

***AGROBACTERIUM-MEDIATED
TRANSFORMATION OF PEA
(*Pisum sativum* L.)***

THESIS

By

**SURESH KUMAR RAJORIYA
(A-2009-30-05)**

Submitted to



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in

partial fulfillment of the requirements for the degree

of

**MASTER OF SCIENCE IN AGRICULTURE
(DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY)
(AGRICULTURAL BIOTECHNOLOGY)**

2012

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*Is there anything I can say,
anything I can give
or do for you.....*

*Because all that I'm
all that I have
I owe to you.....*

Affectionately Dedicated

To

My Revered Parents

&

Major Advisor



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CERTIFICATE – I

This is to certify that the thesis entitled “***Agrobacterium*-mediated transformation of pea (*Pisum sativum* L.)**” 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 **Mr. Suresh Kumar Rajoriya (A-2009-30-05)** son of **Shri Nathu Ram Saini** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

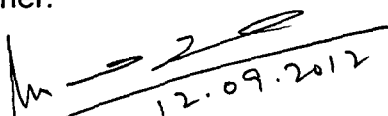
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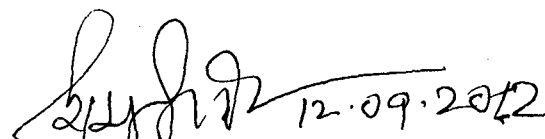

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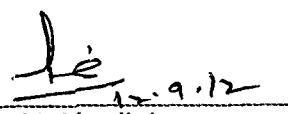
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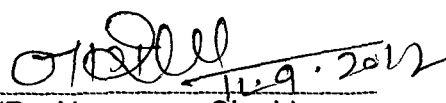
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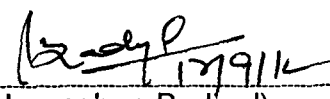
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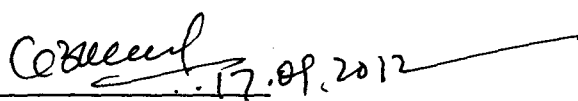
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ACKNOWLEDGEMENTS

In this highly complex society, no work can be accomplished by a single individual but it needs inspiration and sincere gratitude of intellectuals as well as the grace of that Almighty. With limitless humility, I would like to praise and thank the merciful, compassionate "God", who bestowed me with health, tenacity and courage enough to go through this crucial juncture.

I am grateful to "God", for bestowing me with affectionate parents, whose love, dedication & inspiration encouraged me to undergo higher studies. Their sacrifice, heartiest blessings & firm faith have made this documentation a puny remuneration to translate their and my dreams into reality.

With the overwhelming sense of legitimate pride and genuine obligation which gives me exuberant pleasure and privilege to express my eternal gratitude to my Advisor Dr. Kamal Dev Sharma, Associate Professor, Department of Agricultural Biotechnology, CSKHPKV, Palampur and Chairman of my Advisory Committee for his guidance, keen interest, encouragement and parental affection during whole course of my study and particularly at times of research and preparation of this manuscript.

No expression of thanks will be sufficient without recognition of intelligent and professional dexterity of the Members of my Advisory Committee, Dr. R. K. Kapila, Dr. Nageswer Singh and Dr. Jagmohan Badiyal for their valuable guidance, utilitarian exhortation and constructive suggestions.

I emphatically express my thanks to Dr. T. R. Sharma, Head of Department, of Agricultural Biotechnology for his suggestions and kind cooperation during the period of research work. I am also thankful to the esteemed faculty members of the Department of Agricultural Biotechnology, Dr. R. K. Chahota, and Dr. Rajive Rathore for their cordial co-operation extended towards the course of this study.

I sincerely thank Dr. K. K. Katoch, Dean, Postgraduate Studies, CSKHPKV, Palampur and other University authorities for providing necessary facilities to complete this study.

I am also grateful to Dr. S. K. Sharma, Vice-Chancellor, Dr. S. P. Sharma, Director of Research, Dr. P. K. Sharma, Dean, COA, Dr. Sanjay Chadda, warden, Shivalik P.G. hostel and Kamlesh Singh, S.W.O. for facilitating the timely completion of this work.

I feel it great to mention my special thanks to Dr. S. R. Thakur, Dr. Sanjay Kumar, Dr. Amita Bhattacharya, Dr. Kamal Mohini, and Dr. Ashu Gulati for their inspirable guidance during course work.

Heartfelt thanks are also due to the laboratory and field staff of the Department of Agricultural Biotechnology, Raghu, Sunil, Atul, Mukesh, Pooja and Baldev for their cordial help extended during the study.

I am also thankful to the help rendered by the office staff of the Department of Agricultural Biotechnology, Ajay Walia and Prakash during the course of study.

I can hardly overlook the co-operation, timely help and moral support extended by my seniors especially Abhishek, Praveen, Swaranjali, Bhavana, Ekta, Renu, Prashant, Vivek, Jintu, Vishal Sharma, Rohit, Munish, Prabhakar, Anil, Lokinder, Anil Jakhar, Samuel,

Sharvan and my friends Mamta, Subhas, Prakesh, Swaroop, Suspendra, Harish, Praveen, Praveen kumar, Sourabh, Kuldip, Ramesh, Shobit, Ajay, Sumit Sharma, Santhil who boosted me & made this place a home away from home.

It would be inappropriate if I commit a mistake not to mention name of my juniors Tushar Pote, Dilip, Amrita, Sharda Sharma, Harsha Hegde, Hemendra, Shruti Vishal, Ajit, Omkar, Vikram, Girdhar Gopal, Rohit, Navneet, Savneet, Ravi, Sandeep, Sunil, Pawan, Simantha, Vimlesh, Karan, Gajanan, Lokesh, Mukul, and Lalit who kept me in exalted state during moments of despondency.

In particular, I sincerely thank Department of Biotechnology, Government of India, for providing me the Fellowship during the course of my studies.

Mr. Ajay Walia deserves special thanks for his untiring efforts in bringing the manuscript to this form.

Last, but not the least I place on record the untiring support and encouragement provided by my grand-parents, my parents, my brothers, my sisters and all my relatives for pursuance of masters research and thanks are not enough to acknowledge this.

My special thanks to dear sister Anita and to all my well wishers, whose guidance helped me in completing this thesis.

Lastly, it is worth mentioning the beautiful state Himachal Pradesh and pleasant town Palampur for some unforgettable experiences and memories.

Acknowledgements are inherently endless & incomplete, and I request indulgence from many friendly & helpful people whom I could not name here, due to paucity of space.

Needless to say, all omissions and errors are mine.

Place : Palampur

Dated : 19 July, 2012

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LIST OF ABBREVIATIONS USED

Sr. No.	Abbreviation	Meaning
1	%	percent
2	BAP	6-benzylaminopurine
3	bp	base pair
4	CTAB	hexadecyl-trimethyl-ammonium bromide
5	dATP	deoxyadenosine triphosphate
6	dCTP	deoxycytosine triphosphate
7	dGTP	deoxyguanosine triphosphate
8	DNA	deoxyribonucleic acid
9	dNTP	deoxynucleotide triphosphate
10	EDTA	ethylenediamine tetra acetic acid
11	GUS	beta-glucuronidase
12	g	gram
13	h	hour
14	kb	kilobase
15	l	litre
16	M	molar
17	mg	milligram
18	min	minute(s)
19	ml	milliliter
20	μ M	micromolar
21	$^{\circ}$ C	degree Celsius
22	2,4-D	2,4-dichlorophenoxy acetic acid
23	IAA	indole-3-acetic acid
24	IBA	indole-3-butyric acid
25	Kin	kinetin
26	NAA	naphthalene-1-acetic acid
27	PCR	polymerase chain reaction
28	pH	puissance de hydrogen (ion concentration)
29	ppm	parts per million
30	rpm	revolutions per minute
31	s	second
32	Taq	<i>Thermusaquaticus</i>
33	TAE	Tris acetate EDTA buffer
34	TE	Tris EDTA buffer
35	CsTLP	<i>Camelia sinensis</i> thaumatin like protein
36	Tris	tris (hydroxy methyl) amino methane
37	UV	ultraviolet
38	V	volts
39	YEMA	yeast extract mannitol agar
40	YEMB	yeast extract mannitol broth

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Title of the thesis : *Agrobacterium*-mediated transformation of
pea (*Pisum sativum* L.)
Name of the student : Suresh Kumar Rajoriya
Admission number : A-2009-30-05
Major discipline : Agricultural Biotechnology
Minor discipline(s) : Biochemistry
Date of thesis submission: 18 July, 2012
Total pages of the thesis: 93
Major Advisor : Dr Kamal Dev Sharma

ABSTRACT

Pea is an important pulse crop of Himachal Pradesh (HP). Efficient direct regeneration and transformation protocols were developed for 'Azad P1', a widely grown pea cv in HP. Of the three explants (embryos, cut embryos and cotyledonary nodes) and three media evaluated, the cotyledonary node explants and MS + 20 μ M BAP + 0.01 μ M NAA were the best for direct shoot induction. This medium was also the best for shoot elongation. Reduction in amount of media salts/vitamins, ammonical form of nitrogen and sucrose reduced the number of shoots per explant. Using this protocol, 46.63 shoots per explant with an average length of 9.82 cm were generated in 60 days. The shoots were grafted onto root stocks of the same cv. The cotyledonary nodes were transformed using *Agrobacterium tumefaciens* strain GV3102 that harboured binary vector pCAMBIA 1302. The pCAMBIA 1302 had a copy of pathogenesis related (thaumatin like) protein gene from tea (*Camellia sinensis* L.) and a plant selectable marker gene (*hpt*). Fifteen mg l^{-1} hygromycin B was the optimum lethal dose for cotyledonary nodes. The effect of acetosyringone concentration, co-cultivation temperature and injury to explants on transformation efficiency was studied. Optimum concentration of acetosyringone was 50 μ M, co-cultivation temperature was 24°C and injury to explants with silicon carbide enhanced transformation efficiency. Growing co-cultivated explants without selection for two weeks prior to exposure at 15 mg l^{-1} hygromycin for 30 days enhanced transformation efficiency. Using the present protocol, 4.33 transformants per 100 explants were obtained. The transformation was confirmed by PCR using *CsTLP* specific primers.

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INTRODUCTION

1. INTRODUCTION

Pea (*Pisum sativum* L., family Leguminosae), a cool season grain legume is one of the most important pulse crops in the world. It is cultivated over an area of 6 million hectare with a production of 11.25 million ton of dry pea and 7.59 million ton of green pea (FAO 2005). Important pea growing countries in the world are Canada, France, China, Russia, India, Ethiopia and USA out of which, India ranks first in green peaproduction and fifth in dry pea production (FAO 2005). In India, pea is grown over an area of 313.5 thousand hectare with green pea production of about 2.56 million ton (Directorate of agriculture, HP 2008). In comparison to other pulses in India, dry pea ranks 6th in production after chickpea, pigeon pea, mungbean, uradbean and lentil. In Himachal Pradesh, it is grown as an offseason crop in an area of 18.93 thousnad hectare with a total green pea production of 202.521 thousand ton (Directorate of agriculture, HP 2008).

Pea seed is very nutritious and contains good amounts ofproteins, carbohydrates, vitamins and minerals. Nutritional values of different nutrients in raw green pea seed per 100 g is given in Table 1.1 (USDA Nutrition Database 2007).

Table 1.1 Amount of different nutrients in raw green pea seed (per 100 g).

Nutrient	Quantity	Nutrient	Quantity	Nutrient	Quantity
Carbohydrate	14.5 g	Vitamin B2	0.1 mg	Calcium	25 mg
Fat	0.4 g	Vitamin B3	2.1 mg	Iron	1.5 mg
Protein	5.4 g	Vitamin B5	0.1 mg	Magnesium	33 mg
Vitamin A	38 µg	Vitamin B6	0.2 mg	Phosphorus	108 mg
β-carotene	449 µg	Vitamin B9	65 µg	Potassium	244 mg
Vitamin B1	0.3 µg	Vitamin C	40 mg	Zinc	1.2 mg

Pea is also susceptible to several biotic and abiotic stresses. Major biotic stresses in Himachal Pradesh are wilt/root rot, powdery mildew, ascochyta blight and rust. These stresses either alone or together or in combination with abiotic stresses cause heavy losses in pea production. Of late, wilt/root rot caused by *Fusarium oxysporum* f. sp. pisi, *F. solani* f. sp. pisi, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* has become a serious disease of pea in India and Himachal Pradesh (Sharma 2000; Rana *et al.* 2009). Management of the wilt/root rot by use of chemicals is difficult as the pathogens perpetuate in soil. The deployment of resistance genes is also not possible because more than one pathogen usually inhabit soil in a locality and can cause simultaneous infections. Alternatively, the pathogenesis related protein (PR) genes or the transcription factors may be exploited for possible management of these diseases. The PR genes or transcription factors provide resistance by enhancing defence mechanisms of plants. These genes can be inserted into pea genome through *Agrobacterium tumefaciens*. Some success have already been achieved in transformation of the pea, however, no efforts so far have been made in India in this direction. Because of higher yield per unit area, pea is also considered an ideal crop for the production of antibodies (Perrin *et al.* 2000). Novoplant GmbH (Gatersleben), a biotechnology company working on the development of recombinant proteins for animal health, has already obtained the German Federal Office of Consumer Protection and Food Safety (BVL) approval to perform a field trial with antibody producing peas in 2007. Development of an antibody in transgenic pea has already been achieved. However, the transformation efficiency of pea remains very low (Orczyk and Orczyk 2000) and is influenced by factors such as *Agrobacterium* strain, explants, cocultivation treatment, cocultivation temperature and acetosyringone concentration etc. (Svabova *et al.* 2005; Grant *et al.* 2003). Thus, there is a need to improve transformation efficiency in pea.

In view of the above, the present proposal has been formulated with the following objectives:

- i. Transformation of pea using *Agrobacterium tumefaciens*
- ii. Confirmation of putative transformants by PCR analysis



REVIEW
OF
LITERATURE

2. REVIEW OF LITERATURE

Literature relevant to the research problem has been reviewed in the following text.

2.1 Pea tissue culture

Plant regeneration *in vitro* follows two pathways, organogenesis and embryogenesis. In organogenesis shoot buds are formed by meristemic activity while in embryogenesis a single cell undergoes differentiation to produce somatic embryos. Such morphogenesis or differentiation can be direct from explant or indirect through dedifferentiated callus (Chandra and Pental 2003). The urgent need of many identical uniform individuals of a selected genotype has led to the development of *in vitro* culture technique as a source of vegetative propagation, in contrast to conventional methods, that take a long time for multiplication (Nin *et al.* 1996). Plant growth regulators are involved in developmental transitions that include flowering, embryogenesis, dormancy, adjustments in growth rates and stomatal conductance (Bardford and Trewavas 1994). Bud growth, shoot and root regeneration, root and stem hair formation, vascular tissue differentiation, abscission, ripening and flowering are examples of threshold (all or non) phenomena that can be regulated by an inducing stimulus in a dose-dependent fashion (Trewasa 1991). Plants often measure environmental time for days, weeks, months or years before initiating a change in programme. Plant hormones can influence the rates at which physiological processes occur naturally (Cooke and Smith 1990).

Pea is less responsive to tissue culture especially the plantlet regeneration. The plant regeneration efficiency *in vitro* is genotype dependent in pea and some genotypes are more responsive than others (Emmanouil *et al.* 2004). For Azad P1, the variety used for plantlet regeneration and transformation in the present study, tissue culture protocols leading to plant regeneration are not available.

2.1.1 Effect of salt and vitamin concentration, explant type, genotypes and growth regulators on *in vitro* regeneration in pea

There is no report on impact of salt and vitamin concentrations on direct shoot induction and development from pea, however, impact of explant type, genotypes and growth regulators has been studied (Loiseau *et al.* 1998; Griga 1998; Griga 2002; Ochatt *et al.* 2000; Mroginski and Kartha 1981; Malik and Saxena 1992; Franklin *et al.* 2000; Emmanouil *et al.* 2004; Esposito *et al.* 2012; Doorne *et al.* 1995; Pniewski *et al.* 2003).

The suitability of the explants (cotyledonary node and shoot tip) for multiple shoot induction was studied by Franklin *et al.* (2000). Cotyledonary node and shoot tip explants of pea excised from 15 day old aseptic seedlings were cultured on MS medium supplemented with 2.0 mg l⁻¹ BAP. For further production of multiple shoots from the original explants, parent explants were transferred to fresh culture medium every 28 days. The cotyledonary nodes produced more number of shoots (32) than shoot tips (7). In several other studies, different explants have been used, for example, Malik and Saxena (1992) and Mroginski and Kartha (1981) used immature leaflets, Emmanouil *et al.* (2004) used nodal tissue, Jackson and Hobbs (1990) used cotyledonary nodes. Comparison of shoot/plantlet induction per explant to identify the better explant has not, however, been made in these studies.

Genotype appears to have impact on somatic embryo, shoot and callus formation in pea. Doorne *et al.* (1995) studied 16 cultivars of pea for the formation of somatic embryos. The cultivars Stehgolt, Maro and Progretra showed the highest tendency to form somatic embryos (25%) while Alaska, Rondo and Ascona did not show any embryo production. Emmanouil *et al.* (2004) used four pea cultivars ('Espace', 'Classic', 'Solara', and 'Puget') to regenerate plants from meristematic tissues initiated from nodal tissues on MS media supplemented with growth regulators. The regeneration efficiency of Puget cultivar was superior to other three cultivars as it produced maximum number of shoots and had maximum survival percentage (Emmanouil *et al.* 2004). The impact of genotype on callus induction was also evident (Sharma and Kaushal 2004). Between the two cultivars studied, Lincoln was superior to Palampriya for callus induction.

Plant growth regulators affect different aspects of tissue culture including callus induction, multiple shoot formation (direct or indirect) or germination of somatic embryos. *In vitro* regeneration (indirect through callus phase) of plants from immature leaflets of 3 day-old pea (cv. Century) seedlings was studied under defined nutritional, hormonal and environmental conditions (Mroginski and Kartha 1981). Immature leaflets isolated from the second and third apical leaves of aseptically germinated seeds were cultured on MS medium supplemented with different concentrations of NAA (0.1, 1 and 10 μM) and BAP (1 and 10 μM) in various combinations. Although the number of calli producing shoots was maximal at 10 μM levels of NAA and BAP, multiple shoot regeneration was predominant at a combination of 0.1 μM NAA and 10 μM BAP. IAA and Kin, both at 10 μM , also induced shoot regeneration. Multiple shoots were produced from cotyledonary nodes on MS medium containing 1 mg l^{-1} BAP (Jackson and Hobbs 1990). The number of buds formed could be increased by scraping the nodes before culture or by increasing the cytokinin concentration.

It was also shown that cytokinin concentrations above 5 mg l^{-1} increasingly produced shoots that were vitrified and difficult to root (Jackson and Hobbs 1990). With all 6 genotypes tested, developing buds were visible by 5 days after culture and elongated shoots could be removed after 21 days. Histological studies indicated that the buds and shoots were formed from superficial layers of tissue. Shoot induction from pea seeds was best on 25 μM concentration of BAP and the higher concentrations of BAP (50 and 75 μM) shoot induction was low (Esposito *et al.* 2012).

The complete protocol for regeneration and long-term micropropagation of several Polish cultivars of pea was elaborated by Pniewski *et al.* (2003). The shoots were most likely regenerated via *de novo* organogenesis. The adventitious buds formed in callus derived from cotyledons tissue adjacent to axillary meristems of immature embryos. All calli regenerated several shoots per explants on MS supplemented with 4.5 mg l^{-1} of BAP. The plantlets were subsequently micropropagated with slightly higher efficiency and preserving a

good viability over the long-term culture on a medium containing 2 mg l^{-1} than medium containing 4.5 mg l^{-1} of BAP (Pniewski *et al.* 2003).

In a five-step procedure, plants were regenerated from meristematic tissue initiated from nodal tissue by Emmanouil *et al.* (2004). In step 1, stem tissue with one node (1 cm size) was subcultured on medium containing thidiazuron (TDZ). As a result multiple shoots were produced, appearing normal or swollen at their bases. The multiple shoots were subcultured in the same medium, resulting in the formation of a green hyperhydric tissue in the swollen bases of the multiple shoots, which is fully covered with small buds [bud-containing tissue (BCT)]. In step 2, BCT fragments were isolated and subcultured in the same medium and, as a result, they were able to reproduce themselves in a cyclic fashion. In step 3, subculture of BCT on medium supplemented with a combination of gibberelic acid (GA), BAP and NAA, resulted in the formation of shoots, which were rooted in step 4 on medium supplemented with 0.5 mg l^{-1} NAA, IAA or IBA. In step 5, *in vitro* plants were transferred to the greenhouse for acclimatisation and further development. The effect of BAP in combination with other growth regulators on callus induction was also demonstrated by making 14 combinations with different concentrations of 2, 4-D, NAA, Kinetin and IAA (Sharma and Kaushal 2004). The best growth regulator combination was 2, 4-D + NAA + BAP 0.5 mg l^{-1} each for callus induction.

Apart from other growth regulators, TDZ was also used in pea tissue cultures. A system of *in vitro* clonal propagation based on somatic embryogenesis from shoot apical meristems was developed in pea (Griga *et al.* 1998). The conversion rate of somatic embryos was favoured by $10 \text{ }\mu\text{M}$ concentrations of TDZ (Griga *et al.* 1998). Axenic seedling cultures of garden pea were established by culturing mature seeds on MS medium supplemented with TDZ (Malik and Saxena 1992). Of various cytokinins or compounds with cytokinin-like activity (Kin, TDZ, Zeatin) tested for inducing shoot formation in pea seeds cultures, TDZ was the most effective. Pea seedlings exhibited a unique pattern of shoot formation which was accomplished in two distinct phases. Multiple shoots developed within a week, from the nodal and basal regions of the

primary epicotyl in a medium that contained 5-50 μ M TDZ. When these seedlings were exposed for a prolonged time period (3-4 weeks) to the same medium, numerous shoot buds emerged *de novo* from the base and/or from the upper part of multiple shoots. These shoots had no apparent vascular connection with parent tissues. The inductive capability of TDZ was then tested in several other genotypes of pea and multiple shoots developed after 1 week of seed culture on media that contained 1-50 μ M TDZ. However, *de novo* differentiation of shoot buds occurred in cultures exposed to TDZ for 4-6 weeks, only from nodal and adjacent areas. Secondary shoot formation occurred frequently in all of the species tested. Developing shoots were able to form roots and eventually whole plants on a modified MS medium containing 2.5 μ M NAA.

2.1.2 Effect of concentration of ammonium nitrate and sucrose on direct shoot induction in pea

In general, supplementation of cultures with nitrate as the sole source of nitrogen inhibits or reduces the cell or shoots growth (Gamborg 1970; Shanjani 2003; Rasmia *et al.* 2011) and supplementation of tissue culture media with ammonical form of nitrogen is essential for growth of tissue cultures. Soybean suspension cultures were grown on different nitrogen sources in the form of nitrate and ammonium as the sole nitrogen source or combined in different concentrations. Soybean cells growth was stunted on medium with high concentration of ammonium but soybean cells did not grow on nitrate unless medium was supplemented with ammonium or glutamate (Gamborg 1970). Similarly, Shanjani (2003) reported that nitrogen plays a major role in growth and differentiation in callus cultures of *Juniperus excelsa*. Both form and amount of nitrogen had significant effects on rate of cell growth and differentiation. Calli and adventitious buds were initiated from excised shoot cuttings. Full strength MS inhibited active callus production that could be minimized by lowering the amount of ammonium or total nitrogen. Medium with half strength MS nitrogen stimulated better callus production (65%) that can be increased by glutamine supplementation (80%). Nitrogen supplementation only as nitrate produced no callus (Shanjani 2003).

The amount of ammonical nitrogen in the medium has varied impact on tissue cultures of different plant species. Rasmia *et al.* (2011) studied the growth and development of date palm plantlets on MS media containing four different concentrations of ammonium nitrate (full, one half, one fourth and one eighth). The half strength of ammonium nitrate (825 mg l^{-1}) yielded longest shoot and roots and the greatest number of leaves and root per plantlet was obtained from ammonium nitrate concentration of 206.25 mg l^{-1} (one eighth of full concentration; Rasmia *et al.* 2011). In contrast to this, *in vitro* flowering and fruiting in green pea was more at half strength (825 mg/l) ammonium nitrate (Franklin *et al.* 2000). The impact of different concentrations of ammonium on shoot induction in pea has not been studied so far.

Though, effect of sucrose concentrations on direct plant regeneration in pea has not been studied, 30 g l^{-1} was found optimum for flower induction and pod formation *in vitro* (Franklin *et al.* 2000). At lower concentrations ($0, 15 \text{ g l}^{-1}$), no flower and pod formation was observed whereas at a higher concentration (50 g l^{-1}) the reduction was very high. In other plant species also, the supplementation of cultures with lower or higher concentrations of sucrose either reduces or sometime inhibits the growth of cells or shoots (Komatsuda *et al.* 1992; Nhut *et al.* 2001; Sharma *et al.* 2008; Gauchan 2012). In *Lilium longiflorum*, four different concentrations of sucrose ($10, 20, 30$ and 40 g l^{-1}) in half MS basal medium were tested for efficient shoot regeneration by young stem culture (Nhut *et al.* 2001). The highest (8.9) number of shoots were recorded at 30 g l^{-1} concentration of sucrose. While number of shoots were low at 10 g l^{-1} (2.2) 20 g l^{-1} (5.9) and 40 g l^{-1} (7.1) concentration of sucrose. The effect of sucrose on maturation of soybean somatic embryos was investigated for efficient culture. Somatic embryos produced on medium with a low sucrose concentration (5 g l^{-1}), then these were grown for 2 weeks on MS medium containing 5 g l^{-1} or 30 g l^{-1} sucrose and then for another 5 weeks on MS medium containing $5\text{--}90 \text{ g l}^{-1}$ sucrose. The highest increase in fresh weight of somatic embryos was obtained in the treatment of transferring from 30 g l^{-1} sucrose (2 weeks) and 60 g l^{-1} (5 weeks). Similarly in maize, the effect of various concentrations of different

sugars was studied for root and shoot induction from embryos. The seedling development was induced on 1/2 MS basal media with the application of fructose, maltose and sucrose at different concentrations (2.5, 5, 7.5, 10, 25, 50, and 100 g l^{-1}) of each. The maize appears to have lower requirements of sucrose for shoot development as maximum shoot length from the embryo was recorded at 5 g l^{-1} (Gauchan 2012). The shoot length was comparable up to 10 g ml^{-1} sucrose, and it was reduced significantly at 25 g l^{-1} or 50 or 100 g l^{-1} . In contrast to this, shoot length was recorded less at 2.5 g l^{-1} than at 10 g l^{-1} concentrations of sucrose.

The sucrose requirements for tissue cultures of some other plant species are comparatively high. For example, shoot formation from saffron corms and shoot length was more at higher (50 g l^{-1}) sucrose concentration (Sharma *et al.* 2008). The induction of corms from tissue culture derived shoots was still higher with 80 g l^{-1} being the optimum concentration (Sharma *et al.* 2008).

2.1.3 Effect of growth regulators on elongation of *in vitro* regenerated shoots

The impact of growth regulators on shoot elongation had also been observed (Pniewski *et al.* 2003; Franklin *et al.* 2000), however, there is no study on effect of salt concentration on direct shoot induction in pea. Pniewski *et al.* (2003) developed shoots from callus cultures of several Polish cultivars of pea. The shoots were cultured on MS media supplemented with 4.5 mg l^{-1} BAP and 0.02 mg l^{-1} NAA and MS media supplemented with 2.0 mg l^{-1} NAA. The shoot multiplication was more on MS media supplemented with 4.5 mg l^{-1} BAP and 0.02 mg l^{-1} NAA. The shoots were further transferred on MS media supplemented with 0.02 mg l^{-1} BAP for shoot elongation. The elongated shoots were also grafted on to root stocks of pea plants growing in the greenhouse.

Franklin *et al.* (2000), regenerated multiple shoots from cotyledonary node and shoot tip explants of pea excised aseptically from 15 day old seedlings on MS medium with different concentrations of BAP (0.0, 0.5, 1.0, 2.0, 5.0 and 10 mg l^{-1}). For further production of multiple shoots, cultures were transferred to fresh culture medium every 28 days. BAP in the medium induced multiple shoots;

however, the efficiency differed with its concentration and explant types. Among the explants tested, the cotyledonary node produced the maximum number of shoots (32). Each shoot tip produced an average of 7 shoots, in media with 2.0 mg l⁻¹ BAP. Cotyledonary node explants produced multiple shoots in response to a range of BAP concentrations (0.5–5.0 mg l⁻¹) in the medium, but not at a higher BAP concentration of 10.0 mg l⁻¹. Regenerated shoots could elongate in the same medium but the number of elongated shoots was restricted to 1–2 in each culture.

2.2 Genetic transformation

The discovery of the gene transfer mechanism between *Agrobacterium* and the plant kingdom (Chilton *et al.* 1977) has led to its use for facilitating the insertion of desirable genes into the plant genome, irrespective of the origin of these genes. Transformation systems are now available for a large number of plant species and examples of transgenic plant species for pests, viral, fungal, herbicide resistance and environmental stress tolerance exist. Several procedures have been reported to accomplish gene transfer. Gene transfer can be achieved mainly through two broad methods; direct DNA transfer and vector mediated transfer.

2.2.1 Direct DNA transfer

Physical as well as chemical methods have been developed to facilitate DNA delivery across the plasma membrane and cell wall, which led to both stable and transient gene expression. The first report on direct delivery of DNA molecules into plant protoplasts was documented by Davey *et al.* (1989).

Microinjection, sonication, electroporation and biolistic mediated transfer are the main procedures to let desired DNA molecules enter any living cell; plant, animal, or microbial (Fromm *et al.* 1985; Miki *et al.* 1987; Christou *et al.* 1987; Sarangi *et al.* 1991; Klein *et al.* 1985). Microinjection involves immobilization of protoplasts and micro injecting DNA directly into the nucleus. Miki *et al.* (1987) reported transformation efficiencies of 12–66 per cent in tobacco. In the sonication process, ultra sound waves are used to facilitate uptake of nucleic

acids into plant cells and protoplasts. The electroporation method was originally developed to introduce DNA into prokaryotic cells (Fromm *et al.* 1985). This has been successfully used for transformation of wide range of crop species such as pigeonpea (Christou *et al.* 1987; Fromm *et al.* 1985). Sarangi *et al.* (1991) isolated protoplast from mesophyll tissues and purified protoplasts were electroporated with plasmid DNA carrying a chimeric gene for *neomycinphosphotransferase (npt-II)* in pigeonpea.

Another method of physical transfer of DNA is through biolistics. Klein *et al.* (1985) reported a method where *in vitro* cultured cells were bombarded with tungsten particles coated with DNA. Microprojectile mediated transformation of pre-existing meristem, followed by micropropagation can also be employed as it has several advantages over *de novo* organogenesis. It can be used in species where *de novo* organogenesis does not occur, or it is very difficult to achieve. Further, it is less subject to somaclonal variation and less time consuming than regeneration from callus (Christou *et al.* 1990). Transgenic tobacco carrying coding sequences for *npt-II* and *gus* were produced following micro-projectile bombardment of tobacco leaves and subsequent culture (Tomes *et al.* 1990). Interestingly, micro-projectile bombardment of plant tissues has been used to increase transformation frequency by *Agrobacterium tumefaciens* (Bidney *et al.* 1992). Cotyledons of immature peanut (*Arachis hypogaea* L.) zygotic embryos were transformed *via* particle bombardment with a plasmid containing a chimeric *hph* gene conferring resistance to hygromycin. Under continuous selection, hygromycin resistant plantlets were regenerated from somatic embryos and were recovered from nearly 1.6 per cent of bombarded cotyledons (Deng *et al.* 2001).

Pea is relatively less studied for genetic transformation. The first report on pea transformation was by Puonti-Kaerlas *et al.* (1990) and De Kathen and Jacobsen (1990). Most of the pea transformation studies used *Agrobacterium tumefaciens* transformation system and there are only a few reports on direct DNA transformation (Molnár, 1999). Molnár (1999) used a pea genotype called 'Akt'. The Akt has good *in vitro* regeneration capability. Plasmids pRT103

carrying *gus* (β -glucuronidase-*gus* gene), pFF19H (hygromycin resistance gene, *hph*) and pD555 (mannitol phosphate dehydrogenase -mt1D enzyme gene) were introduced into the target tissues (plumule, radicle, callus). The plumules and radicles bombarded with plasmid DNA carrying the *hph* selectable marker gene were cultured on the same composition of selective B5 medium, which was used in the three days pre-culture, containing 50 mg l⁻¹ hygromycin B.

2.2.2 *Agrobacterium tumefaciens* mediated DNA transfer in plants

Agrobacterium mediated DNA transfer is the most common and widely used method for transformation of dicotyledonous plants. The first transgenic *Nicotiana tabaccum* plants were produced via *Agrobacterium* mediated transformation (Horsch *et al.* 1984). Although members of *Agrobacterium* have a wide host range, the ability of the bacterium to produce a compatible reaction varies widely with the host plant species and even with genotypes within a species (Barton *et al.* 1987). Horsch *et al.* (1985) considered this the simplest method available for transferring genes into intact plant tissues, host range being its limitation. Further discoveries in this field indicated that even in cereals and non-host species *Agrobacterium* mediated DNA transfer system could work with the aid of a phenolic compound called acetosyringone (Chang and Chan 1991) or specific *Agrobacterium* strain.

A. tumefaciens is a gram-negative bacterium, which can induce crown gall disease in plants by transferring a discrete portion of its plasmid DNA (Chilton 1977; Zambryski 1983; Klee and Rogers 1989; Binns and Thomshow 1988). Two important regions essential are; oncogenic T-DNA and the virulence (*vir*) genes, which encode proteins for T-DNA transfer. T-DNA transfer is dependent on a direct repeat, flanking sequence of 25 bp. The T-DNA is capable of inducing tumours in transformed plant, accompanied by products of three genes, which code for an auxin (Schroder *et al.* 1984; Thomshow *et al.* 1984), a cytokinin (Akiyoski *et al.* 1984) and an opine compound.

Agrobacterium tumefaciens plasmid transfers only T-DNA (Hooykaas and Schilperoort 1992; Zupan and Zambrysky 1995). The gene which is to be transferred to the host plant is intentionally placed into the T-DNA region

(Zambryski *et al.* 1983). Prior to placement of gene in T-DNA region, the oncogenic T-DNA region is deleted from Ti plasmid to prevent the overproduction of phytohormones, which induce tumours and interfere with normal plant development. The disarmed strain as it is called has only the *vir* region. Many Ti plasmids of *Agrobacterium* strains have been disarmed and are used as a vehicle for gene transfer; e.g., pLBA 4404 (Hoekama *et al.* 1983), pGV3850 (Zambryski *et al.* 1983). Alterations in the virulence and effective host range of commonly used *Agrobacterium* strains have been reported by Hood *et al.* (1986).

2.3 *Agrobacterium* mediated *in vitro* transformation of pea

First study on experimental introduction of crown gall tumors in pea seedlings *in vitro* was reported by Manigault and Kurkdjian (1967) followed by Kurkdjian *et al.* (1968). Puonti-Kaerlas *et al.* (1989) were the first to get successful infection and transformation by non-oncogenic vector where they used four strains of *A. tumefaciens* (A281, C58, GV3101 and B6S3) and five plasmids (pGV3304, pGV2298, pGV3851, Ri15864 and pGV3850) in two cultivars. Among four strains, A281 was the most virulent.

Mroginski and Kartha (1981) and Rubluo *et al.* (1984) used various explants of pea for plant regeneration via embryogenesis and organogenesis after transformation by *A. tumefaciens*. Subsequently, cotyledonary nodes (Jackson and Hobbs 1990), hypocotyls (Nielesen *et al.* 1991) and embryos (Kysely *et al.* 1987) of pea were used for transformation. Schroeder *et al.* (1993) cocultivated longitudinal slices of immature embryos of cultivars Greenfeast and Rondo and used phosphinothricin as selection agent. The efficiency of transformation was 1.5 to 2.5%. Devis *et al.* (1993) obtained transgenic plant by targeting highly regenerable lateral cotyledonary meristems capable of rapidly producing transformants independent of callus phase. Phosphinothricin was used at lower concentration (2.5 mg l⁻¹) for first three weeks followed by an increase in level (5 mg l⁻¹) for the remainder of the selection period. Grant *et al.* (1995) used immature cotyledon explants and *Agrobacterium* strain AGL1 was used to introduce a herbicide resistance gene in four cultivars of pea. They developed a reliable pea transformation system.

The scientists also focused on the specificity of strains towards genotype and the type of explants used. Hobbs *et al.* (1989) used 13 genotypes of varied morphology and geographical origin and three *Agrobacterium* strains (C58, A281, and Ach5). It was found that the pea genotypes differed in their susceptibility to different *Agrobacterium* strains. However, out of three strains, Ach5 was found to be the most promising. Filippone and Lurquin (1989) tested two *A.tumefaciens* strains (6044 and 6048) and three type of explants (epicotyls segment, cotyledon node bud and apical meristem). The *A.tumefaciens* strain 6044 was the most efficient in all three types of explants used as compared to other strains. Puonti-Kaerlas *et al.* (1990) used two cultivars of pea and recovered transgenic plants after co-cultivation of explants from shoot culture and epicotyles and a lengthy callus phase. The primary regenerants that produced viable seed were tetraploid (Puonti-Kaerlas *et al.* 1992) probably due to the long callus phase required in the procedure (Grant *et al.* 1995). Grant *et al.* (2003) compared the efficacy of two *Agrobacterium* strains, for producing transgenic pea in three cultivars. The temperature at which co-cultivation occurred, was an important factor to increase the efficiency of pea transformation (21-25). The strain KYRT1 was found to be best as compared to other *Agrobacterium* strains.

Once the regeneration potential of pea was proved conclusively, attempts were made to increase the efficiency of transformation. Although sufficient protocols for pea transformation have been elaborated, the regeneration of transgenic pea still is not a routine. Two main factors were found necessary to improve the pea transformation efficiency, i. an efficient regeneration system and ii. an appropriate *Agrobacterium* strain (Pniewski and Kapusta 2005). Pniewski and Kapusta (2005) used three hypervirulent strains (AgL₁, EHA105 and AGL₀). AgL₁ was the best among three in all nine genotypes of pea they studied. Orezyk and Orezyk (2000) assessed the influence of factors such as different strains of *Agrobacterium* (octopine LBA4404, nopoline C58C and hypervirulent EHA105) and selective agents (hygromycin B 100 mg l⁻¹, methotrexate 1 mg l⁻¹, phosphinothricin 2 mg l⁻¹ increased to 4 mg l⁻¹ after four week, acetosyringone 50 µg l⁻¹ and methylation inhibitor 5-azacytidin) on the expression of induced transgene. The hypervirulent strain EHA105 was found to be the most efficient and 8.2% explants regenerated transformed plants.

Krejci *et al.* (2007) tested and modified three methods of transformation in pea, i. transformation of stem segments, ii. transformation of axillary bud, and iii. transformation of embryonic segments using hypervirulent strain of *A. tumefaciens* (EHA105). Svabova and Griga (2008), studied the effect of various chemicals like acetosyringone, L-cystine, dithiothreitol, glutathione, cellulose and pectinase, light regimes (16/8 light/dark photoperiod, continuous light and continuous dark) applied during co-cultivation of pea explants with *Agrobacterium* strain EHA105 containing two plasmids (Pgt89 and Pbin19). These plasmids carried reporter *Gus-int* gene, and *bar* or *nptII* selectable marker gene, respectively. They used cotyledonary node explants of three dry seed pea cultivars. Based on these results improved cocultivation step for pea consists of 48 hour co-cultivation at 20°C, with 50 mg l⁻¹ L-cystine and 100 µM acetosyringone, 16/8 light/dark photoperiod.

Svabova *et al.* (2005) were the first to describe a genotype independent protocol of *Agrobacterium* mediated transformation of pea *in vitro* and *in vivo*. They used six cultivars and hypervirulent strain EHA105. Both methods but largely *in vivo* approach was genotype independent, resulting in efficient and reliable transformation system in pea.

2.3.1 Factors affecting *Agrobacterium* mediated transformation in pea

For optimization of the transformation protocol, interaction between *Agrobacterium tumefaciens* and plant is probably the most important aspect to be considered. Many factors influencing *Agrobacterium* mediated transformation of dicotyledonous plants have been elucidated. The factors like genotype, explant type, explant source, *A. tumefaciens* strain, co-cultivation treatment, acetosyringone concentration, co-cultivation temperature and injury to explants prior to inoculation might influence pea transformation.

2.3.1.1 Effect of explant type on transformation efficiency

Explant type is also a very important factor affecting plant transformation. Earlier immature embryos were reported as an appropriate source of explants and different types of embryo-derived explants such as slices of embryo axis

(Schroeder *et al.* 1993; Polowick *et al.* 2000) and basal parts of the embryo (Grant *et al.* 1995), were also used. In addition to embryos, cotyledons (Orczyk and Orczyk 2000), or cotyledonary nodes (De Kathen and Jacobsen 1990; Davies *et al.* 1993) were also tested for transformation efficiency. Krejci *et al.* (2007) found embryo axis to be unsuitable for transformation, as it died soon, especially on the selection medium, whereas cotyledon slices formed callus but had a very limited regeneration capacity. Conceivably, intact cotyledons could regenerate more efficiently than thin slices, as previously reported (Grant *et al.* 1995; Orczyk and Orczyk 2000).

Another explant used was axillary buds (Bean *et al.* 1997). The sterilised seeds of the variety Puget were imbibed in sterile distilled water for 24 h on a shaker at 150 rpm and then they were germinated for 48 h in moistened sterile Agrop-erlite. After germination, the seeds were inoculated, co-cultivated and transferred onto the solid B5 medium with 300 mg l⁻¹ of augmentin, 4.5 mg l⁻¹ BAP and 0.1 mg l⁻¹ IBA, pH 5.7. For the first 3 weeks, the co-cultivated explants were kept without selection pressure to help full development of the regenerating shoots from the wounded axillary buds. These regenerated shoots were excised and transferred onto a selection medium containing 2.5 mg l⁻¹ of PPT (Phosphinothricin). After another 3 weeks 5 mg l⁻¹ of PPT was added to the medium. The histochemical method was used for continuous analyses of the GUS activity in co-cultivated explants (samples were taken from the neighbourhood of the inoculated buds immediately after co-cultivation) as well as in the regenerated shoots. The putative transformants were rooted, acclimatised and transferred to *ex vitro* conditions.

The transformation of embryonic segments method is based on the work of Schroeder *et al.* (1993). The dry mature seeds were sterilised and imbibed for 24 h in sterile distilled water on a shaker at 150 rpm. After removal of the testa and one cotyledon the exposed embryo was cut longitudinally into halves and the greater part of the radicle was removed. These embryonic segments were immersed in a liquid 1/2 MS medium, the *Agrobacterium* suspension was added and the segments were co-cultivated for 1 h on a shaker at 150 rpm. After co-

cultivation the segments were dried on sterile filter paper and placed onto the solid co-cultivation medium P1 (MS with B5 vitamins) without antibiotics for another 48–72 h. The explants were then rinsed in a liquid 1/2 MS medium with 500 mg l⁻¹ of augmentin and transferred to the regeneration medium P1 with 300 mg l⁻¹ of augmentin, 2 mg l⁻¹ BAP and 2 mg l⁻¹ NAA. When the shoots were more than 10 mm long, they were gradually cut off from the original explants and then cultivated to a stage when they were capable of rooting or grafting. Samples were taken during regeneration to test the presence of GUS using histochemical method II. At the stage of short shoots selection was applied by adding a 2.5–5 mg l⁻¹ concentration of PPT to the selection medium (P2 with 4.5 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA).

2.3.1.2 Temperature during co-cultivation

Temperature effects on plant transformation have been studied by a few groups (Dillen *et al.* 1997; Chakrabarty *et al.* 2002; De Clercq *et al.* 2002), after conjugal transfer of Ti plasmid was shown to be sensitive to temperature (Fullner *et al.* 1996). Dillen *et al.* (1997) tested the effect of temperature on transgene expression in two plant systems, *Phaseolus acutifolius* and *Nicotiana tabacum*. The optimal temperature for *Phaseolus* callus transformation was 22°C and for tobacco was 19 to 22°C. *Phaseolus* callus was incubated with bacteria at different temperatures from 15 to 29°C for 3 days. Expression of GUS at 25°C was lower than at 22°C.

Very low levels of GUS expression were detected at 27°C and no expression at 29 and 15°C. They showed highest GUS expression at 22°C co-cultivation. They reported similar results with the tobacco experiment. In both studies, GUS expression was dramatically reduced when the temperature was increased from 22°C to 25°C. GUS expression was low at 27°C and undetectable at 29°C. Chakrabarty *et al.* (2002) evaluated a number of factors that increased the *Agrobacterium* mediated-transformation efficiency of hypocotyl explants of cauliflower. They showed that co-cultivation at 22°C resulted in higher GUS expression than at 28°C. De Clercq *et al.* (2002) also reported that co-cultivation at 22°C resulted in higher GUS expression than temperatures above 22°C in *Phaseolus acutifolius*.

Variable co-cultivation temperatures have been used by various workers (Schroeder *et al.* 1993; Bean *et al.* 1997; Orczyk and Orczyk 2000; Pniewski and Kaputa 2005; Svabova *et al.* 2005; Richter *et al.* 2006; Svabova *et al.* 2008). Bean *et al.* (1997) used a co-cultivation temperature of 20°C under 16-h photoperiod, Svabova *et al.* (2005) used 4°C for seeds and 24°C for cotyledonary node, Svabova *et al.* (2005) applied 20±2°C. While Svabova *et al.* (2008) and Bean *et al.* (1997) used 16L/8D photoperiod, Svabova *et al.* (2005) co-cultivated explants under dark conditions.

2.3.1.3 Injury to explants

Since, injury is known to enhance *Agrobacterium* penetration leading to higher transformation efficiencies in plant tissues and rubbing of tissues with silicon carbide (carborundum powder) is a way to induce injuries in plant tissues. It is the most common method used to wound tissues for viral infections under artificial conditions or even introduce the naked DNA into the cells (Kaeppler *et al.* 1990; Kaeppler *et al.* 1992). Since, the injury increased the transformation efficiency, thus, wounding regenerable differentiating tissues by carborundum rubbing provides a simple and efficient way for pea transformation mediated by *Agrobacterium*.

While no report on pea transformation using injury either by silicon carbide or other means is available, Chen *et al.* (1996) described an efficient *Agrobacterium*-mediated transformation method in papaya based on wounding of cultured embryogenic tissues with carborundum in liquid phase. The embryogenic tissues were vortexed with 600 mesh carborundum in sterile distilled water for 1 min before treating with the *A. tumefaciens*. The average successful rate of transformation was 15.9% of the zygotic embryos treated, about 10–100 times higher than the available methods previously reported. Similarly, Curuk *et al.* (2005) transformed the recalcitrant melon (*Cucumis melo*L.) by wounding cotyledon explants by vortexing with carborundum prior to inoculation with *Agrobacterium tumefaciens*. Only treatments where explants were vortexed with carborundum produced transgenic melon shoots.

2.3.1.4 Acetosyringone concentration

Despite the fact that successful pea transformation was reported 18 years ago (Puonti-Kaerlas *et al.* 1990), the efficiency of transformation protocols is still relatively low (in the range 0.1–6.5% – Davies *et al.* 1993; Schroeder *et al.* 1993; Bean *et al.* 1997; Jones *et al.* 1998; Polowick *et al.* 2000; Grant *et al.* 2003; exceptionally over 10% Orczyk and Orczyk 2000). Particularly, co-cultivation additives have not been studied systematically. Only sporadic data on acetosyringone use are available (De Kathen and Jacobsen 1990; Lulsdorf *et al.* 1991; Grant *et al.* 1995; Orczyk and Orczyk 2000; Svabova *et al.* 2005, 2008). In addition to acetosyringone, a range of chemical substances with a different nature and action were used for co-cultivation with the aim of enhancing the final efficiency of the transformation protocol. Generally, these substances work through an induction of virulence (acetosyringone), a reduction of the resistance of the recipient cell (thiol compounds, anti-oxidants), or a facilitation to penetrate through the cell wall (mechanically or chemically) (Dandekar and Fisk 2005; Opabode 2006).

Chang and Chan (1991) found that filtrate from potato suspension culture was essential for *Agrobacterium* mediated *in vitro* transformation of soybean. They also showed that potato suspension cultures contained many phenolic compounds including acetosyringone and sinapic acid (SA). Acetosyringone is a phenolic compound released by wounded cells that belong to the substances with potential to induce virulence (*vir*) genes of *Agrobacterium*. There were several positive results of acetosyringone on transformation efficiency recorded in several leguminous species (Santarem *et al.* 1998; Trinh *et al.* 1998; Yamada *et al.* 2001; De Clercq *et al.* 2002; Ko and Korban 2004). Acetosyringone is the only co-cultivation additive tested with various effects in pea transformation. Droste *et al.* (2000) used 100 μ M of acetosyringone in order to increase the virulence of *Agrobacterium tumefaciens*. Orezyk and Orezyk (2000) reported that acetosyringone had no apparent influence on efficiency of transformation in pea (*Pisum sativum*) with hypervirulent EHA 105 strain; however it did affect the rate of transformation when moderately virulent C58C1 was used.

Svabova *et al.* (2005; 2008) used sonication followed by co-cultivation on solid co-cultivation medium (5.5% plant agar) with three (0, 100 and 200 μM) acetosyringone concentrations to achieve pea transformation. The 100 μM acetosyringone significantly increased intensity of GUS Expression value, while 200 μM did not differ from control.

A negative effect of 50 μM acetosyringone was reported by De Katheren and Jacobsen (1990). De Clercq *et al.* (2002) exhibited no difference with control. In contrast to other crops, including some legumes, it seems that pea is more sensitive to acetosyringone and there is a relatively narrow acetosyringone concentration range (50–100 μM) stimulating vir gene of *Agrobacterium*. The results of Svabova *et al.* (2005; 2008) are contradictory to those of Orczyk and Orczyk (2000) where acetosyringone treatment did not improve the transformation efficacy in hypervirulent *A. tumefaciens* strain EHA105. Svabova *et al.* (2005; 2008) also recorded distinct results for pre-cultivation treatment of *Agrobacterium* with acetosyringone which was ineffective in peas, but positive in french bean (De Clercq *et al.* 2002). The outcomes consonant with Svabova *et al.* (2005; 2008) data were also reported by Townsend and Thomas (1996) in soybean.

2.3.2 Selectable markers in pea transformation

A marker gene allows the preferential growth of transformed cells in the presence of a selective agent. Many genes for resistance to antibiotics and herbicide have been used as selectable markers. Selection efficiency depends on the size of exposed tissue, developmental stage of the plant cells, regeneration response and concentration of the selective agent.

Neomycin phosphotransferase type-II (*npt-II*) gene has been extensively used in transformation protocols of many plant species than any other selectable markers. Originally isolated from bacterial transposon Tn5, *npt-II* provides resistance to certain aminoglycoside antibiotics such as kanamycin, paramomycin and geneticin, of which kanamycin is the most widely used antibiotic. Constitutive promoters such as the nopaline synthase (*nos*) or cauliflower mosaic virus (CaMv) 35S regulatory sequence has been fused with

npt-II (Fraley *et al.* 1983) and later cloned into plant transformation and expression vectors (An *et al.* 1985; Bevan 1984; Klein *et al.* 1985). Kanamycin selection has proven to be the most suitable; however, the concentration required for effective selection is species specific. Species like *Cajanus cajan* (Geetha *et al.* 1999; Lawrence and Koundal 2001), chickpea (Fontuna *et al.* 1993; Gray *et al.* 1993; Kar *et al.* 1996; Ramana *et al.* 1996; Srinivasan *et al.* 1991; Surya Prakash *et al.* 1992) and pea (De Kathen and Jacobson 1990; Lulsdorf *et al.* 1991; Polowick *et al.* 2000; Grant *et al.* 1998; Davies *et al.* 1993; Svabova *et al.* 2005) are selected at low concentration of Kanamycin (25-100 mg l⁻¹) whereas *Phaseolus aconitifolius* need relatively higher concentration (300 mg l⁻¹) of kanamycin (Dillen *et al.* 1997).

Another widely used and more effective selectable marker is hygromycin phosphotransferase (*hpt*, *aph-IV* or *hph*) which confers resistance to the aminoglycoside antibiotic hygromycin. Hygromycin allows clear discrimination between transformed and non-transformed tissues and do not regenerate albinos as is the case with kanamycin (Ayres and Park 1994). The *hpt* gene is considered as a very efficient marker gene for selection after *Agrobacterium* mediated transformation (Aldemita and Hodges 1996; Dong *et al.* 2001; Hiei *et al.* 1994; Rashid *et al.* 1996; Kumar *et al.* 2003; Singh *et al.* 2004).

Selection of transformed pea has been accomplished with both antibiotic and herbicide resistance genes. Antibiotic selection has been accomplished using *npt-II* for kanamycin resistance and *hpt-II* hygromycin resistance. Kanamycin has been used successfully at concentrations of 50 mg l⁻¹ (De Kathen and Jacobson 1990; Lulsdorf *et al.* 1991; Polowick *et al.* 2000), 75 mg l⁻¹ (Grant *et al.* 1998) and 100 mg l⁻¹ (Davies *et al.* 1993; Svabova *et al.* 2005). Hygromycin has been used at concentration of 5-10 mg l⁻¹ (De Kathen and Jacobson 1990; Grant *et al.* 1995; Bean *et al.* 1997; Jones *et al.* 1998), 15 mg l⁻¹ (Puonti-Kaerlas *et al.* 1992) and 25 mg l⁻¹ (Lulsdorf *et al.* 1991).

Selection using herbicide resistance genes has been accomplished using *ahs3r* for resistance to chlorsulfuron and *bar* for resistance to Basta. Herbicide resistance to Basta conferred by the *bar* gene has been successfully used at

concentrations of 10 mg l⁻¹ (Polowick *et al.* 2000) and 15 mg l⁻¹ PPT (Schroeder *et al.* 1993). The only report using the *ahas3r* gene for resistance to chlorsulfuron used at concentrations of 10 mg l⁻¹ (Polowick *et al.* 2000).

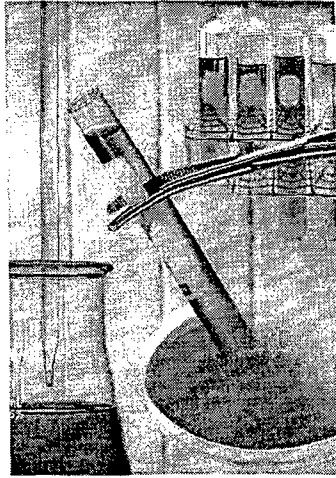
2.4 Confirmation of the transformation through DNA-based methods

The standard polymerase chain reaction (PCR) technique using transgene or marker specific primers has been extensively used to ascertain the presence of transgene. The PCR technique is the quickest method available for checking the presence of DNA sequences of interest in the target species and it has been employed in pea (Richter *et al.* 2006; Savabova *et al.* 2005; Pisniewski *et al.* 2000), pigeonpea (Lawrence and Koundal 2001) chickpea (Mikail *et al.* 2008) and several other crops.

The robustness of PCR technique is low and final confirmation of transformation relies on the restriction fragment length polymorphism (RFLP) and dot blot methods (Perrin *et al.* 2000). Dot blots technique has been used in tobacco to confirm the transfer of *glucanase* and *chitinase* genes (Dinesh 1998). RFLP based on Southern blot is used to confirm the stable integration of DNA in the genome, and to determine the copy number. Southern blot is carried out as a final confirmation and it is routinely used to know the presence of transgene in pigeonpea (Geetha *et al.* 1999; Lawrence and Koundal 2001; Kumar *et al.* 2003; Singh *et al.* 2004) with radioactive probes.

2.5 Grafting of transformed pea shoots to get complete plants

Bean *et al.* (1997) concluded that the rooting of putative transformants using hormone induction was slow, erratic and generally unreliable, taking between 6 and 12 weeks, with the majority of the putative transformed shoots failing to successfully produce roots. The extended culture period also resulted in the production of phenotypically abnormal plants which showed dwarfing, early prolific flowering with reduced seed set, and premature senescence. These problems were overcome by grafting the putative transformed shoots onto pea cv. Puget rootstocks. Successful grafting could be achieved within 1 week, and from 22 putative transformants initially grafted, 21 were successful, giving over 95% efficiency. All of the grafted plants were phenotypically normal (Bean *et al.* 1997).



MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

The material used and the techniques adopted during the course of present investigations are described in this chapter.

3.1.1 Laboratory Facilities and Equipment

All the experiments were conducted at the Department of Agricultural Biotechnology, College of Agriculture, CSK HP Agricultural University, Palampur during 2009-2012. Equipment used for the research is listed in alphabetical order: autoclave, electric heater, electrophoresis kit, electrophoresis power supply, eppendorf micro centrifuge, gel documentation unit, incubator shaker, laminar hood, microwave oven, millipore water purification system, PH meter, refrigerators, spectrophotometer, centrifuge, thermocycler, ultra low temperature freezer and weighing balances.

3.1.2 Chemicals

Chemicals used for the research are listed according to the alphabetical order of suppliers: Hi Media Laboratories Pvt. Ltd.: ethanol (EtOH), methyl alcohol (MeOH), agarose and tris, yeast extract manitol broth medium, dimethyl sulfoxide (DMSO), ammonium nitrate (NH_4NO_3), calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), potassium phosphate (KH_2PO_4), potassium nitrate (KNO_3), boric acid (H_3BO_3), cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), manganese sulfate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$), potassium iodide (KI), sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) ethylenediamine tetraacetic acid disodium salt ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$), sodium hydroxide (NaOH), Bacto agar, ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), chloroform, carbenicillin, glycerol, mannitol, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium dodecylsulfate (SDS), sodium phosphate monobasic (NaH_2PO_4), isoprophyl alcohol, acetosyringone, ammonium chloride (NH_4Cl), 6-benzylaminopurine (BAP), carbenicillin, ethylenediaminetetraacetic acid (EDTA), ethidium bromide (EtBr), hygromycin-B,

indole 3-acetic acid (IAA), Indole-3-butyric acid (IBA), kanamycin, β -mercaptoethanol, *myo*-inositol, rifampicin, α -naphthalene acetic acid (NAA), nicotinic acid, pyridoxine, sucrose, (NaI), Tween-20, thiamine, timentin and hydrochloric acid (HCL). Promega: adenosine 5.-triphosphate (ATP), deoxyribonucleotidetriphosphate (dNTP), Taq DNA polymerase, 10x Taq assay buffer and magnesium chloride (MgCl_2).

3.1.3 Experimental Material

A popular variety of green pea in the Himachal Pradesh named Azad P1 was used for transformation. The seed of the Azad P1 was procured from the Department of Vegetable Sciences of the University and used for transformation. The seed was multiplied in the fields and polyhouses of the Department of Agricultural Biotechnology, CSK HPKV, Palampur.

Glassware like bottles, culture tubes, conical flasks, Petriplates, beakers, pipettes, etc., was of Borosil make. Before washing, glassware was rinsed in water followed by overnight soaking in a detergent solution. Thoroughly washed glassware was rinsed in distilled water and dried in a hot air oven. The forceps, scalpels etc, were also cleaned and dried. Cleaned glassware was sealed with aluminium foil, Petriplates (with blotting/filter papers inside) wrapped in paper covers, and were autoclaved at 121.6°C at 1.5 kg/cm^3 for 15 minutes. All the chemicals and plant growth regulators were of analytical grade and were procured from standard chemical manufacturing companies.

All the tissue culture and transformation experiments were carried out aseptically in Laminar Air Flow hoods (Klenzaid). For tissue cultures, scalpel blades, forceps etc were flame sterilized. The tissue cultures were incubated in a growth chamber at $25\pm 1^\circ\text{C}$ and 16h/8h light/dark cycle. The light intensity (716 lux) was provided using white fluorescent tubes.

3.2 Tissue culture

3.2.1 Explants

Three explants viz., embryos, cut embryos and cotyledonary nodes from pea seeds were tested for direct shoot induction on different media. Seeds of Azad P1 were water soaked for one day. These were surface sterilized by

immersion in 70% (v/v) ethanol for 30 second followed by that in 0.1% (v/v) mercuric chloride for 5 min and 4–5 washings with sterile distilled water. Then cotyledons were opened and embryos were harvested. For cut embryo explants, each embryo was separated into two halves with scalpel blade. Surface sterilized seeds of Azad P1 were cultured onto water agar medium (8 g l^{-1} agar). The flasks were incubated for one week at $25 \pm 1^\circ\text{C}$ and 16h/8h light/dark cycle at 716 lux). After a week the germinated seedlings were harvested, shoot tips and epicotyls of germinated seedlings were cut with scalpel blade and remaining 5–7 mm segment comprising cotyledonary node (herein called cotyledonary node explant) was isolated (see Fig 4.3). The explants were placed onto different media in subsequent experiments for shoot induction and transformation.

3.2.2 Media used

The Murashige and Skoog's (1962) (MS) basal medium was used for all plant tissue culture experiments (Appendix 1). For shoot induction from the explants, three different media treatments were used (MS, $1/2\text{MS} + 20 \mu\text{M}$ BAP + $0.01 \mu\text{M}$ NAA, and $\text{MS} + 20 \mu\text{M}$ BAP + $0.01 \mu\text{M}$ NAA). For shoot elongation, five different media (MS, $1/2\text{MS} + 20 \mu\text{M}$ BAP + $0.01 \mu\text{M}$ NAA, $\text{MS} + 20 \mu\text{M}$ BAP + $0.01 \mu\text{M}$ NAA, $\text{MS} + 8.8 \mu\text{M}$ BAP, and $\text{MS} + 0.08 \mu\text{M}$ BAP) were used. Shoot elongation was studied only for the shoots developed from cotyledonary node explants.

The shoot regeneration media were supplemented with 3% (w/v) sucrose. The pH of all the media was adjusted to 5.8 using 1 N HCl or 1 N NaOH. The media were solidified using 0.8% (w/v) agar and 75 ml of the medium was poured into each of the 250 ml flasks. The media were autoclaved at 121°C for 20 min. The cultures were incubated at $25 \pm 1^\circ\text{C}$ under a 16/8 h light/dark photoperiod regime provided by white fluorescent light at 716 Lux.

Four concentrations i.e. no ammonium nitrate, one fourth (412.5 mg l^{-1}), one half (825 mg l^{-1}) and full ammonium nitrate (1650 mg l^{-1}) compared to MS basal supplemented with $20 \mu\text{M}$ BAP and $0.01 \mu\text{M}$ NAA were tested to study the effect of ammonical nitrogen on shoot induction from cotyledonary node cultures. All other parameters except ammonium nitrate were kept constant in this experiment.

Five different concentrations of sucrose (15, 30, 50, 75 and 100 g^l⁻¹) in the MS salts supplemented with 20µM BAP and 0.01µM NAA were tested to study the effect of sucrose concentration on direct shoot induction from cotyledonary nodes in pea variety Azad P1. All other parameters except sucrose were kept constant in this experiment.

3.2.3 Preparation of stock solutions of MS medium, plant growth regulators, antibiotics and acetosyringone

Murashige and Skoog (1962) basal medium (MS) supplemented with different growth regulators was used for the studies.

i. Preparation of stock solutions of MS basal medium

Composition and method of preparation of stock solutions is given below

SS-I (20X), g/l

FLASK-1

1. Weigh the quantity of each constituent as follows:

MS major salts	mg/1 L medium	1000 ml stock (20X)
1. NH ₄ NO ₃	1650 mg	33 g
2. KNO ₃	1900 mg	38 g
3. MgSO ₄ .7H ₂ O	370 mg	7.4 g
4. KH ₂ PO ₄	170 mg	3.4 g

FLASK-2

MS major salts	mg/1 L medium	1000 ml stock (20X)
CaCl ₂ .2H ₂ O	440 mg	8.8 g

- 2. Dissolve the contents of flasks 1 and 2 separately in about 400 ml of double distilled water
- 3. Mix the solutions of flasks 1 and 2 and make final volume to 1000 ml in one litre measuring cylinder with double distilled water and store in dark bottle (Marked MS-1) at 4°C in a refrigerator

SS-II (40X) mg/200ml

MS minor salts	mg/1 L medium	200 ml stock (40X)
1. H_3BO_3	6.2 mg	248 mg
2. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3 mg	892 mg
3. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6 mg	344 mg
4. KI	0.83 mg	33 mg
5. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25 mg	10 mg
6. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025 mg	1 mg
7. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025 mg	1 mg

Dissolve all micronutrients in double distilled water and make the final volume to 200ml. Mark the container as MS-II and store at 4°C.

SS-III (40X) Iron EDTA solution 200ml Stock

Dissolve 1492 mg of $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (Ethylenediaminetetra acetic acid, disodium salt) in 75 ml dH_2O . Dissolve 1112 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 75 ml dH_2O . Boil $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ solution and add to it, FeSO_4 solution gently by stirring. Make the final volume to 200 ml.

SS-IV (40X), VITAMIN STOCK SOLUTION

MS Vitamins	mg/1 L medium	200 ml stock (40X)
1. Thiamine (HCl)	0.5 mg	20 mg
2. Nicotinic acid	0.5 mg	20 mg
3. Glycine	2.0 mg	80 mg
4. Pyrodoxine (HCl)	0.5 mg	20 mg
5. Myi-inositol	100 mg	4 g

The growth regulator stocks were prepared as 1000 ppm in a volume of 100 ml. The growth regulators were dissolved in minute amounts of appropriate solvents (Table 3.1) followed by addition of distilled water to a volume of 100 ml. The growth regulator benzyle aminopurine (BAP) was added to the media before autoclaving. Heat-labile growth-regulators, naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) were filter sterilized through a bacteria-proof membrane (0.22 µm) filter (Millipore) and added to the autoclaved medium after it has been cooled to less than 60°C. The growth-regulator stocks were stored at 4°C.

Table 3.1 Plant growth regulators used in the present study, nature, molecular weight and solubility

Plant Growth Regulator	Nature	Molecular Weight	Stock (1000 ppm)	Solubility
Benzyl aminopurine	Autoclavable	225.2	50 mg/50 ml	1N NaOH
Naphthalene acetic acid	Autoclavable	186.2	25 mg/25 ml	Ethanol
Indole-3-acetic acid	Autoclavable	175.18	25 mg/25 ml	Ethanol
Indole-3-butyric acid	Autoclavable	203.23	25 mg/25 ml	Ethanol

Filter sterilized kanamycin (Hi-media) stocks of 25 mg/ml, timentin (Hi-media) stocks of 150 mg/ml, carbenicillin (Hi-media) stocks of 250 mg/ml and hygromycin (Hi-media) stocks of 25 mg/ml were prepared in double distilled sterile water and filter sterilized (pore size 0.22 micron, Make Millipore). Rifampicin (Hi-media) stocks of 25 mg/ml and acetosyringone (Hi-media) stocks of 50 μ M were prepared in DMSO (Dimethyl sulfoxide) and filter sterilized. The antibiotic and acetosyringone stocks were stored in a refrigerator (Electrolux) at 4°C. The antibiotics and acetosyringone were added to the media after autoclaving in the pre-cooled (50°C-60°C) media.

3.2.4 Preparation of MS medium from stock solutions

To prepare one litre of MS basal medium, 50 ml of major salt stock, 5 ml of minor salt stock, 5 ml of vitamin stock and 5 ml of iron stock were added to 700 ml of distilled water. Subsequently, sucrose 30 g l^{-1} , if otherwise not mentioned in the text, was added and dissolved completely by mild heating on a flame. The volume of the medium was adjusted to 900 ml with distilled water and its pH was adjusted to 5.6-5.8 with 1N HCl or 1N NaOH. Distilled water was added to make the final volume to one litre. The agar (8 g l^{-1}) was added and the medium was heated gently to melt agar. The melted media was poured in to flasks (250 ml capacity, 75 ml per flask; 150 ml capacity, 50 ml per flask). The flasks were plugged with non-absorbent cotton plugs. The media were autoclaved as described in the preceding text. Thermolabile compounds wherever needed were added after filter sterilization. The media were pre-cooled to 50- 60°C prior to addition of thermolabile compounds.

3.3 Transformation

3.3.1 Gene used for transformation, *Agrobacterium tumefaciens* strain and plasmid vector

Agrobacterium strain GV3101 harbouring binary vector pCAMBIA 1302 (Fig. 3.1) with Thaumatin like protein (TLP) gene from tea (*Camellia sinensis* L.), a selectable marker gene aminoglycoside 3'-phosphotransferase (*nptII*) for bacterial selection and a selectable marker gene hygromycin phosphotransferase (*hpt*) for transformed plant selection were used. In addition to this, pCAMBIA 1302

also harboured a reporter gene, green florescent protein (gfp), under cauliflower mosaic virus (CaMV) 35S promoter. The Gene for *CsTLP* was inserted between *NcoI* and *SpeI* restriction sites in pCAMBIA 1302 binary vector. The gene construct was procured from IHBT, Palampur.

3.3.2 Hygromycin sensitivity test for pea

The *hpt* is the most popular selectable marker gene used in plant transformation. For successful selection, the target plant cells must be susceptible to antibiotics. In this experiment hygromycin sensitivity test was carried out to find out the optimum concentration of hygromycin required for selection of putative transformants. Cotyledonary nodes were cultured on MS media supplemented with 20 μ M BAP and 0.01 μ M NAA and various levels of hygromycin (0, 5, 10, 15, 20, 25 and 50 mg l^{-1}). Lethal dose was identified at shoot-bud initiation and shoot elongation.

3.3.3 Maintenance and growing of *A. tumefaciense*

The *A. tumefaciens* strain GV3101 harbouring pCAMBIA 1302 was maintained on solid yeast extract mannitol agar (YEMA) medium containing 50 mg l^{-1} of kanamycin and 25 mg l^{-1} of rifampicin at 28°C for two days in incubation chamber. Sub culturing was done every fortnight in fresh YEMA (Appendix 2) medium containing kanamycin and rifampicin. The liquid yeast extract manitol broth (YEMB) medium was then inoculated with *Agrobacterium* and cultured overnight at 28°C with 150 rpm shaking, and bacteria were subsequently transferred onto the YEMB medium at a ratio of 1 : 100 and grown up to OD600 $\approx$ 0.6–0.8. Then the culture was centrifuged at 6000 rpm and 4°C for 10 minutes, and the bacterial pellet was resuspended in the MS liquid solution consisting of MS salts. The volume of MS liquid solution was calculated to prepare the bacterial suspension of a density $\approx 10^8$ cells/ml. *Agrobacterium* suspension was then poured off into 150 ml flasks and used for explant inoculation.

3.3.4 *Agrobacterium* mediated *in vitro* transformation

Cotyledonary node explants were harvested as described in section 3.2.1. The explants were immersed in *Agrobacterium tumefaciens* suspension for 45 minutes with gentle shaking. The *Agrobacterium* treated explants were blotted dry using sterile blotting papers and transferred onto culture medium MS+20 μ M BAP+0.01 μ M NAA for two days for co-cultivation.

After co-cultivation, explants were washed 3–4 times in sterile distilled water to remove bacteria followed by another washing with MS salts containing 500 mg/l carbenicillin and 300 mg l⁻¹ timentin. Subsequently, the explants were dried using sterile blotting papers and transferred onto culture medium (MS+20 μ M BAP+0.01 μ M NAA) containing 300 mg l⁻¹ timentin. For first 2 weeks, co-cultivated explants were kept without selection pressure to help full development of the regenerating shoots. After two weeks, the cultures were transferred onto a selection medium (MS+20 μ M BAP+0.01 μ M NAA+300 mg l⁻¹ timentin+15 mg l⁻¹ hygromycin-B) for selection of putative transformants. Shoots were maintained on selection medium for one month. The cultures were sub-cultured on selection medium after every ten days.

Regeneration of whole plants occurred in two stages using the current methodology. Shoots were generated from explants. Well developed shoots were subsequently grafted on to the rootstocks of Azad P1 in the greenhouse (Figure 4.4). Induction of roots is rather time consuming and can take as long as 4-6 weeks, whereas grafting can be accomplished immediately. Both systems will incur some loss, but grafting can shorten the time required to produce T1 seeds by several weeks.

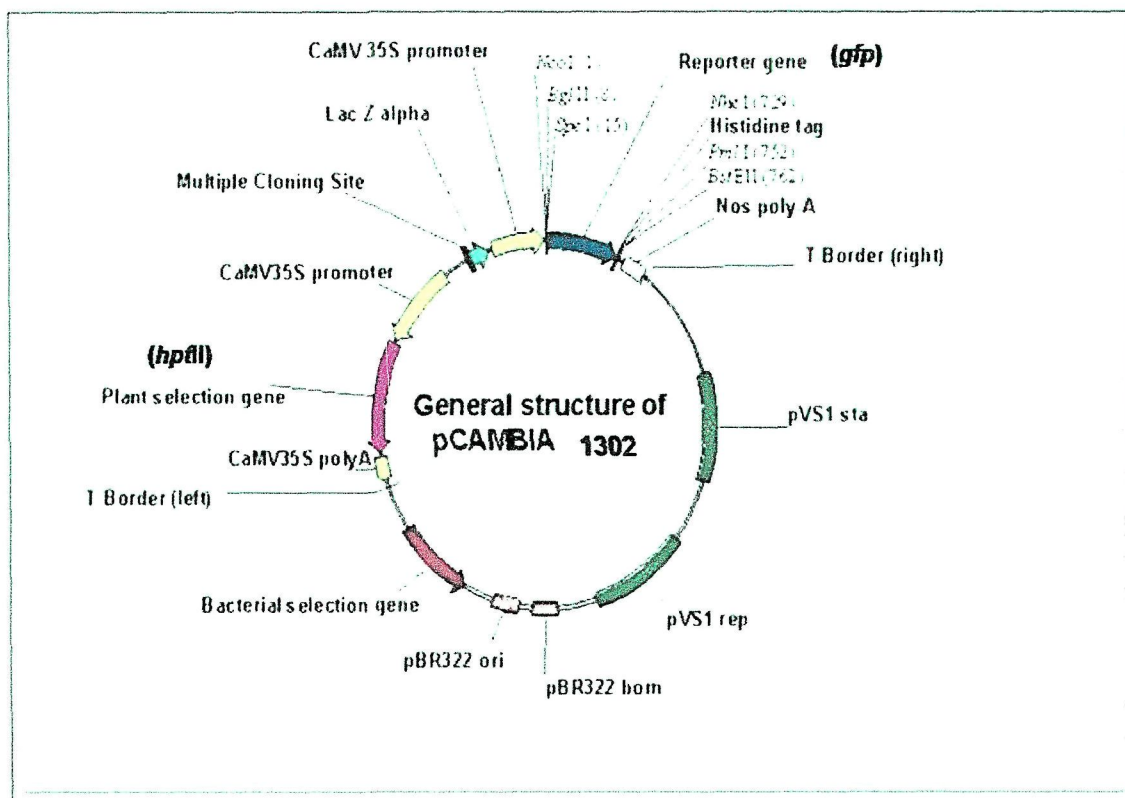


Fig 3.1 Vector map of pCambia 1302.

3.3.5 Standardization of transformation protocol for pea cv. Azad P1

3.3.5.1 Standardization of acetosyringone concentration

Use of virulence inducing agents such as acetosyringone is considered to be an important variable in *Agrobacterium* mediated transformation. To know the effect of acetosyringone, five concentrations viz., 0 μM , 10 μM , 20 μM , 50 μM and 100 μM were used in *Agrobacterium* suspension. Inoculum was prepared from overnight grown *Agrobacterium* strain GV3101 harbouring plasmid pCambia 1302. The cultures were centrifuged at 10000 rpm for 10 minutes and the pellets were dissolved in liquid MS solution. To this solution acetosyringone (0, 10 μM , 20 μM , 50 μM and 100 μM) was added. Explants were immersed in *Agrobacterium* suspension for 45 min with intermittent shaking, blotted dried and co-cultivated for 2 days on MS with 2 mg l^{-1} BAP. The 50 μM acetosyringone was the optimum level for maximum transformation in pea and hence, in subsequent experiments, 50 μM acetosyringone was used during co-cultivation.

3.3.5.2 Standardization of co-cultivation temperature

Co-cultivation temperature is an important variable in *Agrobacterium*-mediated transformation. Five co-cultivation temperatures (4°C, 18°C, 20°C, 22°C and 24°C) were tested. Cotyledonary nodes after inoculation were co-cultured in a medium described above at different temperatures. After co-cultivation, the Cotyledonary nodes were maintained as described in section 3.3.4.

3.3.5.3 Effect of silicon carbide (carborundum powder) on transformation efficiency of Azad pea 1

Wounding of tissues is known to enhance *Agrobacterium* penetration leading to higher transformation efficiencies. In this experiment, the explants were rubbed with carborundum powder prior to inoculation with *Agrobacterium*. The transformation efficiency was compared with control i.e. explants treated with *Agrobacterium* without using carborundum.

3.3.5.4 Grafting of putative transformants

Grafting of putative transformed shoots was achieved using the rootstock of Azad P1 seedlings. Seeds were sown in compost containing 30% added grit, and maintained at 25±1°C with a 16-h photoperiod. Grafting was performed approximately 2 weeks after sowing the seed. The shoot tip of the recipient seedling was excised using a horizontal cut through the stem, midway between the second and third nodes from the base of the seedling. A vertical incision was made in the stem of the rootstock to have a 'v' shape cut. The stem of the putative transformants was also cut in a 'v' shape between two nodes. The 'v' shape of the shoot was inserted into the 'v' shape of the stem of the recipient rootstock and the graft bound using Stericrepe tape. The shoot was protected from excessive water loss by covering with a Richardson jar. Shoot growth commenced within 7 days, after which plantlets were re-potted into fresh compost with 30% added grit.

3.4 Confirmation of presence of transgene

The control and putatively transformed plants were tested for the presence of *CsTLP* gene by polymerase chain reaction (PCR) using gene-specific primers. The detailed procedure is given below.

3.4.1 Extraction of genomic DNA

- Tissue from young pea leaves was macerated in liquid nitrogen using pestles and mortars at room temperature without buffer for 15 sec.
- The powdered tissue was transferred to the 1.5 ml microfuge tubes to which 0.4 ml extraction buffer (Appendix 3) was added and sample was vortexed for 5 sec.
- The 5 µl RNase A (1 mg/ml) was added to the DNA and incubated at 37°C for half an hour. The solution was centrifuged at 10000 rpm for 10 minutes and 400 µl of supernatant was transferred to fresh Eppendorf tube.
- Supernatant was mixed with 400 µl isopropanol and incubated at room temperature for 5 minutes.
- The mixture was centrifuged at 10,000 rpm, the supernatant was discarded and remaining pellet was dried in a laminar airflow hood. The dried pellet was dissolved in 100 µl 1x TE (Appendix 4), and stored at 4°C until further use.
- The DNA was quantified using a spectrophotometer by recording the absorbance at 260 nm and 280 nm and quality was assessed using the agarose (1.8%) gel electrophoresis.

3.4.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in a volume of 50 µl in 0.2 ml PCR tubes. The PCR reaction mixtures consisted of 50 ng of template DNA (DNA of putative and the control plants), two *CsTLP* gene specific primers (reverse and forward) 0.06 µl, 0.8µl of dNTPs, 0.5 µl (1 Unit) of Taq DNA polymerase and Magnesium Chloride (MgCl₂) (Appendix 5).

CsTLP genespecific primers pair:

pCamTLP L: 5'-TACCATGGTAATGAGCTTCCCCAAAAGCCTC-3',

pCamTLP R: 5'-GCACTAGTAGGGCAGAAGGTAATAGCATAATTG-3'

PCR amplification conditions were as follows: The temperature and number of PCR cycles in each step of PCR are given in Table 3.2. PCR consists of a series of 30 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps. Most protocols call for cycles that have three temperature steps. The cycling is often preceded by a single temperature step (called hold) at a high temperature (94°C), and followed by one hold at the end for final product extension or brief storage.

1. Initialization step: This step consists of heating the reaction to a temperature of 94°C, which was held for 4 minutes.
2. Denaturation step: This step is the first regular cycling event and was consisted of heating the reaction to 94°C for 10 seconds. It causes DNA to melt by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA.
3. Annealing step: The reaction temperature was lowered to 58°C for 30 seconds allowing annealing of the CsTLP gene primers to the single-stranded DNA template.
4. Extension/elongation step: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. A temperature of 72°C for 1 min was used as extension/elongation step.
5. Final elongation: This single step was performed at a temperature of 72°C for 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA was fully extended.
6. Final hold: This step at 4°C for an indefinite time was employed for short-term storage of the reaction.

Table 3.2PCR steps with reaction conditions.

Stage	Temperature (°C)	Duration	Cycles
Initial denaturation	94	4 minutes	1
Denaturation	94	10 seconds	30
Annealing	58	30 seconds	
Extension	72	1 minute	
Final extension	72	5 minutes	1
Hold	4	-	-

After the completion of required number of cycles, the samples were stored at 4°C in a refrigerator for agarose gel for electrophoresis.

The *A. tumefaciens* strain GV3101 was also used directly to test the presence on *CsTLP*. The experiment was carried out using the PCR procedure as outlined above except the DNA sample. Ten µl of 24 h old bacterial culture was harvested and transferred to the 0.2 ml PCR tubes. The tubes were heated to 95°C in a PCR for 10 minute. Four µl of the sample was then harvested and used for PCR reaction.

For PCR using isolated plasmid (pCAMBIA1302), 5 µl (25 ng) of the plasmid DNA was used.

3.4.3 Separation of PCR products

The PCR products were separated electrophoretically using 1.8 per cent agarose gels and 1X TAE buffer (pH-8.0) (Appendix 6-8). The ethidium bromide (staining dye) was added to the gel at the time of gel preparation. Fifteen µl of PCR amplified products from each reaction was mixed with 3 µl of loading dye (Bromophenol blue) (Appendix 9) and the samples were loaded into the wells of the gel. The gels were run at 60V for 2 h. After the electrophoresis, the DNA bands were visualized under UV transilluminator and pictures were captured using gel documentation unit (Bio Rad).

3.4.4 Isolation of plasmid DNA from *Agrobacterium* by alkali lysis method

- A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation loop and was grown overnight in 1 ml YEM broth containing kanamycin (50 mg l^{-1}) and rifampicin (25 mg l^{-1}) in a sterile 10 ml culture tube.
- Overnight grown culture (1 ml) was added to 5 ml YEM broth containing kanamycin (50 mg l^{-1}) and rifampicin (25 mg l^{-1}) and again grown overnight on shaker (150 rpm) at 28°C .
- About 1.5 ml of culture was taken and centrifuged at 10000 rpm for 2 minutes at 40°C and supernatant was discarded.
- The pellet was resuspended in 100 μl of resuspension solution (Appendix 10). The contents were mixed thoroughly by gentle vortexing.
- About 200 μl of lysis buffer (Appendix 10) was added, vortexed for few minutes. The contents became clear indicating lysis of the bacteria.
- To this 150 μl of neutralization solution (Appendix 10) was added and kept at -20°C for 20 minutes.
- The mixture was thawed for 3-5 minutes and centrifuged for 15 minutes at 10000 rpm.
- Supernatant was transferred to a fresh tube and 500 μl of phenol was added and mixed gently to form emulsion. After a brief vortexing, mixture was centrifuged for 5 minutes at 10000 rpm. The supernatant was transferred to fresh 1.5 ml tube.
- 600 μl of CIA (chloroform: isoamyl alcohol:: 24:1) was added to each tube followed by gentle mixing and centrifugation for 5 minutes at 10000 rpm.
- The aqueous layer was transferred to fresh 1.5 ml tube.
- To this 200 μl of 3M sodium acetate and 600 μl of isopropanol were added. The contents were spun at 10000 rpm for 10 minutes and supernatant was decanted (Appendix 10).

- The pellet was washed with 500 µl of 70% ethanol for 5 minutes.
- The tube was spun at 10000 rpm for 5 minutes and ethanol was decanted. The tubes were dried under sterile hood and the pellet was dissolved in 50 µl of TE buffer (Appendix 4) and stored at -20°C till further use.

3.5 Data recording and statistical analysis

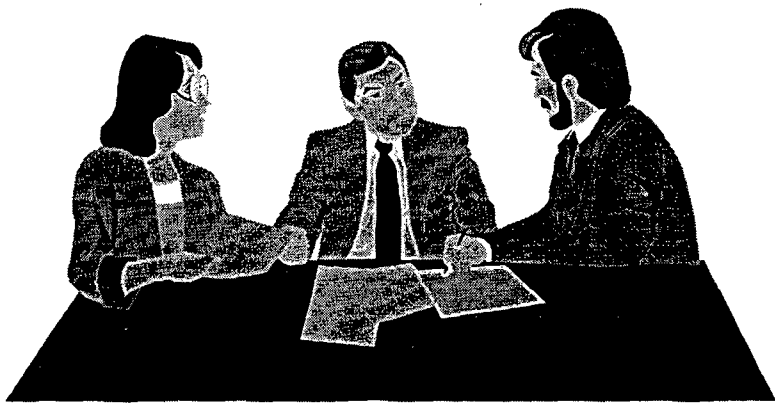
The experiment on effect of media components and explant type were conducted in a factorial completely randomized design (FCRD). There were five replications with 40 explants per replication. All other experiments were conducted in a completely randomized design (CRD). The experiment on shoot elongation had five replications (40 explants per replication) whereas those on effect of sucrose concentration on shoot induction had 4 replications (40 explants each), effect of ammonium nitrate on shoot induction had 3 replications (40 explants each), hygromycin B sensitivity test had 3 replications (40 explants each), effect of acetosyringone on transformation efficiency and effect of temperature and injury to explants by silicon carbide had 3 replications (100 explants per replication).

The data in the experiment on effect of explant and media on shoot induction and other parameters, and effect of ammonium nitrate and sucrose were recorded after 30 days. The data on shoot elongation were recorded after 30 days of culture of shoot bunches on the shoot elongation media. Prior to transfer of shoot bunches onto shoot elongation media, the cultures were maintained on shoot induction medium for 60 days with one subculture after 30 days. The data on inhibitory effect of hygromycin B on shoot induction from cotyledonary nodes was recorded after 10 and 20 days whereas the number of putative transformants was recorded after 45 days (15 days culture on non-selective medium, 30 days culture on selective medium) of culture of transformed explants onto the media. The observations were recorded for the number of explants forming shoots, shoots/explant, shoot length/explant, explants forming roots and roots length /explant. For hygromycin experiments, the explants forming shoots or roots at particular concentration were recorded. The per cent

putative transformants were calculated by survival of shoots on hygromycin medium with number of shoots cultured on hygromycin medium.

$$\text{Per cent putative transformants} = \frac{\text{No. of shoots survived on hygromycin medium}}{\text{No. of shoots cultured on hygromycin medium}} \times 100$$

The statistical analysis of data was done using Microsoft-Excel 2007. All data were subjected to ANOVA, and means were compared using Fisher's least significant difference (LSD) test ($\alpha = 0.05$).



RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

In the present study, Azad P1 variety of green pea, a widely grown variety in the Himachal Pradesh was evaluated for shoot regeneration from embryo, cut embryo and cotyledonary node explants. The best explant for shoot regeneration (cotyledonary nodes) was further used to standardize *Agrobacterium* mediated transformation protocol. For this study *Agrobacterium tumefaciens* strain GV3101 containing pCambia 1302 plasmid was used. The plasmid harboured a thaumatin like protein (*CsTLP*) gene from *Camellia sinensis* (L.) O. Kuntze, a small tree belongs to the family theaceae. *CsTLP* (thaumatin-like protein) belongs to the pathogenesis-related proteins (PR) family. The members of *TLP* are known to induce tolerance response to stresses, pathogenic as well as abiotic stimuli. Several *TLPs* exhibit antifungal activity *in vitro* and some have been shown to have β -glucanase activity. Also, a *TLP* that functions as antifreeze protein has been isolated from winter rye during cold acclimation. Aminoglycoside 3'-phosphotransferase (*npt-II*) gene as selectable marker for *Agrobacterium tumefaciens* and hygromycin phosphotransferase (*hpt*) as plant selectable marker gene were inserted into the binary vector pCambia 1302. PCR analysis using a *CsTLP* gene marker was also carried out to confirm transformation of pea.

The results obtained on different aspects of the present investigation are described under the following heads:

4.1 *In vitro* regeneration

4.1.1 Effect of salt and vitamin concentration, explants type and growth regulators on direct shoot induction

4.1.2 Effect of salt concentration and growth regulators on elongation of shoots

4.1.3 Effect of ammonium nitrate concentration on direct shoot induction

4.1.4 Effect of sucrose concentration on direct shoot induction

4.2 Standardization of *Agrobacterium* mediated transformation of pea

4.2.1 Hygromycin sensitivity test

4.2.2 Standardization of acetosyringone concentration

4.2.3 Effect of temperature and injury to explant on transformation

4.2.4 Confirmation of presence of transgene in putative transformants

4.3 Pea transformation protocol developed for cv. Azad P1

4.1 *In vitro* shoot regeneration

4.1.1 Effect of salt and vitamin concentration, explants type and growth regulators on direct shoot induction

Three explants viz., embryos, cut embryos and cotyledonary nodes from seeds of pea cultivar Azad P1 were evaluated for shoot induction on three different media (MS, $\frac{1}{2}$ MS+20 μ M BAP+0.01 μ M NAA, and MS+20 μ M BAP+0.01 μ M NAA). The media have different concentrations of salts, vitamins and growth regulators. The data were recorded on explants forming shoots or roots, shoots per explant and average shoot/root length after 30 days of culture (Table 4.1). Analysis of variance for explants forming shoots, number of shoots per explant, average shoot length, explants forming roots and average root length involving different media and explant is presented in Tables 4.2 and 4.3. Both the factors i.e. media and explant type had significant effect on explants forming shoots, number of shoots per explant and shoot length but in case of explants forming roots and root length only media had significant effect but not the explant type.

Among the three explants, cotyledonary nodes were superior to others for shoot induction (Table 4.1, Figs 4.1, 4.2, 4.3). Hundred per cent cotyledonary nodes generated multiple shoots on all the media except MS basal whereas the shoot induction frequency varied from 70.86% to 88.99% for other two explants. While, multiple shoots were not induced on MS basal medium from all the explant types, the number of shoots generated from cotyledonary nodes as well as shoot length were more on $\frac{1}{2}$ strength as well as full strength MS media supplemented with 20 μ M BAP+0.01 μ M NAA compared to other two explants.

Table 4.1 Effect of salt and vitamin concentration, explants type and growth regulators on direct shoot induction from pea variety Azad P1.

Medium	Explant	Explants forming shoots (%) ± SE	Shoots/explant (no.)± SE ¹	Average shoot length (cm)± SE ¹	Explants forming roots (%) ± SE ⁴	Average root length (cm) ± SE ⁴
MS basal	Cut embryo	70.86 ±4.08	1±0	4.20±0.54	100±0 (10.04)	9.35±0.12 (3.22)
	Embryo	84.66±3.59	1±0	4.69±0.33	100±0 (10.04)	9.26±0.21 (3.20)
	cotyledonary node	100 ±0	1±0	5.40±0.42	100±0 (10.04)	2.78±0.22 (1.94)
	Average	85.17	1	4.77	100	7.13
1/2MS+GR ²	Cut embryo	71.99±2.70	1.96±0.22	2.28±0.41	0±0 (1.00)	0±0 (1.00)
	Embryo	87.99±4.02	1.62±1.23	3.26±0.52	0±0 (1.00)	0±0 (1.00)
	cotyledonary node	100±0	3.34±.08	4.63±0.35	0±0 (1.00)	0±0 (1.00)
	Average	86.66	2.30	3.38	0	0
MS+GR ³	Cut embryo	76.72±3.95	4.68±0.17	4.46±0.43	0±0 (1.00)	0±0 (1.00)
	Embryo	88.99±3.33	3.1±0.13	5.93±0.38	0±0 (1.00)	0±0 (1.00)
	Cotyledonary node	100±0	9.84±0.25	10.47±0.25	0±0 (1.00)	0±0 (1.00)
	Average	88.90	5.87	6.95	0	0

¹ Shoots ≤ 2 mm were not included. Data were scored after one month of culture of explants on the medium.

² ½ MS basal medium + 20 µM BAP+0.01 µM NAA

³ MS+20 µM BAP+0.01 µM NAA

⁴Transformed values within parenthesis. The data were transformed as square root of value+1. Values with same letter are statistically similar.

Table 4.2 ANOVA for explants forming shoots (%), number of shoots per explant and shoot length per explant (cm) involving different media and explants in pea variety Azad P1.

Source	d.f. ²	Explants forming shoots % ¹			Shoots/explant(no)			Shoot Length (cm)		
		MS	CD	CV	MS	CD	CV	MS	CD	CV
Media	2	25.9	NS		94.44	0.24		48.52	0.66	
Explant	2	2694.7	5.26		32.88	0.24		39.92	0.66	
Media x explant	4	18.25	NS		16.74	0.41		8.94	1.15	
Error	36	50.57		8.22	0.10		10.66	0.80		17.86

¹MS: mean square, CD: critical difference, CV: coefficient of variation

²d.f: degree of freedom

Table 4.3 ANOVA for explants forming roots (%) and root length per explant (cm) involving different media and explants in pea variety Azad P1.

Source	d.f.	Explants forming Roots %			Root length (cm)		
		MS	CD	CV	MS	CD	CV
Media	2	50000.0	0.23		254.27	0.17	
Explant	2	0.00	NS		23.67	0.17	
Media x explant	4	0.00	NS		23.67	0.30	
Error	36	0.01		0.00	0.57		10.06

¹MS: mean square, CD: critical difference, CV: coefficient of variation

²d.f: degree of freedom

On ½ MS+20 µM BAP+0.01 µM NAA, the number of shoots per explants and shoot length from cut embryos were 1.96 and 2.28 cm respectively, from embryos the values were 1.62 and 3.26 cm, respectively whereas from cotyledonary nodes these were 3.34 and 4.63 cm, respectively. In comparison to this, on MS+20 µM BAP+0.01 µM NAA, shoots/explant and shoot length from cut embryos were 4.68 and 4.46 cm respectively, from embryos the values were 3.1 and 5.93 cm, respectively and from cotyledonary nodes these were 9.84 and 10.47 cm, respectively. The shoot formation from embryos, cut embryos and cotyledonary node explants on three media is given in Figs 4.1, 4.2, 4.3, 4.4 & 4.5. Clearly, the cotyledonary nodes were the best explant for shoot induction from pea cultivar Azad P1. Among the three explants used, cut embryos and cotyledonary nodes led to direct shoot induction. On the other hand, callus development from some embryo explants was observed (Fig. 4.2). The calli induced by embryos turned green and subsequently developed shoots.

Medium also affected shoot induction from the three explants (Table 4.1). On MS basal medium, multiple shoots were not induced. All the three explants developed single plantlet per explant on this medium (Fig 4.1 and 4.2). On MS+20 μ M BAP+0.01 μ M NAA and $\frac{1}{2}$ MS+20 μ M BAP+0.01 μ M NAA, multiple shoots were regenerated (Fig 4.1, 4.2, 4.3, 4.4 and 4.5). The shoots were devoid of roots. Concentration of media salts and vitamins affected the shoot induction and development from all the three explants. Reducing the concentration of salts and vitamins to half decreased shoots per explant as well as shoot length, however, it did not affect the number of explants forming shoots. For example, the frequency of cotyledonary node explants forming shoots was 100% on all media except MS basal. Similarly for cut embryos, frequencies varied from 70.86% to 76.72% and for embryos from 84.66% to 88.99%. For cotyledonary nodes, the number of shoots per explant on MS+20 μ M BAP+0.01 μ M NAA was 9.84 compared to 3.34 on half strength of salts and vitamins. Similarly, shoot length was 4.63 cm on half strength MS plus growth regulators compared to 10.47 cm on full strength MS plus growth regulators. Even from embryos and cut embryos, the values of these parameters in full strength media were twice than those obtained at half strength MS (Table 4.1). The data clearly showed that high concentrations of MS salts and vitamins favoured shoot induction in pea cv. Azad P1.

Maximum number of shoots per explant (9.84) and shoot length (10.47 cm) were observed from cotyledonary nodes cultured on MS+20 μ M BAP+0.01 μ M NAA. Thus, the best explant for shoot induction and development from pea cultivar Azad P1 was cotyledonary nodes and the best medium was MS+20 μ M BAP+0.01 μ M NAA. Multiple shoots have already been regenerated from cotyledonary nodes of pea (Franklin *et al.* 2000). While, Franklin *et al.* (2000) found 2.0 mg l^{-1} BAP to be the best for multiple shoot induction, present study showed that a combination of BAP (4.5 mg l^{-1}) and NAA (0.002 mg l^{-1}) was best for direct multiple shoot induction from cotyledonary nodes. Franklin *et al.* (2000) further demonstrated that very high BAP concentration was detrimental to multiple shoot induction. Cotyledonary node explants produced shoots

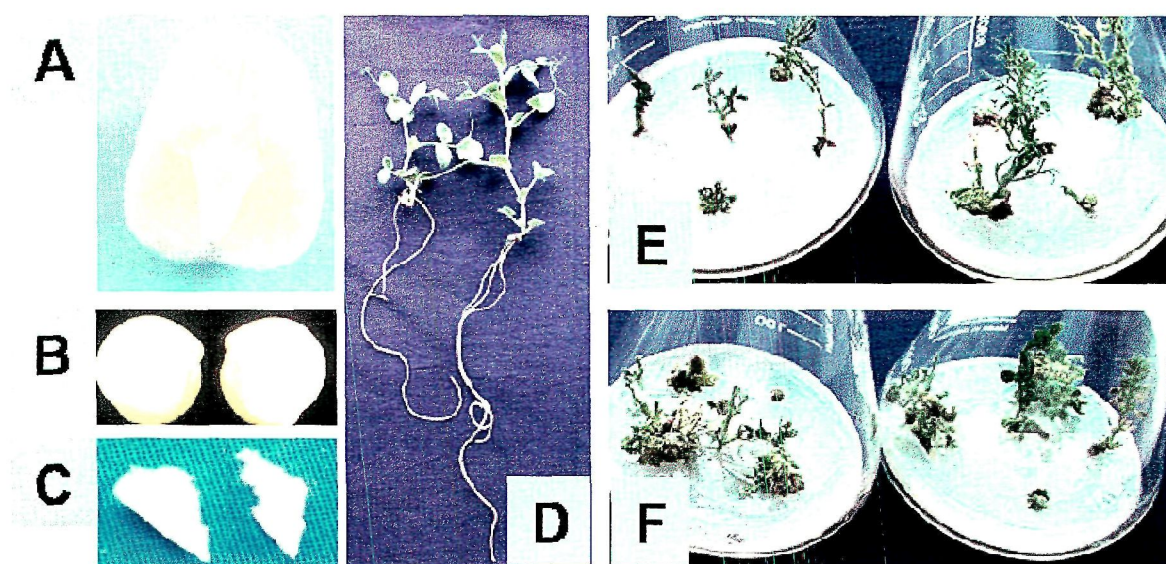


Fig 4.1 Effect of different media on shoot/plant regeneration from cut embryos of *Pisum sativum* cultivar Azad P1. A, B, C) Isolation of cut embryos, A) seed after 24 h of water soaking, B) cotyledonary halves after embryo removal, C) cut embryos, D) cut embryos formed plants with well developed shoots and roots on MS basal medium, E, F) shoot formation on E) $\frac{1}{2}$ MS + 20 μ M BAP + 0.01 μ M NAA and F) MS + 20 μ M BAP + 0.01 μ M NAA.

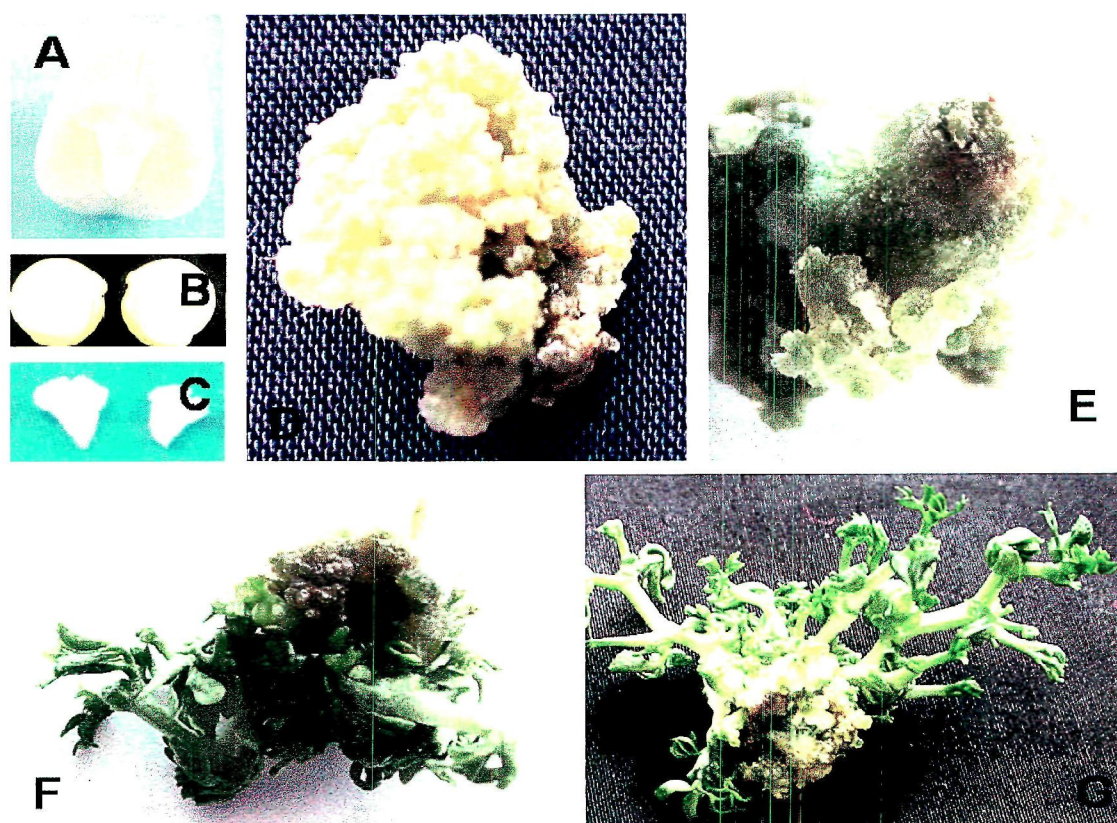


Fig 4.2 Shoot induction from *Pisum sativum* cultivar Azad P1 embryo explants on MS medium supplemented with 20 μM BAP+0.01 μM NAA. A, B, C) Isolation of embryos, A) seed after 24 h soaking in water, B) cotyledons without embryo C) embryos, D) callus induction, E) callus with green cells, F) shoot induction, G) well developed shoots.

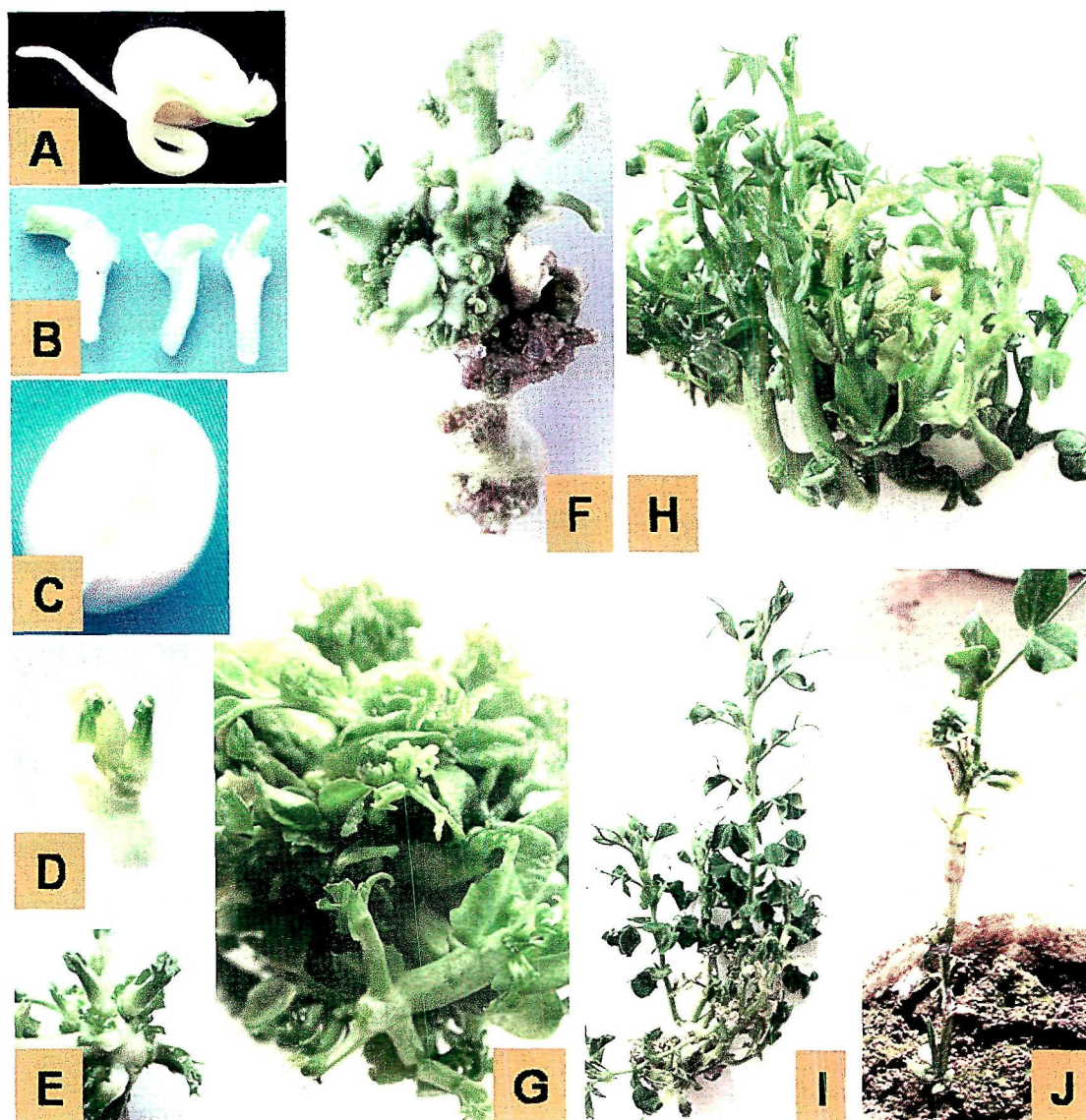


Fig 4.3 Shoot induction from cotyledonary nodes of *Pisum sativum* cultivar Azad P1 on MS medium supplemented with 20 μM BAP+0.01 μM NAA. A) Germinated seed showing shoot and root, B) cotyledonary node explants C) seed after harvesting of cotyledonary node D, E, F) cotyledonary node showing direct shoot induction G, H) multiple shoots from single explant, I) shoot elongation on MS medium supplemented with 0.02 μM BAP, J) tissue culture derived shoot grafted on root stock of pea cultivar Azad P1.

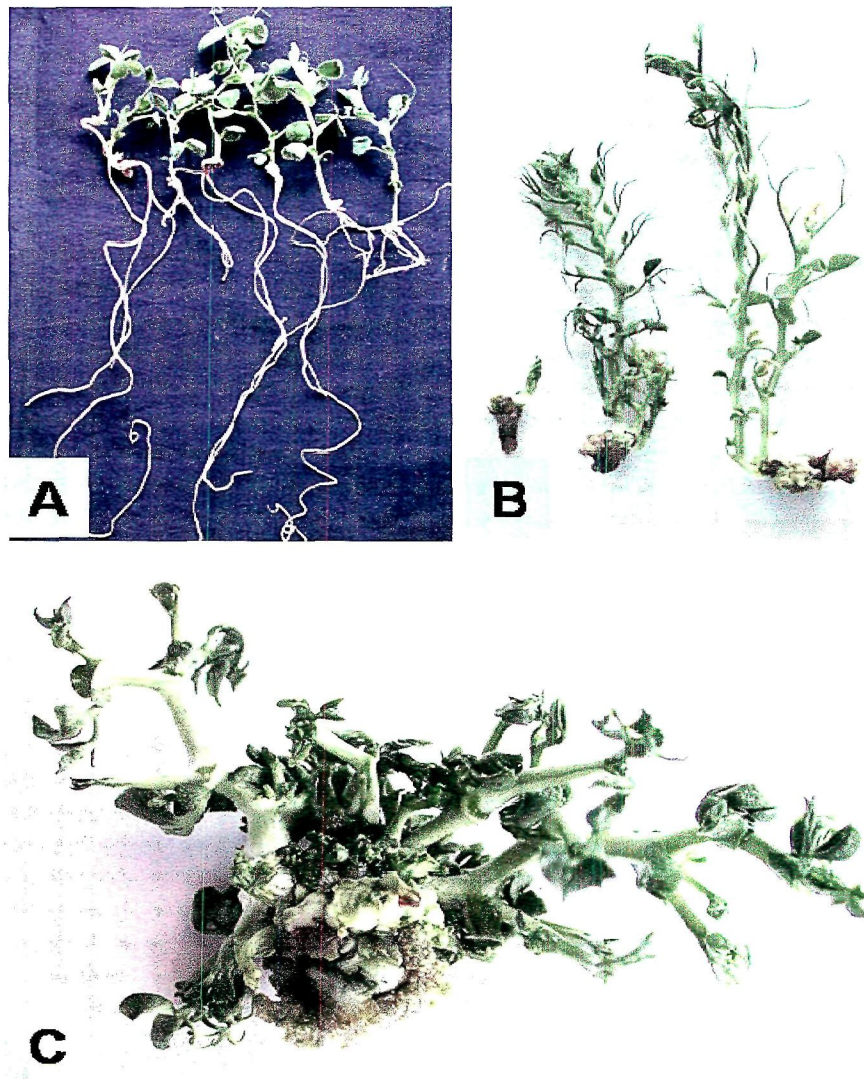


Fig 4.4 Effect of different media on shoot/plant regeneration from embryos of *Pisum sativum* cultivar Azad P1 A) embryos formed plants with well developed shoots and roots on MS basal medium, B, C) shoot formation on B) $\frac{1}{2}$ MS + 20 μ M BAP+0.01 μ M NAA and C) MS + 20 μ M BAP+0.01 μ M NAA.

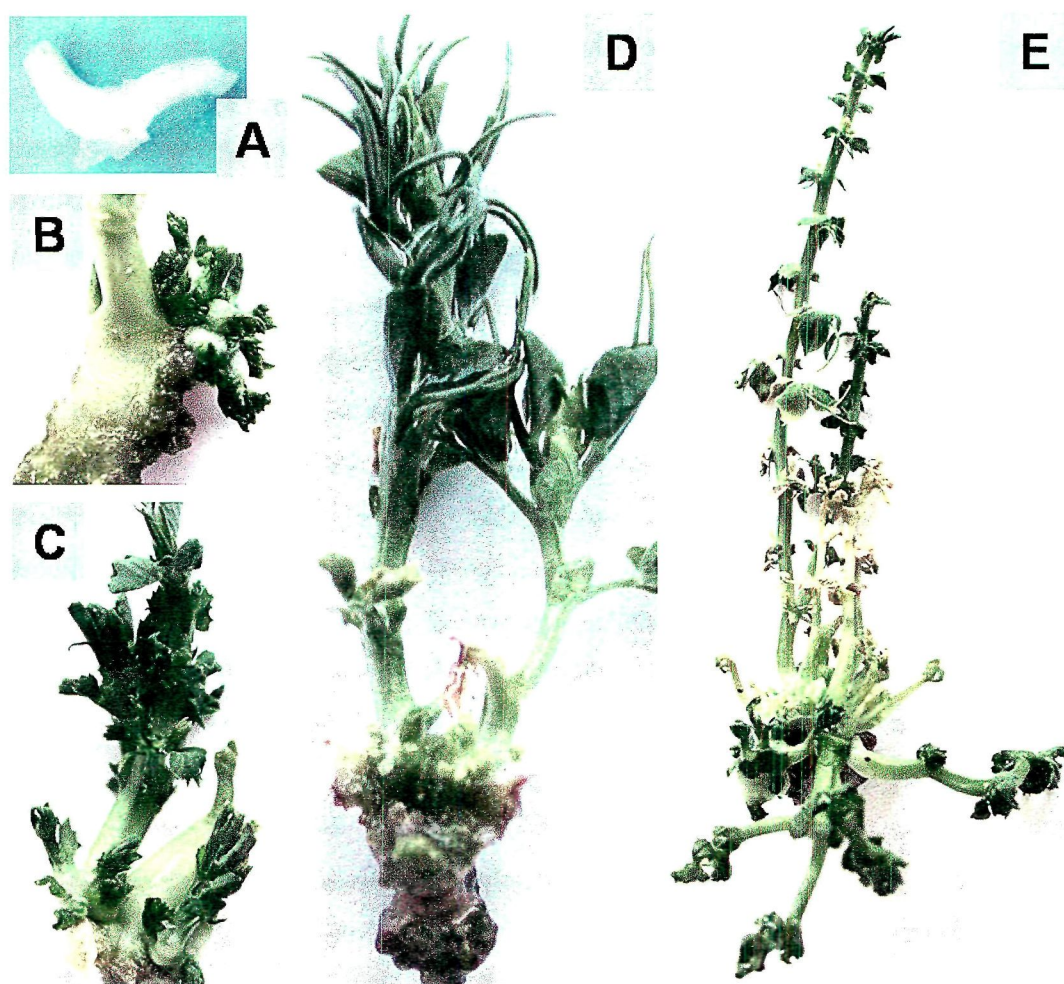


Fig 4.5 Shoot induction from cotyledonary nodes of *Pisum sativum* cultivar Azad P1 on half strength MS medium supplemented with 20 μ M BAP+0.01 μ M NAA. A) cotyledonary node explant B) shoot induction C) multiple shoots D, E) shoot elongation on MS medium supplemented with 0.02 μ M BAP, E) well developed shoots.

at or below 5.0 mg l^{-1} BAP but not at 10.0 mg l^{-1} . In another study, Jackson and Hobbs(1990)used 1 mg^{-1} BAP in MS medium to induce multiple shoots from cotyledonary nodes. It was also shown that cytokinin concentrations above 5 mg/litre increasingly produced shoots that were vitrified and difficult to root. With all 6 genotypes tested, developing buds were visible by 5 days after culture and elongated shoots were removed after 21 days(Jackson and Hobbs 1990). Histological studies indicated that the buds and shoots were formed from superficial layers of tissue. Shoot induction from pea seeds require almost similar ($25 \text{ } \mu\text{M}$) levels of BAP as has been used ($20 \text{ } \mu\text{M}$) in the present study (Esposito *etal.* 2012). The higher concentrations of BAP (50 and $75 \text{ } \mu\text{M}$) were not desirable as at these concentrations shoot induction was low (Esposito *etal.* 2012).

The explant type as well as growth regulators affected frequency of shoot regeneration, no. of shoots per explant, shoot length per explants as has been observed in the present study (Franklin *etal.* 2000; Jackson and Hobbs 1990; Esposito *etal.* 2012; Emmanouil *etal.* 2004; Doorne *etal.* 1995; Malik and Saxena 1992; Griga *etal.* 1998; Pniewski *etal.* 2003; Sharma and Kaushal 2003).

In the present study, the shoots developed via the callus phase in case of embryo explants. Calli can regenerate shoots either via organogenesis or embryogenesis. While we used a two step procedure i.e. induction of calli, shoot regeneration and grafting of *in vitro* shoots. Emmanouil *etal.* (2004) used a five-step procedure to regenerate plants from meristematic tissues initiated from nodal tissue in four pea cultivars ('Espace', 'Classic', 'Solara', and 'Puget'). In step 1, bud containing tissue (BCT) was initiated from one-node explants. In step 2, the BCT was isolated, purified and maintained. Regeneration of shoots from BCT and rooting were steps 3 and 4, respectively and step 5 was plant acclimatization and further development. TDZ was essential for shoot induction. Another difference between the two studies was use of growth regulators. While Emmanouil *etal.* (2004) used low concentrations of TDZ, we used BAP and NAA. Emmanouil *etal.* (2004) further demonstrated that lower concentrations of TDZ

favoured shoot induction but not the higher ones. At TDZ concentration of 2.2–8.8 mg/l, only about half of the BCT explants initiated new BCT but at a lower concentration, all BCT explants initiated new BCT and shoots. TDZ was also used to induce multiple shoots from the nodal and basal regions of the primary epicotyl (Malik and Saxena 1992).

Doorne *etal.* (1995) developed shoots from somatic embryos of pea. They concluded that *in vitro* somatic embryo formation was dependent on the culture conditions and explant used as well as the genotype. For their study, they used 16 cultivars of pea and demonstrated that among nodes, shoot apices and seed; the nodes were the best explant for somatic embryo production. In another study, a system of *in vitro* clonal propagation based on somatic embryogenesis from pea shoot apical meristems was also developed (Griga *etal.* 1998). The conversion rate of somatic embryos was favoured by low concentrations of TDZ (Griga *etal.* 1998).

Impact of growth regulator 'BAP' on shoot induction from callus cultures was also demonstrated. The callus cultures derived from cotyledon tissue adjacent to axillary meristems of immature embryos when cultured on to MS supplemented 4.5 mg l^{-1} of BAP regenerated several shoots per explants (Pniewski *etal.* 2003). Effect of different growth regulators on *in vitro* regeneration of plants from immature pea leaflets was also studied (Mroginski and Kartha 1981). The leaves first developed a primary callus which within 45 to 90 days of culture formed shoots. Multiple shoot regeneration was maximum at a combination of $0.1 \text{ } \mu\text{M}$ NAA and $10 \text{ } \mu\text{M}$ BA. The effect of BAP in combination with other growth regulators on callus induction from leaf, epicotyle and root of two pea cultivars was also demonstrated (Sharma and Kaushal 2003). The best growth regulator combination was 2, 4-D + NAA + BAP 0.5 mg l^{-1} each using leaf explants.

4.1.2 Effect of salt concentration and growth regulators on elongation of shoots

After 60 day of culture of cotyledonary nodes on MS+ $20 \text{ } \mu\text{M}$ BAP+ $0.01 \text{ } \mu\text{M}$ NAA, bunches of multiple shoots were observed. Majority of the shoots developed on this medium were shorter in length and inadequate for rooting or grafting. These shoots bunches were divided into four equal parts and

transferred on to the shoot elongation media (MS, ½ MS+20 µM BAP+0.01 µM NAA, MS+20 µM BAP+0.01 µM NAA, MS+8.8 µM BAP and MS+0.08 µM BAP). The MS basal medium and that MS supplemented with 0.08 µM BAP were equally effective for number of shoots per explant (25.08 and 25.47, respectively), however, shoot were longer (11.53 cm) in MS medium supplemented with 0.08 µM BAP than those obtained at MS medium (4.58 cm, Table 4.4). Enhancing BAP concentration to 8.8 µM increased the shoots per explant (30.77) as well as shoot length (10.6 cm). The best medium for shoots/explant as well as shoot length was MS+20 µM BAP+0.01 µM NAA where 46.63 shoots per explant with an average shoot length of 9.82 cm were generated (Fig 4.4). Reducing the salt and vitamin concentration to half in this medium (Fig 4.5) reduced drastically the shoots per explant (34.93) and shoot length (3.61). For shoot length, the media MS+20 µM BAP+0.01 µM NAA, MS+8.8 µM BAP and MS+0.08 µM BAP were statistically similar. Like shoot induction, the shoot elongation also required high concentration of salts and vitamins in the medium and reduction in these parameters severely reduced the shoot induction and elongation frequencies.

Table 4.4 Effect of salt concentration and growth regulators on elongation of tissue culture derived shoots of pea variety Azad P1. The shoots were induced on MS+20 µM BAP+0.01 µM NAA from cotyledonary nodes and transferred to shoot elongation medium after 60 day of culture.

Sr. No	Medium	Shoots/explant (no.)± SE ¹	Average shoot length (cm)± SE ¹
1.	MS	25.08 ± 0.95 ^a	4.58±0.17 ^a
2.	½ MS+20 µM BAP+0.01 µM NAA	34.93±1.01 ^c	3.61±0.14 ^a
3.	MS+20 µM BAP+0.01 µM NAA	46.63±0.96 ^d	9.82±0.30 ^b
4.	MS+ 8.8 µM BAP	30.77±0.97 ^b	10.60±0.26 ^b
5.	MS+0.08 µM BAP	25.47±0.96 ^a	11.53±0.39 ^b
	CD (p≤ 0.05)	0.82	2.86
	CV	7.22	6.66

¹Values with same letter are statistically similar.

The impact of growth regulators on shoot elongation had also been observed (Pniewski *et al.* 2003; Franklin *et al.* 2000). There is no study on effect of salt concentration on direct shoot induction in pea. Pniewski *et al.* (2003) developed shoots from callus cultures of several Polish cultivars of pea. These shoots were elongated on MS medium supplemented with 0.02 mg/l BAP. This medium was also included in our study, however, a combination of 4.5 mg/l BAP and 0.01 mg/l NAA was far more superior to 0.02 mg/l BAP for shoots per explant. Apart from shoot number, shoot elongation was numerically better but statistically similar for both the growth regulator combinations.

The cotyledonary explants were also used in a previous study where a maximum of 32 shoots were produced per cotyledonary node explant on MS medium supplemented with 2.0 mg/l⁻¹ BAP (Franklin *et al.* 2000). By changing the growth regulators in the medium, the efficiency of multiple shoot induction was increased considerably (46.63 per explant). While in the present study, 4.5 mg/l⁻¹ BAP and 0.01 mg/l⁻¹ NAA was used, Franklin *et al.* (2000) used 2.0 mg/l⁻¹ BAP. The number of shoots per explant (46.63) generated in the present study using 4.5 mg/l⁻¹ BAP + 0.002 mg/l⁻¹ NAA for shoot induction and 2.0 mg/l⁻¹ BAP for shoot elongation was about 45% more than that obtained by Franklin *et al.* (2000; 32 shoots/explant) or by us in the present study (30.77 shoots/explant). Thus the present protocol is far more efficient than that of Franklin *et al.* (2000).

4.1.3 Effect of ammonium nitrate concentration on direct shoot induction

Four concentrations i.e. no ammonium nitrate, one fourth to that in MS medium (412.5 mg/l⁻¹), one half (825 mg/l⁻¹) and full ammonium nitrate (1650 mg/l⁻¹) were tested to study the effect of ammonical nitrogen on shoot induction by cotyledonary nodes of pea variety Azad P1. All other parameters except ammonium nitrate were kept constant in this experiment. The medium used was MS supplemented with 20 µM BAP, 0.01 µM NAA and 30 g/l⁻¹ sucrose. In medium devoid of ammonium nitrate, the frequency of shoots per explant (4.96) as well shoot length (4.2 cm) were reduced to half compared to full strength of ammonium nitrate (shoots per explant 10.26, shoot length 10.8 cm; Table 4.5). It is pertinent to mention here that there was also another source of nitrogen in the

medium i.e. potassium nitrate. Addition of one fourth amount of ammonium nitrate did not enhance the number of shoots per explant, it however, increased shoot length by about two times (8.44 cm compared to 4.2 in medium with no ammonium nitrate). At half strength of ammonium nitrate, the shoot length (10.85 cm) was similar to full strength (10.8 cm) however, number of shoots per explant (8.89) was less (10.26 at full strength).

Table 4.5Effect of concentration of ammonium nitrate on direct shoot induction from pea variety Azad P1. The culture medium used was MS+20 µM BAP+0.01 µM NAA.

Sr. No	Ammonium nitrate concentration (mg l ⁻¹)	Shoots/explant (no.) ± SE ¹	Average shoot length (cm) ± SE ¹
1.	0	4.96±0.58 ^a	4.2±0.27 ^a
2.	412.5	5.73±0.55 ^a	8.44±0.33 ^b
3.	825	8.89±0.55 ^b	10.85±0.35 ^c
4.	1650	10.26±0.48 ^b	10.80±0.51 ^c
	CD (p≤ 0.05)	1.23	1.54
	CV	7.64	10.99

¹Values with same letter are statistically similar.

Hence, 1650 mg l⁻¹ ammonium nitrate was required for generation of maximum shoots per explant as well as average shoot length. In general, supplementation of cultures with nitrate as the sole source of nitrogen inhibits or reduces the cell or shoot growth (Gamborg 1970; Shanjani 2003; Rasmia *etal.* 2011). The soybean cells did not grow on nitrate unless medium was supplemented with ammonium or glutamate (Gamborg 1970). Similarly, callus cultures of *Juniperus excelsa* did not grow on nitrate (Shanjani 2003). However, adventitious buds were formed on nitrate. Though, ammonium is required for successful tissue cultures, its full concentration (1650 mg l⁻¹) inhibited callus induction from *Juniperous excelsa* and reducing ammonium amount to half (825

mg l⁻¹) could induce callus cultures (Shanjani 2003). In date palm plantlets, half strength of ammonium nitrate (825 mg l⁻¹) yielded longest shoots and roots and the greatest number of leaves and root per plantlet was obtained from ammonium nitrate concentration of 206.25 mg l⁻¹ (one eighth of full concentration; Rasmia *et al.* 2011). Pea appears to have higher requirements of ammonium nitrate for shoot development as number of shoots per explant and shoot length was more at 1650 mg l⁻¹ (full strength) ammonium nitrate (present study). In contrast to this, *in vitro* flowering and fruiting was maximum at half strength (825 mg l⁻¹) ammonium nitrate (Franklin *et al.* 2000).

4.1.4. Effect of sucrose concentration on direct shoot induction

Tissue cultures are usually supplemented with suitable energy sources such as glucose, maltose, sucrose, lactose, fructose or sucrose in combination with any of the other sugars, or mannose for tissue growth *in vitro*. A preferred sugar is sucrose at a level of about 5 to 50 g l⁻¹, with molar equivalents of other sugars also being suitable. The carbohydrates are not only the sources of energy but these also maintain the osmotic balance and provide necessary carbon skeleton to the growing cells/tissues.

Of the five different concentrations of sucrose (15, 30, 50, 75 and 100 g l⁻¹) in the MS salts supplemented with 20 µM BAP and 0.01 µM NAA tested to study the effect of sucrose concentration on direct shoot induction from cotyledonary nodes in pea variety Azad P1, 30 g l⁻¹ was the best with 10.55 shoots per explant and 10.56 cm shoot length (Table 4.6). The sucrose concentration lower to this or higher than this decreased the number of shoots per explant as well as shoot length. At 15 g l⁻¹ sucrose, the frequency of shoots per explant (5.45) was reduced by almost half compared to that (10.55) obtained at 30 g l⁻¹, whereas the reduction in shoot length was comparatively less (8.73 cm at 15 g l⁻¹, 10.56 at 30 g l⁻¹). At 50 g l⁻¹, 75 g l⁻¹ and 100 g l⁻¹ sucrose, the reduction in shoots per explant as well as shoot length was more prominent (Table 4.6).

Table 4.6 Effect of sucrose concentration on direct shoot induction from cotyledonary nodes of pea variety Azad P1. The culture medium used was MS+20 μ M BAP+0.01 μ M NAA.

Sr. No	Sucrose concentration (gl^{-1})	Shoots/explant (no.) $\pm$ SE ¹	Average shoot length (cm) $\pm$ SE ¹
1.	15	5.45 $\pm$ 0.19 ^b	8.73 $\pm$ 0.34 ^c
2.	30	10.55 $\pm$ 0.48 ^c	10.56 $\pm$ 0.46 ^d
3.	50	3.72 $\pm$ 0.18 ^a	4.56 $\pm$ 0.40 ^b
4.	75	3.80 $\pm$ 0.34 ^a	5.34 $\pm$ 0.40 ^b
5.	100	2.92 $\pm$ 0.21 ^a	3.20 $\pm$ 0.20 ^a
	CD ($p \leq 0.05$)	1.12	0.91
	CV	11.51	11.54

¹Values with same letter are statistically similar.

Thus, 30 gl^{-1} sucrose is the optimum concentration for achieving maximum shoots per explant as well as longer shoots. Though, effect of sucrose concentrations on direct plant regeneration in pea has not been studied, similar level (30 gl^{-1}) was found optimum for flower induction and pod formation *in vitro* (Franklin *et al.* 2000). At lower concentrations (0, 15 gl^{-1}) no flower and pod formation was observed whereas at a higher concentration (50 gl^{-1}) the reduction was very high. In other plant species also, the supplementation of cultures with lower or higher concentrations of sucrose either reduced or sometime inhibited the growth of cells or shoots (Komatsuda *etal.* 1992; Nhut *et al.* 2001; Gauchan 2012). In *Lilium longiflorum*, four different concentrations of sucrose (10, 20, 30 and 40 gl^{-1}) in half MS basal medium were tested for efficient shoot regeneration by young stem culture (Nhut *et al.* 2001). The highest (8.9) number of shoots were recorded at 30 gml^{-1} concentration of sucrose. The number of shoots was low at 10 gl^{-1} (2.2), 20 gl^{-1} (5.9) and 40 gl^{-1} (7.1) sucrose. The highest increase in fresh weight of somatic embryos of soybean was obtained in 2 weeks by transferring those onto media containing 30 gl^{-1} sucrose (Komatsuda *etal.* 1992). The somatic embryos when transferred on media supplemented with lower or higher concentration of sucrose reduced fresh weight of the somatic embryos.

In contrast to this, maize appears to have lower requirements (5 g l^{-1}) of sucrose for shoot development from the embryo and their elongation (Gauchan 2012) compared to crops like pea. Higher concentrations of sucrose did not favour shoot elongation. The shoot length was significantly less at 25 g l^{-1} , 50 g l^{-1} and 100 g l^{-1} compared to that obtained at 5 g l^{-1} .

The sucrose requirements for tissue cultures of some other plant species such as saffron are comparatively high (Sharma *et al.* 2008). The shoot formation from saffron corms and shoot length was more at 50 g l^{-1} sucrose. Corms formation from tissue culture derived shoots was maximum at 80 g l^{-1} sucrose (Sharma *et al.* 2008).

4.2 Standardization of *Agrobacterium* mediated transformation of pea

A. tumefaciens mediated transformation is routine for several plant species, however, there are many with inefficient *Agrobacterium* mediated transformation protocols. Pea is one such species where transformation frequencies remain low. In *Agrobacterium* mediated transformation, the tissue culture response of the variety or genotype in question, *Agrobacterium* strain used for transformation, acetosyringone concentration, cocultivation temperature and injury to explant are the major factors deciding the transformation efficiencies. Despite some studies on pea transformation, the effect of acetosyringone concentration, cocultivation temperature and injury to explant on T-DNA delivery into host cell have not been studied in detail. Different studies used different concentration of acetosyringone and co-cultivation temperatures without optimising these factors for maximum efficiency (Puonti-Kaerlas *et al.* 1990; De Kanthen and Jacobsen 1990; Grant *et al.* 1995; Bean *et al.* 1997; Jones *et al.* 1998; Orczyk and Orczyk 2000). Apart from this, the inhibitory concentration of plant selective agents varies with the variety/genotype used for transformation.

4.2.1 Hygromycin sensitivity test

Seven different concentrations (0, 5, 10, 15, 20, 25 and 50 mg l^{-1}) of hygromycin B were tested to find the optimum level of this antibiotic that was

inhibitory to shoot induction/development from cotyledonary nodes of pea variety Azad P1. The hygromycin B was added to the MS medium supplemented with 20 µM BAP and 0.01 µM NAA. The data were recorded after exposure periods of 10 and 20 days, respectively. As low as 5 mg l⁻¹ of the antibiotic suppressed the number of explants forming shoots as well as number of shoots per explants (Tables 4.7, 4.8, Fig 4.6). After an exposure of 10 days at 20 mg l⁻¹, none of the explants formed shoots, however, at 15 mg l⁻¹, 33.33% explants developed shoots. Another exposure of 10 days at 15 mg l⁻¹ killed all the shoots that had emerged earlier indicating that extended exposure of lower concentrations was also lethal (Fig 4.7). Thus, the hygromycin concentration as well as exposure period dictated survival of cotyledonary explants or shoots emerged from these explants.

Table 4.7 **Effect of different concentrations of antibiotic hygromycin B on shoot induction from cotyledonary nodes of pea variety Azad P1. The culture medium used was MS+20 µM BAP+0.01 µM NAA. The data were recorded after exposure period of 10 days.**

Sr. No	Hygromycin concentration (mg l ⁻¹)	Explants forming shoots (%) ± SE ¹	Shoots/explant (no.) ± SE ¹	Explants forming roots (%) ± SE ¹	Roots/explant (no.) ± SE ¹
1.	0	100±0.0 ^d (10.04)	1.93±0.24 ^e (1.70)	76.66±1.92 ^f (8.81)	1.16±0.08 ^d (1.47)
2.	5	96.77±3.22 ^d (9.88)	1.63±0.23 ^d (1.61)	60±3.85 ^e (7.80)	1.03±0.03 ^d (1.42)
3.	10	56.88±3.27 ^c (7.60)	0.76±0.08 ^c (1.32)	39.99±1.92 ^d (6.39)	0.46±0.06 ^c (1.21)
4.	15	33.33±1.92 ^b (5.85)	0.20±0.05 ^b (1.09)	20.00±1.92 ^b (4.57)	0.26±0.26 ^b (1.11)
5.	20	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	37.77±2.93 ^c (6.21)	0.46±0.06 ^c (1.21)
6.	25	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)
7.	50	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)
	CD (p≤ 0.05)	0.36	0.12	0.50	0.14
	CV	4.02	5.63	5.65	6.77

¹Transformed values within parenthesis. The data were transformed as square root of value+1. Values with same letter are statistically similar.

Varied concentrations of hygromycin ranging from 5 mg l⁻¹ to 25 mg l⁻¹ have been used for selection of transformants. While De Kanthen and Jacobsen (1990), Grant *et al.* (1995), Bean *et al.* (1997), Jones *et al.* (1998) used 5-10 mg l⁻¹ hygromycin, the concentrations used by Puonti-Kaerlas *et al.* (1992) was 15 mg l⁻¹ and that by Lulsdorf *et al.* (1991) was 25 mg l⁻¹. It appeared that hygromycin concentration depends upon the variety/genotype used for transformation.

Table 4.8 Effect of different concentrations of antibiotic hygromycin B on direct shoot induction from cotyledonary nodes of pea variety Azad P1. The culture medium used was MS+20 µM BAP+0.01 µM NAA. The data were recorded after exposure period of 20 days.

Sr. No	Hygromycin concentration (mg l ⁻¹)	Explants forming shoots (%) ± SE ¹	Shoots/explant (no.) ± SE ¹	Explants forming roots (%) ± SE ¹	Roots/explant (no.) ± SE ¹
1.	0	100±0.0 ^d (10.04)	7.93±0.24 ^c (2.98)	85.33±0.38 ^d (9.29)	1.30±0.05 ^c (1.52)
2.	5	29.83±0.58 ^c (5.55)	0.8±0.05 ^b (1.34)	21.33±0.77 ^c (4.72)	0.53±0.03 ^b (1.24)
3.	10	10.77±0.44 ^b (3.43)	0.4±0.05 ^b (1.18)	14.99±0.96 ^b (3.99)	0.30±0.05 ^b (1.14)
4.	15	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)
5.	20	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)
6.	25	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)
7.	50	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)	0±0.0 ^a (1.00)
	CD(p≤ 0.05)	0.95	0.59	0.17	0.39
	CV	1.66	2.48	3.12	2.00

¹Transformed values within parenthesis. The data were transformed as square root of value+1. Values with same letter are statistically similar.



Fig 4.6 Effect of hygromycin concentration on shoot induction from cotyledonary node explants of pea cultivar Azad P1. The concentrations were A) 5 mg l⁻¹, B) 10 mg l⁻¹, C) 15 mg l⁻¹, D) 20 mg l⁻¹, E) 25 mg l⁻¹, F) 50 mg l⁻¹. The picture was taken after hygromycin exposure of explants for 10 days.

4.2.2 Standardization of acetosyringone concentration

Acetosyringone ($C_{10}H_{12}O_4$) is a phenolic compound. Historically, this substance is best known for its involvement in plant-*Agrobacterium* recognition. It increases *Agrobacterium* mediated transformation efficiency and is especially useful when hypovirulent strains of the bacterium are used. In the present study, strain GV3101 of *A. tumefaciens* was used. To know the effect of acetosyringone on transformation efficiencies by this strain, five concentrations (0 μ M, 10 μ M, 20 μ M, 50 μ M and 100 μ M) of the chemical were used.

While no transformant was observed at 0 μ M acetosyringone, the transformation efficiency was maximum (3.33) at 50 μ M acetosyringone (Table 4.9, Fig 4.7). Addition of 10 μ M acetosyringone during inoculation increased the transformation efficiency to 1.00. Further increase in acetosyringone concentration to 20 μ M doubled the transformation efficiency (2.0) from that (1.0) observed at 10 μ M. After a peak at 50 μ M, the transformation efficiency was lowered to 2.33 at 100 μ M acetosyringone, a level statistically equal to that observed at 20 μ M. Thus, the optimal concentration for transformation of pea variety Azad P1 using *A. tumefaciens* strain GV3101 was 50 μ M. In all the subsequent experiments, 50 μ M acetosyringone was used.

Acetosyringone is the only co-cultivation additive tested in pea transformation and in different experiments its different concentrations were used. While Lulsdorf *et al.* (1991) and Svabova *et al.* (2005) used 100 μ M acetosyringone, Orczyk and Orczyk (2000), Kathen and Jacobsen (1990) used 50 μ M and Bean *et al.* (1997) used 20 μ M. To optimize concentration of acetosyringone for pea transformation, Svabova *et al.* (2008) used three concentrations (0 μ M, 100 μ M and 200 μ M). The 100 μ M acetosyringone significantly increased intensity of GUS Expression value, while 200 μ M did not differ from control. We tested three concentrations between 0 μ M and 100 μ M (10 μ M, 20 μ M and 50 μ M) and found that 50 μ M is the optimum level for pea transformation.

Efficacy of acetosyringone also varies with the *A. tumefaciens* strain used. Orczyk and Orczyk (2000) found a relation between acetosyringone treatment and *Agrobacterium* strain used. No effect on transformation efficiency was recorded with the hypervirulent EHA105 strain, while acetosyringone did affect the rate of transformation when the moderately virulent C58C1 strain was used.

Table 4.9 **Effect of acetosyringone concentration on transformation of pea cultivar Azad P1 (temperature 24°C, replications: 3, explants per replication: 100).**

Sr.No	Acetosyringone concentration (µM)	Expants (%) generating shoots on selective media ±SE ¹
1	0	0.00±0.00 ^a (1.00)
2	10	1.00±0.57 ^b (1.38)
3	20	2.00±0.57 ^c (1.71)
4	50	3.33±0.33 ^d (2.07)
5	100	2.33±0.33 ^c (1.23)
	CD(p≤ 0.05)	0.41
	CV	14.35

¹Transformed values within parenthesis. The data were transformed as square root of value+1. Values with same letter are statistically similar.

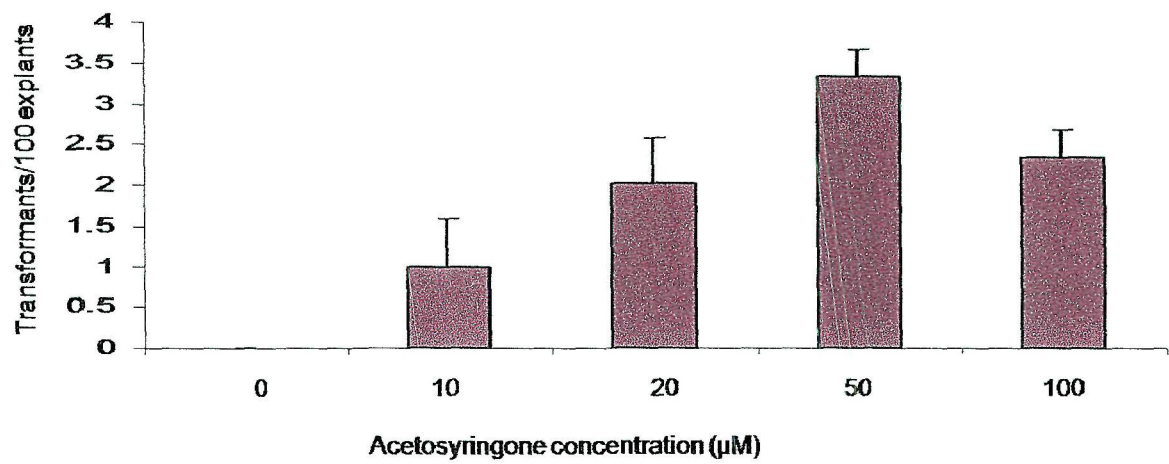


Fig 4.7 Effect of acetosyringone concentration on development of putative transformants from cotyledonary nodes of pea cultivar Azad P1. The explants were inoculated with *Agrobacterium tumefaciens* harbouring binary vector pCAMBIA 1302 for one hour in the presence of acetosyringone.

4.2.3 Effect of co-cultivation temperature and injury to explant on transformation

Once the *Agrobacterium* cells attach to the host cells, the temperature plays vital role in infection. In addition to this, injury to explants also facilitates attachment and infection of host cells. For infection, inoculation is followed by the co-cultivation step which is usually carried out in dark for a period ranging from 2-3 days. In the present study, inoculated explants were incubated at five co-cultivation temperatures (4°C, 18°C, 20°C, 22°C and 24°C). At each temperature, explants treated either with silicon carbide (to have injury to explant cells) or with no silicon carbide treatment were co-cultivated. At 4°C, 1.33% explants showed transformation and transformation efficiency increased to 1.66% in injured explants (Table 4.10, Fig 4.8). With increase in co-cultivation temperature, the transformation efficiency increased and a peak was observed at 24°C. The transformation efficiencies were comparable at 22°C (3.33%) and 24°C (3.66%). Injury to explants increased transformation efficiency compared to un-injured ones. At 24°C, 3.66% un-injured explants were transformed, however, the injury increased the transformation efficiency to 4.33%. Similarly, at 22°C injury enhanced transformation efficiency to 4.0% from 3.33%.

Table 4.10 Effect of temperature and injury to explant on transformation (%) in pea cultivar Azad P1. The explants were injured using silicon carbide (acetosyringone concentration: 50 mg/l, replications: 3, explants per replication: 100)

Sr. No	Temperature °C	As ¹	As+SiC ¹
1	4	1.33±0.33 ^a (1.52)	1.66±0.33 ^a (1.66)
2	18	2.33±0.33 ^b (1.80)	2.00±0.57 ^a (1.72)
3	20	2.66±0.66 ^b (1.90)	3.00±0.57 ^b (1.98)
4	22	3.33±0.33 ^c (2.07)	4.00±0.57 ^c (2.23)
5	24	3.66±0.33 ^c (2.15)	4.33±0.33 ^c (2.31)
	CD	0.42	0.40
	CV	12.28	11.31

¹Transformed values within parenthesis. The data were transformed as square root of value+1. The values with the same letter are statistically non-significant.

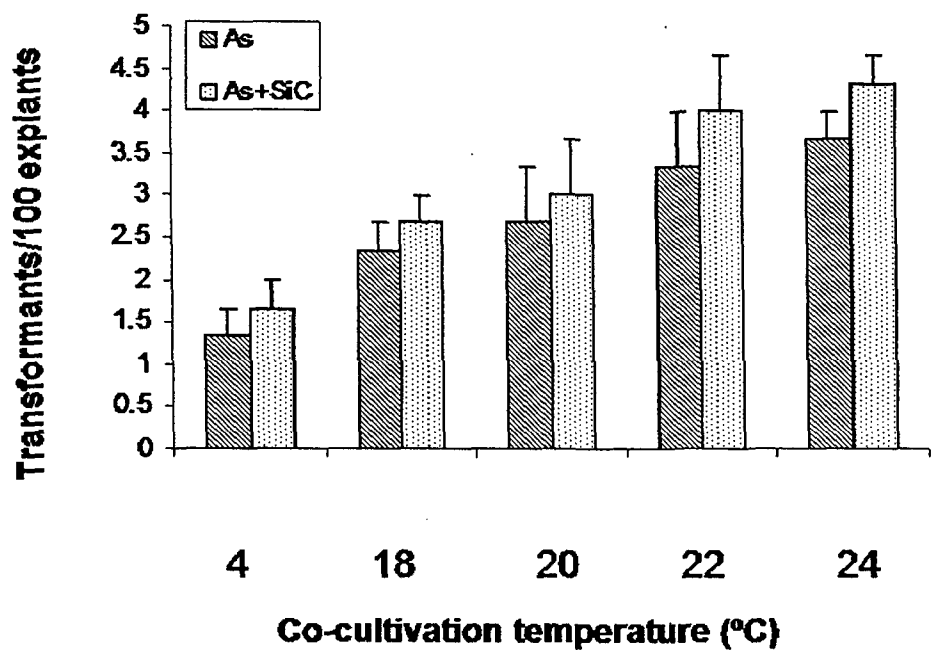


Fig 4.8Effect of co-cultivation temperature and silicon carbide (corborundum powder) rubbing on development of putative transformants from cotyledonary nodes of pea cultivar Azad P1. The explants were treated with silicon carbide powder prior to inoculation with *Agrobacterium tumefaciens* for one hour in the presence of acetosyringone (50 μ M). These were subsequently co-cultivated for two days at different temperatures. As: acetosyringone, SiC: silicon carbide.

Variable co-cultivation temperatures have been used by various workers (Schroeder *etal.* 1993; Bean *etal.* 1997; Orczyk and Orczyk 2000; Pniewski and Kaputa 2005; Svabova *etal.* 2005; Richter *etal.* 2006). Bean *etal.* (1997) used a co-cultivation temperature of 20°C under 16-h photoperiod, Savabova *et al.* (2005) used 4°C for seeds and 24°C for cotyledonary node, Savabova *et al.* (2008) applied 20±2°C. While Savabova *etal.* (2008) and Bean *etal.* (1997) used 16L/8D photoperiod, Savabova *etal.* (2005) co-cultivated explants under dark conditions.

Since, wounding of tissues is known to enhance *Agrobacterium* penetration leading to higher transformation efficiencies and rubbing of tissues with carborundum powder (Silicon cabide) is one of the common ways to induce injuries in plant tissues. It is the most common method used to wound tissues for viral infections under artificial conditions or even introduce the naked DNA into the cells (Kaeppler *etal.* 1990; Kaeppler *etal.* 1992). Since, injury increased the transformation efficiency (present study), thus, wounding regenerable differentiating tissues by carborundum rubbing provides a simple and efficient way for pea transformation mediated by *Agrobacterium*.

While no report on pea transformation using injury either by silicon carbide or other means is available, Chen *etal.* (1996) described an efficient *Agrobacterium*-mediated transformation method in papaya based on wounding of cultured embryogenic tissues with carborundum in liquid phase. The embryogenic tissues were vortexed with 600 mesh carborundum in sterile distilled water for 1 min before treating with the *A. tumefaciens*. The average successful rate of zygotic embryo transformation was 15.9%, about 10–100 times higher than previous reports. Similarly, Curuk *etal.* (2005) transformed the recalcitrant melon (*Cucumis melo* L.) by wounding cotyledon explants by vortexing with carborundum prior to inoculation with *Agrobacteriumtumefaciens*. Only treatments where explants were vortexed with carborundum produced transgenic melon shoots.

4.2.4 Confirmation of presence of transgene in putative transformants

Untransformed control and putatively transformed plants were tested for the presence of thaumatin like protein (*CsTLP*) gene by polymerase chain reaction (PCR). For transformation confirmation, *CsTLP* gene-specific primers were used. *A. tumefaciens* containing Ti plasmid with *CsTLP* gene, the isolated Ti plasmid with *CsTLP* gene and untransformed pea plants acted as controls (Fig 4.9). The *CsTLP* gene-specific band was detected in positive controls i.e. *A. tumefaciens* containing Ti plasmid with *CsTLP* gene and the isolated Ti plasmid with *CsTLP* gene. The *CsTLP* amplification product was absent in negative control i.e. untransformed pea plants. Of the four putative transformants, only one (see lane 6 of Fig 4.9) amplified the *CsTLP* gene indicating that this plant is a transformant with *CsTLP* gene.

The putative transformants were selected on MS medium containing 20 μ M BAP, 0.01 μ M NAA and hygromycin B as selective agent (Fig 4.10), however only one plant was confirmed to be the true transformant. This indicated that the occurrence rate of false positives using hygromycin B was high. In most cases, survival of shoots/explant on selection medium (hygromycin or kanamycin) is not a proof of transformation. Yet, it narrows down the number of individuals to be tested for the presence of transgene. Differential exposure of *in vitro* cultures to the selective agent, residual resistance of the plant tissue to the selective agent and the concentration applied might affect the occurrence of false positives. The low frequency of PCR positive plants among the selected shoots from tissue culture selection regimes is dependent on several factors such as tissue type, size of explant, chemical properties and concentration of selection agent and time of application (Bowen 1993).

PCR has been used widely for confirmation of transgene in pea (Richter *et al.* 2006; Savabova *et al.* 2005; Wisniewski *et al.* 2000), pigeonpea (Lawrence and Koundal 2001), chickpea (Mikail *et al.* 2008) and several other crops.

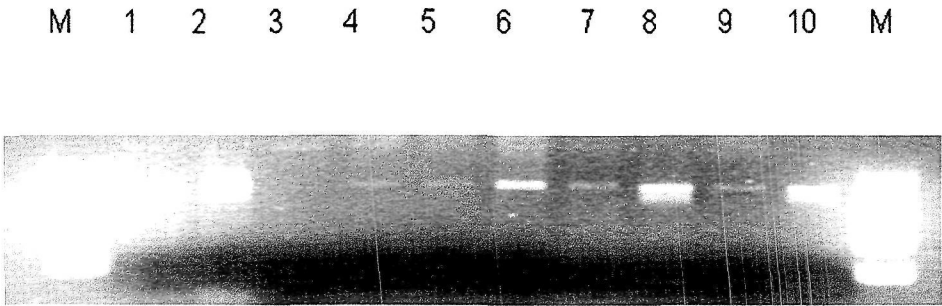


Fig 4.9 Confirmation of insertion of thaumatin like protein gene (*CsTLP*) in the putative transformants. The putative transformants were subjected to PCR using *CsTLP* gene specific primers. M: molecular weight marker, 1, 8, 10: Ti Plasmid with *CsTLP* insert, 2: *Agrobacterium tumefaciens* containing TLP transformed Ti Plasmid, 3, 9: control pea, 4-7: putative transformants, 4: T1, 5: T2, 6: T3, 7: T4.



Fig 4.10 Putative transformants on hygromycin (15 mg l^{-1}) containing MS medium supplemented with $20 \mu\text{M}$ BAP+ $0.01 \mu\text{M}$ NAA. Only a few explants regenerated shoots on hygromycin containing medium. A) Control without hygromycin, B) *Agrobacterium* inoculated and co-cultivated explants with dead shoots on hygromycin containing medium, C, D) *Agrobacterium* inoculated and co-cultivated explants showing shoot formation on selective medium.

4.3 Pea transformation protocol developed for cv. Azad P1

Based on the information obtained from all parameters that we standardized in the present study, the transformation protocol for pea variety Azad P1 can be outlined as below.

Cotyledonary node explants from seven days old germinated seedlings of pea were injured by rubbing with 400 mesh silicon carbide (spherical) powder. The injured explants were inoculated with *Agrobacterium tumefaciens* strain GV3101 (O.D. 600 nm) in the presence of 50 μM acetosyringone. The GV3101 harbours a pCAMBIA Ti plasmid that carried a *CsTLP* gene. The inoculation was carried out for 45 min at 75 rpm. The inoculated explants were transferred onto MS medium supplemented with 20 μM BAP and 0.01 μM NAA and co-cultivated in dark at 24°C for two days. After co-cultivation, the explants were washed in sterile distilled water to remove the bacteria followed by another washing with MS salts containing 500 mg l^{-1} carbenicillin and 300 mg l^{-1} timentin. Subsequently, the explants were dried using sterile blotting papers and transferred onto culture medium (MS+20 μM BAP+0.01 μM NAA) containing 300 mg l^{-1} timentin. For first 2 weeks, no selection pressure was applied after which cultures were transferred onto a selection medium (MS+20 μM BAP+0.01 μM NAA+ 300 mg l^{-1} timentin+15 mg l^{-1} hygromycin B). Selection pressure was applied for one month with sub-culturing after every ten days. Hygromycin resistant shoots were transferred onto shoot elongation medium (MS+20 μM BAP+0.01 μM NAA) without selection pressure. Well developed shoots after one month were grafted on to the rootstocks of Azad P1 (Fig 4.3). The tissue cultures were maintained at $25\pm 1^\circ\text{C}$ for 16h/8h light/dark cycle if otherwise not mentioned. The transgenic shoot development protocol for pea cv Azad P1 is outlined in Fig 4.11.

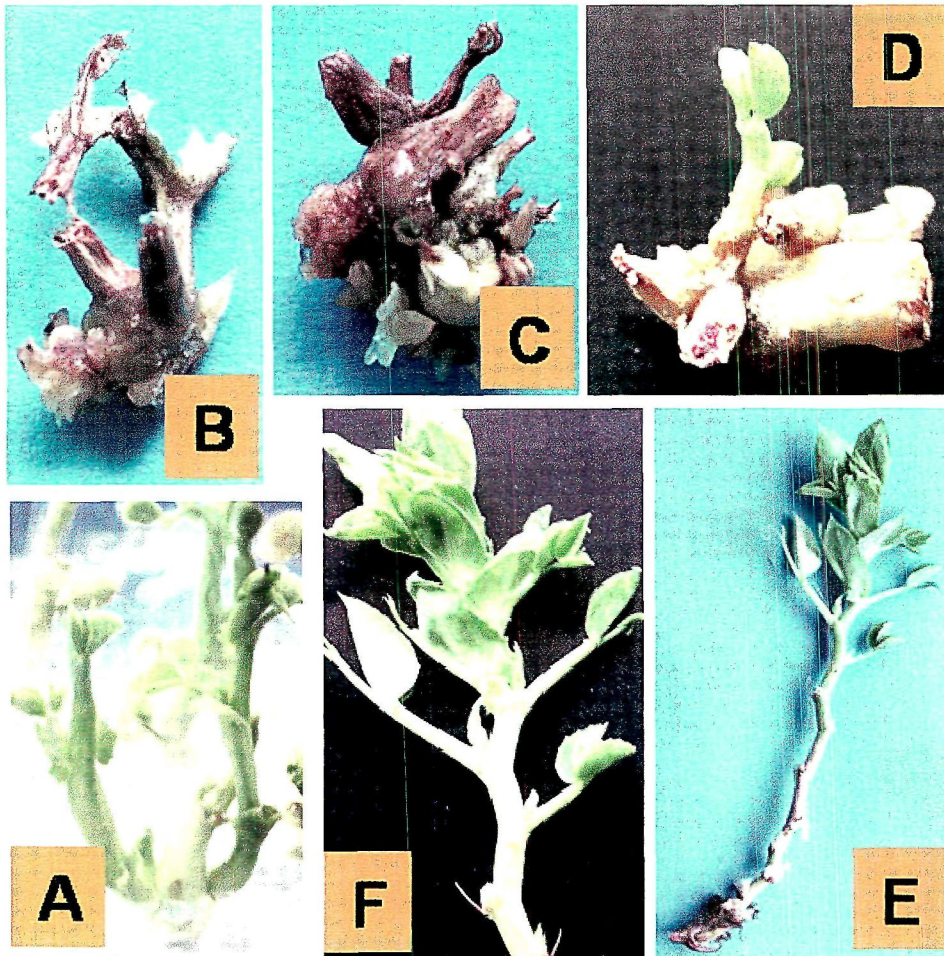
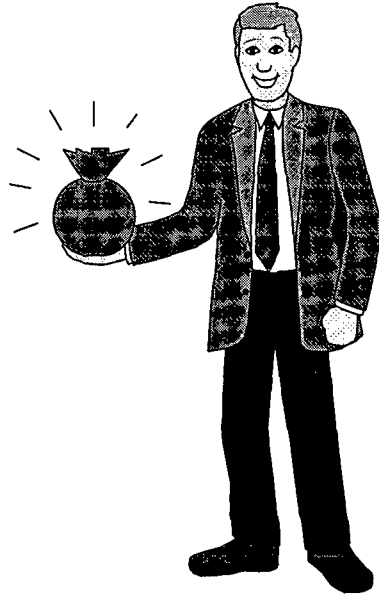


Fig 4.11 Transformation protocol for pea cultivar Azad P1. The cotyledonary nodes were co-cultivated with *Agrobacterium tumefaciens* and (A) grown on non-selective MS medium for 15 days supplemented with 20 μM BAP+0.01 μM NAA+300 mg l^{-1} timantin. After 15 days, these were transferred on to selective medium (MS + 20 μM BAP+0.01 μM NAA+300 mg l^{-1} timantin+15 mg l^{-1} hygomycin B). (B, C) majority of the explants died within 20 days of culture on selective medium. (D) Some explants developed one or two shoots on selective medium. (E, F) The putative transformants developed shoots on selective medium.



SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

Efficient tissue culture and *Agrobacterium*-mediated transformation protocols were developed for green pea (*Pisum sativum*) Variety Azad P1. For transformation, *A. tumefaciens* strain GV3101 and binary vector pCAMBIA 1302 were used. The pCAMBIA 1302 harboured a pathogenesis related protein gene called thaumatin like protein (*CsTLP*) gene from *Camellia sinensis* (L).O. Kuntze. The *CsTLP* gene governs resistance against stresses in plants. The selectable marker genes in pCAMBIA 1302 were aminoglycoside 3'-phosphotransferase (*nptII*; for *A. tumefaciens*) and hygromycin phosphotransferase (*hpt*; for plant).

Salt and vitamin concentrations, explants type and growth regulators affected shoot induction from pea explants (embryo, cut embryo and cotyledonary node). Three media were tested for shoot induction efficiency from these explants. Of these media (MS, ½ MS+20 µM BAP+0.01 µM NAA, MS+20 µM BAP+0.01 µM NAA), MS basal developed single plantlet per explant whereas other two media developed multiple shoots. The number of shoots per explant as well as shoot length was less at half strength of MS salts and vitamins compared to full strength. Among the explants, cotyledonary nodes and among the media MS supplemented with 20 µM BAP+0.01 µM NAA was the best for shoot induction. Five media (MS, ½ MS+20 µM BAP+0.01 µM NAA, MS+20 µM BAP+0.01 µM NAA, MS+8.8 µM BAP and MS+0.08 µM BAP) were tested for shoot elongation and the best medium was MS+20 µM BAP+0.01 µM NAA. The incubation period of explants on shoot induction media was 60 days with one subculture after 30 days. After induction, the shoot bunches were divided into four parts and transferred on to shoot elongation medium where these were incubated for 30 days. The incubation conditions for tissue cultures were, temperature: 25±1°C, light; 16h/8h light/dark cycle, light intensity: 716 lux.

Using the present protocol, hundred percent cotyledonary nodes developed shoots with a frequency of 46.63 shoots per explant and an average shoot length of 9.82 cm. Impact of ammonical form of nitrogen and sucrose concentration on shoot induction was also evaluated. The optimum level of

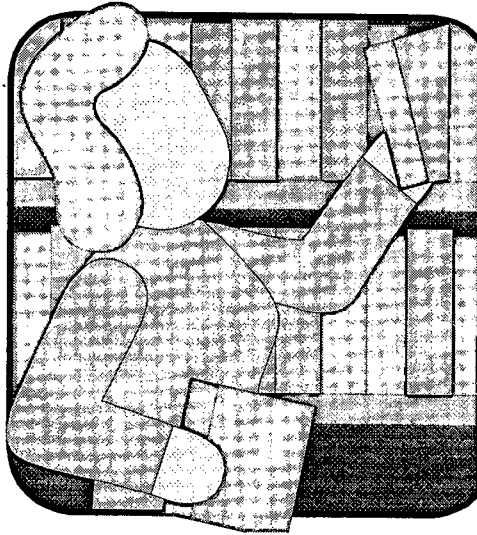
ammonium nitrate was 1650 mg l^{-1} whereas that of sucrose was 30 g l^{-1} . Reducing the concentration of these chemicals reduced shoot length as well as shoots per explant. Like shoot induction, shoot elongation also required high concentration of salts and vitamins.

Hygromycin sensitivity test for cotyledonary node explants showed that 15 mg l^{-1} was the optimum concentration at which either the explants did not induce shoots or the already developed shoots died. The concentration as well as exposure period of hygromycin dictated the survival of cotyledonary explants. At 15 mg l^{-1} , some explants formed shoots, these shoots, however, died when exposure period was increased to 20 from 10 days.

To maximize the transformation efficiency, the effect of acetosyringone, co-cultivation temperatures and injury to explant was studied. In addition to this, effect of transfer of explants to selective medium immediately after co-cultivation or after 15 days of growth on non-selective medium was also studied. Growth of explants on non-selective medium for 15 days after co-cultivation enhanced the generation of transformants.

Acetosyringone was found to be necessary for pea transformation using *A. tumefaciens* strain GV3101. At zero concentration of acetosyringone, no transformants were generated. The optimum concentration of acetosyringone was $50 \text{ } \mu\text{M}$ and increasing it to $100 \text{ } \mu\text{M}$ reduced the transformation efficiency. The optimum co-cultivation temperature was found to be 24°C and injury to explants using silicon carbide prior to inoculation enhanced the transformation efficiency. Using the present protocol, 4.33 putative transformants per 100 explants were generated. True transformants were identified using PCR with *CsTLP* gene specific primers.

The protocol for transformation of pea cv Azad P1 developed here could be used to develop agronomically superior pea. It can also be utilized for functional characterization of genes with no known biological or molecular function.



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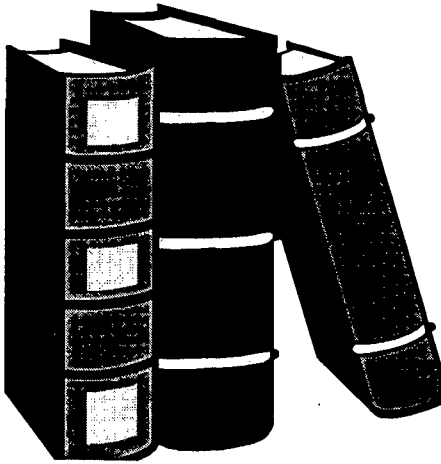
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APPENDICES

Appendix 1. Composition of MS medium (Murashige and Skoog, 1962)

Components	Concentration (mg/l)
NH ₄ NO ₃	1650
KNO ₃	1900
CaCl ₂ .2H ₂ O	440
MgSO ₄ .7H ₂ O	370
KH ₂ PO ₄	170
H ₃ BO ₃	6.2
MnSO ₄ .4H ₂ O	22.3
ZnSO ₄ .4H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
FeSO ₄ .7H ₂ O	27.8
Na ₂ EDTA.2H ₂ O	37.3
Myo-inositol	100
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Nicotinic acid	0.5
Glycine	2.0
Sucrose	30 g/l
Agar	8 g/l

Appendix 2. Composition of Yeast extract Mannitol agar medium (YEMA)

Chemical	Amount (per litre)
Mannitol	10g
Yeast extract	1g
KH ₂ PO ₄ (2%)	10 ml
K ₂ HPO ₄ (2%)	10 ml
MgSO ₄ .7H ₂ O (1M)	0.8 ml
CaCl ₂ . 2H ₂ O (1M)	0.4 ml
Agar	16 gm
Water	1000 ml

Appendix 3. Plant DNA extraction buffer (CTAB buffer)

CTAB	2% (w/v)
Tris-HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	20 mM
NaCl	1.4 M
PVP	1% (w/v) (MW 40,000)

Appendix 4. Composition of TE buffer (10:1)

- 10 mM Tris (pH 8.0)
- 1 mM EDTA (pH 8.0)

Appendix 5. Composition of PCR reaction

Contents	Amount taken
Autoclaved dH ₂ O	34.5 µl
10X PCR buffer	5.0 µl
dNTP mix (10mM)	0.8 µl
Primer forward (10 µM)*	0.6 µl
Primer reverse (10 µM)*	0.6 µl
MgCl ₂	3.0 µl
Taq DNA polymerase	0.5 µl
DNA template (30 ng)	5.0 µl
Total	50.0 µl

Appendix 6. Preparation of Agarose gel

The gel assembly was cleaned, dried, assembled and a comb with requisite number of wells was placed in the gel tray. Agarose (0.9 g) was suspended into 50 ml of 1X TAE buffer (**Appendix 7**). The mixture was heated in a microwave and allowed to cool at room temperature. When the solution was luke warm, it was poured into the casting tray and allowed to set for one hour. Once set, the comb was removed, the gel along with casting tray was placed in the electrophoresis tank.

Appendix 7. 50X TAE buffer

Tris base	242.1 gm
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
The volume was adjusted to 1 litre with dH ₂ O.	

Appendix 8. Loading of samples on the gel and gel electrophoresis

DNA or PCR amplified product was mixed with 1X (of 6X) gel loading dye (**Appendix 9**). The DNA sample and gel loading dye was mixed well using a pipette and loaded into the wells of the gel. The gel was run at 60 V (DNA migrates from cathode to anode, so the wells should always be towards the cathode). Monitor the movement of the tracking dye. When tracking dye has covered the desired distance was, the gel was stained with ethidium bromide (0.5 µg/ml), de-stained with water for 30 min. The banding pattern and intensity of the bands was observed under UV transilluminator of the Gel Documenetation Unit.

Appendix 9. 6X Gel loading dye

Glycerol	30%
Bromophenol blue	0.25%
Xylene cyanol	0.25%

Appendix 10. Composition of Plasmid extraction solutions

Resuspension buffer: 50 mM Tris·HCl, pH 8.0
 10 mM EDTA;
 100 µg/ml RNase A

Add 5 mg/ml lysozyme was added to solution I before use.

Lysis Buffer: 10N NaOH
 10% SDS

Working solution: 0.8 ml 10 N NaOH + 4 ml 10% SDS + 35.2 ml of sterile distilled water.

Neutralization solution: Stock solution (stored at 4°C)

3.0 M Potassium acetate

Brief Biodata of student

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