DISCUSSION
5. DISCUSSION

In the present study transgenic sweet sorghum in the background of SSV 84 was developed using well established and field evaluated stem borer resistant transgenic event (NRCSCRY1B event19) carrying cry1B gene in the background of M35-1. The developed transgenic F1s and BC progenies were screened for the presence and inheritance of cry1B gene. The positive F1 and BC progenies were genotyped in every generation using SSR markers for the recovery of recurrent parent background and phenotyped for the important traits of recurrent parent. The transgenic progenies in BC3F2 generation were evaluated for the cry1B gene insertion and integration in sorghum genome through PCR analysis and Southern blot analysis and quantified the expressed Cry1B protein. The BC3F2 progenies were subjected to insect bioassays to assess the levels of resistance to spotted stem borer in laboratory and glasshouse conditions are discussed below.

Sorghum is a multipurpose staple crop and the species shows greater diversity. Genetic engineering technology can assist the production of agronomically desirable sorghum plants that exhibit increased resistance to biotic and abiotic stress and enhanced nutritional qualities. But only few laboratories in the world are addressing sorghum crop improvement through novel method (Girijashankar and Swathisree, 2009). So far, limited numbers of genes conferring agronomical advantages have been introduced through Agrobacterium and particle bombardment method of transformation and created genetic variations. However, it is limited to a few varieties. So as to create genetic variations in all recalcitrant lines and over the extent, new breeding approaches are required (Brennan and Martin, 2007 and Visarada et al., 2009).

It is now well established that introgression of transgenic gene from one genetic background to another genetic background influenced by several factors. Among these factors, selection of crossing parents, gene that controlling targeted resistance, breeding
approach, stable integration and heritability, foreground selection and background selections played major role. A survey of information from the early work on sorghum backcross breeding also revealed the importance of these things and their effects on trait and variety development (Ramsey and Kernodle, 2009 and Ngugi et al., 2010). Among these, transferred genes conferring resistance over the generations and recovery of recurrent parent genetic background is found to be critical in effecting entire backcross program. Foreground and background screening and heritability of resistance have been taken into consideration in the present study also.

The major obstacle in transformation breeding is transgene silencing (Emani et al., 2002). They reported methylation based transgene silencing of *uidA* and *bar* genes in transgenic sorghum. Casas et al. (1993, 1997) and Zhu et al. (1998) also observed random silencing of *uidA*, *chitinase* gene respectively at different growth stages of primary transgenic plants and in some of their progeny. Krishnaveni et al. (2004) observed loss of transgene expression later the loss of the transgene in sorghum. This occurs through various means which parallels natural gene inactivation mechanisms. We neither observed transgene silencing nor expression loss of *cry1B* gene till BC$_3$F$_2$ in all three events. It may be pertinent here to cite the suggestion of Girijashankar and Swathisree, (2009) that the generation and transgene screening of more number of transgenic breeding events should be the prime focus to meet the goal of stable integration of transgene into elite sorghum genome. This intern can form the platform for the segregation and bioassay studies that can eventually lead towards the successful release of transgenic variety. Since the present study satisfies these requirements, stable integration and stability of *cry1B* gene across the generations have been achieved.

In order to achieve stable introgression of the *cry1B* gene into sweet sorghum SSV 84 genetic background, all progenies were tested for presence of *cry1B* in each generation during backcross program. Initially at first filial generation all plant were tested
phenotypically and genotypically. Having awns, luster and pithy midrib are predominant characters for donor parent M35-1. Presence of awns is dominant over awn less, pithy midrib is dominant over juicy midrib and non lustrous is dominant over lustrous. These three traits were initially used as morphological markers for screen F1 hybrids (Table 4.3). These hybrids were further confirmed for the presence of 992 bp of cry1B gene in all the F1s of 10 crosses was developed. The F1 hybrids representing the above characters also showed clear heterozygous bands with SSR marker (Xtxp 265) indicating that the all F1 hybrids obtained were found to be positives with combined effects. The results in the present study suggest that transgenic M35-1 carrying cry1B gene is noticed to be a better donor parent for gene introgression to SSV84 genetic background.

To establish the transgenic status of the primary transformants, the inheritance pattern of cry1B gene was analyzed in all backcross generation from BC1F1 to BC3F1. The seed collected from the positive plant from each cross of 10 crosses were allowed to germinate and the resultant progenies were analyzed for the segregation of transgene. PCR analysis for the cry1B gene in the plants of each backcross generation revealed a Mendelian segregation of transgene in 1:1 ratio, clearly indicating the stable incorporation of T-DNA into the sorghum nuclear DNA (Table 4.4, Table 4.7 and Table 4.10). 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 Hind III digested genomic DNA from randomly selected plant progenies of three transgenic events (BC₃F₂-1, BC₃F₂-5 and BC₃F₂-9) in BC₃F₂ generation exhibited a single band of 4.2 Kb size T-DNA carrying ubiquitin promoter, cry1B gene and nos terminator, suggesting the stable integration of the cry1B gene in sorghum genome (Plate 4.16).

Immunoassays can be used for the detection and quantification of GMO, based on the quantum of the protein product expression due to the newly introduced genes. A number of ELISAs for different B. thuringiensis toxins has been developed for the determination of these toxins in soil (Tapp and Stotzky, 1995 and Hori et al., 2000), commercial Bt formulations (Takahashi et al., 1998) and transgenic cotton (Sims and Berberich, 1996). All these assays use polyclonal antibodies (PAbs) raised either in rabbits (Tapp and Stotzky, 1995 and Hori et al., 2000) or goats (Sims & Berberich, 1996) or the monoclonal antibody based commercial kits available for quantification of Bt protein.

In the present study, an established indirect ELISA protocol was used with polyclonal rabbit anti-Cry1B antibody for the quantification of Cry1B protein in sorghum transgenic plants and the detection limit of purified Cry1B protein was determined as 1.56 ng ml⁻¹. The limit of detection of other published immunoassays for Bt toxins are given in other units, for example, ng toxin per microgram soluble protein (Vazquez et al., 1996) or ng of toxin per g dry weight soil (Palm et al., 1994) and had difficulty to compare. However, the detection limit of the assay is in the range as expected for a sandwich-type enzyme-linked immunoassay.

In the present study the expression levels of Cry1B protein were in the range of 0.7-0.9 µg g⁻¹ fresh weight tissue in transgenic BC₃F₂-1 event plant progenies, in transgenic BC₃F₂-5 event plant progenies Bt protein was ranged between 0.6-0.8 µg g⁻¹ fresh weight tissue and with BC₃F₂-9 event plant progenies Bt protein is ranged between 0.8-1.1 µg g⁻¹ fresh weight tissue.
The major stumbling block of the introgression is linkage drag where large amounts of donor parent chromosomal material can remain around a target gene even after many generations may cause deleterious effects on final yield and/or quality. Markers can aid selection for target gene/alleles that are not easily assayed in individual plants, minimize linkage drag around the target gene, and reduce the number of generations required to recover a very high percentage of the recurrent parent genetic background (Hospital, 2001 and Hospital and Charcosset, 1997). More efficient procedures are needed to transfer desirable traits to avoid unwanted phenotypic alterations often associated with conventional breeding strategies in plants. Molecular markers can be used to monitor the transfer of the donor segment to the recipient genome during backcrossing, efficient use of molecular markers in backcross breeding to introduce of desirable genes from one line into another was explained earlier in number of crops (Smith et al., 1987; Hillel et al., 1990, 1993; Groen and Timmer-Mans, 1992; Hospital et al., 1992; Groen and Smith, 1995; Peter et al., 1996 and Dekkers and Hospitals, 2002). The present study satisfies these requirements, since the co-dominant microsatellite markers distributed over the chromosomes were used for screening of backcross progenies and achieved maximum of 88 to 92% of recovery in selected BC$_3$F$_2$ introgression lines.

In the present study, the plant progenies of three backcrossed events (BC$_3$F$_1$-1-2, BC$_3$F$_1$-5-11 and BC$_3$F$_1$-9-12) showed 92% recovery of recurrent parent (SSV 84) background were advanced to BC$_3$F$_2$ and subjected to insect bioassays in laboratory (leaf-disc bioassay) as well as at glasshouse (whole-plant bioassay) conditions to assess the level of resistance afford by the transgenic plants developed in sweet sorghum background through backcross breeding against spotted stem borer (*Chilo partellus*).

During leaf disc bioassay, sixty transgenic plants from each backcrossed events (BC$_3$-1, BC$_3$-5 and BC$_3$-9) selected along with non-transformed recurrent parent SSV84 as a control were subjected insect bioassay by releasing 10 larvae of *Chilo partellus* in 1st
instar stage and data was recorded after 5 days of larval infestation. The stem borer caused 30-80% of leaf damage in BC3-1 event plant progenies followed by 30-80% in BC3-5 and 20-80% in BC3-9 event progenies, whereas leaf damage was 70-80% in non-transformed control plants (Plate 4.10). The percentage of larval mortality observed BC3-1 plant progenies was 30-100% followed by 30-100% in BC3-5 and 20-100% in BC3-9 event progenies and no larval mortality observed in non-transformed control plants. Finally a total of 10 transgenic plants from BC3-1 event, 12 from BC3-5 event and 14 from BC3-9 event showed resistance to spotter stem borer during leaf-disc bioassay in terms of less feeding (20-30%) and 100% larval mortality in BC3F2 generation stage were selected.

During whole plant bioassay of BC3F2 plants, the percentage of leaf damage caused by *Chilo partellus* was scored visually using 1-9 scale and the damage was in the range of 30-80% in BC3-1, 30-80% in BC3-5, 20-80% in BC3-9 transgenic event progenies and 90% in non-transformed control recurrent parental lines (Plate 4.11). The percentage of deadheart formation by stem borer larvae was 41% in BC3-1, 33% in BC3-5, 26% in BC3-9 transgenic event progenies and 90% in non-transformed control (Plate 4.12). The stem borer larvae caused exit holes during the whole plant bioassay was counted was 0-7 in BC3-1, 0-8 in BC3-5, 0-5 in BC3-9 transgenic event progenies and 8-9 in non-transformed control. The percentage of stem tunneling caused by stem borer larvae during the assay was in the range of 0-40% in BC3-1, 0-40% in BC3-5, 0-30% in BC3-9 transgenic event progenies and it was 30-40% in non-transformed control (Plate 4.14).

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 mainly in segregating generation of 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 transgenic 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 (mpi), the report concluded that cry1Ac gene did not confer complete resistance against first instar neonate larvae of C. partellus; only two of the T1 transgenic sorghum plants had detectable Cry protein levels in the leaves i.e., 1 and 8 ng per gram of leaf tissue.

In rice, Nayak et al. (1997) reported that cry1Ac gene under control of maize ubiquitin-1 promoter expressed the Bt crystal protein in a range of 58–240 ng per milligram of total soluble protein conferring resistance to yellow stem borer. Wu et al. (1997) reported that
Cry1A(b) protein showed enhanced insecticidal activity against yellow stem borer with mortality rates reaching up to 100%. Cheng et al. (1998) reported that 3% of the total soluble Cry1Ac and Cry1Ab protein were highly toxic to striped stem borer and yellow stem borer. Datta et al. (1998) reported that 10-200 ng of Cry1Ab protein is capable of conferring 100% larval mortality on the yellow stem borer in cut-stem bioassays. Maqbool et al. (1998) reported 0.01-1.0% of total soluble Cry2A protein conferring resistance to yellow stem borer. Breitler et al. (2001) reported that cry1B gene under control of the mpiC1 promoter expressed the δ-endotoxin to levels of up to 0.2% of total soluble protein, and these plants exhibited 100% resistance to second-instar larvae of striped stem borer (Chilo suppressalis Walker). Gong-yu et al. (2001) reported that 0.01% of the total soluble fused Cry1Ab/Cry1Ac protein conferring resistance to four lepidopteran species (striped stem borer (Chilo suppressalis), pink stem borer (Sesamia inferens), leaf folder (Cnaphalocrocis medinalis) and green semilooper (Naranga anescens)).

Khanna and Raina (2002) reported that 0.1% total soluble Cry1Ac protein showing 100% mortality to yellow stem borer; Chen et al. (2005) reported that Cry2A protein concentration of four homozygous transgenic lines ranged from 9.65-12.11 µg g⁻¹ of leaf fresh weight conferring resistance to lepidopteran pests and Ramesh et al. (2004) reported that 0.0188- 0.0835 ng mg⁻¹ Cry1Ac and Cry1Ab protein conferring resistance to yellow stem borer.

In maize, Bohorova et al. (1999) leaf bioassay presented varying levels of resistance to southwestern corn borer of transgenic tropical maize carrying cry1Ac gene. Ty et al. (2005) reported that 44.0-69.8 µg g⁻¹ Cry3Bb1 protein expressed conferring resistance to corn root worm.

In rice, Breitler et al. (2000) reported that synthetic cry1B gene under the control of ubiquitin promoter expressed at a level ranging from 0.01-0.4% of the total soluble
proteins; some transgenic were fully protected from attack of third and fourth instar of striped stem borer larvae over subsequent generations. In maize, Bohorova et al. (2001) reported that the maize plants that carried cry1B gene under the control of ubiquitin promoter conferred resistance to the southwestern corn borer and sugarcane borer.

Mugo et al. (2001) screened different lines of Bt maize transgenics with different Bt gene constructs (cry1Ac driven by the maize ubiquitin promoter, cry1B driven by rice actin, and maize ubiquitin promoter, cry1Ab driven by the maize pollen-specific promoter, cry1B-1Ab driven by the rice actin promoter and cry1E driven by the rice actin promoter) for activity against Kenyan maize stem borers and found that cry1B under the control of maize ubiquitin promoter was more active against Chilo partellus and Chilo orichalcococliellus.

Marker-assisted selection (MAS) was used for introgressing cry1B gene conferring Bt resistance and conventional phenotypic selection was also deployed in the present study for recovery of SSV84 quality parameters. This approach provides three distinct advantages compared to the practice of over conventional breeding or the deployment of MAS alone. Firstly, it helped in reducing the cost of MAS through the progressive reduction in the number of individuals subjected to the final marker analysis by stepwise screening for resistant phenotype, desirable agronomic and grain quality characteristics. Secondly, it reduced the time period required for the recovery of desirable recombinants to a considerable extent. Thirdly, in contrast to a stringent backcross breeding, phenotypic analysis during two and three backcrosses enabled selection of recombinants of desirable plant type, grain type possessing Bt resistance by followed selection of additional desirable traits from the donor besides Bt resistance at each stage (BC1F1, BC2F1 and BC3F1) of breeding material. Joseph et al. (2003) had also made similar efforts earlier in rice to introgress BB resistance into a Basmati variety Pusa Basmati 1 and Basavaraj et al. (2009) achieved highest of 71.0% to 81.0 % RP genome recovery at BC1F1, 87.7% to 92.2 % at
BC$_2$F$_1$ and 92.3% to 95.6% at BC$_2$F$_2$ generation while pyramiding $Xa21$ and $xa13$ genes into the genetic background of Pusa 6B, a maintainer line for aromatic rice hybrid Pusa RH10 by using this methodology.

The selected backcross derived lines with the grain qualities along with other morphological features like number of leaf, leaf area, midrib color, plant height, days to fifty percentage of flowering, stem girth, brix, peduncle length, panicle length, number of nodes per panicle, primary branches for panicle, panicle weight, grain yield and test weight of the ILs almost similar to their recurrent parents were subjected to background selection. More or equal recovery of the recurrent parent (RP) genome was achieved as theoretically expected in the introgression lines at each generation and for each event. These introgression lines possessing $Bt$ resistance, SSV84 morphological and grain quality features along with high RP genome recovery were forwarded to next generation for further selection and evaluation. The higher RP genome recovery in present study was achieved due to stringent selection criterion imposed at each cycle of selection (Table 4.5, Table 4.8, Table 4.11 and Table 4.15).

In conclusion, the present study is an evident that the large number of sorghum introgression lines with genetic background of SSV84 carrying the $Bt$ insecticidal protein gene $cry1B$ under the control of $ubiquitin$ promoter have been produced through marker assisted backcross breeding (MABB). These results have demonstrated that feasibility and effectiveness $Bt$ sorghum introgression lines conferring near-complete resistance against striped stem borer ($Chilo partellus$). These results further represent the first report on the development of stem borer resistant introgression lines, obtained by marker assisted backcross breeding in sorghum. Finally 36 transgenic plants of which 10 from BC$_3$-1 (BC$_3$F$_2$-1-5, BC$_3$F$_2$-1-8, BC$_3$F$_2$-1-12, BC$_3$F$_2$-1-15, BC$_3$F$_2$-1-20, BC$_3$F$_2$-1-31, BC$_3$F$_2$-1-33, BC$_3$F$_2$-1-40, BC$_3$F$_2$-1-43 and BC$_3$F$_2$-1-49), 12 from BC$_3$-5 (BC$_3$F$_2$-5-7, BC$_3$F$_2$-5-13, BC$_3$F$_2$-5-23, BC$_3$F$_2$-5-28, BC$_3$F$_2$-5-33, BC$_3$F$_2$-5-34, BC$_3$F$_2$-5-36, BC$_3$F$_2$-5-39, BC$_3$F$_2$-5-39, BC$_3$F$_2$-5-152


41, BC3F2-5-45, BC3F2-5-47 and BC3F2-5-48) and 14 from BC3-9 (BC3F2-9-6, BC3F2-9-7, BC3F2-9-9, BC3F2-9-11, BC3F2-9-17, BC3F2-9-21, BC3F2-9-24, BC3F2-9-29, BC3F2-9-32, BC3F2-9-37, BC3F2-9-38, BC3F2-9-43, BC3F2-9-45 and BC3F2-9-48) transgenic events showing resistance to spotted stem borer during the insect bioassays were selected based on less leaf feeding, no dead heart formation, no exit holes and no stem tunneling. Thus, these lines can be considered as near isogenic lines of SSV 84 with conferred stem borer resistance. Lines developed in the present study would provide suitable experimental system to undertake detailed analysis of distinctness, uniformity and stability for further development.