Marker-assisted Screening of Cotton Cultivars for Bacterial Blight Resistance Gene

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Abstract: *Bacterial blight or leaf blight is a common disease of cotton in almost all cotton growing countries, including Tanzania. Bacterial blight is caused by infection of plants with the bacteria (Xanthomonas axonopodis pv. malvacearum) and the use of resistant cultivars is the most effective long-term strategy to manage the disease. The strategy starts with identification of resistant individuals, which can be identified either phenotypically by inoculation or by use of molecular markers linked to genes that confer resistance. The B12 gene is known to confer a high level of resistance to all Xanthomonas axonopodis pv. malvacearum races found in USA and Africa. Four Brazilian and three local cultivars were screened for the presence of SSR (CIR246) and SNP NG0207155 markers linked to B12. The SNP marker showed the greatest frequency of resistance-linked alleles in the cultivar UK08 (85.71%) followed by UK91 (75%),UKM08 and Ipê (25%), Araça(8.33%),Aroeira (7.1%) and the least in Cedro (0%). Comparable results were recorded for SSR marker where the cultivar UK08 presented relatively higher frequency of resistance alleles (85.71%) of samples tested followed by UK91 (68.75%), UKM08 (25%), Ipê, Aroeira and Araça (8%) and the last was Cedro (0%). The results suggest the potential utility of Tanzanian germplasm in breeding for resistance to Xanthomonas axonopodis pv. malvacearum race 18 and the need to purify the same germplasm by marker assisted selection.*

Key words: Bacterial Blight, Cotton, Resistant cultivars, Tanzania.

INTRODUCTION

Cotton is an annual plant that belongs to the family Malvaceae, order Malvales of the genus *Gossypium*. The genus *Gossypium* includes about 50 species of which 45 are diploid (2n = 2x = 26) and 5 are allotetraploid (2n = 4x= 52). Out of these only four species are cultivated, two old world species (*G. arboretum* L. and *G. herbaceum* L.) and two New World species (*G. hirsutum* L. and *G. barbadense* L.) with the former being common to most countries including Tanzania (Iqbal. *et al*, 2001). Cotton is a self-pollinated crop as its pollen is relatively large and heavy, and not easily dispersed by wind (Jenkins 1992). However, cross-pollination occurs at

low rate especially when the insect pollinators are present, a cross pollination rate of 50% has been reported in areas where insect population is high (Xiao *et al*., 2009).

Cotton is ranked second in value among export crops with substantial smallholder farmer involvement in Sub-Saharan Africa after cocoa and its production is widely spread across the continent (Tschirley *et al.,* 2006). In Tanzania it is a source of income for more than 500,000 smallholder farmers and its contribution to the national export earnings is about US$ 92 million (15% of the total national exchange earnings) (TCB, 2010).

Bacterial blight of cotton is one of the major diseases of cotton that occurs in most of the cotton growing areas of the world. It is caused by *Xanthomonas axonopodis* pv. *malvacearum*. In cotton, up to 30% yield has been reported to be lost in Africa and Asia due to this disease (Thaxton and El-Zik, 2001). According to International Cotton Advisory Committee (2003), the incidence of the disease in Tanzania especially in the Western Cotton Growing Area (WCGA) is up to 25% and one cultivar UK 91 had some level of resistance to bacterial blight but none of the commercially available varieties has complete resistance to this disease. In Brazil, the disease is common but resistant cultivar and seed treatment has resulted into good disease management (Uitdewilligen, 2008).

The pathogen (*Xanthomonas axonopodis* pv. *malvacearum*) enters the host plant through either the stomata or wounds and creates water-soaked lesions on leaves, stems and bolls, followed by premature leaf senescence and reduced lint yield (Rungis *et al.*, 2002).

Hunter *et al.* (1968) identified 19 physiological races of *Xanthomonas axonopodis* p v. *malvacearum* and later studies by Verma and Singh (1974) revealed more races that increased the number to 32 races. These races are distributed in different cotton growing countries. Race 1 is widespread in Australia, India and USA whereas races 2 to 5 were recorded in USA and India, race 6 in Nigeria, Zimbabwe and India, and race 18 in Australia, USA, Africa and Nicaragua. Some highly virulent races from Africa and other parts of the world have been reported to break the resistance of cotton cultivars (El-Zik and Thaxton, 1994). Six races (namely 1, 2, 8, 21, 26 and 32) were identified in Syria (Abdo-Hasan, 2002) and four additional races (namely 3, 4, 11, and 28) were recorded between 2003 and 2006 (Abo-Hasan, unpublished data). Information on the specific race of *Xanthomonas axonopodis* pv. *malvacearum* prevalent in Tanzania is lacking, but it is believed to be race 18 which has also been reported in neighbouring countries including Uganda (Akello and Hillocks, 2002). Race 18 is the most virulent and prevalent in almost all cotton producing areas of the world (Brown, 2001).

Measures currently used to manage the disease include enforcing sanitary practices during ginning and seed processing, planting of acid-delinted and fungicide-treated seeds, and destruction of residues from the previous crop, crop rotation and use of resistant varieties. Of all these measures the deployment of resistant varieties is the most effective and economical means to control the disease and minimize yield loss

(Xiao *et al.*, 2009). Sources of stable resistance to all races of *Xanthomonas axonopodis* pv. *vasinfectum* are available (Brinkerhoff *et al.*, 1984) except for new races.
Recent studies by Haidar *et al.,* 2007 and Xiao *et al.,* (2009) have revealed monogenic inheritance of bacterial blight in cotton and the possibility of introgressing resistance into elite cultivars through backcrossing. Identification and selection of resistant germplasm is a vital stage in breeding for disease resistance. This can be done either by artificial inoculation or by use of molecular markers (marker assisted selection). Unlike phenotype based selection, marker assisted selection can be done at any stage of plant growth, it is cost effective and has faster turnaround of information (Xiao *et al*, 2009).

At least 22 resistance genes in cotton that confer varying degrees of resistance to various races of the pathogen (*Xanthomonas axonopodis* pv. *malvacearum*) have been reported. Of these 22 genes, only one gene (designated *B12*) confers a high level of resistance to all *Xanthomonas axonopodis* pv. *malvacearum* races found in USA and Africa (Wallace and El-Zik, 1990). Molecular markers associated with this gene have been identified for use in screening for resistant germplasm. Xiao *et al.* (2009) further reported three SSR markers (BNL3545, BNL3644 and CIR246) closely linked to the B*12* resistance gene in upland cotton (*Gossypium hirsutum L.*). The same authors identified three Single Nucleotide Polymorphic (SNP) markers associated with haplotypes for cotton bacterial blight resistance. These markers are useful in selection and are available that can be used for screening resistant genotypes in breeding populations.

Much success has been achieved in breeding for material blight resistance in cotton and previous studies suggest the possibility of introgressing resistance trait into elite adapted cultivars through crossing (Haidar *et al.*, 2007). Some Brazilian cultivars have also shown resistance to diseases including bacterial blight (Cia *et al.,* 2008; Uitdewilligen, 2008). Such cultivars may provide potential source of resistance to local cultivars. This study under collaboration between Embrapa in Brazil, the University of Dar es Salaam and the Lake Zone Agricultural Research Institute screened selected cultivars from Brazil and some local cultivars for the SNP and SSR markers linked to bacterial blight resistance gene (*B12*).

MATERIAL AND METHODS

Plant materials

Table 1: Plant materials used in the study with their origins

S/No	Name of Cultivar	Origin
1.	UK91	Tanzania
2.	UK08	Tanzania
3.	UKM08	Tanzania
4.	Cedro	Brazil
5.	Aroeira	Brazil
6.	Araça	Brazil
7.	Ipê	Brazil

All Brazilian cultivars were selected on basis of their performance; including disease resistance.

Molecular markers/primers, DNA isolation and amplification

A pair of SSR (CIR246) primers and one SNP (NG0207155) marker were used in this study. The SNP marker was designed from the Genbank SNP accession numbers NG0207155 for bacterial blight resistance. The sequence of SSR (CIR246) primer pair is shown below:

Forward primer	TTAGGGTTTAGTTGAATGG
Reverse primer	ATGAACACACGCACG

Tissues for DNA isolation were obtained from young leaves of the individually tagged Tanzanian and Brazilian cotton plants grown in the field at the age of three weeks after germination. Sampling was done by punching two discs of the same leaf into a single well of a 96-wells plate and dried using silica gel. The samples were analyzed for the SSR and SNP at Embrapa, Brazil and DNA Landmarks, Canada respectively.

For the marker NG0207155, DNA isolation was performed using the DNA Landmarksô standard micro-extraction protocol for all samples at DNA Landmarks, Canada. DNA concentrations were measured using Hoechst dye and the quality of the DNA samples was checked on a 0.8% agarose gel (1 column per plate). Once the DNA quality passed the quality control, the DNA samples were then used for PCR amplification. For the SSR marker DNA isolation was done using the Diversity Array Technology (DArT) protocol and DNA concentration was checked using a Nanodrop 2000 (a micro volume UV-Vis spectrophotometer) then diluted to 10ng/µL for PCR amplification.

The SNP genotyping of all cotton samples was carried out using the DNA Landmark standard protocol. Genotyping data analysis was done with the Sequenom Mass ARRAY® Typer 4.0 software.

For the SSR, the PCR mixture (5 εl) contained 1 εl template DNA (10ng/ εl), 2.5 εl of 2x QIAGEN PCR Master Mix (HotStarTaq DNA Polymerase, PCR Buffer and dNTP Mix), 0.5 εl of Q solution, 0.06 εl of forward and reverse primers and 0.88 εl of sterile double distilled water. Amplification was performed in 0.2 ml (each tube) thin walled PCR plates (96 tubes / plate) in a thermal cycler (Applied Biosystems). The amplification temperatures were: 95^{o}C for 15 minutes (activation) followed by 94^{o}C for 1 minute and 30 seconds then annealing at 55^{o}C for 1 minute followed by extension at 72^{o}C for 1 minute for 40 cycles. The amplicons were genotyped using Genetic Analyzer (Applied Biosystems Inc.) and scoring was done using GeneMapper software.

Allele frequency was calculated as the proportion of all alleles at the locus in each population.

RESULTS

Of the 136 samples of individual plants evaluated, 26.47% showed the presence of the SNP allele associated with bacterial blight resistance gene (*B12*) at NG0207155 locus while 73.53% did not present such allele. The greatest frequency of resistance-linked allele was presented by the cultivar UK08 (85.71%) followed by UK91 (75%), UKM08 and Ipê (25%), Araça (8.33%), Aroeira (7.1%) and the least was Cedro (0%) (**Figure 1a**)

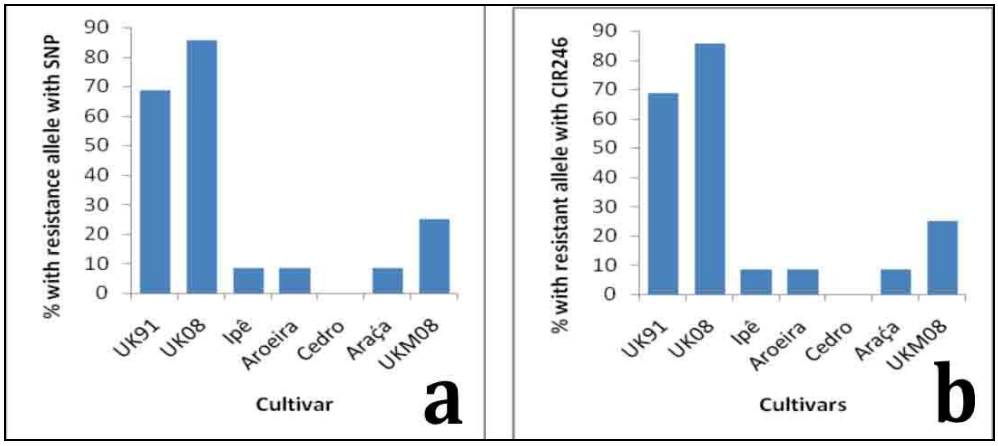

Figure 1(a) Percentage of samples with resistance allele for bacterial blight using NG0207155 marker. (**b)** Percentage of samples with resistance allele for bacterial blight using CIR246 marker.

All the plants that had resistance allele at NG0207155 presented similar results at CIR246 locus (**Figure 1b**) i.e. the two markers used provided consistent results as per two figures above.

DISCUSSION

Existing studies in cotton show that there are variations in resistance to bacterial blight infections among cotton cultivars and that identification of resistant germplasm that are adapted to the local environment is very important in breeding for disease resistance (Xiao *et al.,* 2009). Genotyping elite lines using known markers that flank resistance genes is a useful strategy to identify putative resistant lines. In this study, the local cultivars UK08, UK91 and UKM08 had relatively higher frequencies of alleles linked to a gene for bacterial blight resistance relative to Brazilian cultivars. This strongly suggests that disease resistance is an inherent trait and varies not only among cultivars of the same species but also among individual plants of the same cultivar. Xiao *et al.* (2009) reported similar results when studying bacterial blight resistance among sixteen cultivars using molecular markers. The findings from this study are also consistent with those of Bayles and Verhalen (2007) who used artificial inoculation and observed that cotton cultivars of different origins vary in their response to bacterial blight resistance.

It has also been shown that cotton is not a restricted self-pollinated crop species and has a cross-pollination rate of about 50% depending on insect population in an open field (Xiao *et al.,* 2009). Therefore, available varieties are not true inbreeds, but are relatively heterogeneous in most cases. The presence of a mixture of individual

plants with both resistant and susceptible alleles within the same cultivar as observed in this study strongly suggests that the cultivars screened are not pure inbreeds.

It has further been reported that theresistance of cotton cultivars to *Xanthomonas axonopodis* pv. *malvacearum* race 18 is controlled by one gene *B12* (Rungis *et al*., 2002 and Xiao *et al*., 2009) which is inherited in Mendelian fashion and the presence of resistant and susceptible plants within the same variety implies that continued cross pollination and/or mixing of resistant and susceptible seeds could result in large number of susceptible individuals in the population. This phenomenon might account for the observed incidence of bacterial blight of cotton in the WCGA as the cultivar UK91 was previously known to be resistant to the same disease (ICAC, 2003) but have since shown susceptibility as recorded from field observations. Purification of such variety by marker assisted selection is therefore strongly recommended.

Use of molecular markers, in particular the SSR marker CIR246 and/or the SNP NG0207155 have proven useful indicators for selecting for resistant plants thus facilitating purification of varieties for resistance to bacterial blight through selection of individual plants with resistance gene and subsequently confirming the same with artificial inoculation. The two markers CIR246 and NG0207155 as described by Xiao *et al.* (2009) are associated with a gene for resistance to *Xanthomonas axonopodis* pv. *malvacearum* race 18 which is the most prevalent and virulent in many cotton growing countries of the world (Wallace and El-Zik, 1990). The markers screened out plants that possessed links to the resistance gene and thus are potentially resistant to the pathogenic race (18). Individual plants that had both the SNP and SSR markers should be inoculated with the pathogen to confirm their resistance from which selection can be done.

Molecular markers have long been used for selecting individual plants with desired traits including disease resistance in cotton as they can be applied at any growth stage, have high turnaround information and are less costly (Xiao *et al.,* 2009). Plants with resistance allele at CIR246 locus were confirmed with SNP marker NG0207155 showing that they possess a resistance gene (*B12*). Use of molecular markers is however challenged by mutation of the pathogen, a situation whereby a pathogen can reproduce on plants which were previously resistant to other races making such plants susceptible to the new race (Xiao *et al.,* 2009). For this reason, artificial inoculation may be needed to confirm resistance of the plants identified in this study as possessing resistance gene before they are used for breeding.

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