DISCUSSION

5. DISCUSSION

Transgenic technology is an important tool for introduction of useful genes from alien sources into cultivated genotypes of a crop species. In the present study, *Bt* transgenic sorghum plants were produced through *Agrobacterium*-mediated procedure by introducing synthetic *cry1Aa* gene under the control of maize ubiquitin (*ubi*) promoter using shoot apical meristems of sorghum genotype M35-1. The *Bt* transgenics developed in the present study were evaluated for resistance to the spotted stem borer (*Chilo partellus*) in laboratory and glasshouse conditions by leaf and whole-plant bioassays are discussed below.

5.1. *In Vitro* REGENERATION SYSTEM IN SORGHUM

In vitro regeneration in sorghum is reported from long time using several explants such as immature embryo, mature embryos, immature inflorescence, protoplast, and leaf. Of all the explants, immature embryo and immature inflorescence derived calli have served as the best explants for *in vitro* regeneration. Nevertheless, availability of these explants is limited for a particular period in a year. Besides, usage of these explants has rapid induction of phenolic compounds with a significant negative impact on *in vitro* regeneration response (Cai *et al.*, 1987). Therefore, in the present study, shoot apices from 5-7 day old germinated sorghum seedlings were successfully used as explants. The isolated shoot apices were manipulated to follow the embryogenic pathway bypassing callus production phase. Use of shoot apices from *in vitro* germinated seedlings facilitates the year-round availability of explants (Zhong *et al.*, 1998). Therefore, this system could be efficiently used for genetic transformation due to increased frequencies of multiple buds and somatic embryos capable of regeneration in a relatively short period.

By striking optimal balance between 2,4-D (to avoid callus formation) and TDZ (to initiate regeneration), the isolated shoot apices were manipulated to follow either organogenic or embryogenic pathway. Such morphogenic plasticity of the shoot meristem

was reported by Zhong *et al.* (1998) by using shoot apices of sorghum seedlings obtained from 18 commercial cultivars along with a portion of mesocotyl, leaf primordia, leaf sheath and one or two leaf pairs. Similar kind of results were reported by Sai Kishore *et al.* (2006) as production of multiple shoot buds within 3-4 weeks using TDZ along with BAP and 2,4-D, easy handling of shoot tips compared to callus mediated organogenesis responding in less number with the same time. Direct shoot production without callus mediation in higher number (7.5 to 12) were obtained by Ganeshan *et al.* (2003) in barley using mature embryo explant when application of TDZ as the sole growth regulator at a concentration of 4.5 μM. However, in the present system, such heterogeneity of the explant was minimized by further isolation of shoot apices devoid of much of mother tissues from shoot apices, which also resulted in enhancing the rate of multiplication.

Plant growth regulators controlled the morphogenic competence, pathway and speed of regeneration from isolated shoot apices. The importance of auxin to cytokinin ratio in the control of regeneration is well known (Skoog and Miller, 1957). Such a process depends on cell or tissue competence (Christianson and Warinck, 1988). In earlier reports using shoot apex as an explant (Bhaskaran and Smith, 1988; Bhasakaran *et al.,* 1988; El'konin *et al.,* 1984; Lusardi and Lupotto, 1990 and Seetharama *et al.,* 2000), embryogenic callus formation preceded the process of somatic embryogenesis. However, to minimize the generation of somoclonal variants commonly associated with callus-mediated regeneration, it is desirable to establish direct organogenesis or somatic embryogenesis without going through a callus formation phase.

Similar kind of regeneration systems have been reported earlier in corn (Zhong *et al.,* 1992), oat (Zhang *et al.,* 1996), sorghum (Zhong *et al.,* 1998), and pearl millet (Devi *et al.,* 2000) and wheat (Ahmad *et al.,* 2002). Further, a rapid and high frequency regeneration system will enhance the transformation potential as seen in genetic transformation of oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.) by Zhang *et al.,* 1999 and in maize (*Zea*

mays L.) by Zhong *et al.* (1996). The system described here meets the all above-mentioned criterion. The sorghum genotype M35-1 used in the present study showed uniform regeneration frequency. A similar result has been achieved in previous studies and has proven to be genotype independent with a marginal difference in induction responses (Harshavardhan *et al.*, 2002 and Girijashankar *et al.*, 2005). The results of the present study are in agreement with the findings of Zhong *et al.* (1998).

During the first 2 weeks of culture, there was a significant expansion of shoot meristem region. At this stage, the bulged shoot meristem was trimmed and leaf primordia discarded. If the primordial leaves were not retained with the meristem during the first 2 weeks, meristems did not bulge, and there was no multiple bud formation. Earlier studies, it has been demonstrated that if the apical dominance of existing buds is eliminated either physically or by using high cytokinin levels in the medium, a large number of shoots could arise *in vitro* (Polisetty *et al.*, 1997). In our study, use of BAP and TDZ (which shows strong cytokinin properties) when combined with the technique of trimming leaf initials during *in vitro* culture, lead to such a rapid response.

TDZ is a substituted phenyl urea developed primarily as a cotton defoliant. It also exhibits strong cytokinin like activity in various cytokinin bioassays (Mok *et al.*, 1982). TDZ also efficiently stimulated cytokinin-dependent shoot regeneration from a wide variety of plants (Malik and Saxena, 1992 and Huetteman and Preece, 1993). The precise mechanism of action of TDZ is not yet known. However, two hypotheses have been proposed by Huetteman and Preece (1993). First, TDZ could directly promote growth due to its own biological activity in a way similar to that of cytokinins. Second, it might affect accumulation of endogenous cytokinins (*via* a reduction in the rate of degradation) or increases the synthesis of endogenous cytokinins.

The study demonstrated that the addition of TDZ in the induction medium was effective for multiple bud formation from bulged meristems. The multiple bud formation

was a branch-point from where it can lead to either organogenic or embryogenic pathway depending on the manipulation of plant growth regulators in the culture media. Zhong *et al.* (1998) obtained direct somatic embryogenesis from shoot apices on medium supplemented with BAP and 2,4-D. In our experiments with isolated shoot apices, use of the above medium was always accompanied with a certain degree of callus formation. In the case of isolated shoot apices with high meristematic activity, it was observed that a weak auxin like NAA was more effective; in fact, it was more favored for signaling somatic embryogenesis, than a strong auxin like 2,4-D. Moreover, replacement of NAA with 2,4-D induced somatic embryogenesis without any callus formation. Specific combinations of NAA and BAP were found to be highly effective for inducing somatic embryogenesis in previous study by Tetu *et al.* (1990) in pea (*Pisum sativum*), and in tobacco (Gill and Saxena, 1993).

TDZ altered the shoot regeneration process from organogenesis to embryogenesis in leaf disc cultures of tobacco (Gill and Saxena, 1993). In the present study, though TDZ was used till the somatic embryo induction medium (Table 4.3) along with BAP and 2,4-D, it was not used during the later 4 weeks of culture (during plant regeneration phase). Till this step, it was a common route for both organogenic and embryogenic pathways. This suggests that probably the carry-over effect of TDZ (present during initial 8 weeks) must have promoted the induction of direct somatic embryogenesis. Similar role of TDZ in somatic embryogenesis has also been reported in woody species like *Rubus* (Fiola *et al.*, 1990) and *Vitis vinifera* (Matsuta and Hirabayashi, 1989).

Ideally any regeneration system for genetic transformation should be supported by a detailed anatomical study to establish the nature of regeneration. The findings from Harshavaradhan *et al.* (2002) have clearly shown that shoot apices can be altered to follow either organogenic or somatic embryogenic pathways of regeneration. The results reported by them are in agreement with the conclusion drawn by Zhong *et al.* (1998) from their

electron microscopic studies. Similar nature of regeneration has been reported in *Cicer arietinum L.* (Sagare *et al.,* 1995). Above all, these anatomical studies have paved way for choosing the appropriate stage for transformation to minimize the recovery of chimeras following transformation.

All the processes described above during meristem isolation, the age of the seedling significantly influenced the induction potential of the isolated shoot apices (Harshavardhan *et al.* 2002). When *in vitro* response from explants at seven stages (derived from sets of seven seedlings age from 4 to 10 d) were compared, the induction response showed a steep increase between day 4 or 5 reaching a maximum with explants from 7-day-old seedlings. The induction response declined with the older seedlings. Therefore, explants from 5-7 day old seedlings are optimum for isolated shoot meristem culture of sorghum. Age-dependent variation in *in vitro* responses was linked to difference in endogenous auxin (Cassels *et al.,* 1982) or cytokinin levels (Josephina *et al.,* 1990). Narasimhulu and Chopra, (1988) reported that genetic and environmental factors alter the levels of endogenous hormones, which determine the *in vitro* responses. In contrast to this, Sai Kishore *et al.* (2006) reported the high transient *gus* expression in 3 day old shoot apices of M35-1, RS29 and CSV14 genotypes; *gus* expression decreased slowly with increase in the age of the seedling. However, the survival of plants was low in 3 d old shoot apices compared to 5-7 d old-shoot apices. This difference was due to the presence of higher number of actively dividing cells and recovery after transformation. However, low transient *gus* expression in 7-day old shoot apices may also be due to the physiological condition of the seeds and the varaitaion in the seed germination protocol followed.

The electron microscopic study of the actively dividing sorghum seedlings has revealed that the age of the seedlings is directly in relation to number of actively dividing cells that have greater potential for *in vitro* response (Aravindalakshmi, 2004). In sorghum 7-day old seedlings have more actively dividing cells and are better suited for production

of multiple shoot buds; therefore, such seedlings were used for isolation of shoot meristem and multiple shoot induction medium in our study.

In the current study, isolated shoot apices from seven-day-old germinated seedlings served as explants for *Agrobacterium* mediated transformation. Shoot apices were selected as highly uniform and meristematic tissues as they are desirable for efficient genetic transformation and to minimize formation of chimeras and somoclonal variants. However, transformation percentages were reported to be high with immature embryos than with the shoot tips (Harshavardhan *et al.,* 2002 and Tadesse *et al.,* 2003). Bombardment of shoot apices has already been reported (Able *et al.,* 2001, 2004; Jeoung *et al.,* 2002 and Tadesse *et al.,* 2003). Harshavardhan *et al.* (2002) found that shoot apices followed by immature inflorescence and immature embryos showed maximum receptivity for transgene (measured as percentage of bombarded explants showing GUS spots). Same study has resulted in establishment of a novel and efficient regeneration protocol from shoot apices of germinated seedlings, apart from establishing callus based regeneration systems from immature embryos and immature inflorescence, using three popular sorghum varieties (M35-1, 296B and BTx623). However, in most of the grasses including sorghum, young tissues close meristematic state such as seedling; shoot apices, immature inflorescence, and immature embryo have proven highly responsive in *in vitro* culture (Jogeswar *et al.,* 2007, Smith and Bhaskaran, 1986, Zhong *et al.,* 1995 and Tomes, 1985).

5.2. GENETIC TRANSFORMATION IN SORGHUM

Worldwide, sorghum producers face major threat of insect pests and the most destructive pests are the lepidopteran stem borer (*Chilo partellus*), the dipterans, midge (*Stenodiplosis sorghicola*) and shoot fly (*Atherigona soccata*). Building up resistance to these pests through conventional breeding is limited due to lack of reliable resistance sources. Insecticidal crystal proteins (Cry) from *Bacillus thuringiensis* have been found to be very effective against the lepidopteran and dipteran insects (Hofte and Whiteley 1989).

Successful transfer of *Bt* genes conferring resistance to stem borers have been reported in maize and rice.

In sorghum, three different methods of genetic transformation have been reported *viz*., i) protoplast-mediated, ii) particle bombardment-mediated and iii) *Agrobacterium*-mediated. Most of the reports in sorghum focused on production of transgenics with agronomically important genes such as the rice chitinase that confer resistance against stalk rot (Zhu *et al*., 1998 and Krishnaveni *et al*., 2001), *dhdps-race1* mutated gene that encodes insensitive form of the dihydrodipicolinate synthase, the key regulatory enzyme of the lysine pathway (Tadesse and Jacob, 2004).

The important aspect of successful genetic transformation is the production of a large number of regenerable cells that are accessible to the gene transfer methods and which continue to proliferate during the stringent selection conditions. Further, gene transfer into potentially regenerable cells may not help in the recovery of transgenic plants if the capacity for efficient regeneration is short lived (Birch, 1997) and the transformation efficiency is proportional to the efficiency of the tissue culture and gene transfer systems (Hiei *et al*., 1994; Ishida *et al*., 1996 and Bower *et al*., 1996).

Various explants and methods have been successfully used for genetic transformation of cereal crops. Shoot apical meristems explants were found to have a major advantage in genetic transformation; successful transgenics were developed with shoot apical meristems by different transformation methods in maize, wheat, oat, barley, pearl millet and sorghum. To date, none of successful transgenic developed in sorghum have received from laboratory to the field evaluation stage.

5.3. *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION

Most of the transformation studies prefer *Agrobacterium* mediated genetic transformation vowing to the availability of the well described pathway of T-DNA transfer. The method is also believed to result in transgenic plants single or plants with lesser transgene copy

number. *Agrobacterium* method of transformation has been studied in sorghum for quite a long period with most of the reports available in standardization of protocol. The reasons are obvious, such as to obtain an higher transformation percentage than those are reported, having their own protocol with set of conditions, testing the transient genes available with genotype of interest for the method, and most of all for achieving more deeper insight into the technique.

The most widely used selectable marker in sorghum transformation is the *bar* gene of *Streptomyces hygroscopicus*, encoding the enzyme phosphinothricin acetyltransferase (PAT) that confers resistance to the herbicide phosphinothricin. Most of the studies in sorghum were carried by using callus derived explant. A single exception is the report of Girijashankar *et al*. (2005) used shoot apical meristem. In the present study, experiments were carried out with phosphinothricin to determine the lethal dose by culturing shoot apices from normal plants. The shoot apices could tolerate a concentration of 1.5 mg l^{-1} (LD_{50}), but higher concentrations of beyond 3.0 mg l^{-1} caused complete bleaching of explants (LD_{100}).

Among the reporter genes used in sorghum, *gus* gene is the most widely used in transformation of sorghum. The second most used gene being the green-fluorescent protein (*gfp*) gene; for the advantage of non-destructive study and recovery of samples after infection and further steps (Able *et al.,* 2001; Jeoung *et al.,* 2004; Gao *et al.,* 2005a,b; Gurel *et al.,* 2009; Sridhar *et al.,* 2010 and Guoquan liu *et al*., 2012). In the present study, optimization of the *Agrobacterium*-mediated genetic transformation was done using *EHA 105* strain of *Agrobacterium* carrying pCAMBIA1300 construct and shoot apice explants. The factors such as *Agrobacterium* concentration (OD_{600}), infection timing (min), co-cultivation period (days) and efficiency of antibiotics were standardized. These optimized parameters were used for effective production of transgenic sorghum plants using *Ubiquitin* promoter controlled *cry1Aa* gene. A final concentration of 200 µM

acetosyringone was included in the experiments as the presence is shown critical in *Agrobacterium* mediated transformation of monocotyledons (Hiei *et al.,* 1994). However, variations in the usage of acetosyringone concentrations are reported in sorghum vowing to explant type and sensitivity; as low as 100 µM by Zhao *et al.* (2000), 200 µM by Carvalho *et al.* (2004), 300 µM by Jeoung *et al.* (2002), 100 µM, and 300 µM by Arlene *et al.* (2006).

In the present study, transient *gus* expression in shoot apices after infection and co-cultivation was monitored for the effective transformation of shoot apices. Highest expression (90%) of *gus* expression was observed at 1.0 OD_{600}, 30 min and 5 days of co-cultivation, but the survival of the shoot apices after this period would be very meagre. This was due to overgrowth of *Agrobacterium.* From the 1.0 OD_{600} *Agrobacterium* concentration experiments with different infection timing and co-cultivation period, there was very less variation (most were >85 %) in the *gus* expression. In contrast to this, in 0.5 OD_{600} *Agrobacterium* concentration experiments the highest *gus* expression (37%) was at 30 min infection and 5 days co-cultivation period and 33% *gus* expression was observed in 30 min and 3 days co-cultivation period. Both the experiments, highest *gus* expression was observed at higher values of infection time and co-cultivation period and death of explants were often high after treatment. Thus, an optimum *gus* expression at 1.0 OD_{600} *Agrobacterium* concentration with 15 min infection time and 3 days co-cultivation was used for the further transformation studies with *cry 1Aa* gene. Even though, Gao *et al.,* (2005a) suggests the use of bacterial culture of 0.2 to 0.3 $OD._{600}$ for easy removal of *Agrobacterium* in immature embryo explants infected, it is not universally applicable. This variation exists due to the differential susceptibility of the explants for the bacterium.

Visarada *et al.* (2003) compared the transient *gus* expression in freshly isolated immature embryos and shoot apices after infecting with scrapped and resuspended cultures

of *Agrobacterium.* Cent percent transient *gus ex*pression was observed in freshly isolated immature embryos compared to shoot apices (93%).

In a study by Zhao *et al.,* (2000) even with double *Agro*-infection transgenic plants were produced. This may be due to use of very high dilutions of *Agrobacterium* suspension to safeguard the explants.

Several modifications in protocols have been followed including physical treatments such as blotting of plant tissues after antibiotic washes on every alternate day, with different culture medium, use of antibiotic media covered with Whatman filter paper for subculturing explants. After co-cultivation with bacterium, the overgrowth of bacterium can be controlled by using media adjuvant such as lysozyme, $AgNo_3$, altering pH. However, this approach was not tried in our study. Pu and Goodman (1992) reported the damage of plant tissue caused by *Agrobacterium*-infection. Getting rid of the *Agrobacterium* post co-cultivation period is a major limitation in this method. *Agrobacterium* infection in sorghum has resulted release of phenolic compounds from infected explants were overcome effectively by use of PVPP and use of special M11 medium (Zhao *et al.*, 2000; Gao *et al.,* 2005 and Arlene *et al.,* 2006).

Moderate concentration of antibiotic in the medium reduces the excessive growth of *Agrobacterium* to controllable level and allows healthy development and regeneration of plants. Teixeira Silva and Fukai (2001) reported that too low concentration of a control agent resulted in excessive growth of *Agrobacterium* and reduction in shoot development. The efficacy of antibiotics used for the control of *Agrobacterium* after co-cultivation period steps were studied. This resulted in moderate use of antibiotics (100 mg l^{-1} of carbenicillin and 300 mg $l^{-1}$where healthy growth of explants was supported. Even the bacterium was controlled to considerable extent; the regeneration of the explant was not supported at the higher concentrations of cefotaxime (400 and 500 mg l^{-1}).

An antibiotic susceptibility study with two strains (EHA101 and LBA4404) of *A. tumefaciens* and 10 different antibiotics, carbenicillin was chosen as the most effective antibiotic against EHA101 strain, while cefotaxime was best against LBA4404 (Shackelford and Chlan, 1996). Similar conclusion was drawn in another study testing 6 antibiotics (Okkels and Pedersen, 1988). The antibiotics carbenicillin and cefotaxime when used singly at 1-4 µg ml^{-1} concentration were controlling the growth of Gram-positive bacteria, while 4-6 µg ml^{-1} concentration were required to control Gram-negative bacteria. The above studies have been confirmed with the shoot tip cultures of *Drosera*, *Syngonium, Nephrolepsis,* and *Spathiphyllum* (Kneifel and Leonhardt, 1992). These authors concluded that the practice of using two antibiotics was more effective for bacterial elimination than using single antibiotic (Leifert *et al.*, 1992 and Horsch and King, 1983).

5.3.1. Selection and regeneration of putative transgenics

Effectiveness of selection agent after co-cultivation is an important factor for possible elimination of escapes, and for efficient recovery of the transformed plants. Phosphinothricin was used as selection agent at a concentration of 5.0 mg l^{-1} by Zhao *et al*. (2000); Emani *et al*. (2002); Rathus *et al*. (1996) and Indra *et al*. (2010) used 2.0 mg l^{-1}, Lu *et al*.(2009) used 2.5 mg l^{-1}, Casas *et al*. (1993); Jeoung *et al*. (2004) and Groot boom *et al* (2010) used 3.0 mg l^{-1} of Bialophos. Zhu *et al*. (1998) used 1.0-2.0 mg l^{-1} Basta and Gray *et al*. (2004) used and 2.0 mg l^{-1} Basta in their callus derived cultures and Girijashankar *et al*. (2005) used 1.0-2.5 mg l^{-1} Basta in shoot meristem cultures for selection of transformants.

In the present study, putative transgenic plants were regenerated successfully with a three-stage selection strategy. Selection was imposed after four weeks of *Agrobacterium* infection, i.e., during shoot-bud initiation; this allowed the recovery of the meristems from the infection and helped in the unhindered proliferation of the transformed cells.

The *Agrobacterium* infected shoot apical meristems were first selected on 3.0 mg l^{-1} PPT throughout the selection process. At first selection during shoot bud induction around 42% of infected explant was reduced; this was followed by second selection at this stage many of the non-transformed explants were eliminated. Finally at the third stage of selection with 3.0 mg L^{-1} PPT in the medium for regeneration of putative transformants, all most all the false positives were eliminated.

Increasing the concentration of the selection agent was reported to increase the recovery of transformants (Casas *et al*. 1993 and Zhao *et al*. 2000). To minimize the chances of escapes, Casas *et al*., (1993) used selection agent *Bialophos* also in the rooting medium. However, Casas *et al*., (1997) reduced the selection pressure at this stage to facilitate better plant recovery. Mythili *et al*., (2004) reported that Basta at a concentration of 3.0 mg l^{-1} resulted in abnormal root formation; this problem was overcome by avoiding selection agent in the rooting medium. Therefore, no selection was imposed during rooting of the regenerated shoots in the present study.

A total of 8 putative transgenic plants could regenerate from 450 isolated shoot apical meristems infected, with a transformation frequency of 1.8% as reported in the present study (Table 4.6). Other reports suggest different frequencies of *Agrobacterium* transformation: 5.4-6.3% by Zhoa *et al*., (2000), 0.8-3.5% by Carvalho *et al*., (2004), 2.88-3.30% by Gao *et al*., (2005), 0.3-4.5% by Arlene *et al*., (2006), 5% by Tuong-Van *et al*., (2007) 7.0% by Gurel *et al*., (2009), 0.4-0.7% by Lu *et al*., (2009), 1.9% by Zhang *et al*., (2009), 1.9% by Indra *et al*., (2010), 1-2% by Shridhar *et al*., (2010) and 4% by Kumar *et al*., (2011) in sorghum.

5.4. MOLECULAR ANALYSIS

Stable integration and efficient expression of the transgene are the vital points in the production of transgenic plants. Molecular characterization of putative transgenic plants involves the use of several techniques such as PCR, Southern analysis, RT-PCR, qPCR,

quantitave RT-PCR, Western analysis, Northern analysis and ELISA. Commonly use method of confirmation remains as PCR and Southern analysis. Thus, these two methods were deployed to confirm the presence and insertion of the *cry1Aa* gene in the putatively transgenic sorghum plants. Sequentially once the transgenics were conformed through PCR, Southern analysis was performed and results were analyzed accordingly.

When the 8 independent transgenic events (T_0) obtained in the present study were subjected to PCR analysis for *Cry1Aa* and *bar* genes, only 3 transgenic events showed positive amplification for both the genes. These results indicate the higher efficiency of PPT selection procedure and occurrence of fewer escapes during selection.

Southern analysis of the same PCR positive plants designed for knowing the integration (BamHI and EcoRI digestion) of the gene and sites of insertion (*SmaI* digestion) resulted with identification of 3 positive plants. All the three plants were positive for integration with the corresponding 1.86 kb band (Plate 4.6). The results of integration ($\geq$ 12 kb) were correlating cent percent with the site of insertion analysis resulting in all three plants with single copy insertions (Plate 4.7). The results obtained indicate that the events have originated from different transformation events even when originating from same set of explants.

To establish the transgenic status of the primary transformants, the inheritance pattern of *cry1Aa* gene was analyzed in T_1 generation. Seeds obtained from the selfed primary transformants of three PCR and southern positive events (A1, A4 and A6) were allowed to germinate and the T_1 plants were analyzed for the segregation of transgene. PCR analysis for the *cry1Aa* gene of T_1 plants revealed a Mendelian segregation of transgene in 3:1 ratio, clearly indicating the stable incorporation of T-DNA into the sorghum nuclear DNA (Table 4.8). Earlier reports involving study of inheritance and expression of the transgene have confirmed that transgene behaves in hemizygous manner of segregation in 3:1 dominant: recessive in selfed progeny (Kim *et al*. 1999; Maqbool and Christou, 1999 and

Zhang *et al*. 1996) or 1:1 segregation in backcross experiments with non-transformed plants. Aberrations in the inheritance ratios of *Cry* protein within individual lines were reported by Sachs *et al*., (1996).

Stable genomic integration of the introduced transgene is traditionally analyzed by Southern hybridization (Southern, 1975). However an analysis using only this technique sometimes does not provide sufficient information pertaining to the expression of the transformed event. In the present study, Southern hybridization by *BamHI and EcoRI* digested genomic DNA from randomly selected twelve plant progenies in T_1 generation exhibited a single 1.86 kb band in all the plants, suggesting the stable integration of the *cry1Aa* gene in sorghum genome (Plate 4.15). Expression analysis of these plants was further confirmed through RT-PCR. RT-PCR analysis of twelve A4-15 event plant progenies in T_2 generation carrying *cry1Aa* gene revealed that the transgene was successfully transcribed (Plate 4.17).

5.5 INSECT BIOASSAYS

In the present investigation, sorghum transgenic plant event-A4 produced by introducing synthetic *Bt cry1Aa* gene and evaluated for resistance to the spotted stem borer (*Chilo partellus)* in laboratory and glasshouse conditions by leaf disc, and whole plant bioassays.

5.5.1. Leaf-disc bioassay of plant progenies of transgenic event-A4

The leaf-disc bioassay of plant progenies of transgenic event-A4 in T_1 generation revealed considerable variation in the performance of segregating individual plants in terms leaf damage (20-80%) and larval mortality (20-100%) when compared to non-transformed control plants which recorded 70-80% leaf damage and 100% larval survival.

During T_2 generation plant progenies of transgenic event- A4-15 showed the percentage of leaf damage was 20 to 40% in transgenic plants compared to 70-80% in non-

transgenic control. Larval mortality ranged from 40 to 100% in transgenic plants, while no larval mortality was recorded in non-transgenic control plants (Table 4.9).

5.5.2. Whole plant bioassay of plant progenies of transgenic event-A4

The whole-plant bioassay of T_1 generation transgenic progenies of event-A4 recorded 20-80% leaf damage caused by stem borer larvae, 23% deadheart formation, 0-7 exit holes per plant, 0-30% stem tunneling and 0-7 survived larvae when compared to the non-transformed control plants it was 70-80% leaf damage; 90% plants with deadhearts; 8-9 exit holes per plant; 30-40% stem tunneling.

The plant progeny of transgenic event-A4-15 showed resistance in T_1 generation was selected and advanced to T_2 generation. During T2 generation in whole plant bioassay the percentage of leaf damage caused by stem borer larvae was 20-40% in transgenic plants while it was 70-80% in non-transgenic control plants. The percentage of deadheart formation in transgenic plants was 20-40% in non-transformed control, 90%. The number of exit holes counted was 0-5 in transgenic plants (8-9 in control). The stem tunneling was 0-20% in transgenic plants (30-40% in control). A total of 20 promising transgenic plants in T_2 generation showed complete resistance to stem borer in whole plant bioassays were selected (Table 4.10).

In the present study, the variation observed in the insect bioassays between transgenic plant progenies originating from same transgenic event could be due to the segregation and position effect of the transgene; the plants which did not cause much larval mortality could have influenced the growth of the larvae when fed continuously on these plants. This was observed in T_1 generation transgenic plant progenies. The effect of sub-lethal dose of *Bt* protein on larval development was observed in laboratory (leaf disc) and glasshouse (whole plant) bioassays, where there was a reduction in larval weights in T_1 plants. The larvae that survived on transgenic *Bt* sorghum plants were smaller, and their development was curtailed when compared to non-transformed control plants. Same kind of effect of

insecticidal protein on larval development was also observed in cotton (Sims *et al*. 1996 and Meyers *et al*. 1997) and corn (Horner *et al*. 2003). Sub-lethal effects of MON 810 *Bt* corn resulted in prolonged larval and pre-pupal development, smaller pupae and reduced fecundity of *H. Zea* (Horner *et al*. 2003). Larvae of *H. zea* fed on *Bt* plants, weighed significantly less and generally exhibited slower development and were susceptible to chemical insecticides than those fed on non-*Bt* cotton plants (Brickle *et al*. 2001). Similar observations like developmental changes were recorded when *Helicoverpa Zea* individuals surviving sub-lethal exposure to δ-endotoxin of *Bt* exhibited fitness disadvantages; their development was prolonged, larval weights decreased and fecundity was reduced (Hornby and Gardner, 1987).

The results obtained in this study, were compared with the other cereals, where transgenic plants constitutively expressing *Bt* genes with resistance to stem borers have been reported. Till date no substantial information on *Bt* sorghum transgenics resistant to spotted stem borer is available, except one report of Girijashankar *et al*. (2005) on the expression of synthetic *cry1Ac* gene under the control of wound inducible promoter from a maize protease inhibitor gene (*mp*i), the report concluded that *cry1Ac* gene did not confer complete resistance against first instar neonate larvae of *C. partellus; o*nly two of the T_1 transgenic sorghum plants had detectable *Cry* protein levels in the leaves, 1, and 8 ng per gram of leaf tissue.

From the results of the present study, it is evident that the large number of sorghum transgenic plants of genotype M35-1 carrying the modified *Bt* insecticidal protein gene *cry1Aa* under the control of ubiquitin promoter have been produced by using optimized *Agrobacterium*-mediated transformation procedure. These results have demonstrated that feasibility and effectiveness *Bt* sorghum transgenic plants conferring near-complete resistance against striped stem borer (*Chilo partellus*).