ROLE OF POLYPHENOLS IN *IN VITRO* RESPONSE OF EXPLANTS DURING *AGROBACTERIUM* MEDIATE GENETIC TRANSFORMATION

A THESIS

By

JYOTSNA DAYMA

Submitted to

CHAUDHARY SARWAN KUMAR
HIMACHAL PRADESH KRISHI VISHVAVIDYALAYA
PALAMPUR 176 062 (H.P.) INDIA

IN

Partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE
(AGRICULTURAL BIOTECHNOLOGY)

2009

UNIVERSITY LIBRARY
C.S.K.H.P. KRISHI VISHVAVIDYALAYA
PALAMPUR

Acc. No. 105062
Class No. 633.083 Book No. D27R
Author. Dayma, J.J. et al
Title Role of polyphenols in vitro response of genotypes for transfo

Borrower's No.	Due Date	Borrower's No	Due Date

KRISHI VISHVAVIDYALAYA
LIBRARY, PALAMPUR (H.P.)
Accession No. 105062
Date 12.11.09 Price
Source CSK HPKV, Palampur
Class No. Book No.
Checked by
633.083
D27R
13/11/09

AFFECTIONATELY

DEDICATED TO

MY

ALMIGHTY

Dr. Amita Bhattacharya
Scientist E-2

Division of Biotechnology
Institute of Himalayan Bioresource Technology (IHBT), Palampur-176062 (HP) INDIA

CERTIFICATE I

This is to certify that the thesis entitled **"Role of polyphenols in *in vitro* response of explants during *Agrobacterium* mediated genetic transformation"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the discipline of **Agricultural Biotechnology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Ms Jyotsna Dayma (A-2007-30-03)** daughter of **Smt. Gaytri Dayma** and **Shri Satya Narayan Dayma** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Amita Bhattacharya

(Amita Bhattacharya)
Chair Person
Advisory Committee

Place: Palampur
Dated: 10/9/, 2009

CERTIFICATE II

This is to certify that the thesis entitled **"Role of polyphenols in *in vitro* response of explant during *Agrobacterium*-mediated genetic transformation"** submitted by **Ms Jyotsna Dayma (A-2007-30-03)** daughter of **Smt. Gaytri Dayma** and **Shri Satya Narayan Dayma** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the discipline of **Agricultural Biotechnology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

(Dr. Amita Bhattacharya) 26/10/09
Chair Person,
Advisory Committee

External Examiner

(Dr. S.R. Thakur)
Member

26/10/2009
(Dr. Nageswer Singh)
Member

26-10-09
(Dr. B.D. Kalia)
Dean's nominee

Head of the Department 26.10.2009

26/11/09
Dean, Postgraduate Studies

ACKNOWLEDGEMENTS

The foremost thanks, with all humility to the almighty that directed me and blessed with courage and strength to go through this study successfully.

There is no limit of ecstasy in bringing out this piece of work in light which was carried out in the supervision of Dr. Amita Bhattacharya, whose benign and discreet vetting of my research problem, through supervision, encouragement, salubrious criticism, competent guidance and above all her most humanitarian behavior, benevolent patronage and crisp scientific temperament led me on to achieve my all destination successfully in spite of all my lapses. I am thankful to Dr. Nageswer Singh Department of Biochemistry and Dr. B. D. Kalia, Dean's Nominee, the member of my advisory committee for my valuable and expects guidance during the course of this investigation.

I am grateful to Dr. P.S. Ahuja, Director, Institute of Himalayan Bioresoruce Technology and Dr. O.P. Sharma Dean, College of Agriculture, for providing me the opportunity and necessary facilities to ventures into the fascinating world of biotechnology.

It is also a matter of great pleasure for me to express my sincere regards to Dr. S.R. Thakur, Head of the Department of Biotechnology, for helping me during my whole tenure of study.

I am equally grateful to faculty members of Department of Biotechnology, Late Dr. P. Plaha, Dr. T.R. Sharma, Dr. K.D. Sharma and Dr. Rajeev Rathour for their cordial cooperation extended towards the completion of this study.

I am immensely indebted to Dr. Ashu Gulati, Dr. Anil Sood, Dr. Madhu, Dr. Sinha, Scientists, Institute of Himalayan Bioresoruce Technology, for their valuable help, constructive criticism and inspiring guidance during the period of study.

I emphatically express my venerable thanks to the Department of Biotechnology Government of India, New Delhi for providing financial assistance in the form of scholarships.

Thanks to Dr. Pradeep K. Sharma, Dean, Postgraduate Studies and officers for providing academic financial and necessary facilities during the course of study.

I am highly thankful to Mr. Shekhavat ji for better availability of experimental materials and also Mr. Pabitro Gyian for excellent photography of my work.

I acknowledge with cordial thanks for the help and cooperation received from the office and lab staff, Walia sir, Mukesh sir and all the staff members and Raghubir bhaiya, Praveen sir, Sanjay sir, Sandeep, Surendar, Shushil and Tilak bhaiya.

I express my deep gratitude for the help love and support of my seniors and friends Devindar Kaur, Priyanka, Shveta, Prachi Sharma, Mahesh Duriseti, Jadav Murlidhar, Pratibha Mishra, Ruchi, Savita, Neha, and Pooja specially Manu Sharma, whose eternal affection along with direct and indirect help led me on to achieve my destination successfully.

Every effort is motivated by an ambition and all ambitions have an inspiration behind. I owe this inspiration to my elder brother Aneesh and sister-in law Manju for this encouragement and unwavering faith, without which this treatise would have remained an ambition only.

It is with personal touch of emotion that I seize this opportunity to express my heartfelt and affectionate gratitude to my Maa and Papa for their sempiternal encouragement over at the cost of their own comforts.

Last but not least, I must admit that all the errors in the thesis are mine.

Place: Palampur
Dated:10/9/2009

Jyotsna Dayma
(Jyotsna Dayma)

TABLE OF CONTENTS

ABBREVIATIONS USED

%	Percent
µg	Microgramme
µl	Microlitre
°C	Degree calcius
bp	Base pair
cm	centimeter
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
E	East
e.g.	exempli gratia (for example)
EDTA	Ethylene diamine tetra-acetic acid
et.al.	idest (that is)
g	Gram
gus	β- glucuronidase gene
HCl	Hydrochloric acid
L-glutamine	Laevo glutamine
LB	Luria Broth
MS	Murashige & Skoog's medium
OD	Optical density
ONPG	O-nitrophenol- β-D-galactoside
psi	Ponds square inch
rpm	Revolution per minute
hr	Hour(s)
M	Molar
min	Minute(s)
ml	Milliliter
mM	Millimolar
N	North
pH	Power of hydrogen ions
PVP	Polyvinyl pyrrolidone
SDS	Sodium dodecyl sulphate
sec	Second(s)
TAE	Tris-acetate EDTA
UV	Ultra-violet
v/v	Volume by volume
vir	Virulence
w/v	Weight by volume
YMB	Yeast Mannitol Broth

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Department of Agricultural Biotechnology
College of Agriculture,
CSK Himachal Pradesh Krishi Vishvavidyalaya
Palampur – 176 062

Title of the thesis	:	**"Role of polyphenols in *in vitro* response of explant during *Agrobacterium* mediated genetic transformation."**
Name of the student	:	**Jyotsna Dayma**
Admission No.	:	**A-2007-30-03**
Major Advisor	:	**Dr. Amita Bhattacharya**
Major discipline	:	**Agricultural Biotechnology**
Minor discipline	:	**Biochemistry**
Date of thesis submission	:	**September, 2009**
Total pages in thesis	:	**51**

ABSTRACT

Plant polyphenols are a versatile group of secondary metabolites that function as defense compounds in plants. The present study investigated and confirmed that high contents of polyphenols of several dicot plant species are responsible for the resistance to *Agrobacterium*-mediated-genetic-transformation. Leaf explants of high polyphenol containing plants viz. *Elaeocarpus sphericus, Podocarpus macrophyllus, Rosa* sp, *Podophyllum hexandrum, Malus domesticus* and five important cultivars of tea (i.e. TV1, T-78, U9, U10 and KJ) were transformed using the virulent strain GV 3101 of *Agrobacterium tumefaciens*. GUS expression was directly used to measure *Agrobacterium's* ability to transform these explants. Present study also correlated the effects of freshly extracted leaf polyphenols and PPO activities with *Agrobacterium* growth. The study also revealed that higher concentrations rather than the type of polyphenols posed a 'major hindrance' to *Agrobacterium* infection. However, the growth suppressing effects of leaf polyphenols was largely overcome by 2-amino glutarimide prepared by over autoclaving of L-glutamine. 2-amino glutarimide in the co-cultivation medium was successful in facilitating *Agrobacterium* infection of the leaves of all the studied plants, irrespective of their polyphenol contents. The gene fusion assays with *Lac* Z and *vir A, G* and *pinF* genes for this compound showed that it can function where *Agrobacterium's* own phenol detoxifying mechanism failed. Vegetative growth of *Agrobacterium* was crucial for attaining an optimal infecting density, where 5-oxoproline formed due to normal autoclaving served as a quorum signal. However, the study confirmed that 2-amino glutarimide is not a *vir* inducer by using. TLC and NMR techniques were applied to confirm the xenobiotic detoxification of polyphenols by 2-amino glutarimide and the actual mechanism for polyphenol quenching by 2-amino glutarimide was proposed. The mechanism was further proven by a 'colour based kit' that showed the quenching of toxic polyphenols.

Jdayma
10/09/09
Signature of student with date

Amita Bhattacharya
10/9/09
Signature of Major Advisor

Countersigned
11.9.2009
Head of Department

Chapter-1

1. INTRODUCTION

Genetically modified (GM) crops have a great potential for improving crop quality and productivity and also in meeting the ever increasing demands of man for food, clothes, medicine, environment etc. With man's persistent efforts at meeting the emerging challenges of the world, it is expected that by 2020, almost all crops would be modified using biotechnological technique (Verma, 2004). Although GM crops can be produced both by direct delivery and *Agrobacterium* methods, the latter is generally preferred over the former. Despite several advantages like lower degree of transgene re-arrangement, cost effectiveness and simplicity of usage (Hamilton *et al.,* 1997), the *Agrobacterium* method of gene transfer is highly constrained by its limited range of specific hosts (Potrykus 1990). Thus, while most dicots are susceptible to *Agrobacterium* and were successfully transformed, monocots remained largely resistant until new and innovative interventions were made in the last decade. It was only when this method of gene transfer was extended to newer crops related to other aspects of human needs like clothing, shelter, wood, environment etc. that even some dicot plants were reported to be highly resistant to *Agrobacterium* infection. The limited host range specificity and the differential ability of *Agrobacterium* to infect cultivars or genotypes even within a particular species was also reported (Hawes et al., 1989; Biao et al.). The major reasons for this resistance was attributed to plants' natural defense mechanisms, including 'surface characteristics' and 'wound response' (Kumar et al., 2004).

One common defense mechanism employed by almost all plants is polyphenol exudation and oxidation. It is by this mechanism that plants ward off most pathogen/pest attacks. Polyphenols generally does this by interfering with the physiology and biochemistry of invading microorganisms. Thus, recent reports from our laboratory hypothesized that *Agrobacterium* infection of even dicot explants was resisted mainly by the presence of toxic amounts of plant polyphenols (Kumar et al., 2003; Sandal et al., 2006). Plant polyphenols belong to the family of 'tannins' and are basically defense compounds (Haslam. 1988). On the other hand, *Agrobacterium* is a gram negative soil

phytopathogen belonging to the family Rhizobiaceae. Wild type *A. tumefaciens* and *A. rhizogenes*, the two most popular species of *Agrobacterium* actually cause neoplastic diseases (crown gall and hairy root, respectively) in plants. Thus, it is but natural for plants to produce polyphenols to resist *Agrobacterium* infection. Probably, the resistance created by polyphenols is so strong in some plants, that even the detoxifying mechanisms like the *virH* and *pinF* genes fail to function in *Agrobacterium*.

Almost all plants produce polyphenols, albeit at lesser or higher amounts. However, they are present in surprisingly high concentrations in plant parts that are exposed to maximum biotic and abiotic stresses. Thus, the wood, bark, leaves, fruits and seeds of plants with predominantly woody habit contain toxic concentrations of polyphenols (Kolodziej, et al., 2005). Such explants are generally unsuitable for *Agrobacterium* mediated transformation. More so, because polyphenols are highly susceptible to oxidation as they serve as substrate for the enzyme polyphenol oxidase (PPO) present in plant vacuoles. Polyphenol oxidation as promoted by distressed plant/explants results in the formation of a brown colored product that causes tissue necrosis. This oxidized product severely hampers *in vitro* plant regeneration and is also highly toxic to invading microorganisms, probably including *Agrobacterium*. This necessitates the evolution of innovative steps to overcome such problems.

In the recent past, Saini, 2007 from our laboratory had hypothesized that bactericidity created by extremely high levels of polyphenols could be overcome by quenching them with 2-amino glutarimide (formed by autoclaving L-glutamine). She also hypothesized that once the toxic polyphenol levels were reduced due to quenching by 2-amino glutarimide, *Agrobacterium* was free to infect the recalcitrant and resistant explants of tea cultivars. If this was true, then 2-amino glutarimide probably had the potential of bringing about successful gene transfer in all high polyphenol containing recalcitrant plant species. Therefore, it was necessary to confirm these hypotheses using explants from a number of high polyphenol containing recalcitrant plants.

In view of these probabilities and hypotheses, the objectives of the present thesis were as follows:

(i) To establish the relationship between polyphenols and *Agrobacterium*

(ii) To evolve innovative steps to overcome the recalcitrance of high polyphenol containing explants to *Agrobacterium* infection.

(iii) To ascertain the mechanism of action governing recalcitrance and genetic transformation of explants containing high contents of polyphenol.

Chapter-2

Review of Literature

2. REVIEW OF LITERATURE

Ever since Haberlandt first demonstrated how to culture single plant cells in 1902, explants or in other words cells, tissues and organs have been cultured *in vitro* for a variety of purposes. These range from mass multiplication of elites to production of genetically modified crop plants. The *in vitro* response of 'explants' is particularly, important in all areas of plant biotechnology. However, the desired explant response is often hampered by tissue browning and necrosis due to polyphenol exudation and oxidation. Consequently, the ultimate objective of plant regeneration, multiplication, conservation or improvement using biotechnological tools is often defeated. Thus, polyphenols, their production, exudation and oxidation deserved detailed reviewing in the present thesis.

2.1 Plant polyphenols

Polyphenols belong to the family of 'tannins' that constitute one of the most ubiquitous groups of all plant phenolics. Although they are universally present in the plant kingdom, their quantity may vary from plant to plant and explant to explant (Olhoft. 2004). Since basically these are defense compounds, their levels are particularly, high in plant parts that are exposed to maximum biotic and abiotic stresses. Thus, the wood, bark, leaves, fruits and seeds of plants with predominantly woody habit contain toxic concentrations of polyphenols (Kolodziej, *et al.*, 2005). These compounds exhibit a remarkably wide range of biological activities ranging from antimicrobial, anti-tumour and enzyme inhibiting properties to their ability to interact with pharmacological targets (Haslam, *et al.*, 1989; Huang *et al.*, 1992; Haslam, 1996). Thus, it is a small wonder that polyphenols have attracted considerable attention from scientists of various fields

2.1.1 Chemistry of polyphenols

Natural polyphenols may vary from simple molecules such as phenolics acids to highly polymerized compounds like the condensed tannins (Harborne, 1980). However, like all phenolic compounds, the basic structure of polyphenols comprises of an aromatic ring with one or more hydroxyl substituents. Based on a variety of simple polyphenolic

units derived from the secondary metabolites of the shikimate pathway (Dewick 1995), polyphenols have been divided into three major groups i.e., tannins, lignins and flavonoids. All the three groups have great importance in different fields.

The largest and best studied polyphenols are the flavonoids, which include several thousand compounds. Among these, the flavonols, flavones, catechins, flavanones, anthocynidins, and isoflavanoids are important (Jennifer *et al.*, 1995). On the other hand, the most abundant polyphenols or the condensed tannins are virtually found in all plants. Tannins comprise of up to 50% of leaf dry weight. They were once believed to function only as anti-herbivore and anti-pest compounds, but more and more ecologists now recognize them as important controllers of decomposition and nitrogen cycling processes (Tian *et al.*, 1992; Gamba *et al.*, 2005). With increasing concerns about global warming, a better understanding of the role of polyphenols as regulators of carbon cycling is gaining popularity.

2.1.2 Medicinal effects of polyphenols on humans

Polyphenols have a number of applications in the field of nutrition, health and medicine (Bravo, 1998; Chung *et al.*, 1998; Crozier *et al.*, 2000). The potential beneficial effects of polyphenols on human health are mostly because of their powerful 'free radical scavenging' as well as strong antioxidant activities (Haslam, 1998; Yokozawa, 1998; Pietta, 2000; Rice-Evans, 2001; Higdon, 2003). It is because of these same properties that polyphenols have generated considerable interests in the field of food and nutrition (Okuda 1992; Lin *et al.*, 1996; Bravo 1998;, Pietta *et al.*, 1998; Batchvarov *et al.*, 2001; Scalbert *et al.*, 2002). Presently, their anti-mutagenic and anti-carcinogenic (Stavric *et al.*, 1992; Lin *et al.*, 1996; Friedman 1997; Weisburger *et al.*, 1998) activities have generated new interests. Lin *et al* (1996) discovered that the green tea polyphenols are strong inhibitors of DNA synthesis in tumor cell and peroxyl-radical generation. Moskaug *et al* (2005) further showed that flavanoids increase the expression of γ-glutamylcysteine synthetase thereby, helping in cellular antioxidant defense mechanism as well as xenobiotic detoxification. However, Frei (2006) reported that flavanoids have little or no direct antioxidant value in the human body. They are poorly absorbed by human body and the ones that are absorbed are quickly metabolized and excreted. Rather,

Filippos *et al* (2007) concluded that human body see these flavanoids as xenobiotics and trigger their detoxification and elimination by activating specific enzymes (Bravo, 1998).The phase II enzymes that are involved in the process also help in eliminating a number of mutagens and carcinogens from the body. Several types of polyphenols (phenolic acids, hydrolysable tannins, and flavonoids) have been shown to have anticarcinogenic and anti-mutagenic effects. Polyphenols probably interfere in the steps leading to the development of malignant tumors by inactivating carcinogens, inhibiting the expression of mutant genes and enzymes involved in the activation of pro-carcinogens. However, some polyphenols have been reported to be mutagenic in microbial assays. These are known to act as co-carcinogens or promoters of skin cancer in the presence of other carcinogens (Chung *et al.*, 1998).

Other useful applications of polyphenols have been shown by Frankel *et al.*, (1993). Polyphenols can also inhibit LDL oxidation *in vitro.* Moreover, LDL from volunteers supplemented with red wine or red wine polyphenols showed reduced susceptibility to oxidation (Fuhrman *et al.*, 1995; Nigdikar *et al.*, 1998). Thus, it was concluded that polyphenols not only impart protection from LDL oxidation *in vivo* but also have significant consequences in atherosclerosis. These also protect DNA from oxidative damage and age-related development of some cancers (Halliwell, 1999). Flavonoids also have anti-thrombotic and anti-inflammatory effects (Gerritsen *et al.*, 1995; Muldoon and Kritchevsky, 1996).

In addition, polyphenols provide flavor, color and taste to edible plants/plant parts (Haslam *et al.*, 1979 and Billot 1990) and are also known as 'vitamin P'. Min and Peigen (1991) showed the 'vitamin P' effect of tea polyphenols wherein, the catabolism of catecholamines slowed down and capillaries were strengthened.

2.1.3 Polyphenols as anti-herbivore compounds

Generally, physical damage or wound in plants induce the deposition of brown colored phenolic acids which cross link to proteins, carbohydrates and other cell wall components. This decreases the palatability and digestibility of the tissues in which it gets deposited thereby, hindering the post-absorptive utilization of nutrients (Mole *et al*, 1990;

Tugwell *et al.*, 1992). The anti-nutritional value of tannins was reviewed by Chung *et al.* (1998) where even their carcinogenic nature was reported. However, later it was found that the carcinogenic activity of tannins was more due to the associated compounds rather than tannins themselves. Polyphenols were also confirmed to be anti-nutrient by animal nutritionists. Even most reviews concluded that the impact of phenolic activity on herbivores was usually negative.

2.1.4 Allelopathic effects of plant polyphenols

'Allelopathy' and allelopathic processes are suggested to exist between different or same plant species or individuals in the ecosystem. Allelopathy is the combination of processes involving the production of bioactive molecules, their modification, entry into the environment and finally their interaction with plants so as to compete for successful establishment. Hegazy *et al.* (2004) reported that the allelopathic effect of *Nymphaea lotus* rhizome in rice fields was a major cause of crop failure and reduction in grain yield. The degree of inhibition was dependent on the type of extract and its concentration. The phenolic fractions extracted from rhizomes exhibited highest inhibitory effects. Ishidori *et al.* (2005) reported the phytotoxic effects of 15 phenolic compounds of low molecular weights on the seed germination of *Cucumis sativum, Lepidium sativum* and *Sorghum bicolor.* Similarly, in 2008, Oueslati studied the role of phenolic acids in the autotoxicity of four barley varieties using radical growth bioassay. Inhibition of barley radical growth was also positively correlated with the total phenolics depending on the growth season. Furthermore, Shankar *et al.* (2009) studied the allelopathic effects of phenolics and terpenoids extracted from *G. arborea* on the germination of black gram and green gram and found that *G. arborea* extracts inhibited the germination, seedling growth and total protein content in both these crops.

2.1.5 Polyphenols influence *in vitro* response of plants

Polyphenols also influence plant growth regulation, cell differentiation and organogenesis (Mato *et al.*, 1988). There are two opinions regarding the effect of phenolics on plant growth and development (Lorenzo *et al.*, 2001). Thus, both positive and negative effects of phenolics on explant response have been reported. In tissue

culture studies, phenolics and their oxidized products inhibit *in vitro* proliferation (Arnaldos *et al.*, 2001). The major reason for this is tissue browning and blackening. On being damaged due to surface sterilization and explant processing, the phenolic compounds of the tissues come in contact with the vacuolar polyphenol oxidase enzymes and are readily oxidized by air. The oxidized phenolic compounds in turn inhibit enzyme activity, cause darkening of the culture medium and subsequent lethal browning of explants (Laukkanen *et al.*, 1999). Concentration of tissue phenolics is frequently affected by several internal and external factors (Zapprometov *et al.*, 1989). Light, nutrients, temperature, carbohydrate supplies all influence the phenolic composition of tissues (Lux-Endrich *et al.*, 2000; Kefeli *et al.*, 2003).

2.1.6 Plant polyphenols as anti-phytopathogenic compound

Anti-phytopathogenic property of polyphenolic compounds is well documented (Chung *et al.*, 1998; Suksamrarn, *et al.*, 2003; Musyimi *et al.*,2008). Thus, inhibitory roles of condensed and hydrolysable tannins on the digestive and others enzymes of fungi is not surprising (Goel *et al.*, 2005). In a soil survey also, the important role of plant polyphenols in the ecology and biology of nematophagous and entomopathogenic fungi was aptly demonstrated (Lopez 1997). The report on significant decrease in the fungal succession and colonization in the presence of polyphenols released from *Eucalyptus* and *Camellia japonica* leaves further supports this fact (Canhoto *et al.* 1999; Koide *et al.* 2005).

Plant polyphenols are toxic to phytopathogenic bacteria and have bactericidal activity (Capassor 1994; Capasso *et al.*, 1995; Karou *et al.*, 2005 and Hori *et al.*, 2006). Actually, the polyphenols interfere with the physiology and biochemistry of invading microorganisms. The antimicrobial activity of *Sempervivum* leaves against six of the seven selected micro organisms was reported by Abram *et al.*, (1999). *Hypericum* extracts contained 3.2% tannic compounds, 0.64% phenolic compounds and 2.4% organic acids and all of these had the potential to be used as preservatives (Tolkunova *et al.*, 2002). Green tea polyphenols had anti-microbial activity against *Staphlylococcus* and *Streptococcus* mutants (An-Bougjeun, 2004).

Anitviral activity of blackberry polyphenols against *Pseudomonas aeruginosa* bacteriophage was also demonstrated by Corao *et al.*, (2002).

2.1.7 Polyphenols as chemo-attractant compounds

Plant polyphenols do not always act as antimicrobial compounds. The entire diversity of plant phenolic compounds can also serve as a rich source of developmental signals or chemoattractants in the establishment of specific symbiotic interactions between various microbes and plants (Parke *et al.*, 1985). For example, the root cells of many leguminous plants secrete flavonoid compounds which function as a signal molecule for the induction of nodulation genes and initiation of the legume-rhizobia symbiosis (Peters *et al.*, 1986; Redmond *et al.*, 1986; Zaat *et al.*, 1987; Shaw *et al.*, 2006). On the other hand, plant flavonoids and monocyclic phenolic compounds like acetosyringone, hydroxyl- acetosyringone (Bolton *et al.*, 1996; Song *et al.*, 1990; Goodwin *et al.*, 1991) trigger the expression of virulence genes (*vir*A and *vir*G) in the plasmids of *Agrobacterium* (Stachel *et al.*, 1986; Lee *et al.*, 1995). While plant families highly susceptible to *Agrobacterium* are typical polyphenol producers and accumulators, families that are insensitive to *Agrobacterium* do not accumulate polyphenols (Cleene, 1986). In 1987, Parke *et al.*, studied a number of monophenolic compounds and found that acetosyringone, catechol, gallate, β-resorcylate and protocatechuate are all chemoattractants for the *Agrobacterium tumefaciens,* strain A348. In 1988, Ashby *et al.*, analyzed twelve phenolic compounds with related structures for their ability to induce virulence genes of *A. tumefaciens* C58C. They divided the phenolic compounds into three groups: compounds that act as strong *vir* inducers and chemoattractants, those that were weak *vir* inducers and also weak chemoattractants and finally *vir* noninducer and nonattractants.

In 1995, Joubert *et al.*, established the essentiality of a phenolic function for virulence induction by testing the corresponding ß-glucosides and glucosyl esters from acids. They found that these glucosides led to a decreased toxicity to the bacteria. While the presence of an fl-glucosylated phenol function led to the total loss of *vir* induction, the corresponding esters, particularly the glucosyl sysingate ester were good *vir* inducers. Since then, synthesis of a number of phenolics inducers was initiated. Dye *et al.* 1997

synthesized three phenol amides derived from syringic acid and found that introduction of an amide group in syringic acid strongly enhanced the *vir* gene inducing activity. Particularly, 4-(3,5-dimethoxy)-4-hydroxy-phenyl-but-3- en-2-one was very effective in inducing higher virulence by about 1.5-2 times than acetosyringone (AS). When many derivatives of acetosyringone were used for *Agrobacterium*-mediated transformation, those with two methoxy groups and an acetyl function were more effective. Several alkylsyringamides were also identified as powerful inducers of *vir* genes where ethylsyringamide was stronger than syringic acid (Berthelot 1998).

2.2 Biotechnological implications of plant-*Agrobacterium* interactions

Plant polyphenols influence the success of plant biotechnological applications including *Agrobacterium* mediated transformation in hitherto known and unknown ways. Of these, interactions as to how the plants and *Agrobacteria* resist each other are important. Each of the unique defense mechanisms that are used by the plants and *Agrobacteria* have therefore, been reviewed in the present study. Such interactions are highly complex involving constant exchange of chemical signals between the plants and microorganisms leading to monitoring of events and responses important to these interactions (Petra and Verma 1990). Weakening of defense in either of the partners finally result in susceptibility or resistance of plants to *Agrobacterium* infection.

2.2.1 *Agrobacterium* mediated genetic transformation

Agrobacterium is a gram negative soil-borne plant pathogen of family Rhizobiacae. It elicits neoplastic growths called as crown gall tumor or hairy roots that affect most dicotyledonous plants. Although plants represent the natural hosts for *Agrobacterium*, this microorganism can also genetically transform a wide range of other eukaryotic species ranging from yeast (Bundock *et al.*, 1995; Piers *et al.*, 1996; Sawasaki *et al.*, 1998) to mushrooms (de Groot *et al.*, 1998; Chen *et al.*, 2000), filamentous fungi (de Groot *et al.*, 1998; Gouka *et al.*, 1999), phytopathogenic fungi (Rho *et al.*, 2001; Rolland *et al.*, 2003) and even human cells (Kunik *et al.*, 2001). Most functions for *Agrobacterium*-host cell interaction and DNA transfer are encoded by a large (200-kb) tumor-inducing (Ti) plasmid that resides in the bacterial cell. Of the different regions of

the Ti-plasmid, the *vir* (virulence) region and the T-DNA delimited by the T-DNA borders are the two most important genetic components (Chilton *et al.*, 1977; Citovsky *et al.*, 1992; Zupan *et al.*, 2000; Gelvin, 2000; 2003). These borders comprise of two 25-bp imperfect direct repeats at its ends (Sheng and Citovsky, 1996). The *vir* region comprises of seven major loci, *virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*. These encode for most of the protein machinery (Vir proteins) required for the transport of T-DNA. Only after the induction of *vir* genes, the T-DNA borders are nicked by the bacterial VirD2 endonuclease (Wang *et al.*, 1987). As a result transferable single-stranded (ss) copy of the bottom strand of the T-DNA designated as the T strand is generated (Stachel *et al.*, 1986). Interestingly, the T strand does not travel alone but is directly associated with two *Agrobacterium* proteins, VirD2 and VirE2 that forms a transport or 'T' complex (Zupan and Zambryski, 1997). In the T-complex, one molecule of VirD2 is covalently attached to the 5' end of the T strand, whereas VirE2, a ssDNA binding protein protects the T strand from plant nucleases during their plant intercellular movement (Zupan and Zambryski, 1997; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). On entering the plant cell, the T-DNA is imported into the nucleus by the help of plant as well as *Agrobacterium* nucleolar localization signals (NLS). Integration of T-DNA finally takes place through illegitimate recombination (Tinland, *et al*, 1995). As a result, *Agrobacterium* takes over the plant machinery, produces tumorous growth on plants and uses the host amino acids and sugars to produce opines as important sources of nitrogen and energy.

Opines are low molecular weight compounds and their biosynthesis is catalyzed by specific enzymes encoded by genes contained in the T-DNA of the Ti-plasmid.

Based on the type of opines produced and catabolized, *Agrobacteria* are classified into different strains. The type of opine synthesised or catabolized by the bacteria also determines the host and strain specificity of *Agrobacterium* e.g. The nopaline type of opine produced by *Agrobacterium tumefaciens* C58 in tumors formed on cherry, blackberry, grape and plum (Escobar *et al.*, 2003). Although initially, only a few 30 opines were identified, newer and newer opines are continuously being added to the existing list. Some examples of these are the heliopine produced by octopine type strains A6, A136, E9, A652 and 1590-1 and the vitopines of the grapevine strains S4 and T2.

These are identical to the synthetic N^2-(1′*R*-carboxyethyl) glutamine (Chilton *et al.*, 2001). Tumors produced by strains S4 and T2 do not contain octopine or lysopine, but they do contain heliopine and the new opine ridéopine identified as *N*-(4′-aminobutyl)-glutamic acid.

Catabolism or utilization of opines is strictly coordinated with conjugation of *Agrobacterium*. Thus, *Agrobacterium* always ensures that genes encoding opine biosynthesis should match the genes that catabolise them.

Although only the wild-type T-DNA carries *onc* and *op* genes, any DNA placed between the T-DNA borders are also transferred to the host plant (Zambryski, 1992; Sheng and Citovsky, 1996; Gelvin, 2000; Tzfira *et al.*, 2000).

Binding of *Agrobacterium* to a plant cell (up to 200/cells can attach) and transfer of T-DNA to the host genome (multiple T-DNA can be transferred) are two crucial steps for *Agrobacterium tumefaciens* mediated genetic transformation in plants (Wei *et al.*, 2000). Actually these two steps are the key to the variations observed in the range of specific hosts.

2.2.2 Plants resist *Agrobacterium* by producing inhibitory polyphenols

The efficiency of *Agrobacterium* mediated genetic transformation depends not only on the successful recognition and colonization of plant cells by the *Agrobacterium,* but also on the response of the plant cells to the process of infection (Zambryski, 1988; Binns, 1990). Plant cells generally respond to invading pathogens by first recognizing and then activating the defense signal transduction. This in turn leads to the hypersensitive necrotic responses (Lamb *et al.*, 1989; Mehdy, 1994; Dangl *et al.*, 1996; Hammond-Kosack and Jones, 1996; Blumwald *et al.*, 1998; Somssich and Hahlbrock, 1998; Richter and Ronald, 2000). Generally, hypersensitive reaction (HR) is characterized by a rapid, localized cell death around the infection site and the accumulation of antimicrobial agents (Hammond-Kosack and Jones, 1996; Richter and Ronald, 2000). It was earlier reported that even though a number of bacterial proteins are introduced into the host cell, *Agrobacterium* does not induce the hypersensitive response in target plants (Robinette and Matthysse, 1990). In reality however, the process of

Agrobacterium-mediated T-DNA transfer is associated with a high degree of tissue necrosis and poor survival rate of target plants (Pu and Goodman, 1992; Deng *et al.*, 1995; Perl *et al.*, 1996; Mercuri *et al.*, 2000; Chakrabarty *et al.*, 2002; Das *et al.*, 2002). Thus, the possibility of plant's hypersensitive reaction to *Agrobacterium* infection was suggested. Furthermore, Ditt *et al.*, 2001, demonstrated that plants can modulate their gene expression in response to *Agrobacterium* infection, and the latter can actually trigger the plant defense machinery.

Production of plant polyphenols in response to pathogen infection has already been reviewed above. Since *Agrobacterium* is nothing but a soil phytopathogen, plants exude high concentrations of polyphenols, and at times, these concentrations are either bacteriostatic or bactericidal (Sheng and Citovsky, 1996; Sandal *et al*, 2006). Many plants with high contents of polyphenols are thus, resistant to *Agrobacterium tumefaciens* infection (Fukai 1991; Fortin *et al.*, 1992; Biao *et al.*, 1998; Sandal *et al*, 2007; Ozyigit *et al.*, 2007). Of these, the tea polyphenols are noteworthy. Particularly, the catechols and catechins are maximally inhibitory (Capassor 1994; Sandal *et al.*, 2002). High contents of polyphenols do bring about major reduction in the efficiency of the *Agrobacterium* mediated transformations (Biao *et al.*, 1998; Sandal *et al.*, 2006).

2.2.3 *Agrobacterium* resistance mechanism against plant polyphenols

Defense mechanisms that are common to most bacteria are also employed by *Agrobacterium* to survive and break down the defenses of the host plant. Two mechanisms that have recently emerged as crucial for the success of *Agrobacterium* infection of resistant hosts are: (i) xenobiotic detoxification and (ii) quorum signaling.

2.2.3.1 Xenobiotic detoxification

Xenobiotics are compounds foreign to an organism's normal biochemistry. Their detoxification comprise of a set of metabolic pathways that modify the chemical structure of the 'xenobiotic in question'. The detoxification pathways are actually biotransformation processes are present in all major groups of organisms including bacteria (Tschech *et al.*, 1987; Coleman, 1997; Ashraf, *et al.*, 2001 Rousseaux, *et al.*, 2001). They even bring about detoxification of poisonous compounds. Reactions in these

pathways are of significant interests for drug metabolism particularly, in the understanding of multi-drug resistance in infectious diseases and cancer chemotherapy. When some drugs serve as substrates or inhibitors of enzymes involved in xenobiotic metabolism, they commonly result in hazardous drug interactions. These pathways are also important in environmental sciences.

Since the metabolism of xenobiotics is basically an enzyme driven biotransformation process, it is but natural that organisms would have specific genes to drive these processes. It is well established that many bacteria can catabolize phenolics compounds including some that serve as *vir* gene inducers (Delneri *et al.*, 1995; Harwood and Parales, 1996). Such genes are also expected to be present in *Agrobacterium.* Thus, some or possibly all Ti plasmids of *Agrobacterium tumefaciens* encode *virH* (originally known as *pinF*). Expression of *virH* is induced by a family of phenolic compounds that also induce all other operons within the *vir* regulon. The *virH* gene encodes two proteins vir H1 and H2 that are responsible for detoxifying the xenobiotic phenolic compounds (Kalogeraki et la., 1999). Both these proteins resemble each other as well as a large number of enzymes of the cytochrome P450 family (Kanemoto *et al.*, 1989). Some *A. tumefaciens* have also been shown to break down several phenolic compounds including para-hydroxybenzoate and protocatechuate via its chromosomally encoded ß-ketoadipate pathway (Parke 1995; 1997). In another study, Morris and Morris, (1990) and Castle *et al.*, (1992) also found that some strains converted the phenylpropanoid glucosides called coniferin to an active *vir* gene inducer via the ß-glucosidase gene.

Recently, both virH1 and virH2 proteins have been found to metabolize some or all phenolic compounds. Thus, when Brencic *et al.*, 2004 tested 16 vir-inducing phenolics, they found that each of them was metabolized by VirH2-dependent O-demethylation activity. They also found that VirH2 protein converts ferulic acid, a potent *vir* gene inducer, to the non-inducer caffeate by O-demethylation of a methoxyl group. Further, on testing both wild-type and isogenic strains lacking virH1 and virH2, only the wild-type strains converted virtually all ferulic acid to caffeic acid. Moreover, VirH2 was essential for the O-demethylation reaction. Although ferulic acid was far more toxic to the wild-type strain than caffeic acid, the wild-type strain was more resistant to ferulic

acid than the *virH* mutant. However, O-demethylation rates differed enormously with substrates. The strongest *vir* gene inducers such as acetosyringone were demethylated extremely slowly. Compounds containing two methoxyl groups were demethylated at both positions. In general, phenolic inducers were more toxic than their demethylated counterparts. In another study, Kalogeraki (2002) found that virtually all phenolic inducers inhibited growth in the sensitive virH2 mutant as compared to the wild type. This indicated that VirH2 detoxifies as well as mineralizes many phenolics.

2.2.3.2 Quorum sensing

The other unique defense mechanism employed by most gram negative bacteria including *Agrobacterium* is 'quorum sensing'. Quorum sensing refers to the 'bacterial cross-talk' or bacterial cell to cell communication process for monitoring population density under stressful environmental conditions (Brencic *et al.* 2004). Cross talk is brought about through the secretion and sensing of small signaling molecules or 'quoromones'. Accumulation of quoromones in the growth medium help the bacteria to grow to a desired density and thereby, perform a particular function like bioluminescence, infection etc. (Zhang *et al.*, 2002). During unfavorable or stressful conditions, bacteria fail to do this function (for example, infecting hosts) unless the desired density is attained.

In *Agrobacterium tumefaciens*, TraR, the quorum sensing transcriptional regulator and its inducing ligand 3-oxo-octanoyl-L-homoserine lactone actually control the conjugal transfer of the tumor-inducing Ti plasmid. This occurs when the *tra* gene is activated in response to pheromones (Piper *et al.*1993; Fuqua *et al.*, 1994; Farrand, 1998; Winans *et al.*, 1999).

Acyl HSLs or 3-oxo-octanoyl-L-homoserine lactone are diffusible pheromones produced by a variety of gram-negative bacteria. The release and subsequent detection of acyl HSLs enable the bacteria to monitor their own population density (Fuqua *et al.*, 1998, Fuqua *et al.*, 1994). At low population densities, acyl HSLs are rapidly dissipated by diffusion into the surrounding environment. The contribution of each signal-producing cell is additive. Thus, with increasing population density, the relative acyl HSL

concentration is elevated until an inducing concentration is reached and a programmed set of adaptive responses are stimulated. Acyl HSLs synthesis in most cases is mediated by TraI proteins that resemble the LuxI protein of *Vibrio fischeri*. However, unlike most LuxR-type proteins, the activity of TraR is directly influenced by several other regulatory proteins. The TraI protein, encoded on the Ti plasmid, directs the synthesis of 3-oxo-C8-HSL (Fuqua *et al*., 1999, Fuqua *et al*., 1994).

TraR and the receptors of quorum or Acyl HSLs signals are Acyl-HSL dependent transcriptional regulators that resemble the LuxR protein of *V. fischeri*. TraR is also considered as the primary virulence factor responsible for the crown gall disease of plants. This regulatory system enables *A. tumefaciens* to express its conjugal transfer regulon preferentially at high population densities only. Genetic and biochemical evidences have shown that interaction of TraR with 3-oxo-C8-HSL results in the formation of stable TraR dimers. These in turn bind with the *tra* boxes and activate the transcription process (Zhu *et al*, 1999; Qin *et al*, 2000).

TraR activity is antagonized by TraM, a second Ti plasmid-encoded protein. Both TraM and TraR are thought to form an anti-activation complex that prevents TraR from recognizing its target DNA binding sites. The inhibition of the TraR protein activity by TraM is an absolute requirement for the normal operation of the *A. tumefaciens* quorum sensor.

In conclusion it can be stated that at present, a detailed understanding of only these two defense mechanisms i.e. xenobiotic detoxification and quorum sensing are available. However, there may be many more mechanisms governing plant-*Agrobacterium* interactions that are yet to be unraveled. An in-depth knowledge of overall defense mechanisms would usher the genetic transformation of various hitherto recalcitrant crops. Indeed the world would become substantially as well as sustainably capable of meeting the emerging demands of human beings.

Chapter-3

Materials and Methods

3. MATERIALS AND METHODS

The present study was carried out at Division of Biotechnology , Institute of Himalayan Bioresource technology (CSIR) Palampur in collaboration with the Department of Biotechnology, College of Agriculture, CSKHPKV, Palampur.

3.1 Plant materials

The role of polyphenols on '*in vitro* response of explants during *Agrobacterium* mediated transformation' was studied. Plant species known to contain high contents of polyphenols were selected. Thus, *Elaeocarpus sphericus* (rudraksh), *Podocarpus macrophyllus* (podocarpus, yew-pine, Japanese yew), *Podophyllum hexandrum* (Indian may apple), *Rosa* sp. (wild rose), growing in the main campus of Institute of Himalayan Bioresource Technology (IHBT), Palampur (1290m amsl, 32°N and 76°E), India were marked for experimental purposes. Along with these, five cultivars of *Camellia sinensis* (tea) i.e., T-78, TV-1, UPASI-9, UPASI-10 and Kangra Jat growing in the Tea Experimental Farm, IHBT were also tagged.

Randomly collected, newly emerged, fresh, tender leaves from all these plants were used as explants in the present study. The leaves of *Malus domesticus* (apple) root stock MM106 and B9 served as control for all the experiments.

3.2 Sterilization of explants

All the leaves were first washed with aqueous solution of the detergent, Teepol (v/v) using a sable hair brush and then rinsed 4-5 times in distilled water until Teepol was removed. Based on earlier findings, these were treated with Bavistin (0.2%; w/v) and streptomycin (0.02%; w/v) for 15 and 30 minutes and washed again with distilled water. Finally, the explants were surface sterilized using $HgCl_2$ (0.04%; w/v) for 2 minutes followed by half a minute treatment with 70% ethanol (v/v) under the laminar air flow hood. All traces of mercuric chloride and ethanol were finally removed by rinsing several times with autoclaved distilled water. Observations were recorded on browning, necrosis and freshness of the leaves.

3.3 *Agrobacterium tumefaciens* mediated transformation

3.3.1 Strain and plasmid

A disarmed *Agrobacterium tumefaciens* strain GV3101 carrying pBI121, a derivative of the binary vector pBin19 (Jefferson, 1987) was used. It harbours a *gus* reporter gene driven by CaMV 35S promoter and NOS terminator and (ii) a selection marker gene *nptII* driven by NOS promoter and terminator. The T-DNA region of the pBI121 based plasmid used in the present study is shown in **(Fig. 1).**

For routine experiments a loopful of culture was taken from a glycerol stock and streaked on 1.5% agar solidified Yeast Mannitol Broth (YMB) **(Appendix-1.3.1)** in 9 cm Petri dishes. This was incubated for 48 hrs in dark at 28°C after which a single cell colony was picked and inoculated into 20 ml of liquid YMB containing the selection antibiotics, 50 µg/ml kanamycin and 25 µg/ml rifampicin (**Appendix-1.5**). This was incubated as shake culture at 150-200 rpm and 28±1°C in dark for 16-18 hrs. This freshly grown culture served as the 'starter culture'.

3.3.2 Transformation process

The leaf explants, surface sterilized as described in **Section 3.2** were subjected to the 'process of transformation' using fresh overnight grown starter culture of GV3101 strain of *A. tumefaciens*. The starter culture obtained as above was further grown in 100 ml liquid YMB until an OD A_{600} nm = 0.6 was obtained (this OD was equivalent to a cell density of 1 $X10^9$ cells/ml). The bacterial cells were pelleted by centrifugation at 6000 rpm and 25°C for 20 minutes. The supernatant containing the bacterial cell debris and antibiotics was discarded, whereas, the pellet was retained by suspending it in fresh YMB. The optical density (absorbance) of the suspension was measured at 600 nm using a spectrophotometer (ELCOS SL 150). The cell density was finally adjusted to $1X10^9$ cells/ml by diluting it as below:

a X OD obtained X 1 X 10^9 cells/ml x **3** = 1 X 10^9 cells/ml x **b** ml

Where,

a = amount of bacterial suspension to be taken from the overnight grown bacterial culture in order to obtain an optical cell density of 1 X 10^9 cells/ml

3= correction factor

b= total volume of culture required for genetic transformation

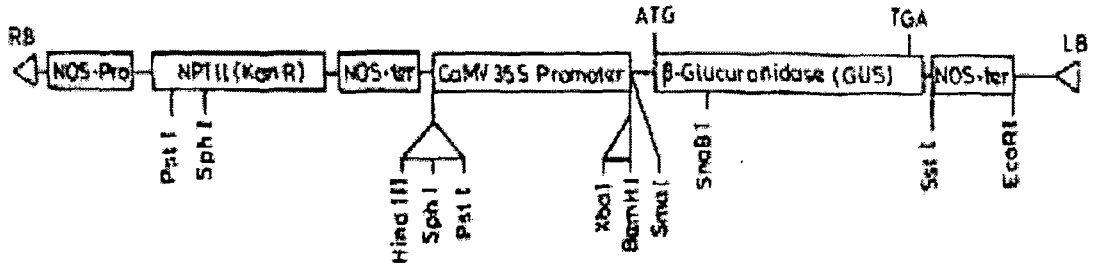


Fig. 1 Construct map of binary vector pBI121

The leaf explants surface sterilized for 15 min were immersed in the bacterial culture diluted as above. After 15 minutes of immersion, the leaf explants were blotted on sterile Whatman No.1 filter paper to remove excess bacteria from the surface. These were then co-cultivated on 0.8% agar solidified hormone free basal Murashige and Skoog's MS medium (MS0) at 25°C for 1, 2, 3, 5, 10, 15 and 20 days in dark. The leaves of apple rootstock B9 and MM106 were used as positive control, whereas, MSO without *Agrobacterium* was used as negative control. The experiment was repeated thrice, and three replicates with three explants each were taken per treatment. Observations were recorded on percent explants showing *Agrobacterium* growth after different days of co-cultivation.

The hormone free basal MS medium (MSO) was prepared as described in **(Appendix-1.3.3).** The pH of the medium was adjusted to 5.6, prior to the addition of agar. The agar containing MS medium was then autoclaved at 121°C and 15 psi for 20 minutes.

3.4 GUS expression

Histo-chemical GUS (β-glucuronidase) assay was carried out following the method of Jefferson *et al.,* (1987). After 1, 2, 3, 5, 10, 15 and 20 days of co-cultivation, leaf explants were randomly selected and immersed in GUS (5-bromo-4-chloro-3-

indolyl-ß-D glucuronide) assay buffer (**Appendix-1.4.1**) contained in 3 cm petri plates (Hi-media India). The leaves were vacuum infiltrated in a vacuum desiccator unit for 15 minutes and incubated overnight at 37°C in dark. After incubation, the explants were bleached in 70% ethanol until the green colored chlorophyll was removed. GUS expression per leaf explant was scored as blue spots and/or sectors and observations were photographed using a Nikon camera.

3.5 Extraction and estimation of total polyphenol in the studied plant species

The inhibitory effect of leaf polyphenols on *Agrobacterium* infection was studied. The method of Swain and Hillis (1959) was used to determine the total phenol contents in the leaves of each of the plant species **(Section 3.1).**

1. 1 g of leaf tissue was homogenized in 0.3N HCl in absolute methanol (2:1) and centrifuged at 10,000 rpm for 20 minutes at 5°C.
2. The supernatant was collected, and the residue was centrifuged again after the addition of 0.3N HCl as in step 1.
3. The supernatants were pooled, evaporated to dryness (50°C), and eluted in 5 ml distilled water.
4. 0.5 ml Folin's Phenol reagent was added to 3 ml sample (0.1 ml elute and 2.9 ml distilled water), and vortexed for 3 minutes followed by the addition of 1 ml of 35% sodium carbonate.
5. The reaction mixture was incubated in dark for 1 hr and the optical density (OD) was measured at A_{630} nm against a blank using a spectrophotometer. The blank comprised of the reaction mixture without the tissue sample.
6. A standard graph prepared with catechol was used to determine the total phenol contents of the samples as below:

   1OD was equivalent to 0.138 mg

   Therefore, x OD = x X 0.138 mg

0.1 ml of elute had x X 0.138 mg of polyphenol

5 ml of elute had 5 X x X 0.138 mg of polyphenol

Three replicates of each sample were taken and the experiment was repeated twice. The mean of the concurrent readings were finally used to estimate the total polyphenol content of the leaves expressed as mg/g fresh weight.

3.6. Effect of leaf polyphenols on *A. tumefaciens* growth

3.6.1 *A. tumefaciens* growth measured by spectrophotometer method

The effect of total polyphenols **(Section 3.5)** was tested on the growth of *A. tumefaciens*. For this, different concentrations of total leaf polyphenols (0.125, 0.25, 0.5 and 1.0 mg/ml) were separately added to fresh overnight (16-18 hrs) grown starter culture of *A. tumefaciens*. The polyphenol supplemented bacterial cultures were further grown as described in **Section 3.3.2.** and absorbance (OD) of 1 ml aliquot was measured at 600 nm at regular intervals of 0, 12, 16, 18 and 24 hrs using a spectrophotometer (ELCOS SL 150). The observations recorded after 18 hrs of incubation were finally tabulated.

3.6.2 Colony forming unit(s)

Furthermore, in order to confirm that the higher OD actually represented living bacterial cells, cfus at each growth interval were also calculated. Thus, 6 µl fresh, overnight grown bacterial cultures were taken and plated on agar (1.5%) solidified YMB containing different concentrations of total leaf polyphenol (0.125, 0.25, 0.5, 1.0 ml) in 9 cm Petriplates. These were incubated at 28°C in dark, and the colony forming units (cfus) were counted after 36-48 hrs.

3.7 PPO activity in leaf explants of different plant species

The effect of oxidized leaf polyphenols of the different plants on *Agrobacterium* infection was tested. The polyphenol oxidase activity (PPO) of the leaves was determined using the method of Moore and Flurkey (1990). For this, 5 g of leaf tissue was homogenized in 20 ml of extraction buffer (**Appendix-1.4.2)** using a mortar and pestle

and centrifuged at 10,000 rpm and 4°C for 10 min. The supernatant that was obtained served as the PPO enzyme extract. A total volume of 1 ml reaction mixture was prepared at room temperature. The reaction mixture contained 50 mM citrate buffer (pH 5.0), 50 mM catechol and 0.2 ml aliquot of the enzyme extract. After 30 seconds, the change in absorbance at 650 nm was monitored with time and an absorbance of 1 was taken as one unit of PPO activity.

3.8 Effect of L-glutamine on *Agrobacterium tumefaciens* growth and genetic transformation of high polyphenol containing leaf explants

3.8.1 *Agrobacterium* growth in response to L-glutamine

The effect of different concentrations of un-autoclaved and autoclaved (20 and 40 min) L-glutamine was tested on the sensitivity and growth of *Agrobacterium* (**Table 1**). These were separately added to fresh starter cultures **(Section 3.3.1)** and growth as well as colony forming units (cfus) were compared against control (*Agrobacterium* grown without un-autoclaved or autoclaved L-glutamine). *Agrobacterium* growth was measured in terms of (i) OD at A_{600} after regular intervals of 0, 12, 18 hrs **(Section 3.6.1)** and also (ii) cfus at 36-48 hrs **(Section 3.6.2)**. Cultures with only 200 μM acetosyringone but not L-glutamine served as the positive controls. On the other hand, those without either L-glutamine or acetosyringone were the negative controls.

3.8.2 *A. tumefaciens* growth in response to the leaf polyphenols and autoclaved L-glutamine

The interaction of leaf polyphenols, autoclaved L-glutamine and *A. tumefaciens* growth was studied. For this, different concentrations of total leaf polyphenols (0.125, 0.25, 0.5 and 1.0 mg/ml) along with L-glutamine concentrations **(Table 1)** were separately added to fresh overnight (16-18 hrs) grown starter cultures. The polyphenol supplemented bacterial cultures were further grown as described in **Section 3.3.2.** and absorbance (OD) of 1 ml aliquot was measured at 600 nm after regular intervals of 0, 12, 16, 18 and 24 hrs using a spectrophotometer (ELCOS SL 150).

3.8.3 Colony forming unit(s)

Furthermore, in order to confirm that the higher OD actually represented living bacterial cells, cfus at each growth interval were also calculated as described in **Section 3.6.2.** However, in this case, the bacterial cultures were plated on agar (1.5%) solidified YMB containing different concentrations of leaf polyphenols and also L-glutamine in 9 cm Petriplates.

Table: 1 Different combinations and concentrations of L-glutamine used to understand the role of amino acid derivative on *Agrobacterium* growth and transformation

Amino acid	Autoclaving time	Conc. mg/ml
L- glutamine	No autoclaving	0.5
		1.0
		2.0
		3.0
		5.0
L- glutamine	20 min	0.5
		1.0
		2.0
		3.0
		5.0
L- glutamine	40 min.	0.5
		1.0
		2.0
		3.0
		5.0

3.8.2 Interaction of *A. tumefaciens* and high polyphenol containing leaf explants in presence of autoclaved L-glutamine

High ploypehnol containing leaves of different plants were transformed as described in **Section 3.3.2** using the strain mentioned in **Section 3.3.1.** However, co-cultivation media containing L-glutamine autoclaved for 20 or 40 min were used for this experiment. For positive control, leaves of apple root stocks, B9 and MM106 were used.

Agrobacterium infection of the explants was confirmed by GUS histo-chemical assay as described in **Section 3.4.**

3.9 Role of polyphenols and autoclaved L-glutamine as *vir* inducer

The *vir* inducing ability of L-glutamine autoclaved for 40 min was tested. For this, the β-galactosidase assay was conducted using *E. coli* cultures harbouring constructs with *LacZ*: *vir* gene fusions. The cultures obtained from Prof. Eugene Nester and Prof. S. Gelvin were grown overnight in liquid Luria Broth (LB) **(Appendix-1.3.2)** containing the selection antibiotic kanamycin (50 μg/ml) at 37°C in dark.

3.9.1 β-galactosidase assay

The β-galactosidase assay was performed with overnight grown *E. coli* cultures harbouring *LacZ: vir* gene fusions as follows:

1. The cultures were incubated on ice for 20 minutes to stop all further growth.
2. One ml of each of the cultures was then centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant was discarded but the pellet was suspended in 2 ml chilled Z-buffer **(Appendix -1.4.3)**.
3. The OD of the pellet suspended in Z buffer was measured against blank at 600 nm (the blank comprised of only Z-buffer without bacterial suspension). After adjusting the OD to 0.6 (equivalent to 1 X 10^9 cells/ml), the bacterial suspension was further diluted up to 1 ml in Z-buffer.
4. To 1 ml of the diluted bacterial culture, 100 μl of chloroform and 50 μl of 0.1% SDS were added and vortexed for 5 minutes.

5. The tubes containing the reaction mixture were equilibrated by incubating in a water bath at 37°C for 5 minutes.

6. The reaction was started by adding the substrate i.e., 0.2 ml ONPG (Sigma). This was prepared by dissolving 4 mg ONPG in 1 ml phosphate buffer, pH 7.

7. The initial time was recorded.

8. After the addition of ONPG, the tubes were further incubated at 37°C for 20 minutes in a water bath until a straw colored product developed.

9. The reaction was stopped by adding 0.5 ml of 1 M sodium carbonate and vortexed for 1 minute.

10. After vortexing, 1 ml of the above reaction mix was transferred into an eppendorf and spun at 10,000 rpm for 5 minutes.

11. The absorbance of each tube was recorded at 420 and 550 nm and the enzyme activity was calculated as per the following equation:

$$\text{Miller units} = 1000 \times [\text{OD } A_{420}\text{ nm} - (1.75 \times \text{OD } A_{550}\text{ nm})] / (T \times V \times \text{OD at } A_{600}\text{ nm})$$

- OD at A_{600} nm reflected the bacterial cell density
- T = time of the reaction in minutes
- V = volume of the culture used in the assay in ml

3.9.2. Effect of leaf polyphenols on *vir* genes of *Agrobacterium*

The effect of leaf polyphenols of different plant species on *virA* and *G* and also the phenol detoxifying *pin F* genes was studied using β-galactosidase enzyme activity. For this, β-galactosidase enzyme activity of reaction mixtures containing each of the extracted leaf polyphenols (0.125, 0.25. 0.5, and 1 mg/ml) was assayed as described in **Section 3.9.1.**

3.9.3 Effect of interaction of autoclaved L-glutamine and leaf polyphenols on *vir* gene induction

The ability of 2 g/l L-glutamine autoclaved for 40 min in counteracting the *vir* suppressing effect of leaf polyphenols was studied. For this, the β-galactosidase enzyme activity of reaction mixtures containing 2 g/l L-glutamine autoclaved for 40 min and leaf polyphenols (0.125, 0.25. 0.5, and 1 mg/ml) was assayed as described in **Section 3.9.1.**

3.10 Development of a kit to detect the formation of 2-amino glutarimide after autoclaving L-glutamine for 20 and 40 minutes

Total polyphenols were extracted from the leaves of Kangra jat cultivar of *C. sinensis* as described in **Section 3.5**. Then 2 g/l each of L-glutamine autoclaved for (i) 20 and (ii) 40 minutes was added to 100 ml of the extracted polyphenols and visual changes in color due to the reaction was recorded after 5 minutes.

3.11 Chemical confirmation of xenobiotic detoxification of toxic tea leaf polyphenols by autoclaved L-glutamine

The chemical identity of the compounds that were formed after autoclaving L-glutamine for 20 and 40 minutes were first tested with TLC and then re-confirmed using NMR. Then both TLC and NMR were used to prove the quenching mechanism proposed by Saini, 2007 for xenobiotic detoxification of tea leaf polyphenols by L-glutamine, autoclaved for 40 min.

3.11.1 Thin layer Chromatography

L-glutamine samples autoclaved for 20 and 40 minutes, with and without catechins of tea leaves were subjected to Thin Layer Chromatography (TLC). TLC plates were prepared from a slurry of silica gel in water and baked at 300°C for 5-10 minutes in hot air oven. 5µl samples (containing about catechins and 2-amino gluterimide) were applied using a standard Hamilton syringe on TLC silica gel plate as spots, with 1-cm space between the spots and at a distance of 2 cm from the bottom edge of the plate. Plates were developed in commercially available vertical TLC chambers (CAMAG, Switzerland) with chamber saturation using 1-butanol, acetone, acetic acid, water (7:7:2:4) and toluene, acetone, formic acid (9:9:2) as solvent mixture for amino acid and catecin analysis respectively. The required time of development was about 35 min and

the TLC run was performed at room temperature (28±2 °C) and 60±5 % relative humidity. Subsequent to development, TLC plates were dried in warm air oven. The total length of the run was about 9 cm. Amino acids were visualized by spraying with a solution ninhydrin reagent (2% ninhydrin in acetone) and catechin reagent (0.1% catechins reagent in acetone). The plates were dried at 110°C and individual amio acids become detectable as blue/violet spots. While the plates were dried at 100°C and individual catechins become detectable as yellow spots. And visual changes in color and Rf values of the spots were noted.

3.11.2 Nuclear Magnetic Resonance

Aqueous solutions of L-glutamine (50 mg/ml) were first autoclaved at 121°C under a high pressure of 15 psi for 20 and 40 minutes. The changes in chemical structure of L-glutamine due to autoclaving was determined using NMR (^{1}H and ^{13}C).In another study, catechin (1 mg/ml acetone) was added to each of the samples, atuoclaved for 20 and 40 minutes. The L-glutamine and catechin mixtures were then evaporated to dryness. Then 0.6 ml duterated acetone was finally added to the dried samples. These were then subjected to NMR (^{1}H and ^{13}C) to confirm the quenching of catechins by L-glutamine autoclaved for 40 minutes.

Chapter-4

Results

4. RESULTS

The response of explants obtained from high polyphenol containing plants to *Agrobacterium* growth and transformation was studied. The salient observations are discussed as under.

4.1 Explant response to surface sterilization

When leaf explants of different plant species were treated with Bavistin (0.2%; w/v) and streptomycin (0.02%; w/v) for 15 and 30 minutes, variable responses were observed (**Table 2**). Treatment for 15 min was better than 30 min as 80-100% explants remained fresh and green, irrespective of plant species. On the other hand, 50 and 60% of explants turned brown after 30 minutes treatment in *C. sinensis* and *E. sphaericus,* respectively.

Table 2: Response of leaf explants of different plant species to duration of surface sterilization

Plant species	(%) Response of leaf explants after treatment for			
	15 min		30 min	
	Fresh & green	Browning	Fresh & green	Browning
E. sphaericus	80	20	40	60
C. sinensis	80	20	50	50
P. macrophyllus	100	-	80	20
Wild Rose	100	-	90	10
M. domesticus	100	-	90	10
P. hexandrum	100	-	100	-

4.2 *Agrobacterium tumefaciens* mediated transformation

Even after 30 days of co-cultivation, *A. tumefaciens* failed to infect the leaves of any of the studied plant species **(Plate 1)**. Rather, the explants turned brown and finally died. The only exception was *Podophyllum hexandrum* where *A. tumefaciens* growth was observed within 15 days **(Plate 2)**.

4.2.1 GUS expression in the *A. tumefaciens*-infected leaves

No GUS expression was observed in the leaves of any of the studied plant species. The only exception was *P. hexandrum*, where expression in the form of a small blue sector was recorded on the leaf blade after 15 days of co-cultivation **(Plate 2)**.

4.3 Estimation of total phenol in the leaves of different plant species

Considerable differences in the total polyphenol content of the studied plant species were observed **(Fig 2)**. Highest polyphenol content was observed in *E. sphaericus* (75.45 mg/g f. wt.) followed by *C. sinensis* (50.38 to 70.45 mg/g f. wt.) and *P. macrophyllus* 48.58 mg/g f. wt.). When the polyphenol content of all the studied plants was compared, the lowest content was recorded in *P. hexandrum* (23.23 mg/g f. wt.). On the other hand, moderate amounts of total polyphenol was recorded in the leaves of *Rosa* sp. (wild rose) and the *M. domesticus* (apple rootstocks). Significant variations in polyphenol contents were also observed in the leaves of different cultivars of *C. sinensis*.. While the highest content was recorded in T-78 (70.45 mg/g f. wt.) followed by TV-1 (69.16 mg/g f. wt.), the lowest contents were recorded in Kangra Jat (50.38 mg/g f.wt.).

4.4 Effect of leaf polyphenols on *A. tumefaciens* growth

The leaf polyphenols from different plants affected the growth of the *Agrobacterium* strain tested. However, the growth was affected to various extents by the polyphenols from different plant species and their concentrations **(Table 3)**. Irrespective of plant species, maximum increase in growth was observed at 0.25 mg/ml of polyphenols. Any increase (1.0 mg/ml) or decrease (0.5 mg/ml) in the polyphenol concentrations impacted the *Agrobacterium* growth negatively.

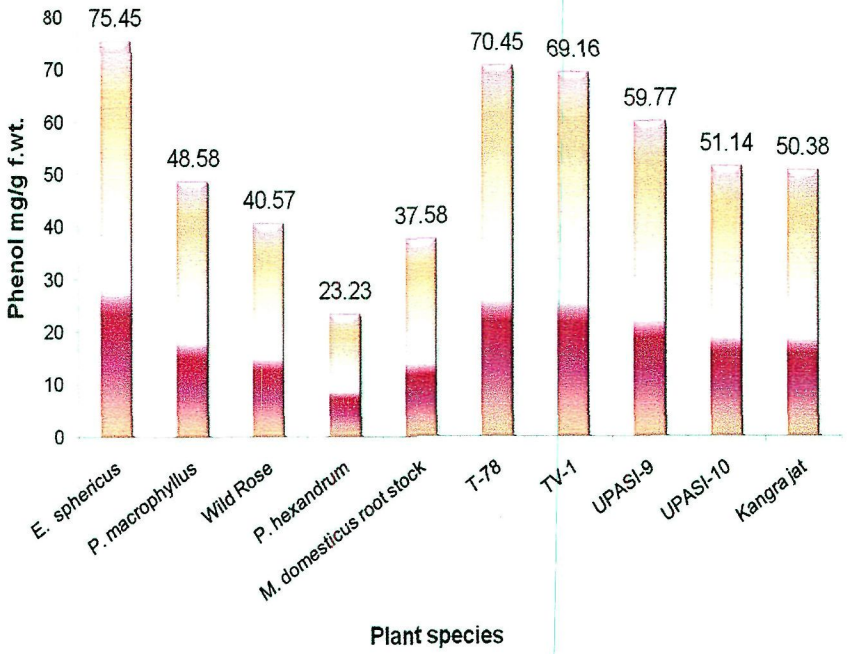


Fig: 2 Total polyphenol contents in leaves of different plant species

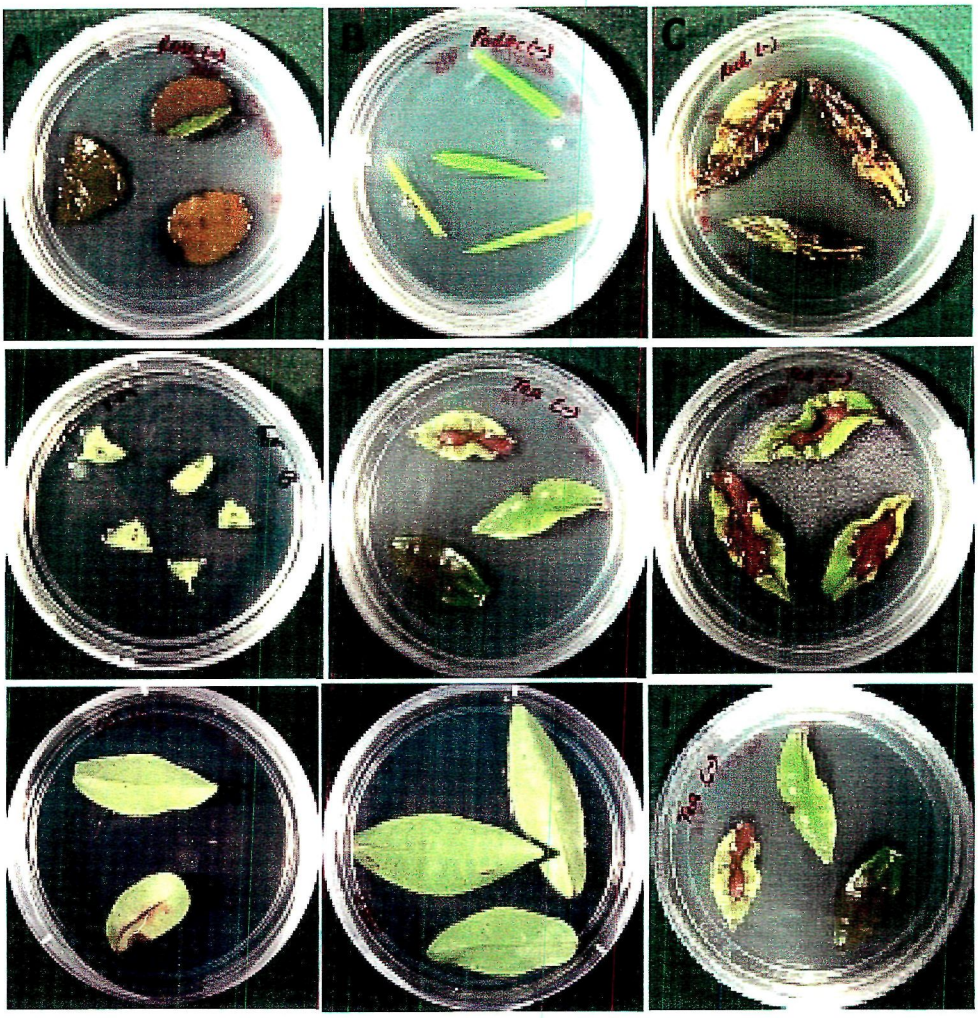
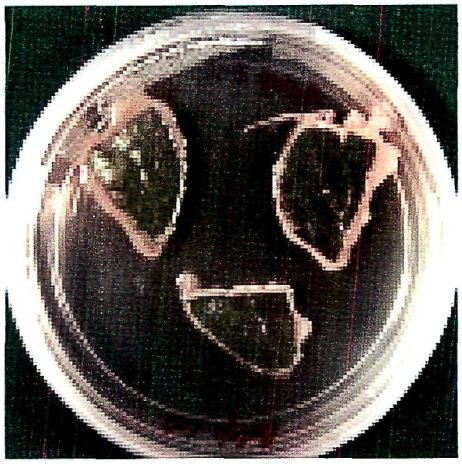


Plate 1 : No *Agrobacterium* growth on leaf explants of (A) *Rosa* sp (B) *P. macrophyllus* (C) *E. sphaericus* (D) *M. domesticus* and *C. sinensis* cvs (E) T-78 (F) TV-1 (G) UPASI-9 (H) UPASI-10 (I) K J, after 20 days of co-cultivation.

A



B

Plate2 : (A) *Agrobacterium* growth on leaf explants of *P. hexandrum* after 15 days of co-cultivation (B) GUS expression.

When the growth in response to polyphenols from different plant species was compared, maximum increase (3.80 fold) was observed in case of *P. hexandrum* followed by *Rosa* sp. (2.52 fold) and *P. macrophyllus* (2.05 fold). The minimum increase in growth was observed when polyphenols from *E. sphaericus* (1.17 fold) and the *C. sinensis* cultivars, TV1 (1.18 fold) and T-78 (1.19 fold) of were used.

Different cfu counts of *Agrobacterium* were recorded in response to the studied plant polyphenols after 48 hrs **(Table 4).** Highest cfus equivalent to control (>500) was recorded when *P. hexandrum* polyphenol at a concentration of 0.125 mg/ml was used. With an increase in the polyphenol concentration however, a decrease in cfu counts was recorded, irrespective of plant species,. Minimum (7 cfu) was recorded in response to *E. sphaericus* polyphenol at 0.25 mg/ml. No cfus were recorded either at 0.5 and 1.0 mg/ml polyphenol from *E. sphaericus* and *C. sinensis* cultivar, T-78 or 1.0 mg/ml polyphenol from TV-1.

4.5 PPO activity in leaf explants of different plant species

The specific PPO activity in the leaves of different plant species was directly related to the polyphenol contents of the leaf tissue (**Fig. 3**). Thus, while the highest specific activity (0.75 A/min/mg) was observed in *E. sphaericus* with a polyphenol content of 75 mg g^{-1} f. wt., the lowest activity was observed in *P. hexandrum* (0.30 A/min/mg) with the lowest polyphenol content of 23.23 mg g^{-1} f. wt.

4.6 Effect of L-glutamine on *A. tumefaciens* growth

Irrespective of concentrations, L-glutamine autoclaved for 40 minutes was more effective in promoting *A. tumefaciens* growth as compared to the one autoclaved for 20 minutes or L-glutamine that was un-autoclaved **(Fig. 4)**. While the increase in case of autoclaved L- glutamine (20 min) was only 1.2 fold, that in case of 40 min autoclaved L-glutamine was 3.30 fold. Irrespective of the duration of autoclaving, a maximum increase was observed at 2.0 g/l L-glutamine autoclaved either for 20 or 40 minutes. Despite increasing the L-autoclaved glutamine concentration, stable growth of *A. tumefaciens* with no further increase was observed. No increase in growth was observed when un-autoclaved L-glutamine was used.

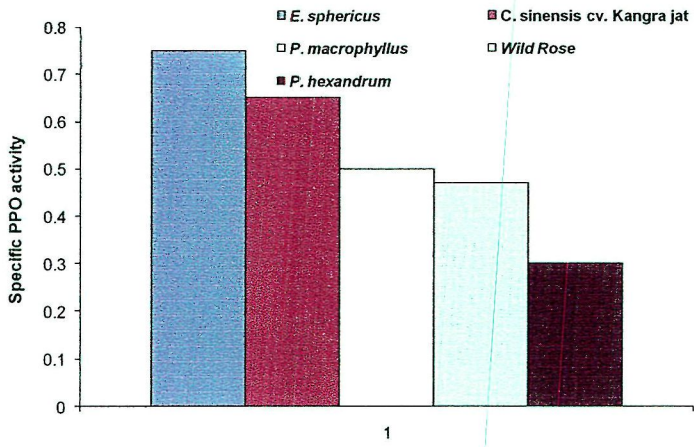


Fig: 3 Specific PPO activity in different plant species

Table 3: ***A. tumefaciens*** **growth in response to leaf polyphenols from different plant species after 18 hrs of incubation at 28°C**

Plant species	Polyphenol content of leaves mg/g f. wt.	Polyhenol conc. (mg/ml)	Fold increase in growth of *A. tumefaciens* after 18hrs
E.sphaericus	75.45	0.125	1.16
		0.250	1.17
		0.500	1.10
		1.000	1.08
P.macrophyllus	48.58	0.125	2.00
		0.250	2.05
		0.500	1.95
		1.000	1.92
Rosa sp.	40.57	0.125	2.50
		0.250	2.52
		0.500	2.47
		1.000	2.43
P. hexandrum	23.23	0.125	3.79
		0.250	3.80
		0.500	3.75
		1.000	3.72
C. sinensis cvs: T-78	70.45	0.125	1.17
		0.250	1.19
		0.500	1.11
		1.000	1.07
TV-1	69.16	0.125	1.17
		0.250	1.18
		0.500	1.10
		1.000	1.08
UPASI-9	59.77	0.125	1.19
		0.250	2.00
		0.500	1.18
		1.000	1.16
UPASI-10	51.14	0.125	2.00
		0.250	2.02
		0.500	1.99
		1.000	1.97
Kangra Jat	50.38	0.125	2.00
		0.250	2.03
		0.500	1.98
		1.000	1.95

Table 4: cfus in response to leaf polyphenols from different plant species after 48 hrs of incubation at 28°C

Plant species	Phenol content mg/g f. wt.	Polyphenol conc. mg.ml	cfu
Control	-	-	>500
E. sphaericus	75.45	0.125	8
		0.250	7
		0.500	None
		1.000	None
P. macrophyllus	48.58	0.125	119
		0.250	112
		0.500	70
		1.000	50
Rosa sp.	40.57	0.125	250
		0.250	245
		0.500	180
		1.000	150
P. hexandrum	23.23	0.125	>500
		0.250	450
		0.500	365
		1.000	350
C. sinensis cvs: T-78	70.45	0.125	12
		0.250	10
		0.500	None
		1.000	None
TV-1	69.16	0.125	20
		0.250	18
		0.500	10
		1.000	None
UPASI-9	59.77	0.125	58
		0.250	56
		0.500	40
		1.000	10
UPASI-10	51.14	0.125	68
		0.250	63
		0.500	55
		1.000	15
Kangra Jat	50.38	0.125	73
		0.250	70
		0.500	60
		1.000	20

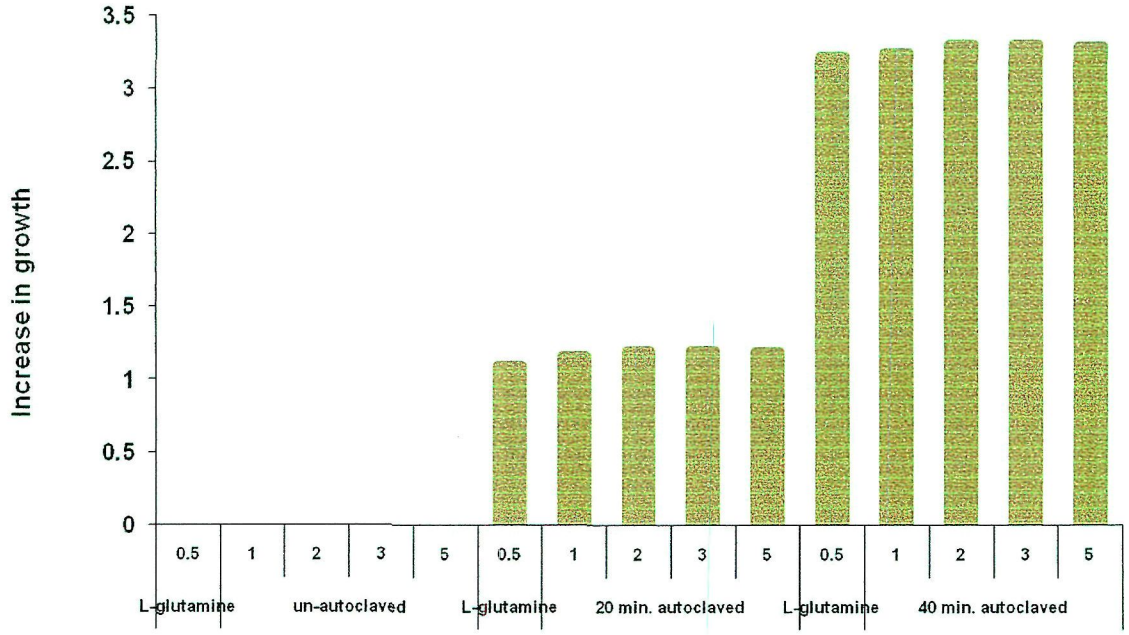


Fig: 4 Fold increase in *A. tumefaciens* growth in response to L-glutamine concetration

Irrespective of the concentrations tested, the cfu counts in response to L-glutamine autoclaved for 40 min were next only to those of positive control i.e., acetosyringone (**Table 5**). As in case of growth curve, *Agrobacterium* growth was maximum in response to 2.0 g/l L-glutamine autoclaved for 40 min. On the other hand, the cfu counts in response to un-autoclaved L-glutamine and 5.0 g/l L-glutamine autoclaved for 20 minutes was at par with negative control.

4.7 *A. tumefaciens* growth in response to leaf polyphenol and L glutamine autoclaved for 40 minutes

A maximum increase in growth was observed in the presence of L-glutamine autoclaved for 40 minutes at 0.25 mg/ml of polyphenols, irrespective of plant species. However, the growth decreased at 0.5 and 1.0 mg/ml leaf polyphenol (, for all the plant species despite the presence of autoclaved L-glutamine **(Table 6)**.

Differences in *Agrobacterium* growth was however, recorded in response to leaf polyphenols from different plant species in the presence of autoclaved L-glutamine **(Table 6)**.Maximum increase in *Agrobacterium* growth (4.81 fold) was observed in case of *P. hexandrum* leaf polyphenols followed by those from *P. macrophyllus* (2.52 fold) and *Rosa* sp. (3.52 fold). On the other hand, growth was minimum when leaf polyphenols from *E. sphaericus* (2.14 fold) and the *C. sinensis* cvs. T- 78 (2.17 fold) and TV-1 (2.20 fold) were used.

A significant increase in the cfus was observed when 2.0 g/l L-glutamine autoclaved for 40 minutes was added to the culture medium along with polyphenol at different concentrations **(Table 7)**. This increase was higher than that obtained on medium containing only polyphenols from different plant species **(Tables 4).** When the *Agrobacterium* growth in response to 0.5 and 1 mg/ml polyphenols from different plant species were compared, a minimum of only 3 colonies was recorded at 0.5 mg/ml polyphenol from *E. sphaericus* followed by the *C. sinensis* cultivar T- 78 with 9 colonies. However, the cfus in response to 0.125 to 0.5 mg/ml polyphenols from *P. hexandrum* was at par with control.

4.8 *A. tumefaciens* mediated transformation of high polyphenol containing leaf explants using autoclaved L-glutamine

No *Agrobacterium* growth was observed in the leaf explants of any of the studied plants when the co-cultivation medium contained L-glutamine, either un-autoclaved or autoclaved for 20 minutes. However, *Agrobacterium* growth varied between the leaf explants of different plants when L-glutamine, autoclaved for 40 min was present in the co-cultivation media .

Table 5: cfus in response to autoclaved and un-autoclaved L-glutamine

Samples	Conc mg/ml	cfu
Control (none)		>500
Acetosyringone		Infinite (+++)
L-glutamine (un-autoclaved)	0.5	>500
	1.0	
	2.0	
	3.0	
	5.0	
L-glutamine (autoclaved for 20 min.)	0.5	>500
	1.0	>500
	2.0	Infinite +
	3.0	Infinite
	5.0	>500
L-glutamine (autoclaved for 40 min.)	0.5	Infinite ++
	1.0	
	2.0	
	3.0	
	5.0	

Table 6 : Effect of leaf polyphenol and autoclaved L-glutamine (40 minutes) on the growth of *A. tumefaciens* after 18 hrs.

Plant species	Polyphenol content of leaves mg/g f. wt.	Conc. of Polyphenol mg/ml	Fold increase in growth of *A. tumefaciens* after 18 hrs.
E. sphaericus	75.45	0.125	2.14
		0.250	2.15
		0.500	1.98
		1.000	1.95
P. macrophyllus	59.77	0.125	2.75
		0.250	2.78
		0.500	2.70
		1.000	2.67
Rosa sp.	51.14	0.125	3.51
		0.250	3.52
		0.500	3.48
		1.000	3.45
P. hexandrum	50.38	0.125	4.80
		0.250	4.81
		0.500	4.78
		1.000	4.77
C. sinensis cvs: T-78	48.58	0.125	2.15
		0.250	2.17
		0.500	2.10
		1.000	2.07
TV-1	40.57	0.125	2.18
		0.250	2.20
		0.500	2.15
		1.000	2.10
UPASI- 9	23.23	0.125	2.20
		0.250	2.24
		0.500	2.18
		1.000	2.12
UPASI-10	70.45	0.125	2.60
		0.250	2.65
		0.500	2.56
		1.000	2.53
K J	69.16	0.125	2.61
		0.250	2.64
		0.500	2.57
		1.000	2.53

Table 7: cfus in response to leaf polyphenols from different plant species and L-glutamine autoclaved for 40 min.

Plant species	Polyphenol content mg/g f. wt.	Polypenol conc. mg/ml	cfu
None			>500
E. sphaericus	75.45	0.125	15
		0.250	10
		0.500	3
		1.000	None
P. macrophyllus	48.58	0.125	150
		0.250	147
		0.500	120
		1.000	70
Rosa sp	40.57	0.125	300
		0.250	290
		0.500	270
		1.000	250
P. hexandrum	23.23	0.125	>500
		0.250	>500
		0.500	450
		1.000	396
C. sinensis cvs. T-78	70.45	0.125	20
		0.250	18
		0.500	9
		1.000	None
TV-1	69.16	0.125	28
		0.250	26
		0.500	19
		1.000	12
UPASI-9	59.77	0.125	75
		0.250	71
		0.500	30
		1.000	22
UPASI-10	51.14	0.125	88
		0.250	85
		0.500	70
		1.000	43
K J	50.38	0.125	89
		0.250	87
		0.500	75
		1.000	50

Thus, *Agrobacterium* growth was recorded in *M. domesticus* root stock and *P. hexandrum* within 3 days of co-cultivation. Even the leaves of wild rose and *P. macrophyllus* showed *Agrobacterium* growth within 5 and 7 days respectively **(Plate 3 & Table 8).** Although delayed, growth was also recorded in *C. sinensis* and *E. sphaericus* after 10-15, and 20 days, respectively **(Plate 3).**

Table 8: *A. tumefaciens* growth on high polyphenol containing leaf explants from different plant species

Sample	*Agrobacterium infection*							
	Day 1	**Day 2**	**Day 3**	**Day 5**	**Day 7**	**Day 10**	**Day 15**	**Day 20**
E. sphaericus	-	-	-	-	-	-	-	+
C.sinensis cvs. KJ	-	-	-	-	-	+	+	+
T-78	-	-	-	-	-	-	-	+
TV-1	-	-	-	-	-	-	+	+
U-9	-	-	-	-	-	+	+	+
U-10	-	-	-	-	-	+	+	+
P. macrophyllus	-	-	-	-	+	+	+	+
Rosa sp.	-	-	-	+	+	+	+	+
P. hexandrum	-	-	+	+	+	+	+	+
M. domesticus	-	-	+	+	+	+	+	+

(+ = present; - = absent)

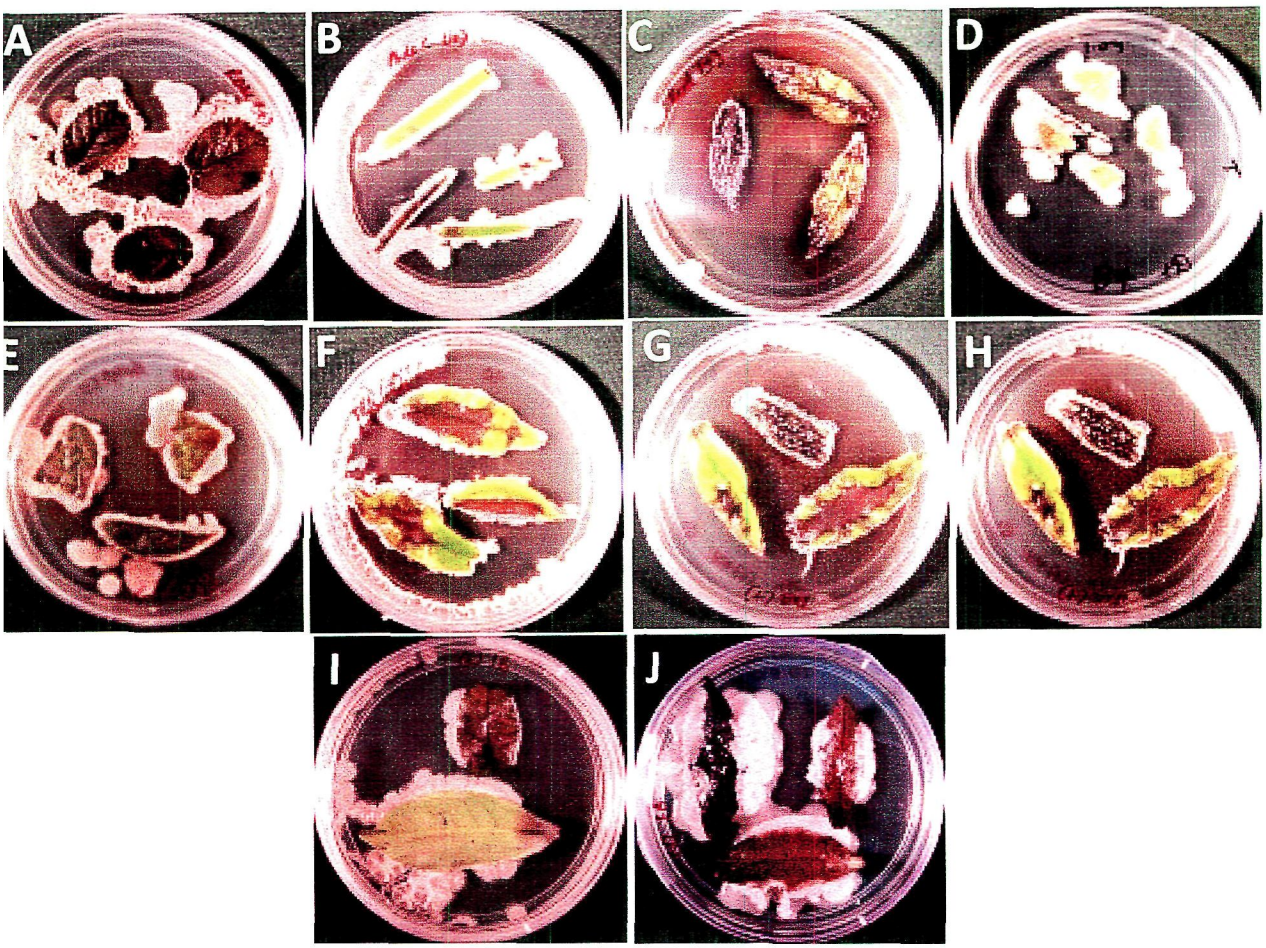


Plate3: *Agrobacterium* growth on recalcitrant leaf explants of different plant species (A) *Rosa* sp after 5 days (B) *P. macrophyllus* after 7 days (C) *E. sphaericus* after 20 days (D) *M. domesticus* after 3 days (E) *P. hexandrum* after 3 days and *C. sinensis* cvs (F) T-78 after 20 days (G) TV-1 after 15 days (H) UPASI-9 after 10 days (I) UPASI-10 after 10 days (J) KJ after 10 days on media supplemented with L-glutamine autoclaved for 40 min

4.8.1 GUS expression on *A. tumefaciens* infected leaves of different plants

GUS expression in the form of blue colored spots/sectors was recorded on the leaf blade after co-cultivation for different durations on medium containing L-glutamine autoclaved for 40 minutes **(Plates 4).** However, the explants showing GUS expression varied between the species **(Table 9).**

Table 9: GUS expression on leaves of different plant species

Plant species	**GUS expression**
E. sphaericus	Small light blue sector
C. sinensis	
T-78	Small blue sector
TV-1	Intense blue spots at margins of leaf
UPASI-9	Intense blue spots at margins of leaf
UPASI-10	Intense blue spots at margins of leaf
KJ	Intense blue sector at margin of leaf
P. macrophyllus	Large intense blue sectors
Rosa sp.	Almost entire leaf intense blue
P. hexandrum	Entire leaf intense blue
M. domesticus	More than half of leaf intense blue

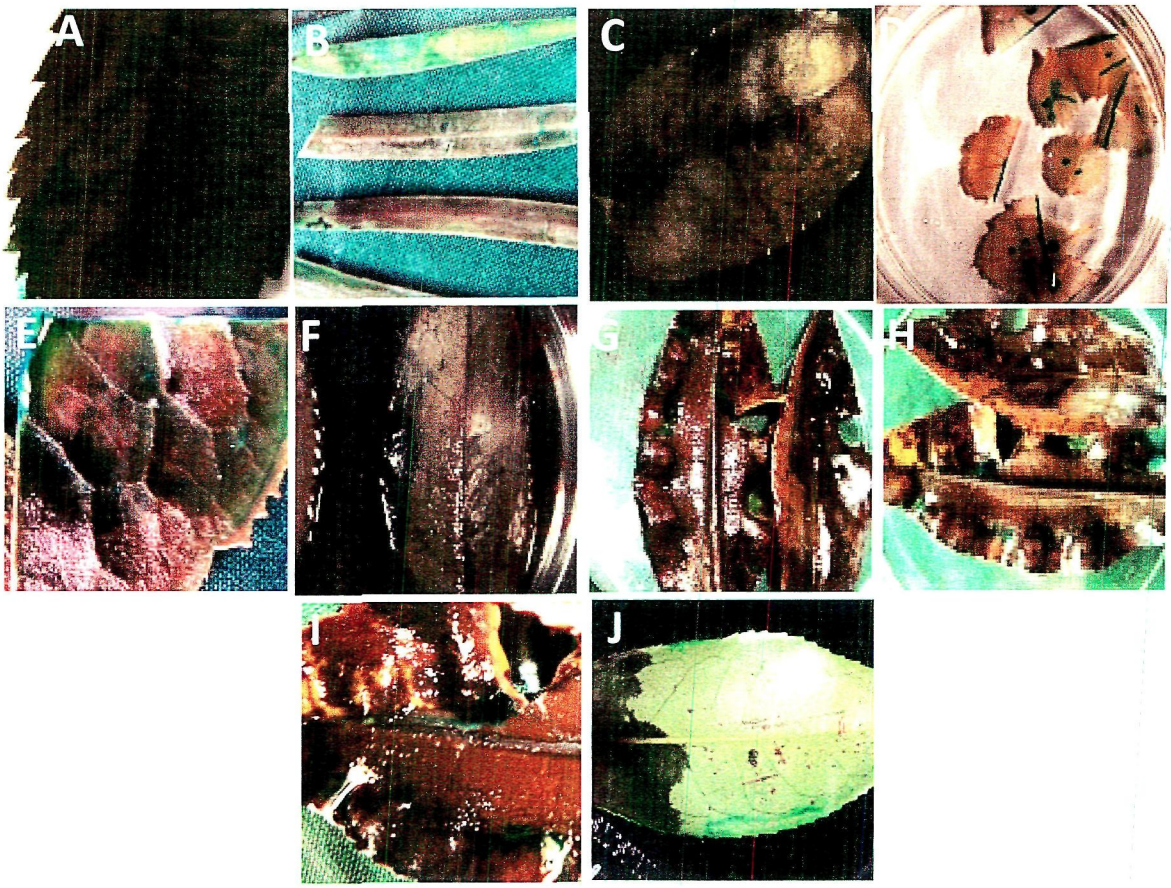


Plate 4: Blue colored spots/sectors on high polyphenol containing, recalcitrant leaf explants of different plant species indicating variable GUS expression in A) *Rosa* sp (B) *P. macrophyllus* (C) *E. sphaericus* (D) *M. domesticus* (E) *P. hexandrum* and *C. sinensis* cvs (F) T-78 (G) TV-1 (H) UPASI-9 (I) UPASI-10 (J) KJ

4.9 Induction of *Agrobacterium* virulence (*vir*) genes in response to leaf polyphenols and autoclaved L-glutamine (40 min.)

4.9.1 In the presence of acetosyringone (a known inducer of *vir* genes), a significant increase in the reporter β-galactosidase activity was observed. This increase was invariably higher than those where polyphenols extracted from leaves of different plant species were added to the assay mixture. Furthermoe, aA decline in the reporter β-galactosidaes activity was observed with increasing concentrations of leaf polyphenols **(Figs. 5, 6 & 7).**

When the reporter β-galactosidase activity was compared with polyphenols from different plant species, maximum activity was observed in case of *P. hexandrum* and the minimum in case of *E. sphaericus.* When 0.125 mg/ml polyphenol from *P. hexandrum* was added to the assay mixture, the activities of the three strains pSM229 (harbouring *vir pinF* gene), E488 (harbouring *vir A* gene), and E498 (harbouring *vir G* gene) at were 161.272, 165.529 and 169.296 Miller units, respectively **(Figs. 5, 6 & 7)**. However, the β-galactosidaes activities at the same concentration (0.125 mg/ml) of polyphenol from *E. sphaericus* were 132.24, 136.24 and 140.0 Miller units for the three strains pSM229, E488, E498, respectively. Maximum β-galactosidaes activity was recorded in case of E498 strain, irrespective of polyphenol concentration or plant species.

4.9.2 Effect of autoclaved L-glutamine on *vir* gene induction in the presence of leaf polyphenols

Even in the presence of 2.0 g/l L-glutamine autoclaved for 40 minutes, the reporter β-galactosidase activity continued to follow the same trend as in the ones with polyphenols but without autoclaved L-glutamine **(Fig 8, 9& 10)**. Thus, highest activity was observed in case of acetosyringone. Maximum activities (152.592, 161.681 and 162.473 Miller units) were also observed when 0.125 mg/ml polyphenols from *P. hexandrum* were used for the strains pSM229, E488, E498, respectively. On the other hand, the lowest activities (123.562, 132.650 and 133.442) were observed for the pSM229, E488, E498 strains, respectively when 0.125 mg/ml polyphenols from *E. sphaericus* were used. In general, significant suppression of the reporter β-galactosidase activity was observed in the presence of autoclaved L-glutamine (40 min.) as compared to the ones containing only leaf polyphenols but not autoclaved L-glutamine.

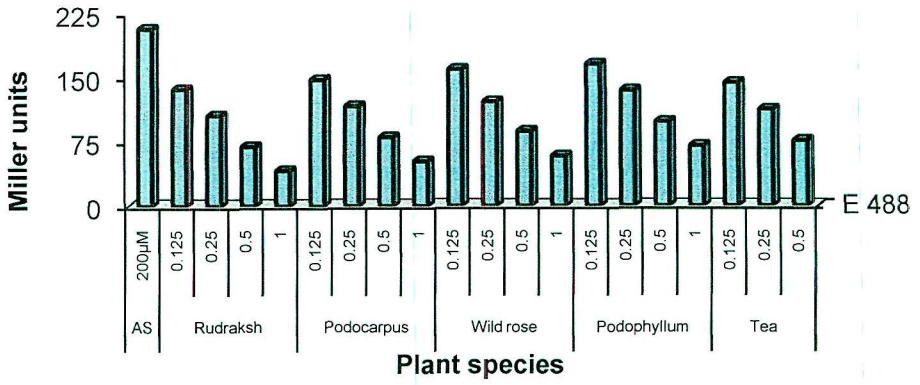


Fig. 5 ***Vir A*** **gene activity in activity in E 488 strain in response to leaf polyphenols of different plant species**

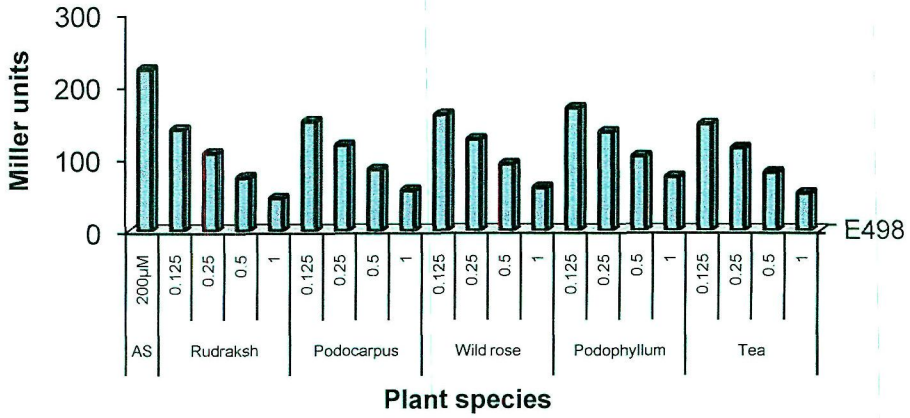


Fig.6 ***Vir G*** **gene activity in activity in E 498 strain in response to leaf polyphenols of different plant species**

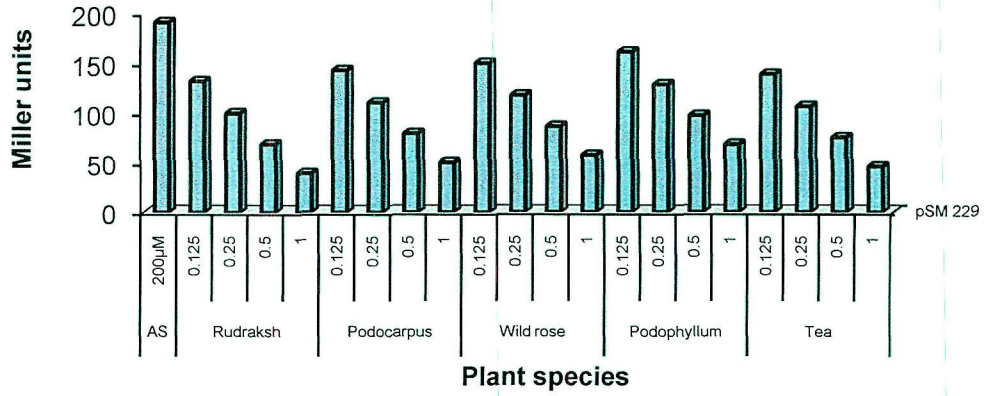


Fig.7 ***Vir pinF*** **gene activity in pSM229 strain in response to leaf polyphenols of different plant species**

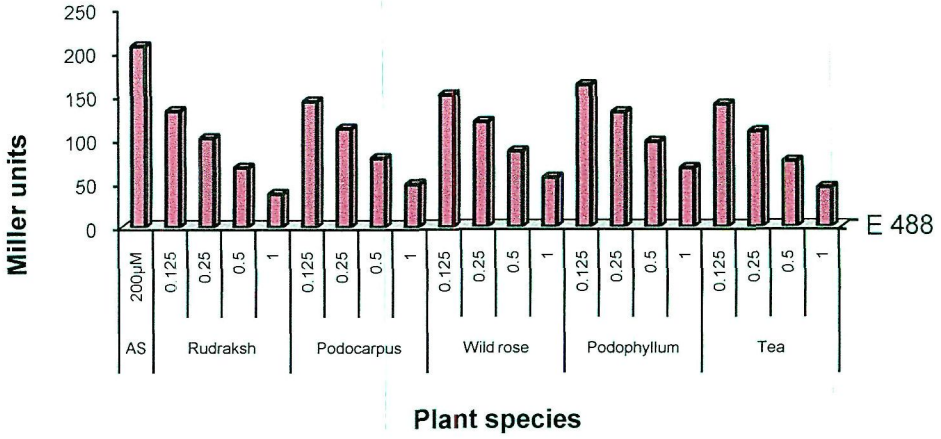


Fig.8 ***Vir A*** **gene activity in activity in E488 strain in response to leaf polyphenols of different plant species and autoclaved L-glutamine**

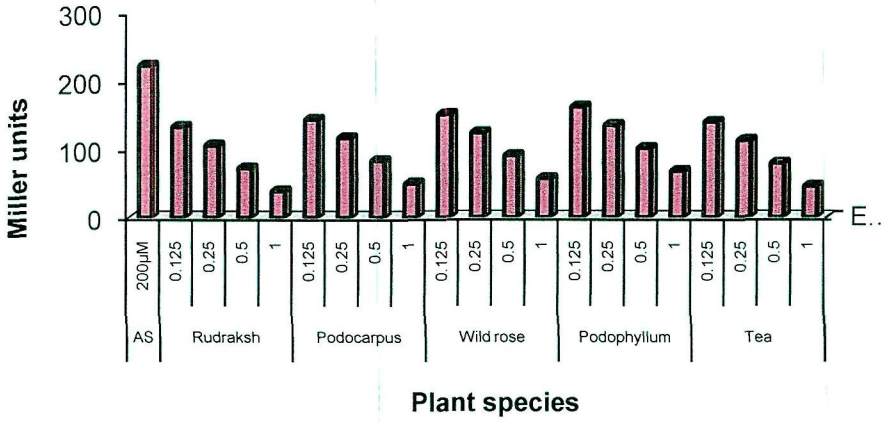


Fig.9 ***Vir G*** **gene activity in activity in E498 strain in response to leaf polyphenols of different plant species and autoclaved L-glutamine**

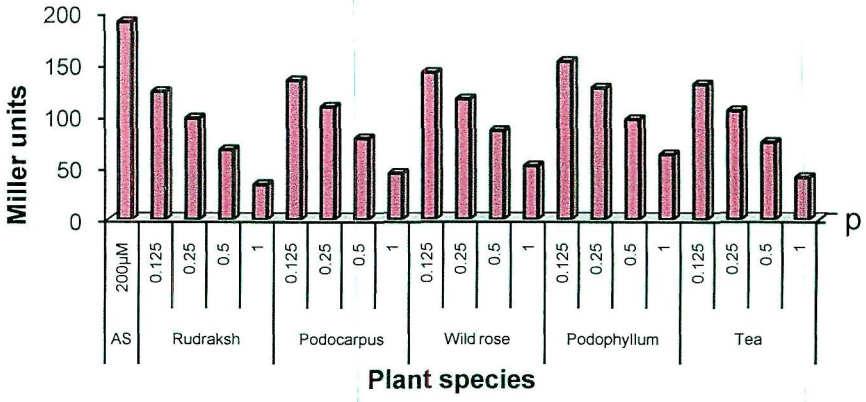


Fig.10 ***Vir pinF*** **gene activity in activity in pSM 229 strain in response to leaf polyphenols of different plant species and autoclaved L-glutamine**

4.10 Kit for the detection of 2-amino glutarimide formation after autoclaving L-glutamine for 20 and 40 minutes

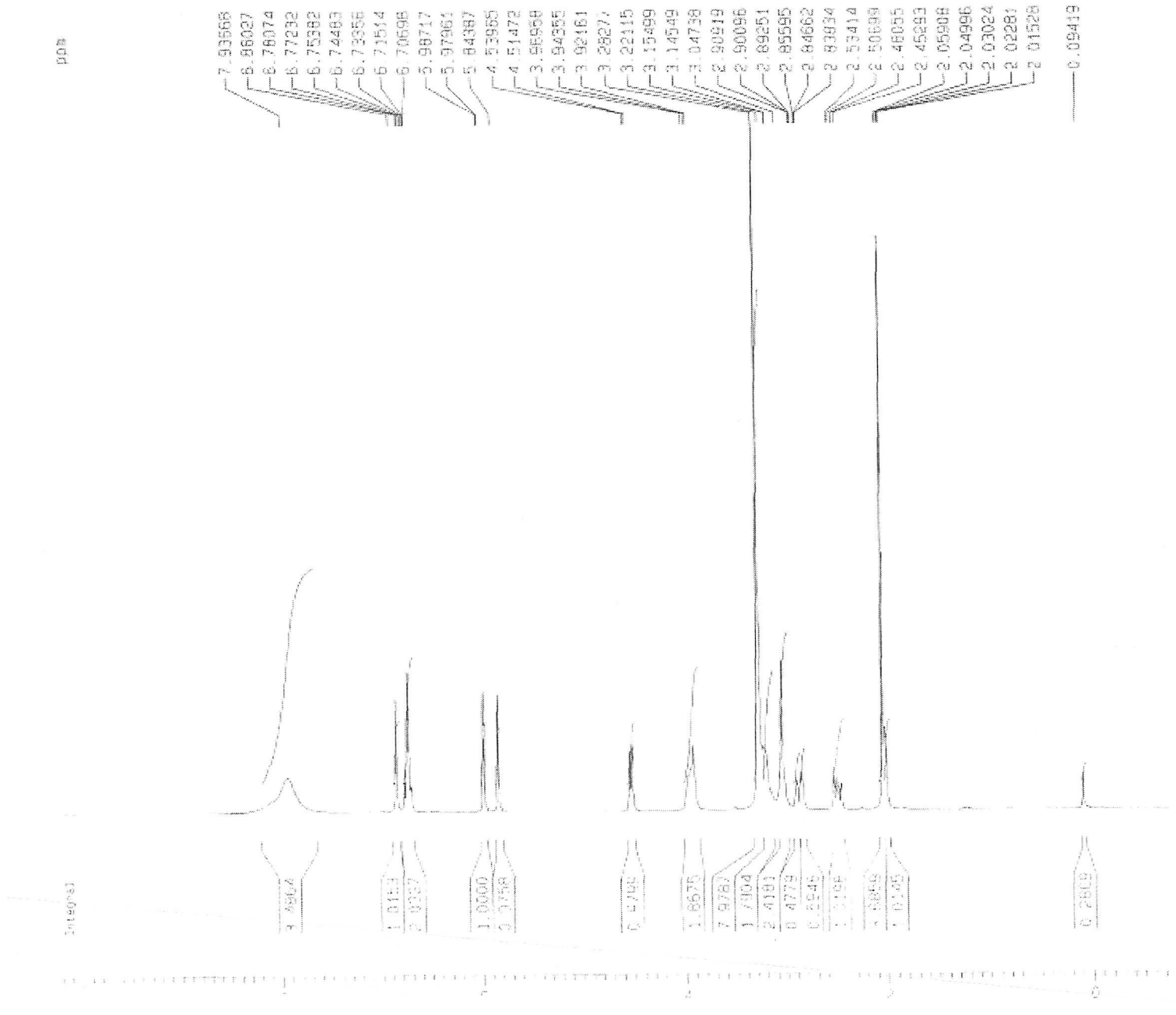
Distinct colour was observed when 2 g/L of L-glutamine was autoclaved for 20 and 40 min. and added separately to 100 ml of tea polyphenols. While the colour changed from green to brown in case of L-glutamine autoclaved for 20 min, no change was observed in the one autoclaved for 40 min. Rather it remained as green as pure tea polyphenols where no autoclaved (20 and 40 min) L-glutamine was added **(Plate 5).** However, after 24 hrs, the colour started changing to brown indicating that the compound formed after 40 min were unstable after 24 hrs.

Thin layer chromatographic confirmation of the role of 2-amino glutarimide

The TLC plates spotted with L-glutamine autoclaved for 40 min showed pink spots when dried at 110°C, whereas, the freshly prepared un-autoclaved L-glutamine gave blue/violet spots with an Rf value of 0.411 On the other hand, TLC plates spotted with individual catechins gave yellow spots with an Rf value of 0.637 when dried at 100°C. The TLC plates when spotted with a mixture of catechins and autoclaved L-glutamine (40 min.) gave a dark pink colour with Rf value of 0.209. This indicated some reaction between the catechins and L-glutamine autoclaved for 40 min .

Nuclear Magnetic Resonance

When L-glutamine was autoclaved for 20 min qualitative and quantitative differences were observed in the 1H and 13C NMR spectra of catechin+2-amino glutarimide, catechin+5-oxo proline and catechin standard. The NMR spectra indicated interaction between catechin and 2-amino glutarimide.



1H

Current Data Parameters

NAME	cate
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20090820
Time	14.34
INSTRUM	av300
PROBHD	5 mm PHBBO BB-
PULPROG	zg30
TD	65536
SOLVENT	Acetone
NS	32
DS	2
SWH	5095.204 Hz
FIDRES	0.077780 Hz
AQ	5.4657536 sec
RG	45.3
DW	83.400 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

======== CHANNEL f1 ========

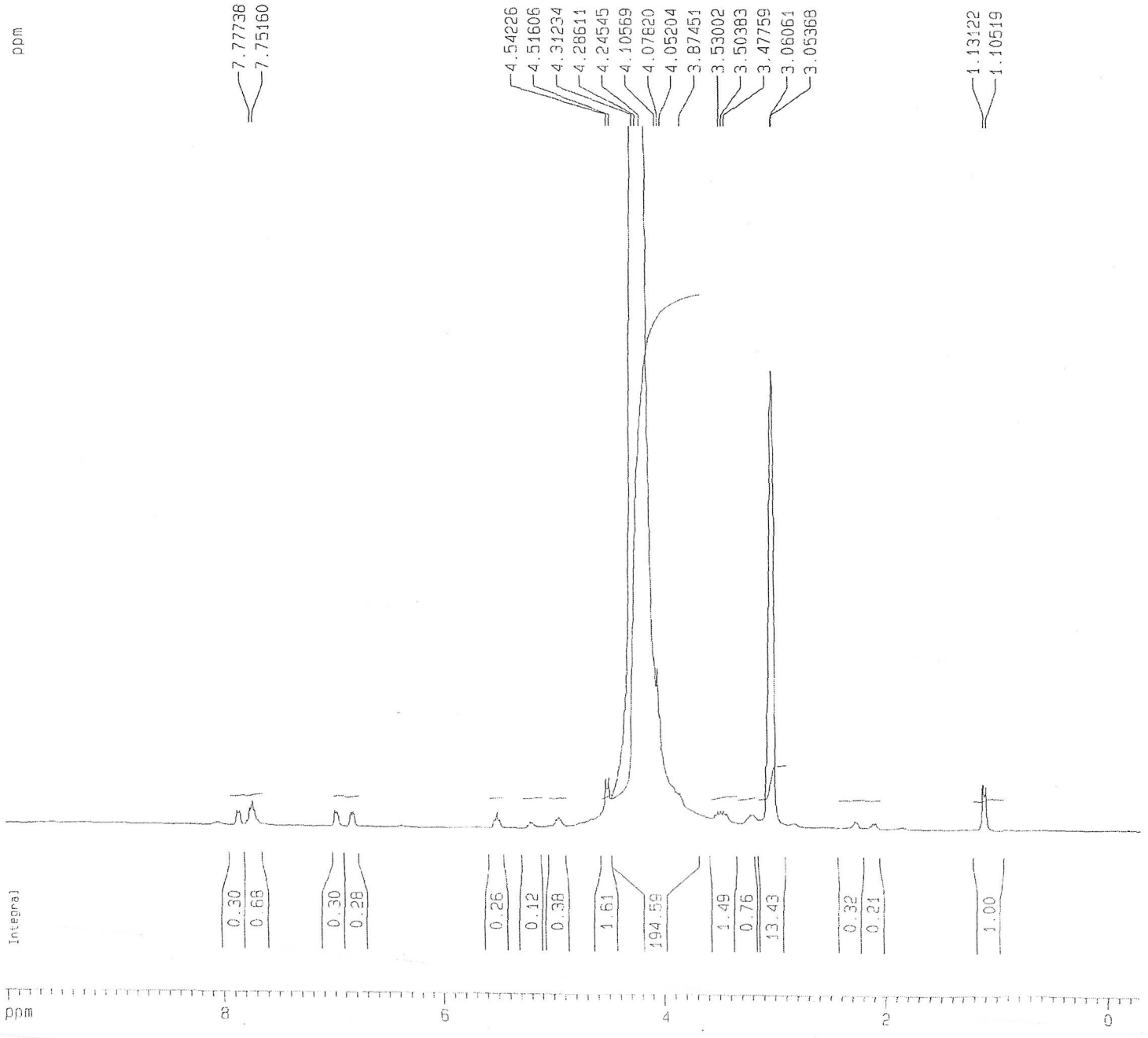
NUC1	1H
P1	12.50 usec
PL1	2.00 dB
SFO1	300.1321809 MHz

F2 - Processing parameters

SI	32768
SF	300.1300243 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
CY	12.50 cm
F1P	10.221 ppm
F1	3067.64 Hz
F2P	0.702 ppm
F2	-225.73 Hz
PPMCM	0.54800 ppm/cm
HZCM	164.66850 Hz/cm



1H

Current Data Parameters

NAME	cate2am
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20090729
Time	14.41
INSTRUM	av300
PROBHD	5 mm PHBBO BB-
PULPROG	zg30
TD	65536
SOLVENT	Acetone
NS	16
DS	2
SWH	5995.204 Hz
FIDRES	0.091480 Hz
AQ	5.4657526 sec
RG	101.6
DW	83.400 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

======== CHANNEL f1 ========

NUC1	1H
P1	12.50 usec
PL1	2.00 dB
SFO1	300.1321009 MHz

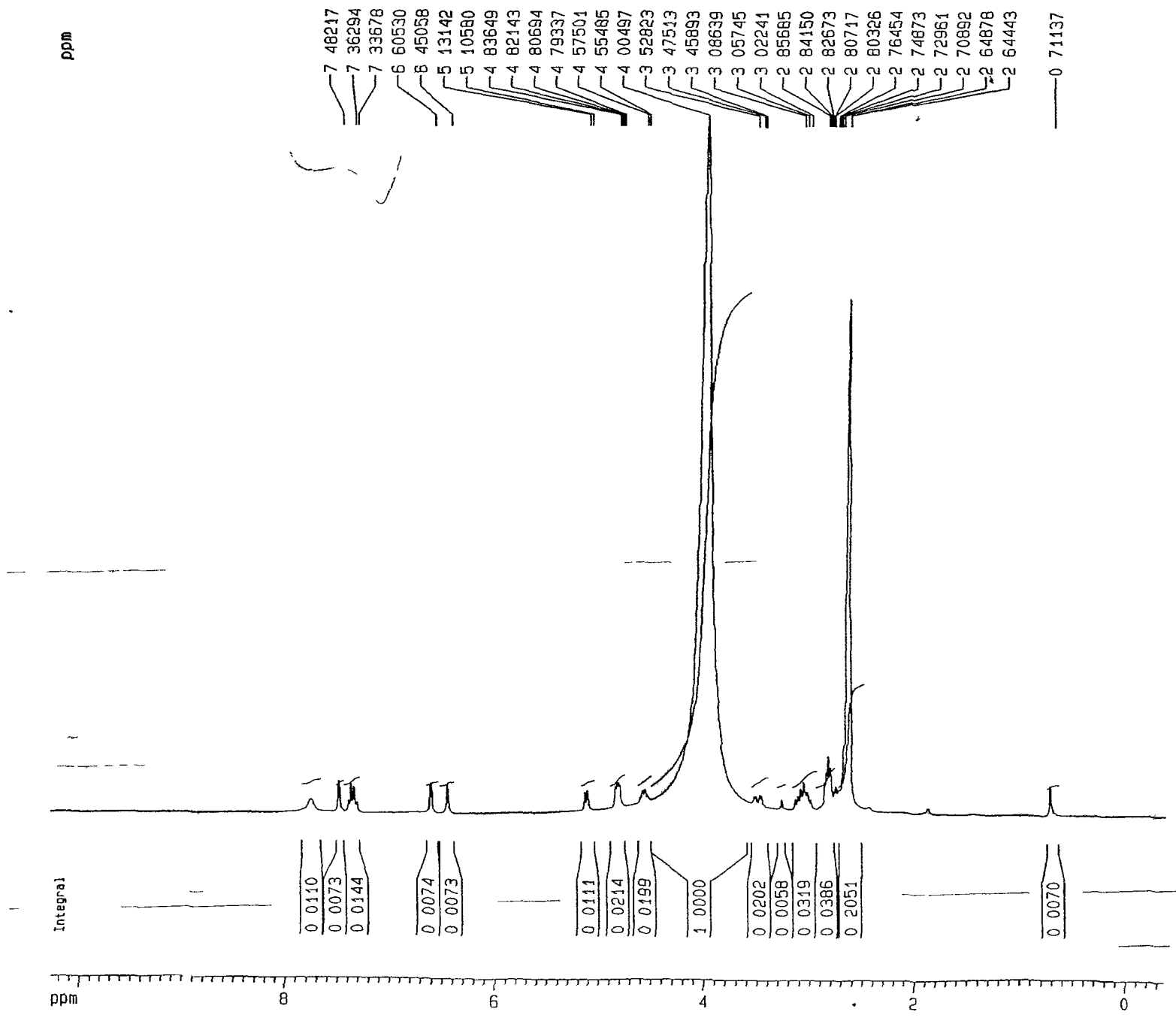
F2 - Processing parameters

SI	32768
SF	300.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
CY	100.00 cm
F1P	10.035 ppm
F1	3011.78 Hz
F2P	-0.284 ppm
F2	-85.19 Hz
PPMCM	0.51594 ppm/cm
HZCM	154.84850 Hz/cm

1D



1H

Current Data Parameters

NAME	cate6
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20090722
Time	16 48
INSTRUM	av300
PROBHD	5 mm PHBBO BB-
PULPROG	zg30
TD	65536
SOLVENT	Acetone
NS	16
DS	2
SWH	5995 204 Hz
FIDRES	0 091480 Hz
AQ	5 4657526 sec
RG	128
DW	83 400 usec
DE	6 00 usec
TE	300 0 K
D1	1 00000000 sec

======== CHANNEL f1 ========

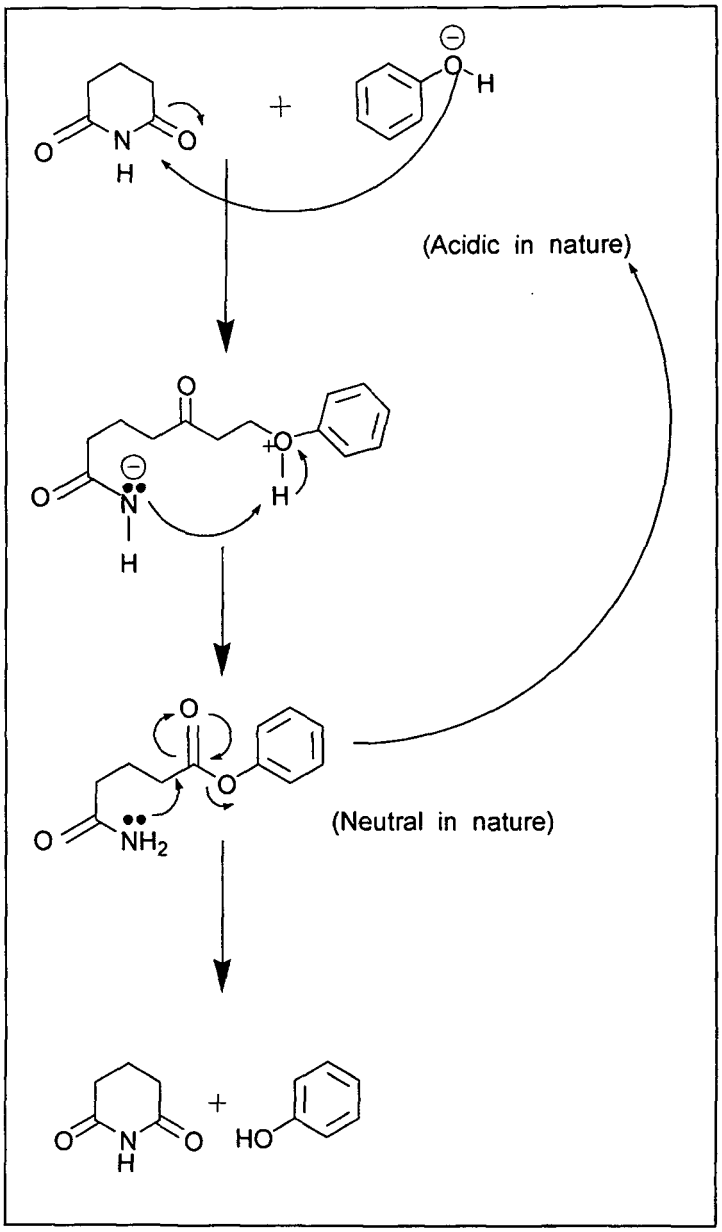
NUC1	1H
P1	12 50 usec
PL1	2 00 dB
SFO1	300 1321009 MHz

F2 - Processing parameters

SI	32768
SF	300 1300000 MHz
WDW	EM
SSB	0
LB	0 30 Hz
GB	0
PC	1 40

1D NMR plot parameters

CX	20 00 cm
CY	12 50 cm
F1P	10 276 ppm
F1	3084 12 Hz
F2P	-0 378 ppm
F2	-113 32 Hz
PPMCM	0 53268 ppm/cm
HZCM	159 87210 Hz/cm



Proposed mechanism for polyphenol quenching by 2-amino glutarimide

Plate 5 : A kit for the confirmation of compounds synthesized by autoclaving L-glutamine for 20 and 40 min (A) Leaf extract (B) Leaf extract+ 5-oxoproline (C) Leaf extract+ 2, amino glutarimide

Chapter-5

Discussion

5. DISCUSSION

The hypothesis that polyphenols and their oxidized products influence the *in vitro* response of plants/explants and also inhibit *Agrobacterium* growth and infection was investigated and proven in the present study. Such response under *in vitro* conditions is particularly, crucial because it often inhibits the regeneration of tissue cultured plants. It also requires extensive manipulations and interventions (Largimini, 1991). Explants experience different degrees of stresses during their excision from *in vivo* plants and surface sterilization (Bolwell et al., 1995; Baker and Orlandi, 1995; Doke et al., 1996; McKersie and Leshem, 1994 Yahraus et al., 1995).This triggers a 'defense response' and in turn induces the production as well as oxidation of polyphenols. Thus, in the present study, it was a small wonder that high polyphenol containing leaf explants of *E. sphaericus, P. macrophyllus, C. sinensis, Rosa* sp. and *M. domesticus* turned brown during the process of surface sterilization and/or co-cultivation. Consequently, *Agrobacterium* infection was also inhibited.

Plant polyphenols are basically heterogeneous group of defense compounds that are biosynthesized in response to different biotic and abiotic stresses ((Mondolot *et al.*,2006, Cheruiyot *et al.*, 2007). Being universally or widely distributed among plants, these are known to impart protection against herbivores, microbes, viruses and competing plants (Wink, 1999). Polyphenols also exhibit a remarkably wide range of biological activities including the antimicrobial, antitumour, immunomodulatory and enzyme inhibitory properties (Haslam *et al.*,1989; Huang *et al.*, 1992: Haslam *et al.*,1996). Thus, it is but natural that plants or their healthy parts would ward off *Agrobacterium*, a commonly occurring soil phytopathogen from infecting them. The easiest way to do so would be to exude large amounts of bactericidal or bacteristatic polyphenols or produce harmful oxidized products. Physical damage or wound induces the oxidation of polyphenols and, consequent deposition of brown colored phenolic acids or quinones (Ozawa *et al.*,1987). These are known to be highly detrimental to the growth of microbes including *Agrobacterium.* The oxidation of phenols to quinones is generally catalyzed by

the nuclear encoded copper-containing enzyme 'Polyphenol oxidase or PPO' and is widely distributed in plants (Kim et al., 2001). Such inhibition of *Agrobacterium* mediated genetic transformation by host plant polyphenolic compounds is not unnatural. This is because several of the tropical crops that have proven recalcitrant are actually high polyphenol containing ones (Biao et al 1998; Brencic et al 2004; Sandal et al., 2007; Sood et al., 2009).

PPO is localized in the plastids, whereas, its' phenolic substrates are mainly present in the vacuoles (Lobreaux et al., 1995). PPO-mediated browning reaction occurs only when the sub-cellular compartmentation is lost due to damages like wounding (Kim et al., 2001). The quinones that are formed due to polyphenol oxidation by PPO activity are highly toxic and generally affect the tissues negatively. Further polymerization of quinones cause discoloration of the medium and death of the cultured explants (Ozyigit et al., 2007). However, metabolized phenols may affect tissue culture systems positively with auxin metabolism and hence promote rapid cell division and synthesis of cell wall and other related components (Ozyigit et al., 2007). PPO is also important in pigment formation, oxygen scavenging, pseudocyclic phosphorylation in chloroplasts, and defense mechanism against insects and plant pathogens (Tang and Newton, 2004; Kefeli et al., 2003; Yoruk and Marshall, 2003). Since the activity of PPO is totally dependant on the availability of tissue polyphenol (Sood et al., 2009), the leaf explants with higher polyphenol content (*E. sphaericus*) showed higher PPO activity and no *Agrobacterium* infection (Fig.3 and Plate 1). On the other hand, the low polyphenol containing *P. hexandrum* leaves with low PPO activity were susceptible to *Agrobacterium* infection (Fig.3 and Plate 2). Interestingly, even the *P. hexandrum* leaf polyphenols were inhibitory at higher concentrations (Table 3).

Thus, it was proposed that the high contents of polyphenols in the recalcitrant leaf explants were bactericidal and prevented the *Agrobacterium* from attaining an infecting density. The importance of appropriate cell density for successful *Agrobacterium* mediated transformation is a well documented fact (Amoah *et al.*,2001.). Thus, when the growth curves in response to different concentrations of exogenously added leaf polyphenols were studied, suppression of *Agrobacterium* growth at higher concentrations

of polyphenols but normal growth in their absence was recorded (Table 3&4). *Agrobacterium* also failed to grow on the explants with high polyphenols and PPO activity (Plate 1). Consequently, it was concluded that polyphenols and their oxidation products were indeed bactericidal and prevents the *Agrobacteria* from growing to a density that is normally observed during the log phase (i.e., 18hrs, in the present study). Since *Agrobacteria* failed to attain an appropriate density, it failed to infect the resistant explants and no GUS expression was observed Rather, the high contents of polyphenols and PPO activity led to subsequent browning and death of the explants (Fig. 2&3 and Plate 1). Probably, the phenol detoxifying genes of *Agrobacterium* also fail to function normally at high concentrations of polyphenols present in plants like *E. sphaericus, P. macrophyllus* and *C. sinensis.* Indeed, the *pinF or H* gene and *lacZ* fusions showed significant suppression of ß-galactosidase activity in the presence of leaf polyphenols from resistant leaf explants. In contrast, the activity was higher when these leaf polyphenols were absent. On the other hand, no suppression of ß-galactosidase activity in the presence of *P. hexandrum* leaf polyphenol indicated that the function of *virH* gene of *Agrobacterium* was not disturbed. Thus, it could easily detoxify the polyphenols present in the *P. hexandrum* leaf explants (Fig 5,6&7).

Generally, the problem of polyphenol oxidation and browning of explants under *in vitro* conditions is solved by using charcoal, PVP or antioxidants in the culture medium (Pan and Staden 1998; Madhusudhan *et al.*,2004; Abdelwahd *et al.*,2008). However, these were ineffective in overcoming the inhibitory effects of polyphenols on *Agrobacterium* infection (Kumar 2003). The same year, Sandal (2003) from this laboratory demonstrated the use of 2-amino glutarimide or over autoclaved L-glutamine for successful *Agrobacterium* infection of the Kangra jat cultivar of tea. She proposed that since *Agrobacterium* cell density increases in response to 2-amino glutarimide, a tilt in the ratio of *Agrobacterium* cell density and toxic polyphenol exudates render the polyphenols in the co-cultivation medium, non toxic. When this hypothesis was tested using several high polyphenol containing plants in the present study, this was indeed proven true. Thus, when an optimized dose of 2-amino glutarimide was added to the *Agrobacterium* culture along with toxic levels of leaf polyphenols from different plants,

the suppressing effect of polyphenols on *Agrobacterium* growth was alleviated. As a result, an increase in growth was observed. In contrast, significant inhibition of *Agrobacterium* growth by high concentrations of polyphenols without 2, amino glutarimide was recorded (Table 6&7). Moreover, this compound was also effective in bringing about *Agrobacterium* infection in all the plants when added to the co-cultivation medium (Plate 3). Consequently, strong GUS expression was recorded in all the studied plants. Interestingly, the intensity of GUS expression was also dependant on the polyphenol content of the explant. Thus, while high expression was observed in *P. hexandrum*, that in *E. sphaericus* was low.

Plausible reasons for the successful *Agrobacterium* mediated genetic transformation of recalcitrant species by 2, amino glutarimide was also proposed. Saini (2007) and Sandal et al. (2007) from this laboratory considered the toxic polyphenols as 'xenobiotics' for *Agrobacterium*, and 2 amino glutarimide as the 'detoxifier'. Saini (2007) further proposed a mechanism of polyphenol detoxification by exogenously added 2-amino glutarimide. 'Xenobiotics' are compounds foreign to an organism's normal biochemistry and may often include certain drugs and poisons (Parvez and Ressis 2001). A set of metabolic pathways that modify the chemical structure of the 'xenobiotic' in question constitute the detoxification process. Considered to be of ancient origin, these pathways are present in all major groups of organisms including bacteria (Tschech and Fuchs 1987; Coleman 1997; Rousseaux et al 2001). Even certain biotransformation processes bring about the xenobiotic detoxification of poisonous compounds (Richardson,1996 and Loannides, 2001). At times, the intermediates in xenobiotic metabolism can themselves have toxic effects.

Xenobiotic metabolism is divided into three phases (Richardson,1996). In phase I, enzymes such as cytochrome P450 oxidase introduce reactive or polar groups into xenobiotics. These modified compounds are then conjugated to polar compounds in phase II reactions. These reactions are catalyzed by transferase enzymes such as glutathione S-transferase. Finally, in phase III, the conjugated xenobiotic is further processed, recognized by efflux transporters and pumped out of cells. These pathways are

particularly important for applications in environmental science because xenobiotic metabolism of microorganisms can determine whether a pollutant will persist in the environment or be broken down during bioremediation. In the present study, however, the phenol detoxifying gene i.e., *vir H* or *pinF* of *Agrobacterium* failed to remove the toxic polyphenols from its environment. Rather, such mechanism was externally facilitated by 2, amino glutarimide. Glutarimides are particularly, known to have xenobiotic properties (Kalgutkar et al 2002). Thus, Saini (2007) proposed that the potent moiety with –N-H group of 2, amino glutarmide formed due to complete cyclization of L-glutamine during autoclaving for 40 minutes reacts with the –OH group of polyphenol. Binding of one free radical of glutarimide and one highly reactive positively charged radical of epicatechin through the release of a water molecule results in the formation of concatamers which in turn reduces the overall concentration of the polyphenols. This thereby, renders the polyphenols either non toxic or less toxic to the *Agrobacterium.* As a result, the recalcitrant explants become susceptible to *Agrobacterium* infection. The present study was also successful in proving that even in presence of bactericidal leaf polyphenols, *Agrobacterium* growth could be actually enhanced to an infecting density by 2, amino glutarimide (2.0 g/l).

Experiments on *Agrobacterium* mediated transformation of high polyphenol containing leaf explants of *E. sphaericus, P. macrophyllus, C. sinensis, Rosa* spp. and *M. domesticus* also showed that the explants were not as damaged in the presence of 2, amino glutarimide as in its absence. TLC and NMR further confirmed the quenching ability of 2, amino glutarimide. The xenobiotic epicatechins molecules in the polyphenol exudates from tea leaf explants were indeed quenched if 2, amino glutarimide was present in the co-cultivation medium. As most of the polyphenols in the leaf exudates were quenched, the *Agrobacteria* grew to an optimal density for cell-cell contact. Consequently, even the resistant/recalcitrant explants were infected and GUS expression was obtained in all of them. The observation that maximum and fastest *Agrobacterium*

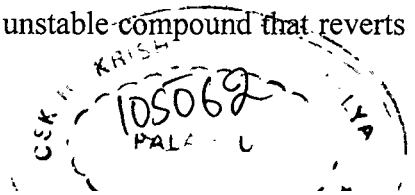
growth in case of *P. hexandrum* but slowest and lowest growth in *E. sphaericus* probably indicated that while all or most of the polyphenols were quenched in the former, some remained in case of the latter. Browning of *E. sphaericus* leaves during transformation as compared to healthy green leaves in *P. hexandrum* appears to further confirm this assumption.

An important matter of concern i.e., the *vir* inducing ability of 2 amino glutarimide was also refuted in the present study. That this compound is certainly not a '*vir* inducer' was proven using the gene fusion cassettes of *virA*, *G* and phenol detoxifying *vir* genes (*pinF or H*) and *lacZ*. The gene fusion assays showed significantly lower β-galactosidase activity when 2 amino glutarimide was added to the assay mixture along with polyphenols as compared to the ones containing only polyphenol. Lower β-galactosidase activity in case of polyphenol and polyphenol + 2 amino glutarmide positively confirmed that 2–amino glutarimide as well as used concentration of leaf polyphenols were non-inducers of *vir* genes. When either or both polyphenol or 2, amino glutarimide were absent, the β-galactosidase activity was highest in presence of acetosyringone, the popular inducer. Thus, it could be concluded that while leaf polyphenol suppressed the induction of *vir* genes, 2, amino glutarimide suppressed their induction further. The mean suppression in the former was about 30 fold and that in the latter was 41 fold. Consequently, highest β-galactosidase activity was recorded in case of *P. hexandrum* with lowest polyphenol and lowest activity in *E. sphaericus* with highest polyphenol.

Another observation that emerged during the course of the study in this laboratory was that incomplete autoclaving of L-glutamine yields a partially cyclized compound i.e., 5-oxoproline (Sandal 2003). Interestingly, the findings of the present study showed that this compound did not have the ability to quench the xenobiotic leaf polyphenols. Rather, the compound has a close resemblance with opines having lactam rings i.e., agropine and agropinic acid or the lactonized forms of mannopine and mannopinic acid. The latter are again the condensation products of glutamine/glutamic acid and mannose (Dessaux *et al.*, 1998)). While lactonization of opines make them specific substrates for only

Agrobacterium, lactones like the 'acyl homoserine lactone' serve as its quorum signal (Zhang *et al.*, 2001 and Brencic *et al.*, 2004). In the present study, the 5-oxoproline which was found to induce higher *Agrobacterium* growth than untreated control (Fig.4) probably served as either 'opine' or 'quorum sensing signal (QS)'. However, this enhanced induction of *Agrobacterium* growth by 5-oxoproline was always lower than that of 2, amino glutarimide. One possible explanation for this observation is that while 5-oxoproline cannot quench toxic polyphenols from the environment of the *Agrobacterium*, it can probably act as QS for the induction of vegetative growth. Thus, in the present study, 5-oxoproline triggered *Agrobacterium* growth to a level that is optimum for infection, once the toxic levels of polyphenols were reduced from the environment by phenol quenching ability of 2, amino glutarimide.

One matter of concern that was further addressed in the present study is that *Agrobacterium* do not induce vir genes or infect its host plants when they are in a state of vegetative growth. However, it is now known that the att J and M genes regulate the start and end of vegetative growth in response to quorum signals (Zhang, *et al.,* 2002). Probably, while the attJ gene continued to maintain vegetative growth in presence of 5-oxoproline, the attM gene provided the signal for vegetative growth to stop and the process of T-DNA transfer to start. Since the two compounds i.e., 5-oxoproline and 2 amino glutarimide are formed due to autoclaving, the presence of both in a single sample was not impossible. Therefore, it was extremely crucial to have proper identification steps or methods for these compounds. In this regard, a kit based on colour changes was developed in the present study to determine the formation of the two compounds by complete or incomplete autoclaving of L-glutamine. The kit reproducibly shows that while polyphenols extracted from fresh leaves remained green in presence of 2, amino glutarimide for 24 hrs, that in presence of 5-oxo-proline turned brown, thereby, confirming the synthesis of the two compounds by autoclaving. The ability or failure to quench polyphenols was hypothesized to be the reason for the colour change in the kit. 2-amino glutarimide is an unstable compound that reverts back to its more stable form i.e.,



5-oxoproline after some time. As a result, they probably lose their quenching ability after 24hrs. However, this did not hamper the transformation process probably because 24 hrs duration was sufficient enough for establishing the 'cell-cell contact between the *Agrobacteria* and the host plant' and for initiating genetic transformation.

In conclusion, the study confirmed that the (i) plants/explants containing high polyphenols are recalcitrant to *Agrobacterium* infection, (ii) polyphenols and their oxidized products do inhibit *Agrobacterium* growth, (iii) the phenol detoxifying genes of *Agrobacteium* fail to detoxify the bactericidal levels of polyphenols (iv) the bactericidal levels of these polyphenols can be quenched or detoxified by 2-amino glutarimide formed by autoclaving L-glutamine for 40 minutes but not by 5-oxoproline, (v) *Agrobacterium*, like most gram negative bacteria, probably employs its unique quorum sensing system once the toxicity of the tea polyphenols is reduced by 2-amino glutarimide(vi) finally, neither 5-oxoproline nor 2 amino glutarimide are vir inducers.

Chapter-6

Summary and Conclusions

6. SUMMARY AND CONCLUSIONS

The present study investigated and confirmed the hypothesis that extremely high contents of polyphenols are responsible for the resistance of several dicot plant species to *Agrobacterium*-mediated-genetic-transformation. During surface sterilization, genetic transformation and co-cultivation, large amounts of these explant polyphenols come in contact with the vacuolar PPO. As a result, brown coloured toxic compounds are produced in the presence of air. Since these compounds are known to interfere with the physiology and biochemistry of invading microorganisms, their role in *Agrobacterium* growth and transformation was investigated in the present thesis.

For this, supposedly high polyphenol containing leaves of different dicot plants were first selected. These were then transformed using the virulent strain, GV3101 of *Agrobacterium tumefaciens* harbouring the construct, pBI121 with *gus* reporter gene. Expression of *gus* gene was used to directly measure *Agrobacterium's* ability to transform supposedly high polyphenol containing explants of *Elaeocarpus sphericus, Podocarpus macrophyllus, Rosa* sp. *Podophyllum hexandrum* and five important cultivars of tea (i.e. TV1, T-78, U9, U10 and KJ). Since there are a number of reports on successful transformation of *Malus domesticus,* their available rootstocks B9 and MM106 were used as controls. None of these explants with exception to *P. hexandrum* showed *Agrobacterium* growth. Even in *P. hexandrum,* the growth was delayed by 15 days. In *gus* gene assays also, only the *P. hexandrum* leaves showed GUS expression in the form of small blue sectors on the leaf blades.

Studies were also conducted to correlate the effects of freshly extracted leaf polyphenols and PPO activities with *Agrobacterium* growth. Irrespective of plant species, susceptibility of explants to *Agrobacterium* infection appeared to depend on PPO activity and their substrate (i.e. leaf polyphenols in this study). Thus, the *E. sphaericus* leaves contained the maximum amounts of polyphenols, showed maximum PPO activity and were most resistant to *Agrobacterium* infection. In contrast, the *P. hexandrum* leaves

possessed the minimum contents of polyphenols, showed lowest PPO activity and were reasonably susceptible to *Agrobacterium* infection. These leaves also showed earliest *Agrobacterium* growth and infection. Invariably, explants with high polyphenols and PPO activity turned brown and necrotic during the process of surface sterilization and co-cultivation, and failed to show any signs of *Agrobacteirum* infection. Strong GUS expression in *P. hexandrum* leaves within 15 days but no expression in *E. sphaericus* leaves further suggested that exudation and oxidation of polyphenols was indeed a major obstacle in *Agrobacterium* mediated genetic transformation of plants.

Distinct suppression of *Agrobacterium* growth by leaf polyphenols from different plant species was actually confirmed through spectro-photometric and plate count methods. These studies also showed that concentrations rather than the type of polyphenols posed a 'major hindrance' to *Agrobacterium* infection. Thus, irrespective of plant species, *Agrobacterium* growth was inhibited. The *P. hexandrum* leaf polyphenols were the only exception which posed no inhibition to *Agrobacterium* growth at concentrations below 1.0 mg/ml. Although these *Agrobacterium* growth at concentrations below 1.0 mg/ml. Although these polyphenols supported *Agrobacterium* growth that was at par with control at 0.125 mg/ml, it proved growth inhibitory at 1.0 mg/ml. However, irrespective of plant species, this growth suppressing effects of leaf polyphenols was largely overcome by 2-amino glutarimide. Particularly, 2 g/l 2-amino glutarimide was optimized as the most effective concentration for promoting *Agrobacterium* growth to an optimal density. This was evident from bacterial growth curves as well as cfu plate counts. The presence of 2 g/l 2-amino glutarimide in the co-cultivation medium was also successful in facilitating *Agrobacterium* infection of the leaves of all the studied plants, irrespective of their polyphenol contents. This was further evident from the GUS expression of the leaves. However, the intensity of the GUS expression was dependent on the polyphenol content of the leaves. Thus, while highest expression was observed in the *P. hexandrum* leaves, lowest expression was observed in *E. sphaericus* leaves.

The present study also confirmed the earlier findings of this laboratory that over-autoclaving of L-glutamine for 40 min. resulted in complete cyclization. As a result, an unstable compound with distinct fragrance and colour was produced. On the other hand,

autoclaving for 20 min. resulted in complete cyclization and formation of a stable compound. These were identified as '2-amino glutarimide' and 5-oxo-proline using 1H and 13C NMR. The hypothesis that 2-amino glutarimide is a potent xenobiotic detoxifier of bactericidal polyphenols was also confirmed. TLC and NMR of the interaction between 1 mg/ml pure catechins and 2 g/l 2-amin glutarimide was used for this confirmation. On the other hand, 5-oxoproline lacked the polyphenol quenching ability. Rather, based on the structural similarities between 5-oxoprolinea nd opines-with lactum rings, it was hypothesized that this compound probably served as a quorum signal. Since the two compounds were produced by complte and incomplete autoclaving, a colour based diagnostic kit was developed for detecting their formation.

An important cause of concern that 2-amino glutarimide and 5-oxo-proline are not *vir* inducers was also clarified using the gene fusion assay of *Lac* Z and *vir A, G* and *pinF* genes. Comparatively lower β-galactosidase activity in the presence of 2-amino glutarimide in assay mixtures with and without leaf polyphenols confirmed this observation. Differences in β-galactosidase activity in response to polyphenols specific to a plant indicted strong interactions between the *vir* genes and the amount of polyphenols. Furthermore, suppressed β-galactosidase activity of *LacZ:pinF* gene indicted that *Agrobacterium's* own phenol detoxifying mechanism failed to function in the presence of toxic or bactericidal levels of polyphenols.

In conclusion, the present study showed that supplementation of 2-amino glutarimide to the co-cultivation medium is an innovative approach for overcoming the resistance of high polyphenol containing leaf explants to *Agrobacterium* infection.

KAISM
105062
PAL

Literature Cited

LITERATURE CITED

Abdelwahd R, Hakam N, Labhilili M and Udupa SM. 2008. Use of an adsorbent and antioxidants to reduce the effects of leached phenolics in *in vitro* plantlet regeneration of faba bean. *African Journal of Biotechnology* 7 : 997-1002.

Abram V and Donko M. 1999. Tentative identification of polyphenols in *Seupervivum tectorum* and assessment of the antimicrobial activity of *Sempervivum* L. *Journal of Agriculture and Food Chemistry* 42 : 485-489.

Amoah BK, Wu H, Sparks C and Jones HD. 2001. Factors influencing *Agrobacterium*-mediated transient expression of *uid*A in wheat inflorescence tissue. *Journal of Experimental Botany* 52 :1135-1142

An-Bong J, Kwak JH, Son JH, Park JM, Lee JY, Jo CR and Byun MW. 2004. Biological and anti-microbial activity of irradiated green tea polyphenols. *Food Chemistry* 88 : 549-555.

Arnaldos TL, Munoz R, Ferrer MA and Calderon AA. 2001. Changes in phenol content during strawberry (*Fragaria x ananasa*, cv. Chandler) callus culture. *Physiol. Plant* 113 : 315-322.

Ashby AM, Watson MD, Loake GJ and Shaw CH.. 1988. Ti-plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58C toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. *Journal of Bacteriology* 170 : 4181-4187.

Ashraf M, Mazhar H, Naqvi M and Mohsin I. 2001. Microbial biofilms for environmental waste management: an overview. *The Nucleus* 38 : 131- 135.

Baker CJ and Orlandi EW. 1995. Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* 33: 299–321.

Batchvarov V and Marinova G. 2001. Healthy ingredients in malt, hops and beer. *Khranite Inovkusova promishlenost* 50 : 15-17.

Berthelot K, Buret D, Guerin B, Delay D, Nergel J and Delmotte FM. 1998. *Vir* gene inducing activity of hydroxylcinnamic acid amides in *Agrobacterium tumefaciens. Journal of Phytochemistry* 49 : 1537-1548.

Biao X, Toru K, Jain X and Yougyan B. 1998. Effect of polyphenolic compounds in tea transformation. In American Society of Plant Physiologists, *Plant Biology* Abstr. No. 314.

Binns AN. 1990. *Agrobacterium* mediated gene delivery and the biology of host range limitations. *Physiol. Plant* 79: 135-139.

Blumwald E, Aharon GS and Lam BCH. 1998. Early signal transduction pathways in plant–pathogen interactions. *Trends in Plant Sciences* 3 : 342-346.

Bolton GW, Nester FW and Gordon MP. 1986. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science* 232 : 983-985.

Bolwell GP, Butt VS, Davies DR and Zimmerlin A. 1995. The origin of the oxidative burst in plants. *Free Rad. Res.* 23: 517–532.

Bravo L. 1998. Polyphenol: chemistry, dietry sources, metabolism and nutritional significance. *Nutr. Rev.* 56 : 317-333.

Brencic A, Eberhard A and Winanas SC. 2004. Signal quenching, detoxification and mineralization of VirH2 protein of *Agrobacterium tumefaciens. Molecular Microbiology* 51 : 1103-1115.

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJJ. 1995. Transkingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae. EMBO J* 14 : 3206–3214.

Canhoto C and Graca MAS. 1999. Leaf barriers to fungal colonization and shredders (*Tipula lateralis*) consumption of decomposing *Eucalyptus glofulus. Microb. Ecol.* 37 : 163-172.

Capasso R, Evidente A, Schivo L, Orru G, Marcialis MA and Cristinzio G. 1995. Antibacterial polyphenols from olive oil waste mill waste waters. *Journal of Applied Bacteriology* 79 : 393-398.

Capassor R, Evidentge A, Tremblay E, Sala A, Santoro C, Cristinzio G and Scognamiglio F. 1994. Direct and mediated effect on *Bactrocera oleae* of natural polyphenols and some of related synthetic compounds: structure activity relationships. *Journal of Chemical Ecology* 20 : 1189-1199.

Castle LA, Smith KD and Morris RO. 1992. Cloning and sequencing of an *Agrobacterium tumefaciens* β-glucosidase gene involved in modifying a *vir*-inducing plant signal molecule. *Journal of Bacteriology* 174 : 1478-1486.

Chakrabarty R, Viswakarma N, Bhat SR, Kirti PB, Singh BD and Chopra VL. 2002. *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *Journal of Biosciences* 27: 495-502.

Chen X, Stone M, Schlagnhaufer C and Romaine CP. 2000. A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. *Applied Environmental Microbiology* 66: 4510–4513.

Cheruiyot EK, Mumera ML, Ne'gtich KW, Hassanali A and Wachira F. 2007. Polyphenols as potential indicators for drought tolerance in Tea (*Camellia sinensis* L.). *Biosci. Biotechnol. Biochem.* 71 (9): 2190-2197.

Chilton MD, Drummond MH, Merio DJ, Sciaky D, Montoya AL, Gordon MP and Nester EW. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 11: 263–271.

Chung KT, Wong TY, Wei C, Hunag YW and Lin Y. 1998. Tannins and human health: a review. *Crit. Rev. Food Sci. Nutr.* 38(6): 421-464.

Citovsky V, McLean BG, Greene E, Howard E, Kuldau G, Thorstenson Y, Zupan J and Zambryski PC. 1992a. *Agrobacterium*-plant cell interaction: induction of *vir* genes and T-DNA transfer. *In* DPS Verma, ed, Molecular Signals in Plant-Microbe Communications. CRC Press, Inc., Boca Raton FL, pp 169–198.

Coleman JDD. 1997. Detoxification of Xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science* 2: 144-151.

Crozier A, Burns J, Aziz AA, Stewart AJ, Rabiasz HS, Jenkins GI, Edwards CA and Lean Me J. 2000. Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. *Biological Research* 33: 79-88.

Dangl JL, Dietrich RA and Richberg MH. 1996. Death doesn't have no mercy: Cell death programs in plant–microbe interactions. *Plant Cell* 8: 1793--1807.

Das DK, Reddy MK, Upadhyaya KC and Sopory SK. 2002. An efficient leaf-disk culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). *Plant Cell Rep.* 20: 999-1005.

de Groot MJ, Bundock P, Hooykaas PJJ and Beijersbergen AG. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16:839–842; erratum de Groot MJ, Bundock P, Hooykaas PJJ, Beijersbergen AG (1998) Nat Biotechnol 16: 1074.

deCleene M. 1986. The susceptibility of plants of *Agrobacterium*: A discussion of the role of phenolic compounds. *FEMS Microbiology Review* 54: 1-8.

de-Corao MG, Marquina DMA, Sosa M, Araujo L and Buitrago D. 2002. Antiviral activity of black berry (*Rubus fruticosus* B.) using gel electrophoresis. *Revista Latinoamericana-de Quimica* 30 : 17-23.

Delneri D, Degrassi G, Rizzo R. and Bruschi CV. 1995. Degradation of trans-ferulic and p-coumeric acid by *Acine tobacter* calcoaceticus DSM 586. *Biochim. Biophys. Acta.* 1244: 363-367.

Deng W, Pu XA, Goodman RN, Gordon MP and Nester EW. 1995. T-DNA genes responsible for inducing a necrotic response on grape vines. *Mol. Plant-Microbe Interact.* 8: 538-548.

Dessaux YA, Petit Farrand SK and Murphy PJ. 1998. Opines and opine-like molecules involved in plant-*Rhizobiaceae* interactions, 173-197. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The *Rhizobiaceae*: molecular biology of model plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Dewick PM. 1995. The biosynthesis of shikimate metabolites. *Natural Product Reports* 12:579-607.

Ditt R, Nester EW and Comai L. 2001. The plant gene expression response to *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* 98: 10954-10954.

Doke N, Miura Y, Sanchez LM, Park HJ, Noritake T, Yoshioka H and Kawakita K. 1996. The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defense – a review. *Gene* 179: 45–51.

Dye F, Berthelot K, Griffon B, Delay D and Delmotte FM. 1997. Alkylsyringamide, new inducers of *Agrobacterium tumefaciens* virulence genes. *Journal of Biochemistry* 79: 3-6.

Farrand SK. 1998. In the Rhizobiaceae: molecular biology of model plant-associated bacteria (Spaink, H. P., Kondorosi, A., and Hooykaas, P. J. J., eds), pp. 199-233, Kluwer Academic Publishers, Dordrecht.

Fortin C, Nester EW and Dion P. 1992. Growth inhibitors and loss of virulence in cultures of *Agrobacterium tumifaciens* treated with acetosyringone. *Bacteriology* 174: 5676-5685.

Frankel E, Kanner J, German JB, Parks E, Kinsella J. 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* 341:454-457.

Friedinan M. 1997. Chemistry, Biochemistry and dietary role of potato polyphenols. A review J. Agric. *Food Chemistry* 45(5): 1523-1540.

Fuhrman B, Lavy A and Aviram M. 1995. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am. J. Clin. Nutr.* 61:549-554.

Fukai K, Ishigami T and Hara Y. 1991. Antibacterial activity of tea polyphenols against phytopathogenic bacteria. *Agricultural and Biology Chemistry* 55: 1895-1897.

Fuqua C and Eberhard A. 1999. *In*: Cell-Cell Signaling in Bacteria. Eds. Winans, S. C., and Dunny, G. M.:211-230, ASM Press, Washington, D.C.

Fuqua C and Winans SC. 1996. Localization of OccR-activated and TraR-activated promoters that express two ABC-type permeases and the *traR* gene of Ti plasmid pTiR10. *Molecular Microbiology* 20: 1199-1210.

Fuqua WC and Winans SC. 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *Journal of Bacteriology* 176: 2796-2806.

Fuqua WC and Winans SC. 1994. Greenberg EP: Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* 176: 269-275.

Gamba C, Piovanelli C, Papini R, Pezzarossa B, Ceccarini L and Bonari E. 2005. Soil microbial characteristics and mineral availability as affected by olive oil waste applied to cultivated soil. *Comm. in Soil Science and Plant Analysis* 36 (718): 937-950.

Gelvin SB. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* 51: 223–256.

Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiological and Molecular Biological Review* 67: 16–37.

Gerritsen ME, Carley WW, Ranges GE, Shen CP, Phan SA, Ligon GF and Perry CA. 1995. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *American Journal of Pathology* 147: 278-292.

Goel G, Puniya AK, Aguilar CN and Singh K. 2005. Interaction of gut microflora with tannins in feeds Naturwissenschaften 92: 497-503.

Goodwin I, Todd G, Ford-Loyd B, and Newbery HJ. 1991. The effects of acetosyringone and pH on *Agrobacterium* mediated transformation vary according to plant species. *Plant Cell Reports* 9: 671-675.

Gouka RJ, Gerk C, Hooykaas PJ, Bundock P, Musters W, Verrips CT, de Groot MJ. 1999 Transformation of *Aspergillus awamori* by *Agrobacterium tumefaciens*-mediated homologous recombination. *Nat Biotechnol*ogy 17: 598–601.

Halliwell B. 1999. Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. *Nutr. Rev.* 57:104-113.

Hamilton CM. 1997. A binary-BAC system for plant transformation with high molecular weight DNA. *Gene* 200: 107-116.

Hammond-Kosack KE and Jones JDG. 1996. Resistance gene–dependent plant defense responses. *Plant Cell* 8: 1773—1791.

Harborne JB. 1980. Plant phenolics. *In*: Bell Ea, Charlwood BV (eds) Encyclopedia of Plant Physiology, Chapter 8. Secondary Plant Products, Springer-Verlag, Berlin Heidelberg New York: 329-395.

Harwood CS and Parales RE. 1996. The beta-ketoadipate pathway and the biology of self-identity. *Annual Review of Microbiology* 50: 553-590.

Haslam E. 1998. Practical polyphenolics: from structure to molecular recognition and physiological action. *J. Nat. Prod.* 61: 1454-1455.

Hawes MC, Robbs SL and Pueppke SG. 1989. Use of a root tumorigenesis assay to detect genotypic variation in susceptibility of 34 cultivars of *Pisum sativum*. *Plant Physiology* 90: 180–184.

Higdon JV and Frei B. 2003.Crit. Rev. Food Science and Nutrition. 43 : 89–143.

Hori Y, Sato S and Hatai A. 2006. Antibacterial activity of plant extracts from azuki beans (*Vigna angularis*) *in vitro*. *Agricultural and Biology Chemistry* 55 : 1895-1897.

Huang MT, Ho CT and Lee CY. 1992. ACS Symposium Series 507, Oxford University Press, Washington.

Isidori M, Lavorgna M, Nardetti A, Parrella A. 2005. Model study on the effect of 15 phenolic olive mill waste water constituents on seed germination and *Vicbrio fischeri* metabolism. *Journal of Agricultural and Food Chemistry* 53: 8414-8417.

Jefferson RA. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5: 387–405.

Jefferson RA, Kavanagh TA and Bevan MW. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907.

Jennifer R. 1995. Jamison Clinical Guide to Nutrition and Dietary Supplements in Disease Management, ISBN 0-443-07193-4: 525.

Joubert P, Daniel B, Wadouachi A, Chateau S, Sangwan RS and Sangwan-Norree BS. 2004. Effect of phenolic glycosides on *agrobacterium tumefaciens virH* gene induction and plant transformation. *J. Nat. Prod.* 4 : 348-351.

Kalgutkar AS, Dalvie DK, O'Donnell JP, Taylor TJ, Sahakian DC. 2002. On the diversity of oxidation bioactivation reaction on nitrogen containing xenobiotics. *Curr.Drug Metab.* 3: 379-424.

Kalogeraki VS, Zhu J, Eberhard A, Madsen EL and Winans SC. 2002. The phenolic *vir* gene inducer ferulic acid is O-demethylated by the VirH2 protein of an *Agrobacterium tumefaciens* Ti plasmid. *Molecular Microbiology* 34 (3): 512–522.

Kanemoto RH, Powell AT, Akiyoshi DE, Regier DA, Kerstetter RA, Nester EW. 1989. Nucleotide sequence and analysis of the plant inducible locus *pin F* from *Agrobacterium tumefaciens. J. Bacteriol.* 171: 2506-2512.

Karou D, Dicko MH, Simpore J and Traore AS. 2005. Antioxidant and antibacterial activities of polyphenols from ethnomedicinal plants of *Burkina faso. African Journal of Biotechnology* 4(8): 823-828.

Kefeli VI, Kalevitch MV and Borsari B. 2003. Phenolic cycle in plants and environment. *J. Cell Mol. Biol.* 2: 13-18.

Kim JY, Sea YS, Kim JE, Sung SK, Song KJ and An G. 2001. Two polyphenol oxidases are deferentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple. *Plant Science* 161: 1145–1152.

Koide K, Osono T and Takeda H. 2005. Fungal succession and decomposition of *Camellia japonica* leaf litter. *Ecological Research* 20(5): 599-609.

Kolodziej H, Burmeister A, Trun W, Radtke OA, Kiderlen AF, Ito H, Hatano T, Yoshida T and Foo LY. 2005. Tannins and related compounds induce nitric oxide synthase and cytokines gene expressions in *Leishmania major*-infected macrophage-like RAW 264.7 cells. *Bioorganic & Medicinal Chemistry* 13: 6470-6476.

Kumar N. 2003. Studies on recalcitrance off leaf explants to *Agrobacterium*-mediated genetic transformation during the production of transgenic tea. M.Sc. Thesis, Himachal Pradesh Krishi Vishwavidhyalaya, Palampur.

Kumar N, Pandey S, Bhattacharya A and Ahuja PS. 2004. Do leaf surface characteristics affect *Agrobacterium* infection in tea (*Camelia sinensis* (L.) O. Kuntze). *Journal of Biosciences* 29: 309-317.

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C and Citovsky V. 2001. Genetic transformation of HeLa cells by *Agrobacterium*. Proc Natl Acad Sci USA 98: 1871–1876.

Lagrimini LM. 1991. Wound-induced deposition of polyphenols in transgenic plants over expressing peroxidase. *Plant Physiology* 96: 577-583.

Lamb CJ, Lawton MA, Dron M and Dixon RA. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* 56: 215-224.

Laukkanen H, Haggman H, Kontunen-Soppela S, Hohtola A. 1999. Tissue browning of *in vitro* cultures of Scots pine: Role of peroxidase and polyphenol oxidase. *Physiologica Plantarum* 106: 337-343.

Lee Y, Jin S, Sim W and Nester EW. 1995. Genetic evidence for direct sensing of phenolic compounds by the Vir A protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Science, 92 : 12245-12249.

Lin LY, Juan M, Chen YL, Liang YC and Lin JK. 1996. Composition of polyphenols in fresh tea leaves and associations of their oxygen radical absorbing capacity with antiproliferative actions in fibroblast cells. *Journal of Agriculture Food Chemistry* 44 : 1387-1394.

Loannides C. 2001. Enzyme systems that metabolize drugs and other xenobiotics. John Wiley and Sons. ISBN 0-471-89466-4.

Lobreaux S, Thoiron S, Briat JF. 1995. Induction of ferritin synthesis in maize leaves by an iron-mediated oxidative stress. *Plant Journal* 8: 443-448.

Lopez V, Lorca L and Bernabeu CO. 1997. Growth inhibition of nematophagous and entomopathogenic fungi by leaf litter and soil containing phenols. *Mycology Research* 101: 691-697.

Lorenzo JC and Angeles ML. 2001. Sugarcane microporopogation and phenolic excretion. *Plant Cell, Tissue Organ Culture* 65: 1-8.

Lotito SB and Frei B. 2006. Consumption of flavonoid-rich foods and increased plasma anti-oxidant capacity in humans: cause, consequences, or epiphenomenon. *Free Radic. Biol. Med.* 41 : 1727-1746.

Lux-Endrich A, Treutter D and Feucht W. 2000. Influence of nutrients and carbohydrate supply on the phenol composition of apple shoots cultures. *Plant Cell, Tissue Organ Culture* 60: 15-21.

Madhusudhan R, Ishikawa T, Sawa Y, Shigeoka S and Shibata H. 2003. Characterization of an ascorbate peroxidase in plastids of tobacco by-2 cells. *Physiol. Plant.* 117: 550–557.

Mato M, Rua M and Ferro E. 1988. Changes in levels of peroxidases and phenolics during root formation in *Vitis* cultured *in vitro*. *Physiologica Plantarum* 5: 473-479.

McKersie BD and Leshem YY. 1994. Stress and Stress Coping in Cultivated Plants. Kluwer Academic Publishers, Dordrecht.

Mehdy MC. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiology* 105: 467-472.

Mercuri A, Benedetti LD, Burchi G and Schiva T. 2000. *Agrobacterium*-mediated transformation of African violet. *Plant Cell Tissue and Organ culture* 60: 39-46.

Min Z and Peigen X. 1991. Quantitative analysis of the active constituents in green tea. *Phytother. Research* 5: 239-240.

Mole S, Rodger JC, Morell CJ and Butler LG. 1990. Herbivore growth reduction by tannins : use of Waldbauer ratio technique and manipulation of salivary protein production to elucidate mechanisms of action. *Biochem. Syst. Ecol.* 18: 183-197.

Mondolot L, Fisca P, Buatois B, Talansier E, De Kochko A and Campa C. 2006. Evolution in Caffeoylquinic Acid content and histolocalization during *Coffea canephora* leaf development. *Annals of Botany* 98: 33-40.

Moore BM and FIurkey WH. 1990. Sodium dodecyl sulfate activation of a plant polyphenol oxidase. *Journal of Biological Chemistry* 265: 4982-4988.

Moore LW, Chilton WS and Canfield ML. 1997. Diversity of Opines and Opine-Catabolizing Bacteria Isolated from Naturally Occurring Crown Gall Tumors *Applied and Environmental Microbiology* 63: 201–207.

Morris JW and Morris RO. 1990. Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii.* Proc. Natl. Acad. Sci. USA 87: 3614-3618.

Moskang J, Carlsen H, Myhrstad MCW and Blomhoff R. 2005. Polyphenols and glutathione synthesis regulation. *American Journal of Chin. Nutrition* 81 : 277-283.

Muldoon MF, Kritchevsky SB. 1996. Flavonoids and heart disease. *Brit. Med. J.* 312: 458-459.

Musyimi DM, Ogur JA and Muema PM. 2008. Phytochemical compounds and antimicrobial activity of extracts of Aspilia plant (*Aspilia mossambicensis*) (Oliv) wild. *International Journal of Botany* 4: 56-61.

Nigdikar SV, Williams NR, Griffin BA, Howard AN. 1998. Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation *in vivo*. *Am. J. Clin. Nutr.* 68: 258-265.

Okuda T, Yoshida T and Hatano T. 1992. Antioxidant effects of tannins and related polyphenols. *ACS symp. ser.* 507 : 87-97.

Olhoft P. and David A. 2004. Method to enhance *agrobacterium*-mediated transformation of plants. United States Patent 6759573.

Oueslati O, Benhammouda M, Ghorbel M, Gazzeh ME and Kremer RJ. 2008. Role of phenolic acids in expression of barley (*Hordeum vulgare*) autotoxicity. *Allelopathy Journal* 23(1) : 157-166.

Ozawa T, Lilley TH and Haslam E. 1987. Polyphenol interactions: astringency and the loss of astringency in ripening fruit. *Phytochemistry* 26: 2937-2942.

Ozyigit I, Kahraman MV and Ercan O. 2007. Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.). *African Journal of Biotechnology* 6 : 3-8.

Pan MJ and J Staden. 1998. The use of charcoal in *in vitro* culture – a review. *Plant Growth Reg.* 26:155–163.

Parke D. 1995. Supraperonic clustering of *pca* genes for catabolism of phenolic compound, protocatechuate in *Agrobacterium tumefaciens. J. Bcateriol.* 177: 3808-3817.

Parke D. 1997. Acquisition, recognition and merger of genes: novel management of the β-ketoadipate pathway in *Agrobacterium tumefaciens. FEMS Microbiol. Lett.* 146: 3-12.

Parke D, Oruston LN and Nester NW. 1987. Chemotaxis of plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti-plasmid in *A. tumefaciens. Journal of Bacteriology* 169 : 5336-5338.

Parke D, Rivelli M and Ornston LN. 1985. Chemotaxis to aromatic and hydroaromatic acids: Comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolei. Journal of Bacteriology* 163: 417-422.

Parke D, Rivelli M and Ornston LN. 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii. Journal of Bacteriology* 163: 417-422.

Parvez H and Ressis C. 2001. Molecular Responses to Xenobiotics. Elsevier. ISBN 0-345-42277-5.

Perl A, Lotan O, Abu-Abied M, Holland D. 1996. Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): The role of antioxidants during grape-*Agrobacterium* interactions. *Natur. Biotechnol.* 14: 624-628.

Peters NK and Verma DPS. 1990. Phenolic compounds as regulators of gene expression in plant-microbe interaction. *Molecular Plant-Microbe Interaction* 3: 4-8.

Peters NK, Frost JW and Long SR. 1986. Plant flavones luteolin, induces expression of *Rhizobium meliloti* nodulation gene. *Science* 233 : 977-980.

Piers KL, Heath JD, Liang X, Stephens KM and Nester EW. 1996. *Agrobacterium tumefaciens*-mediated transformation of yeast. Proc. Natl. Acad. Sci. USA 93: 1613–1618.

Pietta PG. 2000. Flavonoids as Antioxidants : *Review.J. Nat. Prod.* 63: 1035–1042

Piper KR, Beck Von Bodman S, Hwang I, Farrand SK. 1999. Hierarchical gene regulatory systems arising from fortuitous gene associations: Controlling quorum sensing by the opine regulon in *Agrobacterium. Molecular Microbiology* 32: 1077-1089.

Potrykus I. 1990. Gene transfer to cereal: an assessment. *Nature Biotechnology* 8: 535–542.

Pu XA and Goodman RN. 1992. Induction of necrosis by *Agrobacterium tumefaciens* on grape explants. *Physiol. Mol. Plant Pathol.* 41: 245—254.

Qin Y, Luo ZQ, Smith AJ, Gao P, Beck Von Bodman S and Farrand SK. 2000. *EMBO J.* 19, 5212-5221.

Redmond JW, Batley MA, Djordjevic RW, Kuempel PL and Rolfe BG. 1986. Flavones induce expression of nodulation gene in *Rhizobium. Nature* 323 : 632-635.

Rho HS, Kang S, Lee YH. 2001. *Agrobacterium tumefaciens*-mediated transformation of plant pathogenic fungus, *Magnaporthe grisea. Mol. Cells* 12: 407–411

Rice-Evans CA. 2001. Hesperetin Glucuronide, a Photoprotective Agent Arising from Flavonoid Metabolism in Human Skin Fibroblasts. *Curr. Med. Chem.* 8: 797–807.

Richardson M. 1996. Environmental Xenobiotics. Taylor & Francis Ltd. ISBN 0-748-40399-X.

Richter TE and Ronald PC. 2000. The evolution of disease resistance genes. *Plant Mol. Biol.* 42: 195–204.

Robinette D and Matthysse AG. 1990. Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas* syringae pv. phaseolicola. *Journal of Bacteriology* 172: 5742- 5749.

Rolland S, Jobic C, Fevre M, Bruel C. 2003. *Agrobacterium*-mediated transformation of *Botrytis cinerea*, simple purification of monokaryotic transformants and rapid conidia-based identification of the transfer-DNA host genomic DNA flanking sequences. *Curr Genet.*

Rousseaux S, Hartmann A and Soulas G. 2001. Isolation and characterization of new gram negative and gram positive atrazine degrading bacteria from different French soils. *FEMS Microbiol Ecol.* 36: 211-222.

Saini U. 2007. Development of transgenics for *osmotin* and *chitinase* genes in *Camellia sinensis* (L.) O. kuntze. Ph. D. Thesis. Guru Nanak dev University, Amritsar (P.B.).

Sandal I, Bhattacharya A, Gulati, Ravinderanath SD and Ahuja PS. 2002b. A thermolabile caffeine fraction of tea leaves – a substitute of acetosyringene for *Agrobacterium* mediated genetic transformations. Patent field in US : 022INF 2002/US.

Sandal I, Bhattacharya A, Kumar A, Gulati A, Ravindranath SD and Ahuja PS. 2002a. Tea leaf extract as a potent bacterial agent for *Agrobacterium tumefaciens* mediated genetic transformations. Patent filed in US and PCT : 041NF 2002/US.

Sandal I, Saini U, Lacroix B, Bhattacharya A, Ahuja PS and Citovesky V. 2006. *Agrobacterium* – mediated genetic transformation of tea leaf polyphenols without loss of bacterial virulence. *Plant Cell Report* 2: 169-176.

Sawasaki Y, Inomata K, Yoshida K. 1998. Trans-kingdom conjugation between *Agrobacterium tumefaciens* and *Saccharomyces cerevisiae*, a bacterium and yeast. *Plant Cell Physiology* 37: 103–106.

Scalbert A, Morand C, Manach C and Remesy C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomedicine and Pharmacotherapy* 56 : 276-282.

Shankar SRM, Girish R, Karthik N, Rajendran R and Mahendran VS. 2009. Allelopathic effects of phenolics and terpenoids extracted from *Gmelina arborea* on germination of black gram (*Vigna mungo*) and green gram (*Vigna radiate*). *Allelopathy Journal* 23(2) :323-332.

Shaw CH, Ashby AM, Brown AP, Royal C and Loake GJ. 1988. *Vir* A and *Vir* G are the Ti-plasmid functions require for chemotoxis of *Agrobacterium tumefaciens* towards acetosyringone. *Molecular Microbiology* 2 : 418-423.

Sheng and Citovsky V. 1996. *Agrobacterium* – plant cell interaction : Have virulence proteins, will travel. *Plant Cell Report* 8 : 1699-1710.

Somssich IE, Hahlbrock K. 1998. Pathogen defense in plants – a paradigm of biological complexity. *Trends in Plant Science* 3, 86--90.

Song YN, Shifuya M, Ebizuka Y and Sankawa V. 1990. hydroxyacetosyringone is the major virulence gene activating factor in Belladouna hairy root cultures and inositol enhances its activity. *Chemical Pharmaceutical Bulletin* 38 : 2063-2065.

Sood P, Sandal I, Saini U, Sharma S, Dayma J, Kumar N, Bhattacharya A, Gulati A, Singh B, Kaul VK, Ravindranath SD, Sood A and Ahuja PS. 2009. Plant resistance against *Agrobacterium* mediated transformation: a case of host-pathogen interaction. Abstract no.147. National Symposium on plant Propogation, Consevation, Modification and Characterization.

Stachel SE, Messens E, Van Montagu M, Zambryski PC. 1985. Identification of the signal molecules produced by wounded plant cell that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318: 624–629

Stachel SE, Timmerman B and Zambryski PC. 1986. Generation of single stranded T-DNA molecules during the initial stages of T-DNA transfer for *Agrobacterium tumefaciens* to plant cells. *Nature* 322: 706–712.

Stavric B, Matula T, Klassen R, Downie RH, Wood RJ. 1992. Effect of flavnoids on mutagenicity and bioavailability of xenobiotics in foods. *ACS Symp. Ser.* 507 : 239-249.

Suksamrarn S, Suwannapoch N, Phakhodee W, Thanuhiranlert J, Ratananukul P, Chimnoi N, Suksamrarn A. 2003. Antimycobacterial activity of prenylated xanthones from the fruits of Garcinia mangostana. *Chem Pharm Bull* (Tokyo). 51(7): 857-859.

Swain T and Hillis WE 1959. The phenolic constituents of *Prunus domesticus* I. The quantitative analysis of phenolic constituents. *Journal of Plant Science of Food and Agriculture* 10: 63-68.

Tang W, Newton RJ. 2004. Increase of polyphenol oxidase and decrease of polyamines correlate with tissue browning in Virginia pine (*Pinus virginiana* Mill.). Plant Sci. 167: 621-628.

Tian G, Kang BT and Brussaard L. 1992. Biological effects of plant residues with contrasting chemical compositions under humid tropical conditions-decomposition and nutrient release. *Soil Biology and Biochemistry* 24(10) : 1051-1060.

Tinland B, Hohn B. 1995. Recombination between prokaryotic and eukaryotic DNA: integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. *Genet Eng.* 17: 209–229.

Tolkunova NN, Chueva YN and Bidiuk AY. 2002. The influence of extracts of medicinal plants on the development of micro organisms. *Pishchevaya Promyshlennost* 8 : 70-71.

Tschech A, Fuchs G. 1987. Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. *Arch. Microbiol.* 148: 213-217.

Tugwell S and Branch GM. 1992. Affects of Herbivore Gut Surfactants on Kelp Polyphenol Defenses. *Ecology* 73 : 205-215.

Tzfira T, Rhee Y, Chen MH and Citovsky V. 2000. Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. *Annual Review of Microbiology* 54: 187–219.

Verma A, Abbott L, Werner D and Hampp R. 2004. Chapter I. Root colonization following seed inoculation. *In*: Plant Surface Microbiology. *Publisher Springer*: 13

Wang K, Stachel SE, Timmerman B, Van Montagu M, Zambryski PC. 1987. Site-specific nick occurs within the 25 bp transfer promoting border sequence following induction of vir gene expression in *Agrobacterium tumefaciens*. *Science* 235: 587–591.

Wei I, Guanggin and Guochang Z. 2000. *Agrobacterium* mediated transformation : state of the art and future prospect. *Chinese Science Bulletin* 45 : 1537-1546.

Weisburger JH, Rivenson A, Aliaga C, Reinhardt J, Kelloff GJ, Boone CW, Steele VF, Balentine DA, Pittman B and Zang E. 1998. Effect of tea extracts, polyphenol and epigallocatechin gallate on azoxymethane induced colon cancer. *Proc. Soc. Exp. Biol. Med.* 217 : 104-108.

Winans SC, Zhu J and More MI. 1999. in Cell-Cell Signaling in Bacteria (Dunny,G. M., and Winans, S. C., eds), pp. 117-128, ASM Press, Washington D.C.

Wink M. 1999. Biochemistry, role and biotechnology of secondary metabolites. *Annu. Plant Rev.* 3: 1–16.

Yahraus T, Chandra S, Legendre L and Low PS. 1995. Evidence for a mechanically induced oxidative burst. *Plant Physiology* 109: 1259–1266.

Yokozawa T, Chen CP, Dong E, Tanaka T, Nonaka I and Nishioka I. 1998. *Biochem. Pharmacol.* 56 : 213–222.

Yoruk R, Marshall MR. 2003. Physiochemical properties and function of plant polyphenol oxydase: a review, *J. Food Chem.* 27: 361-422.

Zaat SAJ, Wijffelman CA, Spaink HP, Van Brussel AAN, Okker RJH and Lugtenberg BTJ. 1987. Induction of the nod A promoter of *rhizobium leguminosarum* symplasmid pRLIJI by plant flavanones and flavones. *Journal of Bacteriology* 169 : 198-204.

Zambryski P. 1988. Basic processes underlying *Agrobacterium* mediated DNA transfer to plant cells. *Annu. Rev. Genet.* 22 : 1-30.

Zambryski PC. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu Rev Plant Physiol Plant Mol Biol* 43: 465–49.

Zapprometov M. 1989. The formation of phenolic compounds in plant cell and tissue cultures and possibility of its regulation. *Adv. Cell. Cult* 7: 240-245.

Zhang H, Wang L and Zhang L. 2002 Genetic control of quorum-sensing signal turnover in Agrobacterium tumefaciens. *PNAS* 99(7) : 4638–4643.

Zhang Z, Pierson LS 3rd. 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* 67: 4305-4315.

Zhu J, Winans SC. 1999. Auto inducer binding by the quorum-sensing regulator TraR increases affinity for target promoters in vitro and decreases TraR turnover rates in whole cells. Proc. Natl. Acad. Sci. USA. 96: 4832-4837.

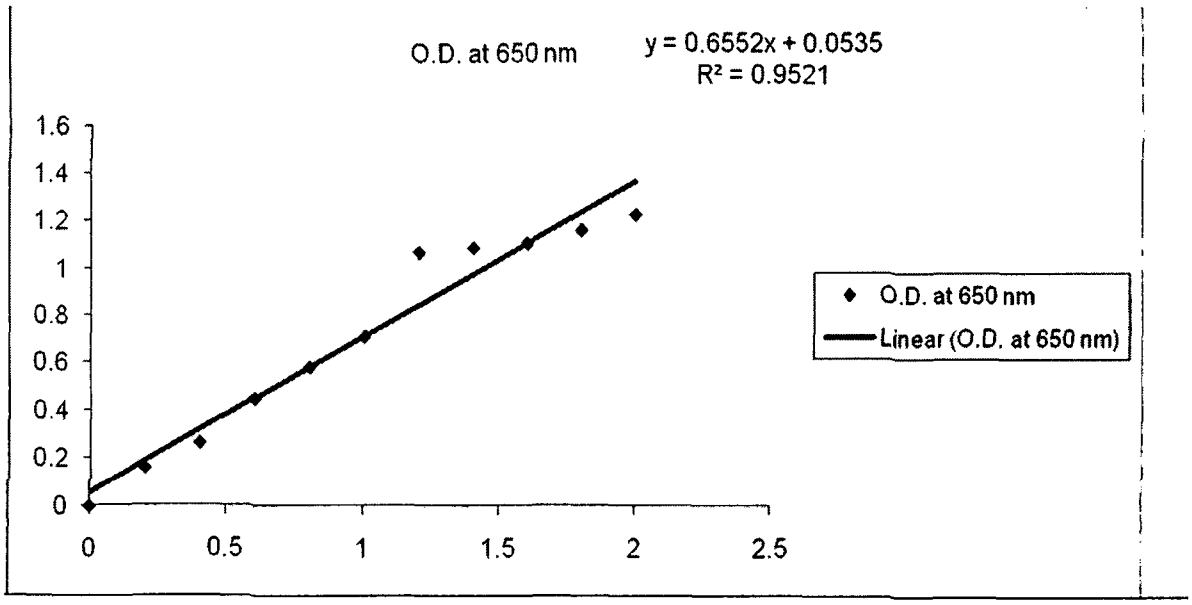
Zupan J. and Zambryski PC. 1997. The *Agrobacterium* DNA transfer complex. *Crit. Rev. Plant. Sci.* 16: 279–295.

Zupan J, Muth TR, Draper O and Zambryski PC. 2000. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant. J.* 23: 11–28.

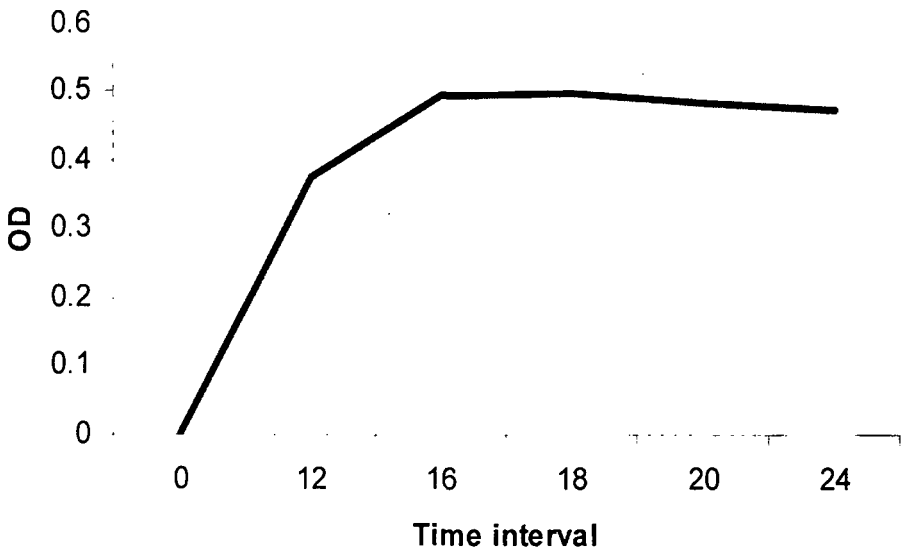
Appendices

Appendix

1.1 Standard curve for determination of phenol prepared from catechol



1.2. Standard growth curve of *Agrobacterium tumefaciens* strain GV3101



1. 3. Broths and media

1.3.1. Yeast Mannitol Broth (YMB)

Components	Conc. (g/l)
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract	0.4
Mannitol	10
Agar	1.5 %

1.3.2. Luria Bertani Broth (LB)

Components	Conc. (g/l)
Trypton	10
Yeast extract	5
NaCl	10
Agar	1.5 %

1.3.3. Preparation of Murashige and Skoog's (MS) medium

Components	Conc. (mg/l)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.4H_2O$	370
KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Meso-inositol	100
Pyridoxine-HCl	0.5
Thiamin-HCl	0.1
Nicotinic acid	0.5
Glycine	2.0
Sucrose	30 g/l
Agar	8 g/l

Agar added for solid medium. Depending upon the solubility of the salts, separate stocks are made for Ammonium Nitrate, Potassium Nitrate, Vitamins, major elements and micro elements.

1.4. Buffers

1.4.1. GUS assay buffer

Components	Conc.	Stock	Amount taken for 10 ml
Phosphate Buffer	0.1 M	0.5 M	2 ml
EDTA (pH 8)	10 mM	0.5 M	0.2 ml
Potassium ferrocyanide	10 mM	0.1 M	1 ml
Potassium ferricyanide	1 mM	0.1 M	0.1 ml
Trypton X	0.1 %	-	0.01 ml
X-gluc	1 mM	-	

1.4.2. Enzyme extraction buffer

Components	Conc.	Stock	Amount taken for 20 ml
Tris-HCl (pH 7.2)	50 mM	1 M	1 ml
Sorbitol	0.4 M	1 M	8 ml
NaCl	10 mM	1 M	0.2 ml

1.4.3. Z-buffer

Components	Conc.	Stock	Amount taken for 100 ml
$Na_2HPO_4.7H_2O$	60 mM	1 M	6 ml
$NaH_2PO_4.H_2O$	40 mM	1 M	4 ml
KCl	0.01 M	1 M	1 ml
$MgSO_4$	0.001 M	1 M	0.1 ml
β-mercaptoethanol	0.05 M	-	100 mg

1.5. Antibiotics

Antibiotic	Conc. (µg/ml)	Stock (mg/10ml)	Amount taken (µl/ml)
Rifampicin	25	250	1
Kanamycin	50	500	1

1.6. Filter sterilization of antibiotics

Heat labile antibiotics get destroyed during autoclaving. Therefore, they are sterilized by filtration through a filter unit containing a filter membrane of 0.22 µm.

Filtration is done inside the laminar air flow hood. And finally, antibiotics are stored at 4°C for further use. As these are photolabile, care is taken to avoid exposure to light by covering the vial containing the antibiotics with aluminum foil.

1.7. Storage of bacterial cultures

1.7.1 Glycerol stock for long term storage

50% glycerol stock (v/v) in water was prepared and then autoclaved at 15 psi, 121°C for 20 min for sterilization. Then 400 µl glycerol stock and 600 µl bacterial cultures were taken in cryo-vials (4:6 ratio). These were then mixed thoroughly by vortexing, immediately plunged into liquid nitrogen and finally stored at -80°C for further use.

1.7.2. Storage as plate cultures

Agar solidify (1.5 %) YMB /LB plates with specific bacterial selection antibiotics were prepared. With the help of pipette tip a single colony of the bacterium (*Agrobacterium/ E. coli*) was spread on the surface of plates. These were then incubated at 28°C (for *Agrobacterium*) or 37°C (for *E. coli*) for further growth. Once the colonies were formed the plates were stored at 4°C for further use.

1.8. β-galactosidase assay

β-galactosidase encoded by the *lacZ* gene of *lac* operon in *E. coli* cleaves lactose to glucose and galactose so that that they can be used as energy sources. The synthetic compound o-nitrophenyl-β-D-galactosidase (ONPG) is also recognized as a substrate and can be cleaved to yield galactose and o-nitrophenol to yield a yellow colored product. When ONPG is in excess over the enzyme in a reaction, the production of o-nitrophenol per unit time is proportional to the concentration of β-galactosidase. This yellow colored product can be used to determine the enzyme concentration.

KRISH
105062

Brief Resume of Student

Name: Jyotsna Dayma

Mother's Name: Smt. Gaytri Dayma

Father's Name: Sh. Satya Narayan Dayma

Date of Birth: 08/12/1983

Permanent Address: H.No. 4/16 Swami Vivekanand Nagar Kota, Rajasthan 324005

Academic Qualifications:

Qualification	**Year**	**School/Board /University**	**Marks(%)/ Division**	**Major Subjects**
10^{th}	1999	C.B.S.E.	72	Eng,Math,Science,S.Sc.,Hindi,
12^{th}	2001	C.B.S.E.	67	P.C.M.B.
B.Sc.	2007	G.B.P.U.A&T.	77.9	Agriculture
M.Sc.	2009	C.S.K.H.P.K.V.	75	Agricultural Biotechnology

Theses Title in M.Sc.: Role of polyphenols in i*n vitro* response of explants during *Agrobacterium* mediated genetic transformation.

Scholership: DBT Scholership