tuber discs	LBA4404	No preculture	MS medium+1 mg L^{-1} IAA+0.2 mg L^{-1} GA3+0.5 mg L^{-1} BA+2 mg L^{-1} zeatin riboside	MS medium+1 mg L^{-1} IAA+0.2 mg L^{-1} GA$_3$+0.5 mg L^{-1} BA+2 mg L^{-1} zeatin riboside+75 mg L^{-1} kanamycin+400 mg L^{-1} carbenicillin	Highest transformation efficiency was observed	Si et al. 2003

andigena sp. line 7540	Leaf	GV2260	No preculture	MS medium	Step 1: MS medium+ 5mg L^{-1} NAA+0.1 mg L^{-1} BA+250 mg L^{-1} cefotaxime+50 mg L^{-1} kanamycin Step 2: MS medium+2.2 mg L^{-1} zeatin riboside+0.02 mg L^{-1} NAA+0.15 mg L^{-1} GA$_3$+250 mg L^{-1} cefotaxime+50 mg L^{-1} kanamycin	Higher transformation efficiency	Banerjee et al. 2006
Desiree	Internodes	LBA4404	No preculture	MS medium+ 0.2 mg L^{-1} NAA+0.02 mg L^{-1} + GA 3+ 2.5 mg L^{-1} zeatin riboside	Step1: MS medium+ 0.2 mg L^{-1} NAA+0.02 mg L^{-1} + GA$_3$+ 2.5 mg L^{-1} zeatin riboside+500 mg L^{-1} cefotaxime Step2: MS medium +0.02 mg L^{-1} NAA+ 0.02 mg L^{-1} GA$_3$+ 2 mg L^{-1} zeatin riboside+500 mg L^{-1}cefotaxime +50 mg L^{-1} kanamycin	Regeneration of transformed shoots	Millan 2006
Desiree	Internodes	LBA4404	No preculture	MS medium+ 0.2 mg L^{-1} NAA+0.02 mg L^{-1} + GA 3+ 2.5 mg L^{-1} zeatin riboside	Step1: MS medium+ 0.2 mg L^{-1} NAA+0.02 mg L^{-1} + GA$_3$+ 2.5 mg L^{-1} zeatin riboside+500 mg L^{-1} cefotaxime Step2: MS medium +0.02 mg L^{-1} NAA+ 0.02 mg L^{-1} GA$_3$+ 2 mg L^{-1} zeatin riboside+500 mg L^{-1}cefotaxime +50 mg L^{-1} kanamycin	Regeneration of transformed shoots	Millan 2006

Dihaploid genotypes 178/10, 224/1, 227/5, Desiree, Agave and Delikat Norika, 'Baltica'	Leaf, stem	LBA4404	MS medium+ 10 mg L^{-1} NAA+ 10 mg L^{-1} zeatin riboside	MS medium+0.2 mg L^{-1} NAA+0.02 mg L^{-1} GA_3+ 2 mg L^{-1} zeatin riboside	MS medium+0.05 mg L^{-1} biotin, 40 mg L^{-1} adenine, 0.02 mg L^{-1} GA3, 0.02 mg L^{-1}+NAA+2.0 mg L^{-1} zeatin riboside+250 mg L^{-1} cefotaxime+50 mg L^{-1} kanamycin	Regeneration of shoots from transformed callus	Rakosy-Tican et al. 2007
Superior, Bintje, Atlantic, Shepody and Russet Burbank	Leaf, stem	GV3101	MSMO salts+ 0.04 % adenine sulphate+ 0.05 % MES +0.05 % PVP +2 mg L^{-1} 2-4-D+0.8 mg L^{-1} zeatin riboside	MSMO salts+ 0.04 % adenine sulphate+ 0.05 % MES +0.05 % PVP +2 mg L^{-1} 2-4-D+0.8 mg L^{-1} zeatin riboside	Step1: Preculture medium+100 mg L^{-1} kanamycin+300 mg L^{-1} claforan Step2: Preculture medium devoid of 2,4-D+100 mg L^{-1} kanamycin+300 mg L^{-1} claforan	Improved transformation efficiency was observed	Chakravarty and Wang-Pruski 2010
Cardinal, Heera	Leaf internode	LBA4404	MS medium	MS medium	Step1: MS medium+2 mg L^{-1} BA+5 mg L^{-1} NAA+200 mg L^{-1} cefotaxime Step2: MS medium+2 mg L^{-1} BA+5 mg L^{-1} NAA+5-20 mg L^{-1} kanamycin+100 mg L^{-1} cefotaxime	Higher transient GUS expression was recorded	Khatun et al. 2012

Jowon, Atlantic	Leaf stem	GV3101	MS medium +30g L^{-1} sucrose+ 10 mg L^{-1} BA+10mg L^{-1} NAA	MS medium +30g L^{-1} sucrose+2mg L^{-1} 2,4-D	Step1: MS medium+30 g L^{-1} sucrose+0.01mg L^{-1} NAA+0.1 mg L^{-1} GA3+2 mg L^{-1} zeatin+0.5 mg L^{-1} PPT+500 mg L^{-1} carbenicillin Step2: MS medium+16g L^{-1} glucose+0.02 g L^{-1} NAA+0.15 mg L^{-1} GA_3+0.5 mg L^{-1} PPT+500 mg L^{-1} carbenicillin	Highest transformation efficiency on this medium	Han et al. 2015
Desiree	leaf	C58C1	No preculture	MS medium+500 mg L^{-1} MES +2 mg L^{-1} 2, 4-D+0.5 mg L^{-1} zeatin	Step1: MS medium+500 mg L^{-1} MES +2 mg L^{-1} 2, 4-D+0.5 mg L^{-1} zeatin+100 mg L^{-1} kanamycin+300 mg L^{-1} timentin Step2: MS medium+500 mg L^{-1} MES +2 mg L^{-1} GA_3+0.5 mg L^{-1} zeatin+100 mg L^{-1} kanamycin+150 mg L^{-1} timentin	Rapid production of Transgenic plants	Craze et al. 2018
Desiree, Ranger Russet, Umatilla Russet, Alturas, and Yukon Gold	Internode	GV3101, LBA4404	No preculture	MS salt+1 mg mL^{-1} BA+1 mg mL^{-1} NAA	MS salt+ 1 mg mL^{-1} trans zeatin riboside+50 µg mL^{-1} timentin+ kanamycin	Transformed shoot regeneration	Bruce and Shoup Rupp 2019

Kufri Chipsona 1	Leaf, Internode	EHA105, LBA4401	MS+10 µM $AgNO_3$ +10 µM BA+15 µM GA_3	MS+10 µM $AgNO_3$+10 µM BA+15 µM GA_3+100 µM acetosyringone	MS+10 µM $AgNO_3$+10 µM BA+15 µM GA_3+200 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin	Increased rate of transient GUS expression	Kaur et al. 2020
Asterix, Diamant	Internodes, tuber disc	LBA4404	No preculture	MS+ 4.0 mg L^{-1} BAP+1 mg L^{-1} IAA+100 µmol acetosyringone	MS + 4 mg mL^{-1} BAP+ 1 mg mL^{-1} IAA+ 200 mg L^{-1} kanamycin +300 mg L^{-1} carbenicillin.	Transgenic shoots were developed	Mollika et al. 2020
Lady Olympia, cv. Granola, cv. Agria, cv. Désirée, and cv. Innovator	Leaf, internode	LBA4404	No Preculture	MS medium +100 µM acetosyringone	MS medium+2 mg mL^{-1} BA+0.2 mg L^{-1} NAA+2 mg mL^{-1} trans-zeatin +0.1mg L^{-1} GA_3+100 mg L^{-1} kanamycin+300 mg L^{-1} duocid	Shoots were developed from transformed callus	Bakhsh 2020
Spunta	Leaf	EHA105	No preculture	MS medium+0.2 mg L^{-1} NAA+0.02 mg L^{-1} GA_3+2.5 mg L^{-1} zeatin riboside	Step 1: MS medium+0.2 mg L^{-1} NAA+0.02 mg L^{-1} GA_3+2.5 mg L^{-1} zeatin riboside+2.5 mg L^{-1} cefotaxime+100 mg L^{-1} ampicillin Step 2: MS medium+0.2 mg L^{-1} NAA+0.2 mg L^{-1} GA_3+2.5 mg L^{-1} zeatin riboside+400 mg L^{-1} cefotaxime+100 mg L^{-1} ampicillin+82 mg L^{-1} hygromycin	Transgenic shoots were developed from callus	Decima et al. 2020

optimised shoot organogenesis protocol for callus induction and regeneration of transgenic lines. Moreover, it has been reported that successful genetic transformation is not only dependent on the shoot regeneration protocol but also upon many other factors, mainly explant type, preculture period, infection time, *Agrobacterium* strain, co-cultivation period, and antibiotics in the selection medium (Heeres et al. 2002; Aggarwal et al. 2011; Mehrota and Goyal 2012; Kaur et al. 2020). Antibiotics play an important role in the selection of transformants and subsequent elimination of *Agrobacterium* from culture after co-cultivation (Aggarwal et al. 2011; Kaur et al. 2020). In majority of the studies, kanamycin is used for the selection of transformants (Craze et al. 2018; Bruce and Shoup Rupp 2019; Kaur et al. 2020; Mollika et al. 2020; Banfalvi et al. 2020; Kaur et al. 2022); however, ampicillin and hygromycin have also been used in some studies (Song et al. 2003; Decima et al. 2020; Listanto et al. 2020). Antibiotics such as carbenicillin cefotaxime, timentin, and duocid reportedly eradicate *Agrobacterium* from the culture (Craze et al. 2018; Bruce and Shoup Rupp 2019; Kaur et al. 2020; Mollika et al. 2020; Bakhsh 2020). Several studies have demonstrated the use of various explants such as leaf (Craze et al. 2018; Bakhsh 2020; Kaur et al. 2020; Decima et al. 2020), internodes (Kaur et al. 2020; Bakhsh 2020; Bruce and Shoup Rupp 2019; Mollika et al. 2020;) and tuber disc (Ishida et al. 1989; Kumar 1995; Si et al. 2003; Mollika et al. 2020); however, it has been reported that the susceptibility of tissues, organ and cell types within a plant to *Agrobacterium* may vary, thereby affecting the transformation efficiency. In addition, *Agrobacterium* strain LBA4404 was utilised in the majority of the studies. Studies have also emphasised the significance of preculture and co-cultivation for the efficient transfer of T-DNA into plant genomes. Furthermore, all *A. tumefaciens*-mediated genetic transformation protocols are cultivar-specific (De Block 1988; Ishida et al. 1989; Dale and Hampson 1995). Therefore, utilising the same transformation protocol for every cultivar will result in lower transformational frequencies, which is a bottleneck for efficient crop improvement programs (Trujillo et al. 2001; Chakravarty et al. 2007; Han et al. 2015). Thus, to optimise genetic transformation efficiencies, all factors must be optimised for each cultivar to ensure efficient delivery of the desired transgene.

Chapter 3

Materials and Methods

3.1 Plant material and culture conditions

In this study, cultures of *Solanum tuberosum* L. cvs. **‘Kufri Pukhraj’** and **‘Kufri Chipsona 1’** were used. These cultivars were chosen because of their economic importance (cv. ‘Kufri Chipsona 1’is used in the process industry, and cv. ‘Kufri Pukhraj’ is a table variety) and susceptibility to late blight (cv. ‘Kufri Chipsona 1’ is moderately resistant, and cv. ‘Kufri Pukhraj’ is susceptible). Shoot cultures of these two cultivars were available at the Plant Tissue Culture Laboratory of TIFAC-CORE, Thapar Institute of Engineering & Technology, and were being maintained on basal MS medium (Appendix I). These cultures were grown at 25 ± 1 °C with 16 h light/8 h dark cycle under 42 µmol m^{-2} s^{-1} light intensity provided by white fluorescent lights (Philips India Ltd., Mumbai).

3.2 Chemicals, glassware, and plasticware

All plant tissue culture grade chemicals such as macronutrients, micronutrients, plant growth regulators, other chemicals and single-use disposable sterilization filters (0.22 µm) were procured from HiMedia Laboratories Mumbai (India). All molecular biology grade chemicals such as deoxynucleoside triphosphates (dNTPs), Taq polymerase, restriction enzymes, T4 DNA ligase, and cDNA synthesis kit were procured from Thermo Fisher Scientific India Pvt. Ltd. Mumbai (India). The plasmid isolation and gel purification kits were purchased from HiMedia Laboratories Mumbai (India). Glasswares such as Erlenmeyer flasks, beaker, measuring cylinder, and test tubes were procured from Borosil Glass Works Limited, India. Culture bottles of 300 mL capacity were procured from Kasablanka Corporation, Mumbai (India). Plasticwares such as Petriplates, Eppendorff tubes, micropipette tips, and measuring cylinders were purchased from Tarsons Products Private Limited (India).

3.3 Medium preparation and shoot multiplication

The shoot cultures of *Solanum tuberosum* L. cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ were retrieved from the plant tissue culture lab of TIFAC-CORE, Thapar Institute of Engineering & Technology, and multiplied on basal MS medium, 3% (w/v) sucrose, additionally supplemented with 10 µM $AgNO_3$ (MS1 medium) (Kaur et al. 2017) to achieve fully expanded leaves. The pH of the medium was adjusted in a range between 5.75-5.80 with the help of 0.1 N HCL or NaOH. The medium (30 mL) was poured into 300 mL culture bottles containing 210 mg agar (0.7% (w/v) agar and subjected to autoclave at 121°C for 20 min at 15 psi. The nodal segments of actively growing shoot cultures of selected cvs. were excised and inoculated on MS1 medium. Cultures were incubated at 25 ± 1 °C and were regularly sub-cultured every 21 days.

3.4. Effect of antibiotics on shoot regeneration

Leaf and internodal explants of 21day old microshoots were cultured on MS medium supplemented with 10 µM $AgNO_3$, 10 µM 6-benzyl adenine (BA) and 15 µM gibberellin acid (GA_3) (MS2 medium; Kaur et al. 2017) and further supplemented with different concentrations (0, 100, 200, 300, 400, 500 mg L^{-1}) of cefotaxime, carbenicillin, and cephalexin. Filter-sterilized antibiotics were added to autoclaved medium when it had cooled down to less than 45 °C. Leaf explants were cultured facing the adaxial surface towards the medium, and internodes were cultured horizontally on the surface of the medium. Results were recorded after 5-6 weeks for scoring the percent explants showing shoot regeneration and the mean number of shoots per explant.

Table 3.1 The equations used for calculating the various shoot regeneration parameters

Parameters	***Equation***
Shoot regeneration percentage (%)	$\frac{\text{No. of explants showing shoot organogenesis}}{\text{Total no. of explants inoculated}} \times 100$
Mean number of shoots per explants	$\frac{\sum A_1 + A_2 + A_3 \dots . A_n}{n}$
Where A_1, is no. of shoots induced in explant 1 and n is the number of explants	

3.5 Effect of sucrose on shoot regeneration

Leaf and internodal explants from 21 days old micro shoots were inoculated on MS2 medium supplemented with 100 mg L^{-1} cefotaxime antibiotic (MS3 medium; Guleria and Kumar 2021) and further supplemented with different concentrations of sucrose (30, 60, 90, 120, 150 mM). Leaf explants were cultured facing the adaxial surface towards the medium, and internodes were cultured horizontally on the surface of the medium. The percentage of explants showing shoot regeneration and the mean number of shoots per explant were recorded after 4-5weeks of culture. These two parameters were calculated using equations described in Table 3.1.

For biochemical and gene expression studies, separate experiments were performed, and pooled samples were harvested after different days of culture (0, 7, 14, 21, and 28 days) for each sucrose concentration. The samples were dried in the oven at 80 °C for 2 weeks or with liquid N_2 and subsequently stored at − 80 °C in a deep freezer till analyses.

3.6 Biochemical assays

3.6.1 Estimation of soluble sugars

Oven-dried samples of about 50 mg were extracted in 8 mL of 80% (v/v) aqueous ethanol for 3 h. The extract was centrifuged at 10,000 ×g for 10 min at 4 °C. The supernatants were collected and the extraction process was repeated two times. Finally, the supernatants collected at each extraction process were pooled and the final volumes were made up to 25 mL for the estimation of soluble sugars. The pellets were used for the estimation of starch content.

For the estimation of total soluble sugar, the protocol given by Dubois et al. (1956) was followed. The reaction mixture consisted of 500 µL extract, 10 µL of phenol reagent (90% w/v aqueous phenol), 5 mL concentrated sulphuric acid, water was added to make up a volume of 6 mL. The resultant mixture was incubated for 10 min before taking absorbance at 485 nm using a UV-Vis spectrophotometer (U-2900, Hitachi, Tokyo, Japan). The concentration of the total soluble sugar was calculated by standard curve of D-glucose expressed in mg g^{-1} dry weight (DW).

For the estimation of reducing sugars, a protocol of Sumner and Howell (1935) was followed. The reaction mixture was prepared by adding 500 µL extract to 1 mL of dinitrosalicylic acid (DNSA) reagent (43.8 mM DNSA, 0.4 M NaOH, 1.06 M sodium/potassium tartarate) and 500 µL dH_2O. The mixture was boiled for 10 min in a water bath and then cooled to room temperature. The absorbance was recorded at 560 nm and concentrations of reducing sugars were calculated using D-glucose as standard and expressed in mg g^{-1} DW.

3.6.2 Estimation of starch

A pellet obtained during the extract preparation of soluble sugar was used for determining the starch content. The pellet was dissolved in 1mL solution of 80% (v/v) dimethyl sulfoxide and 8 N hydrochloric acid in a ratio of 8:2, respectively. The mixture was incubated at 60 °C for 30 min, then cooled to room temperature. After centrifugation (13,000 ×g for 10 min), the supernatant was collected and used as an extract to estimate starch content by the method of Lebon et al. (2016). A 100 µL extract was added to 100 µL Lugol’s solution (38.3 mM KI and

2.8 mM I_2 in 0.25 M HCL). After 15 min, the absorbance was recorded at 620 nm and concentrations were calculated using starch as standard and expressed in mg g^{-1} DW.

3.6.3 Enzyme activity

3.6.3.1 Extract preparation

For the preparation of enzyme extract, the modified protocol of Nakamura and Yuki (1992) was followed. Samples (1 g FW each) were ground in a mortar pestle and 10 mL of chilled buffer (8 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 100 mM Tris HCL; pH 6.5) was added to these, the homogenates were then centrifuged (10,000 ×g, 4 °C for 15 min). The supernatants were used for the estimation of total soluble protein content and enzyme activities, whereas the pellets were washed and suspended in a chilled buffer for the estimation of cell wall-bound invertase activity. All the procedures were performed at 0-4 °C. For protein estimation, the above extracts (5 mL) were mixed with an equal volume of 20% (w/v) chilled trichloro-acetic acid (TCA) and incubated at 4 °C for 4 h. The mixtures were centrifuged for 15 min (10,000 ×g, 4 °C). The pellets were dissolved in a 3 mL of 1 N NaOH solution and used for estimation of total soluble proteins (Lowry et al. 1951). To 500 µL of dissolved pellet, 5 mL of solution C (Appendix II) was added and incubated at room temperature for 10 min. To this mixture, 0.5 mL of solution D (Appendix II) was added and again incubated at room temperature for 30 min. Absorbance was taken at 750 nm. Total soluble proteins were estimated using standard curve prepared by taking known concentration of Bovine serum albumin.

3.6.3.2 Acid invertase

The protocol given by Dreier et al. (1998) was followed to estimate the activity of cell wall-bound and cytosolic acid invertase. For cell wall-bound invertase, pellets dissolved in chilled buffer were used as an enzyme extract, whereas for cytosolic invertase, the supernatants were used as an enzyme extract as mentioned above. For estimation of enzyme activity, 100 µL of the extract was added to a reaction mixture containing 400 µL of acetate buffer (0.2 M; pH 4) and 800 µL sucrose (0.225 M). The reaction mixture was incubated at 30 °C for 30 min and stopped by the addition of 1mL DNSA reagent (43.8 mM DNSA, 0.4 M NaOH,1.06 M sodium/potassium tartrate). Glucose (0.5 µmol) was added to avoid oxygen interference at lower reducing sugar concentration, and the reaction mixture was boiled for 10 min and cooled to room temperature. The activity of invertase was calculated as a mean of absorbance at 560 nm and expressed in U mg^{-1} protein where 1unit of enzyme is defined as the μmol glucose formed mg^{-1} protein min^{-1}.

3.6.3.3 Sucrose synthase

The activity of sucrose synthase was measured by the protocol of Kubo et al. (2001). An aliquot 200 μL of enzyme extract was added to 50 μL reaction mixture (0.05 M Tris HCl, pH 7.5 containing 50 mM sucrose). The resultant solution was then incubated for 30 min at 25 °C, and the reaction was terminated by adding 250 μL of 1 N NaOH and then boiling for 10 min. The blank contained 1 N NaOH in place of the enzyme extract. After the assay, hexoses were destroyed by heating at 100 °C for 10 min. An aliquot of 50 μL was added to 3 mL of 0.15% anthrone reagent prepared in 13.7 M H_2SO_4. The mixture was incubated at 40 °C for 20 min, and the absorbance was measured at 620 nm after cooling to room temperature. The activity of sucrose synthase was calculated as a mean of absorbance at 620 nm and expressed in U mg^{-1} protein where 1unit of enzyme is defined as the μmol sucrose hydrolyzed mg^{-1} protein min^{-1}.

3.6.3.4 α- amylase

To estimate the activity of α-amylase modified protocol of Salas and Cardemil (1986) was followed. The 1 mL of enzyme extract was heated at 70 °C for 15 min (to inactivate the activity of β- amylase) followed by centrifugation (10,000 ×g, 15 min). After centrifugation, 100 μL of supernatant was suspended in a reaction mixture containing 0.15% (w/v) starch, 20 mM $CaCl_2$, and 50 mM NaOH prepared in 50 mM sodium acetate buffer; pH 4.8. The reaction mixture was incubated at 37 °C for 60 min, and then stopped by the addition of 800 μL of Lugol's solution. The activity of α- amylase was calculated as a mean of absorbance at 620 nm and expressed in U mg^{-1} protein where 1unit of enzyme is defined as the μmol starch hydrolysed mg^{-1} protein min^{-1}.

3.6.3.5 β-amylase

The activity of β-amylase was measured by the modified protocol of Okamoto and Akazawa (1979). An enzyme extract of 200 μL was suspended in 1% (w/v) starch and 0.78 mM EDTA prepared in 50 mM citrate buffer (pH 3.6; to inhibit the activity of α- amylase) followed by incubation at 20 °C for 60 min. The reaction was stopped by the addition of 400 μL of DNSA reagent (43.8 mM DNSA, 0.4 M NaOH, 1.06 M sodium/potassium tartrate). The reaction mixture was incubated at 95 °C for 5 min, and absorbance was measured at 540 nm. The activity of β-amylase was calculated as a mean of absorbance at 540 nm and expressed in U

mg^{-1} protein where 1unit of enzyme is defined as the μmol maltose formed mg^{-1} protein min^{-1}.

3.7 Gene expression studies of sucrose and starch metabolizing enzymes by quantitative Real-time PCR (qRT-PCR)

3.7.1 RNA isolation

Total RNA was extracted from each sample according to the protocol given by Chang et al. (1993). Briefly around 100 mg of plant tissue was grounded in liquid N_2 and transferred to DEPEC treated centrifuge tube. The samples were then suspended in 1 mL prewarm CTAB extraction buffer (60 °C) (Appendix II) followed by the addition of 40 μL of β-mercaptoethanol. Incubation was performed for 20 min at 65 °C. The chloroform: isoamyl (24:1) extraction was done twice, followed by centrifugation at 12000 ×g for 10 min. To the aqueous phase 1/4th volume of $LiCl_2$ (10 M) was added and kept overnight at 4 °C for precipitation of RNA. After overnight incubation, RNA was pelleted by centrifugation at 12000 ×g for 30 min at 4 °C, the pellet was washed twice with 75% ethanol and centrifuged (12000 ×g at 4 °C for 10 min). The pellet was dissolved in 25 μL DEPEC treated water and stored at -80 °C. The quality of total RNA was determined on 2% (w/v) agarose gel stained with 0.5 μg mL^{-1} ethidium bromide (EtBr) and quantity was measured using nanodrop spectrophotometer.

3.7.2 cDNA preparation and qRT-PCR

The first strand cDNA was synthesised using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Mumbai) using oligo dT_{18} primer. The reaction mixture was prepared according to the manufacturer's protocol briefly by adding 2 μg of template RNA and 1 μL of oligo dT primer (100 μM) followed by the addition of water to make final volume 12 μL. Incubation was done at 65 °C for 5 min to denature RNA and then cooled on the ice. The remaining components the of kit were added in the following order which includes 4 μL 5X reaction buffer, 1 μL ribolock RNAase inhibitor (20 U μL^{-1}), 1 μL dNTP mix (10 mM), 1 μL RevertAid M-MuLV RT (200 U μL^{-1}) and final volume made upto 20 μL. The reaction mixture was incubated at 42°C for 60 min and the reaction was terminated by heating at 70 °C for 5 min and used directly for qRT-PCR.

For Real time qRT-PCR studies, 10 μL reaction mixture consisting of 5 μL Maxima SYBR Green/ROX qPCR master mix, 3 μL cDNA (100X diluted), 1 μL of each forward and reverse primer (0.2 μM) and the remaining volume made upto 10 μL with nuclease-free water. A list of primer pairs used for different genes is mention in Table 3.2. Quantitative RT-PCR was performed with amplification conditions; initial denaturation at 94 °C for 2 min, 40 cycles for denaturation at 94 °C for 15 s, annealing at 54 °C for 30 s, and extension at 72 °C for 20 s in a Realplex 2.2 real-time PCR system (Eppendorf AG, Hamrung). Amplified products were separated on 2% (w/v) agarose gel. The gel was visualised and photographed using a UV transilluminator 2000 (Bio-Rad, USA). The relative fold expression of selected genes of sucrose and starch metabolism was calculated using $2^{-\Delta\Delta Ct}$ method with respect to *β-actin*, elongation factor *(EF)-1α*, exocyst component *Sec3A* as reference genes (Schmittgen and Livak 2008).

$\Delta\Delta C_t = (C_{target} - C_{reference}) - (C_{control} - C_{reference})$

where C_{target} is the threshold value of a particular gene at a particular treatment. $C_{control}$ is the threshold value of a particular gene in the absence of treatment, $C_{reference}$ is the average threshold value of three different reference genes.

Table 3.2 Sequence of primer pairs used for qRT-PCR analysis of different gene

Gene	***Sequence (5'-3')***	
Sucrose synthase	Forward Reverse	GTTGGCGGAGATCGAAGGAA AATGCAGGCTGAACGAAAGC
Acid invertase	Forward Reverse	TTCAGTACCCGTGTTGGACG ATCTTGACGGAGGCAGTCAC
α-amylase	Forward Reverse	TGGATCAACTCAGGCGCATT TGAACGGCTGTGTATGCCTT
β-amylase	Forward Reverse	CTTTTGCACAAGCCAAGCCA TGGCCTGTTCAAGTTCCCTC
β-actin	Forward Reverse	AGGAGCATCCTGTCCTCCTAA CACCATCACCAGAGTCCAACA
(EF)1-α	Forward Reverse	GATGGTCAGACCCGTGAACA CCTTGGAGTACTTCGGGGTG
Sec3A	Forward Reverse	GCTTGCACACGCCATATCAAT TGGATTTTACCACCTTCCGCA

3.8 Clonal fidelity of regenerated lines

3.8.1. DNA isolation

Total DNA was isolated from the microshoots of the mother plant and regenerated shoots according to the CTAB method described by Doyle and Doyle (1990). Leaf samples (1.5-2.0 g FW) were crushed to a fine powder in liquid nitrogen and transferred to a 50 mL polypropylene tube. A prewarmed CTAB extraction buffer (60 °C) (Appendix II) along with 0.2% (v/v) β-mercaptoethanol was added followed by incubation at 60 °C for 1 h. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the slurry and mixed properly. Centrifugation was done at 5000 ×g for 10 min at 4 °C. To supernatant, 0.66 volume of cold isopropanol was added and DNA was precipitated followed by incubation at -20 °C for 1 h. Again, centrifugation was done at 10000 ×g for 15 min and the pellet was dissolved in 1 mL TE buffer. For purification, 2 µL RNAase enzyme (10 µg mL^{-1}) was added to the above-isolated DNA followed by incubation at 37 °C for 1 h. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifugation was done at 10000 ×g for 10 min at 4 °C. After this, an aqueous layer was pipette out into a fresh vial and 0.3 volume of 3 M sodium acetate and 0.6 volume of chilled isopropanol was added followed by incubation at -20 °C for 1 h. Centrifugation was done at 10000 ×g for 10 min and the pellet was dissolved in 30 µL TE buffer.

3.8.2 Qualitative and quantitative estimation of isolated DNA

For qualitative estimation, agarose gel electrophoresis was performed. A 0.24 g of agarose (0.8% w/v) was dissolved in 30 mL of 0.5 X Tris-acetate EDTA (TAE) buffer. The agarose was dissolved by boiling in a microwave oven for 1 min. The molten agarose was cooled to room temperature and 1 µL EtBr (0.5 µg mL^{-1}) was added. The agarose mixture was poured into the gel casting tray after inserting the comb and allowed to solidify. Later on, the comb was removed and the gel casting tray was loaded to an electrophoretic tank containing 0.5 X TAE buffer. A 2 µL of DNA sample was mixed with 1 µL bromophenol dye (6 X) and loaded into the well. The lid of the electrophoretic tank was closed and voltage (50 V) was applied for 30-45 min. Once the loading dye has travelled the appropriate distance the power was turned off and the gel was visualized under a UV transilluminator. The quantity of isolated DNA was measured using Nanodrop 1000$^{\text{TM}}$ UV/VIS spectrophotometer (Thermo Fisher Scientific, USA). Before measurement, the optical surfaces of the instrument were wiped off with lab wipes. ND1000 software installed on the PC was opened and under the nucleic acid section, dsDNA was selected. After setting instrument zero with blank (1 µL TE buffer), the concentration of isolated DNA samples was measured.

3.8.3 Amplification with RAPD and ISSR molecular marker

The clonal fidelity was checked using RAPD and ISSR markers. A total of 10 RAPD and 10 ISSR primers were tested, and those showing amplification were selected for the analysis. All DNA samples were diluted to a concentration of 40 ng uL^{-1} before amplification. PCR amplifications were performed in 20 µL reaction volume consisting of 2 µL PCR buffer (10 X), dNTPs (100 µM), primer (10 µM), template DNA (40 ng), Taq DNA polymerase (1 U) and Milli-Q water to make up the volume to 20 µL. Samples were amplified using gradient PCR (Applied Biosystems Veriti® 96-well thermal cycler: Life Technologies, Singapore) (Kaur et al. 2017). Amplification conditions were initial denaturation at 94 °C for 4 min, 41 cycles of denaturation at 94 °C for 45 s, annealing at 38 °C for 1 min for RAPD (55 °C for ISSR), extension at 72 °C for 2 min and final extension at 72 °C for 5 min. Amplified products were separated on agarose gel 1% (w/v) containing 1 µL of ethidium bromide (EtBr; 0.5 µg mL^{-1}) and visualized under a UV transilluminator (Gel Doc Mega: Biosystematics USA) and photographed.

3.9 Isolation of *Phytophthora infestans*

Late blight infected leaf samples were collected from potato fields of Thapar Institute of Engineering and Technology. A single lesion part of the infected leaf was cut into small pieces and washed with autoclaved dH_2O followed by surface cleaning using Tween 20 detergent. Explants were then washed with autoclaved dH_2O for 5-6 times to completely remove the detergent, blot dried on sterilized tissue paper and inoculated on water agar plates (2% w/v agar) supplemented with 30 µg mL^{-1} rifamycin and 10 µg mL^{-1} natamycin (Sobkowiak and Sliwka 2017). After 3-4 days, mycelium growth was observed and the single hyphal tip was transferred to potato dextrose agar (PDA) medium (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, 15 g L^{-1} agar) supplemented with above mentioned antibiotics or on disinfected potato tuber slices placed in moist chamber. Cultures were specifically maintained at a temperature of 17 °C in dark conditions and various growth parameters such as colony colour and hyphae growth patterns were observed.

3.9.1 Morphological and molecular confirmation

A small part of mycelium was scraped from PDA plate and transferred to a clean microscopic slide containing a drop of dH_2O. A cover slide was placed and the slide was observed at 10X and 40X magnification under compound microscope. Various morphological parameters such as the shape and size of spores, sporangium, and hyphae patterns were observed. Images were captured using Nikon digital camera (Nikon Corporation, Japan) attached to a microscope and NIS element imaging software suite.

To confirm the identity of *P. infestans*, the internal transcribed spacer (ITS) region was amplified. For that, a hyphae plug was inoculated in potato dextrose broth (PDB) (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, pH-5.1) medium amended with 30 µg mL^{-1} rifamycin and 10 µg mL^{-1} natamycin antibiotics. The culture was grown for 4-5 weeks at 17 °C, and filtered to isolate DNA using CTAB method described by Doyle and Doyle (1990). A typical 20 µL reaction consisted of 2 µL PCR buffer (10X), dNTPs (100 µM), primer (10 µM), template DNA (40 ng), Taq DNA polymerase (1U) and Milli-Q water to make up the volume. Universal primer pair forward (5'-GCGTTGGGACTCCGGTCTGAGC -3') and reverse (5'-CGCCACAGGAGGAAAATCAC -3') was used for amplification of ITS region. Samples were amplified using Veriti® 96-well thermal cycler (Life Technologies, Singapore). Amplification conditions performed were initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. Amplified products were separated on agarose gel 1.2% (w/v) containing 1µL EtBr (0.5 µg mL^{-1}) and visualized under a UV transilluminator (Gel Doc Mega: Biosystematics USA). The amplified band was gel-purified sequenced and annotated using Biosoft software. The nucleotide sequence obtained after annotation was subjected to BLAST analysis to find the closely related sequence. Finally, a phylogenetic tree was constructed using the neighbor-joining method with MEGA6 software (Tamura et al. 2013) by adjusting the bootstrap value up to 1000 replications.

3.10 Evaluation of potato cultivar for late blight resistance

The cultures of Indian potato cultivars 'Kufri Pukhraj' (KP), 'Kufri Chipsona 1' (CS-1), 'Kufri Jyoti' (KJ), and 'Kufri Surya' (KS) were evaluated for late blight susceptibility.

3.10.1 Hardening of plants in the transgenic green house

After 15-20 days of culture on MS1 medium, the well-rooted plantlets were transferred to protrays (49.5×29.3 cm; having 209 cells of 1.8 cm diameter each) filled with coco-peat and soil (1:1) mixture for 15 days. These trays were kept in the transgenic greenhouse at optimum temperature (25±1 °C) with relative humidity of >80%. After 1 week, the plantlets were sprayed with basal MS medium (devoid of organic supplements) to ensure proper growth. After 2 weeks, the plantlets in pro-trays were planted in plastic poly bags. Once the plants were fully grown, the detached leaf and whole plant assays were performed to check the susceptibility of potato cultivars (Kaur et al. 2020). Based on the disease incidence and disease severity parameters the susceptible cultivars were selected for present study.

3.10.2 Preparation of *P. infestans* sporal suspension

A mycelium plug of *P. infestans* was scraped from the PDA plates and inoculated on fully grown leaves of susceptible cultivars placed in a moist chamber at 17 °C. After 7-10 days, sporangium was harvested by flooding the mycelium with 100 mL of cold sterile dH_2O. The sporal suspension was filtered with mesh 40 and further diluted to get desired sporal density (5×10^4 spores mL^{-1}) by adding sterile dH_2O (Karki and Halterman 2021). The sporal density within the suspension was determined using a cell counting chamber. After that, the sporal suspension was incubated at 4 °C for 5-6 h to release zoospores.

3.10.3 Detached leaf assay

Healthy fully grown leaves of ‘KP’, ‘CS-1’, ‘KJ, and ‘KS’ were excised and placed in a moist chamber. Sporal suspension of 10 µL (5×10^4 spores mL^{-1}) was placed on the abaxial sides of leaves and incubated at 20 °C. After inoculation, leaves were monitored for brown and water-soaked lesions, and lesion diameter was measured to check the disease resistance (Namukwaya 2015).

3.10.4 Whole plant assay

The healthy fully grown plants in a transgenic greenhouse were evenly sprayed with *P. infestans* sporal suspension using a spray pump. The temperature of the transgenic house was maintained at 17 ℃ with a relative humidity of 90-95% using humidifiers. Symptoms were recorded at 5 and10 days post inoculation (dpi) to record disease incidence and disease severity.

Table 3.3 The equations used to record disease incidence and disease severity

Parameters	**Equation**
Disease incidence	$\frac{\text{No. of plants infected}}{\text{Total no. of plants}} \times 100$
Disease severity	$\frac{\text{No. of leaves showing disease per shoot}}{\text{Total no. of leaves}} \times 100$

3.11 Generation of RNAi construct(s) expressing hpRNAs

3.11.1 Selection and amplification of target sequence

The nucleotide sequence of four cellulose synthase (*CesA*) genes of *P. infestans* available on National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) under accession number >EF563993.1 (*CesA1*), >EF563994.1 (*CesA2*), >EF563995.1 (*CesA3*), >EF563996.1 (*CesA4*) were downloaded. These were aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/) software to find the conserved region. The region was then matched with the potato, potato *CesA* genes, and other available potato genes to find the non-homologous sequence for the construction of two hp-RNAi construct(s). The selected target sequences (~200bp) were amplified in sense and antisense orientation with specific primers and cloned in the hp-RNAi plasmid. For sense *AscI* and *SwaI* whereas for antisense *BamHI* and *XbaI* restriction enzymes were used. A list of the target sequences and primer pairs has been enlisted in the Table 3.4. Amplification of both sense and the antisense fragments was carried out in 20 µL reaction volume comprised of 2 µL PCR buffer (10X), dNTPs (100 µM), primer (200 µM), template DNA (40 ng), Taq DNA polymerase (1U) and Milli-Q water to make up the volume. The amplification conditions followed and the size of the amplified band is described in the Table 3.5. The amplified products were gel purified using HiPurA® quick gel purification kit (Himedia) and sequenced to confirm the correct identity of the sequence. The nucleotide sequence obtained was subjected to BLAST analysis to find the closely related sequence.

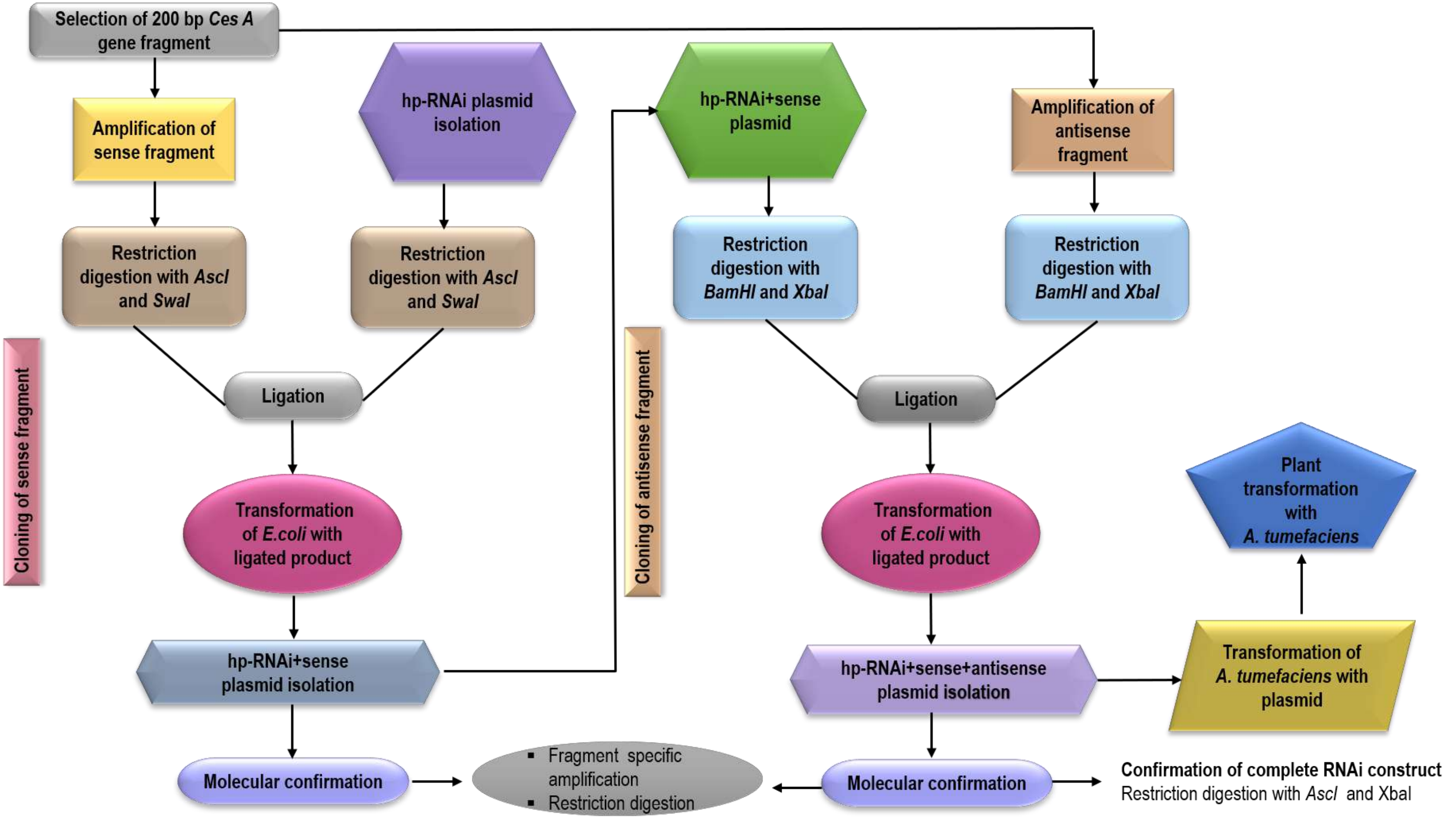


Fig 3.1. Flow chart for development of hp-RNAi construct(s)

Table 3.4 The amplified target sequences and corresponding primer pairs used to amplify sense and antisense fragments

Construct-I	
Target Sequence 5’GATGGCTGCTGATGGTGTCTCGCGTCTCATCGACTCTGGCGCTGTGCTGTCGA CTGTGGCCTACACGTCGATCCAGGACGGCGGCAAGGCGGCAATGGACGGCATC GACGCTAGCGCCCAGACGCTCATGTTCGTGATCTGGGGCGCAGTCTTCGTGGTC GGCGGCCTCAACCAGCTGTCGATGCGCTGGGG 3’	
Sense	*Forward:* 5’*AGGCGCGCCT*GATGGCTGCTGATGGTGTCTC 3’ *AscI* site: AGG/CGCGCCT *Reverse:* 5’ *GCTTAATTTAAAT*CCCCAGCGCATCGACAGC 3’ *SwaI* site: GCTTAATTT/AAAT
Antisense	*Forward:* 5’*CGGGATCCCG*CCCCAGCGCATCGACAGC 3’ *BamH1* site: CGG/GATCCCG *Reverse:* 5’*GCTCTAGAGC*GATGGCTGCTGATGGTGTCTC 3’ *XbaI* site: GCT/CTAGAGC
Construct-II	
Target Sequence 5’GGTGTTCTCTACTATCTTGGTATGTGGAAGCCCGTGCGTCGTGGCGCCCACTA CTTCGACGAGTCGAGCCGCCCGTCCCGGATGATCTGTGGCCCAAGGTCGATGTG CTGTTGTGTCACTACTCTGAGCCGGCTGAAGAGACGATCGACACGTTGATGGCC TGTATGAACCTGCAGTACCCTCCGCAC 3’	
Sense	*Forward:* 5’ *AGGCGCGCCT* GGTGTTCTCTACTATCTTGGTATG 3’ *AscI* site: AGG/CGCGCCT *Reverse:* 5’ *GCTTAATTTAAAT* GTGCGGAGGGTACTGC 3’ *SwaI* site: GCTTAATTT/AAAT
Antisense	*Forward:* 5’*CGGGATCCCG* GTGCGGAGGGTACTGC 3’ *BamH1* site: CGG/GATCCCG *Reverse:* 5’*GCTCTAGAGC* GGTGTTCTCTACTATCTTGGTATG 3’ *XbaI* site: GCT/CTAGAGC

Table 3.5 Various amplification parameters used to amplify sense and antisense fragments and the size of the amplified fragments

Steps	Temperature	Time	Cycles	Size
Initial denaturation	94 °C	4 min	1	***Construct I*** Sense: 215 bp Antisense: 212 bp ***Construct II*** Sense: 211 bp Antisense: 208 bp
Denaturation	94 °C	45 s	30	
Annealing	Construct I: 68 °C Construct II: 55 °C	45 s		
Extension	72 °C	45 s		
Final Extension	72 °C	5 min	1	

3.11.2 Hairpin (hp)-RNAi plasmid

The hp-RNAi plasmid was kindly provided by Prof. M V Rajam; Delhi University. The size of the plasmid is 12,274 bp (Fig. 3.2). The T-DNA region of the plasmid contains a cauliflower mosaic virus (CaMV) 35S promoter, an octopine synthase gene (OCS) terminator, multiple cloning sites, a kanamycin resistance gene as a selection marker, and a CHSA intron, which is a non-translatable sequence that facilitates stabilization of inverted-repeat transgenes by producing a hairpin loop structure after transcription.

3.11.3 Isolation of hp-RNAi plasmid

The plasmid DNA was isolated based on the alkaline lysis method (Birnboim and Doly 1979). A transformed colony of *Escherichia coli* (*E. coli*) DH5α cells (containing hp-RNAi plasmid) were inoculated into 3 mL of luria broth (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride, pH-7) containing 50 µg mL^{-1} kanamycin antibiotic. The culture was incubated at 37 °C overnight on a rotary shaker at 120 rpm. A 2 mL of the above bacterial culture were taken in each tube and these tubes were centrifuged (8000 ×g; 5 min) and bacterial pellets were resuspended in 200 µL of ice-cold Solution I (50 mM glucose; 25 mM Tris HCL (pH 8); 10 mM EDTA, pH 8). Further 200 µL of freshly prepared Solution II (0.2 N NaOH; 1% SDS) was added and mixed by gentle inversion of the tubes (5 times). The tubes were kept on ice for 5 min. Finally, 300 µL of ice-cold Solution III (3 M Potassium acetate) was added and mixed gently. The tubes were kept on ice for 10 min and centrifuged (12000 ×g; 10 min).

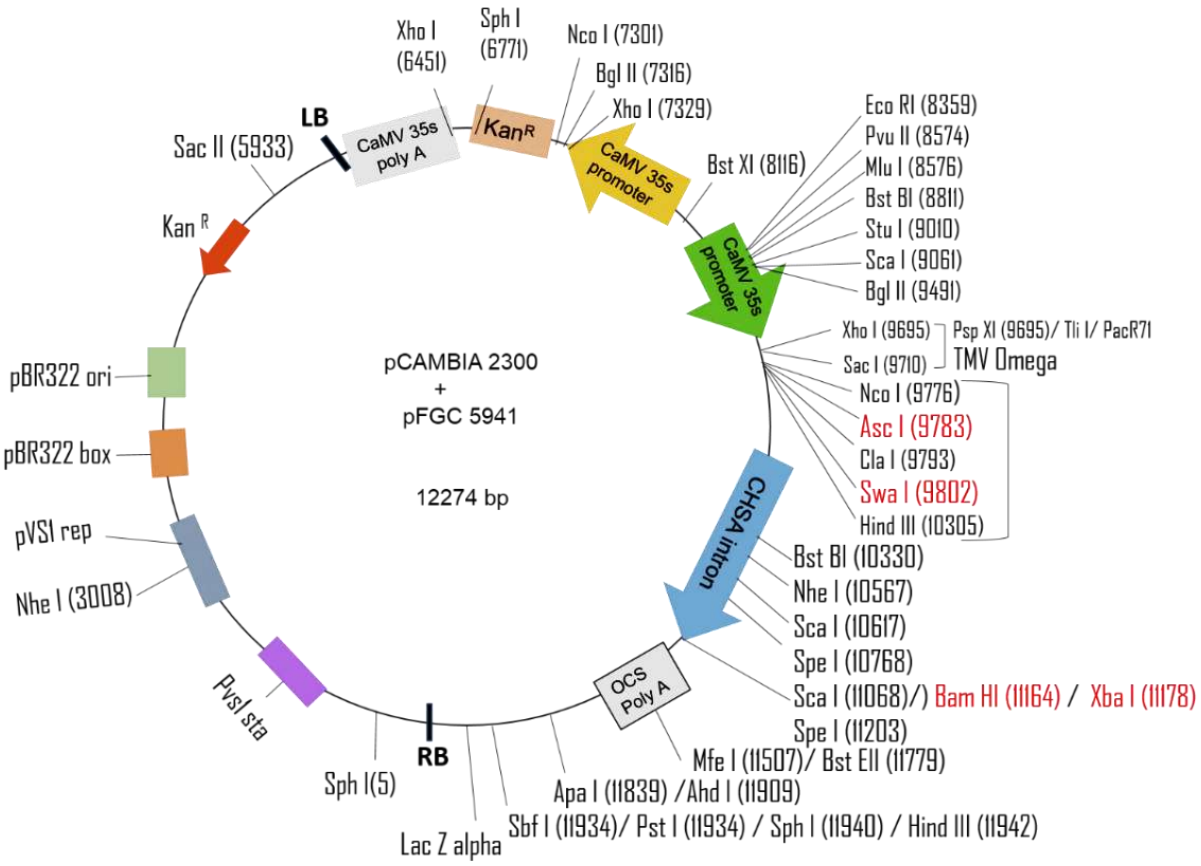


Fig. 3.2 Schematic diagram of hp-RNAi plasmid

To upper aqueous phase 2 µL RNAase enzyme (10 mg L^{-1}) was added and incubated at 37 °C for 1 h. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifugation was done (10000 ×g; 10 min; 4 °C). To aqueous phase 0.7 volume isopropanol was added and incubated at -20 °C for 30 min, followed by centrifugation (12000 ×g; 10 min). The DNA pellets were washed with 500 µL EtOH (70%) and centrifuged (10000 ×g; 10 min). Finally, the pellets were resuspended in 30 µL TE buffer and stored at 4 °C for further use.

The quality of isolated plasmid was checked on agarose gel 1% (w/v) containing 1µL of EtBr (0.5 µg mL^{-1}) and visualized under a UV transilluminator (Gel Doc Mega: Biosystematics USA) and photographed. The concentration was determined using Nanodrop spectrophotometer.

3.11.4 Cloning of sense fragment

The strategy involved first cloning of sense fragment in hp-RNAi plasmid followed by cloning of antisense fragment in a plasmid containing sense fragment. For cloning of the sense fragment, the hp-RNAi plasmid and sense fragment were digested with *AscI* and *SwaI* restriction enzymes. Digestion was performed sequentially for each restriction enzyme at different incubation temperatures: 37°C for *AscI* and 30°C for *SwaI*.

Table 3.6 Restriction digestion of hp-RNAi plasmid

Components	**Volume**
Nuclease free water	12.7 µL
Tango buffer (10X)	2 µL
Plasmid (1 µg)	5 µL
AscI (10 U μL^{-1})	0.2 µL
Total volume	20 µL
Incubation at 37 °C for 1 h	
Tango buffer	2.5 µL
SwaI (10 U μL^{-1})	0.4µL
Incubation at 30 °C for 1 h	

Table 3.7 Restriction digestion of sense fragment

Components	**Volume**
Nuclease free water	10 µL
Tango buffer (10X)	2 µL
Sense (0.5-1µg)	8µL
Asc I (10 U μL^{-1})	0.2 µL
Total volume	20 µL
Incubation at 37 °C for 1 h	
Tango buffer	2.5 µL
Swa I (10 U μL^{-1})	0.4µL
Incubation at 30 °C for 1 h	

Restriction digestion was confirmed on 1.2% (w/v) agarose gel and the restricted products were gel purified using HiPurA® quick gel purification kit (Himedia). Further, the gel-purified restricted hp-RNAi plasmid and sense fragment were used for setting up the ligation reaction using the given formula

$$\frac{\text{Vector amount (ng)} \times \text{ size of insert (kb)}}{\text{size of vector (kb)}} \times \text{molar ratio of} \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Table 3.8 Ligation reaction for ligating restricted hp-RNAi plasmid and sense fragment

Components	**Volume**
Nuclease free water	4.5 µL
10X ligation buffer	1 µL
Insert DNA (at 3:1 molar excess over vector)	1 µL
Plasmid DNA (40-100 ng)	2.5 µL
T4 DNA ligase (5U/ µL)	1 µL
Final volume	10 µL
Incubation at 16 °C overnight	

The given ligated mixture was used to transform the DH5α cells of *E. coli*.

3.11.5 Transformation of *E. coli* (DH5α) cells with ligated mixture

Competent cells of *E. coli* were prepared by inoculating a single colony of untransformed *E. coli* strain DH5α into 25 mL of LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10g L^{-1} sodium chloride, pH-7). The culture was incubated for 24 h at 37 °C on a rotary shaker. After 24 h of incubation, 200 µL of actively growing culture was inoculated into 25 mL of fresh LB medium followed by incubation for 2 h at 37 °C till the $O.D_{600}$ reached 0.5-0.7. The culture was transferred to an ice-cold 50 mL polypropylene tube followed by incubation on ice for 10 min. Centrifugation was done at 3000 ×g for 5 min at 4°C. The cells were pelleted and suspended in 10 mL of ice-cold 100 mM $CaCl_2$ and stored on ice for 15 min. Centrifugation was done at 3000 ×g for 5 min at 4°C. To the cell pellet 1 mL of ice-cold $CaCl_2$ was added and stored on ice for 12-24 h or incubated for 3-4 h to achieve transient competence.

Competent cells of *E. coli* were transformed using the heat shock method (Singh et al. 2010). A 5 µL of plasmid (~100-300 ng) was suspended in 100 µL of competent cells. The cells were kept on ice for 30 min. Immediately, the cells were placed in a preheated water bath at 42 ℃ for 90 s and rapidly transferred on ice for 2-3 min. A 1mL of LB medium was added and cells were incubated at 37 ℃ for 45 min. The cells were centrifuged at 3000 ×g for 5 min and the pellet was suspended in 500 µL of LB medium. The suspension of cells was spread on luria agar (LA) plates containing 50 µg mL^{-1} kanamycin. Plates were incubated at 37 ℃ for 24 h and observed colonies were grown in LB medium containing 50 µg mL^{-1} kanamycin for plasmid isolation.

3.11.6 Molecular confirmation of cloning of sense fragment in hp-RNAi plasmid

To confirm the integration of the sense strand in a hp-RNAi plasmid, sense-specific amplifications as described in section 3.11.1 and restriction digestion of a modified plasmid (hp-RNAi+sense plasmid) was performed as described in section 3.11.4 with *AscI* and *SwaI* restriction enzymes to release the fragment.

3.11.7 Cloning of antisense fragment

After confirming the integration of the sense fragment in the hp-RNAi+sense plasmid, the cloning of the antisense fragment in the hp-RNAi+sense plasmid was proceeded. The modified hp-RNAi+sense plasmid and antisense fragment were digested with *BamHI* and *XbaI* restriction enzymes to produce compatible sticky ends.

Table 3.9 Restriction digestion of hp-RNAi+sense plasmid

Components	**Volume**
Nuclease free water	14 µL
Tango buffer (10X)	2 µL
Plasmid (1µg)	3.5 µL
BamHI (10U/ µL)	0.2 µL
XbaI (10U/ µL)	0.2 µL
Total volume	20 µL
Incubation at 37 °C for 1 h	

Table 3.10 Restriction digestion of antisense fragment

Components	**Volume**
Nuclease free water	13 µL
Tango buffer (10X)	2 µL
Antisense fragment (0.5-1 µg)	5 µL
BamHI (10U/ µL)	0.2 µL
XbaI (10U/ µL)	0.2 µL
Total volume	20 µL
Incubation at 37 °C for 1 h	

As mentioned above, the restricted products were confirmed on 1.2% (w/v) agarose gel, gel purified, and ligated. The ligated mixture was further used to transform the *E. coli* cells as described in section 3.11.5. The transformed colonies were selected on luria agar (LA) plates containing 50 µg mL^{-1} kanamycin and plasmid was isolated as described in section 3.11.3.

3.11.8 Molecular confirmation of cloning of antisense fragment in hp-RNAi+sense plasmid

To confirm the integration of the antisense fragment in a hp-RNAi+sense plasmid, antisense-specific amplifications as described in section 3.11.1 and restriction digestion of a modified plasmid (hp-RNAi+sense+antisense plasmid) was performed as described in section 3.11.7 with *BamHI* and *XbaI* restriction enzymes to release ~200 bp fragment.

Further, the complete hp-RNAi construct (T-DNA region of hp-RNAi+sense+antisense plasmid) was finally confirmed by restriction digestion with *AscI* and *XbaI* enzymes.

3.11.9 Transformation of *Agrobacterium tumefaciens* with hp-RNAi+sense+antisense plasmid

For the preparation of competent cells, a single colony of *A. tumefaciens* disarmed strain LBA4404 was picked and inoculated into 25 mL of Yeast Extract Peptone (YEP) medium (10 g L^{-1} peptone, 10 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, pH-7) containing 15 µg mL^{-1} rifampicin. The culture was incubated at 28 ℃ for 24 h on a rotary shaker. After 24 h of incubation, 100 µL of actively growing culture was inoculated into 25 mL of fresh YEP medium followed by incubation for 3-4 h at 28 °C till the $O.D_{590}$ at 0.5-0.7 was attained. The culture was transferred to a sterile ice-cold 50 mL polypropylene tube followed by incubation at ice for 10 min. The culture was centrifuged at 3000 ×g for 5 min at 4°C. The cells were pelleted and suspended in 1 mL of ice-cold 20 mM $CaCl_2$ and stored on ice for 15 min.

Competent cells of *A. tumefaciens* were transformed using Freeze and thaw method (Holsters et al. 1978). An aliquot of 100 µL of competent cells was mixed with a plasmid (~ 100-300 ng). The content was snap-frozen in liquid nitrogen for 2 min and thawed at room temperature for 2 min. A freshly prepared 1 mL of YEP medium was added and incubated at 28 °C for 4 h. The culture was centrifuged at 3000 ×g for 5 min and the pellet of cells was suspended in 500 µL of YEP medium. The suspension of cells was spread on YEP-agar plates containing 15 µg mL^{-1} rifampicin and 50 µg mL^{-1} kanamycin. Plates were incubated at 28 °C for 24-36 h and observed colonies were maintained in a YEP medium containing 15 µg mL^{-1} rifampicin and 50 µg mL^{-1} kanamycin antibiotic. The transformed bacterial culture was used for the transformation of plants.

3.12 Optimisation of *Agrobacterium* mediated genetic transformation

For, optimisation, the *A. tumefaciens* strain LBA4404 harboring binary vector pBI121 was used. The pBI121 vector contains the *uidA* reporter gene and a *nptII* selection marker gene (Fig. 3.3). Many factors involved in *Agrobacterium* infection and shoot regeneration efficiency influence transformation efficiency. Using transient GUS expression, the critical parameters such as preculture period, bacterial density, co-cultivation period, infection time, and acetosyringone concentration on *Agrobacterium*-mediated genetic transformation protocol were standardized.

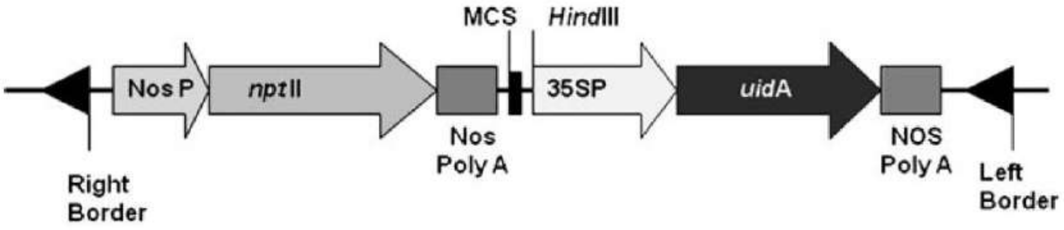


Fig. 3.3 T-DNA region of binary vector pBI121 plasmid (Tripathi et al. 2011)

3.12.1 Determination of kanamycin sensitivity of explants

Leaf and internodal explants of both the cultivar ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ were excised and inoculated on MS3 medium (Guleria and Kumar 2022) further supplemented with different concentrations of kanamycin (0, 25, 50, 75, 100, 125 mg L^{-1}). The stock of kanamycin was filter sterilized (0.22 µm; Millipore India) and added to the autoclaved medium after it had cooled down to 45 °C. The concentration at which all the explants died was selected for screening of transformed tissue.

3.12.2 Factors affecting genetic transformation

A single bacterial colony of *Agrobacterium* strain LBA4404 containing pBI121 plasmid was picked up from the plate and inoculated in yeast mannitol broth (YMB) medium containing 50 µg mL^{-1} kanamycin and 15 µg mL^{-1} rifampicin and grown overnight at 28 °C (120 rpm).

The leaf and internodal explants were excised from the 21-day old microshoots of cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ and cultured on MS3 medium (Guleria and Kumar 2022) for different intervals of time (0,1, 2, 3, 4 days). Following infection with *Agrobacterium* (OD_{590} ~0.2, 0.4, 0.6, 0.8, 1.0) for different time period (5, 10, 15, 20, 25 min), these explants were then co-cultivated on MS2 medium containing acetosyringone concentration (0, 50, 100, 150, 200 µM) for different interval of time (1, 2, 3, 4, 5 days). The explants were then properly washed, blot dried and cultured on MS3 medium+100 mg L^{-1} kanamycin. The transient GUS expression was recorded after 3 days using histochemical GUS assay.

3.12.3 Histochemical analysis of explants

For transient GUS expression, 100 explants from each experiment were randomly collected and immersed in histochemical GUS solution containing 1 mM X-Gluc, 0.1% (v/v) triton X-100, 10 mM disodium ethylenediaminetetraacetic acid (Na2EDTA), 0.5 mM each of potassium ferrocyanide and potassium ferricyanide in 100 mM sodium phosphate buffer adjusted to pH 7. After overnight incubation at 37 °C, explants were washed with 70% (v/v) ethanol to remove chlorophyll. Explants showing blue colouration were scored for GUS expression. The optimised factor conditions showing highest transient GUS expression were further selected and incorporated in genetic transformation of the explants.

3.13 Development of transgenic lines containing hp-RNAi construct(s)

The above optimised *Agrobacterium*-mediated genetic transformation protocol was employed for development of transgenic lines. The leaf and internodal explants were excised from the 21-day old microshoots of cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ and precultured on MS3 medium for 2 days. After 2 days, the explants were slightly injured with a scalpel and infected with *Agrobacterium* strain LBA4404 harboring plasmid (hp-RNAi+sense +antisense) (OD_{590} at 0.6) for 15 min followed by transfer to co-cultivation medium (MS4 medium) for 2 days. After co-cultivation, explants were washed with sterile distilled water, blot dried, and transferred to selection-cum-regeneration medium (MS5 medium). Every 15 days, the explants were transferred to a fresh selection- cum-regeneration medium. After 4-5 subculture cycles on selection-cum-regeneration medium, the developed transgenic lines were further maintained on multiplication medium (MS1 medium).

Table 3.11 The different modified MS medium standardized in this study

Name	Medium composition
MS1 medium	basal MS +10 µM $AgNO_3$
MS2 medium	basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3
MS3 medium	basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3+100 mg L^{-1} cefotaxime
MS4 medium	basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3 +100 µM acetosyringone
MS5 medium	basal MS +10 µM $AgNO_3$+10 µM BA+15 µM GA_3+100 mg L^{-1} cefotaxime+100 mg L^{-1} kanamycin

3.13.1 Molecular analysis of transgenic lines

Genomic DNA was isolated from the transgenic and non-transgenic shoots of both the cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ using CTAB method as described in section 3.8.1. The integration of the hp-RNAi construct (T-DNA region) within transgenic lines was confirmed by amplification of sense and antisense fragments using specific primers mentioned in Table 3.4. Amplification with respective primers using modified plasmid DNA is considered as positive control, and with non-transgenic shoot as negative control. The PCR amplification reaction volume and conditions was followed same as mentioned in section 3.11.

The integration of *nptII* was confirmed through amplification using *nptII* gene specific primers. For this, the amplification with bacterial DNA was taken as positive control and with non-transgenic shoot as negative control. *16s rRNA* amplification was also carried out to rule out the possibility of bacterial existence in cultures. A 20 µL reaction volume contains 2 µL of PCR buffer (10X), dNTPs (100 µM), primer (0.2 µM), template DNA (40 ng), Taq DNA polymerase (1U) and Milli-Q water to make up the volume. A list of primers used to amplify *nptII* and *16s rRNA* gene is mentioned in Table 3.12. Amplification conditions were initial denaturation at 94 ºC for 4 min; 30 cycles of 94 °C for 45 s, 64 °C for *nptII* (58 °C for *16s rRNA*) 45 sec and 72 °C for 1 min; with final extinction at 72 °C for 5 min. The amplified products were separated on a 1% (w/v) agarose gel stained with EtBr (0.5 µg mL^{-1}) and viewed under a UV transilluminator.

Table 3.12 List of primers pairs used to amplify *nptII gene* and *16s rRNA*

Gene		**Sequence (5'-3')**
nptII	*Forward*	ATCGGGAGCGGCGATACCGTA
	Reverse	GAGGCTATTCGGCTATGACTG
16s rRNA	*Forward*	AGAGTTTGATCCTGGCTCAG
	Reverse	ACGGGCGGTGTGTTC

3.14 Evaluation of transgenic lines against *Phytophthora infestans*

3.14.1 Acclimatisation of plants in the transgenic green house

For this, the transgenic lines expressing hp-RNAi construct(s), vector control, and non-transgenic plants of both the cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' were grown on MS1 medium and acclimatized in soil as described in section 3.10.1.

3.14.2 Detached leaf assay

Healthy fully grown leaves of transgenic lines of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' harboring hp-RNAi construct(s), vector control and non-transgenic plants were excised and placed in a moist chamber. Sporal suspension of *P. infestans* was prepared as mentioned in section 3.10.2. A 10 µL sporal suspension (5×10^4 spores mL^{-1}) was placed on the abaxial sides of leaves and incubated at 20 °C. After inoculation, leaves were monitored for brown and water-soaked lesions, and lesion diameter was measured to check the disease resistance (Namukwaya 2015).

3.14.3 Whole plant assay

The fully grown plants in a transgenic greenhouse were evenly sprayed with *P. infestans* sporal suspension using a spray pump. The temperature of the transgenic house was maintained at 17 °C with a relative humidity of 90-95% using humidifiers. Symptoms were recorded at 5 and10 days post inoculation (dpi) to record disease incidence and disease severity using formula mentioned in Table 3.3.

3.15 Biochemical assays

During disease progression, the activity of antioxidant enzymes such as superoxide dismutase, peroxidase, ascorbate peroxidase, and catalase was studied. Leaf samples were collected every 24 h for 10 days from uninoculated and inoculated plants with *P. infestans* to perform the following assays.

3.15.1 Preparation of protein extract

For the preparation of protein extract 1 g (FW) of leaf, samples were homogenized in 10 mL of the extraction buffer (50 mM sodium phosphate buffer (pH 6.8) containing 100 mM sucrose, 200 mg L^{-1} polyvinylpyrrolidone, and 1 g L^{-1} cysteine hydrochloride). The homogenate was centrifuged (12000 ×g, 15 min, 4 °C), the supernatant was retained and the total volume was made up to 10 mL using extraction buffer. This extract was used for the estimation of total soluble protein content and enzyme activity assay.

3.15.2 Enzymatic Activities

The superoxide dismutase activity was estimated using a method described by Beauchamp and Fridovich (1971). The reaction mixture consisted of 50 mM phosphate buffer, pH 7.8 (2.186 mL), 1 M methionine (39 µL), 1 mM nitro blue tetrazolium (225 µL), 3 mM EDTA-disodium (100 µL), crude protein extract (150 µL) and 0.2 mM riboflavin (30 µL) and placed under fluorescent lights for 20 min. A reaction mixture without any enzyme extract served as the control. The absorbance was recorded at 560 nm against a blank (non-irradiated), and one unit of superoxide dismutase activity was expressed as the amount of enzyme required for 50% inhibition of the photoreduction of NBT and expressed as U mg^{-1} protein.

$$\text{Superoxide dismutase} = \frac{\left(\frac{A_{control} - A_{sample}}{A_{control}}\right)}{50\% \times V \times P} \times T$$

T is total reaction volume in mL, V is sample volume in mL, and P is the soluble protein content in mg mL^{-1}.

The peroxidase activity was determined according to the method given by McEwen Jr (1971), the reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7 (2.75 mL), 1% (w/v) o-dianisidine solution (100 µL) and 1% (v/v) hydrogen peroxide (50 µL). The reaction was initiated by adding 100 µL of enzyme extract. Change in absorbance was recorded at 470 nm for 3 min. The peroxidase activity was expressed as U mg^{-1} protein where 1 unit of enzyme is defined as the amount of enzyme required for oxidation of the o-Dianisidine oxidation per min.

$$\text{Peroxidase activity} = \frac{\frac{\Delta A}{\Delta t} \times T}{\xi \times V \times P}$$

$\Delta A/\Delta t$=change in absorbance per min, T is total reaction volume (mL), ξ is extinction coefficients (mM^{-1} cm^{-1}), V is sample volume (mL) and P is the soluble protein content (mg mL^{-1}).

The catalase (CAT) activity was determined using the method given by Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7 (2.85 mL), 30% (v/v) hydrogen peroxide (50 µL), and crude protein extract (100 µL). A reduction in the absorbance at 240 nm was recorded for 3 min against control (reaction mixture without protein extract). The catalase activity was expressed as U mg^{-1} protein where one unit of catalase is defined as the amount of enzyme required for decomposing 1 µmol H_2O_2 under standard conditions.

The ascorbate peroxidase activity was estimated by protocol given by Nakano and Asada (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7 (2.825 mL) containing 10 mM EDTA (30 µL), 100 mM ascorbate (15 µL), and 30% (v/v) hydrogen peroxide (30 µL). The reaction was started by adding enzyme extract (100 µL) into the reaction mixture and a decrease in absorbance at 290 nm was recorded at 3 min. The activity was expressed as U mg^{-1} protein where one unit of enzyme is defined as the amount of enzyme required to oxidize 1 µmol of ascorbate in 1 min.

The extinction coefficients used for the calculation of antioxidant enzyme activity were: Catalase=39.4 mM^{-1} cm^{-1}, Ascorbate peroxidase= 2.8 mM^{-1} cm^{-1}, and Peroxidase=11.3 mM^{-1} cm^{-1}.

3.16 Statistical analysis

Unless otherwise mentioned, experiments were repeated three times and performed in triplicates. Means of each experiment were compared and subjected to either one-way or two-way analysis of variance (ANOVA) using GraphPad Prism Version 5.0 and Mean values were compared by Duncan’s Multiple Range Test (DMRT) and LSD at $P<0.05$ using CoStat 6.4 software. (CoHort Software, Pacific Grove U.S.A). For biochemical analyses, three composite samples were withdrawn for analysis, Data were subjected to Two-way analysis of variance (ANOVA) at $P<0.05$ using GraphPad Prism Version 5.0 (GraphPad Software Inc., San Diego CA) and graphs were designed using Origin pro 18 software (Origin Lab, Northampton, Massachusetts, USA).

Chapter 4

Results

Chapter 4

The effect of β-lactam antibiotics on shoot organogenesis of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'

Abstract

In this chapter, the effect of β-lactam antibiotics viz. cefotaxime, carbenicillin, and cephalexin was investigated on shoot organogenesis of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. Leaf and internodal explants from 21-day-old microshoots were cultured on pre-standardized medium (basal Murashige and Skoog (MS) containing 10 µM $AgNO_3$+10 µM 6-benzyladenine (BA)+15 µM gibberellin A3 (GA3) (MS2 medium), which was further supplemented with different concentrations (0-500 mg L^{-1}) of various β-lactam antibiotics. These antibiotics promoted shoot organogenesis in both cultivars at lower concentrations, except cephalexin, which completely inhibited shoot organogenesis in cv. 'Kufri Chipsona 1'. Cefotaxime (100 mg L^{-1}) induced shoots in a maximum number of explants, with a maximum number of shoots per explant. The promotion of shoot organogenesis from internodal explants was significantly higher than that of leaf explants. Cefotaxime at all concentrations (100–500 mg L^{-1}) increased shoot organogenesis in all explants and cultivars. The regenerated shoots tested clonally uniform among themselves and also to that of the mother plant by random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers.

4.1 The effect of β-lactam antibiotics on shoot organogenesis

In the beginning, the size of the explant (internodes, leaf) increased, when inoculated on MS2 medium supplemented with different concentrations (0-500 mg L^{-1}) of β-lactam antibiotics (cefotaxime, carbenicillin and cephalexin). After 7 days of inoculation, thickening of the overgrown tissues on explants was observed, which later developed into the nodular callus, and after 25 days, adventitious shoot buds emerged (Fig 4.3 and 4.6). The efficiency of shoot organogenesis was assessed in terms of percent explants showing shoot organogenesis and mean number of shoots per explant as compared to control MS2 medium.

In cv. 'Kufri Pukhraj', incorporation of cefotaxime, carbenicillin, and cephalexin increased the percent of explants showing shoot organogenesis in both the explants. Cefotaxime at 100 mg L^{-1} induced shoot organogenesis in maximum number of explants (internode 85.41%; leaf 65.27%). However, carbenicillin improved shoot organogenesis at 300 mg L^{-1} (internode 77.77%; leaf 37.47%) while the incorporation of cephalexin improved shoot organogenesis up to 100 mg L^{-1} (internode 73.61%; leaf 36.10%). A further increase in these antibiotics (cefotaxime and carbenicillin) reduced the response from both the explants, which was still higher than the control (internode 37.34%; leaf 20.6%) (Figs. 4.1).

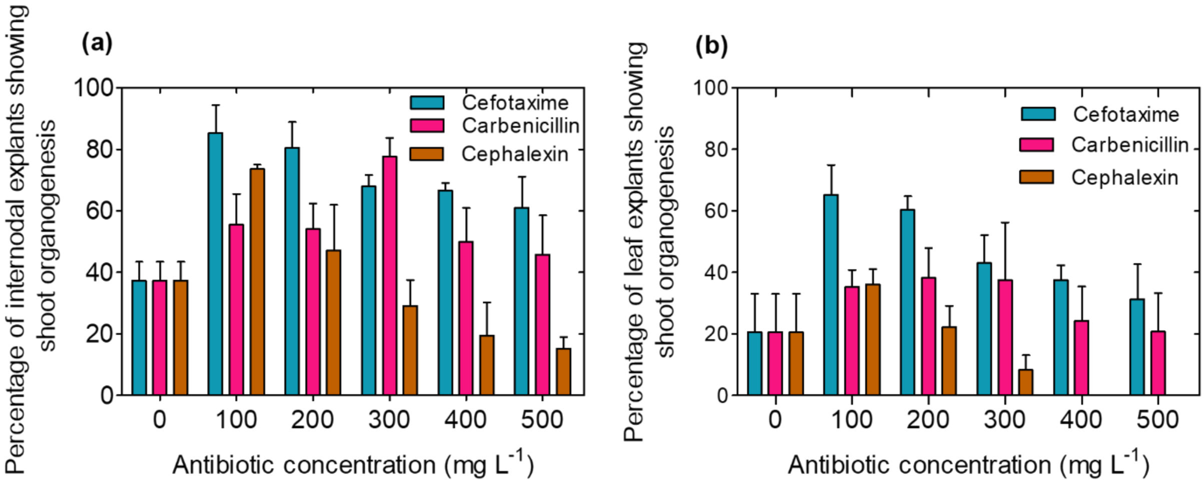


Fig. 4.1 The effect of different concentrations of β-lactam antibiotics on percent explants showing shoot organogenesis **(a)** internodal and **(b)** leaf of cv. 'Kufri Pukhraj'. Data were recorded after 6 weeks of culture and analysed by Two-way Analysis of variance (ANOVA) with a significance level < 0.05

It was noticeable that cephalexin at higher concentrations (>100 mg L^{-1}), reduced the shoot organogenesis (Fig. 4.1a, b) and at >300 mg L^{-1} it completely inhibited the shoot organogenesis in leaf explant (Figs. 4.1b, and 4.3).

A similar trend of antibiotics in case of number of shoots regenerated per explant was recorded and a maximum number of shoots per explant was recorded on a medium containing 100 mg L^{-1} cefotaxime. The number of shoots were higher from internodal explants (6.48) as compared to leaf (3.31) (Fig. 4.2a and b). The ANOVA for shoot organogenesis of cv. 'Kufri Pukhraj' reveals statistically significant differences between the antibiotics, the concentrations of the antibiotics, and some of their interactions (Table 4.1).

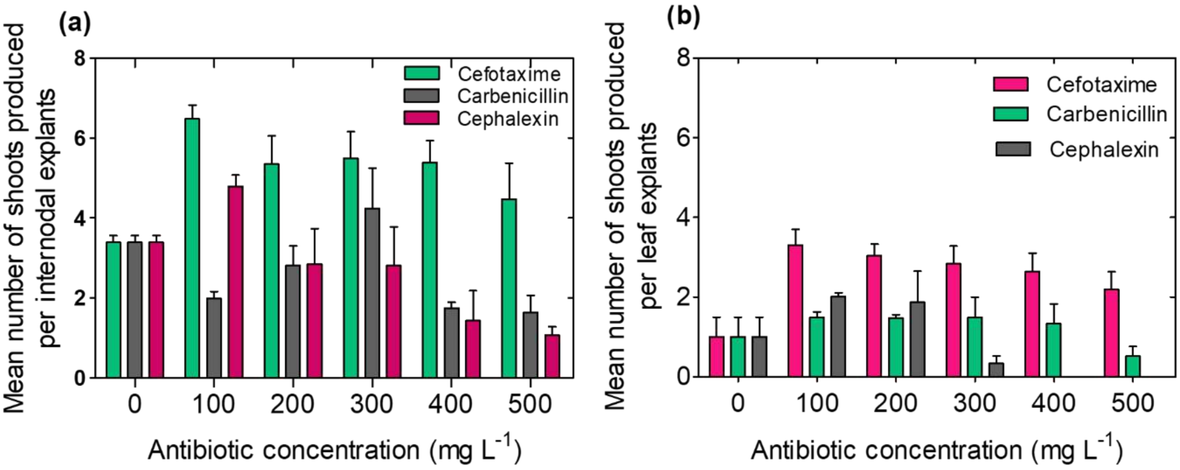


Fig. 4.2 The effect of different concentrations of β-lactam antibiotics on mean number of shoots regenerated per explant **(a)** internodal and **(b)** leaf of cv. 'Kufri Pukhraj'. Data were recorded after 6 weeks of culture and analysed by Two-way Analysis of variance (ANOVA) at a significance level < 0.05

The ANOVA for cv. 'Kufri Pukhraj' reveals statistically significant differences between the antibiotics, the concentrations of the antibiotics, and some of their interactions (Table 4.1). Based on the results, it was observed that in cv. 'Kufri Pukhraj', among explants the internodes gave better shoot organogenesis response than leaf explants on medium supplemented with β-lactam antibiotics.

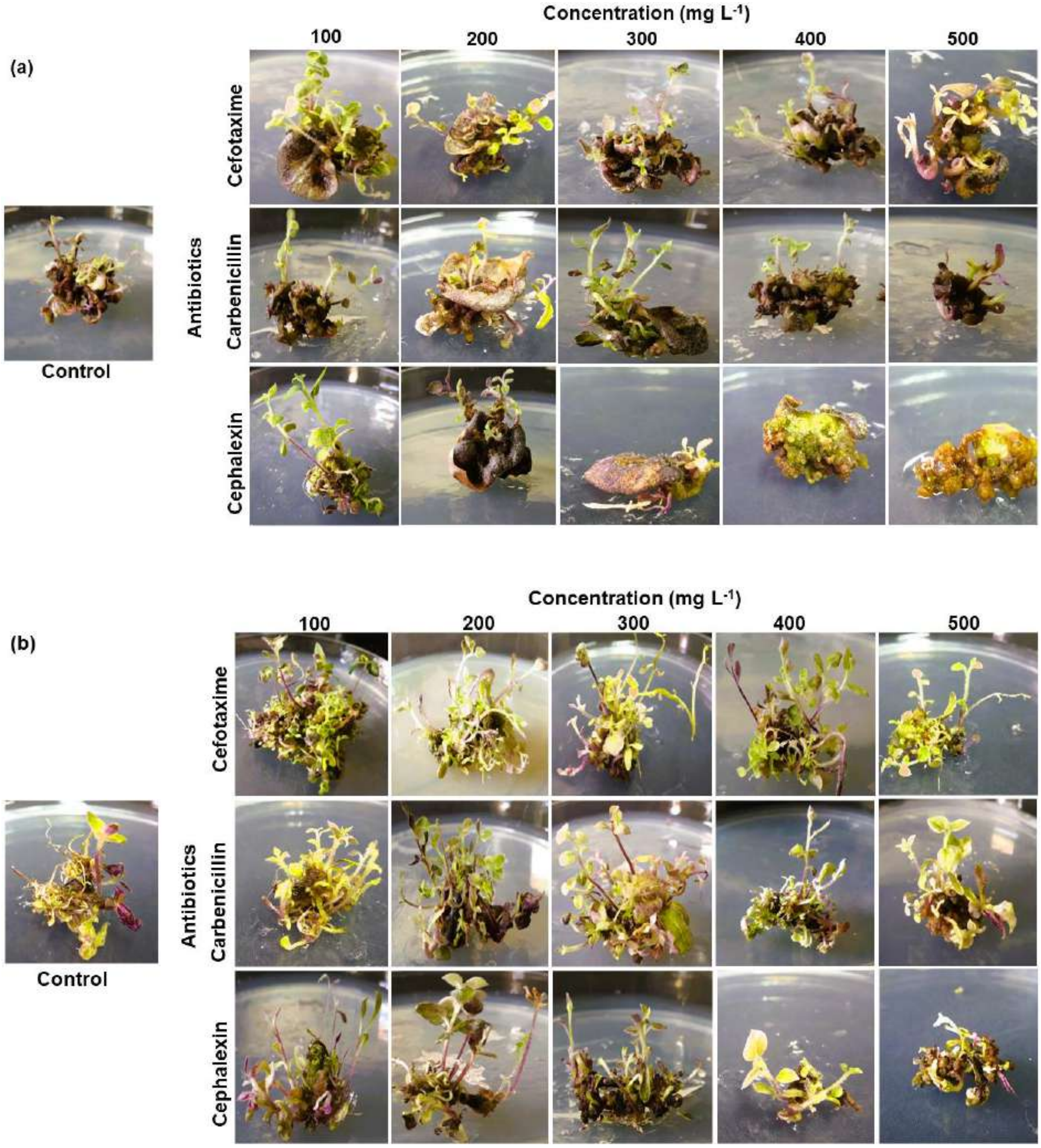


Fig. 4.3 The effect of cefotaxime, carbenicillin and cephalexin on shoot organogenesis of potato cv. 'Kufri Pukhraj'. **(a)** leaf **(b)** internodal explants cultured on MS2 medium (basal MS +10 µM $AgNO_3$ +10 µM BA + 15 µM GA_3) supplemented with different concentrations of antibiotics (100-500 mg L^{-1}). Control is the explants cultured on MS2 medium lacking antibiotics

Table 4.1 Two-way ANOVA table of cv. 'Kufri Pukhraj' for the percentage of explants showing shoot organogenesis and the mean number of shoots per explant

Source	***Df***	***Sum of square***	***Mean square***	***F value***	***P value***
Leaf					
Percentage of explants showing shoot organogenesis					
Between antibiotics	2	7302	3651	13.09	0.0001
Between concentrations	5	6109	1222	4.380	0.0032
Interaction	10	2430	243.0	0.871	0.5673
Errors	36	10040	278.9		
Mean number of shoots					
Between antibiotics	2	26.71	13.35	28.15	0.0001
Between concentrations	5	14.58	2.915	6.148	0.0003
Interaction	10	10.38	1.038	2189	0.0419
Errors	36	17.08	0.4744		
Internodes					
Percentage of explants showing shoot organogenesis					
Between antibiotics	2	7873	3936	18.26	0.0001
Between concentrations	5	7931	1586	7.357	0.0001
Interaction	10	6018	601.8	2.791	0.0115
Errors	36	7762	215.6		
Mean number of shoots					
Between antibiotics	1	70.10	35.05	34.17	0.0001
Between concentrations	5	26.98	5.397	5.260	0.0010
Interaction	5	33.32	3.332	3.247	0.0044
Errors	36	36.93	1.026		

In cv. ‘Kufri Chipsona 1’, from both explants (internode and leaf) shoot organogenesis was also observed on MS2 medium supplemented with cefotaxime and carbenicillin, but not on medium supplemented with cephalexin (Fig. 4.6a, b). In this cultivar, addition of cefotaxime was also found to be beneficial for shoot organogenesis at all the concentrations, whereas carbenicillin improved shoot organogenesis at lower concentrations (< 300 mg L^{-1}), and at higher concentration (> 300 mg L^{-1}) it was inhibitory (Figs. 4.4a, b). Percent explants showing shoot organogenesis were maximum on medium containing 100 mg L^{-1} cefotaxime (internode 75%; leaf 62.5%) and carbenicillin (internode 66.66%; leaf 50%).

As previously stated, all concentrations of cephalexin completely inhibited shoot organogenesis in both the explants; however, the explants developed nodular calluses.

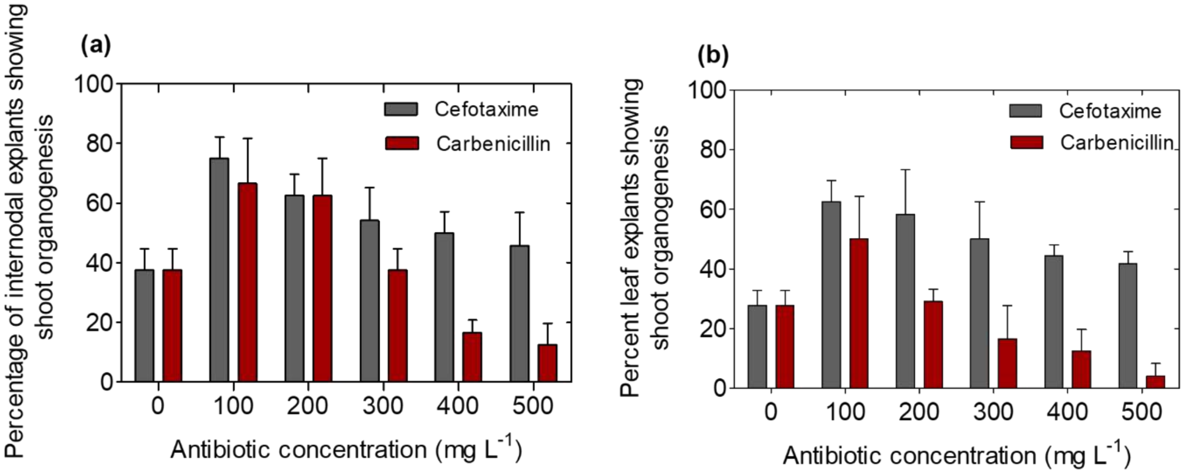


Fig. 4.4 The effect of different concentrations of β-lactam antibiotics on percent explants showing shoot organogenesis **(a)** internodal and **(b)** leaf of cv. ‘Kufri Chipsona 1’. Data were recorded after 6 weeks of culture and analysed by Two-way Analysis of variance (ANOVA) with a significance level < 0.05

In this cultivar, a similar trend in case of number of shoots regenerated per explant was recorded, and a maximum number of shoots per explant was recorded on a medium containing 100 mg L^{-1} cefotaxime. The number of shoots were higher from leaf explants (6.38) as compared to internodes (5.19) (Fig. 4.5a and b). The ANOVA for shoot organogenesis of cv. ‘Kufri Chipsona 1’ reveals statistically significant differences between the antibiotics, the concentrations of the antibiotics, and some of their interactions (Table 4.2).

Comparing the two cultivars, higher shoot organogenesis response was observed in cultivar 'Kufri Pukhraj' as compared to cultivar 'Kufri Chipsona 1'. Amongst the explants, internodal explants showed better shoot organogenesis than the leaf explants (Figs. 4.3 and 4.6).

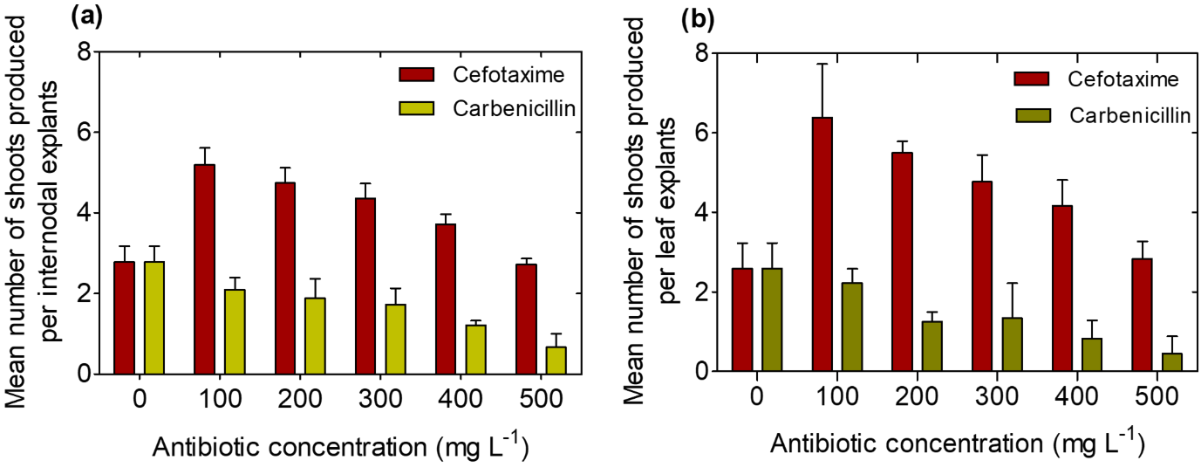


Fig. 4.5 The effect of different concentrations of β-lactam antibiotics on mean number of shoots regenerated per explant **(a)** internodal and **(b)** leaf of cv. 'Kufri Chipsona 1'. Data were recorded after 6 weeks of culture and analysed by Two-way Analysis of variance (ANOVA) with significance level < 0.05

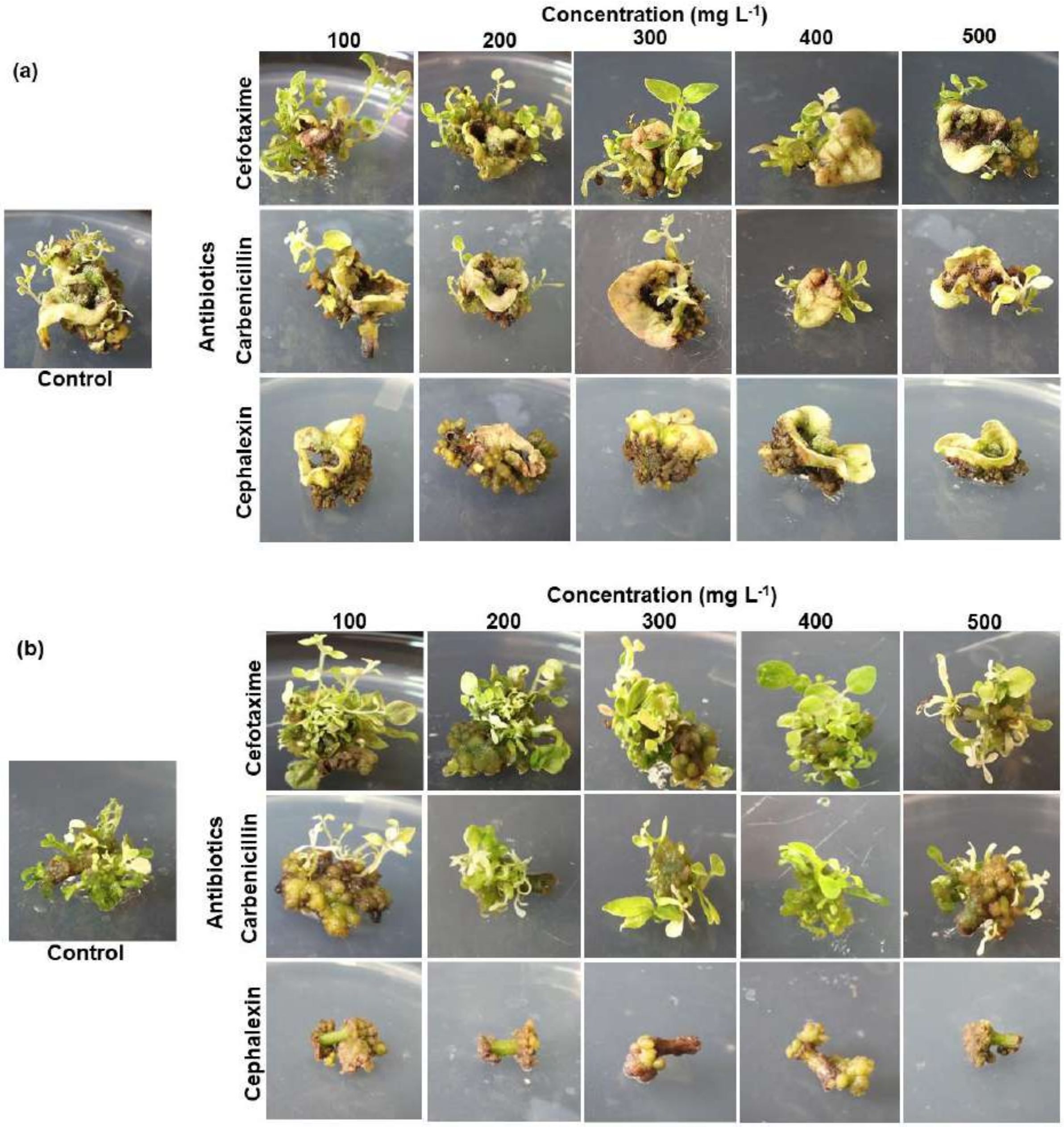


Fig 4.6 The effect of cefotaxime, carbenicillin, and cephalexin on shoot organogenesis of potato cv. 'Kufri Chipsona 1' **(a)** leaf **(b)** internodal explants cultured on MS2 medium (basal MS +10 µM $AgNO_3$ +10 µM BA + 15 µM GA_3) supplemented with different concentrations of antibiotics (100- 500 mg L^{-1}). Control is the explants cultured on MS2 medium lacking antibiotics

Table 4.2 Two-way ANOVA table of cv. 'Kufri Chipsona 1' for percentage of explants showing shoot organogenesis and mean number of shoots per explant

Source	*Df*	*Sum of square*	*Mean square*	*F value*	*P value*
			Leaf		
Percentage of explants showing shoot organogenesis					
Between antibiotics	1	5216	5216	22.39	0.0001
Between concentrations	5	4624	924.8	3.970	0.0091
Interaction	5	1601	320.2	1.375	0.2687
Errors	24	5590	232.9		
Mean number of shoots					
Between antibiotics	1	77.21	77.21	59.90	0.0001
Between concentrations	5	24.40	4.880	3.786	0.0114
Interaction	5	18.85	3.769	2.924	0.0336
Errors	24	30.93	1.289		
			Internodes		
Percentage of explants showing shoot organogenesis					
Between antibiotics	1	2101	2101	8.345	0.0081
Between concentrations	5	8420	1684	6.690	0.0005
Interaction	5	1753	350.7	1.393	0.2622
Errors	24	6042	251.7		
Mean number of shoots					
Between antibiotics	1	43.08	43.08	118.7	0.0001
Between concentrations	5	14.31	2.863	7.889	0.0002
Interaction	5	9.53	1.908	5.258	0.0021
Errors	36	36.93	1.026		

4.1.2 Clonal fidelity of regenerated shoots

As mentioned above the best shoot organogenesis was achieved from internodal explants when cultured on MS2 medium containing 100 mg L^{-1} cefotaxime. The shoots were further multiplied and maintained on MS1 medium to ascertain the clonal fidelity of regenerated shoots vis-a-vis the mother plant. The RAPD and ISSR based molecular markers were used to test the clonal fidelity (Table 4.3). The RAPD amplification profiles indicated that out of 10 RAPD primers, only 8 primers amplified scorable bands (amplicon size 250 to 1000 bp) in cv. 'Kufri Pukhraj' and in cv. 'Kufri Chipsona 1' 6 RAPD primers amplified the scorable bands (amplicon size 250 to 1000 bp) (Table 4.3).

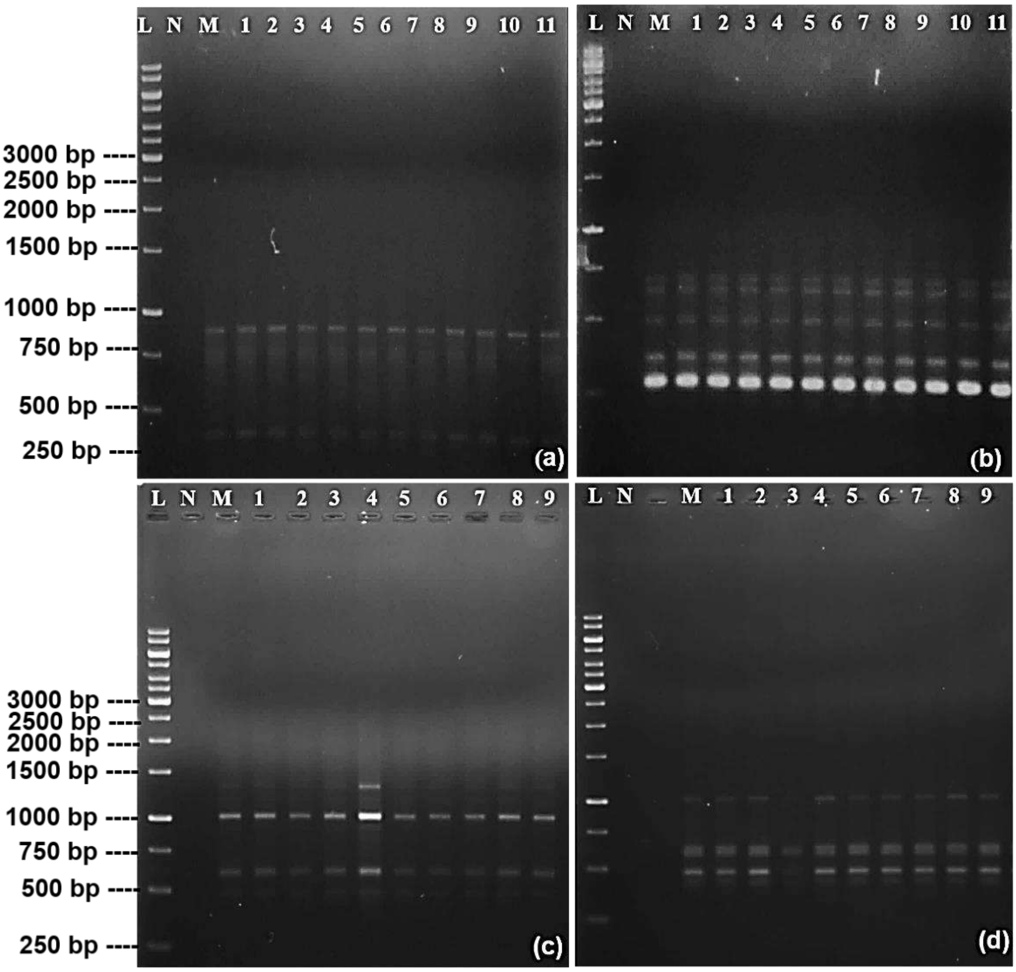


Fig. 4.7 RAPD and ISSR of regenerated shoot lines (lane1-11) and mother plants (lane M) of cv. 'Kufri Pukhraj' **(a)** RAPD-1 primer and **(b)** ISSR-25 primer and cv. 'Kufri Chipsona 1' regenerated lines (lane 1-9) and mother plant (lane M) using **(c)** RAPD-1primer and **(d)** ISSR-23 primer. Lane L represents 1kbp DNA marker, whereas lane N is negative control

Table 4.3 List of RAPD and ISSR primers, their sequences, and number of fragments amplified by each primer in cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'and size range of amplified fragments

RAPD Primer	*Sequence (5'→3')*	*Number of fragments amplified*	*Size range (bp)*	*ISSR Primer*	*Sequence (5'→3')*	*Number of fragments amplified*	*Size range (bp)*
			Kufri Pukhraj				
RAPD1	AGCGCCATTG	3	250-500	***ISSR9***	$(GC)_8T$	-	-
RAPD3	AGGGCGTAAG	1	500	***ISSR10***	$(GC)_8A$	-	-
RAPD6	GGACCCAACC	3	500-1000	***ISSR11***	$(GC)_8AT$	-	-
RAPD8	TCTGGTGAGG	3	250-1000	***ISSR19***	$(AT)_8GC$	-	-
RAPD15	GGGGTGACGA	3	250-500	***ISSR20***	$(AT)_8$	-	-
RAPD18	GAGAGCCAAC	2	500-750	***ISSR21***	$(GA)_8TG$	3	250-500
RAPD20	ACCCGGTCAC	2	250-1000	***ISSR22***	$(GA)_8C$	2	250
RAPD28	GTGACGTAGG	3	250-750	***ISSR23***	$(GA)_8CT$	4	250-750
RAPD29	GGGTAACGCC	-	-	***ISSR24***	$(GA)_8CA$	3	250-750
RAPD30	GTGATCGCAG	-	-	***ISSR25***	$(GA)_8CC$	5	250-1000
			Kufri Chipsona 1				
RAPD1	AGCGCCATTG	5	250-1000	***ISSR4***	$(AC)_8(GC)_2$	3	250-500
RAPD2	CTTCCCCAAG	2	750-1000	***ISSR5***	$(AC)_8$	-	-
RAPD3	AGGGCGTAAG	1	1000	***ISSR6***	$(CA)_8TG$	4	250-750
RAPD4	CTGGGGGACT	-	-	***ISSR13***	$(CT)_8A$	3	250-750
RAPD6	GGACCCAACC	1	500	***ISSR20***	$(AT)_8$	-	-
RAPD8	TCTGGTGAGG	-	-	***ISSR21***	$(GA)_8TG$	3	250-1000
RAPD11	TTGCACGGG	2	250-500	***ISSR22***	$(GA)_8C$	4	250-750
RAPD13	CTCTGGAGAC	-	-	***ISSR23***	$(GA)_8CT$	4	250-750
RAPD21	CAGGCCCTTC	3	500-1000	***ISSR24***	$(GA)_8CA$	4	250-1000
RAPD36	AGCCAGCGAA	-	-	***ISSR25***	$(GA)_8CC$	1	250

The ISSR amplification profiles indicated that out of 10 ISSR primers, only 5 primers amplified a scorable bands in cv. ‘Kufri Pukhraj’, whereas in cv. ‘Kufri Chipsona 1’, 8 primers amplified scorable bands (amplicon size 250-1000 bp) (Table 4.3). All bands scored using RAPD and ISSR primers were monomorphic (Fig. 4.7). This indicates the absence of polymorphism and compared to the mother plant ensured the true-to-type nature of regenerated shoots.

Chapter 5

The effect of sucrose on shoot organogenesis of potato cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’

Abstract

The chapter describes the effect of sucrose on the shoot organogenesis of two potato cvs. ‘Kufri Pukhraj’and ‘Kufri Chipsona 1’. Internodal and leaf explants of both the cultivars were cultured on MS3 medium (MS medium containing 10 µM $AgNO_3$ + 10 µM BA + 15 µM GA_3 + 100 mg L^{-1} cefotaxime) supplemented with different sucrose concentrations (30, 60, 90, 120 and 150 mM). It was observed that sucrose at 60-90 mM induces higher shoot organogenesis and number of shoots per explant, whereas lower and higher concentrations inhibited the same. Maximum shoot organogenesis was achieved from internodal explants of cv. ‘Kufri Pukhraj’ (84.44%) followed by ‘Kufri Chipsona 1’ (65.55%) on a medium containing 60 mM sucrose. It was noticed that the effect of sucrose on leaf explants was less pronounced than internodal explants. During the process of shoot organogenesis on medium containing different sucrose concentration, changes in the activities of sucrose metabolising enzymes (acid invertase and sucrose synthase) and starch metabolising enzymes (α-amylase and β-amylase) were also studied following 7, 14, 21 and 28 days of culture on medium containing different sucrose concentrations. Maximum activity of cell wall-bound invertase, cytosolic invertase, and sucrose synthase was recorded on day 14, whereas the maximum activity of α-amylase and β-amylase was recorded on day 21 on a medium containing 60 mM sucrose. The total soluble sugars, reducing sugars, and starch content were also higher on day 14 on the same medium composition. Real-time PCR (RT-PCR) studies were also performed to track the expression levels of invertase, sucrose synthase, α -amylase, and β-amylase genes. The analyses revealed upregulation of invertase (4.19-fold) and sucrose synthase (4.39-fold) on day 14, whereas on day 21, α-amylase and β-amylase expression of 5.07 and 4.43-fold respectively was observed. It seems that sucrose influences shoot organogenesis by altering the expression of these key genes and has a significant impact on enzymatic activities.

5.1 Effect of sucrose on shoot organogenesis

Swelling at the cut ends of the explants was observed after 7 days of culture, which progressed to nodular structures by day 14 on all sucrose concentrations (Fig. 5.1, 5.3). Between 21 and 28 days, adventitious shoot emergence was observed. The shoot organogenic response of cultivars and explants (internode and leaf) varied with sucrose concentrations (Figs. 5.2, 5.4).

In cv. 'Kufri Pukhraj' highest shoot organogenesis was observed at 60 mM sucrose from internodal explants (84.44%), with 6.02 shoots per explant (Fig. 5.2a, b), whereas 64.44% of the leaf explants showed shoot organogenesis with 3.70 shoots per explant (Fig. 5.2c, d).

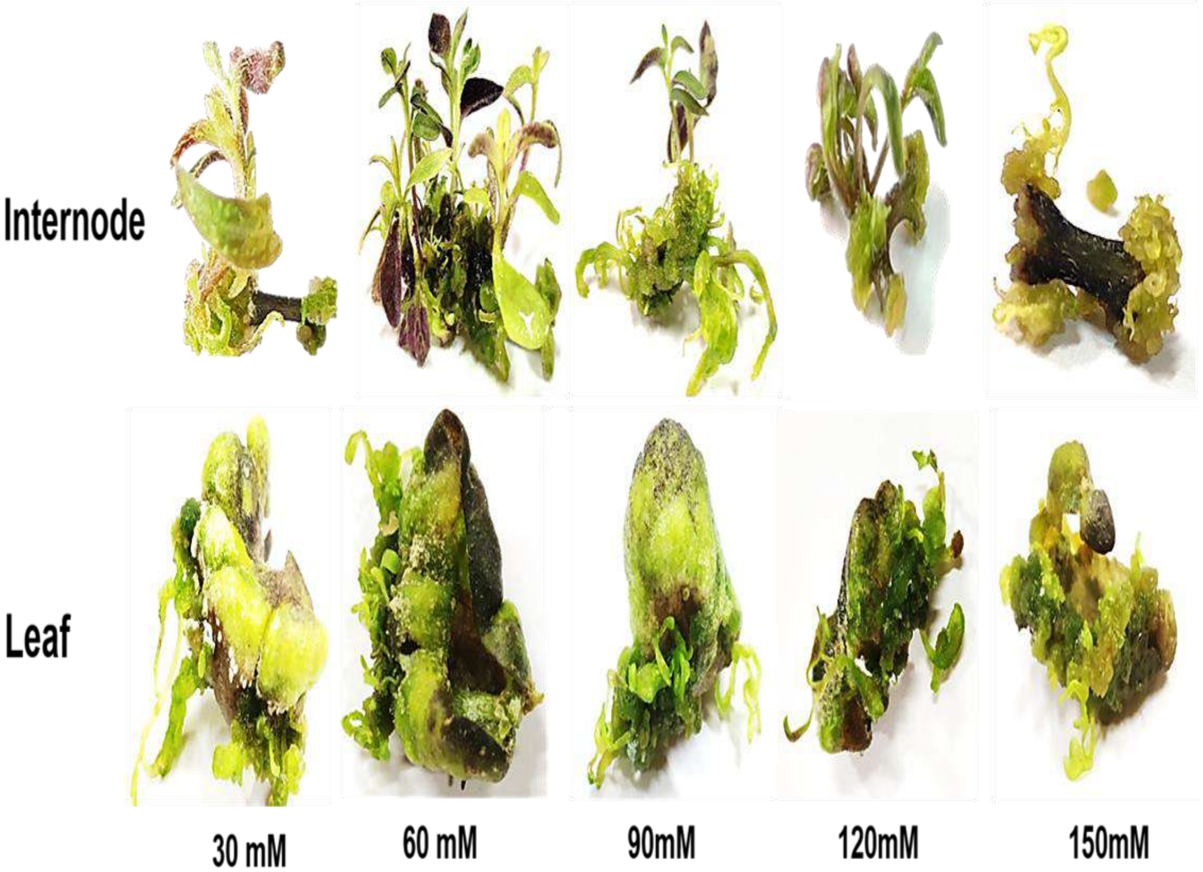


Fig. 5.1 The effect of different sucrose concentrations on shoot organogenesis from leaf and internodal explants of potato cv. 'Kufri Pukhraj' cultured on MS3 medium (MS medium containing 10 µM $AgNO_3$+ 10 µM BA + 15 mM GA_3 + 100 mg L^{-1} cefotaxime) supplemented with different concentrations of sucrose (30-150 mg L^{-1})

At sucrose concentrations of 30 mM, both explant types showed a lower shoot organogenic response. A further increase in sucrose concentration beyond 90 mM leads to a decrease in the shoot organogenic response from both the explants. The lowest shoot organogenesis response 26.66% (internode) and 11.11% (leaf) was observed in cv. 'Kufri Pukhraj' at 150 mM sucrose. One-way ANOVA analysis revealed a significant effect of sucrose concentrations on percent shoot organogenesis and number of shoots in internodal (p value = 0.0005; 0.0004) and leaf explants (p value = 0.0001; 0.0001), respectively.

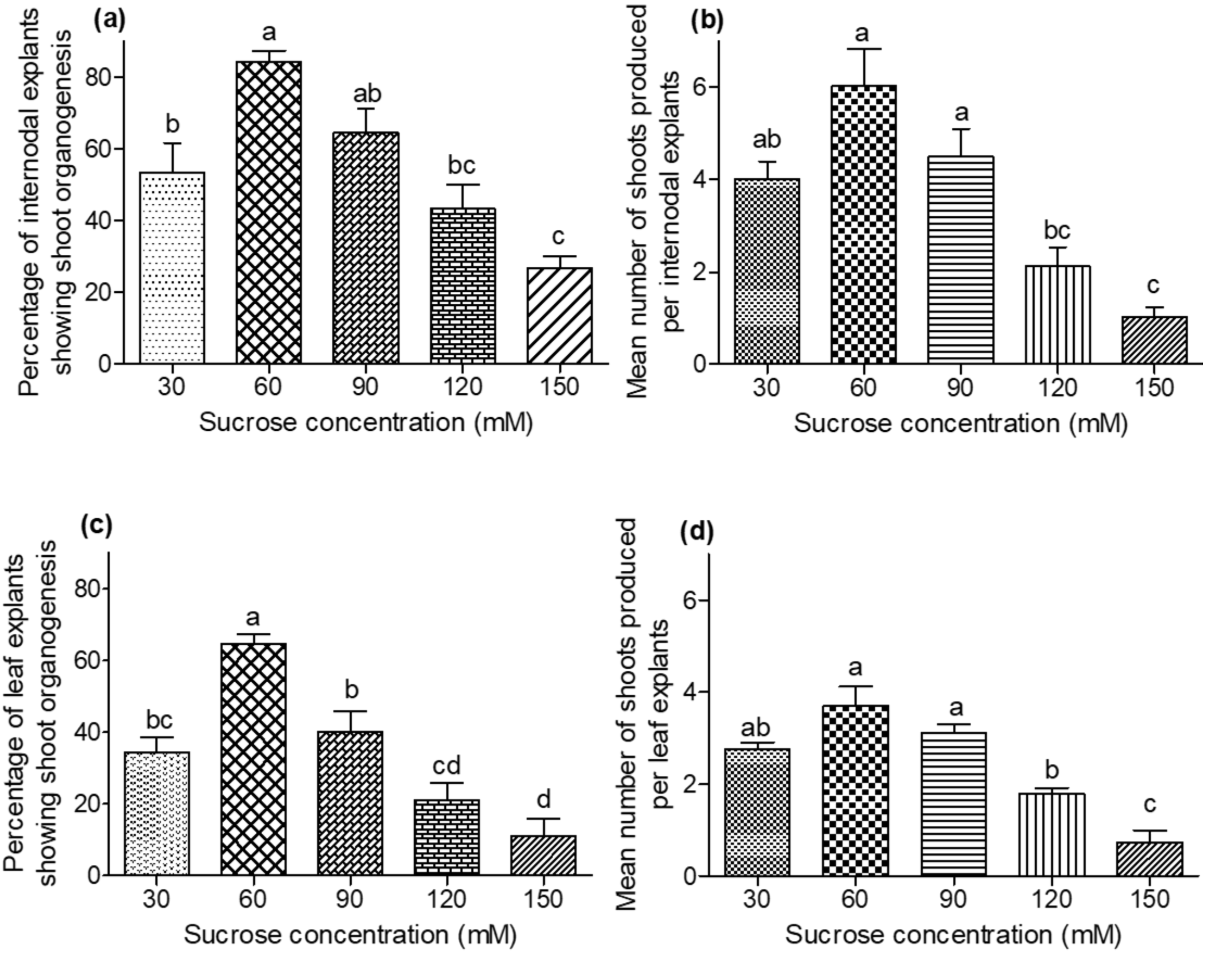


Fig. 5.2 The influence of different sucrose concentrations on percent explants showed shoot organogenesis and mean number of shoots produced per internodal **(a-b)** and leaf **(c-d)** explants of 'Kufri Pukhraj'. Results were scored after 5-6 weeks and analysed by One way Analysis of variance (ANOVA) with significance level < 0.05 by LSD

In cv. ‘Kufri Chipsona 1’, a similar trend was also noticed with a maximum response recorded at 60 mM sucrose concentration. At this concentration, the highest shoot organogenesis rates of 65.55% (internodes) (Fig. 5.4a, b) and 50.00% (leaves) (Fig. 5.4c, d) were recorded, with a 4.50 and 2.86 number of shoots per explant, respectively. Moreover, with increasing concentration of sucrose decrease in shoot organogenic response was observed. At the highest sucrose concentration (150 mM), the lowest shoot organogenesis was observed with 20% in internodes and 10% in leaf explants of ‘Kufri Chipsona1’.

Fig. 5.3 The effect of different sucrose concentration on shoot organogenesis from internodal and leaf explants of potato cv. ‘Kufri Chipsona 1’ cultured on MS3 medium (MS medium containing 10 µM $AgNO_3$ + 10 µM BA + 15 mM GA_3 + 100 mg L^{-1} cefotaxime) supplemented with different concentrations of sucrose (30-150 mg L^{-1})

One-way ANOVA analysis showed significant differences among sucrose concentrations for percent shoot regeneration and mean number of shoots in internodal (p value = 0.0004; 0.0025) and leaf explants (p value = 0.0106; 0.0180) respectively.

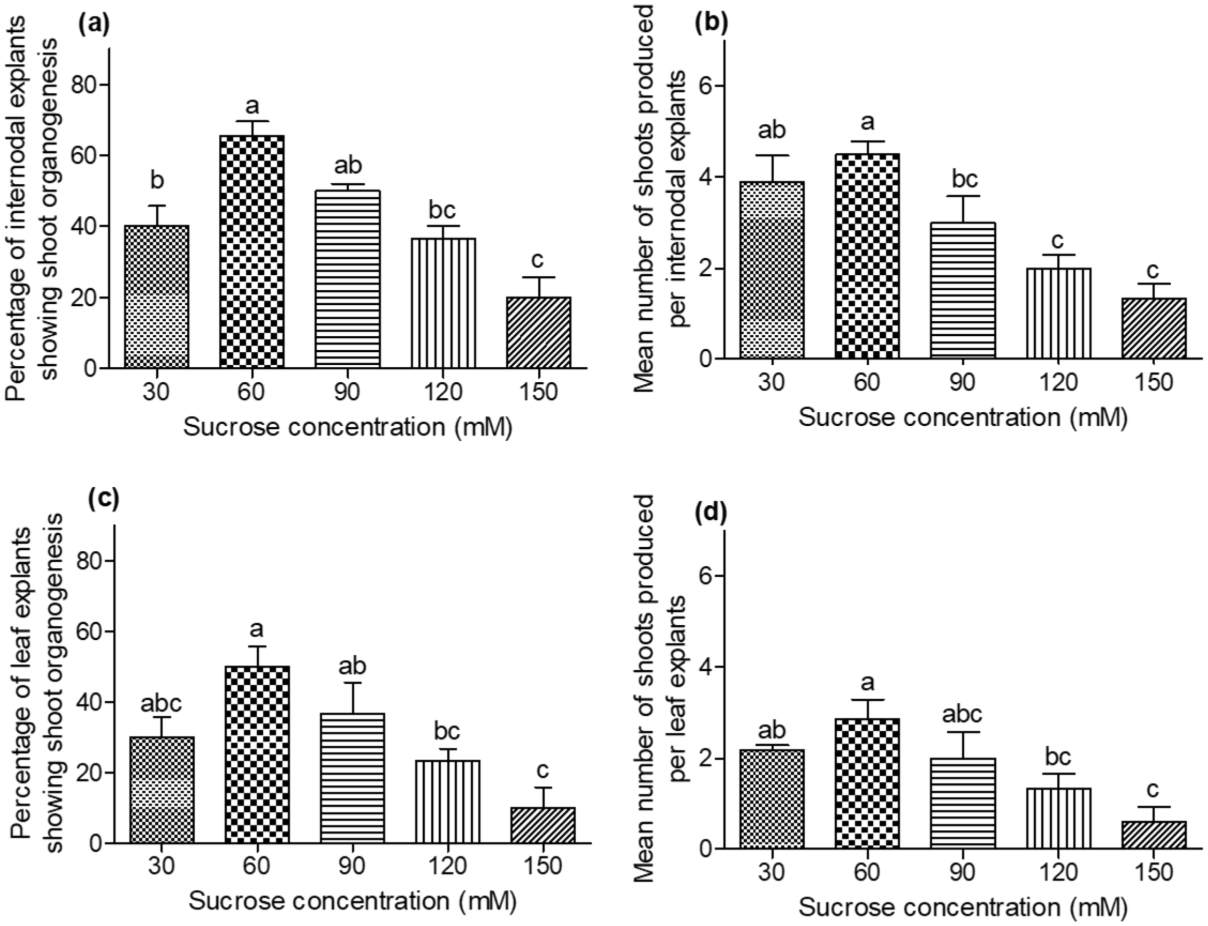


Fig. 5.4 The influence of different sucrose concentrations on percent explants showing shoot organogenesis and mean number of shoots produced per internodal **(a-b)** and leaf **(c-d)** explants of 'Kufri Chipsona 1'. Results were scored after 5-6 weeks and analysed by One way Analysis of variance (ANOVA) with significance level < 0.05 by LSD

On comparing the shoot organogenesis response of both the cultivars and explants; the internodal explants of cv. 'Kufri Pukhraj' showed higher shoot organogenic potential. Therefore, for enzymatic activity and gene expression studies, the internodal explants of cv. 'Kufri Pukhraj' were selected.

5.2 Biochemical analysis

5.2.1 Total soluble and reducing sugar

A significant effect of different sucrose concentrations on total soluble sugar and reducing sugar was observed (Table 5.1). Maximum total soluble sugar content was recorded as 150 mg g $^{-1}$ DW (Fig. 5.5a), whereas maximum reducing sugar content was 60.41 mg g $^{-1}$ DW (Fig. 5.5b) in internodes of cv. 'Kufri Pukhraj' on medium supplemented with 60 mM sucrose after day 14. The contents of total and reducing sugars declined significantly after day 14 on all the concentrations. A minimum total soluble sugar (87.01 mg g–1 DW) and reducing sugars (39.40 mg g $^{-1}$ DW) were recorded on day 28 on a medium containing 150 mM sucrose (Fig. 5.5a, b).

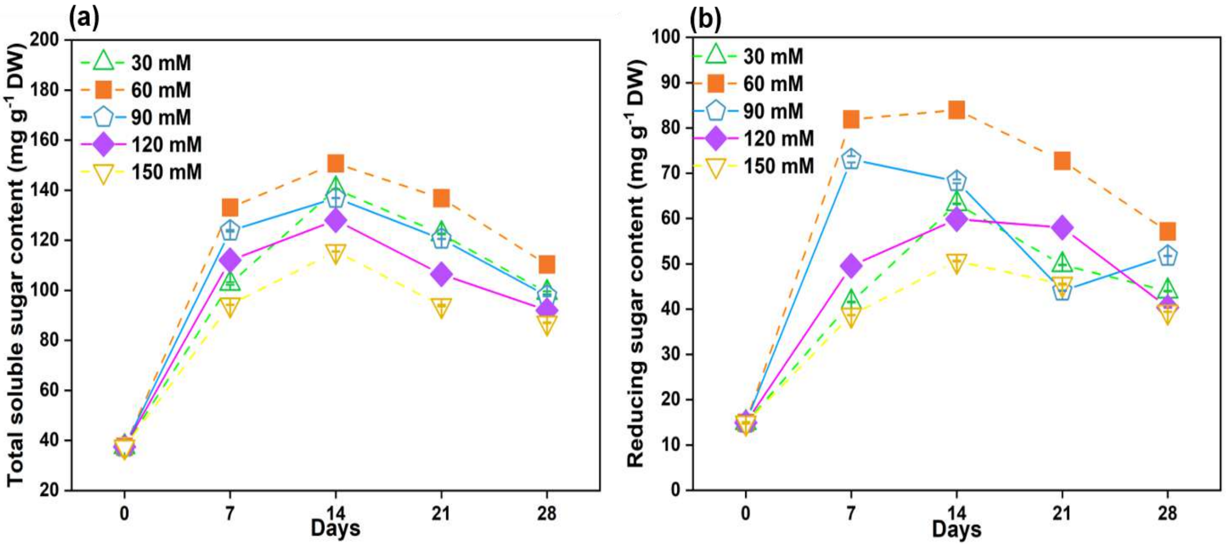


Fig. 5.5 Changes in **(a)** total soluble sugar, and **(b)** reducing sugar in internodal explants on MS3 medium containing different concentrations of sucrose (30- 150 mg L $^{-1}$) after different days of culture (0,7,14, 21, 28 days). Results were analysed by Two-way Analysis of variance (ANOVA) with $p<0.05$

5.2.2 Starch content

A significant effect of sucrose concentration on the starch content of cultures was also observed (Table 5.1). The starch content also increased in all sucrose concentrations up to 14 days (Fig. 5.6) and maximum starch content was recorded (60.41 mg g $^{-1}$ DW) on day 14 in a medium supplemented with 60 mM sucrose concentration (Fig. 5.6). Subsequently with the increase in sucrose concentration and after 14 days of culture, starch content declined in all media combination. After 28 days, much lower starch content (26.33 mg g $^{-1}$ DW) was observed in cultures on a medium containing 150 mM sucrose.

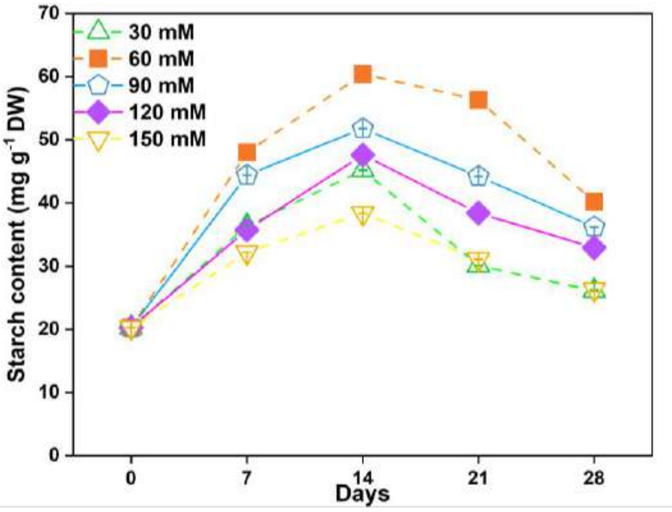


Fig. 5.6 The changes in starch content of internodal explants on MS3 medium containing different concentrations of sucrose (30-150 mg L^{-1}) after different days of culture (0,7,14, 21, 28 days). Results were analysed by Two-way Analysis of variance (ANOVA) with $p<0.05$

Table 5.1 Two-way ANOVA table of total soluble sugar, reducing sugar and starch content present in internodal explants

Source	***Df***	***Sum of square***	***Mean square***	***F value***	***P value***
Total soluble sugar					
Between concentrations	4	6404	1601	1601	<0.0001
Between days	4	82880	20720	7656	<0.0001
Interaction	16	2790	174.4	64.42	<0.0001
Error	50	135.3	2.706		
Reducing sugar					
Between concentrations	4	5219	1305	11980	<0.0001
Between days	4	22630	5658	51940	<0.0001
Interaction	16	3494	218.4	2005	<0.0001
Error	50	5.446	0.1089		
Starch content					
Between concentrations	4	2304	576.1	564200	<0.0001
Between days	4	6716	1679	1645000	<0.0001
Interaction	16	864	54	52890	<0.0001
Error	50	0.05105	0.001021		

5.2.3 The activity of sucrose metabolising enzymes

The cleavage of sucrose by acid invertase may occur either by cytosolic invertase or cell wall-bound invertase. Changes in the activity of acid invertase and sucrose synthase were measured as the amount of glucose formed or sucrose degraded in a particular reaction, respectively. The cytosolic invertase activity was highest (1.9 U mg $^{-1}$ protein) on day 14 of culture on a medium containing 60 mM sucrose (Fig. 5.7a). On the same medium, a sharp decrease of cytosolic invertase activity was observed up to day 28, with 0.59 U mg $^{-1}$ protein.

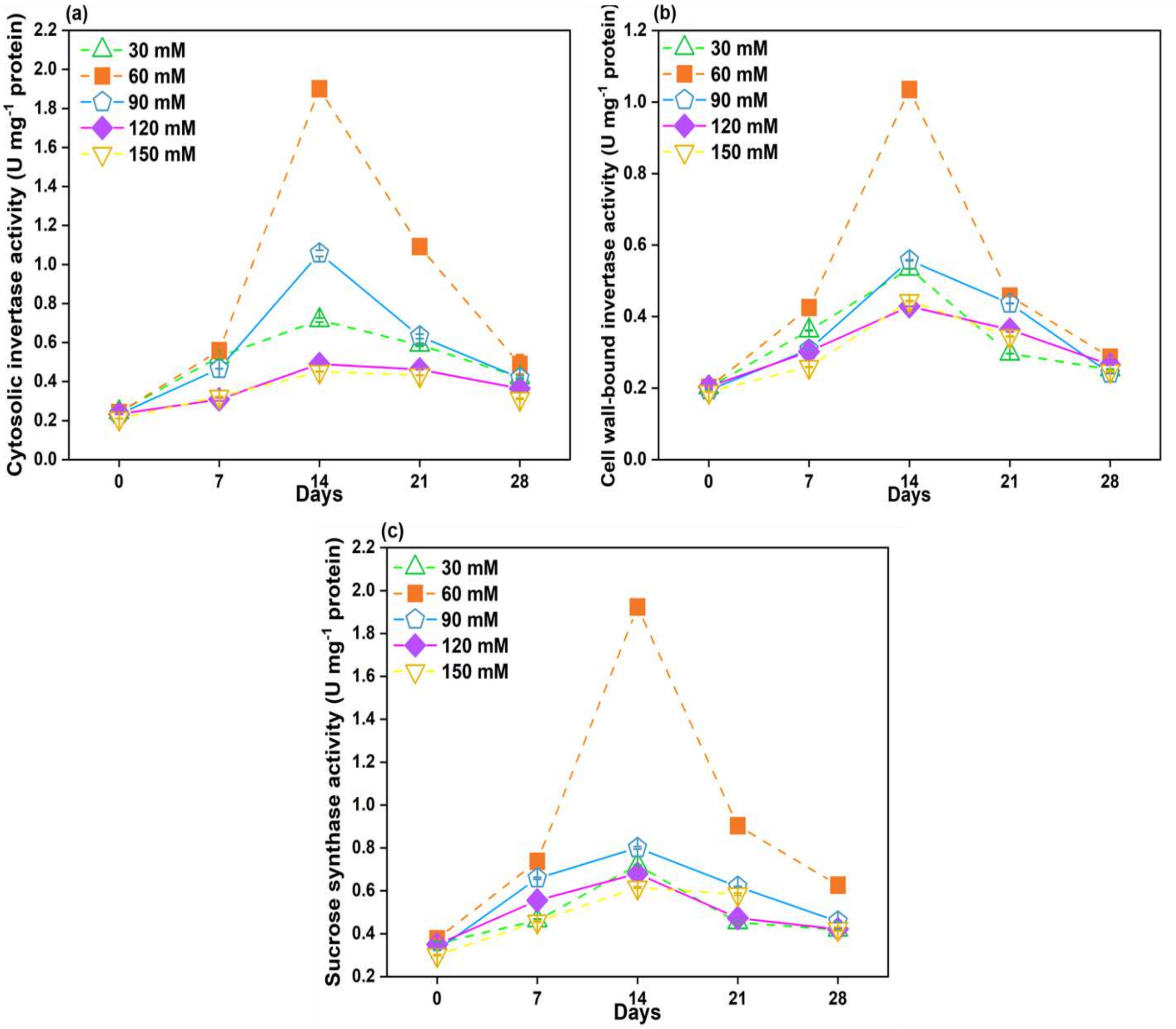


Fig. 5.7 The changes in the activities of **(a)** cytosolic **(b)** cell wall bound invertase **(c)** and sucrose synthase after 7, 14, 21, 28 days of culture on MS3 medium containing different sucrose concentration (30-150 mM) during shoot organogenesis from internodal explants of cv. 'Kufri Pukhraj. Results were analysed by Two-way Analysis of variance (ANOVA) with p<0.05

The highest activity of cell wall-bound invertase (1 U mg $^{-1}$ protein) (Fig. 5.7b) was observed on day 14, which gradually decreased to 0.2 U mg $^{-1}$ protein on day 28 on a medium containing 60 mM sucrose. However, the activity of cell wall-bound invertase was recorded lower than cytosolic invertase (Fig. 5.7a, b).

In the case of sucrose synthase, observed activity showed a similar trend to cytosolic invertase. Maximum activity (1.92 U mg^{-1} protein) (Fig. 5.7c) was observed on day 14 on a medium containing 60 mM sucrose, which declined to 0.62 U mg $^{-1}$ protein on day 28 on the same medium. The significant effect of different sucrose concentrations on activity of cytosolic invertase, cell wall-bound invertase and sucrose synthase was observed (Table 5.2).

5.2.4 The activity of starch metabolising enzymes

The activity of α-amylase was measured as the amount of starch degraded, and the activity of β-amylase as the amount of maltose formed. The activity of α-amylase sharply increased from day 7 to day 14 and finally reached 1.6 U mg $^{-1}$ protein (Fig. 5.8a) on day 21, whereas β-amylase had its highest activity (0.96 U mg $^{-1}$ protein) on day 21 on a medium containing 60 mM sucrose (Fig. 5.8b). The significant effect of different sucrose concentration on activity of α-amylase and β-amylase was observed (Table 5.2).

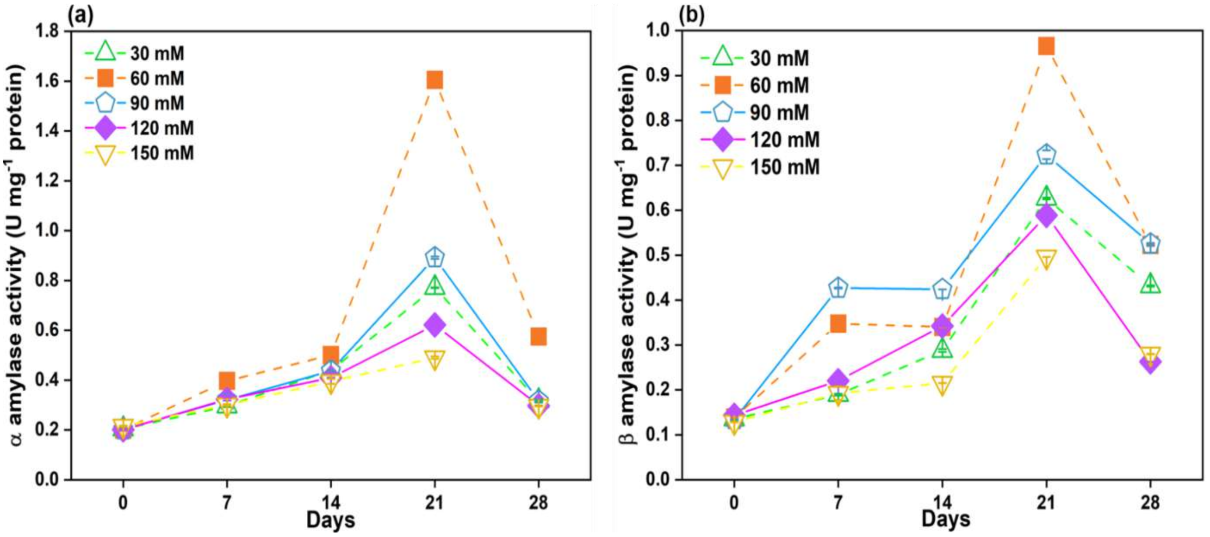


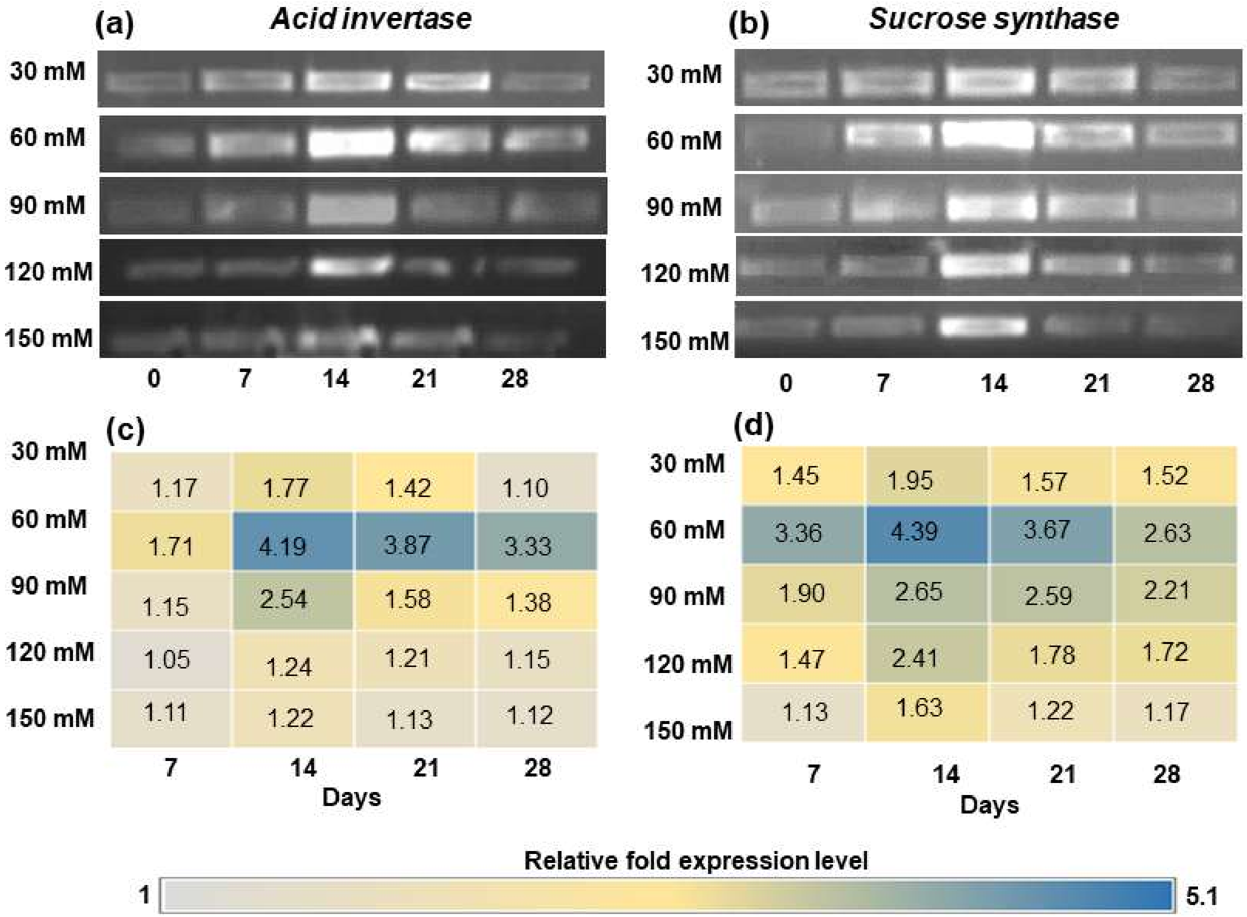
Fig. 5.8 The changes in the activities of **(a)** α-amylase, **(b)** β-amylase after 7, 14, 21, 28 days of culture on MS3 medium containing sucrose concentration (30-150 mM) during shoot organogenesis from internodal explants of cv.'Kufri Pukhraj'. Results were analysed by Two-way Analysis of variance (ANOVA) with $p<0.05$

Table 5.2 Two-way ANOVA table of changes in the activities of sucrose and starch metabolising enzymes during shoot organogenesis from internodal explants of cv. 'Kufri Pukhraj'

Source	***Df***	***Sum of square***	***Mean square***	***F value***	***P value***
Cytosolic Invertase					
Between concentrations	4	2.721	0.6803	73.43	<0.0001
Between days	4	4.136	1.035	111.7	<0.0001
Interaction	16	2.704	0.6803	73.43	<0.0001
Error	50	0.4632	0.009264		
Cell wall bound invertase					
Between concentrations	4	0.3264	0.0816	4061	<0.0001
Between days	4	1.424	0.3559	17710	<0.0001
Interaction	16	0.5279	0.03299	1642	<0.0001
Error	50	0.0010	0.00002		
Sucrose synthase					
Between concentrations	4	2.079	0.5198	14.68	<0.0001
Between days	4	3.075	0.7688	21.71	<0.0001
Interaction	16	2.224	0.139	3.924	0.0001
Error	50	1.771	0.03542		
α-amylase					
Between concentrations	4	0.9412	0.2353	714.6	<0.0001
Between days	4	3.967	0.9917	3012	<0.0001
Interaction	16	1.541	0.09634	292.6	<0.0001
Error	50	0.01646	0.0003		
β-amylase					
Between concentrations	4	0.4587	0.1147	2508	<0.0001
Between days	4	2.449	0.6122	13390	<0.0001
Interaction	16	0.3294	0.02059	450.4	<0.0001
Error	50	0.002286	0.00004		

5.3 Gene expression of sucrose and starch metabolism

The expression of selected genes involved in sucrose and starch metabolism during shoot organogenesis was also studied in internodal explants of cv. ‘Kufri Pukhraj’ cultured on a MS3 medium containing different concentrations of sucrose (30-150 mM) after 0,7,14, 21, and 28 days of culture. The qRT-PCR analyses revealed the change in the expression level of these genes from shoot induction to shoot emergence. The highest relative expression of all the selected genes was observed on a medium containing 60 mM sucrose which favoured shoot organogenesis. The study revealed the upregulation of acid invertase and sucrose synthase gene on day 14 with a subsequent decrease in the expression level on day 28 (Fig. 5.9). The increase in expression levels (about 4.19-fold) on day 14 was recorded in the case of acid invertase (Fig. 5.9a, c). However, 4.39-fold increase in the expression of the sucrose synthase gene was recorded on day 14 (Fig. 5.9b, d), which also gradually declined to 2.63-fold on day 28. On the other hand, on the same medium, a gradual increase in the expression level of starch metabolising enzymes was recorded. The relative expression of α-amylase increased from 2.44 to 4.92-fold from day 7 to day 14 and finally reached a peak (5.0-fold) on day 21 (Fig. 5.10a, c). However, in the case of β-amylase the fold expression increases from 1.36 to 4.43-fold from day 7 to day 21 (Fig. 5.10b, d).



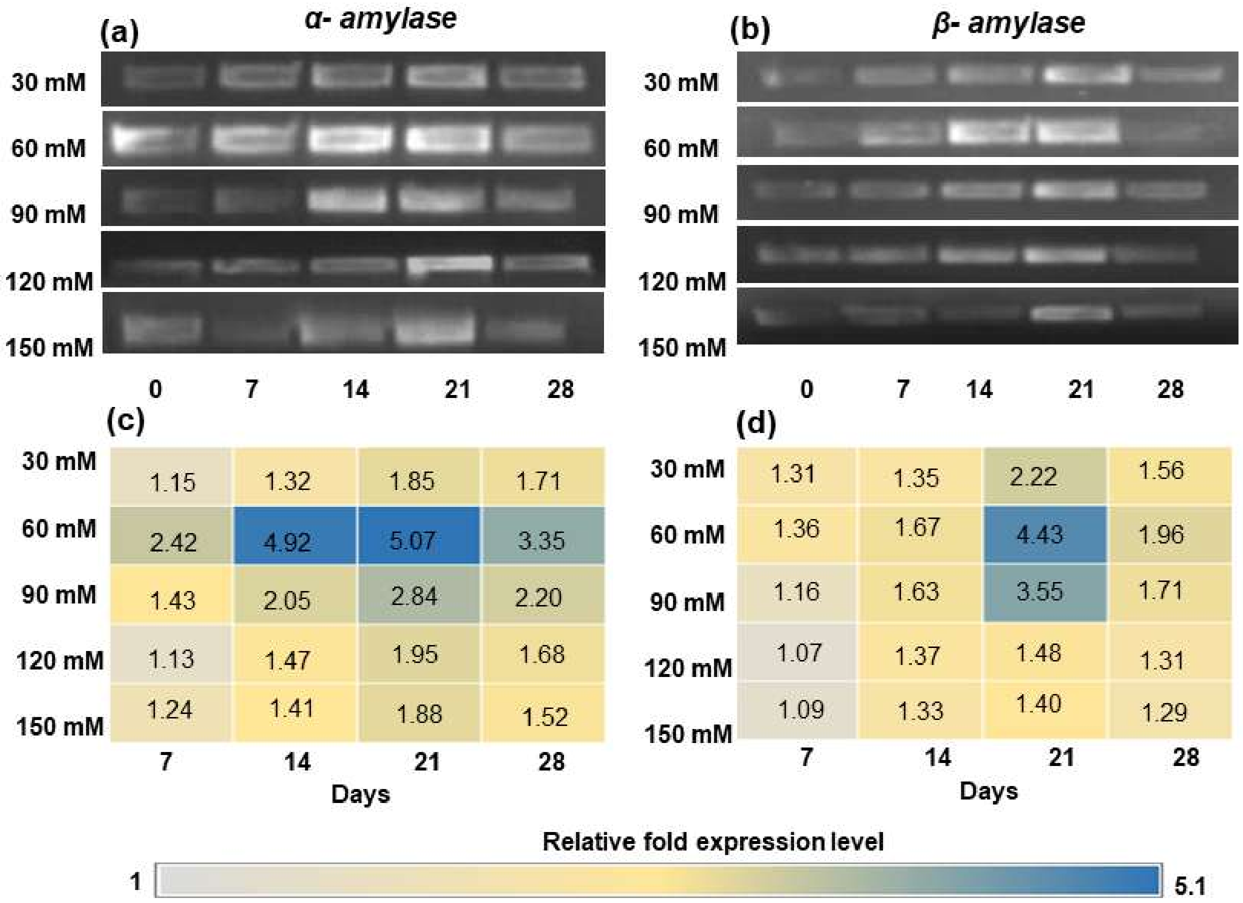
(c)

	7	14	21	28
30 mM	1.17	1.77	1.42	1.10
60 mM	1.71	4.19	3.87	3.33
90 mM	1.15	2.54	1.58	1.38
120 mM	1.05	1.24	1.21	1.15
150 mM	1.11	1.22	1.13	1.12

(d)

	7	14	21	28
30 mM	1.45	1.95	1.57	1.52
60 mM	3.36	4.39	3.67	2.63
90 mM	1.90	2.65	2.59	2.21
120 mM	1.47	2.41	1.78	1.72
150 mM	1.13	1.63	1.22	1.17

Fig. 5.9 The expression profiles of sucrose metabolising enzymes in internodal explants of cv. ‘Kufri Pukhraj’ after 0,7,14, 21, 28 days of culture on shoot organogenesis medium (MS3 medium) supplemented with different concentrations of sucrose (30-150 mg L^{-1}). **(a, b)** is the gel image showing expression level of acid invertase and sucrose synthase and **(c, d)** heat map of data derived through qRT-PCR analysis



(c)

	7	14	21	28
30 mM	1.15	1.32	1.85	1.71
60 mM	2.42	4.92	5.07	3.35
90 mM	1.43	2.05	2.84	2.20
120 mM	1.13	1.47	1.95	1.68
150 mM	1.24	1.41	1.88	1.52

(d)

	7	14	21	28
30 mM	1.31	1.35	2.22	1.56
60 mM	1.36	1.67	4.43	1.96
90 mM	1.16	1.63	3.55	1.71
120 mM	1.07	1.37	1.48	1.31
150 mM	1.09	1.33	1.40	1.29

Fig. 5.10 The expression profiles of starch metabolising enzymes in internodal explants of cv. 'Kufri Pukhraj' after 0,7,14, 21, 28 days of culture on shoot organogenesis medium (MS3 medium) supplemented with different concentrations of sucrose (30-150 mg L^{-1}). **(a, b)** is the gel image showing expression level of α-amylase and β-amylase and **(c, d)** heat map of data derived through qRT-PCR analysis

Chapter 6

Isolation of *Phytophthora infestans* and screening of potato cultivars against late blight

Abstract

In this chapter, the late blight susceptibility of Indian potato cvs. ('Kufri Pukhraj' (KP), 'Kufri Chipsona 1' (CS-1), 'Kufri Jyoti' (KJ), and 'Kufri Surya' (KS)) was investigated. A pure culture of *Phytophthora infestans* was isolated from the infected plants and microscopic examination confirmed it as an aseptate long-branched hyphae with lemon-shaped sporangia. Further confirmation of *Phytophthora infestans* was done based on molecular taxonomical tools such as amplification and sequencing of ITS region. The phylogenetic analysis indicated that the isolated culture is closely related to *P. infestans* strain ALI-786. The inoculation of *P. infestans* on potato cvs. resulted in varying levels of resistance to late blight. The detached leaf assay revealed significant differences in lesion diameters between all four potato cvs. The leaves of cv. 'Kufri Pukhraj' developed large irregular lesions when inoculated with a sporal suspension of *P. infestans*, whereas the leaves of cvs. 'Kufri Jyoti' and 'Kufri Chipsona 1' developed smaller lesions. Furthermore, no lesions were found on the leaves of cv. 'Kufri Surya'. In a whole plant assay, the potato cv. 'Kufri Pukhraj' showed maximum susceptibility to late blight (primarily disease severity and incidence), while cvs. 'Kufri Chipsona 1' and 'Kufri Jyoti' showed moderate resistance. It was notable that cv. 'Kufri Surya' was completely resistant to infection and showed no signs of infection.

6.1 Isolation and identification of *Phytophthora infestans*

6.1.1 Collection of samples from infected plants

Late blight-infected leaf samples were collected from potato fields at the Thapar Institute of Engineering and Technology, Patiala (Punjab). The samples were collected in January and February. In this month the weather conditions were ideal for *P. infestans* infection. Infected plants were identified by typical black/brown lesions on the leaf and stem parts. Lesions observed were irregular and water-soaked on the leaves. The sporangia and sporangiophores were produced on the lower surface of the infected leaves and were observed as white sporulation at the margins of lesions and along the petiole. Small portions of the leaf samples were taken and examined under a microscope first, and after confirming the presence of *P. infestans* hyphae and spores, the samples were processed for isolation.

6.1.2 Isolation and morphological identification of isolated culture

The leaf samples with lesions were cut into small parts and surface disinfected as described in section 3.9. To prevent the growth of other contaminating fungi and bacteria, the samples were inoculated on a 2% (w/v) water agar medium amended with rifamycin and natamycin antibiotics. After 3-4 days, the mycelia that had grown on the leaf were transferred to thin potato slices and placed in a moist chamber. Furthermore, a small portion of these were placed on a PDA medium amended with both the antibiotics. Both potato slices and PDA plates showed white-colored cottony growth after 5-7 days. Small hyphae were carefully picked and examined under the microscope. The hyphae were aseptate, long, branched, and transparent. They were densely packed with the nuclei in the cytoplasmic matrix. The spores were semi-papillate and lemon-shaped, measuring 30-50 µm in length.

6.1.3 Molecular identification of *Phytophthora infestans*

Further, the identity of isolated *P. infestans* was confirmed by amplifying the genomic DNA with *P. infestans* specific primers. A 600 bp band was amplified and sequenced. The nucleotide sequence obtained after sequencing was subjected to BLAST analysis that resulted in a list of other reported *P. infestans* ITS sequences, showing significant alignments. These sequences were selected, aligned with clustalW, and subjected to phylogenetic analysis by the neighbor-joining method through MEGA 6 software. The strength of internal branches was tested by

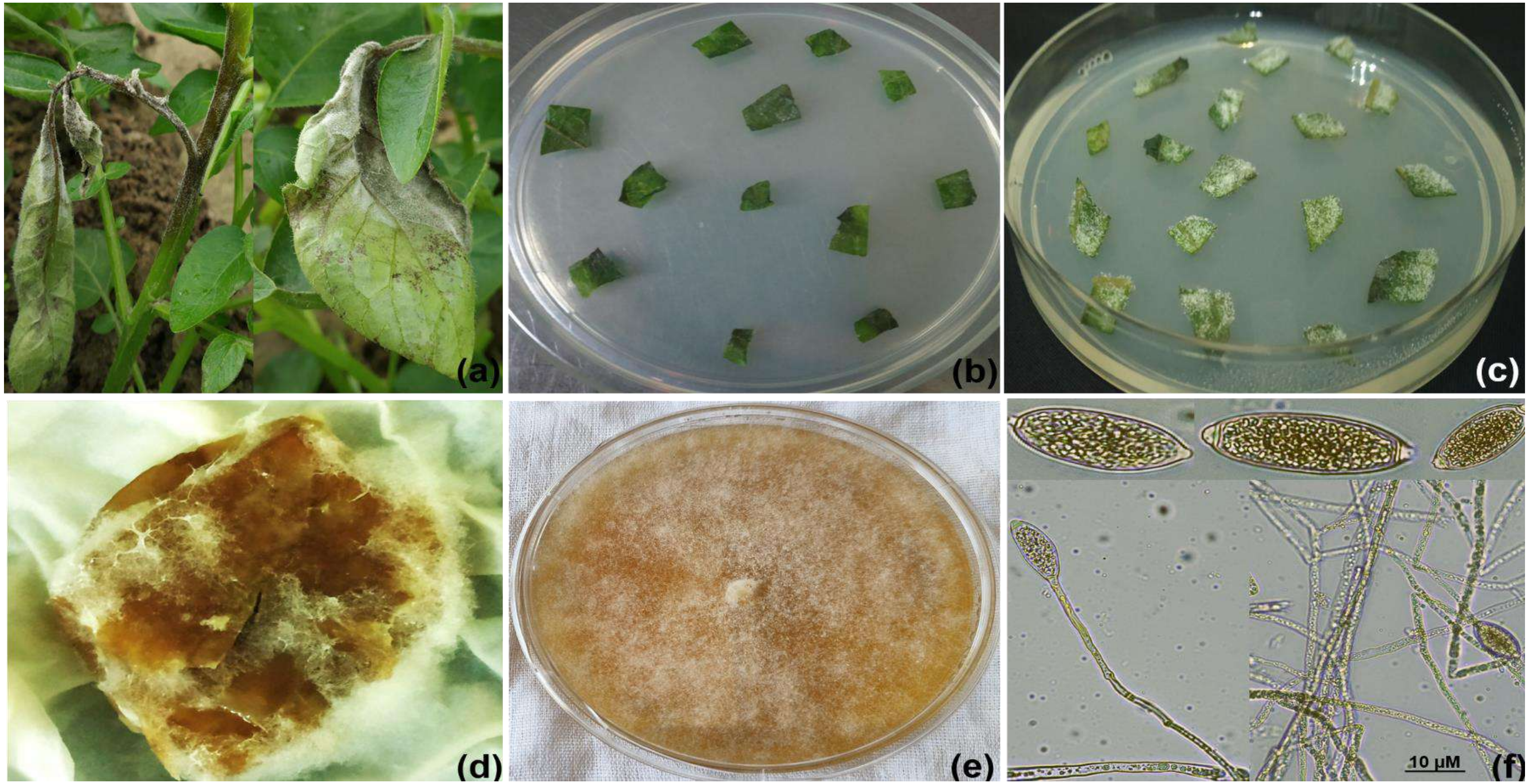


Fig. 6.1 Isolation of *P. infestans* **(a)** infected potato plant **(b)** infected leaf parts placed on water agar plates supplemented with 30 µg mL^{-1} rifamycin and 10 µg mL^{-1} natamycin **(c)** mycelium growth on leaf samples placed on water agar plates after 2 days **(d)** maintenance of pure culture of *P. infestans* on potato tuber slice and **(e)** on PDA plates containing 30 µg mL^{-1} rifamycin and 10 µg mL^{-1} natamycin **(f)** Microscopic visualisation of lemon shaped spores, sporangium and aseptate hyphae of *P. infestans* at 40 X

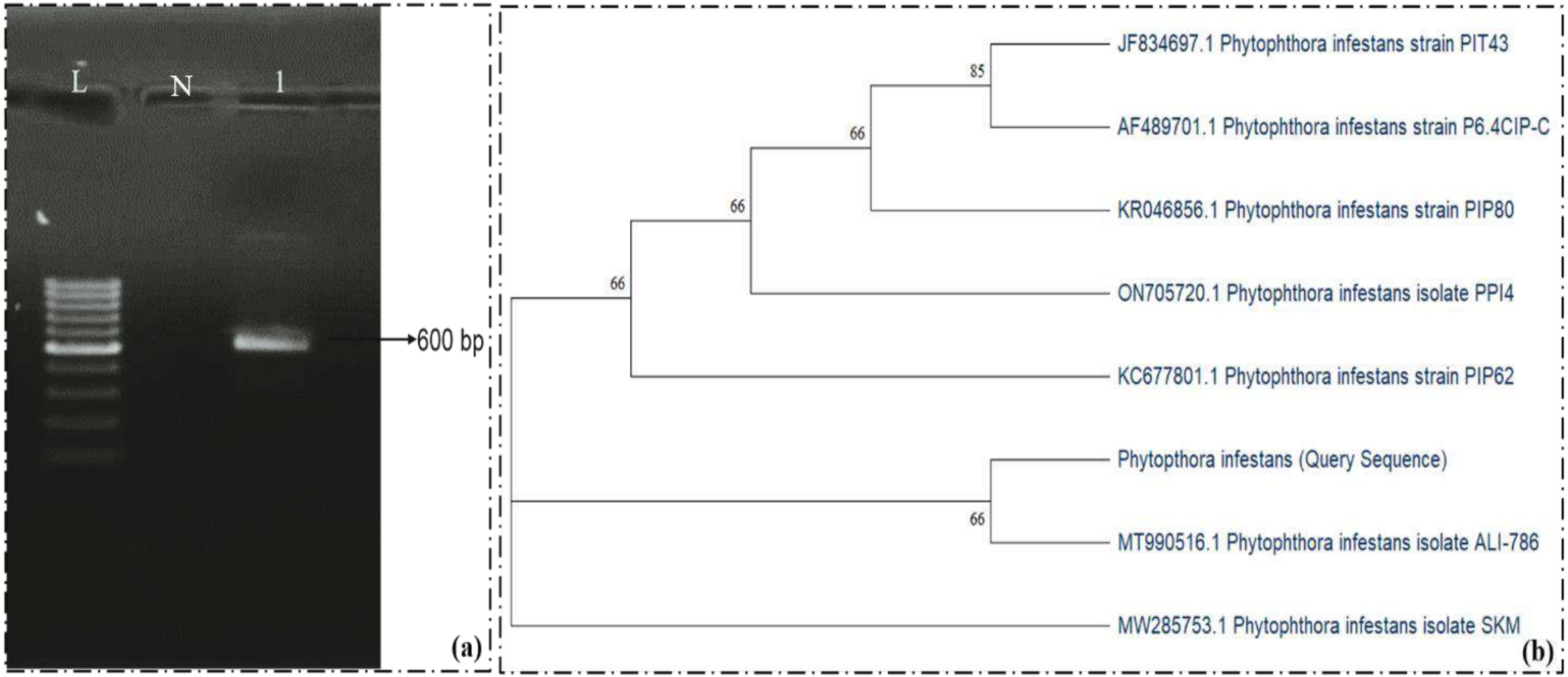


Fig. 6.2 (a) Molecular confirmation of isolated *Phytophthora infestans* by (Lane 1) amplification of 600 bp fragment from genomic DNA of isolated *P. infestans* with specific primers, L is the 100 bp ladder and N is the negative control **(b)** is the phylogenetic tree constructed using neighbour joining method of MEGA6 software after blast analysis of sequencing results

Table 6.1 BLAST analysis of nucleotide sequences of isolated *P. infestans* ITS region

Accessions	Description	Similarity (%)	E-value
JF834697.1	*Phytophthora infestans strain* PIT43 18S ribosomal RNA gene	92.77	0
AF489701.1	*Phytophthora infestans* strain P6.4CIP-C internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	96.07	0
KR046856.1	*Phytophthora infestans* strain PIP80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	96.07	0
ON705720.1	*Phytophthora infestans* isolate PPI4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	96.07	0
KC677801.1	*Phytophthora infestans* strain PIP62 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	96.07	0
MT990516.1	*Phytophthora infestans* isolate ALI-786 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	96.07	0
MW285753.1	*Phytophthora infestans* isolate SKM internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	96.07	0

bootstrap analysis from 1000 bootstrap replications. The phylogenetic analysis indicated that the isolated culture is closely related to *P. infestans* strain ALI-786.

6.2 Susceptibility of potato cultivars to late blight

The susceptibility of potato cultivars ‘Kufri Pukhraj’ (KP), ‘Kufri Chipsona 1’ (CS-1), ‘Kufri Jyoti’ (KJ), and ‘Kufri Surya’ (KS) to late blight disease was investigated. Detached leaf and whole plant assay were performed after inoculating the respective potato cultivars with *P. infestans* sporal suspension. In detached leaf assay, a drop of sporal suspension was placed on the leaves of respective cultivars placed in a moist chamber.

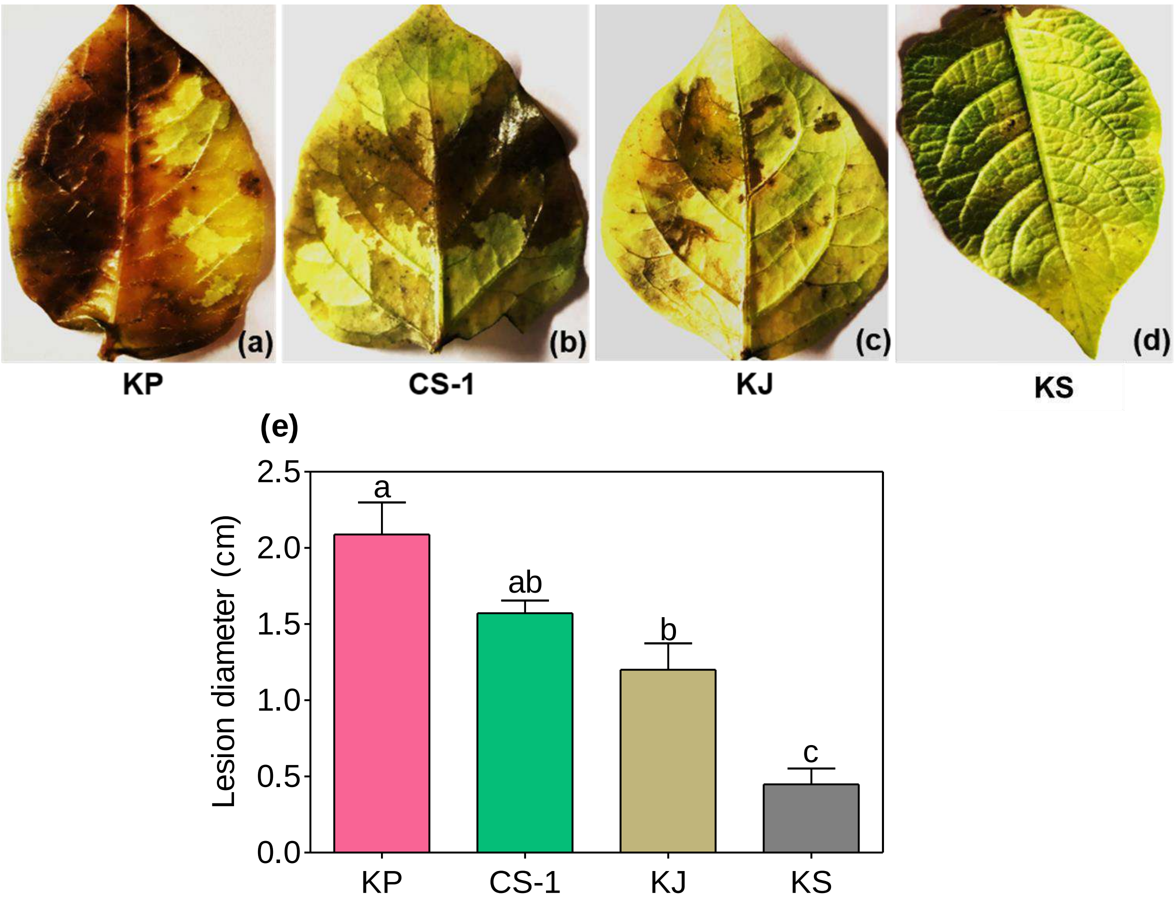


Fig. 6.3 Detached leaf assay performed on leaves of potato cvs. **(a)** Kufri Pukhraj’ (KP), **(b)** ‘Kufri Chipsona 1’ (CS-1), **(c)** ‘Kufri Jyoti’ (KJ), and **(d)** Kufri Surya (‘KS’) by inoculating with *P. infestans* sporal suspension **(e)** is the lesion diameter recorded after 10 days post inoculation, analysed by One way Analysis of variance (ANOVA) with significance level < 0.05 by LSD. Data was shown as Mean± Standard error

The lesion diameter was recorded on 10 days post-inoculation (dpi) of *P. infestans* sporal suspension. The results revealed a significant difference in potato cultivar's lesion diameters (P value=0.0004). The largest lesion diameter was found on the leaves of cv. 'Kufri Pukhraj' (2.08 cm), followed by cv. 'Kufri Chipsona1' (1.57 cm). However, in cv. 'Kufri Jyoti' lesion size (1.2 cm) was smaller as compared to cvs. 'Kufri Chipsona 1' (1.57 cm), and 'Kufri Pukhraj' (2.08 cm) at 10 dpi. On the other hand, the leaves of cv. 'Kufri Surya' developed a smaller lesion diameter (0.45 cm).

The whole plant assay revealed a similar pattern of susceptibility and resistance to late blight in potato cultivars. The late blight symptoms were first observed on the leaves of cv. 'Kufri Pukhraj', while it developed slowly in cvs. 'Kufri Chipsona 1', 'Kufri Jyoti', and 'Kufri Surya'. This demonstrated that these cultivars differ in their resistance level to late blight disease. Initially, the disease symptoms were not visible at 3 dpi but at 5 dpi the symptoms were visible as an irregular small lesion. The results were recorded as a measure of disease incidence and disease severity at 5 and 10 dpi. In cv. 'Kufri Pukhraj' the disease was progressing faster. At 5 dpi the 68.75% 'Kufri Pukhraj' plants were infected. Moreover, the lesions were also large. In cv. 'Kufri Chipsona 1' 56.25% plants were infected and the lesions were comparatively smaller than as observed in cv. 'Kufri Pukhraj'. Similarly, like 'Kufri Chipsona 1' smaller lesions were observed in cv. 'Kufri Jyoti' but only 43.75% of plants were infected. It was worth noting that only 6.25% of 'Kufri Surya' plants were infected with late

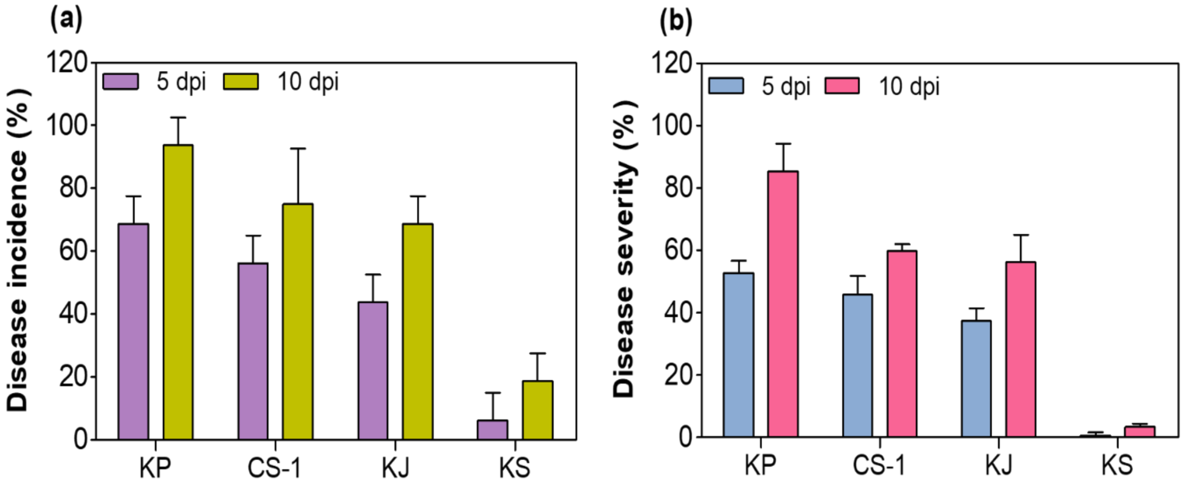


Fig. 6.4 Differences in the **(a)** disease incidence and **(b)** severity of Indian potato cvs. 'Kufri Pukhraj' (KP), 'Kufri Chipsona 1' (CS-1) and 'Kufri Jyoti' (KJ) and 'Kufri Surya' (KS) recorded on 5 and 10 days post inoculation and analysed by Two-way Analysis of variance (ANOVA) with significance level < 0.05

blight. However, at 10 dpi the infection progressed further and resulted in 93.75% of cv. ‘Kufri Pukhraj’ plants infected with late blight. In cvs. ‘Kufri Chipsona 1’ and ‘Kufri Jyoti’ 75% and 68.75% of plants were infected, respectively. It was noteworthy that disease was limited to only 18.75% of cv. ‘Kufri Surya’ plants at 10 dpi.

Additionally, the disease severity was also calculated and it was recorded that 52.77% of cv. ‘Kufri Pukhraj’ leaves were affected with late blight followed by 45.83% of cv. ‘Kufri Chipsona 1’ leaves. A similar pattern was also observed in disease severity where 37.49% of cv. ‘Kufri Jyoti’ leaves, and 0.694% of cv.‘Kufri Surya’ leaves were infected at 5 dpi. However, the severity of the disease also increased with days and resulted in 85.41% leaf damage in cv. ‘Kufri Pukhraj’ at 10 dpi. However, in cvs. ‘Kufri Chipsona 1’, ‘Kufri Jyoti’, and ‘Kufri Surya’ the leaf damage restricted up to 59.83%, 56.25%, and 3.46% respectively at 10 dpi. Considering these results, the cv. ‘Kufri Pukhraj’ is referred to as late blight sensitive while ‘Kufri Chipsona 1’ and ‘Kufri Jyoti’, are referred to as moderately resistant and ‘Kufri Surya’ as a resistant potato cultivar, respectively.

Table 6.2 Two-way ANOVA table of disease incidence and severity in potato cultivars infected with *P. infestans* sporal suspension

Source	*Df*	*Sum of square*	*Mean square*	*F value*	*P value*
			Disease incidence		
Between days post inoculation (dpi)	1	1650	1650	15.36	0.0044
Between cultivars	3	10420	3473	32.33	<0.0001
Interaction	3	107.4	35.81	0.3333	0.8018
Error	8	859.4	107.4		
			Disease severity		
Between days post inoculation (dpi)	1	1161	1161	40.71	0.0002
Between cultivars	3	9866	3289	115.3	<0.0001
Interaction	3	459.0	153.0	5.362	0.0256
Error	8	228.3	28.53		

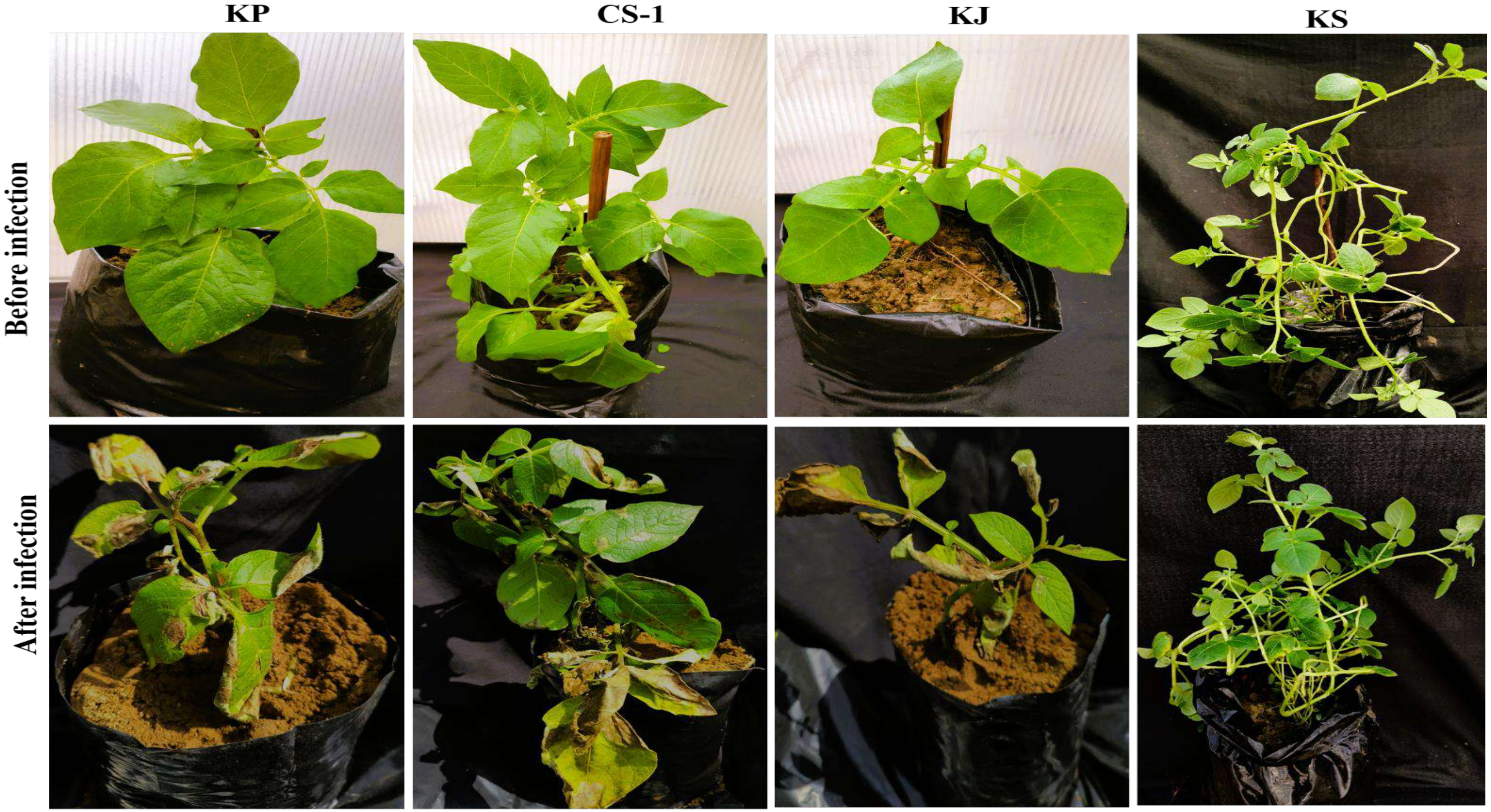


Fig. 6.5 Plants of potato cvs. Kufri Pukhraj' (KP), 'Kufri Chipsona 1' (CS-1), 'Kufri Jyoti' (KJ), and 'Kufri Surya'(KS) before and after infection with sporal suspension of *P. infestans*

Chapter 7

Development of RNAi construct(s) for silencing of cellulose synthase gene in *P. infestans*

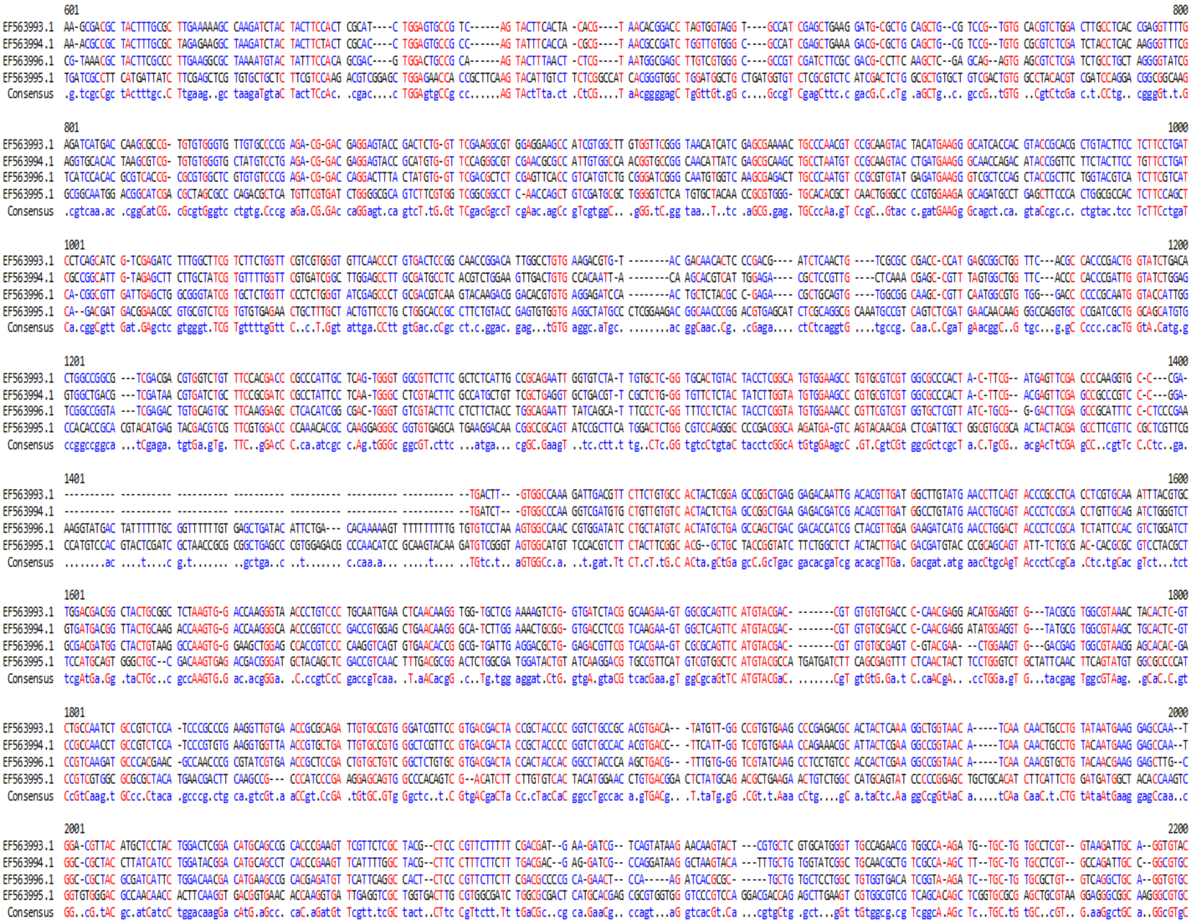
Abstract

In this chapter, the RNAi construct(s) targeting the cellulose synthase gene (*CesA*) of *P. infestans* was developed. Based on the differences in the N terminal region of the *CesA* gene of *P. infestans* and potato, a target sequences of ~200 bp was selected which is non-homologous to the potato *CesA* gene sequence. The target sequences were amplified in sense and antisense orientation with required overhangs and further restriction digested with *AscI, SwaI* (for sense), and *BamHI, XbaI* (for antisense) restriction enzymes. The cloning was performed in a stepwise manner, first the sense fragment was cloned in the hp-RNAi plasmid, and then to that plasmid, the antisense fragment was cloned. The cloning of sense and antisense was confirmed by amplification and restriction digestion of respective fragments. The modified hp-RNAi plasmid carrying sense and antisense fragment showed the expected fallout (~200 bp) through restriction digestion with *AscI, SwaI,* and *BamHI, XbaI* respectively. Moreover, amplification with modified plasmid using specific primers also showed the expected amplified band of sense and antisense fragment~200 bp.

7.1 Development of RNAi construct I containing Pi*CesA3* gene fragment in sense and antisense orientation

7.1.1 Selection of target sequence for Pi*CesA3*-RNAi construct I

FASTA sequences of cellulose synthase (*CesA*) genes of *P. infestans* were downloaded from the NCBI (>EF563993.1, >EF563994.1, >EF563995.1, >EF563996.1). These sequences were aligned to find the conserved region. The region was then matched with the potato, potato *CesA* genes, and other available potato genes to find the level of homology within sequence for the construction of RNAi construct I. A region of lower homology at the N-terminal were selected.



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           601                                                                                                                                                                                                            800
EF563993.1 AA-GCGACGC TACTTTGCGC TTGAAAAAGC CAAGATCTAC TACTTCCACT CGCAT----C TGGAGTGCCG TC------AG TACTTCACTA -CACG----T AACACGGACC TAGTGGTAGG T----GCCAT CGAGCTGAAG GATG-CGCTG CAGCTG--CG TCCG--TGTG CACGTCTGGA CTTGCCTCAC CGAGGTTTTG
EF563994.1 AA-ACGCCGC TACTTTGCGC TAGAGAAGGC TAAGATCTAC TACTTCTACT CGCAC----C TGGAGTGCCG CC------AG TATTTCACCA -CGCG----T AACGCCGATC TGGTTGTGGG C----GCCAT CGAGCTGAAA GACG-CGCTG CAGCTG--CG TCCG--TGTG CGCGTCTCGA TCTACCTCAC AAGGGTTTCG
EF563996.1 CG-TAAACGC TACTTCGCCC TTGAAGGCGC TAAAATGTAC TATTTCCACA GCGAC----G TGGACTGCCG CA------AG TACTTTAACT -CTCG----T AATGGCGAGC TTGTCGTGGG C----GCCGT CGATCTTCGC GACG-CCTTC AAGCTC--GA GCAG--AGTG AGCGTCTCGA TCTGCCTGCT AGGGGTATCG
EF563995.1 TGATCGCCTT CATGATTATC TTCGAGCTCG TGTGCTGCTC TTCGTCCAAG ACGTCGGAGC TGGAGAACCA CCGCTTCAAG TACATTGTCT TCTCGGCCAT CACGGGTGGC TGGATGGCTG CTGATGGTGT CTCGCGTCTC ATCGACTCTG GCGCTGTGCT GTCGACTGTG GCCTACACGT CGATCCAGGA CGGCGGCAAG
 Consensus .g.tcgcCgc tActttgc.C Ttgaag..gc taagaTgtaC TactTCcAc. .cgac....c TGGAgtgCCg cc......AG TActTta.ct .CtCG....T aAcggggagC TgGttGt.gG c....GccgT CgagCttc.c gacG.C.cTg .aGCTg..c. gccG..tGTG ..CgtCtcGa c.t.CCtg.. cgggGt.t.G

           801                                                                                                                                                                                                           1000
EF563993.1 AGATCATGAC CAAGCGCCG- TGTGTGGGTG TTGTGCCCCG AGA-CG-GAC GAGGAGTACC GACTCTG-GT TCGAAGGCGT GGAGGAAGCC ATCGTGGCTT GTGGTTCGGG TAACATCATC GAGCGAAAAC TGCCCAACGT CCGCAAGTAC TACATGAAGG GCATCACCAC GTACCGCACG CTGTACTTCC TCTTCCTGAT
EF563994.1 AGGTGCACAC TAAGCGTCG- TGTGTGGGTG CTATGTCCTG AGA-CG-GAC GAGGAGTACC GCATGTG-GT TCCAGGGCGT CGAACGCGCC ATTGTGGCCA ACGGTGCCGG CAACATTATC GAGCGCAAGC TGCCTAATGT CCGCAAGTAC CTGATGAAGG GCAACCAGAC ATACCGGTTC TTCTACTTCC TGTTCCTGAT
EF563996.1 TCATCCACAC GCGTCACCG- CGCGTGGCTC GTGTGTCCCG AGA-CG-GAC CAGGACTTTA CTATGTG-GT TCGACGCTCT CGAGTTCACC GTCATGTCTG CGGGATCGGG CAATGTGGTC AAGCGAGACT TGCCCAATGT CCGCGTGTAT GAGATGAAGG GTCGCTCCAG CTACCGCTTC TGGTACGTCA TCTTCGTCAT
EF563995.1 GCGGCAATGG ACGGCATCGA CGCTAGCGCC CAGACGCTCA TGTTCGTGAT CTGGGGCGCA GTCTTCGTGG TCGGCGGCCT C-AACCAGCT GTCGATGCGC TGGGGTCTCA TGTGCTACAA CCGCGTGGG- TGCACACGCT CAACTGGGCC CCGTGGAAGA GCAGATGCCT GAGCTTCCCA CTGGCGCCAC TCTTCCAGCT
 Consensus .cgtcaa.ac .cggCatCG. cGcgtGggtc ctgtg.Cccg aGa.CG.GAc caGGagt.ca gtcT.tG.Gt TCgacGgccT cgAac.agCc gTcgtggC.. .gGG.tC.gg taa..T..tc .aGCG.gag. TGCccAa.gT CcgC..Gtac c.gatGAAGg Gcagct.ca. gtaCcgc.c. ctgtac.tcc TcTTCctgaT

           1001                                                                                                                                                                                                          1200
EF563993.1 CCTCAGCATC G-TCGAGATC TTTGGCTTCG TCTTCTGGTT CGTCGTGGGT GTTCAACCCT GTGACTCCGG CAACCGGACA TTGGCCTGTG AAGACGTG-T --------AC GACAACACTC CCGACG---- ATCTCAACTG ----TCGCGC CGACC-CCAT GAGCGGCTGG TTC---ACGC CACCCGACTG GTATCTGACA
EF563994.1 CGCCGGCATT G-TAGAGCTT CTTGCTATCG TGTTTTGGTT CGTGATCGGC TTGGAGCCTT GCGATGCCTC ACGTCTGGAA GTTGACTGTG CCACAATT-A --------CA AGCACGTCAT TGGAGA---- CGCTCCGTTG ----CTCAAA CGAGC-CGTT TAGTGGCTGG TTC---ACCC CACCCGATTG GTATCTGGAG
EF563996.1 CA-CGGCGTT GATTGAGCTG GCGGGTATCG TGCTCTGGTT CCCTCTGGGT ATCGAGCCCT GCGACGTCAA GTACAAGACG GACACGTGTG AGGAGATCCA --------AC TGCTCTACGC C-GAGA---- CGCTGCAGTG ----TGGCGG CAAGC-CGTT CAATGGCGTG TGG---GACC CCCCGCAATG GTACCATTGG
EF563995.1 CA--GACGAT GACGGAACGC GTGCGTCTCG TGTGTGAGAA CTGCTTTGCT ACTGTTCCTG CTGGCACCGC CTTCTGTACC GAGTGTGGTG AGGCTATGCC CTCGGAAGAC GGCAACCCGG ACGTGAGCAT CTCGCAGGCG CAAATGCCGT CAGTCTCGAT GAACAACAAG GGCCAGGTGC CCGATCGCTG GCAGCATGTG
 Consensus Ca.cggCgtt Gat.GAgctc gtgggt.TCG TgttttgGtt C..c.T.Ggt attga.CCtt gtGac.cCgc ct.c.ggac. gag...tGTG aggc.aTgc. ........ac ggCaac.Cg. .cGaga.... ctCtcaggtG ....tgccg. Caa.C.CgaT gAacggC..G tgc...g.gC Cccc.cacTG GtA.Catg.g

           1201                                                                                                                                                                                                          1400
EF563993.1 CTGGCCGGCG ---TCGACGA CGTGGTCTGT TTCCACGACC CGCCCATTGC TCAG-TGGGT GGCGTTCTTC GCTCTCATTG CCGCAGAATT GGTGTCTA-T TGTGCTC-GG TGCACTGTAC TACCTCGGCA TGTGGAAGCC TGTGCGTCGT GGCGCCCACT A-C-TTCG-- ATGAGTTCGA CCCCAAGGTG C-C---CGA-
EF563994.1 GTGGCTGACG ---TCGATAA CGTGATCTGC TTCCGCGATC CGCCTATTCC TCAA-TGGGC CTCGTACTTC GCCATGCTGT TCGCTGAGGT GCTGACGT-T CGCTCTG-GG TGTTCTCTAC TATCTTGGTA TGTGGAAGCC CGTGCGTCGT GGCGCCCACT A-C-TTCG-- ACGAGTTCGA GCCGCCCGTC C-C---GGA-
EF563996.1 TCGGCCGGTA ---TCGAGAC TGTGCAGTGC TTCAAGGAGC CTCACATCGG CGAC-TGGGT GTCGTACTTC CTCTTCTACC TGGCAGAATT TATCAGCA-T TTCCCTC-GG TTTCCTCTAC TACCTCGGTA TGTGGAAACC CGTTCGTCGT GGTGCTCGTT ATC-TGCG-- G-GACTTCGA GCCGCATTTC C-CTCCCGAA
EF563995.1 CCACACCGCA CGTACATGAG TACGACGTCG TTCGTGGACC CCAAACACGC CAAGGAGGGC GGTGTGAGCA TGAAGGACAA CGGCCGCAGT ATCCGCTTCA TGGACTCTGG CGTCCAGGGC CCCGACGGCA AGATGA-GTC AGTACAACGA CTCGATTGCT GGCGTGCGCA ACTACTACGA GCCTTCGTTC CGCTCGTTCG
 Consensus ccggccggca ...tCgaga. tgtGa.gTg. TTC..gGAcC C.ca.atcgc c.Ag.tGGGc ggcGT.cttc ...atga... cgGC.GaagT ..tc.ctt.t tg..CTc.GG tgtcCtgtaC tacctcGGcA tGtgGAagcC .GT.CgtCGt ggcGctcgcT a.C.TgCG.. acgAcTtCGA gCC..cgtTc C.Ctc..ga.

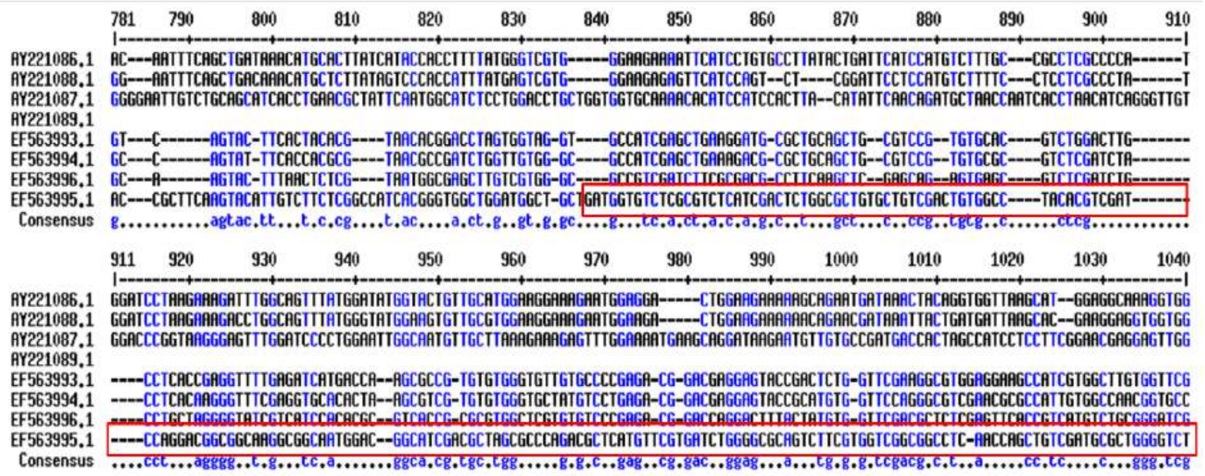
           1401                                                                                                                                                                                                          1600
EF563993.1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --TGACTT-- -GTGGCCAAA GATTGACGTT CTTCTGTGCC ACTACTCGGA GCCGGCTGAG GAGACAATTG ACACGTTGAT GGCTTGTATG AACCTTCAGT ACCCGCCTCA CCTCGTGCAA ATTTACGTGC
EF563994.1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --TGATCT-- -GTGGCCCAA GGTCGATGTG CTGTTGTGTC ACTACTCTGA GCCGGCTGAA GAGACGATCG ACACGTTGAT GGCCTGTATG AACCTGCAGT ACCCTCCGCA CCTGTTGCAG ATCTGGGTCT
EF563996.1 AAGGTATGAC TATTTTTTGC GGTTTTTTGT GAGCTGATAC ATTCTGA--- CACAAAAAGT TTTTTTTTTG TGTGTCCTAA AGTGGCCAAC CGTGGATATC CTGCTATGTC ACTATGCTGA GCCAGCTGAC GACACCATCG CTACGTTGGA GAAGATCATG AACCTGGACT ACCCTCCGCA TCTATTCCAC GTCTGGATCT
EF563995.1 CCATGTCCAC GTACTCGATC GCTAACCGCG CGGCTGAGCC CGTGGAGACG CCCAACATCC GCAAGTACAA GATGTCGGGT AGTGGCATGT TCCACGTCTT CTACTTCGGC ACG--GCTGC TACCGGTATC TTCTGGCTCT ACTACTTGAC GACGATGTAC CCGCAGCAGT ATT-TCTGCG AC-CACGCGC GTCCTACGCT
 Consensus ........ac ....t....c g.t....... ..gctga..c ..t....... c.caa.a... .....t.... ..TGtc.t.. aGTGGCc.a. ..t.gat.Tt CT.cT.tG.C ACta.gCtGa gcC.GcTgac gacacgaTcg acacgTTGa. Gacgat.atg aacCtgcAgT AccctCcgCa .Ctc.tgCac gTct...tct

           1601                                                                                                                                                                                                          1800
EF563993.1 TGGACGACGG CTACTGCGGC TCTAAGTG-G ACCAAGGGTA ACCCTGTCCC TGCAATTGAA CTCAACAAGG TGG-TGCTCG AAAAGTCTG- GTGATCTACG GCAAGAA-GT GGCGCAGTTC ATGTACGAC- -------CGT GTGTGTGACC C-CAACGAGG ACATGGAGGT G---TACGCG TGGCGTAAAC TACACTC-GT
EF563994.1 GTGATGACGG TTACTGCAAG ACCAAGTG-G ACCAAGGGCA ACCCGGTCCC GACCGTGGAG CTGAACAAGG GCA-TCTTGG AAACTGCGG- GTGACCTCCG TCAAGAA-GT GGCTCAGTTC ATGTACGAC- -------CGT GTGTGCGACC C-CAACGAGG ATATGGAGGT G---TATGCG TGGCGTAAGC TGCACTC-GT
EF563996.1 GCGACGATGG CTACTGTAAG GCCAAGTG-G GAAGCTGGAG CCACCGTCCC CAAGGTCAGT GTGAACACCG GCG-TGATTG AGGACGCTG- GAGACGTTCG TCACGAA-GT CGCGCAGTTC ATGTACGAC- -------CGT GTGTGCGAGT C-GTACGAA- --CTGGAAGT G---GACGAG TGGCGTAAGG AGCACAC-GA
EF563995.1 TCCATGCAGT GGGCTGC--C GACAAGTGAG ACGACGGGAT GCTACAGCTC GACCGTCAAC TTTGACGCGG ACTCTGGCGA TGGATACTGT ATCAAGGACG TGCCGTTCAT GTCGTGGCTC ATGTACGCCA TGATGATCTT CAGCGAGTTT CTCAACTACT TCCTGGGTCT GCTATTCAAC TTCAGTATGT GGCGCCCCAT
 Consensus tcgAtGa.Gg .taCTGc..c gccAAGTG.G ac.acgGGa. .C.ccgtCcC gaccgTcaa. .T.aACacgG .c..Tg.tgg aggat.CtG. gtgA.gtaCG tcacGaa.gT ggCgcaGtTC ATGTACGaC. .......CgT gtGtG.Ga.t C.caACgA.. .ccTGGa.gT G...tacgag TggcGTAag. .gCaC.C.gt

           1801                                                                                                                                                                                                          2000
EF563993.1 CTGCCAATCT GCCGTCTCCA -TCCCGCCCG AAGGTTGTGA ACCGCGCAGA TTGTGCCGTG GGATCGTTCC GTGACGACTA CCGCTACCCC GGTCTGCCGC ACGTGACA-- -TATGTT-GG CCGTGTGAAG CCCGAGACGC ACTACTCAAA GGCTGGTAAC A-----TCAA CAACTGCCTG TATAATGAAG GAGCCAA--T
EF563994.1 CCGCCAACCT GCCGTCTCCA -TCCCGTGTG AAGGTGGTTA ACCGTGCTGA TTGTGCCGTG GGCTCGTTCC GTGACGACTA CCGCTACCCC GGTCTGCCAC ACGTGACC-- -TTCATT-GG TCGTGTGAAA CCAGAAACGC ATTACTCGAA GGCCGGTAAC A-----TCAA CAACTGCCTG TACAATGAAG GAGCCAA--T
EF563996.1 CCGTCAAGAT GCCCACGAAC -GCCAACCCG CGTATCGTGA ACCGCTCCGA CTGTGCTGTC GGCTCTGTGC GTGACGACTA CCACTACCAC GGCCTACCCA AGCTGACG-- -TTTGTG-GG TCGTATCAAG CCTCCTGTCC ACCACTCGAA GGCCGGTAAC A-----TCAA CAACGTGCTG TACAACGAAG GAGCTTG--C
EF563995.1 CCGTCGTGGC GCGCGCTACA TGAACGACTT CAAGCCG--- CCCATCCCGA AGGAGCAGTG GCCCACAGTC G--ACATCTT CTTGTGTCAC TACATGGAAC CTGTGACGGA CTCTATGCAG ACGCTGAAGA ACTGTCTGGC CATGCAGTAT CCCCCGGAGC TGCTGCACAT CTTCATTCTG GATGATGGCT ACACCAAGTC
 Consensus CcGtCaag.t GCcc.Ctaca .gcccg.ctg ca.gtcGt.a aCCgt.CcGA .tGtGC.GTg Ggctc..t.C GtgACgaCTa Cc.cTacCaC ggccTgccac a.gTGACg.. .T.taTg.gG .CGt.t.Aaa cCtg....gC a.taCtc.Aa ggCcgGtAaC a.....tCAa CaaC.t.CTG tAtaAtGaag gagCcaa..c

           2001                                                                                                                                                                                                          2200
EF563993.1 GGA-CGTTAC ATGCTCCTAC TGGACTCGGA CATGCAGCCG CACCCGAAGT TCGTTCTCGC TACG--CTCC CGTTCTTTTT CGACGAT--G AA-GATCG-- TCAGTATAAG AACAAGTACT ---CGTGCTC GTGCATGGGT TGCCAGAACG TGGCCA-AGA TG--TGC-TG TGCCTCGT-- GTAAGATTGC A--GGTGTAC
EF563994.1 GGC-CGCTAC CTTATCATCC TGGATACGGA CATGCAGCCT CACCCGAAGT TCATTTTGGC TACG--CTTC CTTTCTTCTT TGACGAC--G AG-GATCG-- CCAGGATAAG GCTAAGTACA ---TTTGCTG TGGTATCGGC TGCAACGCTG TCGCCA-AGC TT--TGC-TG TGCCTCGT-- GCCAGATTGC C--GGCGTGC
EF563996.1 GGC-CGCTAC GCGATCATTC TGGACAACGA CATGAAGCCG CACGAGATGT TCATTCAGGC CACT--CTCC CGTTCTTCTT CGACGCCCCG CA-GAACT-- CCA-----AG ATCACGCGC- -----TGCTG TGCTCCTGGC TGTGGTGACA TCGGTA-AGA TC--TGC-TG TGCGCTGT-- GTCAGGCTGC A--GGTGTGC
EF563995.1 GGTGTGGGAC GCCAACAACC ACTTCAAGGT GACGGTGAAC ACCAAGGTGA TTGAGGTCGC TGGTGACTTG CGTGGCGATC TGGCGCGACT CATGCACGAG CGCGTGGTGG GTCCCGTCCA GGACGACCAG AGCTTGAAGT CGTGGCGTCG TCAGCACAGC TCGGTGCGCG AGCTGCGTAA GGAGGGCGGC AAGGGCGTGC
 Consensus GG..cG.tAC gc.atCatcC tggacaagGa cAtG.aGcc. caC.aGatGt Tcgtt.tcGC tact..CTtc CgTtctt.Tt tGaCGc..cg ca.GaaCg.. ccagt...aG gtcacGt.Ca ...cgtgCtg .gct...gGt tGtggcg.cg TcggcA.AGc Tc..TGC.tG tGC..cGT.. G.aaGgctGC a..GGcGTgC
```

Fig. 7.1 Multiple sequence alignment of cellulose synthase (*CesA*) genes of *P. infestans* to find the conserve region



```
            781    790       800       810       820       830       840       850       860       870       880       890       900       910
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY221086.1  AC---AATTTCAGCTGATAAACATGCACTTATCATACCACCTTTTATGGGTCGTG-----GGAAGAAAATTCATCCTGTGCCTTATACTGATTCATCCATGTCTTTGC---CGCCTCGCCCCA------T
AY221088.1  GG---AATTTCAGCTGACAAACATGCTCTTATAGTCCCACCATTTATGAGTCGTG-----GGAAGAGAGTTCATCCAGT--CT----CGGATTCCTCCATGTCTTTTC---CTCCTCGCCCTA------T
AY221087.1  GGGGAATTGTCTGCAGCATCACCTGAACGCTATTCAATGGCATCTCCTGGACCTGCTGGTGGTGCAAAACACATCCATCCACTTA--CATATTCAACAGATGCTAACCAATCACCTAACATCAGGGTTGT
AY221089.1
EF563993.1  GT---C------AGTAC-TTCACTACACG----TAACACGGACCTAGTGGTAG-GT----GCCATCGAGCTGAAGGATG-CGCTGCAGCTG--CGTCCG--TGTGCAC----GTCTGGACTTG-------
EF563994.1  GC---C------AGTAT-TTCACCACGCG----TAACGCCGATCTGGTTGTGG-GC----GCCATCGAGCTGAAAGACG-CGCTGCAGCTG--CGTCCG--TGTGCGC----GTCTCGATCTA-------
EF563996.1  GC---A------AGTAC-TTTAACTCTCG----TAATGGCGAGCTTGTCGTGG-GC----GCCGTCGATCTTCGCGACG-CCTTCAAGCTC--GAGCAG--AGTGAGC----GTCTCGATCTG-------
EF563995.1  AC---CGCTTCAAGTACATTGTCTTCTCGGCCATCACGGGTGGCTGGATGGCT-GCTGATGGTGTCTCGCGTCTCATCGACTCTGGCGCTGTGCTGTCGACTGTGGCC----TACACGTCGAT-------
   Consensus  g...........agtac.tt...t.c.cg....t.ac....a.ct.g..gt.g.gc....g...tc.a.ct.a.c.a.g.c..t...gct...c..ccg..tgtg..c......ctcg............

            911    920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY221086.1  GGATCCTAAGAAAGATTTGGCAGTTTATGGATATGGTACTGTTGCATGGAAGGAAAGAATGGAGGA-----CTGGAAGAAAAAGCAGAATGATAAACTACAGGTGGTTAAGCAT--GGAGGCAAAGGTGG
AY221088.1  GGATCCTAAGAAAGACCTGGCAGTTTATGGGTATGGAAGTGTTGCGTGGAAGGAAAGAATGGAAGA-----CTGGAAGAAAAAACAGAACGATAAATTACTGATGATTAAGCAC--GAAGGAGGTGGTGG
AY221087.1  GGACCCGGTAAGGGAGTTTGGATCCCCTGGAATTGGCAATGTTGCTTAAAGAAAGAGTTTGGAAAATGAAGCAGGATAAGAATGTTGTGCCGATGACCACTAGCCATCCTCCTTCGGAACGAGGAGTTGG
AY221089.1
EF563993.1  ----CCTCACCGAGGTTTTGAGATCATGACCA--AGCGCCG-TGTGTGGGTGTTGTGCCCCGAGA-CG-GACGAGGAGTACCGACTCTG-GTTCGAAGGCGTGGAGGAAGCCATCGTGGCTTGTGGTTCG
EF563994.1  ----CCTCACAAGGGTTTCGAGGTGCACACTA--AGCGTCG-TGTGTGGGTGCTATGTCCTGAGA-CG-GACGAGGAGTACCGCATGTG-GTTCCAGGGCGTCGAACGCGCCATTGTGGCCAACGGTGCC
EF563996.1  ----CCTGCTAGGGGTATCGTCATCCACACGC--GTCACCG-CGCGTGGCTCGTGTGTCCCGAGA-CG-GACCAGGACTTTACTATGTG-GTTCGACGCTCTCGAGTTCACCGTCATGTCTGCGGGATCG
EF563995.1  ----CCAGGACGGCGGCAAGGCGGCAATGGAC--GGCATCGACGCTAGCGCCCAGACGCTCATGTTCGTGATCTGGGGCGCAGTCTTCGTGGTCGGCGGCCTC-AACCAGCTGTCGATGCGCTGGGGTCT
   Consensus  ....cct...agggg..t.g...tc.a.......ggca.cg.tgc.tgg.....g.g.c..gag..cg.gac..ggag...a...tg.g.g.tcgacg.c.t..a.....cc.tc....c...ggg.tcg
```

Fig. 7.2 Multiple sequence alignment of cellulose synthase (*CesA*) gene of *P. infestans* with cellulose synthase (*CesA*) gene of potato. The highlighted red part is the selected fragment for making RNAi construct I

7.1.2 Cloning of Pi*CesA3* gene fragment in sense orientation

The selected target sequence (~200bp) was amplified in sense and antisense orientation (Fig. 7.3) with specific primers containing specific overhangs of restriction enzymes for sense (*AscI* and *SwaI)* and antisense (*BamHI* and *XbaI*) fragments. The amplified fragments were sent for sequencing to confirm the correct nucleotide sequence and cloned in a desired orientation in the hp-RNAi plasmid.

For cloning of sense fragment, *AscI* and *SwaI* restriction enzymes were used. The amplified sense fragment with overhangs and the hp-RNAi plasmid were restriction digested with *AscI* and *SwaI* to generate compatible ends (Fig. 7.4 and Fig. 7.5). The digested sense DNA fragment and hp-RNAi plasmid were ligated to get the circular plasmid containing sense DNA fragment (referred as hp-RNAi+sense). The modified plasmid containing sense was transformed in *E. coli* DH5α cells. The hp-RNAi+sense plasmid showed the expected fallout (~200 bp) of sense upon digestion with *AscI* and *SwaI* (Fig.7.6b). Moreover, amplification of modified plasmid with specific primers showed the expected band of ~200 bp (Fig. 7.6a).

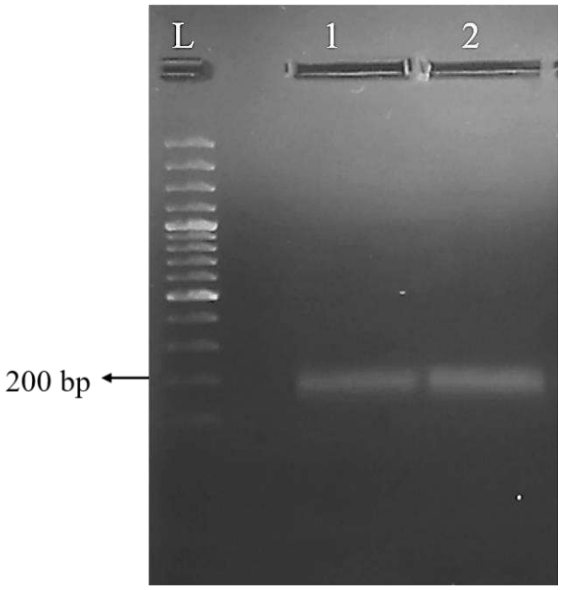


Fig. 7.3 Amplification of sense (lane1) and antisense fragment (lane 2) where L is the 100 bp plus ladder

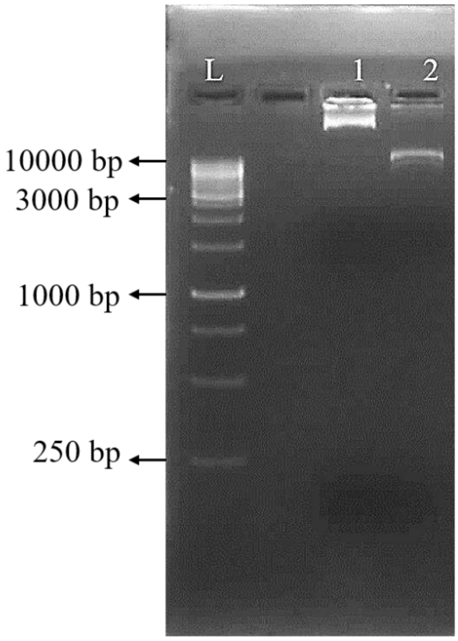


Fig. 7.4 Restriction digestion of hp-RNAi plasmid, lane 1 is an undigested plasmid, lane 2 is digested plasmid with *AscI* and *SwaI* restriction enzymes and L is the 1kb ladder

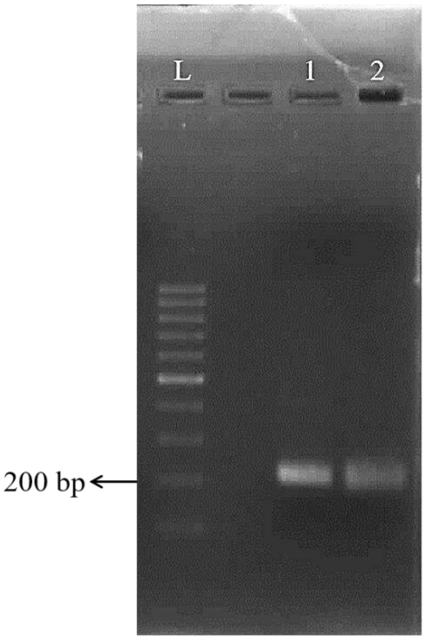


Fig. 7.5 Restriction digestion of sense strand, lane 1 is the undigested sense fragment, lane 2 is digested sense fragment with *AscI* and *SwaI* restriction enzymes and L is the 100 bp ladder

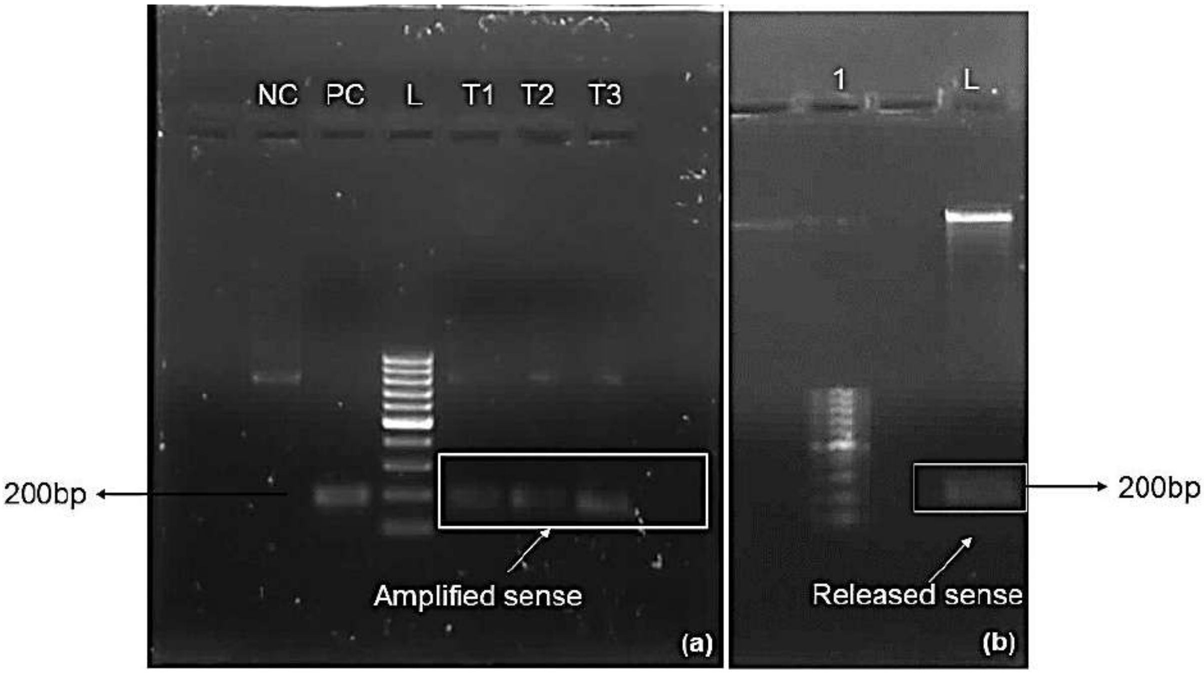


Fig. 7.6 Molecular confirmation of cloning of sense fragment into hp-RNAi plasmid **(a)** amplification of transformed plasmid with sense primer (T1, T2, T3), NC: amplification of untransformed plasmid with sense primer, PC: positive control, L is the 100 bp ladder **(b)** restriction digestion of transformed plasmid with *AscI* and *SwaI* to release 200 bp sense fragment

7.1.3 Cloning of Pi*CesA3* gene fragment in antisense orientation

The amplified antisense DNA fragment with *BamHI* and *XbaI* overhangs and hp-RNAi+sense plasmid was restriction digested with the corresponding enzymes to generate compatible ends (Fig.7.7 and Fig. 7.8). The restriction digested antisense DNA fragment was ligated to hp-RNAi+sense plasmid and transformed in *E. coli* DH5α cells. The hp-RNAi+sense plasmid carrying antisense fragment (hp-RNAi+sense+antisense) showed the expected fallout (~200 bp) through restriction digestion with *BamHI* and *XbaI* (Fig. 7.9b). Moreover, amplification with modified plasmid also showed the expected band of ~200 bp (Fig. 7.9a).

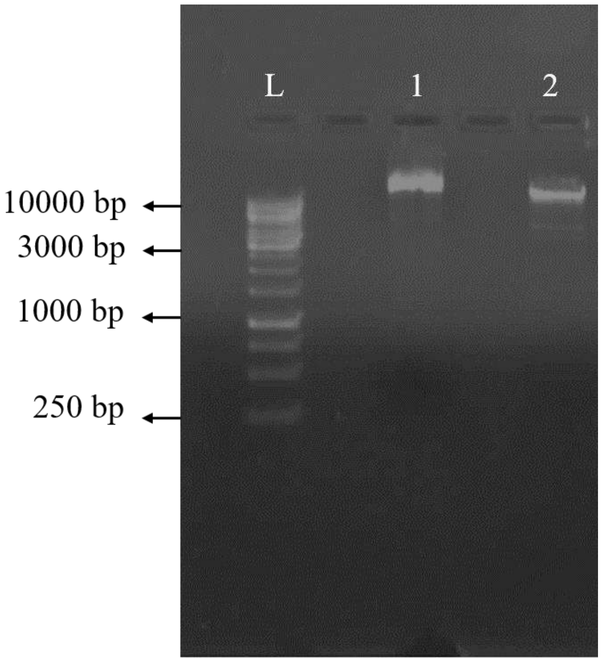


Fig. 7.7 Restriction digestion of hp-RNAi+sense plasmid, lane 1 is an undigested plasmid, lane 2 is digested plasmid with *BamHI* and *XbaI* restriction enzymes and L is the 1kb ladder

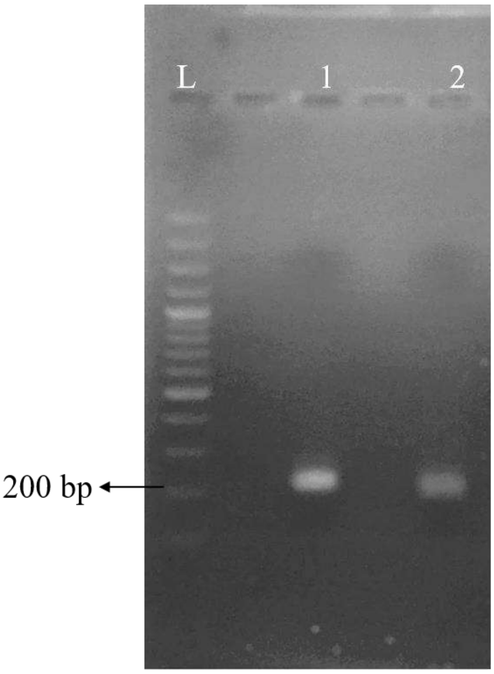


Fig. 7.8 Restriction digestion of antisense fragment, lane 1 is undigested antisense fragment, lane 2 is digested antisense fragment with *BamHI* and *XbaI* restriction enzymes and L is the 100 bp plus ladder

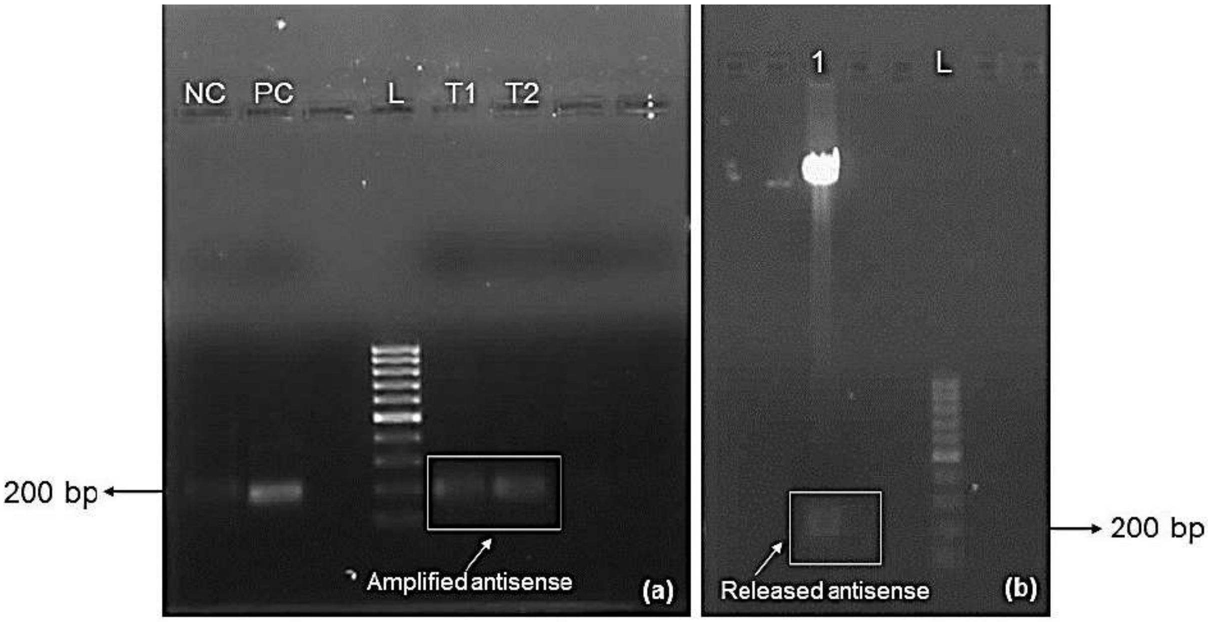


Fig. 7.9 Molecular confirmation of cloning of antisense fragment into hp-RNAi+sense plasmid **(a)** amplification of transformed plasmid with antisense primer (T1, T2), NC: amplification of untransformed plasmid with antisense primer, PC: positive control, L is the 100 bp ladder **(b)** restriction digestion of transformed plasmid with *BamHI* and *XbaI* to release 200 bp antisense fragment

7.1.4 Molecular confirmation of complete Pi*CesA3*-RNAi construct I in hp-RNAi plasmid

After cloning of sense and antisense fragments in hp-RNAi plasmid, the plasmid was again confirmed for complete Pi*CesA3*-RNAi construct I by restriction digestion of region starting from cloned sense to antisense fragment by *AscI* and *XbaI* restriction enzymes. This resulted in the expected fallout of the 1798 bp fragment (Fig. 7.10). The modified hp-RNAi plasmid harboring the Pi*CesA3*-RNAi construct I was mobilized in *A. tumefaciens* strain LBA4404 to transform the selected potato cultivars.

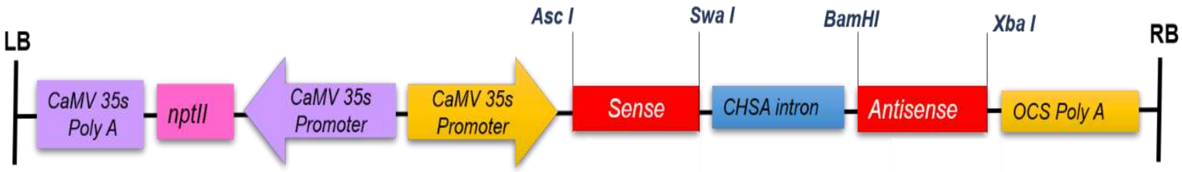


Fig. 7.10 T-DNA region of hp-*CesA3* RNAi construct

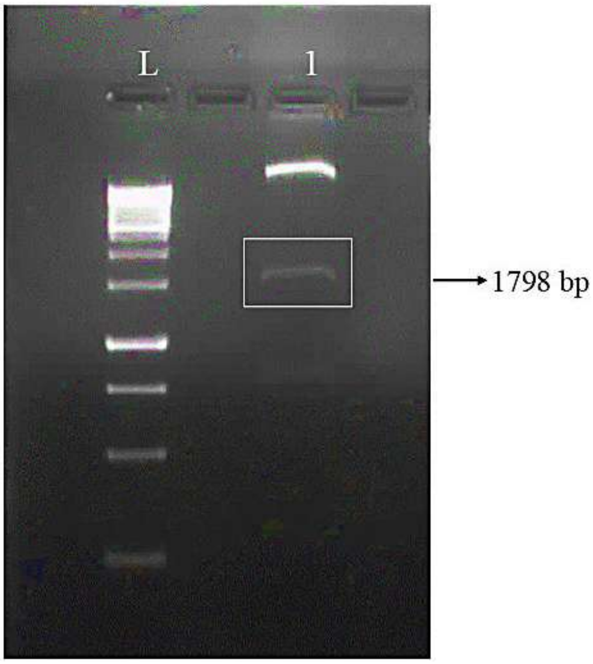


Fig. 7.11 Restriction digestion of hp-RNAi+sense+antisense plasmid with *AscI* and *XbaI* to release 1798 bp fragment, L is the 1kb ladder

7.2 Development of RNAi construct II containing Pi*CesA2* gene fragment in sense and antisense orientation

7.2.1 Selection of target sequence for Pi*CesA2*-RNAi construct II

As mentioned in section 7.1.1 the FASTA sequences of cellulose synthase (*CesA*) genes of *P. infestans* were downloaded from the NCBI (>EF563993.1, >EF563994.1, >EF563995.1, >EF563996.1). These sequences were aligned to find the conserved region (Fig.7.1). The region was then matched with the potato, potato *CesA* genes, and other available potato genes.

7.2.2 Cloning of Pi*CesA2* gene fragment in sense orientation

The selected target sequence (~200bp) was amplified in sense and antisense orientation (Fig. 7.13) with specific primers containing specific overhangs of restriction enzymes for sense (*AscI* and *SwaI)* and antisense *(BamHI* and *XbaI)* fragments. The amplified fragments were sequenced to confirm the correct nucleotide sequence and cloned in a desired orientation in the hp-RNAi plasmid.

For cloning of sense fragment, *AscI* and *SwaI* restriction enzymes were used. The amplified sense fragment with overhangs and the hp-RNAi plasmid were restriction digested

```
            1431                                                                                                                             1560
AY221086.1  TGGATCCCCT AAAAGAACCT CCACTTATTA CTGCAAATAC TGTTCTCTCC ATTCTCGCTT GTGACTATCC AGTGGACAAG GTTTCGTGCT ATGTCTCTGA TGATGGTGCT GCCATGCTTA CTTTTGAGGC
AY221088.1  TGGATCCTTT GAAAGAGCCT CCACTTATTA CTGCAAATAC TGTTCTCTCC ATTCTTGCTG TGGATTATCC GGTGGATAAG GTTTCATGCT ATGTCTCTGA CGATGGTGCT GCCATGCTTA CTTTTGAGGC
AY221087.1  TGGATCCTTT GAAGGAGCCT CCTCTTGTTA CAGCAAATAC TGTCCTGTCC ATTCTTGCAG TTGACTATCC TGTTGATAAG GTGTCCTGTT ATGTGTCTGA TGATGGTGCT GCCATGTTGA CATTTGAAGC
AY221089.1  TGGATCCTTT GAAAGAACCT CCTCTCATTA CTGCAAACAC AGTGTTGTCC ATCCTTGCTG TTGATTATCC TGTGGACAAG GTCTCATGCT ATGTGTCAGA TGATGGTTCA GCAATGCTAA CGTTTGAAGC
EF563993.1  ATTG-CCGCA GAATTGGTGT CTATT--GTG ----CTC-GG TGCACTGTAC TACCTCGGCA TGTGGAAGCC TGTGCGTCGT GGCGCCCACT A-----C-TT CG--ATGAGT TCGA-CCCCA AGGTGC-C--
EF563994.1  CTGT-TCGCT GAGGTGCTGA CGTTC--GCT ----CTG-GG TGTTCTCTAC TATCTTGGTA TGTGGAAGCC CGTGCGTCGT GGCGCCCACT A-----C-TT CG--ACGAGT TCGA-GCCGC CCGTCC-C--
EF563996.1  TACC-TGGCA GAATTTATCA GCATT--TCC ----CTC-GG TTTCCTCTAC TACCTCGGTA TGTGGAAACC CGTTCGTCGT GGTGCTCGTT AT----C-TG CG--G-GACT TCGA-GCCGC ATTTCC-CTC
EF563995.1  AGGA-CAACG GCCGCAGTAT CCGCT--TCA TGGACTCTGG CGTCCAGGGC CCCGACGGCA AGATGA-GTC AGTACAACGA CTCGATTGCT GG----CGTG CGCAACTACT ACGA-GCCTT CGTTCCGCTC
 Consensus  tgga.cc.ct Gaa..a.tct cc.ct..tta ..g.ct..gg tgt.ctgt.C ..cctcGgta tg..gaa.cC .GTgcatcg. gtcgc.tgcT at....C.t. cG..g.tact .CgA.gcc.a c.tT.c.c.c

            1561                                                                                                                             1690
AY221086.1  TCTCTCTGAA ACATCTGAGT TCGCAAGGAA ATGGGTCCCA TTCTGCAAAA AGTTCAACAT AGAGACTCGA GCGCCGGAGT GGTATTTTTC TCTGAAGGTT GATTATCTGA AAAATAAAGT A--CACCCAT
AY221088.1  ACTCTCTGAA ACATCTGAGT TTGCAAGGAA ATGGGTCCCA TTCTGCAAGA AATTCAGCAT AGAGCCTCGA GCCCCGGAGT GGTATTTTGC TCAGAAGGTT GATTATTTGA AGAACACAGT A--AATCCAT
AY221087.1  CCTATCTGAA ACAGCAGAGT TTGCAAGGAA ATGGGTTCCT TTCTCTAAGA AGTACAGCAT AGAGCCACGA GCTCCAGAGT GGTATTTTTC TCAGAAGGTT GACTACTTGA AGGATAAAGT T--CAAACAT
AY221089.1  CCTTTCAGAG ACCGCAGAGT TTGCAAGGAG ATGGGTTCCC TTCTGCAAGA AATTCAGCAT TGAGCCTCGG GCCCCTGAAT TTTATTTTGC TCAAAAGATA GATTATTTGA AGGACAAGGT T--CAGCCTT
EF563993.1  -CGA------ ---------- ---------- ---------- ---------- ---------- ---------- -------TGA CTT---GTGG CCAAAGATTG ACGTTCTTCT GTGCCACTAC T-----CGGA
EF563994.1  -GGA------ ---------- ---------- ---------- ---------- ---------- ---------- -------TGA TCT---GTGG CCCAAGGTCG ATGTGCTGTT GTGTCACTAC T-----CTGA
EF563996.1  CCGAAAAGGT ATGACTATTT TTTGCGGTTT TTTGTGAGCT GATACATTCT GA---CACAA AAAGTTTTTT TTTTGTGTGT CCTAAAGTGG CCAACCGTGG ATATCCTGCT ATGTCACTAT G-----CTGA
EF563995.1  GTTCGCCATG TCCACGTACT CGATCGCTAA CCGCGCGGCT GAGCCCGTGG AGACGCCCAA CATCCGCAAG TACAAGATGT CGGGTAGTGG CATGTTCCAC GTCTTCTACT TCGGCACGGC TGCTACCGGT
 Consensus  .cta.c.g.. ac..c..a.t tt....g.a. .tggg...c. ........g. a......ca. ..agc..... ......gtgt ..tat.gTgg ccaaa.g.t. g..T.cTt.t a.g.cAc.gt t...a.c.gt

            1691                                                                                                                             1820
AY221086.1  CATTTGTGAG GGAACGTCGT GCTATGAAGA GAGATTATGA AGAATTCAAG GTTCGGATAA ATGGGCTGGT TGCCACAGCA CAAAAGGTTC CTGAAGATGG CTGGACCATG CAAGATGGAA CGCCTTGGCC
AY221088.1  CATTTGTGAG GGAACGCCGT GCCATGAAGA GAGACTATGA AGAATTTAAG GTTCGGATAA ATGGATTGGT TTCCATTGCA CAAAAGGTTC CTGAGGATGG TTGGACCATG CAAGACGGAA CTCCTTGGCC
AY221087.1  CATTTGTAAA AGAACGCAGG GCAATGAAGA GGGAGTATGA AGAGTTCAAA ATTCGCATCA ATGCCCTCGT TGCAAAAGCT CAAAAGGTCC CTGAAGAAGG ATGGATTATG CAAGATGGTA CMCCATGGCC
AY221089.1  CTTTTGTGAA AGAGCGCAGG GCAATGAAGA GGGAATATGA AGAATTTAAA ATACGTATCA ATGCTCTTGT TGCAAAAGCA CAAAAAATGC CTGAAGAAGG TTGGACAATG CAAGATGGAA CAGCTTGGCC
EF563993.1  GCCGGCTGAG ---------- -GAGACAATT GACACGTTGA TGGCTTGTAT GAAC-CTTCA GTACCCGCCT CACCTCGTG- CAAA-----T TTACGTGCTG ---GACGACG --G----CTA CTGCGGCTCT
EF563994.1  GCCGGCTGAA ---------- -GAGACGATC GACACGTTGA TGGCCTGTAT GAAC-CTGCA GTACCCTCCG CACCTGTTG- CAGA-----T CTGGGTCTGT ---GATGACG --G----TTA CTGCAAGACC
EF563996.1  GCCAGCTGAC ---------- -GACACCATC GCTACGTTGG AGAAGATCAT GAAC-CTGGA CTACCCTCCG CATCTATTC- CACG-----T CTGGATCTGC ---GACGATG --G----CTA CTGTAAGGCC
EF563995.1  ATCTTCTGGC TCTACTACTT GACGACGATG TACCCGCAGC AGTATTTCTG CGAC-CACGC GCGTCCTACG CTTCCATGC- AGTGGGCTGC CGACAAGTGA ---GACGACG --GGATGCTA CAGCTCGACC
 Consensus  ..cttcTga. ....c..... g.a.acaAt. ga.acg.tGa aGaattt.a. g.aC.catca .tgccCtcct c.cc.atgc. caaa...t.c ctg.a..tgg ...GAcgAtG ..ggatg.tA C.gc..ggCc
```

Fig. 7.12 Multiple sequence alignment of cellulose synthase (*CesA*) gene of *P. infestans* with cellulose synthase (*CesA*) gene of potato. The highlighted black part is the selected fragment for development of RNAi construct II

with *AscI* and *SwaI* to generate compatible ends. The digested sense DNA fragment and hp-RNAi plasmid were ligated to get the circular plasmid containing sense DNA fragment (referred as hp-RNAi+sense). The modified plasmid containing sense was transformed in *E. coli* DH5α cells. The hp-RNAi+sense plasmid showed the expected band of ~200 bp (Fig. 7.14a) upon amplification with specific primers. Moreover, the hp-RNAi+sense plasmid also showed the expected fallout (~200 bp) of sense upon digestion with *AscI* and *SwaI* (Fig.7.14b).

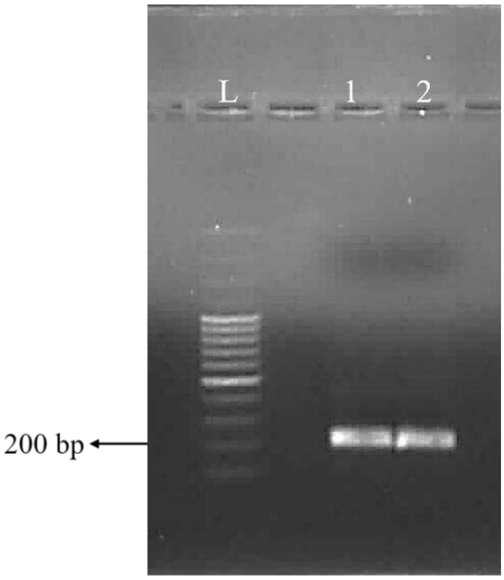


Fig. 7.13 Amplification of sense and antisense strand lane 1 is sense and lane 2 is antisense strand, where L is the 100 bp ladder

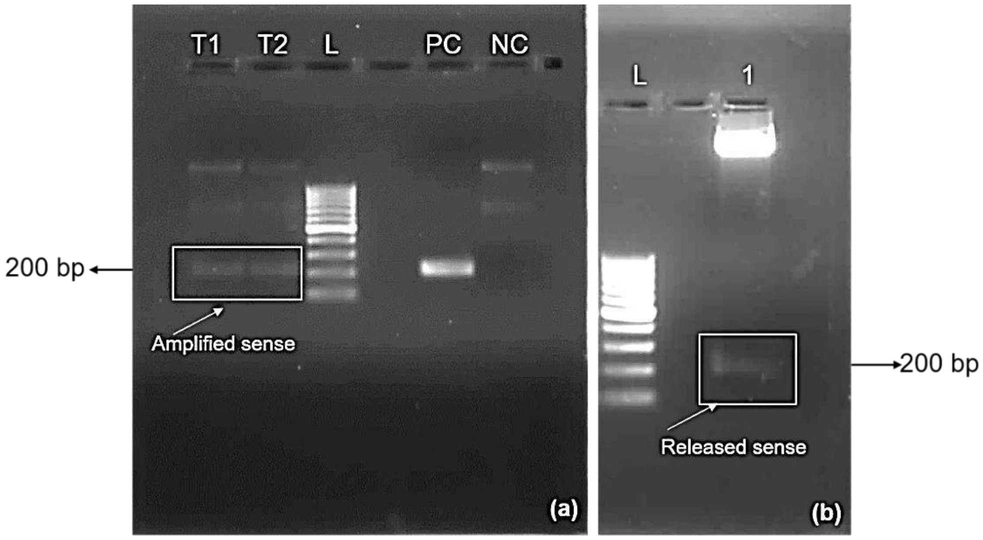


Fig. 7.14 Molecular confirmation of cloning of sense fragment into hp-RNAi plasmid **(a)** amplification of transformed plasmid with sense primer (T1, T2), NC: amplification of untransformed plasmid with sense primer, PC: positive control, L is the 100 bp ladder **(b)** restriction digestion of transformed plasmid with *AscI* and *SwaI* to release 200 bp sense fragment

7.2.3 Cloning of Pi*CesA2* gene fragment in antisense orientation

The amplified antisense DNA fragment with *BamHI* and *XbaI* overhangs (Fig. 7.13) and hp-RNAi+sense plasmid was restriction digested with the corresponding enzymes to generate compatible ends. The restriction digested antisense DNA fragment was ligated to modified hp-RNAi+sense plasmid, and transformed in *E. coli* DH5α cells. The modified hp-RNAi+sense plasmid carrying antisense fragment (referred as hp-RNAi+sense+antisense) showed the expected fallout (~200 bp) through restriction digestion with *BamHI* and *XbaI* (Fig. 7.15b). Moreover, amplification with modified plasmid also showed the expected band of ~200 bp (Fig. 7.15a).

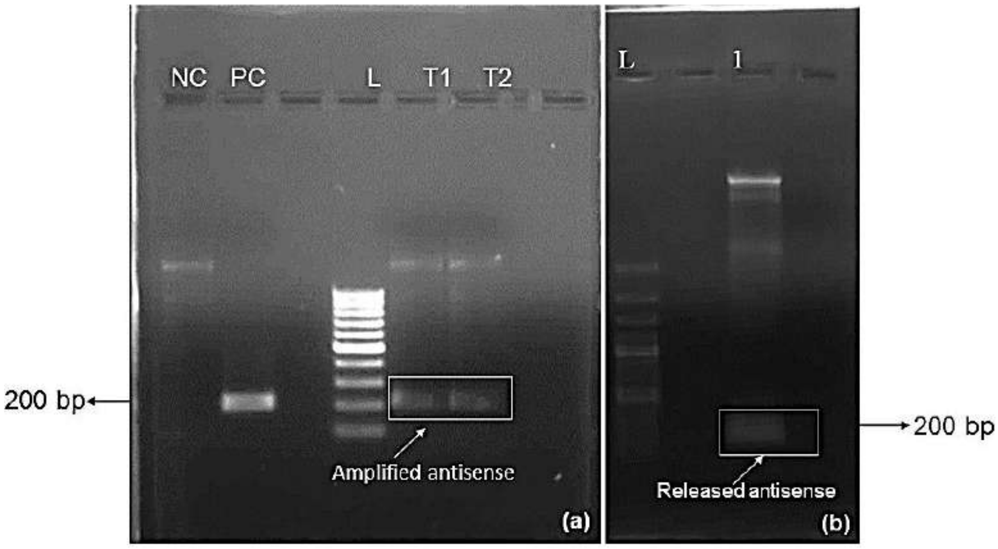


Fig. 7.15 Molecular confirmation of cloning of antisense fragment into hp-RNAi+sense plasmid **(a)** amplification of transformed plasmid with sense primer (T1, T2), NC: amplification of untransformed plasmid with antisense primer, PC: positive control, L is the 100 bp ladder **(b)** restriction digestion of transformed plasmid with *BamHI* and *XbaI* to release 200 bp antisense fragment

7.2.4 Molecular confirmation of Pi*CesA2-RNAi* construct II in hp-RNAi plasmid

After cloning of sense and antisense fragments in hp-RNAi plasmid, the plasmid was again confirmed for complete Pi*CesA2*-RNAi construct II by restriction digestion of region starting from cloned sense to antisense fragment by *AscI* and *XbaI* restriction enzymes. This resulted in the expected fallout of the 1798 bp fragment (Fig. 7.17). After confirmation the hp-RNAi plasmid harboring the Pi*CesA2-RNAi* construct II was mobilized to *A. tumefaciens* strain LBA4404 to transform the selected potato cultivars.

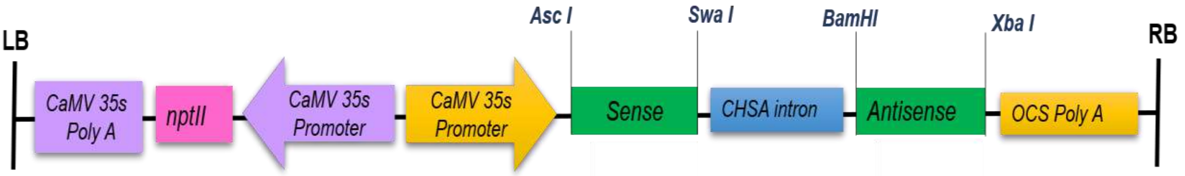


Fig. 7.16 T-DNA region of hp-*CesA2* RNAi construct

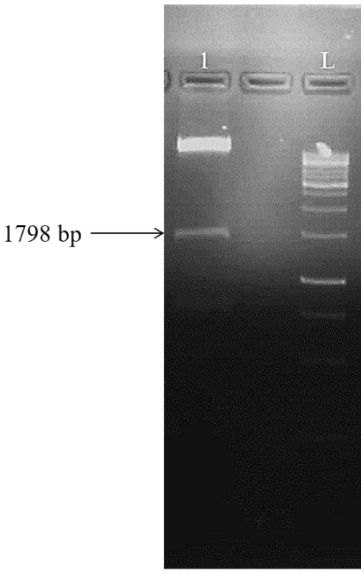


Fig. 7.17 Restriction digestion of hp-RNAi+sense+antisense plasmid with *AscI* and *XbaI* to release 1798 bp fragment, L is the 1kb ladder

Chapter 8

Agrobacterium-mediated genetic transformation of potato cultivar(s) using RNAi construct(s) and their molecular characterization

Abstract

In the present study, the optimisation of key parameters for the *Agrobacterium*-mediated genetic transformation were carried out. The highest transient GUS expression was recorded when the explants were precultured for 2 days on a preculture medium (MS3 medium) and then infected with *A. tumefaciens* (OD_{590}-0.6) for 15 min. The infected explants co-cultivated for 2 days on MS4 medium. After washing with autoclaved distilled water, the explants were cultured on regeneration-cum-selection medium (MS5 medium). The optimised *Agrobacterium*-mediated genetic transformation protocol was then used to get transgenic lines of the cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' expressing Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II. Within 7 days of culturing on regeneration-cum-selection medium, the untransformed explant died, and the transformed sectors of explants remained green. After 14-20 days, growth in these green sectors started which further progressed to callus and subsequently regenerated shoots. Later on, the regenerated shoots were multiplied and maintained on MS1 medium+100 mg L^{-1} kanamycin for further analysis. The PCR analysis of regenerated shoots confirmed the presence of respective sense and antisense fragments, *nptII* gene, and absence of *16s rRNA* gene.

8.1 Determination of optimum concentration of kanamycin for selection of transformants

The sensitivity of leaf and internodal explants to the kanamycin was determined for the selection of transformants. For that, the explants were cultured on a MS3 medium containing different concentrations of kanamycin (0-125 mg L^{-1}) for 25 days. It was recorded that in the absence of kanamycin, the explants started showing callus initiation from the cut ends of the internode and leaf explants in cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. Similar results were also observed on lower concentrations of kanamycin (25-50 mg L^{-1}). However, it was noteworthy that on lower concentration of kanamycin (25-50 mg L^{-1}) some of the explants regenerated shoots in both cultivars. Furthermore, the survival of both explants decreases with an increase in concentration (75-125 mg L^{-1}). The explants started to show browning at higher concentrations (100-125 mg L^{-1}). The survival rate decreases drastically from 80 to 43.33% in cv. 'Kufri Pukhraj' and from 90 to 40 % in cv. 'Kufri Chipsona 1' when the concentration of kanamycin was increased from 25 to 75 mg L^{-1} kanamycin. The leaves cultured on a MS3 medium containing 100 mg L^{-1} and 125 mg L^{-1} kanamycin did not survive and all turned brown. Therefore, the 100 mg L^{-1} kanamycin concentration was selected to be incorporated in the selection medium for the selection of transformants during genetic transformation experiments.

8.2 Optimization of factors affecting *Agrobacterium*-mediated genetic transformation

The *Agrobacterium tumefaciens* strain LBA4404 harboring binary vector 'pBI121' was used for the genetic transformation of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' using internode and leaf explants (Kaur et al. 2020). The key parameters involved in *Agrobacterium*-mediated genetic transformation such as preculture period, bacterial culture density, infection time of *Agrobacterium*, co-cultivation period, and concentration of acetosyringone in the co-cultivation medium were optimized for *Agrobacterium*-mediated genetic transformation of selected potato cultivars.

8.2.1 The effect of preculture

In cv. 'Kufri Pukhraj' the transient GUS expression was 55% (internode) and 51.66% (leaf) in explants directly infected with *A. tumefaciens*. The transient GUS expression of explants precultured for 2 days on MS3 medium increased to 74.99% (internode) and 66.66% (leaf). Further increase in the preculture to 2 days reduced the transient GUS expression to 46.66% (internode) and 41.66% (leaf) (Table 8.1).

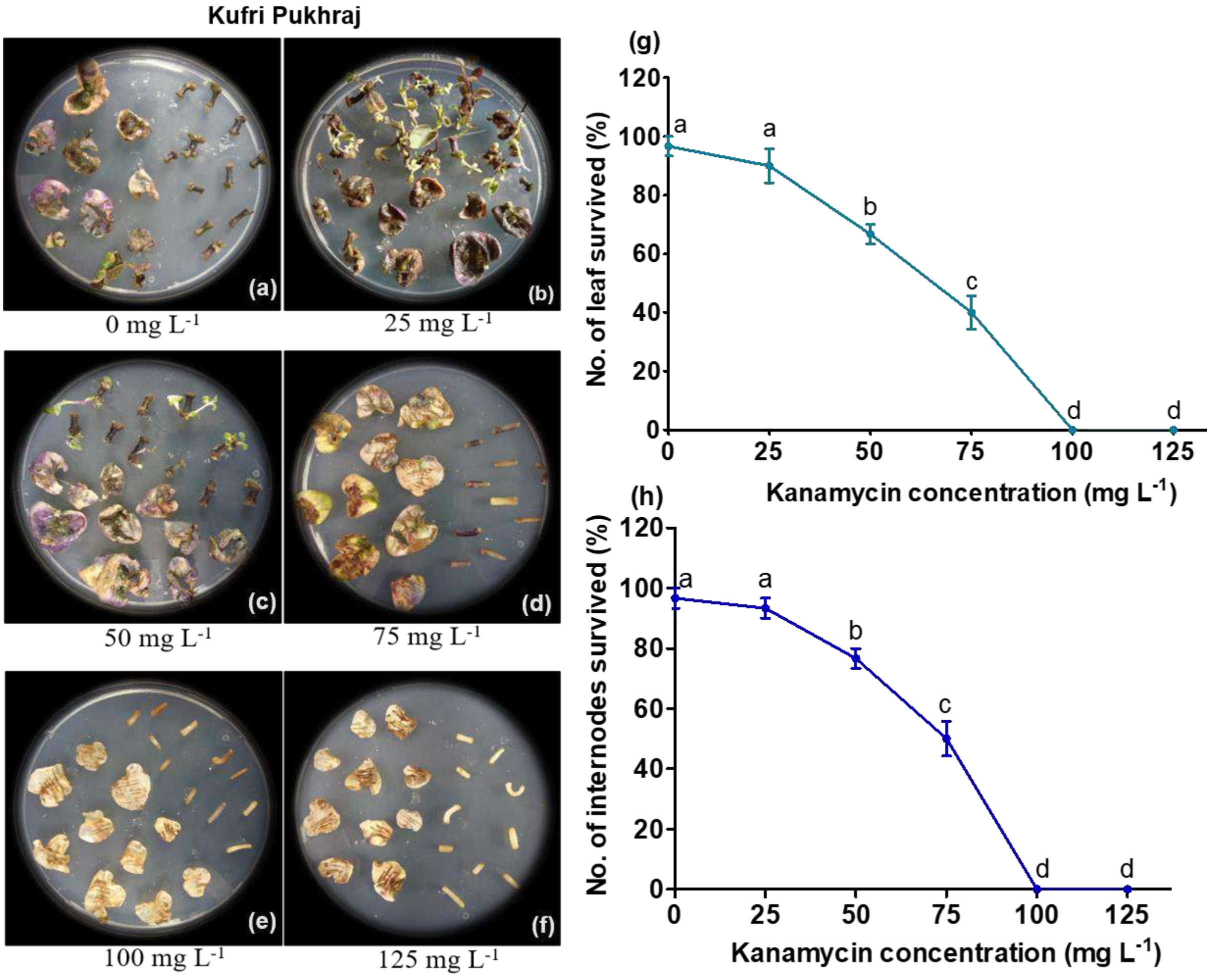
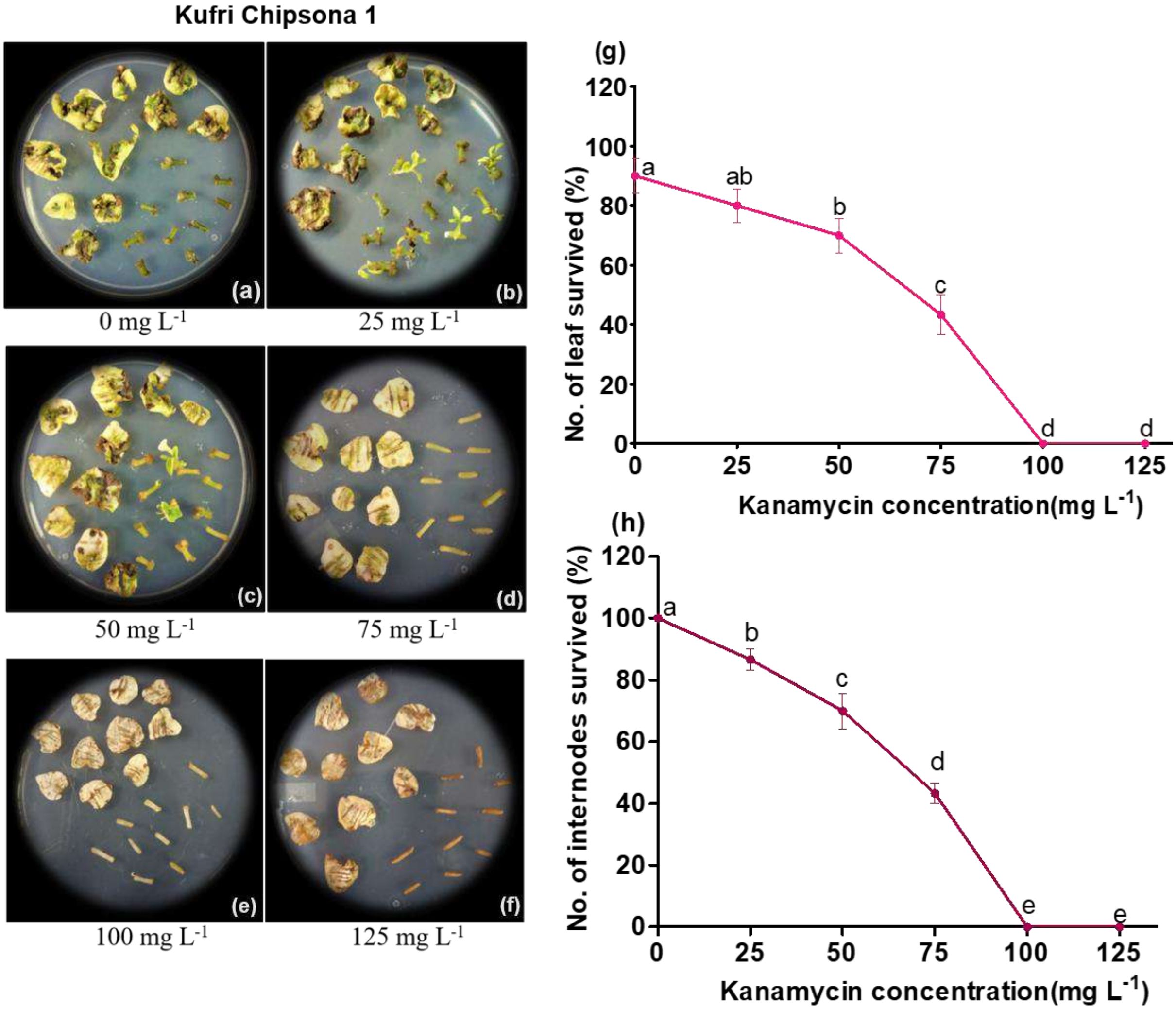


Fig. 8.1 The effect of various concentrations of kanamycin (0-125 mg L^{-1}) on the survival of leaf and internodal explants of cv. 'Kufri Pukhraj'. Data were recorded after 25 days of culture. Mean values followed by different lower-case letter are significantly different at $p < 0.05$ by DMRT

In cv. 'Kufri Chipsona 1' the transient GUS expression was 54.99% (internode) and 36.66% (leaf) in explants directly infected with *A. tumefaciens*. However, the transient GUS expression of explants precultured for 2 days on MS3 medium increased to 72.19% (internode) and 51.66% (leaf). Further increase in the preculture to 2 days reduced the transient GUS expression to 58.33% (internode) and 33.33% (leaf) (Table 8.2).

8.2.2 The effect of bacterial culture density and infection time

To determine the culture density of *A. tumefaciens* for infection, explants were infected with different bacterial suspensions of OD$_{590}$ (0.2, 0.4, 0.6, 0.8, and 1.0) for different infection times (5, 10, 15, 20, and 25 min).



0 mg L^{-1} 25 mg L^{-1} 50 mg L^{-1} 75 mg L^{-1} 100 mg L^{-1} 125 mg L^{-1}

Fig. 8.2 The effect of various concentrations of kanamycin (0-125 mg L^{-1}) on the survival of leaf and internodal explants of cv. 'Kufri Chipsona1'. Data were recorded after 25 days of culture. Mean values followed by different lower-case letter are significantly different at $p<0.05$ by DMRT

In cv. 'Kufri Pukhraj' the highest transient GUS expression was 76.66% (internodes) and 69.99% (leaf) when infected with bacterial suspension of OD_{590} of 0.6 (Table 8.1). Similarly in cv. 'Kufri Chipsona 1' the highest transient GUS expression was 71.66% (internodes) and 58.33% (leaf) when infected with bacterial suspension of OD_{590} of 0.6 (Table 8.2).

The rate of transient GUS expression also varied significantly with infection time. The optimal infection time was found to be 15 min showing transient GUS expression in 76.66% (internode) and 68.33% (leaf) explants of cv. 'Kufri Pukhraj' and 68.33% (internode) and 54.99% (leaf) explants of cv. 'Kufri Chipsona 1' (Table 8.1 and Table 8.2). The increase in infection time beyond 15 min reduced the transient GUS expression.

8.2.3 The effect of co-cultivation

The effect of co-cultivation period on transient GUS expression in infected explants was studied for 1-5 days. The highest transient GUS expression was observed after 2 days of co-cultivation (71.66% internode; 69.99 % leaf) in cv. ‘Kufri Pukhraj’ whereas in cv. ‘Kufri Chipsona 1’ highest transient GUS expression was 66.66% (internode) and 54.99 % (leaf). (Table 8.1 and Table 8.2). Increasing the co-cultivation from 1 to 2 days significantly increased the GUS expression. A further increase in co-cultivation period to 3 days reduced the GUS expression.

8.2.4 The effect of acetosyringone

A significant variation in transient GUS expression was observed with the incorporation of acetosyringone in the co-cultivation medium. In the absence of acetosyringone, the transient GUS expression was found to be 41.66% (internode) and 43.33% (leaf) in cv. ‘Kufri Pukhraj’ whereas in cv. ‘Kufri Chipsona 1’ the transient GUS expression was 56.66% (internode) and 40% (leaf). It was observed that the transient GUS expression increased to 73.33% (internode) and 68.33% (leaf) in cv. ‘Kufri Pukhraj’ whereas in cv. ‘Kufri Chipsona 1’ 69.99% (internode) and 61.66% (leaf), when explants were co-cultivated on MS4 medium (Table 8.1 and Table 8.2).

Table 8.1 The effect of various factors (preculture, bacterial density, infection time, co-cultivation period, and concentration of acetosyringone) on the transient GUS expression in leaf and internodal explants of cv. 'Kufri Pukhraj' infected with *A. tumefaciens* (LBA4404) harboring pBI121

Kufri Pukhraj		
Factors	**Transient GUS expression**	
	Leaf	**Internode**
Preculture Period (days)		
0	51.66^{bc}	55^{bc}
1	58.33^{ab}	65^{ab}
2	**66.66^{a}**	**74.99^{a}**
3	48.33^{bc}	61.66^{b}
4	41.66^{c}	46.66^{c}
Bacterial Density (OD)		
0.2	48.33^{c}	54.99^{c}
0.4	59.99^{b}	65^{bc}
0.6	**69.99^{a}**	**76.66^{a}**
0.8	48.33^{c}	66.66^{ab}
1.0	46.66^{c}	56.66^{bc}
Infection Time (min)		
5	46.66^{bc}	55^{b}
10	56.66^{ab}	59.99^{b}
15	**68.33^{a}**	**76.66^{a}**
20	51.66^{bc}	61.66^{b}
25	43.33^{c}	51.66^{b}
Co-cultivation Time (days)		
1	58.33^{ab}	68.33^{a}
2	**69.99^{a}**	**71.66^{a}**
3	51.66^{bc}	64.99^{ab}
4	43.33^{bc}	58.33^{b}
5	35^{c}	50^{c}
Acetosyringone Conc. (µM)		
0	43.33^{b}	41.66^{d}
50	56.66^{ab}	64.99^{ab}
100	**68.33^{a}**	**73.33^{a}**
150	44.99^{b}	63.33^{bc}
200	41.66^{b}	55^{c}

Table 8.2 The effect of various factors (preculture, bacterial density, infection time, co-cultivation period, and concentration of acetosyringone) on the transient GUS expression in leaf and internodal explants of cv. 'Kufri Chipsona 1' infected with *A. tumefaciens* (LBA4404) harboring pBI121

Kufri Chipsona 1		
Factors	**Transient GUS expression**	
	Leaf	**Internode**
Preculture Period (days)		
0	36.66^{c}	54.99^{c}
1	46.66^{ab}	68.33^{b}
2	**51.66^{a}**	**72.19^{a}**
3	38.33^{bc}	64.99^{b}
4	33.33^{c}	58.33^{c}
Bacterial Density (OD)		
0.2	33.33^{c}	56.66^{bc}
0.4	44.66^{b}	64.99^{ab}
0.6	**58.33^{a}**	**71.66^{a}**
0.8	43.33^{b}	51.66^{c}
1.0	38.33^{bc}	44.99^{c}
Infection Time (min)		
5	41.66^{b}	53.33^{bc}
10	50^{a}	58.33^{b}
15	**54.99^{a}**	**68.33^{a}**
20	41.66^{b}	51.66^{bc}
25	35^{b}	46.66^{c}
Co-cultivation Time (days)		
1	41.66^{b}	61.66^{ab}
2	**54.99^{a}**	**66.66^{a}**
3	40.33^{bc}	53.33^{bc}
4	36.66^{bc}	45^{c}
5	34.99^{c}	33.33^{d}
Acetosyringone Conc. (µM)		
0	40^{bc}	56.66^{ab}
50	51.66^{ab}	58.33^{ab}
100	**61.66^{a}**	**69.99^{a}**
150	45^{bc}	51.66^{bc}
200	36.66^{c}	40^{c}

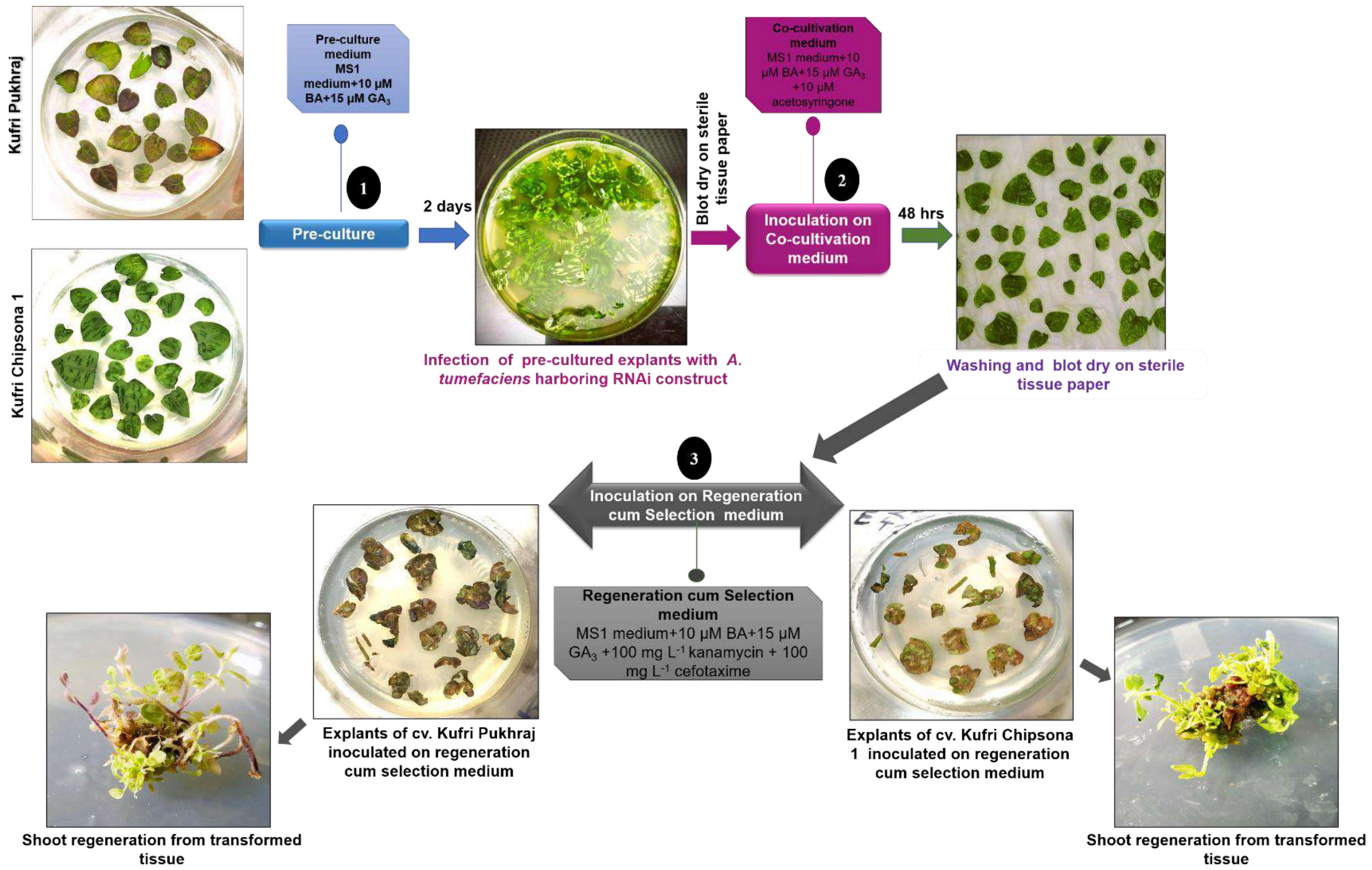


Fig. 8.3 Transformation of potato cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ with *A. tumefaciens* harbouring RNAi construct and subsequent regeneration of transgenic lines

8.3 *Agrobacterium*-mediated genetic transformation of potato cultivars with modified hp-RNAi plasmid harbouring Pi*CesA*-RNAi constructs

The genetic transformation of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'with plasmid harboring hp-Pi*CesA3* RNAi construct I and hp-Pi*CesA3* RNAi construct II was carried out using optimized genetic transformation protocol. The explants were precultured for 2 days on a preculture medium (MS3 medium) and then infected with *A. tumefaciens* harbouring hp-RNAi+sense+antisense plasmid (OD_{590}-0.6) for 15 min. The infected explants were co-cultivated for 2 days on MS4 medium. After washing with autoclaved distilled water, the explants were cultured on regeneration-cum-selection medium (MS5 medium). Within 10-15 days, the death of the untransformed parts of explant commenced with yellowing of tissue. However, transformed part of the tissue was remained as green within the explants. After 3-4 weeks of growth, well-developed kanamycin-resistant tissue had grown from the transformed cells. However, no growth was observed in control explants not infected with *Agrobacterium*, or co-cultivated with *Agrobacterium* without the binary vector. The putatively transformed tissue was carefully picked up and subcultured to a fresh regeneration-cum-selection medium (MS5 medium) for further growth.

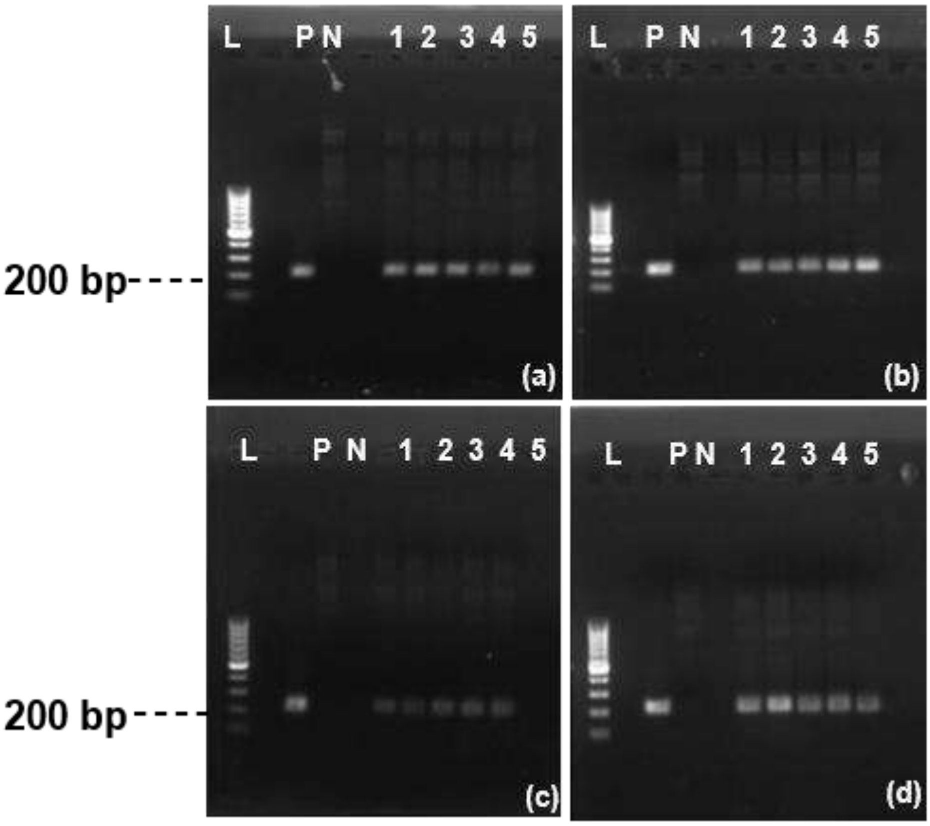
The transformed tissue started developing callus within 2-3 weeks followed by shoot regeneration. Later, the regenerated kanamycin resistant shoots with lengths 1-2 cm were cut from the calli and cultured on MS1 medium containing 100 mg L^{-1} kanamycin. A total of 10 transgenic lines were developed, 5 for cv. 'Kufri Pukhraj' and 5 for cv. 'Kufri Chipsona 1' with Pi*CesA3*-RNAi construct I. However, 11 transgenic lines were developed with Pi*CesA2*-RNAi construct II, 4 for cv. 'Kufri Pukhraj' and 7 for cv. 'Kufri Chipsona 1'. All transgenic lines continued to grow with normal morphology and initiated roots on the MS1 medium in the presence of 100 mg L^{-1} kanamycin. The transgenic shoots showed normal and similar growth patterns as those of their mother plant.

8.4 Molecular confirmation of transgenic lines

8.4.1 Amplifications of sense and antisense fragments

The genomic DNA of transgenic shoots expressing of Pi*CesA3*-RNAi construct I, Pi*CesA2*-RNAi construct II and non-transgenic shoots from potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' was isolated using the method described in section 3.8.1.

Genomic DNA amplifications were performed with sense and antisense specific primers of respective RNAi constructs (Table 3.4, 3.5). In this study, the modified plasmid DNA (hp-RNAi+sense+antisense) served as a positive control, while the DNA from non-transgenic shoots served as a negative control. All the transgenic lines, including the positive control, showed amplification for sense and antisense fragments (~200 bp) for their respective RNAi construct. On the other hand, non-transgenic shoots showed no amplification.



s

Fig. 8.4 Amplification of **(a)** sense and **(b)** antisense fragments from transgenic lines of cvs. 'Kufri Pukhraj' and **(c)** sense **(b)** antisense fragments from transgenic lines of cv. 'Kufri Chipsona 1' harboring Pi*CesA3*-RNAi construct I

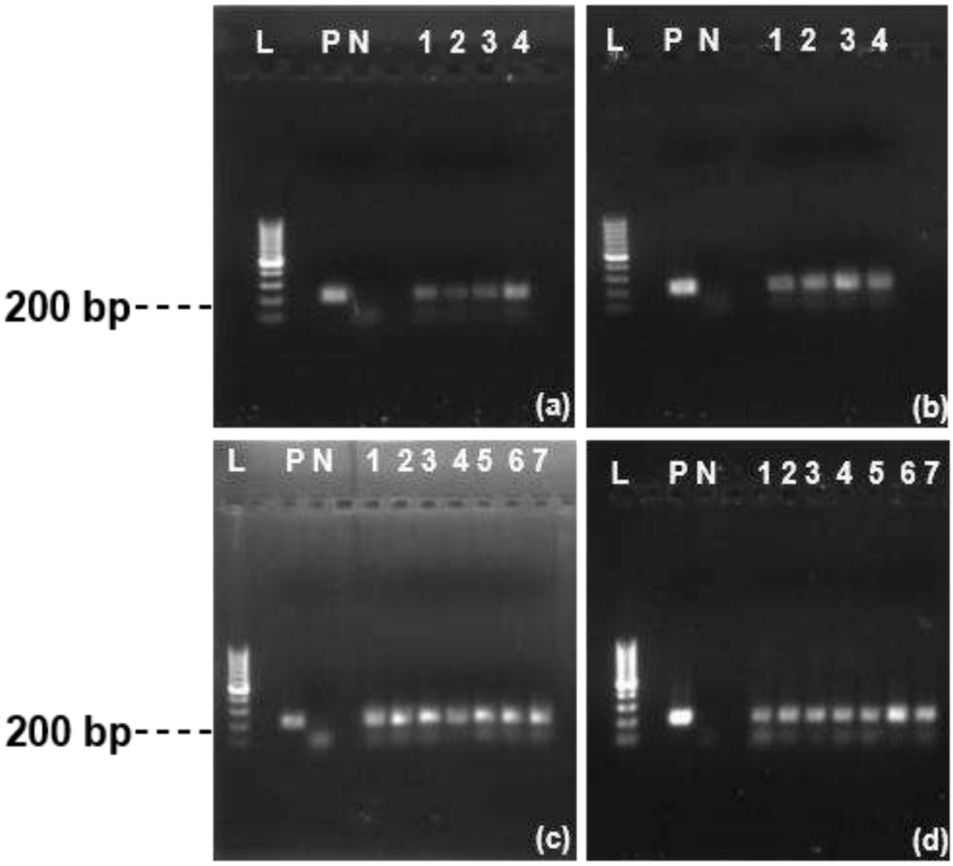


Fig. 8.5 Amplification of **(a)** sense and **(b)** antisense fragments from transgenic lines of cvs. 'Kufri Pukhraj' and **(c)** sense **(b)** antisense fragments from transgenic lines of cv. 'Kufri Chipsona 1' harboring Pi*CesA2*-RNAi construct II

8.4.2 Amplification of *nptII* gene and *16s rRNA* gene

The genomic DNA amplification with the transgenic lines and plasmid DNA (positive control) with *nptII* gene-specific primers yielded a fragment of 750 bp. However, the non-transgenic shoots (negative control) showed no amplification. Furthermore, the transgenic lines were tested for bacterial contamination using *16s rRNA* specific primers. The absence of amplification of a DNA fragment specific to the *16s rRNA* from the genomic DNA of transgenic lines indicated that bacteria is completely eliminated from these cultures. Therefore, this confirmed the integration of RNAi constructs in the DNA of the transgenic lines.

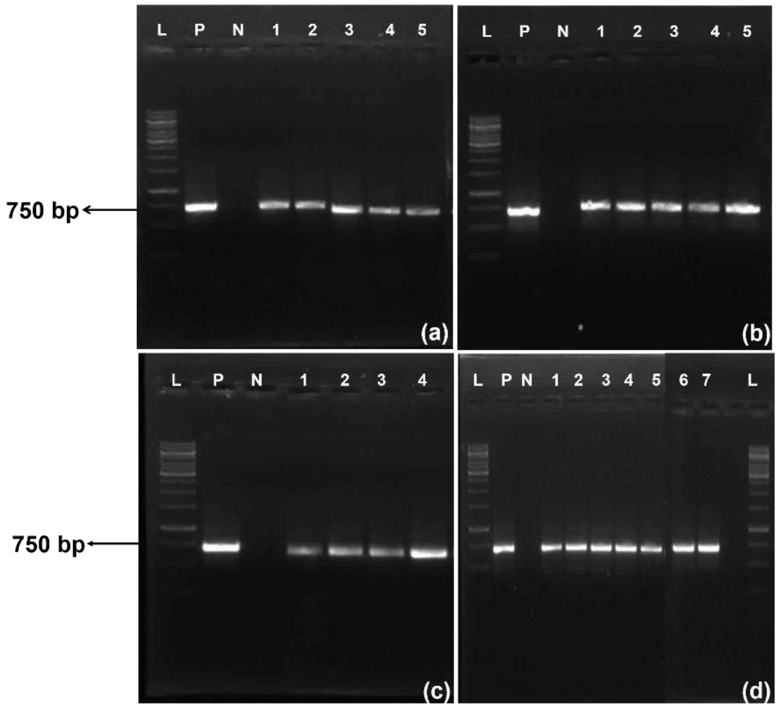


Fig. 8.6 Amplification of 750 bp fragment (*nptII* gene) from transgenic lines of **(a, c)** cvs. 'Kufri Pukhraj' and **(b, d)** 'Kufri Chipsona 1', L is the 1kb ladder, P is the positive and N is negative control

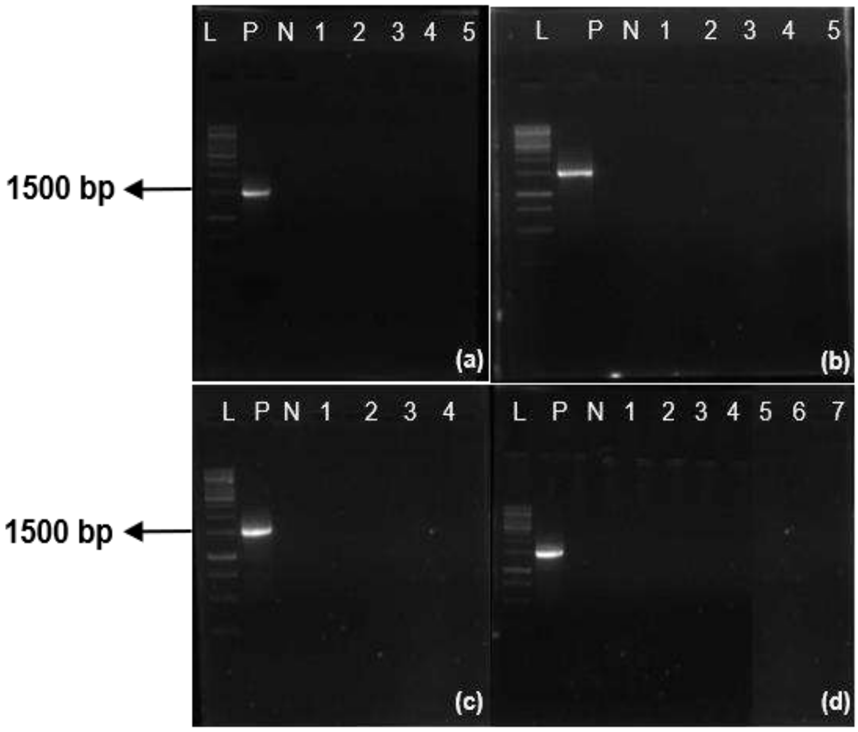


Fig. 8.7 Amplification of 1500 bp fragment (*16s rRNA*) from transgenic lines of **(a, c)** cvs. 'Kufri Pukhraj' and **(b, d)** 'Kufri Chipsona 1', L is the 1kb ladder, P is the positive and N is negative control

Chapter 9

Evaluation of transgenic lines for late blight resistance

Abstract

The present chapter focused on the acclimatization of transgenic and non-transgenic plants of cv. 'Kufri Pukhraj'(KP) and 'Kufri Chipsona 1'(CS-1) and subsequent establishment in the green house. The fully grown plants were further evaluated for late blight resistance mainly by detached leaf and whole plant assays. In detached leaf assay, larger lesions were recorded on the control leaves of cv. 'Kufri Pukhraj' (1.54-1.68 cm) and cv. 'Kufri Chipsona 1' (1.35-1.43 cm) when inoculated with a sporal suspension of *P. infestans* at 10 dpi. However, the smaller lesion diameter was recorded on leaves of transgenic line 2 (0.30 cm), line 5 (0.35 cm), line 4 (0.37 cm) (Pi*CesA3*-RNAi construct I), and line 1(0.35 cm) (Pi*CesA2*-RNAi construct II) of cv. 'Kufri Pukhraj' whereas in cv. 'Kufri Chipsona 1' smaller lesion diameter was recorded on leaves of line 3 (0.37 cm) (Pi*CesA3*-RNAi construct I) and line 1 (0.32 cm), line 2 (0.31cm), line 3 (0.38) and line 4 (0.30 cm) (Pi*CesA2*-RNAi construct II). In whole plant assay, significantly lower disease incidence (DI) and disease severity (DS) was recorded in transgenic lines. In cv. 'Kufri Pukhraj' transgenic line 4 (50% DI; 38.88 % DS) harbouring Pi*CesA3*-RNAi construct I, and line 4 (43.75% DI; 30.55% DS) harbouring Pi*CesA2*-RNAi construct II showed the lowest disease incidence and severity whereas in cv. 'Kufri Chipsona 1' line 3 (37.5 % DI; 37.49 % DS) harbouring Pi*CesA3*-RNAi construct I, and line 1 (43.75% DI; 38.88% DS), line 7 (43.75% DI; 37.49% DS) harbouring Pi*CesA2*-RNAi construct II showed lowest disease incidence and severity. The response of plants to late blight was further studied by estimation of antioxidant enzyme (superoxide dismutase, peroxidase, ascorbate peroxidase and catalase) activity in inoculated and non-inoculated transgenic and control plants. It was recorded that in inoculated plants the enzyme activity significantly increases. The highest activities of superoxide dismutase, peroxidase, ascorbate peroxidase and catalase were recorded at 5 dpi, and afterward, it decreased at 10 dpi. Significant differences were recorded in the antioxidant enzyme activity between non-inoculated and inoculated plants after 5 and 10 dpi.

9.1 In vitro multiplication and acclimatization of plants

Transgenic and non-transgenic plants of cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ were multiplied on MS1 medium (Fig. 9.1a-d). The plantlets with well-developed roots were transferred to pro trays filled with cocopeat-soil mixture (1:1) and maintained in the transgenic green house facility for 15-20 days (Fig. 9.1e). The survival rate of plantlets was recorded as 90-95%. The plantlets were next transferred to the poly bags filled with farm soil and compost (1:1) into the transgenic greenhouse (Fig. 9.1f-g). The plantlets grew normally and morphologically identical to the mother plant (untransformed).

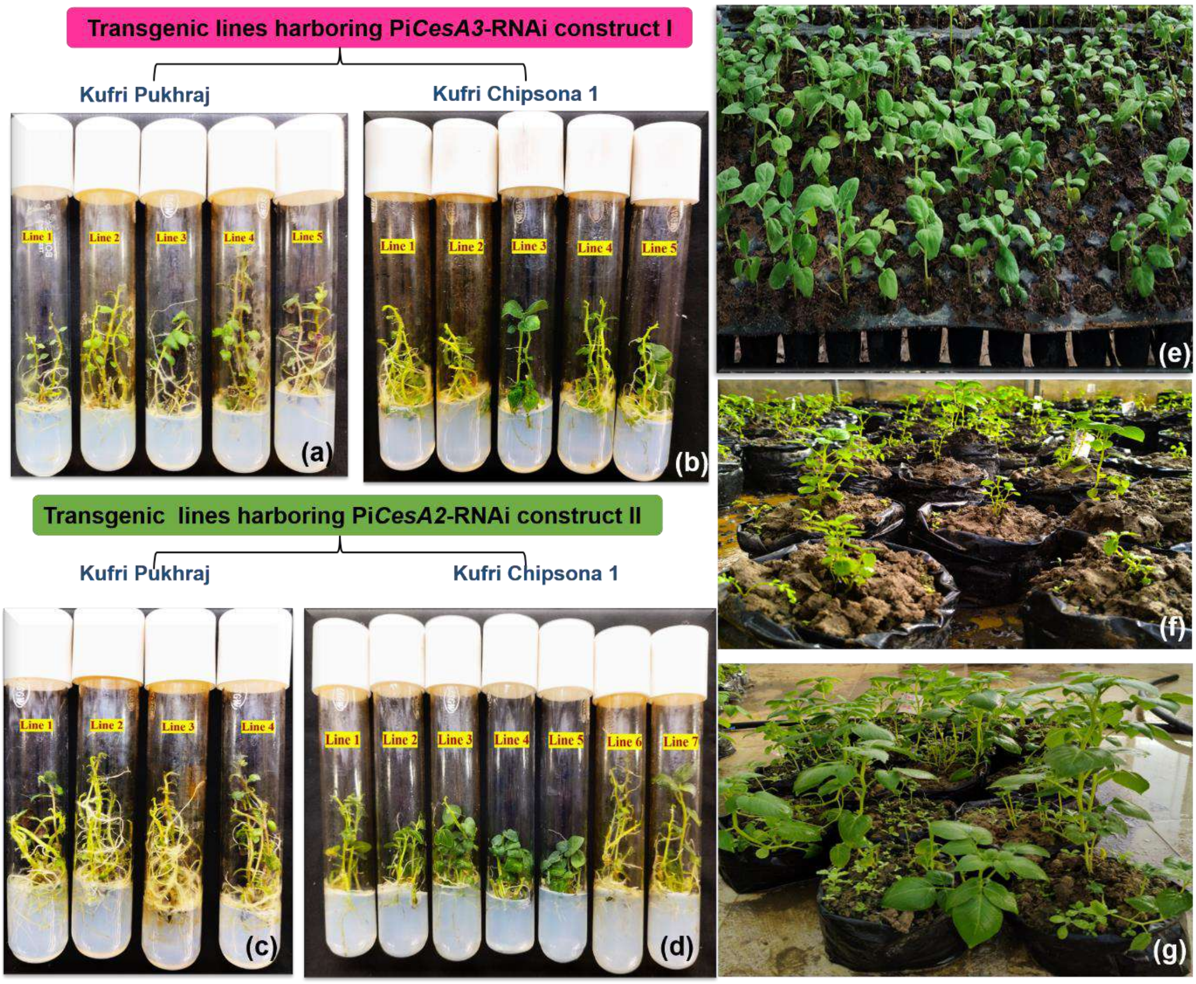


Fig. 9.1 Multiplication of transformed lines of cvs. **(a, c)** Kufri Pukhraj and **(b, d)** Kufri Chipsona 1 harboring Pi*CesA3*-RNAi cassette I and Pi*CesA2*-RNAi cassette II on multiplication medium (MS1 medium+100 mg L^{-1} kanamycin) **(e)** transgenic lines were further acclimatized in poly trays **(f-g)** followed by transfer on poly bags

9.2 Evaluation of transgenic lines for late blight resistance

The evaluation of transgenic lines against late blight resistance was performed through detached leaf and whole plant assay.

9.2.1 Detached leaf assay

The assay was performed to determine whether the regenerated transgenic lines of both the cultivars expressing Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II have some degree of resistance to late blight. For that the leaves of 6-week-old transgenic lines of cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ were inoculated with the sporal suspension of *P. infestans*. The leaves of non-transgenic plants (NT) and those transferred using unmodified hp-RNAi plasmid (referred as vector control (VC)) of cvs. ‘Kufri Pukhraj’ and ‘Kufri Chipsona 1’ were used as controls. The inoculated leaves were visually observed and the lesion diameter was measured 10 days post-inoculation (dpi).

In cv. ‘Kufri Pukhraj’, large lesions were recorded on the leaves of control plants when inoculated with a sporal suspension of *P. infestans* at 10 dpi (Fig. 9.3). The lesion size increased steadily and ranged from 1.54-1.68 cm. However, the transgenic lines expressing Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II showed varied degree of resistance to *P. infestans*. Smaller lesions were observed on leaves of transgenic lines than those of leaves from control plants (Fig. 9.3). The leaf samples of transgenic lines 2, 5, and 4 (‘Kufri Pukhraj’; Pi*CesA3*-RNAi construct I) showed smaller lesions ranging from 0.30-0.37 cm (Fig. 9.2a). Similar observations were also recorded from transgenic leaf samples expressing Pi*CesA2*-RNAi construct II, where smaller lesions were recorded in line 1 (0.35 cm) followed by line 4 (0.44 cm) inoculated with a sporal suspension of *P. infestans* (Fig. 9.2b). Transgenic lines 1, 3 (Pi*CesA3*-RNAi construct I) and lines 2, and 3 (Pi*CesA2*-RNAi construct II) showed the development of lesion diameter larger than other lines where the diameters were much restricted (line 1, 4 and 5 of Pi*CesA3*-RNAi construct I; line1 and 4 of Pi*CesA2*-RNAi construct II).

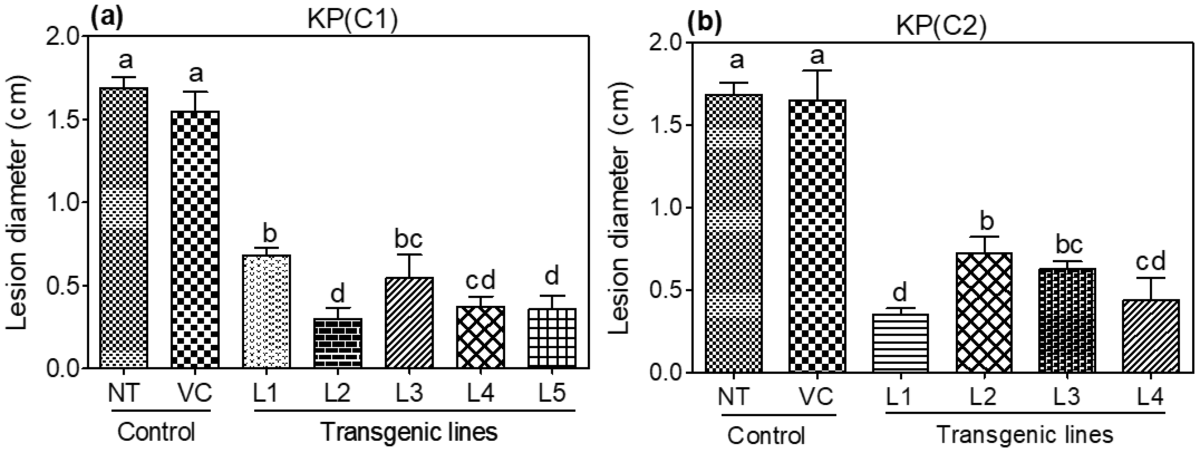


Fig. 9.2 Lesion diameter after 10 days post inoculation of sporal suspension of *P. infestans* on leaves of control and transgenic plants of cv. 'Kufri Pukhraj'developed using **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi cassette II and data were analysed by One way Analysis of variance (ANOVA) with significance level <0.05 by LSD and shown as Mean± Standard error

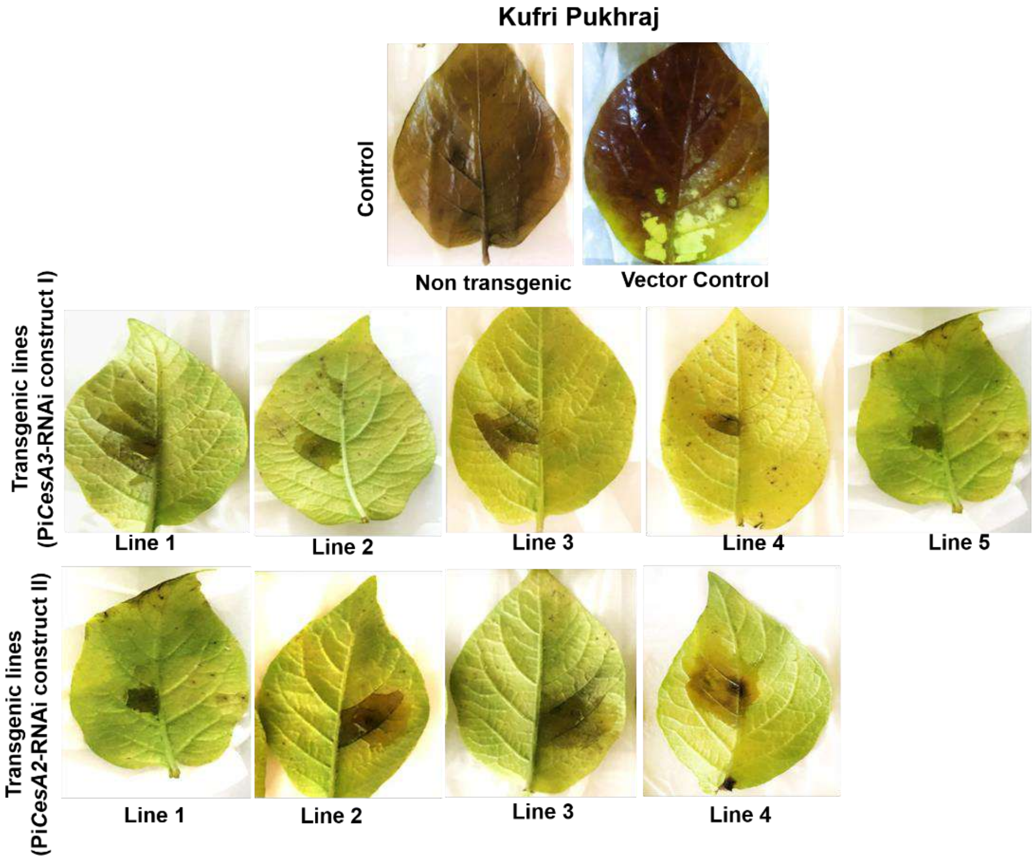


Fig. 9.3 Detached leaf assay performed on leaves of control and transgenic plants of cv. 'Kufri Pukhraj' harboring Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II. Leaves of non-transgenic and vector containing plants are control. Results were recorded after 10 days of inoculation of sporal suspension of *P. infestans*

Furthermore, in cv. 'Kufri Chipsona 1', the leaves from control plants showed lesion diameter in a range of 1.35-1.43 cm at 10 dpi. The transgenic lines obtained using Pi*CesA3*-RNAi construct I showed lesion diameter ranging from 0.37-0.75 cm (Fig. 9.4a and Fig. 9.5). The leaves of transgenic line 3 showed smallest lesion diameter of 0.37 cm and leaves of transgenic line 4 showed maximum lesion diameter of 0.75 cm. The lesion diameter of the transgenic lines 4, 2, 1, and 3 (Pi*CesA2*-RNAi construct II) ranged from 0.30-0.38 cm, whereas it reached 0.45 to 0.58 cm in lines 5 and 4 respectively (Fig. 9.4b and Fig. 9.5). One-way ANOVA analysis showed that the results were statistically significant at $p<0.05$. Thus, it can be concluded that leaf samples of transgenic lines of both cultivars resulted in increased resistance to *P. infestans* as compared to control leaf samples.

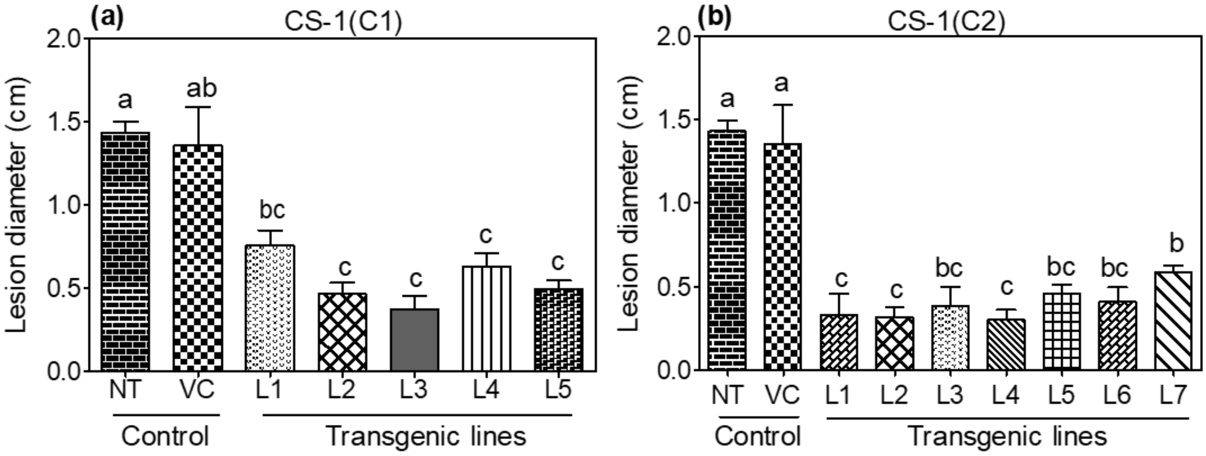


Fig. 9.4 Lesion diameter after 10 days post inoculation of sporal suspension of *P. infestans* on leaves of control and transgenic plants of cv. 'Kufri Chipsona 1' developed using **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II and data were analysed by One way Analysis of variance (ANOVA) with significance level <0.05 by LSD and shown as Mean± Standard error

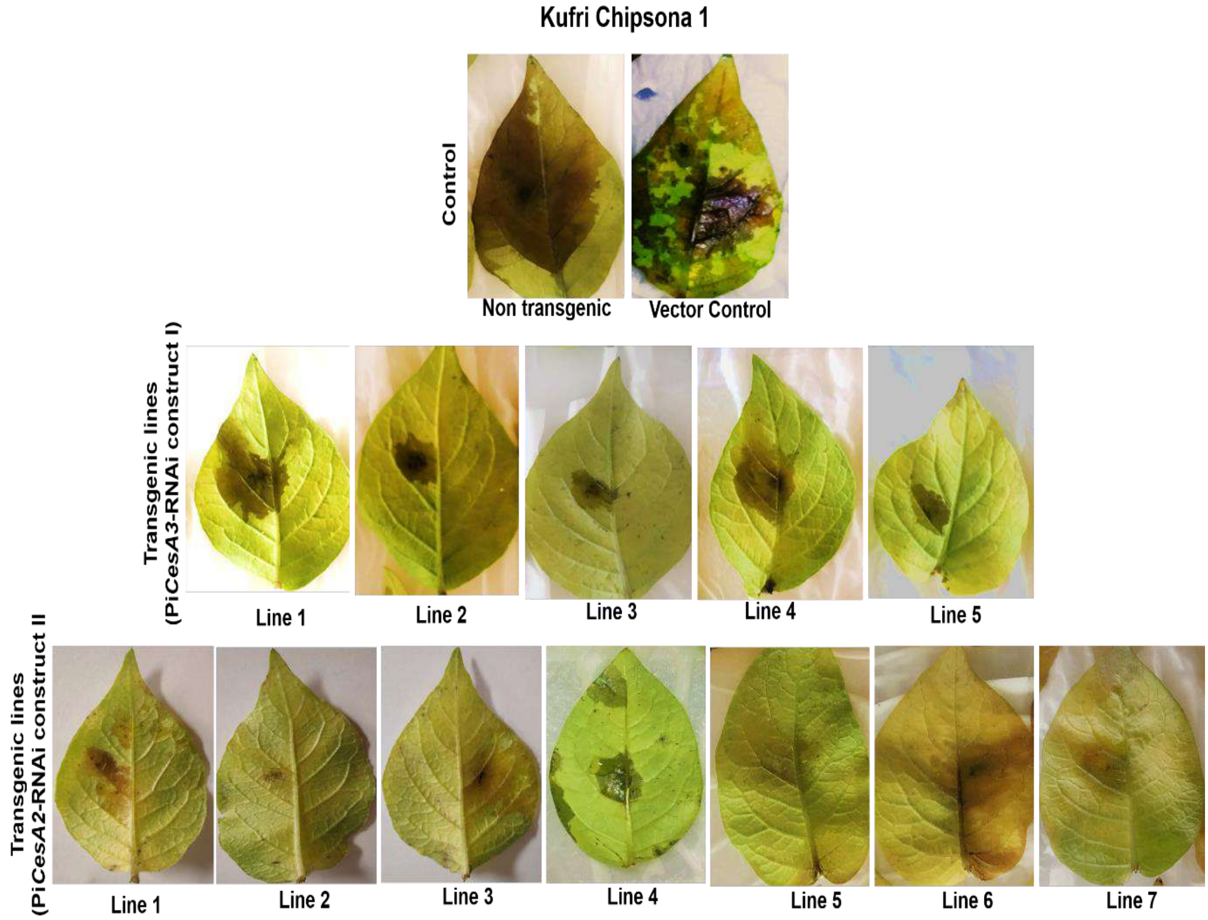


Fig. 9.5 Detached leaf assay performed using leaves of control and regenerated transgenic plants of cv. 'Kufri Chipsona 1' harboring Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II. Leaves of non-transgenic and vector-containing plants served as control. Results were recorded after 10 days of inoculation of sporal suspension of *P. infestans*

9.2.2 The whole plant assay

For the whole plant assay, the transgenic lines and control plants were sprayed with *P. infestans* sporal suspension. Disease symptoms started in the form of small lesions on the lower leaves at 3 dpi. The lesions were evident on control plants but these were slow to develop in transgenic lines. Initially, the lesions were small and light brown but with time these started enlarging and turned dark brown. The lesions were irregular necrotic or brown surrounded by collapsed pale or chlorotic tissue. In whole plant assays, results were recorded as disease incidence and disease severity.

In cv. 'Kufri Pukhraj', control plants sprayed with *P. infestans* sporal suspension showed a disease incidence (DI) in the range of 68.75-75% at 5 dpi which increased up to 93.75% at 10 dpi. The DI was found to be significantly lower in plants of all transgenic lines than those of control plants. In transgenic lines developed using Pi*CesA3*-RNAi construct I, minimum disease incidence of 37.5% was recorded in line 5 followed by lines 2 and 4 (43.75%) whereas lines 1 and 3 recorded with 56.25% and 62.5% higher disease incidence at 5 dpi. At 10 dpi it was noteworthy that 68.75% higher disease incidence was recorded in transgenic lines 1, and 3 whereas, in other transgenic lines (line 2, 4 and 5) it was limited to the range of 50-62.5%. A minimum disease incidence of 50 % was recorded from transgenic lines 4 and 5 at 10 dpi (Fig. 9.6a and Table 9.1).

In the transgenic lines developed using Pi*CesA2*-RNAi construct II, minimum disease incidence was recorded in lines 1(31.25%), 4 (31.25 %), and 3 (37.5%) at 5 dpi. However, at 10 dpi, it reaches 50%, 43.75%, and 62.5% respectively. Moreover, the higher disease incidence was recorded in line 2 (81.25%) at 10 dpi (Fig. 9.6b and Table 9.1).

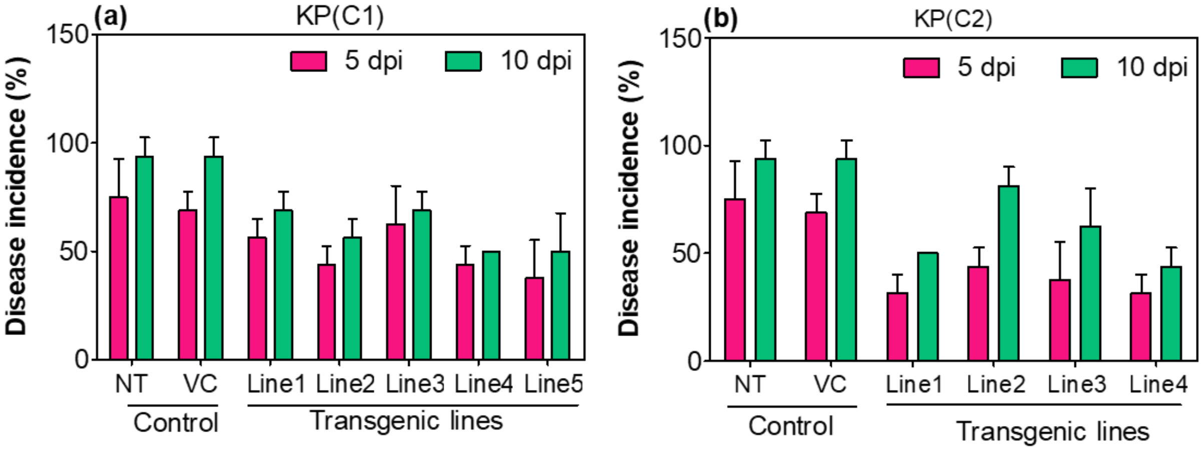


Fig. 9.6 Disease incidence recorded in transgenic vis-à-vis control plants of cv. 'Kufri Pukhraj' harboring **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II. Untransformed and vector-containing plants are control. Data were recorded at 5 and 10 dpi of *P. infestans* inoculation

Disease severity (DS) was also found to vary significantly between the transgenic and control plants (Table 9.1). A maximum of 63.88% DS was observed from control plants at 5 dpi. However, significantly lower DS was recorded in all transgenic lines of Pi*CesA3*-RNAi construct I ranging from 21.52-28.47% at 5 dpi. The severity of the disease further progressed to 38.88-64.69% at 10 dpi significantly lower than the control plants (91.66 %). Minimum disease severity was recorded in transgenic line 4 (38.88 %) followed by line 5 (45.13%) and line 2 (47.91%) while higher disease severity was recorded in line 3 (64.69 %) of transgenic lines developed using Pi*CesA3*-RNAi construct I at 10 dpi (Fig. 9.7a).

In transgenic lines developed using Pi*CesA2*-RNAi construct II, minimum disease severity was recorded in a range of 11.11-19.19% at 5 dpi, while it was further limited to a range of 30.55-45.83% at 10 dpi (Fig. 9.7b). It was noteworthy that the disease severity was minimal in transgenic lines harboring Pi*CesA2*-RNAi construct II than Pi*CesA3*-RNAi construct I at 10 dpi. Overall, the transgenic lines (Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II) of cv. 'Kufri Pukhraj' were showing lower disease severity than control plants (91.66 %).

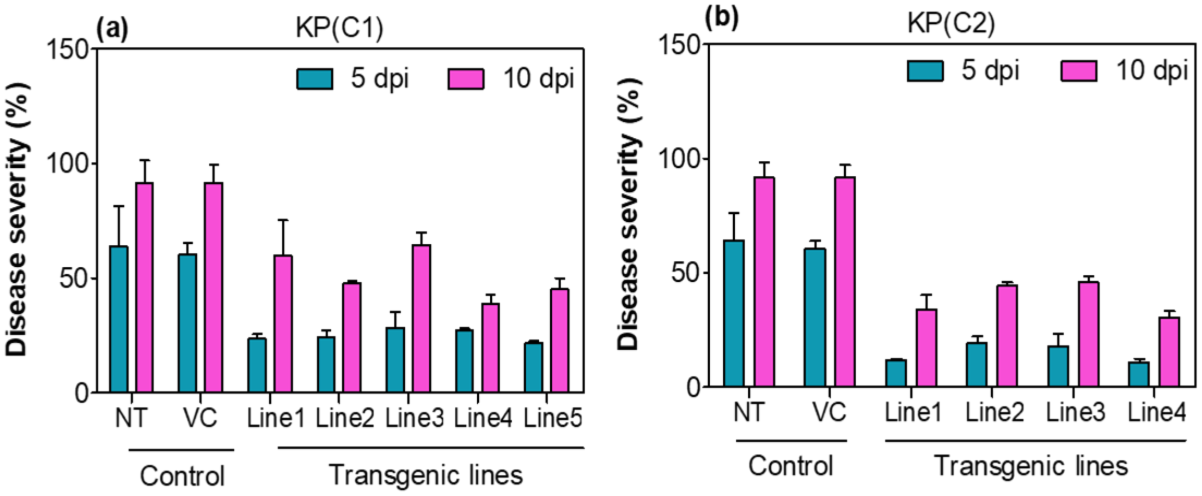


Fig. 9.7 Disease severity recorded in transgenic and control plants of cv. 'Kufri Pukhraj' harboring **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II. Non transgenic and vector-containing plants served as controls. Results were recorded at 5 and 10 dpi of *P. infestans* inoculation

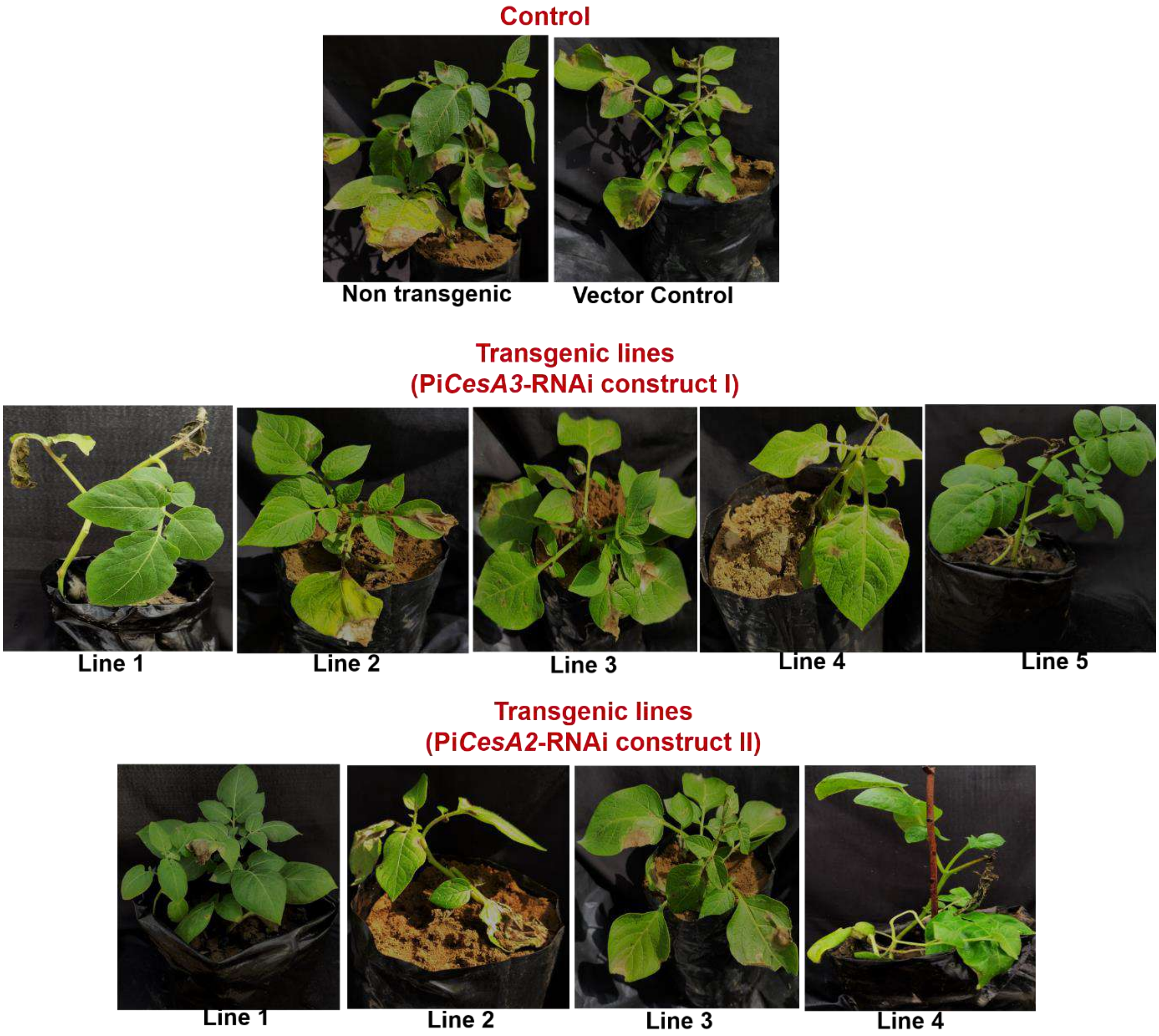


Fig. 9.8 The whole plant assays performed on fully grown transgenic and control plants of cv. 'Kufri Pukhraj' harboring Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II at 10 days post inoculation

Table 9.1 Two-way ANOVA table of disease incidence and disease severity of transgenic and non-transgenic plants of cv. 'Kufri Pukhraj' harboring Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II

Source	*Df*	*Sum of square*	*Mean square*	*F value*	*P value*
Kufri Pukhraj (PiCesA3-RNAi construct I)					
Disease incidence					
Between days post inoculation (dpi)	1	1256	1256	9.00	0.0096
Between cultivars	6	6362	1060	7.600	0.0009
Interaction	6	267.9	44.64	0.320	0.9157
Error	14	1953	139.5		
Disease severity					
Between days post inoculation (dpi)	1	5172	5172	83.38	<0.0001
Between cultivars	6	9139	1523	24.56	<0.0001
Interaction	6	441.1	73.52	1.185	0.3685
Error	14	868.3	62.02		
Kufri Pukhraj (PiCesA2-RNAi construct II)					
Disease incidence					
Between days post inoculation (dpi)	1	3151	3151	24.20	0.0004
Between cultivars	5	8125	1625	12.48	0.0002
Interaction	5	364.6	72.92	0.5600	0.7289
Error	12	1563	130.2		
Disease severity					
Between days post inoculation (dpi)	1	3938	3938	68.24	<0.0001
Between cultivars	5	13760	2753	47.70	<0.0001
Interaction	5	91.01	18.20	0.3154	0.8943
Error	12	692.6	57.71		

In cv. 'Kufri Chipsona 1', disease incidence (DI) in transgenic lines (Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II) and control plants vary significantly (Table 9.2) as mentioned in the case of cv. 'Kufri Pukhraj' (Table 9.1). The transgenic lines expressing Pi*CesA3*-RNAi construct I, minimum disease incidence was recorded in lines 3 and 5 (25%) followed by lines 1, 2 (31.25%), and line 4 (37.5%), while in control plants it ranges from 50-56.25 % at 5 dpi. At 10 dpi the disease incidence in control plants reaches a maximum of 68.75-75% whereas minimum disease incidence was recorded in line 3 (37.5%) followed by line 5 (43.75%) while the maximum among transgenic lines was recorded in line 1 (56.25 %) at 10 dpi (Fig. 9.9a and Table 9.2).

The transgenic lines expressing Pi*CesA2*-RNAi construct II, minimum disease incidence was observed in lines 1, 3, and 7 (31.25%), and the maximum was recorded in line 6 (56.25%) at 5 dpi. At 10 dpi, 43.75 % of disease incidence was recorded in lines 1, and 7 which is the minimum, while 62.5 % of higher disease incidence was recorded in lines 3 and 6 which was lower than the disease incidence recorded in control plants (68.75-75%) (Fig. 9.9b and Table 9.2).

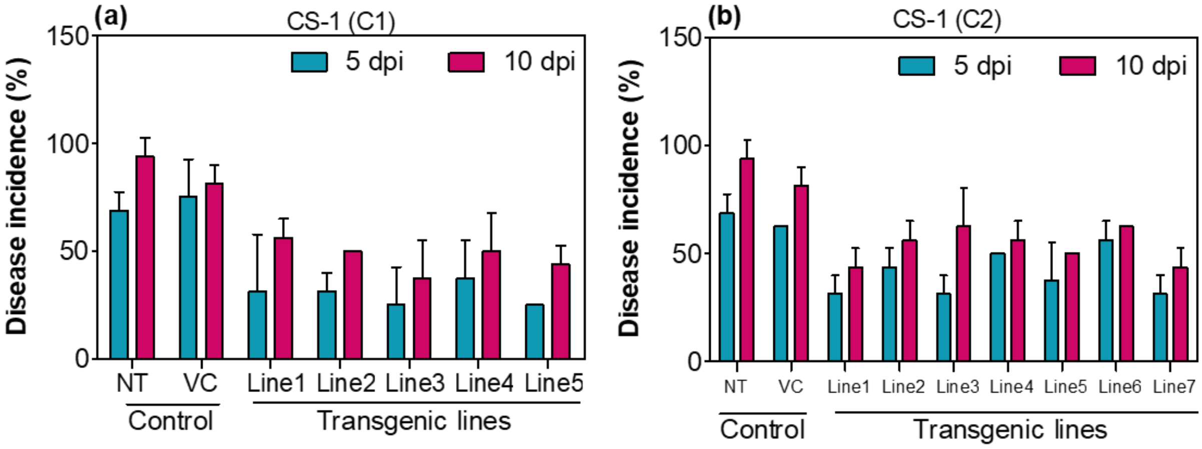


Fig. 9.9 Disease incidence recorded in control and transgenic plants of cv. 'Kufri Chipsona 1' harboring **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II. Non transgenic and vector-containing plants are control. Results were recorded at 5 and 10 dpi of *P. infestans* inoculation

The disease severity (DS) of control plants was recorded in a range of 51.38-61.8% at 5 dpi. However, it was recorded lower in all transgenic lines expressing Pi*CesA3*-RNAi construct I ranging from 12.49-19.44% at 5 dpi. After 5 dpi, the disease progressed further and reached 48.60 % in line 4 at 10 dpi, which was higher among other lines (37.49-46.32%) (Fig. 9.10a and Table 9.2).

In transgenic lines developed using Pi*CesA2*-RNAi construct II, similar results were observed. The transgenic lines were showing minimum disease severity (11.8 -18.74%) at 5 dpi. Further, disease severity was recorded in a range of 37.49-49.99% at 10 dpi. The minimum disease severity was recorded in line 1 (38.88%) and line 7 (37.49%) and the maximum was recorded in line 3 (49.99%) which was lower than the control (61.8-74.99%) (Fig. 9.10b and Table 9.2).

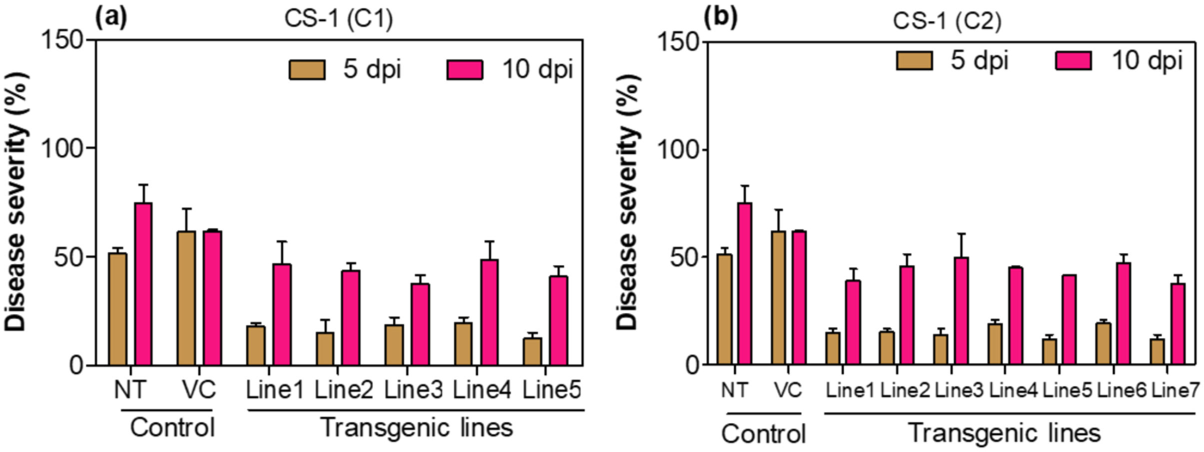


Fig. 9.10 Disease severity recorded in transgenic and control plants of cv. Kufri Chipsona 1' harboring **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II. Non transgenic and vector-containing plants served as controls. Results were recorded at 5 and 10 dpi of *P. infestans* inoculation

Therefore, it can be concluded that all the transgenic lines were able to resist the disease up to some extent till 5 days after inoculation, after which the disease started progressing further to some extent. The disease incidence and severity were found to increase significantly between the days of inoculation.

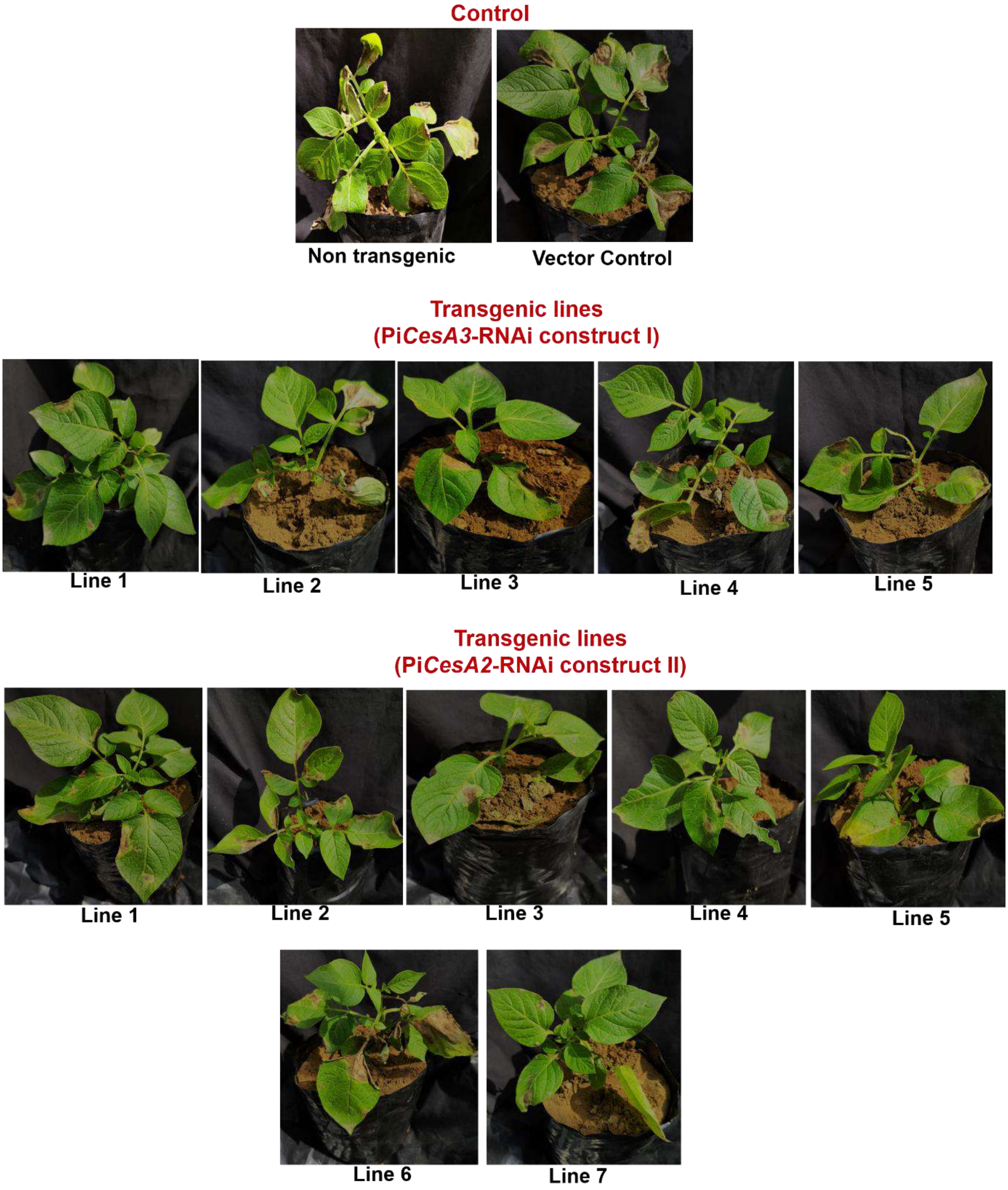


Fig. 9.11 The whole plant assays performed on fully grown transgenic and control plants of cv. ‘Kufri Chipsona 1’ harboring Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II at 10 days post inoculation

Table 9.2 ANOVA table of disease incidence and disease severity of transgenic and non-transgenic plants of cv. ‘Kufri Chipsona 1’ developed using Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II

Source	*Df*	*Sum of square*	*Mean square*	*F value*	*P value*
Kufri Chipsona 1 (PiCesA3-RNAi construct I)					
Disease incidence					
Between days post inoculation (dpi)	1	2015	2015	10.31	0.0063
Between cultivars	6	10110	1685	8.629	0.0005
Interaction	6	290.2	48.36	0.2476	0.9523
Error	14	2734	195.3		
Disease severity					
Between days post inoculation (dpi)	1	3518	3518	51.54	<0.0001
Between cultivars	6	6130	1022	14.97	<0.0001
Interaction	6	672.4	112.1	1.642	0.2081
Error	14	955.6	68.26		
Kufri Chipsona 1 (PiCesA2-RNAi construct II)					
Disease incidence					
Between days post inoculation (dpi)	1	2101	2101	24.20	0.0001
Between cultivars	8	7222	902.8	10.40	<0.0001
Interaction	8	555.6	69.44	0.8000	0.6104
Error	18	1563	86.81		
Disease severity					
Between days post inoculation (dpi)	1	5590	5590	115.5	<0.0001
Between cultivars	8	7082	885.2	18.29	<0.0001
Interaction	8	815.1	101.9	2.105	0.0904
Error	18	871.1	48.40		

9.3 Antioxidant enzyme activity

The activity of four antioxidant enzymes namely superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase, was studied in inoculated and uninoculated control and transgenic plants. It was found that there were no significant differences in the enzyme activities of uninoculated control and transgenic plants. However, significant differences in the activities of enzymes were observed between the inoculated control and transgenic plants (Table 9.3, 9.4).

In cv. ‘Kufri Pukhraj’ the catalase activity of uninoculated control and transgenic plants was in a range of 18.49-20.68 U mg^{-1} protein, 21-24.12 U mg^{-1} protein (Pi*CesA3*-RNAi construct I) and 21.97-25.12 U mg^{-1} protein (Pi*CesA2*-RNAi construct II). After inoculation, in transgenic lines of Pi*CesA3*-RNAi construct I, the catalase activity significantly increases as compared to control plants. The highest catalase activity (42.86 U mg^{-1} protein) was recorded in transgenic ‘line 4’of Pi*CesA3*-RNAi construct I at 5 dpi. Later, this reduced to 36.97 U mg^{-1} protein at 10 dpi (Fig 9.12a and Table 9.3).

While in transgenic plants of Pi*CesA2*-RNAi construct II, the highest activity was also recorded in ‘line 4’ (49.45 U mg^{-1} protein) at 5 dpi which further reduced to 37.22 U mg^{-1} protein at 10 dpi (Fig. 9.12b and Table 9.3).

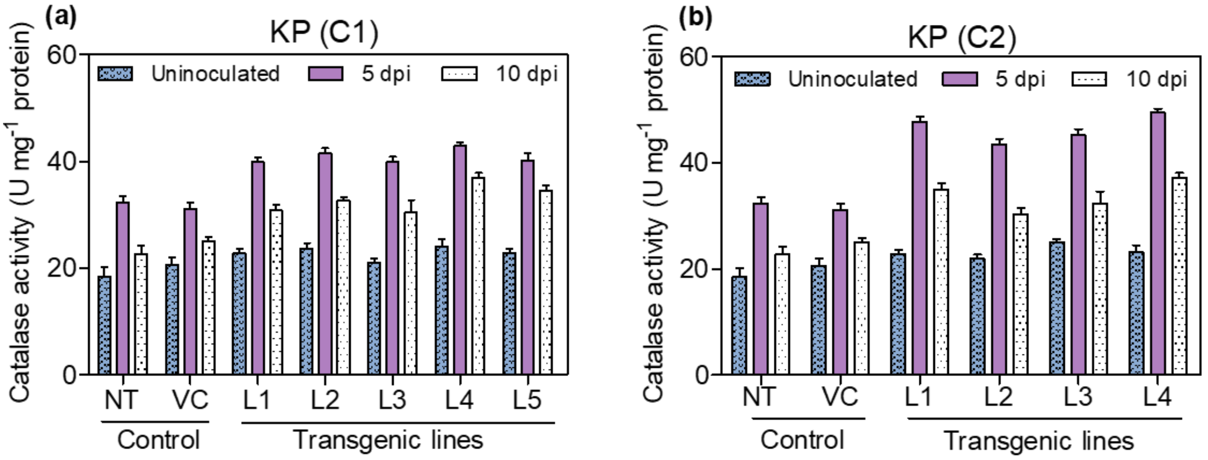


Fig. 9.12 Changes in the catalase activity in inoculated and non-inoculated control and transgenic lines of cv. ‘Kufri Pukhraj’ **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

In case of superoxide dismutase activity, again no significant differences were recorded in uninoculated control (2.35-2.51 U mg^{-1} protein), transgenic plants of Pi*CesA3*-RNAi construct I (2.42-3.44 U mg^{-1} protein) and Pi*CesA2*-RNAi construct II (2.63-2.88 U mg^{-1} protein). After infection significant differences were recorded in the enzyme activity. In case of transgenic plants developed with Pi*CesA3*-RNAi construct I, the highest activity was recorded in 'line 4' (12.67 U mg^{-1} protein), which reduces to 9.48 U mg^{-1} protein at 10 dpi whereas in transgenic plants of Pi*CesA2*-RNAi construct II, highest activity was recorded in 'line 4' at 5 dpi (14.52 U mg^{-1} protein), which reduces to 12.37 U mg^{-1} protein at 10 dpi (Fig 9.13 a, b and Table 9.3).

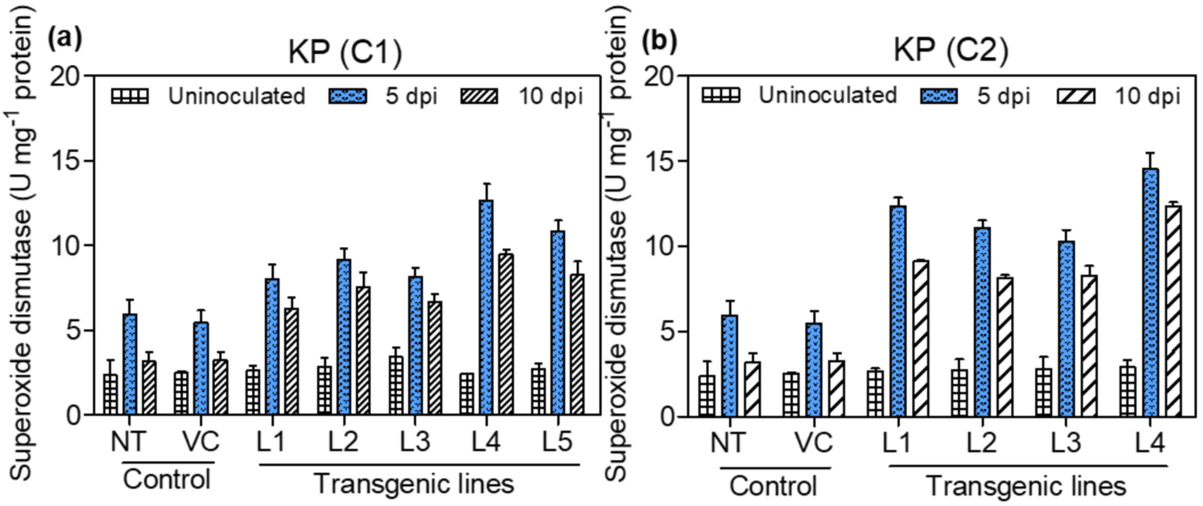


Fig. 9.13 Changes in the superoxide dismutase activity in inoculated and non-inoculated control and transgenic lines of cv. 'Kufri Pukhraj' **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

Likewise, the activity of peroxidase in uninoculated control plants ranges from 42.30-44.85 U mg^{-1} protein whereas in transgenic plants of Pi*CesA3*-RNAi construct I as 40.18-45.74 U mg^{-1} protein and Pi*CesA2*-RNAi construct II as 40-75-48.96 U mg^{-1} protein. After inoculation, the highest activity of peroxidase in 'line 4' of both the RNAi constructs was recorded. In case of Pi*CesA3*-RNAi construct I it was as 85.39 U mg^{-1} protein and in case of Pi*CesA2*-RNAi construct II, the activity of peroxidase was 89.14 U mg^{-1} protein at 5 dpi, which further reduced to 75.03 U mg^{-1} protein at 10 dpi (Fig. 9.14a, b and Table 9.3).

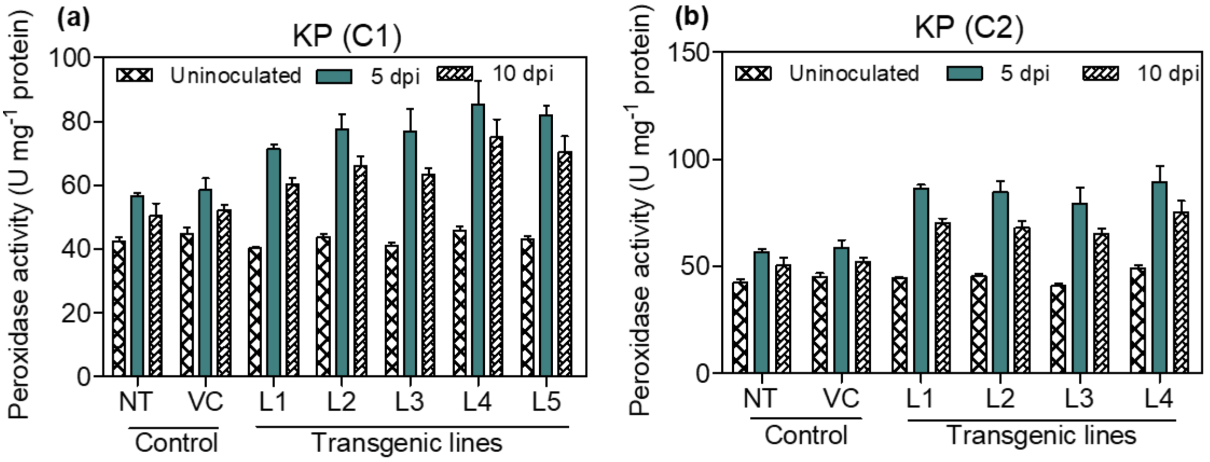


Fig. 9.14 Changes in the peroxidase activity in inoculated and non-inoculated control and transgenic lines of cv. 'Kufri Pukhraj' **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

Further, the activity of ascorbate peroxidase in uninoculated control plant was 65.89-67.36 U mg^{-1} protein, transgenic lines of Pi*CesA3*-RNAi construct I showed 63.24-73.56 U mg^{-1} protein and in transgenic lines of Pi*CesA2*-RNAi construct II activity was 60.22-75.73 U mg^{-1} protein, these values were not significant. However, after inoculation the significant differences were recorded among control and transgenic lines. The highest activity of ascorbate peroxidase was recorded as 128.17 U mg^{-1} protein in transgenic 'line 4' of Pi*CesA3*-RNAi construct I at 5 dpi which reduced to 120.45 at 10 dpi.

While in transgenic plants of Pi*CesA2*- RNAi construct II, the highest activity was recorded as 125.64 U mg^{-1} protein in 'line 4' at 5 dpi, which reduced to 112.07 U mg^{-1} protein at 10 dpi (Fig.9.15a, b and Table 9.3).

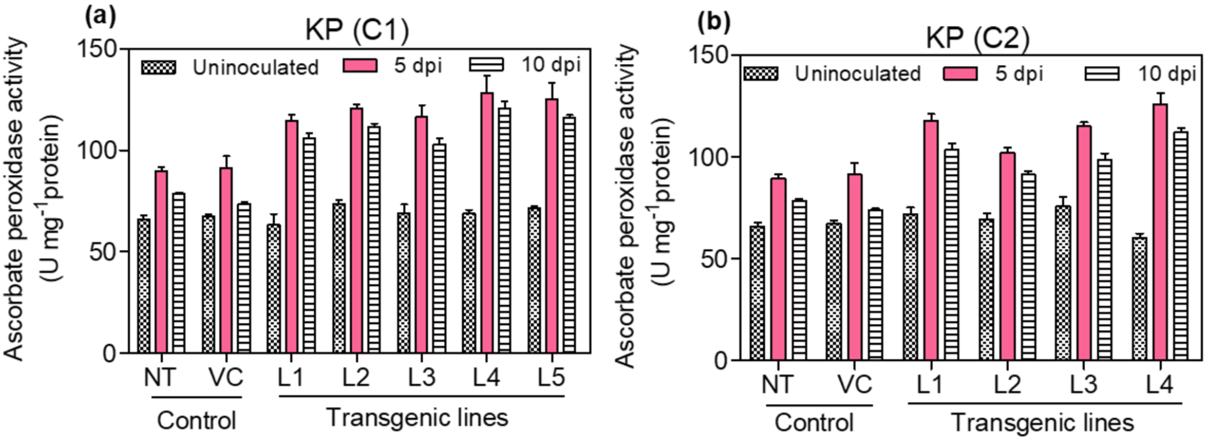


Fig. 9.15 Changes in the ascorbate peroxidase activity in inoculated and non-inoculated control and transgenic lines of cv. ‘Kufri Pukhraj’ **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

Table 9.3 Two-way ANOVA table on antioxidant enzyme activities (catalase, superoxide dismutase, peroxidase, and ascorbate peroxidase) in inoculated and non-inoculated control and transgenic lines (Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II) of cv. ‘Kufri Pukhraj’

			Kufri Pukhraj (PiCesA3-RNAi construct I)						
Source	***Df***	***Sum of square***	***Mean square***	***F value***	***P value***	***Sum of square***	***Mean square***	***F value***	***P value***
			Catalase activity				***Superoxide dismutase***		
Between days post inoculation (dpi)	2	2787	1393	1035	<0.0001	374	187	479.1	<0.0001
Between cultivars	6	768.8	128.1	95.15	<0.0001	149	24.85	63.68	<0.0001
Interaction	12	143.3	11.94	8.868	<0.0001	74.74	6.228	15.96	<0.0001
Error	42	56.56	1.347			16.39	0.3903		
			Peroxidase activity				***Ascorbate peroxidase***		
Between days post inoculation (dpi)	2	9501	4751	387.8	<0.0001	21760	10880	724.2	<0.0001
Between cultivars	6	2548	424.6	34.66	<0.0001	7557	1259	83.86	<0.0001
Interaction	12	1217	101.4	8.278	<0.0001	3015	251.2	16.73	<0.0001
Error	42	514.5	12.25			630.8	15.02		
			Kufri* Pukhraj *(PiCesA2-RNAi construct II)						
			Catalase activity				***Superoxide dismutase***		
Between days post inoculation (dpi)	2	3450	1725	1142	<0.0001	493.4	246.7	755.9	<0.0001
Between cultivars	5	1178	235.5	155.9	<0.0001	267.6	53.52	164	<0.0001
Interaction	10	314.4	31.44	20.81	<0.0001	117.6	11.76	36.04	<0.0001
Error	36	54.39	1.511			**11.75**			
			Peroxidase activity				***Ascorbate peroxidase***		
Between days post inoculation (dpi)	2	8928	4464	333.4	<0.0001	13660	6831	712.4	<0.0001
Between cultivars	5	3339	667.7	49.86	<0.0001	4446	889.2	92.73	<0.0001
Interaction	10	1390	139	10.38	<0.0001	2559	255.9	26.69	<0.0001
Error	36	482.1	13.39			345.2			

Similarly, in cv. 'Kufri Chipsona 1' there were no significant differences in the enzyme activities of uninoculated control and transgenic lines. However, significant differences in the activities of enzymes were observed in inoculated control and transgenic plants. In cv. 'Kufri Chipsona 1' the catalase activity of uninoculated control plants was in a range of 23.36-24.51 U mg^{-1} protein, in transgenic lines of Pi*CesA3*-RNAi construct I as 22.30-26.52 U mg^{-1} protein and Pi*CesA2*-RNAi construct II as 23.38-36.62 U mg^{-1} protein. In inoculated transgenic plants of Pi*CesA3*-RNAi construct I, the highest catalase activity was recorded as 56.95 U mg^{-1} protein in 'line 3' whereas in Pi*CesA2*-RNAi construct II as 64.02 U mg^{-1} protein in 'line 7' at 5 dpi. Later, this was reduced to 42.20 U mg^{-1} protein in transgenic lines of Pi*CesA3*-RNAi construct I and to 50.86 U mg^{-1} protein in transgenic lines of Pi*CesA2*-RNAi construct II at 10 dpi (Fig. 9.16a, b and Table 9.4).

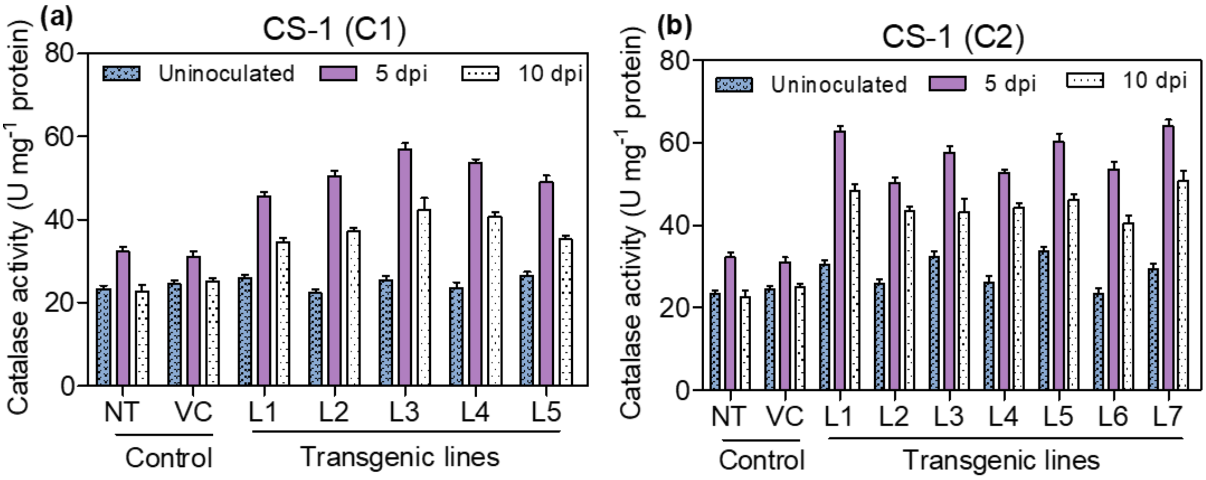


Fig. 9.16 Changes in the catalase activity in inoculated and non-inoculated control and transgenic lines of cv. 'Kufri Chipsona 1' **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

The activity of superoxide dismutase in uninoculated control plants was recorded as 3.30-3.52 U mg^{-1} protein, in transgenic plants of Pi*CesA3*-RNAi construct I as 3.41-3.95 U mg^{-1} protein, and in Pi*CesA2*-RNAi construct II as 3.01-3.78 U mg^{-1} protein. After inoculation, the highest activity in transgenic lines of Pi*CesA3*-RNAi construct I was recorded in 'line 3' as 15.96 U mg^{-1} protein whereas in transgenic 'line 7' of Pi*CesA2*-RNAi construct II as 14.11 U mg^{-1} protein at 5 dpi which reduced to 13.70 U mg^{-1} protein and 12.69 U mg^{-1} protein respectively at 10 dpi (Fig. 9.17a, b and Table 9.4).

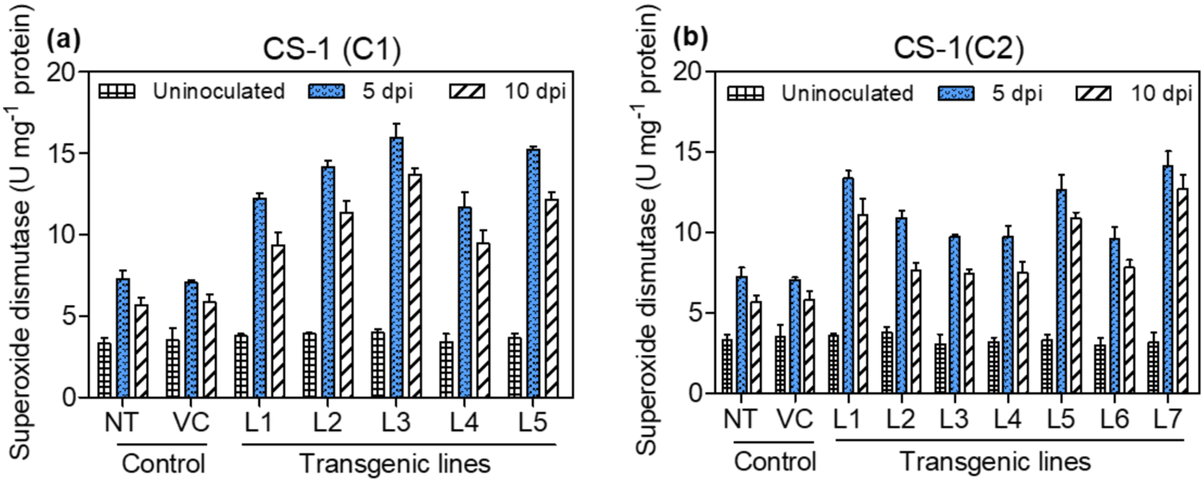


Fig. 9.17 Changes in the superoxide dismutase activity in inoculated and non-inoculated control and transgenic lines of cv. 'Kufri Chipsona 1' **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

Likewise, the activity of peroxidase in uninoculated control plants was in the range of 50.27-53.97 U mg^{-1} protein, in transgenic plants of Pi*CesA3*-RNAi construct I as 49.55-59.92 U mg^{-1} protein and Pi*CesA2*-RNAi construct II as 50-76-56.70 U mg^{-1} protein. The highest activity of peroxidase in inoculated transgenic lines of Pi*CesA3*-RNAi construct I was recorded in 'line 3' as 95.33 U mg^{-1} protein at 5 dpi which marginally reduced to 88.83 U mg^{-1} protein at 10 dpi

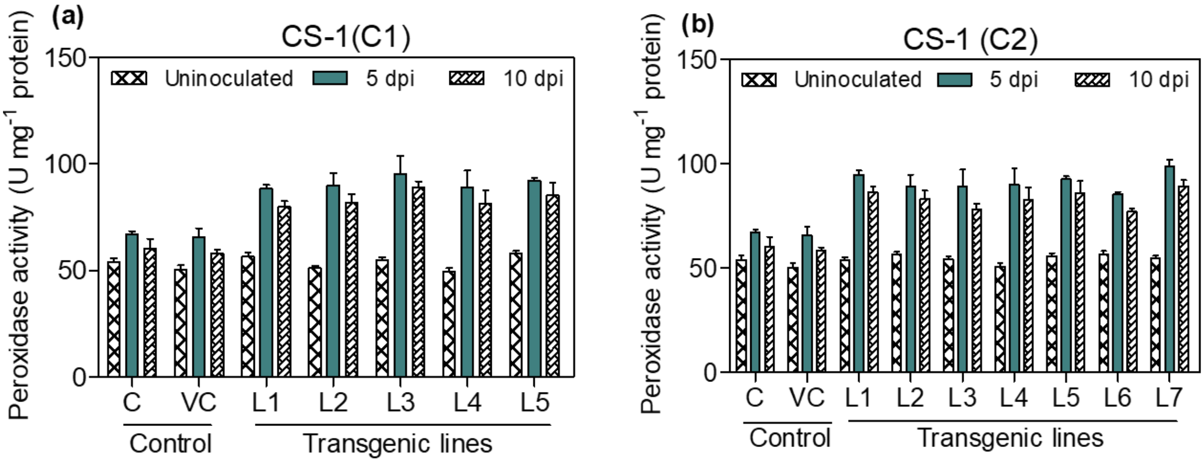


Fig. 9.18 Changes in the peroxidase activity in inoculated and non-inoculated control and transgenic lines of cv. 'Kufri Chipsona 1' **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

whereas in transgenic lines of Pi*CesA2*-RNAi construct II, the highest activity was recorded in line 7 as 98.56 U mg^{-1} protein at 5 dpi which further reduced to 89.32 U mg^{-1} protein at 10 dpi (Fig. 9.18a, b and Table 9.4).

However, the activity of ascorbate peroxidase in uninoculated control plants was in range of 71.98-76.41 U mg^{-1} protein, in transgenic lines of Pi*CesA3*-RNAi construct I as 69.83-74.09 U mg^{-1} protein, and Pi*CesA2*-RNAi construct II as 70.38-79.95 U mg^{-1} protein. In inoculated transgenic plants of Pi*CesA3*-RNAi construct I, the highest activity was recorded in 'line 3' as 132.77 U mg^{-1} protein at 5 dpi, which reduced to 117.91 U mg^{-1} protein at 10 dpi. Moreover, in transgenic plants of Pi*CesA2*-RNAi construct II, the highest activity was recorded in 'line 7' as 134.49 U mg^{-1} protein at 5 dpi which was reduced to 120.34 U mg^{-1} protein at 10 dpi (Fig. 9.16a, b and Table 9.4).

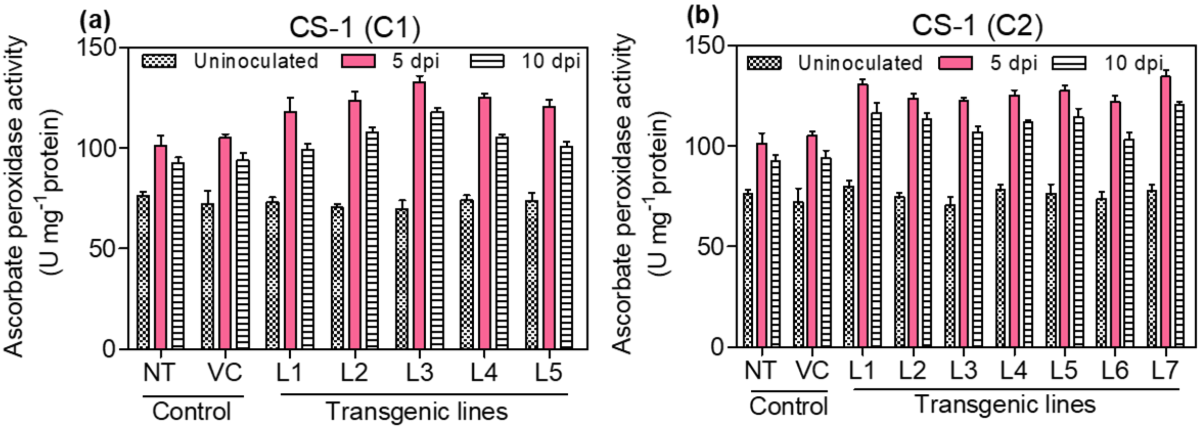


Fig. 9.19 Changes in the Ascorbate peroxidase activity in inoculated and non-inoculated control and transgenic lines of cv. 'Kufri Chipsona 1' **(a)** Pi*CesA3*-RNAi construct I and **(b)** Pi*CesA2*-RNAi construct II during disease development. The results were analyzed by two-way ANOVA at 0.05 significant level

Table 9.4 Two-way ANOVA table on antioxidant enzyme activities (catalase, superoxide dismutase, peroxidase, and ascorbate peroxidase) in inoculated and non-inoculated control and transgenic lines (Pi*CesA3*-RNAi construct I and Pi*CesA3*-RNAi construct II) of cv. 'Kufri Chipsona 1'

Kufri Chipsona 1 (PiCesA3-RNAi construct I)									
Source	***Df***	***Sum of square***	***Mean square***	***F value***	***P value***	***Sum of square***	***Mean square***	***F value***	***P value***
			Catalase activity				***Superoxide dismutase***		
Between days post inoculation (dpi)	2	4678	2339	1462	<0.0001	768.6	384.3	13505	<0.0001
Between cultivars	6	1906	317.7	198.6	<0.0001	281.6	46.94	164.9	<0.0001
Interaction	12	977.2	81.43	50.90	<0.0001	122.6	10.22	35.89	<0.0001
Error	42	67.20	1.600			11.96	0.2847		
			Peroxidase activity				***Ascorbate peroxidase***		
Between days post inoculation (dpi)	2	10670	5333	330.6	<0.0001	22210	11110	847.6	<0.0001
Between cultivars	6	3989	664.8	41.21	<0.0001	1983	330.5	25.22	<0.0001
Interaction	12	1587	132.3	8.198	<0.0001	1759	146.6	11.19	<0.0001
Error	42	677.6	16.13			550.4	13.11		
Kufri Chipsona 1 (PiCesA3-RNAi construct II)									
			Catalase activity				***Superoxide dismutase***		
Between days post inoculation (dpi)	2	7736	3868	1743	<0.0001	737.6	368.8	1153	<0.0001
Between cultivars	8	4925	615.6	277.4	<0.0001	195.4	24.42	76.37	<0.0001
Interaction	16	1361	85.08	38.34	<0.0001	103.2	6.450	20.17	<0.0001
Error	54	119.8	2.219			17.27	0.3198		
			Peroxidase activity				***Ascorbate peroxidase***		
Between days post inoculation (dpi)	2	14740	7369	554.8	<0.0001	30080	15040	1312	<0.0001
Between cultivars	8	4579	572.4	43.10	<0.0001	3988	498.5	43.48	<0.0001
Interaction	16	1831	114.4	8.616	<0.0001	1505	94.06	8.204	<0.0001
Error	54	717.2	3.28			619.1	11.46		

Chapter 10

Discussion

The worldwide cultivation of potato is threatened by late blight disease caused by *P. infestans*. It is the most dreaded biotic stress of potato, which affects both quality and overall yield (Arora et al. 2014). *P. infestans* can infect potato crops at all developmental stages, causing worldwide economic losses accounting for 6 billion dollars annually (Xiao et al. 2019). Since its emergence, it has become a significant threat to global food security, causing about 25 % damage to potato yield each year (Mhatre et al. 2020). In an attempt to improve resistance in potato cultivars, various management strategies were adopted. Improving resistance with breeding programs by exploiting *R*-genes is a time-consuming process (Barrel et al. 2013; Dong and Zhou 2022). Moreover, the differences in the ploidy levels of potato is also a serious issue affecting breeding programmes. Further, fungicides are the most widely used method to control late blight, but it is not safe for the environment and human health. Moreover, it is also associated with the emergence of new strains of *P. infestans* (Majeed et al. 2017). Therefore, for effective late blight management, developing genetically modified crops is considered the method of choice. This can be accomplished through genetic engineering, involving the integration of transgene(s) (Del Mar Martinez-Prada et al. 2021). Attempts have been made to integrate and express resistance (*R*) genes, pathogenesis-related proteins, transcription factor (s), small G proteins, antioxidant enzymes, antimicrobial proteins, and cytotoxic compounds in potato cultivars (Liu et al. 1994; Yu et al. 1999; Moon et al. 2009; Vossen et al. 2016; Tian et al. 2023).

The technology also provides other new approaches for attaining late blight resistance in potato cultivars. RNAi is the one such approach that has a sequence-dependent mode of action for disease resistance (Saurabh et al. 2014). It has a great potential to protect plants from different pathogens such as fungi (Mamta et al. 2016), bacteria (Yanagihara et al. 2006), viruses (Tan and Yin 2004), insects (Gordon and Waterhouse 2007), nematodes (Banerjee et al. 2017) and weeds (Zabala-Pardo et al. 2022). The RNAi concept relies on the delivery of small RNA molecules (dsRNA/siRNA) to the pathogen, where these RNAs inhibit the growth of the pathogen by silencing targeted essential genes (Saurabh et al. 2014; Xu et al. 2019). Delivering small dsRNA molecules to a pathogen is crucial to the success of the RNAi in crop protection. This can be accomplished via host-induced gene silencing (HIGS) approach, which corresponds to the in-planta expression of siRNA targeting key genes of the pathogen (Gebremichael et al. 2021). In the past decade, however, HIGS has been widely utilised for developing disease resistance in plants (Jahan et al. 2015; Cheng et al. 2015; Mamta et al. 2016; Forster and Shuai 2020; Yang et al. 2021; Adeyinka et al. 2023; Guo et al. 2023).

In view of the specificity of HIGS, the present study was aimed to silence the cellulose synthase gene of *P. infestans*. Cellulose is the major component of the *P. infestans* cell wall, and it plays an important role in the infection process by forming a structure called appressorium (Grenville-Briggs et al. 2008). It was therefore assumed that inhibiting the cellulose synthesis in *P. infestans* would prevent the appressorium formation, which is required for penetration to the host cell and for the establishment of an infection. So, attempts were made to develop two RNAi constructs for silencing the *CesA* gene of *P. infestans*.

Furthermore, putting the concept into practice necessitates the development of transgenic lines expressing siRNAs specific to *P. infestans CesA* gene. An efficient plant regeneration through shoot organogenesis and/or somatic embryogenesis and an efficient system of gene transfer are required (Gelvin 2003; Ebrahimzadegan and Maroufi 2022; Khatun et al. 2012). The regeneration of transgenic lines from a limited number of transformed cells is influenced by many factors like genotype, explant, macro and micronutrients, carbon source, plant growth regulators (PGRs), antibiotics, light and temperature (Anjum and Ali 2004; Bell et al. 2009; Tiwari and Singh 2010; Sridhar and Naidu 2011; Abrahamian and Kantharajah 2011; Parris et al. 2012; Rawat et al. 2013; Kaur et al. 2017; Martinez et al. 2021; Guleria and Kumar 2022; Cavallaro et al. 2022). Therefore, the optimisation of factors for high frequency plant regeneration (through shoot organogenesis and/or somatic embryogenesis) is a prerequisite for efficient *Agrobacterium*-mediated genetic transformation of potato. Therefore, attempts were also made to optimise the shoot organogenesis protocol in target cultivar(s).

10.1 Selection of potato cultivar for the present study

Screening of the potato cultivars reveals a susceptibility or resistance level of a particular cultivar against late blight resistance. In this study, four Indian potato cultivars, 'Kufri Pukhraj', 'Kufri Chipsona 1', 'Kufri Jyoti' and 'Kufri Surya' were evaluated for their resistance against late blight disease. The disease resistance in plants is widely assessed using detached leaf and whole plant assays (Dufera 2014; Kaur et al. 2020; Karki and Halterman 2021; Kaur et al. 2022). These assays particularly involved the use of sporal suspension of *P. infestans* (pathogen) on plants and test the ability of the *P. infestans* to infect potato plants (Namukwaya 2015). In literature, it has been reported that the maintenance *P. infestans* on an artificial medium may lead to loss of virulence (Loesch et al. 2010; Ansari and Butt 2011; Attah et al. 2021). The virulence can be preserved by maintaining the pathogen on the host tissue (Attah et al. 2021). Therefore, to prepare sporal suspension for evaluation, the *P. infestans* were inoculated on the leaves of susceptible potato cultivars. The actively growing *P. infestans* on the leaf was then utilised to perform detach leaf and whole plant assays. Both the assays revealed the variation in the resistance level of the cultivars. The lesions observed on the leaves of cv. 'Kufri Pukhraj' were larger. The lesions on cvs. 'Kufri Chipsona 1' and 'Kufri Jyoti' were smaller as compared to cv. 'Kufri Pukhraj'. However, in cv. 'Kufri Surya', there were no signs of infection. In the whole plant assay, the disease incidence and disease severity also followed the same trend and was highest in cvs. 'Kufri Pukhraj' followed by 'Kufri Chipsona 1', Kufri Jyoti' and Kufri Surya. The variation in the resistance level of these four cultivars could be explained as differences in the genetic backgrounds of the cultivars especially related to resistance genes, and also due to the differences in defense response.

Therefore, based on the results, the cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' were regarded as susceptible and moderately resistant to late blight respectively.

10.2 The effect of β-lactam antibiotics in improving shoot regeneration

The plant regeneration process from cell/tissue is primarily regulated by plant growth regulators (PGRs) and their concentration (Grafi 2004; Su et al. 2011; Khan et al. 2019). In addition to PGRs, many other factors have also been implicated, and recently, the role of β-lactam antibiotics has been recognised (Costa et al. 2000; Aggarwal et al. 2010; Haddadi et al. 2015; Guleria and Kumar 2021). Earlier, the effect of the β-lactam antibiotics on shoot organogenesis had been reported in some plant species (Nauerbey et al. 1997; Tereso et al. 2006). It was reported that the β-lactam antibiotics are metabolised to phenylacetic acid (PAA), which is known to possess auxin-like activity (Meng et al. 2014), which could impact the shoot organogenesis (Tambarussi et al. 2015). Moreover, different plant tissues/organs contain different levels of endogenous PGRs, and interaction of PAA with endogenous PGRs is reported to maintain the balance of auxin levels and could lead to inhibition or stimulation of shoot organogenesis in different genotypes (Souza et al. 2003; Tambarussi et al. 2015).

In the present investigation, cefotaxime significantly promoted shoot organogenesis in both the explants of both the cultivars, namely 'Kufri Pukhraj' and 'Kufri Chipsona 1'. Increased shoot organogenesis was observed at all concentrations (100-500 mg L^{-1}) of cefotaxime when compared with other antibiotics and control, but the best response was observed at 100 mg L^{-1} cefotaxime in both the cultivars and explants, which could result from metabolised product of cefotaxime as mentioned above. These results are in line with earlier reports indicating the positive effect of cefotaxime on shoot organogenesis in both monocot and dicot plants (Borrelli et al. 1992; Torregorsa et al. 2000; Yu et al. 2001; Aoshima 2005; Kaur et al. 2008; Mittal et al. 2009). It is important to note that cefotaxime at a concentration of 300 mg L^{-1} is optimal for the removal of *Agrobacterium* after co-cultivation (Tiwari et al. 2018; Kaur et al. 2020). There are also many reports of the inhibitory effect of cefotaxime on shoot organogenesis at higher concentrations (>300 mg L^{-1}) (Predieri et al. 1989; Yepes and Aldwinckle 1994; Meng et al. 2014). Moreover, the effect of antibiotics on shoot regeneration has been found to be concentration dependent, and the inhibitory effects of higher concentration are frequently observed (Nauerby et al. 1997; Naing et al. 2014), which is also dependent on the specific antibiotic, genotype and explant used (Tran and Sanan-Mishra 2015; Guleria and Kumar 2021; Varlamova et al. 2021)

Carbenicillin, a β-lactam antibiotic of the carboxypenicillin group, is also routinely used for the elimination of *Agrobacterium* from cultures after co-cultivation during plant transformation and also a wide range of endogenous bacteria within the plants in tissue culture (da silva and Fukai 2001; Liang et al. 2019). In this study, the beneficial effect of carbenicillin on shoot organogenesis was observed in both cultivars, especially at lower concentrations. A concentration of 300 mg L^{-1} carbenicillin was found to be beneficial for shoot organogenesis in cv. 'Kufri Pukhraj', whereas 100 mg L^{-1} was optimum in the case of cv. 'Kufri Chipsona 1'. A further increase in the concentration of carbenicillin inhibited shoot organogenesis. Such genotype-dependent effect of carbenicillin on plant morphogenesis has been reported earlier (Qin et al. 2011). The stimulatory effect of carbenicillin on shoot organogenesis has been reported in many other plant species such as *Carica papaya*, *Dianthus caryophyllus*, *Pinus taeda* and *Buddleia* species (Yu et al. 2001; Estopa et al. 2001; Wei et al. 2003; Dai and Castillo 2007). In contrast to this, inhibition of shoot organogenesis by carbenicillin has also been observed in some of the plant species (Yepes and Aldwinckle 1994; Nauerbey et al. 1997).

Like cefotaxime, cephalexin is also a β-lactam antibiotic of the cephalosporin group, which can also be used for the elimination of *Agrobacterium* from the culture. There are very limited reports on the effect of cephalexin on shoot organogenesis (Aggarwal et al. 2011; Kaur et al.

2020). In general, an inhibitory effect of cephalexin was observed on shoot organogenesis, and this was also found to be cultivar and explant dependent. The exact reason of inhibition is not yet clear, but the possible reason could be the increase in endogenous auxin concentration, as mentioned above. At 100 mg L^{-1}, a marginal improvement of shoot organogenesis was observed in cv. 'Kufri Pukhraj', but a further increase in concentration directly inhibited the shoot organogenesis. In contrast, 'Kufri Chipsona 1' explants failed to regenerate shoots at all the concentrations tested, indicating that the effect of cephalexin is genotype and explant dependent.

10.3 The effect of sucrose in improving shoot regeneration

Studies have focused on the role of factors which modulate in vitro growth and development along with many other processes in plants (Roychoudhury and Aftab 2021; Kaur et al. 2022; Bull and Michelmore 2022). Among these factors, sucrose has been considered important in regulating different morphogenetic events in cultures (Kumar et al. 1999, 2002; Deljou et al. 2007). Sucrose has been reported to act as a signalling molecule for many morphogenetic and metabolic processes (Cosic et al. 2021), as well as an osmotic agent, besides working as a carbon source (Kumar et al. 1999). Due to the lack of photosynthesis, cultures need exogenous sucrose (carbon source) in the medium to meet the energy demands (Jo et al. 2009). The shoot organogenesis requires either direct meristemoid formation through the callus phase (Lee and Huang 2013), which needs induction, and the role of sucrose, besides other molecules in induction, is now being established. It has been reported that shoot organogenesis can be significantly enhanced by manipulating the concentrations of exogenous sucrose in a medium (Kumar et al. 2002; Lee and Huang 2013). Therefore, in the present study, the effect of different concentrations of sucrose on shoot organogenesis from different explants of potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1', along with various biochemical parameters and gene expression were studied.

The present study revealed the significant effect of sucrose concentrations on shoot organogenesis ($P < 0.001$) and concentrations of 60-90 mM were found optimum for shoot organogenesis from both the explants of cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. Similar results emphasising the requirement of sucrose for shoot organogenesis were obtained earlier also (Kumar et al. 1999; Jo et al. 2009; Fatima and Anis 2011, 2012; Praveena and Veeresham 2014; Saema et al. 2015; Naz et al. 2015; Cosic et al. 2021). The observed inhibitory effect of higher concentrations of sucrose on shoot organogenesis could result from increased osmotic potential, as mentioned earlier (Jo et al. 2009; Kim et al. 2016; Cosic et al. 2021). In the present study, the lower response of shoot organogenesis was also observed at 30 mM sucrose, which could be due to the higher energy requirement. Therefore, in the present study 60-90 mM sucrose was found to be optimal for shoot organogenesis in both the cultivars of potato.

The interactions between sugars and plant growth regulators were observed to play an important role in shoot organogenesis (Gibson 2004; Lee and Huang 2013). Many researchers have reported that sucrose and its products, such as glucose, might act synergistically with plant growth regulators during morphogenesis (Kushwah and Laxmi 2014; Barbier et al. 2015; Sakr et al. 2018; Cosic et al. 2021). Many enzymes and sucrose transporters were reported to be involved in the uptake, transport, storage and metabolism of sucrose (Cosic et al. 2021). Among these, acid invertase, sucrose synthase (sucrose metabolism) and α- and β-amylases (starch metabolism) are the key enzymes (Ansari et al. 2013; Wu et al. 2014; Stein and Granot 2019). Therefore, in the present study, levels of soluble sugars and starch vis-a-vis the above enzymes were studied during shoot organogenesis. The maximum activity of acid invertase (cell wall-bound and cytosolic) and sucrose synthase was observed on day 14 on a medium containing 60 mM sucrose. Similar results were also observed in tobacco by Naidu and Kishor (1995). The increase in activity of these enzymes indicates the rapid conversion of sucrose into glucose and fructose. Although levels of total soluble sugars, reducing sugars and starch were higher on day 14 of culture on all media combinations, the maximum levels were observed on a medium containing 60 mM sucrose. It has been reported that glucose is the key influx molecule for respiration to produce energy (Li and Sheen 2016; Yetkin-Arik et al. 2019). Moreover, reducing sugars have been reported to interact with DNA and modulate the expression of various genes involved in the metabolism of cytokinin, perception and signalling (Kushwah and Laxmi 2017; Jiao et al. 2019; Cosic et al. 2021). Therefore, sucrose signalling and interaction with cytokinin might be key factors influencing shoot organogenesis. Further, a sharp increase in activities of sucrose synthase and cytosolic invertase was observed from 7 to 14 days of culture in a medium containing 60 mM sucrose, which then declined rapidly during subsequent periods. This sharp rise in the activities of these enzymes is possibly involved in the rapid utilisation of sucrose, reflected in the higher levels of soluble and reducing sugars during this stage of early shoot organogenesis.

It has been reported that organ initiation is an energy-requiring process; therefore, the accumulation and utilisation of sugars is critical during shoot organogenesis (Ross et al. 1973; Huang and Liu 2002; Blanc et al. 2002). In this study, maximum starch accumulation was also observed in cultures on a medium containing 60 mM sucrose during the early stages of shoot organogenesis (day 14). The exact role of the starch accumulation during this period of shoot initiation is not very clear; however, it can be suggested that it acts as an energy reservoir during shoot organogenesis (Carciofi et al. 2012; MacNeill et al. 2017). The starch accumulation during shoot differentiation has been reported by many researchers (Abe et al. 1996; Blanc et al. 2002). It may also be suggested here that rapid starch synthesis plays an essential role in maintaining the tissue's sugar homeostasis and osmotic potential (Thorpe et al. 1986; Carciofi et al. 2012). These results are in line with many earlier reports (Thorpe and Murashige 1968; Huang and Liu 2002; Sane et al. 2006; Shang et al. 2009; Carciofi et al. 2012). A sharp decrease in starch content was observed from day 14 to day 21, which could be due to a sharp rise in the activity of starch metabolising enzymes (α and β-amylase) during this period (Fig. 5d, e). This is another sign of starch utilisation during the early stages of shoot growth phase. These results corroborate the findings of many earlier studies (Naidu and Kishor 1995; Kumar et al. 2017; Huang and Liu 2002). It was noticeable that an uninterrupted supply of free sugar is essential for organ initiation and early growth. This supply is most likely provided by the hydrolysis of sucrose and starch into simpler sugars by the respective enzymes. A significant effect of sucrose concentration on the activity of sucrose synthase, invertase, α- and β-amylase was also observed ($P < 0.0001$).

Furthermore, it has also been reported that sucrose acts as a signalling molecule and plays an essential role in modulating the expression of sucrose-specific genes (Sakr et al. 2018). In the present studies, the data on sucrose utilisation vis-a-vis activities of related enzymes could be related to the expression of genes involved in sucrose and starch metabolism. The qRT-PCR analysis has shown that the expression levels of acid invertase and sucrose synthase were strongly enhanced on day 14 after culture, whereas the expression level of α and β-amylase increased during early shoot growth, i.e., on day 21 on a medium containing 60 mM sucrose. This expression pattern seems to be linked with various molecular processes involved in shoot organogenesis. Furthermore, at higher sucrose concentrations, the expression of these genes was lowest, which in turn may have inhibited shoot organogenesis. Similar results were also been reported earlier (Cosic et al. 2021). This is because, during high sucrose demand, the genes involved in the hydrolysis and transport of sucrose are upregulated (Verma et al. 2019). On the contrary, when sucrose is not required, genes involved in conversion of sucrose into storage are upregulated to maintain a balance (Rolland et al. 2006; Sakr et al. 2018).

10.4 Optimisation of *Agrobacterium*-mediated genetic transformation protocol

In addition to regeneration protocol, many factors involved in genetic transformation such as preculture period, bacterial density, infection time, co-cultivation period and acetosyringone concentration in the medium, influence the transformation efficiency. The transformation efficiency varies with the type of genotype used (Aggarwal et al. 2011; Girijashankar 2011), and optimising these factors for a particular genotype is important to undertake genetic transformation. The first step towards the *Agrobacterium* mediated genetic transformation is the selection of antibiotic marker to be used for screening of transgenic cells for shoot regeneration. The selection of antibiotic marker for the transformation experiment depends on the binary vector and the presence of antibiotic resistant gene on the T-DNA region. In the majority of the cases, the binary plasmid exploits the *nptII* gene as a selection marker, which confers resistance to the aminoglycoside group of antibiotics such as kanamycin, neomycin, etc. (Yenofsky et al. 1990). The optimisation of concentration before the genetic transformation is a crucial step for screening of transformed cells. In the present study, the 100 mg L^{-1} kanamycin concentration was selected for screening of transformed shoots (Fig. 8.1 and Fig. 8.2). These results are consistent with previous findings in *Carrizo citrange*, *Solanum tuberosum* and *Hevea brasiliensis*, where 100 mg L^{-1} kanamycin concentration was optimised to select the transformed shoots (Dutt et al. 2010; Kaur et al. 2020; Udayabhanu et al. 2022). The sensitivity of explants to particular kanamycin concentrations depends upon the genotype and cultivar. Many studies have optimised the kanamycin concentration at 30 mg L^{-1} (Veale et al. 2012), 50 mg L^{-1} (Nisha et al. 2003; Karishnan et al. 2008; Pandey et al. 2010; Aggarwal et al. 2011) and 60 mg L^{-1} (Ebrahimzadegan and Maroufi 2022; Polowick and Yan 2023) in different plant species. It has been reported that kanamycin induces chlorosis in explants by inhibiting protein synthesis in chloroplast and mitochondria (Kaur et al. 2020; Chen et al. 2020).

The preculture of explants on a cytokinin-auxin rich medium also improves the efficiency of transformation, which could be due to the acquation of competence by cells to promote infection as well as enter into desired path of regeneration (Xing et al. 2007; Aggarwal et al. 2011). The 2-day preculture period of explants on MS3 medium prior to infection resulted in the highest transient GUS expression of explants compared to explants that were directly infected (Table 8.1, Table 8.2). According to reports, preculture treatment induces dedifferentiation of cells and serves as a rejuvenating treatment for explants. The newly formed juvenile cells are more prone to *Agrobacterium* infection than differentiated old cells (Sangwan et al. 1992; Kuta and Tripathi 2015; Xing et al. 2007). In this study, however, an increase in the preculture period beyond 2 days decreased the transient GUS expression. This may have been caused by the activation of the plant’s defense system, which led to a reduction in transformation efficiency. In contrast, some studies have reported better transformation efficiency of explants when they are not precultured on a medium before infection (Banerjee et al. 2006; Millam 2006; Mollika et al. 2020; Bakhsh 2020; Decima et al. 2020).

The culture density of bacteria used to infect explants also affects the transient GUS expression. In the present study, explants infected with *Agrobacterium* suspension of 0.6 OD_{590} showed the highest GUS expression (Table 8.1, Table 8.2). These results are in line with the previous findings of Aggarwal et al. 2010; Aggarwal et al. 2011; Kaur et al. 2020, indicating that the growth of *A. tumefaciens* has a substantial impact on transformation efficiency. It has been reported that the lower growth of *Agrobacterium* is associated with its low ability to infect the explant, and higher growth of *Agrobacterium* leads to the death of leaf explants due to bacterial overgrowth (Maheshwari and Kovalchuk 2016). In contrast, bacterial densities of 0.2 to 0.3 (Chakravarty and Wang-Pruski 2010; Craze et al. 2018), 0.8 (Bakhsh 2020) and 1.0 (Borna et al. 2010) have also been reported by researchers for higher transformation efficiency.

The genetic transformation is also dependent upon infection time (Niu et al. 2000; Aggarwal et al. 2011; Bhatt et al. 2021). A 15 min of *A. tumefaciens* infection to explants resulted in the highest transient GUS expression (Table 8.1, Table 8.2). These results are in line with other reports highlighting the role of infection time on genetic transformation (Banerjee et al. 2006; Kaur et al. 2020). However, many researchers have reported higher transformation efficiency with 20 min (Bakhsh 2020), 30 min (Beaujean et al. 1998; Craze et al. 2018; Mollika et al. 2020), 40 min (Sarkar and Mustafa 2002), and 60 min (Borna et al. 2010) of infection time in different potato cultivars.

The optimum co-cultivation period is an additional step for the effective delivery of T-DNA into plant cell (Godwin et al. 1991; Zuker et al. 1999; Zambre et al. 2003; Tournier et al. 2003; Li et al. 2017). It has been observed that the addition of the phenolic compound called acetosyringone in co-cultivation medium acts as a signal for *Agrobacterium* for activation of the *vir* genes, stimulation, and production of the T-DNA thus affecting T-DNA delivery (Gelvin 2003; Lacroix and Citovsky 2019). A co-cultivation medium supplemented with 100 µM acetosyringone increased the transient GUS expression (Table 8.1, Table 8.2). The same concentration of acetosyringone has been optimised for *Solanum tuberosum* for better transformation efficiency (Bakhsh 2020; Kaur et al. 2020; Cordeiro et al. 2023). However, few studies have reported the use of 50 µM (Veale et al. 2012), 150 µM (Chakravarty and Wang-Pruski 2010) and 200 µM (Tran and Sanan-Mishra 2015; Mba'u et al. 2018) acetosyringone concentration that improved the transient GUS expression of explants.

Moreover, the 2 days co-cultivation period showed the highest transient GUS expression (Table 8.1, Table 8.2). However, increasing the co-cultivation period beyond 2 days resulted in bacterial overgrowth that inhibited normal growth, causing the leaf explants to turn brown and die. These findings are consistent with previous results in many other plants (Niu et al. 2000; Banerjee et al. 2006; Chakravarty and Wang-Pruski 2010; Aggarwal et al. 2011; Craze et al. 2018; Kaur et al. 2020; Li et al. 2022). However, many researchers have reported a co-cultivation period of 3 days (Beaujean et al. 1998; Borna et al. 2010; Bruce and Shoup Rupp 2019; Mollika et al. 2020). Therefore, the optimised *Agrobacterium* mediated genetic transformation protocol was used for the transfer of RNAi construct in selected potato cultivars.

10.5 Development of RNAi construct targeting *CesA* gene of *P. infestans*

The identification of the target gene for potential control of *P. infestans* through RNAi-based silencing is the most predominant factor. The RNAi-based silencing has been proven to be an effective tool when genes essential for *P. infestans* growth and infection process are selected (Jahan et al. 2015; Sanju et al. 2015; Thakur et al. 2015; Sanju et al. 2016). In the present study, two RNAi constructs specifically targeting the *CesA* genes of *P. infestans* were developed. *CesA* genes are reportedly involved in the synthesis of cellulose, which is a major component of *P. infestans* cell wall (Grenville-Briggs et al. 2008; Blum et al. 2010; Pang et al. 2020). Targeting the *CesA* gene of the *P. infestans* was, therefore considered. Selection of a fragment for RNAi construct specifically targeting the *CesA* gene of *P. infestans* and not the potato was also a major challenge. In literature, the differences in the N-terminal ends of *CesA* genes of *P. infestans* and potato were reported (Grenville-Briggs et al. 2008). Thus, ~200 bp fragments of *P. infestans CesA* genes that were non-homologous to potato *CesA* genes were identified for the development of RNAi constructs for silencing of the *CesA* gene of *P. infestans*. In a similar study of silencing the *CesA2* gene of *P. infestans,* researchers have reported a reduction in *P. infestans* DNA content over time on *CesA2* silenced transgenic plant leaves compared with the wild type (Jahan et al. 2015). In literature, the silencing of the cell wall-associated genes, such as chitin synthase (Cheng et al. 2015), glucan synthase gene (Yang et al. 2021), pectinase (Jahan et al. 2015), chitinase gene (Mamta et al. 2016; Adeyinka et al. 2023; Guo et al. 2023) have been proved effective against the various pathogens in many plant species. The HIGS approach involves the designing of the RNAi construct to express a self-complementary sequence homologous to the target gene in the form of hairpin RNA (hpRNA). Consequently, the selected fragments were amplified and cloned in sense orientation at one end of the intron and in antisense orientation to the other side of an intron in the hp-RNAi plasmid. Other researchers have adopted the similar strategy for developing the RNAi construct (Eschen-Lippold et al. 2012; Jahan et al. 2015; Sanju et al. 2015; Thakur et al. 2015; Sanju et al. 2016; Sun et al. 2016; Jiang et al. 2023). The successful integration of sense and antisense fragments by the specific amplifications and subsequent restriction digestion was confirmed. This is comparable to many studies confirming the cloning of gene fragments (Kaur et al. 2020; Ismail et al. 2020; Singh et al. 2020; Tetorya and Rajam 2021).

10.6 Development of transgenic lines containing RNAi constructs

The development of transgenic lines involved the expression of developed RNAi constructs in potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. In the present study, the *Agrobacterium* mediated genetic transformation method was used to develop transgenic lines. It offers numerous benefits, including low copy number transgene integration, transgene stability in the host genome, and efficient transformation (Dai et al. 2001; Travella et al. 2005; Ebrahimzadegan and Maroufi 2022). As discussed earlier, the method involves the optimisation of regeneration protocol and various factors involved in genetic transformation. For achieving genetic transformation, the explants were precultured for two days and infected with a bacterial density of *Agrobacterium* ($OD_{590} \approx 0.6$) for 15 min, and co-cultivation on acetosyringone supplemented medium (Fig. 8.3). The screening of transformed and untransformed explant tissue after the co-cultivation period is the most critical step in genetic transformation method. In the present study, the transformed explants were selected on 100 mg L^{-1} optimised kanamycin concentration. It was observed that on an MS3 medium containing 100 mg L^{-1} kanamycin, the untransformed sectors of explants turned colourless, and the transformed ones showed callus initiation. Subsequently, from the callus, the putatively transformed shoots regenerated on MS3 medium containing 100 mg L^{-1} kanamycin. The selection of the particular antibiotic for screening of transformed tissue is reported to depend on the choice of the plasmid and the antibiotic resistant gene present in the T-DNA region. Moreover, the growth of the transformed shoots is also reported to depend upon the concentration of the antibiotic. The same process of selection and regeneration of transformed shoots has been reported by many researchers (Banerjee et al. 2006; Tran and Sanan-Mishra 2015; Bakhsh 2020; Kaur et al. 2020). A total of five putatively transgenic shoots were regenerated expressing Pi*CesA3*-RNA construct I each for cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' whereas with Pi*CesA*2-RNA construct II, four transgenic shoots for cv. 'Kufri Pukhraj' and seven for 'Kufri Chipsona 1' were regenerated in the present study. The transformation efficiency is dependent on the cultivar and is also associated with low transformation rates. The transgenic shoots were maintained and multiplied on medium containing kanamycin antibiotics to completely rule out the chance of escaped shoots. The PCR amplification of *16s rRNA* gene from transgenic shoots was performed, which confirm the absence of bacteria (Fig. 8.8). The transgenic shoots were further confirmed for the presence of sense and antisense fragments (Fig. 8.5, 8.6) and *nptII* gene (Fig. 8.7). The similar approach has been employed by many researchers, where specific amplification confirmed the integration of transgene in many plants (Aggarwal et al. 2011; Mamta et al. 2016 Kaur et al. 2020; Singh et al. 2020; Tetorya et al. 2021) The phenotypical observation of transgenic plants revealed their similarity to control plants with no noticeable morphological changes with respect to growth and development. This is probably due to the selection of *CesA* gene fragments of *P. infestans* that did not have sequence homology with the *CesA* gene sequence of potato.

10.7 Evaluation of transgenic lines against *P. infestans*

Further, the transgenic lines were evaluated for disease related bioassays to determine the effect of Pi*CesA*-RNAi constructs on late blight resistance. The evaluation of disease resistance in transgenic lines is widely assessed using detached leaf and whole plant assays (Dufera 2014;

Kaur et al. 2020; Karki and Halterman 2021; Kaur et al. 2022). The detached leaf assay involved the inoculation of a drop of sporal suspension on a detached leaf placed in a moist chamber. This led to the development of visible necrotic lesions on leaves of transgenic and control plants, as also reported by other researchers during late blight disease development (Kitazawa and Tomiyama 1969; Vleeshouwers et al. 2000). The lesions observed on the leaves of transgenic plants were smaller as compared to control plants; showing the improved resistance to *P. infestans* in transgenic plants (Fig. 9.3, Fig. 9.5). Similar, results have been reported by other researchers, where transgenic plants developed smaller lesions than non-transgenic plants (Jo et al. 2014; Sun et al. 2016; Rajesh et al. 2016; Chowdhury et al. 2017; Kaur et al. 2020; Beliaev et al. 2021).

Further, the whole plant assay necessitates the spraying of sporal suspension on the entire plant. This involves the scoring of disease incidence and disease severity during disease development (Kaur et al. 2020). It was observed that both the factors (disease incidence and severity) were significantly lower in transgenic plants than in control plants (Table 9.1, Table 9.2). The rate of disease progression was higher in the control plants as compared to transgenic plants. Other researchers have also reported similar findings stating the higher tolerance of transgenic plants of potato against *Fusarium oxysporum, P. infestans, Alternaria solani* rather than control plants (Majeed et al. 2018; Chung et al. 2019; Kaur et al. 2020; Beliaev et al. 2021). The late blight resistance of transgenic lines expressing the Pi*CesA*-RNAi constructs explains the applicability of HIGS approach for achieving disease resistance. A similar approach has been used by other researchers for disease resistance against *P. infestans*, *Fusarium graminearum, Fusarium oxysporum, Magnaporthe oryzae, Bremia lactucae, Rhizoctonia solani, Puccinia striiformis, Chilo partellus, Helicoverpa armigera* in potato, tomato, tobacco, arabidopsis, lettuce, rice, wheat, maise and tall fescue (Govindarajulu et al. 2015; Jahan et al. 2015; Hu et al. 2015; Mamta et al. 2016; Zhou et al. 2016; Yang et al 2021; Adeyinka et al. 2023).

It can be assumed that the developed Pi*CesA3*-RNAi construct I and Pi*CesA2*-RNAi construct II have been expressed in plants, and the siRNAs have targeted the sequence specific degradation of the *CesA3* and *CesA2* gene of *P. infestans*. It has been reported that *CesA3* and *CesA2* genes get highly upregulated in comparison to other *CesA1 and CesA4* genes of *P. infestans* during cyst germination and appressoria formation. Therefore, silencing of these genes may have interfered in the formation of normal appressorium, which in turn has reduced the disease progression in transgenic lines. Moreover, different transgenic lines have showed different levels of resistance against *P. infestans*, and this may be due to the position effect and copy number of the transgene (Mamta and Rajam 2017). Further, the transgenic lines expressing Pi*CesA2*-RNAi construct II showed a higher resistance to late blight than the transgenic lines expressing Pi*CesA3*-RNAi construct I. This could be due to the efficacy and effectiveness of the selected *CesA* gene fragment in inducing the mRNA degradation of the target *CesA* gene of *P. infestans*. It was also observed that the resistance level in transgenic lines was also observed as cultivar specific in addition to Pi*CesA*-RNAi constructs. In transgenic lines of cv. 'Kufri Chipsona 1', higher resistance to late blight disease, was recorded other than the cv. Kufri Pukhraj. The variation in the resistance level of transgenic lines of cultivars could be due to the differences in the genetic backgrounds of both the cultivars especially related to resistance genes (Asakaviciute et al. 2009). Moreover, the pathogenicity of the prevalent *P. infestans* isolates also affects the disease resistance levels among the potato cultivars (Kirk et al. 2001; Andersen and Ospina-Giraldo 2011).

The antioxidant defense response of potato cultivars during infection was also reported to contribute to variation in disease tolerance (Kuzniak and Skłodowska 2005; Misra et al. 2008; Kaur et al. 2022). Therefore, the activity of superoxide dismutase, peroxidases, ascorbate peroxidase and catalase activity during disease development in infected and non-infected transgenic and control plants of cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'was studied. In the present study, there were no significant differences in the enzyme activities of non-infected transgenic and control plants. However, significant differences in the enzyme activities were evident between the infected transgenic and control plants. It has been reported that during infection acquired defense response gets activated, which results in the production of superoxide ($^{\bullet}O_2^-$) burst and contributes to a hypersensitive response (Tian et al. 2021). The $^{\bullet}O_2^-$ burst is regulated by superoxide dismutase by dismuting superoxide-free radicles into H_2O_2 (Del Rio et al. 2016; Wang et al. 2021). The higher superoxide dismutase activity of infected transgenic and control plants increases as compared to non-infected plants. The highest superoxide dismutase activity was observed in transgenic lines, and the lowest activity was observed in the control plants of cvs. 'Kufri Pukhraj' (Fig. 9.13) and 'Kufri Chipsona 1' (Fig. 9.17). The higher superoxide dismutase activity contributes to reduced superoxide accumulation, which lowers cell damage (Fortunato et al. 2015). These results are consistent with other reports in cotton, wheat, strawberry, tomato, potato and soybean (Kuzniak and

Skłodowska 2005; Ehsani-Moghaddam et al. 2006; Debona et al. 2012; Fortunato et al. 2015; Kaur et al. 2022).

The released H_2O_2 plays an important role in defense, strengthening of cell walls, plant development and signalling (Schmidt et al. 2016; Niu and Liao 2016). However, it has been reported that elevated levels of H_2O_2 are toxic to cells (Mittler 2002; Almagro et al. 2009). Therefore, the excess of H_2O_2 is detoxified by ROS scavengers such as peroxidases, ascorbate peroxidases, and catalase. In the present study, the activity of peroxidases (Fig. 9.14, Fig. 9.18) and ascorbate peroxidases (Fig. 9.15, Fig. 9.19) was higher on day 5 of infection, followed by a decrease on day 10 of infection studies. However, the antioxidant activities of transgenic lines were significantly high compared to control plants (Fig. 9.14, Fig. 9.18). These antioxidants may protect cells from the cytotoxicity of H_2O_2. A similar observation has been recorded by many other researchers (Sasaki et al. 2004; Lavania et al. 2006; Diaz-Vivancos et al. 2006; Debona et al. 2012; Kaur et al. 2022).

Moreover, the study has also found increased catalase activity in transgenic lines on day 5 of infection, followed by a decrease on day 10 (Fig. 9.12, Fig. 9.16). These findings are also consistent with previous studies in plum, mung bean and potato, where reduced catalase activity is linked to oxidative stress (Hernandez et al. 2001; Hameed et al. 2017; Kaur et al. 2022).

A significant variation in the superoxide dismutase, peroxidases, ascorbate peroxidases and catalase activities witnessed throughout the experimentation revealed their crucial role in providing late blight tolerance to the potato cultivars.

Chapter 11

Conclusion and future perspectives

Conclusion

Potato is the third most important food crop with high nutritional and economic value. It has been designated a food security crop to feed the world's population. Many efforts have been made to increase its production, yield, and nutrient value, but the crop's susceptibility to many diseases including late blight has impacted its production globally over the years. Effective management strategies have been developed, but the *P. infestans* rapid genome evolution is the primary concern; overcome all control strategies. In the past, the host-inducing gene silencing approach has been the most preferred and proven method to protect crops from various diseases. It involves the silencing of pathogen genes essential for growth and infection cycle. Therefore, in the present study *CesA* gene of *P. infestans*, necessary for the synthesis of cellulose (major cell wall component), is silenced through host-induced silencing approach. The work involved designing of two RNAi constructs containing the non-homologous fragment of *CesA* gene of *P. infestans* to that of potato followed by their incorporation into selected potato cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. The developed transgenic lines were then evaluated for their resistance against *P. infestans*.

Based on the work conducted in this thesis, the following conclusions are drawn:

- Among four Indian potato cvs. ('Kufri Pukhraj', 'Kufri Chipsona 1', 'Kufri Jyoti' and cv. 'Kufri Surya'), the cvs. 'Kufri Pukhraj' and Kufri Chipsona1' were identified as susceptible and moderately resistant to late blight respectively, and selected for further study.
- Addition of β-lactam antibiotics (100 mg L^{-1} cefotaxime) improved shoot regeneration in cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1'. Moreover, sucrose concentration in the range of 60-90 mM in the medium was also beneficial for shoot regeneration. Among the cultivars and explants, internodes of cv. 'Kufri Pukhraj' showed maximum shoot regeneration.
- Higher genetic transformation efficiency was achieved by preculturing of explants for two days and infected with bacterial density of *Agrobacterium* ($OD_{590} \approx 0.6$) for 15 min, and co-cultivation on acetosyringone supplemented medium, leading to maximum transient GUS expression.
- The expression of hairpin RNA specific to non-homologous regions of *P. infestans CesA* genes to that of potato in sense and antisense orientation in cvs. 'Kufri Pukhraj' and 'Kufri Chipsona 1' conferred resistance to late blight in transgenic lines.

- The transgenic lines developed using Pi*CesA2*-RNAi construct II showed higher late blight resistance than those developed using Pi*CesA3*-RNAi construct I.
- Among cultivars, the transgenic lines of cv. 'Kufri Chipsona 1' showed higher late blight resistance.
- The higher activity of antioxidant enzymes during infection corresponds to the higher tolerance level of transgenic lines to late blight.

Future scope of the work

The research shows the silencing of *CesA* genes of *P. infestans* reduces the disease progression in potato cultivars. The developed RNAi constructs can be incorporated into other potato cultivars or plants to address the issue of late blight disease. Combining multiple RNAi transgenes, preferably those that target single copies of the genes involved in the infection process, would likely result in broad-spectrum disease resistance. In addition, understanding the potential siRNA transport from potato to *P. infestans* will open up alternative revenues to control this important plant pathogens. Thus, host-induced gene silencing approach can be utilised for disease management in many other plant species.

Chapter 11

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Appendix I

1. Murashige and Skoog's medium (1962)

Components	Amount (mg L^{-1})
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
Micronutrients	
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2Fe-EDTA	30
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	3% w/v
Agar-Agar	0.8% w/v

2. CTAB buffer

Ingredient	Quantity
CTAB	2.0% w/v
EDTA	20 mM
Tris-HCL (pH 8.0)	100 mM
NaCl	1.4 M
β-mercaptoethanol	0.2 % v/v

Prepared by dissolving 20 g of CTAB in 600 mL of warm distilled water. 81.82g of NaCl, 40 mL of 0.5 M EDTA (pH 8.0) and 100 mL of 1 M Tris HCl (pH 8.0) were also added. Total volume was adjusted to 1L.

3. 10X TAE buffer

Ingredient	Quantity
Tris Base	48.5 G
Glacial Acetic acid	11.4 mL
0.5 M EDTA (pH 8.0)	20 mL

48.5 g of Tris Base was dissolved in 800 mL of distilled water.11.4 mL of glacial acetic acid and 20 mL of 0.5 M EDTA (pH 8.0) were added and the total volume was adjusted to 1 L with the help of distilled water.

4. TE buffer

Prepared by dissolving 10 mL of 1 M Tris HCL (pH 7.4) and 2 mL of 0.05 M EDTA (pH 8.0) and total volume was adjusted to 1 L with Distilled water.

5. Solution A (2 % (w/v) Na2CO$_3$ solution)
Prepared by adding 2 g of Na_2CO_3 in 100 mL 0.1N NaOH.

6. Solution B (0.5 % (w/v) $CuSO_4.5H_2O$)

Prepared by dissolving 1 g disodium tartarate and 0.5 g CuSO4.5H2O in 80 mL distilled water and final volume was made to 100 mL.

7. Solution C
Prepared by mixing the 50 mL of solution A and 1mL of solution B.

8. Solution D (1N Folin Reagent)

Prepared by diluting 10 mL folin ciocalteu phenol reagent with 10 mL of distilled water.