African Journal of Biotechnology

Volume 16 Number 10, 8 March 2017

ISSN 1684-5315

Academic Journals

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African Journal of Biotechnology

Table of Content: Volume 16 Number 10 8 March. 2017

ARTICLES

academicJournals

Vol. 16(10), pp. 434-449, 8 March, 2017
DOI: 10.5897/AJB2016. 15601
Article Number: 5A9FDDB63087
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular characterization of introduced sugarcane genotypes in Ethiopia using inter simple sequence repeat (ISSR) molecular markers

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Received 5 August, 2016; Accepted 6 January, 2017

The major thrust of sugarcane (*Saccharum* spp.) variety improvement programs is to increase sugar yield in addition to biomass and energy cane for biofuel production. However, due to its genetic complexity, sugarcane has received very little research interest, despite its economic importance, and molecular marker techniques are being developed only in recent times. Furthermore, until the present date few molecular studies were carried out to evaluate sugarcane germplasm in Ethiopia. Therefore, this study was conducted at Addis Ababa University, Genetic Research Laboratory to evaluate molecular genetic diversity and establish relationships among genotypes and populations of introduced sugarcane in Ethiopia using ISSR molecular markers. Genomic DNA was extracted from silica-gel-dried leaf samples of 82 sugarcane genotypes according to cetyltrimethylammonium bromide (CTAB) method. A total of 149 scorable and reproducible bands were generated using 12 ISSR primers among which 124 were polymorphic and attributed to percentage of polymorphic loci (PPL) = 83.22%, Nei's gene diversity (h) = 0.31, and Shannon's information index (I) = 0.45 at genotypic level. The number and percentage of polymorphic loci of the marker ranged from 7 to 16 and 70 to 90.91%, respectively. Intra-population diversity based on percentage of polymorphic loci ranged from 28.86 to 47.65% with mean of 38.35%, Nei's gene diversity of 0.097 to 0.171 with mean of 0.137, Shannon's information index of 0.147 to 0.255 with mean of 0.205. Partitioning the genetic variation by AMOVA further revealed that 63.56% of the total genetic variation occurred among genotypes within population and 36.4% among population. All diversity index parameters confirm that the highest diversity was obtained from those that were obtained from France and Cuba whilst the lowest was from those of Barbados and South Africa. From Jaccard's pairwise similarity coefficient, pairwise comparison of the seven populations of sugarcane, the genetic identity values were ranged from 0.532 to 0.700 with mean of 0.616. With all clustering analysis, most of the genotypes clustered to their respective geographical origins as there was some mixture of genotypes from different origins in the different clustering groups. The level of polymorphism observed proved that the ISSR marker system was robust at amplifying markers on sugarcane. Thus, ISSR markers detected a range of diversity from investigated sugarcane genotypes with their unique identity that deserve conservation attention and improvement programs. This molecular-based genetic information can be used for establishing proper identity of the genotypes, strategic conservation of these germplasm resources, and future improvement work of the sugarcane crop through selecting the appropriate parents in their breeding programs to maximize sugar yield and maintaining genetic diversity.

Key words: Ethiopia, genetic diversity, inter simple sequence repeat (ISSR), sugarcane.

INTRODUCTION

Sugarcane (*Saccharum* spp.) belongs to the genus *Saccharum* of the family Poaceae within the tribe Andropogoneae (Phillips, 1995). The center of origin of sugarcane was in dispute until Artschwager and Brandes (1958) exhaustively surveyed the evidence and concluded that New Guinea is the undoubted center of origin of the species and it represents the generally accepted view today on existence of wild relative as main criteria (Brandes, 1956; Artschwager and Brandes, 1958; Daniels and Roach, 1987; Grivet et al., 2006) and it was then dispersed in the Pacific and mainland Asia and elsewhere globally during human migrations (Artschwager and Brandes, 1958). Sugarcane is an economically important commercial crop that accounts for 70% of the world's sugar production (Cunff et al., 2008) and it recently has gained increased attention because of the potential of its by-products; ethanol, molasses, and bagasse, as important renewable biofuel sources. In Ethiopian context, sugarcane and its valuable products have significant contribution to the overall economic development of the country through satisfying the local demand for sugar supply and seizing a remarkable market share at the international level. It has also been reported that sugarcane has a C_4 carbohydrate metabolism which allied with its perennial nature, and that makes it one of the most productive cultivated plants (Cunff et al., 2008). In recent investigation however, sugarcane probably has the most complex genome structure than all other crop genomes, mainly due to its very high degree of polyploidy and interspecific origin (D'Hont, 2005). Thus, due to the complexities associated with this crop, not many investigations were made regarding the genetics and inheritance of agronomically important traits. However, the developments in the field of genetics and breeding both at classical and molecular level have helped us to make a quantum leap in the area of sugarcane improvement (Swapna and Srivastava, 2012).

Sugarcane production in Ethiopia started in 1954 when the Wonji Sugar Factory was commissioned by Dutch Company, HVA (Handlers Vereeniging Amsterdam) in 1951. Experiences of the existing sugar factories in Ethiopia showed that because of the presence of large areas of suitable low lands, adequate water resources, favorable climate, and suitable soil types are all proven to be highly conducive for sugarcane development and productivity (EIA, 2008). As a result, an average sugarcane production per hectare per month of the land under irrigation is very high as compared to other countries, that is, 9-11 tons in Ethiopia against 6 to 8 tons elsewhere (EIA, 2008). This would make Ethiopia a very attractive location for private investors to invest in production and processing of sugarcane. Ethiopian Sugar Corporation is therefore working vigorously to raise the production of sugar in the country. However, the per capita annual consumption of sugar in Ethiopia is one of the lowest in the world with about 5.1 kg as estimated by International Sugar Organization (ISO, 2009). This is considered very low as compared to 16.3 kg per capita of African average (ISO, 2009). Among others, very low sugar yield of sugarcane in Ethiopia is the major reason for lower per capita consumption. The lower sugarcane productivity could also be attributed mainly due to lack of stable high yielding and novel improved varieties and other biotic and abiotic factors. Additionally, it has also been reported that due to the genetic complexity, narrow genetic base, susceptibility to various diseases or pests, and long breeding/selection cycle make the improvement of this crop through conventional breeding laborious and ineffective (Lakshmanan et al., 2005).

Moreover, in spite of its commercial importance, only few molecular characterization studies of exotic sugarcane materials have so far been carried out in Ethiopia (e.g. Tena et al., 2014). The government of Ethiopia has an ambition to make the total sugarcane plantation area reach 386000 ha at the end of the second growth and transformation (2015/2016 to 2020) plan (GTP) (ESC, 2015). Therefore, at the end of the GTP sugar production will be boosted from the current level of 0.3 to 3.9 million tons, ethanol production will be 31,341 m^3, and the factories will contribute 448 megawatt electric power to the national grid (ESC, 2015). For this big dream to become realized the contribution of improved sugarcane varieties is very paramount. Since the demand for new superior improved varieties that can adapt different agro-ecologies of sugarcane plantations and withstand various biotic and abiotic factors is expected to rise ever. Therefore, as the first step in this endeavour is to conduct perfect assessment of genetic diversity as it helps in the selection of desirable genotypes and introgressing desirable genes from diverse germplasm into the available genetic base. In Ethiopia, many of the sugarcane varieties have been introduced from many source countries to satisfy varietal requirements of sugarcane plantations in various regional states of the country. In spite of long history of introduction, no depth and enough systematic efforts have been made to characterize the available genotypes. Thus, this necessitated characterizing exotic sugarcane genotypes using molecular marker technique which could provide baseline information for future breeding and improvement of the crop.

It is well known that rapid advancements in molecular genetics and emerging modern biotechnology innovations

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Table 1. Number of introduced genotypes of sugarcane used for molecular diversity analysis.

S/N	Population	Letter code	Year of introduction	No. of genotypes
1	Barbados	B	Before 2000	13
2	Cuba	C	2012	11
3	France	F	2012	22
4	India	I	Before 2000	9
5	South Africa	SA	Before 2000	8
6	Sudan	S	2012	10
7	USA	U	2012	9
	Total			**82**

would play a significant role in the future sugarcane crop improvement programs and offer many new opportunities to develop it as a new generation of industrial crop (Lakshmanan et al., 2005). Thus, molecular marker techniques are one part of the modern biotechnological tools that are an accurate, fast, reliable, and cost effective genetic analysis technique to distinguish between sugarcane varieties, verify pedigrees, and estimate level of genetic diversity. Moreover, such a technique would provide impetus for the development of new and superior varieties. Therefore, molecular characterization of available sugarcane germplasm provides essential information on the extent of genetic diversity among parental lines and the difference in genetic background among germplasm for breeders to utilize it in sugarcane improvement programs (Singh et al., 2010). Ming et al. (2006) also pointed out that the knowledge on phylogenetic relationship among genotypes of sugarcane or germplasm collection will guide parental selection for the development of new varieties. Tiliye et al. (2007) reported that several molecular marker systems have been developed to measure genetic diversity in plant species. In sugarcane, several different molecular markers have been used in previous studies that have examined genetic diversity and relationships among cultivars from different regions worldwide. These include Restriction Fragment Length Polymorphism (RFLP) (Lu et al., 1994; Grivet et al., 1996; Jannoo et al., 1999), Random Amplified Polymorphic DNA (RAPD) (Nair et al., 1999), Amplified Fragment Length Polymorphism (AFLP) (Lima et al., 2002), Simple Sequence Repeat (SSR) (Chen et al., 2009; Glynn et al., 2009; Pandey et al., 2011; Smiullah et al., 2013), and Inter Simple Sequence Repeat (ISSR) (Srivastava and Gupta, 2008; Da Costa et al., 2011).

In the present study, inter simple sequence repeat (ISSR) markers were used for the investigation in an attempt to assess their potential as molecular marker system to evaluate the genetic diversity among and within sugarcane genotypes in Ethiopia. The application of ISSR marker is found to be quick and cost-effective based on polymerase chain reaction (PCR) amplification of inter-microsatellite sequences to target multiple loci in the genome (Kassahun et al., 2014) and in this technique; no DNA sequence information for primer designing is needed (Zietkiewicz et al., 1994). The analytical procedures include PCR, only low quantities of template DNA are required (5 to 50 ng per reaction). ISSRs are randomly distributed throughout the genome and are a multi-locus marker system so that it can randomly sample the whole genome and provides multiple fragments. Moreover, ISSR is a very potential DNA marker system in discriminating within and among population genetic variation without prior DNA sequence information especially for crops that are very difficult to classify/identify morphologically (Aga et al., 2005; Tiliye et al., 2007; Gezahegn et al., 2010; Bekele et al., 2014). Therefore, this study was initiated with the aim of evaluating the level and patterns of molecular genetic diversity and establishing relationships among introduced genotypes and populations of sugarcane in Ethiopia using Inter Simple Sequence Repeat (ISSR) markers.

MATERIALS AND METHODS

Plant materials

A total of 82 sugarcane genotypes from seven populations were collected from Sugar Corporation, Research and Development germplasm conservation garden found at Wonji. These genotypes were previously introduced to Ethiopia from various countries abroad namely Barbados, Cuba, France, India, South Africa, Sudan, and USA (Table 1 and Supplementary Table 1) for the purpose of commercial sugarcane production. Genotype selection was made taking into consideration the variation in place of origin source countries and based on their nature of utilization/current status. Young leaf samples of each genotype were taken from randomly selected three sugarcane plants in the field. The leaf samples were dried with silica gel in the ratio 1:5 in plastic bag and put separately until genomic DNA extraction.

DNA extraction

As reported by Gilbert et al. (1999), bulk sampling approach was chosen as it permits representation of a sample by optimum number of plants to yield optimal amounts of DNA. Therefore, in this study, approximately equal number of three silica-gel-dried leaf tissue per sample was bulked and ground with Mix and Mill (MM

400) grinding machine.

DNA was extracted from silica-gel-dried leaf tissue using cetyltrimethylammonium bromide (CTAB) method employing second extractions to yield optimal amounts of DNA (Borsch et al., 2003). DNA concentration and quality was analyzed using nanodrop measurement (NanoDrop^{TM}2000/2000c) with a range of 53.70 to 1960.70 ng/µL concentration and 1.78 to 2.01 ratio purity. The genomic DNA was also tested using 1% agarose gel by applying 2 µL genomic DNA loaded after mixing with 2 µL 6X loading dye to check the concentration and quality of DNA on gel matrix using gel documentation system (Biosens SC750) under UV light. Genomic DNAs were then diluted for ISSR-PCR to an approximate concentration of 20 ng/µL.

ISSR primer selection and PCR conditions

A total set of 15 ISSR primers were acquired from Genetic Research Laboratory (originally obtained from University of British Colombia, Primer kit UBC 900) of the Microbial, Cellular and Molecular Biology Department, College of Natural Sciences, Addis Ababa University. These primers were selected based on published experimental results in sugarcane (Da Costa et al., 2011) and related *Saccharum* species. For an efficient molecular characterization of sugarcane genotypes, initially two individuals were randomly selected from three representative populations to screen the primers for their polymorphism and reproducibility at 45, 48, 53 and 55°C annealing temperature. Band intensity and reproducibility of all conditions were compared and optimized. Therefore, based on amplified PCR products of six representative sugarcane samples, eight di-, one tri-, one tetra-, and two penta-nucleotide primers were finally selected for further molecular analysis based on their reproducibility and polymorphism (Table 2). The selection includes a spectrum of primers with different repeat motifs.

The PCR was conducted in Biometra 2003 T3 Thermo cycler machine. PCR amplification was carried out in a 26 µL final reaction volume containing 2 µL diluted template DNA, 16.2 µL ddH_2O (ultra pure), 1 µL dNTPs (20 mM each), 2.5 µL Taq reaction buffer (10X buffer), 3.5 µL $MgCl_2$ (25 mM), 0.4 µL primer (20 pmol/µL), and 0.4 µL Taq DNA polymerase (5 u/µL).Then, 12 ISSR primers were selected having good band intensity and reproducible results with PCR condition operated at 94°C initial denaturation for 4 min followed by 40 cycles of 94°C denaturation for 15 s, 45°C/48°C/53°C/55°C for 1 min primer annealing [depending on primer guanine-cytosine (GC) content and melting temperature], 72°C for 1 min and 30 s extension and final extension at 72°C for 7 min for Taq DNA polymerase at 99°C lid temperature. PCR amplification products were loaded onto 1.72% agarose gels in TBE buffer. The first lane was loaded with 100 bp DNA ladder by loading 2 µL (peq gold range mix) with loading dye in that well as a size standard and the last lane was loaded by a control, and visualized using gel documentation system (Biosens SC750) under ultraviolet light after staining in 10 mg/mL ethidium bromide.

Band scoring and data analysis

The ISSR band profiles were considered as dominant markers and each locus was counted as bi-allelic locus with one amplifiable and one null allele (Aga et al., 2005; Tiliye et al., 2007; Gezahegn et al., 2010). Thus, ISSR bands were treated as an independent locus and polymorphic bands were scored as binary data; (1) band present or (0) band absent for all the 82 sugarcane individual samples. However, there were some samples that did not yield ISSR-PCR products for several of the primers, and were therefore excluded from data analysis. Thus, only scorable bands were considered for diversity analysis.

POPGENE version1.32 software (Yeh et al., 1999) was used to calculate the following genetic diversity parameters: Percent of polymorphic loci (PPL), Nei's (1973) gene diversity (h), and Shannon's information index (I). Thus, estimations of within and among population genetic diversity as percent polymorphism was calculated for each population based on the banding profile using POPGENE version1.32 software (Yeh et al*.,* 1999). Analysis of molecular variance (AMOVA) was used to estimate genetic variance components using Areliquin version 3.01 (Excoffier et al., 2006) computer software to describe the genetic structure and partitioning the total variation to different hierarchical levels. Jaccard's similarity coefficient (1908) was calculated using NTSYS-PC version 2.02 (Rohlf, 2000). The unweighted pair group method with arithmetic mean (UPGMA; Sneath and Sokal, 1973) was then used to compare genotypes with NTSYS-PC version 2.1 (Rohlf, 2000). In addition, neighbour-joining (NJ; Saitou and Nei, 1987) was used to compare genotypes and evaluate patterns of genotype clustering with Free Tree 0.9.1.50 (Pavlicek et al., 1999). To further examine patterns of genetic relationship among individual genotypes principal coordinate analysis (PCoA) was performed using PAST version 1.18 software (Hammer et al., 2001) and STATISTICA version 6.0 software (Hammer et al., 2001).

RESULTS

Level of polymorphism

A total of 149 clear and scorable bands were amplified using 12 ISSR primers, with an average of 12.41 bands per primer. From the total amplified loci (149), 124 were polymorphic among the sugarcane genotypes, with an average of 10.33 ISSR polymorphic loci per primer. All primers amplified fragments, with a number of amplicons varying from 10 (primers 811 and 812) to 18 (primer 880) fragments, with molecular weight ranging from 200 to 4000 bp. The number of polymorphic loci ranged from 7 (primer 812) to 16 (primer 880) attributing to 83.22% total polymorphism. Percent of polymorphic loci (PPL) varied from 70 (primer 812) to 90.91% (primers 810 and 824). Primer 812 resulted in the least polymorphic loci (7) with 70% of polymorphism overall individual comparison, while primer 810 and 824 generated the highest percent of polymorphic loci (90.91%) with 10 polymorphic loci out of 11 (Table 2).

Genetic diversity

Within populations, the number and percentage of polymorphic loci (data generated by all primers) ranged from 43 (28.86%) for introduced genotypes from Barbados to 71 (47.65%) for France with a mean of 38.35% which indicates genotypes from France and Barbados as having highest and lowest genetic diversity, respectively (Table 3). Based on the analyses of the only eight di-nucleotide primers, the highest polymorphism was observed in France and India populations (48.39%) followed by genotypes from Cuba (46.24%) with the lowest recorded in Barbados (34.41%). The penta-nucleotide repeat primers (primers 880 and 881) revealed highest polymorphism in Sudan genotypes with 61.29%

Table 2. List of all ISSR primers used in the analysis, primer sequence, estimated band size, number of amplified loci (NAL), number of polymorphic loci (NPL), and percentage of polymorphic loci (PPL).

Primer code	Sequence (5'-3')	Band size (bp)	NAL	NPL	PPL
810	GAGAGAGAGAGAGAGAT	750-4000	11	10	90.91
811	GAGAGAGAGAGAGAGAC	1000-4000	10	9	90.00
812	GAGAGAGAGAGAGAGAA	300-1000	10	7	70.00
815	CTCTCTCTCTCTCTCTG	300-2000	14	12	85.71
822	TCTCTCTCTCTCTCTCA	200-1000	13	11	84.62
824	TCTCTCTCTCTCTCTCG	1500-4000	11	10	90.91
834	AGAGAGAGAGAGAGAGYT	1000-4000	13	10	76.92
848	CACACACACACACACARG	750-3500	11	9	81.82
866	CTCCTCCTCCTCCTCCTC	400-1000	11	9	81.82
873	GACAGACAGACAGACA	300-1000	14	10	71.43
880	GGAGAGGAGAGGAGA	750-4000	18	16	88.89
881	GGGTGGGGTGGGGTG	200-1500	13	11	84.62
Mean		620-2583	12.41	10.33	83.14
Overall		200-4000	149	124	83.22

Y = Pyrimidine (C or T), R = Purine (A or G).

Table 3. Percentage of polymorphic loci (PPL) and number of polymorphic loci (NPL) in studied sugarcane populations.

Population	di-primers	Percentage of polymorphic Loci (PPL)			Overall PPL	NPL
		Tri-primer	Tetra-primer	Penta-primers	All primers	All primers
Barbados	34.41	18.18	14.29	22.58	28.86	43
Cuba	46.24	18.18	21.43	58.06	44.30	66
France	48.39	45.45	64.29	38.71	47.65	71
India	48.39	18.18	35.71	25.81	40.27	60
S. Africa	36.56	36.36	21.43	22.58	32.21	48
Sudan	39.78	9.09	14.29	61.29	39.60	59
USA	35.48	18.18	28.57	45.16	35.57	53
Mean	41.32	23.37	28.57	39.17	38.35	57.14
Overall	83.87	81.82	71.43	87.10	83.22	124

followed by genotypes from Cuba with 58.06% of polymorphism. The overall level of polymorphism for only single tri-nucleotide primer (primer 866) and tetra-nucleotide primer (primer 873) attributed to 81.82 and 71.43%, respectively and the only eight di-nucleotide primers contributed 83.87% of polymorphism for all sugarcane populations, while the two penta-nucleotide primers contributed 87.10% of polymorphism (Table 3).

Based on the overall dataset, Nei's gene diversity (h) and Shannon's information index (I) ranged from 0.097 and 0.147 to 0.171 and 0.255 for Barbados and France with a mean of 0.137 and 0.205, respectively (Table 4). Nei's gene diversity (h) value generated by only eight di-primers for France and India genotypes were highest with values of 0.173 followed by Cuba genotype with a value of 0.168. The lowest gene diversity was observed in Barbados (0.115). Based on this dataset, Shannon's information index (I) for France population was highest with a value of 0.260 followed by India and Cuba populations with value of 0.259 and 0.249, respectively, but the least Shannon index was observed in population of Barbados (0.174) (Table 4). The overall percent of polymorphism (PPL), gene diversity (h), and Shannon's information index (I) for all study populations of sugarcane with 12 ISSR primers of the current study was 83.22%, 0.310, 0.458, respectively (Tables 3 and 4).

Partitioning genetic diversity

Partitioning of genetic diversity by analysis of molecular variance (AMOVA) using grouped populations revealed that out of the total genetic diversity, most of the ISSR diversity was distributed between individual genotypes within the populations (61.66%), with the remaining diversity being distributed among populations within

Table 4. Nei's gene diversity (h) and Shannon's information index (I) in studied sugarcane populations.

Population	Nei's gene diversity (h)				Overall (h)	Shannon's information index (I)				Overall (I)
	Di-primers	Tri-primer	Tetra-primer	Penta-primers	All primers	Di-primers	Tri-primer	Tetra-primer	Penta-primers	All primers
Barbados	0.115	0.075	0.053	0.072	0.097	0.174	0.109	0.079	0.111	0.147
Cuba	0.168	0.087	0.095	0.209	0.164	0.249	0.122	0.136	0.308	0.241
France	0.173	0.146	0.22	0.145	0.171	0.260	0.223	0.334	0.217	0.255
India	0.173	0.054	0.144	0.108	0.148	0.259	0.086	0.212	0.157	0.221
S. Africa	0.125	0.156	0.076	0.092	0.116	0.189	0.225	0.116	0.135	0.174
Sudan	0.136	0.031	0.044	0.251	0.144	0.205	0.048	0.069	0.364	0.214
USA	0.119	0.046	0.111	0.168	0.123	0.181	0.076	0.164	0.249	0.185
Mean	0.144	0.085	0.106	0.149	0.137	0.216	0.127	0.158	0.220	0.205
Overall	0.306	0.248	0.260	0.365	0.310	0.455	0.387	0.384	0.526	0.458

Table 5. Analysis of molecular variance (AMOVA) results for seven populations of introduced sugarcane genotypes based on 149 ISSR bands generated by 12 primers.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation index (F_{ST})	P-value*
With grouping						
Among groups	3	366.553	0.825 Va	3.46		0.00
Among populations within groups	3	268.471	8.328 Vb	34.88	0.383	0.00
Within populations	75	988.821	14.72 Vc	61.66		0.00
Total	81	1623.846	23.874	100		
Without grouping						
Among populations	6	114.252	1.44530 Va	36.44	0.364	0.00
Within populations	75	189.065	2.52087 Vb	63.56		0.00
Total	81	303.317	3.96617	100		

groups (34.88%) and among groups (3.46%) (Table 5). Similarly, partitioning of genetic diversity by analysis of molecular variance without grouping populations revealed that out of the total genetic diversity, most of the ISSR diversity is due to differences between individual genotypes within the populations/country based group (63.56%), while the remaining is due to differences among populations (36.44%) with significant components of molecular variation (*P*= 0.00) (Table 5).

Genetic similarity and distance

Jaccard's similarity coefficient-based pairwise comparisons of the seven populations of sugarcane similarity ranged from 0.532 to 0.700 with mean of 0.616. The highest genetic similarity was found between Sudan and South Africa populations (0.700). However, the maximum genetic distance was observed between population from USA and Barbados followed by USA and Cuba with similarity coefficient of 0.532 and 0.543, respectively (Table 6).

Cluster analysis

UPGMA and NJ-based tree construction methods was used to construct dendrogram on the basis of Jaccard's similarity coefficient to explore the relationship among genotypes of sugarcane based on 149 ISSR bands amplified by a total of 12 ISSR primers (eight di-, one tri-, one tetra-, and two penta-nucleotide primers). The dendrogram derived from UPGMA-based cluster analysis of the whole ISSR data with 82 sugarcane genotypes showed two major distinct clusters (major-I and II) and two sub-clusters (sub-I and II) within each major cluster at around 0.60 similarity coefficient and with similarity values ranging from 0.59 to 0.90 with mean of 0.74 for all investigated individuals (Figure 1). The first major cluster (major-I) was dominated by individuals from Barbados (B), Cuba (C), India (I), and some of individuals from France (F) and they formed their own separate groups within this cluster. The second major cluster (major-II) was comprised individuals from France (F), South Africa (SA), Sudan (S), and USA (U) and clearly formed their own separate groups within the clusters. Individual-based

Table 6. Similarity matrix among the sugarcane populations obtained by Jaccard's similarity coefficient.

Population	Barbados	Cuba	France	India	South Africa	Sudan	USA
Barbados	1.000						
Cuba	0.621	1.000					
France	0.589	0.625	1.000				
India	0.614	0.615	0.617	1.000			
South Africa	0.584	0.569	0.609	0.555	1.000		
Sudan	0.562	0.568	0.646	0.601	0.700	1.000	
USA	0.532	0.543	0.595	0.594	0.651	0.653	1.000

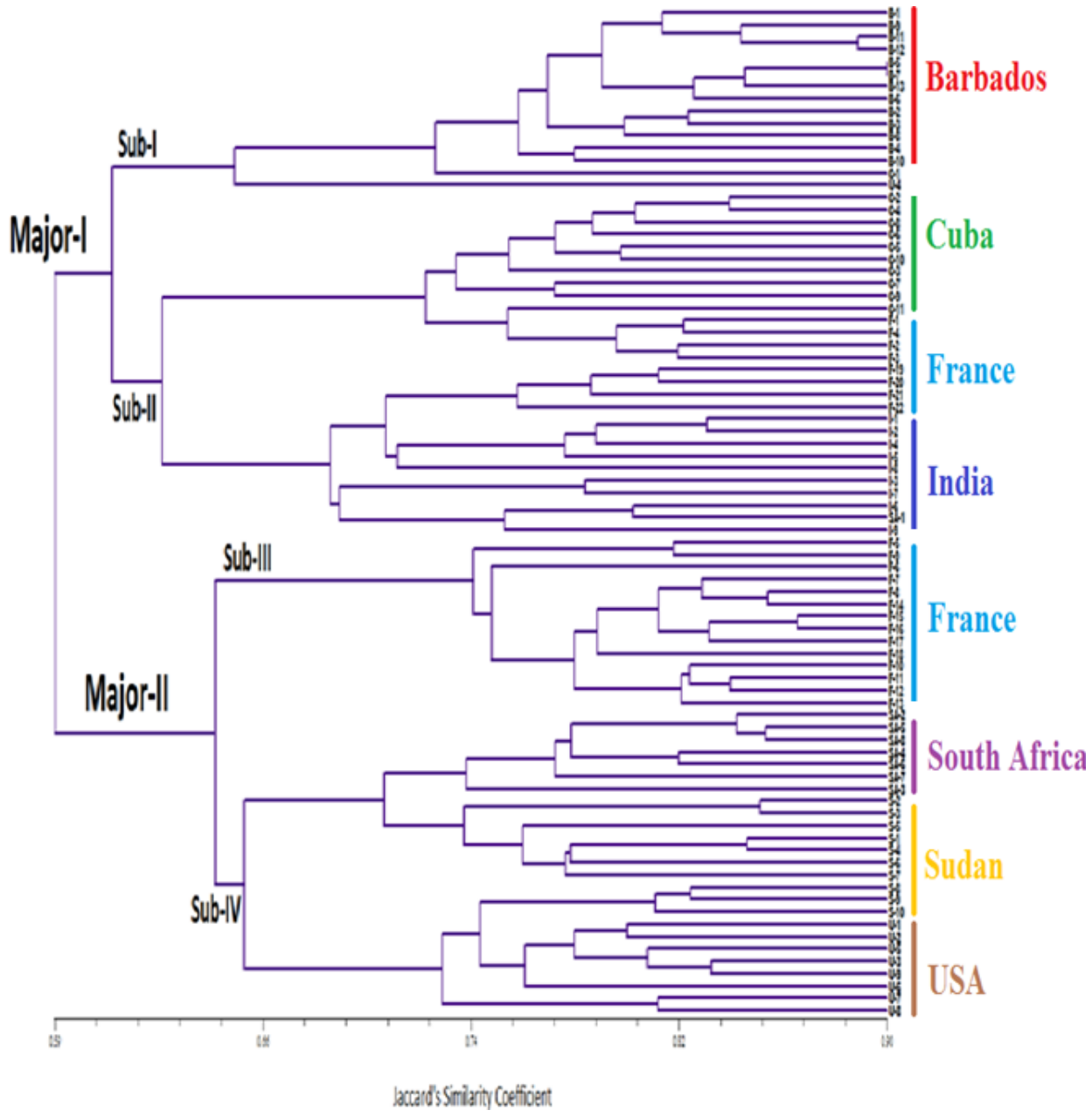

Figure 1. Dendrogram obtained with UPGMA based on Jaccard's similarity coefficients for 82 genotypes of sugarcane using 12 ISSR primers. B = Barbados, C = Cuba, F = France, I = India, SA = South Africa, S = Sudan, U = USA.

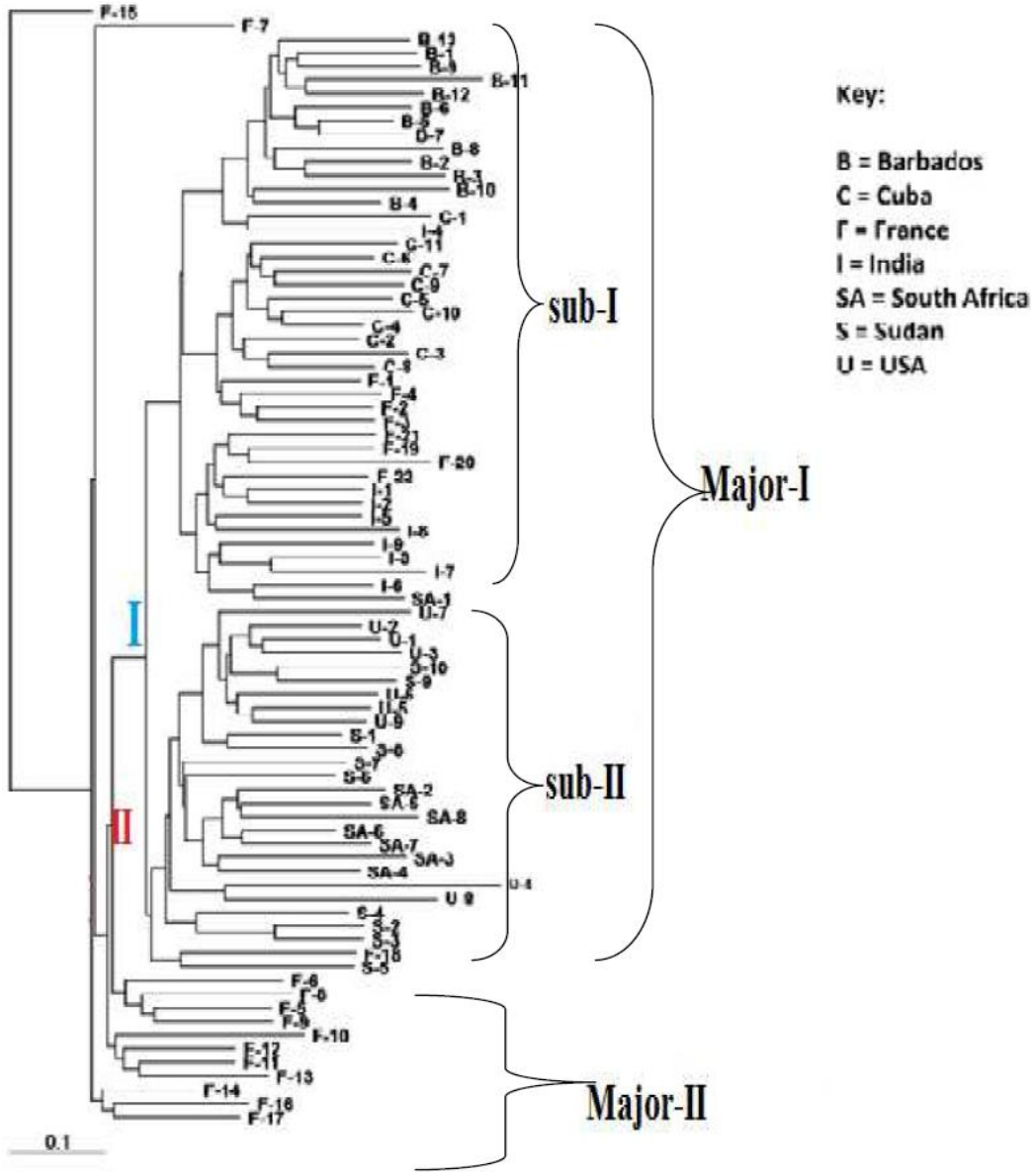

Figure 2. NJ based analysis of 82 genotypes of sugarcane using 12 ISSR primers after pairwise comparisons using Jaccard's similarity coefficient.

UPGMA clustering of an overall analysis showed strong clustering of individuals with respect to their populations/ origins except few intermixed individuals from one another. Clustering analysis based on Neighbor Joining (NJ) also resulted in similar clustering patterns as that of UPGMA but moderate intermixing were observed in NJ cluster analysis (Figure 2).

Principal coordinate analysis

Principal coordinate analysis (PCoA) was used to reveal patterns of variation among sugarcane genotypes on the basis of Jaccard's similarity coefficient using PAST and STATISTICA software packages. Based on the overall ISSR data obtained, principal coordinate analysis was performed utilizing the first three coordinates having eigen-values of 4.45, 3.04, and 2.31, and which accounted for 7.81, 5.35 and 4.06% of the variation, respectively, cumulatively explaining 17.22% by employing PAST version 1.18 software (Hammer et al., 2001). It was used to show the grouping of genotypes using both two dimensional and three dimensional PCoA analyses.

The analysis of this study showed that the genotypes of the seven sugarcane populations examined tend to form

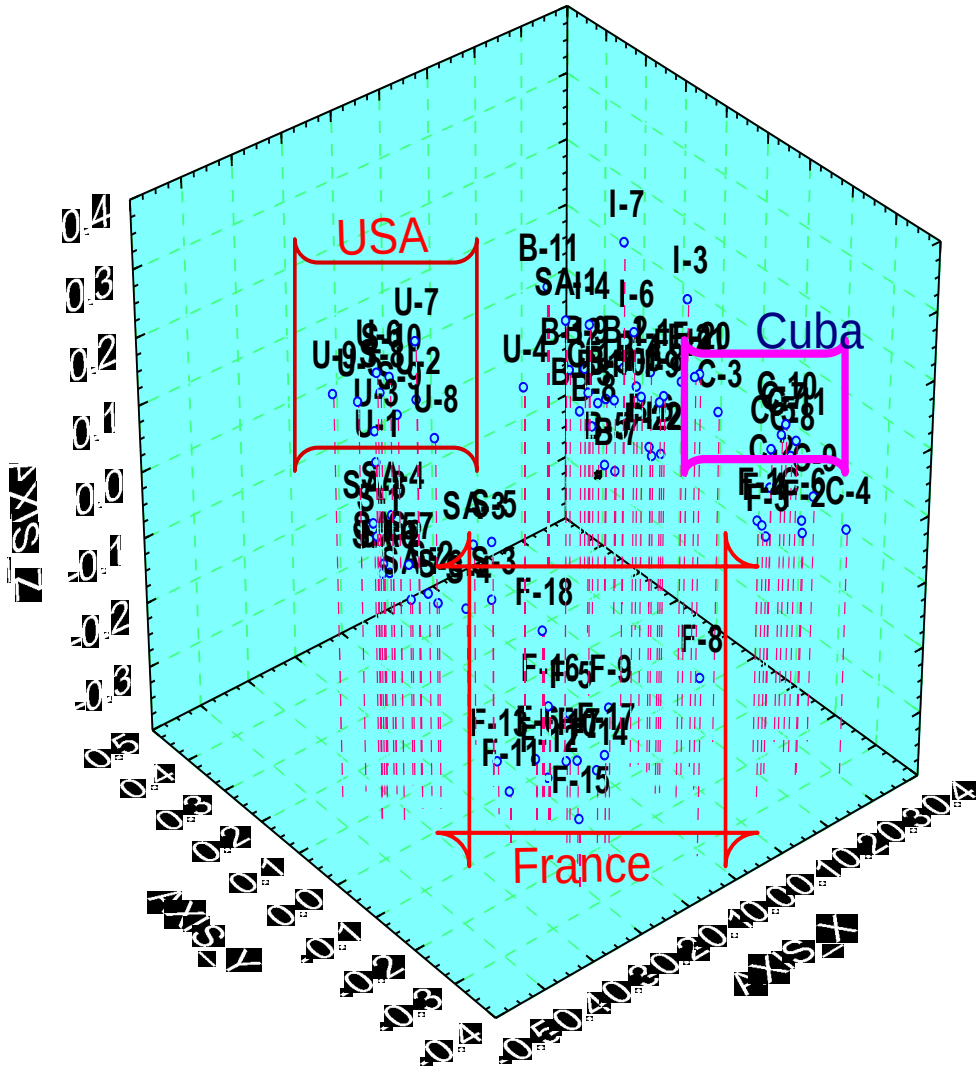

Figure 3. Three dimensional (3D) representation of principal coordinate analysis of genetic relationships among 82 genotypes of sugarcane as revealed by ISSR marker generated by 12 primers. B = Barbados, C = Cuba, F = France, I = India, SA = South Africa, S = Sudan, U = USA.

grouping based on their geographic region. With an overall analyses, the two dimensional (2D) representation of PCoA revealed somewhat poor patterns of grouping on the basis of population and the individuals were intermixed from one another (Figure not shown). However, the three dimensional (3D) representation showed better grouping patterns than two dimensional (2D) and most of the genotypes were made some sort of clear grouping pattern with respect to their population/origin. Thus, genotypes from France (F), Cuba (C), and USA (U) formed their own grouping on 3D PCoA (Figure 3). Likewise, genotypes from Barbados (B) and France (F) showed clear grouping on 2D analysis (Figure not shown). Similar to 2D PCoA analysis, individuals of India (I), South Africa (SA), and Sudan (S) populations were intermixed with other populations in 3D PCoA.

DISCUSSION

Genetic diversity and utility of ISSR marker in sugarcane

ISSR markers have high resolution power in fingerprinting and diversity analysis of sugarcane (Srivastava and Gupta, 2008; Da Costa et al., 2011). In addition to the advantages (inexpensive, easy to generate), ISSR markers are more powerful and efficient in detecting polymorphisms within and among populations and/or species. The present study also utilized the ISSR markers to investigate genetic diversity and relationships within and among the seven groups of introduced sugarcane genotypes in Ethiopia. This report makes this study the first investigation to assess molecular genetic diversity

within and among introduced sugarcane genotypes using ISSR molecular markers from Ethiopia. Molecular marker-based analysis of genetic diversity in plant species have become an important tools in crop improvement and conservation purpose (Weising et al., 2005).

In terms of percentage polymorphism, gene diversity, and Shannon index, per classes of primer; penta-nucleotide primers were found to be superior (PPL = 87.10%, h = 0.365, I = 0.526) followed by the di-nucleotide primers (PPL = 83.87%, h =0.306, I = 0.455). These all clearly indicate that the extent and distribution of penta- and di-nucleotide repeats polymorphism in sugarcane genome used for this study is very high and every individual sugarcane genotype is almost unique for these primers. Among the di-nucleotide primers, primer 810 and 824 with (GA)8 and (TC)8 repeats anchored with (T) and (G) respectively, detect high level of poly-morphism (90.91%) among the sugarcane populations followed by primer 811 (90.00%) with (GA)8 repeats anchored with (C). Similar finding has been reported by Reddy et al. (2002), generally primers with (AG), (GA), (CT), (TC), (AC), and (CA) repeats show higher polymorphism in plants than primers with other di-nucleotide repeats, while tri- and tetra-nucleotide repeats are less frequent in plants and their use in ISSR analysis is lower than the di-nucleotide repeats. Furthermore, the choice of appropriate primer repeat motives in ISSR fingerprint is critical to detect high level of polymorphism and reveal relationship within and among populations. Blair et al. (1999) and Reddy et al. (2002) reported that the (AG) and (GA) based ISSR primers have been shown to amplify clear bands in rice. Similarly, Joshi et al. (2000) also reported comparative assessments of ISSR primer repeat patterns of di-, tri-, tetra- and penta-nucleotides in the genus *Oryza* and found that di-nucleotide repeats of (AG)8 and (GA)8 with a number of anchors gave the best polymorphic and informative patterns.

In general, the present study showed that the ISSR markers detected high level of polymorphism, gene diversity, and Shannon's information index at genotypic level (PPL = 83.22%, h = 0.310, and I = 0.458). This indicates the existence of high level of genetic diversity within and among investigated sugarcane genotypes in Ethiopia. Similar findings were reported for other accessions during the evaluation of the genetic diversity of sugarcane varieties from India with ISSR markers by Srivastava and Gupta (2008) with 78.48% of the bands being polymorphic. Moreover, Smiullah et al. (2013) detected 85.25% polymorphism with SSR markers on sugarcane accession from Pakistan. However, the result obtained in our study bear higher polymorphism levels than those previously reported by other authors using RAPD markers (Nair et al., 2002; Kawar et al., 2009).

For achieving improved productivity in sugarcane, it is essential to maintain a high degree of genetic diversity among the commercial varieties and breeding populations since sugarcane is a clonally propagated crop, and creation of new genotypes is done through sexual crossing (Que et al., 2014). Thus, choosing parental lines is the most crucial step in any sugarcane improvement program. In the present study, the ISSR markers found to be an appropriate molecular marker system for generating the detailed intraspecific genetic diversity data to evaluate the extent and distribution of genetic diversity within and among sugarcane genotypes introduce from abroad. Therefore, with regard to intra varietal diversity, France genotypes have been found to be a more diverse population as compared to other populations studied in the overall analysis (PPL = 47.65%, h = 0.171, I = 0.255) followed by Cuba population (PPL = 44.30%, h = 0.164, I = 0.241), while the least variable population was from Barbados (PPL = 28.86%, h = 0.097, I = 0.147). This could be associated with the original sources of genotypes and wider genetic bases among individuals used for breeding program in France. Moreover, the introduced genotypes in Ethiopia are from various breeding institutes globally with different genetic origin. In this study, the Nei's gene diversity (h = 0.31) is relatively closer to what was recently found in sugarcane varieties by Que et al. (2014) in their effort to study genetic diversity within a Chinese local sugarcane germplasm based on start codon targeted (SCoT) polymorphism (h = 0.35) but much higher than which was reported by You et al. (2013) (h = 0.17) using SSR markers. Similarly, Lu et al. (1994) and Janno et al. (1999) also reported that due to its polyploid nature, interspecific origin, and vegetative mode of propagation, high levels of heterozygosity were detected among sugarcane cultivars using RFLP markers. Similar patterns of molecular diversity were also detected on sugarcane cultivars using AFLP markers (Lima et al., 2002), SSR markers (Pandey et al., 2011; Tena et al., 2014), and ISSR markers (Srivastava and Gupta, 2008; Da Costa et al., 2011). However, Rodriguez et al. (2005) reported in a contrast way and found that high percentage of genetic similarity among the sugarcane cultivars with AFLP markers and suggested that the sugarcane germplasm collections from Mexico present a genetically narrow base. This is likely due to the fact that their parental breeding lines, used to develop these cultivars, are very close to each other. Abdalla et al. (2001) also stated that hybridization event followed by selfing would be predicted to decrease the number of polymorphic loci in subsequent generations by 50%. To this respect, D'Hont et al. (1995) suggested that because of the small number of parental breeding lines of these species used in the primary crosses of breeding program, the genetic base of commercial hybrid varieties appears to be narrow and could be the reason for the present slow progress in sugarcane breeding.

However, in this study, the ISSR fingerprint identified genetically unique genotypes from studied populations that are potentially important source of diverse sugarcane germplasm along with divergent individuals. This opens

up an excellent opportunity for their utilization in sugarcane breeding programs since intercrossing of elite hybrids is a main component in varietal development and such studies based on molecular marker information can be helpful in selecting diverse parental combinations giving maximum diversity with respect to sugar content. In general, the high level of genetic diversity detected by ISSR markers in our study was expected due to the use of newly released hybrid varieties from different countries of sugarcane breeding institutes and also considering allopolyploid nature of sugarcane, generally attributed to the interspecific hybridization crosses used during breeding programs that generated the actual breeding varieties (Da Costa et al., 2011; Pandey et al., 2011). The nature of ISSR, targeting regions especially rich in microsatellites may also justify the higher level of genetic polymorphism, since those regions are known to accumulate a larger number of mutations in DNA during replication and unequal crossing-over (Schlotterer and Tautz, 1992). The complexity of the sugarcane genome and the abundance of microsatellite repeats make also this a priority (Pandey et al., 2011).

Genetic differentiation and population structure

The genetic structure of plant populations is usually determined by the interactions of various factors, including the long-term evolutionary history of the species (habitat fragmentation and population isolation), genetic drift, mating system, gene flow, mutation, and selection (Schaal et al., 1998; Weising et al., 2005). Genetic differentiation among populations is often estimated with G_{ST} according to Nei (1973). However, a multi-locus approach, AMOVA is nowadays even more widely used than G_{ST} for the partitioning of genetic variation within and among populations (Excoffier et al*.,* 1992). In the present study, the estimate of population differentiation in sugarcane using F_{ST} and G_{ST} was 0.36 and 0.55, respectively. However, the value of G_{ST} may not be as strong as F_{ST} as pointed out by Culley et al. (2002) since G_{ST} is dependent on sample sizes and number of populations, in addition to its reliance on Hardy-Weinberg genotype proportions, and conditions that may be violated when analyzing small isolated populations, while the value of F_{ST} from AMOVA is more recommended in various literatures (Laurentin, 2009) particularly for multi-locus markers. Therefore, using both F_{ST} and G_{ST} the present study revealed relatively high level of genetic differentiation among populations. This could be associated with mutation at ISSR primers annealing site, random genetic drift, and differential selection pressure by the environment on the loci assessed.

Estimates of genetic differentiation among populations of sugarcane cultivars from China based on AMOVA derived by analyzing SSR markers revealed that the majority of variation detected in the SSRs was within populations (90.5%) with only 9.5% of the variation was attributed to differences among populations (You et al., 2013). Que et al. (2014) also reported that a high level of genetic variation was found within populations rather than among populations of sugarcane using start codon targeted (SCoT) polymorphism. Similar finding was reported by Glynn et al. (2009) using SSR marker on sugarcane cultivars from USA, the values of AMOVA across the populations indicated that intrapopulation genetic variation (96.6%) was much higher than that of interpopulation (3.4%). The AMOVA results obtained in our study do not contradict with the above findings. It is a prevalent view that outcrossing and long-lived perennial species retain higher genetic variation within populations than among populations. Thus, sugarcane is one of a cross-pollinating and long-lived perennial species (Berding et al., 2004). In accordance with this, the AMOVA analysis obtained in this study revealed higher within population genetic variation (63.56%) than among population genetic variation (36.44%). Similarly, for grouping, AMOVA showed a higher genetic variation within populations (61.66%) but lower variation exists among populations within groups (34.88%) and among groups (3.46%). Therefore, the majority of the ISSR diversity was distributed among individual genotypes within the populations. This is likely due to the fact that the gene flow obtained largely from the interspecific crosses involving *Saccharum officinarum* and the wild species *S. spontaneum* in sugarcane breeding program since their interspecific F1 hybrids could form complete pairing in meiosis thus resulting in gene flow between them. However, this limited gene flow among populations could be attributed to geographical isolation/barrier, clonal way of propagation, and limited number of parental clones used in crossing programs (Nair et al., 1999, 2002; Selvi et al., 2003; Berding et al., 2004; Rodriguez et al., 2005). It is also believed that the high genetic diversity observed within sugarcane populations studied, might be due to preferential adaptive gene complexes being evolved during long evolutionary period in the regions adapted to environmental changes. Moreover, introduction of genotypes from various breeding institutions and lineages could be another explanation for the observed variation.

Genetic relationships among sugarcane genotypes in Ethiopia

Clustering analyses was employed to better visualize the genetic relationships of the 82 genotypes of sugarcane in Ethiopia that originated from different countries abroad. The UPGMA analysis in this study revealed that most of the genotypes that originated from the same geographic region showed close genetic relationships and were grouped into the same cluster. This result in agreement with the studies of Chen et al. (2009) who used SSR markers and found that based on Jaccard's similarity coefficient 40 sugarcane cultivars from different

geographical regions tend to cluster with their respective origins. In NJ analysis, the majority of the genotypes of the respective population were also observed to form similar grouping patterns with that of UPGMA but there were moderate intermixing with other group and also some of the individuals from each population appear to have longer branches which indicate that they are more variable than others. This is in accordance with Weising et al. (2005), UPGMA assumes a rigid molecular clock, which means that the evolutionary rates along all branches of the tree need to be identical, while neighbor joining (NJ) algorithm produces additive trees and does not assume identical evolutionary rates along all branches.

In general, clustering parameters (PCoA, NJ, and UPGMA) revealed some intermixed individuals from one population to another and vice versa. This type of admixture may be resulted from the involvement of sugarcane genotypes in breeding programs for improving some of the traits of commercially exploited varieties so that to achieve such objectives there might be parental breeding lines exchange throughout sugarcane growing regions in the world. On the other hand, sugarcane growing countries might be use the cross progenies that are released by Center for Research in Regional Planning and Development (CRAD) in France and Indian Institute of Sugarcane Research (IISR). As a general point of view, it was not surprising that geographically different populations made a cluster with other populations since most of the commercial cultivars of sugarcane that were bred after the turn of the 20th century are interspecific hybrids between *S. officinarum* and *S. spontaneum* (D'Hont et al., 1996). Thus, there might be the cross progeny clustered with their progenitor parents from other regions or the parents may have clustered with their cross progeny from distantly related populations. This is in agreement with the results obtained by microsatellite markers (Selvi et al., 2003) wherein the highest genetic similarity was observed between the cross progeny and their progenitor parents and they cluster in the same group despite of their geographic origin. It has also been reported that one best explanation for the high levels of similarity within sub-groups and groups is that the lineages have been subjected to a greater degree of intercultivar gene flow during sugarcane breeding program (Rodriguez et al., 2005).

Conclusion

The present study is the first of its kind that utilized ISSR molecular marker technique to assess genetic diversity and establishes relationships among introduced geno-types of sugarcane in Ethiopia. However, the scanty information available on the genetic diversity within and between sugarcane varieties has been based mainly on morphological characteristics. This necessitated the estimation of genetic diversity based on molecular markers which could provide more accurate information to sugarcane breeder. The level of polymorphism observed proved that the ISSR marker system was robust at amplifying markers on sugarcane and did so according to the complex genome structure interconnecting with interspecific origin. Moreover, ISSR markers could potentially be useful in the characterization and management of germplasm where the aim is to enhance the germplasm for specific traits. Thus, future sugarcane breeding efforts involving crosses between and within the groups identified in this study may provide useful strategies for combining beneficial genes and alleles in developing improved sugarcane varieties to satisfy varietal requirements of different agro-ecologies of sugarcane plantations in various regional states of the country.

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to express their utmost gratitude to: Ethiopian Sugar Corporation, Research and Development Directorate for their financial support and for kindly providing plant material with passport data and also Addis Ababa University for their research funding; Mr. Feyissa Tadesse, Director of Varietal Development and Research at Wonji Sugar Factory, for his unlimited cooperation and for the success of this research work.

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Supplementary Table 1. Lists of sugarcane genotypes studied with their origin, year of introduction and nature of utilization/current status.

S/N	Genotype code	Country of origin	Year of introduction	Nature of genotype
1	B52/298	Barbados	Before 2000	Commercial
2	B4906	Barbados	Before 2000	Candidate
3	B59212	Barbados	Before 2000	Old
4	B863/349	Barbados	Before 2000	Old
5	B60/263	Barbados	Before 2000	Old
6	B52/219	Barbados	Before 2000	Old
7	B45/54	Barbados	Before 2000	Old
8	B5736	Barbados	Before 2000	Candidate
9	B5490	Barbados	Before 2000	Candidate
10	B58230	Barbados	Before 2000	Candidate
11	B59/04	Barbados	Before 2000	Candidate
12	B60/63	Barbados	Before 2000	Candidate
13	B50210	Barbados	Before 2000	Candidate
14	C92/26	Cuba	2012	Advanced breeding lines
15	C323/68	Cuba	2012	Advanced breeding lines
16	C98/128	Cuba	2012	Advanced breeding lines
17	C85/102	Cuba	2012	Advanced breeding lines
18	C132/81	Cuba	2012	Advanced breeding lines
19	C90/530	Cuba	2012	Advanced breeding lines
20	C88/556	Cuba	2012	Advanced breeding lines
21	C92/514	Cuba	2012	Advanced breeding lines
22	C89/147	Cuba	2012	Advanced breeding lines
23	C87/51	Cuba	2012	Advanced breeding lines
24	C88/356	Cuba	2012	Advanced breeding lines
25	FG03/291	France	2012	Advanced breeding lines
26	FG04/798	France	2012	Advanced breeding lines
27	FG04/420	France	2012	Advanced breeding lines
28	FG06/806	France	2012	Advanced breeding lines
29	PSR07/84	France	2012	Advanced breeding lines
30	Vmc96/22	France	2012	Advanced breeding lines
31	FG04/641	France	2012	Advanced breeding lines
32	FG04/896	France	2012	Advanced breeding lines
33	Vmc95/252	France	2012	Advanced breeding lines
34	FG06/750	France	2012	Advanced breeding lines
35	FG04/275	France	2012	Advanced breeding lines
36	PSR97/105	France	2012	Advanced breeding lines
37	Vmc96/47	France	2012	Advanced breeding lines
38	FG04/622	France	2012	Advanced breeding lines
39	FG06/747	France	2012	Advanced breeding lines
40	Vmc9660	France	2012	Advanced breeding lines
41	FG04/607	France	2012	Advanced breeding lines
42	Vmc96/62	France	2012	Advanced breeding lines
43	FG06/755	France	2012	Advanced breeding lines
44	Vmc96/55	France	2012	Advanced breeding lines
45	FG05/424	France	2012	Advanced breeding lines
46	Vmc95/243	France	2012	Advanced breeding lines
47	Co680	India	Before 2000	Commercial
48	Co1148	India	Before 2000	Candidate
49	Co678	India	Before 2000	Candidate

Supplementary Table 1. Contd.

50	Co434	India	Before 2000	Old
51	Co0238	India	Before 2000	Advanced breeding lines
52	Co421	India	Before 2000	Commercial
53	Co449	India	Before 2000	Commercial
54	Co810	India	Before 2000	Candidate
55	Co740	India	Before 2000	Commercial
56	N52/219	South Africa	Before 2000	Commercial
57	N14	South Africa	Before 2000	Commercial
58	N55/805	South Africa	Before 2000	Old
59	NCo334	South Africa	Before 2000	Commercial
60	NCo376	South Africa	Before 2000	Candidate
61	N53/216	South Africa	Before 2000	Commercial
62	N11	South Africa	Before 2000	Old
63	NCo310	South Africa	Before 2000	Old
64	2/333	Sudan	2012	Advanced breeding lines
65	2/111	Sudan	2012	Advanced breeding lines
66	2/999	Sudan	2012	Advanced breeding lines
67	Q217	Sudan	2012	Advanced breeding lines
68	2/888	Sudan	2012	Advanced breeding lines
69	2/555	Sudan	2012	Advanced breeding lines
70	Q200	Sudan	2012	Advanced breeding lines
71	2/777	Sudan	2012	Advanced breeding lines
72	2/222	Sudan	2012	Advanced breeding lines
73	2/444	Sudan	2012	Advanced breeding lines
74	CP00/1527	USA	2012	Advanced breeding lines
75	CP97/1944	USA	2012	Advanced breeding lines
76	CP97/1989	USA	2012	Advanced breeding lines
77	CP47/193	USA	2012	Advanced breeding lines
78	CP00/1301	USA	2012	Advanced breeding lines
79	CP61/39	USA	2012	Candidate
80	CP00/1302	USA	2012	Advanced breeding lines
81	CP60/23	USA	2012	Candidate
82	CP04/1566	USA	2012	Advanced breeding lines

Candidate variety = refers to varieties introduced and currently under final trial for verification to get released as a certified variety. Advanced breeding lines = these are breeding lines at the final selection stages when introduced and currently are in trials. Commercial variety = any variety under cultivation; Old or obsolete variety = any variety currently not in production.

academicJournals

Vol. 16(10), pp. 450-462, 8 March, 2017
DOI: 10.5897/AJB2016.15771
Article Number: CC2870663091
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Refinement of protocol for rapid clonal regeneration of economical bamboo, *Bambusa balcooa* in the agroclimatic conditions of Bihar, India

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Received 8 November, 2016; Accepted 6 January, 2017

Bambusa balcooa **is a commercially important bamboo. The present study was undertaken for refinement of protocol for the rapid and mass production of this species of bamboo in agroclimatic condition of Bihar. Micro-clonal propagation techniques have been employed for the study. This technique is the only method for the large scale production of *B. balcooa.* Explants were collected from Bamboo setum from TNB College campus. Combined effect of 6-benzylaminopurine (BAP) and Kinetin (Kn) (BAP 2 mg/l + Kn 0.5 mg/l) resulted in 85% bud breakage during initiation of cultures. One remarkable feature that was observed is that microshoots in medium without supplementation of naphthalene acetic acid (NAA) had necrotic shoots and they had less multiplication rate in liquid media. High multiplication rate with comparatively longer shoots were observed in liquid media. NAA (2.5 mg/l) along with half strength of MS showed 100% rooting but it was less than 50% when Murashige and Skoog (MS) media was supplemented with indole 3-butyric acid (IBA). Remarkably, in half strength of MS, clumps dried at first within 7 days in rooting media, however, new green healthy shoots proliferated later (after 21 days). The data obtained from this study has set a refined protocol for the clonal regeneration of *B. balcooa* for the state of Bihar. The current study will help to produce this species of bamboo on large scale, and thereby help to boost rural economy of Bihar.**

Key words: *In vitro* regeneration, clonal propagation, bamboo, *Bambusa balcooa,* micropropagation.

INTRODUCTION

Bamboos are one of the fastest growing mono-cotyledonous plants which have multipurpose utility. They produce huge amounts of biomass. They are universal in occurrence except in European continent (Nadgauda et. al., 1993). Being a substitute of timber, they have attracted the growers and scientists widely throughout the world. They are preferred due to their high agroclimatic suitability, as renewable source of energy and also as they do help to prevent soil erosion (Gantait et. al., 2016).

After the creation of Jharkhand state, Bihar is left with a

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6.87% green cover only causing a drastic change in climate due to human consumption (India State of Forest report, 2009). Earlier, Bihar used to receive an annual rainfall of 1200 to 1500 mm annually which has now come down to 800 to 1000 mm only for last 11 years. It is believed that bamboos can help man to mitigate global warming as they are one of the fastest growing plants and also they have comparatively better CO_2 sequestration abilities (Nasreen et. al., 2015). They can fix atmospheric carbon in organic form to soil. Under the "Hariyali Mission (a mission for Green Cover)", Department of Environment and Forest, Bihar Government is striving to increase green cover by 19% consequent upon which there is a target to plant massive quality bamboo plantlets in wastelands, marginal lands and also in agroforestry. Among the 136 species of bamboos in India, *Bambusa balcooa* is the strongest and multipurpose bamboo (Sharma and Sarma, 2011). Some of the morphologically distinguishable features of this species are like they have short internodes (20 to 24 cm), nodes being swollen (8 to 15 cm in diameter), at the lower nodal region prominent brown hairs remain present as well as white rings mostly present at the nodal rings of young culms. On leaf sheaths mostly brownish hairs remain present; however, culm sheath auricles may remain present or absent. Flowering cycle of this species is of 35 to 45 years and after that plants die without seed setting. It is being used in construction, ladders, boats, rickshaw hood frames, to weave mats and baskets, pulp and paper, making handicraft, biofuels/bioenergy and also as bamboo chips. Additionally, tender shoots are edible as vegetable and pickles.

Considering the versatile use of *B. balcooa*, it is an important desirable species for cultivation. Plant tissue culture is the only technique which can be utilized to solve the challenges of rapid and mass regeneration of this species (Kaur et. al., 2014). The offset and rhizome cuttings used for vegetative propagation being bulky are troublesome to handle (Singh et. al., 2013). They have very low rooting frequency and also culms/rhizome is susceptible of getting desiccated, which thereby restricts their large scale production (Vishwanath et al., 2012). They also show high season specificity owing to the morpho-physiological state of the plant. This method can thus be tried only to a limited scale. In addition, *in vivo* technique requires large number of bamboo culms for macropropagation in order to meet high demand of the species. Hence, *in vitro* propagation is the only option for bulk production of *B. balcooa*. There are few earlier reports on clonal propagation of *B. balcooa* through modal shoot proliferations, however, all such studies have either one or other limitations like lack of information about explant treatments, suitable season for initiation, frequency of multiplication/rooting or discussed with complex hardening procedures (Das and Pal, 2005a, b; Mudoi and Borthakur, 2009; Negi and Saxena, 2011, Wei et al., 2015).

Figure 1. Clump of *Bambusa balcooa*.

Our study demonstrates the various critical factors effecting *in vitro* propagation and also the comparative measures to overcome those factors as well as an efficient method for micropropagation of *B. balcooa* in agroclimatic conditions of Bihar. Explants were excised every month to evaluate the influence of seasons on micropropagation. The various sizes of explants were selected to find out the most suitable for the climate of Bihar. Mean and standard deviations of the data were analysed.

MATERIALS AND METHODS

Aseptic culture establishment

The newly grown culm and culm branches (2 to 3 years old) were collected periodically from actively growing branches of *B. balcooa* planted in the PTC lab premises, TNB College campus, Bhagalpur, Bihar, India (Figure 1). Collections were made during four seasons (spring, summer, rainy and winter).

Surfaces of shoot segments were swabbed with 70% ethanol.

Table 1. Seasonal variations in bud initiation for establishment of aseptic culture (Oct 2015 to Sept 2016).

Season	Date of explant collection	Number of shoots	Shoot length (cm)	Initiation of bud (%)	Contamination (%)
Autumn + Winter	08-Oct	3.2 ± 0.03	1.33 ± 0.06	45	41
	15-Oct	2.8 ± 0.05	1.9 ± 0.01	42	31
	10-Nov	2.3 ± 0.02	2 ± 0.4	35	25
	25-Nov	2.1 ± 0.04	1.2 ± 0.05	20	36
	09-Dec	1.1 ± 0.05	1.5 ± 0.03	10	33
	23-Dec	1.5 ± 0.09	1.2 ± 0.06	12	41
	07-Jan	1.8 ± 0.02	1.6 ± 0.03	15	39
	16-Jan	1.9 ± 0.01	1.1 ± 0.07	18	44
Spring + Summer	03-Feb	3.2 ± 1.0	2.3 ± 0.03	48	35
	17-Feb	7.2 ± 0.09	3.8 ± 0.04	72	25
	05-Mar	7.8 ± 0.06	6.9 ± 0.02	89	15
	25-Mar	11.3 ± 0.01	7.2 ± 0.02	85	10
	20-Apr	14.6 ± 0.03	5.6± 0.05	91	12
	07-May	20.1 ± 0.8	3.9 ± 0.07	92	18
	21-May	25.1 ± 0.7	4.2 ± 0.06	78	13
	06-Jun	30.6 ± 0.4	3.5 ± 0.04	75	20
	23-Jun	15.6 ± 0.7	3.2 ± 0.04	72	15
Rainy season	16-Jul	14.1 ± 0.9	5.8 ± 0.06	74	83
	29-Jul	15.2 ± 1.0	5.4 ± 0.05	79	90
	07-Aug	10.7 ± 2.0	5.2 ± 0.03	81	92
	23-Aug	9.3 ± 0.5	5.7 ± 0.05	89	85
	06-Sep	8.4 ± 0.8	5.9 ± 0.04	69	87
	25-Sep	8.9 ± 0.9	5.3 ± 0.03	65	81

Using scalpel and leaf sheath were removed carefully without giving any injury to the buds.

Seasonal variation and explant collection

Collections of explants were made throughout the year from October 15 to September 16 in order to ascertain the most suitable season/period for establishment of cultures. The initiation and contamination percentage in different seasons were also monitored (Table 1).

Processing and surface sterilization of explants

Single node shoot segments of variable length and diameter were fine cut at both ends using sharp blade secateurs. For surface sterilization of explants disinfectants were used in variable combinations (Table 2).

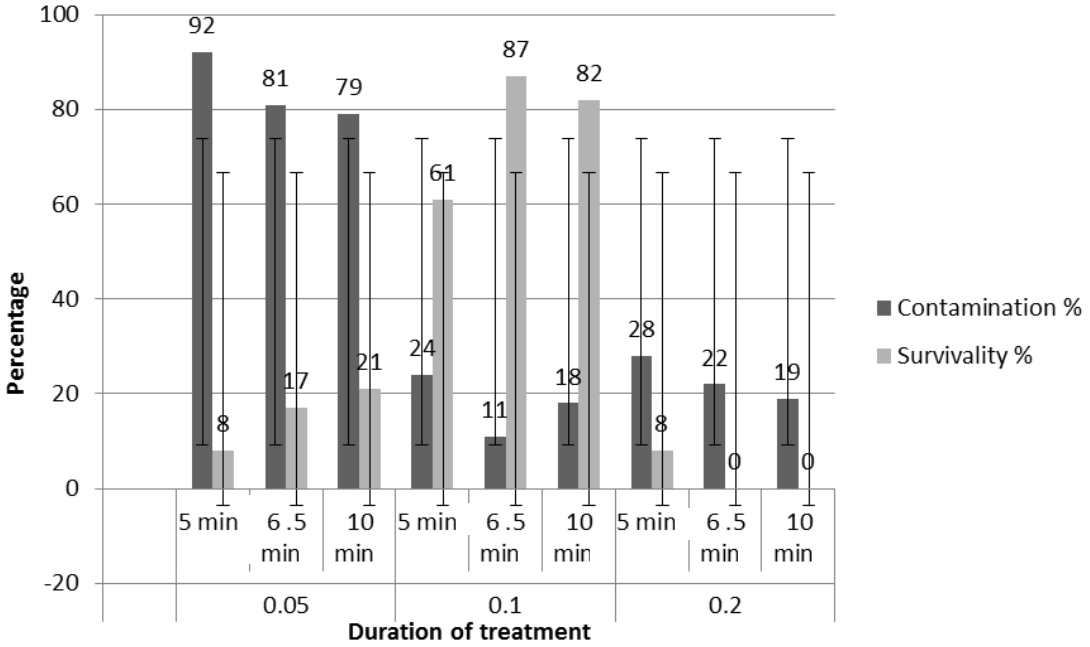
Procedure I

Explants (nodal segments) were first dipped in 0.1% (v/v) Tween 20 solution (5 to 10 min) and they were shaken continuously to remove dust and dirt particles from the surface. Then after, they were washed 5 to 6 times with distilled water. The explants were subsequently treated with fungicide (Bavistin) (1%) for 10 min followed by 70% ethanol (35 s) and surface sterilization was made with different concentrations of $HgCl_2$ (0.05, 0.1, and 0.2%) at different intervals (5 to 10 min) (Graph 1).

Table 2. Explant treatment procedure and percentage survival.

Treatment No.	Contamination (%)	Survivality (%) of explant
Treatment 1	11	83
Treatment 2	10	88
Treatment 3	13	81
Treatment 4	68	45
Treatment 5	90	10
Treatment 6	87	15

Treatment 1: Tween 20 (5-10') ->Bavistin 0.1% (10') -> Ethanol 70% (30 sec.) -> 0.1 % mercuric chloride (5'); Treatment 2: Bavistin 0.1% + Indofil 0.1% (1 hour) -> Ethanol 70% (35 sec.) ->+ Tween 20 + mercuric chloride 0.1% (6'); Treatment 3: Tween 20 (5-10') ->Bavistin 0.1% + Indofil 0.1% (1 hour) + Ethanol 70% (30 sec.) -> mercuric chloride 0.1% (6'); Treatment 4: Tween 20 (5-10') ->Bavistin 0.1% (10') -> Ethanol 70% (35 sec.) ->NaOCl, 0.1% (6'); Treatment 5:5 – 10 drops Savlon (5-10') -> Ethanol 70% (35 sec.) -> 0.1 % mercuric chloride (6'); Treatment 6:5-10 drops Teapol (5-10') -> Ethanol 70% (30 sec.) + 0.1% mercuric chloride (6').



Graph 1. Effect of duration of mercuric chloride treatment on contamination and survival rate of explants (0.05, 0.1 and 0.2 are expressed in %).

Procedure II

In this case explants were first dipped in (0.1%, v/v) Bavistin and (0.1%, v/v) Indofil solution for 1 h followed by treatments with (70%) ethanol for 35 s and (0.1%) mercuric chloride along with (0.1%, v/v) Tween 20 solutions for 6 min.

Procedure III

Explants were first washed with (0.1%, v/v) Tween 20 for 5 to 10 min and subsequent treatments were same as mentioned earlier followed in Procedure II.

Procedure IV

In this case explants were treated with sodium hypochlorite (0.1%) instead of mercuric chloride for 6 min. However, other processes were the same as in Procedure I.

Procedure V and VI

Savlon and Teepol (10 drops each for 5 to 10 min) for washing of explants were used.

Different surface sterilization techniques as mentioned earlier were compared in order to evaluate the successful establishment of cultures under aseptic conditions. The percentage contamination following different sterilization procedures was also examined. Percentage survival rate of explants was also monitored.

Shoot initiation

For shoot initiation of *B. balcooa*, MS liquid medium supplemented with different concentrations of hormones, additives (ascorbic acid 50 mg/l + citric acid 25 mg/l + cysteine 25 mg/l) with 3% sucrose (Table 3) were used and the pH of medium was adjusted to 6.2 prior to autoclaving. In order to obtain high frequency of multiple shoot induction, explants of different sizes (2 to 5 cm) and diameter (2 to 4 mm) were cultured (Graph 2) in different combinations of growth regulators utilizing solid and liquid media. Percentage shoot induction and number of shoots per explants were calculated after three weeks duration.

Effects of plant growth regulators (PGRs)

To determine the most suitable hormonal combinations for explant establishment and bud proliferation, sterilized explants were cultured on MS media supplemented with different cytokinins (6-benzylaminopurine (BAP), Kinetin (Kn) and thidiazurone (TDZ)) of different concentrations (1, 2, and 5 mg/l) along with naphthalene acetic acid (NAA; 0.25 mg/l) (Graph 3) (Wei et. al., 2015). Each set was of 30 sample size and the experiment was performed in triplicate.

All the cultures were grown under a photoperiod of 16 h a day (illuminated by 40 watt cool white fluorescent tubes of 1200 lux). The explants were regularly transferred to fresh media within 12 days in order to avoid any browning of explants.

Shoot multiplication

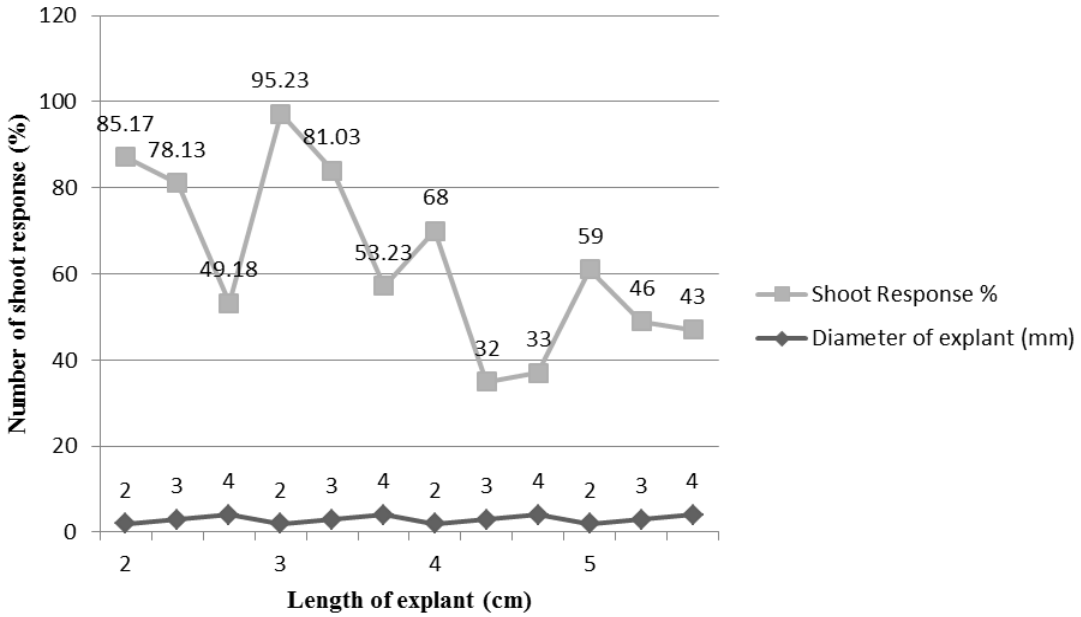
For multiplication of shoots and shoot clumps were excised from the explants and were subsequently sub-cultured on fresh medium along with additives and different combinations of phytohormones. 5 to 8 clumps of shoots were taken in each cycle. Each time sub-culturing was made after 10 days.

Effect of PGRs

In order to obtain high multiplication rate and better shoot length,

Table 3. Number of shoots, shoot length and percentage response of multiplication in liquid and agar gelled media at different phytohormonal concentrations.

Combination of PGRs	Number of shoots		Shoot length (cm)		Response % for bud sprouting	
	Solid	Liquid	Solid	Liquid	Solid	Liquid
MS + BAP (1 mg/l)	5	12	3.4 ± 0.02	5.6 ± 0.06	50	55
MS + BAP (2.5 mg/l)	8	18	2.5 ± 0.9	4.5 ± 0.05	41	48
MS + BAP (5 mg/l)	6	10	1.5 ±0.02	2 ± 0.01	40	50
MS + BAP (2 mg/l) + Kn (0.5 mg/l)	6	8	1.4 ±0.01	2 ± 0.03	35	30
MS + NAA (0.1 mg/l) + BAP (1 mg/l)	11	20	4.1 ± 0.3	6.2 ± 0.07	65	67
MS + NAA (0.1 mg/l) + BAP (2.5 mg/l)	16	25	3.5 ± 0.04	5.2 ± 0.03	70	79
MS + NAA (0.25 mg/l) + BAP (1 mg/l)	8	13	3 ± 0.3	5 ± 0.1	60	64
MS + NAA (0.25 mg/l) + BAP (2.5 mg/l)	10	18	1.9 ± 0.9	2.5 ± 0.3	48	41
MS + Add + BAP (1 mg/l)	13	22	2.1 ± 0.07	5.1 ± 0.4	80	81
MS + Add + BAP (2.5 mg/l)	14	26	3.4 ± 0.9	4.7 ± 0.3	82	84
MS + Add + NAA (0.1 mg/l) + BAP (1 mg/l)	21	30	4.2 ± 0.02	6.8 ±0.8	92	95
MS + Add + NAA (0.1 mg/l) + BAP (2.5 mg/l)	29	35	3.9 ± 0.08	6.4 ± 0.3	90	96
MS + Add + NAA (0.25 mg/l) + BAP (1 mg/l)	16	19	3.5 ± 0.3	5.6 ± 0.5	72	78
MS + Add + NAA (0.25 mg/l) + BAP (0.25 mg/l)	12	15	2.6 ± 0.7	3.9 ± 0.3	69	72
MS + Add + Glutamine + NAA (0.25 mg/l) + BAP (2.5 mg/l)	25	14	2.1 ± 0.2	2.1 ± 0.2	82	64

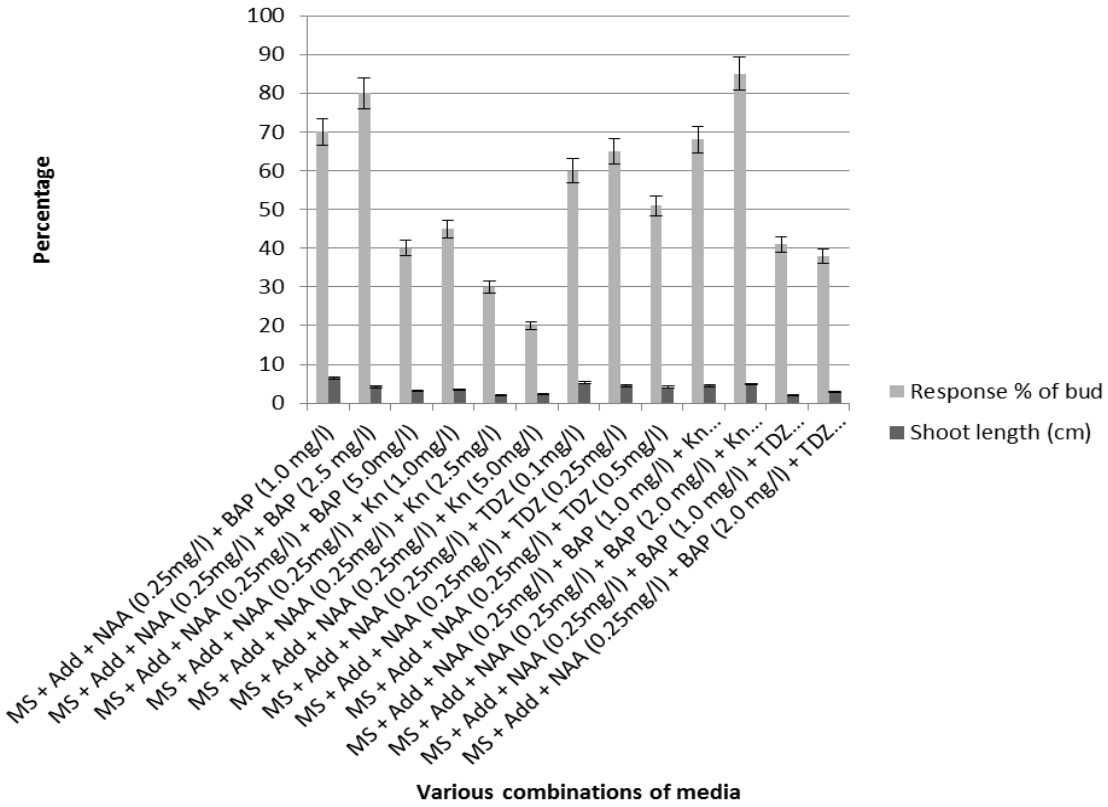


Graph 2. Effect of diameter and length of explants on percentage response and contamination.

various cytokinins BAP (1, 2, and 5 mg/l), Kn (1, 2.5, and 5 mg/l) and TDZ (0.1, 0.25, and 0.5 mg/l) in combination with NAA (0.1 mg/l) were tested in MS liquid medium supplemented with additives (ascorbic acid, citric acid and cysteine) (Graph 3). Altogether 13 treatments were made and each set consisted of 30 samples and the experiment was made in triplicate. Sub-culturing of *in vitro* shoots was carried out at an interval of 10 days or at least before the medium started turning brown (browning problems appeared due to phenolic exudates). The rate of multiplication was calculated by counting the number of shoots produced after each cycle divided by the number of shoots involved in regeneration.

Effect of liquid vs. agar gelled medium

To compare the liquid vs. agar gelled medium for shoots multiplication and subsequent growth, MS liquid and agar gelled media (0.6% w/v) with hormones (NAA 0.1 mg/l and BAP 2.5 mg/l)



Graph 3. Effect of different concentrations and combinations of growth regulators on shoot induction.

supplemented with or without additives were tested (Table 3). Influence of NAA in combination with BAP on both the media (solid or liquid) was also examined.

Effect of glutamine

Effect of glutamine in solid and liquid media was also tested. Glutamine (25 mg/l) was added to MS media to determine effect on shoot multiplication.

***In vitro* rooting**

Five to eight shoot clumps of *B. balcooa* were inoculated in nutrient MS media (half strength) for *in vitro* rooting. Multiplied shoot cultures after fifth cycle onwards were tested for the *in vitro* rooting.

Effect of various auxins on in vitro rooting

Clumps bearing 5 to 8 shoots were transferred to MS solid media containing different concentrations (1 and 2.5 mg/l) of auxins, namely, indole 3-acetic acid (IAA), indole 3-butyric acid (IBA) and NAA (Graph 4). Each hormone concentration was tested in triplicate with 100 experimental plants. Rooting response and rooting percentage on supplied medium were observed.

Effect of nutrient media

Full and half strength of MS nutrient media for *in vitro* rooting were also tested. Observations were made after 7 and 21 days for calculation of rooting percentage.

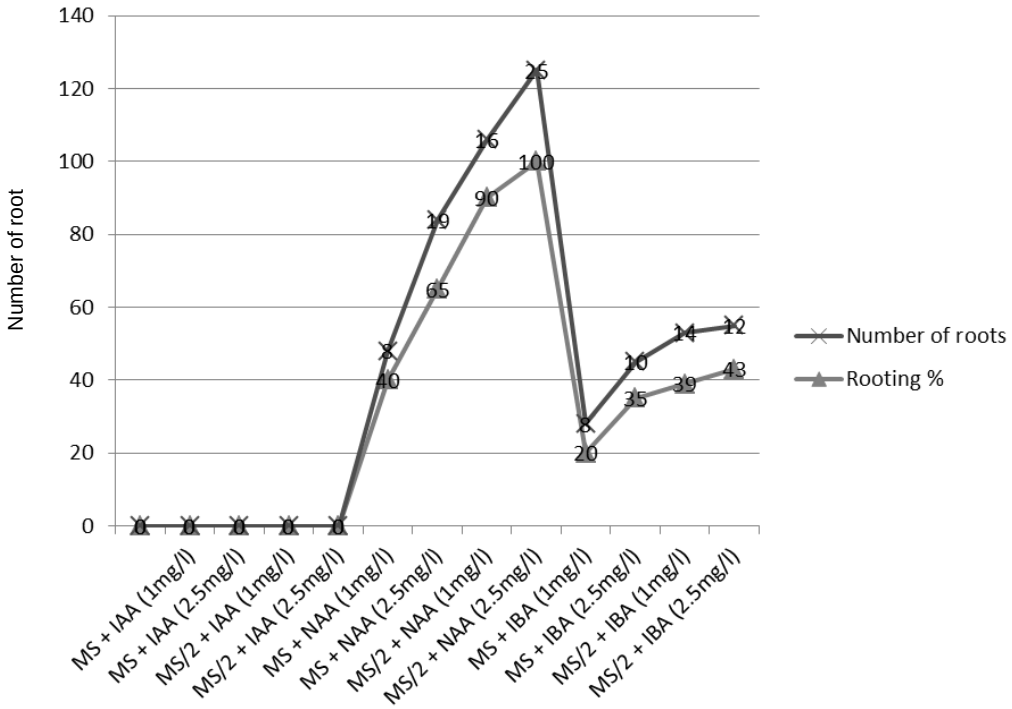
Hardening

In vitro rooted shoots were carefully taken out from culture bottles and were washed with water. They were then transferred to seedlings trays containing different types of transplanting media, namely, sand: soilrite (1:1), sand: soilrite: cocopit (1:1:1), cocopit, cocopit: vermicompost (2:1) as well as vermicompost. Initially, plantlets were kept under poly-tunnel for 3 weeks in green house at high temperature (30 ± 5°C) and relative humidity (80 ± 5%). Later on, plants were transferred to mother bed (sand: soil: FYM, 1:1:1) in agro shade net house for 3 to 4 weeks and then they were transferred to polybags containing soil and FYM (1:1). Plantlets were irrigated on alternate day and sprayed with MS/4 basal salts at an interval of 15 days.

RESULTS AND DISCUSSION

Aseptic culture establishment

Seasons had pronounced effect on *in vitro* shoot proliferation of *B. balcooa* (Agnihotri and Ansari 2000 in *B. vulgaris)*.. The establishment of pure cultures was influenced with the season of explant collections. In our study, spring and summer season (February to June) were comparatively better period for collection of explants, when percentage of successful initiation of



Graph 4. Effect of various auxins in different strength of MS media on *in vitro* rooting.

cultures was high (48 to 92%) (Table 1). Initiation response of explants in rainy seasons had also marked effect (65 to 89%), however, the levels of contamination were comparatively high (81 to 92%). Shoot length in average was high (5.2 to 5.9 cm) in rainy season followed by spring/summer (2.3 to 7.2 cm). Due to high levels of contamination in rainy season, there were difficulties in establishment of pure cultures. It has been reported that during the summer bamboo outgrows pathogen which in turn results in viable bud response and culture establishment. Successful establishment of aseptic cultures and bud response depend on the level of contamination, physiological state of explants and also the season of collection (Saxena and Bhojwani, 1993; Ramanayake and Yakandwala, 1997; Sanjaya et al., 2005; Mishra et al., 2008; Singh et. al., 2012, Nadha et. al., 2013).

During the growing season of bamboo there is higher secretion of endogenous phytohormones which result in corresponding higher growth of the developing seedling (Cinivora and Sladsky, 1990). Earlier, Negi and Saxena (2011) have observed high aseptic cultures along with 90% bud response during July to October. However, in Bihar, July to September is the rainy season. Cultures established during those periods remain prone to microbial contamination, thereby, diminished the chances to procure aseptic cultures (Msogoya et. al., 2012; Torres et. al., 2016)). Higher level of contaminated axillary bud was also observed by Mishra et al. (2008) during the rainy season. Influence of seasons on bud breakage in *Dendrocalamus giganteus* and *Berberis vulgaris* has also been reported (Ramanayake et al., 1995). They observed seasonal effect on bud initiation and found that February to March was the congenial period for obtaining axillary buds for cultures development (Mudoi et. al., 2009).

Surface sterilization

In the present studies, six different procedures for treatment of explants prior to initiation (Table 2) were used. In procedure I, explants were treated with Bavistin (0.1%) for 10 min; however, in procedures II and III, explants were treated with Bavistin (0.1%) for 1 h. In procedures II and III instead of Bavistin, we used Indofil fungicide (0.1%). The percentage contamination was quite less (10%), however, the survival rates of explants were maximum (88%) when the explants were treated with Bavistin (0.1%) and Indofil (0.1%) together for 1 h (as in procedure II) (Table 2). While utilizing procedure IV, we used sodium hypochlorite instead of mercuric chloride, but in that case the percentage survival was quite less (45%) (Shroti et. al., 2012). Following procedures IV and V, we treated explants with Savlon and Teapol but in those cases microbial contaminations were quite high (87 to 90%).

It was observed, out of six ways of surface sterilization, explants first treated with (0.1%) Bavistin and (0.1%)

Indofil for 1 h followed by surface sterilization with ethanol 70% (35 s) and mercuric chloride 0.1% (6 min) along with Tween 20, were the most suitable way for aseptic sterilization and establishment of cultures (as in procedure II) resulting in 88% of uncontaminated explants.

Different procedures for surface sterilization of explants have been reported by earlier workers. Negi and Saxena (2011) utilized savlon and teepol for sterilization of explants and establishment of aseptic culture. In another experiment while culturing the nodal segments of *B. balcooa,* washing with Cetavelon (5 min) followed by 3 to 4 washing with water and subsequent treatment with 1% Bavistin (fungicide) for 5 to 7 min to avoid the problem of fungal contamination, has been reported by Arya et al. (2008). Another worker treated explants of *B. balcooa* with fungicide (Bavistin1%) for 2 to 3 min and then the surface sterilization was made with 0.1% mercuric chloride (12 to 15 min) (Arya et al., 2006).

Different concentrations (0.05, 0.1 and 0.2%) have been observed of $HgCl_2$ for different time durations of sterilization of explants (Graph 1). With lower concentrations (0.05%) of $HgCl_2$, there were comparatively very high levels of microbial load. At higher levels (0.2%) of mercuric chloride treatments, the explants became black, dried and also their survival rate was negligible. High percentage of $HgCl_2$ treatment for more duration (10 min) had adverse impact on bud proliferation. However, 0.1% $HgCl_2$ concentration proved to be comparatively better responsive for establishing aseptic cultures as in that case chances of survival of cultures were more (87%). Previous workers have also recommended the similar concentrations of $HgCl_2$ as an effective sterilizing agent for bamboo species (Saxena and Bhojwani, 1991; Arya et al., 2001).

Another important aspect for establishment of aseptic culture was the diameter and length of explants. Graph 2 showed shoot initiation response, while utilizing different lengths and diameters of explants. Explants size of 3 cm with diameter of 2 mm revealed better response (95%) in shoot initiation. Higher length and diameter (5 cm and 3 to 4 mm) were least responsive (32 to 46%). This might be attributed to high rate of endogenous phenolics secreted in the medium resulting thereby in inhibition in shoot proliferation and shoot length. High rate of response in culture initiation with medium sized explants might be due to minimum leaching of phenolics in the medium (Mudoi et. al., 2013). Explants of 2 to 3 mm diameter and 2.5 to 3.5 cm in length in case of *Pseudoxytenthera stocksii* (Sanjaya et al., 2005; Somashekhar et al., 2008) and 2 to 3 cm in length in case of *B. balcooa* (Arya et al., 2006; Arya et al., 2008) have been reported by previous workers as desirable size for initiation of cultures.

Duration of ethyl alcohol (70%) treatment also affected the degree of aseptic culture establishment. When treated with 70% ethyl alcohol for 30 s, explants showed comparatively high level of contamination. Treatment of explants with ethyl alcohol (70%) for 35 s was found most responsive.

Figure 2. Initiation of *B. balcooa*.

Shoot initiation

Among the various concentrations and combinations of phytohormones (growth regulators) tried in our experiment (Graph 3), the combined effect of BAP and Kn (MS + Add + NAA 0.25 mg/l + BAP 2 mg/l + Kn 0.5 mg/l) resulted in 85% bud breakage (Venkatachalam et. al, 2015). BAP alone showed 80% bud response (Figure 2).

A similar combined effect of two cytokinins (BAP and Kn) has been demonstrated previously in *B. balcooa* (Negi and Saxena, 2011; Das and Pal, 2005a, b), *Bambusa tulda* (Saxena, 1990), *D. giganteus* (Arya et al., 2006), *Dendrocalamus strictus* (Chowdhury et al., 2004) and *Bambusa nutans* (Choudhary et al., 2016). Cytokinin BAP was found to be most effective for shoot initiation through axillary bud proliferation. In shoot initiation experiment, we used auxin (NAA 0.25 mg/l) along with different combinations of cytokinins (BAP, Kn, and TDZ). However, Arya et al. (2008), while working on *Dendrocalamus asper* observed that there was no significant increase in shoot proliferation if auxin either NAA or IAA were added along with BAP. Increased levels of BAP, Kin and TDZ showed poor response in bud

initiation. High level of cytokinins perhaps induce programmed cell death in cell cultures resulting in yellowing of leaves and reduced root mass in intact plants (Carimi et al., 2003).

Due to exudation of phenolic compounds, it was required to transfer proliferated bud into new fresh media (after 8 to 12 days) in order to avoid the browning and leaching problems (Das and Pal, 2005a, b; Arya et al., 2008; Saxena and Bhojwani, 1993; Negi and Saxena, 2011).

Uses of Kn (1, 2 and 2.5 mg/l) showed higher number of shoots, however, growth of seedling was stunted. Contrary to other cytokinins in TDZ, there was less number of shoots, but their size was longer. Combined effect of BAP and TDZ had non-significant response.

Solid and liquid media

MS agar gelled media resulted in dwarf and lower number of shoots (2 to 3 shoots per explant). This may be due to leaching and browning problems (Somshekhar et al., 2008). Arya and Sharma (1998) also observed leaching of exudates (phenolic) and poor growth of shoot in agar gelled medium.

However, Nadgir et al. (1984) while working on *D. strictus* found agar gelled medium comparatively better media for multiple shoot induction from nodal segments of mature plants and they observed shoots (2 to 3) within 3 to 4 weeks. High rate of shoot initiation in liquid medium compared to agar gelled medium may be attributed to easy availability and faster uptake of nutrients in liquid medium (Figure 3). Liquid cultures generally grow faster, required less hardening time (Bonga and Vongvaleskar, 1992).

Liquid media showed 90% response within 3 to 4 days, however, in semisolid media cultures responded 70% within 6 to 7 days.

Shoot multiplication

The initiated microshoot clumps were separated from the explant and transferred to the semisolid and liquid media supplemented with different concentrations of phytohormonal compositions. As obvious (Table 3), variable effects in shoot multiplication were observed, while using different concentrations and combinations of growth regulators. BAP was found most important cytokinins for shoot multiplication in case of *B. balcooa.* High rate multiplication of shoot of *B. balcooa* in MS media (with BAP) has also been reported by previous workers (Das and Pal, 2005a, b; Mudoi and Borthakur, 2009; Arya et al., 2006). Kn alone did not show significant response in shoot multiplication (Kumar and Banerjee, 2014). When Kn (alone) was used in multiplication media, the clumps turned dry and brown. High efficacy BAP over Kn in relation to shoot

Figure 3. Shoot proliferation in *B. balcooa*.

multiplication has also been reported by previous workers (Bonga and Von Aderkas,1992; Saini et. al., 2016). In case of shoot multiplication, combined effect of BAP in combination with Kn and BAP in combination with TDZ did not show significant effect. However, the strong cytokinin, TDZ was found suitable for shoot multiplication.

The media supplemented with growth regulator as 1 and 2 mg/l of BAP showed remarkable effect. In case of NAA (0.1 mg/l) + BAP (1 mg/l), the shoot length was longer (6.2 cm) as compared to BAP (2 mg/l) + Kin (0.5 mg/l), where the average length was 4.9 cm. MS media was also observed with and without additives to minimize the cost of production. The media lacking additive turned brown after five days due to phenolic secretion and later showed the symptoms of necrosis of shoot clump. On the other hand, MS supplemented with additive (MS + Add + NAA 0.1 mg/l + BAP 2.5 mg/l or 1 mg/l) showed better response (Table 3). MS media supplemented with additives (Ascorbic acid, Cysteine, Citric acid) showed positive response in shoot multiplication. This might have been due to minimized phenolic exudation (Negi and Saxena, 2011; Devi and Sharma, 2009). Glutamine supplemented either in liquid or solid media had no effect on proliferation of microshoots. One remarkable feature that was observed was that microshoots in medium without supplement of NAA showed necrotized shoots and they had less multiplication rate in liquid media.

Figure 4. Multiplication stage of *B. balcooa*.

A comparative study was done for shoot multiplication rate in liquid and semi-solid media. High multiplication rate with comparatively longer shoots were observed in liquid media (Figure 4). However, in semi-solid media, low multiplication rate with dwarf shoots were developed. After 2 to 3 cycles of multiplication, there was drastic reduction in multiplication rate as well as in shoot length. Our findings are in conformity with the previous works (Negi and Saxena, 2011; Arya et al., 2008).

BAP (2 mg/l) in combination with Kn (0.5 mg/l) had high response in the axillary bud breakage, however, in the same combination, cytokinins were least effective for shoot multiplication. After 2 to 3 cycles in the same media, the cultures started drying of leaves and turned pale brown.

Rooting

In vitro root initiation depends upon various auxin concentrations in the media. Rooting was maximum in half strength of MS media supplemented with NAA (2.5 to 5 mg/l). The root induction experiment was carried out in two strengths of MS media (MS, MS/2) along with variable concentrations of auxins (Graph 4). In full strength MS media, the percentage of root induction was low compared to MS/2 strength.

While considering different type of auxins (IBA, NAA and IAA), pronounced root induction frequency was observed in media containing different concentrations of NAA (1 and 2.5 mg/l). Auxins (NAA and IBA) were responsive in root induction; however, in IAA there was no root induction either in full strength or half strength of MS. NAA (2.5 mg/l) along with half strength of MS showed 100% rooting (Figure 5), but it was less than 50% when MS media was supplemented with IBA. Remarkably, clump become dry after seven days when MS strength was lower (half), however, after 21 days new green healthy shoots proliferated from old clumps with profuse growth of roots. In full strength of MS media,

Figure 5. *In vitro* rooting in MS containing NAA (2.5 mg/l).

Figure 6. Profuse root growth.

clumps were healthy in appearance even after 7 days, but the rooting response was poor. This might be due to sufficient uptake of nutrients by cultures in full strength MS media (Negi and Saxena, 2011).

It was observed that 28 days was enough for procuring well-developed rooted clump (Figure 6), however, earlier workers have reported 40 days duration for sufficient rooting in cultures.

Varied rooting experiments have also been done by earlier workers using different auxins. Saxena (1990) working on *B. tulda* suggested to supplement Coumarin in rooting media. Similarly, Ramanayake and Yakandwala (1997) working on *D. giganteus* and Sood et al. (2009) on *Drosera hamiltonii* reported high frequency of rooting when IBA was used in combination with Coumarin. In case of *D. strictus*, up to 90% rooting was reported in medium containing IBA (Mishra et al., 2008; Arya et al., 2001). However, well-developed roots with

Figure 7. Plantlets in green house.

healthy shoots were observed in half strength MS medium containing NAA (3.0 mg/l) (Goyal and Sen, 2016). IBA supplemented medium showed only 40% rooting frequency with poorly developed roots. Thus, it was studied that IAA was ineffective in root initiation.

Hardening

After attaining the height of 5 cm, rooted plantlets were needed to be hardened and acclimatized. Among the various rooting mixtures with different ratios of sand, soil, cocopit, vermicompost, and soilrite tested (Ray and Ali, 2016), we observed suitably well-developed healthy plants in cocopit and vermicompost (2:1) and also there was low rate of mortality. Healthy rooted plantlets were transferred to seedling trays containing different types of transplanting media like sand (1:0), soilrite (1:0), sand: soil (1:1), cocopeat: vermicompost (2:1) and cocopit (1:0). Root trainers containing potting media were sprayed with MS/4 nutrient media and were maintained in the mist chamber for 2 to 3 weeks. During hardening, use of medium with reduced mineral salts forced the regenerated plantlets to rely at their own photosynthetic apparatus for nutrition (Kozai et al., 1988). Combination of cocopit and vermicompost was found congenial for survival and growth of plantlets (Figure 7). Singh et al. (2012) have reported high survivality rate in combination of Dune sand + vermicompost for hardening of *D. hamiltonii*. Soilrite, perlite, vermiculite and compost as potting mixture in seedling trays were used by earlier

Figure 8. Seedlings in polybag in net house.

workers (Mishra et al., 2011) and soilrite was found most suitable for hardening of *B. tulda* seedlings.

Initial application of MS/2 minerals to the plantlets was found essential for better hardening and acclimatization. After 3 weeks, plants from the trays were transferred to mother bed made of the mixture of sand: soil: cow dung (Figure 8).

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

Authors are thankful to Dept. of Environment and Forest, Govt. of Bihar for financial support (Sanction no. : Yo.Wa.13/14-2227/2014).

Abbreviations

PGR, Plant growth regulators; **MS,** Murashige and Skoog; **BAP,** 6-benzylaminopurine; **Kn,** kinetin; **TDZ,** thidiazurone; **IAA,** indole 3-acetic acid; **IBA,** indole 3-butyric acid; **NAA,** naphthalene acetic acid.

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Vol. 16(10), pp. 463-469, 8 March, 2017
DOI: 10.5897/AJB2016.15659
Article Number: 37DCD4663094
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Banana influences on differential expression of hypersensitive response and pathogenicity gene f (*hrpf*) in *Xanthomonas campestris* pv. *musacearum*

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Received 9 September, 2016; Accepted 2 February, 2017

Banana *Xanthomonas* wilt is a devastating disease of cultivated banana in East and Central Africa, manifesting as initial leaf wilting, premature fruit ripening and eventual death of all infected plants leading to total yield loss. In order to contribute towards development of effective disease control options, *hrpf* expression by *Xanthomonas campestris* pv *musacearum* (*Xcm)* during establishment of *Xanthomonas* wilt infection was determined. To successfully initiate infection, plant pathogenic bacteria deliver effector proteins into host cells using specialized protein transport system such as the Type III secretion system (TTSS). It is a syringe needle-like translocation apparatus essential for delivery of effector proteins into the host cells and hypersensitive response and pathogenicity gene f (*hrpf*) encodes one of the structural proteins for effector protein delivery. In this study, *hrpf* expression by *Xcm* during establishment of *Xanthomonas* wilt infection was determined in minimal medium amended with extracts from susceptible banana genotypes and banana host plants. Total RNA was isolated from *Xcm* recovered from inoculated plants and also from minimal medium amended with banana extracts; cDNA synthesised and *hrpf* amplified by PCR using gene specific primers. Findings showed that *Xcm* multiplied in susceptible host banana and minimal medium amended with their extracts but not in resistant *M. balbisiana* and its extract. *hrpf* gene was thus amplified from cDNA samples of susceptible banana genotypes and their extracts suggesting its expression and involvement in the successful establishment of *Xanthomonas* wilt disease by *Xcm*.

Key words: *Xanthomonas campestris* pv. *musacearum* (*Xcm)*, Type three secretion system (TTSS), hypersensitive response and pathogenicityf (*hrpf*) gene, banana.

INTRODUCTION

Banana *Xanthomonas* wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* (*Xcm)* is a rapidly spreading disease that leads to total destruction of cultivated banana in East and Central Africa (Tushemereirwe et al., 2003). Typical BXW symptoms are leaf wilting, premature fruit ripening and eventual

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death of infected plants (Karamura et al., 2008). All indigenous banana varieties that are widely grown by the farmers are susceptible to *Xcm (*Ssekiwoko et al*.,* 2006). If left uncontrolled, BXW epidemic is projected to cause economic loss of $2 to $8 billion within a period of one decade. The loss of such magnitude ultimately endangers food security and livelihood of more than 80% of the population that is dependent on banana (Tushemereirwe et al., 2003). With continued devastation of banana by *Xcm*, there has been increasing need to understand molecular mechanisms of the infection process and bacteria effector proteins involved with the aim of developing appropriate control measures.

Most plant pathogenic bacteria are equipped with injectosomes for delivery of effector proteins to the interior of host cells for example *Pseudomonas syringae*, *Erwinia amylovora*, *Pantoae* agglomerans members of injectosome family *Hrp*1 and *Burkholderia Pseudomallei*, *Ralstonia solanacearum* and *X. campestris* which belong to injectosome family *Hrp*2 (Galan and Collmer, 1999). The TTSS that is encoded by the hypersensitive response and pathogenicity (*hrp*) genes is used to secrete and translocate effector proteins from bacteria into host cells. Basically, the TTSS is a syringe needle-like translocation apparatus, made up of inner and outer membrane rings and a protruding filament called pilus which functions as a conduit to guide the translocation of effector proteins to the interior of the host cells (Jin et al*.,* 2001; Webber et al*.,* 2005). After delivery into the host, effector proteins manipulate host cell functions and break host defences, allowing bacteria to multiply to cause disease development (Alfano and Collmer, 2004; Yang and White, 2005; Grant et al., 2006; Gurlebeck et al., 2006; White et al., 2009; Kay and Bonas, 2009; Buttner and Bonas, 2010). However, resistant and non-host plants recognise specific effector proteins triggering the hypersensitive response (HR). HR is a rapid, local, programmed cell death that is induced upon recognition of the pathogen and concomitant with the inhibition of pathogen growth within the attacked resistant plants. In resistant hosts, these effector proteins show an 'avirulent' activity limiting the pathogen's host range.

A single *Xanthomonas* genome encodes 20-30 TTSS effector proteins and this varies between species and strains. The variance in TTSS effector proteins produced among diverse species and strains of bacteria is believed to be the main determinant for host range of a given pathogen (Zou et al., 2006). 26 functional TTSS effector (*hrp)* candidate genes within the NCBI data base of *Xcm* and *X. vasicola* pv *vasicolorum (Xvv*) were identified although only 15 coded for products related to the *hrp* cluster (Aritua et al., 2008; Data not published). Six *hrp* genes (*hrpA- hrpF)* were identified and isolated from *X. campestris* pv*. vesicatoria* (*Xcv)* and *X. oryzae* pv oryzae (*Xoo*) where they were responsible for pathogenicity and induction of hypersensitive response *HR* (Li et al., 2011). Of all the *hrp* genes, *hrpf* was found to be indispensible for type III secretion *in vitro* and essential for introduction of virulence factors into the plant by *Xcv* (Zou et al., 2006). It was upon this background that *in vitro* study of minimal medium amended with crude extracts from different banana genotypes was designed to establish whether chemical compounds in these extracts could influence *Xcm* growth and therefore expression of *hrpf*. If *Xcm* growth *in vitro* has a bearing on the rate of *hrpf* expression both *in vitro* and *in planta*, it would then mean that different banana genotypes have different genes/compounds that determine either susceptible or resistant reaction. The study therefore, correlates growth of *Xcm in vitro* to *hrpf* expression and compares it with wilt disease development and *hrpf* expression *in planta*. While most bananas are susceptible, *M. balbisiana*, a wild relative is resistant (Ssekiwoko et al., 2006) and yet it is not known if the different banana genotype responses to *Xcm* infection are influenced by expression of *hrpf*. In addition, the general belief that for successful host colonization *Xanthomonads* express *hrpf,* a member of the *hrp* gene cluster which encodes the TTSS had not been confirmed for *Xcm*/banana pathosystem.

MATERIALS AND METHODS

In vitro culture and recovery of *Xcm*

In vitro studies were conducted in various liquid media including minimal medium (containing 20 mM NaCl, 10 mM $(NH_4)_2PO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4 and 10 mM sucrose in 1 L of distilled water). Also studies were extended to minimal medium amended with banana extracts from *M. balbisiana* (BB) resistant to *Xcm,* Psangawak (ABB) popularly called "Kayinja in Uganda" which is very susceptible to both tool and insect transmitted *Xcm* and 'Saba' (ABB); susceptible mainly to insect transmitted *Xcm*. Minimal medium pH was adjusted to 7.4. To prepare the banana extracts, 10 g of leaf tissues harvested from three months old tissue culture plants were crushed in a blender, mixed with 100 ml of minimal medium and filter sterilised using 0.2 micro filters. 30 ml of each amended extract was dispensed in sterile 100 ml conical flasks.

Xcm from a preserved culture at National Agricultural Research Laboratories (NARL) was used as inoculum. A single colony of pure culture grown on Yeast Peptone Glucose Agar (YPGA) medium for 48 h at 28ºC (Figure 4) was multiplied on the same medium by sub culturing. The cells were mixed with sterile water in a MacConkey bottle to form a suspension and optical density (OD) adjusted to a concentration of 0.1 at OD_{600} ($1x10^8$ CFU).

30 ml of amended and 30 ml of non-amended (control) culture media were inoculated with 0.5 ml of *Xcm* suspension, and incubated at 28°C on an orbital shaker at 100 rpm. A completely randomised design was adopted for the experiment where each treatment (media type) was replicated four times and the experiment repeated 3 times. Bacterial growth was determined at 24, 36, 48, and 60 h after inoculation by spectrophotometry. In addition, cells were recovered respectively after each time period, pelleted and immediately frozen in liquid nitrogen and stored at -80ºC awaiting RNA extraction.

Plant inoculation and recovery of *Xcm*

Three months old tissue cultured banana plantlets of Psang Awak,

Saba and *M. balbisiana* were inoculated with 0.5ml of bacterial suspension at the leaf petiole of the youngest leaf using syringe and needle. They were allowed to grow in pots in a screen house. *Xcm* was recovered after 7, 14 and 21 (for Pisang Awak and Saba) but 32 and 40 days after inoculation for *M. balbisiana* corresponding with *Xcm* incubation period in it. To recover *Xcm*, leaves were surface sterilised with 70% ethanol, sliced into small pieces and suspended in 30 ml of sterile water to allow *Xcm* to ooze out for 1 h (Angela and Rosato, 2003; Gottig et al., 2008). Suspended cells were recovered by centrifugation at 6000 rpm and 25°C and immediately frozen at -80ºC awaiting RNA extraction. Meanwhile plants were observed for 40 days for symptom development and disease progression.

RNA extraction, cDNA synthesis and PCR

Bacterial cells recovered from minimal medium with or without extracts and inoculated plants were washed twice with TE buffer (1 M Tris Hcl, pH 7.4 and 0.5 M EDTA, PH 8.0) to remove salts and *Xanthan* gum. RNA was extracted following the phenol-SDS method for bacterial total RNA extraction as described by Mahuku (2004), with slight modifications. 440 µl of phenol emulsion/phenol saturated buffer containing 200 µl of phenol, 240 µl of CTAB extraction buffer (1 M Tris HCl, pH 8.0; 0.5 M EDTA; pH 8.0; NaCl 81.76 g; β-mercaptoethanol 0.1% pH 8.0; PVP-40, 20 g) was used for RNA isolation. Cells in a falcon tube were chilled in liquid nitrogen and later ground to a powder in a mortar with a pestle. The powder was recovered in sterile 1.5 ml Eppendorff tubes and mixed with 440 µl of hot phenol saturated extraction buffer. This was followed by vortexing for 5 min and then incubated for 15 min at 65°C with mixing by inversion every after 5 min and finally left to cool at room temperature for 2 min. The suspension was then cleaned with 200 ml of 24:1 of chloroform iso amyl alcohol with mixing by inversion in order to break the cell walls. Contents were centrifuged for 10 min at 12000 rpm at room temperature and the supernatant pipetted into new sterile Eppendorff tubes and step above repeated in order to extract as much RNA as possible. The aqueous phase was collected and precipitated with $1/10^{th}$ volume of ammonium acetate (10 M) and an equal volume of ice-cold isopropanol at room temperature and allowed to rest for 15 min. The RNA pellet was recovered by centrifugation at 12000 rpm for 10 min. The pellet was washed with 500 µl of 70% ethanol by centrifugation for 3-5 min, air dried for 40 min and re-suspended in 40 µl of diethylpyrocarbonate (DEPC treated/nuclease free water). The resuspended RNA was then kept at -80°C for downstream applications.

RNA synthesised was DNase treated using the ready to use master mix kit (Bioneer, 2012) as follows; 2 µl RNA was pipetted into sterile Eppendorff tubes and mixed with 1 µl of 10× reaction buffer, 1 µl of DNase1 (1 U/µl) and 6 µl of DEPC-treated water making up a total reaction volume of 10 µl. The reaction was incubated at 37°C for 30 min and then 1 µl of 25 mM EDTA (EDTA is an exonuclease inhibitor, DNase1 is a 5' exonuclease inhibitor) or 1 µl of DNase stop solution was added in each tube. The reaction was again incubated at 65°C for 10 min to heat inactivate the DNase1 then placed on ice for 1 min. The effectiveness of DNase treatment was analyzed on 1% agarose gel electrophoresis to confirm that all residual DNA was removed before cDNA was synthesized from total RNA.

The cDNA was synthesized using the ready to use master mix kit (Bioneer, 2012) following the manufacturer's instructions. The reaction was constituted by; 2 µl of 4 µM RNA sample, 4 µl of 10X reaction buffer, 2 µl of 10 µM dNTPs, 2 µl of random oligo nucleotide primers, 0.25 µl of 40 U/µl RNasin, 1 µl of 10 U/µl reverse transcriptase. These were mixed and topped to 20 µl with sterile de-ionised water. The reaction mixture was incubated at 42°C for 60 min and terminated at 70°C for 15 min using the Mygenie 96 thermo cycler (Bioneer) to inactivate the reverse transcriptase**.** Tubes were later chilled on ice for 1 min and the reaction collected by brief centrifugation in a micro centrifuge. The cDNA synthesized was immediately used for PCR.

PCR was conducted using the constitutively expressed 16s rRNA gene primers (forward 5' TGGTAGTCCACGCCCTAAACG 3' and reverse 5'CTGGAAAGTTCCGTGGATGTC 3' (Gottig et al., 2008) and the *Xcmhrpf* gene specific primers Forward GACGAGTGGAAGGAATTTGC, and Reverse ACATGTCCCCACCTTGAATC (designed at NARL Kawanda by Aritua Valentine). For the PCR reactions, the following components were mixed on ice, 2 µl of 0.5 µM RNA sample, 2 µl of oligonucleotide primers (forward and reverse), 10 µl of a Bioneer PCR premix (containing 0.25 mM dNTPS, 1X reaction buffer, 2 mM $MgCl_2$) and topped up with 6 µl RNase-free water to make up to 20 µl total reaction mixture. PCR conditions for the constitutively expressed 16s rRNA were; initial denaturation of 94^oC for 2 min; then 30 cycles run each at cycle denaturation of 94°C for 1 min, primer annealing of 54°C for 1 min and extension of 72°C for 1 min and a final extension of 72°C for 10 min. PCR conditions for *Xcmhrpf* gene were; initial denaturation temperature of 95°C for 5 min, then 35 cycles each run at cycle denaturation of 94°C for 20 s, primer annealing of 60°C for 40 s, an extension of 72°C for 1 min and a final extension of 72°C for 10 min.

RESULTS

Effect of crude banana leaf extracts on medium colonisation by *Xcm*

The *Xcm* colonisation of minimal medium containing sucrose as carbon source was determined by measurement of turbidity or optical density using a spectrophotometer. Colonisation varied significantly with crude leaf extracts from different banana genotypes. *Xcm* colonisation followed a normal growth curve except for the medium amended with *M. balbisiana* leaf extracts that was barely detectable (Figure 1). Colonisation was more in minimal medium with leaf extracts of banana genotypes 'Kayinja' and 'Saba' than that without extracts or that with extracts of *M. balbisiana.* Colonisation of minimal medium amended with 'Kayinja' and 'Saba' leaf extracts or that without extracts increased exponentially with time to reach a peak 24 h after inoculation and thereafter declined steadily. Between 0 and 10 h there was no significant difference in colonisation of minimal medium without extracts and that with leaf extracts of 'Kayinja' and 'Saba'. Between 10 and 24 h, colonisation among all the media evaluated was significantly different. Optimum colonisation of medium without extracts and that with extracts of 'Kayinja' and 'Saba' was reached between 20 and 30 h after inoculation. Colonisation steadily declined at about 24 h after inoculation. Colonisation of those media did not decline further or increase 60 h after inoculation.

Banana bacterial wilt (BBW) symptoms on infected banana plants

BBW symptoms incited on infected banana plants varied

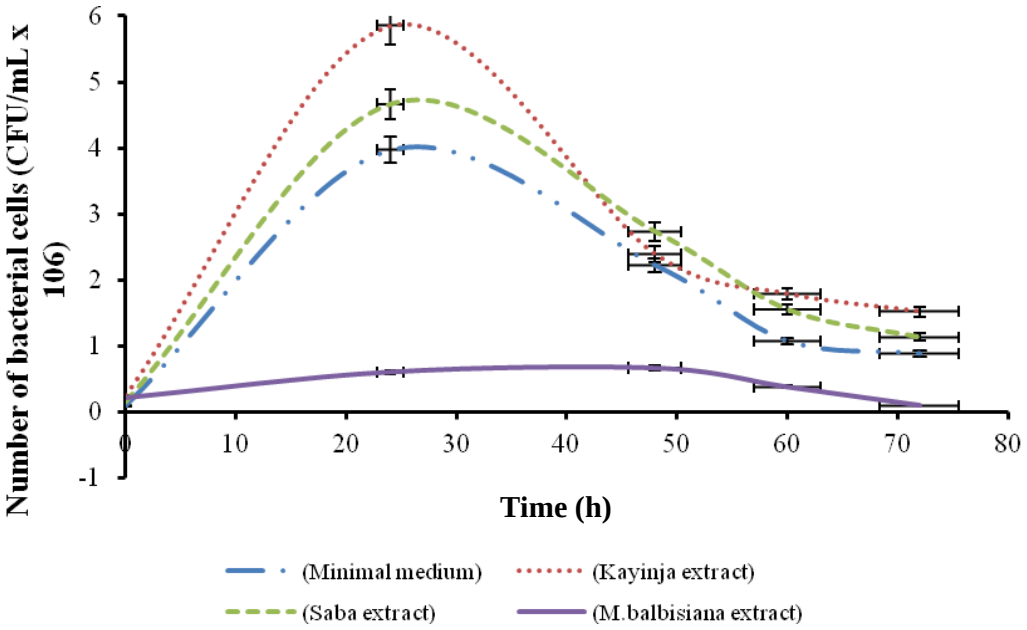

Figure 1. Growth curve of *Xcm* during colonisation of minimal medium amended with or without leaf extracts from susceptible and resistant banana genotypes. Error bars represent 95% confidence interval.

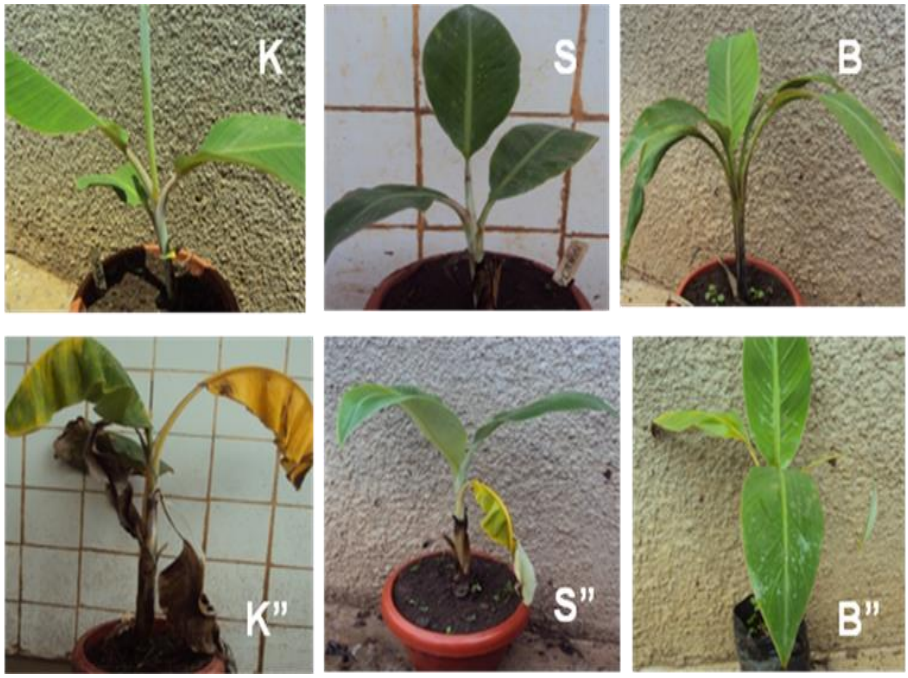

Figure 2. Banana bacterial wilt symptoms on infected plants of genotypes Kayinja (K"), Saba (S") and *M. balbisiana* (B") inoculated with *Xcm* (Bottom). Negative control reference plants of these genotypes; Kayinja (K), Saba (S) and *M. balbisiana* that were inoculated with water (Top) did not develop BBW symptoms throughout the observation period.

with the genotype inoculated (Figure 2). Typical BBW symptoms that progressed up to leaf wilting and eventual plant death after 32 days were incited on banana cv Kayinja. BBW symptom development on genotype Saba

Figure 3. Colony characteristics of 48 hr old YPGA culture of Bacteria re-isolated from inoculated plants was typical of *Xcm*.

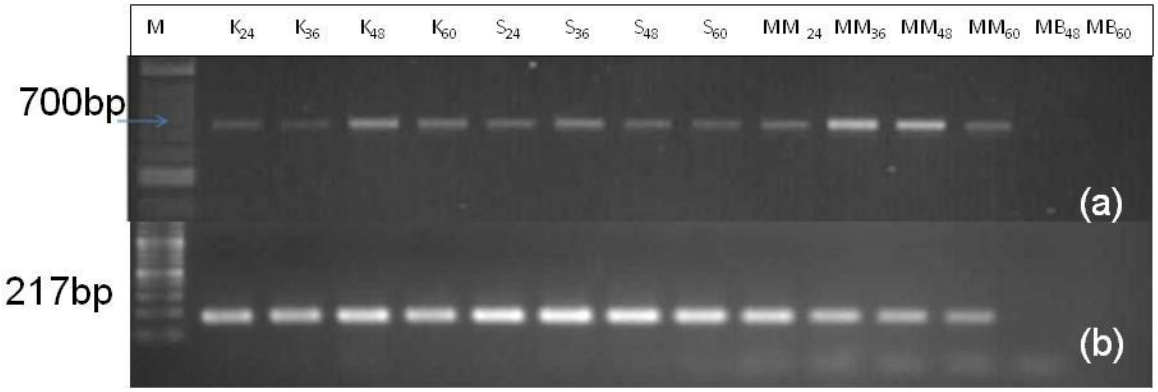

Figure 4. RT-PCR amplification products obtained with primers (a) specific for detection of 700 bp fragment of *hrpf* expressed by *Xcm* after 24, 36, 48 and 60 h of multiplication in minimal medium without (MM_{24}, MM_{36}, MM_{48}, MM_{60}) or with leaf extracts from susceptible banana cv Kayinja (K_{24}, K_{36}, K_{48}, K_{60}), moderately resistant banana genotype Saba (S_{24}, S_{36}, S_{48}, S_{60}) and resistant banana genotype *M. balbisiana* (MB_{48}, MB_{60}). Internal quality control RT-PCR was with primers (b) specific for detection of 217 bp fragment of 16S rRNA housekeeping gene. Lane M represents 100kb Molecular weight DNA marker.

was delayed but eventually infected plants also died. The BBW symptoms on genotype *M. balbisiana* took more than 35 days to develop and were restricted to the inoculated leaf only and symptoms later cleared. Also bacteria that showed yellow, mucoid, shiny, smooth colony growth characteristics on YPGA media were re-isolated from potted symptomatic plants of genotypes 'Kayinja', and 'Saba' (Figure 3). Ooze of bacterial cells was not recovered from the genotype *M. balbisiana.* Downstream h*rpf* expression studies during infection of banana performed utilizing bacterial ooze from inoculated plants were thus only carried out for banana genotpes 'Kayinja' and 'Saba'.

Detection of h*rpf* expression during infection of banana

RT-PCR internal positive control was carried out by detecting 217 bp fragment of *Xcm* 16S rRNA housekeeping gene. The fragment was detected from *Xcm* cDNA synthesized for samples that were obtained at 7, 14, and 21 h after inoculation of banana genotypes 'Kayinja' and 'Saba' (Figure 4). The intensity of 217 bp fragment band on 1% agarose gel was the same for all the samples analyzed. Intensity of 700 bp fragment of *hrpf* detected for *Xcm* samples isolated from both minimal medium and during infection of banana genotypes

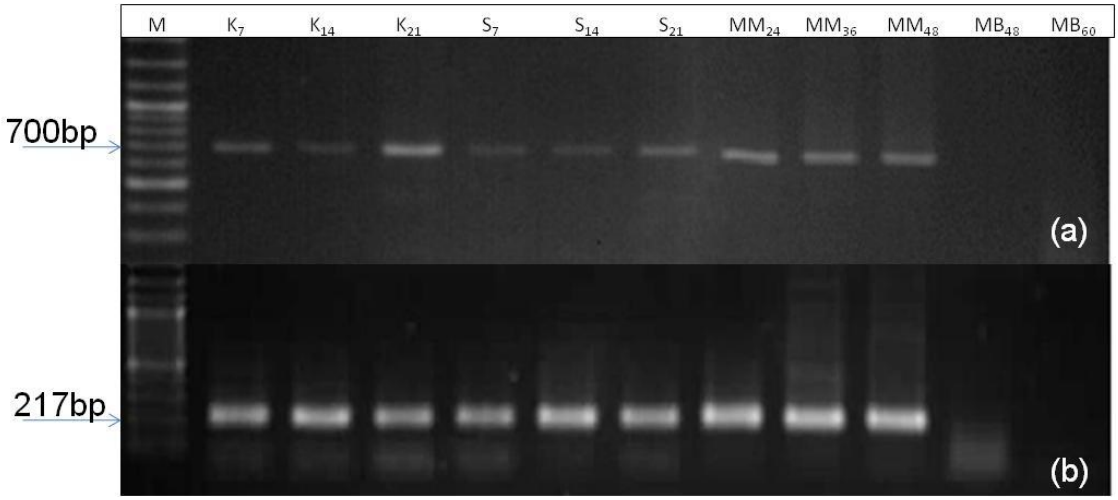

Figure 5. RT-PCR amplification products obtained with primers (a) specific for detection of 700bp fragment of *hrpf* expressed by *Xcm* during infection of susceptible banana cv Kayinja (K_7, K_{14} and K_{21}) and moderately resistant banana genotype Saba (S_7, S_{14} and S_{21}) at 7, 14, and 21 days post inoculation. Expression was not detected in resistant banana genotype *M. balbisiana* (MB_{48} and MB_{60}). Internal quality control RT-PCR was with primers (b) specific for detection of 217 bp fragment of 16s rRNA housekeeping gene. Lane M represents 100 kb Molecular weight DNA marker.

'Kayinja' and 'Saba' varied with time. Band of 700 bp fragment detected was more intense for samples of *Xcm* recovered at 36 and 48 h from minimal medium amended with leaf extracts of Kayinja and Saba (Figure 4) and 21 days after inoculation than that for the samples isolated 7 and 14 days during infection of banana (Figure 5). Meanwhile, intensity of 700 bp fragment band detected for *Xcm* samples from minimal medium without leaf extracts was uniform (Figure 5).

DISCUSSION

This study sought to determine the *Musa* genotype effects on *in vitro* multiplication of *Xcm* and expression of *hrpf* gene during infection. *Hrpf* is indispensible for translocation of virulence factors into plant host cells (Buttner and Bunas, 2010). In this study, *Xcm* colonization of minimal medium followed the normal growth curve pattern (Figure 1). Colonisation was significantly affected by banana genotype leaf extracts used for amendment of minimal medium. Leaf extracts of banana genotypes 'Kayinja' and 'Saba' that are susceptible to BBW (Figure 2) enhanced medium colonization by *Xcm* while leaf extracts *of M. balbisiana* a wild relative of banana that is resistant to BBW (Figure 2) suppressed medium colonization by *Xcm*. Recovery of *Xcm* cells from minimal medium amended with leaf extracts of the resistant genotype *M. balbisiana* was also impossible. On the other hand *Xcm* cells were recovered from medium amended with leaf extracts of susceptible banana genotype'Kayinja' and 'Saba'. Susceptible banana genotype extracts support multiplication and resistant genotypes suppress multiplication of *Xcm.* Since the leaf extracts were in form of a homogeneous solution, it suggested that *M. balbisiana* extracts most probably contained inhibitory chemical compounds and metabolites that could have significantly diminished ability of *Xcm* multiplication *in vitro*. Similar inhibitory compounds and metabolites are not found in susceptible banana genotypes like 'Kayinja' and 'Saba'. *Xcm* multiplication also depended on degree of banana genotype susceptibility because *in vitro* multiplication was more in minimal medium amended with leaf extracts of the highly susceptible genotype, 'Kayinja' than in minimal medium amended with leaf extracts of moderately tolerant banana genotype 'Saba' suggesting that the level of production of those compounds and metabolites may vary with degree of banana genotype susceptibility. Already, Yulu et al. (2013) reported, that there was no detectable expression of genes controlling ethylene production during infection of *M. balbisiana* by *Xcm* but that expression of those genes was relatively high in 'Saba' and 'Kayinja'. This study also seems to support the possibility that resistance to *Xcm* in banana is a heritable attribute since *Xcm* cells were not recovered from artificially inoculated *M. balbisiana* plants which was not the case for susceptible banana genotypes Kayinja and Saba in pot trials. The intensity on 1% agarose gel, of 700bp fragment of *hrpf* (Figures 4 and 5) varied with degree of susceptibility of banana genotype and duration of infection also suggesting there was differential expression of *hrpf* gene in *Xcm* during medium colonization and infection of banana. *Hrpf* and other

members of the *hrp* gene cluster are known to be critical in the initial infection process by bacterial pathogens because they are used to construct bacterial pili (needle/injectisome) through which virulence factors are introduced into plant (host) cells (Cornelis et al*.,* 2009). *Hrpf* gene that encodes the TTSS was previously predicted to be associated with *Xcm*/banana infection (Aritua et al*.,* 2007). The findings of this study suggest that *Xcm* expresses this gene during infection of susceptible banana plants. The *hrpf* that was detected in *Xcm* during infection of banana was consistent with similar studies involving interaction of *X. oryzae* pv. *oryzae* with rice (Zou et al*.,* 2006) and *X. campestris* pv. *vesicatoria* in pepper and tomato (Schulte and Bonas, 1992).

This finding establishes information that may be useful in the development of genetically modified banana expressing RNAi molecules for silencing of expression of *hrpf* gene in *Xcm* and thus decreasing pathogen capacity to cause infection.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to National Agricultural Research Organisation (NARO), Biosciences East and Central Africa (BECA) who sponsored this work up to completion. Also, appreciation goes to Dr. Velentine Aritua who provided primers and initially assisted in developing the concept; Supervisor - Dr. Charles Changa Mwesigye, research mentor and Dr. Geoffrey Tusiime - author's University supervisor. The entire National Banana Research Programme team then headed by Dr. Wilberforce Tushemereirwe but now Dr. Jerome Kubiriba are duly acknowledged and most importantly God for enabling this research.

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academicJournals

Vol. 16(10), pp. 470-482, 8 March, 2017
DOI: 10.5897/AJB2016.15486
Article Number: 735516363098
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Production and characterization of bacterial cellulose before and after enzymatic hydrolysis

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Received 23 May, 2016; Accepted 26 August, 2016

Bacterial cellulose (BC) is produced by bacteria such as *Gluconacetobacter xylinus*. It has unique structural and mechanical properties and is highly pure as compared to plant cellulose. In this study, BC was produced in Hestrin-Schramm (HS) medium. *G. xylinus* produced an average dry yield of 1.4 ± 0.09 g/L BC after 9 days of fermentation period. Scanning Electron Microscopy (SEM) analysis confirmed BC to be highly structured while Fourier Transform infrared spectroscopy (FTIR) analysis indicated that the absorption peaks at 3000 – 3700, 2800 – 2970 and 1023 – 1024 cm^{-1} were derived from –OH bending, -CH stretching and C-C/C-OH/C-H ring vibrations. X-Ray Diffraction (XRD) revealed a high purity of BC indicating type I cellulose with high crystalline nature. The solid state ^{13}C Nuclear magnetic resonance (NMR) spectroscopy of the untreated BC sample also indicated high crystallinity while the cellulase treated BC sample was different, but dominated by a polysaccharide signal between 55 and 110 ppm. The results suggest the prospects of the production and recycling of biopolymers from bacterial sources.

Key words: Bacterial cellulose, Scanning Electron Microscopy (SEM), Fourier Transform infrared spectroscopy (FTIR), X-Ray Diffraction (XRD), Nuclear magnetic resonance (NMR), cellulase enzyme.

INTRODUCTION

Cellulose is the most abundant macromolecule on earth and is one of the major components of the dry mass in materials such as cotton (90%) and wood (50%), (Hon, 1994; Keshk, 2014). The biosynthesis of cellulose is not exclusively found in plants alone but can be found in a variety of microorganisms such as algae and bacteria (Brown, 2004). Bacterial cellulose (BC) also known as microbial cellulose and bacterial nanocellulose is produced by bacteria such as *Gluconacetobacter xylinus* (*G. xylinus*) (originally known as *Acetobacter xylinum*). *G. xylinus* acquires glucose and other organic substrates converting them into pure cellulose with unique structural and mechanical properties which are highly pure because of a lack of contaminating lignin and hemicellulose as compared to natural plant cellulose (Gayathry and Gopalaswamy, 2014; Son et al., 2001). BC is secreted as

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an extracellular insoluble biofilm and forms a gelatinous mat on the air liquid interface due to its comparatively low density to water. Biosynthesis of BC requires a carbon source that is converted into Glucose-6-Phosphate, a metabolic intermediate for the formation of precursor substrate uridine diphosphoglucose (UDPGlc), which act as D-glucose donor to cellulase synthase to facilitate the polymerization of a β (1→4) D-glucan chain (Rose et al., 1991). Many other carbon sources that are alternative to glucose have been reported in the production of BC by *G. xylinus* (Keshk, 2014; Mikkelsen et al., 2009; Sheykhnazari et al., 2011).

Cellulose synthase (CS) complexes are coded by the CS operon which codes for four different subunits, *bcsA*, *bcsB*, *bcsC* and *bcsD*; *bcsA* function in the acceleration of cellulose synthesis by combining with cyclic-di-GMP, while *bcsB* is involve in inner membrane attachment and the catalysing unit. Though the primary function of *bcsC* and *bcsD* are still not known, they are thought to be involved in the aggregation of each cellulose chain (Amano et al., 2005; Krystynowicz et al., 2005; Wong et al., 1990). Through extensive inter- and intra- fibrillar hydrogen bonding of the hydroxyl and esters groups, the sub-elementary fibre aggregates and crystallizes to form micro-fibrils (Hon, 1994; Ross et al., 1991). Depending on the formation and organization of the chain, cellulose is assembled in a parallel fashion known as type I cellulose which has high crystalline structural morphology with increased strength and stability, or into type II cellulose which is composed of antiparallel chains and results in an amorphous structure (Sarkar and Perez, 2012). Though many other microbes produce cellulose, those produced by *G. xylinus* have better quality and are more stable, and are also produced in a larger quantity (Ross et al., 1991). Intriguingly, *G. xylinus* strains which are known to be efficient producers of bacterial cellulose can also produce β-glucosidase. β-glucosidase is a rate limiting factor during enzymatic hydrolysis of cellulose; this is due to the fact that endoglucanase and exoglucanase which play an important role during cellulose hydrolysis are often inhibited by accumulation of cellobiose (Harhangi et al., 2002). Although these enzymes are known for their hydrolytic capabilities, they have been reported to play a role in the enhancement and production of BC (Koo et al., 1998; Standal et al., 1994; Tonouchi et al., 1995). ß-glucosidases have the capability to catalyse and polymerise cellulose chains (Kono et al., 1999) and the encoding gene is thought to be located downstream of the cellulose synthase CS operon in *G. xylinus* (Tajima et al., 2001; Tonouchi et al., 1997).

This study provides for the first time a fully detailed characterisation of pure bacterial cellulose before and after treatment with a commercially available cellulase enzyme. The BC samples will be characterised by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, Differential scanning calorimeter (DSC), Thermogravimetric analysis or thermal gravimetric analysis (TGA) and Nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Microorganism

Gluconacetobacter xylinus 639 (*G. xylinus*) used in this study was obtained from the organisms collection centre of Microbiology laboratory, University of Wolverhampton, Wolverhampton United Kingdom.

Culture medium and growth conditions for BC production

The Schramm and Hestrin medium (HS medium) (Hestrin and Schramm, 1954) with initial pH adjusted to 6.0 using acetic acid was used to produce BC. HS medium was made up of (g/l): glucose – 20, bactopeptone – 5, yeast extract – 5, disodiumpohosphate – 2.7 and citric acid – 1.15. Inoculum was prepared by transferring two colonies of *G. xylinus* from an HS agar plate into a 250 ml conical flask containing 100 ml sterile HS medium. The culture was incubated at 25°C on a water rotary shaker for 24 h at 150 rpm. After 24 h, the cellulose pellicle formed on the surface of the culture broth was vigorously shaken in order to remove active cells embedded in the cellulose membrane. 6 ml of the cell suspension was introduced into 94 ml of fresh SH medium. The culture was cultivated statically at 30°C for 9 days. The synthesized cellulose was harvested and then purified by boiling in 1% NaOH for 2 h and next it was thoroughly washed in tap water until the product was transparent while the second sample was washed with distilled water only.

Cellulase pretreatment of BC

Cellulase C1184 from *Aspergillus niger* (Sigma-Aldrich, UK) which catalyzes the hydrolysis of endo-1,4-β-D-glycosidic linkages in cellulose was used in the hydrolysis of the BC. Five gram (5 g) BC was incubated for 12 h with 2 mg/ml cellulase enzyme at 37°C. To inactivate the enzyme adsorbed to the BC, 1 M NaOH (pH 13.3) was used to treat the hydrolysed BC for 10 minutes. The enzymatically pre-treated BC was then thoroughly washed with distilled water until pH 7.2 was achieved. The treated BC sample was freeze dried and stored at room temperature for further analysis.

Characterization of bacterial cellulose

To study conformational characteristics, BC polymer was characterized using microscopic and spectroscopic methods. The microscopy method used was SEM. The spectroscopy techniques used included FTIR, XRD, DSC and Thermogravimetric analysis or thermal gravimetric analysis (TGA).

Scanning electron microscopy (SEM)

The BC fibril (cellulase treated and untreated BC) was characterized using SEM (Zeiss Evo 50 fitted with an Oxford EDX, Zeiss, UK). Scanning electron microscopy reveals information about the BC chemical composition, crystalline structure and the external morphology of the sample. A thin layer of freeze dried BC was gold coated using an ion sputter-coater to improve the conductivity of the samples and the quality of the SEM images (emscope SC 500).

The gold coated sample was viewed and the images were digitally captured.

Fourier transform infrared spectroscopy (FTIR)

To study conformational characteristics of BC obtained from HS medium, polymer was analysed by FTIR spectrometer (Genesis II with DuraScope, Mattson Instruments UK) in a transmittance mode at wavelength ranging from 4000 to 400 cm^{-1}. The FTIR provided information on chemical structures and physical characteristics of the BC produced. BC samples were initially freeze dried and made into powder. A little quantity of the sample (or cellulase treated BC), just enough to cover the disc hole was placed on the disc. The disc was pressed and the scan of BC was operated on the personal computer (PC) which further generates the IR spectrum. A detector monitors the wavelength range and transmits the signal to a computer which translates the signal into an absorption spectrum. A standard cellulose material C6288 (Sigma Aldrich UK) was used as a reference.

X-Ray diffraction (XRD)

X-Ray diffraction (XRD) is a rapid analytical technique that is used in the qualitative identification of crystalline phases by their diffraction pattern. In this study, XRD analysis was utilised to characterize the crystallinity index and the degree of polymerisation of the BC sample. This was performed using the Empyrean PANalytical diffractrometer (Philips model PW1770) at the University of Wolverhampton. BC was ground and homogenised before analysis. The sample was then placed in the sampling tray where the surface was smoothed to eliminate surface irregularities. The X- ray diffraction spectra were recorded using an Empyrean PANalytical diffractrometer system in steps of 0.1° using Cu Kα radiation as X-ray source at 25°C. X- ray diffraction spectra were recorded using diffractometer at a plate current intensity of 40 mA and an accelerating value of 40 kV. Scans were performed over the 5.0064 – 79.9904 [^{o}2Th] range using step 0.0130 in width.

Solid state nuclear magnetic resonance (SSNMR) spectroscopy

Solid-state NMR spectra were obtained at the EPSRC UK, National solid-state NMR service at Durham. Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique that is used to determine the content and purity of a sample as well as its molecular structure. The solid state ^{13}C NMR spectra were measured on a Varian VNMRS 600 spectrometer. When untreated and cellulase treated BC samples were irradiated with a radio frequency, absorption of the resonant energy between adjacent energy levels occurs. The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy was emitted at the same frequency. The precise frequencies at which the spin-active BC resonates was picked up and displayed by the NMR spectrometer.

Differential scanning calorimeter (DSC)

DSC is a thermos-analytical technique used to study what happens to polymers when they are heated, that is thermal transitions of a polymer. Two aluminium pans are used; sample is placed in one pan while the second pan which serves as a reference pan is left empty. The two pans are heated at specific rate, say 20°C per minute. For untreated BC, 8.2220 mg of BC from the compression moulded products was loaded into aluminium pans. DSC measurement were taken with a TA-DSC 2010 apparatus (TA Instruments, Newcastle, DE, USA), under the nitrogen atmosphere (flow = 50 mL/min). The instrument was calibrated with high purity indium. The melting temperature (T_m) was taken as the peak temperature maximum of melting endotherm. In this study, T_g was taken as the midpoint of the step-transition.

A first temperature program was conducted to record Tg and Tm. After heating to 200°C at 20°C/min in order to erase thermal histories the BC samples were cooled down to -100°C at a cooling rate of 30°C/min. During the first heating run the broad endotherm could probably be connected with solvent or water was observed. A second heating scan was conducted from -70 to 280°C at 20°C/min and two thermal transitions were recorded. The third heating scan was conducted again from -30 – 3500°C at 20°C/min and melting transition at 103.21°C and Tg at 38°C was recorded. For cellulase treated BC, 14.2590 mg BC was used and the same procedure was carried out as described above with only first and second scan rounds using same conditions.

Thermogravimetric analysis or thermal gravimetric analysis (TGA)

TGA is a thermos-analytical technique in which changes in weight are measured as a function of increasing temperatures. To determine the thermal stability of untreated and cellulase treated BC, approximately 3,4830 mg of the untreated BC and 5,6700 mg of the cellulase treated BC were placed in aluminium pans. Raw and derivative weight data were used to determine decomposition, temperatures and associated weight losses. TGA analysis was performed with a TGA/DSC1 Mettler-Toledo thermal analyser at a heating rate of 10°C/min in a stream of nitrogen (60 mL/min). The obtained TGA data were analysed using the Mettler-Toledo star system SW 9.30.

RESULTS

In this study, BC was obtained as a thick layer from HS medium and the average wet yield of cellulose by *G. xylinus* was found to be 90.48 ± 12.14 g/l with an average dry yield of 1.4 ± 0.09 g/l. The BC samples used for analysis were harvested from a single flask of HS media containing multiple layers of BC (Figure 1). The multiple layers of BC were thought to be caused by the formation of a new pellicle due to the preceding pellicle sinking.

This unique opportunity of formation of new pellicle due to preceding pellicle sinking eliminates much variability that can affect the quality of the BC thus allowing a better comparison among samples. All samples were similar in appearance and thickness. Samples were processed with distilled water (dH_2O), dH_2O + NaOH, cellulase pretreatment and were freeze dried before the IR analysis.

Fourier Transform infrared spectroscopy (FTIR) Analysis

Table 1 is a summary table of corresponding wave numbers derived from the FTIR analysis associated with interpreted bonds and functional groups (Dai and Fan, 2010; Fan et al., 2012; Nam et al., 2011).

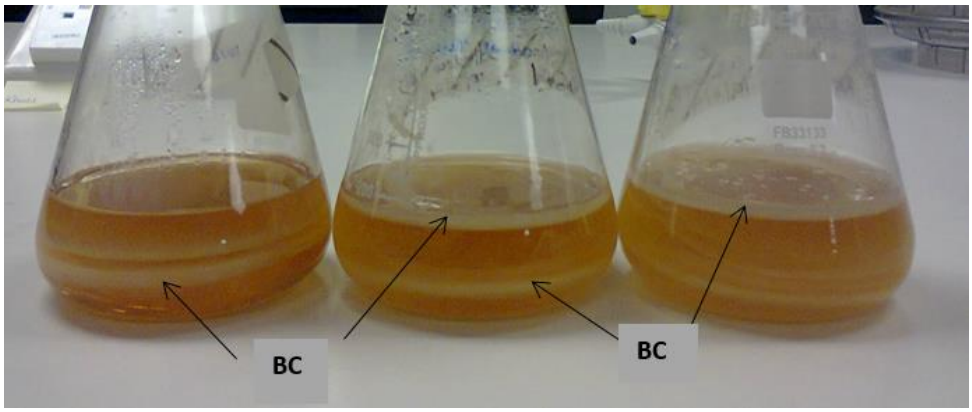

Figure 1. Layers of bacterial cellulose formed by *G. xylinus* 639 in triplicate.

Table 1. Summary table of corresponding wave numbers derived from the FTIR analysis associated with interpreted bonds and functional groups (Dai and Fan, 2010; Fan et al., 2012; Nam et al., 2011).

Wavenumber peaks (cm^{-1})	Type	Type of vibration	Functional group
3328	Broad	Stretching	-OH (H-bonded)
2950	Narrow	Stretching	C-H
1620	Narrow	Bending	-OH of absorbed water
1380		bending	Planar CH
1153		Stretching	Asymmetrical C-O-C
1020		vibration	C-C, C-OH, C-H ring and side group

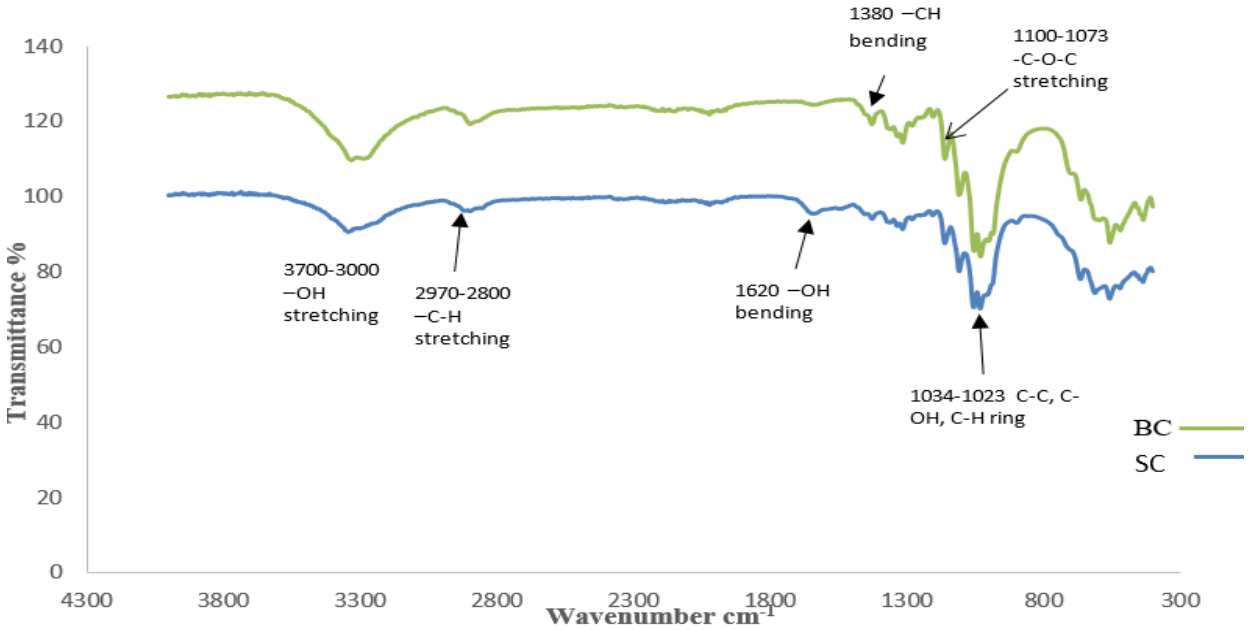

Figure 2. FTIR spectrum of freeze-dried BC and standard cellulose. SC: Standard cellulose (Sigma-Aldrich, UK), BC: Bacterial cellulose.

Figure 2 shows the FTIR spectra in 4300-300 cm^{-1} region of BC and standard cellulose from Sigma-Aldrich. Both diffraction curves are of typical cellulose structure with a few exceptions of the BC spectra from the

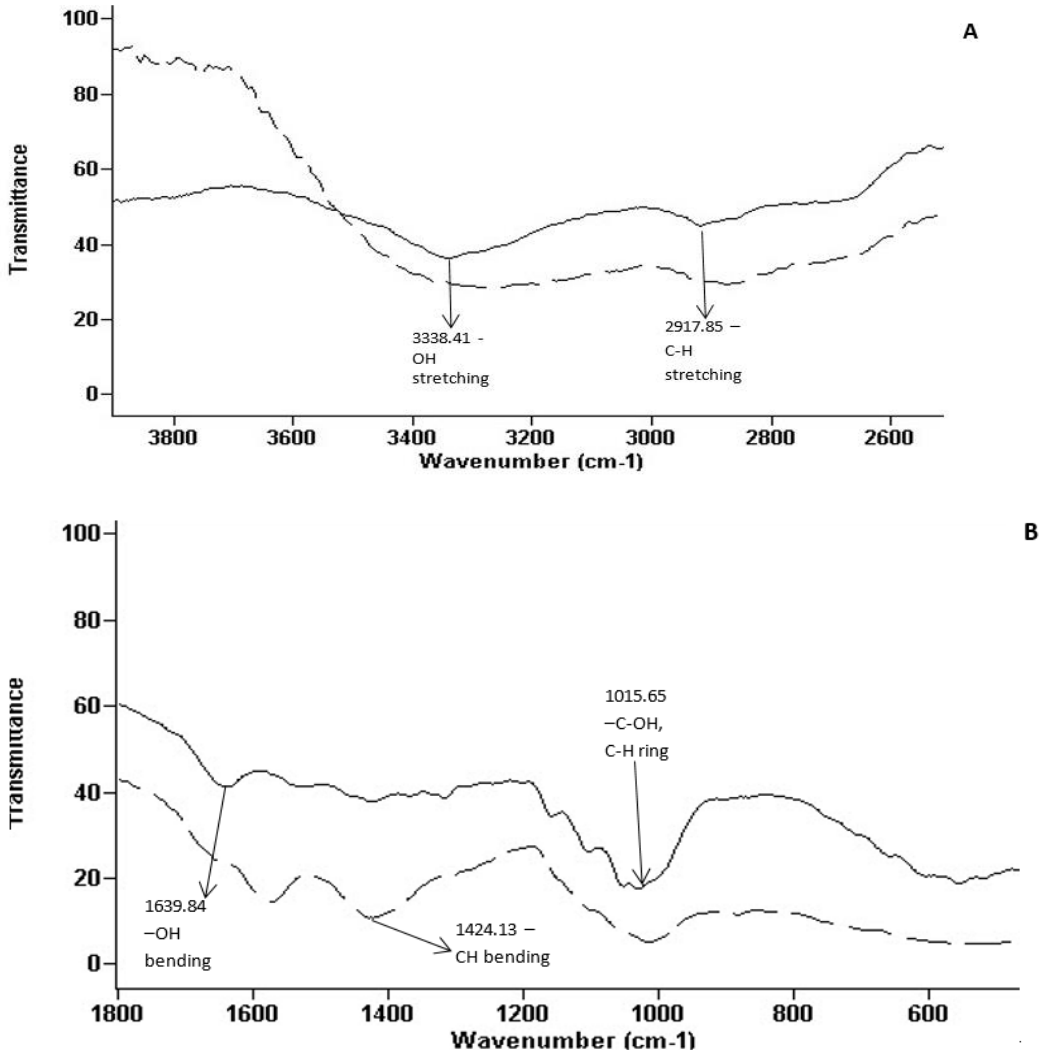

Figure 3. FTIR spectrum of BC before and after pretreatment with cellulases. A: 3800 – 2600 cm $^{-1}$ region; B: 1800 – 600 cm $^{-1}$ region. - - - - - Treated BC, Untreated BC.

standard cellulose spectra. FTIR spectra in 1620 - 1380 cm^{-1} region show the band of the NaOH washed BC to be shifted. The bands at 37000 - 3000, 2970 - 2800, 1100 - 1073 and 1034 -1023 cm $^{-1}$ are similar.

Figure 3 (A and B) show the FTIR spectra in 3800 – 600 cm^{-1} region of BC and cellulase hydrolyzed BC which were employed to characterized the structure of the BC material before and after treatment with cellulase enzyme. The spectra shown in Figure 3A show the absorption spectra band at 3338.41 and 2917.85 cm^{-1} for –OH stretching and –C-H stretching respectively. The absorption spectra band at 1015.65 cm $^{-1}$ (Figure 3B) is assigned as C-C, C-OH, C-H ring and C-O-C stretching at the β-(1→4)-glycosidic bond/linkage in cellulose (Nelson and O'Connor, 1964) while the absorption spectra band at 1424.13 cm^{-1} is assigned to the CH_2 scissoring motion in cellulose (Spiridon et al., 2011). Similarly, the absorption spectra band at 1639.84 cm^{-1} is assigned to the –OH bending. As shown in Figure 3B, the absorption spectra band at 600 -1000 cm^{-1} was strong for untreated BC but somehow weak for the treated BC material.

SEM analysis

SEM analysis was carried out with 4 different samples, of BC after freeze drying. Figure 4A show the SEM micrographs of unwashed BC microfibrils and the bacterial cells enmeshed in it. Figure 4B is SEM images of threadlike cellulosic microfibrils without visible bacterial cells. The fibrils are tightly packed with dendritic nodes present which are thought to be regions of amorphous cellulose (Liu et al., 2013). The tightly packed fibrils conferred morphological features similar to pure microcrystalline cellulose. There were also regions of

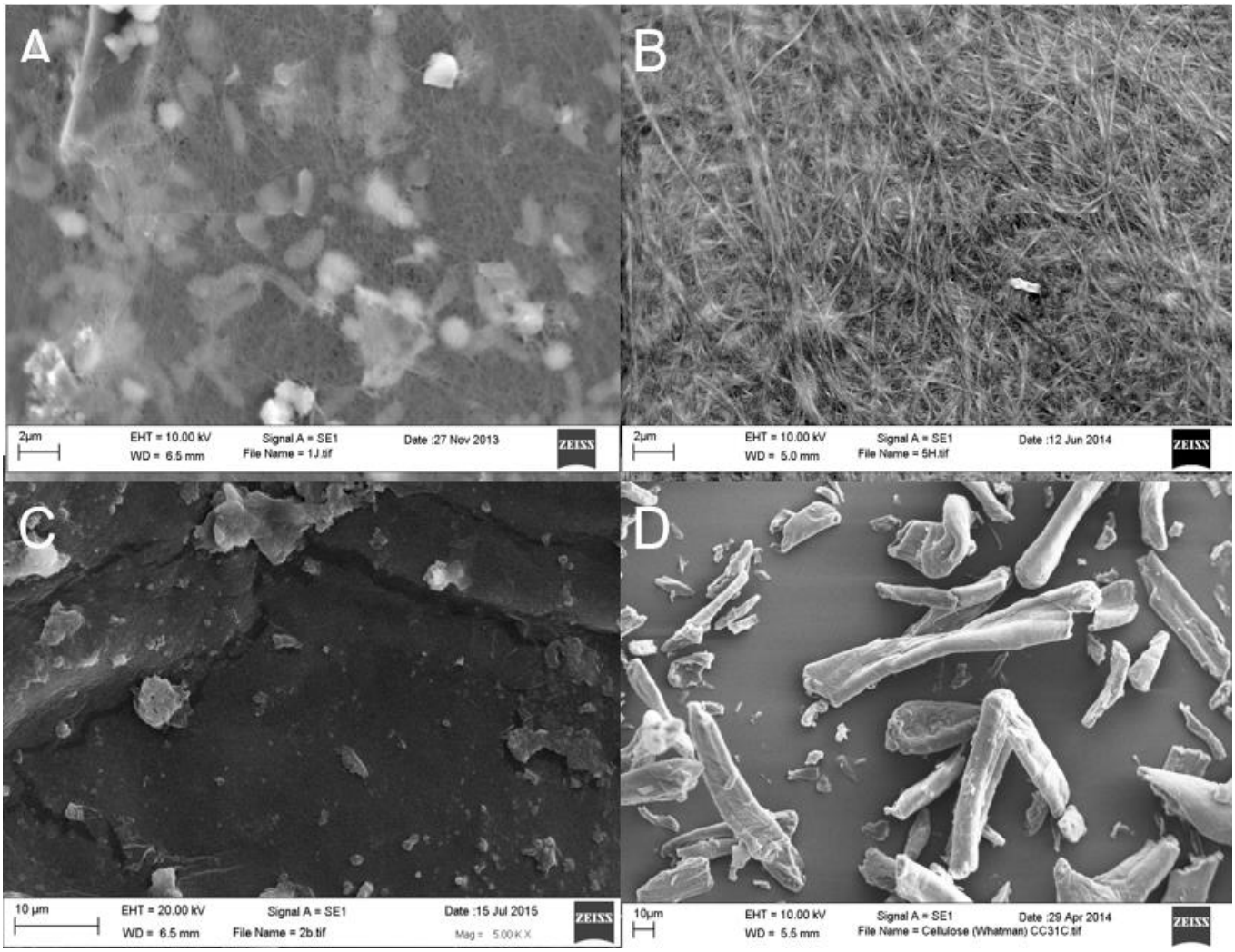

Figure 4. SEM collection of BC micrograph. A: Rod-shaped cells of *G. xylinus* (approx 1.5-2 µm in length) entangled in BC, B: Cellulosic fibrils of BC, C: Cellulase treated BC (2 mg/ml), D: The non-microbial cellulose sample from Sigma-Aldrich.

highly crystallized cellulose which contained fibrils that are more defined and orientated. Figure 4C show the SEM images of cellulase treated BC that was hydrolyzed to sugar monomers. The micrograph shows that some microfibrils are separated while others cluster together. Figure 4D show the SEM image of the non-microbial cellulose sample (Sigma-Aldrich) which differs considerably from the BC; and exhibits a discontinuous and fragmented formation. The non-microbial cellulose shows cellulose sheets having large gaps between the fibers and the fibers are not intertwined closely, while the BC has much smaller fibers which are closely intertwined.

XRD analysis

A typical X- ray diffractogram obtained from the BC sample demonstrates two characteristic clearly resolved peaks (Figure 5). The crystallinity index was estimated based on the diffractogram. X-ray diffractogram investigations indicated that majority of the cellulose was type-1 cellulose.

Solid state nuclear magnetic resonance (SSNMR) spectroscopy

To determine the content and purity of untreated and cellulase treated BC, solid state ^{13}C NMR was carried out using Varian VNMRS 600 spectrometer. Figure 6 shows the spectrum from an untreated BC sample. The BC untreated is dominated by a set of signals consistent with cellulose. Judging from the intensity of 89 ppm peak to the broader one at 84 ppm, the BC is quite highly crystalline.

Figure 7 shows the spectrum of cellulase treated BC

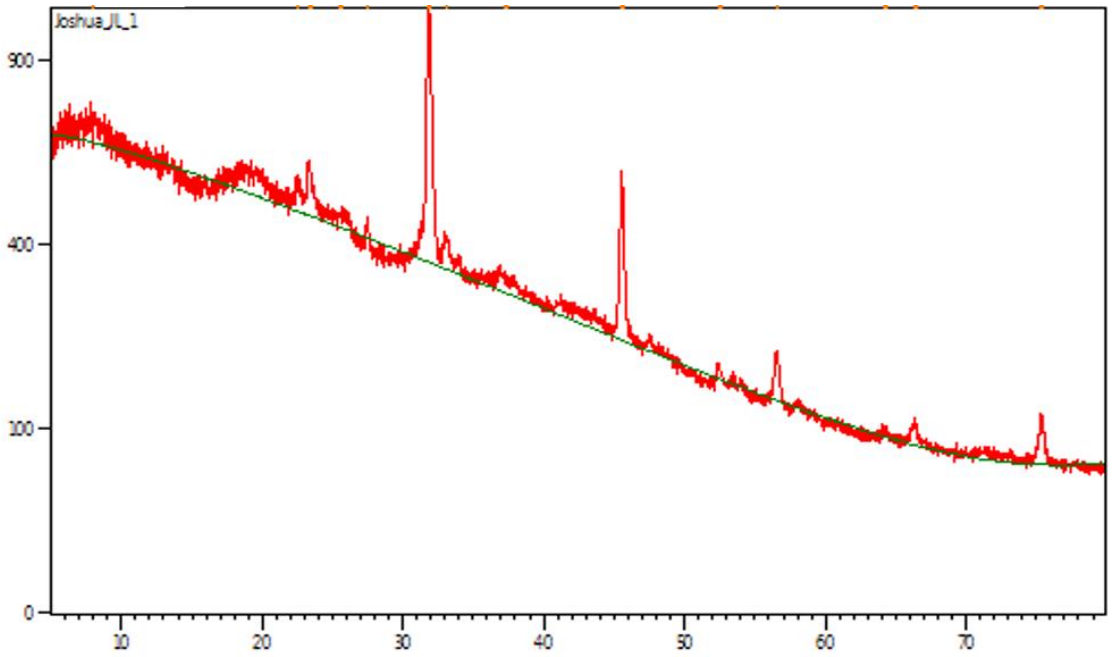

Figure 5. XRD diffraction result of untreated bacterial cellulose (BC).

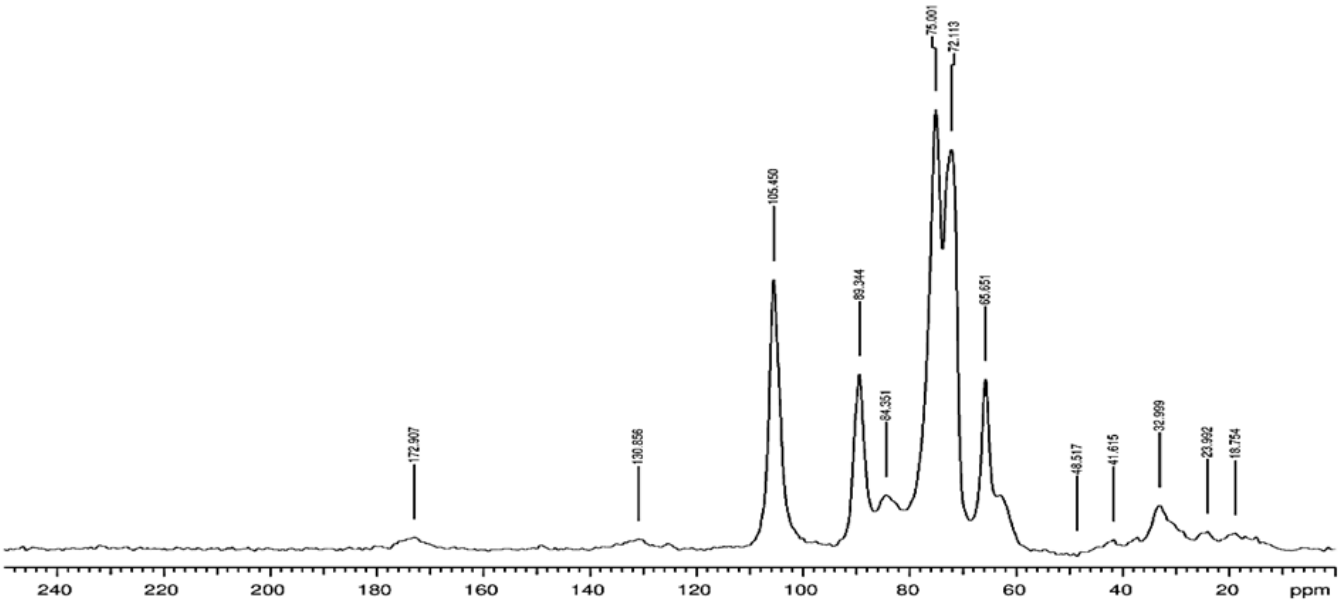

Figure 6. ^{13}C SSNMR spectrum of untreated bacterial cellulose (BC).

sample which is different from the untreated BC. The spectrum is still dominated by the polysaccharide signal between 55 and 110 ppm but it is much less well defined than for the untreated BC sample. The spectrum also show some other signal at the low-frequency end of the spectrum and four sharp lines at the high frequency end that were not present in the untreated BC sample.

Differential scanning calorimeter (DSC)/Thermogravimetric analysis (TGA)

To determine the thermal transitions of untreated and cellulase treated BC, glass transition (Tg) and melting temperature (Tm) were evaluated. The DSC of the untreated BC indicated that the Tg and Tm were around

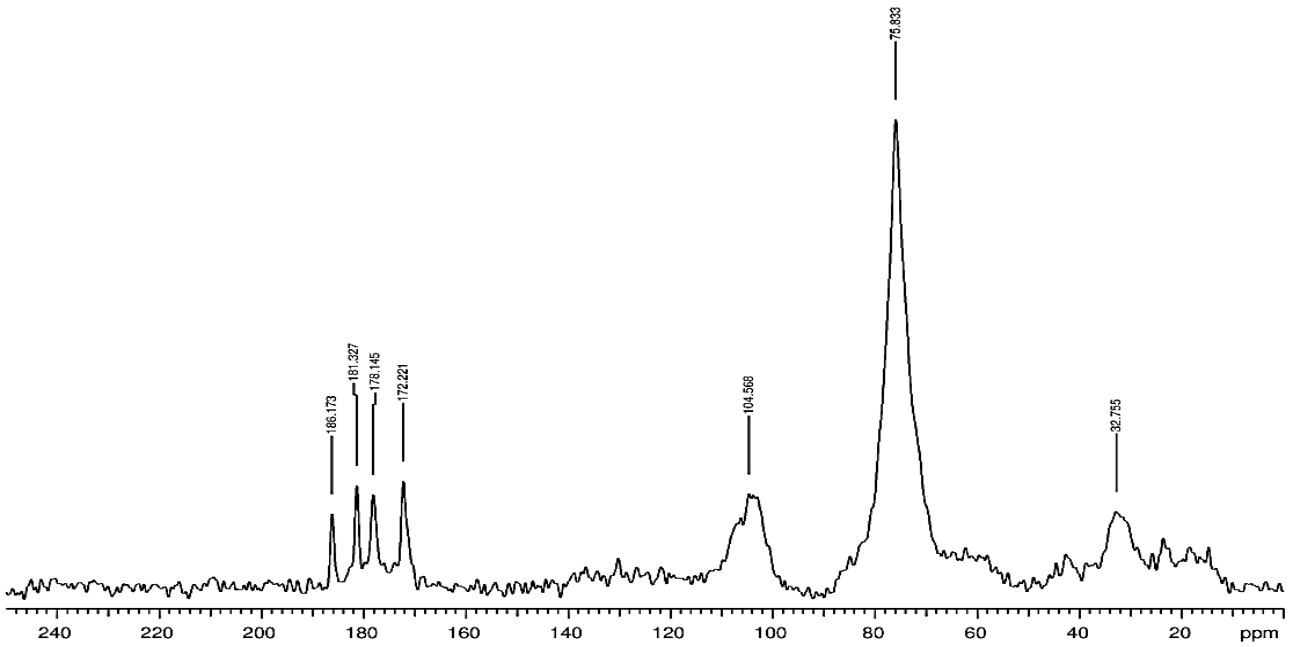

Figure 7. ^{13}C SSNMR spectrum of cellulase treated bacterial cellulose (BC).

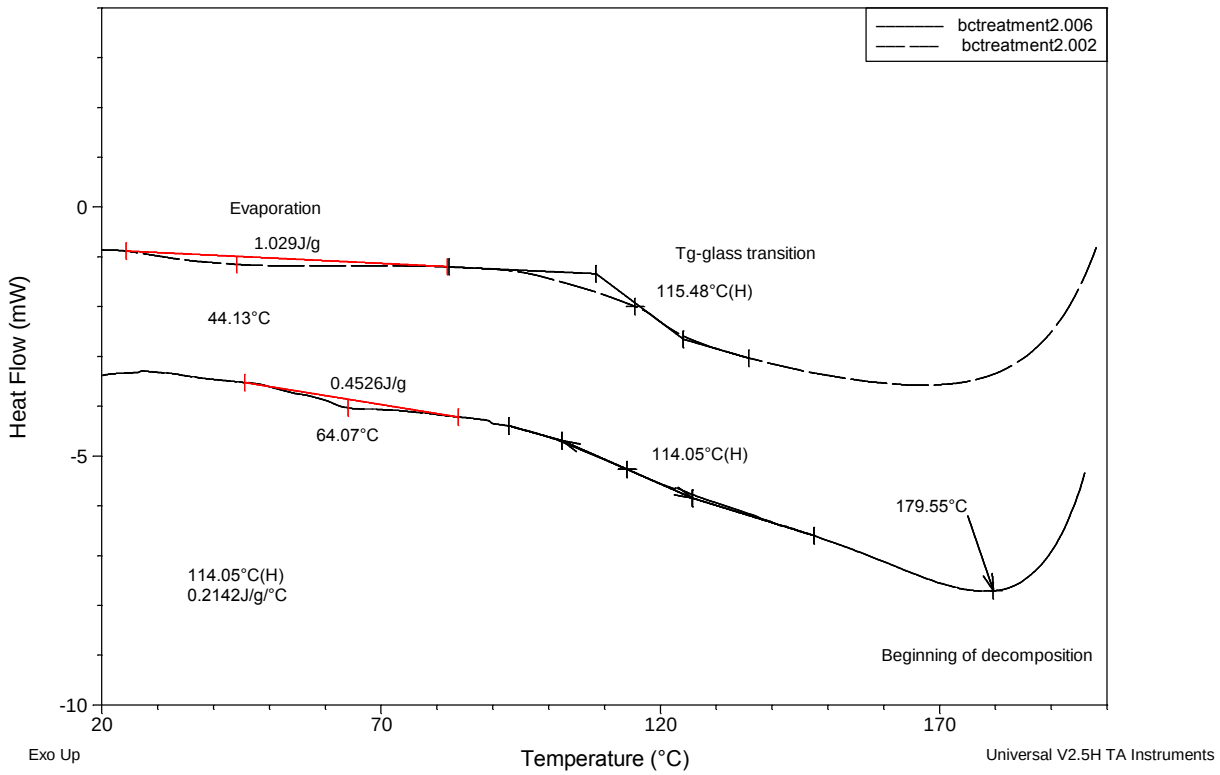

Figure 8. DSC thermogram of cellulase treated BC illustrating the determination of Tg and Tm.

38 and 117.31°C respectively (Figure 8). The BC sample was stable up to 200°C above which decomposition started with a pronounced disintegration at 338.13°C. The initial BC sample varied greatly between 10 to 200°C

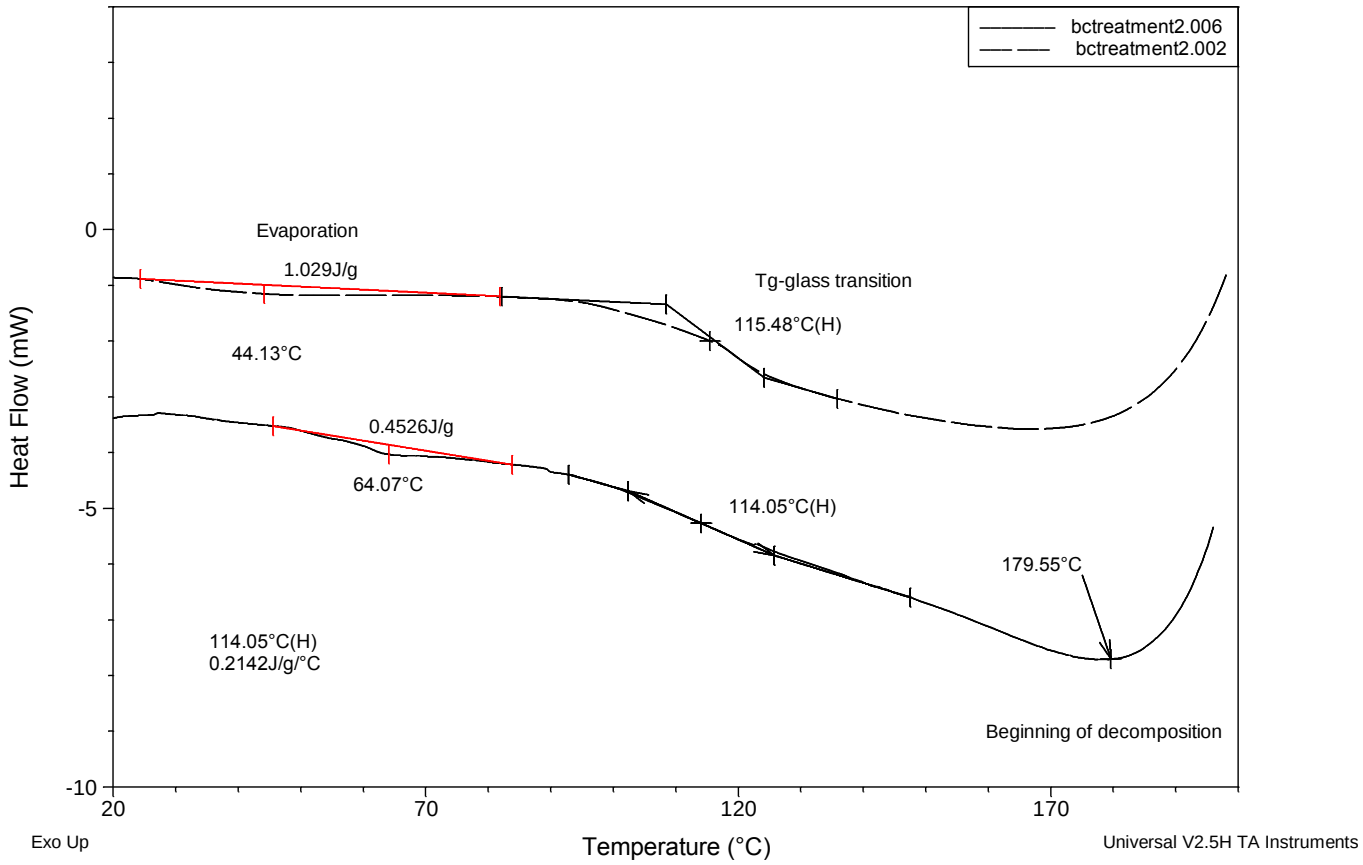

Figure 9. DSC thermogram of cellulase treated BC illustrating the determination of Tg and Tm.

as a result of evaporation due to water contents. On the other hand, the Tg of cellulase treated BC samples were around 115°C (Figure 9). Melting transition was not observed. A small endotherm probably due to solvent evaporation was observed at 44-60°C. There was quick evaporation of the sample which was stable up to around 150°C above which disintegration began and there was pronounced decomposition at 170°C.

The TGA curve for untreated BC sample shows bound water for the untreated BC sample to be evaporated at approximately 50°C with degradation temperature after 200°C (data not shown). The TGA of the cellulase treated sample shows a fast evaporation of the sample at a temperature of 30-80°C which could be ascribed to the presence of moisture and other materials used in the treatment of BC sample. The treated sample was stable until about 150°C before degradation. The maximum mass loss for the cellulase treated BC sample was much higher than that of the untreated BC sample.

DISCUSSION

After nine days of static fermentation, bacterial cellulose (BC) was obtained as a thick layer on the surface of HS medium. The dry yield of BC produced by *G. xylinus* was found to be1.4 ± 0.09 g/L. The thickness of the wet BC was 12 mm and the thickness of the dry sheets was 0.18 mm. The yield results of BC production in this study is lower than the previous work of Carreira et al. (2011) and Gayathry and Gopalaswamy (2014) who reported BC production with a weight of 2.60 and 11 g/L respectively. Differences in yield may reflect strain differences.

Figure 2 shows the FTIR absorption spectrum of standard cellulose and BC. The FTIR results as deduced from Table 1 indicated many similarities between the bacterial cellulose and the standard cellulose. Results indicated that all samples contain functional groups that were associated with cellulose, such as: 3700-3000 cm^{-1} hydrogen bonding -OH bending, 2970-2800 cm^{-1} -CH symmetrical stretching and 1034 - 1023 cm^{-1} C-C, C-OH and C-H ring side group vibrations, which is typically widely reported for bacterial cellulose (Fan et al., 2012; Trovatti et al., 2011).

The broad absorption spectra in the region 3600 - 3000 cm^{-1} (-OH stretching, H-bonded) indicates a shallower peak for the treated BC sample. Treatment with cellulase enzyme is thought to disrupt the β (1→4) linkages

between the cellulose chains (Fan et al., 2012). Hydrolysis of the β-linkages can lead to changes in the molecular, supramolecular and morphological level orientation (Fan et al., 2012) which can have a variety of effects on the physical properties of the BC such as crystallinity, water holding capacity and mechanical strengths. The spectra in Figures 2 and 3 (A and B) indicate that the untreated BC possess type I cellulose. The type I cellulose was transformed into type II cellulose or amorphous cellulose after enzymatic hydrolysis (Figure 3A and B).

The -OH bending of adsorbed water was observed at 1639.84 cm^{-1} for untreated BC sample. This result is in agreement with those previously reported (Okiyama et al., 1992) whereby 0.3% of the 90% of water (by mass) is held through the extensive hydrogen bonding network facilitated by the hydroxyl groups and ester bonds. In untreated BC samples, the intensity for adsorbed water is comparably less intense than those found in cellulase treated BC sample spectra. The FTIR absorption spectra for both treated and untreated BC show characteristic peaks around 1000 – 1200 cm^{-1} (Langkilde and Svantesson, 1995; Zhbankov et al., 2000) and the band around 1424.13 cm^{-1} in treated BC can be ascribed to CH shake vibrations in cellulose (Spiridon et al., 2011). Generally, the results of the FTIR investigation in Figure 3A and B showed that the peaks of BC samples were lower after enzymatic hydrolysis indicating that the BC was degraded. The band at 1400-1600 cm^{-1} for the treated BC sample could be attributed to the absorbed water bending vibrations after enzymatic hydrolysis.

Confirmation of the general structure of the untreated and cellulase treated BC was carried out using the SEM analysis. BC images from SEM micrographs (Figure 4) revealed complex meshed cellulose ribbons that interweaved among each other to form the BC membrane. Figure 4A is an SEM image of BC washed with dH_2O showing distinct rod-shaped cells that were immobilized and entangled by the fine cellulose ribbons. The length of each cell is roughly 1 to 2 µm. The regions containing thicker cellulose ribbons displayed no obvious sign of cells, which suggests that the cells were covered by the successive formation of cellulose and that these fine fibrils were in the early stages of cellulose matrix formation. The absence of other cell types indicates that *G. xylinus* is the only producer of the BC pellicle. Interestingly, samples that were subjected to repeated and prolonged washing with dH_2O showed no presence of cells within their matrix thus suggesting that thorough washing of BC with dH_2O is effective in removing cells and other cellular debris.

Figure 4B shows the SEM image of threadlike cellulosic microfibrils of BC. White dendritic nodules were observed throughout the structure of the BC, forming junctions, diverging microfibers from the center which were then incorporated to the surrounding meshwork. The dendritic nodes which were widespread throughout the sample in a variety of shape and sizes were amorphous in nature indicating the presence of type II cellulose (Sarkar and Perez, 2012). Whilst washing of BC with dH_2O was effective in removing cells, treatment of the sample with NaOH before washing with dH_2O may be successful in the context of sterility. A dramatic morphological change on BC microfibrils was observed after hydrolysis (Figure 4C) indicating that BC microfibrils were hydrolyzed to sugar monomers.

The XRD analysis result gave an insight into the physical properties of the BC produced. The bacterial cellulose diffractogram reveals two principal diffraction peaks at 15° and 29° confirming the presence of type-1 cellulose. Previous studies indicated that a high intensity at diffraction plane 14.5° and 22.6° indicates the presence of cellulose type-1 (Czaja et al., 2004; Moosavi-Nasab and Yousefi, 2011; Sheykhnazari et al., 2011).

The ^{13}C SSNMR spectrum of untreated BC is presented in Figure 6. The broader peak was due to the crystallite surfaces and the amorphous domains while the comparatively sharp peak was due to a crystalline region (Shezad et al., 2010). The NMR spectrum of the BC is in agreement with those reported in the literature for BC and cellulose (Shezad et al., 2010; Zhao et al., 2007). Although the SEM analysis (Figure 4 C) show a dramatic changes on cellulose microfibrils of cellulase treated BC sample, the NMR data (Figure 7) were still dominated by polysaccharide signals. The NMR result of the cellulase treated BC show that BC apparent crystallinity was alternated by hydrolysis which suggests that BC could be a valuable raw material for fermentable sugars.

The thermal behaviour evaluated by DSC and TGA shows the thermal stability of BC. DSC measures the heat absorbed or released by a material as a function of temperature or time. The Tg of the untreated BC was found to around 37.14°C which was found to be lower than the value 44.28°C reported by Mohite and Patel (2014) but higher than the value of 13.94°C reported by George et al. (2005). The higher the Tg of a material, the better the advantage because minimal aging is expected at a storage temperature which is below Tg (Bechard et al., 1995). The thermal stability of BC up to 200°C could be attributed to its crystallinity and high molecular weight (Chen et al., 2009) while the low thermal stability of treated BC could be as a result of enzyme hydrolysis yielding low molecular weight oligosaccharides. The results indicated that the cellulase treated BC sample was hydrolysed hence the low Tm and decomposition temperatures compared to the untreated BC samples.

Thermal degradation temperature of a sample is influenced by the following factors: sample size, moisture content of sample and the stability of functional groups. Other features include experimental factors such as nitrogen flow rate and the heating rate (Chang et al., 2010; Roman and Winter, 2004). The TGA results (Figures 10 and 11) confirm the evaporation in both untreated and cellulose treated BC samples which are

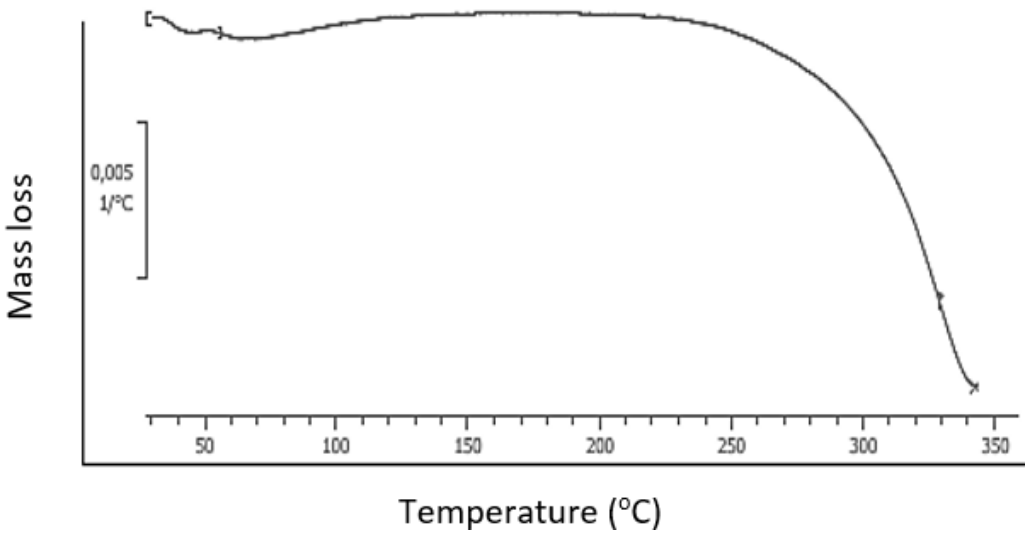

Figure 10. TGA thermogram of untreated BC sample.

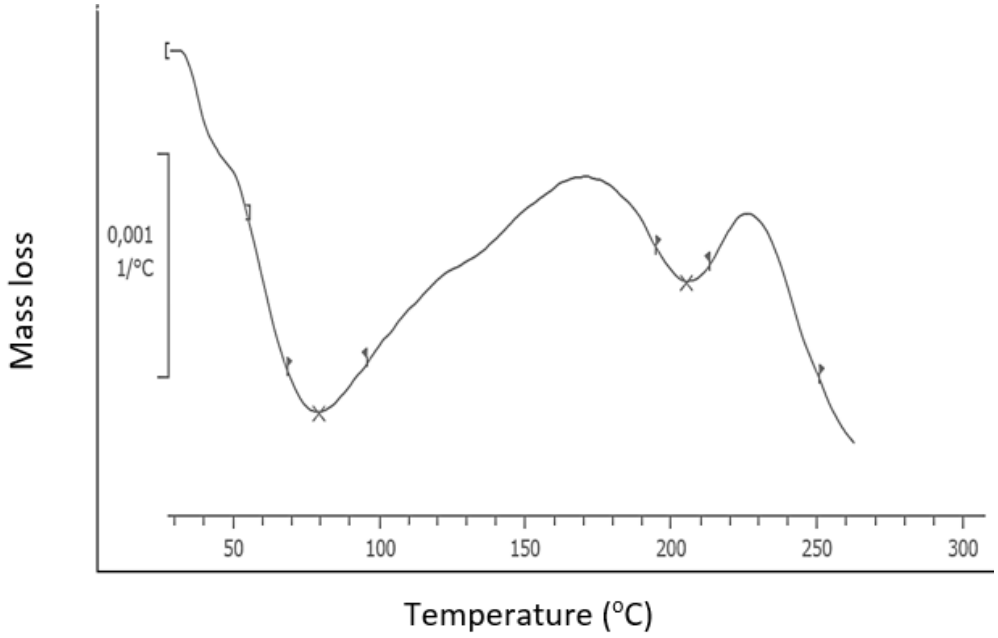

Figure 11. TGA thermogram of cellulase treated BC sample.

caused as a result of solvent or the presence of water in the BC samples. The probable presence of water could be as a result of sample exposure which thereby absorbs atmospheric moisture. The results from the TGA analysis also confirm the degradation of untreated BC after 200°C and that of treated BC after 150°C. The fast degradation of cellulase treated BC in DSC analysis was found to be consistent with the observed degradation in TGA analysis.

Conclusion

G. xylinus is considered to be the bacterial species with the most commercial interest (Andrade et al., 2010) and BC produced by *G. xylinus* could be of commercial interest and open new avenues in the field of degradable polymers. This study provides for the first time a fully detailed characterisation of pure bacterial cellulose before and after treatment with a commercially available

cellulase enzyme. This suggests an attractive prospect for the production and recycling of biopolymers from bacterial sources.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

ACKNOWLEDGMENTS

This work was supported financially by the Petroleum Technology Development Fund (PTDF) of Nigeria. Solid-state NMR spectra were obtained at the EPSRC UK, National solid-state NMR service at Durham, UK. We thank Professor Marek Kowalczuk for the help in the development of this work.

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Vol. 16(10), pp. 483-489, 8 March, 2017
DOI: 10.5897/AJB2017.15925
Article Number: 918D29563127
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular characterization of *Chenopodium quinoa* Willd. using inter-simple sequence repeat (ISSR) markers

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Received 1 February, 2017; Accepted 17 February, 2017

Quinua (*Chenopodium quinoa* Willdenow) is a pseudocereal of Amaranthace family which originated from the Andes of South America. Quinoa is an interesting plant whose capacity to tolerate adverse environmental factors and exceptional nutritional qualities warrant further research in all fields of plant biology, agronomy, ecology and biotechnology. Presently, it is an underutilized crop, which has the potential become a major crop. It has increases in importance in the world due to the nutritional quality of its grains and crop adaptability to diverse climatic conditions. In Colombia, more accurately in the Department of Nariño, Cauca, Cundinamarca y Boyacá currently Quinua has had a huge boost due to their agronomic potential and different benefits derived from the production, processing and marketing of its products. The objective of this research was to characterize the genetic diversity of a collection of 82 materials of with seven microsatellite markers [inter-simple sequence repeats, (ISSRs)]. The analysis by the coefficient of Nei-Li at the level of similarity of 0.65 divided the population into four groups according to the site of origin of the materials. The value of average heterozygosity was 0.38 which is considered low compared to other studies of genetic diversity in *Chenopodium*. Molecular Analysis of Variance (AMOVA) and Fst demonstrate the existence of genetic variability at the intraspecific level that should be used in breeding programs of the species lead to obtaining new and better materials of quinua.

Key words: Genetic diversity, microsatellites, Andean cereal.

INTRODUCTION

Quinua (*Chenopodium quinoa* Willd.) is a pseudocereal of the Amaranthace family which originated from the Andes of South America where it has been cultivated since more than 5,000 (Adolf et al., 2012). Quinua is an allotetraploid (2n=4x=36)y, thus exhibits disomic inheritance for most qualitative traits (Maughan et al., 2004); their seeds, and to some extent its leaves, are traditionally used for human and livestock consumption in

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the Andean region and have exceptional nutritional qualities (Lamothe et al., 2015; Yasui et al., 2016). Moreover, the species, being adapted to the harsh climatic conditions of the Andes (De Jesus Souza et al., 2016), exhibits remarkable tolerance to several abiotic stresses such as frost (Jacobsen et al., 2005), salinity (Shabala et al., 2013) and drought (Jacobsen et al., 2012). Production of quinoa has, until now, been prevalently conducted in Bolivia and Peru and still its productions are very small in other Andean countries like Ecuador, Chile, Argentina, and Colombia. Production in Peru, Ecuador, and Bolivia has increased from 1980 to 2011 by approximately 300%, with the largest increase (from ca. 9 to 38 metric tons) in the latter country (FAOSTAT, 2015).

Cultivated quinua display a genetic diversity, mainly represented in an ample range of characters like plant coloration, flowers protein content, seeds, saponin content and leaves calcium oxalates content, which allows obtaining a wide range of adaptability to agroecological conditions (Rodríguez et al., 2009). Within the diversity centers, the center of Perú (Huancayo, Ayacucho, Cajamarca), the Ecuadorian Mountain range, the Argentine Northeast, the South of Chile and of Colombia (Pasto, Nariño and Cundinamarca) are identified (Jacobsen, 2003). The adaptation capacities of quinua are huge since we can find varieties developed from sea level up to 4,000 m above and from 40°S to 2°N of latitude (Zurita et al., 2014). The genetic bases of several quinua traits was identified several decades ago (Lescano-Rivera, 1980), but the first true genetic descriptions more recently provided the starting point for improvement of quinua. Several genetic tools have been developed, and today molecular markers are an effective way to enhance breeding efficiency (Ruíz et al., 2014).

Quinua is one of the Andean crops with little research in the area of genetics and plant breeding, although, it has a high variability in characteristics such as plant color, flowers, nutritional contents and metabolites of interest (Bazile et al., 2014). Collecting, conservation and characterization studies are necessary for the development of strategies to improve of this species. At the international level, approximately 16,263 *Chenopodium* accessions are collected worldwide, which have been preserved and characterized in part by institutions mainly from Bolivia, Perú, United States and India (Rojas et al., 2015). In Colombia, Corpoica Tibatitatá reports a germplasm bank with 28 accessions of quinua (Rojas et al., 2015), however, small collections are conserved in the main producers departments. In the country, the characterization of this plant genetic resources only morphoagronomic studies developed by Torres et al. (2008) in the Savannah of Bogotá.

Molecular markers are also employed for the genetic characterization of *Chenopodium* germplasm. They have been used to differentiate genotypes under environmental conditions that confounded their phenotypes (Nolasco et al., 2013). Simple sequence repeats (SSR) are one of the frequently used molecular markers for genotyping crops (Jarvis et al., 2008). A number of research studies have demonstrated the use of SSRs and ISSRs to detect polymorphism and diversity in quinua (Costa, 2014; Lu et al., 2015; Fuentes et al., 2009) related species like amaranth (Jimenez et al., 2013; Oduwaye et al., 2014) and others (Morillo et al., 2015, 2016; Dotor et al., 2016). However, inter-simple sequence repeat (ISSR) markers are simpler to use than SSR technique (Oduwaye et al., 2014; Morillo et al., 2015). The use of ISSR does not require prior knowledge of the target sequences flanking the repeat regions, is not expensive and is relatively easy to score manually compared to SSR.

In order to establish a strategy and management plant for phytogenetic resources for quinua, it is necessary to begin studies on morphological, agronomic, physiological and molecular characterizations to know the genetic diversity, to generate basic information necessary to obtain sustainable solutions for the problems of low levels of technology in production, common in quinua cultivation, such as lack of homogeneity in organoleptic characteristics in materials used in beverages and flour, genetic transformation that allows for a shorter growth cycle for production in less time and tolerance to pests and diseases through plant breeding (Zurita et al., 2014). Yazici and Bilir (2017) reported that genetic knowledge is one of the important tools used for different purposes such as gene conservation, managing of genetic resources, evolutional and genetic management of populations for plant breeding. Within this context, this research aimed to molecularly characterize quinua materials using inter-simple sequence repeat (ISSR) markers or random amplified microsatellite markers (RAM) to reveal genetic polymorphism in quinua.

MATERIALS AND METHODS

Plant

A total of 81 individuals of quinua belonging to genebank from Secretary of Agriculture of Government of Boyacá, Colombia, were evaluated in the Molecular Biology Research Laboratories, Gebimol and Bioplasma, of Pedagogical and Technological University of Colombia, Tunja located at 2,820 msnm with average temperature of 13°C (Table 1).

Molecular characterization

For DNA extraction, the Dellaporta et al. (1983), protocol was used. The total DNA was visualized with 0.8% agarose gels, stained with Z-Vision, with a Maxicell EC-340 Primo Gel Electrophoresis System chamber. In order to determine the DNA concentration of each accession, a dilution curve with DNA from bacteriophage Lambda with an initial concentration of 20 ng/µl was made. The quantified DNA was diluted in HPLC type water to a total volume of 100 µl to 10 ng/µl and stored at -20°C.

For analysis, seven ISSR primers synthesized by Technologies

Table 1. Quinua materials used for the assessment of genetic diversity with Inter-Simple Sequence Repeat (ISSR).

Origin	Quantity	Height (msnm)	Latitude	Longitude	Average temperature (°C)
Nariño	48	2,817	3°17´20" N	77°21´28"	13
Soracá	13	2,942	5°30´02" N	73°19´59"	13
Tunja	11	2,822	5° 32′ 25″ N	73° 21′ 41″	13
Perú	4	3,259	12°04´15" N	75°12´24"	12
Chocontá	2	2,689	5° 8′ 48″ N	73° 40′ 57″	13
Siachoque	2	2,753	5°30´47" N	73°14´39"	13
Cauca	1	2,750	2°27´00" N	76°37´00"	14

Table 2. Primers used in the ISSR technique.

Markers	Sequence (5´to 3´)
CCA	$DDB(CCA)_5$
CGA	$DHB(CGA)_5$
ACA	$BDB(ACA)_5$
AG	$HBH(AG)_7A$
CT	$DYD(CT)_7C$
TG	$HVH(TG)_7T$
CA	$DBDA(CA)_7$

Inc. were used (Table 2). The amplification reaction was prepared in a sterile microcentrifuge tube (1.5 ml) to a final volume of 25 µl. The reaction mixture was prepared with 1X buffer, 1.5 mM $MgCl_2$, 0.2 M dNTPs, 1U Taq Polymerase, 2 µM primer and 10 ng genomic DNA.

The following designations are used for degenerated sites: H (A/T/C); B (G/T/C); V (G/A/C) and D (G/A/T).

The amplification was carried out in a thermocycler PTC 100 Programmable Thermal Controller (MJ. Research, Inc). Initial denaturation was at 95°C for 5 min; denaturation at 95°C for 30 s, annealing temperature of 50°C (AG and CA primers), 55°C (CCA, TG and CT primers) and 58°C (CGA primers) for 45 s, an extension of 72°C for 2 min, 37 cycles of denaturation, and finally, extension at 72°C for 7 min.

Amplified products were separated by electrophoresis in polyacrylamide gels 37:1 (acrylamide: bisacrylamide) at 7% and 150 V for 1 h in a small DNA Sequencing System chamber (Fisher Biotech FB-SEQ-3545). The staining was carried out using silver salts.

Statistical analysis

An absence (zero) and present (one) binary matrix was generated. The genetic similarity between individuals was calculated using the similarity coefficient of Nei and Li (1979). The cluster analysis was conducted by the UPGMA method and a dendrogram was generated using the statistical package NTSYS (Numerical Taxonomy System for Personal Computer, PC version 2.02). To evaluate the genetic diversity, unbiased heterozygosity and percentage of polymorphic loci were estimated using the statistical package TFPGA (Tools For Population Genetic analysis, version 1.3, 1997). Unbiased statistical f with a confidence interval of 95% was determined.

RESULTS AND DISCUSSION

The main objective of this study was to analyze the molecular diversity of 81 materials of quinua using ISSR markers. In the analysis with the Nei-Li coefficient, at a similarity level 0.65, the population was distinguished into four groups based mainly on geographical origin of materials (Figure 1). The first group contains quinua materials collected in different municipalities from Department of Nariño, it presented genetic distances ranging from 0.80 to 0.92; they are highly homogeneous materials, which can be attributed to domestication processes, constant exchange of seeds between farmers of the producer areas, mating system and bottleneck events through which this species has passed; it has led to the loss of genetic diversity (Zurita et al., 2014).

At 0.60 of similarity, group II was found with materials collected in the producing areas of Department of Nariño, to genetic distance of 0.91 with respect to group I, which showed high degree of relationship among the quinua materials in the same region. In groups III and IV, a much laxa distribution of individuals from the different evaluation sites was observed, revealing the genetic flow between them, with genetic distances within groups more than that 0.80; it could be beneficial for breeding programs that implement hybridization strategies (Bhargava et al., 2016). On the other hand, it is also possible to observe the degree of consanguinity between quinua (*C. quinoa*) and other related species such as kiwicha (*Amaranthus* species) and even with Peruvian materials, reaffirming the existence of a continuous seed exchange between farmers and researchers.

In general terms, the clusters corresponded to the geographic site where quinua materials were collected; this had already been reported in other studies of genetic diversity using different types of markers (Maughan et al., 2012; Oduwaye et al., 2014; Rajkumari et al., 2015). Studies carried out in other Andean countries had also reported low variability in local varieties of quinua; it was to be expected from the selection processes carried out by breeders. Thus, genetic diversity declined after systematic selection from farmers or breeders (Bazile et

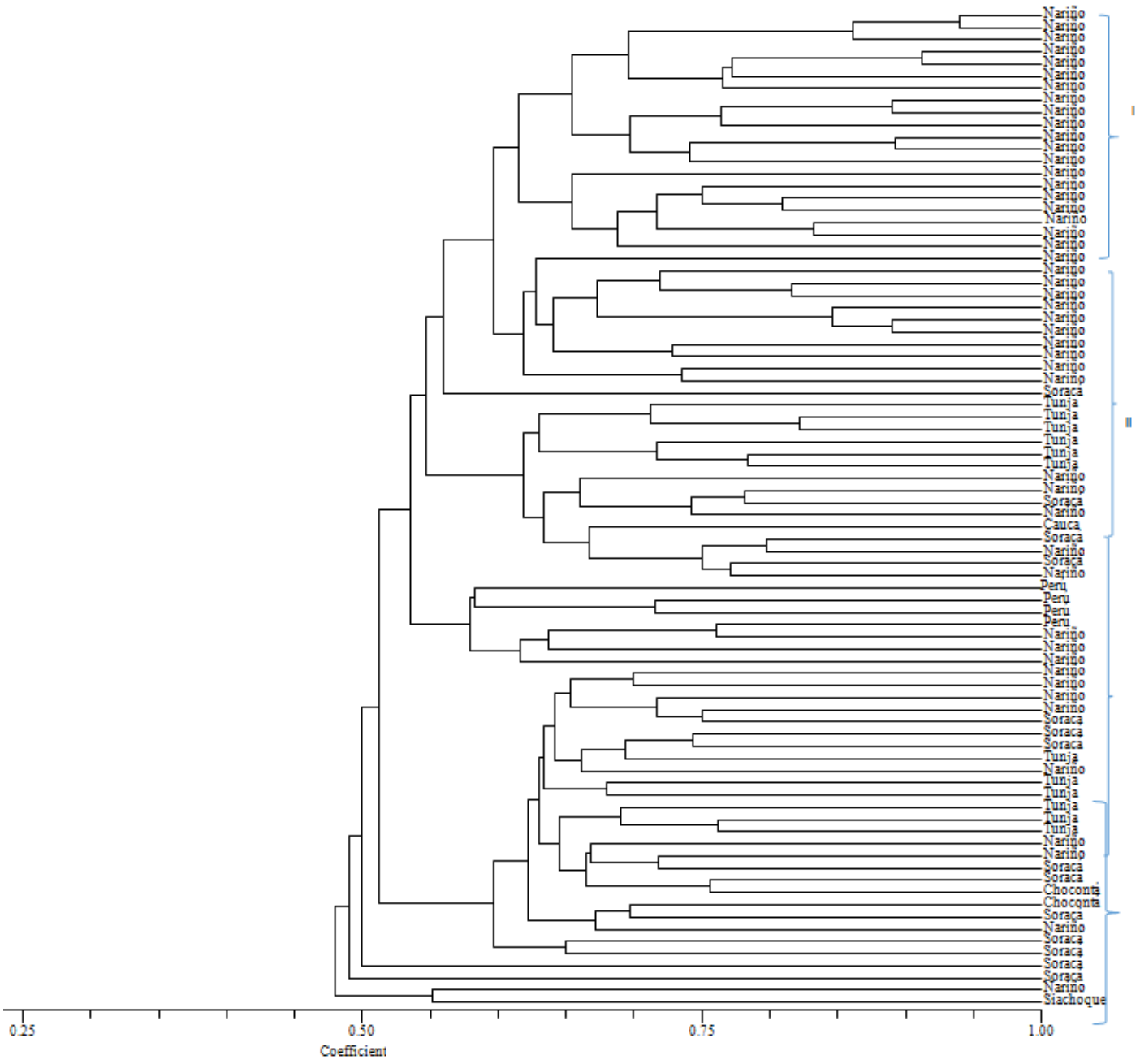

Figure 1. Dendrogram of quinua materials based on the Nei-Li similarity coefficient and calculated with seven ISSR markers with UPGMA classification method, SAHN and TREE of NTSYS-pc version 1.8 (Exeter Software, NY, USA).

al., 2014). Considering both the conditions under which quinoa is cultivated and its genetic variability, the plant has a remarkable adaptability to different agro-ecological zones. This adaptability is of great importance for the diversification of future agricultural systems; however, there is an urgent need to strengthen the breeding programs in quinoa (conventional as well as biotechnological) for its genetic improvement and conservation. The high nutritional quality and multiple uses in food products make quinoa ideal also for utilization by the food industry. Other potential uses are the medicinal and nutraceutical properties, due to its high phenolic acid content (Tuisima and Fernández, 2014).

In this study, a total of 178 bands were generated, with 99% polymorphism. The number of bands varied from 20 (CGA) to 35 (ACA), with molecular weights between 350 and 2700 pb and polymorphic loci percentages between 95 and 100% (Table 2). The number of bands and the

Table 3. Parameters of genetic diversity estimated in quinua materials evaluated.

Primer	Number of loci	Estimated He	% Polymorphic loci (95%)	Fst	SD
ACA	35	0.38	100.00	0.27	0.04
AG	30	0.38	100.00	0.09	0.03
TG	22	0.37	100.00	0.33	0.05
CT	24	0.35	100.00	0.21	0.03
CA	25	0.41	100.00	0.27	0.03
CCA	22	0.38	95.45	0.18	0.06
CGA	20	0.42	100.00	0.26	0.04
Total	178	0.38	99.44	0.23	0.02

Table 4. Analysis of molecular variance (AMOVA) for the formed groups.

Source	Dl.	SS	MS	Est. Var.	Percentage
Among Pops	5	749,154	149,831	9,091	22
Within Pops	76	2432,200	32,003	32,003	78
Total	81	3181,354	-	41,094	100

percentages of polymorphism found in this study are suitable for estimating genetic parameters when compared with others species that used ISSR markers (Rodríguez and Isla, 2009; Nolasco et al., 2013; Suresh et al., 2014). Heterozygosity values ranged between 0.35 (CT) and 0.42 (CGA). Taking into account the definition of Ott (1992), that consider marker like polymorphic, if H is greater than or equal to 0.1 and highly polymorphic if it is greater than or equal to 0.7, because ISSR are polymorphic markers that are useful for the discrimination of closely related quinua individuals (Oduwaye et al., 2014; Suresh et al., 2014; Lu et al., 2015). The TG marker made the greatest contribution to the observed variation with a 0.33 Fst which means it can be useful for the differentiation of materials of the genus *Chenopodium* in intra - interspecific genetic diversity studies (Table 3).

It was also identified that the CA, CGA and TG repeats are the most frequent repeated sequences in the quinoa genome compared to CCA and AG. Results are similar to those found by Jarvis et al. (2008), who developed a linkage map using SSR markers, AFLPs, the protein storage region in seed (11S) and the nucleolar organizing region (NOR) and in other related species such as amaranth (Jimenez et al., 2013; Oduwaye et al., 2014).

The average estimated heterozygosity value and percentage of polymorphic loci for the total population was 0.38 and 99%, respectively. The coefficient of genetic differentiation (Fst) obtained in evaluating 82 quinua materials with seven ISSR markers was 0.23 with a standard deviation of 0.02 (Table 3). According to Wright (1978), values of 0.25 show high genetic differentiation, which may be reflected in the high degree of domestication that these materials have suffered, since most of them are commercial varieties.

Genetic diversity studies using microsatellite markers in different *Chenopodium* species have shown a higher heterozygosity than this study, which may have been due to the nature of markers, genome coverage and reproductive factors (Self-pollination, cross pollination, bee pollination, seed dispersal, exchange of genetic information at intra and interspecific level between wild and ancestral relatives), which subject these species to their natural environment (Costa et al., 2012; Suresh et al., 2014; Vía and Fernández, 2015). With these molecular tools, it has been possible to identify differences at the genome level and similarities that are associated with morphological characteristics such as grain and panicle color, phenology and geographic distribution (Ruíz et al., 2014). In contrast, genetic diversity parameters found in this study show that quinua materials evaluated are very homogeneous which corroborates the results obtained in the dendrogram and genetic distances estimation and coincides with researches carried out by the Chilean and Peruvian breeding programs (Bazile et al., 2014; Vía and Fernández, 2015).

The molecular variance analysis AMOVA showed that the genetic variation observed in the evaluated quinua materials was mainly within groups, with 78% (Table 4). This high variation could indicate the presence of higher levels of subdivision and hierarchy. The remaining 22% was due to the component of genetic variance between the groups, which was significant ($P \leq 0.001$); such genetic variation between groups might be used for conservation and breeding of this species. Similar results have been reported in other studies of genetic diversity in the genus *Chenopodium* using microsatellite markers (Rodríguez and Isla, 2009; Suresh et al., 2014; Oduwaye et al.,

2014).

Considering both the conditions under which quinoa is cultivated and its genetic variability, the plant has a remarkable adaptability to different agro-ecological zones. This adaptability is of great importance for the diversification of future agricultural systems; however, there is an urgent need to strengthen the breeding programs in quinoa (conventional as well as biotechnological) for its genetic improvement and conservation. The high nutritional quality and multiple uses in food products make quinoa ideal also for utilization by the food industry. Other potential uses are the medicinal and nutraceutical activity due to its high phenolic acid content. With management strategies of quinoa cultivation among the farmers as well as the consumers, proper marketing and efficient post-harvest technologies, quinoa has the potential to become an important industrial and food crop of the 21st century (Tuisima and Fernández, 2014).

Conclusions

ISSR markers allowed the determination of the genetic variability in quinoa materials by grouping them according to the geographical location of origin.

It determined the existence of a moderate genetic variability due to the reproduction processes of the species as well as the spatial-temporal dynamics to which these materials are subjected in their natural environment and the constant exchange of seed between the Andean farmers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 16(10), pp. 490-498, 8 March, 2017
DOI: 10.5897/AJB2016.15209
Article Number: F66CD4A63132
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Micropropagation of Peach, *Prunus persica* (L.) BATSCH. cv. Garnem

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Received 6 January 2016; Accepted 9 June, 2016.

The present study aimed to develop micropropagation procedure for *Prunus persica* cv. Garnem. Shoot induction and multiplication was found from nodal explants cultured on MS medium fortified with different concentrations of two cytokinins (6-benzyladeninepourine and Kinetin) alone and in combination with 0.5 mg/l gibberellic acid and 0.01 mg/l indole-3-butyric acid. Microplantlets were cultured to root on ½ strength MS medium supplemented with different concentrations of indole-3-butyric acid and α-naphthalene-acetic acid alone. 6-Benzyladeninepourine (0.5 mg/l) with indole-3-butyric acid (0.01 mg/l) and gibberellic acid (0.5 mg/l) was found to be the optimum combination treatment for shoot initiation (100%). 6-Benzyladeninepourine (2.0 mg/l) with indole-3-butyric acid (0.01 mg/l) and gibberellic acid (0.5 mg/l) was found to be optimum for maximum shoot number per explant (7.67). The maximum rooting (42.86%), maximum root number/shoot (6.33) and longest rooting (7.17 cm) were found at 1.5 mg/l indole-3-butyric acid. α-naphthalene-acetic acid was found to be not effective. The plantlets were acclimatized in the glasshouse and survival percentage was 73.3% at potting mixture of autoclaved river sand, forest soil and manure in a 2:1:1 (v/v/v) ratio.

Key words: Acclimatization, auxin, cytokinin, micropropagation, *Prunus persica*.

INTRODUCTION

Peach (*Prunus persica* L. Batsch) belongs to the *Prunoideae,* a subfamily of *Rosaceae,* with 8 basic and 16 somatic chromosome numbers (2n = 16) (Hesse, 1975). *Prunus* includes several approximately 400 species adapted primarily to the temperate regions of the northern hemisphere (Krussmann, 1986). China is the native home for peach, which was domesticated there 4000-5000 years ago (Aranzana et al., 2010).

The introduction of temperate fruits especially of peach to Ethiopia and North Africa took place during the era of exploration and colonization in sixteenth and seventeenth century by Europeans (Scorza and Sherman, 1996). Because of its early introduction, peach is relatively well established in many highland areas and is introduced

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much earlier than apple and plum. From recently (2011) introduced, Garnem is one of the peach rootstock cultivars introduced from Spain to the country. The growth, productivity, and longevity of a peach tree are greatly influenced by the selection of an appropriate rootstock. The cultivar Garnem (*Prunus persica* cv. garnum) is highly immune to root-knot nematode *Meloidogyne javanica* (Treub.) Cjotwood (Pinochet et al., 1999), the most vigorous, and iron chlorosis resistant rootstock (Jimenez et al., 2011). It is a Spanish rootstock and is ranked third following GF677, and Seedling (Reighard, 2011). Jimenez et al. (2011), noted that Garnem significantly affects tree size, as measured by Trunk Cross Sectional Area (TCSA). In 2009 the 'Calrico' scion when grafted on Garnem and PADAC 9970-23 rootstocks exhibited higher yield per tree bigger fruit size and showed the higher TCSA values (239.6 and 239.0 cm^2, respectively). Garnem is the most invigorating rootstock, in agreement with genotype description (Felipe, 2009) and field evaluation (Zarrouk et al., 2005). However, it is difficult to be multiplied on mass scale through cuttings because of very low rooting percentages (Ammer, 1999). The use of conventional propagation methods in peach is quite difficult because it results low multiplication rate and also quite difficult in peach (Stylianides et al., 1989).

Recently, breeding practices in *Prunus* have been advanced by the development and application of micropropagation (Martinez-Gomez et al., 2005). There has been no report on micropropagation of *P. persica* cv. garnum in Ethiopia. The purpose of this study was therefore to find out the following objectives: to assess the effects of different types and concentrations of cytokinin on shoot induction and multiplication; to assess the effects of different types and concentrations of auxin on root induction; and to acclimatize *in vitro* seedlings.

MATERIALS AND METHODS

The study was conducted at the Plant Tissue Culture Laboratory, National Agricultural Biotechnology Laboratory, Holeta Agricultural Research Center from October 2013 to January 2014.

The nutrient media used was Murashige and Skoog (1962). To prepare a ready use MS media, the MS stock solutions were mixed along with plant growth regulators (PGR) and sucrose (3%). Magnetic stirrer was used to mix the solution well. The pH of the medium was adjusted at 5.75 using 0.1N NaOH or 0.1N HCl before adding 0.4% agar (Agar-Agar, Type 1), and heated till the agar melts properly. About 10 and 50 ml of the medium was dispensed in each culture tube (150 mm long and 25 mm diameter) and jar (250 ml). The culture tubes and jars containing the medium were plugged tightly with non-absorbent cotton wool plugs and autoclavable lids prior to autoclaving at 121°C with 0.15 KPa pressure for 20 min. Since, Gibberellins are rapidly degraded by elevated temperatures, and the biological activity of a freshly prepared solution of GA_3 was reduced by more than 90% as a result of autoclaving (Van Bragt and Pierik, 1971), it was sterilized by filtration through a sieve or a filtration assembly using filter membranes of 0.22 to 0.45 µm size. The filter sterilized GA_3 was then added into the autoclaved MS media before solidified.

Young and healthy shoots (4-6 cm long), containing axillary buds (third, fourth and fifth nodes; from shoot apex), were excised and collected from three years old Garnem fruit crop by cutting with sterile scissor and used as explant. Actively growing shoots (juvenile plants) were taken as they are more responsive to shoot regeneration and proliferation than shoot explants from adult forms as reported by Naghmouchi et al. (2008). The explants were washed with tap water 3-5 times followed by liquid soap for 30 min with agitation to physically remove most microorganisms, and treated in 70% ethanol for 30 s and then in NaOCl (0.25% w/v) containing one drop of 'Tween 20' per 50 ml solution for 15 min.

Shoot initiation

Sterilized explants' were cultured in test tubes containing MS (Murashige and Skoog, 1962) medium fortified with 3% sucrose, 0.4% agar (Agar-Agar, Type 1) and varying level of BAP (SIGMA) (0.5, 1.0, 2 and 4 mg/l) alone and in combination with 0.01 mg/l IBA (HIMEDIA) and 0.5 mg/l GA_3. In addition, Kn (UNI-CHEM) (0.5, 1.0, 2 and 4 mg/l) alone and in combination with 0.01 mg/l IBA and 0.5 mg/l GA_3 (Table 1) was the other treatment combination for the initiation experiment. MS medium without Plant Growth Regulators (PGRs) was used as control. For each initiation treatment seven test tubes were lined up randomly in Completely Randomized Design (CRD) with three replications. All test tubes with cultured explants were properly sealed with cotton and parafilm and maintained in the growth room at standard conditions (22 ± 1°C and 16/8 h light/dark using cool-white fluorescent lamps (photon flux density, 40 µmol m^{-1} s^{-1} irradiance) and relative humidity (RH) of 70-80%. Number of days to shoot initiation and number of explants initiated were recorded after four weeks of culturing, and shoot initiation percentage was computed.

Shoot multiplication

To avoid the carry over effect of shoot initiation media on shoot multiplication, initiated shoots were maintained on PGRs free MS medium with 1 g/l activated charcoal for two weeks. Aseptically initiated 1.0-1.5 cm long shoots with 7- 10 nodes were trimmed at both sides and placed horizontally and lightly pressed into the medium in 200 ml jars each with 50 ml MS medium supplemented with 3% sucrose, 0.4% agar and varying levels of BAP (0.5, 1.0, 2 and 4 mg/l) alone and in combination with 0.01 mg/l IBA, 0.5 mg/l GA_3. The other treatment combination was Kn (0.5, 1.0, 2 and 4 mg/l) alone and in combination with 0.01 mg/l IBA, 0.5 mg/l GA_3 (Table 1). MS medium without PGRs was used as control. For each treatment four jars (each with 5 shoots) were lined up randomly in CRD with three replications. All shoots were incubated on multiplication medium for 6 weeks then data on number of shoot per explant, number of leaves per shoot, and shoot length (cm) were recorded.

Rooting of shoots

Microshoots 1.5 cm long were cultured on half MS medium supplemented with 3% sucrose, 0.4% agar, 1 mg/l activated charcoal and different concentrations of IBA (0.75, 1.5, 3.0 and 6.0 mg/l) and NAA (0.75, 1.5, 3.0 and 6.0 mg/l) (Table 2). The MS medium without PGRs was used as control. For each treatment seven test tubes, each with one plantlet, were lined up randomly in CRD with three replications. After the shoots were incubated on the rooting medium for 4 weeks, data on number of rooted microshoots, number of roots per microshoot, and average root length (cm) were recorded.

Table 1. Treatment combination for initiation and shoot multiplication experiments.

Cytokinin	IBA	GA_3	Treatment code	
			Initiation	Multiplication
BAP				
0	0	0	IT01	MT01
0.5	0	0	IT02	MT02
1	0	0	IT03	MT03
2	0	0	IT04	MT04
4	0	0	IT05	MT05
0.5	0.01	0.5	IT06	MT06
1	0.01	0.5	IT07	MT07
2	0.01	0.5	IT08	MT08
4	0.01	0.5	IT09	MT09
Kn				
0.5	0	0	IT10	MT10
1	0	0	IT11	MT11
2	0	0	IT12	MT12
4	0	0	IT13	MT13
0.5	0.01	0.5	IT14	MT14
1	0.01	0.5	IT15	MT15
2	0.01	0.5	IT16	MT16
4	0.01	0.5	IT17	MT17

Table 2. Treatment combination for the rooting experiment.

Treatment code	IBA
RT1	½ MS + 0
RT2	½ MS + 0.75
RT3	½ MS + 1.5
RT4	½ MS + 3.0
RT5	½ MS + 6.0
	NAA
RT6	½ MS + 0.75
RT7	½ MS + 1.5
RT8	½ MS + 3.0
RT9	½ MS + 6.0

Acclimatization

Plantlets with well-developed shoots and roots were transplanted on a tray containing a mixture of autoclaved river sand, forest soil and manure in a 2:1:1 (v/v/v) ratio and transferred to greenhouse for hardening. The transplanted plantlets were kept in greenhouse under shade of polyethylene sheets and red cheese cloth, to reduce light intensity and maintain moisture, for one week and were sprayed with water two to three times every day. After 15 days, percentage of plantlets that were successfully acclimatized was recorded.

Statistical analysis

Shoot length, root number and root length data were transferred into square root value for statistical analysis. Rooting percentage data was also transferred into arc sine value for statistical analysis. Significance of the treatment effects was determined by analysis of variance SAS computer software (version 9.1), employing a completely randomized design. Variations among treatment means were assessed by Duncan's Multiple Range Test (DMRT) ($P = 0.05$) (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Shoot Initiation

Analysis of variance revealed that concentration of BAP and Kn alone and in combination with IBA and GA_3 had highly significant effect ($P \leq 0.0001$) on days for shoot initiation, shoot initiation percentage and percent of usable shoots (vigor) (Table 3).

The nodal explants started to initiate after a week of culturing on most of the media. Shoot initiation was observed in all treatments including the control, hormone free MS medium (81% initiation) (Table 4), indicating that the Garmem cultivar has enough endogenous cytokinin and auxin combination for initiation. However, the length of initiated shoots and usable initiated shoots, differ in different treatments (Figure 1). Maximum shoot initiation

Table 3. ANOVA for treatment effects on days for initiation, percent shoot initiation, % usable shoots, shoot number per explant, shoot length and leaf number per shoot.

Source of variation	DF	Mean square of					
		Days for initiation	% shoot initiation	% usable shoot	Shoot no/ explant	shoot length	leaf no/ shoot
Treatments	16	6.09***	389.11***	1949.02***	19.79***	0.05***	15.83***
error	34	0.19	48	27.94	0.23	0.001	0.25
R^2		0.94	0.79	0.97	0.97	0.96	0.97
CV (%)		3.51	8.71	20.3	15.08	2.70	7.21

***, highly significant (P≤ 0.0001) at α=0.05 significant level; R^2 = coefficient of determination; CV = coefficient of variation; DF = Degree of freedom

Table 4. The effect of BAP and Kn alone and in combination with GA_3 and IBA on shoot initiation percentage, percent of usable shoot initiation, mean number of days for shoot initiation, shoot number per explant, shoot length and leaf number per shootlet.

Treatments			Shoot initiation (%)	Usable shoot initiation (%)	days for initiation	Shoot no/ explant	Shoot length	Leaf no/ shoot
BAP	GA_3	IBA						
0	0	0	81±8.24^{cde}	0 ± 0^{e}	11.3±0.6^{e}	1.3±0.6^{e}	0.50 ± 0^{i}	4.7±0.6^{f}
0.5	0	0	81±8.24^{cde}	14.26±0^{d}	12.3±0.6^{d}	4.3±0.6^{c}	0.73±0.06^{fg}	7.7±0.58^{d}
1	0	0	85.7± 0^{bcd}	28.6 ± 0^{c}	10.3±0.6^{f}	6.0 ± 0^{b}	0.87±0.06^{cde}	8.0 ± 0^{cd}
2	0	0	85.7± 0^{bcd}	52.4±8.2^{b}	11.0±0^{ef}	7.3±0.6^{a}	0.67 ±0.06^{gh}	8.0 ± 0^{cd}
4	0	0	76.2±8.2^{def}	0 ± 0^{e}	9.0 ± 0^{g}	2.7±0.6^{d}	0.53± 0.06^{i}	5.3±0.58^{f}
0.5	0.5	0.01	95.2±8.3^{ab}	76.2±8.2^{a}	13.3±0.6^{bc}	4.7±0.6^{c}	1.0 ± 0^{ab}	9.3±0.6^{ab}
1	0.5	0.01	100± 0^{a}	81±8.24^{a}	13.3±0.6^{bc}	5.7±0.6^{b}	0.93±0.12^{bc}	8.7±0.6^{bc}
2	0.5	0.01	85.7 ± 0^{bcd}	57.14±0^{b}	14.3±0.6^{a}	7.7±0.6^{a}	0.80 ± 0^{def}	8.3±0.6^{cd}
4	0.5	0.01	71.43± 0^{efg}	28.57±0^{c}	13.3±0.6^{bc}	6.3±0.6^{b}	0.83±0.06^{cdef}	8.0 ± 0^{cd}
Kn	**GA_3**	**IBA**						
0.5	0	0	66.7±8.3^{fg}	9.5±8.2^{de}	13.3±0.6^{bc}	1.7±0.6^{e}	0.77±0.06^{efg}	8.7±0.6^{bc}
1	0	0	81±8.24^{cde}	14.26±0^{d}	13.00±0^{cd}	1.3±0.6^{e}	0.90±0.1^{bcd}	7.7±0.6^{d}
2	0	0	90.5±8.3^{abc}	28.6 ± 0^{c}	13.00 ±0^{cd}	1.0 ± 0^{e}	1.10 ± 0.2^{a}	9.7±0.6^{a}
4	0	0	81±8.2^{cde}	14.26±0^{d}	13.3±0.6^{bc}	1.0 ± 0^{e}	0.57±0.06^{ih}	6.7±0.6^{e}
0.5	0.5	0.01	52.4± 8.2^{h}	14.26±0^{d}	12.3±0.6^{d}	1.0 ± 0^{e}	0.50 ± 0.06^{i}	5.3±0.6^{f}
1	0.5	0.01	76.2±8.2^{def}	9.5±8.2^{de}	13.00±0^{cd}	1.3±0.6^{e}	0.73±0.06^{fg}	6.7±0.6^{e}
2	0.5	0.01	76.2±8.2^{def}	9.5±8.2^{de}	14.0± 0^{ab}	1.3±0.6^{e}	0.73±0.06^{fg}	6.3±0.6^{e}
4	0.5	0.01	66.7±8.3^{gh}	4.8±8.2^{de}	14.0± 0^{ab}	0 ± 0^{f}	0 ± 0^{j}	0 ± 0^{g}
CV (%)			8.74	20.3	3.51	6.82	2.64	3.55

For each parameter, values followed by different letter are significantly different according to Duncan Multiple Range Test at α=0.05 significant level; CV = coefficient of variation.

on a culture medium augmented with BAP alone was 85.7% and its maximum usable shoot was 52.4%.

The shoot initiation percentage was increased when BAP was combined with 0.5 mg/l GA_3 and 0.01 mg/l IBA. The maximum shoot initiation percentage was 100%, with 81% usable shoot (Figure 2, IT07) on MS medium containing 1.0 mg/l BAP + 0.5 mg/l GA_3 and 0.01 mg/l IBA. However, this treatment was at par with 0.5 mg/l BAP + 0.5 mg/l GA_3 and 0.01 mg/l IBA (Table 4). The shoot initiation percentage was declined when BAP concentrations were decreased and increased in concentration lower and higher than 1 mg/l. The addition of GA_3 and IBA on BAP fortified MS medium increased shoot initiation percentage than BAP alone supplemented MS medium. The combinations of BAP, GA_3 and IBA at definite proportions were very critical and found to be essential in *P. persica* for shoot initiation from nodal explants.

The maximum shoot initiation percentage was recorded on the culture medium containing Kn alone, and it was 90.5% with 28.6% usable shoots. Shoot initiation percentage was declined when Kn concentrations were lower and higher than 2 mg/l. The addition of 0.5 mg/l GA_3 and 0.01 mg/l IBA on MS medium containing Kn did

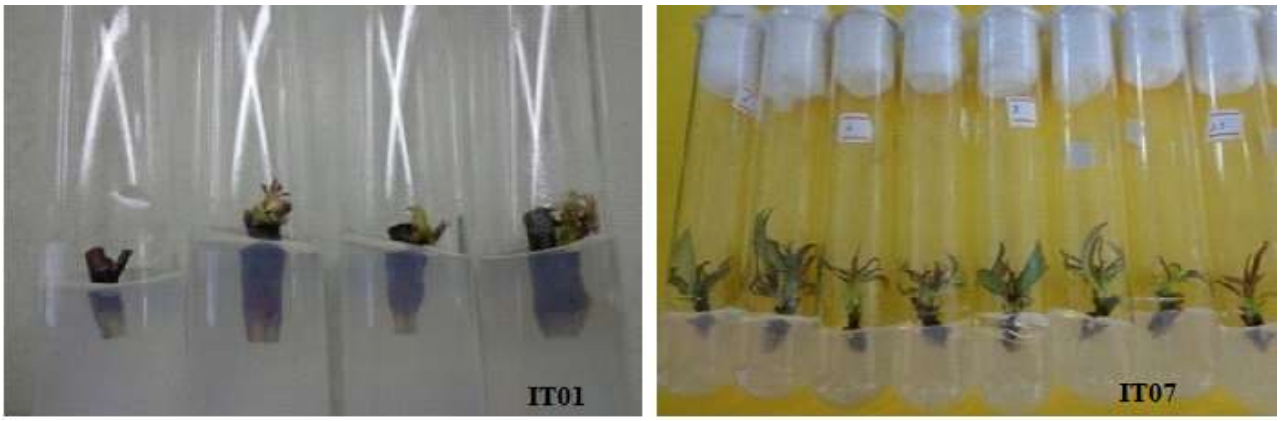

Figure 1. The effect of BAP in combination with GA_3 and IBA on shoot initiation of nodal explants. Note: IT01 = hormone free (control); and IT07 = 1.0 + 0.5 + 0.01 mg/l BAP + GA_3 + IBA.

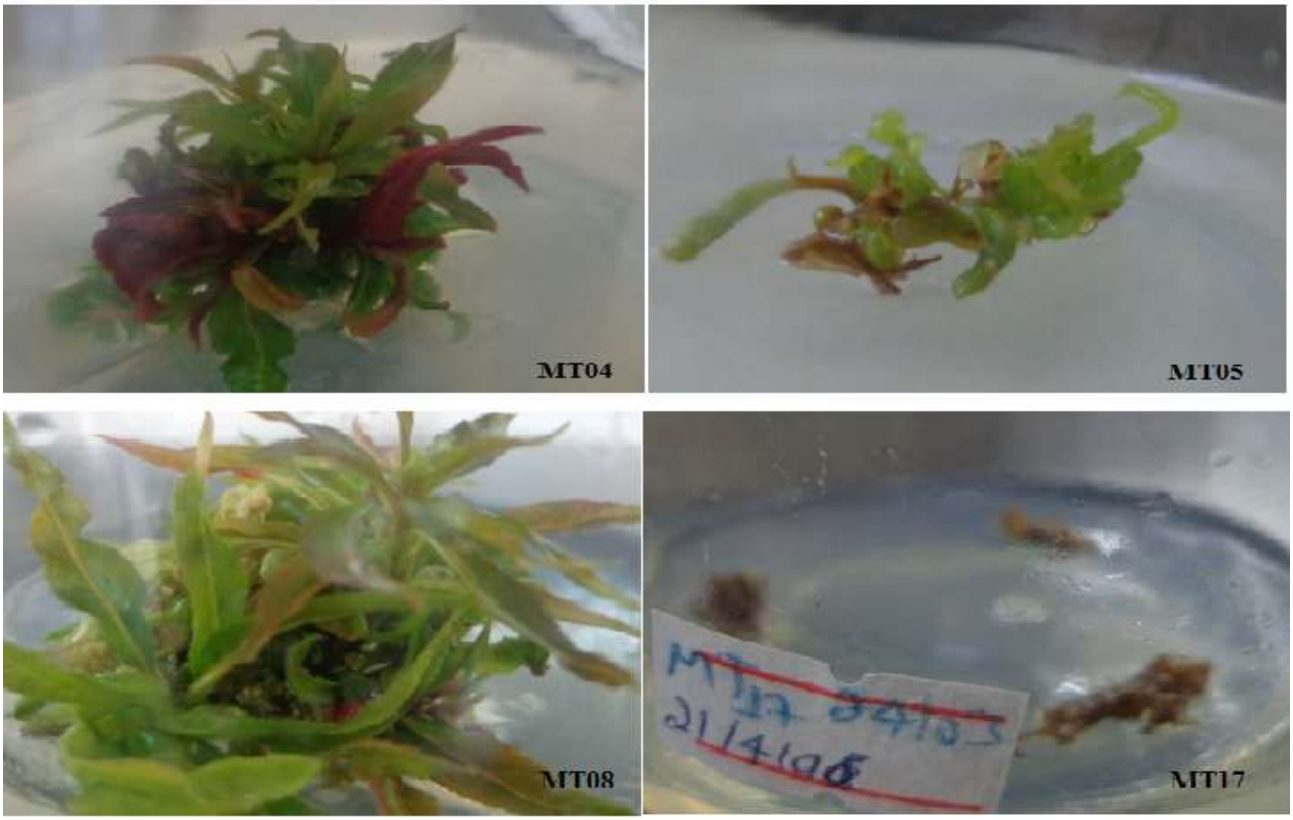

Figure 2. The effect of BAP and Kn alone and in combination with GA_3 and IBA on shoot number per explant, shoot length and leaf number per shoot. Note: MT04 = 2.0 mg/l BAP; MT05 = 4.0 mg/l BAP; MT08 = 2.0 + 0.5 + 0.01 mg/l BAP + GA_3 + IBA; and MT17 = 4.0 + 0.5 + 0.01 mg/l Kn + GA_3 + IBA.

not produce better shoot initiation than Kn alone. The best shoot initiation on culture medium fortified with Kn in combination with GA_3 and IBA was 76.2% with 14.3% usable shoots initiation. Shoot initiation of 81% was recorded, on hormone free culture medium although their length was too short (<0.5 cm) (Figure 2, IT01), and these shoots were dried when sub-cultured. Most of the initiated shoots from the culture medium fortified with BAP in combination with GA_3 and IBA were longer than those shoots regenerated on the culture medium fortified

with BAP and Kn alone and Kn in combination with GA_3 and IBA (Table 4). Kinetin showed poor response to shoot regeneration as compared to BAP. Subbu et al. (2008) reported that BAP (82%) was more effective than Kn (16%) on shoot regeneration of *Saraca asoca*. Mansseri-Lamrioui et al. (2011) also described that 2ip and Kn had lower results in comparison with BAP on *Prunus avium*.

The synergistic effect of BAP, gibberellic acid and auxin has been demonstrated in many plants including *Prunus persica x P. amygdalus* (Fotopoulos and Sotiropoulos, 2005) who noted that BAP and GA_3 in combination produced shoots possessing better macroscopic appearance. Vaghari-Azar et al. (2012) also reported that the addition of GA_3 to the culture medium showed a positive influence on shoot length, confirming the results reported by Dejampour et al. (2007) on peach x almond and apricot x plum inter-specific hybrids. Also, Tsipouridis and Thomidis (2003) reported that exogenous application of GA_3 cause a significant increase in sprouting of resetting plantlets of GF677 (peach x almond hybrid). The hormonal balance: BAP (2 mg/l), IBA 0.1 mg/l and GA_3 0.1 mg/l with 1 mM phloroglucinol showed its positive influence in *P. avium* (Hammatt and Grant, 1997). On the other hand, studies by Nagaty (2012) reported a different result where media containing 3.6 µM TDZ with 2.5 µM IBA was more effective than other TDZ levels in inducing shoot initiation in some other *Prunus* spp. This difference might be due to the genotype effect as reported by various authors (Hammatt and Grant, 1998; Ainsley et al., 2000; Gentile et al., 2002; Perez-Tornero et al., 2000).

The current study revealed that 0.5 and 1.0 mg/l BAP in combination with 0.5 mg/l GA_3 and 0.01 mg/l IBA gave maximum shoot initiation percentage (95.2-100%, respectively), and maximum percentage of usable shoots (76.2- 81%, respectively) which are at par. Therefore, 0.5 mg/l BAP in combination with 0.5 mg/l GA_3 and 0.01 mg/l IBA is recommended for maximum percentage of shoot initiation and usable shoot.

Shoot multiplication

Analysis of variance (Table 3) revealed that the treatment had highly significant effect on mean number of shoots, length of shoot, and mean number of leaves. The combination of IBA, GA_3 and BAP was more effective on shoot multiplication compared to BAP alone. Data indicated that the maximum mean number of shoots (7.3 - 7.7) per explant was found on MS medium containing 2 mg/l BAP alone, and in the combination of 2 mg/l BAP, 0.5 mg/l GA_3 and 0.01 mg/l IBA (Table 4). There was no significance difference regarding shoot number between these two treatments. However, 2 mg/l BAP, 0.5 mg/l GA_3 and 0.01 mg/l IBA showed better number of shoots (7.7) and shoot length (8.0 cm) as compared to 2 mg/l BAP. This is consistent with the findings of Demsachew (2011) on *Malus domestica* cvs. MM106 and Anna. Hammatt and Grant (1997) also reported that the hormonal balance: BAP (2 mg/l), IBA 0.1 mg/l and GA_3 0.1 mg/l with 1 mM phloroglucinol showed positive influence in *P. avium* multiplication. There was no sign of shoot multiplication on medium supplemented with 4.0 mg/l Kn, 0.5 mg/l GA_3 and 0.01 mg/l IBA. All samples cultured in this medium were dried (Figure 2, MT17). The addition of IBA and GA_3 to MS medium fortified with Kn did not produce better shoot number per explant as compared to those explants cultured on MS medium fortified with Kn alone. Shoot multiplication on Kn supplemented MS medium did not differ from shoot multiplication on control MS medium. This indicates that the use of Kn for *P. persica* cv. Garnem micropropagation is not advisable.

Further increase in the concentration of BAP beyond the optimal level reduced the number of shoots indicating an upper limit in concentration. When the concentration of BAP was increased to 2 - 4 mg/l in multiplication medium, the shoots were turned very dwarf and bushy. They also, callused and became to red color (Figure 2, MT04). Generally, higher concentration of BAP had suppressive effect on morphogenesis of *P. persica* (Figure 2, MT05). This is in harmony with the findings of Tiwari et al. (2002). Who noticed that high BAP concentration had an inhibiting effect on further shoot multiplication and growth. Ramage and Williams (2004) also reported that stunted plants were associated with increased exogenous BAP concentration.

The type and concentration of cytokinin influenced shoot multiplication. Among the two cytokinins (BAP/Kn) in combination with GA_3 and IBA, BAP was better and more effective than Kn, for shoot multiplication. This result is in agreement with previous reports of Kalinina and Brown (2007) and Mansseri-Lamrioui et al. (2011) who found multiple shoot formation in nine ornamental *Prunus* species using 1 mg/l BAP, among the studied cytokinins suck as 2ip, Kn and BAP. Muna et al. (1999) and Pruski et al. (2000) also reported that BAP could be used successfully to induce shoot multiplication in *Prunus* spp. The reason for the effectiveness of the BAP may lie in its ability to get metabolized in plant tissues or its ability to induce other natural endogenous hormones for initiation, proliferation and elongation of shoots (Zaerr and Mapes, 1982).

The maximum shoot length (1.1±0.2 cm) was found at 2.0 mg/l Kn and the minimum (0.5 cm) at the control treatment (Table 4). The maximum and minimum number of leaves (9.7±0.6, 4.7±0.6) per shoot was recorded at 2.0 mg/l Kn and control treatment respectively (Table 4). The number of leaves per shoot was increased with the increase in shoot length. The shoot length was higher in the Kn supplemented MS medium than BAP supplemented MS medium. This is consistent with the findings of Ndoye et al. (2003) who stated that Kn promoted shoot elongation in *Balanite aegyptiaca* but led

Table 5. ANOVA for the effect of IBA and NAA on rooting %, root number per shoot and root length.

Source of variation	DF	Mean square of		
		Rooting %	Root number per shoot	Root length
Treatments	8	528.199***	1.2***	1.37***
error	18	1.376	0.036	0.06
R^2		1.0	0.99	0.99
CV (%)		2.5	3.96	6.54

***, highly significant (P≤ 0.0001) at α=0.05 significant level; R^2 = coefficient of determination; CV = coefficient of variation; DF = Degree of freedom.

Table 6. The effect of IBA and NAA on rooting %, root number per shoot and root length.

IBA	NAA	Rooting (%)	No of roots/shoot	Root length (cm)
0	0	0^b	-	-
0.75	0	0^b	-	-
1.5	0	42.86^a	6.3	7.2
3	0	0^b	-	-
6	0	0^b	-	-
0	0.75	0^b	-	-
0	1.5	0^b	-	-
0	3	0^b	-	-
0	6	0^b	-	-
CV (%)		2.5	3.96	6.54

to a decline in shoot multiplication.

Hormone free MS medium produced less number of shoots per explant indicating that hormones are needed to power shoot multiplication. Hence, 2.0 mg/l BAP in combination with 0.5 mg/l GA_3 and 0.01 mg/l IBA is recommended for shoot multiplication of *P. persica* cv. Garnum, since this combination treatment produces the higher number of shoot per explant as well as comparable elongated shoots than the other treatments (Figure 2, MT08).

Root induction

Analysis of variance revealed that concentration of IBA and NAA had highly significant effect (P≤0.0001) on rooting %, root number per shoot and root length (Table 5). The results presented in Table 6 showed that the addition of 1.5 mg/l IBA with 1 g/l AC was the only treatment that initiated roots. The percentage of rooting (42.86%), number of roots per shoot (6.3) and root length (7.2 cm) were recorded (Table 6 and Figure 3A). Earlier studies on peach by Alanagh et al. (2010), reported that the highest rate of rooting (up to 40%) and the maximum number of roots per shootlet (2.62±0.56) were obtained in the induction medium supplemented with 2 mg/l IBA. The influence of IBA on root induction has been reported in many plants and proved effective as compared to NAA (Benelli et al., 2001; Tanimoto, 2005; Ansar et al., 2009).

The application of NAA resulted in no rooting of Garnem shoots. This might be explained due to the NAA resistance to degradation by the auxin-oxidase enzyme (Smulders et al., 1990). Nissen and Sutter (1990) have shown that, in tissue culture, media IAA is rapidly photo-oxidized (50% in 24 h), while IBA is oxidized slowly (10%) and NAA is very stable. Slow movement and delayed degradation of IBA may be the reason for the better performance as compared to IAA and NAA. IBA may also enhance rooting via increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA (Krieken et al., 1993).

Acclimatization

Well-developed rooted plantlets were gently removed from the culture tubes, washed to remove adhered agar and traces of the medium to avoid contamination. Then, they were transferred to plastic planting tray containing a mixture of autoclaved river sand, forest soil and manure in a 2:1:1 (v/v/v) ratio and transferred to greenhouse for hardening. After 15 days the survival rate was 73.3% (Figure 3 D). This result agrees with the findings of Demsachew (2011), who reported 65.7% acclimatization

Figure 3. Root formation after 30 days from cultivation on rooting medium containing 1.5 mg/l IBA (A), Polythelene sheet and cheese close covered shoots (B), shoots at the time of transplantation (C), and shoots after 15 days (D).

in apple cv. Anna. Deepa et al. (2011) also reported that the well rooted plantlets transferred to pots containing a mixture of sterilized sand, soil and vermiculate in a 2:1:1 (v/v/v) ratio. After 2 weeks, the acclimatized plantlets were looked healthy with vigorous growth and 70% survival rate was recorded.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

We are indebted to Haramaya University and Holeta Agricultural Research Center, National Agricultural Biotechnology Laboratory for funding and facilitating the working environment.

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