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Review

Y-Chromosome short tandem repeat, typing technology, locus information and allele frequency in different population: A review

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Chromosome Y microsatellites seem to be ideal markers to delineate differences between human populations. They are transmitted in uniparental and they are very sensitive for genetic drift. This review will highlight the importance of the Y-Chromosome as a tool for tracing human evolution and describes some details of Y-chromosomal short tandem repeat (STR) analysis. Among them are: microsatellites, amplification using polymerase chain reaction (PCR) of STRs, separation and detection and advantages of X-chromosomal microsatellites.

Key words: Forensic, population, review, STR, Y-chromosome.

INTRODUCTION

Microsatellites are DNA regions with repeat units that are 2 to 7 bp in length or most generally short tandem repeats (STRs) or simple sequence repeats (SSRs) (Ellegren, 2000; Imad et al., 2014). The classification of the DNA sequences is determined by the length of the core repeat unit and the number of adjacent repeat units. It may contain several hundred to thousands (Butler, 2012) of these. Tandem repeats occur in the form of iterations of repeat units of almost anything from a single base pair to thousands of base pairs. Mono-, di-, tri- and tetranucleotide repeats are the main types of microsatellite, but repeats of five (penta-) or six (hexa-) nucleotides are usually classified as microsatellites as well. DNA can be used to study human evolution. Besides, information from DNA typing is important for medico-legal matters with polymorphisms leading to more biological studies (Walkinshaw et al., 1996). Since the STR markers are important for human identification purposes (Rui et al., 1996) the number of repeats can be highly variable among individuals and can be used for identification purposes. There are three types of repeat patterns for STRs. Two or more adjacent simple repeats

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Abbreviations: STR, Short tandem repeat; PCR, polymerase chain reaction; SSRs, simple sequence repeats; PAGE, polyacrylamide gel electrophoresis; CE, capillary electrophoresis; SNP, single nucleotide polymorphism; Y-DNA, Y-chromosome DNA; mtDNA, mitochondrial DNA.
Figure 1. Relative positions of 23 Y-STR loci available in the PowerPlex® Y23 System. The six new loci are shown in bold font. PAR1 and PAR2 are pseudo-autosomal regions on the tips of the Y chromosome that recombine with the X chromosome. The shaded region around AMEL Y can sometimes be deleted, causing loci such as DYS458 to be missing from an otherwise full Y-STR profile. DYS391 is located a sufficient distance away to avoid deletions affecting AMEL Y (Butler, 2012).

are considered a compound repeat. Units of similar length are called simple repeats (Budowle, 1995; Butler et al., 2009; Mohammed and Imad, 2013).

Chromosome Y microsatellites or short tandem repeats (STRs) seem to be ideal markers to delineate differences between human populations for several reasons: (i) They are transmitted in uniparental (paternal) fashion without recombination, (ii) they are very sensitive for genetic drift, and (iii) they allow a simple highly informative haplotype construction (Kayser et al., 1997). Also for forensic applications, this ability to differentiate distinct Y chromosomes makes Y-STRs an advantageous addition to the well characterized autosomal STRs. For a number of forensic applications, Y-STRs could be preferred to autosomal STRs. Especially in rape cases where (i) the differential extraction was unsuccessful, (ii) the number of sperm cells is very low, (iii) due to vasectomy epithelial cells instead of sperm cells from the ejaculate of the perpetrator have to be analyzed, and (iv) the perpetrator, due to a familial relationship shares many autosomal bands with the victim, Y-STRs could provide crucial evidence. Also, in the case of male-male rape or rape cases with multiple perpetrators Y-STRs could lead to essential qualitative evidence. In all such cases Y-STRs facilitate a simple and reliable exclusion of suspects.

Unlike autosomal STR markers, Y-STR markers are linked on the same chromosome and there is no genetic recombination between the markers (Figure 1). Therefore, unlike for autosomal STRs, the Hardy-Weinberg equation is not suitable for determining the frequency of a genotype from the frequency of the alleles at each locus (Beleza et al., 2003; Dupuy et al., 2004; Imad et al., 2015a; Mohammed et al., 2015). To determine the frequency of a particular Y-STR profile, the profile must be searched against different databases for a possible match, and these databases must be large enough to accurately represent the frequencies of the haplotypes present in the population of interest. Thus, as more Y-STR samples are typed and contributed to a database the more useful the database will become. However, at present, the databases are much too small to enable forensic scientists to attain the level of
discrimination provided by autosomal STR analysis (Ballantyne et al., 2010; Imad et al., 2015b; Muhanned et al., 2015).

AMPLIFICATION USING POLYMERASE CHAIN REACTION OF STRs

A billion copies of a given target sequence can be provided by Polymerase Chain Reaction (PCR) in a fast in vitro DNA synthesis process. A DNA polymerase may duplicate to result in specific DNA markers to be surface. dNTPs, Mg++ and a thermal stable DNA polymerase, (usually Taq polymerase) are five main chemical components. During the cycling of temperatures, the primers are designed to hybridize to the specific markers (e.g. STR loci) along the length of the template. A special DNA polymerase that is heat stable is used to copy and amplify the genetic markers using the remaining components after the DNA strands are separated and the primers bind to the template. This happens for a given thermal cycle (Del et al., 2009; Nadine et al., 2010). To analyze the DNA, the process of 28 to 32 heating and cooling cycles, is increased. The amplification of multiple samples can be done at one time. In fact in 3 h 96 samples can be amplified in this manner. The thermal samples contain many sample wells that allows this to happen when several different loci are simultaneously amplified in a single tube when multiple PCR occurs. It has been found lately that even 15 autosomal short tandem repeats (STRs) have been done at one time using DNA from a very small amount of contaminated sample.

SEPARATION AND DETECTION

After PCR there must be a process of separation and detection of the amplified products. A number of ways can be used to carry out the typing. Among them are (1) PolyAcrylamide Gel Electrophoresis (PAGE) followed by silver staining or if the primers are fluorescently tagged, detection by fluorescent gel scanners and (2) Capillary Electrophoresis (CE) with laser induced fluorescence. Based on the fact that it is automated this method has become popular. No gel is used and samples can be inserted mechanically. The resolution of the higher molecular weight loci is usually better than in the PAGE methods since the DNA traverses the entire length of the capillary. There are several components that impact DNA separations within CE systems. Among these are the polymer used for enabling the separation, the capillary, the electrophoresis buffer, and the field strength (John et al., 2004). The objective of the exercise is to introduce a different dye onto the 5’-(nonreactive) end of each primer or set of primers (Giusti and Adriano, 1993). The properties of these dyes are quite unusual. Although fluoresce in different regions of the spectra they all excited by a single argon-ion laser tuned to 488 nm. To determine which dye is present, based on the emission of each fragment as it passes the detector window, a multiwavelength analyzer, such as a charged coupled device (CCD) camera, can then be used. The advantage of this method allows the analysis of fragments of DNA that overlap in size as long as they are labeled with different colors, which fluoresce at different wavelengths.

The STR fragments in the sample are amplified using primers with fluorescent tags in the most commonly used analytical method for detecting STRs. There is fluorescent dye in every new STR fragment made in a PCR cycle. When light is shown over it, each dye will emit a different color. Using electrophoresis in automated “genetic analyzer” machinery the fragments are separated according to their length. This technology is developed as a by-product of the technology developed for the Human Genome Project. That first carried out to sequence most of the entire genome. In these machines an electric field is used to extract DNA fragments placed at one end of the tube through the entangled polymer or comparable sieving medium. This is done using a long, narrow tube (a “capillary”). The bigger or bulkier fragments move slowly in the medium as compared to the smaller ones. Sending a laser beam through small glass window in the tube causes it to fluoresce at specific wavelength as the tagged fragments pass under the light. A kind of electronic camera records the intensity of light emitted by the dye. This can be translated into a graph, which shows a peak as an STR flashes by. Firstly, a short allele will pass by the window and fluoresce first. Later a longer fragment will come by, and this will show another peak on the graph.

HAPLOTYPE DIVERSITY FOR Y-CHROMOSOMAL STR IN DIFFERENT POPULATIONS

Observed Alleles, PCR Product Sizes, Repeat Structure and PCR Primer Sequences have been tabulated (White et al., 1999). The observed numbers of haplotypes and their frequencies have been tabulated (Table 1). Haplotypes detected in this study group have been compared with seven other populations: German (n = 88), Indian (n = 25), Chinese (n = 36), Italians (n = 100) (Manfred et al., 2001), Mozambican (n = 112) (Alaves et al., 2003), Japanese (n = 161) (Hara et al., 2007) and Turkish (n = 280) (Henke et al., 2001). Our data have also provided additional information to the framework of variation involving seventeen Y-STR loci as well as a further contribution to the Y-STR database for Iraq population. This supports the observations by others (Jorde et al., 2000) that, especially among European populations. Y-STRs are very powerful in the detection of genetic differences between male populations, compared with autosomal STRs. This can be attributed to the
greater sensitivity of nonrecombining Y-chromosomal markers to founder effects and genetic drift. A similar conclusion was reached by Forster et al. (2000), on the basis of a phylogenetic approach only. The use of Y-STRs allows the simple construction of highly variable haplotypes. With these haplotypes, it is possible to analyze differences in population structure by a comparison of haplotype diversity and of the number of population-specific haplotypes.

In the study of molecular evolution, a haplogroup is a group of similar haplotypes that share a common ancestor with a single nucleotide polymorphism (SNP) mutation. Haplogroups pertain to deep ancestral origins dating back thousands of years. The most commonly studied human haplogroups are Y-chromosome or (Y-DNA) haplogroups and mitochondrial DNA (mtDNA) haplogroups, both of which can be used to define genetic populations. Y-DNA is passed solely along the patrilineal line, from father to son, while mtDNA is passed down the matrilineal line, from mother to both daughter and son. The Y-DNA and mtDNA may change by chance mutation at each generation. Therefore pattern H77 shared in three unrelated males in this study may be descended from same ancestry. According to a 2000 study of Y-chromosome sequence variation human Y-chromosomes trace ancestry to Africa, and the descendants of the derived lineage left Africa and eventually were replaced by archaic human Y-chromosomes in Eurasia. The study also shows that a minority of contemporary East Africans and Khoisan are the descendants of the most ancestral patrilineages of anatomically modern humans that left Africa 35,000 to 89,000 years ago (Collins et al., 1998). Other evidence supporting the theory is that variations in skull measurements decrease with distance from Africa at the same rate as the decrease in genetic diversity. Human genetic diversity decreases in native populations with migratory distance from Africa, and this is thought to be due to bottlenecks during human migration, which are events that temporarily reduce population size (Manica et al., 2007; Phillips et al., 2011). Regarding forensic applications, the unique pattern of Y-DNA in the rest of samples in present study makes it useful as powerful tool for discrimination individuals in crimes, rapes and paternity or log dead people such as in mass graves.

**Conflict of interests**

The authors did not declare any conflict of interest.

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**REFERENCES**


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Suitability and use of two molecular markers to track race-specific resistance *striga gesnerioides* in cowpea (*Vigna unguiculata* (L.) Walp.)

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Full Length Research Paper

The obligate root parasitic weed *Striga gesnerioides* poses a severe constraint to cowpea productivity in the dry savannahs of West and Central Africa, where cowpea is a major crop. At least seven races of *S. gesnerioides* have been identified within the cowpea-growing regions of West and Central Africa, based on host differential response and genetic diversity analysis. Molecular markers linked to resistance to different races of *S. gesnerioides* have been identified. It was desirable to demonstrate the applicability and efficiency for use in marker-assisted selection (MAS) to fast-track the development of cowpea for resistance to *S. gesnerioides*. The objective of the study was to determine the suitability of two molecular markers in tracking race-specific *S. gesnerioides* resistance in cowpea (SG3), the predominant race found in Nigeria. F₂ mapping populations and recombinant inbred lines (RILs) derived from the cross involving IT97K-499-35 and a susceptible local landrace (Borno Brown), and another resistant parent B301 with the same susceptible land race (Borno Brown) were assayed using two linked markers. Genetic analysis showed that resistance to *S. gesnerioides* in cowpea is qualitatively inherited with single dominant gene action. Two SCAR markers, 61RM2 and C42-2B were validated in the same F₂ populations and subsequent recombinant inbred lines (RILs). The two markers were able to discriminate between resistance and susceptibility and the genotypic score was quite similar to the phenotypic score with the markers score showing greater efficiency in selection than phenotypic score. The 61RM2 had two bands in resistant cultivars and amplified a ~450 bp fragment with marker efficiency of 98% while C42-2B amplified a single ~250 bp fragment with marker efficiency of 96% in resistant cultivars and absent in susceptible cultivars. The genetic distance between 61RM2 and phenotypic score was 3.5 cM while that of C42-2B and phenotypic score was 8.5 cM. The two marker data set were significantly correlated with the phenotypic data (r=0.95). Based on the tight linkage with the resistant locus, 61RM2 was found to be a utility marker to initiate MAS in cowpea breeding for resistance to *S. gesnerioides*.

**Key words:** Cowpea, Striga, molecular marker, genetic distance, race-specific, obligate parasitic weed, Vigna unguiculata.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most important grain legumes grown in tropical and subtropical regions of the world, primarily in sub-Saharan Africa (Singh, 2005; Timko et al., 2007; Ehlers and Hall, 1997). The majority of cowpea is grown by poor farmers in West and Central Africa, where its grain is highly valued for food, and the fodder as source of animal feed (Langyintuo et al., 2003). Cowpea is a food legume of...
Cowpea production is constrained by a wide range of biotic and abiotic factors. Among the major biotic constraints are the obligate root-parasitic weeds *Striga gesnerioides* and *Alectra vogelii* of the Orobaceae family. *S. gesnerioides*, in particular, causes extensive damage to cowpea in the Sudano-Saharan belt of West and Central Africa (Parker, 2009) where its damaging effects are compounded by drought (Obilana, 1987). Successful parasite establishment creates a strong sink for nutrients to the detriment of the host, leading to drastic growth reduction (Keyes et al., 2001; Joe et al., 2006). Yield losses range from 83 to 100% in severely infested fields (Emechebe et al., 1997; Omoigui et al., 2011). Farmers with crop fields severely infested with *Striga* often resort to abandoning their fields, contributing to an already severe non availability of farm lands. In northeast Nigeria, where cowpea is the most important legume crop, Dugje et al. (2006) reported that more than 97% of cowpea fields in the dry savannas were infested with *S. gesnerioides*, leading to serious crop losses. Therefore, the rapid spread of this parasitic weed to new regions would constitute a severe threat to cowpea production in those areas, and the virulence of the different races of *S. gesnerioides* further compounds the problem. The damage to host is already done before the *S. gesnerioides* shoots emerge from the soil. Control of *S. gesnerioides* is difficult to achieve due to the number of seeds of the parasite and their viability in the soil for over 20 years (Ouedraogo et al., 2012). The *Striga* seed germinate in response to the specific stimulants exuded by the host's roots (Worsham, 1987). Several methods are available for the control of *Striga* in cowpea. However, the use of resistant cultivars is considered the most practical, sustainable and effective method to control the parasite. The lack of broad or horizontal resistance, however, is one of the biggest problems when trying to develop resistant cultivars across different races. 

Cowpea cultivar with complete resistance to *Striga* stimulates germination and permit attachment of *Striga* radicles to their root but the haustorium development is inhibited. This mechanism involves the plant recognizing parasite virulence effectors, usually through intracellular resistance proteins (R-proteins), causing effector-triggered immunity (ETI). ETI corresponds to what is classically referred to as gene-for-gene, vertical or race-specific resistance (Flor, 1955; Dodds and Rathjen, 2010). Resistance to *Striga* generally follows a qualitative mode of inheritance where resistant and susceptible reactions are clearly differentiated.

Recently, there are increasing interests in studies aiming at the molecular characterization of the plant-parasitic weed interaction and its resistance through expression analysis of genes, proteins and metabolites involved in these processes (Dos Santos et al., 2003; Castillejo et al., 2004). The availability of molecular markers tightly linked to *S. gesnerioides* resistance genes opens up the possibility of applying Marker-Assisted Selection (MAS) to cowpea breeding. To-date, limited information is available on large scale implementation of marker assisted selection (MAS) in cowpea breeding programs. Heritable sources of resistance in cowpea to both *S. gesnerioides* and *A. vogelii* have been reported (Timko and Singh, 2008). However, most of these resistant lines have poor agronomic characteristics and therefore, their direct use is limited. These germplasm are being used as donor parent to introgress resistant gene(s) into local adapted cowpea cultivars, but the delivery of improved varieties to the farmers is slow. Among the limitations to successful development of improved *Striga*-resistant cowpea is the fact that *S. gesnerioides* is variable in its parasitic abilities, showing both host and cultivars-specific selectivity. At least seven distinct races of *S. gesnerioides* (designated SG1 through SG7) have been identified throughout West Africa (Lane et al., 1997a, b; Botanga and Timko, 2006). Most cowpea plants are susceptible to *Striga* parasitism, although some local landraces have been identified that show resistance to one or more of the known races (Timko et al., 2007), with resistance being conferred by a single dominant gene (Aggarwal et al., 1984; Atopkile et al., 1995). Vos et al. (1995) and others have been able to map several of the race-specific resistance genes to two linkage groups on the cowpea genome via the application of amplified fragment length polymorphism (AFLP) markers (Ouedraogo et al., 2001, 2002; Boukar et al., 2004). The *S. gesnerioides* race SG1 and SG3 resistance genes Rsg2-1, Rsg1-1 and Rsg4-3, present in the resistant cowpea lines B301, IT82D-849 and TVu14676, respectively, were mapped to LG1. Whereas, the *S. gesnerioides* race SG1 resistance genes Rsg3-1 and Rsg2-1 present in Suvita-2 (Gorom local) and IT81D-994, respectively, were mapped to LG6 (Ouedraogo et al., 2001, 2002). One of the *Striga* resistance genes, RSG3-B301 has been cloned and shown to be effective only to SG3 race (Li and Timko, 2009).

Over the years, significant progress has been made by national and international centres toward developing

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Striga-resistant lines in different breeding programs, but the constraint of pyramiding these resistant genes still lingers. To alleviate these constraints and for other reasons (for example, speeding breeding efforts, possibility of identifying other races of the parasite's development), MAS has been proposed as an alternative solution for pyramiding resistance genes (Haley et al., 1994; Ouedraogo et al., 2001). Several molecular marker technologies have been exploited for MAS. Amplified fragment length polymorphism (Vos et al., 1995), combined with bulked segregant analysis (BSA) (Michelmore et al., 1991), have been used to discover markers closely associated with economically important traits in many crop species including cowpea.

Studies conducted by Ouedraogo et al. (2001) using these techniques (AFLP and BSA) identified three markers tightly linked to the resistance gene Rsg2, effective against S. gesnerioides race 1 from Burkina Faso, and present in IT82D-B49; and six AFLP markers associated with the resistance gene Rsg4, effective against S. gesnerioides race 3 from Nigeria, and present in TVu 14676. Two of the markers, E-AAC/M-CAA300 and E-ACA/M-CAT150, were linked to Rsg2 and Rsg4, respectively. One of the AFLP markers has been converted into a SCAR marker, 61R and an improved SCAR 61RM2 (Ouedraogo et al., 2012). Both were also reported to be linked to race 3 resistances. These two markers were dominant markers with wider applications. However, Boukar et al. (2004) also reported a SCAR marker for race 3 resistance that is co-dominant in nature.

Even though a single gene controlling resistance has been identified in the parasite, the transfer of the gene and genes pyramiding through marker assisted backcrossing (MAB) is the most effective and efficient way to develop stable and durable Striga resistant cultivars. The identification of markers for major gene in one segregating population does not mean that the same marker work well for similar genes in other segregating populations. Such findings represent an important advance in the genetic analysis of the character. The recent development of tightly linked markers in cowpea for resistance to Striga provides the opportunity to initiate molecular study to this important trait. As a contribution to the development and implementation of MAS approaches, our study was to investigate the efficiency of one SCAR marker and another gene specific marker that are tightly linked to the S. gesnerioides SG3 and SG5 resistance, respectively, in discriminating between resistance and susceptible individuals in genetic populations produced from a cross between improved and local cultivar.

MATERIALS AND METHODS

Plant materials, development of advanced populations, and phenotypic screening for Striga resistance

Seeds of the cowpea genotypes used in this study were obtained from the International Institute of Tropical Agriculture (IITA), Kano Station, Nigeria. The Striga resistant lines B301 and IT97K-499-35 have been previously described (Singh et al., 2006).

Development of genetic populations

The populations used for this study were developed from the cross Borno Brown x IT97K-499-35 and Borno Brown x B301. The resulting F1 plants were allowed to self-pollinate yielding two F2 populations. The backcross populations were also developed from the respective crosses. The F2 seeds were planted in 13 cm diameter pots containing about 1 L unsterilized sieved sand and top soil (sand loam) mixture (1:1 vol/vol) previously inoculated uniformly with about 2000 S. gesnerioides seeds as described (Singh and Emchebe, 1990; Atopoke et al., 1995). Two hundred F2 and the corresponding F1 and two parents were planted in plastic pot. At four to five weeks after planting (WAP), the pots were scored for day of first Striga emergence and scoring continued on a daily basis until termination of the experiment at 75 days after planting. Striga shoot count was done at 7, 8, 9 and 10 (WAP). After the last Striga shoot count at 10 weeks, the soil was washed off the plant roots after submerging each pot in a 20 L bucket of water for about 5 min. The roots of each plant that had entangled were gently separated from the other and carefully freed from any remaining soil. The cowpea plant root was examined closely for Striga attachment. Plants allowing attachment, haustoria development, and emergence of Striga were categorized as susceptible. Those without any attachment and free of infection were categorized as resistant.

Progeny testing

The resistant F2 plants were further classified into homozygotes and heterozygotes by a progeny testing. For this analysis, 16 individuals from each of 30 randomly selected F1 families were rescreened for Striga resistance.

DNA extraction

Young leaves from two weeks old plants were collected from clearly labeled plants. A total of 100 F2 plants from each cross were sampled. Genomic DNA was isolated from each plant on FTA paper matrix and processed as previously described by Omoigui et al. (2012).

PCR amplification of genomic DNA

To amplify regions of genomic DNA, 25 µl of PCR mixture was added to a tube containing a processed 1.2 mm FTA disc in 0.2 ml PCR tube. 25 µl PCR ready mix (Sigma-Aldrich) had 18 µl of sterilized water, 2.5 µl of dNTPs mix, 2.5 µl 10 x PCR buffer, 0.05 µl of Taq DNA polymerase and 1 µl each of the forward and reverse primers (synthesized by Integrated DNA Technologies, Coralville, IA). Each of the DNA samples was amplified using two different primer pairs, 61RM2 (M2F: 5'-gattgtggttgccttaag-3'; M2R: 5'-ggttcatgtgagcttcttt-3') and C42-2B (C42-2BF: 5'-cgtttctgattgtgagcttcttt-3'; C42-2BR: 5'-caagctcatctcatctgtg-3'). 61RM2 primer is a modification of the 61R marker linked to S. gesnerioides (Ouedraogo et al 2012); C42-2BR is a primer developed by Gowda, BS and Timko MP (Manuscript in Preparation).

Amplification was performed in a heated lid thermal cycler programmed at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57.5°C for 30 s, and...
extension at 72°C for 1 min followed by final extension of 10 min at 72°C to ensure completion of the final amplification products. For C42-2B marker, a similar procedure was followed but the annealing temperature used was 67.5°C. The F2 families were successfully characterized for resistance to S. gesnerioides using the BIONER AccuPower PCR premix.

Gel electrophoresis of PCR products

After performing the PCR, 20 µl of each of the PCR products were electrophoresed on a 2% agarose gel pre-stained with ethidium bromide as previously described (Omoigui et al., 2012). Representative gels were photo-documented and the presence and absence of the polymorphic bands associated with 61RM2 and C42-2B were scored, and these data, along with phenotypic segregation, were used for linkage analysis.

Genetic analysis

Goodness-of-fit of observed to expected segregation ratios was tested by chi-square.

Marker analysis

Linkage analysis between either of the markers 61RM2/C42-2B to the phenotypic score was performed using the computer-aided program Joint MAPMAKER/EXP version 3.0 (Lander et al., 1987) and the Kosambi’s mapping function (Kosambi, 1943) for correcting the recombination values to CM distances.

The genetic distance matrices test of the markers to each other was computed using NTSYSpc (Exeter Software, Setauket, New York, USA) as follows:

\[ D = \frac{1 - N_{xy}}{N_x + N_y - N_{xy}} \]

Where, \( D_{xy} \) = the genetic distance between marker “x” and marker “y”; \( N_x \) = the number of bands shared by marker “x” and marker “y”; \( N_x \) = the number of bands in marker “x”; \( N_y \) = the number of bands in marker “y”.

RESULTS

Phenotypic data

Emergence of Striga on susceptible plants began at 29 days after planting (DAP). At about 70 DAP, several Striga plants had emerged in those pots containing susceptible plants and the differences in level of susceptibility was quite clear. Striga emergence was delayed up to 75 days in some pots involving the cross, Borno Brown x IT97K-499-35. Symptoms of infection such as leaf chlorosis, stunted growth, and partial defoliation were quite visible even before the parasite emerged above soil level. Some plants developed symptoms, but Striga did not emerge from the soil. Some of the highly susceptible plants that had emerged Striga at 29 days after planting died before reaching the reproductive phase. Classification of individual plants into resistant and susceptible groups on the basis of emerged Striga was complicated because some pots had unemerged Striga but the plant showed Striga symptom. Thus, the segregation ratios were ascertained only after plant roots were carefully washed off soil and the parasite’s attachment had been observed before classifying cowpea plants as resistant or susceptible. The differences between plants were clear and easily noticed so that a resistant plant would be completely free of attached Striga while a heavily Striga attached plants were termed susceptible.

Striga infestation on plants derived from cross Borno Brown × IT97K-499-35 showed that the number of attached Striga, including those which emerged, ranged from 1-6 Striga on susceptible individual plant. Resistance to Striga in the resistant parents ‘B301 and IT97K-499-35’ was characterized by a lack of attachment of the parasite to the roots. This result confirmed that both cultivars exhibited vertical resistance as they were completely resistant to Striga. A total of 60 plants of each of Borno Brown were screened in the study, all of which were heavily attacked by Striga. All the 16 F1 hybrid plants derived from the cross had neither emerged Striga nor haustoria’s attachment. All the plants were as resistant as their resistant parent (IT97K-499-35) indicating the complete dominance of resistance over susceptibility.

Segregation in the F2 population yielded 231 resistant plants (no Striga emergence or attachment) and 69 susceptible plants with either fully emerged Striga or with Tubercle attachment or both (Table1). The observed segregation ratio of resistant versus susceptible in this cross fits closely to a 3:1 (resistant: susceptible) expected ratio (\( \chi^2 = 0.65; p = 0.05 \)), thus, indicating the inheritance of resistance by a single dominant gene conferring resistance to S. gesnerioides.

The segregation for Striga in the cross derived from Borno Brown × B301 followed the same pattern conformed with the 3:1 R:S segregation ratio. The 20 plants of the susceptible parental line (Borno Brown) were severely infested with Striga; most of the plants died before reaching reproductive stage due to severe attack by the parasite. All the 16 F1 hybrid plants derived from the cross had neither emerged Striga nor haustorial attachment. All these plants were as resistant as their resistant parent (B301) indicating the complete dominance of resistance over susceptibility. Segregation in the F2 yielded 217 resistant plants (no Striga merge or attachment) and 83 susceptible plants with either fully emerged Striga or with haustoria attachment or both. The number of Striga count including those with haustoria’s attachment and emerged Striga on susceptible plants ranged from 1 to 10. The observed segregation ratio of resistant versus susceptible plants in this cross fits closely to a 3:1 (resistant: susceptible) expected ratio (\( \chi^2 = 1.137; p = 0.05 \)), thus, indicating the inheritance of resistance by a single dominant gene.
The F3 progeny testing of 30 families randomly selected from seeds of F2 resistant plants showed that 10 families bred true for Striga resistance while 20 families segregated for resistance and susceptibility. The genetic analyses conformed to the 1: 2 non-segregating: segregating families (p= 0.999) (Tables 2 and 3). This further confirmed the inheritance of resistance by a single dominant gene for the cross. The 20 families segregating for Striga yielded 140 resistant plants and 53 susceptible plants which fits closely to a 3:1 ratio ($X^2$ = 0.623; p = 0.42). This analysis further confirmed that single dominant gene confers resistance to S. gesnerioides in IT97K-499-35 and B301.

**Marker analysis**

The two primers used were very informative as they showed polymorphism between the parents and were subsequently used to screen the individual F2 plants of the two segregating populations. The two markers (61RM2 and C42-2B) analysis, conducted on the F2 populations derived from the crosses Borno Brown x IT97K-499-35 and Borno Brown x B301, showed that the bands were clearly readable for the different markers used. The result of the analysis of marker segregation in the cross derived from Borno Brown x IT97K-499-35 is presented in Figure 1.

The electrophoresis of the PCR product generated polymorphic bands that were highly reproducible and scoreable. Of the 100 F2 individual plants screened with 61RM2, amplification of product was detected in both susceptible and resistant lines. In the resistant lines, two bands appeared consistently, which is a characteristic of 61RM2 dimorphic marker (Figure 1). The 61RM2 marker amplified a 450 bp fragment similar to that reported in mapping population for Striga race 3. 61RM2 which has a characteristic dimorphic banding pattern with one band similar in both the resistant and susceptible genotypes and a lower fragment that was polymorphic, being present only in resistant genotypes but absent in susceptible genotypes. The inability of the marker to differentiate between homozygotes and heterozygotes resistance indicates the dominant nature of the marker (Figure 1).

On the other hand, the primer C42-2B has a monomorphic banding pattern and identifies resistant lines with a definite single band while susceptible line had no band (Figure 2). In the screening of the parental cultivars with 61RM2 marker, all the 12 plants of the susceptible parent (Borno brown) showed a single band while those of IT97K-499-35 showed double bands similar to that observed in the segregating F2 resistant lines for 61RM2. As it was observed with the phenotypic score, all the F1 plant DNA samples were also resistant using the markers. Scoring of the F2 segregation shows that of the 100 plant DNA analyzed, 81 of them showed double bands while 19 plants showed single band which closely fit the expected genetic ratio of 3:1. The phenotypic score data did not significantly deviate from the expectation of random segregation when compared with the marker score 61RM2 tagging SG3 resistance gene.

Percent co-segregation of the marker between susceptible and resistant relative to the phenotypic data was found to be 98% with less than 2% recombinants. The genetic distance of the marker using Joint MAPMARKER was found to be 3.4 cM from the resistance gene indicating a tight linkage. However, the genetic distance from the phenotype score was found to be 5.9 cM (Figure 3). The markers were also used to validate an F2 population derived from the resistant parent B301 crossed to a susceptible parent Borno Brown, and similar band patterns to that of the first population was observed. The electrophoresis of the PCR product generated polymorphic bands that were reproducible and scorable. A double band was present in resistant lines while a single band was showed by the susceptible line for 61RM2.

In the resistant lines, two bands appeared consistently, which is a characteristic of a dimorphic marker. The presence of a double band indicates resistance while presence of a single band indicates susceptibility. All the susceptible plants showed the 500 bp amplification product.

The resistant plants showed 450 bp band in addition to 500-bp band (homozygous or heterozygous). Linkage analysis performed by Mapmarker confirmed the association of these markers with the resistant gene. Their flanking status was maintained and their distance from the resistance gene were 3.4 and 8.7 cM for the markers 61RM2 and C42-2B, respectively, which were similar to that obtained from the first population (Figure 4).

<table>
<thead>
<tr>
<th>Population</th>
<th>Generation</th>
<th>Total no. of plants</th>
<th>No. of plants</th>
<th>Genetic ratio</th>
<th>X-value</th>
<th>Pr&gt;ChisSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borno Brown</td>
<td>Parent 1</td>
<td>20</td>
<td>1</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IT97K-499-35</td>
<td>Parent 2</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borno Brown x IT97K-499-35</td>
<td>F1</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borno Brown x IT97K-499-35</td>
<td>F2</td>
<td>300</td>
<td>231</td>
<td>69</td>
<td>3:1</td>
<td>0.65</td>
</tr>
</tbody>
</table>

**Table 1. Segregation analysis of Striga resistance in a cross between Borno Brown x IT97K-499-35**
Table 2. Marker segregation analysis of F₂ and F₃ progeny testing of the population derived from the cross Borno Brown × IT97K-499-35.

<table>
<thead>
<tr>
<th>Population</th>
<th>Generation</th>
<th>Total number of plants</th>
<th>Number of plants</th>
<th>Genetic ratio</th>
<th>χ²-value</th>
<th>Pr&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borno Brown</td>
<td>Parent 1</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IT97K-499-35</td>
<td>Parent 2</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borno Brown x IT97K-499-35</td>
<td>F₁</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borno Brown x IT97K-499-35</td>
<td>F₂</td>
<td>100</td>
<td>82</td>
<td>18</td>
<td>3:1</td>
<td>2.61</td>
</tr>
<tr>
<td>Borno Brown x IT97K-499-35</td>
<td>F₃</td>
<td>30 families</td>
<td>10 homozygote</td>
<td>20 heterozygote</td>
<td>1:2</td>
<td>0.00</td>
</tr>
<tr>
<td>Non-segregating family =10</td>
<td></td>
<td></td>
<td>88</td>
<td>87</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Segregating family=19 =20</td>
<td></td>
<td></td>
<td>193</td>
<td>140</td>
<td>53</td>
<td>3:1</td>
</tr>
</tbody>
</table>

Table 3. Marker segregation analysis of F₂ and F₃ progeny testing of the population derived from the cross Borno Brown × B301.

<table>
<thead>
<tr>
<th>Population</th>
<th>Generation</th>
<th>Total number of plants</th>
<th>Number of plants</th>
<th>Genetic ratio</th>
<th>χ²-value</th>
<th>Pr&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borno Brown</td>
<td>Parent 1</td>
<td>20</td>
<td>1</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B301</td>
<td>Parent 2</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borno Brown x B301</td>
<td>F₁</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borno Brown x B301</td>
<td>F₂</td>
<td>100</td>
<td>78</td>
<td>22</td>
<td>3:1</td>
<td>0.48</td>
</tr>
<tr>
<td>Borno Brown x B301</td>
<td>F₃</td>
<td>30 families</td>
<td>11 homozygote</td>
<td>19 heterozygote</td>
<td>1:2</td>
<td>0.1503</td>
</tr>
<tr>
<td>Non-segregating family =11</td>
<td></td>
<td></td>
<td>108</td>
<td>107</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Segregating family=19 =20</td>
<td></td>
<td></td>
<td>183</td>
<td>134</td>
<td>49</td>
<td>3:1</td>
</tr>
</tbody>
</table>

Progeny testing with DNA marker

The identification of heterozygous F₂ plants was done through their F₃ progeny testing by growing 30 families obtained from F₂ plants carrying the dominant allele for *Striga*. Of the 30 families analyzed, 10 families breed true for *Striga* resistance while 20 families segregated for *Striga*. The proportions of the non-segregating to segregating families fit the theoretical 1:2 ratio as confirmed by chi-square test, with probability values of p=0.34.

Relative efficiency of 61RM2 and C42-2B in detecting SG3 race

These two primers generated polymorphisms that were linked to SG3 resistance in coupled phase. They only segregated with the dominant allele. Both markers are dominant markers. Comparison of the similarity values obtained with the two DNA markers used in the genetic populations gave highly significant correlations with the phenotypic score (Figure 3). This very strong correlation between the phenotype and the markers suggests that a locus with a major influence on *Striga* was closely linked to 61RM2 on the linkage group. Thus, these markers, especially the 61RM2, can be used in screening segregating populations for *Striga* resistance instead of the phenotypic screening that is laborious and environmentally influenced. C42-2B similarity values (Table 4) correlated with those obtained using phenotype in the different population but these correlations should be interpreted carefully due to the low number of amplification products obtained with C42-2B and the discrepancies observed in the F₂
segregating population. For example, in some cases 61RM2 identified some lines as susceptible but phenotypic data and C42-2B marker analysis classified them as resistant. When the seeds of these lines were screened in the F₃ families, they were found to support many haustorial attachments. The linkage to the resistance gene in 61RM2 was found to be 3.4 cM while that of C42-2B was found to be 5.4 cM. 61RM2 showed consistent value of 99, 97, and 98%, respectively in detecting SG3 resistance (Figure 3). On the other hand, C42-2B marker showed inconsistency in the different populations (98, 95, and 96%). Similarly 61RM2 had a relatively sharper product resolution and a tight linkage with the resistant gene locus compared with C42-2B. In this study, 61RM2 and C42-2B were found to be reliable markers for race 3 resistance. However, in IT97K-499-35, both markers are on the same side of the SG3 resistance gene, while in B301, the markers are (Figure 3) flanking

**Figure 1.** A 2% agarose gel electrophoretic analysis of PCR amplified product using 61RM2 marker for F₂ progenies derived from Borno brown x IT99K-499-35. All F₂ lines are resistant except line 7 which is smeared. R and S indicate Resistant and Susceptible respectively. P₂, IT97K-499-35; P₁, Borno brown; C, Control without genomic DNA template; L, 100 bp ladder.

**Figure 2.** A 2% agarose gel electrophoretic analysis of PCR amplified product using C42-2B marker for the F₂ progenies derived from Borno brown x B301. All F₂ lines are resistant except line 2 which is susceptible as indicated by the absence of band. R and S indicate resistant and susceptible respectively. P₂, IT03K-338-1; P₁, Borno brown; C, Control without genomic DNA template, L, 100 bp ladder.
DISCUSSION

Reaction of parental lines to *Striga*

The results of the pot culture screening revealed that the cultivars B301 and IT97K-499-35 have resistance to the Prevalent race of *Striga* (SG3) from the northeast of Nigeria. Resistance to *Striga* of these lines was characterized by a lack of parasite emergence and attachment on the root. Also the F1 hybrids resulting from crosses between these lines with the susceptible cultivar (Borno Brown) shows lack of parasite attachment which indicate the complete dominance of resistance over susceptible. These observations further confirm the earlier report of Singh and Emechebe, (1991), Atokple et al. (1993) and Carsky et al. (2002). The local variety (Borno Brown) used in this study showed high levels of susceptibility to *Striga*, exemplified by several parasite attachments on the root with only one plant free of parasite attachment probably due to escapism.

Segregation analysis of the F2 progenies of the two different genetic populations of susceptible × resistant cowpea crosses used in the present study showed that a single dominant gene conferred resistance to the *S. gesnerioides* collected from Maiduguri, Nigeria. The entire F1 hybrids were resistant indicating a complete dominance of resistance over susceptibility. Segregation analysis fits expected genetic ratio of 3:1 confirming a monogenic dominant inheritance. This finding is consistent with the earlier work of Singh and Emechebe, (1990) and Atokple et al. (1993) who reported that a single dominant gene in cultivar B301 confers resistance to *S. gesnerioides* race 3 predominant in Nigeria.

The results obtained from the F3 progeny testing further revealed that inheritance of *Striga* resistance in each of the population derived from Borno Brown x B301 and Borno Brown x IT97K-499-35 is controlled by a single dominant gene. Single dominant gene inheritance of resistance to *S. gesnerioides* in B301 has been earlier reported using similar populations derived from a cross involving B301 and another susceptible cultivar (Lane and Bailey, 1992; Atokple et al., 1995), and a gene symbol *Rsg1* (resistance to *S. gesnerioides*) was proposed for this trait. The result of the pot culture techniques was also confirmed using two molecular markers (61RM2 and C42-2B) earlier reported to be linked to *Striga* race 1 and 3 (Oudraogo et al., 2012 and Gowda et al., unpublished). The highly significant correlation between the phenotypic scoring and the markers indicates that the results of the phenotypic marker and genotypic marker are the same. The slight differences observed are well within the range expected from sampling, as shown by a relatively small deviation of the samples. There was a clear segregation pattern in the F2 populations and the F3 families. The F2 populations segregated in the ratio 3:1 (resistant 75% and 25% susceptible), which suggest the inheritance of a single dominant gene for resistance. This was further confirmed in the F3 progeny testing by growing 30 families randomly selected from F2 plants which were resistant to *Striga* and have large seed size for each cross. The proportion of segregating to non-segregating F3 families obtained fits the theoretical 1:2 ratio as confirmed by chi-square test, with probability values of p=0.999 and p=0.69 for the cross derived from Borno Brown x IT97K-499-35 and Borno Brown x B301 crosses, respectively. This result shows that selection for resistance in F2 population is effective. However, the genetic diversity for resistance established by this study is therefore particularly significant in that it promises a long lasting protection against *Striga*. If more virulent races of these parasites
Figure 4. Dendogram for 100 lines derived from a NYST cluster analysis using Dice similarity coefficient based on three markers.

Table 4. Spearman rank correlation coefficient of marker similarity matrix

<table>
<thead>
<tr>
<th>Population</th>
<th>DNA marker</th>
<th>Phenotype marker</th>
<th>Correlation Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borno Brown x IT97K-499-35</td>
<td>61RM2</td>
<td></td>
<td>0.8346 (0.0001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C42B</td>
<td></td>
<td>0.8382 (0.0001)</td>
<td></td>
</tr>
<tr>
<td>Borno Brown x B301</td>
<td>61RM2</td>
<td></td>
<td>0.8207 (0.0001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C42B</td>
<td></td>
<td>0.91437 (0.0001)</td>
<td></td>
</tr>
</tbody>
</table>

Correlation values above 0.8 are considered to be good association (Rohlf, 1993). Values in parenthesis are level of significance expressed as probability with the appropriate complementary agronomic practices of an integrated control package.

Marker screening

The two markers used to screen the F2 segregating population showed polymorphisms with both the parents and the segregating populations. About 3% of the generated polymorphic fragments showed segregation distortion from the expected ratio of 3:1. Many authors have also reported segregation distortion of molecular markers in relation to a phenotype in F2 mapping populations in other crops such as sunflower (Berry et al., 1995), rice (McCouch et al., 1998), lettuce (Landry et al., 1991), common bean (Paredes and Gepts, 1995). Based on linkage analysis using Joint Mapmaker, the two markers were identified to be associated with the gene conferring resistance to race 3 (Nigerian race) present in B301 and IT97K-499-35.

Earlier study conducted by Ouèdraogo et al. (2001) using similar marker E61R found that E61R marker was linked to the resistance gene race 3 (Rsg3) present in B 301 from Nigeria. The 61RM2 and 61R markers were to emerge, the strategy of sequential release of resistant varieties with varying backgrounds would have to be relied upon. The commercial use of varieties resistant to Striga is expected to offer both economical and practical method of control. Host plant resistance is used as a component in an integrated program of pest control in several crops. For Striga, the complete resistance available in cowpea however, should be used

were to adhere to the complete resistance available in cowpea
reported to be effective in identifying resistance to Striga race 1 and 3 (Timko et al., 2007; Ouedraogo et al., 2012). If the genes conferring resistance to *S. gesnerioides* were clustered within the cowpea genome, the markers identified will be immediately useful in the analysis of other populations of cowpea segregating for other genes with race-specific resistance to *S. gesnerioides* (Ouedraogo et al., 2001). The two molecular markers systems used in this study discriminated the segregating population for resistance and susceptibility to *Striga*. However, 61RM2 which had a relatively tight linkage with the resistance gene gave a better resolution among the segregating populations and was consistent in detecting resistance in the two genetic populations indicating the relative efficiency of the marker compared to C42-2B. The results reported here corroborate earlier findings of Boukar et al. (2004). In another study, both markers, E61R-M2 and C42-2B, have been shown to be effective in identifying resistance to *Striga* race 1 and 3 (Timko et al., 2007).

The resolution of a map and the ability to determine marker order are largely dependent on population size, the larger the mapping population the better. Markers linked at a distance less than 5 cM to the target gene can be used for effective indirect selection (Weber and Wrickle, 1994 cited by Brahm et al. 2000). The efficiency of MAS can be increased by employing markers flanking the gene of interest. This has been demonstrated for cowpea resistance gene in cowpea (Boukar et al., 2004), and for the common bean (Kelly and Miklas, 1998). The finding of flanking markers around the resistance gene is an important factor that can increase the efficiency of this indirect selection. When a marker used for selection is not tightly linked to the gene of interest, cross-over will occur between the marker and the gene of interest. This will lead to a high percentage of false-positive/negative selections in the screening process. Procuñer et al. (1997) reported, however, that when flanking markers are used simultaneously, error due to cross-over will be reduced. Thus, the dominant nature of the SCAR markers used in the present study is an important factor for reliability in the linkage analysis. The reliability of the dominant markers has also dispelled the fear reported by other workers that the use of dominant markers in linkage analysis of an F2 population can lead to errors (Beaumont et al., 1996).

The two markers used in the present study, have been identified to be linked to *S. gesnerioides* resistance and were reported to be effective in identifying resistance to race SG1, SG3 and SG5 (Timko and Gowda personal communication) suggesting that the *Striga* gene in the study could belong to linkage group 1. It is worth mentioning here that these two markers were efficient in characterizing the populations for resistance to *S. gesnerioides* although the efficiency of the two markers differs. From the results obtained, 61RM2 appeared to be very useful marker tool in characterizing populations for resistance to *S. gesnerioides* race 3. The marker provides 2 reproducible bands and the process is fairly simple to carry out and easy to score.

**Marker efficiency**

The linkage analysis performed by Joint Mapmaker showed the association of these markers with the *Rsg*-3. Their flanking status was maintained at a distance from the resistance gene of 2.5 and 4.5 cM for marker 61RM2 and C42-2B, respectively, which was similar to that reported for E61R by Ouedraogo et al. (2002, 2012). Markers linked at a distance less than 5 cM to the target gene, as those obtained in the present study, can be effectively used for indirect selection (Weber and Wrickle, 1994). In particular, the significant correlations of 61RM2 and C42-2B matrices with that of the phenotypic score commonly used in the classical genetic analysis indicate that these markers are suitable for this kind of genetic study. Thus, these markers can be recommended for commercial use in screening genetic populations for *Striga* resistance.

61RM2 was the most tightly-linked marker compared to C42-2B. Percent co-segregation of 61RM2 between susceptible and resistant was found to be consistent with a high value 98. However, the advantages of these two markers indicate that both 61RM2 and C42-2B can be used in different genetic populations. Depending upon the parents used in the mapping population, the arrangement of markers and map distance between markers may vary as evidenced in the present study (Figures 3 and 4). The marker results reported herein are comparable with similar studies in *Striga* with reference to percentage polymorphism and number of amplified DNA fragments. The marker score was significantly correlated with the phenotypic score. This is in accordance with the findings of Sato and Takeda (1995), where correlation coefficients of 0.77 and 0.78 were obtained, thus, indicating that the results of the phenotypic marker and genotypic marker are the same, and either method can be used to screen populations for resistance to *Striga*. However, the efficiency of 61RM2 in screening genetic populations for SG3 resistance was quite high and reliable and appeared to be the most efficient marker compared to C42-2B which was specifically developed for SG5 resistance (Gowda personal communication). However, the advantage of genotypic marker over the conventional marker is the speed in detecting resistant and susceptible lines thus shortening the breeding period and avoids the laborious screening period for *Striga* resistance. It also eliminates the effect of genotype x environment interaction. The RIL derived from the crosses were evaluated in *Striga* hot spot field. Those lines that were selected based on marker result were completely free of *Striga* infestation on the field while some of the lines identified to be resistance based on
phenotypic pot screening were found to be susceptible when planted on heavily *Striga* infested field. This makes genetic marker a reliable stable tool for selection and for characterization of cowpea populations for resistance to *Striga*.

**Conclusion**

The results of this study shows that characterization of cowpea lines for *Striga* resistance using molecular markers linked to the trait is feasible and more reliable. The findings of this study have shown that the derived SCAR-Marker 61RM2 was found to be reliable in discriminating between resistant and susceptible individuals in a segregating population other than the population it was originally developed for, thus showing its wider application. The only weakness in the markers is that they are both dominant markers.

**ACKNOWLEDGMENT**

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**Conflict of interests**

The authors did not declare any conflict of interest.

**REFERENCES**


**In vitro** embryo rescue and plant regeneration following self-pollination with irradiated pollen in cassava (*Manihot esculenta* Crantz)

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Cassava is a highly heterozygous species; hence, current methods used in classical cassava breeding cannot match the urgent need to high yielding varieties. Recently, progress was made through androgenesis and gynogenesis as pathways for raising doubled cassava haploid lines to overcome problems associated with cassava’s inherent reproductive biology, but these efforts were limited (no candidate cassava plantlets were regenerated). For the first time, this study shows that pollen irradiation coupled with self-pollination and embryo rescue regenerated 62 candidate cassava plantlets. Plants of an elite cassava variety, Nase14, served as a mother plant and as the pollen donor for the irradiation. Irradiation dosages of 50 to 250 Gray studied across five pollination events and 300 or 500 Gray in one pollination event caused a reduction in pollen germination up to 67.0%. By 15 days after pollination (DAP) with irradiated pollen, up to 89.7% of the pollinated flowers had aborted. By embryo rescue time (42 DAP), significant differences were observed in number of fruits, seeds and embryos generated, with the non-irradiated pollen treatments having significantly higher numbers. Sixteen (16) heterozygous SSR markers in the parent and ploidy analysis showed that none of the regenerated plants was haploid or homozygous. However, the plantlets resulting from pollination with non-irradiated pollen had 56.2% homozygous loci, while progeny derived from irradiated treatments had frequencies of homozygous loci between 28.1 and 55.0%. This is the first time to use irradiated pollen in cassava as a pathway to generate candidate plantlets as an initial step in double haploid production.

Key words: Cassava, doubled haploids, embryo rescue, plant regeneration, pollen germination, pollen irradiation.

**INTRODUCTION**

Cassava (*Manihot esculenta* Crantz) is the world’s second most important root crop that is grown in over 100 countries in Africa, Asia, the Pacific, and South America (FAOSTAT, 2014). Cassava is becoming popular for
three main reasons: (i) Its yields are the highest with respect to the amount of starch per unit area when compared to other staples; (ii) cassava starch is amenable to diverse food and non-food uses; and (iii) it has a flexible harvesting schedule that is suitable for most rural and subsistence farmers. Also, the high resilience of cassava and its adaptation to climatic changes when other major food staples fail is an attribute for sustainable food security (Burns et al., 2010; Jarvis et al., 2012).

Globally cassava yields have increased by ca. 1.8% per annum over the last decade to 12.8 tons per hectare, resulting into a global cassava harvest of more than 280 million tons in 2012, reflecting a 60% increase since 2000 (FAO, 2013). This output is set to accelerate further due to enhanced recognition of cassava’s potential. Therefore, the current and anticipated demands for cassava starch necessitate concerted and systematic efforts tailored to the development of higher yielding cassava varieties (Aerni, 2006; Bull et al., 2011).

Although conventional breeding may still deliver high yielding varieties (Ceballos et al., 2004; 2012), systematic progress in the genetic improvement of cassava is challenged by the heterozygous nature of the crop (Kawano et al., 1978; Meireles da Silva et al., 2003; El-Shakaway 2004; Bull et al., 2011). Similar limitations in maize (Zea mays) were overcome by introducing inbreeding and use of inbreds over a century ago (East, 1908; Shull, 1908; 1909; Crow, 1998). Inbred lines (or pure, homozygous lines) have two copies of the same genome, such that after self-pollination (same genotype is both male and female); all the offsprings are identical to the parent. Similarly, double haploids (DHs) (genotypes formed when haploid cells undergo chromosome doubling), eliminate heterozygosity and achieve approximate complete homozygosity in one generation, compared to many generations in conventional inbreeding (Jain et al., 1996; Maluszynski et al., 2003a, b). The success attained in maize hybrid breeding (Crow, 1998) spurred the use of inbred lines or doubled haploids for several other economically important crop species such as cucumber, flax, oil palm, barley, solanaceous crops (Magoon and Khanna, 1963; Maluszynski et al., 2003a, b; Nasertorabi et al., 2012; Nasution et al., 2013; reviewed in Andersen, 2005; Dunwell, 2010; Germanà, 2011).

Based on the fact that cassava is outcrossing and monoeious, utilization of heterozygous parents to generate F1’s for onward evaluation and selection is the norm since the inception of cassava breeding (reviewed in Ceballos et al., 2004; 2012). However, several advantages in using inbred progenitors in cassava have been reported (Ceballos et al., 2012). In maize, genetic homogeneity and inbred lines can be obtained in one generation through use of doubled haploid inducers, which are attained by crossing target lines with a DH inducer line and thereafter selection among progeny individuals that are doubled haploids (Kebede et al., 2011).

The use of DH inducer technology boosted maize breeding as inbred lines are readily tested and used within a shorter time. In contrast, cassava has a long breeding cycle (that is, generation of sexual seeds) of more than a year, followed by five to six years of evaluation and selection to identify best performing genotypes (Kawuki et al., 2011). Therefore, conventional breeding approach has limited genetic precision particularly when responding to emerging threats such as cassava brown streak and cassava mosaic diseases in Africa and thus remains limited to match the emerging demands for cassava resources (Aerni, 2006; Bull et al., 2011; Ceballos et al., 2012).

Doubled haploids (DHs) are a preferred method for developing homozygous lines in a timely fashion (Jain et al., 1996; Maluszynski et al., 2003a, b; Wedzony et al., 2009). DHs can be produced by tissue culture methods that use immature pollen (microspores) or anthers (androgenesis), ovules (gynogenesis), immature embryos from distant crosses and after pollination with irradiated mature pollen to produce a fully homozygous plant in a short time, enabling the production of pure lines from a donor plant within one generation (reviewed in Forster et al., 2007; Wedzony et al., 2009). The outstanding contributions of DH technology have been reported in several crops, especially in Brassica spp., maize, barley and wheat (Alison et al., 2011). The possibility to raise DH lines in cassava has just been investigated by Perera et al (2014a, b) focusing on androgenesis, gynogenesis and crosses with castor bean (Ricinus communis L.). While all the three methods gave promising results in terms of ability to generate calli, neither of them has so far made a breakthrough to generate cassava plantlets (Perera et al., 2014a, b). Moreover, there is no optimized method to regenerate microspore or anther-derived embryos and plants.

The use of irradiated pollen in combination with embryo culture is an efficient method for induction of haploids in several plants notably plum (Prunus domestica L; Peike et al., 2000), oil pumpkin (Curcurbita pepo; Košmrlij et al., 2013) and melon (Cucumis melo; Sari et al., 1994; Nasertorabi et al., 2012). In cassava, embryo rescue methods are available (Fregene et al., 1999; Akinbo et al., 2010; Yan et al., 2014), which further motivates the use of the pollen irradiation technology. Recently, considerable progress was made for early embryo rescue (two to three weeks after pollination) in cassava by...
cassava crosses (Restrepo, 2014). Taken together, the prevailing challenges in cassava breeding arising from cassava reproductive biology can be overcome using DH technology to facilitate attainment of homozygosity in a short time.

Regardless of the method chosen for doubled haploid production, fast and inexpensive identification of candidate plants and the development of accurate methods or tools chromosomal duplication are critical. Flow cytometry is widely used to evaluate DNA content and determination of the efficiency of haploid induction (Nasution et al., 2013).

Other tools used in the identification of haploids include molecular markers, especially microsatellites (simple sequence repeats, SSR), due to their stability and co-dominance, allowing the separation of homozygotes from heterozygotes (F.) (Drumeva et al., 2005; Belicuas et al., 2007).

The aim of this study was to regenerate cassava plantlets from embryos after self-pollination with irradiated cassava pollen of cassava genotype Nase 14. To our knowledge, this is the first attempt of using irradiated pollen to generate plantlets for on-ward development of doubled haploid cassava lines.

**MATERIALS AND METHODS**

**Plant material**

A cassava variety, Nase 14, was used in this study. Isolated plots of ca. 1.5 acres (0.60 ha) were established at National Crops Resources Research Institute (NaCRRRI), Namulonge, Uganda (~200 m away from the nearest cassava plants of another genotype. The variety Nase 14 was selected because it flowers profusely and is highly responsive to tissue culture (unpublished personal observation); it is thus likely that its explants or progeny will respond well in tissue culture during downstream applications. Mature male flowers (unopened buds) were harvested from cultivar Nase 14 and subjected to gamma irradiation.

**Pollen irradiation**

Harvested mature male flowers were irradiated using a Co\(^{60}\) gamma ray source (GWGP-80, Cobalt-60, Nuclear Power Unit Institute, China, installed at Mulago Hospital, Kampala, Uganda). This irradiation was performed in small cardboard boxes (5 x 7 x 2 cm). The irradiation source had a half-life T\(_1/2\) of 5.26 years and the mean energy of 1.2 MV with the maximum source to surface distance of 80 cm. The field size was set using a hand control panel. The treatment couch table was adjusted by moving it vertically, laterally and horizontally to position the samples in the center of the radiation beam.

Based on the fact that this was a pioneering pollen irradiation experiment in cassava, there was no prior knowledge of the most appropriate dosage to be used. Therefore, the most frequently used ranges in other crops were used (Peixe et al., 2000; Nasetortari et al., 2012), taking keen interest in dosage rates that have been translated into approximately 74, 148, 221, 295 and 369 min of exposure, respectively. At one point, dosages of 300 Gy (443 min) and 500 Gy (738 min) were also used. Non-irradiated male flowers/buds (0 Gy treatments) were included in the experiment as controls.

For each irradiation treatment, ~150 male buds were detached from the plants in the morning (0630 h local time) including buds for the control treatment and placed in (90 x 30) mm Petri dishes. Each irradiation treatment was represented by five Petri dishes, with each Petri dish having ~40 male buds. Only mature male buds (just before anthesis) were irradiated (treatments running from 0800 to 1500 h) and their extracted pollen used for pollination in the evening (1700 to 1830 h). Thus, pollen detachment from mother plants, irradiation and pollination were all done on the same day (within 13 h). Pollination with non-irradiated pollen was also done in the evening, as stated above.

**Pollen germination**

Pollen was cultured on modified Brewbaker and Kwack (1963) medium containing 100 mg/l boric acid; 300 mg/l calcium nitrate; 200 mg/l magnesium sulfate; 100 mg/l potassium nitrate; and 5% sucrose adjusted to pH 5.8. Petal-like bracts were removed from the male flowers and pollen was brushed into the medium. Thereafter, the pollen was incubated in an oven maintained at 40°C and 100% relative humidity. Aniline blue was used for staining, prepared by dissolving 1 g of aniline blue in 100 ml of 85% ethyl alcohol. Aniline blue stains callose that is located in the inner layer of the pollen tube, and although it is not commonly used to stain pollen from the genus Manihot; evidence showed that it is an effective method for observing and studying pollen tube development, pollen morphology, longevity, viability and cross-ability in species of Manihot and other genera (Fang, et al., 2015; Vieira et al., 2015). Pollen germination and viability were assessed after 24 h using a sample of 300 pollen grains per treatment. Three replications were performed per treatment. Pollen was considered to have germinated when pollen tube length was at least equal to or greater than the grain diameter. Pollen viability and germination ability were observed by direct microscopy through an inverted microscope (Nikon Alphaphot-2 YS2, Japan) equipped with a mercury lamp of 100 W and an excitation UV light.

**Pollination with irradiated pollen**

Female flowers were bagged for one day (with white interwoven a nylon bag that is routinely used in cassava pollinations) to avoid undesirable pollinations. Only bagged and opened (receptive) female flowers were pollinated. Pollination was done by hand by brushing the stigmas with irradiated pollen, and thereafter the pollinated flowers were re-bagged for three to seven days to eliminate contamination by foreign pollen. This is the same protocol used in controlled pollinations in cassava (Kawano, 1980). Control pollinations with non-irradiated pollen were performed at the same time. All pollinations experiments were conducted in the field at NaCRRRI as described above. The cassava field was laid out in plots such that each irradiation dosage rate was represented by five Petri dishes with each Petri dish containing ~40 male buds. Only mature male buds (just before anthesis) were irradiated (treatments running from 0800 to 1500 h) and their extracted pollen used for pollination in the evening (1700 to 1830 h). Thus, pollen detachment from mother plants, irradiation and pollination were all done on the same day (within 13 h). Pollination with non-irradiated pollen was also done in the evening, as stated above.

**In vitro embryo rescue**

Fruits were harvested 42 DAP, washed under running tap water, and surface-sterilized for 20 min with 15% sodium hypochlorite (NaOCl) containing two to three drops of Tween 20. Thereafter, the
fruits were rinsed three times with sterile distilled water in a laminar flow hood as previously described (Fregene et al., 1999). Some fruits did not have seeds. Setted seeds were excised from the fruits under sterile conditions in a laminar flow hood and the immature embryos were isolated under a stereomicroscope using sterile forceps.

The immature embryos were each separately cultured in culture tubes containing 15 ml of solid medium (M6) containing modified Murashige and Skoog (MS) medium; 2% sucrose, 1 mg/l GA3, 0.7% agar, adjusted to pH 5.8. This method was adopted from Catano et al. (1993). Immature embryos were cultured for approximately one month in the growth room with a temperature of 28±2°C for 16 h in the light and 8 h of darkness. Embryos that had experienced reasonable growth (with 1-2-cm long shoots) were transferred to baby jars containing 40 ml of solid medium of MS supplemented with 2% sucrose, vitamins and 0.3% phytagel adjusted to pH 5.8. Subsequently, each plantlet was sub-cultured by putting the single stem node cutting on new medium containing solid MS-medium with 2% sucrose. The generated plantlets were subjected to microsatellite genotyping and ploidy analysis.

Microsatellite genotyping

To ascertain zygosity status of the 62 plantlets generated from the rescued embryos, DNA was extracted using the Qiagen kit (Qiagen, Venlo. The Netherlands), following manufacturer’s instructions. The DNA was assayed using 16 genomic simple sequence repeat markers (SSRs; EME425, NS158, NS169, SSRY100, SSRY103, SSRY106, SSRY135, SSRY148, SSRY179, SSRY181, SSRY19, SSRY20, SSRY215, SSRY240, SSRY28 and SSRY59) (Mba et al., 2001). All these markers/loci were heterozygous in the mother genotype (Nase 14), and thus deviations from the original heterozygous state to homozygous state (for the generated plantlets) would indicate attainment of homozygosity. The increased homozygosity attained through inbreeding could be inferred from the plantlets derived from the non-irradiated treatments. Amplifications with SSR primers were performed in 10 µl reactions containing 50 ng of DNA, 1 pmole of each primer, 1x Taq DNA Polymerase buffer, 2 mM MgCl2, 0.2 mM dNTPs and 0.375 U Taq DNA Polymerase (New England Biolabs Inc.). The PCR cycling parameters were 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55-57°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 30 min. Differently-sized amplification products at the different loci and variable fluorescent labels [(NED, 6-FAM, PET and VIC (by manufacturers, MWG-Biotech)] on the forward primer allowed multiplexing of amplicons from the same individual.

For each co-loading set, 1 to 2 µl depending on amplification efficiency, of the different amplicons were mixed and briefly vortexed. Aliquots of 1 µl of the mixture were added to 9 µl of a master-mix containing Hi-Di formamide and GeneScan 500 LIZ size standard (1 ml of Hi-Di and 12 µl of 500-LIZ). Amplicons were denatured at 95°C for 3 min, and subjected to capillary electrophoresis using the ABI 3730 DNA sequencer (Applied Biosystems) and allele calls made using GENEMAPPER® software version 3.7 (Applied Biosystems). All genotyping was done at the Biosciences Eastern and Central Africa hub in Nairobi, Kenya. The DNA of the heterozygous Nase 14 was used as a control. In addition, genetic relationships among the generated plantlets were calculated using the simple matching Euclidean distance (D2) followed by cluster analysis using the weighted neighbour-joining algorithm. Relationships were visualised as a dendrogram. This analysis was done using PowerMarker version 3.25 (Liu and Muse, 2005).

Ploidy analysis

Eight-week old plantlets (since initiation onto embryo rescue medium) were obtained from rescued embryos and subjected to ploidy analysis using flow cytometry technique according to Otto (1990). For each plantlet, the fresh innermost part of approximately 25 mg of a young leaf tissue was cut with a sharp razor blade in a glass Petri dish containing 0.5 ml of cold OTTO1 buffer (0.1 M citric acid monohydrate and 0.5% Tween-20). The contents were then passed through a nylon filter of 50 µm pore size. The filtrate was centrifuged at 1000 rpm for 5 min, and the pellet was re-suspended in OTTO 1 buffer. Thereafter, 400 µl of OTTO II buffer (containing 0.4 M anhydrous NaHPO4, 4 µg/ml of DAPI (4,6-diamidino-2-phenylindole), and 1µl/ml mercaptoethanol) was added and the samples run for analysis. Diploid mother plants of cassava (cv. Nase 14) were used as internal controls for ploidy determination. The channels or peak mean of diploid cassava were used to compute a ratio that could be used to discriminate the ploidy levels of the samples.

RESULTS

Effects of radiation on pollen, fruits, and embryos

Pollination germination done 13 h after irradiation treatments showed that irradiation doses used did not prevent pollen germination. Microscopic observations also indicated that pollen grains germinated across all irradiation doses (Figure 1). However, the frequency of pollen germination was reduced for irradiated pollen as compared to fresh or non-irradiated pollen (Table 1). Indeed, compared to fresh pollen, the 13 h old non-irradiated pollen (0 Gy) had the lowest reduction in pollen germination (24%) while the irradiated pollen registered reductions of up to 67% with 200 Gy irradiation dose (Table 1).

Five sets of pollinations with irradiated pollen were undertaken with the number of pollinated female flowers ranging from 494 (for irradiation dosage of 250 Gy) to 595 pollinations (for dosage of 50 Gy) (Table 1). A total of 660 pollinations were made with non-irradiated pollen (Table 1). Thus, across the five irradiation sets, mean numbers of pollinations made with irradiated pollen were 98.8 (for 250 Gy), 108.2 (for 200 Gy), 112.4 (for 150 Gy), 117.2 (for 100 Gy), 119 (for 50 Gy), and 132 pollinations (for non-irradiated pollen) (Table 1). By 15 DAP, most of the pollinated flowers (fruits) had aborted (Table 1). The highest abortion (89.7%) was observed in flowers pollinated with 250 Gy, while the least abortion rate (72.6%) was found in flowers pollinated with non-irradiated pollen (Table 1).

At the time of embryo rescue (42 DAP) (Figure 2A-E), less than 10 fruits per irradiation treatment were still surviving following pollination with irradiated pollen (Table 1). Fruit length and width did not differ significantly (P > 0.05) across the irradiation doses. However, average fruit length (1.4 cm) and fruit width (1.2 cm) were consistently lower than those recovered from non-irradiated pollen treatments, which were 1.6 and 1.5 cm, respectively (Table 1). Significant differences (P < 0.05) were observed in number of fruits, seeds and embryos generated with the non-irradiated pollen treatments having significantly higher numbers (Table 1).
Figure 1. *In vitro* germination of pollen grains of cassava (*M. esculenta* Crantz) cv. Nase 14 after irradiation with various dosages. Pollen tubes (thick thread-like structures) emerging out of the germinating pollen grains (darkened circular structures) are shown. Upper panel (A-F) shows germination without aniline blue staining, while the lower panel (G-L) shows germination with aniline blue staining. The dosage used was 0 Gy (no irradiation, A and G), 50 Gy (B and H), 100 Gy (C and I), 150 Gy (D and J), 200 Gy (E and K), and 250 Gy (F and L). Images for dosage of 300 and 500 Gy are not shown.

Under natural conditions, the cassava pistil is trilocular with a single ovule in each locule, producing a maximum of three seeds from each fruit at maturity (Ogburia et al., 2000). However, for all pollinations undertaken with irradiated pollen, some fruits did not contain seeds while some recovered seeds did not contain embryos. For example, at 42 DAP in 100 Gy treatment, an average of 9.8 (ca. 10) fruits were rescued containing 21.2 (ca. 21) seeds (instead of the expected maximum 30 seeds in a trilocular pistil) from which only 5.4 (ca. 5) embryos were recovered (Table 1). On the other hand, the non-irradiated pollen treatment had on average 26.2 (ca. 26) fruits at 42 DAP from which 64.8 (ca. 65) seeds were recovered and 45.6 (ca. 47) embryos were rescued (Table 1, Figure 2D-F). On average, fruits resulting from pollinations with irradiated pollen had 1.9 seeds per fruit, while those from non-irradiated pollen had 2.5 seeds per fruit (Table 1). For the seeds that had an embryo, one embryo was found for each seed. Two weeks after embryo rescue treatments, the first leaf appeared (Figure 2F) and by eight weeks, a total of 62 cassava plantlets were recovered from embryo rescue procedures (Figure 2H-J).

**Ploidy and zygosity analysis**

The regenerated 62 plantlets (Figure 2I-J) were subjected to zygosity and ploidy analysis. Each plantlet was considered a different genotype and thus analyzed separately. On calibration using diploid Nase 14 as the internal control, the channel or peak mean was 78.8 (Table 2). Ideally, a haploid plant should have half of this mean (that is, channel mean of 39.4). The ratio of channel means of mother plant, Nase 14, were all above 39.4 (Table 2), indicating that none of the plantlets was haploid.

When analyzed with 16 SSR markers that are heterozygous in the mother plant (Nase 14), none of the 62 plantlets was completely homozygous (Supplementary Table S1). However, varying levels of homozygosity were observed. For instance, the four plantlets resulting from 150 Gy had homozygous loci in a frequency varying between 6.0% (one locus out of 16 loci) and 50.0% (eight loci out of 16 loci), while the four plantlets from 250 Gy had homozygous loci varying between 12.5 and 68.0% (Table 2, Supplementary Table S1). The five plantlets from 0 Gy had percentages of homozygous loci ranging from 43.0 to 68.0% (Table 2). On average the highest homozygosity was observed from plantlets from 0 Gy and the lowest was from plantlets from 200 Gy (Table 2). These results suggest that the plantlets obtained may have originated in self-pollinations rather than through parthenogenesis as it was hoped.

**DISCUSSION**

This study reports for the first time in the history of cassava breeding the use of irradiated pollen and *in vitro* embryo rescue procedures to regenerate cassava plantlets as candidate materials for the development of doubled haploids. Irradiation dosages 50 to 250 Gy were studied across five pollination events, while dosages 300 and 500 Gy were used in one pollination event. Although irradiation reduced pollen germination (between 30-67%), none of the irradiation dosages was able to completely inhibit pollen germination completely. Non-irradiated 13 hour-old pollen showed a 24% reduction in pollen germination, a finding that has strong implications towards ongoing efforts to address flowering and fruit-set bottlenecks in cassava crossing nurseries. Pollination with irradiated pollen and subsequent embryo rescue resulted in the recovery of 62 diploid cassava plantlets.
Table 1. Influence of irradiation dosage on cassava pollen germination, mean number of fruits, seeds, and embryos generated following pollination with irradiated pollen.

<table>
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<tr>
<th>Sample Identity</th>
<th>Total no. of pollen grains</th>
<th>No. of pollen grains with pollen tube</th>
<th>Percent reduction in pollen germination</th>
<th>Total pollinations</th>
<th>Mean pollinations</th>
<th>Percent abortion, 15 DAP</th>
<th>Average no. of fruits at 42 DAP</th>
<th>Fruit length (cm)</th>
<th>Fruit width (cm)</th>
<th>No. of seeds</th>
<th>Average no. of seeds per fruit</th>
<th>No. of embryos</th>
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<tr>
<td>Fresh pollen</td>
<td>898</td>
<td>242.3 (80.8)</td>
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<tr>
<td>Dose 0 Gy</td>
<td>890</td>
<td>184 (61.3)</td>
<td>24.1</td>
<td>660</td>
<td>132.0</td>
<td>72.6</td>
<td>26.2</td>
<td>1.7</td>
<td>1.6</td>
<td>64.8</td>
<td>2.5</td>
<td>45.6</td>
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<tr>
<td>Dose 50 Gy</td>
<td>880</td>
<td>167 (55.7)</td>
<td>31.1</td>
<td>595</td>
<td>119.0</td>
<td>85.0</td>
<td>8.2</td>
<td>1.3</td>
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<td>16.2</td>
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<tr>
<td>Dose 100 Gy</td>
<td>815</td>
<td>90.6 (30.2)</td>
<td>62.6</td>
<td>586</td>
<td>117.2</td>
<td>81.1</td>
<td>9.8</td>
<td>1.7</td>
<td>1.3</td>
<td>21.2</td>
<td>2.2</td>
<td>5.4</td>
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<td>Dose 150 Gy</td>
<td>520</td>
<td>140.6 (46.9)</td>
<td>42.0</td>
<td>562</td>
<td>112.4</td>
<td>85.4</td>
<td>5.6</td>
<td>1.2</td>
<td>1.0</td>
<td>9.2</td>
<td>1.6</td>
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<td>Dose 200 Gy</td>
<td>161</td>
<td>80.0 (26.7)</td>
<td>67.0</td>
<td>541</td>
<td>108.2</td>
<td>81.8</td>
<td>6.2</td>
<td>1.3</td>
<td>1.2</td>
<td>8.6</td>
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<td>4.0</td>
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<tr>
<td>Dose 250 Gy</td>
<td>852</td>
<td>170.6 (56.9)</td>
<td>29.0</td>
<td>494</td>
<td>98.8</td>
<td>89.7</td>
<td>7.2</td>
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\( ^* \) Numbers in parentheses indicate percent germination based on counts of 300 pollen grains across replicates. Assessments of number of fruits, fruit length, fruit width, number of seeds and embryos rescued, all done at 42 DAP. Data for dosages 300 and 500 Gy are not presented.

Figure 2. Embryo rescue at 42 days after pollination (DAP) and regeneration of cassava plantlets. A and B: Opened and unopened cassava locules containing one embryo each for extraction; C: Freshly extracted embryo; D, E and F: Embryo germination after three days, 1 week, and 2 weeks of culture, respectively; G and H: cassava plantlets after 4 and 16 weeks of culture, respectively, from the time embryo rescue was done; I and J: cassava plantlets from embryos rescued from irradiated pollinations for three and eight weeks, respectively, of being hardened in the screen house. A total of 62 cassava plantlets were successfully regenerated from the rescue embryos.
that are currently undergoing in vitro growth to allow future field evaluations and characterization.

Successful in vitro pollen germination in the family Euphorbiaceae is rare with limited success (for example, Orrego and Hershey, 1984; Mbahe et al., 1994; Chavarriaga-Aguirre and Halsey, 2005; Viera et al., 2012); however, efficient pollen germination was achieved in our study using modified Brewbaker and Kwack (1963) medium at pH 5.8. These results annul an earlier hypothesis by Viera et al. (2012) claiming the presence of a specific substance found in the stigma but not in the medium that induces pollen germination, which opens up opportunities to study the impact of irradiation in cassava. The effects of ionizing radiation on pollen germination have been studied in a number of plants (Visser and Oost, 1981; Pooler and Scorza, 1997; Musial and Pryzwarra, 1998; Yue and Zou, 2012; Košmrlj et al., 2013). These studies generally concluded that irradiation results in a reduction of pollen germination. Similarly, our study shows that pollen germination in cassava is possible up to 500 Gy, although there was a general reduction in the frequency of pollen germination with irradiated pollen. Studies in peach (Prunus persica) have shown that pollen germination is still possible at 1500 Gy (Pooler and Scorza 1997). Furthermore, huge differences in pollen germination frequency between non-irradiated pollen (that is, freshly collected pollen and 13 h old pollen) indicate that viability of cassava pollen after shedding drops rapidly, something that is indirectly implied by poor fruit set observed in cassava crossing nurseries (Nassar and Ortiz, 2006). Future experiments will reduce the time between pollen collection and pollination; develop protocols for preserving pollen viability, and to determine a clear dose response. Nonetheless, irradiation may have had an effect on the pollen as revealed by the zygosity patterns of the generated plantlets. In addition, source activity (for example, Gy/min) along with total radiation is an important factor to take into account. Ideally, future studies should use high-activity sources that will require a short time of exposure to achieve the desired and probably higher levels of radiation.

Pollinations with irradiated pollen ranged between 494 (for 250 Gy) to 595 (for 50 Gy), potentially translating to 494 and 595 fruits, respectively. However, by 15 DAP over 80.0% of the fruits had aborted (highest abortion in the 250 Gy treatments). By embryo rescue time at 42 DAP, fewer fruits and seeds were obtained from irradiated treatments as compared to the non-irradiated treatments (P < 0.05). Due to the fact that this was the first time to use irradiated pollen in cassava, no direct comparisons can be made. Studies on other plants, notably peach (Prunus persica) have reported fruit set of less than 15 % for pollinations with pollen irradiated with 530, 820 and 5000 Gy (Pooler and Scorza, 1997). This suggests that more than 85% fruits or seed loss is observed following pollination with irradiated pollen. In kiwifruit (Actinidia deliciosa), the number of abnormal seeds drastically increased in fruits when pollinations with irradiated pollen were performed, that is by 68% for 700 Gy and 74% for 900 Gy (Musial and Pryzwarra, 1998). Related studies show that the number of seeds generated by pollination with irradiated pollen is a function of genotype (Nasertorabi et al., 2012), while the frequency of haploids generated using irradiated pollen is a function of the recipient female parent and the pollen donor (Košmrlj et al., 2013). Also, haploid production from the same genotype is not consistent across different pollination events with irradiated pollen (Košmrlj et al., 2013). Our study consisted of only one cassava genotype, Nase 14. This decision was made in order to get an initial insight based on one genotype to guide follow-up studies on the use of irradiated pollen on many other cassava genotypes.

High fruit abortion was observed in all cases resulting from pollination with irradiated pollen, irrespective of low (50 Gy) or five-fold higher doses (250 Gy). Each irradiation

<table>
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<th>Dosage</th>
<th>No. of plants</th>
<th>No. of homozygous loci</th>
<th>Mean (%) homozygous loci</th>
<th>Channel mean</th>
<th>Mean ratio of sample to diploid mother plant</th>
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<tr>
<td>Dose 0 Gy</td>
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<td>16</td>
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<td>78.8</td>
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*aCassava mother genotype that was considered under this study. Number of homozygous loci out of the 16 loci that were assayed in each of the samples.
dose was associated with a significantly lower number of seeds that had no embryos. In kiwifruit, seeds resulting from pollination with irradiated pollen at 700 and 900 Gy had 90% of the seeds containing only endosperm and 9.6% seeds containing both endosperm and embryos (Musial and Pryzwara, 1998). On the other hand, in plum (Prunus domestica), there was a continuous fruit drop in number from 30 to 70 DAP due to absence or occurrence of fertilization, followed by a rapid rejection of the male genome (Peixe et al., 2000).

Fruit abortion can be a result of rapid collapse of the zygote due to problems related to mitosis in irradiated pollen (Peixe et al., 2000), a phenomenon that could also explain the high abortion rates observed in our study. It is also possible that the aborted cassava fruits had fragile haploid embryos, but this was not determined under this study. However, without systematic efforts to rescue and analyze ploidy and zygosity levels of the embryos, it is rather difficult to make accurate judgments on what is happening in vivo. In cassava elite lines HMC-1, SM1219-9 and MCOL1505, natural open pollination by insects showed that the ideal stage for embryo rescue was 32 to 36 DAP (Yan et al., 2014) compared to 42 DAP in our study. Thus, our current efforts are tailored towards optimizing embryo rescue protocols for cassava embryos that are 10-14 DAP, a period associated with high fruit abortion as observed in our study. This similar strategy could be helpful in rescuing cassava embryos from wild crosses, which are also associated with significantly high abortion rates (Restrepo, 2014). These efforts are critical as the lack of normal fertilization process (due to the irradiation of pollen) would prevent the normal development of endosperm which would result in the abortion of the haploid embryos that would otherwise develop.

Ploidy and zygosity analysis of the 62 plantlets rescued from embryos at 42 DAP indicated that none was haploid, suggesting that the irradiated pollen from each of the dosages was able to germinate on stigma, grow within the style, reach the embryo sac, and thereafter fertilize the egg cell. Alternatively, the development of normal diploid zygotes may have originated in pollinations happening after the bags covering the flowers have been removed. It is because of the interest to develop doubled haploids through different approaches that recent studies have demonstrated that stigmas remain viable for up to three days after anthesis (unpublished data). This information was not available at the time this current study was conducted. Thus, no parthenogenesis was initiated in the recovered plantlets. It is also likely that the irradiation doses partially affected the pollen sperm nuclei but did not affect its capacity to fertilize the egg cell. In kiwifruit, the frequency of abnormal seeds was shown to increase following pollination with irradiated pollen (Musial and Pryzwara, 1998). On the other hand, it is also possible that parthenogenesis may have been induced but the resulting embryos would have failed to develop fully as reflected by the high fruit abortion. In kiwifruit, parthenocarpic fruits were obtained at varied doses, that is, at 200 Gy (hexaploids), 500 to 900 Gy (hexaploid and triploids), and at 1500 Gy (only triploids) with embryo rescue done between 50 and 72 DAP (Musial and Pryzwara, 1998). In cassava, the deployed irradiation doses (50 to 500 Gy) were probably not sufficient. Thus, follow-up studies could consider exploring higher irradiation doses that should be accompanied by optimal protocols for embryo rescue within the first 14 days after pollination. Equally important is the need to have short time requirements for attaining irradiation doses and undertaking subsequent pollinations.

The possibility that some mutations or aberrations occurred in the pollen grains and were transferred to the progeny is implied by the genetic diversity observed among the S1-inbreds as revealed by heterozygosity data. For example, the S1-inbreds resulting from pollination with non-irradiated pollen had 56.2% homozygous loci (implying that some 43.8% could potentially be heterozygous). On the other hand, S1-progeny derived from pollinations with irradiated pollen had an average frequency of homozygous loci between 28.1% (100 and 200 Gy) to 55.0% (50 Gy). This translates to about 71.9 to 45.0% loci being in a heterozygous state. One generation of inbreeding of a completely heterozygous plant is expected to generate 50.0% loci in a homozygous state and the other 50.0% loci in heterozygous state. Indeed, the results from the non-irradiated control approximate this expectation with 56.2% loci being in homozygous state, but not the results of the irradiation treatments (Table 2, Supplementary Table S1). This suggests that new genetic variability may have resulted from radiation-induced mutations; however, we plan to fully characterize these plantlets and to establish whether or not novel traits have been added to the already existing native traits in the mother parent.

Taken together, this study provides evidence that pollen germination in cassava though reduced is not completely inhibited by irradiation dosages of up to 500 Gy, which opens up opportunities to explore the effects of higher irradiation dosages. Despite the high incidences of fruit abortions and seeds without embryos after self-pollination with irradiated pollen, the successful regeneration of 62 diploid cassava plantlets via embryo rescue demonstrates a major advancement towards the development of doubled haploid plants via irradiated pollen for pure line breeding schemes in cassava. As such, the placement of cassava in the current and future agriculture merits adoption of this technology to generate genotypes that match emerging needs. Current follow-up studies are aimed at weaning and hardening of the plantlets to allow field evaluations, self-pollination experiments, implementing these procedures to other genotypes, exploring higher irradiation doses by use of high activity sources, and rescuing embryos at earlier stages of development.
Conflict of interests
The authors did not declare any conflict of interest

ACKNOWLEDGEMENTS
The funds for undertaking this work were provided by the International Centre for Tropical Agriculture (CIAT). We thank personnel of Mulago Hospital, Kampala, Uganda, who assisted with the gamma irradiation. We thank Mr. Moses Mugisha of the International Institute of Tropical Agriculture (IITA-Uganda) for technical assistance in ploidy analysis. We also thank each of the six anonymous reviewers for their helpful comments that improved this manuscript and Professor Silvester Nyakaana (Makerere University) for his critical comments and assistance in the English editing of the manuscript.

REFERENCES


**Supplementary Table S1:** Genotypes of 62 cassava plantlets assayed with 16 heterozygous SSR loci.

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Adventitious shoots induction and plant regeneration from cotyledons of watermelon (*Citrullus lanatus* L.)

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A highly efficient regeneration system is a prerequisite step for successful genetic transformation of watermelon cultivars (*Citrullus lanatus* L.). The objective of this study was to establish efficient *in vitro* plant regeneration for three watermelon cultivars. To achieve optimal conditions for adventitious shoot induction, the 5-day-old explants (cotyledon base portion, apical portion and hypocotyl) of three cultivars were placed on MB$_5$ media supplemented with different concentrations and combinations of growth regulators (1.0 to 10.0 mg L$^{-1}$ 6-benzyladenine (BA) and 0 to 1.0 mg L$^{-1}$ indole acetic acid (IAA)); the explants from seedling of different development stages (0 to 10 d) were cultured on MB$_5$ medium containing 2.0 mg L$^{-1}$ BA and 0.2 mg L$^{-1}$ IAA for investigating the effect of age on adventitious shoots initiation; besides, 5-day-old seedlings were grown on optimal regeneration medium supplemented with different concentrations of kanamycin for screening the lowest lethal concentration for adventitious shoots. The results show that the basal region of cotyledon showed higher frequency of shoot formation (79.17-83.33%) than the apical region (5.23-8.25%); high percentage of shoots regeneration was induced from 5-day-old cotyledons base portion cultured on MB$_5$ containing 1 or 2 mg L$^{-1}$ BA; the 100 mg L$^{-1}$ kanamycin proved to be the optimal concentration for screening the transformants. Our results provide an efficient stable regeneration system for genetic transformation of watermelon.

**Key words:** Watermelon (*Citrullus lanatus*), cotyledon, growth regulator, kanamycin, regeneration.

**INTRODUCTION**

Watermelon (*Citrullus lanatus* L.), one of the most important vegetable crops, is eaten chiefly as a fresh fruit, because of its rich carbohydrates, vitamins and minerals (Cho et al., 2008; Huang et al., 2011; Yu et al., 2011; Guo et al., 2013). It originated from tropical and subtropical Africa (Cho et al., 2008). However, it is susceptible to a number of diseases (Kim et al., 1998; Compton and Gray, 1999), insect pest (Coffey et al., 2015) and other environmental factors, which lead to reductions in crop yield and quality. To decrease the
The effects of these disadvantage factors, annual field rotations, frequent chemical sprays, and new elite cultivars (Compton and Gray, 1999) are required. The annual field rotation needs lots of land, and the chemical sprays can cause environmental pollution. Thus, breeding elite cultivars of watermelon is the most effective way to improve crop yield and quality.

Although removing unfavorable traits and finding material sources through traditional breeding methods are genetically agreeable (Compton and Gray, 1993a), introducing resistant genes into commercial cultivars by traditional breeding mechanisms is not very efficient. Therefore, introduction of foreign genes has a potential for the improvement of watermelon. Recently, success of transformation has been reported in watermelon using transgenic technology (Choi et al., 1994; Chen et al., 1998; Ellul et al., 2003; Akashi et al., 2005; Park et al., 2005; Cho et al., 2008; Huang et al., 2011; Yu et al., 2011; Lin et al., 2012). Furthermore, the success of genetic manipulation using transgenic technology mainly depends on an efficient regeneration system.

Plant regeneration is a prerequisite step for genetic transformation and is usually influenced by biotic factors such as genotype and explant type, and abiotic factors such as culture media and environmental conditions (Liu et al., 2010). Most of the regeneration occurs via organogenesis, and successful plant regeneration from watermelon mature cotyledons (Srivastava et al., 1989; Dong and Jia, 1991; Compton and Gray, 1993b; Tabei et al., 1993; Chaturvedi and Bhatnagar, 2001; Pirinç et al., 2003; Li et al., 2011; Zhang et al., 2014), immature cotyledons (Compton and Gray, 1993a), petiole (Jeyakumar et al., 2014) and hypocotyl (Srivastava et al., 1989) have been reported. However, these reports of shoot regeneration from watermelon cotyledons are contradictory with regards to genotype, age of explant, optimum growth regulator combinations and concentrations, conditions of rooting and acclimatization, and may not be suitable for many commercial cultivars. Therefore, it is necessary to establish an efficient protocol for regeneration for a wide range of commercial cultivars.

The objectives of this study were to establish efficient plant regeneration system by testing the effects of the type and age of the explants and growth regulator concentrations on plant regeneration in three commercial cultivars. This tissue culture system would be used for the transformation of watermelon by Agrobacterium tumefaciens.

**MATERIALS AND METHODS**

**Plant materials and adventitious shoots introduction**

Three watermelon (Citrus vulgaris L.) cultivars, ‘Heihai’, ‘Tiancheng’ and ‘Jimanduo’, were used throughout this study. After the mature seeds coats were removed by a forceps, the seeds were surface-sterilized in 70% (v/v) ethanol (Shenggong China) for 1 min, in 0.1% (m/v) HgCl₂ (Shenggong China) for 5 min, and then washed 3-5 times in sterilized distilled water. The sterilized decoated seeds were placed on 1/2-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) under dark conditions at 25 ± 1°C for germination. The hypocotyls and cotyledons were removed by a scalpel from 5-day-old seedlings and then the cotyledons were cut crosswise and lengthwise into cotyledon base portion and apical portion. The different parts of cotyledons and the hypocotyls segments were set as explants. Then, explants were placed on MB₅ media (MS salts, B₅ vitamins (Gamborg et al., 1968), 30 g L⁻¹ sucrose (Shenggong China) and 7 g L⁻¹ agar (Shenggong China)) supplemented with 1, 2, 3, 4, 5, 6, 7, and 10 mg L⁻¹ N₆-benzyladenine (BA, Invitrogen USA) in combination with 0, 0.2, 0.5, and 1 mg L⁻¹ indole-3-acetic acid (IAA, Invitrogen USA) for shoot induction.

**Optimal seedling age for adventitious shoots regeneration**

To investigate the effect of age of the seedlings on adventitious shoots initiation, the basal region of cotyledon of three cultivars from seedling of different ages (0, 3, 5, 7, and 10 days) were cultured on MB₅ medium containing 2 mg L⁻¹ BA and 0.2 mg L⁻¹ IAA.

**Rooting and plant acclimatization**

After four weeks in shoots regeneration media, regenerated multiple shoot buds from cotyledon base portion were sub cultured onto MB₅ medium supplemented with 2.0 mg L⁻¹ BA, 1.0 mg L⁻¹ IAA and 2.0 mg L⁻¹ Gibberellic acid (GA₃, Invitrogen USA) for shoot elongation. The resulting elongated shoots (about 2-2.5 cm) were separated into signal one and transferred to rooting medium, which consisted of 1/2-strength MS medium supplemented with 0.2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D, NAA, Invitrogen USA). Regenerated plantlets with well-developed roots were gently washed with tap water to remove the attached medium from their roots and then transplanted to flowerpots (14 × 16.5 cm, Shenggong China) in greenhouse, one plant per pot. All pots contained equal quantities of the nutrient soil (1 vermiculite: 1 perlite: 2 garden nutrient soils, by volume).

**Determination of optimal kanamycin concentrations for shoot bud induction**

To determine the lowest lethal concentration of kanamycin (Invitrogen USA) for adventitious shoots induction, the cotyledon base portion of 5-day-old seedlings was used as explants. The explants of three cultivars were grown on optimal regeneration medium supplemented with different concentrations of kanamycin (0, 50, 75, 100 and 125 mg L⁻¹). The explants were maintained for four weeks without subculture.

For all in vitro, the pH of the medium was adjusted to 5.85 before autoclaving; the medium was then autoclaved for 20 min at 1.1 kg cm⁻² and 121°C. Cotyledon were cultured in petri plates (90×16 mm) containing 20-25 ml medium for shoot induction and adventitious shoots were cultured in culture bottle (90×60 mm) that contained 30 ml medium and five adventitious shoots for elongation and rooting. The cultures were maintained at 24 ± 1°C under a 16 h photoperiod of 40-50 µmol· m⁻²· s⁻¹ irradiance provided by cool white fluorescent lamps. Cultures were observed weekly, and the adventitious bud primordia and shoots were counted at the same time. Each treatment was arranged in a completely randomized design (CRD) with 4 replicates and 8 explants per replicate. The number of shoot and roots initiated per explants were calculated after 1 month. The data were analyzed by analysis of variance (ANOVA) and Duncan’s multiple range tests (p<0.05) with the SPSS software (SPSS Inc, Chicago, IL, USA).
RESULTS

Shoot regeneration

The sterilized decoated seeds were cultured on ½ strength MS medium under dark conditions for 5 days and then the radicles (Figure 1A) were carefully removed. Cotyledons and hypocotyls on regeneration medium expanded in size during the first few days of culture. There were significant differences in shoot induction of different regions of cotyledons and hypocotyls. Green meristematic protrusions, which resembled young shoot apices, were observed at the enlarged cotyledon base portion. Then multiple shoot buds differentiated directly from per cotyledon base portion within 10-20 days (Figure 1B). Calli were present in all cotyledons apical portion of three cultivars in induction medium (Figure 1C), but only 5.23-8.25% shoot buds differentiated from callus. Analysis showed that the cotyledon apical portion gave the lower frequency of shoots induction for ‘Heihaier’, ‘Tiancheng’ and ‘Jinmanduo’ (5.25, 5.23 and 8.25%, respectively). However, white loose calli were formed from the hypocotyls but no shoot buds differentiated from callus (Figure 1D). At the same time, some regenerated shoot from cotyledon base portion appeared to have a hyperhydric phenotype (Figure 1E).

Effect of genotypes on shoot induction

The regenerated shoots were obtained from the three cultivars. The cotyledon base portions of the three watermelon genotypes gave shoot differentiation frequencies of 8.33-83.33% on MB5 medium supplemented with BA in combination with IAA (Figure 2). The highest shoot induction frequencies were the same for ‘Heihaier’ (83.33%) and ‘Tiancheng’ (83.33%) but these were higher than ‘Jinmanduo’ (79.2%) and they were no significant differences in shoot induction. The genotypes also differed in the time required for the initiation of shoot buds and the number of shoots produced. The cotyledons from ‘Heihaier’ and ‘Jinmanduo’ showed faster morphological response than the cotyledons from ‘Tiancheng’.

Effect of BA and IAA concentration on shoot induction

The cotyledon base portion of ‘Heihaier’ gave high shoot regeneration frequencies on MB5 medium containing 2.0 mg L⁻¹ BA or 2.0 mg L⁻¹ BA and 0.2 mg L⁻¹ IAA (83.33
Figure 2. Effects of different concentrations of BA and IAA on adventitious shoots formation from cotyledon base portion explants of the three watermelon cultivars (*Citrullus lanatus* L.). Adventitious shoots of three watermelon cultivars. The shoot differentiation frequency is expressed as the percentage of the cotyledon base portion forming shoots in 5 weeks. Error bars with standard error.

and 70.0%, respectively). Low concentrations of BA (1.0-3.0 mg L\(^{-1}\)) in shoot induction medium were effective for shoot initiation and small multiple buds were usually formed on medium which contained low concentrations of IAA (0.2 mg L\(^{-1}\)). However, shoot regeneration with high concentrations of BA (4.0-10.0 mg L\(^{-1}\)) showed lower frequencies (16.67-54.17%) than for low concentrations of BA, whether we added the IAA or not, and the number of shoot buds per explant also decreased. Furthermore, these shoots were difficult to elongate. 'Tiancheng' and 'Jinmanduo' showed similar responses to the BA in combination with IAA, and the optimal shoot regeneration medium are MB\(_5\) medium supplied with 1 mg L\(^{-1}\) BA (Figure 2).

**Effect of age of donor seedlings on shoot induction**

The shoot differentiation frequencies of 'Heihaier', 'Tiancheng' and 'Jinmanduo' varied remarkably with the different ages of seedlings at MB\(_5\) medium containing 2 mg L\(^{-1}\) BA and 0.2 mg L\(^{-1}\) IAA. The shoot differentiation frequencies of three varieties ('Heihaier', 'Tiancheng' and 'Jinmanduo') increased with extension of culture time (0, 3 and 5 days); 5-day-old seedlings had the highest frequencies, which were 1.28-, 2.55- and 1.25-folds of 3-day-old seedlings, respectively.

However, shoot regeneration ability of the cotyledonary explants decreased dramatically when the age of donor seedlings exceeded seven days (Table 1). At the same
Table 1. Effect of age of cotyledon base portion explants on shoot induction of three watermelon cultivars (Citrullus lanatus L.).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Age of explants (d)</th>
<th>No. of explants cultured</th>
<th>No. of explants forming adventitious buds</th>
<th>Induction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>32</td>
<td>10</td>
<td>31.25 ± 3.61 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32</td>
<td>18</td>
<td>56.25 ± 3.61 b</td>
</tr>
<tr>
<td>'Heihaier'</td>
<td>5</td>
<td>32</td>
<td>25</td>
<td>71.88 ± 3.13 a</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>32</td>
<td>17</td>
<td>53.13 ± 5.98 b</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>32</td>
<td>6</td>
<td>18.75 ± 3.61 d</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>32</td>
<td>6</td>
<td>18.75 ± 3.61 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32</td>
<td>9</td>
<td>28.13 ± 3.13 c</td>
</tr>
<tr>
<td>'Tiancheng'</td>
<td>5</td>
<td>32</td>
<td>23</td>
<td>71.87 ± 3.13 a</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>32</td>
<td>15</td>
<td>46.88 ± 3.13 b</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>32</td>
<td>6</td>
<td>18.75 ± 3.61 c</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>32</td>
<td>11</td>
<td>34.38 ± 3.13 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32</td>
<td>20</td>
<td>62.50 ± 5.10 b</td>
</tr>
<tr>
<td>'Jimanduo'</td>
<td>5</td>
<td>32</td>
<td>25</td>
<td>78.13 ± 3.13 a</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>32</td>
<td>16</td>
<td>50.00 ± 5.10 b</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>32</td>
<td>8</td>
<td>25.00 ± 5.10 c</td>
</tr>
</tbody>
</table>

Date represents mean ± standard error for four replicates (sixteen explants each). Values with different lowercase letters within each variable have significant difference at p < 0.05 (Duncan’s multiple range test).

time, with the increase of seedling age (more than seven days), the number of adventitious buds derived from every explant also decreased gradually (less than 3). These results indicate that the selection of younger aseptic seedling was one of the keys to increasing the budding rate. Thus, 5-day-old seedlings proved to be the optimal source for culture.

Shoot elongation

It was important that the shoot buds were transferred to elongation medium in time. Shoot buds could elongate normally when they were subcultured on MB5 medium supplemented with 2 mg L⁻¹ BA, 1 mg L⁻¹ IAA and 2 mg L⁻¹ GA₃ after four weeks on regeneration medium (Figure 1F). If the cotyledons with multiple shoot buds were maintained on regeneration medium for longer than four weeks, only a few shoot buds could elongate and the rest of shoot buds showed abnormal morphology, such as yellow color, swollen and brittle buds, and some callus formed on the wound. At the same time, about 3-6 shoots can be obtained from each explants of the cultivars used in this study.

Rooting of shoot

For the induction of root, elongated shoots from the three cultivars were excised and cultured on ½ strength MS medium supplemented with 0.2 mg L⁻¹ NAA. The roots were observed after about two weeks (Figure 1G). In rooting medium, 94.8 (55/58), 95 (57/60) and 94.3% (50/53) of green shoots from 'Heihaier', 'Tiancheng' and 'Jinmanduo' rooted. After three weeks, rooted plantlets were transferred to greenhouse, only 70% of the plantlets survived and their leaves turned dark green in one week (Figure 1H).

Effects of kanamycin on adventitious buds induction

Kanamycin had a great influence on organ differentiation for watermelon cotyledon (Figure 3). Adventitious buds from three watermelon cotyledon base portion were insensitive to low concentrations (0-75 mg L⁻¹) of kanamycin, and the range of induction rate for 'Heihaier', 'Tiancheng' and 'Jinmanduo' were 15.63-71.88, 18.75-71.88 and 15.63-78.13%, respectively. However, the frequency of shoot regeneration decreased rapidly with increasing kanamycin concentration and the shoots were not formed from cotyledon base portion cultured at 100 mg L⁻¹ kanamycin (Table 2). Undifferentiated cotyledon became yellow and necrosis after 1 month.

DISCUSSION

Three cultivars received positive results on MB5 medium supplemented with low BA concentration alone (1.0 or
Figure 3. Effect of different concentrations of kanamycin on adventitious shoots formation from cotyledon base portion explants of the three watermelon cultivars (Citrus lanatus L.). Adventitious shoot was determined after 4 weeks of cultivation on induction medium. The antibiotic concentrations were 0, 50, 75, 100 and 125 mg L\(^{-1}\) kanamycin from left to right.

Table 2. Effect of kanamycin concentrations on shoot regeneration from cotyledon base portion explants of three watermelon cultivars (Citrus lanatus L.)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Kanamycin concentrations (mg L(^{-1}))</th>
<th>No. of explants cultured</th>
<th>No. of explant forming adventitious buds</th>
<th>Induction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Heihaier'</td>
<td>0</td>
<td>32</td>
<td>25</td>
<td>71.88 ± 3.13 a</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>32</td>
<td>9</td>
<td>28.13 ± 3.13 b</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>32</td>
<td>5</td>
<td>15.63 ± 3.13 c</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>32</td>
<td>1</td>
<td>3.13 ± 3.13 d</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>32</td>
<td>0</td>
<td>0 ± 0 d</td>
</tr>
<tr>
<td>'Tiancheng'</td>
<td>0</td>
<td>32</td>
<td>23</td>
<td>71.88 ± 3.13 a</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>32</td>
<td>10</td>
<td>31.25 ± 3.61 b</td>
</tr>
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<td>75</td>
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<td>6</td>
<td>18.75 ± 3.61 c</td>
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<td></td>
<td>100</td>
<td>32</td>
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<td>3.13 ± 3.13 d</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>32</td>
<td>0</td>
<td>0 ± 0 d</td>
</tr>
<tr>
<td>'Jimanduo'</td>
<td>0</td>
<td>32</td>
<td>25</td>
<td>78.13 ± 3.13 a</td>
</tr>
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<td></td>
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<td>75</td>
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<td>15.63 ± 3.13 c</td>
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<td>100</td>
<td>32</td>
<td>0</td>
<td>0 ± 0 d</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>32</td>
<td>0</td>
<td>0 ± 0 d</td>
</tr>
</tbody>
</table>

Values with the different lowercase letters within each variable have significant difference at p < 0.05 (Duncan’s multiple range test). Note: Date represent mean ± standard error for four replicates (eight explants each).

2.0 mg L\(^{-1}\)). This phenomenon suggests that low concentration of BA is necessary for shoot induction and differentiation from the cotyledon of watermelon. Srivastava et al. (1989) induced adventitious shoots from cotyledon in watermelon by low BA concentration (1.0 mg L\(^{-1}\)) and similar results were reported in many studies (Compton and Gray, 1993b; Choi et al., 1994; Pirinc et al., 2003; Huang et al., 2011; Yu et al., 2011; Choi et al., 2012). These reports showed that the high frequency of shoot regeneration in watermelon required a low concentration of BA (BA: 1.0-4.0 mg L\(^{-1}\)). However, Blackmom and Reynold (1982) induced adventitious shoots from cotyledon of watermelon by using the combination of 10 mg L\(^{-1}\) 2ip and 0.1 mg L\(^{-1}\) NAA, and similar results were reported (Dong and Jia, 1991; Tabei et al., 1991). They thought high concentration of cytokinin (BA: 5-10 mg L\(^{-1}\)) were prerequisite for inducing adventitious shoots, which was very different from our result. We observed that high concentration of BA (over 3 mg L\(^{-1}\)) resulted in very low shoot differentiation and low concentration of IAA (0.2 mg L\(^{-1}\)) was helpful for shoot induction and differentiation. Besides, a higher concentration of auxin inhibits shoot formation and promotes callus proliferation (Tabei et al., 1991).

In this study, the shoot formation frequency of the three cultivars was different between the basal region and apical...
region of cotyledon. The basal region had higher frequency (79.17-83.33%) than the apical region (5.23-8.25%) after four weeks culture on regeneration medium. Similar results were reported by Monacelli et al. (1988), Tabei et al. (1993) and Košmrlj et al. (2015). These phenomena suggested that the proximal end of each cotyledon segment regenerated adventitious shoots more effectively than the distal end and the basal region showed polarity. Compton and Gray (1993b) obtained adventitious shoots only on the proximal region of cotyledons and suggested that the competence for adventitious shoot formation in watermelon was restricted to the proximal region of the cotyledons. Besides, it had the highest regeneration rate (89.67%) when entire cotyledon was used as explants (Li et al., 2011). However, Choi et al. (1994) indicated that the distal half cotyledonary explants produced more shoots than the proximal half. They thought cells competent for shoot formation are not localized at one site of the cotyledon in these cultivars and light was essential for adventitious shoot formation, because no cotyledonary explants of two cultivars (‘Sweet Gem’ and ‘Gold Medal’) formed shoots in the dark (Choi et al., 1994). In this study, many shoots appeared at the proximal end of the basal region. Our results supported that the proximal end of basal region had high potential for shoot formation.

The age of donor seedlings is one of the key factors for adventitious bud induction in C. lanatus sp. and Cucurbita sp. (Zhang et al., 2008). The numerical results indicated that cotyledon from 5-day-old seedlings were the most sensitive to shoot formation (Dong and Jia, 1991; Choi et al., 1994; Pirinç et al., 2003) and similar results were obtained in our study. However, different optimal age of cotyledons for adventitious bud induction had been reported; such as two days (Compton and Gray, 1993c), three days (Tabei et al., 1993; Ellul et al., 2003; Krug et al., 2005; Yu et al., 2011), seven days (Huang et al., 2011) and 7-10 days (Cho et al., 2008). A possible explanation is that type and concentration of disinfectant, sterilization time and seed storage time could affect the physiological status of mature seeds.

Although natural aromatic cytokinins such as BA and kinetin were most commonly used in regeneration systems, it might have several side effects, such as difficulties in rooting, hyperhydricity, stunted shoots and callus formation (Magyar-Tábori et al., 2010). The undesirable effects are attributed to either its N7- and N9-glucosylation or to conjugation with alanine, which results in biologically inactive but chemically stable derivatives and enabling slow release of active compounds (Werbrouck et al., 1995). In this study, we also found that there was hyperhydricity phenomenon in watermelon tissue culture, but lower concentration of BA can reduce the number of hyperhdyric adventitious shoots. Yu et al. (2011) indicated that SH vitamins with 50 mg L⁻¹ thiamine HCl could overcome the hyperhydric phenotype and effectively reduce the percentage of hyperhydricity in watermelon. However, the reason SH vitamins affected hyperhydricity is still not clear. Besides, it has been reported that hyperhydricity would be prevented by adding silicon to the culture medium (Sivanesan et al., 2010) and Ag⁺ (Vinoth and Ravindran, 2015), decreasing the ratio of NH₄⁺: NO₃⁻ (Ivanova and Van Staden, 2009), increasing agar concentration (Brand, 1993), and using techniques such as bottom cooling (Saher et al., 2005).

Watermelon regenerated seedling is prone to etiolation and senescence, which might be caused by rapid growth of many adventitious buds. Through tests we found that shortening subculture times could get good regeneration plants. Another noteworthy result is that the multiple shoots regenerated from watermelon explants must be transferred to elongation medium, because prolonged culture on shoot induction medium not only stimulates callus formation but also produces abnormal shoots. Similar phenomenon was found in other watermelon cultivars (Chaturvedi and Bhatnagar, 2001). One of the most commonly used selection marker genes for screening transgenic plants is nptII, which encodes a phosphotransferase capable of phosphorylating aminoglycoside antibiotics, including kanamycin, genetin, neomycin and paromomycin (Yoshikura, 1989). To date, kanamycin has been commonly used for the selection of nptII transformed plants of watermelon (Huang et al., 2011; Yu et al., 2011) and melon (Choi et al., 2012). The optimal concentrations of kanamycin used to suppress non-transgenic watermelon adventitious shoots were different, such as 40 mg L⁻¹ (Park et al., 2005), 100 mg L⁻¹ (Choi et al., 1994; Huang et al., 2011; Yu et al., 2011), 125-175 mg L⁻¹ (Ellul et al., 2003) and 200 mg L⁻¹ (Huang et al., 2011; Yu et al., 2011). Similar phenomenon was reported by Choi et al. (2012). Our results indicated that differentiation of adventitious buds was suppressed with 100 mg L⁻¹ kanamycin. Thus, 100 mg L⁻¹ kanamycin proved to be the optimal concentration for screening the transformants.

In conclusion, our results clearly demonstrate that high frequency in vitro plant regeneration of watermelon can be obtained by the proper combination of explant age, types and concentrations of plant growth hormones. The system will be potentially useful in the transformation of watermelon via Agrobacterium-mediated or micro particle bombardment.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (No.31272188), the Natural Science
Plant watermelon (Citrullus colocynthis and Citrullus vulgaris) and resquencing of 20 diverse accessions. Nat. perhydricity in termelon rootstock resistant to CGMMV (C. lanatus) germplasm. HortScience 151 Cucumis iruzian ES (1989). Tissue culture and Citrullus lanatus ion to the endoreduplication pattern and Cucumber. J. Cancer 43x67


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Physicochemical, antioxidant, and sensorial properties of peach snacks prepared from different cultivars

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High potentiality of medicinal benefits of peach have increased its demand, however, supply of fresh fruit to meet the demand is challenging as it grows in specific climatic regions and in particular season. Preparation of varieties of processed peach products could be a good option to supply in lean season. Objective of the study was to assess the quality characteristics of peach snacks prepared from 11 different cultivars. We investigated the physicochemical (soluble solid, titratable acidity, hardness and dry yield), antioxidant (DPPH radical scavenging capacity and total phenolics content) and sensorial properties (color, flavor, texture, sweetness and overall acceptance) of peach snacks prepared from 11 peach cultivars. Peach snacks of different cultivars evaluated in this study showed substantial variations in antioxidant capacity, physicochemical and sensorial properties. Some of the samples showed higher physicochemical properties while the others contained better antioxidant capacity or sensory properties. Results of this study reveal that quality peach snacks having different properties could be prepared by drying thin slices of fresh fruits of different cultivars. However, supplementary studies on cost effective techniques of peach snacks preparation and variation in nutritive and medicinal properties of processed products could increase the application of the findings of this experiment.

Key words: Antioxidant, peach snack, physicochemical, sensory property.

INTRODUCTION

Peach (Prunus persica L. Batsch) is a cool season fruit that could be cultivated in different parts of the world. It is regarded as an important economic crop and also recommended for its various health benefits (Yang et al., 2011). Peach contains caffeoylquinic acid, one of the bioactive polyphenols with significant antioxidant activity and important beneficial effect in human health (Luo et al., 2008). Antioxidants present in peach scavenge the reactive oxygen species in human blood plasma and thus provide a potential protection against various chronic diseases with dietary consumption of peach (Tsantili et al., 2010).
As the fruit is grown only in a particular season and specific climatic regions, supply of fresh fruit to a large extent is challenging in spite of its big demand from across the world. On the other hand, some consumers decline to buy fresh fruits as they find the internal quality poor when fruit is consumed fresh (Byrne, 2002). In such a scenario, high quality dried fruits could be a good option to replenish the supply of health benefits of peach during off-season and in those areas where peach is not produced.

High potentiality of medicinal benefit (Manzoor et al., 2012) of some fruits including peach has increased their demand; however, some of the processing related problems have raised concerns among large number of consumers. In order to maintain the quality in some dried fruits, sulfur fumigation is often applied to prevent quality deterioration and fungal attacks (Miller, 1984). However, the hazardous nature of sulfur raises a health risk and food safety concerns (Islam and Hoque, 2013) as the traces of sulfur dioxide remained in food causes asthma or allergic reaction to some people.

Different cultivars of peach have different fruit quality and nutritional composition. Various preferences of consumers have raised the interest in breeding programs worldwide to focus on fruit quality and nutritional composition (Wolfe et al., 2008). Some of them are devoted to produce cultivars with excellent taste, high sugar levels, and balanced sugar/acid ratios (Esti et al., 1997). Other breeding programs are focused to the identification and quantification of phenolic compounds (Cantin et al., 2009). High quality dried fruits could be a good option to replenish the supply of health benefits of peach during off-season and in those areas where peach is not produced.

Very limited studies regarding the potential use of dried peach snacks have been reported. In the context of big scope of dried fruits of peach as fresh fruits are not available year round in the all parts of the world and different quality and nutritional composition of the cultivars, the objective of this study was to evaluate the physicochemical, antioxidant, and organoleptic properties of peach snacks prepared from 11 peach cultivars. This work will benefit the preparation of good quality processed dry peach snacks.

MATERIALS AND METHODS

Sample and chemicals

Fruits of 11 peach cultivars namely; Hikawa Hakuo, Yume Fuji, Kunika, Jinmi, Kawanakajima Hakoutou, Baekhyang, Changhowon Hwangdo, Wolbongosaeng, Daemyung, Red Start and Shuho, grown at Cheongdo Peach Experiment Station of Cheongdo-city in Korea, were manually harvested 80-85 days after flowering and transported to the laboratory. Fruits were thoroughly washed with tap water and kept for surface drying at room temperature. Seed was separated from flesh and 5 mm thick slices of flesh were prepared manually using knife. The slices were delivered to quick freezing (~40°C) for 12 h then kept for freeze drying to prepare peach snacks. The freeze-dried snacks were packed into air-tight plastic bags until further analysis. All the chemicals and reagents used in the study were of analytical grade.

Rheological measurement of hardness and weight

Hardness of peach snacks was measured using a rheometer (COMPAC-100, Sun scientific Co., Japan) under the following operational conditions: test type, mastication; probe, 25 mm aluminium cylinder probe; load cell, 2.0 kg and table speed, 60 mm/min. Fresh and dry weight of peach slices were measured to observe the dry yield of peach snacks of different cultivars.

Standard chemical analysis

Titratable acidity (TA) and soluble solid (SS) content of peach snacks of 11 cultivars were analyzed to determine their chemical properties. Peach snacks were ground into flour (60-mesh). Measurement of titratable acidity was carried out by titrating 10 mg of ground peach snack to 100 mL of deionized water and expressed as percentage citric acid. Soluble solid content was determined using a refractometer (RX-5000c, Atago, Tokyo, Japan) and expressed as °Brix.

Determination of total phenolic compounds

Total phenolic compounds in ground peach snacks were analyzed following Folin-Ciocalteu method (Singleton, 1999). The reagents were allowed to react for 60 min at room temperature and absorbance was measured at 750 nm using ELISA microplate reader (Infinite F50, Teco, Switzerland). Standard calibration curve was drawn using gallic acid and results were expressed as μg/g on a dry weight basis of sample. The values are presented as means of triplicate experiments.

Determination of DPPH radical scavenging activity

Antioxidant capacity of ground peach snack was quantified by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method as described by Blois (1958). The DPPH solution was prepared at the concentration of 4×10⁻⁴ mol/L in methanol. Ground sample (1 g) was extracted in 10 mL of absolute methanol and hot water (100°C) at room temperature for 12 h. The supernatant was centrifuged (1660×g) at room temperature for 10 min and filtered through 2-μm syringe filter. A 0.1-ml of sample extract was mixed with 2.9 mL of freshly prepared DPPH solution. The mixture was incubated in the dark at room temperature for 30 min and absorbance (Abs) was measured at 517 nm by ELISA microplate reader (Sunrise basic, Tecan, Austria). The control was prepared without mixing any sample extract, and methanol was used for the baseline correction. Values are presented as means of three replications. The radical scavenging activity was calculated using the following equation:

\[ \text{DPPH radical scavenging activity (%) } = \frac{(\text{Control Abs } - \text{Sample Abs})}{\text{Control Abs}} \times 100 \]

Sensory properties evaluation

Sensory properties of peach snacks were evaluated for color, taste (sweetness, astringency), texture, and overall acceptance on 5-scale basis: 1 = very bad, 2 = bad, 3 = moderate, 4 = good, 5 = very good. All the sensory properties were evaluated by 20 volunteer panelists (10 women and 10 men) selected in Kyungpook.
Table 1. Physicochemical properties of 11 peach snacks.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Soluble solid (°Brix)</th>
<th>Titratable acidity (%)</th>
<th>Hardness (Kg/Ø 5 mm)</th>
<th>Dry snack yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH</td>
<td>55.5±0.1^i</td>
<td>0.14±0.02^j</td>
<td>0.72±0.02^k</td>
<td>9.5±0.1^l</td>
</tr>
<tr>
<td>YF</td>
<td>65.8±0.2^d</td>
<td>0.28±0.02^g</td>
<td>0.49±0.04^f</td>
<td>9.9±0.1^j</td>
</tr>
<tr>
<td>KK</td>
<td>63.0±0.1^i</td>
<td>0.37±0.04^d</td>
<td>0.67±0.01^i</td>
<td>10.0±0.1^l</td>
</tr>
<tr>
<td>JM</td>
<td>68.6±0.1^d</td>
<td>1.02±0.05^i</td>
<td>0.72±0.02^h</td>
<td>11.0±0.03^g</td>
</tr>
<tr>
<td>KH</td>
<td>69.1±0.2^d</td>
<td>0.19±0.01^g</td>
<td>2.62±0.01^a</td>
<td>12.0±0.02^d</td>
</tr>
<tr>
<td>BH</td>
<td>75.6±0.1^c</td>
<td>1.59±0.13^g</td>
<td>1.40±0.03^d</td>
<td>13.0±0.02^c</td>
</tr>
<tr>
<td>CH</td>
<td>77.0±0.1^b</td>
<td>0.70±0.23^f</td>
<td>1.19±0.01^g</td>
<td>13.6±0.1^b</td>
</tr>
<tr>
<td>WB</td>
<td>72.8±0.2^d</td>
<td>1.31±0.15^e</td>
<td>1.22±0.01^f</td>
<td>13.0±0.1^c</td>
</tr>
<tr>
<td>DM</td>
<td>78.9±0.1^b</td>
<td>0.23±0.02^e</td>
<td>1.73±0.02^c</td>
<td>15.0±0.3^a</td>
</tr>
<tr>
<td>RS</td>
<td>70.0±0.1^e</td>
<td>5.40±0.16^g</td>
<td>1.34±0.01^h</td>
<td>9.2±0.2^g</td>
</tr>
<tr>
<td>SH</td>
<td>70.0±0.1^e</td>
<td>2.10±0.17^f</td>
<td>2.03±0.02^d</td>
<td>12.0±0.1^d</td>
</tr>
</tbody>
</table>

HH, Hikawa Hakuho; YF, Yume Fuji; KK, Kunika; JM, Jinmi; KH, Kawanakajima Hakutou; BH, Baekhyang; CH, Changhowon Hwangdo; WB, Wolbongjosaeng; DM, Daemyung; RS, Red Start; SH, Shuho. Values are means±SD of three replications. The values followed by different superscript within a column indicate significant difference (p<0.05).

Statistical analysis

Data were analyzed using the statistic version 4.0 package (Analytical Software, AZ, USA) to generate one-way or two-way analysis of variance (ANOVA) when needed and the significant differences between means were identified using the Tukey’s mean test (p<0.05).

RESULTS

Peach snacks of different cultivars evaluated in this study showed substantial variations in antioxidant capacity, physicochemical, and sensorial properties as mentioned by other authors (Byrne et al., 1991; Cantín et al., 2009a; 2009b).

Physicochemical properties

SS contents of peach snacks of different cultivars were significantly different except Red Start and Shuho. Daemyung (78.9±0.1 °Brix) cultivar showed the highest SS content whereas that of Hikawa Hakuho (55.5±0.1 °Brix) was the lowest. With respect to TA, considerable variations among cultivars were found, with the lowest value for Hikawa Hakuho (0.14±0.02% of citric acid) and the highest value for Red Start (5.40±0.16% of citric acid) (Table 1). The highest hardness value was observed in Kawanakajima Hakutou (2.62±0.01 Kg/Ø 5 mm) followed by Shuho (2.03±0.02 Kg/Ø 5 mm). Cultivar Yume Fuji (0.49±0.04 Kg/Ø 5 mm) contained the lowest hardness value. Dry snacks yield was the highest in Daemyung (15.0±0.3%) and the lowest in Hikawa Hakuho (9.5±0.1%) and Red Start (9.2±0.2%) (Table 1). In general, dry snack yield was higher in cultivars with higher SS content except for Red Start (70.0±0.1 °Brix and 9.2±0.2% dry yield) which had equal SS value to Shuho (70.0±0.1 °Brix) but significantly low dry yield (12.0±0.1%).

Antioxidant capacity

Total phenolic compounds and DPPH radical scavenging activity were considered to evaluate the antioxidant capacity of peach snacks of different cultivars. Peach snacks of some of the cultivars showed variability in their antioxidant capacity in hot water extract and methanol extract (Table 2). Total phenolic content of Hikawa Hakuho (1.44±0.05 µg GAE/mg on a dry basis) was the highest in hot water extract whereas Wolbongjosaeng (1.41±0.02 µg GAE/mg on a dry basis) showed the lowest value. On the other hand, Hikawa Hakuho (1.52±0.07 µg GAE/mg on a dry basis) was found to possess the lowest and Kunika (3.12±0.07 µg GAE/mg on a dry basis) the highest value in methanolic extract. The DPPH radical scavenging capacity was the highest in Changhowon Hwangdo (11.00±0.37%) and Red Start (10.50±0.50%) and the lowest in Yume Fuji (3.50±0.31%) as the extraction was carried out in hot water whereas the highest value for the same was found in Red Start (19.00±0.60%) and the lowest in Baekhyang (8.00±0.36%) when the extraction was made in methanol.

Sensory characteristics

Sensorial characteristics of peach snacks varied slightly among cultivars (Table 3). The highest and lowest values
Table 2. Total phenolic content and DPPH radical scavenging activity of 11 peach snacks in hot water and methanol extracts.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total phenolics (µg GAE/mg on a dry basis)</th>
<th>DPPH (% on a dry basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HWE</td>
<td>ME</td>
</tr>
<tr>
<td>HH</td>
<td>1.44±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.52±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>YF</td>
<td>1.42±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.90±0.08&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>KK</td>
<td>1.42±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.12±0.07&lt;sup&gt;m&lt;/sup&gt;</td>
</tr>
<tr>
<td>JM</td>
<td>1.42±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.61±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>KH</td>
<td>1.42±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.84±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BH</td>
<td>1.43±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.32±0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CH</td>
<td>1.42±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.06±0.03&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>WB</td>
<td>1.41±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.29±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM</td>
<td>1.42±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.37±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>RS</td>
<td>1.43±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.02±0.05&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH</td>
<td>1.42±0.02&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.59±0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>H, Hikawa Hakuho; YF, Yume Fuji; KK, Kunika; JM, Jinmi; KH, Kawanakajima Hakutou; BH, Baekhyang; CH, Changhowon Hwangdo; WB, Wolbongjosaeng; DM, Daemyung; RS, Red Start; SH, Shuho. GAE = Gallic acid equivalent; HWE = hot water extract; ME = methanol extract. Values are means±SD of three replications. The values followed by different superscript within a column indicate significant difference (p<0.05).

Table 3. Sensory characteristics of 11 peach snacks.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Color</th>
<th>Flavor</th>
<th>Texture</th>
<th>Sweetness</th>
<th>Overall acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH</td>
<td>3.9±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.7±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>YF</td>
<td>4.5±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KK</td>
<td>2.7±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.8±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.5±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8±0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.5±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>JM</td>
<td>3.1±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.9±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.0±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KH</td>
<td>3.8±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0±0.1&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>4.1±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BH</td>
<td>2.7±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.8±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.0±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.4±0.2&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>CH</td>
<td>4.6±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.2±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>WB</td>
<td>2.6±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM</td>
<td>2.8±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.1±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RS</td>
<td>2.0±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.1±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5±0.1&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>1.9±0.2&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH</td>
<td>2.0±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.7±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.6±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.7±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.6±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>H, Hikawa Hakuho; YF, Yume Fuji; KK, Kunika; JM, Jinmi; KH, Kawanakajima Hakutou; BH, Baekhyang; CH, Changhowon Hwangdo; WB, Wolbongjosaeng; DM, Daemyung; RS, Red Start; SH, Shuho. Values are the means±SD of triplicate experiments (n=10) based on 5-point scores (very poor, 1; poor, 2; fair, 3; good, 4; very good, 5). The values followed by different superscript within a column indicate significant difference (p<0.05).

for color were found in Yume Fuji (4.5±0.1), Changhowon Hwangdo (4.6±0.1), Red Start (2.0±0.3) and Shuho (2.0±0.3), respectively. The flavor value was the highest for Hikawa Hakuho (3.6±0.1), Yume Fuji (3.6±0.2), Kawanakajima Hakutou (3.4±0.1), Baekhyang (3.6±0.2) and Changhowon Hwangdo (3.5±0.1) and the lowest for Shuho (1.7±0.2). Hikawa Hakuho (4.1±0.2), Yume Fuji (4.0±0.1), Jinmi (3.9±0.2), Kawanakajima Hakutou (4.1±0.1), and Changhowon Hwangdo (4.0±0.1) had the highest and Shuho (1.6±0.1) had the lowest values for texture. The highest sweetness value was obtained for Jinmi (4.1±0.2) and Kawanakajima Hakutou (4.0±0.1) while the lowest for Red Start (1.5±0.1) and Shuho (1.7±0.2). Finally, the highest overall acceptance scores were for Yume Fuji (4.9±0.1) and the lowest for Shuho (1.6±0.1).

**DISCUSSION**

Significant differences in SS content were found in the dried peach snacks made of different cultivars. These
variations enrich the scope of preparing varieties of snacks from them since SS content is a key quality trait in peaches and nectarines as it is reported that consumer acceptance and satisfaction are related to these traits. However, this relationship varies with cultivar as no standard SS content satisfies consumer, moreover, is affected by other quality traits, such as TA (Crisosto and Crisosto, 2005).

A large number of reports showed a beneficial effect of phenol antioxidants on heart disease and cancer. Phenolic content found in these peach snacks are higher than those previously reported (Gil et al., 2002; Celia et al., 2009) which might be due to the difference in the nature of sample: dry fruit sample was used in the present study instead of fresh one as did in previous studies. However, the proportion of variation in antioxidant capacities between hot water and methanol among different cultivars were not clearly understood.

Differences in overall acceptance of peach snacks in the present study could be considered to prepare highly acceptable product as statistics showed that many food products, even when developed from a sound scientific point of view, encounter poor market acceptance (Hilliam, 1998). Approximately 75% of newly launched food products suffer from poor liking and are withdrawn from the food market during their first two years (Menrad, 2003). The variation in physicochemical, antioxidative, and sensorial traits of different peach snacks might be due to the genotypic variation among cultivars as pomological characteristics of fruits are strongly affected by the genotypes and environments (Moghaddama et al., 2013; Mratnic et al., 2011).

In conclusion, high potentiality of medicinal benefits of peach have increased its demand, however, supply of fresh fruit to meet the demand is challenging as it grows in specific climatic regions and in particular season. Preparation of varieties of processed peach products could be a good option to supply in lean season. This study, portrayed that peach snacks having different physicochemical, sensorial, and antioxidant properties could be prepared by drying thin slices of fresh fruits of different cultivars. However, further study on cost effective techniques of peach snacks preparation could increase the application of the findings of this experiment.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES


Full Length Research Paper

Enrichment and isolation of microbial strains degrading bioplastic polyvinyl alcohol and time course study of their degradation potential

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Polyvinyl alcohol (PVA) degrading bacterial strains were isolated from various environmental sites rich in plastic wastes by using the enrichment culture technique. Among the various isolated strains, the selected potent PVA degrading bacterial strains were tentatively characterized as Bacillus and Pseudomonas sp. The time course of the PVA degradation potential of the characterized strains in growth media containing PVA as a major carbon source was evaluated using the spectrophotometric assay method. This was done by determining the residual PVA remaining in the culture media, increase in cell growth and change in pH of the media over a period of twenty days. The ultimate biodegradation (mineralization) of PVA to its mineral constituents CO₂ and H₂O was determined by the CO₂ evolution test. The strain characterized as Bacillus sp. showed 65% of PVA degradation as determined by spectrophotometric assay and 45.4% of mineralization of PVA over a period of 20 days. The strain characterized as Pseudomonas sp. showed 42% of PVA degradation as determined by spectrophotometric assay and 28.9% of mineralization of PVA over a period of 20 days.

Key words: Polyvinyl alcohol (PVA) degrading bacteria, isolation, ultimate biodegradation, mineralization.

INTRODUCTION

Use of biodegradable, or single use disposable items as a replacement of inert synthetic plastics is gaining wide scale recognition due to its potential in overcoming, or at least reducing issues associated with the management of the post consumption status of synthetic plastics waste. Among the many others biodegradable polymers polyvinyl alcohol (PVA) is attracting increasing attention for its application in the production of environmental-friendly plastic items (Corti et al., 2002; Tang and Alavi, 2011; Qui and Netravali, 2012). PVA is a vinyl polymer where the main chain is joined by only one carbon-carbon linkage (-CH₂-CHOH-)ₙ. This linkage is the same as those of typical plastics, such as polyethylene, polypropylene and polystyrene. PVA is a water soluble polymer but also has thermo plasticity and can be molded in various shapes such as container and films (Shimao,
Biodegradability of this material is mainly due to the presence of the hydroxyl group, which leads to its water solubility and susceptibility to oxidation (Kawai, 1995). Due to these properties, biodegradable plastic based on PVA gains popularity among biodegradable plastics, and is widely used in packaging and agricultural mulch film (Bastioli et al., 1993). Also, types of plastic formulated with PVA are available in the market with several trade names such as Akwa Tears, Alcotex, Alvyl, Aracet, Cipoviol, Celvol, Elvanol, Gelvatol, Ivalon, Solvar, Sumitex, Vinol, etc. Moreover, new and high production capacities of PVA-based plastics are opening in the Republic of Korea, India and South-East Asia (Flieger et al., 2003). In particular, four major segments of PVA consumption include wrap sizing, paper coating, adhesives, films and biodegradable PVA items for example mulching films, laundry bags, etc. (Chiellini et al., 1999).

Widespread application of this polymer as biodegradable plastic has evoked research to understand the fate of this polymer in nature due to microbial degradation. Several groups of scientists have reported microbial degradation of PVA. Suzuki et al. (1973) showed for the first time that PVA was completely degraded and utilized by the bacterial strain *Pseudomonas O-3*, which can use it as the sole source of carbon and energy. An overview of literature cited on PVA biodegradation indicated that many of the research groups reported members of the genus Pseudomonadaceae as the key group providing useful organisms for accomplishing PVA degradation (Hoffmann et al., 2003).

The enrichment culture technique employing PVA as the sole source of carbon and energy is the most extensively used method for isolation of these groups of microorganisms. In the present study, isolation of PVA degrading microbial strains using the enrichment culture technique was carried out from various samples collected from different sites polluted with plastic waste in Mumbai, India. The major objective of the work was to isolate potent PVA degrader which can then be selectively applied to enhance biodegradation of PVA accumulated after its post-consumption. The potential PVA degraders were selected from various isolates and further characterized. The rate of PVA degradation by selected strains in PVA containing growth media were evaluated using spectrophotometric assay and the CO₂ evolution test.

**MATERIALS AND METHODS**

**Collection of microbial source samples from different environmental sites**

Samples from various sites were collected to be used as a microbial source for enrichment of PVA degrading microorganisms. Soil samples were collected from the garden area near the campus of the University of Mumbai, India, and from a dumping ground used to dump municipal solid waste, near Kalyan city, India. Industrial effluent samples were collected from effluent drainage near Gharda Chemicals Ltd, Dombivli, India and from the sea creek, near Century Rayon Ltd. Shahad, India. The sea sediments and sea water samples were collected from sites highly polluted with plastic waste from one of the beaches of Mumbai, India.

**Polymer sample**

PVA (M.W. 125000) in powdered form was purchased from S. D. Fine Chemicals, Mumbai, India.

**Enrichment of PVA degrading microbial stains**

Environmental samples collected from different sites rich in plastic waste were used as a source of microbial inoculum for enrichment culture. Each solid sample 1 to 2 g and liquid sample 1 ml was diluted to 10 and 9 ml using normal saline and used as inoculum for the enrichment culture in the ratio of 1 ml to 100 ml of mineral salt vitamin media (MSV). 1000 mL MSV medium in distilled water contained: PVA, 5.0 g; (NH₄)₂SO₄, 1.0 g; KH₂PO₄, 1.0 g; K₂HPO₄, 8.0 g; MgSO₄.7H₂O, 0.2 g; NaCl, 0.1 g; CaCl₂.2H₂O, 0.02 g; FeSO₄, 0.01 g; Na₂MoO₄.2H₂O, 0.5 mg; MnSO₄, 0.5 mg; Inositol, 0.2 mg; p-amino benzoic acid, 0.2 mg; pyridoxine, 0.4 mg; thiamine, 2.0 µg; biotine, 2.0 µg; vitamin B, 120.5 µg; DW, 1000 ml; pH 7. The medium used was the same one that was used previously by Suzuki et al. (1973). A solid medium was prepared by adding 20 g of agar agar powder to 1000 ml of the MSV medium before autoclaving.

**Isolation and characterization of PVA degrading microbial strains**

A portion of enrichment culture was diluted adequately with sterile saline and spread on the nutrient agar plates. Incubation was carried out at 30°C for 48 h. Individual colonies formed on nutrient agar were picked and tested for their ability to grow on a solid MSV medium containing emulsified PVA, where the medium was fortified with a supplement and without any supplement such as 0.1% yeast extract and 0.1% glucose. Pure cultures of the PVC degrading bacteria were obtained by repeated sub-culturing of the isolated colonies on the same medium. The selected isolates were assigned codes such as PVA 1, PVA 2 etc. for further study. The selected isolates were characterized up to species level using Bergey's manual of determinative bacteriology (Krieg and Holt, 1984).

**Optimization of media used for degradation study**

For isolation and degradation studies, various compositions of the MSV containing PVA as a primary source of carbon and energy were supplemented with an additional co-substrate, such as yeast extract and glucose at a final concentration of 0.1%.

**Preparation of microbial cell suspension for various degradation studies**

The suspension of microbial cells used in various degradation studies was grown in a nutrient broth for 18 h at 30°C. The cells were harvested from the culture by centrifugation at 4500 rpm for
Determination of PVA degradation by spectrophotometric assay

The kinetics of PVA degradation by the selected isolated strain was studied by determining the residual concentration of PVA left in the growth media at regular intervals of time using spectrophotometric assay. The test was performed in a 500 ml Erlenmeyer flask containing 250 ml of MSV-PVA media containing 0.5% PVA supplemented with 0.1% yeast extract (Hi Media India). 5% of washed microbial cell suspension (approximately 3.1 x 10^6 cells/ml) was used as an inoculum for the test. The test flasks were incubated at 30°C in a shaker incubator (Neolab India) at 180 rpm along with control flasks. Two controls were maintained in the present experiment, one with MSV-PVA medium without test cultures and a second MSV medium without PVA and inoculated with a suspension of the test culture. At each test interval, the cell growth was estimated by reading the optical density at 600 nm. Simultaneously, 10 ml of the sample was taken from each culture flask for analysis of residual PVA concentration. Samples were centrifuged at 4500 rpm for 20 min at 4°C (Sorvall RC 5B Plus, Kendo, Newtown, USA). The resulting supernatants were filtered sterilized through the Sartorius filter (Sartorius, Germany) and the filtrates were used for analysis of residual PVA concentration. Controls were treated in the same way. Absence of microbial contaminants was checked by optical microscopy before any determinations were made. After appropriate dilution, residual PVA concentration in the culture filtrate was estimated according to Finley (1961) using spectrophotometric assay. The assay is based on a green color produced by the reaction of PVA with iodine in the presence of boric acid.

In a properly diluted 1 ml of culture filtrate 0.75 ml of 4% boric acid and 0.15 ml of potassium iodide (KI) solution (12.7 g of iodine and 25 g KI were dissolved in distilled water and a 1000 ml solution was made). The solution was mixed well and allowed to equilibrate for 30 min at room temperature. The mixture was diluted to a volume of 2.5 ml with distilled water and analyzed at 660 nm using Shimadzu UV-Vis double beam spectrophotometer (Shimadzu Ltd.). The blank was treated in the same way. The amount of PVA in the filtrate was determined using a standard calibration curve. The calibration curve was prepared by using a range of standard PVA solution from 10-100 μg/ml in distilled water. Color development and absorbance measurements were carried out following procedure described above. A standard calibration curve was plotted as absorbance 600 nm against PVA concentration. All the measurements were made in triplicate.

**RESULTS**

**Isolation of PVA degrading microbial strains**

PVA degrading microbial strains were isolated using the enrichment culture technique. Samples collected from different microbial sources were subjected to repeated enrichment in MSV-PVA containing PVA as the sole source of carbon and energy. From enrichment cultures incorporated with PVA and incubated for two weeks at 30°C, 30 morphologically different bacterial isolates were obtained on the nutrient agar. These strains were then tested for their ability to grow on MSV-PVA without any supplementation and with supplements. Among these thirty isolates, only fourteen strains were able to grow on MSV agar medium containing PVA. Table 1 shows the total number of organisms isolated from a different source on the MSV-PVA agar medium without supplementation and with supplements from an enrichment culture containing PVA as the enrichment substrate. Table 1 reveals that the PVA degrading microorganism inhabited different environments. However, industrial effluent samples harbored the maximum number of microorganisms as compared to the other microbial sources. Observation from Table 1 also reveals that only three strains were able to grow on the MSV-PVA agar medium without any supplementation. The maximum number of isolates could grow in the presence of the yeast extract. The 14 isolates obtained were numbered from strain PVA-1 to PVA-14. Screening of 14 different PVA degrading isolates for their PVA degradation potential was carried out by determining the extent of PVA degraded by an individual isolated strain in the MSV-PVA medium supplemented without and with 0.1% yeast extract and 0.1% glucose. PVA degradation in various degradation media was determined by analyzing the residual PVA concentration remaining in

PVA biodegradation was determined by the CO₂ production test as per the general guidelines of ISO 14855 (1999) and ASTM D5338 (1998). The medium used for assay of CO₂ production was the same as that given in the OECD (2001) guidelines for the testing of chemicals. The apparatus bio meter flask used in the present study was as described by Reich and Bartha (1977), and Yabannavar and Bartha (1993, 1994). For measurement of CO₂ production, the main compartment of the bio meter flask was amended with 100 ml of mineral medium with 0.1% of PVA along with 5% washed cell suspension of the selected isolated strain. The CO₂ produced during the metabolic activity was absorbed in a solution of barium hydroxide Ba (OH)₂ and subsequently determined by titration using 0.05N HCl where the amount of CO₂ produced was calculated from the amount of residual base remaining in the absorption tube. The mineralization was expressed as a percentage of the theoretical CO₂ (ThCO₂) produced, computed from the total carbon content of the samples. During the test period, flasks were incubated at room temperature in the dark. The stopcock was periodically opened for exchange of air. At each test interval (1, 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70), Ba (OH)₂ from the side arm was removed for analysis of residual Ba (OH)₂. The amount of un-reacted Ba(OH)₂ in the sample was with 0.05 N HCl control containing an inoculated medium without any test compounds, was evaluated for CO₂ production to determine endogenous metabolism of the test culture. A control, containing an un-inoculated medium with test substance was also used for determining the CO₂ evolved due to non-biological degradation. The amount of CO₂ evolved from the control flask was subtracted from the corresponding experimental flask. The percentage biodegradation was calculated from the cumulative amount of CO₂ released during the entire test period.
the culture broth at the end of the test period. Figure 1 shows the extent of PVA degradation by an isolated strain in three different media viz. MSV-PVA medium without any supplementation, MSV-PVA medium supplemented with glucose and MSV-PVA medium supplemented with yeast extract by fourteen isolated strains after two week’s incubation at 30°C. The degradation potential is expressed in terms of percentage (%) PVA degradation. The results illustrated in Figure 1 clearly indicate that all the isolates except PVA-10 showed degradation of PVA in a mineral medium without any supplement. As seen in the figure, it was observed that organisms responded differently to the medium composition with respect to their degradation potential and there existed no general optimal medium for all the different organisms. Among fourteen isolates tested, strain PVA-4 and PVA-7 showed the maximum extent of PVA degradation in all the three media. At the end of the test period, strain PVA-4 showed the maximum 51% PVA degradation in the MSV-PVA medium supplemented with yeast extract, whereas strain PVA-7 showed 41% of PVA degradation in the MSV-PVA medium supplemented with yeast extract. From the data, it was established that strains PVA-4 and 7 exhibited the highest overall PVA degradation capabilities and were therefore selected for further investigation. Figures 2 and 3 show photographs of strain PVA-4 and strain PVA-7 isolated on the MSV-PVA medium. As seen in Figure 2, strain PVA-4 forms medium sized (1-2 mm), circular, convex and cream colored colonies with regular edges on the mineral medium after 72 h of incubation at 30°C. Strain PVA-7 (Figure 3) forms small, circular, convex and colorless colonies with regular edges on the mineral medium incorporated with PVA after 5 days of incubation at 30°C.

Characterization of strain PVA-4 and PVA-7

The morphological, cultural and biochemical characteristics of strains PVA-4 and PVA-7 that are depicted in Tables 2 and 3, respectively, reveal that these isolates seem to be similar to the strain which belonged to Bacillus and Pseudomonas sp. respectively, after referring to the 8th edition of Bergey's Manual of Determinative Bacteriology.

<table>
<thead>
<tr>
<th>Microbial source</th>
<th>Number of microorganisms isolated on PVA medium supplemented</th>
<th>PVA as enrichment substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without supplement</td>
<td>With yeast extract</td>
</tr>
<tr>
<td>Garden soil</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Dumping ground</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Effluent I*</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Effluent II*</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Sea sediment</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

I* and II* represents effluent samples collected from different sources.

Time course degradation study of PVA by identified strains

The time course of PVA degradation by strain PVA-4 characterized as Bacillus sp. in the MSV-PVA medium containing 0.5% PVA and 0.1% yeast extract was monitored by determining the residual PVA concentration remaining in the culture medium, the increase in cell growth and change in pH of the culture media at regular test intervals as elucidated in Figure 4. As seen in Figure 4, the growth of strain PVA-4 increased with the decrease in PVA concentration. As such, the initial lag phase was not observed in the growth curve. After, 12th days, the cell growth reached its maximum and about 0.1% PVA remained in the medium which corresponds to 65% degradation. At the end of 20th day, PVA was completely exhausted from the medium. The pH of the culture medium decreased during the initial incubation period from 7 to 6.1. Afterward, a sudden rise in pH from 6.1 to 7.3 was observed until the 12th day onward. Again a drop in pH was observed until the end of the test period. In the control flask, the pH remained nearly throughout the test period.

Figure 5 elucidates the time course of PVA degradation by strain PVA-7 characterized as Pseudomonas sp. in the MSV-PVA medium measured as residual PVA (R-PVA), increase in cell growth (CG) measured as OD 600 nm, and change in pH of the test medium over a period of twenty days. As seen in Figure 5, the growth of strain PVA-7 increased with the decrease in PVA concentration during test period. PVA concentration in the liquid culture started to decrease steadily after an initial lag phase of 2-3 days. The exponential phase lasted until the 14th day of inoculation, during which cell growth reached to its maximum and about 0.3% of the PVA remained in the medium.
Figure 1. Degradation of PVA by 14 isolated strains in MSV-PVA medium without supplement and with supplements after two weeks at 30°C (MSV-PVA medium with 0.1% Glucose or yeast extract, pH 7, stirred at 150 rpm).

Figure 2. Isolated colonies of strain PVA-4 on MSV-PVA agar plate (the plates were incubated at 30°C for 72 h).

Figure 3. Isolated colonies of strain PVA-7 on MSV-PVA agar plate (the plates were incubated at 30°C for 5 days).
Table 2. Morphological, cultural and biochemical characteristics of strain PVA-4.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristics of Strain PVA-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Size</td>
<td>2-3 µm</td>
</tr>
<tr>
<td>Form</td>
<td>Rods</td>
</tr>
<tr>
<td>Arrangement</td>
<td>Single, in pairs and in chains</td>
</tr>
<tr>
<td>Capsule</td>
<td>Present</td>
</tr>
<tr>
<td>Spore</td>
<td>Present (central)</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>Size</td>
<td>4 to 5 mm</td>
</tr>
<tr>
<td>Shape</td>
<td>Irregular</td>
</tr>
<tr>
<td>Surface</td>
<td>Dull</td>
</tr>
<tr>
<td>Elevation</td>
<td>Flat</td>
</tr>
<tr>
<td>Edge</td>
<td>Wavy</td>
</tr>
<tr>
<td>Consistency</td>
<td>Smooth</td>
</tr>
<tr>
<td>Opacity</td>
<td>Opaque</td>
</tr>
<tr>
<td>Color of colony</td>
<td>Buff colored</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>+</td>
</tr>
<tr>
<td>Acid from arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Acid from xylose</td>
<td>+</td>
</tr>
<tr>
<td>Acid from mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Catalse</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Degradation of tyrosin</td>
<td>-</td>
</tr>
<tr>
<td>De-amination of phenylalanine</td>
<td>-</td>
</tr>
<tr>
<td>Growth in NaCl (range)</td>
<td>2 - 10%</td>
</tr>
<tr>
<td>Hydrolysis of casein</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of gelatin</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 40°C</td>
<td>+</td>
</tr>
</tbody>
</table>

-, Negative reaction; +, positive reaction.

culture broth which corresponds to 42% of the PVA degradation. No further degradation of PVA was observed during the stationary phase of growth. The pH of the culture media got lowered from 7 to 5.8. In the control flask, a slight change in pH from 7 to 6.8 was recorded.

**CO₂ production test**

Mineralization of PVA into its mineral constituents viz, CO₂ and H₂O was investigated using strain PVA-4 and PVA separately in the mineral medium over a period of 20 days, where PVA was used as the sole source of carbon and energy. The net CO₂ production over a period of 20 days by the two strains is shown in Figure 6 and the percentage biodegradation by two strains is shown in Figure 7. Complete conversion of 100 mg of PVA with 54.5 mg of total organic carbon (TOC) could have yielded 19.98 mg of theoretical CO₂. The observed net CO₂ production from the Bio meter flask inoculated with strain PVA-4 was found to be 9 mg in twenty days which corresponds to 45.4% of biodegradation (mineralization) of the PVA during test period. As was observed, net CO₂ production from the Bio meter flask inoculated with strain PVA-7 was found to be 5.8 mg during the 20 days of the test period, which corresponds to 28.9% of mineralization of added PVA in the medium.
Table 3. Morphological, cultural and biochemical characteristics of strain PVA-7.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type of characteristics of Strain PVA-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Gram negative</td>
</tr>
<tr>
<td>Size</td>
<td>2-3 µm</td>
</tr>
<tr>
<td>Form</td>
<td>Small rods</td>
</tr>
<tr>
<td>Morphological characteristics</td>
<td></td>
</tr>
<tr>
<td>Arrangement</td>
<td>Single</td>
</tr>
<tr>
<td>Capsule</td>
<td>Present</td>
</tr>
<tr>
<td>Spore</td>
<td>Absent</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>Size</td>
<td>1-2 mm</td>
</tr>
<tr>
<td>Shape</td>
<td>Circular</td>
</tr>
<tr>
<td>Surface</td>
<td>Smooth</td>
</tr>
<tr>
<td>Cultural characteristics on nutrient agar at 30°C for 24 h</td>
<td></td>
</tr>
<tr>
<td>Elevation</td>
<td>Convex</td>
</tr>
<tr>
<td>Edge</td>
<td>Entire</td>
</tr>
<tr>
<td>Consistency</td>
<td>Mucoid</td>
</tr>
<tr>
<td>Opacity</td>
<td>Opaque</td>
</tr>
<tr>
<td>Color on nutrient agar</td>
<td>Greenish</td>
</tr>
<tr>
<td>Oxidation/fermentation test</td>
<td>Oxidative</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>-</td>
</tr>
<tr>
<td>Acid from arabinose</td>
<td>-</td>
</tr>
<tr>
<td>Acid from xylose</td>
<td>-</td>
</tr>
<tr>
<td>Acid from mannitol</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
</tr>
<tr>
<td>Arginine hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of gelatin</td>
<td>+</td>
</tr>
<tr>
<td>Indol test</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red test</td>
<td>-</td>
</tr>
<tr>
<td>Vogues Proskaur test</td>
<td>-</td>
</tr>
<tr>
<td>Biochemical characteristics</td>
<td></td>
</tr>
<tr>
<td>Growth on TSI slant</td>
<td>Alkaline slant, alkaline butt, no gas, and no H₂S production</td>
</tr>
<tr>
<td>Growth on Cetrimide agar</td>
<td>+</td>
</tr>
</tbody>
</table>

-, Negative reaction; +, positive reaction.

DISCUSSION

The present study describes enrichment, isolation and characterization of microbial strains degrading PVA. Enrichment was performed by providing PVA as the sole source of carbon and energy, using samples collected from different sites polluted by plastic waste. Two potential PVA degraders were then selected among various isolates and further characterized as Bacillus and Pseudomonas sp. Several earlier attempts were carried out to isolate the PVA degrading microbial strain. Sakazawa et al. (1981) reported isolation of a symbioant mix culture of PVA degrading strains, Pseudomonas putida VM15A and Pseudomonas sp. VM 15C, where, the latter was a PVA degrader and the former supplied PQP (Pyrroloquinoline), a growth factor. Larking et al. (1999) reported degradation of PVA by Pycnoporus cinnabarinus after pretreatment with Fenton’s reagent. Ishigaki et al. (1999) reported isolation of Pseudomonas vesicularis var. povaloyticus strain from an activated sewage sludge sample. Mori et al. (1996) isolated Bacillus megaterium that degrades PVA. Kim et al.
**Figure 4.** Time course of PVA degradation by strain PVA-4 identified as *Bacillus subtilis* (MSV-PVA medium with 0.5% PVA and 0.1%; pH, 7; incubated at 30°C aerobically in a shaker incubator).

**Figure 5.** Time course of PVA degradation by strain PVA-7 identified as *Pseudomonas aeruginosa* (MSV-PVA medium with 0.5% PVA and 0.1%; pH 7; incubated at 30 °C aerobically in a shaker incubator).
**Figure 6.** Net CO$_2$ evolution during breakdown of PVA by *Bacillus subtilis* and *Pseudomonas aeruginosa* over a period of 20 days measured in a Bio meter flask (mineral medium with 0.1% PVA; pH, 7; incubation in dark at 30°C).

**Figure 7.** Percentage biodegradation of PVA by *Bacillus subtilis* and *Pseudomonas aeruginosa* over a period of 20 days measured in a Bio meter flask (Mineral medium with 0.1% PVA; pH, 7; incubation in dark at 30°C).
(2003) reported isolation of a novel strain of Sphingomonas sp. SA3 and its symbiotic strain SA2 where, the former strain consisted of PVA degraders and the latter was the growth factor promoter. Recently, Maiti et al. (2013) and Yuich et al. (2014) reported isolation of fungal strains Penicillium daleae, Aspergillus flavus and Thalassospira pavaliytica from compost and marine environment.

In the current work, the time course degradation of PVA by the identified PVA degrading bacterial strains was monitored by determining the residual PVA left in the media using spectrophotometric assay. The results obtained with the degradation study of PVA of Bacillus and Pseudomonas sp. show that the concentration of PVA decreases when the bacterial cell density increases. This means that PVA degradation and growth of bacteria are interrelated. For other PVA degrading bacteria, the same relationship has been reported (Kim et al., 2003 and Suzuki et al., 1973).

In the present work, it was observed that during breakdown of PVA by Bacillus sp., the pH of the culture medium decreased during the initial incubation period. Afterwards, a sudden rise in pH was observed for a few days and again a drop in pH was observed at the end of the test period. This characteristic pattern of the change in pH of the culture medium was also reported by Larking et al. (1999) during the breakdown of PVA by Pycnophorus cinnabarinus. The initial decrease in pH of the medium may be due to the formation of a low molecular weight acidic intermediate such as carboxylic acid, a degradation product of PVA, whereas the sudden increase in pH was due to consistent utilization of an acidic intermediate by the strain. A further drop in pH also suggests an active degradation of PVA, which results in accumulation of a large amount of acidic products. The drop in pH of the culture medium during a breakdown of PVA by the Pseudomonas species was also reported by Suzuki et al. (1973) and Sakazawa et al. (1981).

Mineralization of PVA expressed in terms of percentage biodegradation of PVA by both Bacillus and Pseudomonas sp. reached about 45.4% in 20 days and 28.9% in 20 days of incubation period, respectively. Lee and Kim (2003) reported 75% mineralization of PVA by the symbiotic strain Cardiobacterium sp. SB98 and unidentified bacterium (SB69), as well as by Achromobacter and cholinophagum SB98 after 46 days, where the initial PVA concentration was 0.01%. Thus, our findings are comparable to the earlier studies. The described work was further extended by developing the strain for enhanced degradation of PVA by employing genetic engineering techniques. The results of this study are beyond the scope of the present work and hence not included here. However, the data obtained in the current work has a valuable application in understanding biodegradation of PVA and depict the usefulness of application of polyvinyl alcohol for making bio plastic material which is completely degradable in the natural environment as compared to a non-biodegradable synthetic polymer by different group of microbial consortia.

Conclusion

The PVA degrading microbial strain was isolated from different environmental samples. The isolated strains were from the group of Bacillus and Pseudomonas sp. The various degradation tests carried during the present study indicated that the strain showed higher rate of degradation in the presence of various co-substrates which may be due to enhancement in biomass formation due to an additional carbon source.

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aerobic compost environment. International Biodeterioration & Biodegradation. 82:9-12.
Full Length Research Paper

Phenotypic characters of yeasts isolated from kpete-kpete, a traditional starter of a Benin opaque sorghum beer

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Opaque sorghum beers are the most consumed African alcoholic beverages. Tchoukoutou is one of the Benin opaque sorghum beers. Its fermentation process is carried out using a traditional starter called kpete-kpete. The present study characterized and identified the yeasts isolated from kpete-kpete. A total of 24 samples of kpete-kpete were collected from eight different commercial processing sites in Northern Benin. The mean values of the pH, titrable acidity, dry matter content and refractive index for all samples were respectively 3.58; 0.07% as lactic acid; 16.61% and 7.0. The mean counts of yeasts was 9.24 log cfu/ml. Based on their phenotypic characters and their assimilation profiles, 49 yeasts were isolated and found to belong to five genera with seven species. Seventy one percent (71%) of the isolates were identified as Saccharomyces cerevisiae.

Key words: Sorghum beer, tchoukoutou, kpete-kpete, yeast, Saccharomyces cerevisiae.

INTRODUCTION

Fermented beverages play a major role in the diet of African people. The most studied African alcoholic beverages are opaque beers often produced from sorghum (Chamunorwa et al., 2002; Jespersen, 2003; Maoura et al., 2005; Lyumugabe et al., 2010; Lyumugabe et al., 2012). Opaque sorghum beers are consumed at various festivals and African ceremonies (for example, marriage, birth, the handing over of a dowry, etc.) and constitute a source of economic return for the women beer producers. They are known as tchoukoutou in Benin, dolo in Burkina-Faso, pito in Ghana and burukutu, otika or sekete in Nigeria, Impeke in Burundi (Odunfa, 1985; Sanni and Lönner, 1993; Kayode et al., 2005). These beers are very rich in calories, B-group vitamins

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and essential amino acids such as lysine (Lyumugabe et al., 2012) and inexpensive. Therefore, they are largely consumed by the poorest people and contribute to their dietary needs (Kayodé et al., 2012). Due to their low alcohol content (2-3% v/v) and the large quantity of suspended solids (5-7%), many consumers consider fermented sorghum beers to be more a food than a beverage (Pattison et al., 1998). The beers are mostly produced at household level or at small industrial scale with varying quality and stability (Sanni and Lönn, 1993; Zulu et al., 1997). Basically, the processing of African opaque sorghum beers involves malting, souring, boiling, mashing, straining and alcoholic fermentation (Kayode et al. 2005; Odunfa, 1985; Haggbblade and Holzapfel, 1989). Depending on country and region, variations occur in the beer process (Jespersen, 2003). The fermentation remains a critical step in the process (Kayode et al., 2012). Beneficial effects of fermentation include improvement of flavor and texture, reduced loss of raw materials, reduced cooking time, improved bioavailability of micronutrients and elimination of toxic and anti-nutritional factors (Sanni and Lönn, 1993; Iwuoha and Eke, 1996; Padmaja, 1995; Sindhu and Khetarpaul, 2001).

In Benin, Lactic acid bacteria and yeasts have been reported (Kayodé et al., 2007) to be the major microorganisms involved in the fermentation of tchoukoutou. Kpete-kpete is the traditional starter used for the fermentation of tchoukoutou. Based on its fermenting properties, producers use it to ferment the sorghum wort during the manufacturing process. It is generally harvested from the bottom of a previous fermenting beer resulting from 13 to 14 h overnight fermentation. However, the microorganisms contained in kpete-kpete used for the fermentation of tchoukoutou have not yet been investigated. Especially, works reporting on the species of yeasts contained in such starter are hard to come by. The increasing interest for moving from uncontrolled conditions towards regulated processing conditions, and thus ensuring quality safety and product stability, makes the application of starter cultures and thereby identification and classification of the strains involved necessary (Van der Aa Kühle et al., 2001).

The present study was conducted to determine the physicochemical and microbiological characteristics of the traditional starter kpete-kpete, and to identify the different species of yeasts involved using phenotypic analysis tools.

MATERIALS AND METHODS

Sampling

Twenty four (24) samples (500 mL) of kpete-kpete, the traditional starter of tchoukoutou, were collected from eight of the most important production sites of tchoukoutou in northern Benin. The processors (one per site) were selected on the basis of their rich beer brewing tradition. The samples were collected in screw-capped bottles, packed in an insulated icebox, transported to the laboratory and analyzed immediately for microbiological analysis (Hounhouigan et al., 1993).

Physico-chemical analysis

Dry matter was determined according to the AACC method (AACC, 1984). The pH was determined using a digital pH meter (HI 8418; Hanna instruments, Limena, Italy) calibrated with buffers at pH 4.0 and 7.0 (WTW, Weilheim, Germany). The titratable acidity, expressed as lactic acid, was performed by using the method described by Nout et al. (1989). The refractive index was measured using a refracto meter (Soeleam 9596, France).

Enumeration of yeasts

Duplicate samples of “kpete-kpete” (10 mL) were diluted in 90 mL sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, and 1000 mL distilled water, pH = 7.0) and homogenized with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated. Total count of yeasts was determined on oxytetracycline glucose yeast extract agar (OGYA, Oxoid CM 0545, Basingstoke, Hampshire, England) containing oxytetracycline (Hounhouigan et al., 1993). Forty nine (49) yeast strains were obtained and subjected to morphological, fermentation and assimilation tests. Prior to these tests, a preliminary microscopic confirmation was performed.

Identification of yeast

The identification of yeast strains was performed according to the method described by Yarrow (1998) and Kurtzman et al. (2011). The isolates from eight representative sites were purified by successive sub-culturing on oxytetracycline glucose yeast agar (OGYA, CM0545, Basingstoke Hampshire, England) made selective by addition of oxytetracycline. Preliminary confirmation was based on microscopic observation. The isolates were tested for the fermentation of sucrose, lactose, glucose and raffinose, as well as the assimilation of selected nitrogen sources that is, nitrate, ethylamine, L-lysine, cadaverine and creatine. The assimilation of carbon sources was performed using API 20 C AUXstrips (BioMérieux, Lyon, France) according to the manufacturer’s instructions. The diazonium blue B reaction, a test to differentiate between ascomycetous and basidiomycetous yeasts, was performed as described by Kurtzman et al. (2003).

Data analysis

For the analytical data, mean values as well as standard deviation are reported. The data were analysed using the statistical program, SPSS 11.0. The on-line available software (http://www.cbs.knaw.nl) of Centraal bureauvoor Schimmel cultures (Central Bureau of Fungal Cultures), Utrecht, the Netherlands was used for identification of yeasts.

RESULTS AND DISCUSSION

Physico-chemical characteristics and yeast content of kpete-kpete

The mean value of yeast counts was 9.24 log cfu/mL
Table 1. Physicochemical and microbiological characteristics of the traditional starter kpete-kpete.

<table>
<thead>
<tr>
<th>Samples origin</th>
<th>Yeasts (log cfu/g)</th>
<th>pH</th>
<th>Titratable acidity (%) lactic acid</th>
<th>Dry matter (%)</th>
<th>Refractive index</th>
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</thead>
<tbody>
<tr>
<td>Boukombé</td>
<td>9.53±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.22±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.18±1.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Djougou</td>
<td>9.51±0.59&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.44±0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.07±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.50±4.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Natitingou</td>
<td>8.64±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.56±0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.08±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.78±4.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Toucountouna</td>
<td>9.88±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.71±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.95±3.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tchaourou</td>
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<td>3.54±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>5.0±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Parakou</td>
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<td>3.77±0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.10±2.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.0±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pérèrè</td>
<td>9.11±0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.58±0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.08±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.39±1.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>N'Dali</td>
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<td>3.79±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.61±4.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Means</td>
<td>9.24</td>
<td>3.58</td>
<td>0.07</td>
<td>16.61</td>
<td>7.0</td>
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<td>CV (%)</td>
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<td>5.26</td>
<td>14.79</td>
<td>15.64</td>
<td>20.44</td>
</tr>
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</table>

<sup>a</sup>Coefficient of variation, <sup>b</sup>Values with the same letter in the same column are not significantly different (P<0.05)

The yeast concentration in *kpete-kpete* is higher than the counts of yeast (7.8–8.5 log cfu/g) in the sorghum beer *tchoukoutou* as reported by Kayodé et al. (2006). That difference could be due to the fact that there is a significant difference between the dry matter of both products: 16.6% for the kpete-kpete and 10.0% for *tchoukoutou* (Kayodé, 2006). Data from Table 1 show there is a significant difference (p<0.05) between the counts of yeasts from the eight communities. Previous studies performed on African opaque sorghum beers established that the frequencies of microbial species vary according to the region and the ingredients used for the brewing (Demuyakor and Ohta, 1991; Ekundayo, 1969; Paparusi et al., 1973; Nout, 1980; Odunfa, 1985; Sanni and Lönnér, 1993; Sefa-Dedeh et al., 1999).

Mean values of pH was 3.58. Data analysis showed there is a significant (p<0.05) difference between samples from the various production sites. However, there is no significant difference (p<0.05) for the titratable acidity, dry matter and refractive index between the samples from the eight areas. On the basis of the titratable acidity, the dry matter and the refractive index, the starters collected from various sites appear to be similar. However, the different starters seem different on the basis of their yeasts content and their pH values.

Phenotypic characteristics of yeasts isolates

Results (Table 2) show that a minority of the strain could ferment raffinose (24.5%), whereas the great majority fermented sucrose (85.7%) and glucose (100%); but none of the isolates could ferment lactose. These results are in close agreement with data reported by Kayode et al. (2011) for yeast strains isolated from tchoukoutou. The nitrogen assimilation test revealed that a minority of the isolates assimilated ethylamine (16.3%), L-lysine (32.7%) and cadaverine (42.9%) whereas the majority assimilated sulfate of ammonium (67.3%) and nitrate (55.1%). The diazonium blue B test (Table 2) revealed that 22.4% of the isolates were basidiomycetous whereas 77.6% were ascomycetous. On the basis of their fermentation profile and the nitrogen assimilation pattern, the 49 yeasts could be grouped into 18 distinct clusters. 14.3% were in the first cluster, 8.2% were in the second cluster, 8.2% in the fourth cluster and the rest are distributed in the 15 other clusters.

Assimilation profile and identification of yeasts isolates

On the basis of their assimilation of carbon compounds, seventeen assimilation profiles were distinguished (Table 3). The majority of yeasts assimilated glucose (100%), galactose (93.9%) and maltose (79.6%); many assimilated acetyl-glucosamine (51%), saccharose (46.9%), palatinose (30%), glycerol (32.7%), xylose (36.7%), lactic acid (38.8%), xylene (36.7%), glycerol (32.7%), palatinose (30.6%); some of yeasts assimilated potassium gluconate (26.5%) methyl-D-glucopyranoside (22.4%), lactose (20.4%), sodium glucuronate (18.7%), trehalose (18.4%), mannitol (18.4%), levulinic acid (18.4%); only a few yeasts could assimilate erythritol (2%), sorbose (4.1%), potassium-2-cetoglucuronate (6.1%), arabinose (6.2%), glucosamine (8.2%). None of them assimilated actidione, cellobiose, rhamnose (Table 3). Based on their phenotypic characteristics, the 49 yeasts were found to belong to five genera and seven species of yeasts. These are *Saccharomyces cerevisiae* (71.4%), *Sporobolomyces odoratus* (12.2%), *Candida pseudoharragii* (6.1%), *Candida heliconiae* (4.1%), *Schizosaccharomyces octosporus* (2%), *Schizosaccharomyces pombe* (2%) et *Zygosaccharomyces rouxii* (2%) (Figure 1). The diversity of yeast strains contained in "kpete-kpete" can be
Table 2. Phenotypic characters of yeasts isolated from traditional starter kpete-kpete.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Isolates numbers</th>
<th>Fermentation</th>
<th>Assimilation of nitrogen source</th>
<th>DBB test²</th>
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<tr>
<td></td>
<td></td>
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<td>Lac</td>
<td>Suc</td>
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<tr>
<td>I</td>
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<td>II</td>
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<tr>
<td>XVIII</td>
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<tr>
<td>Frequency (%)</td>
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<td>24.5</td>
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1Glu = glucose, Lac = lactose, Suc = sucrose, Raf = rafinose, Nit = nitrate, Eth = ethylamine, Lys = L-lysine, Cad = cadaverine, SAM = sulfate of ammonium ²DBB = diazonium blue B.

Table 3. Assimilation profiles of yeasts isolated from traditional starter kpete-kpete.

<table>
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<tr>
<th>Parameter</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>E</th>
<th>f</th>
<th>g</th>
<th>h</th>
<th>i</th>
<th>j</th>
<th>k</th>
<th>l</th>
<th>m</th>
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<th>o</th>
<th>p</th>
<th>q</th>
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<td>D-glucose</td>
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explained by the fact that in Africa, traditional fermented products result from spontaneous fermentation and as a result, both desirable and non-desirable strains are present in the product (Lyumugabe et al., 2012).

*S. cerevisiae* was found as being the predominant yeast strain in the traditional starter *kpete-kpete*. These findings are in accordance with previous studies. Konlani et al. (1996) found that *S. cerevisiae* accounted for 55-90% of yeast population in samples of sorghum beer originated from Togo and Burkina Faso. Likewise, Demuyakor and Ohta (1991); Van der Aa Kühle et al. (2001); Sanni and Lönner (1993), have respectively reported the predominance of *S. cerevisiae* in the traditional sorghum beers from Nigeria, Burkina-Faso and Ghana. Also, in an opaque sorghum beer from Northern Ghana, Glover et al. (2005) identified 72% of 247 isolates as *S. cerevisiae* on the basis of their carbon and nitrogen compounds assimilation profiles. Moreover, N’guessan et al. (2011) investigated 240 yeast strains isolated from fermenting sorghum wort inoculated with yeast. In this study 87.36% of strains are found to be *S. cerevisiae*. To be accepted as *S. cerevisiae*, the isolate should be able to assimilate glucose, sucrose, maltose, raffinose and ethanol (Vaughan-Martini and Martini, 1998). In the present study, some of the isolates could not assimilate all of these sugars. In spite of that, they were identified as *S. cerevisiae*. Many isolates from Ghanain and Burkina-Faso sorghum beers were identified as *S. cerevisiae* by Demuyakor and Ohta (1991) and Van der Aa Kühle et al. (2001), and yet, these microorganisms showed carbon assimilation profiles different from the taxonomical key proposed by Vaughan-Martini and Martini (1998). Like in our study, some of the isolates analyzed by thesis authors could not assimilate sucrose, raffinose and
trehalose.

Researches on improvement of traditional sorghum beers revealed that *S. cerevisiae* is of a vital importance for making an effective starter culture. Sefa-Dedeh et al. (1999) used a pure culture of *S. cerevisiae* and a mixture culture consisting of *S. cerevisiae* and other strains such as Kloeckera apiculata and Candida tropicalis, to produce a pito beer containing high ethanol content. Also, Orji et al. (2003) found that *S. cerevisiae* in combination with Lactobacillus plantarum, as a starter culture, also led to the satisfactory production of a pito beer. N’Guessan et al. (2010) successfully used *S. cerevisiae* in combination with *C. tropicalis* as starter cultures for the alcoholic fermentation of the tchapalo beer.

**Conclusion**

*S. cerevisiae* was identified as the predominant specie of yeast in the traditional starter *kpete-kpete* on the basis of the phenotypic characterization. In order to refine the identification process, the molecular characterization is found to be necessary. This would be an important step towards the elaboration of a starter culture for the fermentation of African opaque sorghum beers. Such approach would lead to an improvement of the fermentation process and the quality of local African beers.

**Conflict of Interests**

The author(s) have not declared any conflict of interests.

**REFERENCES**


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