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CAPS Markers TAO1 and TG105 in the Identification of *I2* Resistant Gene in Nigerian Accessions of Tomato, *Solanum lycopersicum* L.

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Abstract

***Fusarium oxysporum* (f.sp). *lycopersici* (*Fol*) is a soil-borne fungus that inhabits most tomato-growing regions of the world, causing vascular wilt disease. In Nigeria, the disease constitutes close to 40% loss in tomato yield annually. Cleaved Amplified Polymorphic Sequence (CAPS) markers *TAO1* and *TG105*, developed elsewhere, were used to identify tomato accessions possessing the gene (*I2*) which confers resistance to *Fol* race 2. In this work, fifty Nigerian accessions of tomato, *Solanum lycopersicum* L., were screened with the two CAPS markers for resistant *I2* gene. The primer pairs for *TAO1* and *TG105* produced amplification at 902 bp in 33 accessions and 450 bp in 38 accessions, respectively. The restriction enzymes *Fok1* and *Hinf1,* for *TAO1* and *TG105* respectively, produced fragments at base pairs indicative of susceptible, homozygous and heterozygous resistant accessions to *Fol.* Restriction fragments from the two markers indicated that 11 accessions were homozygous resistant to *Fol*. Four of these accessions (Delila, Gem Pride, K-Small and Oxheart) occurred in the two molecular markers as homozygous resistant to *Fol*. The combined effect of the two markers enhanced precision in the identification of tomato accessions with resistance status to Fusarium vascular wilt.**

Keywords: CAPS markers, Fusarium wilt, I2 gene, tomato accessions,

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Introduction

Fusarium oxysporum f.sp. *lycopersici* (*Fol*) is a soil-borne fungus which infects susceptible tomato plants through wounds or direct penetration of roots thereby colonizing the xylem vascular tissue. In resistant plants, the fungus is confined to the lower part of the roots, and additional symptoms do not develop (Ori et al*.,* 1997). Entire plants or plant parts above the point of vascular invasion may die within few weeks after infection (Popoola et al., 2012). Three host-specific races (races 1, 2 and 3) of *Fol* have been described and named in their order of discovery (Beckman, 1987). The *I* (immunity to Fusarium wilt) gene for resistance to *Fol* race 1 was reported in the wild tomato *Lycopersicon pimpinellifolium* accession 160 (PI79532) (Bohn and Tucker, 1939). The *I2* gene conferring resistance to *Fol* race 2 was discovered in PI126915, a *Lycopersicon esculentum* × *L. pimpinellifolium* hybrid (Stall and Walter, 1965). The *I3* gene conferred resistance to race 3 *Fol* and was discovered in *L. pennellii* accessions PI414773 (McGrath et al*.,* 1987) and in accessions LA716 (Scott and Jones, 1989). Ori *et al.* (1997) reported two members of a multigene family designated as

I2C-1 and *I2C-2.* They were only able to provide circumstantial evidence of the genes' involvement in Fusarium resistance (Simons et al*.,* 1998).

In Nigeria and most parts of sub-Saharan Africa, *Fol* Race 2 has been implicated in the devastation of tomato field (Osuinde and Ikediugwu, 2002). The disease had been rightly referred to as farmers' nightmare and total loss had been reported in endemic regions of Nigeria and Ghana (Popoola et al*.,* 2011).

The control of *Fol* involves three strategies: husbandry practices, application of agrochemicals and use of resistant varieties (Barone and Frusciante, 2007). Resistant varieties are mostly produced by crossing resistant wild types and existing cultivars developed for their properties like good taste, shape and color. A molecular marker linked to resistance would be useful for tomato improvement programmes (Staniazsek et al., 2007) as it will improve precision and reduce time.

Screening of accessions under field conditions based on phenotype is time-consuming and challenging. Tightly linked molecular markers associated with resistance genes can improve selection efficiency and avoid associated field problems. Cleaved Amplified Polymorphic Sequence, CAPS, is a PCR-based marker system that could identify the resistant and susceptible genotypes and such findings have been consistent with phenotypic data (Dilip and Feng, 2010). TAO1 and TG105 are co-dominant CAPS markers known to be closely linked to the *I2* gene and could easily be used to identify genotypes possessing the *I2* gene, which confers resistance to *Fol* race 2 (Dilip and Feng, 2010; Staniazsek et al*.,* 2007).

Popoola et al*.* (2012) identified some Nigerian accessions of tomato, *Solanum lycopersicum* L., containing homozygous resistant *I2* gene using Cleaved Amplified Polymorphic Sequence (CAPS) marker TAO1. In this paper, we report a more precise identification of tomato accessions that were resistant to Fusarium wilt using two CAPS markers – TAO1 and TG105.

Materials and Methods

Plant material: Fifty accessions of tomato (*Solanum lycopersicum* L.) were used in the amplification experiment with two CAPS markers – TAO1 and TG105. The accessions (Table 1) were mostly indigenous to Nigerian villages and settlements with little information on presence or absence of *I2* resistant gene. Few accessions were sourced from Ghana, Kenya and Italy. Accessions which produced amplicons with either primer were used in the restriction enzyme digestion.

DNA extraction: Fresh young leaves of 50 varieties/lines of tomato were subjected to DNA extraction, using the procedure described by Doyle and Doyle (1987).

Primer sets for TAO1 and TG105 markers: The forward and reverse primers for TAO1 (Staniazsek et al*.,* 2007) were:-

f: 5'-GGGCTCCTAATCCGTGCTTCA-3'

r: 5'-GGTGGAGGATCGGGTTTGTTTC-3'

For TG105, the set of primers as provided by Dr. M. R. Ercolano, Department of Soil, Plant, Environmental and Animal Production Sciences, DISPAPA, University of Naples, Via Universita, 100 80055 Portici, Italy were:-

f : 5'-CTTCAGAATTCCTGTTTTAGTCAGTTGAAC-3'

r : 5'-ATGTCACATTTGTTGCTTGGACCATCC-3'

CAPS reaction (Akkale and Tanyolac, 2009): The CAPS markers were amplified in 25 µl reaction mixture comprising 20 mM Tris-HCl buffer pH 8.4, 50 mM KCl, 0.8 mM $MgCl_2$, 0.1 mM of each deoxynucleotide, 0.12 mM of each primer, 1 U Taq DNA Polymerase and 30 ng genomic DNA sample as template. The PCR parameters were: 94^{o}C for 5 mins, followed by 35 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 1 min and a final extension time of 7 min at 72^{o}C, using a Biometra THERMAL CYCLER T1.

Restriction enzyme digestion and analysis of amplified products (Staniazsek et al., 2007): PCR amplicons were digested with 5 units of *Fok*I and *Hinf1* restriction endonucleases (New England BioLabs Inc®) for TAO1 and TG105 primers, respectively. The digestion was performed in a 20 µl reaction volume containing recommended buffer (NEBuffer 4) for 1 h at 37°C and an enzyme inactivation step was performed for 20 min at 70°C.

Gel electrophoresis and imaging (Popoola et al., 2012): Amplification products were resolved by electrophoresis in 2% agarose gels under 3 V/cm potential and 100 mA currency in TAE buffer for 3 h. Gels were stained with cybergreen for 30 min and visualized under UV light using Kodak 2D Imaging System. Resolution was against 1 kb DNA ladder.

Bioassay for Fusarium wilt in tomato accessions: Fusarium inocula were prepared according to a technique recorded in Popoola et al. (2012). Fusarium wilt tests were conducted on eleven

Table 1: Sources of tomato accessions

	Accessions	Source	S/N	Accessions	Source
1	Roma round	IARɑT, Ibadan, Nigeria	27	K-Small	Nairobi, Kenya
2	Beske	IARɑT, Ibadan, Nigeria	28	FUNAAB BO1	FUNAAB, Nigeria
3	NG/MR/MAY/09/005	NACGRAB, Ibadan, Nigeria	29	FUNAAB BO2	FUNAAB, Nigeria
4	NG/MR/MAY/09/019	NACGRAB, Ibadan, Nigeria	30	FUNAAB SR1	FUNAAB, Nigeria
5	NG/OE/MAY/09/011	NACGRAB, Ibadan, Nigeria	31	FUNAAB SR2	FUNAAB, Nigeria
6	NG/AO/MAY/09/030	NACGRAB, Ibadan, Nigeria	32	K-Kwung	Nairobi, Kenya
7	GH/11/41	Kumasi, Ghana	33	Tomachiva	NIHORT, Nigeria
8	NG/AA/SEP/09/037	NACGRAB, Ibadan, Nigeria	34	Derica Opa	Imo State, Nigeria
9	Delila	JG Bodunde, FUNAAB, Nigeria.	35.	FUNAAB CG2	FUNAAB, Nigeria
10.	Markis	JG Bodunde, FUNAAB, Nigeria.	36	Derica	Imo State Nigeria
11.	Hypee 45	JG Bodunde, FUNAAB, Nigeria.	37.	Roma VF	IAR, Zaria Nigeria
12	Gem Pride	JG Bodunde, FUNAAB, Nigeria.	38	Gboko	Benue State, Nigeria
13	Perfect Pee	Ajara farm settlement, Lagos	39	Mbase local	Imo State, Nigeria
14	F1 Mongal	Ajara farm settlement, Lagos	40	Omo mola	Olorunda Iwoye Nigeria
15	Pure water	Ile-ona, Ekiti State, Nigeria	41	Ibadan Local	Ibadan market, Nigeria
16.	Santana	Ilaro, Imeko, Ogun State	42	Totem	Ibadan market, Nigeria
17.	Tyre type	Ife-Odan, Osun State	43	Lilly of the Valley	Markurdi, Benue State
18.	Hausa type	Ife-Odan, Osun State	44	Oxheart	FUNAAB, Nigeria
19	UC 82 B	Kano market, Nigeria	45	Zeukertruabe	NIHORT, Ibadan
20	Dankukumi	Kano market, Nigeria	46	NHGB/09/111	FUNAAB, Nigeria
21.	UTC18	Kano market, Nigeria	47	NHGB/09/116	FUNAAB, Nigeria
22	Funavto 98	FUNAAB, Nigeria	48	Brandy wine	Gwagwalada, FCT
23	Ontario	Napoli, Italy	49	Roman FN	JG Bodunde, FUNAAB, Nigeria
24	FUNAAB Oblong	FUNAAB, Nigeria	50	Floradade	JG Bodunde, FUNAAB, Nigeria
25	K-Kibirigwi	Nairobi, Kenya			
26	FUNAAB Small	FUNAAB, Nigeria			

Note: FUNAA B = Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.; NIHORT = National Horticultural Research Institute, Ibadan; NACGRAB = National Center for Genetic Resources and Biotechnology, Ibadan

accessions that showed homozygous resistant polymorphic bands. Control resistant and susceptible accessions were also included as positive and negative checks, respectively. Sandy-loam top soil (60% sand, 25% silt and 15% clay) was heat sterilized with the aid of electric soil sterilizer at 65^{o}C

for 90 minutes. Thirty to thirty-five tomato seeds, surface sterilized with sodium hypochlorite, were sown in plastic buckets measuring 28 cm diameter and 30 cm deep, and containing 8 kg sterilized soil.

The roots of 13 to 14-day-old tomato seedlings were washed in tap water and trimmed. The seedlings were inoculated by dipping the roots of each accession for 10 minutes in the fungal spore suspension. Those dipped in sterile water served as control. Seedlings were then transplanted into pots filled with sterilized soil, kept in the screenhouse, and examined for three weeks for visual disease symptoms - wilt and dessication. At 21-days post inoculation, seedlings were cut at 1 cm above soil level to determine vascular discoloration. A seedling was determined to be susceptible if, according to El-Mohtar et al. (2006), wilt symptoms or vascular discoloration were apparent.

Results

Amplification with CAPS Primer TAO1: Amplification with *TAO1* primers showed the presence of 902 bp fragment in thirty-three out of 50 tomato accessions studied (Fig. 1). Advantage of this primer came from the possibility of further subjection of amplicons to restriction enzyme digestion to observe polymorphisms that are characteristic of susceptible and resistant accessions. CAPS markers could also differentiate homozygous resistant accessions from heterozygous resistant ones.

Amplification with CAPS Primer TG105: A 450-bp fragment was produced during amplification with TG105. This was observed in 38 accessions (Fig. 2). Most of the accessions produced bands at both 902 and 450 bp, suggesting an agreement between the two molecular markers. Eight accessions did not produce amplicons at either 902 or 450 bp indicating these eight accessions did not possess *I2* gene at all, either at recessive or dominant level.

Digestion pattern of TAO1 amplicons using Fok1 restriction enzyme: Amplicons were subjected to restriction enzyme digestion to determine susceptibility and nature of resistance - homozygous or heterozygous for *I-2* resistance gene. PCR products from primers of *TAO1* were digested using restriction enzymes *Fok1*. Restriction fragments, 410 and 390 bp, specific to *I2* homozygous resistance were detected in Accession No. 9 (Delila), 12 (Gem Pride), 27 (K-Small) and 44 (Oxheart) (Fig. 3). No heterozygous resistant fragments (bands at 800, 410 and 390 bp) were detected. Other accessions produced restriction fragments at 800 bp, indicating susceptibility to *Fusarium oxysporum* f.sp. *lycopersici.*

Digestion pattern of TG105 amplicons using Hinf1 restriction enzyme: Restriction enzyme *Hinf1* produced more polymorphic bands than *Fok1.* Ten accessions produced fragments at 350 bp only to indicate homozygous resistance to the pathogen (Fig. 4a). The enzyme also produced fragments at 500 and 150 bp for susceptible accession (Fig. 4b). Fragments at base pairs 500, 350 and 150 would indicate heterozygous resistance, but no accession was found with this trait.

Bioassay for Fusarium wilt in tomato accessions: Four accessions (Delila, Gem Pride, K-Small and Oxheart) showed no symptom of wilt at 21 days after inoculation, although they had some manifestation of vascular discoloration. All other accessions had between 7 and 14% of the seedlings showing visual wilt symptoms. The overall performances of the eleven accessions tallied largely with the results of restriction enzyme digestion (Table 2).

Discussion

Molecular markers have been used successfully to identify tomato accessions with resistant genes to Fusarium wilt (Barone and Frusciante, 2007; El Mohtar et al*.,* 2007; Akkale and Tanyolac, 2009; Popoola et al*.,* 2012). This process of identification is crucial in the development of tomato varieties that are resistant to the highly destructive pathogen, *Fusarium oxysporum* f.sp. *lycopersici,* responsible for the vascular wilt of tomato. Developing resistant varieties is the most environmentally sound and effective strategy for the management of the disease (El-Mohtar et al*.,* 2007).

Marker-assisted screening had reduced a bulk of genetic material to manageable size which could be taken to the field for phenotypic or bioassay confirmation (Popoola et al*.*, 2012). Past workers had observed close correlation between the genotypic and phenotypic determination of resistance to disease, and it will not be out of place in the future to settle only for marker-assisted trait determination. Difficulties encountered during bioassay are pointing to that direction. As observed by Alon et al. (1974) and Sarfatti et al. (1989), bioassays for resistance to Fusarium wilt are time-consuming and complicated by variations in host genotype, inoculum concentration, stage of seedling development, soil temperature and soil contamination by other pathogens or races. Out of fifty accessions studied in this work, seventeen produced no fragment at 902 bp when subjected to

pcr amplification with *TAO1* marker. Thirty-three accessions produced amplicons at 902 bp on this marker.

TABLE 2. Bioassay and restriction enzyme analyses for the identification of tomato accessions resistant to Fusarium wilt

	Bioassay (no. out of 30 seedlings)			Lane number and base pairs of restriction fragments		Genotypic classification based on restriction fragments
Accession	Healthy	Vascular discoloration	Wilted	*Fok1*	*Hinf1*	
GH/11/41	22	5	3	-	7(350bp)	HR
Delila	27	3	0	9(410,390 bp)	9(350bp)	HR
Gem Pride	26	4	0	12(410,390bp)	12(350bp)	HR
UTC18	24	4	2	-	21(350bp)	HR
Funavto 98	25	4	1	-	22(350bp)	HR
Ontario	20	6	4	-	23(350bp)	HR
K-Small	27	3	0	27(410,390bp)	27(350bp)	HR
FUNAAB BO1	24	4	2	-	28(350bp)	HR
FUNAAB BO2	26	3	1	-	29(350bp)	HR
Derica Opa	24	4	2	-	34(350bp)	HR
Oxheart	30	0	0	44(410,390bp)	-	HR

HR = Homozygous Resistant

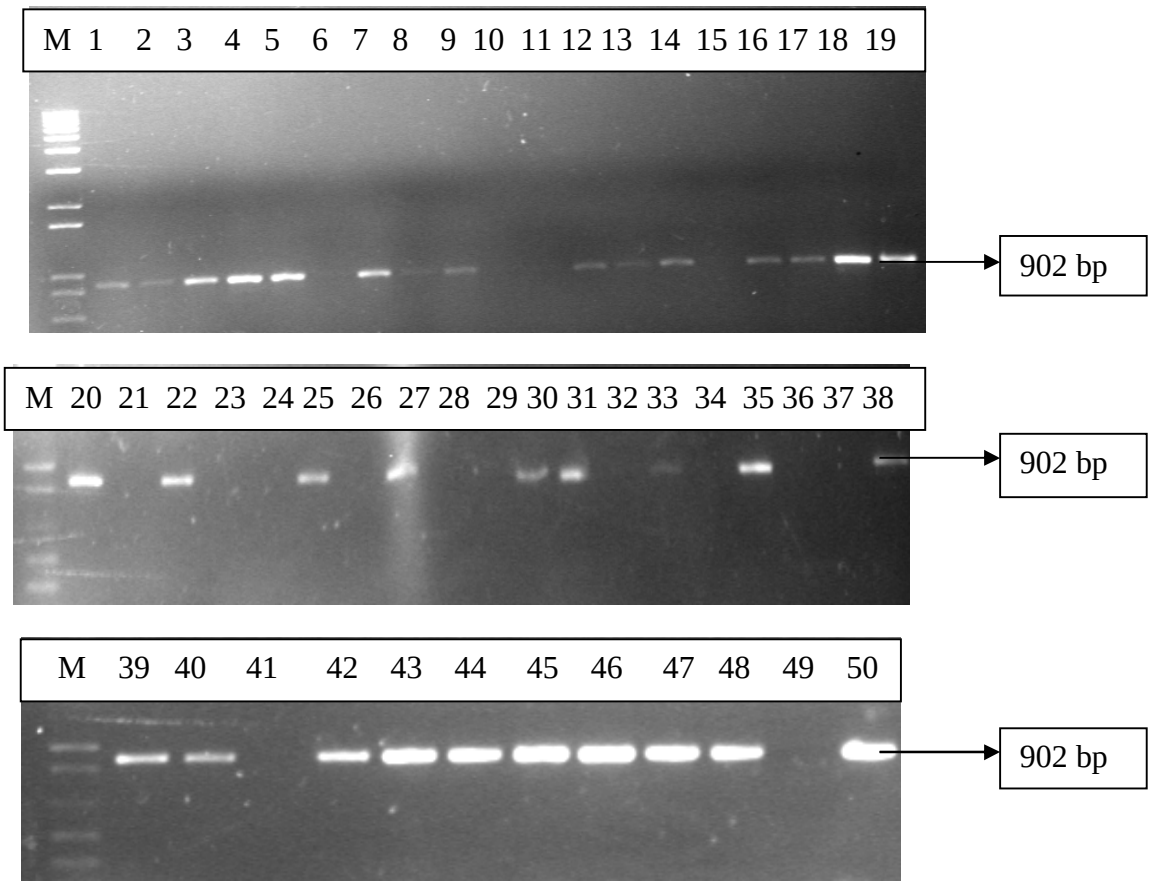

Figure 1. Gel electrophoresis of polymerase chain reaction (PCR)-amplified products of CAPS primer *TAO1* in 2% agarose. Lanes 1 – 50 contained genomic DNA of fifty accessions of tomato and lanes M contained the 1 kb DNA ladder. Thirty-three accessions produced amplicons at 902 bp characteristics of *TAO1*-primed *I2* gene. Seventeen accessions did not produce amplicon.

With the use of *TG105* marker, thirty-eight accessions were amplified at the characteristic 450 bp. The 902- and 450-bp-long fragments in the two markers were found to be polymorphic in ten and four tomato accessions, respectively. Staniazsek et al. (2007) reported that a size of 902-bp-long fragment of *TAO1* marker was polymorphic in both susceptible and resistant tomato plants after further digestion of the 902-bp-amplicon by appropriate restriction enzyme. With restriction enzyme *Fok1*, *I2*-specific restriction fragments were detected in four accessions namely Delila, Gem Pride, K-Small and Oxheart. The fragments were at 410 and 390 bp, suggesting homozygous resistant status. Three of these accessions were equally detected by *Hinf1* in addition to seven more accessions giving a total of ten accessions which produced *I2*-specific restriction fragments at 350 bp corresponding to homozygous resistant genotypes. These fragments showed that both alleles that were related to the *I*-2 gene were present in the accessions.

The two markers (*TAO1* and *TG105*) are complementary and have produced a wider polymorphism, leading to identification of dominant allele of the *I2* gene for resistance to *Fol.* Huang et al. (2005) observed that *TAO1* resided very closely to *TG105*, which was about 0.1 centimorgan (cM) away from *I2.* The homozygous genotypes identified in this work were GH/11/41, Delila, Gem Pride, UTC18, Funavto 98, Ontario, K- Small, FUNAAB BO1, FUNAAB BO2, Derica Opa and Oxheart. Identification of homozygotes for the *I-2* gene is especially important when searching for parent lines for backward integration of the resistant gene to other farmer-preferred varieties.

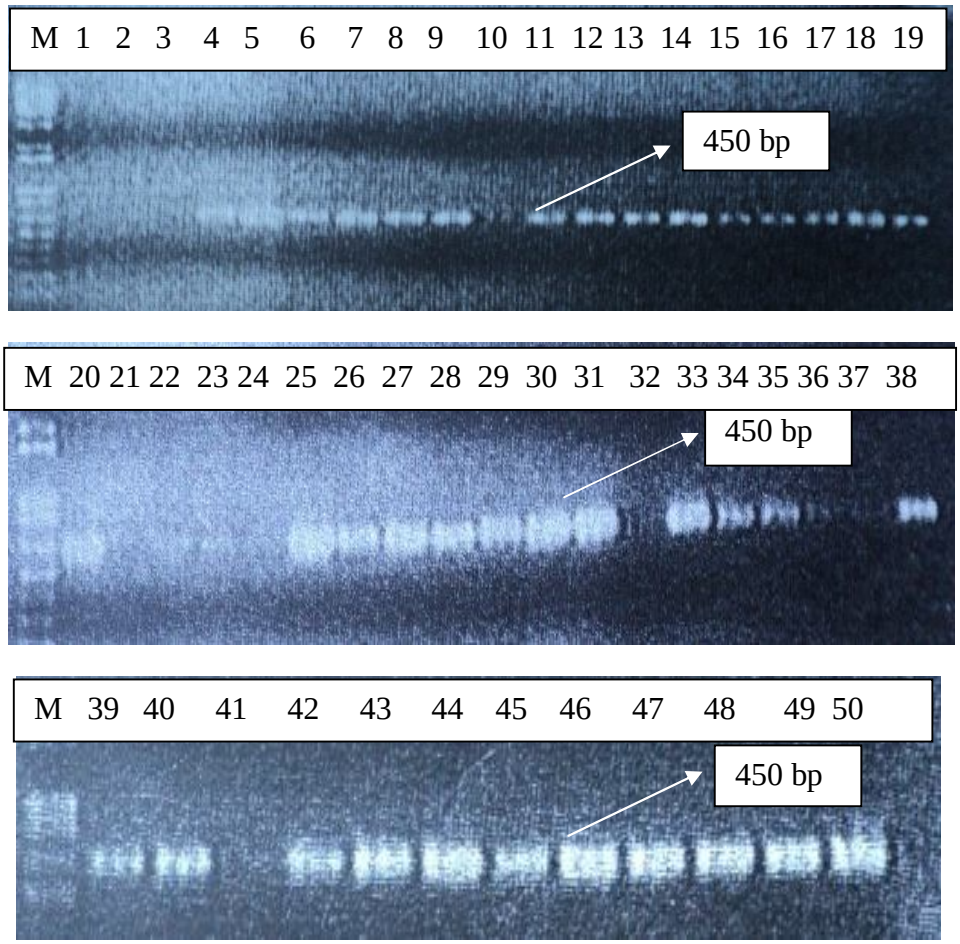

Fig. 2. Gel electrophoresis of PCR-amplified products in 2% Agarose using *TG105* primers. Amplification was at 450 bp. Twelve accessions were not amplified. Lanes 1 – 50 contained genomic DNA of fifty accessions of tomato and lanes M contained the 1 kb DNA ladder.

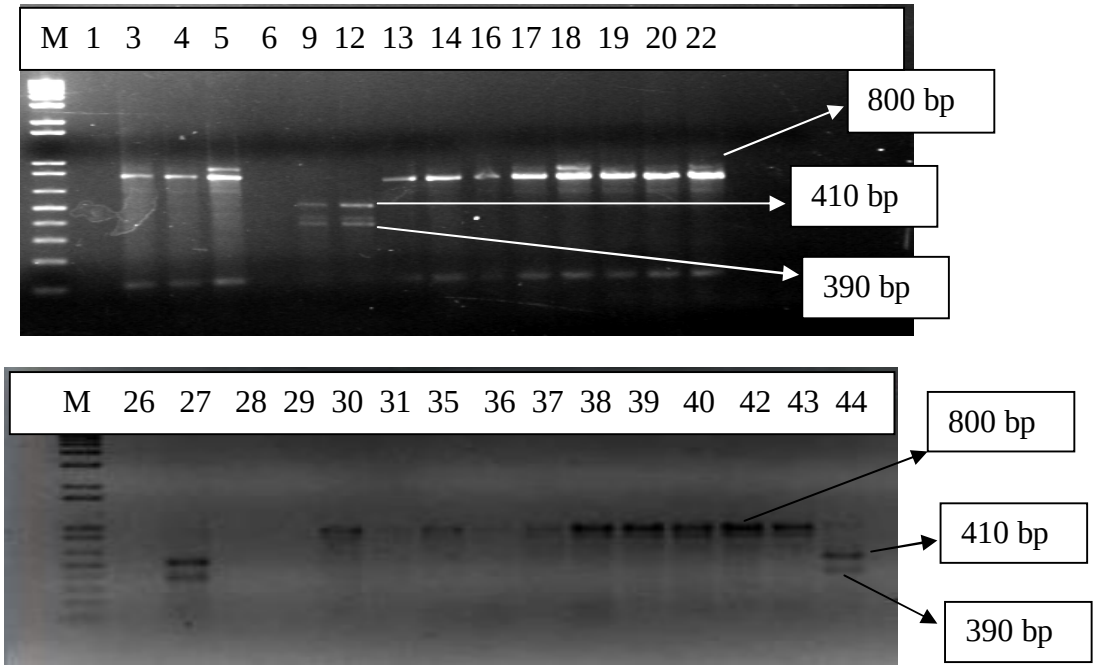

Fig. 3. Restriction enzyme (*Fok1*) patterns of amplicons obtained with CAPS marker *TAO1*. Susceptible accessions amplified at 800 bp while homozygous resistant ones (9, 12, 27 and 44) amplified at 410 and 390 bp. Numbered lanes contained genomic DNA of tomato accessions while lane M contained the 1 kb DNA ladder.

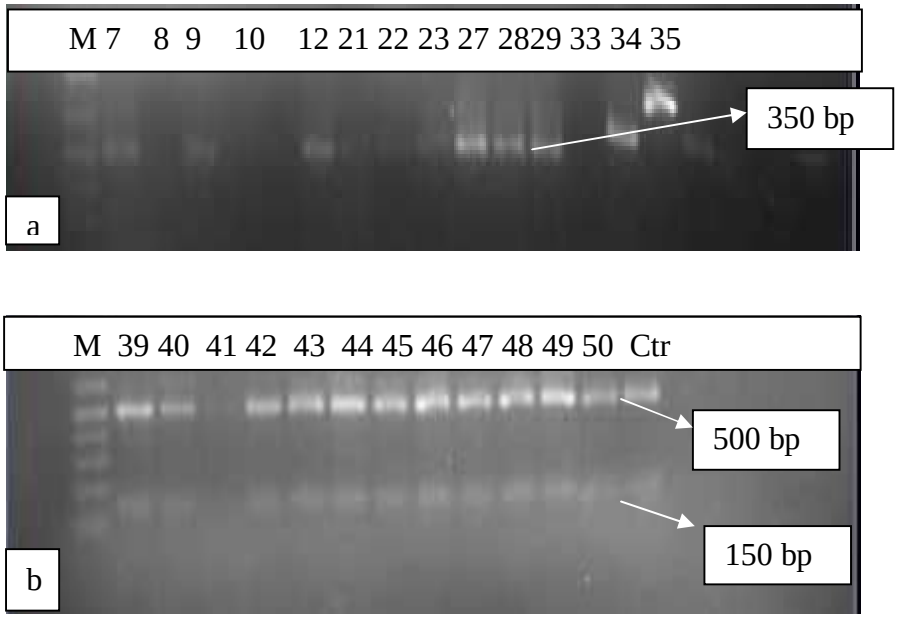

Fig. 4. Gel electrophoresis of *Hinf1* restriction enzyme patterns of CAPS marker TG105 amplicons with homozygous resistant tomato accessions (**a)** showing bands at 350 bp and susceptible ones having bands at 500 and 150 bp **(b).** Numbered lanes contained genomic DNA of tomato accessions while lane M contained 1 kb DNA ladder.

Conclusion

This work had combined the diagnostic potentials of two sets of molecular markers in a process that provided additional advantage of identifying co-dominance through the use of Cleaved Amplified Polymorphic Sequence. This is an advantage over our earlier use of a single molecular marker which was able to identify only four accessions with homozygous resistance to Fusarium wilt. With the two markers, a further identification of seven more accessions had been achieved. With this, we have a wider choice of genotypes in marker-assisted selection of wilt-resistant tomato genotypes for breeding purposes.

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