African Journal of Biotechnology Vol. 10(19), pp. 3659-3670, 9 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1530
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Transgenic sorghum (*Sorghum bicolor L. Moench*) developed by transformation with *chitinase* and *chitosanase* genes from *Trichoderma harzianum* expresses tolerance to anthracnose

Linus Moses Kosambo-Ayoo[1]*, Maram Bader[2], Horst Loerz[2] and Dirk Becker[2]

[1]Kenya Industrial Research and Development Institute (KIRDI), P.O. Box 30650 – 00100 Nairobi, Kenya.
[2]Developmental Biology and Biotechnology, Biocenter Klein Flottbek, University of Hamburg, Ohnhorststrasse 18, 22609 Hamburg, Germany.

Accepted 18 February, 2011

Sorghum (*Sorghum bicolor* (L.) Moench) is an important food and fodder crop. Fungal diseases such as anthracnose caused by *Colletotrichum sublineolum* reduce sorghum yields. Genetic transformation can be used to confer tolerance to plant diseases such as anthracnose. The tolerance can be developed by introducing genes encoding proteins such as chitinases and chitosanases that hydrolyse fungal cell wall. Chitinases endolytically hydrolyse the β-1,4-linkages of chitin whereas, chitosanases hydrolyse the β-1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated chitin polymer. Particle bombardment was used to genetically transform a sorghum genotype, KAT 412, with *chitinase* (*harchit*) and *chitosanase* (*harcho*) genes isolated from *Trichoderma harzianum*. Transgenic sorghum plants, KOSA-1-3, that expressed the two anti-fungal genes were developed. Expression of *harchit* and *harcho* in the transformants was confirmed by quantitative real time PCR. *In planta and ex planta C. sublineolum* infection assays were carried out using one-week old seedlings to determine tolerance to anthracnose. Seedlings from a transgenic line, KOSA-1, were found to be significantly more tolerant to anthracnose than the parent wild type, KAT 412. The transgenic line was further compared with other wild type sorghum cultivars. The comparison revealed a genotype-dependent difference in anthracnose response. The transgenic line KOSA-1 was found to be more tolerant than the sorghum line SDSH 513 but less tolerant than KAT L5. This demonstrated the existence of genetic diversity, which together with the transgenes, could be utilised to pyramid genes for higher tolerance to anthracnose. The two antifungal genes introduced into sorghum genome could be introgressed into other sorghum lines for fungal diseases resistance.

Key words: Transgenic, sorghum, chitinase, chitosanase, *Colletotrichum sublineolum*, anthracnose

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the crops that are traditionally produced in marginal areas, where low soil moisture and high ambient temperatures are the main limiting abiotic factors (Wenzel and Rooyen, 2001; Machado et al., 2002; Gebeyehu et al., 2004). Sorghum is a primary staple in the semi arid tropics of Africa and Asia for over 300 million people (ICRISAT 2010). Sorghum is attacked by fungal, bacterial and viral pathogens causing root, stalk, foliar, panicle and caryopsis diseases (Sutton,1980; Thomas et al., 1996; Bueso et al., 2000; Waniska et al., 2001, Prom et al., 2005). One of the important fungal diseases of sorghum is anthracnose that is caused by *Colletotrichum sublineolum* (Sutton, 1980; Hammer et al., 1988; Nicholson and Epstein, 1991; Ngugi et al., 2002). Genetic transformation can be used to introduce new genes to confer anthracnose tolerance. One of the mechanisms of

*Corresponding author. E-mail: kosambosan@yahoo.com. Tel: 002540728403697. Fax: 0025420607023.

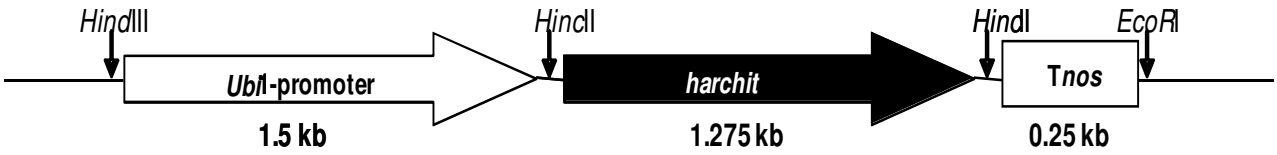

Figure 1. pUbi*HarChit* vector. The transformation vector contains *ubi*1 promoter, *harchit* and T*nos* terminator gene regions and restriction sites used in cloning and southern blot analysis.

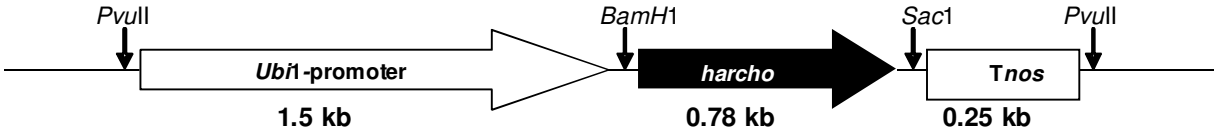

Figure 2. pUbiHarCho vector. The transformation vector contains *ubi*1 promoter, *harcho* and T*nos* terminator gene regions and restriction sites used in cloning and southern blot analysis.

conferring tolerance is to introduce into the genome of plants genes that encode proteins such as, chitinase and chitosanase that target components of cell walls of fungi. Chitinases endolytically hydrolyse the β-1,4-linkages of chitin, while chitosanases hydrolyze the β-1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated chitin polymer (Cabib et al., 1987; Garcia et al., 1994; Lorito et al.,1998; Hendrix and Stewart, 2002). The hydrolysis renders fungal cells osmotically sensitive and ultimately destroys the fungus. In addition, glucosamine oligomers, released from fungal cell walls after hydrolysis with chitinase or chitosanase, are elicitors of plant defence response such as stomata closure (Lee et al., 1999) and cell wall lignifications (Vander et al., 1998). Mycoparasitic fungi such as *Trichoderma harzianum* are natural sources of chitinases and chitosanases (Garcia et al., 1994; Lorito et al., 1998; Hendrix and Stewart, 2002). Efficacy of *T. harzianum* in control of other fungi is attributed to its chitinases and chitosanases, which are considered to be more active and effective on a much wider range of pathogens than corresponding enzymes found in plants (Lorito et al., 1998; Garcia et al., 1994).

The respective genes, *chitinase* (*harchit*) and *chitosanase* (*harcho*) that encode chitinase and chitosanase enzymes have been cloned from *T. harzianum* (Garcia et al., 1994; Lorito et al., 1998; Hendrix and Stewart, 2002). Co-expression of the two genes in sorghum cultivars that are susceptible to anthracnose could result in a synergistic development of disease tolerance. The objective of this investigation was to transform sorghum with *harchit* and *harcho* genes, in order to develop transgenic plants that are tolerant to fungal diseases such as anthracnose. Immature zygotic embryos (IZE) of sorghum were genetically transformed by particle bombardment with plasmids containing *harchit, harcho* and the *pat* gene that encode the herbicide-resistance enzyme, phosphinothricin Acetyl-transferase as the selectable marker. Putative Transgenic plantlets were regenerated from the bombarded IZE through tissue culture. *In planta* and *ex planta* infection assays were carried out to compare the response of the transgenic and wild type sorghum cultivars to *C. sublineolum* infection.

MATERIALS AND METHODS

Plant growth

A collection of sorghum cultivars were cultivated in a greenhouse under the standard conditions consisting of a 16/8 h day/night photoperiod of 220 ± 20 µE/m^2/s at changing day/night temperature of 24/20°C, as reported by Oldach et al. (2001). Seedlings for *ex planta* and *in planta* infection assays were cultivated under controlled conditions (28°C, 220 µE/m^2/s, 16/8 h light/darkness) in a growth chamber.

Pathogen culture

Stock cultures of *C. sublineolum* were maintained on 1/2 potato dextrose agar (PDA) (2.0 g/l potato starch, 10 g/l dextrose, 7.5 g/l agar). Sporulation was induced by growing fungal isolates on 7.25% (w/v) oatmeal agar (OMA) at 26°C with 50 µE/m^2/s backlight illumination for two weeks as described by Boora et al. (1998).

Vectors

The *chitinase (harchit)* (NCBI GeneBank accession AB041751) and the *chitosanase (harcho)* (NCBI GeneBank accession (AY571-342.1) genes were extracted from *T. harzianum* and cloned into the pUbiCas plasmid (Dr. D. Becker, University of Hamburg, Germany, unpublished) and used to transform immature zygotic embryos of sorghum. A 5.766 kbp (pUbiHarchit) vector was constructed by cloning *harchit* into the pUbiCas plasmid under the control of the constitutive ubiquitin1 promoter (*ubi*1 promoter) from maize (*Zea mays*) (Christensen et al., 1992) and the nopaline synthase terminator (T*nos*) from *Agrobacterium tumefaciens* (Figure 1). The chitosanase gene *harcho* was cloned as described for *harchit* to form the vector pUbiHarcho (Figure 2). The p35SAcS vector containing *pat* gene (Strauch et al. 1988) encoding the herbicide-

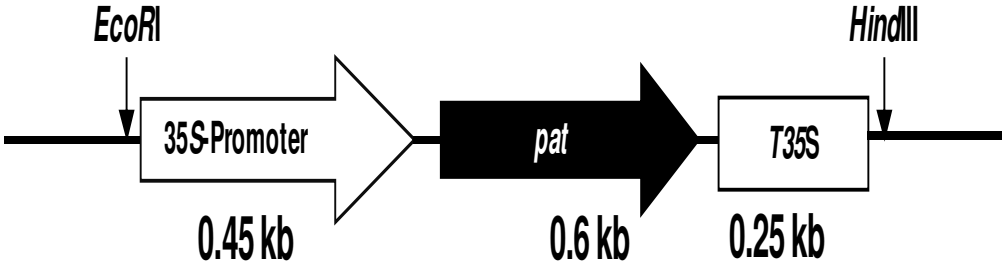

Figure 3. p35SAcS vector. The transformation vector contains CaMV 35S promoter, *pat* and T35S terminator genes and restriction sites used in cloning.

Table 1. *Harchit* and *harcho* southern blot primers sequences showing position and the product length after PCR.

Primer	Forward primer	Primer position	Product length
HarChitDig (5´-Primer):	5´-TCT CTG GCG ATA CCT ACG CT-3´	244-264	332bp
HarChitDig (3´-Primer)	5´-AGA TCG GAC TTC CTT CAG CA-3´	556-576	
HarChoDig (5´-Primer)	5´-TAG TGG CGG AGC TAC T-3´	135-151	381bp
HarChoDig (3´-Primer)	5´- GCT TGC TTT GGG TAA G-3´	500- 516	

resistance conferring enzyme, phosphinothricin acetyltransferase, was used as the selectable marker (Figure 3).

Isolation and genetic transformation of immature zygotic embryos

Immature seeds were harvested from sorghum panicles 14 to 20 days after flowering, washed 3 times with distilled water, sterilized with 2% sodium hypochlorite containing 0.1% MUCASOL® detergent (Brand Gmbh + Co KG, Wertheim, Germany) for 25 min and rinsed with sterile double distilled water. Immature zygotic embryos (IZE) were thereafter, aseptically isolated from the immature seeds under a dissecting microscope. The sorghum IZE was transformed with *harchit* and *harcho*, *pat* genes through particle bombardment with plasmid DNA-coated gold particle using the BIORAD PDS-1000/He particle gun (BioRad, München, Germany). Coating of gold particles with plasmid DNA and bombardment of sorghum IZE were based on the procedure described by Becker et al. (1994). Particle bombardment was carried out with helium gas at 1350 psi under a partial vacuum of 27 mmHg according to Brettschneider et al. (1997).

Tissue culture of IZE

The bombarded IZE were cultured in a regime of MS-based (Murashige and Skoog, 1962) callus induction (CIM), regeneration (REM) and rooting media (ROM), containing macroelements, microelements, vitamins, amino acids, recommended additives and plant hormones (Oldach et al., 2001). For induction of calli formation, sorghum IZE were cultured in darkness for two weeks at 26°C on CIM containing 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/ml 6-benzylaminopurine (BAP). Calli were thereafter, transferred onto a regeneration medium (REM) with 1 mg/l of BAP and cultured under 16/8 h light/darkness cycle at 26°C. The calli were cultured on REM for two weeks without selection and thereafter sub-cultured onto REM with selection containing 1 mg/l of BAP and 2 mg/l of the herbicide BASTA for 4 to 10 weeks. Calli and young regenerants were sub-cultured every 2 to 3 weeks onto fresh media. Regenerants with shoots were sub-cultured onto hormone-free ½ MS rooting media with 2 mg/l BASTA selection. Regenerants were transferred to the greenhouse upon attaining a height of 7 to 10 cm.

Progeny segregation

Segregation of the transgenes in sorghum progeny was evaluated from the expression of the herbicide resistance gene. T_1 seedlings were sprayed twice with 200 and 300 mg/l of the herbicide, BASTA, at 7 and 14 days after germination, respectively. Surviving and dead seedlings were then, enumerated and computed to establish the segregation ratio.

DNA isolation and southern blot analysis

Genomic DNA was extracted from sorghum leaves as described by Dellaporta et al. (1983). The purity, integrity and concentration of the isolated DNA were checked by absorbance spectrophotometry and gel electrophoresis. Digoxigenin-11-dUTP (DIG-dUTP) from Roche DIAGNOSTICS (Roche, Mannheim, Germany) was used to label DNA probes for detection of *harchit* and *harcho* genes in southern blot analysis. DIG-labeling was done through PCR of respective plasmid DNA with *harchit* and *harcho* DIG-primers (Table 1). The PCR reaction mix used for DIG-labeling included 0.3 µM of each Primers; 0.3 mM of each dATP, dGTP, dCTP; 0.2 mM dTTP; 35 µM DIG-dUTP; 2 mM $MgCl_2$, 1x PCR buffer; 1 U Taq-polymerase and 50 ng plasmid DNA. Southern blotting was carried out as described by Sambrook et al. (1989). A quantity of 10 to 25 µg genomic DNA were restricted with respective endonucleases that excise and linearize the gene cassette as shown in vectors in Figures 1 and 2. The enzyme digests were separated in 0.8% agarose gels. DNA was transferred onto HYBOND™ NX nylon membranes (Amersham Life Science, Buckinghamshire, England) by capillary action with 20 x SSC and thereafter, fixed to membrane with 120 mJ using STRATALINKER™ 1800 UV CROSSLINKER

Table 2. qRT-PCR primers of target genes, their respective forward and reverse primers, optimal annealing temperatures, polymerization fragment length, primers efficiencies and NCBI accession numbers.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Fragment length (bp)	Primer efficiency (%)	NCBI accession
Sorghum actin	AGG CGC AGT CCA AGA GGG GTA	ATG GCG GGG GGT GTT GAA GGT	226	93	X79378
T. harzianum Chitinase (*HarChit*)	ACC CCA ATG CCA CCC CCTTCA A	TAC CGG CCT CCC AGC TTC CAC TT	174	99.37	AB041751
T. harzianum Chitosanase *(HarCho)*	GTG GCC AGA GCG AGA CT	TCA CCC CAG ATA CCA TAG AA	199	79.83	AY571342

(Stratagene, La Jolla, U.S.A.). Hybridisation with DIG-labeled DNA probes (25 ng/ml hybridisation solution) was performed under high stringency conditions at 42°C using DIG EASY HYB solution (Roche, Mannheim, Germany). Chemilumi-nescence detection was performed with CSPD® substrate according to the manufacturer's prescriptions (Roche, Mannheim, Germany).

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from sorghum leaves using the peqGOLD TriFast extraction protocol according to the manufacturer's instruct-tion (PeqLab Biotechnologie, Erlangen, Germany). Extracted 10 µg total-RNA was treated with 10 U of RNA-free DNAse and RNAse inhibitor as described by reagents manufacturer (Fermentas Life Sciences, St. Leon Rot, Germany).

Complementary DNA (cDNA) was synthesized using 18-mer oligonucleotide (Oligo(dT)$_{18}$) primer, dNTPs, RNAse inhibitor and the Moloney murine leukaemia virus reverse transcriptase (M-MuLV) as recommended by the reagents manufacturer (Fermentas Life Science, Leon Rot, Germany).

The sorghum actin gene (NCBI accession no. X79378) was used as the internal standard to quantify the expression of *harcho* and *harchit*. 150 to 200 bp *Harchit* and *Harcho* primers were used in the qRT-PCR. A serial dilution of 1 ng-1 fg of cDNA were used as the templates in a qRT-PCR BIORAD ICYCLER® (Hercules, CA, USA) to determine the efficiencies of the *Harchit* and *Harcho* primers (Table 2). SYBR GREEN 1 with fluorescein (Eurogentec, Seraing, Belgium) was used for detection in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The reaction mixtures and thermo-cycler program were developed from the protocol Descri-bed by the manufacturer (Eurogentec, Seraing, Belgium).

***Ex planta* and *in planta C. sublineolum* infection assays**

Response of transgenic and Wt sorghum to pathogen infection was carried out with conidial suspension of *C. sublineolum* in water containing 0.01% Tween 20 detergent. A conidia-count of 1 x 10^6 conidia/ml was used in all infection experiments. Second leaf of two weeks old sorghum seedling were cut in three equal parts (triplicate) of approximately 3 x 6 cm using a cutting mould, plated on 0.8% neutral agar plates with 40 mg/l of anti-senescence compound, benzimidazole. The leaf segments were infected through point inoculation with 10 µl of conidia and incubated at 26°C under 16/8 h light/dark conditions. *In planta* Infection assay was done by spraying two-leaf stage seedlings with conidia suspension until run-off. The infected seedlings were thereafter, grown in 80% relative humidity (RH), 8/16 h light/dark cycle at 28°C for 48 h and then, under the same RH and 12/12 h cycle for the rest of the experimental period. Responses to infection were quantified at specific time interval between 0-144 HPI by quantifying the timing and severity of symptoms. A sampling size of 30 seedlings was used for both *ex planta* and *in planta* experiments. The number of leaves showing symptoms was enumerated at specific intervals. The infection experiments were repeated three times. Non infected controls were included in all experiments to monitor secondary infection.

RESULTS

Sorghum tissues culture

A collection of Kenyan sorghum genotypes were used in tissue culture (Figure 4a, b). Successful tissue culture of KAT 412, KAT 369X, KAT 487, SDSH 513, GBK 046820, Serena, KAT L5 (Line 5), ICSV III, GBK 046812, GBK 046842 and GBK 046844 was achieved. Genotype dependent variation in the responses of IZE in tissue culture was noted. Differences in the amount of phenolic compounds secreted into the tissue culture media were observed. Cultivars, such as KAT 412, KAT 487 were associated with low levels of secretion and tissue culture pigmentation (Figure 4c) where-as, KAT L5, Serena, GBK 046844 secreted copious amount of phenolic compounds into culture media (Figure 4d). It was also noted that, sorghum lines that secreted copious amount of pigments produced significantly less calli and regenerants than those associated with low level of pigmentation during tissue culture (Table 3). For example, only 0.7% Serena IZE, a cultivar that secreted high levels of phenolics into culture media, developed regenerants that reached the greenhouse stage when compared with 3% in lower phenolics producing line, KAT 412.

Analysis of transgenes integration, inheritance and expression in sorghum

Genetic transformation was attempted in 9 genotypes: Aralba, KAT 412, KAT 487, KAT L5, GBK 046820, ICSV III, SDSH 513 and Serena. From these, one genotype was successfully trans-formed with *harchit, harcho* and the selectable

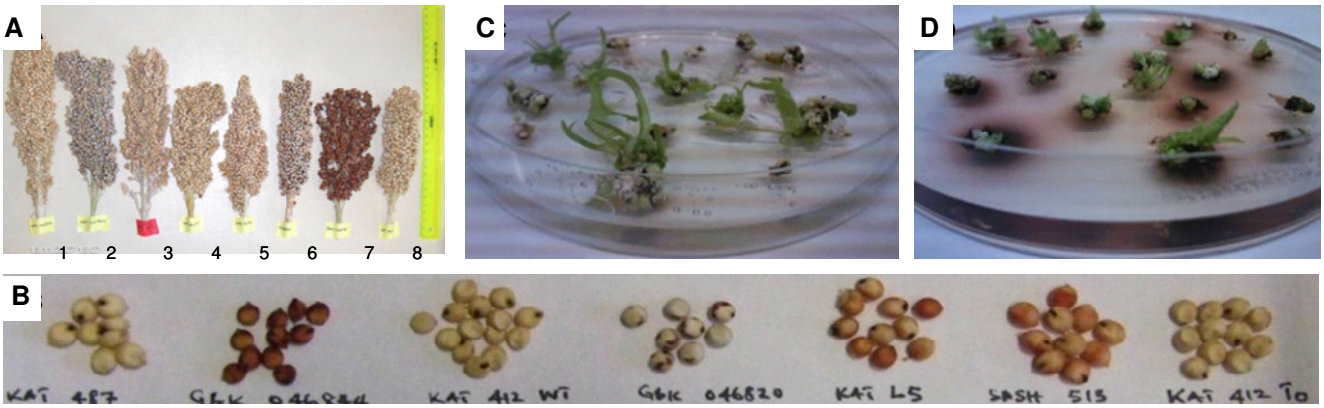

Figure 4. Sorghum kernel, seeds colour and phenolics secretion in regeneration media. A) Assortment of red, brown, cream and white coloured kernel colour of different sorghum cultivars: 1-GBK 046812, 2-GBK 046820, 3-KOSA-1 T_1, 4-KAT 412 Wt, 5-KAT 369X, 6-Gadam, 7-GBK 046844 and 8-KAT 487; B) Seeds of different colours of some of the cultivars studied; C and D) Tissue culture condition in 3 weeks old REM culture of two sorghum line secreting low (C) KAT 487 and high (D) KAT 046844 levels of pigments into the culture medium.

Table 3. Effect of sorghum cultivars on regenerants formation showing correlation between cultivar's grain colour and pigments secretion into culture media and sorghum regenerants formation after particle bombardment: +very low, ++ - low, +++ moderate and ++++ - high phenolics production.

Sorghum line	Grain colour	Amount of phenol in culture	Embryos cultured	Regenerants to greenhouse	Embryos forming regenerants (%)
KAT 412	White	+	3455	105	3.0
Serena	Red	++++	608	4	0.7
KAT 487	White	+	674	8	1.2
SDSH 513	Brown	+++	1746	12	0.7
GBK 046820	Brown	++	600	6	1.0
ICSV	Red	++++	265	2	0.8
KAT L5	Brown	+++	346	3	0.9

marker *pat* gene. Three herbicide resistant sorghum plants, KOSA-1, KOSA-2 and KOSA-3, were successfully regenerated after microprojectile bombardment of IZE of KAT 412 (Figure 5). Stable integration of *harchit* and *harcho* transgenes was confirmed by southern blot and qRT-PCR. The transgenic plants KOSA1-3 showed normal growth, flowered and produced progeny. Three generations of KOSA-1 and one generation of KOSA-2 and KOSA-3 were analysed. KOSA-1 produced fertile T_1, T_2 and T_3 progenies. KOSA-2 and KOSA-3 produced normal T1 progenies.

Progeny segregation ratio was determined from herbicide resistance of T1 seedlings of KOSA-1. Analysis of herbicide resistant T_1 progeny of KOSA-1 showed that, an average of 73.92% were transgenic, which corresponded to a Mendelian segregation ratio of a dominant gene. Southern blot analysis using respective restriction enzymes that excised and linearized the gene cassettes were used to confirm integration and to analyse the number of integrated copies of the *harchit* and *harcho* genes. Molecular analysis of the three primary transformants, KOSA-1, KOSA-2 and KOSA-3, showed that both *harchit* and *harcho* were integrated into the genome of the transgenic plants as shown by the southern blot of *harchit* (Figure 6). Inheritance of *harchit* and *harcho* was confirmed in 3 successive KOSA-1 generations, (T_0 to T_3) (Figure 7). Real time expression of *harchit* and *harcho* in KOSA-1 was quantified by qRT-PCR. Both genes were expressed in all the transgenic seedlings of KOSA-1 analyzed. The expression level oscillated between 0.5 to 2 fold changes (FC) (Figure 8).

Response to *C. sublineolum* infection

Response to fungal infection was studied in the T_1 of the transgenic line, KOSA-1, parent Wt KAT 412 and 6 other Wt sorghum cultivars from Kenya: KAT 487, KAT L5, GBK 042820, GBK 042812, SGSH 513 and Serena. *Ex planta* and *in planta* tests were done. *Ex planta* studies with leaf segments revealed that, there were differences in the timing and severity of the disease

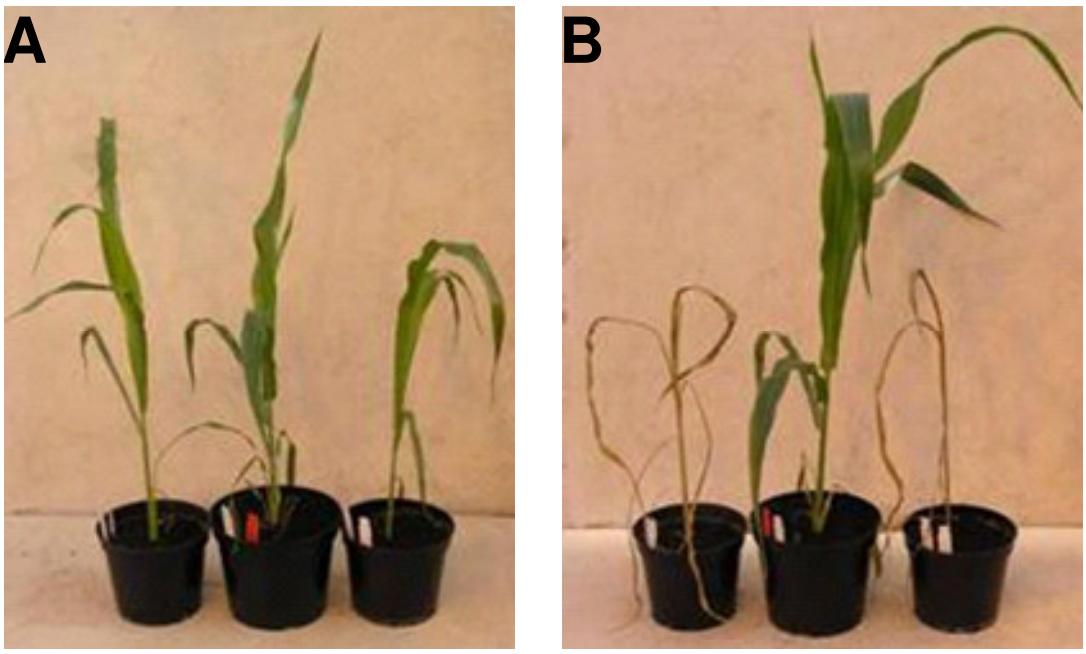

Figure 5. Herbicide resistance test of sorghum. Test shows 3 plants before BASTA spraying (A) and 7 days after spraying (B). The transgenic plant (center, B) was resistant to the herbicide and was later confirmed to contain the transgenes *harcho* and *harchit*.

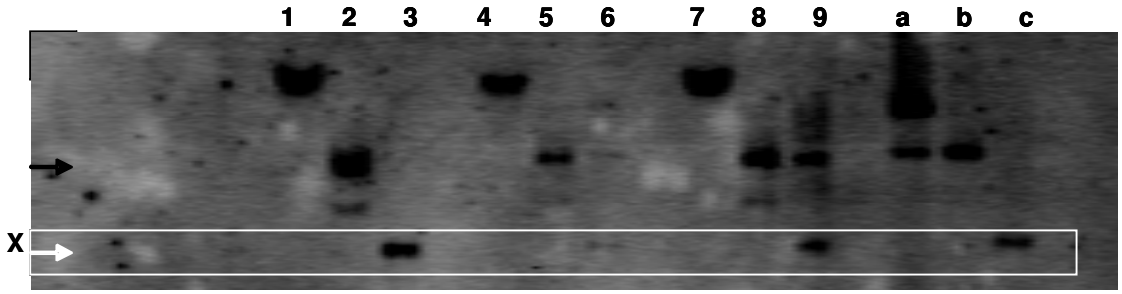

Figure 6. Southern blot of parental transgenic plants. Analysis of gene integration in transgenic sorghum KOSA-1(1 to 3), KOSA-2 (4 to 6) and KOSA-3 (7 to 8) with *HarChit* probes, positive control (a, b, c) with plasmid, pUbiHarChit. All samples consisted of un-restricted DNA (1,4,7,a), linearized (2,5,8,b) with *EcoR*1 and cassette out (3,6,9,c) with *EcoR*1 and *Hind*III double digest. Linearized band (arrow), cassette out band (X - arrow) of the *harchit* was detected in all three transformants. The bands show the presence of *harchit* in all the three transgenes.

symptoms among the cultivars studied. Comparison of the leaf segments of KOSA-1 T_1 and Wt KAT 412 showed that, the transgenic plants developed less and late symptoms upon infection than the parent Wt plants (Figure 9). Comparison of transgenic KAT 412 T_1 with other cultivars established that there were differences among cultivars in the severity of infection symptoms (Figure 9). Leaf segments of seedlings of KAT L5 suffered the least symptoms, while SDSH 513 had the most diseased plants at 48 h post infection (HPI). By 72 HPI, most of the leaves were symptomatic with KAT L5 being the least symptomatic. Infection symptoms developed later in the transgenic KAT 412 T_1 than Wt, SDSH 513, Serena and GBK 046820 but earlier than KAT L5, KAT 487 and KAT 046812. All the leaf segments from all the sorghum lines were symptomatic by day 5 (120 HPI). These observations established that KAT L5 and SDSH 513 were the most and least tolerant lines, respectively. The transgenic KOSA-1 T_1 was more tolerant than the parent wild type, KAT 412 Wt. However, the transgenic line was less tolerant than the sorghum cultivar KAT L5.

Further assays were done with intact seedlings. These *in planta* infection assays were done with two weeks old seedlings. Seedlings were infected with spores of *C.*

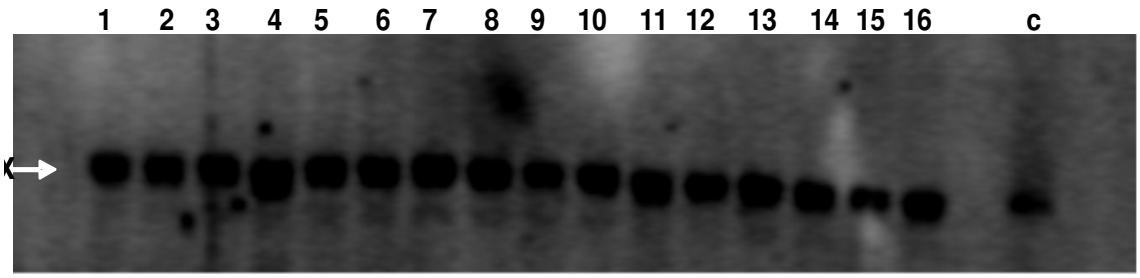

Figure 7. Southern blot of generations of KOSA-1. Blot analysis for gene integration and inheritance of *harchit* gene in KOSA-1 T_o, (1), seedlings of T_1, (2 to 6), T_2 (7 to 11) and T_3 (12 to16) and the positive control pUbiHarChit (c). The DNA of the KOSA-1 T_0-T_3 was restricted with *Hind*III and *EcoR*I enzymes that excised the promoter, *harchit* and *nos* gene cassette. The blots show inheritance of the *harchit* in successive generations.

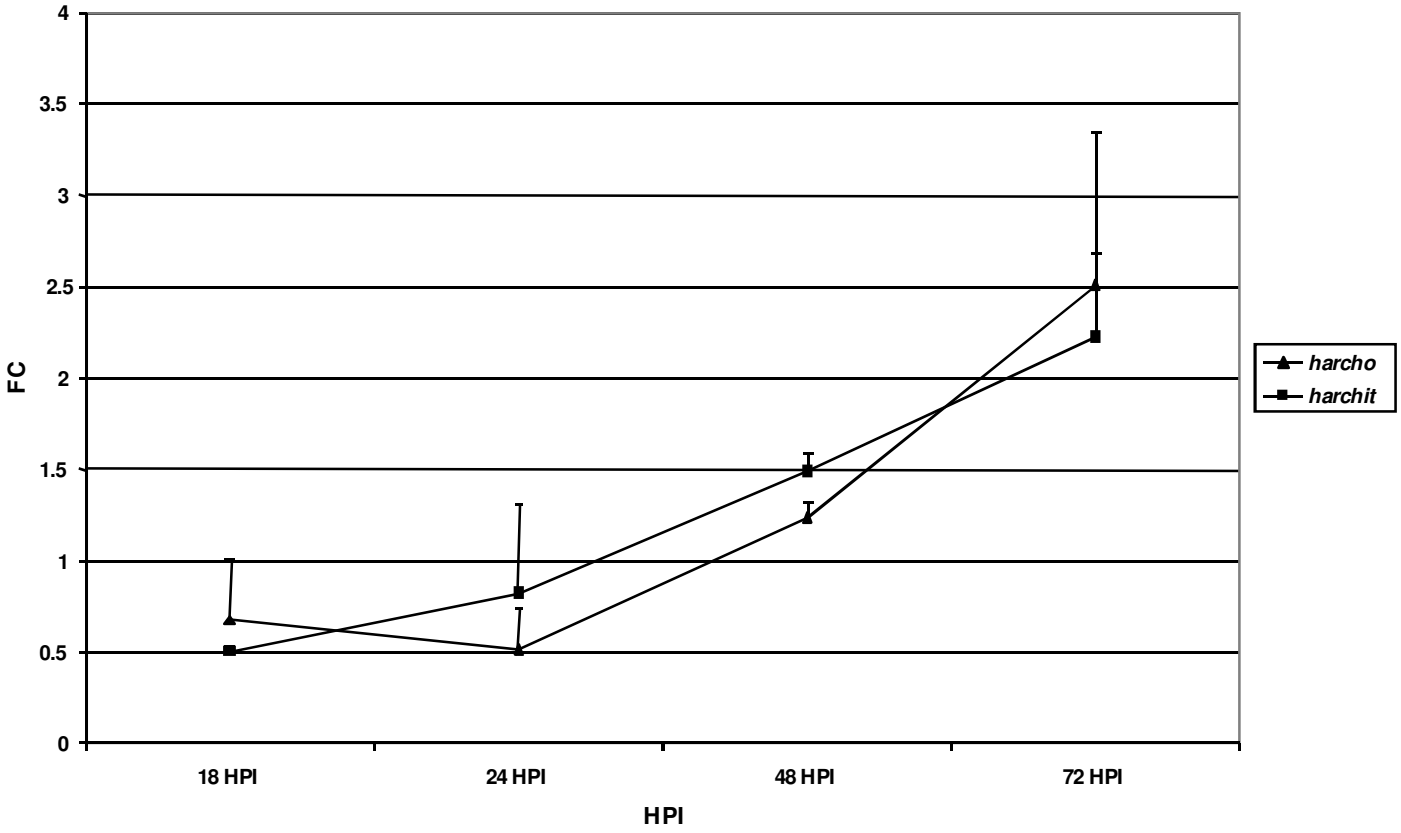

Figure 8. Expression of *HarChit* and *HarCho* after *C. sublineolum* infection. KOSA-1 seedlings were infected with conidia of the pathogen and changes in the expression of the transgenes between 0-72 HPI quantified by qRT-PCR. Both genes were expressed in the seedlings. Expression oscillated between 0.5-2.5 FC (1 FC = no change in gene activity).

sublineolum and symptoms monitored over a period of 144 h after infection. *In planta* assays revealed that, the leaves of the parent KAT 412 Wt developed more and severe lesions than those of the transgenic KAT 412 T_1 (KOSA-1 T_1) seedlings (Figure 10). Comparison of the symptoms development 5 days after infection indicated that the leaves of the transgenic line were healthier than those of KAT 412 Wt (Figure 10). The transgenic sorghum line developed by transformation with *harchit* and *harcho* genes, KOSA-1, was found to be more tolerant to anthracnose than the parent wild type line. Response of the seedlings of the transgenic line to infection by *C. sublineolum* was further evaluated in comparison to sorghum cultivars, SDSH 513 and KAT L5. Disease symptoms in the seedlings of transgenic KOSA-1 were less severe than those observed on SDSH 513. However, seedlings of KAT L5 developed the least symptoms and showed the most tolerance when compared

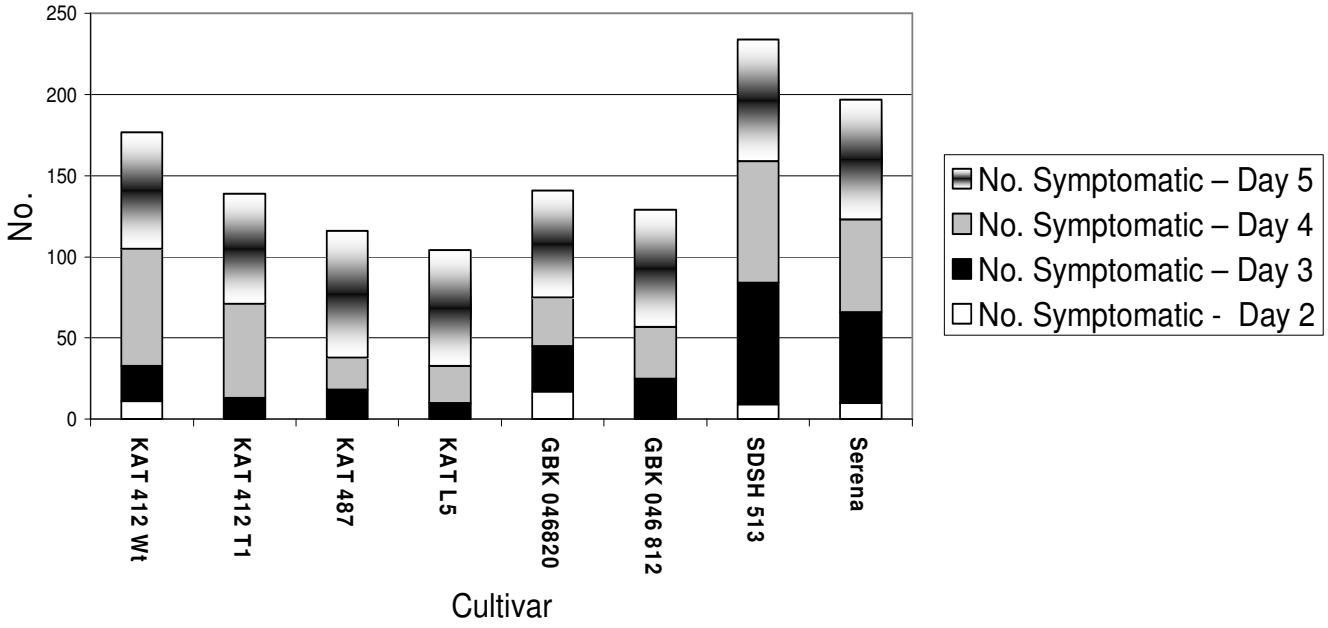

Figure 9. Comparison of *ex planta* response of Kenyan sorghum cultivars. Leaf segments were infected with 10 µl of *C. sublineolum* and the number of segments showing symptoms was enumerated for 5 days. Transgenic plant (KAT 412 T1) was less symptomatic that the wild type KAT 412 Wt. Leaf segments of KAT L5 and SDSH 513 showed least and highest number of infection after 5 days post infection, respectively.

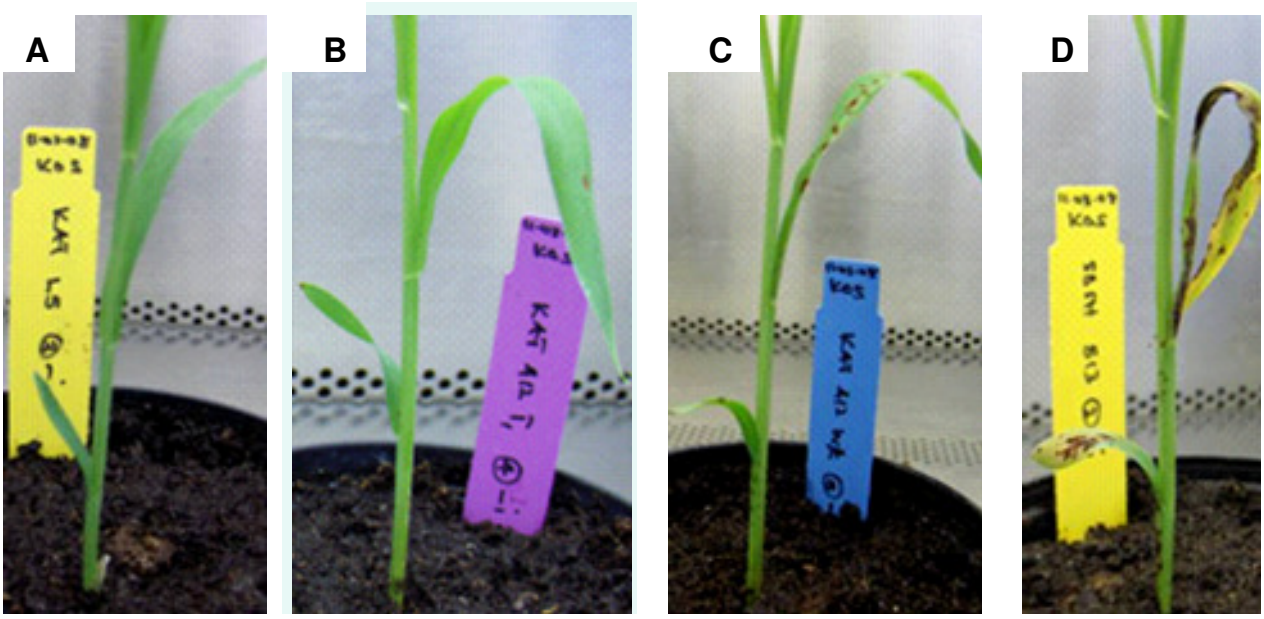

Figure 10. *In planta* sorghum infection assay. Seedlings were spray-infected with *C. sublineolum* conidia. Symptoms development was recorded on the 5th day (120 HPI) after infection. Virtually no symptoms was observed on KAT L5 leaves – resistant (A), few necrotic local lesions ((NLL) on KAT 412 T_1 leaves - tolerant (B), extensive NLL in KAT 412 Wt – less tolerant (C) and extensive NLL on both leaf blades and sheath, chlorosis and discoloration of both leaves of SDSH 513 – susceptible (D).

with SDSH 513, KAT 412 and KOSA-1 (Figure 10). The symptoms were quantified by computing the percentage of leaves showing symptoms every 24 h for 144 HPI. There was variation among cultivar in the percentage of the leaves showing symptoms. Quantification of the symptoms showed no symptoms were observable at 24

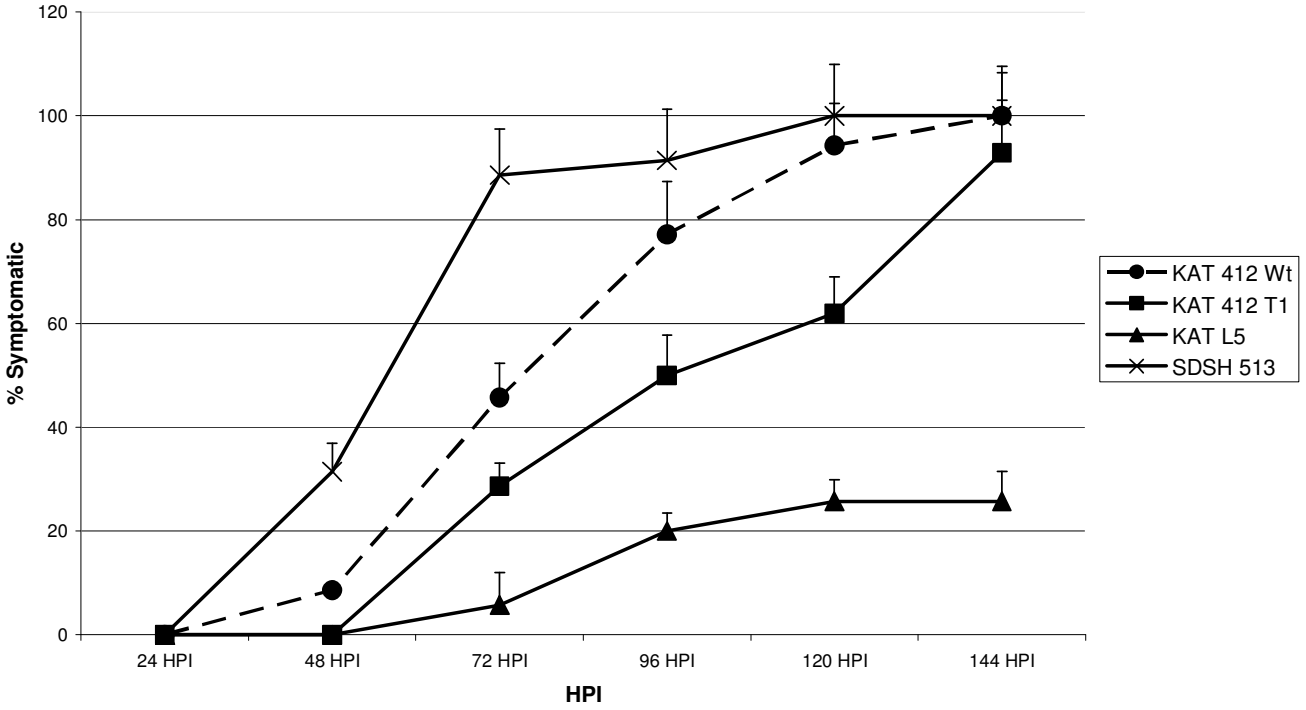

Figure 11. Symptoms development after *in planta C. sublineolum* infection. Sorghum seedlings were infected with conidia of *C. sublineolum* and the proportion of those showing symptoms enumerated between 24-144 HPI. Fewer KAT L5 (25%) developed symptoms later (72 HPI) than SDSH 513 that had more (100%) and sooner (48 HPI) infection. KOSA-1 was symptomatic later (72 HPI) and more tolerant than KAT 412 Wt. The transgenic line was more tolerant than the wild type parent.

Table 4. ANOVA of square root transformation (√0-5+×) of the percentage of symptomatic leaves to establish the effect of cultivar and time on anthracnose symptoms development on four sorghum lines.

Parameter	Sum of squares	Degree of freedom	Mean square	F	Significance
Constant	6732.182	1	6732.182	426099.9	0.00*
Cultivar	603.041	3	201.014	12722.8	0.00*
Time	1920.701	5	384.140	24313.4	0.00*
Cultivar*time	212.329	15	14.155	895-9	0.00*

* - significant at 0.05 confidence limit.

HPI, but an average of 8.57% KAT 412 and 31.43% SDSH 513 were symptomatic at 48 HPI (Figure 11). Quantification of the symptomatic seedlings at 72 HPI showed that, 5.71% KAT L5, 28.57% KOSA-1 T_1, 45.71% KAT 412 and 88.57 DSH 513 were symptomatic. It was notable that the transgenic line showed more tolerance than the parent Wt KAT 412. The percentages were subjected to square root transformation (√0-5+X) and statistically analysed with Newman-Keuls test. Analysis of variance (ANOVA) analyses showed that cultivar and time were statistically significant. The interaction of cultivar and time were also determined to be statistically significant (Table 4). This study established that, the transgenic line KOSA-1 was more tolerant to anthracnose than the wild type KAT 412 parent line. It was also observed that, the wild type genotype KAT L5 was the most tolerant while SDSH 513 was the least tolerant (Figure 11) of the sorghum lines studied.

DISCUSSION

Tissue culture

A prolific tissue culture system is fundamental for successful regeneration of transgenic plants after genetic transformation. Immature zygotic embryos isolated from sorghum genotypes collected in Kenya were tissue cultured. A modified tissue culture protocol based on Oldach et al. (2001) that comprised of 2 weeks calli induction, 2 to 4 weeks regeneration and 3 to 4 weeks rooting was used. Regeneration of plantlets through

somatic embryogenesis was achieved in all the cultivars cultured. A genotype-dependent difference in IZE morphogenesis during tissue culture, especially in the levels of phenolics secreted onto the culture and the number of plantlets regenerated was observed. The cultivars that secreted high levels of phenolics developed less calli and plantlets. Genotype influences somatic embryogenesis especially the recovery of regenerated plantlets upon tissue culture (Casas et al., 1993; Takashi, 2002). It is observed that sorghum explants, especially from cultivars that are rich in phenolic compounds are recalcitrant to morphogenesis during tissues culture (Cai and Butler, 1990; Casas et al., 1993). Attempts have been made to ameliorate the negative effect of phenolics secretion into culture media. Anti-oxidants and anti-phenolics compounds and formulations such as polyvinyl-pyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP), dithiothreitol, coconut water, silver nitrate and active carbon have been used in sorghum tissue culture with mixed results (Zhao et al., 2000; Takashi, 2002). In this study, regeneration of plantlets was achieved in sorghum lines both containing both low and high level phenolic compounds. This was achieved by limiting injuries to isolated embryos and maintaining the hormonal balance at 2.5 mg/l 2,4 D and 0.1 mg/l BAP in callus induction medium and 1 mg/l BAP in regeneration medium. The significance of the successful tissues culture of both phenolics-rich and poor lines lies in the potential use of the tissue culture protocol to tap into the genetic resource present in recalcitrant sorghum land races. This is important considering the fact that, most of the recalcitrant high tannins and phenolics-rich brown-red sorghum lines are relatively tolerant to biotic and abiotic stress (Jambunathan et al., 1992; Esele et al., 1993; Waniska et al., 2001).

Genetic transformation

KAT 412 was successfully transformed with *harchit, harcho* and *pat* genes. Three fertile, herbicide resistant sorghum plants (KOSA-1, KOSA-2 and KOSA-3) were developed through particle bombardment of IZE of KAT 412. Three generations of KOSA-1 and one generation of KOSA-2 and KOSA-3 showed normal growth and Mendelian segregation for herbicide resistance. Co-integration and expression of the two transgenes were confirmed by southern blot and qRT-PCR analysis. This variation in the expression of the transgenes under a constitutive *ubi* promoter was attributed to the effect of pathogen stress. It is noted that, the activity of *ubi*-promoter is increased by stress factors (Oldach et al., 2001). The observed elevated expression of the trans-genes under a constitutive promoter was associated to stress after *C. sublineolum* infection.

Sorghum is one of the most difficult cereals to transform (O´Kennedy et al., 2006). The genetic transfor-mation of sorghum with *harchit* and *harcho* genes achieved in this study is the first report of successful co-transformation of sorghum through microparticle bombardment with two important anti-fungal genes. This marks a progress in bioengineering sorghum for resis-tance against fungal diseases. The production of transgenic sorghum plants via particle bombardment of IZEs was reported for the first time by Casas et al. (1993) and subsequently, by Zhu et al. (1998), (Tadesse, 2000; Tadesse et al. 2003), Able et al. (2001), Emani et al. (2002) and Girijashankar et al. (2005). Most of the early reports involved transformation with only reporter and marker genes. Casas (1993) introduced the reporter gene *gus* and marker *bar* gene. Transformation of sorghum with an important gene was achieved by introducing a rice *chitinase* (Zhu et al. 1998; Krishnaveni et al., 2000) and a *Bacillus thuringiensis* c*ry1Ac* coding a δ-endotoxin (Girijashankar et al., 2005). Transformation frequency in microparticle bombardment is traditionally low. Success-ful transformation of sorghum has been achieved with low frequencies: 0.008 to 0.33% (Casas et al., 1993, 1997) and 1.5% (Girijashankar et al., 2005). In this inves-tigation, three fertile transformants were developed. The low sorghum transformation frequency could be attributed to various factors that affect the response of sorghum to morphogenesis as well as innate characteristics that make the integration of foreign genes and regeneration of transformed plantlets difficult. The large amount of phenolic compounds produced by sorghum cultures is associated with the low transfor-mation frequency (Cai and Butler, 1990; Casas et al., 1993). Transformation is known to be affected by the physiological condition of the explant (Casas et al., 1993; Emani et al., 2002). Biotic and abiotic stress affects the transformation success (Casas et al., 1993; Emani et al., 2002). Artificial growth conditions in greenhouse marked by low illumination affected the state and quality of the explant used for transformation.

Ex planta assay to fungal infection

Excised leaf segments and seedlings were used in *ex planta* and *in planta* infection assays, respectively. Leaf segments are useful for rapid *in vitro* infection assays (Girgi et al., 2006; Oldach et al., 2001). The transgenic KOSA-1 was found to be more tolerant to *C. sublineolum* than KAT 412, the wild type parent. Comparison was also made between KOSA-1 and selected sorghum cultivars. A statistically significant genotypic difference in response to infection was noted. The transgenic plant was more tolerant to infection than SDH 513. KAT L5 and SDSH 513 were found to be the most tolerant and susceptible cultivars, respectively. This study demonstrated the existence of genetic diversity in the sorghum collection studied that could be exploited for further transformation for anthracnose resistance. A temporal variation in the

development of symptoms was observed. This observation bears a correlation to studies done to profile accumulation of pathogenesis-related secondary metabolites. Young sorghum leaves accumulate a complex of phenolic compounds in response to invasion by both pathogenic and non-pathogenic fungi and the five major components of this complex are the 3-deoxyanthocyanidin flavonoids: apigeninidin, luteolinidin, arabinosyl-5-O-apigeninidin, 7-methylapigeninidin and 5-methoxyluteolinidin (Nicholson et al., 1988; Hipskind et al., 1990; Snyder and Nicholson, 1990; Snyder et al., 1991; Lo et al., 1996; Awika et al., 2004). These responses could form part of the visible manifestation of the response pathogen seen on infected leaves.

Effect of genetic transformation with *harchit* and *harcho*

Comparison of infection assays showed that, introduction of *harchit* and *harcho* significantly protected the transgenic sorghum against *C. sublineolum* under the conditions of this investigation. This demonstrated that, introduction and activation of cell wall degrading proteins such as chitinases and chitosanases can be effective in protecting plants against pathogens. The anti-fungal activity of *harchit* and *harcho* is confirmed in other crops (Lorito et al., 1998; Hendrix and Stewart, 2002). Selected transgenic tobacco and tomato lines containing antifungal endochitinases from the mycoparasitic fungus *T. harzianum* are tolerant to the foliar pathogens *Alternaria alternate*, *Alternaria solani*, *Botrytis cinerea* and the *Rhizoctonia solani* (Lorito et al., 1998). The activity of *harcho* in activating defence mechanism is demonstrated in transgenic tobacco (Hendrix and Stewart, 2002). Chitinases from other sources are also known to be active against fungal pathogens. Zhu et al. (1998) and Krishnaveni et al. (2000) showed the protection of sorghum through the introduction of a rice *chitinase* gene. Barley class II *chitinase* also show activity against fungal pathogens (Punja, 2001). In addition to the chitinase and chitosanase, other antifungal proteins also enhance fungal disease tolerance and resistance in cereals and other crops. Transgenic wheat expressing resveratrol synthase genes *vst1* and *vst2* show a substantial reducetion of disease symptoms caused by *Puccinia recondita f.* sp*. tritici* (Serazetdinova et al., 2005) Other cereals such as pearl millet (*Pennisetum glaucum*) have also been transformed with various antifungal proteins for fungal diseases resistance, for example, antifungal protein (*afp)* from *Aspergillus giganteus* (Girgi et al., 2002; Girgi et al., 2006) and synthetic prawn protein encoding gene (*pin*) (Latha et al., 2006). In wheat, introduction of genes encoding antifungal protein Ag-AFP from the fungus *A. giganteus*, a barley class II chitinase inhibit the spread of *P. recondita f.* sp. *tritici* and *Erysiphe graminis f.* sp*. tritici* (Oldach et al., 2001). This investigation taken into consideration with other sorghum and cereals transformations, demonstrates the potential of enhancing disease tolerance by combining and pyramiding genes encoding antifungal proteins such as chitinase and chitosanase.

REFERENCES

Able JA, Rathus C, Godwin ID (2001). The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. *In vitro* Cell. Dev. Biol. Plant, 37: 341-348.

Awika JM, Rooney LW, Waniska RD (2004). Properties of 3-deoxyanthocyanins from sorghum. J. of Agric. Food Chem. 52(14): 4388-4394.

Becker D, Brettschneider R, Lörz H (1994). Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J. 5: 299-307.

Boora KS, Frederiksen R, Magill C (1998). DNA-based markers for a recessive gene conferring anthracnose resistance in sorghum. Crop Sci. 38: 1708-1709.

Brettschneider R, Becker D, Lörz H (1997). Efficient transformation of scutellar tissue of immature maize embryos. Theor. Appl. Gene. 94: 737-748.

Bueso FJ, Waniska RD, Rooney WL, Bejosano F (2000). Activity of antifungal proteins against mold in sorghum caryopses in the field. J. of Agric. Food Chem. 4: 810-816.

Cabib E (1987). The synthesis and degradation of chitin. In: Meisler A (ed.). Advances in enzymology and related areas of molecular biology. Wiley, New York, pp. 59-101.

Cai T, Butler L (1990). Plant regeneration from embryogenic callus initiated from immature inflorescences of several high-tannin sorghums. Plant Cell Tissue Organ Cult. 20: 101-110.

Casas AM, Kononowicz AK, Zehr UB, Tomes DT, Axtell JD, Butler LG, Bressan RA, Hasegawa PM (1993). Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA, 90: 11212-11216.

Casas AM, Kononowicz AK, Haan TG, Zhang L, Tomes DT, Bressan RA, Hasegawa PM (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. *In vitro* Cell Dev. Biol. Plant, 33: 92-100.

Christensen AH, Sharrock RA, Quail PH (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation, Plant Mol. Biol. 18: 675-689.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19-21.

Emani C, Sunilkumar G, Rathore KS (2002). Transgene silencing and reactivation in sorghum. Plant Sci. 162: 181-192.

Esele JP, Frederiksen RA, Miller FR (1993). The association of genes controlling caryopsis traits with grain mold resistance in sorghum. Phytopathology, 83: 490-495

Garcia I, Lora JM, De la Cruz J, Benítez T, Lobell A, Pintor-Toro JA (1994). Cloning and characterization of a chitinase (CHIT42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr. Genet. 27: 83-89.

Gebeyehu G, Adugna A, Tadesse T, Tesso T, Belete K, Michael H (2004). Development of sorghum varieties and hybrids for dry land areas of Ethiopia and Uganda. J. Agric. Sci. 9: 594-605.

Girgi M, O'Kennedy MM, Morgenstern A, Smith G, Lörz H, Oldach KH (2002) Transgenic and herbicide resistant pearl millet (*Pennisetum glaucum* L.) R. Br. via microprojectile bombardment of scutellar tissue. Mol. Breed. 10: 243-252.

Girgi M, Breese WA, Lörz H, Oldach KH (2006). Rust and downy mildew resistance in pearl millet (*Pennisetum glaucum*) mediated by heterologous expression of the afp gene from *Aspergillus giganteus*. Transgenic Res. 15: 313-324.

Girijashankar V, Sharma HC, Sharma KK, Swathisree V, Prasad LS, Bhat BV, Royer M, Secundo BS, Narasu ML, Altosaar I, Seetharama N (2005). Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*). Plant Cell Rep. 24: 513-522.

Hammer JE, Howard RJ, Chumley FG, Valent B (1988). A mechanism

for surface attachment in spores of a plant pathogenic fungus, Science, 239: 288-290.
Hendrix B, Stewart JMD (2002). Chitosanase may enhance anti-fungal defence response. AAES Research Series 507:2154-217.
Hipskind JD, Hanau R, Leite B, Nicholson RL (1990). Phytoalexin accumulation in sorghum: Identification of an apigeninidin acyl ester. Physiol. Mol. Plant Pathol. 36: 381-396.
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (2010). An Endowment Fund for Sorghum and Pearl Millet Research Enhanced nutritional security, biodiversity and environmental sustainability. www.icrisat.org/Investors/Flyers/59Endowment%20fund_D_scr.pdf. Accessed 14 Jan 2010.
Jambunathan R, Kherdekar MS. Stenhouse JW (1992). Sorghum grain hardness and its relationship to mold susceptibility and mold resistance. J. Agric. Food Chem. 40: 1403-1408.
Krishnaveni S, Jeoung JM, Muthukrishnan S, Liang GH (2000). Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. J. Genet. Breed. 55: 151-158.
Latha AM, Rao KV, Reddy TP, Reddy VD (2006). Development of transgenic pearl millet (*Pennisetum glaucum* (L.) R. Br.) plants resistant to downy mildew. Plant Cell Rep. 29: 927-935.
Lee S, Choi H, Suh S, Doo IS, Oh KY, Choi EJ, Schroeder Taylor AT, Low PS, Lee Y (1999). Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. Plant Physiol. 121: 147-152.
Lo SC, Weiergang T, Bonham C, Hipskind J, Wood K, Nicholson RL (1996). Phytoalexin accumulation in sorghum: Identification of a methyl ether of luteolinidin. Physiol. Mol. Plant Pathol. 49: 21-31.
Lorito M, Woo SL, Garcia I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA, 95(14): 7860-7865.
Machado S, Bynum ED, Archer TL, Bordovsky J, Rosenow DT, Peterson C, Bronson K, Nesmith DM, Lascano RJ, Wilson LT, Segarra E (2002). Spatial and Temporal Variability of Sorghum Grain Yield: Influence of Soil, Water, Pests, and Diseases Relationships. Precision Agric. 3(4): 389-406(18)
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum, 15(3): 473-497.
Ngugi HK, King SB, Abayo GO, Reddy YVR (2002). Prevalence, Incidence, and severity of sorghum diseases in western Kenya. Plant Dis. 86: 65-70.
Nicholson RL, Jamil F, Snyder BA, Lue WL, Hipskind J (1988). Phytoalexin synthesis in the juvenile sorghum leaf. Physiol. Mol. Plant Pathol. 33: 271-278.
Nicholson RL, Epstein L (1991). Adhesion of fungi to the plant surface: Prerequisite for pathogenesis. In: Cole GT and Hoch HC, Eds. The fungal spore and disease initiation in plants and animals. Plenum Press, New York.
Oldach KH, Morgenstern A, Rother S, Girgi M, O'Kennedy MM, Lörz H (2001). Efficient *in vitro* plant regeneration from immature zygotic embryos of pearl millet [*Pennisetum glaucum* (L.) R. Br.] and *Sorghum bicolor* (L.) Moench. Plant Cell Rep. 20: 416-421.
O'Kennedy MM, Grootboom A, Shewry R (2006). Harnessing sorghum and millet biotechnology for food and health. J. Cereal Sci. 44(3):224-235.
Punja Z (2001). Genetic engineering of plants to enhance resistance to fungal pathogens-a review of progress and future prospects. Canadian J. Plant Pathol. 23: 216-235.
Prom LK, Waniska RD, Kollo AI, Rooney WL, Bejosano FP (2005). Role of Chitinase and Sormatin accumulation in the Resistance of Sorghum Cultivars to Grain Mold. J. Agric. Food Chem. 53(14): 5565-5570.
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn, Cold Spring Habour Laboratory, Cold Spring Harbour, New York.
Serazetdinova L, Oldach KH, Lörz H (2005). Expression of transgenic stilbene synthases in wheat causes the accumulation of unknown stilbene derivatives with antifungal activity. J. Plant Physiol. 162(9): p. 985.
Snyder BA, Nicholson RL (1990) Synthesis of phytoalexins in sorghum as a site specific response to fungal ingress. Science, 248: 1637-1639.
Snyder BA, Leite B, Hipskind J, Butler LG, Nicholson RL (1991). Accumulation of sorghum phytoalexins induced by *Colletotrichum graminicola* at the infection site. Physiol. Mol. Plant Pathol. 39: 463-470.
Strauch E, Wohlleben W, Pühler A (1988). Cloning of a phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene, 63: 65-74
Sutton BC (1980). The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata. Commonwealth Mycological Institute, Kew, London, p. 696.
Tadesse YS (2000). Genetic transformation of sorghum (*Sorghum bicolor* (L.) Moench) towards improving nutritional quality. Ph.D. thesis. Vrije Universiteit Brussel Instituut Voor Moleculaire Biologie, Faculteit Wetenschappen, Belgium.
Tadesse Y, Sgi L, Swennen R, Jacobs M (2003). Optimisation of transformation conditions production of transgenic sorghum (*Sorghum bicolor*) via microparticle bombardment. Plant Cell, Tissue Organ Cult. 75: 1-18.
Takashi H (2002). Adventitious shoot regeneration from immature embryos of sorghum. Plant Cell, Tissue Organ Cult. 68(1): 65-72.
Thomas MD, Sissoko I, Sacko M (1996). Development of leaf anthracnose and its effect on yield and grain weight of sorghum in West Africa. Plant Dis. 89: 151-153.
Vander P, Vrum KM, Domard A, Eddine El Gueddari N, Moerschbacher BM (1998). Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. Plant Physiol. 118(4): 1353-1359.
Waniska RD, Venkatesha RT, Chandrashekar A, Krishnaveni S, Bejosano FP, Jeoung J, Javaraj J, Muthukrishnan S, Liang GH (2001). Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold. J. Agric. Food Chem. 49(10): 4732-42.
Wenzel WG, Rooyen PJ (2001). Moisture stress and potential sorghum yield. International Sorghum and Millets Newsletter, 42: 28-29.
Zhao ZU, Cai T, Tagliani L, Miller M, Wang N, Pang H, Rudert M, Schroeder S, Hondred D, Seltzer J, Pierce D (2000) *Agrobacterium*-mediated sorghum transformation. Plant Mol. Biol. 44: 789-798.
Zhu H, Muthukrishnan S, Krishnaveni S, Wilde G, Jeoung JM, Liang GH (1998). Ballistic transformation of sorghum using a rice chitinase gene. J. Genet. Breed. 52: 243-252.