Discussion
5. DISCUSSION

India is predominantly an agriculture-based country with 70% of its population living in rural environment. India must cope with an expanding population projected to grow to 1.3 million by 2025. The growing demand for food has propelled the use of chemical pesticides, which requires application at regular intervals, resulting in increase in the cost of production and environmental degradation. With the huge amount of pesticides going into our environments one can predict the enormous amount of health hazards it will lead to, and also the cost of it will have to be bore by the poor farmer. With majority (60%) of the India’s population dependent on agriculture (ISAAA 2006), along with a gist for accepting newer technologies, development and utilization of advanced agricultural techniques need to be given more importance and priority. In 2006, the record increases in adoption in India for Bt cotton continued with almost a tripling of area from 1.3 million hectares in (2005) to 3.8 million hectares (ISAAA, 2006).

Therefore, the use of biopesticides like transgenic crops in our farming systems will lead to less usage of chemical pesticides at the same time ensuring maximum yield. The serious and urgent concerns about the environment highlighted in the Stern Report (2006) on climate change, has implications for biotech crops. Bt expressing transgenic crops of worldwide important plant species such as rice, potato and corn have already reached the market. Valuable crop species, cultivated on a comparatively smaller scale, are also of interest if a proper transformation and regeneration protocol for transformation with the Bt gene can be standardized.

This study reports the successful transformation of brinjal (*Solanum melongena* L.), one of the important vegetable crops in South Asia and Mediterranean countries. India ranks second in the production of brinjal, second to
China (FAO 2006). The total area under eggplant production in India is 5.8 lakhs hectares with a yield of 98 million tones (FAO 2007). Brinjal is susceptible to numerous diseases and parasites particularly bacterial wilts, nematodes and insects. It exhibits partial resistance to most of these pathogens, but at insufficient levels. Among the insects, the brinjal shoot and fruit borer (*Leucinodes orbonalis* L.) is the most serious pest causing damage to the whole plant as well as the fruits (Krishna *et al.*, 2001). Insertion of resistance gene into the plant to increase yield and fruit quality is the need of the hour.

This study was undertaken to transform brinjal with the Bt insecticidal crystal protein gene *cry1Ab*. Cry1Ab protein renders brinjal resistant to the brinjal shoot and fruit borer (BSFB) (Rao *et al.*, 1999; Kumar *et al.*, 1998). The present study reports the successful genetic transformation of brinjal cv. Arka Keshav through *Agrobacterium tumifaciens*, *Agrobacterium* mediated transformation system being the most preferred system of transformation used in the transformation of several crops. Chugh and Khurana, (2003), reported that transformation efficiency was found better with *Agrobacterium* meditation in wheat. Since there exists a need for optimization of a wide range of culture conditions when working with new genotypes (Holford *et al.*, 1992), factors effecting in vitro transformation and regeneration efficiency were standardized to achieve increased number of transformation events in this variety of brinjal. A successful transformation of plants resistant to the brinjal shoot and fruit borer was achieved; transformation protocol along with the factors affecting transformation and regeneration efficiencies are discussed here under.
5.1 Standardization of an efficient \textit{in vitro} transformation and regeneration protocol for brinjal cv. Arka Keshav

5.1.1 Standardization of seed sterilization and germination

Before starting any experiment it is important to procure viable seeds of the species of interest. A proper seed sterilization regimen has also to be developed. Adequate seed sterilization has to result in maximum \textit{in vitro} grown seedlings without any contamination. In tissue culture, seeds are surface sterilized before sowing in the aseptic medium with chemicals like sodium hypochlorite, mercuric chloride solution and ethanol. The duration of sterilization with these chemicals is crucial and has to be standardized before starting any experiment. Seed treatment with these chemicals for a little lesser duration than the optimum results in seed contamination a little larger duration leads to seed death. A negative correlation exists between seed sterilization duration and seed vigor and a positive correlation between seed vigor and regenerability of explants, suggesting that use of high vigor seed and minimum seed sterilization duration can further improve transformation efficiency (Paz \textit{et al.}, 2004).

In the present study, seeds of brinjal cv. Arka Keshav were imbibed in gibberellic acid (250 ppm) prior to sowing to hasten germination. Seeds were sterilized by rinsing with ethanol (76%) for half a minute followed by 10-15 minutes swirling with sodium hypochlorite solution (4%). Complete seed sterilization, accompanied with maximum seed germination (up to 95%) was obtained. Similar reports of brinjal seeds being sterilized with Sodium hypochlorite solution was reported by Matsuoka and Hinata (1979); Filippone and Lurquin, (1989) and with mercuric chloride solution by Prabhavathi \textit{et al.}, (2002); Sharma and Rajam, (1995).
5.1.2 Standardization of hormonal concentrations

Agrobacterium-mediated transformation is highly dependent upon the competency of the target plant tissues. It is important to develop the capacity of transformed cells to induce cell proliferation and differentiation. This can be done by externally applying phytohormones to the explants. Co-cultivation of citrus explants in a medium rich in auxins caused a significant increase in the rate of actively dividing cells in S-phase, the stage in which cells are more prone to integrate foreign DNA (Pena et al., 2004). Specific hormonal requirements for regeneration have to be determined to obtain maximum in vitro shoot regeneration from explants. Skoog and Miller (1957) were among the first researchers who discovered the hormone-controlled organogenesis in cultured callus tissues of tobacco. Cytokining-auxin interactions either promoted or inhibited the development of shoots and roots in brinjal cultures and this depended on the ratio of the hormones in the media (Kamat and Rao, 1978).

In the present study, several concentrations of BAP-NAA (Benzylaminopurine-Naphthalene acetic acid) were used for both hypocotyl and cotyledonary leaf explants to arrive at a standard combination. The optimum ratio of BAP-NAA varied for both control as well as Agrobacterium treated explants. It was found that BAP (1µM) + NAA (0.1µM) was optimum for shoot regeneration from control hypocotyl explants and in case of control cotyledonary leaf explants BAP (12.5µM) and NAA (0.1µM) gave maximum shoot regeneration. Similar reports in the case of Solanum surattense was observed when maximum efficiency of shoots formation per explant was obtained on medium with 0.1 mg/L NAA and 5 mg/L BAP/Kinetin (Swamy et al., 2006).

However, in this study, BAP (2µM) and NAA (0.05µM) was the optimum hormonal concentration for Agrobacterium treated (co-cultivated) hypocotyl explants. In case of Agrobacterium- treated cotyledonary leaf explants no shoot
regeneration was obtained for any of the hormonal concentrations tested. This may be due to the type of phytohormones used, which might not have induced any morphogenetic response after the bacterial treatment or due to the morphology of the cotyledonary leaf explants, which did not allow it to completely get rid of the bacterium after co-cultivation.

There was a difference in the requirement of phytohormone concentrations in case of control as well as Agrobacterium treated hypocotyl explants. Shoot regeneration of hypocotyl explants after transformation required a little higher concentration of the cytokinin (BAP) accompanied by a little lower concentration of auxin (NAA). This may be due to the influence of Agrobacterium and also due to a stringent kanamycin concentration on which the transformed plants had to grow and survive. Callusing (100%) was observed in all hormonal concentrations for both hypocotyl and cotyledonary leaf explants. For hypocotyl explants callusing was evident as bulged edges, whereas, profuse callusing was observed from cut edges of cotyledonary leaf explants.

Fari et al. (1995) reported that, use of higher concentration of NAA resulted in transgenic brinjal plants from embryogenic calli and lower concentration of NAA resulted in shoot organogenesis through callus formation. Auxins are also reported to increase Agrobacterium competence (Cervera et al., 1998; Pena et al., 2004).

Use of cytokinin alone has also been reported to develop shoots. For instance, highest shoot multiplication rates in Solanum nigrum (Kannan et al. 2006) were observed when explants were grown in the medium supplemented with BAP (2.0 mg/L). Mukherjee et al. (1991) also reported shoot regeneration from medium consisting of only cytokinin. However, Sharma and Rajam (1995b) reported that though cytokinin (BA) alone was used in the medium to induce multiple shoots in brinjal, shoots obtained on medium containing both cytokinin
(BA) in combination with auxin (IAA) could be rooted and regenerated in to whole plants.

In view of the above, in this study, a cytokinin and auxin combination of BAP (2μM) and NAA (0.05μM) respectively was standardized for all the further experiments, which resulted in shoot and root regeneration (sparse) from hypocotyl explants.

Similarly, a cytokinin-auxin combination of BAP and IAA respectively was used by Kumar and Rajam (2005) and Prabhavathi et al. (2002) for transformation of brinjal explants. BAP and NAA gave shoot and root regeneration accompanied by callus formation from embryo cultures of brinjal (Swamy et al., 1988). BAP and NAA along with other hormones were used by Arpaia et al. (1997) for brinjal transformation.

5.1.3 Standardization of explant source

Success of any transformation strategy depends largely upon the regeneration capability of the target explant. It is also one of the important initial steps, on which the experimental results are dependent. For successful transformation of a plant by Agrobacterium tumefaciens it is essential that the explant used in co-cultivation has the ability to induce Agrobacterium tumour-inducing (Ti) plasmid and virulence (vir) genes (Vijayachandra et al., 1995). A significant variation in the ability of different tissues of a species to induce A. tumefaciens vir genes and T-strand generation has been reported. In rice a significant variation in the ability of different tissues to induce A. tumefaciens vir genes and T-strand generation was observed, only scutellum from 4-day-old rice seedlings induced vir genes and generation of T-strands (Vijayachandra et al., 1995).
In this study, more attention was paid on standardization of the explant material and other important parameters related to the explants, which influenced the rate of transformation and regeneration. Influence of explant selection on transformation and regeneration was reported by Sharma and Rajam (1995a). They demonstrated the relative importance of genotype, explant and their interactions for in vitro plant propagation in aubergine. Selection of explant type is the most important parameter essential for obtaining high-frequency advantageous shoot bud induction in chilli (Golegaonkar and Kantharajah, 2006). The effect of explant source on in vitro plant regeneration in brinjal has also been reported by Sihachakr and Ducreux (1987).

Type of explant played an important role in this study. Transformation frequency varied depending on the explant used. Hypocotyl explants gave better frequency of shoot regeneration as compared to cotyledonary leaf explants in both control as well as co-cultivated (with Agrobacterium) explants. This may be due to the reason that at the given phytohormone concentrations, hypocotyl explants differentiated at a higher rate than cotyledonary leaf explants. Dhar and Joshi (2005) reported the existence of high inter-explant variability in response to growth regulators during regeneration of explants of Saussurea obvallata. Another reason for this behavior of the hypocotyl explants may be due to the genetics of the hypocotyls, which makes it more prone to regeneration. Torelli et al. (2004) reported a particular abundance of the corresponding LESK1 gene transcript in tomato hypocotyls; the elevated expression of the gene underlined the higher shoot regeneration capacity of hypocotyl explants.

Although, according to earlier reports regeneration and transformation in brinjal has been carried out using different explants of brinjal like leaf explants (Prabhavati et al., 2002; Magioli et al., 1998) cotyledonary leaf explants (Magioli et al., 1998; Ianamico et al., 1993); whole leaves as explants (Filippone and
Lurquin 1989), and root explants (Franklin and Sita, 2003), in this study hypocotyl explants gave better shoot regeneration frequency as compared to other explants. This is in accordance with the report by Sharma and Rajam (1995a) who demonstrated that hypocotyl cultures of brinjal yielded maximum number of shoots followed by cotyledon and leaf cultures. The experimental result carried out by Kumar and Rajam (2005) also demonstrated that hypocotyl explants of brinjal were superior to leaf and cotyledonary explants in terms of percent explants showing GUS expression; however, hypocotyl explants did not show good regeneration on selection medium. Filippone and Lurquin (1989) reported highest transformation frequency with cotyledonary leaf explants of brinjal. Whereas, Arpaia et al. (1997), reported that though maximum kanamycin resistant calli was obtained with cotyledonary explants of brinjal, hypocotyl derived calli showed a better morphogenetic response. Differences in results may be due to genotypic variation within species, which greatly influences the response of organogenesis to different hormones. It has been reported that differences in regeneration response may occur due to the different genotypes used (Sharma and Rajam, 1995a). In this study hypocotyl segments were used as the source of explants for all further experiments; apart from hypocotyl explants performing better in all regeneration and transformations experiments, the explants were also easy in handling and exhibited convenience in getting rid of excess bacterium after co-cultivation.

5.1.3.1 Direct shoot regeneration

In this study, shoot regeneration from all the treatments was direct, from the bulged cut edges of the hypocotyl explants. However, Arpaia et al. (1997) reported shoot regeneration from brinjal hypocotyl explants through callus (indirect). This may be due to the different growth hormone used and also the genotype used. Variations in vitro responses may also occur due to the different basal media (Bregitzer, 1992) and phytohormones used (Wofford et al., 1992). Similarly, direct regeneration was reported by Akhtar et al., (2006) who developed a simple and
effective direct regeneration protocol using sprout explants of potato cultivars. Direct shoot regeneration of tomato cotyledonary explants was also reported by Bhatia et al. (2005).

In this study, direct shoot regeneration from hypocotyl explants may be due to the concentration of cytokinin-auxin used in the medium (BAP 2µM and NAA 0.05 µM). BAP has earlier been reported to induce in vitro shoot regeneration in brinjal whereas NAA has been reported to induce callus formation and roots and due to a lower concentration of NAA accompanied with a higher concentration of cytokinin in the medium, might have suppressed profuse callusing resulting in direct organogenesis. The concentration of NAA in the medium has been shown to influence the type of regeneration (callus, callus + root and shoot) from brinjal hypocotyl explants (Matsuoka and Hinata, 1976).

5.1.3.2 Polarity in shoot regeneration

In the present study, polarity in shoot regeneration from explants was observed. Shoot regenerated from only one end of the hypocotyl explants and the other end sometimes gave rise to root. Some explants exhibited both shoot organogenesis as well as root organogenesis. In some explants only shoot or only root organogenesis was also evident. Similar results were obtained in brinjal by Matsuoka and Hinata (1979), who reported shoot regeneration from morphologically upper end of the hypocotyl explants and root regeneration from morphologically lower end of the explants. Shoot bud regeneration accompanied by rooting from hypocotyls explants of brinjal has also been reported by Kamat and Rao (1978). Similarly, Picoli et al. (2002a) reported shoot as well as root organogenesis from brinjal hypocotyl explants. This polarity in shoot and root organogenesis may not be related to the external application of phytohormones; it may be due to the abundance of the shoot-producing genes at the morphologically upper end of the explant and lesser at the morphologically lower end where roots
regenerated. Torelli et al. (2004) reported that the polarization observed in morphogenesis of tomato is associated with polarized expression of LESK1 gene, which is elevated at the upper end of the explants, where shoot production occurs, and low at the basal pole, where roots are observed.

In the present study, rhizogenesis observed in all categories of the explants may be due to the auxin (NAA) used in the medium. NAA has been reported to induce rhizogenesis in brinjal explants (Swamy et al. 1988; Kamat and Rao, 1978). When Matsuoka and Hinata (1979) used lower concentrations of NAA, they obtained callus and adventitious roots from the hypocotyl explants of brinjal. Highest root induction with brinjal hypocotyl explants was achieved with IAA (Picoli et al., 2002a).

5.1.4 Factors affecting regeneration and transformation efficiency of hypocotyl explants of brinjal cv. Arka Keshav

5.1.4.1 Effect of size of the explants

Size of the explants plays an important role in increasing the percentage of in vitro morphogenesis. Many reports on regeneration of brinjal explant are evident in which size of the explants in relation to maximum transformation and regeneration frequency is specified (Picoli et al., 2002a; Prabhavathi et al., 2002; Sharma and Rajam, 1995a; Mukherjee et al., 1991 & Kamat & Rao, 1978). In this study, standardization of size of the explant was carried out with control explants. It was demonstrated that when control explants of different sizes (0.5, 1 and 2cms) were cultured, maximum shoot regeneration was evident with 0.5cm and 1cm long explants. Profuse callusing at the cut ends was evident in 0.5cm long explants as compared to 1cm and 2cm long explants. Both shoot as well as root regeneration was evident from all the three categories of the explants. Maximum root regeneration was obtained from 1cm long explants and lowest root regeneration was evident from 0.5cm long explants.
Maximum callus and shoot regeneration was evident from smaller explants (0.5cm) followed by medium (1cm), which may be due to polyamines and also due to a lesser distance between morphologically lower and upper ends of the explants resulting in lesser non-organogenic area (centre). Regions closest to the cut ends (borders) displayed a significantly higher ratio of conjugated to free spermidine and/or putrescine than the non-organogenic regions (centers) of the brinjal cv. Violetta Lunga explant (Scoccianti et al., 2000). Polyamines have been reported to play an important role in cell differentiation and development (Bajaj and Rajam, 1996). Explants with smaller size (0.5cm) possessed lesser non-organogenic region, which enabled most of cells to dedifferentiate (callus) and to utilize phytohormones from the medium. Adventitious roots and shoots were formed along the cut surfaces of brinjal explants. At the cut ends of Arabidopsis thaliana explants, maximum cells differentiated, a process induced by wounding and/or phytohormones (Sangwan et al., 1992). In 0.5 cm long explants maximum shoots and lesser roots were exhibited due to most of the differentiated cells exhibiting shoot morphogenesis in response to the ratio of hormones (BAP 2μM and NAA 0.05μM) in the medium, and lesser root regeneration may also be due to a lesser demarcation between the morphologically upper and lower ends of the explants.

In the medium sized explants (1cm) also, maximum shoot regeneration was evident along with maximum root regeneration as compared to smaller (0.5cm) and the larger (2cms) explants. This may be due to a comparatively lesser non-organogenic area as compared to the larger explants (2cms), accompanied by a distinct demarcation between the morphological upper and the lower ends of the explants, resulting in maximum shoot as well as root regeneration from the morphological upper and the lower ends of explants.
In case of the larger sized explants (2cm), due to the length of the explants, there was a greater region between the morphologically upper and the lower ends resulting in a larger non-organogenic area. Therefore, lesser number of cells dedifferentiated which might have resulted in lower shoot regeneration at the morphologically upper end of the explants and also lower root regeneration at the morphologically lower end of the explants.

Reports on effect of the explant size on in vitro regeneration was reported by Salehi and Khui (1997), they compared the effects of explant length and diameter on in vitro shoot growth and proliferation rate of miniature roses. They obtained best shoot development and proliferation rate from explants with the 9-10mm length and 3.0-3.5mm diameter. Similarly, medium sized explants of length 1-2cm gave better shoot organogenesis in case of Prunus cerasifera (Norton and Norton, 1986). In this study, the differences in the regeneration responses observed among the different sizes of the explants used, were not statistically significant. However, 1cm long hypocotyl explants were used for all experimental purposes, due to convenience in handling.

5.1.4.2 Effect of position of the explant

In this study, effect of explants from different regions of the hypocotyl system on morphogenesis and transformation was investigated. Differences were observed for morphogenetic potential of explants collected from different regions of the hypocotyl. The apical explants gave the highest number of shoots, both in case of control and Agrobacterium treated explants compared to the middle and basal explants. Similarly, significant differences in embryogenic potential within a single brinjal explant (hypocotyls) was reported by Sharma and Rajam (1995a). However, they achieved maximum number of adventitious shoots from the basal segment followed by sub-basal, sub-apical and apical. These differences between
the results may be due to different genotypes used, difference in age of the explants and also difference in the phytohormones used.

In this study, the second highest regeneration frequency was attained with basal hypocotyl explants. This result was in agreement with the result of Sharma and Rajam (1995a), regarding embryogenesis. They observe that terminal hypocotyl segments (apical and basal) yielded more somatic embryos than the medial (sub-apical and sub-basal) segments. These significant differences may occur due to a gradient of phytohormones and/or polyamines existing within the same explant (Ulvskov et al., 1992; Dumortier et al., 1983). This may also be due to the position of the segments along the hypocotyl, which influenced the role of the various phytochromes and the interactions between them (Lercari & Bertram 2004).

Intermediate levels of conjugated and total putrescine and spermidine were reached in tissues with good embryogenic potential; tissues with poor embryogenic potential attained lower or higher levels (Sharma and Rajam, 1995b). Disks from the apical region of brinjal leaves yielded more somatic embryos than those from the basal region. Apical disks showed consistently higher polyamine titers than the basal disks (Yadav and Rajam, 1997).

Maximum root regeneration was evident from the explants from the basal portion of the explants, which may be due to plenty of root initiating gene at the morphological lower end of the hypocotyls where roots regenerated. Maximum explants exhibiting shoot as well as root organogenesis were obtained with explant belonging to the basal portion of the hypocotyls and lower number of explants exhibiting both shoot as well as root organogenesis were evident from explants from the middle portion of the explants as these explants exhibited lower shoot regeneration frequency.
However, when explants were co-cultivated with *Agrobacterium*, maximum shoot and root regeneration was evident from explants belonging to the apical portion of the hypocotyls. This may be due to the actively dividing cells at the apical regions of the hypocotyls as compared to the middle and the basal portions, which provides more number of competent cells to the bacterium thus increasing the probability of transformation and increased regeneration of shoots as well as roots. However Scoccianti *et al.* (2000) reported that, extent and the temporal changes in soluble-conjugate levels of conjugated polyamines differed between root-forming and shoot-forming explants; in the former, accumulation began earlier (within 1 day of culture) and reached the highest levels.

There are plenty of earlier reports on the effect of position of the explants, which reports that maximum shoot regeneration was obtained from apical explants. The transformed cells were always more numerous on the apical side, mainly localized in the intrafasicular cambium and in the immature phloem strands of carrot (*Daucus carota*) root disk (Guivarc *et al.*, 1993). The frequency of organogenesis in banana was dependent on the position from where the explant was cut. Generally, more adventitious shoots were obtained from explants cut from regions close to apical meristem (Li *et al.*, 2006). Among several cowpea explants studied, shoot initiation response was observed from shoot apices of 3-5-day-old seedlings (Mao *et al.*, 2006). Terminal hypocotyl segments of brinjal yielded more shoots as well as somatic embryos as compared to the medial segments (Sharma and Rajam, 1995a).

### 5.1.4.3 Effect of age of the explant

Age of the explants was standardized by using hypocotyl explants from seedling of different ages (5, 7, 10, 15, 20, 25 and 30 days). Callusing was unaffected by the age of the explant, callusing at the cut ends was observed in all categories of the explants. However, shoot regeneration and root regeneration
percentages showed variations among explants of different ages. A significantly higher transformation and shoot regeneration was obtained from explants from 10-day old seedlings; lower regeneration frequencies were obtained from 7 and 5-day-old hypocotyl explants. Nine days and eleven days old explants showed significantly low shoot regeneration percentage as compared to ten days old explants, suggesting that hypocotyl explants from ten days old seedlings had higher differentiation rates. Cell competence for regeneration and for transformation does not always fall into the same cell type/developmental stage and this is one of the main causes of the so-called recalcitrance for transformation of certain plant species (Pena et al., 2004).

An important aspect associated with susceptibility of the explants to *Agrobacterium* infection is the targeting of cells competent for regeneration (Clemente et al., 1995). In this study, age of the hypocotyl explants wherein maximum cells were competent for transformation was standardized. It was evident from the results that age affected the transformation efficiency of the explants. Since *Agrobacterium* may require dedifferentiated tissue before wounding for successful T-DNA transfer (Schlappi and Hohn, 1992), hypocotyls explants from ten-day old seedlings were used for all transformation experiments in this study. The physiological maturity level of the explants, expressed in terms of developmental age was optimum with 10-days old seedling explants. Explants with higher age, exhibit more root regeneration than shoot regeneration. Root organogenesis increased with age (till 20 days). Furthermore, a decline in shoot regeneration capacity was noticed as the age of the seedling hypocotyls increased (after 20 days). This indicates that there is a specific age limit for the seedling hypocotyls where physiological maturity of the explants for transformation and regeneration is optimum. Sinha and Caligari (2004) reported that the physiological maturity level of the explants, expressed in terms of developmental age, was optimum when 14 to 18 day old seedling cotyledons of *Lupinus albus* were used.
rather than more mature ones. Torelli et al. (2004) reported that increase in the rate of regeneration of 8 days old tomato hypocotyls is due to the abundance of callogenesis inducing genes.

Similarly, experiments conducted by Malagon and Alejo (1996) demonstrated that, seedlings at the stage of curved hypocotyl stage (9 days old), were the best explants for shoot regeneration in chili pepper (*Capsicum annum* L.). Optimum plant propagation from cotyledon explants of 8-10 day old seedlings of tomato (*Lycopersicon esculentum*) was obtained by Hamza and Chupeau (1993). Explants from 10 to 15 day-old seedlings of *Saussurea obvallata* showed maximum callus induction (Dhar and Joshi, 2005).

5.1.4.4 Effect of etiolation on explant regeneration

In the present study, experiments on the effect of etiolation on explant regeneration were performed. Regeneration from hypocotyl explants from seedlings germinated and grown in the dark (etiolated) (G2) was compared with the hypocotyls explants from seedlings grown in the light (G1) (non-etiolated). Explants from both G1 and G2 were placed under light (culture room condition) for shoot regeneration. Shoot organogenesis from control G2 explants was better as compared to control G1 explants. This result was in accordance with results obtained by Liu et al. (1998), who used etiolated internodal explants of apple and obtained 7 times the number of shoots as non-etiolated internodal explants.

The stems of plants raised in the dark, elongate much more rapidly than normal, a phenomenon called etiolation. It is a mechanism that increases the probability of the plant reaching the light and is due to higher levels of gibberlic acid (GA) in the etiolated seedling. The increased elongation of dark-grown plants resulted from increased responsiveness of the plant to its endogenous levels of GA1 (Weller et al., 1994). The seedling utilizes the food reserves found in the seed. The etiolated seedling grows until food reserves are available in the seed. A
higher regeneration potential of G2 explants may be because once the G2 explants were exposed to light, the formation of chlorophyll began and this led to the synthesis of nutrients in the explants. This rush in the nutrients in the explants might have led to an increased regeneration frequency observed in the explants.

This was evident when *Agrobacterium* was co-cultivated with G1 and G2 explants. The shoot regeneration frequency of G1 explants increased with increase in preconditioning period till four days after which the frequency decreased, While in case of G2 explants, shoot regeneration frequency increased with increase in the preconditioning period. That is because, as the synthesis of nutrients increased with the increase in the chlorophyll content of the explants (de-etiolation), the regeneration potential of the explants also increased. It has been reported that the relative growth rate of plant cells in vitro is considerably affected by initial cell density (Matsubayashi *et al.*, 2004). Therefore, G2 explants require some time in light to increase their cell densities (de-etiolate) and due to the freshly formed reserves, there might have been a rush of nutrients in the explants leading to increased transformation efficiency.

However, in the present study, transformation and shoot regeneration frequency was higher in G2 explants as compared to G1 explants. This was in accordance with the results obtained by Liu *et al.* (1998) during transformation of apple. They reported that all first internodes from etiolated shoots exhibited GUS-expressing zones and yielded 4-fold as many GUS-expressing zones as commonly used leaf explants from non-etiolated shoots.

Similarly, Karam *et al.* (2000) demonstrated that etiolated explants of *Cyclamen persicum* were superior, they demonstrated that cultures of etiolated petioles were free of contamination, had superior shoot regenerative potential and produced more vigorous callus compared to those of non-etiolated petioles. In Venus fly-trap (*Dionaea muscipula*) etiolated petiole explants exhibited the
greatest percentage of shoot formation (74%) and number of shoots when cultured on media containing 0.022 mg l-1 TDZ. (Teng, 1999). Kamo (1997) worked on the factors affecting Agrobacterium tumefaciens-mediated gusA expression and opine synthesis in gladiolus. They reported that GUS expression in leaves from seedlings grown in the dark, whereas leaves from seedlings grown under a 16-h light photoperiod showed no GUS, indicating the significant effect of etiolation on transient GUS expression mediated by A. tumefaciens.

5.1.4.5 Effect of preconditioning

Conditions such as pre-culture and the co-cultivation period are identified as determinants to achieve high expression levels (Fuentes et al., 2004). Transformation of the T-DNA from Agrobacterium to the plant cell is a crucial step, on which depends the performance quality of the final product (transgenic plants). Wounding of the explant is said to be an important step in Agrobacterium infection.

Some plants species are recalcitrant to Agro infection and there exists reports of such recalcitrant plants being made calcitrant by applying pretreatment to the explants with phytohormones prior to co-cultivation with Agrobacterium (Chateau et al., 2000). In Peach embryo transformation with Agrobacterium, histochemical GUS assay showed that the transient expression rate was positively affected by pre-culture (Wu et al., 2004). Therefore, preparation of the explants prior to co-cultivation (preconditioning) is another important parameter related to successful transformation. Precondition of explants prior to co-cultivation demonstrated that just wounding the explants prior to Agro infection was insufficient for stable integration of T-DNA in to the plant cells.

In the present study, when explants were co-cultivated with Agrobacterium without preconditioning (with phytohormones) resulted in cell death and no shoot regeneration occurred (data not shown). This may be due to a lower availability of
competent cells for the bacterial T-DNA transfer, resulting in cell death due to
*Agrobacterium* overgrowth. Cocoa tissue was frequently destroyed due to
*Agrobacterium* overgrowth following co-cultivation (Mayolo *et al.*, 2003).
*Agrobacterium* requires adequate amount of competent cells available in the
explants for transformation. Here, the competent cell association between the
polysaccharide of the host cell wall and that of the bacterial filament was required
and was frequently observed during the T-DNA transfer (Sangwan *et al.*, 1992),
indicating that transformation required polysaccharide-to-polysaccharide contact.

Therefore, in the present study, preconditioning of G1 and G2 explants with
phytohormones (BAP 2µM and NAA 0.05µM) for 1-5 and 1-6 days respectively
was done prior to *Agrobacterium* co-cultivation. This resulted in an increased
shoot regeneration frequency for both G1 (up to 65%) and G2 (up to 72.5%)
explants. This radical increase in the regeneration frequency after preculture of
explants may be due to an increase in the number of putatively competent cells in
the explants due to phythohormone preconditioning (Sangwan *et al.*, 1992). The
competent cells increased the transformation frequency by increasing the
efficiency of the tobacco explants to induce *Agrobacterium vir* genes (Sunilkumar
*et al.*, 1999). In both cotyledon and root explants of *Arabidopsis thaliana*,
histological enumeration and GUS assays showed that the number of putatively
competent cells was increased by preculture treatment, indicating that cell
activation and cell division following wounding were insufficient for
transformation without phytohormone treatment (Sangwan *et al.*, 1992). Preconditioning of the explants prior to *Agro*-infection has also been reported to
hasten transformation. When Guivarc *et al.* (1993) applied acetosyringone as a pre
treatment to the carrot disks, a decreased in the lag time for the appearance of the
first GUS positive cells (48 hrs) was observed.
In the present study when performances of explants preconditioned for different days on preconditioning medium (PC medium) were compared, there was an increase in the percentage of kanamycin resistant shoot regeneration till four (with G1 explants) and five (with G2 explants) days of preconditioning. G1 explants with five days of preconditioning showed reduced shoot regeneration potential as compared to four days. Carnation node explants precultured for 3 and 5 days before inoculation showed higher transient and stable GUS expression than those precultured for 7 days (Nontaswatsri et al., 2004).

Preconditioning followed by co-cultivation of the explants on medium with phytohormones increases the transformation frequency by influencing the physiology of the explants; it however does not influence the bacterium. When acetosyringone (As) was applied to the bacterium there was no increase in the transformation events but when As was applied as a pretreatment to the carrot disks it strongly increased the number of transformed cells in the target tissues (Guivarc et al., 1993).

In case of G1 explants shoot regeneration frequency after three days of precondition was lesser than the regeneration frequency after two days of preconditioning. This may be due to the reduced transformation rate occurring due to beginning of wound healing. On four days of preconditioning, the regeneration is greater than on three days. This may be due to untransformed cells being regenerated because of wound healing, which may be due to suberin like substance being deposited at the wounded sites as the days of preconditioning increased (by 96 hrs) which acts as a physical barrier to Agrobacterium infection (Davis et al., 1991). Davis et al. (1991) also reported that the highest transformation competency in tomato cotyledonary explants occurred during the first 24 hours. Therefore, in this study, preconditioning of explants was carried out for not more the two days prior to co-cultivation in all transformation experiments.
In case of G2 explants the percentage of putative transformed shoots increased with increase in days of precondition due to the gradual de-etiolating process. However, shoot regeneration after five days of preconditioning decreased, which may be due to wound healing. Root regeneration in case of both G1 and G2 explants exhibited the same pattern as in the case of shoot regeneration. This may be due to the same reasons given in case of shoot organogenesis.

Similarly, Kumar and Rajam (2005); Prabhavathi et al. (2002) and Arpaia et al. (1997) reported two days of preconditioning for brinjal explants on medium with phytohormone prior to Agrobacterium co-cultivation. Several workers have recommended precondition of explants before Agrobacterium treatment; Christopher and Rajam (1997), suggest preculturing of Capsicum sp. explants before co-cultivation. Broccoli hypocotyl explants precultured for 2 days prior to inoculation with A. tumefaciens resulted in improved transformation frequency (Suri et al., 2005).

5.1.4.6 Effect of duration of Agrobacterium co-cultivation

Number of days used for co-cultivation also affects the Agrobacterium mediated transient transformation (Nandakumar et al., 2004). Mullins et al. (2001) reported that the transformation efficiency was correlated with the duration of co-cultivation of fungal spores with A. tumefaciens cells and with the number of A. tumefaciens cells present during the co-cultivation period. In this study effect of Agrobacterium co-cultivation with explants for 24 and 48 hours was tested. Agrobacterium treatment for 48 hours was better than for 24 hours. This may be because the first transient gene expressing cells are observed by the first 24 hours. The first GUS expressing cells were observed as early as 24 hours after co-cultivation of Typha latifolia explants (Nandakumar et al., 2004). Several workers have recommended co-cultivation of explants for two days (Fari et al., 1995 and Lim et al., 1996). Co-cultivation has been reported for explants from 14 hours
(Menendez et al., 1995) to 4 days (Petri et al., 2004) and for calluses up to 6-7 days (Zhang et al., 1997; Suzuki et al., 2001).

Increasing the co-cultivation period may cause extensive stress to the regenerating transformed cells and also may result in *Agrobacterium* over growth on the explants, which may later be difficult to get rid of. Moreover, since transgene expression has been reported to occur as early as 24 hours (Nandakumar et al., 2004) and 48 hours (Guivarc et al., 1993), increasing the co-cultivation period beyond 48 hours is not recommended. Moreover, Sangwan et al. (1992) reported that exposure of explants for 48 hours to *A. tumefaciens* produced neither characteristic stress response nor any gradual loss of viability or cell death.

The preculturing of explants and the composition of the co-cultivation medium are the two factors that greatly influence transformation efficiency (Costa et al., 2002). In the present study co-cultivation of the explants with *Agrobacterium* was done for 48 hours on medium with phytohormones (BAP 2μM and NAA 0.05μM respectively). Preconditioning of the explants before co-cultivation prepares the explants for co-cultivation with *Agrobacterium* by increasing the number of competent cells and also reduces the stress on the explants by providing time between the wounding of the explants and Agro infection. The co-cultivation period also provides some time for the *Agrobacterium* for its T-DNA transfer to the plant cells and the presence of phytohormones in the co-cultivation medium increases the number of dividing cells thus encouraging transformation. A 3-day co-cultivation on a medium rich in auxins improved transformation frequencies of citrange explants, since it increased the number of dividing cells competent for transformation, at the cut ends of the explants (Cervera et al., 1998).
5.1.4.7 Effect of extent of wounding

Wounding of the explant plays an important role in in vitro plant regeneration and initiating the chemotaxis of Agrobacteria towards the wound for T-DNA transfer, by providing rapidly dividing competent cells. Agrobacterium colonizes plant cells via a gene transfer mechanism that results in plant tumorigenesis, virulence genes are transcriptionally activated in the bacteria by plant metabolites released from the wound site. These virulence-inducing chemicals include acetophenones (C6-C2), which may be specific to members of Solanaceae (Spencer and Towers, 1991), whereas, genera belonging to other families commonly produce sets of virulence-inducing C6-C1 and C6-C3 acids, esters and/or benzaldehydes. Therefore, for a successful transformation of plants with Agrobacterium, wounding of the explants is necessary. Increasing the wound on the explant increases Agrobacterium transformation. (Norelli et al., 1996).

Competent cells were mesophyll cells that were dedifferentiating, a process induced by wounding and/or phytohormones (cytokinins and auxins). The cells were located either at the cut surface or within the A. thaliana explants after phytohormone pretreatment (Sangwan et al., 1992).

Several methods of wounding of the explants to increase regeneration and transformation rate have been reported. Increasing the wound area by cutting the explant (hypocotyl) length wise with scalpel (Yu et al., 2002; Takeuchi et al., 1985) cutting calluses into 2-3 pieces before co-cultivation (Nandakumar et al., 2004); wounding of embryogenic tissues with carborundum (Cheng et al., 1996); crush-wounding leaf tissue with forceps (Norelli et al., 1996); shaking with glass beads (Grayburn and Vick, 1995); wounding by cell-wall-digesting enzymes and tissue disruption caused by sonication (Weber et al., 2002; Santarem et al., 1998).

In this study, hypocotyls from both (G1 and G2) were cut (TS) into fragments (1cm long) and used as explants for transformation experiments.
Transformation efficiency of the explants was increased by increasing the wound area of the explants by further splitting the explants longitudinally (LS). Control explants from both G1 and G2 were preconditioned for two days after which the explants were split longitudinally to increase the wound area. When G1 explants were split longitudinally [G1 (LS)] the shoot regeneration frequency increased as compared to G1 (TS), similarly when the etiolated (G2) hypocotyl explants were split longitudinally G2 (LS) and cultured, they showed an increase in the shoot regeneration frequency as compared to G2 TS. These results were in agreement with results obtained by Yu et al. (2002), who found that increasing the wounded area of sweet orange and citrange epicotyl explants by cutting longitudinally into two halves, dramatically enhanced both transformation and regeneration frequencies.

However, root regeneration frequency was highest with G2 explants (both TS followed by LS explants). Explants [G2 (TS)] exhibited more root regeneration frequency as compared to G2 (LS) because, in case of TS explants the polarity of the morphological upper and the lower ends existed and at the lower ends the tendency for root organogenesis is high. In case of G2 (LS) explants, however, this polarity got disrupted due to explants being split longitudinal, thus spreading out both shoot as well as root organogenesis throughout the length of the explants where maximum shoot regeneration occurred due to the hormonal concentration in the medium.

G1 hypocotyl explants split longitudinally showed higher regeneration frequency than the similarly split G2 explants this may be because G2 explants being fragile (etiolated) needed a longer period of preconditioning before being further wounded (longitudinal split) as compared to G1 explants. However, when G1 and G2 explants were split longitudinally and co-cultivated with Agrobacterium, G2 (LS) explants gave better regeneration efficiency than G2
Here, G2 (LS) explants were preconditioned for ten days before being split longitudinally. G2 (LS) explants could be preconditioned for a longer time (10 days) as they were etiolated. Whereas, in case of G1 explants, G1 (TS) performed better than G1 (LS). This is because the preconditioning period of G1 (TS) explants was two days and that of G1 (LS) explants was four days, after which the G1 (LS) explants were split longitudinally and co-cultivated with Agrobacterium. However, G1 (LS) explants might require some preconditioning period before co-cultivation to withstand the stress of increased wound area along with Agrobacterium. Therefore, the lack of preconditioning prior to co-cultivation resulted in the explants undergoing stress when treated with Agrobacterium. However, increase in the wound area of the explants, which have been adequately preconditioned, resulted in increased transformation efficiency. When G1 and G2 explants were split longitudinally and co-cultivated after only two days of preconditioning, a very low regeneration frequency was evident with G2 (LS) explants. This may be due to the co-cultivation of explants before complete de-etiolation. However, in all the treatments G2 explants exhibited maximum root organogenesis, which may be due to an initial higher tendency of the etiolated tissue for in vitro root organogenesis.

Increase in the wound areas of explants increase shoot regeneration frequency. This may be because increase in the wound area exposed more number of putatively competent cells, which had accumulated due to preconditioning of the explants (Sangwan et al., 1992) and also due to the increase in the cell activation and cell division following extensive wounding, which elicits a positive chemotactic reaction of A. tumefaciens (Hernandez et al., 1999). Un-split explants produced direct shoot regeneration (1 shoot per explant) whereas, longitudinally split explants produced multiple shoots (1-3 shoots/explants) which may also be due to interference with auxin transport due to explant wounding which may have stimulated increased shoot induction (Gosukonda et al., 1995).
Increasing the wound area on the explant is reported to tremendously increase the transformation and regeneration frequency. Chemotactic reaction of *A. tumefaciens* was enhanced by extensive wounding of banana explants (Hernandez et al., 1999). An efficient protocol was developed by Beaujean et al., (1998) wherein *Agrobacterium*-mediated transformation of longitudinal cut internodal explants derived from potatoes (*Solanum tuberosum*) yielded an average transformation rate of 90% with even up to nine shoots per explant were obtained. Wounding explants through epidermal peeling with normal horizontal orientation of the explants during incubation also resulted in multiple shoot production (Gosukonda et al., 1995). Another advantage of increasing the wound area by cutting the explants into two halves could be to improved the contact of the tissue to the selection medium thus reducing regeneration of untransformed shoots (escapes). For reducing the number of escapes during *Agrobacterium*-mediated transformation of carnation, Luis et al. (2006) modified selection procedure in such a way that the contact between explants and medium was more intense; this improved selection and decreased the number of escapes.

*Agrobacterium* culture concentration was crucial while co-cultivating the bacterium with explants possessing increased wound area. This was because, due to the increased wound area the *Agrobacterium* growth was evident across the explants and it became a little tedious process to get rid of the bacterium completely. In view of the above, shoot regeneration capacity of G1 and G2 explants (split longitudinally) can be further increased by standardizing factors like, *Agrobacterium* culture concentration, preconditioning period, cefotaxime concentration and by increasing the number of sub-cultures following co-cultivation.
5.1.4.8 Kanamycin resistance

In this study, *Agrobacterium tumefaciens* strain EHA105/LBA 4404/A208 with plasmid pBinBt that contains the constitutive CaMV35S promoter driving the synthetic *crylAb* (Crystal protein gene of *Bacillus thuringiensis* var. Kurstaki) gene and with selectable marker genes for neomycin-phosphotransferase-II (*nptII*) selectable marker cassette was used. When the *crylAb* gene along with the *nptII* gene gets inserted in to the host cells, the *nptII* (selectable marker) gene imparts antibiotic (kanamycin) resistance to the transformed cells and the untransformed cells get killed due to kanamycin. However, an adequate kanamycin concentration in the medium is required to serve this purpose. A critical requirement for the development of plastid transformation technology is the availability of selectable markers (Jayasree *et al.*, 1997).

When the medium contains adequate kanamycin concentration, the transformed cells proliferate into shoots and the untransformed cells/explants get killed. However, selection steps must be revised carefully. Jordan and McHughen, (1998) reported that, although shoots gave rise to kanamycin sensitive progeny most of them probably arose from non-transformed cells, protected from the selective agent by transformed cells in the callus. Therefore, careful analysis is needed to distinguish true transgenics.

In the present study, for the standardization of lethal kanamycin concentration for untransformed cells, control explants were placed on medium with different concentrations of kanamycin (25, 50, 75, 100 and 150 mg/L). Shoot regeneration from these explants was evaluated; in a few kanamycin concentrations (25, 50 and 75mg/L) shoot regeneration was obtained, although in less frequency. 100mg/L and 150mg/L concentrations of kanamycin resulted in all the explants turning pale (dying) and there was no shoot or root regeneration observed from the explants. Root regeneration was not obtained in any of the
kanamycin concentrations. Thus in the present study, kanamycin at 100mg/L concentration was used in the selection medium for all transformation experiments. Earlier works on brinjal (Prabhavathi et al., 2002; Picoli et al., 2002a; Reynolds et al., 1994 and Rotino and Gleddie, 1990) suggested a kanamycin concentration of 100 mg/L.

5.2 Rooting of the putative transformed shoot

In the present study, although shoot as well as roots regenerated on selection medium from hypocotyl explants, only putative transformed shoot were used for further propagation into transgenic plants. The regenerated roots, which could have been putatively transformed, were not used for further propagation. Although, regenerated roots from hypocotyl explants which survived on selection medium could be used for further propagation into transgenic plants. Franklin and Sita (2003) reported Agrobacterium-mediated transformation of brinjal using root explants.

In the present study, rooting of the putative transformed shoots was achieved by following a procedure reported by Prabhavathi et al. (2002) with few modifications. The putative transformed shoots were transferred to shoot proliferation medium (SPM) (MS medium with 2μM BAP + NAA 0.05 μM with cefotaxime 250mg/L and kanamycin 25mg/L). After 2-3 weeks the well-proliferated shoots were transferred onto rooting medium (SPM without kanamycin) and ½ MS medium with 250mg/L cefotaxime. Although, SPM medium (without kanamycin) gave rooting of the putative transformed shoots, rooting was better in ½ MS medium. Therefore, ½ MS medium (with 250 mg/L cefotaxime) was used for rooting of the putative transformed shoots. The rooted plants were subjected to hardening with 1-1.2 % agar in the medium (½ MS with 250mg/L cefotaxime) 2 weeks. The plants were grown on soilrite for 2 weeks and then were potted in the greenhouse.
5.3 Molecular confirmation of the Bt cry1Ab gene integration in the transgenic brinjal cv. Arka Keshav plants

5.3.1 PCR

Plant DNA was used for detection of the transgene integration in the plants. Young leaves from the putative transgenic plants were used for the extraction of genomic DNA. Plant DNA was subjected to PCR analysis for the detection of the presence of the nptII marker gene (750 bp). Out of 27 putative transfomers, 6 plants (T015, T016, T018, T019, T024 and T025) were positive for the nptII gene integration, along with the positive control (plasmid). All these six putative transformed plant were amplified with the cry1Ab gene primer. All the 6 plants and the plasmid were positive for the cry1Ab gene integration, exhibiting a 1000bp band along with the plasmid, thereby confirming the stable integration of the transgene into the tissue resulting in 22.22% transformation efficiency.

Prabhavathi et al. (2002) analyzed the integration of the transgene into brinjal plants through PCR using the nptII gene primer. They obtained about 78% PCR positive plants. Arpaia et al. (1997) used PCR as evidence to confirm the integration of Bt cry3b gene into brinjal genome.

In the present study, putative transformants that did not show the presence of the Bt gene might have been escapes. Although a stringent concentration of kanamycin is used, escapes do occur for reasons not well known. Such results have earlier been reported. Escapes could have occurred due to increased rate of differentiating explant cells due to preconditioning on phytohormone rich medium. The survival of these escapes on selection medium without the T-DNA integration may be due to the interaction between Agrobacterium strain and the genotypes in relation to shoot formation. Zhang et al. (2005) provided evidence that bacterial infection can increase the plant resistance to selective agents like kanamycin, mannose and phosphinothricin (PPT). Also Jordan and McHughen (1998) reported that, although shoots gave rise to kanamycin sensitive progeny, most of them
probably arose from non-transformed cells, protected from the selective agent by transformed cells in the callus.

5.3.2 ELISA

In the present study, detection of transgene protein expression in the transformed plants was done with ELISA using Cry1Ac antibodies. Cry1Ac antibodies have cross reactivity with Cry1Ab proteins (Sardana et al., 1996). Among the six transformed plants four plant showed higher expression of the Cry1Ab toxin T₀19 expressed the highest level of the toxin protein, 160ng/µg of total soluble leaf protein followed by T₀25 (150ng/µg), T₀15 (142 ng/µg) and T₀18 (120 ng/µg). However, line T₀-16 and T₀-24 showed a little lower levels of protein expression (90ng/µg & 87ng/µg respectively). Variations in the level of protein expressed with the same construct in brinjal plants (cv. Pusa Purple Long) was reported by (Kumar et al., 1998). In the present study, all the transformed plants obtained were morphologically identical to the control (untransformed) plants. The transgenic plants flowered and set fruits that were similar (in colour, shape and size) to the flowers and fruits set by the control plants.

5.4 Insect bioassay

After the confirmation of the transgene integration into the transformed plants, insect bioassay was taken up to find out whether the amount of Bt toxin produced by the transformed plants is sufficient to kill the brinjal shoot and fruit borer (BSFB). Explants from young shoots of the six transformed plants and one control plant (non transgenic) were taken from six different shoots per plant. The shoot pieces (1.5-2cms) were placed in Petri plates with moist cotton to avoid dehydration. First instar larva (3-4) of L. orbonalis were placed on each shoot explant. And observations were taken from 24 hrs to 72 hrs.
After 24 hrs larvae of *L. orbonalis* had dug small holes on shoot explants of the control plant. These holes were bored by the 1st instar larvae and these holes were observed on the control explants only. After 48 hrs when the observations were taken larvae were found dead on the surface of few explants from transgenic plants (T₀-15, T₀-18, T₀-19 and T₀-25). It was clearly visible that the larvae were dead as soon as it started feeding on the transgenic tissue. When control as well as transgenic explants were cut open under a stereo-microscope, dead larvae (1st instar) were observe in transgenic tissue (T₀-16 and T₀-24) whereas, the larvae were alive within the control tissue. The larvae found within the control plant tissue were vigorously feeding and had increase in size as compared to the dead larvae found in the transgenic tissue.

This demonstrates that the larva were dead because of the transgenic tissue which was expressing the Bt Cry1Ab toxin in concentrations that were lethal to the larvae. The larvae were dead as soon as they started feeding on the transgenic explant tissue (T₀-15, T₀-18, T₀-19 and T₀-25). When the explants were cut open the larvae found dead inside the transgenic explants were found dead near the cut edges of the transgenic explants (T₀-16 and T₀-24). This may be due to the possibility that, the larvae must have tried entering the explants tissue through the cut edges as there were no bores evident on these explants. Detection of the dead larvae within the transformed tissue at the cut edges also infers that, although the larvae would have tried entering the tissue through the cut edges of the transformed explant, it could not penetrate further due to the toxicity of the transformed tissues. All the larvae were dead (100% mortality) within 24-72 hours. Similarly, Arpaia *et al.* (1997) reported that larvae feeding on transgenic brinjal leaf-discs were mostly dead within 72 hours. However, it is evident from this experiment that all the transformed plants produced the Bt Cry1Ab protein in levels sufficient to kill the larvae of BSFB.
Similarly cry1Ab gene integration into *Brassica campestris* was confirmed by conducting bioassay with larvae of diamondback moth and it was demonstrated that the transgenic plants were resistant to feeding damage (Xiang *et al.*, 2000). Expression of synthetic cry1AB and cry1AC genes in Basmati rice (Ahmad *et al.*, 2002) was confirmed by insect bioassay wherein transgenic lines, showed high levels of resistance to the European corn borer, resistant lines showed 100% mortality. In this study, bioassay results were correlated with the molecular analysis. Insect bioassays indicated the effectiveness of the transgene against infestation by BSFB. When fruits of control (untransformed) and a transgenic plant (T0-25) were infested with the larvae, the larvae infested the control fruits by boring into it. Whereas, there was no infestation seen in the transformed fruit, indicating that the fruits of the transformed plants expressed the transgene in quantities, which were sufficient to kill the larvae of BSFB.