Chapter 5

DISCUSSION

Diseases caused by viruses in plants are a great problem as many viruses are capable of infecting multiple hosts and also classical sources of virus resistance are rare. In this scenario, prospects of generating transgenic plants would broaden the options for viral resistance. Transgenic approaches were shown to produce durable and safe virus resistance in the field, enabling the production of crops that would otherwise not been possible (Fuchs and Gonsalves 2007). Based on the pathogen derived resistance (PDR) first proposed by Sanford and Johnson (1985), various transgenic approaches based on viral genes and sequences were applied to many plant species. A number of plant virus nucleic acid sequences including virus coat protein were found useful in developing virus resistant transgenic plants (Golembowski 1990, Fitch et al. 1990, Lapidot et al. 1993, Beachy et al. 1999, Thomas et al. 2000).

As black pepper is predominantly a vegetative propagated crop, occurrence of stunted disease caused by viruses is a major matter of concern. Viral diseases are known to be spread through the use of infected cuttings for planting and also through insect vectors in the field. None of the cultivars of black pepper grown is resistant to disease and there is no chemical control for viruses. So development of a resistant variety is the only promising strategy. Development of virus resistant genotype through breeding is often restricted by availability of limited germplasm for source of resistance and also due to broad host range of viruses. Hence transgenic approach offers an attractive and potential tool for the development of virus resistant genotype.

Among different genetic transformation methods, *Agrobacterium* mediated gene transfer has remarkable advantage over direct methods, such as defined integration of transgenes, ability to transfer large segments of DNA with minimal rearrangement, stable integration into genomic DNA, simple segregation pattern which include low copy number of transgene leading to fewer problems of co-suppression and instability, no requirement of special equipment, and preferential integration into transcriptionally active regions of the chromosomes (Hiei et al. 1994, Smith and Hood 1995, Gelvin 2005).
5.1 Development of an *Agrobacterium* mediated transformation system for black pepper

5.1.1 Selection of suitable explant for transformation

There has been a limited research on genetic engineering of black pepper. So far there are only two preliminary reports on *Agrobacterium* mediated transformation of black pepper. Sasikumar and Veluthambi (1996) reported transformation of callus derived from cotyledon explants using *Agrobacterium* strain LBA4404 harboring binary vector PGA 472. Sim et al. (1998) reported transformation of leaf explants with *Agrobacterium* harboring GUS construct. Transgeneicity of leaf explants was determined by GUS assay. But both these reports did not report regeneration of transformed tissue.

Most of the difficulties in obtaining transgenic plants are associated largely with regeneration, so the first step for transformation is to establish an efficient *in vitro* regeneration system. Identification of suitable target tissue is the primary requirement to establish an efficient regeneration system. In the present study, five types of black pepper explants (leaf, internode, petiole, embryo along with micropylar region and embryogenic mass) were tested for their regeneration ability in MS and SH media with different hormonal combinations. Among them, only embryo along with micropylar region and embryogenic mass gave successful regeneration while leaf, internode and petiole explants failed to give regeneration. Bhat et al. (1995) also reported a similar trend in their studies on regeneration capabilities of black pepper using roots, node, internode and leaves as explants. They found that except nodal region all other explants failed to give regeneration. Sim et al. (1998) reported callus formation from leaf, petiole and stem explants of black pepper seedlings. But they also failed to get regeneration. Presence of excessive phenolics may be one of the reasons for poor regeneration of black pepper explants. Sai et al. (2006) reported that excessive phenolic production is one of the key limitations in grain sorghum tissue culture. Recalcitrant nature of woody plants to regeneration was reported by other workers also (Toureblanca et al. 2010). However, recently callus induction and shoot regeneration from leaf explants of potted black pepper plants cultured on MS medium supplemented with different plant growth regulators were reported by Ahmad et al. (2010).
Embryo along with the surrounding micropylar tissue was able to exhibit primary somatic embryogenesis but with low frequency (14%) with an average of 4-6 somatic embryos per explant. But embryogenic mass obtained from primary somatic embryo through cyclic secondary embryogenesis was able to give high frequency regeneration of about 75 – 100 plantlets from every 250 mg of embryogenic mass. About 90% of the plantlets rooted without any additional treatment. SH medium without any of the plant growth hormone was used for the entire life cycle of the plants. All regenerated plants showed normal growth and development with uniform appearance like any normal black pepper plants. Nair and Gupta (2006) reported that 20% of germinating black pepper seeds cultured on SH medium without growth regulators produced primary somatic embryos directly from micropylar tissue within 90 days of culture. Embryogenic mass obtained from (25 mg each) cyclic embryogenesis of these primary somatic embryos gave more than 2, 000 well developed plantlets within a period of 60 days. They suggested that embryogenic mass of black pepper obtained through cyclic somatic embryogenesis of primary somatic embryos can be a potential candidate as explant for genetic manipulations of this crop for specific traits including abiotic and biotic stress tolerance.

Leelavathi et al. (2004) used embryogenic calli as explant for transformation and regeneration of cotton and obtained an average of 12 transgenic plants per petri plate of co-cultivated callus. Andrade et al. (2009) reported a high throughput Agrobacterium mediated transformation protocol for stable integration of foreign genes into American chestnut tree genome using embryogenic mass as source of explant. They could achieve an average efficiency of four independent transformation events per 50 mg of target tissue and minimal escapes. Gao et al. (2009) used embryogenic calli of Festuca pratensis Huds. a forage grass for transformation and obtained 40 independent transgenic plants from 45 bialaphos resistant callus lines with an average transformation efficiency of 2%. Many workers reported that regeneration of plants using cyclic secondary embryogenesis is ideal for gene transfer as large numbers of transformed somatic embryos can be obtained by embryo cycling (Merkle et al. 1990, Ellis 1995, Liu et al. 1996). Gelvin (2003) reported that the cells undergoing active vegetative growth would be more susceptible to agrobacterial infections, hence a potential target for transformation. Embryogenic cultures have proven to be useful target tissue for transformation of many agronomic crops as it is actively dividing (Finer and McMullin 1990, Parrott et al. 1994, Wang et al. 1998, Cheng et al. 2004, Leelavathi et al. 2004, Xing et al. 2008, Salaj et al. 2009).
5.1.1.1 Genetic fidelity analysis of embryogenic mass derived (somatic embryo derived) plantlets

In the present study, analysis of 150 scorable bands obtained by 23 random primers (with an average of 6.52 bands per primer) showed that all the 150 markers were monomorphic across all the plantlets and to the parent plant tested indicating that all plantlets were genetically uniform to their parents. Bennici et al. (2004) assessed the genetic stability analysis of organogenesis and somatic embryogenesis derived plants of one fennel donor plant both at nuclear DNA and chloroplast DNA level using RAPD and microsatellite markers respectively, revealed the genetic stability and uniformity of organogenic and embryogenic regenerated fennel plants. Qin et al. (2007) analyzed genetic stability of somatic embryo derived broccoli plantlets using RAPD markers and confirmed the genetic stability of regenerants. Genetic stability of in vitro regenerated plants of *Centaurea ultroaei* was assessed by RAPD analysis and flow cytometry by Mallon et al. (2010) and they concluded that there is no genomic alterations in any of the regenerated plants. Genetic fidelity studies of vanilla plantlets derived through protocorm like bodies using RAPD markers, Retheesh and Bhat (2011) concluded that there was no genetic variation between regenerants and the parent plant from which they originated.

Genetic stability of plantlets obtained in the present study may be due to maternal origin (micropylar region) of somatic embryos and also absence of plant growth promoters in the culture medium. Somaclonal variation due to the presence high concentration of plant growth hormones was reported by Bauru et al. (2006).

5.1.2 Determination of phytotoxic levels of bactericidal and selective antibiotics

5.1.2.1 Effect of carbencillin and cefotaxime on embryo (along with micropylar tissue) and embryogenic mass

Carbencillin and cefotaxime, both β-lactam antibiotics have minimum toxicity on most plant tissue and thus have been widely used in *Agrobacterium* mediated transformation to suppress *Agrobacterium* overgrowth after co-cultivation (Leelavathi et al. 2004, Han et al. 2009). Both the antibiotics were known to have certain effect on regeneration of different
crop plants. Break down product of these antibiotics mimic plant phytohormones which in turn alter auxin/cytokinin ratios in the media and thereby regeneration (Oz et al. 2009).

In the present study embryo along with surrounding micropylar tissue did not show any primary somatic embryogenesis in presence of different concentrations of these antibiotics tested. In the case of embryogenic mass, maximum (108.3) and minimum (8.66) number of plantlets were produced in treatments containing 100 μg/ml of cefotaxime and 500 μg/ml of carbenicillin respectively. Results clearly showed that cefotaxime at 100 μg/ml produced significantly higher number of plantlets (108.3) compared to control (62). In both antibiotics, number of plantlets produced was increased with increasing concentration of antibiotics up to 100 μg/ml, thereafter decrease in number plantlets was seen. Presence of carbenicillin at 100 μg/ml did not show much inhibitory effect on plant growth but this concentration retarded somatic embryo proliferation significantly. Cefotaxime was reported as effectively promoting somatic embryogenesis of *Triticum aestivum* at 60 – 100 mg/l (Mathias and Boyd 1986) and *Dianthus* cultivars at 100 – 500 mg/l (Nakano and Mu 1993). Suzuki et al. (2002) reported growth enhancement of embryogenic calli in to somatic embryos in presence of carbenicillin and cefotaxime in ornamental plant, *Agapanthus*. Cefotaxime enhanced somatic embryo production of *Theobroma cacao* at 100 mg/l (de Mayolo et al. 2003). On the other hand, Li et al. (2007) reported that cefotaxime, even at 100 mg/l (the minimum level effective for bacterial elimination), significantly inhibited shoot regeneration with just 32% of explants developing one or more shoots, as compared to 64% in the absence of antibiotics in London plane tree. Higher concentrations of cefotaxime resulted in a further decline in regenerative growth and 500 mg/l cefotaxime prevented all shoot production from leaf explants.

Decrease in embryo production in the presence of carbenicillin was also observed in *Picea sitchensis* at 500 mg/l (Saima et al. 1995), *Juglans regia* at 100-1000 mg/l (Tang et al. 2000), *Picea omorika* at 500 mg/l (Mihaljevic et al. 2001), *Carica papaya* at 375 and 500 mg/l (Yu et al. 2001). Reduction in shoot induction of chick pea in presence of carbenicillin was reported by Oz et al. (2009).

To find out minimum concentration of cefotaxime and carbenicillin to control *Agrobacterium* over growth from black pepper explants a separate study was conducted. Study with *Agrobacterium* strain EHA 105 harbouring pBI 121 showed that cefotaxime at 25
µg/ml and above or carbenicillin at 50 µg/ml and above were able to control the *Agrobacterium* growth from co-cultivated embryo or embryogenic mass up to 30 days while carbenicillin at 25 µg/ml failed to control the *Agrobacterium* overgrowth. Similar kind of observations was made by Wiebke et al. (2006) also, where they found that cefotaxime was better than carbenicillin for eliminating *Agrobacterium* from soybean somatic embryo clusters. Xia et al. (2006) found that the inhibition of cefotaxime on *Agrobacterium* strain C58 was stronger than that of carbenicillin and the optimum concentration of cefotaxime was 100 mg/l. Oz et al. (2009) reported that cefotaxime was superior over augmentin and carbenicillin in controlling *Agrobacterium* strain KYRT1.

In the present study it was found that cefotaxime at 100 µg/ml was able to control *Agrobacterium* overgrowth and also produced maximum number of plantlets from a given quantity of embryogenic mass. So this concentration of cefotaxime can be used for black pepper transformation experiments. Very low concentration of bactericidal agent results in excessive *Agrobacterium* growth with reduction in regeneration and death of co-cultured explants, a moderate antibiotic concentration (usually species dependent) reduces *Agrobacterium* growth to controllable levels and allows for non-phytotoxic growth and development, too high antibiotic concentration results in both a decrease of *Agrobacterium* growth and plant regeneration (da Silva and Fukai 2001). Bornhoff et al. (2005) reported low plant regeneration rate due to hyper sensitivity like reaction of the plant tissue to *Agrobacterium* in combination with the treatment used for selection during grape transformation.

5.1.2.2 Optimization of minimum lethal concentration of kanamycin for the selection of transformants

Sensitivity of explant to kanamycin was reported to vary with explants stage (Zhang et al. 2001). Keeping this in view, in the present study explants of three developmental stages, embryo along with micropylar tissue, embryogenic mass and fully developed plantlets were used to determine the optimum concentration of kanamycin required for the selection of transformants. Results showed that kanamycin tested at all concentrations inhibited germination of zygotic embryo and primary somatic embryo induction from embryo along with micropylar tissue. But embryogenic mass treated with kanamycin at lower concentrations (10 and 25 µg/ml) survived although there was significant reduction in
number of somatic embryos produced. At 50 μg/ml of kanamycin, blackening around embryogenic mass, at 100 μg/ml complete blackening of embryogenic mass and only a few embryos produced (3 3). At 200 μg/ml of kanamycin, complete blackening of embryogenic mass and only 0 6 embryos were produced. This data is in agreement with the studies of Sasikumar and Veluthambi (1994) where they found that kanamycin at 50 μg/ml and above completely inhibited callus formation of black pepper using cotyledons as explants suggesting that 50 μg/ml of kanamycin is the minimum concentration needed to select transformed tissue. Another study on black pepper transformation, Sasikumar and Veluthambi (1996) found that transformed calli remained fresh and proliferated up to 150μg/ml of kanamycin while above this concentration resulted in callus death after 25 days of culture.

In studies on kanamycin sensitivity of cotton cotyledon, hypocotyl explants, and embryogenic calluses, Zhang et al. (2001) found that kanamycin at 10 mg/l or higher concentrations reduced callus formation, with complete inhibition at 60 mg/l. They also reported that the sensitivity of embryogenic callus and somatic embryos to kanamycin was different during the initiation and development stages. Xing et al. (2008) reported that increased kanamycin concentration in the medium significantly decreased the frequency of embryogenic callus liveliness and 10 μg/ml of kanamycin was chosen as an optimal concentration for the selection of kanamycin resistant callus in variety Xu55-2 of sweet potato. In studies on kanamycin sensitivity in cucumber, Sharma et al. (2009) reported that MS medium supplemented with 100 mg/l of kanamycin arrests the growth and differentiation of shoot tips totally. Rethesh and Bhat (2011) reported that concentrations >100 mg/l of kanamycin inhibited regeneration leading to complete death of protocorm like bodies of vanilla after 30 days of culture.

In the present study effect of kanamycin at plantlet stage showed that plantlet treated with kanamycin up to 50 μg/ml, survived without bleaching. Plantlets treated with 100 and 200 μg/ml of kanamycin started bleaching within 15 days and after 30 days, 60% and 100% of plantlets were bleached respectively. Results indicated that kanamycin at 200 μg/ml would give complete selection of non transformed plants both at embryogenic mass and at plantlet stage. Sarker et al. (2009) reported complete death of non transformed tomato plants at 200 μg/ml of kanamycin while studies on in vitro regeneration and kanamycin sensitivity in
cucumber showed that at 100 mg/l and above, all the explants were bleached out just after
initiating shoot formation and root induction was inhibited completely (Sharma et al 2009)

5.1.3 Step wise increase in kanamycin concentration for selection of transformants

Present study on kanamycin sensitivity of non transformed black pepper plants
indicated that kanamycin at 200 µg/ml would give complete selection of non transformed
plantlets both at embryogenic mass stage and at plantlet stage. Even though selection at 200
µg/ml gave 100% bleaching of non transformed plantlets this concentration also reduced the
growth and survival of transformed plantlets drastically. Thus assuming that such a high
concentration of kanamycin at once may cause sudden death of the tissue or a slow
proliferation a step wise increase in kanamycin concentration for selection of transformants
was followed. Embryogenic mass after co-cultivation with Agrobacterium in the regenation
medium were subjected to selection in three stages with increasing concentration of
antibiotic initially at 25 µg/ml for 3-5 weeks followed by 50 µg/ml for next 30 days and at
plantlet stage it was at 100 µg/ml. This strategy would create an increasingly stringent
environment enabling the transformed embryogenic mass to express effectively the antibiotic
resistance gene and initiate cell division and potentially improving the successful plant
regeneration. From the preliminary experiment it was observed that incooperation of
kanamycin in the liquid culture stage reduced growth, differentiation and rooting of
transformed plantlets drastically. Hence to avoid the potential negative impact, kanamycin
was excluded during this stage of selection. It was reintroduced in to next solid culture stage
which helped to remove escapes if any. In the present study high frequency transformation
was obtained by following step wise selection with increasing concentration of kanamycin.
Out of 108 plants regenerated 92 were found positive in PCR (85%). Previous workers also
reported this kind of step wise increase in kanamycin concentration for selection of
transformants. Cai et al (1999) reported that limiting the period under antibiotic selection and
allowing regeneration of plants in the absence of kanamycin to obtain maximum number of
transgensics. Agro infected globular somatic embryos of Camellia sinensis were initially
placed on multiplication medium containing 50 µg/ml of kanamycin. Further selection in the
maturation and germination medium at an elevated kanamycin level of 75 µg/ml gave an
average of 40% transient expression in GUS histochemical assay (Mondal et al 2001). Akcay
et al (2009) reported a gradual increase in selection pressure with increasing kanamycin
concentrations (1 week at 100, 1 week at 200, and 4 weeks at 300 mg/l) played significant role in increasing transformation efficiency of lentil cotyledonary node tissues with less chimerism. Bull et al. (2009) used step wise increase in hygromycin concentration successfully for transformation of friable embryogenic calli of cassava to get transgenic plantlets. They used hygromycin of 5, 8 and 15 mg/l for selection of transformed embryogenic calli in different stages followed by a hygromycin free medium for plant regeneration. Then the plantlets were transferred to a hygromycin (10 mg/l) containing medium to avoid ‘escapes’. Kung et al. (2010) adopted almost similar strategy where concentration of stepwise increasing kanamycin selection for initial 90 days (50 mg/l for 30 days and 100 mg/l for next 60 days) and then the selected tissues was sub cultured on the kanamycin free medium for embryo development in papaya. The mature somatic embryos formed were cultured on MS medium containing 50 mg/l kanamycin for shoot development. The transformation efficiency obtained with this method was 31.7%. Retheesh and Bhat (2011) also used a step wise increase in kanamycin concentration in their studies on genetic transformation and regeneration of transgenic vanilla plants where selection was done in three stages with increasing concentration of antibiotic, initially at 50 mg/l for 15 days followed by 75 mg/l for another 15 days and finally at 100 mg/l from 30th day of culture and thereafter. This apparently reduced sudden shock to the explants and increased the frequency of transgenic lines as shown by GUS histochemical assay (100%) and PCR (100%)

5.1.4 Development of protocol for transformation using GUS construct and analysis of transformants

After standardizing antibiotic concentration needed for eliminating Agrobacterium after co cultivation and selection of transformants, a protocol was developed for transformation of black pepper using embryogenic mass as explant. The protocol included co cultivation of embryogenic mass with A. tumefaciens EHA 105 followed by selection of transformants using increasing kanamycin concentration at different stages of regeneration. The fully developed plantlets with roots were hardened and established in the glass house. Using this protocol, from one gram of embryogenic mass nine putative transformed plants could be established in the glass house. This is in contrast to the 200 non transformed plants that could be established using one gram of embryogenic mass. Though there are two previous reports of Agrobacterium mediated transformation of black pepper (Sasikumar and Veluthambi 1996, Sim et al. 1998), both these methods did not report regeneration of
transformed tissues. Thus, the present study is the first report of an efficient *Agrobacterium* mediated transformation of black pepper. In the present study, co-cultivated embryogenic mass in selection medium (25 μg/ml of kanamycin) under darkness for 3–5 weeks produced some vigorous growing points. Each growing point was proliferated into embryo clusters of 3–4 plantlets. Regeneration efficiency was higher for embryogenic mass that was not subjected to *Agrobacterium* infection (300–400 plantlets for one g embryogenic mass) compared to embryogenic mass subjected to agro infection (30–40 plantlets for 1 g of embryogenic mass). Such decline in regeneration may be due to certain level of stress to the explants caused by *Agrobacterium* infection and also due to presence of different antibiotics during stages of life cycle of plantlets. A similar kind of reduction in regeneration on agroinfection was reported in other crops like *Cicer arietinum* (Husnain et al. 1997), *Pelargonium* sp (Kishnarah et al. 1997) and *Cajanus cajan* (Singh et al. 2002).

5.1.4.1 Confirmation of transgenicity by assay for GUS activity

The *E. coli* GUS gene encoding β-glucuronidase (GUS) (Jefferson et al. 1987) is widely used as a reporter gene in plant transformation studies (Wyayanto and McHughen 1999) because the gene expression patterns can be quantified by fluorometric and spectrophotometric analysis. Additional advantages of the GUS assay are that it is very straightforward and requires no expensive equipment. The major disadvantage of the GUS assay is that it is lethal for the plant tissues. With these limitations, however, the GUS assay is still one of the most effective reporter gene systems used by the scientists in plant gene expression studies. β-glucuronidase which catalyses conversion of colourless 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA) into blue coloured 5-bromo-4-chloro-3-indole so the plant cells which are transformed with GUS gene will give a blue coloured appearance on GUS assay (Jefferson 1987). Plant cells do not have any endogenous GUS activity so the production of blue colour on staining with X-GlcA indicates the activity of GUS gene. This assay is easy to perform, sensitive, highly reliable and requires no special equipment (Jefferson et al. 1987).

Many workers have used GUS as a reporter gene for standardization of transformation of various plant species such as pea (Svabova et al. 2005), mulberry (Agarwal and Kanwai 2007), London plane tree (Li et al. 2007) and golden pothos (Kotsuka and Tada 2008), vanilla (Rethesh and Bhat 2011).
In the present study GUS assay of transformed embryogenic mass (one month after transformation) showed uniform blue colour in transformed embryogenic mass in contrast to the colourless negative control after overnight incubation with X-GlcA suggesting that the GUS gene is transcribed from the transformed embryogenic mass and dictates the synthesis of functional enzyme. The colourless nature of non-transformed embryogenic mass after staining indicate that there is no detectable endogenous GUS activity in non-transformed control embryogenic mass. Sim et al. (1998) reported GUS activity in leaf explants of black pepper transformed with Agrobacterium (after 10 days in regeneration medium) harbouring GUS construct. Expression of the GUS gene was confirmed by blue coloration of the recipient tissue.

5.1.4.2 Confirmation of transgenecity by PCR analysis of transformed plants

Polymerase chain reaction is a fast and sensitive tool for initial screening of putative transgenics. All reports on production of transgenic plant used PCR for preliminary screening as it is fast and cost effective. Some selective reports include Pisum sativum (Orczyk and Orczyk 2000), Citrus sinensis (Li et al. 2003), Capsicum annum (Lee et al. 2004), Morus alba (Agarwal and Kanwai 2007), Allium sativum (Kene et al. 2010) and Vanilla planifolia (Retheesh and Bhat 2011).

In the present study, PCR analysis was performed for all survived plants after hardening using two sets of primers, specific to NPT II and GUS genes. PCR analysis of 108 kanamycin resistant lines revealed specific predicted amplification products of 980 bp and 660 bp with NPT II and GUS gene specific primers, respectively in 85% plants while no such bands were visible in non-transformed plantlets. This indicated the presence of both the linked genes NPT II and GUS as a single T-DNA in the transformed genome without end deletion.

Although PCR is the most widely used technique for initial screening of transgenic plants, its sensitivity and reliability is not foolproof as reported by many workers (Barik et al. 2005, Hei et al. 1994). Continued presence of recombinant Agrobacterium in transformed tissue might result in false positives during PCR analysis of putative transgenics (Shekhawat et al. 2008). Hence more reliable techniques like dot blot, Southern and northern hybridization are needed for further confirmation of true integration of transgene.
5.1.4.3 Confirmation of transgenicity by dot blot and Southern hybridization

Dot blot, Southern and northern hybridization are valuable tools for analysis of transgenic plants, as it confirms the presence of transgene and copy number in putative transgenic plant. Northern hybridization confirms the expression in transgenic tissue as it detects the presence of transcript in the tissue. Many genetic transformation reports available are supported by dot blot, Southern and northern hybridization such as in *Rosa hybrida* (Li et al 2002), *Capsicum annum* (Lee et al 2004), *Camellia sinensis* (Lopez et al 2004), *Hevea brasiliensis* (Leclercq et al 2010), *Prunus mume* (Gao et al 2010), *Vitis vinifera* (Jardak-Jamoussi et al 2010), *Arachis hypogaea* (Bhatnagar et al 2010) and *Vanilla planifolia* (Retheesh and Bhat 2011).

In the present study, 83% of PCR positive plants showed positive reaction in dot blot also Bhatnagar et al (2010) used dot blot assay to confirm the integration of transgene into peanut genome. They performed dot blot assay with nine selected plants using denatured genomic DNA where five plants (55%), showed integration of the transgene. In the present study seventeen plants were randomly selected for Southern blot hybridization to confirm the incorporation of T-DNA into genomic DNA. Southern analysis confirmed the stable integration of the NPT II gene in six of the putative transgenics indicating that 35% of the dot blot positive plants were positive in Southern as well. One to four bands were seen indicating one to four insertion events in the same genome. Many researchers suggested that plants with 1 or 2 integration events yield high level expression of the transgene while high copy numbers result in unstable or silencing of transgene (Flavell 1994, Vauchelet et al 1998, Kooter et al 1999; Iyer et al 2000). However, Alpeter et al (2005) and Kung et al (2010) reported that higher transgene copy numbers correspond to higher expression. In the present study some PCR positive plants failed to give positive signals in dot blot and Southern. This may be associated with ‘escapes’ produced during transformation which is a major problem in woody plant transformation (Pena et al 1995, Li et al 2003).

Neither the antibiotic resistance gene (NPT) nor the beta-glucuronidase gene (GUS) had any apparent effect on normal development and morphology of the transgenic black pepper plants as all plants maintained in the green house possessed normal growth and morphology with uniform appearance. The protocol presented in this study demonstrates an
efficient way of transformation for black pepper which can be extended to incorporate desirable genes into existing black pepper cultivars. As a growing embryogenic mass forms the starting material for the transformation here, even a single embryogenic line once established, might be used for parallel, multiple transformation experiments.

5.2 Development of gene constructs using CMV and PYMoV sequences in plant transformation vectors

A total of three constructs (portion of ORF III region of PYMoV in sense and antisense orientation, and CMV coat protein in sense orientation) were prepared in the plant transformation vector, pBI121 in the present study. Plasmid pBI121 is one of the most commonly used plant transformation vector that belongs to pBIN series. Plasmid pBI121 was extensively used in standardization of transformation experiments as it has β glucuronidase region which enable to score transgene expression by histo chemical GUS assay at an early stage (Svabova et al. 2005, Agarwal and Kanwar 2007). Also, GUS region in the vector can be replaced with ‘useful genes’ namely conferring for herbicide tolerance, insect and virus resistance (Shade et al. 1994, Grant et al. 1995, Jones et al. 1998, Chowira et al. 1995, Charity et al. 1999) for preparing suitable gene constructs for transformation experiments.

5.2.1 Preparation of gene construct carrying PYMoV sequence in sense and antisense orientation

From the many clones of pPCRScriptAmpSK(+)ORF III available in the laboratory, the one with ORF III in sense orientation was selected for the preparation of gene construct in plant transformation vector pBI 121.

pBI 121 is a low copy plasmid and hence yielded relatively low quantity ranging from 0.25 µg/ml – 0.35 µg/ml of the culture. Presence of RK2 ori as replication origin in plasmid is the reason for low copy number (Bevan 1984). Kues and Stahl (1989) reported the low yield of plasmids that undergo stringent replication. But plasmid pPCRScriptAmpSK(+)ORF III is a high copy number plasmid, so yield was relatively high (3.3 µg/ml of the culture).
Plasmid vectors pPCRScriptAmpSK(+)ORF III and pBI 121 were subjected to restriction digestion using \textit{BamH1} in separate tubes. The cloned region of ORF III harboured a \textit{BamH1} site at 409 bases from its 5’ end. The vector pPCRScriptAmpSK(+)ORF III was cut into two fragments releasing a 409 bp fragment of ORF III and a fragment of about 3 kb containing linearized vector and a portion of ORF III. Similarly \textit{BamH1} digestion linearized plasmid transformation vector pBI 121 as it possess a single \textit{BamH1} site. Alkaline phosphatase treatment of linearized pBI 121 removed its phosphate group and thereby prevented its self-ligation.

Ligation of linearized pBI 121 with ORF III was done and \textit{E. coli} DH 5a cells were transformed using ligated recombinant pBI 121 followed by selection of transformants on LB agar plate containing kanamycin. Non transformed cells could not grow in the medium as they lack kanamycin resistant gene. All the colonies grown on the selection medium were streaked onto a master plate and, presence and orientation (sense or antisense) of insert DNA (transgene) in the binary vector was confirmed by PCR and restriction analysis.

Triparental mating is the most prevalent method for introducing recombinant cell in to \textit{Agrobacterium} (Ditta et al. 1980). It requires three different bacteria, multiple incubation temperatures and long incubation time. Recombinant plasmid in \textit{E. coli} was transferred to \textit{A. tumefaciens} strain EHA 105 with the help of helper plasmid pRK2014. Transformed cells were plated on YEB agar containing 50 μg/ml of each of rifampicin and kanamycin. Recombinant plasmid did not have rifampicin resistance and \textit{Agrobacterium} strain did not have kanamycin resistance. So all the colonies growing on double selection media (rifampicin and kanamycin) were \textit{Agrobacterium} transformed with recombinant plasmid. Recombinant nature of plasmid was further confirmed by PCR and restriction analysis.

5.2.2 \textit{Cucumber mosaic virus} coat protein (CMVCP) in sense orientation

A clone of pPCRScriptAmpSK(+)CMVCP where CMVCP was cloned in sense orientation was used for the preparation of gene construct in plant transformation vector. Plasmid vectors pPCRScriptAmpSK(+)CMVCP and pBI 121 were subjected to double digestion using \textit{BamH1} and \textit{SacI} in separate tubes. The vector pPCRScriptAmpSK(+)CMVCP was cut into two fragments releasing a 657 bp fragment of CMVCP and a fragment of about 3 kb containing linearized vector. Similarly \textit{BamH1-SacI}
double digestion released GUS gene from plasmid transformation vector pBI121 as it was flanked by a BamH1 site at one side and a SacI site at other side. This caused the digestion of pBI121 into two fragments of 1.8 kb GUS fragment and a 12.9 kb vector fragment. These two molecules, GUS removed pBI121 and CMVCP insert had complementary ends that allowed the directional cloning of CMVCP region in sense orientation in pBI121. Recombinant vector was used to transform *E. coli* DH5α cells and recombinant nature and orientation was confirmed both in *E. coli* and *Agrobacterium*. Many previous workers have reported CMVCP based constructs for transformation of different hosts (Cuozzo et al. 1998, Beachy et al. 1990, Fitchen and Beachy 1993, Palukaitis and García-Arenal 2003, Srivastava and Raj 2008, Lee et al. 2009).

### 5.3 Transformation of embryogenic mass with PYMoV sense (pBI121PYMoVS) and antisense (pBI121PYMoVAS) constructs

*Agrobacterium* containing recombinant plasmid pBI121PYMoVS/ pBI121PYMoVAS were grown in YEB medium to mid log phase and diluted to 1/5th with basal SH medium. About one g of embryogenic mass was immersed in *Agrobacterium* suspension with gentle shaking for 2 h followed by incubation in regeneration medium devoid of antibiotics for 2 days and subsequently in selection medium containing kanamycin. The co-cultivated embryogenic mass in selection medium (25 μg/ml of kanamycin) under darkness for 3–5 weeks produced some vigorous growing points. Each growing points were proliferated into embryo clusters of 3-4 embryos. An average of 10 and 9 transformation events per g of embryogenic mass led to 167 and 253 kanamycin resistant plantlets in case of pBI121PYMoVS and pBI121PYMoVAS respectively. Decline in regeneration of transformed tissue compared to non-transformed embryogenic mass may be due to certain level of stress to the explants caused by *Agrobacterium* infection and also due to presence of different antibiotics during different stages of life cycle of plantlets. Such decline in regeneration capabilities of explant after *Agrobacterium* infection was reported by other workers also (Husnain et al. 1997, Krishnaraj et al. 1997, Singh et al. 2002). The transformation efficiency obtained in the present study is on par with reports from other workers also. Suzuki et al. (2002) reported that transformation of three g fresh weight embryogenic calli of *Agapanthus* that resulted into 2 and 18 hygromycin resistant callus lines with *A. tumefaciens* strains EHA101/pIG121Hm and LBA4404/pTOK233, respectively. Leelavathi et al. (2004) obtained an average of 12 kanamycin resistant plants from a single transformation experiment.
using cotton callus. In studies on transformation of *Festuca pratensis* an important cool season forage grass, Gao et al. (2009) were able to obtain 23 bialaphos resistant callus lines which regenerated into 21 independent plantlets from 1,000 embryogenic callus co cultivated with *Agrobacterium*. In the present study after step wise selection with kanamycin, all regenerated 50 plantlets of pBI121PYMoVS and 220 plantlets of pBI121PYMoVAS constructs were hardened in the greenhouse. After hardening for 3-6 months, 60% and 34% plantlets of pBI121PYMoVS and pBI121PYMoVAS constructs survived with an average of 10 and 8.7 transformed plantlets per g of embryogenic mass respectively. While in non transformed control out of 250 plantlets hardened 200 were survived with an efficiency of 80%. Low survival rate of transgenic plant may be due to residual effect different antibiotics applied during different stages of lifecycle of plants.

5.3.1 Confirmation of transgenicity

5.3.1.1 PCR analysis of transformed plants

Good quality genomic DNA was isolated from all regenerated plantlets. PCR performed with two sets of primers each specific for kanamycin region and transgene gave expected amplicons of approximately 980bp and 539bp respectively in the case of both sense and antisense constructs. In the case of pBI121PYMoVS construct all the 30 plants screened were positive in PCR (100%) where as in pBI121PYMoVAS construct out of 78 plants screened, 62 plants tested as positive (79%). All positive plants gave amplification with both the set of primers indicating that both genes are linked and transferred efficiently without end deletion. PCR technique was routinely used by many workers for screening of transgenic plants against DNA viruses for the presence of transgenes as reported in tomato (Praveen et al. 2005, 2006, Lin et al. 2011), common bean (Atagao et al. 1998, Bonfim et al. 2007), and rice (Dai et al. 2008).

5.3.1.2 Dot blot, Southern hybridization and northern hybridization

As PCR is not foolproof for the analysis of putative transformants (Hei et al. 1994, Barik et al. 2005, Shekhawat et al. 2008) more sensitive technique like dot blot, Southern and northern hybridization are needed for further confirmation of true integration of transgene. Some selected reports are *Capsicum annuum* (Lee et al. 2004), *Camellia sinensis* (Lopez et al. 2004), *Hevea brasiliensis* (Leclercq et al. 2010), *Rosa hybrida* (Li et al. 2002), *Prunus*
In the present study, 80% of sense and 86% antisense construct plants were positive in dot blot test. In Southern analysis 20% and 57% of sense and antisense construct plants showed positive signals. No hybridization signal was observed in non-transgenic control plants. In case of sense construct, all Southern positive plants showed single copy integration whereas in the case of antisense construct, one to two integration events were observed. In general, plants with 1 or 2 integration events were reported to yield high level expression of transgene (Flavell 1994, Vaucheret et al 1998, Kooter et al 1999, Iyer et al 2000). But Kung et al (2010) reported that transgenic papaya lines with two or more copies of transgene showed more resistant to *Papaya leaf distortion mosaic virus* than transgenic lines that carried single copy insert. Similarly, experiences with rice, potato and wheat, Altpeter et al (2005) suggested that higher transgene copy numbers correspond to higher expression levels, indicating that the transgenes were expressed efficiently.

Failure of some dot blot positive plants to give positive signal in Southern may be associated with ‘escapes’ produced during transformation. Occurrence of escapes is a major problem in woody plants (Pena et al 1995, Li et al 2003). It is thought that transformed cells on inoculated explants after co-cultivation with *Agrobacterium* could protect non-transformed cells from the selection agents and cause the production of escapes (Dong and McHughen 1993). Iocco et al (2001) reported that, non-transgenic embryos developing before 10–12 weeks could be attributed to the presence of some advanced multicellular embryos at the time of *Agrobacterium* inoculation, giving rise to untransformed or partly transformed multicellular embryos which may be tolerant to the effects of kanamycin. Lopez-Perez et al (2008) also described on the occurrence of escapes in grape vine transformation using embryogenic calli as explants. All plants maintained in the glass house exhibited normal growth and morphology indicating that transformation events did not affect the normal development and morphology of the transgenic black pepper plants.
5.4 Transformation of embryogenic mass with *Cucumber mosaic virus* coat protein (pBI121CMVS) sense construct

As observed in PYMoV, regeneration efficiency of CMVCP transformed tissue was also lower (18 plantlets established in green house from gram of embryogenic mass) compared to the regeneration efficiency obtained with non transformed embryogenic mass (200 plantlets established in green house from one gram of embryogenic mass) Such decline in regeneration capabilities of embryogenic tissue after *Agrobacterium* infection compared to control was reported by previous workers also (Malabadi and Nataraja 2007, Malabadi et al 2008) In the present study an average of 9.7 transformation events per gram of embryogenic mass was obtained which led to an average of 26 kanamycin resistant plantlets from a single transformation experiment This transformation efficiency is on par with reports from other workers also Choi et al (2007) obtained 18 transgenic plants from the infected 644 embryogenic calluses of sweet potato cv Yulmi Malabadi et al (2008) obtained 14 transgenic somatic seedlings per gram fresh weight of khasi pine embryogenic tissue Deng et al (2009) reported that 21 calli of Chinese white poplar infected with *A tumefaciens* harbouring pLFFLPBBM, 6 calli produced 12 somatic embryos and out of these 12 somatic embryos, six embryos germinated to form plantlets

Of the 156 plantlets transferred to green house, 109 plantlets survived with a hardening efficiency of 70% PCR analysis of all 109 plantlets showed 95% of the plants as positive while in dot blot, 53% of plants were positive In Southern analysis, all nine tested plants were positive with one to two copies of transgene Southern analysis of transgenic table grape plants by Lopez-Perez and his associates (2008) revealed up to three integration events in the same genome Lee et al (2009) reported single copy integration of CMVCP gene to chilli pepper using CMVCP transformation system One to three copy integration of CP gene of ZYMV in to oriental melon genome was reported by Wu et al (2009) They also reported that there is no correlation between resistance and copy number of the transgene

5.5 Engineered resistance against viruses

The use of viral CP and other sequences as a transgene for producing virus resistant plants is one of the most spectacular successes achieved in plant biotechnology Numerous crops have been transformed to express viral sequences and have been reported to show high
levels of resistance in comparison to untransformed plants. Powell-Abel et al. (1986) first reported resistance against TMV in transgenic tobacco expressing the TMV CP gene. The resistance was manifested as delayed appearance of symptoms as well as a reduced titre of virus in the infected transgenic plants, as compared to the controls. The success of CPMR has prompted the production of transgenic plants expressing multiple CP genes from more than one virus. Several important crops have been engineered for virus resistance using CPMR approach and released for commercial cultivation. Virus resistant transgenic summer squash (Cucurbita pepo spp. ovifera var. ovifera), and transgenic papaya have been successfully commercialized in United States (Gaba et al. 2004, Johnson et al. 2007, Tennant et al. 2001, Tripathi et al. 2007). However, for DNA viruses there are very limited reports on success of PDR and none have been commercialized so far (Tepfer 2002, Vanderschuen et al. 2007, Prins et al. 2008; Ganesan et al. 2009). With this in view in the present study, transgenic black pepper plants were produced using CMV and PYMoV sequences and their transgenicity confirmed. However, their reaction to respective viruses is yet to be determined.

### 5.6 Future line of work

Transgenic black pepper plants expressing ORF III region of *Piper yellow mottle virus* in sense and antisense orientation and *Cucumber mosaic virus* coat protein in sense orientation produced in this study are maintained in insect proof glass house. After proper establishment, these plants need to be subjected to challenge inoculation with PYMoV and CMV respectively. Presence of virus within the plants may be monitored by visual symptoms and PCR along with inoculated non transgenic control plants. Resistant lines if any should be subjected to repeated subculturing to ensure the stability of the transgene.