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Full Length Research Paper

Microsatellites markers associated with resistance to flower bud thrips in a cowpea F_2 population derived from genotypes TVU-123 and WC36

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Breeding for resistance to flower bud thrips (*Megalurothrips sjostedti*) in cowpea has been hindered by the quantitative nature of resistance. To identify simple sequence repeat (SSR) markers associated with resistance to flower bud thrips that could be used for marker-assisted breeding, a F_2 population was generated from a cross between genotypes TVU-123 (resistant) and WC36 (susceptible). The population was evaluated for thrips damage scores, thrips counts, and pods number per plant under artificial infestation. Sixty-six microsatellites markers were screened between the two parental lines and seven polymorphic markers were used to genotype 100 F_2 plants. Single marker analysis was used to evaluate an association between the markers and traits. Transgressive segregation among the F_2 plants for resistance to flower thrips was observed. A significant negative relationship was observed between thrips damage scores and pods number per plant. Markers CP37/38 and CP215/216 were significantly associated with thrips damage scores and thrips counts, respectively. The two markers explained 7 and 11.2% of the total variation in thrips damage scores and thrips counts with positive and negative effects, respectively. Mainly additive gene effects were observed. A more detailed study using more markers on these loci should provide better understanding of this complex trait.

Key words: Cowpea, single marker analysis, polymorphism, simple sequence repeat (SSR) markers.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.), is one of the most important vegetable legumes in Africa (Olawale and Bukola, 2016). It is grown principally for its grains, fresh leaves and immature pods which are consumed fresh or

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as cooked pods (Dungu et al., 2015). It is an important source of dietary proteins, amino acids, vitamins and minerals for African peoples (Boukar et al., 2016). However, cowpea production is constrained by a complex of insects throughout its life cycle and also during seed storage (Boukar et al., 2016). One of the most devastating of these pests is the cowpea flower bud thrips (*Megalurothrips sjostedti* Trybom), which can inflict substantial yield losses, reaching 100% in cases of severe infestation (Sobda et al., 2017). Thrips nymphs and adults damage the plant by feeding on its flowers, resulting in at best, their distortion and discoloration, and at worst, their abortion and consequent yield reduction (Sani and Umar, 2017). The insects are especially difficult to control because of their wide host range and thrips populations build up rapidly and their ability to fly in mass helps them to spread and form colonies in a new population of host plants in a short period (Sani and Umar, 2017). Currently, the most effective control measure available is to apply repeated doses of insecticide, but even this strategy is not fully effective as the ability of some of the insects to escape the spray by sheltering within the flower can drive the rapid development of insecticide resistance (Mohammad et al., 2018).

The majority of resource-poor farmers are in any case unable to afford the purchase of both the necessary chemicals and effective spraying equipment (Mohammad et al., 2018). A more sustainable approach would be to deploy genetic resistance against infestation, which may be feasible, since several cowpea accessions have been shown to suffer only limited damage when infested by thrips. The resistance to flower bud thrips has been reported to be quantitative, thus controlled by several genes (Omo-Ikerodah et al., 2008). Like most economically important traits, resistance to flower thrips in cowpea is controlled by genes located in regions known as quantitative trait loci (QTLs) (Adetumbi et al., 2016). In dealing with quantitative traits, molecular breeding requires the mapping of QTLs associated with the traits under consideration to enable marker-assisted breeding and individual gene cloning (Muhammad et al., 2018). With the help of molecular markers linked to QTL, the heredity of some related complex traits such as thrips resistance could be tracked (Muhammad et al., 2018). The ability of genetic manipulation through QTL analysis is greatly enhanced, thus improving the accuracy and predictability to select genotypes with superior quantitative trait loci (Muhammad et al., 2018). Information generated on QTL associated with resistance to cowpea flower bud thrips would facilitate the development of molecular marker to be used in breeding for thrips resistant cowpea. However, there is limited information on the molecular genetics of thrips resistance.

Few studies reported the detection of QTL for resistance to cowpea thrips, *M. sjostedti* (Omo-Ikerodah et al., 2008; Sobda et al., 2017) and *Frankliniella* sp. (Muchero et al., 2010). Muchero et al. (2010) identified three QTL for resistance to foliar thrips (*Thrips tabaci* and *Frankliniella schult* Zeiusing) using amplified fragment length polymorphism (AFLP) markers. The QTL were designated *Thr-1, Thr-2* and *Thr-3*, and were identified on linkage groups 5 and 7 on 127 cowpea recombinant inbred population. Huynh et al. (2015) identified one major and one minor QTL conferring aphid resistance on LG7 and LG1, respectively, with both favorable alleles contributed by IT97K-556-6. Omo-Ikerodah et al. (2008) used a cowpea linkage map of AFLP markers to identify QTL for resistance to flower bud thrips (*M. sjostedti*) using a set of 92 recombinant inbred lines (RILs) derived from a cross between 'Sanzi' (resistant) and 'VITA7' (susceptible) lines in Nigeria. Five QTL were identified and arranged according to their contributions to resistance of flower bud thrips in descending order as follows: LG3 (E-ACT/M-CAA376), LG2 (E-ACG/M-CTT2), LG6 (E-AAC/M-CTA120), LG7 (EAAC/ M-CAA155) and LG1 (E-AAC/M-CAA255). The QTL were designated *FTh1, FTh2, FTh3, FTh4* and *FTh5* and the phenotypic variance explained by the QTL were 32.0, 18.4, 12.6, 11.9 and 9.5%, respectively. Sobda et al. (2017) identified three QTL on flower bud thrips using SNP markers on F2 population from Sanzi x VYA. The three QTL for thrips resistance were *Fthp28*, *Fthp87* and *Fthp129*, detected on chromosomes 2, 4 and 6 and explained 24.5, 12.2 and 6.5% of the total phenotypic variation, respectively. Most of these QTL identified, except for Muchero et al. (2010) and Sobda et al. (2017) were mainly based on dominant markers, AFLP markers. According to Kongjaimun et al. (2012), dominant markers are not suitable for marker-assisted selection and comparative genomics studies. In addition, none of these QTL has been validated for maker-assisted selection. Additional identification of the molecular co-dominant markers associated with resistance genes controlling flower thrips would be extremely beneficial because plant breeders could use such markers during preliminary selection process to track the loci in existing population or to pyramid resistance into new populations. Such information would allow much faster progress in breeding for resistance to flower thrips, mostly with respect to the modern plant breeding methods such as marker-assisted selection (MAS). Therefore, the objective of this study was to identify simple sequence repeat (SSR) markers associated with flower thrips resistance in cowpea, in order to provide the basis for marker-assisted selection.

MATERIALS AND METHODS

Mapping population

The parents used in this study were TVU-123 (resistant parent) (IITA, 1996) and WC36 (susceptible parent) (Agbahoungba et al., 2017). TVU-123 (female parent) and WC36 (male parent) were crossed and F_1 seeds were grown in plastic pots to generate 212 F_2

seeds.

Testing for resistance to flower bud thrips

The F_2 and parents seeds were planted in pots of 21 cm diameter and 25 cm in depth filled with 15 kg sterilized topsoil. Each pot contained a single F_2 plant and pots were placed under a cage of 10 m length, 3 m width and 2 m height at Makerere University Agricultural Research Institute of Kabanyolo. Flowers containing flower bud thrips were collected from a susceptible cultivar (WC36) planted in the field and introduced into the screen house 20 days after sowing by dropping 30 flowers in each pot (Omo-Ikerodah et al., 2008; Sobda et al., 2017). Subsequently, flowers loaded with flower bud thrips were introduced into the cage on a daily basis for 15 days until a high population of the insects was achieved. Plants were scored for thrips damage 30 days after planting and at weekly intervals for four weeks. Thrips damage was scored using a 1-9 scale (Jackai and Singh 1988), where 1 = highly resistant and 9 = highly susceptible. The number of nymphs and adults thrips per flower was also recorded 30 days after planting and at weekly intervals for four weeks. The number of pods per plant was recorded once at podding stage.

DNA extraction, purification and quantification

Newly expanded leaves from 2 to 3 weeks old seedlings were collected from 100 F_2 progeny and the parents. The 100 plants were representative of the 212 F2 plants as they were selected based on the phenotypic distribution pattern (highly resistant, moderately resistant, susceptible and highly susceptible) of the F2 population to run a cost effective DNA extraction and F2 genotyping. Total genomic DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) extraction method (Lodhi et al., 1994) and purified using the AccuPrep® PCR purification Kit protocol (Cat.No.K-3034, K.3034-1; www.bioneer.com). DNA concentration was determined at 260 nm using a bio-spectrometer (Nanodrop).

Microsatellite analysis

Sixty-six SSR markers were selected from the cowpea SSR database (http://cowpeagenomics.med.virginia.edu/CGKB/). Sequences were synthesized at the Biosciences Laboratory, Bioneer (South Korea). The primers names, sequences, length and the fragment size are presented in Table 1. The SSR markers were randomly selected from the cowpea database since none of these markers has been associated with any insect pest yet.

PCR amplifications were conducted in a 10 µl reaction volume containing 5 µl premix (PCR mater mix containing 100 mM dNTPs, 0.1 taq polymerase), 0.70 µl of primers (0.35 µl of forward primer and 0.35 µl reverse primer) and 1 µl genomic DNA (20 ng), and diluted with 3.3 µl of water (Cat.No.K-3034, K.3034-1; www.bioneer.com). Amplifications were performed in an Eppendorf Mastercycler (Techne TC-512) with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. Amplification products were resolved for 2 h at 130 V on 2.5% (w/v) agarose gel in 1 × TAE buffer using a gel electrophoresis apparatus (Model V16.2 Gibco BRL, Gaithersburg, MD, USA). Gels were stained with ethidium bromide and visualized using a UV transilluminator (M-15 UVP Upland, CA 91786 USA) and photo-documented with a digital camera. DNA fragment sizes were determined based on a 100 bp DNA standard ladder (Bioneer C&D Center, South Korea).

SSR markers were initially screened for polymorphism between the parental genotypes TVU-123 and WC36. Markers that showed clear polymorphic bands were selected to analyze the F2 population. Each amplified loci was considered as a unit character and was scored as “0”, “1” and “2” where, “0” corresponded to amplified loci in WC36, “2” in TVU-123 and “1” when the amplified loci of both parents are present.

Statistical analysis

The distribution histograms of the phenotypic data (thrips damage scores and thrips counts) were generated on the whole population generated from the cross, TVU-123 x WC36. The relationship between thrips damage scores and number of pods per plant was established using Genstast software (Payne et al., 2009). Chi-squared (χ^2) tests were performed to examine the goodness of-fit between the expected Mendelian ratio for the F2 populations (1:2:1 for the SSR markers based on 100 plants). Single-marker analysis (single-point analyses) was employed to determine markers associated with the phenotypic data using GenStat 12 version software (Payne et al., 2009). Chi-square independence test was used on the thrips damage score because the scores collected were grouped into resistant and susceptible classes. Analysis of variance (ANOVA) was performed on the markers scores for the thrips counts. The ANOVA assumptions have been verified before analyzing the data. Linear regression was also performed to estimate the phenotypic variation arising from the QTL linked to the marker. All phenotype analyses were however performed on untransformed data. Normalizing data through transformation may misrepresent differences among individuals by pulling skewed tails towards the center of the distribution (Omo-Ikerodah et al., 2008).

Recombination frequency between two marker loci ($\hat{r}$) and the estimation of maximum likelihood (LOD) of the recombination frequency was computed using the procedure described by Xu (2013):

$$\hat{r} = \frac{n_r}{n}$$

$$s.e_{(\hat{r})} = \sqrt{\frac{\hat{r}(1-\hat{r})}{2n}}$$

$$\mathrm{L}OD = \frac{\lambda}{2\ln(10)}$$

$$\lambda = -2[L\left(\frac{1}{2}\right) - L(\hat{r})]$$

$$L(\hat{r}) = n_r \ln(\hat{r}) + n_p \ln(1-\hat{r})$$

$$L\left(\frac{1}{2}\right) = -n\ ln2$$

Where, $\hat{r}$ is the estimate of the recombination frequency between two loci, n_r is the number of recombinants, n_p is the number of parental gametes and n is the total number of individuals.

In linkage analysis, a LOD score of 3 or larger is generally taken as evidence of linkage, whereas a LOD score smaller than 3 is not considered as a proof of linkage (Xu, 2013).

RESULTS

Distribution of thrips damage scores, thrips counts and pods numbers for the F2 population

The F2 population displayed a continuous distribution for flower thrips damage scores and thrips counts (Figure 1A and B). The distributions of the thrips damage scores and

Table 1. Primers name, starting and ending points, sequence information and fragment size of cowpea derived microsatellite primers used in this study.

Name	Direction	Start points	End points	Length	Primer sequence	Fragment size
[SSR-6169]	Forward	32	52	20	ACCCAAGGACTTCAAGAGCA	
CP1/CP2	Reverse	613	633	20	CGAGTGCAAGAAATGGTTCA	603
[SSR-6170]	Forward	5	25	20	ACCTGCATTGCCTCATATCC	
CP3/CP4	Reverse	488	508	20	GCTGATTCGGCTTGTTCTTC	505
[SSR-6171]	Forward	22	42	20	ATTCGATCCAACCCAATGAC	
CP5/CP6	Reverse	509	529	20	AGCGAAGGCATGTTCGTAAG	509
[SSR-6172]	Forward	25	45	20	GGAAGACACGCGTTATGGTT	
CP7/CP8	Reverse	575	598	23	TTTTTCCACTAAAAGGTTTGTCA	575
[SSR-6173]	Forward	70	90	20	AGATCCCACGCTGATTATGG	
CP9/CP10	Reverse	606	626	20	ACTTGACGCAGAGCCATCTT	558
[SSR-6174]	Forward	48	68	20	TCCTTAGAGGTCCAGCCAGA	
CP11/CP12	Reverse	568	588	20	GGAGGAAGAGAGCACACACA	542
[SSR-6175]	Forward	37	57	20	GCAAGCTTTTGGAAGTTGGA	
CP13/CP14	Reverse	572	592	20	GGCCAGAAGCATGAATCACT	557
[SSR-6176]	Forward	103	123	20	GCCACAAGTGCTTGAAGTGA	
CP15/CP16	Reverse	622	642	20	CCACGTAACGAGGATCAACA	541
[SSR-6177]	Forward	0	22	22	GTAAGTGGGATTCTTATTGTTG	
CP17/CP18	Reverse	620	642	22	CAAGAACCTTACTCTAGATACC	644
[SSR-6178]	Forward	309	335	26	GAAAAAATCACACACACCAAAATTTG	
CP19/CP20	Reverse	691	715	24	CAATCGACTGATTTCACTTAAGTC	408
[SSR-6179]	Forward	237	264	27	GGATTCAAGAATATTGGTGTTTTCTCC	
CP21/CP22	Reverse	634	660	26	TGCCATCTCTTATCAAGACACTTTAG	425
[SSR-6180]	Forward	268	288	20	CCCCATAAACCATTGCTACG	
CP27/CP28	Reverse	442	462	20	AAGTGTAAGCCTGCCGAAGA	196
[SSR-6181]	Forward	72	92	20	AATGACCCACAAAGCAAAGT	
CP29/CP30	Reverse	352	372	20	TTGGCCCAAAATATCACACA	302
[SSR-6182]	Forward	0	23	23	ATGAACCTACTCCTAAACAGAAC	
CP33/CP34	Reverse	265	290	25	GGATGCATAGAGACTGTCAAAATTA	292
[SSR-6183]	Forward	185	207	22	CCTAAGCTTTTCTCCAACTCCA	
CP35/CP36	Reverse	316	336	20	CAAGAAGGAGGCGAAGACTG	153
[SSR-6184]	Forward	334	354	20	CTGGGACCACTTCCTTTTCA	
CP37/CP38	Reverse	543	563	20	GGATGGCTCCAGAAAGAGTG	231
[SSR-6185]	Forward	385	405	20	CGGAAAAGTAGAGGGCACAG	
CP39/CP40	Reverse	582	602	20	AGAGGTTTGATACGCGCACT	219
[SSR-6186]	Forward	357	377	20	GGGATCATGGGATAGGGATT	
CP45/CP46	Reverse	600	628	28	CTATATTAAATTCCTACATTAGATCAGG	273
[SSR-6187]	Forward	338	358	20	ACCGCCTAACCCAAGAGTTT	
CP47/CP48	Reverse	596	616	20	TGGGACCACTTCCTTTTCAG	280

Table 1. Contd.

[SSR-6188]	Forward	462	482	20	ACCAGGTGCAATGCTTCTCT	
CP51/CP52	Reverse	591	611	20	CCACACCCTGTTCCGTACTC	151
[SSR-6189]	Forward	65	85	20	CTCAATGTCCAACCAGGTCA	
CP55/CP56	Reverse	226	246	20	CAACTCACCAAAGGGAAGGA	183
[SSR-6190]	Forward	171	191	20	CGAGTTGCGATATCTCCCTG	
CP57/CP58	Reverse	593	613	20	CGAAGACGACAACACAGTGG	444
[SSR-6191]	Forward	4	28	24	AAACTGCTAACCAGAAACAGAAAA	
CP59,CP60	Reverse	315	335	20	TGTCAATTTTGTTGGCCTCA	333
[SSR-6192]	Forward	243	263	20	AACGGGTCCTAAACGAATGA	
CP61/CP62	Reverse	476	496	20	ATCCTTGAACTCCGTGTTGC	255
[SSR-6193]	Forward	197	217	20	ACCAAAGCAACACCAACACA	
CP63/CP64	Reverse	383	403	20	GATGTGGGAAGAAGCTGAGG	208
[SSR-6194]	Forward	506	526	20	CACACACAAGGTGGGTCTCA	
CP65/CP66	Reverse	636	656	20	TTTGGGACCGTGTCTTCCTA	152
[SSR-6195]	Forward	398	418	20	GATGCTGGTGCTTGTATGGA	
CP67/CP68	Reverse	559	582	23	TAATTTCTACGCAAGGGAGAGAG	186
[SSR-6196]	Forward	204	224	20	TGAAAGAATCCTCGTCATCG	
CP69/CP70	Reverse	364	384	20	TCAGGTCCAAAGAGCCAAAC	182
[SSR-6197]	Forward	307	327	20	CATGGCTATCATGGGTCCTT	
CP71/CP72	Reverse	488	510	22	TGATGTACGGAGTGAAGGAAGA	205
[SSR-6198]	Forward	485	505	20	TGAAGCAAAGGGAGTTGTGA	
CP73/CP74	Reverse	627	647	20	GAAAGCCCAAAAGGGAAAAA	164
[SSR-6199]	Forward	0	25	25	TGGAAAATTGGTGTTATTAAAGTAT	
CP75/CP76	Reverse	157	177	20	ATGGGGATTTGCTTCCTTGT	179
[SSR-6200]	Forward	370	390	20	CCAGACAGTGCATCCCATAG	
CP77/CP78	Reverse	603	625	22	GCGTTGATTTATGGACATTCAA	257
[SSR-6201]	Forward	540	560	20	TGGGCACTATTCCATGCTTT	
CP79/CP80	Reverse	669	689	20	ATTGCAATATCAGTTTTTTC	151
[SSR-6202]	Forward	48	68	20	ACATGCAAAACGTGAAAGCA	
CP81/CP82	Reverse	288	308	20	GGTTGAGTCGAGGGATTTGA	262
[SSR-6258]	Forward	236	257	21	GGTTTCCTAGTTGGGAAGGAA	
CP201/CP202	Reverse	474	494	20	ATTATGCCATGGAGGGTTCA	260
[SSR-6259]	Forward	143	164	21	CCTTCATAAAGACCACGTCCA	
CP203,CP204	Reverse	337	358	21	TGTTGCTCAAATTTCCAGCTT	217
[SSR-6260]	Forward	10	35	25	AAAGTTTTAATATTACCAACAACAA	
CP205/CP206	Reverse	268	288	20	CAACCAGGCAAATGGAAATC	280
[SSR-6261]	Forward	7	29	22	TTCTGTAACGCCGTTTAAATCA	
CP207/CP208	Reverse	208	228	20	TGCAACTGCAATCCAATGAT	223
[SSR-6262]	Forward	18	42	24	CAAGAAGAGGAAACTGAACTGTGA	
CP209/CP210	Reverse	107	127	20	AGCTTCTTGGTCCTGTTCCA	111

Table 1. Contd.

[SSR-6263]	**Forward**	**503**	**523**	**20**	**GCTGGCTCAACAGTCACCTT**	
CP211/CP212	Reverse	596	615	19	GGGAACCTCCCCTACTGGT	**114**
[SSR-6264]	Forward	30	55	25	AAAAAGGAATTTAACCTTCTAAAAT	
CP213/CP214	Reverse	318	341	23	TTTTTGTGGTAGATTTTATTGCT	313
[SSR-6265]	Forward	221	242	21	CAGAAGCGGTGAAAATTGAAC	
CP215/CP216	Reverse	438	458	20	GCATGTTGCTTTGACAATGG	239
[SSR-6266]	Forward	212	232	20	AAGTTGTTCCACCCCACTGT	
CP217/CP218	Reverse	396	417	21	TTTCCTTCCATTTTCATGGTG	207
[SSR-6267]	Forward	145	169	24	CAAGAAGAGGAAACTGAACTGTGA	
CP219/CP220	Reverse	234	254	20	AGCTTCTTGGTCCTGTTCCA	111
[SSR-6268]	Forward	230	250	20	GCAAAGGGATCACCAAACAT	
CP221/CP222	Reverse	397	414	17	TCGTTCAGTTGAGCCAC	186
[SSR-6269]	Forward	31	51	20	GACCATGGCACAATTCTTCA	
CP223/CP224	Reverse	207	230	23	TTAAGTGAAGCATCATGTTAGCC	201
[SSR-6270]	Forward	116	136	20	TCCTCCCACACTTGGAAATC	
CP225/CP226	Reverse	367	387	20	TATGCGAAAAGGGATTGCTC	273
[SSR-6271]	Forward	262	282	20	CGAAATATGTCCCCAAAACG	222
CP227/CP228	Reverse	462	482	20	TGCGTGGTTGGATAGACTCA	
[SSR-6272]	Forward	163	183	20	GCCAAAAGTTTGGTGCAACT	
CP229/CP230	Reverse	314	334	20	TAGCCCTCGTAAGGAATCCA	173
[SSR-6273]	Forward	528	550	22	CCCCCAGAACAAATAGAAACTC	
CP231/CP232	Reverse	698	721	23	TGAATTTGAAGAAGAGATGGTTG	195
[SSR-6274]	Forward	57	82	25	TCAAATAGAAAGAAAAACAAGAAAT	
CP233/CP234	Reverse	142	162	20	TTCTCAACGTGCTGCTTCTG	107
[SSR-6275]	Forward	100	121	21	CAGGTGAAAAATTGCAAAAGG	
CP235/CP236	Reverse	435	455	20	GGCTGCTTGGAGCTTGTAGA	357
[SSR-6276]	Forward	566	586	20	TCAACGTGGTTTGGAACGTA	
CP237/CP238	Reverse	694	716	22	CGATTAGACTGGTCTTTGCTCA	152
[SSR-6277]	Forward	284	303	19	CACCCCCGTACACACACAC	
CP239/CP240	Reverse	416	439	23	CACTTAAATTTTCACCAGGCATT	157
[SSR-6278]	Forward	4	26	22	TGGCTTGAGTACTCTTGGATCA	
CP241/CP242	Reverse	300	320	20	AGCAACCAAAACACCCAAAA	318
[SSR-6279]	Forward	96	116	20	AGGGCCCTCCAATCTGTTAT	
CP243/CP244	Reverse	428	448	20	TGTCTTTCCCCACTCAATCA	354
[SSR-6280]	Forward	4	26	22	GTTATCAGATCTGGTCAGATGC	
CP245/CP246	Reverse	102	121	19	GAAGAAACCACCCGACCAT	119
[SSR-6281]	Forward	323	343	20	GCATCAATTTGAGCGAGGAT	
CP247/CP248	Reverse	498	518	20	GAGTGACATTTCCGCGTCTT	197

Table 1. Contd.

[SSR-6282]	Forward	352	374	22	CCAAAATTAAAGTGCAAGCTCA	
CP249/CP250	Reverse	431	451	20	TCTTTGGATGGGATGAGAGC	101
[SSR-6283]	Forward	373	393	20	GTGCATCGGGAAAAAGAAAA	
CP251/CP252	Reverse	552	572	20	GAAGCGAGGGAATTATGCAG	201
[SSR-6284]	Forward	38	60	22	GAAAGGGAAGGATTATGGGATA	
CP253/CP254	Reverse	190	210	20	GGCAAATAGCGGGGTAGAGT	174
[SSR-6285]	Forward	4	32	28	AACTATTTTCATCTTAAATATACGTCTT	
CP255/CP256	Reverse	142	166	24	TTCATAACTCTAATTGTCACACCA	164
[SSR-6286]	Forward	131	160	29	AAAAATAGGTAAAATAGGAAGTTACAAAA	
CP257/CP258	Reverse	363	383	20	TGAACCCATTGCACTCTACG	254
[SSR-6287]	Forward	486	506	20	GCCTTTTGGCAACTTCTGAG	
CP259/CP260	Reverse	620	644	24	TGCAAGAGAACATTAAAAAGCCTA	160
[SSR-6288]	Forward	114	137	23	GATGTTGTAGCAGGCTAATTGGA	
CP261/CP262	Reverse	186	207	21	TGGCCAATTGTCCTAAGTTGA	95
[SSR-6289]	Forward	456	476	20	CCCCCAAAGTTGATGAACAC	
CP263/CP264	Reverse	542	563	21	TTGATGGAGTTCGCATCTTCT	109

Source: http://cowpeagenomics.med.virginia.edu/CGKB/.

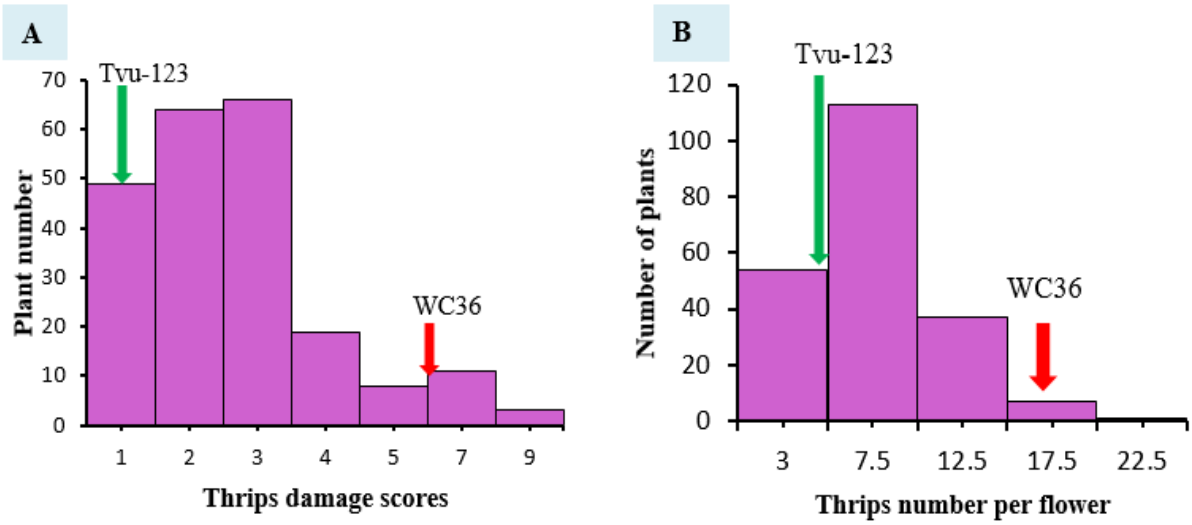

Figure 1. Frequency distribution for flower bud thrips damage and thrips number for the F_2 population derived from the cowpea cross, TVU-123 × WC36.

thrips counts in flower for the 212 F2 plants were significantly different from normal (W statistic = 0.81 and 0.95, P<0.001, respectively). Damage scores and thrips counts for the population tended to be skewed towards the resistant category.

The regression of the flower thrips damage scores and the number of pods produced per plant showed relatively negative relationship, R^2 = 0.21 (P<0.001) with plants having higher damage scores producing fewer pods (Figure 2).

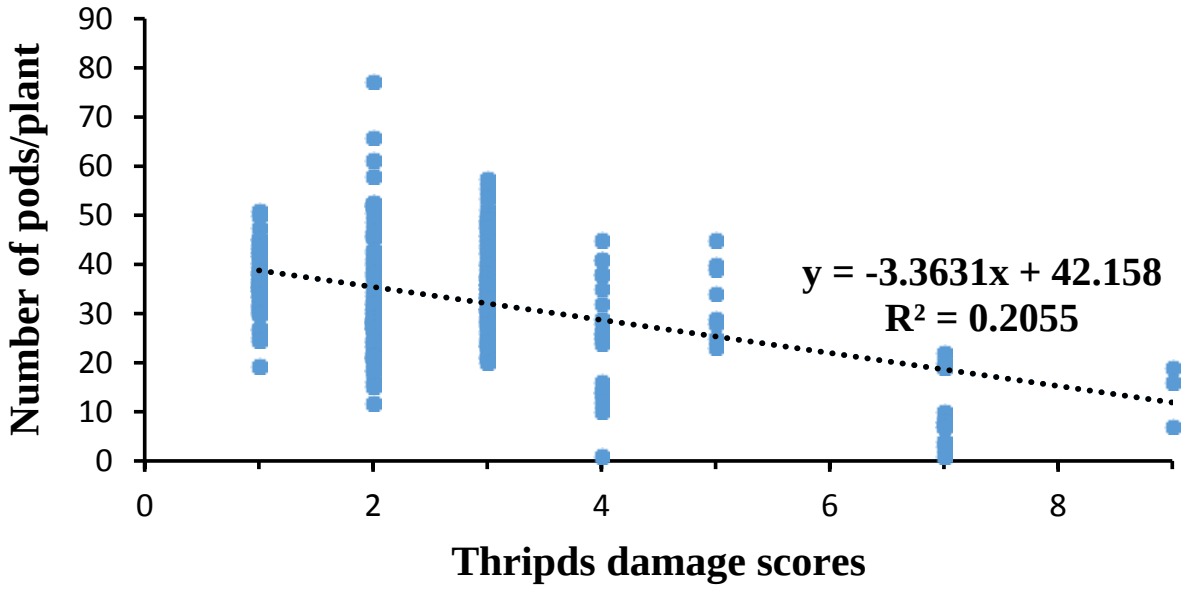

Figure 2. Effect of flower bud thrips on number of pods produced per plant observed in a F_2 population dervied from the cross, TVU-123 x WC36.

SSR markers screening and segregation distortion

Seven SSR markers: CP3/4, CP37/38, CP55/56, CP215/216, CP219/220, CP225/226 and CP239/240, were polymorphic between the two parents (Figure 3). The distribution of different genotypes among the F2 populations showed that except for the SSR marker CP37/38 that showed an excess of the heterozygote genotypes, the other markers showed an excess of the homozygote genotypes for flower thrips resistance alleles among the F2 population (Table 2). The Chi-square analysis showed significant segregation distortion (Table 3) for the SSR markers, except maker CP239/240 that conformed to the 1:2:1 segregation ratio. The electrophoretic profiles for the population are presented in Figure 4.

Marker association analysis with thrips damage score and thrips counts

Maker-traits association data are presented in Table 3. SSR marker CP 37/38 was significantly (χ^2 =11.40, P<0.01) associated with thrips damage scores while the results of the analysis of variance on thrips counts showed that the marker, CP215/216 was significantly (P<0.01) associated. The recombination frequency between two marker loci was 0.34 ± 0.033 with a LOD score of 4.07.

DISCUSSION

Host plant resistance is one of the most important strategies for crop improvement (Omo-Ikerodah et al., 2008). Insect resistance genes have been introduced into several crop varieties and its importance is increasing as insecticides lose efficacy due to pest adaptation or are removed from use to protect the environment and human health (Omo-Ikerodah et al., 2008). In many cases, multiple genes are required for sustained resistance to counter pest adaptation. Thus, maintaining agricultural productivity to meet world food needs depends on access of agricultural scientists, to many sources of host plant resistance genes. Only low levels of resistance to flower bud thrips exist in different cowpea lines and there is need to bring these genes together in a line with good agronomic performance. In this study, the continuous and skewed distribution towards the resistant parent for flower thrips damage scores and thrips counts indicated that resistance of cowpea to flower bud thrips was polygenic and suggested dominance over susceptible parent. Omo-Ikerodah et al. (2008) reported that more than two genes probably control the resistance to flower bud thrips. Similar segregating pattern was reported by Sobda et al. (2017) for the F2 population developed from the genotypes Sanzi and VYA evaluated in Cameroon. In this study, lower damage ratings than the resistant parent was observed for approximately 49 plants from the population suggesting transgressive segregation for resistance. Similar results were reported by Omo-Ikerodah et al. (2008) on Sanzi and VITA 7 in Nigeria. Transgressive segregation for resistance to flower thrips has important breeding implications because it is possible to obtain plants with resistance levels higher than those of the parental lines (Omo-Ikerodah et al., 2008; Muchero et al., 2009a). The level of polymorphism between the

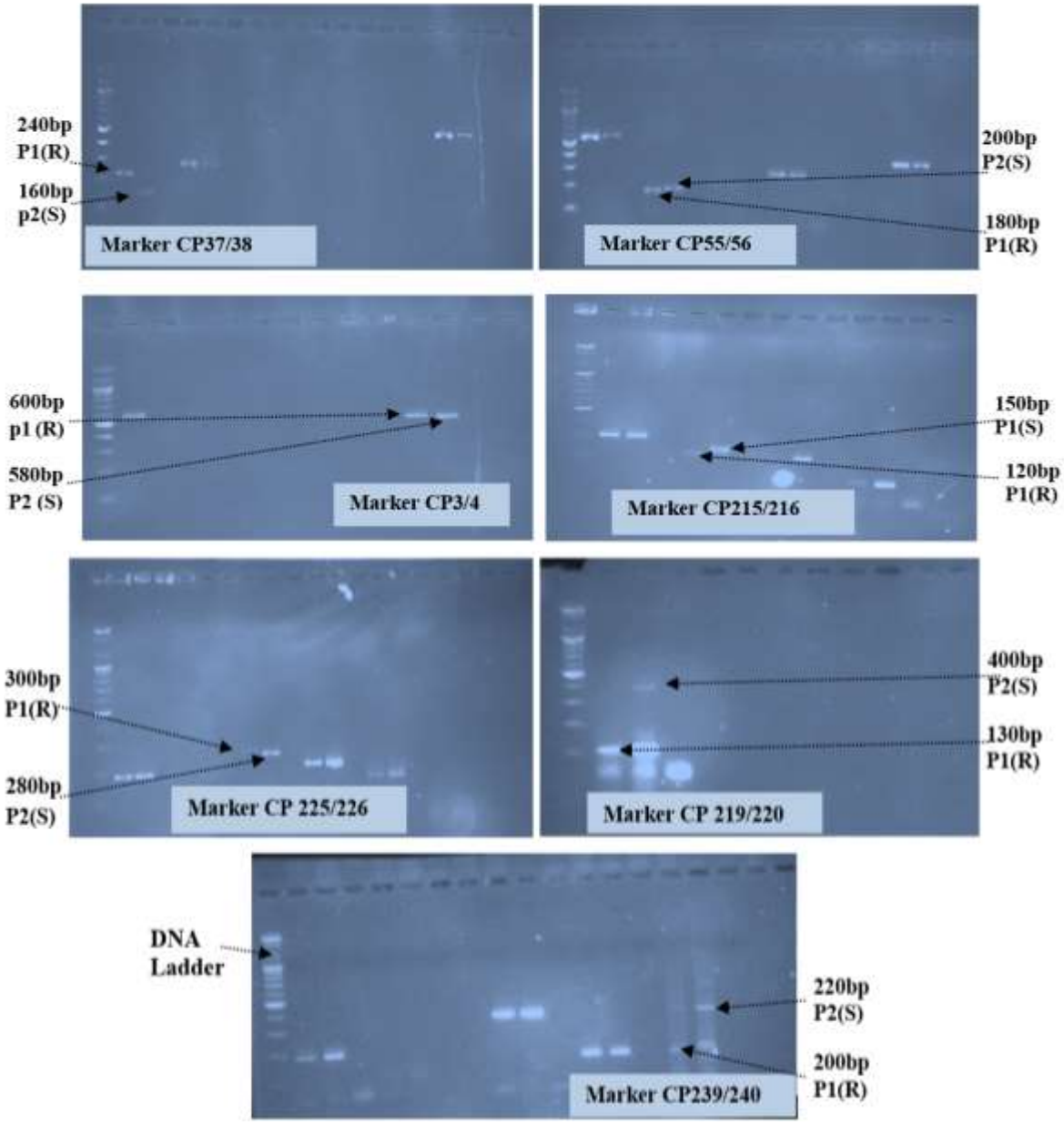

Figure 3. Polymorphic SSR markers screened between the two parents: TVU-123 (resistant) and WC36 (susceptible).

Table 2. Segregation pattern for seven polymorphic SSR markers among F2 progeny.

Markers	[a]Progeny segregation	χ2 (d.f.=2)
CP3/4	39/17/44	44.06***
CP37/38	14/69/17	14.62***
CP55/56	70/13/17	110.94***
CP215/216	28/25/47	32.22***
CP219/220	43/12/45	57.84***
CP225/226	40/5/54	83.14***
CP239/240	19/57/24	2.46[ns]

[a]Female parent/Heterozygote/male parent; χ2, Calculated Chi-square value (Steel et al., 1997) according to the expected Mendelian genotypic segregation ratio 1:2:1. ***Significant segregation distortion at 0.001.

Table 3. χ^2 Independence test and means squares for association of the SSR markers with thrips damage scores and thrips number.

Source of variation		χ^2 independence test (df = 4) on thrips damage scores						
		CP3/4	CP37/38	CP55/56	CP215/216	CP219/220	CP225/226	CP239/240
		0.94^{ns}	15.99**	6.06^{ns}	2.08^{ns}	4.15^{ns}	0.59^{ns}	1.76^{ns}
		Markers means squares for number of thrips/flower						
	DF	CP3/4	CP37/38	CP55/56	CP215/216	CP219/220	CP225/226	CP239/240
Marker classes	2	18.74^{ns}	25.47^{ns}	**58.88^{ns}**	599.62**	52.78^{ns}	41.09^{ns}	46.47^{ns}
Error	97	109.98	109.84	109.15	98	109.28	109.52	109.41
QTL effect			1.64		-1.76			
Additive effect			0.82		11.20			
Dominance effect			-0.93		8.04			
R^2			7.00		11.20			

**Significant at 0.01 probability level; ns, not significant.

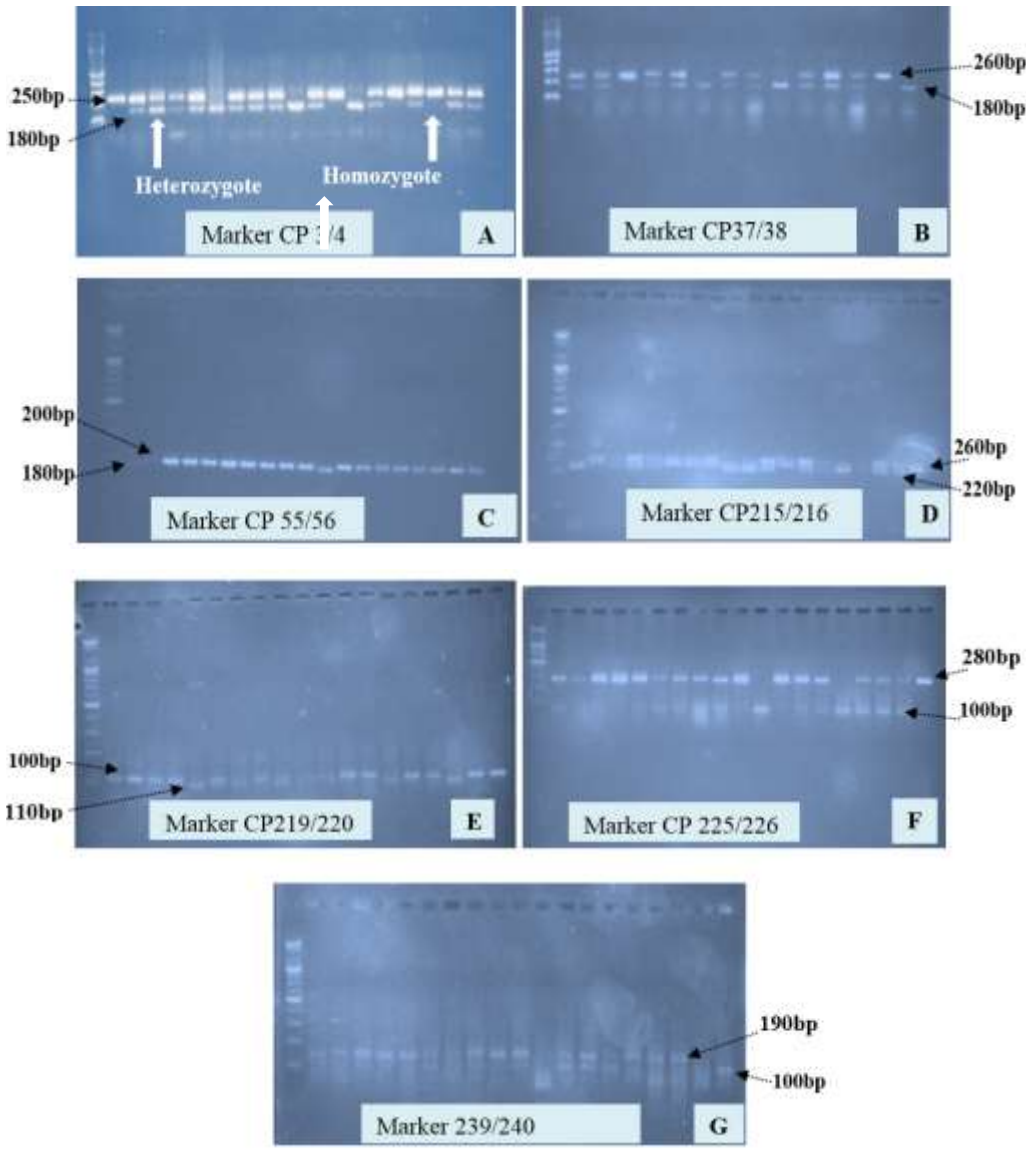

Figure 4. Electrophoretic profiles for 7 SSR markers for 20 genotypes from the TVU-123 × WC36 F_2 population.

two parents as revealed by the cowpea derived microsatellite primers used in this study was low as only 7 of 66 primers showed polymorphic bands (10.61%). Twelve percent polymorphism for SSR primers was reported in cowpea by Diouf and Hilu (2005). Low (4%) level of microsatellite polymorphism in cowpea has been reported in earlier findings (Li et al., 2001; Diouf and Hilu, 2005; Omo-Ikerodah et al., 2008; Uma et al., 2009). The low level of microsatellite polymorphism was attributed to relatively low genetic diversity of cowpea as compared to other crops.

Markers showed significant segregation distortion for the F2 population. Segregation distortion is common phenomenon observed in wide intraspecific crosses of many plants (Song et al., 2006; Kongjaimum et al., 2012). In *Vigna* species, distorted segregation of markers has been reported in genetic maps of cowpea (Xu et al., 2010, 2011).

In this study, the markers CP 37/38 and CP215/216 were identified to be significantly associated with thrips damage score and thrips counts, respectively. These markers explained 7 and 11.2% of the total phenotypic variance in thrips damage scores and thrips counts, respectively, indicating that the markers identified are still far from the genes controlling the resistance to flower thrips. The markers effects observed were low as compared to 77.5 and 43.2% observed by Omo-Ikerodah et al. (2008) and Sobda et al. (2017) in Nigeria and Cameroon, respectively, indicating that the resistance of cowpea to flower thrips is controlled by several genes and the identified markers, were not able to cover most of these genes. Molecular markers with positive as well as negative effects were detected in this study. The positive effects suggested resistance-enhancing QTL originating from the resistance parent TVU-123 and indicated that the alleles at these loci contributed to increase in the resistance genes. The negative effects suggested resistance-reducing QTL originating from the susceptible parent WC36. Allele at this QTL contributed to increase in the susceptibility, suggesting selection against QTL when breeding cowpea for resistance to flower bud thrips. Similar QTLs with effects contrary to the overall effect of the parents have been reported by Omo-Ikerodah et al. (2008), Sobda et al. (2017) on flower thrips and Muchero et al. (2010) on foliar thrips in cowpea. The QTLs detected in this study have mainly additive gene effects. They can therefore, be used for breeding purposes (Acquaah, 2012).

The recombination frequency estimated indicated that the two markers loci are linked (Collard et al., 2005). Close association of these markers with the phenotypic data could facilitate the introgression of this QTL block as a single unit by targeting low recombination rates in breeding progenies (Muchero et al., 2010). This provides opportunity for development of molecular markers for use in marker-assisted selection for resistance against flower thrips. The SSR markers, CP 37/38 and CP215/216 that co-located with thrips damage scores and thrips counts in flower, respectively, are potential candidates for use in developing molecular markers.

Conclusion

The SSR marker, CP37/38, was associated with thrips damage while CP215/216 was associated with thrips counts in flower. The two markers explained 7 and 11.2% of the total variation observed in thrips damage and counts, respectively. The QTLs detected in this study have mainly additive gene effects with positive effect for CP37/38 marker and negative effects for CP215/216 marker. Further research focusing on possible QTL with more SSR markers using recombinant inbreed lines and more integrative approaches to establish position and order of putative QTLs should provide better understanding of this complex trait.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Studies on styrene concentration in drinking water and hot beverages in some settings

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Water bottles and cups composed of polystyrene also contain non-polymerized styrene. Styrene's toxicological profile is associated with several health issues for humans. Mainly, the central and peripheral nervous systems are highly disturbed by styrene ingestion. Styrene is also considered to be a carcinogenic agent and has been linked to cancer. The HPLC method was validated through prepared QC samples. The HPLC method validated over the range (0.2 - 50 ng) with good linearity r^2=0.9998. The validation data proved on average 97.5% accuracy with this method. The analysis further depicted that both sources of water contained styrene; 2.2 and 3.2 ng/mL for fresh and stored water respectively. Styrene was released in larger quantities in boiled water than in cold water. In fresh water, the styrene level was raised by 50% and by 100% for the stored water. On the average, a person may be exposed up to 7 µg/day for cold water, and up to 13 µg/day for hot water. Consequently, we also studied the effect of sugar on bottled water, which showed a 180 and 250% increase on cold and boiled water respectively. Caffeine was also found to increase the leachability of styrene; 150% in case of fresh water and 170% in stored water.

Key words: Styrene, water, hot beverages, high-performance liquid chromatography, fluorescent detector (HPLC-FD).

INTRODUCTION

Polystyrene (PS) is used worldwide as a food packaging material. The non-polymerized styrene monomer migrates from packaging material into our food and beverages every day. There are several known health impacts in connection to exposure to styrene (Muratak et al., 1991; Varner and Berede, 1981; Varner et al., 1983). Styrene, also known as vinylbenzene or ethynylbenzene (Figure 1), is a naturally occurring, colorless liquid that

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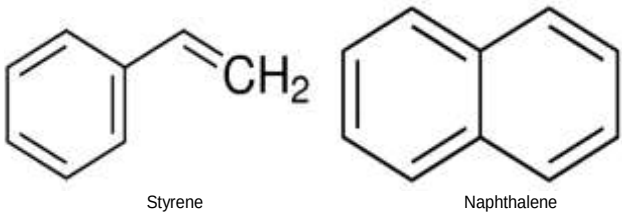

Figure 1. Styrene and Naphthalene chemical structure.

easily evaporates with an unpleasant smell, similar to gasoline. Recycled PS materials leach greater amounts of styrene than virgin PS (Qin-Baol et al., 2017). A recent study urged the need for a policy to specifically address the problems of PS plastic contamination in ocean water environments (Kwon et al., 2018). Exposure to styrene vapors may cause irritation to the throat, eyes, nose and skin. Styrene also has a toxic effect on the liver, and is thought to cause depression on the central nervous system as well as cause neurological impairment (Cohen et al., 2002). Chronic effects of styrene and styrene epoxide (metabolite of styrene) are traced to chromosomal aberrations in lymphocytes, which damage the human liver and nervous system. In recent years, studies of the toxic effect of styrene have given widespread concern on the hematopoietic, central and peripheral nervous systems, ingestion, reproductive organs, and the lymphatic system (Sherrington and Routledge, 2001; Brown et al., 2000).

Early in 1993, the International Agency for Research on Cancer (IARC, 1994) classified styrene as a 'group 2B' carcinogen (possibly carcinogenic to humans) (WHO and IARC, 1993). The carcinogenicity of styrene is not clearly proven, but there are many similar volatile organic chemicals, which serve as carcinogenic agents. The National Toxicology Program (NTP) listed styrene as an anticipated carcinogenic agent to humans. Most of the genotoxic effects associated with exposure to styrene are thought to be caused by 7, 8-oxide (SO). This compound is considered to be a human carcinogen based on sufficient evidence of carcinogenicity in multiple animal species at multiple tissue sites (WHO and IARC, 1993; IARC, 2002). According to a study (ASTDR, 1992), styrene was detected in adipose tissues as well as blood. There is great importance in knowing the toxic effect of styrene and its leachability in food materials and water from PS.

Water is an essential resource that is vitally important for humans and should be consumed while clean and uncontaminated (Cabejskova, 2016). It is needed in everyday life and strongly affects the well-being of each individual. Global consumption of bottled water increased drastically over the last few decades (Cabejskova, 2016). The FDA has determined that the styrene concentration in bottled drinking water should not exceed 0.1 ppm (FDA, 2007). Abdominal discomfort was observed in humans exposed to elevated levels of styrene in drinking water (Arnedo-Pena et al., 2003). Tap water is seen to be safer than bottled water (Ashton, 2014), because polystyrene (PS) and Styrofoam leach styrene into the bottled water containers (Maqbool and Ahmed, 2007). The presence of styrene has been confirmed in drinking water containers made of PS (FDA, 2003; Health Canada, 1993). There are many factors affecting the rate of styrene migration such as quality of plastic, storage time and temperature. The concentration of styrene steadily increased to 69.53 µg/L after one-year of storage (Maqbool and Ahmed, 2007).

Modern high-performance liquid chromatography (HPLC) with UV detection (Bourque et al., 1994; Fujii et al., 1999; Inoue et al., 1991), gas chromatography (GC) with flame ionization detection (Chakroum et al., 2008; Kataoka et al., 1991), and high-performance chromatography with mass spectrometry (Pacenti et al., 2008; Marais and Laurens, 2005) were performed for evaluating styrene. The level of styrene present in food and drinks depend on many factors such as heat, pH, fats, and the time of storage. It is important to accurately evaluate the amount of styrene in drinking water for safe human consumption. Since bottled water is used every day for drink and food preparation in many countries around the world, it is necessary to evaluate how much styrene is consumed on a daily basis and to what extent health might be affected.

Studies on the use of Styrofoam and PS cups (Khaksar and Ghazi-Khansari, 2009) for water revealed that the water was in fact, contaminated with styrene. Those researchers have determined the migration of monomer styrene from GPPS (general purpose polystyrene) and HIPS (high impact polystyrene) cups in hot drinks. It was observed that temperature plays a major role in the leaching of a styrene monomer from GPPS and HIPS as well with a minor difference in the amount measured. Hot caffeinated beverages contain caffeine and sugar; thus, these two factors might be affecting the leachability of styrene from PS containers into cold or hot drinks. This research hypothesized that caffeine and sugar might increase styrene level inside beverages that are served in polystyrene or plastic cubs. These two factors have not yet been investigated. Here, the effect of storage time and heat factors concerning bottled water is also being observed. In addition, we will evaluate the additional contamination of styrene in bottled water versus that served in PS cubs. Furthermore, we will explore the effects of caffeine and sugar in hot drinks.

MATERIALS AND METHODS

Styrene (purity 99%) and naphthalene (purity 99%) analytical grade was obtained from Sigma Aldrich (Steinheim, Germany). High performance liquid chromatography water and methanol were obtained from Sigma Aldrich (Seelze, Germany). Caffeine (purity 99%) of analytical grade was obtained from Sigma Aldrich (Seelze, Germany). Water packed in PS bottles and Styrofoam, PS and paper cups (size 250-ml) and sugar were collected from local market in Madinah, Saudi Arabia.

A Shimadzu ultra-high-performance liquid chromatography (UHPLC, purchased 2016) system (Japan, Kyoto) consisted of Shimadzu Prominence LC equipped with LC-20AD quaternary gradient pump, a Prominence RF-20A fluorescence and Prominence SPD-M-20A Diode Array detector, CBM-20A communication bus module, CTO-20A column oven, a SIL-20AP auto sampler, and Shimadzu LC solution software (ver. 1.21 SP1 from Shimadzu, Japan) was used. All samples and standards were filtered through 0.2 µm (Millipore) filters.

Analytical column

Compounds were separated isocratically on Thermo BDS Hypersil C18 column (150 mm × 4.6 mm, 5 µm). Separation was maintained at ambient temperature (25±2°C).

Mobile phase

This involves a mixture of methanol and water (30:70, v/v). The flow rate was 1.0 mL min^{-1} and detection was adjusted at wavelength λ= 270 nm. The mobile phase was filtered and degassed by sonication using the ultrasonic cleaner (Ultrasons-HD) from Selecta S.A. (Barcelona, Spain). The flow rate was set at 1.0 mL min^{-1} and the HPLC chromatograms was monitored at emission wavelength (λem= 310 nm) after excitation at (λex= 250 nm).

Calibration curve standards and quality controls samples

A stock solution for styrene was prepared in HPLC grade 50/50 methanol/water (styrene free) solution at concentration of 1 mg/mL. Styrene calibration curve solutions were made by diluting the stock solution to six different concentrations (0.2, 1, 5, 10, 25 and 50 ng/mL). Quality control samples were prepared at 0.25, 2.0 and 20 ng/mL from a separate stock solution. Internal standard of the method was naphthalene. Naphthalene solution (1 mg/mL) was prepared and diluted in HPLC grade 50/50 methanol/water (styrene free). This stock solution was further diluted in HPLC grade water to the final concentration working solutions.

Sample collection and preparation

All water samples in PS bottles were collected fresh from the local market and were analyzed on the same day. All samples were assayed for styrene monomer contents in cold and boiled water. Before analysis, pH of bottled water was measured 7.2 to 7.4. Cold and boiled water were directly added to (paper and PS) cups. The boiler, steering rod and HPLC tubes were all made of glass in order to avoid any additional contamination of styrene.

Cups were labeled ahead of time. Cold water and boiled water were transferred to cups (PS and paper) at the same time. Using a stop-watch, solutions in all cups were stirred with the glass rod for exactly 10 min. The samples were then transferred to the HPLC tube and analyzed on the same day. The temperature of the HPLC autosampler was adjusted to 4°C. The amount of sugar used to study its effect was 12 g per cup. The caffeine working solution was prepared in three different concentrations (25, 50 and 100 g).

RESULTS

Chromatographic separation and choosing the proper detector

The fluorescent detector (FD) was used in our experiment due to its high sensitivity to styrene at excitation wavelength (250 nm) and emission wavelength (310 nm) at a concentration range of 0.2 – 50 ng/ml. Also, ultraviolet Diode Array (DAD) wavelength (254 nm) in parallel with FD was used. Diode array detector (DAD) appeared less sensitive and was unable to detect styrene at low concentration ranges levels (Figure 2a and b).

HPLC calibration curve data

A calibration curve was constructed using the six concentrations of standard styrene that ranged from 0.2 – 50 ng/mL. The curve drawn was shown between styrene concentrations versus the measure peak area ratio. Another calibration curve has been drawn between reciprocal of standard styrene concentration on the x-axis and the ratio between peak areas to standard styrene concentration on the y-axis. This curve was found to be more accurate in its use for calculations. Styrene concentrations were calculated by the equation: Y=0.4107+0.234, and r^2=0.999 for determination of styrene concentration.

Limits of quantification and detection

The lower limit of quantification (LLOQ) was defined as the concentration for which both the relative standard deviation (CV%) and the percent deviation from the nominal concentration (dev%) were less than 20%. The upper limit of quantification (ULQ) was defined as the concentration for which both the relative standard deviation and the percent of deviation from the nominal concentration were less than 15% (USFDA, 2001). The detection limit was defined as the signal-to-noise ratio of 3:1.

Accuracy and precision

The results of the method validation are shown in the Table 1. All observed data for inter assay precision were at or below 15%, and in accordance with the FDA guidelines (USFDA, 2001). The method showed good accuracy that ranged from 99.9 to 100.6%. The deviation from nominal concentration ranged from -1.8 to 2.0% for all QC samples. The method's precision was always within 8.6%.

Concentration of styrene in water samples

Table 2 represents the type of water sample in the first column. The amount of styrene monomer released in 1 mL of water was represented in the second column. Column three illustrates the amount of styrene in 200 mL of water (one cup). Sugar increases the release of

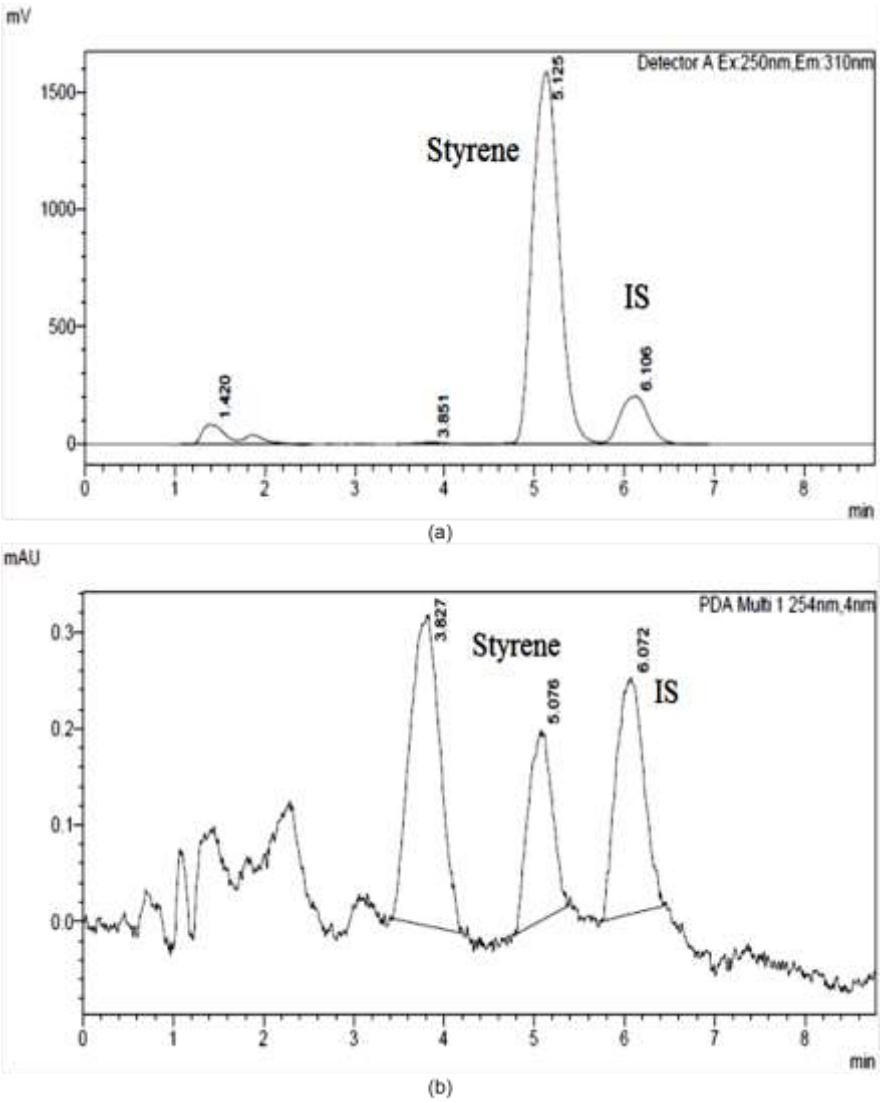

Figure 2. Chromatogram of (a) styrene standard (20 ng/ml) (tR=5.1) and naphthalene internal standard (100 ng/ml) (tR=6.1) using fluorescent detector (λex 250 nm, λem 310 nm) (b) styrene standard (20 ng/ml) (tR=5.07) and naphthalene internal standard (100 ng/ml) (tR=6.07) using UV detector (λ=254).

Table 1. Inter-assay accuracy and reproducibility (n=6).

Nominal concentration (ng/mL)	Back calculated (ng/mL)	Deviation (%)	R.S.D. (%)	Accuracy (%)
0.25	0.2514	2.0	4.0	100.6
2.00	2.0650	0.2	8.6	103.3
20.00	19.9798	-1.8	1.9	99.9

styrene by 150%.

Comparison of styrene level for all samples

A histogram was plotted to represent the comparison between the styrene concentration in all water samples. Figure 3 shows the calculated data for all water samples; stored (old) and fresh, hot and cold, with and without sugar.

Effect of heat and contact time

Heat's effect on styrene concentrations is presented in

Table 2. Concentration of styrene in different water samples.

Water sample	Styrene concentration (n=3) (ng/ml)	Styrene concentration (ng/cup)
Cold fresh water	2.20	440
Boiled fresh water	3.28	656
Cold fresh water with sugar	3.96	792
Boiled fresh water with sugar	8.29	1658
Cold stored water	3.22	644
Boiled stored water	6.47	1294

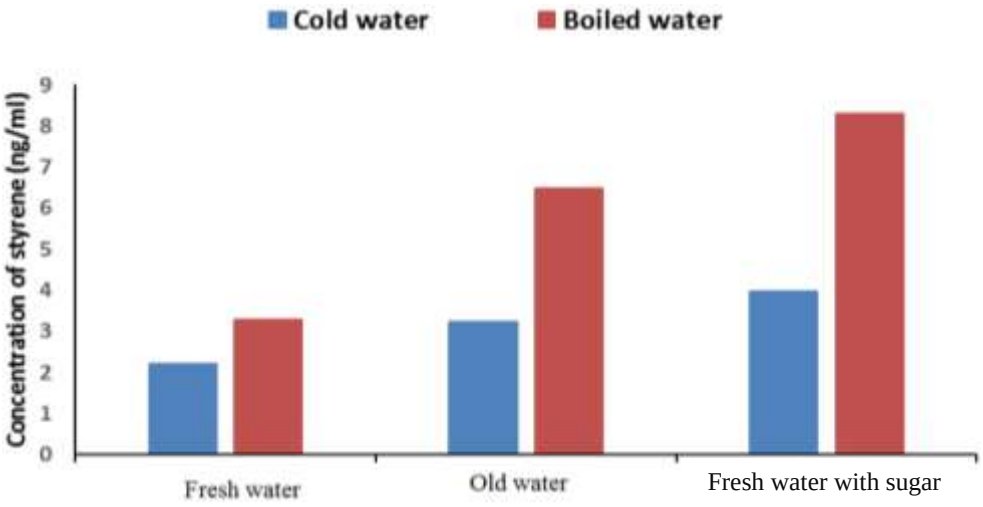

Figure 3. Styrene concentration in different water samples.

Table 3. Heat effect % for both stored and fresh water.

Sample type	Cold water (n=3) (ng/mL)	Boiled water (n=3) (ng/mL)	Difference (ng/mL)	Effect (%)
Fresh water	2.20	3.28	1.08	49.09
Stored water	3.22	6.47	3.25	100.93
Fresh water with sugar	3.96	8.29	4.33	109.34

Table 4. Daily styrene consumption from drinking bottled water.

Sample type	Styrene (n=3) (ng/mL)	Volume of water per (mL/day)	Styrene (ng/day)
Fresh water	2.20	2000	4400
Stored water	3.22	2000	6440

Table 3. The effect of adding sugar on styrene migration in the cold and boiled fresh water is also present. Styrene migration on boiled water increased by about 50% for fresh and 101% for long stored water compared with amount migrated on the cold water for both. Comparing sugar effect on cold and boiled water, styrene migration increases 109% of the cold. This was observed only for fresh water.

The daily consumed styrene from cold and boiled drinking water

The calculated amount of styrene that can be consumed by an individual per day is represented in Table 4. A person who drinks 2 L of cold water per day can absorb styrene up to 4,400 ng of fresh water, and up to 6,440 ng of stored water in polystyrene bottles.

Table 5. Consumed styrene from drinking one cup of hot water.

Sample type	Styrene (n=3) (ng/mL)	Size of one cup (mL)	Styrene (ng/cup)
Fresh water	3.28	200	656
Stored water	6.47	200	1294

Table 6. Consumed styrene from drinking one cup of hot beverage prepared from fresh and old (stored) water.

Caffeine (mg)	Styrene (fresh water) (n=5) (ng/ml)	Amount of styrene/cup (ng)	Styrene (Stored water) (n=5) (ng/ml)	Amount of styrene/cup (ng)
0	3.27	654	6.27	1,254
25	4.68	936	8.31	1,662
50	5.10	1020	9.80	1,960
100	5.57	1,114	11.25	2,486

As shown in Table 5, hot drinks, which were prepared using boiled water, contain more total styrene. Styrene is calculated as ng/cup.

Effect of caffeine

Consumed styrene from drinking one cup of hot beverage prepared from fresh and old (stored) water is illustrated in Table 6. Caffeine clearly increases the amount of leached styrene from cups into the water. Water samples from paper cups containing caffeine were analyzed as well. There was no detected styrene in the samples which proves that no interreference occurred. A caffeine beak was eluted early at 2.1 min (Figure 2a and b). Caffeine increasingly releases styrene by 170% if prepared from fresh bottled and 180% if prepared from stored water.

DISCUSSION

Styrene's toxicological profile is an indication that it poses several health issues for humans. The world's plastic production is consequentially increasing every year (Jambeck et al., 2015). The total global consumption of styrene is approximately 25 million metric tons annually, which estimates about 30 billion in the USD market (Lian et al., 2016). About 1.5 million tons of plastic is produced yearly by the bottled water industry alone. Recently, a Micro-Fourier infrared spectroscopy analysis was carried out on the water of Bohai Sea, China. This study showed that polystyrene was among the main microplastic contaminants in the water (Zhang et al., 2017). The non-plasticizer styrene migration to water starts from day one of the destruction process to the aging of plastics. The presence of unbound low molecular mass compounds considerably increases migration levels (Chakroun et al., 2008). Styrene monomers exist in drinking bottles and cups and other "food-use" items. In most countries around the world, drinking water is delivered to homes through metal pipes. Metal pipes do not leach styrene into the water source. In Madinah, KSA, and in almost all cities in the Gulf Cooperation Council (GCC) countries and around the world, drinking water is packed in plastic containers. The public should be advised to adapt to drinking tap water instead of bottled water (Saylor et al., 2011). It is also observed that all food and drinks prepared in some homes comes from bottled water as well. Moreover, hot drinks are served in polystyrene cups. The extent to which migration occurs depends upon factors such as the contact area, type of plastic, temperature, contact time, solution pH, fat contents and food additives (FDA-Food and Drug Administration).

Because drinking water is packed in polystyrene containers and consumed out of polystyrene cups in some settings, it was hypothesized that leached styrene from the big containers and small cups could be doubling the amount of styrene in our drinks; cold or hot. It is important to evaluate the amount of styrene present in drinking water. In this study, styrene is measured in hot and cold water, as well as fresh and stored water. This work aimed to determine how much total styrene is released in water. To the authors' knowledge, there are no published reports that have calculated the total amount of released styrene in the final consumed drinking water for individual handling water in multiple PS containers. This is the first work of literature that focuses on finding the amount of styrene in bottled water consumed per day by an average person in communities that heavily depend on bottled water. Furthermore, the effect of caffeine and sugar as new factors affecting styrene migration was studied.

The powerful technique of using high performance liquid chromatography (HPLC) has become increasingly popular over the last thirty or forty years. We have chosen to use RP-chromatography separation in our

work and fluorescence detector (FD). As indicated in the experimental section, the methods were well validated assuring styrene measurements with an excellent accuracy. The standard curve linearity over a wide range (0.2 - 50 ng/m) is convenient for the sample analysis. Back calculated accuracy of the three QC's 0.25, 2.0 and 20.0 was 100.4, 100.6 and 91.5% with average 98%. The UHLPC with fluoresce detector in this research proved to have more accuracy and sensitivity than Khakstar and Ghazi-Khanari method whom used UV detector (Khaksar and Ghazi-Khansari, 2009).

The generated results from this study showed that styrene monomer is found in relatively fresh and stored water packed in PS bottles. There is significant increase in the amount of styrene contamination over longer contact periods with the water container. This finding is in agreement with Maqbool and Ahmed (2007). Moreover, additional amounts of styrene were observed when water was boiled and decanted in Styrofoam cups as reported by Khakstar and Ghazi-Khanari (Khaksar and Ghazi-Khansari, 2009).

Data analysis shows that both sources of water contain styrene; 2.2 and 3.2 ng/mL for fresh and stored water respectively. These values are exceeding the permitted level of styrene set by FDA guidelines (FDA, 2007). Through literature, it was documented that the highest rate of migrated styrene in boiled water was done over the first 10 min of exposure. Styrene was released in a higher amount in boiled water than in cold water. This study's data also indicates that in fresh boiled water, styrene levels rose 50 to 100% for the long time stored boiled water.

Studying hot drinks, time of water contact with polystyrene cups was set to be 10 min. Water temperature was also adjusted by boiling for 10 min before transferring to cups containing sugar alone or caffeine alone. When sugar was added to hot or cold drinks, styrene migration increased. Hot drinks containing sugar presented the highest amount of styrene migration in PS cups. Also, normal person daily consumption of SM from hot drinks prepared or served in PS bottled water was calculated. Caffeine doubles the amount of released styrene at 100°C. Finally, the average person can be exposed up to 7 µg/day for cold drink and up to 13 µg/day for hot drink. More research work is needed to study other beverages with different additives and under different conditions.

Conclusion

Successfully, this research was able to measure the heat and time effects on drinking water, as well as study the effect of caffeine and sugar for the first time in releasing styrene from PS cups into our hot beverages. Consequently, sugar showed positive effect in increasing released styrene from PS cups. Coffee is the world's number one drink served with many different additives which could release even more styrene from cups. Caffeine is also found to increase styrene leachability in hot beverages served in PS cubs. Styrene water content is accurately measured using the HPLC method. This research data relied on an accurate and sensitive UHPLC with FD analytical method which was fully validated for ensuring accurate data. The research findings here are important for the public's awareness of using tap water instead of PS cups for hot drinks, especially caffeinated drinks.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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