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León Irapuato,
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Australia*

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*Molecular Mycology and Plant Pathology
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Isfahan
Iran*

Dr. Beatrice Kilel

*P.O Box 1413
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Prof. H. Sunny Sun

*Institute of Molecular Medicine
National Cheng Kung University Medical College
1 University road Tainan 70101,
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Jalan Raja Muda Abdul Aziz
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Ago-Iwoye.
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Dr. Aritua Valentine

*National Agricultural Biotechnology Center,
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*DuPont Industrial Biosciences
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Dr. Sang-Han Lee

*Department of Food Science & Biotechnology,
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Full Length Research Paper

Application of random amplified polymorphic DNA (RAPD) markers to identify *Taxus chinensis* var. *mairei* cultivars associated with parthenogenesis

Yongjun Fei¹, Wei Tang^{1*}, Jinhua Shen¹, Tianjing Zou¹, Rui Qi¹, Bo Xiao¹, Cunyu Zhou¹, Zhixiong Liu¹ and Anna Y. Tang²

¹College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei 434025, People's Republic of China.

²101 Stadium Drive, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, USA.

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The random amplified polymorphic DNA (RAPD) technique has been widely applied to identify different varieties of plants for molecular breeding. However, application of RAPD markers to identify parthenogenesis in plants has not been reported. In this investigation, we used pedigree and RAPD markers to differentiate different *Taxus chinensis* var. *mairei* cultivars that were associated with parthenogenesis. Among 180 and eighty RAPD primers used, 108 primers generated RAPD bands from genomic DNA of *T. chinensis* var. *mairei* cultivars ("Jingzhou" and "Baokang"). Six hundred and thirty-three RAPD loci bands were produced and used to identify eight cultivars with unique banding patterns. Pedigree and RAPD data demonstrated that *T. chinensis* var. *mairei* cultivars with parthenogenesis were separated from others in both "Jingzhou" and "Baokang" cultivars. These results provide evidence for identification of parthenogenesis and confirm that the RAPD technique is especially suitable for identification of *T. chinensis* var. *mairei* cultivars for agricultural purposes.

Key words: Cultivar identification, DNA extraction, parthenogenesis, pedigree, random amplified polymorphic DNA (RAPD), *Taxus chinensis* var. *mairei*.

INTRODUCTION

Molecular markers are of great interest to plant breeders as a source of genetic information on crops and for use in selecting traits to which the markers are linked (Benoit et al., 1997; Mir et al., 2011; Xavier et al., 2011). In classic breeding approach, the breeders expended considerable effort and time in refining the crosses as the tight linkage

or association of the desired characters with the obvious phenotypic characters was never unequivocally established (Bublyk et al., 2013; Degani et al., 1998; Doyle and Doyle, 1990). Compared to classic breeding approach, the advancement in using of molecular markers in plant breeding has become very Common

*Corresponding authors. E-mail: t12y11u28@gmail.com. Tel: +86-13921074062. Fax: +86-716-8066262.

place (Cires et al., 2013; Gidoni et al., 1994). Molecular markers provide advantage to score multiple morphological mutant traits in a single segregating population in which a trait using morphological markers was not practical due to the undesirable pleiotropic effects of many morphological markers on plant phenotype (Graham et al., 1996; Graham and McNicol, 1995). In addition, molecular markers can function as a tag or label for the gene of interest (Graham et al., 1994; Mir et al., 2011; Xavier et al., 2011), important prerequisites exist. In this study, we attempted to use molecular markers to differentiate different cultivars that are associated with parthenogenesis in *Taxus chinensis* var. *mairei*.

The identification of different varieties of plants is a relevant issue especially when it concerns commercially valuable species such as fruits (Levi et al., 1993; MacPherson et al., 1993). Frequently, the varieties obtained through genetic selection for commercial purposes involving high economical interests.

The molecular approach has proved itself an increasingly valuable tool in the identification of plant varieties (Bublyk et al., 2013; Cires et al., 2013; Morell et al., 1995). Some of the commercially desirable ones, such as strawberries, are reproduced by micropropagation; therefore all individuals belonging to a given variety share an identical genome. In this field one of the most successful techniques is random amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al., 1990) which has two main advantages: it allows random sampling of markers over whole genomic DNA and does not require any previous information on the genome of the organism under investigation (Mir et al. 2011; Xavier et al. 2011). RAPD technique has not been used to identify different cultivars that are associated with parthenogenesis in *T. chinensis* var. *mairei*.

T. chinensis var. *mairei* is an important medical plant species in Southern China. Here we present the results of an application of RAPD markers to identify cultivars that are associated with parthenogenesis. The RAPD technique was chosen because it had been successfully applied in crop to estimate genetic distances among varieties (Graham et al., 1996; Benoit et al., 1997) and to genetically characterize different cultivars (Gidoni et al., 1994; Degani et al., 1998). RAPD also proved itself highly effective in this case.

MATERIALS AND METHODS

Plant materials

Eight cultivars of *T. chinensis* var. *mairei* were collected from Jingzhou and Baokang in Southern China (Jingzhou-F, Jingzhou-M, Jingzhou-FM, Jingzhou-P; Baokang-F, Baokang-M, Baokang-FM, Baokang-P). Stems, leaves, and flower cones of *T. chinensis* var. *mairei* were progressively numbered and were used to identify

relationship that is associated with parthenogenesis in *Taxus chinensis* var. *mairei*. All plants were analyzed by RAPD.

DNA extraction

Genomic DNA was extracted as previously described (Tang et al., 2007), using a genomic DNA Isolation kit following the manufacturer's protocol. Small stem, leave, or flower cone pieces were minced by micropestles in the presence of 50 μ L of extraction buffer containing 4% hexadecyl trimethylammonium bromide (CTAB), 1.4 mM NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA and 100 mM Tris-HCl, pH 8. To each minced sample, 350 μ L of the same extraction buffer was added and the samples were incubated for 2 h at 50°C. After incubation three purification steps were performed with equal volumes of phenol, phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), respectively. DNA was precipitated with 2 vols of absolute ethanol and 1/10 vol. 3 mM Na acetate pH 5.2, washed in 70% ethanol, dried and resuspended in TE buffer. The purification procedure was then repeated from the beginning on the previously extracted DNA. This twice-purified DNA finally yielded good amplification products. The amount of DNA was estimated by the minigel method (Maniatis et al., 1982) and the spectrophotometric readings.

RAPD amplification

PCR analysis was performed with a PTC-100TM Programmable Thermal Controller (MJ Research, San Francisco, CA). RAPD amplification reactions were performed in a total volume of 20 μ L with the following final concentrations: A total of 100 ng of genomic DNA was used as a template in a 20 μ L PCR reaction mix. The PCR mixture consisted of 200 μ M each of dATP, dCTP, dGTP, dTTP, 35 pmol of each primer, 2.5 U Taq DNA polymerase (Promega), 1.5 mM MgCl₂, and 5 μ L 10 x buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0 at 25°C, 1% Triton X-100, and 15 mM MgCl₂). Amplifications were carried out in a PTC-100TM Programmable Thermal Controller (MJ Research, San Francisco, CA). The PCR conditions were 95°C for 5 min followed by 29 cycles at 95°C for 60 s, 57°C for 40 s, and 72°C for 90 s. Cycling was followed by a final incubation of 72°C for 10 min. In each thermal cycling a negative control (water instead of template) was included to rule out amplification products due to external contamination. All amplifications were repeated twice for each sample and the PCR products were separated by electrophoresis on 1.0% agarose gels in 1-TAE buffer and were detected by fluorescence under UV light (302 nm) after staining with 0.1% ethidium bromide. A molecular marker of HyperLadder I (Biolone) was used. The results obtained with eight cultivars were confirmed by 180 standard Operon primers (Operon Technologies Inc., Alameda, CA, USA), which yielded satisfying results in the same conditions.

Data analysis

Each gel was run twice and the repeatable bands of each primer were scored as present or absent. The similarity matrix between cultivars was computed using Jaccard's coefficient of similarity (Jaccard, 1908). NTSYS-PC2.1 software was employed for cluster analysis using the data from the similarity matrix and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to construct a dendrogram that represents the genetic relationships among the studied cultivars.



Figure 1. *Taxus chinensis* var. *mairei* cultivars and the development of novel DNA markers for cultivars identification of parthenogenesis. Samples showed in this figure were as follows: (A) 'Jingzhou-M'; (B) 'Jingzhou-P'; (C) 'Jingzhou-F'; (D) 'Jingzhou-FM'. Each of *Taxus chinensis* var. *mairei* illustration shows the positions of stem, flower cone, and leaf in each cultivar.

RESULTS

The *T. chinensis* var. *mairei* cultivars Jingzhou-M, Jingzhou-P, Jingzhou-F, Jingzhou-FM (Figure 1) and Baokang-M, Baokang-P, Baokang-F, and Baokang-FM used in this study were a diverse group of cultivars. *T. chinensis* var. *mairei* Jingzhou-P and Baokang-P are cultivars that are associated with parthenogenesis. Among 180 RAPD primers (OP A1-20, B1-20, C1-20, D1-20, E1-20, F1-20, G1-20, H1-20, and I1-20) used, 108 primers generated RAPD bands (Table 1) and a total of 633 unique bands were obtained. Examples of RAPD gel bands from *T. chinensis* var. *mairei* cultivars Jingzhou-M, Jingzhou-P, Jingzhou-F, and Jingzhou-FM are showed in Figure 2, in which genomic DNA was amplified using primers OP-A09 (Figure 2A) and OP-D10 (Figure 2B).

Examples of RAPD gel bands from *T. chinensis* var. *mairei* cultivars Baokang-M, Baokang-P, Baokang-F, and Baokang-FM are shown in Figure 3, in which genomic DNA was amplified using primers OP-A09 (Figure 3A) and OP-D10 (Figure 3B).

To verify whether RAPD markers can be used to identify *T. chinensis* var. *mairei* cultivars, 633 RAPD bands were used to analyze the genetic relationships among the studied cultivars. The repeatable bands of each primer were scored as present or absent. The similarity matrix between cultivars was computed using Jaccard's coefficient of similarity (Jaccard, 1908) and the UPGMA was used to construct a dendrogram that represents the genetic relationships among the studied cultivars. The 8 analyzed cultivars showed different profiles. The RAPD analyses performed using Opem

Table 1. Nucleotide sequences of selected primers with the number of amplified products and fragment size range (kb)

Primer number	Primer code	Primer sequence (5'-3')	Total band	Monomorphic band	Polymorphic band	Polymorphism (%)	Size range (kb)
1	OP A-01	CAGGCCCTTC	4	3	1	25	0.4-1.9
2	OP A-02	TGCCGAGCTG	6	6	0	0	0.8-3.0
3	OP A-03	AGTCAGCCAC	5	5	0	0	0.8-2.5
4	OP A-04	AATCGGGCTG	3	3	0	0	0.4-1.5
5	OP A-05	AGGGGTCTTG	7	5	2	28.6	0.4-1.8
6	OP A-06	GGTCCCTGAC	2	2	0	0	0.4-1.5
7	OP A-07	GAAACGGGTG	8	7	1	12.5	0.4-1.6
8	OP A-08	GTGACGTAGG	7	6	1	14.3	0.4-1.5
9	OP A-09	GGGTAACGCC	6	6	0	0	0.4-4.0
10	OP A-10	GTGATCGCAG	3	3	0	0	0.4-1.9
11	OP A-11	CAATCGCCGT	6	6	0	0	0.8-3.0
12	OP A-12	TCGGCGATAG	5	5	0	0	0.8-2.5
13	OP A-13	CAGCACCCAC	4	4	0	0	0.4-1.5
14	OP A-14	TCTGTGCTGG	7	7	0	0	0.4-1.8
15	OP A-15	TTCCGAACCC	8	8	0	0	0.4-1.5
16	OP A-16	AGCCAGCGAA	9	9	0	0	0.4-1.6
17	OP A-17	GACCGCTTGT	3	3	0	0	0.4-1.5
18	OP B-01	GTTTCGCTCC	4	3	1	25	0.4-1.5
19	OP B-02	TGATCCCTGG	5	3	2	40	0.4-1.9
20	OP B-03	CATCCCCCTG	2	2	0	0	0.8-3.0
21	OP B-04	GGACTGGAGT	6	5	1	16.7	0.8-2.5
22	OP B-05	TGCGCCCTTC	5	4	1	20	0.4-1.5
23	OP B-06	TGCTCTGCC	4	3	1	25	0.4-1.8
24	OP B-07	GGTGACGCAG	3	3	0	0	0.4-1.9
25	OP B-08	GTCCACACGG	4	3	1	25	0.8-3.0
26	OP B-09	TGGGGGACTC	5	4	1	20	0.8-2.5
27	OP B-10	CTGCTGGGAC	6	4	2	33.3	0.4-1.5
28	OP B-11	GTAGACCCGT	5	4	1	20	0.4-1.8
29	OP B-12	CCTTGACGCA	4	4	0	0	0.4-1.5
30	OP B-13	TTCCCCCGCT	3	3	0	0	0.4-1.6
31	OP B-14	TCCGCTCTGG	6	5	1	16.7	0.4-1.5
32	OP B-15	GGAGGGTGTT	5	5	0	0	0.4-1.5
33	OP B-16	TTTGCCCGGA	7	5	2	28.6	0.4-1.9
34	OP B-17	AGGGAACGAG	3	3	0	0	0.8-3.0
35	OP B-18	CCACAGCAGT	4	3	1	26	0.8-2.5
36	OP B-19	ACCCCCGAAG	5	3	2	40	0.4-1.5
37	OP B-20	GGACCCTTAC	7	6	1	14.3	0.4-1.8
38	OP B-01	GTTTCGCTCC	8	6	2	25	0.4-1.5
39	OP C-11	AAAGCTGCGG	9	6	3	33.3	0.4-1.6
40	OP C-12	TGTCATCCCC	6	6	0	0	0.4-1.5
41	OP C-13	AAGCCTCGTC	7	5	2	28.6	0.4-1.9
42	OP C-14	TGCGTGCTTG	5	5	0	0	0.8-3.0
43	OP C-15	GACGGATCAG	4	4	0	0	0.8-2.5
44	OP C-16	CACACTCCAG	3	3	0	0	0.4-1.5
45	OP C-17	TTCCCCCAG	5	4	1	20	0.4-1.8
46	OP C-18	TGAGTGGGTG	6	4	2	33.3	0.4-1.5
47	OP C-19	GTTGCCAGCC	7	5	2	28.6	0.4-1.6
48	OP C-20	ACTTCGCCAC	8	5	3	37.5	0.4-1.5

Table 1. Contd.

49	OP D-01	ACCGCGAAGG	9	6	3	33.3	0.4-1.5
50	OP D-02	GGACCCAACC	6	5	1	16.7	0.4-1.9
51	OP D-03	GTCGCCGTCA	5	5	0	0	0.8-3.0
52	OP D-04	TCTGGTGAGG	4	4	0	0	0.8-2.5
53	OP D-05	TGAGCGGACA	5	4	1	20	0.4-1.5
54	OP D-06	ACCTGAACGG	6	4	2	33.3	0.4-1.8
55	OP D-07	TTGGCACGGG	7	4	3	42.5	0.4-1.5
56	OP D-08	GTGTGCCCCA	8	6	2	25	0.4-1.6
57	OP D-09	CTCTGGAGAC	5	5	0	0	0.4-1.9
58	OP D-10	GGTCTACACC	3	3	0	0	0.8-3.0
59	OP E-16	GGTGACTGTG	6	5	1	16.7	0.8-2.5
60	OP E-17	CTACTGCCGT	7	5	2	28.6	0.4-1.5
61	OP E-18	GGACTGCAGA	8	6	2	25	0.4-1.8
62	OP E-19	ACGGCGTATG	5	4	1	20	0.4-1.5
63	OP E-20	AACGGTGACC	6	5	1	16.7	0.4-1.6
64	OP F-01	ACGGATCCTG	7	5	2	28.6	0.4-1.5
65	OP F-02	GAGGATCCCT	9	7	2	22.2	0.4-1.5
66	OP F-03	CCTGATCACC	7	5	2	28.6	0.4-1.9
67	OP F-04	GGTGATCAGG	4	4	0	0	0.4-1.5
68	OP F-05	CCGAATTCCC	5	4	1	20	0.4-1.6
69	OP G-11	TGCCCGTCGT	3	3	0	0	0.4-1.5
70	OP G-12	CAGCTCACGA	6	5	1	16.7	0.8-2.5
71	OP G-13	CTCTCCGCCA	7	5	2	28.6	0.4-1.5
72	OP G-14	GGATGAGACC	8	6	2	25	0.4-1.5
73	OP G-15	ACTGGGACTC	9	7	2	22.2	0.4-1.8
74	OP G-16	AGCGTCCTCC	7	6	1	14.3	0.4-1.5
75	OP G-17	ACGACCGACA	6	5	1	16.7	0.4-1.6
76	OP G-18	GGCTCATGTG	4	4	0	0	0.4-1.5
77	OP G-19	GTCAGGGCAA	5	5	0	0	0.4-1.5
78	OP G-20	TCTCCCTCAG	7	5	2	28.6	0.4-1.9
79	OP H-01	GGTCGGAGAA	8	6	2	25	0.4-1.5
80	OP H-02	TCGGACGTGA	9	7	2	22.2	0.4-1.6
81	OP H-03	AGACGTCCAC	7	6	1	14.3	0.4-1.5
82	OP H-04	GGAAGTCGCC	6	5	1	16.7	0.8-2.5
83	OP H-05	AGTCGTCCCC	4	3	1	25	0.4-1.9
84	OP H-06	ACGCATCGCA	7	7	0	0	0.8-2.5
85	OP H-07	CTGCATCGTG	6	5	1	16.7	0.4-1.5
86	OP H-08	GAAACACCCC	4	4	0	0	0.4-1.8
87	OP H-09	TGTAGCTGGG	3	3	0	0	0.4-1.5
88	OP H-10	CCTACGTCAG	7	7	0	0	0.4-1.6
89	OP I-01	ACCTGGACAC	8	7	1	12.5	0.4-1.5
90	OP I-02	GGAGGAGAGG	9	8	1	11.1	0.4-1.5
91	OP I-03	CAGAAGCCCA	7	7	0	0	0.4-1.9
92	OP I-04	CCGCCTAGTC	5	5	0	0	0.4-1.5
93	OP I-05	TGTTCCACGG	4	3	1	25	0.4-1.6
94	OP I-06	AAGGCGGCAG	6	6	0	0	0.4-1.5
95	OP I-07	CAGCGACAAG	8	7	1	12.5	0.4-1.5
96	OP I-08	TTTGCCCGGT	9	7	2	22.2	0.4-1.8
97	OP I-09	TGGAGAGCAG	7	6	1	14.3	0.4-1.5
98	OP I-10	ACAACGCGAG	6	6	0	0	0.4-1.7

Table 1. Contd.

99	OP I-11	ACATGCCGTG	5	5	0	0	0.4-1.5
100	OP I-12	AGAGGGCACA	4	4	0	0	0.4-1.6
101	OP I-13	CTGGGGCTGA	8	8	0	0	0.4-1.5
102	OP I-14	TGACGGCGGT	7	6	1	14.3	0.4-1.9
103	OP I-15	TCATCCGAGG	6	6	0	0	0.4-1.5
104	OP I-16	TCTCCGCCCT	7	7	0	0	0.4-1.6
105	OP I-17	GGTGGTGATG	9	9	0	0	0.4-1.8
106	OP I-18	TGCCCAGCCT	5	3	2	40	0.4-1.5
107	OP I-19	AATGCGGGAG	6	6	0	0	0.4-1.9
108	OP I-20	AAAGTGCGGG	8	6	2	25	0.4-1.5

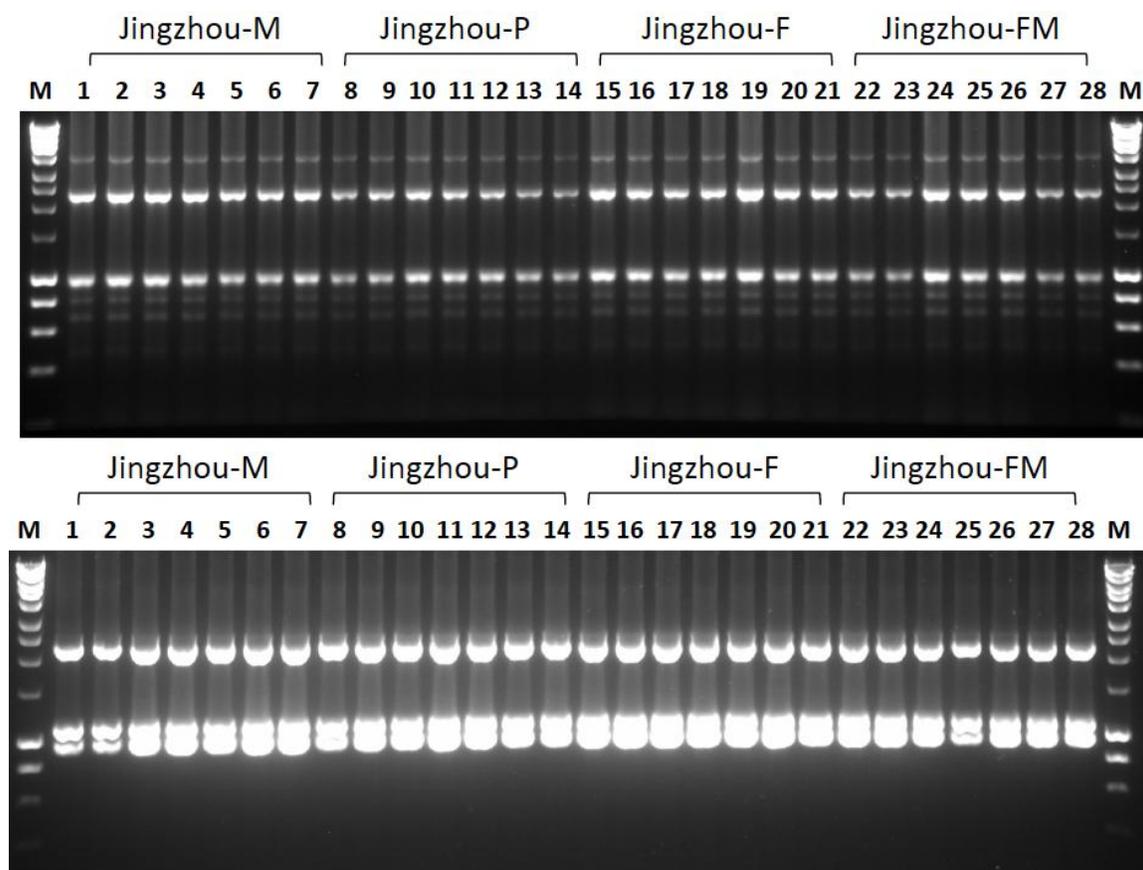


Figure 2. Cultivars-specific patterns detected by primers using the sequence data of each polymorphic band. Representative banding pattern as revealed by primers OP-A09 (A) and OP-D10 (B) (Lanes M: DNA Markers; 1, 8, 15, and 22: PCR bands amplified from DNA of flower cone; 2, 9, 16, and 23: PCR bands amplified from DNA of stem; 3-7, 10-14, 17-21, and 24-28: PCR bands amplified from DNA of leaf in *Taxus chinensis* var. *mairei* cultivars Jingzhou-M, Jingzhou-P, Jingzhou-F, Jingzhou-FM, respectively).

primers suggested that 4 out of 8 cultivars did not belong to the same group, because they showed unambiguous, reproducible and consistent bands not shared by the 'Baohkang' samples (Figures 2 and 3). The remaining 4

cultivars had profiles whose bands were all shared by the 'Jingzhou' pattern. These differences in profiles could be ascribed to genetic information.

The DNA from the above mentioned 8 cultivars was also

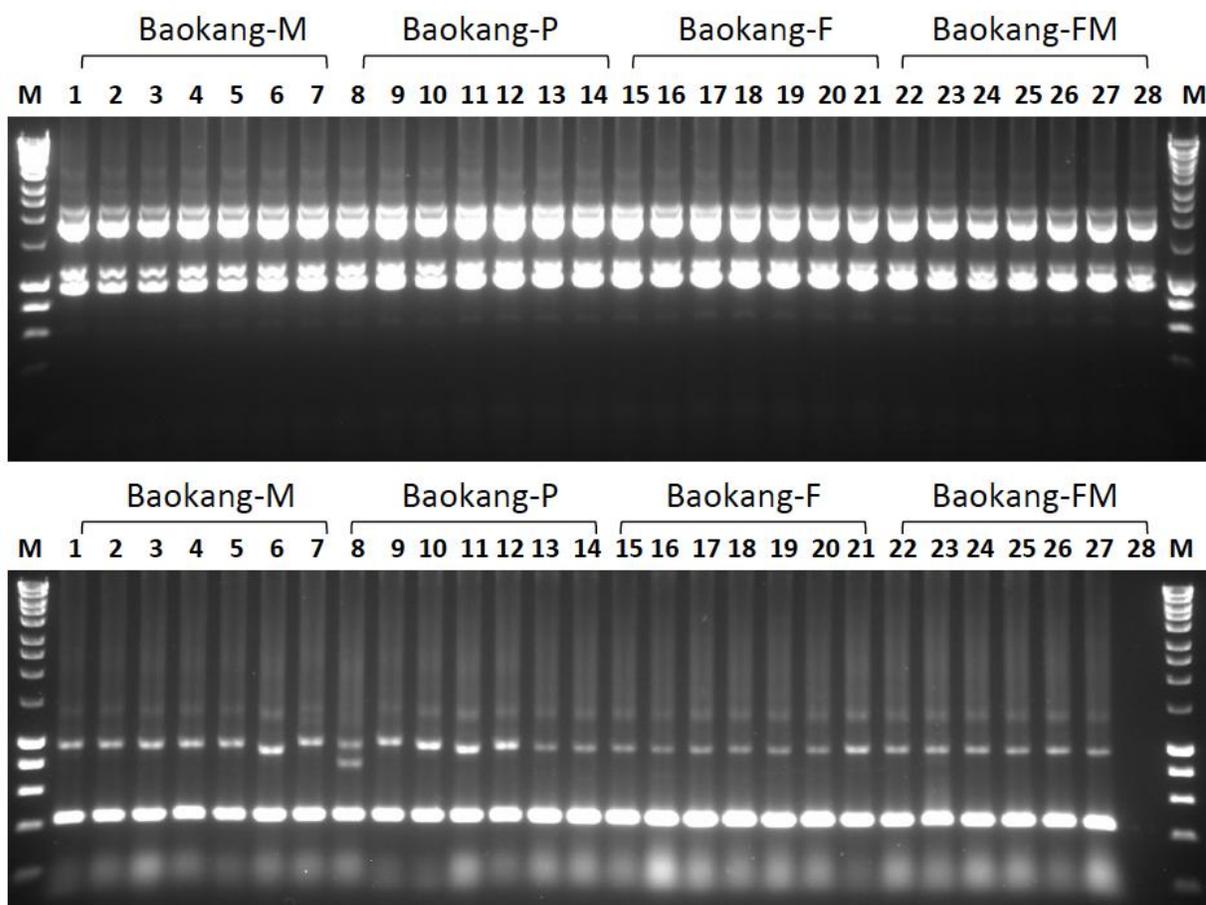


Figure 3. RAPD profiles of *Taxus chinensis* var. *mairei* cultivar (Baokang) with primer. Representative banding pattern as revealed by primers RAPD-OP-H06 (A) and RAPD-OP-I18 (B) (Lanes M: DNA Markers; 1, 8, 15, and 22: PCR bands amplified from DNA of flower cone; 2, 9, 16, and 23: PCR bands amplified from DNA of stem; 3-7, 10-14, 17-21, and 24-28: PCR bands amplified from DNA of leave in *Taxus chinensis* var. *mairei* cultivars Baokang-M, Baokang-P, Baokang-F, Baokang-FM, respectively). Lane 28 in Baokang-FM is the negative control.

re-amplified by Operrn primers with different amounts of template and the results are clearly shared the complete profile, except Jingzhou-P, which showed a slightly different profile from that of Jingzhou-M, Jingzhou-F, and Jingzhou-FM.

By varying template amount, subsequent amplifications with the same primer always yielded a perfectly identical profile to the 'Jingzhou' one. The same results were obtained after re-extracting and re-amplifying DNA from Jingzhou-P. This supports the fact that a critical issue in RAPD experiments is the amount of template employed and, consequently, the correct quantification of extracted DNA. The 4 cultivars showing a profile identical to 'Jingzhou' with Operrn primers Group A, B, C, D, E, F, G, H, and I also yielded profiles identical to 'Baokang' with the previously listed OPA primers. The other 4 cultivars did not show profiles identical to 'Baokang'. The results of RAPD analyses were double-checked.

Cluster analysis of the pedigree data yielded results that were more or less expected (Figure 4). The *T. chinensis* cultivars 'Baokang' and 'Jingzhou' did not join, but 'Jingzhou-M, Jingzhou-P, and Jingzhou-FM' did join 'Jingzhou-F', which also has *T. chinensis* var. *mairei* germplasm in its background. 'Baokang-M', 'Baokang-P', 'Baokang-FM', 'Baokang-F' were all joined together. The Mantel matrix correlation test generated a value of $r = 0.95$, suggesting a very good fit of the data to the resulting dendrogram (Table 2). Overall, 633 RAPD loci were used to calculate the similarity estimates, a number that Fu et al. (2002) deemed to be within an acceptable range. Of this number, most were polymorphic due to the inclusion of the *T. chinensis* var. *mairei*. Eight cultivars were identified specifically within these sets of primers. When compared amongst themselves, the *T. chinensis* var. *mairei* displayed from 74.9% ('Jingzhou-F') to 82.1% (Jingzhou-M) similarity, with the average at 75% for

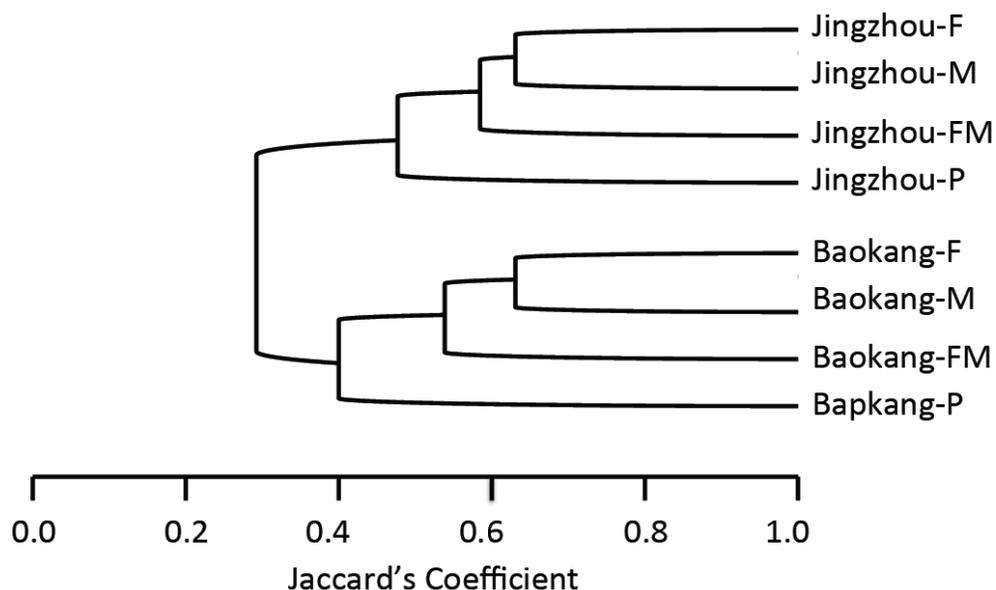


Figure 4. Dendrogram illustrating genetic similarity (Jaccard's coefficient) among eight *Taxus chinensis* var. *mairei* cultivars generated by UPGMA cluster analysis calculated from 633 RAPD bands produced by 108 primers.

Table 2. Genetic similarity matrix of eight *Taxus chinensis* var. *mairei* cultivars generated by 108 RAPD primers

Parameter	Jingzhou-F	Jingzhou-M	Jingzhou-FM	Jingzhou-P	Baokang-F	Baokang-M	Baokang-FM	Baokang-P
Jingzhou-F	1.000							
Jingzhou-M	0.749	1.000						
Jingzhou-FM	0.735	0.796	1.000					
Jingzhou-P	0.821	0.864	0.824	1.000				
Baokang-F	0.769	0.736	0.729	0.754	1.000			
Baokang-M	0.892	0.825	0.736	0.812	0.817	1.000		
Baokang-FM	0.786	0.768	0.815	0.726	0.763	0.789	1.000	
Baokang-P	0.843	0.689	0.739	0.796	0.812	0.816	0.786	1.000

RAPD marker data.

DISCUSSION

RAPD results are reliable for the remaining pairwise comparisons (Welsh and McClelland, 1990; Parent et al., 1993). Pedigree and RAPD similarity matrices produced results that were comparable (Bublyk et al., 2013; Cires et al., 2013; Parent and Pagé, 1992; Trople and Moore, 1999).

In the present investigation, the RAPD results were completely reliable because the percentage of 'Jingzhou' and 'Baokang' identification was 100% (8 out of 8). The results were accepted as strong evidence. This study confirms that the RAPD technique, which is easy, fast

and inexpensive, is especially suitable for identification of *T. chinensis* var. *mairei* cultivars for agricultural purposes. Each primer yields a typical electrophoretic profile and in this way the number of marker bands suitable for variety fingerprinting can be improved simply by performing further amplifications with different primers.

On the other hand, the sensitivity of the RAPD technique requires repeated amplifications with different amounts of template to avoid artifacts and obtain reliable results. The Mantel test revealed a correlation of $r = 0.95$, a very good fit of the similarity matrix data to the cluster analysis. In the RAPD dendrogram, 'Baokang' and 'Jingzhou' was a distinct cluster from the *T. chinensis* var. *mairei* cultivars. This result is likely more accurate than that of the pedigree dendrogram because of the taxonomic designation of 'Jingzhou' as a *T. chinensis* var.

mairei.

However, some discrepancies became evident. 'Jingzhou-P' and 'Baokang-P' are cultivars that are associated with parthenogenesis. Surprisingly, 'Jingzhou-P' and 'Baokang-P' did not join together, and were not in the same cluster. Yet, 'Baokang-F' and 'Baokang-M' did join together, and had the highest similarity percentage of any pair of cultivars (75%). This is comparable to the result in the pedigree similarity matrix (71%). Baokang-M shows more similarity to 'Baokang-F' than to 'Baokang-P'. Thus, the contribution of 'Baokang-M' to both 'Baokang-FM' and 'Baokang-F' could account for their degree of similarity detected in this study. When the matrices of the pedigree data and the RAPD data were tested with the Mantel matrix correlation the result was correlation ($r = 0.95$), therefore the pedigree and RAPD matrices may be considered as relating to one another in a meaningful way.

In conclusion, from the results obtained in this investigation, there is a correlate relationship between the pedigree and RAPD data. The pedigree and RAPD data did correlate for cultivars that shared many of the same founding markers, as the results tended to overestimate relatedness. If a more accurate assessment of pedigree relatedness among cultivars was used, then the results may have been more precise. In general, RAPD marker data proved to be a good method of assessing genetic relatedness among different *T. chinensis* var. *mairei* cultivars that are associated with parthenogenesis. Therefore, RAPD markers can effectively differentiate closely related *T. chinensis* var. *mairei* cultivars involved in parthenogenesis, as well as more distantly related *T. chinensis* cultivars.

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Conflict of Interests

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

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Full Length Research Paper

Genome polymorphism markers and stress genes expression for identifying turf species

Hany M. El-Naggar

Horticulture Department, Alexandria University, Egypt.

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Reactive oxygen species (ROS) are produced in both stressed and unstressed cells. Superoxide dismutase (SOD) and phenylalanine ammonia lyase (PAL) play an important role in the defense against ROS. Eight different turf grass species were used in order to detect their ability to withstand environmental stress through investigating SOD and PAL gene expression and also the genetic relationship among them using random amplified polymorphic DNA (RAPD) molecular markers. The levels of expression of PAL, SOD genes and mRNA varied with the type of turf; both PAL and SOD gene expressions were low in cold season turf grasses (kentucky blue grass and fine fescue), moderate for bermuda hybrids (tifgreen and tifway) and high in *Paspalum vaginatum*. Primer 3 (UBC-245) can be used to distinguish between *Paspalum* species, also between common bermuda (*Cynodon dactylon*) and bermuda hybrids. It was concluded that hot season genera can withstand environmental stress more than cold season ones since they have more SOD and PAL gene expressions. Also, DNA markers can be used to differentiate between different turf genera which are hard to be differentiated morphologically.

Key words: Turf, superoxide dismutase, phenylalanine ammonia lyase, RAPD markers, oligonucleotide primers, bermuda hybrids.

INTRODUCTION

Reactive oxygen species (ROS) are produced in both stressed and unstressed cells. Plants have developed a defense system against ROS for limiting the formation of ROS as well as organizing its removal. In the cell, the superoxide dismutase (SOD) constitute the first line of defense against ROS. SOD is found in plant cell wall, mitochondria, chloroplast, microsomes, gloxysomes, apoplast and cytosol (Ruth et al., 2002). Phenylalanine ammonia lyase (PAL) is the enzyme at the entry-point of

the phenylpropanoid pathway, which yields a variety of phenolic compounds, salicylic acid and lignin with structural and defense-related functions. PAL activity has been considered to be part of a defense mechanism operating in stress-afflicted cells (Dixon and Pavia, 1995; Yang and Shetty, 1998). Identifying turf grass phenotypes based on morphological traits involves a lengthy survey of plant growth that is labor intensive and vulnerable to environmental conditions (Lin and Hong, 1994), also

*Corresponding author. E-mail: hanyelnaggar@hotmail.com. Tel: 002-0100-7340409.

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Abbreviations: SOD, Superoxide dismutase; PAL, phenylalanine ammonia lyase; ROS, reactive oxygen species.

identifying cultivar breeding lines is critical for turfgrass industries to control germplasm quality and protect their rights (Lin and Hong, 1994).

DNA polymorphisms amplified by oligonucleotide primers, 9 or 10 nucleotides in length or longer, were used as genetic markers for fingerprinting (Gustave et al., 1991; Hu and Quiros, 1991). Isoenzyme and protein electrophoresis method that have been developed to identify turfgrass has disadvantages such as the limited amount of polymorphism that can be detected among closely related genotypes, the quality and quantity of isoenzymes and proteins may be subject to variation due to environmental conditions during plant growth and development therefore DNA-based procedures and genetic markers have been proposed for improving turfgrass identification (Lin and Hong, 1994). DNA markers are not typically influenced by environmental conditions and therefore can be used to describe patterns of genetic variation among plant populations (Zabeau and Vos, 1993). The randomly amplified polymorphic DNA (RAPD) markers techniques is quick, easy and requires no prior sequence information; it detects nucleotide sequence polymorphisms using single primer of arbitrary nucleotide sequence (Williams et al., 1990).

Since the identification and differentiation of different turf grass phenotypes based on morphological traits is very difficult and also the mixing of different turf varieties in landscape uses, therefore the main aim of this research was to study the genetic diversity between eight commercial turf grasses in order to distinguish between and to detect the genetic relationship among them using RAPD markers and also to compare between the eight turf grasses in their ability to withstand abiotic and environmental stress through investigating the SOD and PAL gene expressions thus enhancing the selection of drought-tolerant cultivars for landscape uses.

MATERIALS AND METHODS

Plant material

Eight different turf grass species were brought from a private nursery and planted in the greenhouse of the Horticulture department of the Faculty of Agriculture Alexandria University. In 20 cm pots, the grasses were irrigated as necessary to prevent drought stress, mowed twice a week and fertilized weekly with water soluble fertilizer of 20:20:20 (N:P:K). The eight turf species were: Bermuda grass (*Cynodon dactylon*), Tifway (*C. dactylon* x *Cynodon transvalensis*), Tifgreen (*C. dactylon* x *C. transvalensis*), *Paspalum vaginatum*, *Paspalum dilatatum*, St. Augustine grass (*Stenotaphrum secundatum*), fine fescue (*Festuca rubra*) and Kentucky bluegrass (*Poa pratensis*).

RNA extraction and purification for SOD and PAL gene expression

Fresh leaf tissues (100 mg), from the eight turf species, were used for the extraction of total RNA. Total RNA extraction was done using GeneJET RNA Purification Kit (Fermentas) according to the manufacturer's instructions.

The first strand cDNA synthesis

According to the manufacturer's instructions, reverse transcription of the purified RNA was performed by first strand cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Fermentas).

Primer design for the Mn SOD and the PAL gene expression

The sequence of the Mn SOD gene was taken from *Oryza sativa* a related plant from the same family (graminae). The sequence was obtained from the gene bank (www.ncbi.gov) under accession number GQ848046. The primer was designed using www.ncbi.nlm.nih.gov/tools/primer-blast tool. The sequence of the forward primer for the Mn SOD was GTCGCCAACTACAACAAGGC and the reverse primer was TGCAGGTAGTACGCATGCTC with product length of 413 bp. While, the forward primer used for the PAL gene was GGGTCTCTCTACCAGGTGTTAT and the reverse primer sequence was GATCACGTCTTCATTACGACC, each of forward and the reverse primers were used to amplify about 350 bp.

DNA extraction and purification for the RAPD analysis

Total DNA was extracted from the plants using gene jet TM plant genomic DNA purification Mini Kit # K0791, # K0792 from Fermentas. 100 mg of young leaves were taken from each plant and thoroughly washed with water then ethanol to remove dust and other contaminants and then milled under liquid nitrogen. The DNA was extracted using Fermentas plant tissue DNA purification kit according to the manufacturer's instructions. DNA existence was tested using electrophoresis on 1.2% agarose gels. DNA was stored at -20°C for further work.

Primers design for the RAPD analysis

Six oligonucleotide primers, 10 nucleotides in length were brought from Bioneer Korea and used for RAPD analysis for the eight turf grasses (Table 1). Two PCR reactions were performed one for the gene expression and one for the RAPD analysis, each PCR reaction was repeated twice to insure precision. Master Mix (Dream Taq TM green PCR Master Mix (2x) containing (DNA polymerase + optimized green buffer + MgCl₂ and dNTPs.) from Thermo-Scientific was used. For the RAPD analysis, the PCR amplification was performed with initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 30 s extension at 72°C for 30 s, and a final extension at 72°C for 5 min and storage at 4°C using thermal cycler Techne Endurance TC 3000 while, for the Mn SOD and PAL gene expression, the PCR reaction was initial denaturation at 94°C for 3 min, 35 cycles of denaturation 94°C for 30 s, annealing at 55°C for 30 s extension at 72°C for 30 s, and a final extension at 72°C for 5 min and storage at 4°C.

Gel electrophoresis

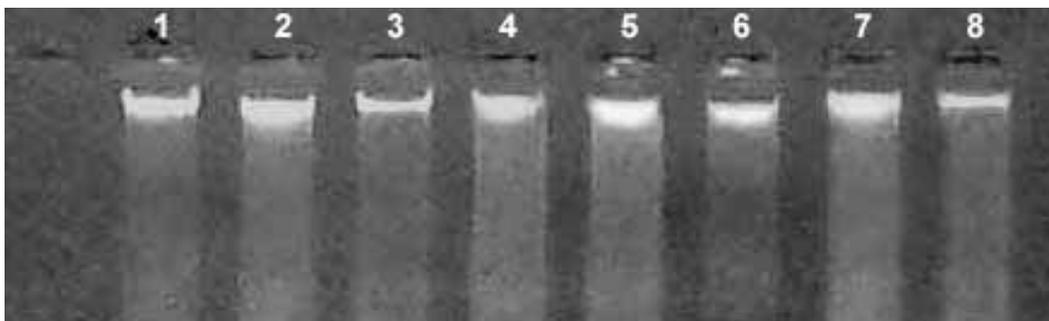
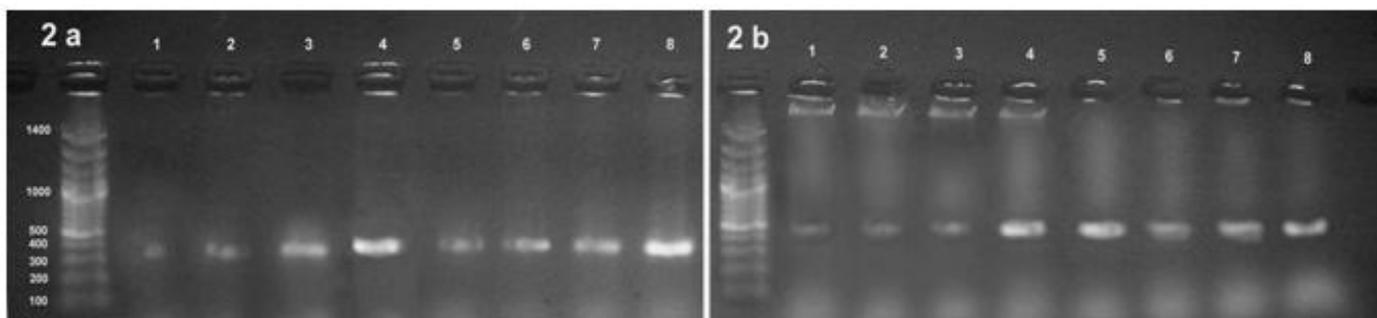
PCR amplified products were subjected to electrophoresis in a 1.5% agarose gel containing Ethidiumbromide in (1x) TBE buffer at 100 V for 40 min using Cleaver submarine electrophoresis unit. Gene ruler 100 bp plus DNA ladder from Fermentas was used to identify the DNA amplified bands.

Data analysis

Gelquant program for quantification of protein, DNA and RNA gel (version 1.8.2) was used for the quantification of bands for the Mn

Table 1. Primers used in RAPD-PCR reactions.

S/N	Primer	Sequence (5'-3')
P1	OPD-05	TGAGCGGACA
P2	OPH-20	GGGAGACATC
P3	UBC-245	CGCGTGCCAG
P4	UBC-261	CTGGCGTGAC
P5	OPC-12	TGTCATCCCC
P6	UBC-231	AGGGAGTTCC

**Figure 1.** Gel electrophoresis of the DNA extracted from leaf samples of the eight turf species. 1, Kentucky bluegrass; 2, fine fescue; 3, St. Augustine; 4, *P. vaginatum*; 5, *P. dilatatum*; 6, Tifgreen; 7, Tifway; 8, common bermudagrass.**Figure 2. (a)** Gel electrophoresis of PCR reaction showing bands amplified between 300 and 400 bp for the PAL gene with different band intensity. **(b)** Gel electrophoresis showing bands between 400 and 500 bp for the MnSOD gene. 1, Kentucky bluegrass; 2, fine fescue; 3, St. Augustine; 4, *P. vaginatum*; 5, *P. dilatatum*; 6, Tifgreen; 7, Tifway; 8, common bermudagrass.

SOD and PAL gene expression. The cluster analysis and polymorphic tree was done by PyElph software system for gel image analysis and phylogenetics (version 2.6.5).

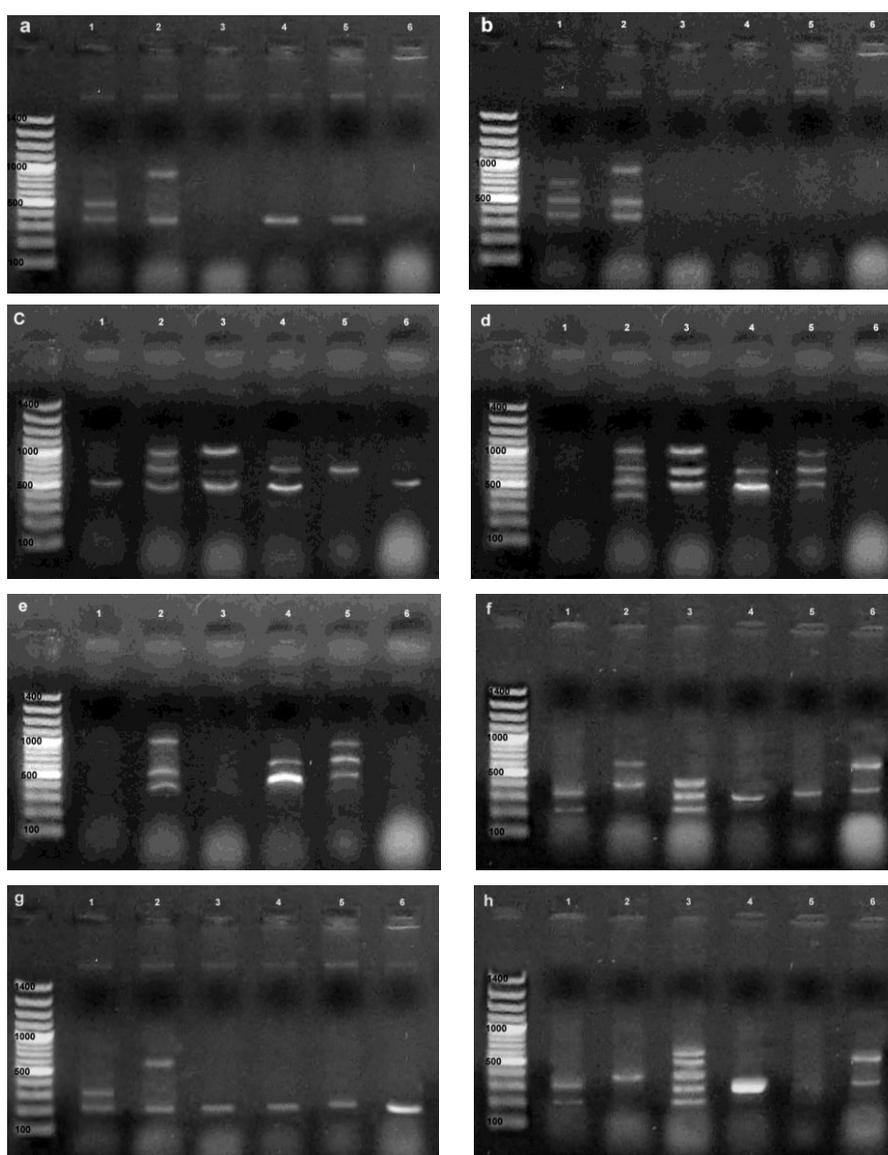
RESULTS

Highly purified DNA extracts of the eight species were used as templates for RAPD-PCR analysis. In all species evaluated, DNA extracted from leaf samples using the described protocol was successfully amplified (Figure 1). The results show bands between 300 and 400 bp which is consistent with the 341 bp of the PAL gene primers

(Figure 2a). Also, bands between 400 and 500 bp were consistent with the 413 bp of the SOD gene primers (Figure 2b). The PAL gene expression was low for Kentucky bluegrass, fine fescue and *P. dilatatum*, moderate for St. Augustine, tifgreen and tifway and high for *C. dactylon* followed by *P. vaginatum* (Figure 2a). For the SOD gene expression, it was low in cold season turf grass (kentucky bluegrass and fine fescue), moderate for the Bermuda cultivars and high in both *Paspalum* species (Figure 2b). Data in Table 2 and Figures 3 shows that the number of amplified fragments differed with different primers. The phylogenetic tree (Figure 4) of the eight turf

Table 2. The number of amplified fragments using different primers for the RAPD analysis.

Primer	Number of fragments							
	Kentucky	Fine fescue	St Augustine	<i>P. vaginatum</i>	<i>P. dilatatum</i>	Tifway	tifgreen	<i>C. dactylon</i>
OPD-05	3	2	1	0	0	2	2	2
OPH-20	3	2	3	3	4	2	2	1
UBC-245	0	0	3	0	3	3	1	5
UBC-261	0	1	2	2	2	1	1	1
OPC-12	0	1	1	4	3	1	1	0
UBC-231	0	0	1	0	0	2	1	2
Total number of bands	6	6	11	9	12	11	8	11



Figures 3. Gel electrophoresis of RAPD-PCR reaction of the eight turf species. a, Kentucky bluegrass; b, fine fescue; c, St. Augustine; d, *P. vaginatum*; e, *P. dilatatum*, f, Tifgreen; g, Tifway; h, common bermudagrass. Lanes from 1 to 6 indicates primers OPD-05, OPH-20, UBC-245, UBC-261, OPC-12, UBC-231 respectively.

enzymes such as SOD is related to induced oxidative stress tolerance in grass. Dombrowski et al. (2008) mentioned that the plants can increase level of tolerance by reprogramming the expression of endogenous genes. Higher level of these antioxidant enzyme activities is considered as one of the tolerance mechanism in most plants (Ashraf, 2009). Bermuda grass species are classified as semi-tolerant to tolerant species to drought stress (Kamal et al., 2012). Common Bermuda grass tolerates drought and salinity stresses by increasing the activity of antioxidant enzymes and could be grown under moderate combined drought and salinity stresses without considerable damage to plants at the physiological or biochemical level. Therefore, common bermuda grass can be recommended for culture in semi-arid areas with limited water resources and areas with semi-saline water resources (Reza and Hassan, 2014). SOD gene expression was low in cold season varieties and this was also found by Zhang and Schmidt (1999) who mentioned that drought stress also had no effect on leaf SOD activity in Kentucky bluegrass.

RAPD is a multiplex marker system that conventionally uses single-primer PCR to amplify random DNA fragments (Kumar et al., 2009). The fragment size produced by primers ranged from 200 to 1000 BPs. The number and size of DNA fragments found in the RAPD profiles varied among turf species. A number of unique DNA fragments were found between the two paspalum species among primer 3 (Figure 3d and e) can be used to distinguish between the two paspalum species. To distinguish between tifway and tifgreen, two extra bands were found in tifway using primer 3 while the same primer gave 5 bands with *C. dactylon* (Figure 3f, g and h). This difference may be due to the difference in the amount of genetic variation that exists between the different species. The primers and conditions for DNA amplification in this study produced reasonably RAPD markers that can be used to identify different turf cultivar. More researches are needed to be done to establish a more broadly applicable DNA fingerprint for identifying different turf grasses.

Conclusion

In general, hot season species have SOD and PAL gene expressions more than cold season ones. Also, oligonucleotide primers can be used to differentiate between different turf varieties which are hard to differentiate morphologically. Primer 3 (UBC-245) can be used to distinguish between *Paspalum* species and also between *C. dactylon* and bermuda hybrids.

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Full Length Research Paper

Combining ability and heterosis for phenologic and agronomic traits in maize (*Zea mays* L.) under drought conditions in the Northern Guinea Savanna of Borno State, Nigeria

D. Aminu¹ Y. M. Garba² and A. S. Muhammad^{1*}

¹Department of Crop Production, Faculty of Agriculture, University of Maiduguri, Borno State, Nigeria.

²College of Education, Hong, P. M. B. 2237 Yola, Adamawa State, Nigeria.

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Five International Institute of Tropical Agriculture (IITA) drought tolerant and open pollinated varieties (OPVs) (lines) and four local varieties with various level of susceptibility to drought (testers) were used. These maize varieties were crossed using a line x tester mating design during the 2007 cropping season to determine the general combining ability (GCA) and specific combining ability (SCA); also the level of heterosis was investigated. Parental lines and hybrids were evaluated in Biu in 2008 and 2009 cropping seasons. Results from the analysis of variance and combining ability shows that there was high and significant level ($P < 0.05$) of genetic variability among the parental lines used and their hybrids in days to tasseling, days to silking, anthesis silking interval (ASI), plant height, ear height, weight of cobs, dehusked cobs and grain yield, thus suggesting the possibility for genetic improvement. The study established that, there were significant ($P < 0.05$) differences of GCA effects of parents and that of SCA effects of hybrids. Estimates of GCA were consistently lower than SCA effects in almost all the traits evaluated. This suggests that high performing hybrids such as EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE, EVDT-99WSTRC0 x EX-DAMBOA YELLOW and TZE-COMP₃DTC₁ x EX-DAMBOA WHITE may be used to develop potential varieties. The parents: EVDT-99WSTRC0, EVDT-99WSTRQPMC0, TZE-COMP₃DTC₁ and EX-BIU WHITE were identified as the best general combiners in terms of GCA for days to tasseling, days to silking, ASI, plant height, ear height, dehusked cobs and grain yield.

Key words: Maize, combining ability, additive, non-additive genetic effects, drought tolerance.

INTRODUCTION

Maize (*Zea mays* L.) is a tropical cereal and belongs to the plant family Gramineae (Poaceae). Globally, maize is

ranked the third most important cereal crop, after wheat and rice (Jaliya et al., 2011). It is one of the most widely

*Corresponding author. E-mail: elsas.sas@gmail.com.

cultivated cereal crops due to its adaptation to a wide range of environments. It is also a major staple food crop in Nigeria and in many developing countries receiving much attention in industrial development.

World maize production was estimated to be 950 million tonnes, for the 2012/2013 season, an increase of 9% from 2011/2012 (Brandt 2013). However, according to International Institute of Tropical Agriculture (IITA, 2011); worldwide maize production is 785 million tonnes with the largest producer, the United States, producing 42%. Africa imports 28% of the required maize from countries outside the continent.

As a result of continuous shortage and unpredictability of rains in the drier areas of the world, possibly due to the effect of climate changes (Sodangi et al., 2011), research attention is being directed toward producing maize hybrids that can withstand moisture stressed ecologies. Drought is one of the most important environmental stresses affecting agricultural productivity worldwide and can result in a considerable yield loss (Ludlow and Muchow, 1990). It is a major abiotic constraint to maize production which is mostly rain fed in Africa. A lack of adequate rainfall can lead to decrease in yield and trigger food shortages. The effect of drought on maize production and food supplies are most severe in the dry savanna zone of West Africa (Fajemisin et al., 1985). This is because rainfall in this region is very unpredictable in terms of timing (may start early or very late in the season), quantity (some times less than 600 mm/annum) and distribution (could be poorly distributed) (Izge and Dugie, 2011).

Combining ability and heterosis concepts have been successfully studied in this work for the production of drought tolerant hybrids. The need for breeding maize crop tolerant to drought conditions is pertinent. The choice for selection and breeding procedure to be used for genetic improvement of crop plants therefore will largely depend on the magnitude of genetic variability and the nature of gene action governing the inheritance of desirable traits. It is eminent for plant breeders to be familiar with the potentials of local materials before embarking on population improvement (Aminu and Izge, 2013). It is also important to have information on the nature of combining ability of parents, their behaviour and performance in hybrid combination (Chawla and Gupta, 1984). Such knowledge of combining ability is essential for selection of suitable parents for hybridization and identification of promising hybrids for the development of improved varieties for a diverse agro-ecology (Alabi et al., 1987). As such, drought tolerance breeding has been used as a tool for identifying traits that are most vital in selection in order to improve crop yield and other yield attributes (Hallauer and Miranda, 1988). Therefore, this study was performed to estimate the general combining ability effect of parents, specific combining effect of hybrids and to determine the high parent heterosis existing among the traits.

MATERIALS AND METHODS

Nursery experiments were conducted at the Faculty of Agriculture, Teaching and Research Farm, University of Maiduguri (latitudes 11° 14'N and longitude 13° 04'E on an altitude of 354 m above sea level) for the initial breeding population (F₁ hybrids). Five drought tolerant and open pollinated varieties (OPVs) were used as parental lines: EVDT-99WSTRC0, TZE-WDTSTRQPMC0, EVDT-99WSTRQPMC0, TZECOMP₃DTC₁ and BG9TZECOMP_{3x4}. These lines were developed at IITA, in Ibadan, Nigeria, from diverse sources of germplasm through evaluation and selection at multiple locations. In addition, four local cultivars susceptible to drought were used as testers: EX-BIU WHITE, EX-BIU YELLOW, EX-DAMBOA WHITE and EX-DAMBOA YELLOW. These local cultivars are those predominantly growing by the farmers in the study areas. The materials were crossed in line by tester mating design during the dry season of 2007 under manual irrigation to generate twenty F₁ hybrids.

The hybrids produced and their parents were evaluated in Biu location in 2008 and 2009 rainy seasons. Biu is located in Northern Guinea Savanna and is characterized by a rainy season period of 130 - 160 days per year, with average annual rainfall ranging between 900 - 1400 mm. Biu geographic locations is latitude 11° 2'N and longitude 13° 2'E on an altitude of 687 m above sea level. The soil type is clay or black cotton vertisols (FAO/UNESCO, 1998). The treatments were laid out in a randomized complete block design (RCBD), with three replications. Each plot size was 5 x 2.75 m (13.75 m²), with four rows spaced at 0.75 x 0.40 m intra-row spacing. The planting was done in August ending (30th August) to subject the genotype to moisture stress.

Every plot was fertilized twice: 10 days after and four weeks after planting, applying 333.33 kg/ha of NPK (15:15:15) fertilizer and 110 kg/ha of urea, respectively. All fertilizers were dibbled by side placement during the application according to Kamara et al. (2004). On every plot, number of plants/plot, days to 50% tasseling, days to 50% silking, ASI, plant height (cm) and ear height (cm) were recorded. The parameters number of cobs per plant, number of cobs harvested per plot, 100 seed weight (g) and grain yield (kg/ha) were also recorded.

The combining ability analysis and the GCA and SCA effects estimation were carried out based on the procedures described by Kempthorne (1957) and Singh and Chaundhary (1985), using SPAR 2.0 Statistical Package for Agricultural Research. The significant differences (P < 0.05) among GCA effects and SCA effects were tested using the formula of Cox and Frey (1984). High parent heterosis was estimated according to Liang et al. (1972).

RESULTS AND DISCUSSION

Analysis of combining ability

The analysis of variance for combining ability and variance in a line x tester for twelve agronomic traits in maize combined across years are presented in Table 1. The results indicate that mean squares due to lines were significant for days to 50% tasseling, days to 50% silking, ear height, weight of cobs and 100 seed weight. The results also reveal that the mean squares were significant in testers for days to 50% tasseling, days to 50% silking, plant height, weight of cobs, dehusked cobs and grain yield. However, the analysis of variance for combining ability showed that the mean squares due to line x tester interaction were significant (p < 0.05) for ASI, plant

Table 1. Analysis of combining ability variance and variance for twelve agronomic traits combined across years.

Source of variation	DF	NSP	DTT	DTS	ASI	PHT	EHT	NCPL	NCPT	WC	DC	HSW	GRY
Line	4	24.02	42.97*	51.58*	0.25	510.77	853.91*	0.36	47.62	627345.31*	3929744.58	19.65*	1317984.41
Tester	3	8.88	138.22**	56.10*	0.56	38399.73**	505.97	0.97	17.82	7206633.30*	119713532.22**	4.92	50280622.81**
Line x tester	12	42.94	10.91	18.06	1.54*	967.02*	1557.75**	0.67	80.45	4275712.82	2194899.58	10.46*	948263.50
Error	56	37.21	20.415	20.25	0.32	423.28	371.13	0.52	48.84	2781073.04	3864283.23	7.42	1368782.44
Variance component estimates													
Line		-0.79	1.34	1.40	-0.06	-10.68	13.71	-0.008	-0.88	31036.25	72285.21	0.19	15405.04
Tester		-1.14	4.24	1.27	-0.09	1254.43	931.94	0.078	41.79	4833748.36	3917287.76	1.65	1644411.98
δ^2_{gca}		-0.14	0.39	0.19	0.03	85.92	65.43	0.005	2.82	336418.46	276110.05	0.13	114804.89
δ^2_{sca}		-1.85	6.27	3.42	0.43	2047.84	1444.69	0.091	66.42	7450987.76	6070852.55	2.05	2576335.30
$\delta^2_{gca/sca}$		0.07	0.06	0.06	0.07	0.04	0.05	0.055	0.04	0.05	0.05	0.06	0.05
Proportional contribution to total variation													
Line		15.06	23.96	34.90	24.44	1.62	3.36	2.17	2.32	2.59	3.93	13.37	3.15
Tester		4.17	18.25	28.46	26.60	7.28	5.86	16.61	12.48	5.91	6.57	16.51	6.79
Line x tester		80.77	57.80	36.64	48.96	91.11	90.78	71.22	85.21	91.51	89.52	70.12	90.06
GCA (line + tester)		19.23	52.21	63.36	51.04	8.90	9.22	28.78	14.80	8.50	10.50	29.88	9.94
GCA/SCA		0.24	0.90	1.73	1.04	0.10	0.10	0.404	0.17	0.09	0.12	0.43	0.11

NSP, Number of stands per plot; ASI, anthesis silking interval; NCPL, number of cobs per plant; DC, dehusked cobs; DTT, days to 50% tasseling; PHT, plant height; NCPT, number of cobs per plot; HSW, 100 seed weight; DTS, days to 50% silking; EHT, ear height; WC, weight of cobs; GRY, grain yield; *Significant; **Highly significant ($P < 0.05$).

height, ear height and 100 seed weight. Therefore, the result shows that both additive and non-additive gene actions were important and responsible for the genetic expression. These results are in agreement with those of Joshi et al. (2002), Asif et al. (2007), Premalatha and Kalamani (2010) and Aminu and Izge (2013). The fact that both additive and non-additive gene actions were important in genetic control of most traits studies means that there is the existence of tremendous amount of variability in the genetic materials evaluated, confirming the results of Izge et al. (2007) and Bello and Olaoye (2009).

The estimates of variance components indicated that the ratio of GCA to SCA variance shows the importance and the predominance of

non-additive genetic effects because the SCA variance was higher than the GCA and most of the ratios were less than unity. However, the results were in agreements with that of Sharma et al. (2004) who found the preponderance of additive genetic effects in the control of traits in maize. The proportional contribution of lines to total variation is lower than the testers for seed weight.

General combining ability effects of parents

The estimate of general combining ability of effects of lines and testers in maize combined across years are presented in Table 2. Line

EVDT-99WSTRCO expressed positive significant GCA effects for almost all the traits except for number of cobs per plant. Therefore, the result indicated it is the highest general combiner. Similarly, TZE-COMP₃DTC₁ exhibited positive GCA effects for anthesis silking interval, number of cobs per plant, number of cobs per plot, weight of cobs, dehusked cobs and grain yield. However, it expressed negative significant GCA effects for plant height and ear height. Parents with high negative GCA effects for days to tasseling, days to silking, plant height and ear height are desirable under drought and windy environment as these parents could escape drought and lodging in stormy areas. Similar results were reported by Izge et al. (2007) and Aminu and Izge

Table 2. Estimate of general combining ability effect for male and female parents for 12 agronomic traits in maize combined across years.

Entry	NSP	DTT	DTS	ASI	PHT	EHT	NCPL	NCPT	WC	DC	HSW	GRY
Male entries												
EVDT-99WSTRC0	12.20**	7.17**	4.92*	2.47*	60.00*	58.16**	0.86	12.35**	4588.68**	4159.68**	12.13*	2656.20**
TZE-WDTSTRQPMC0	12.62**	3.75*	2.33	-1.72*	83.50*	62.14**	-0.63	-14.48**	-4730.57**	-4270.40**	23.54*	-2795.10
EVDT-99WSTRQPMC0	-10.80**	-3.08	-2.83	-1.05*	-13.42	-7.85	-1.34	0.85	268.68	328.02	-1.88*	241.05
TZE-COMP ₃ DTC ₁	0.72	-1.75	-4.75*	5.95**	-77.46*	-43.96*	2.95*	12.77**	4622.85**	3971.35**	0.75	2526.50**
BG 97 TZECOMP ₃ x ₄	-13.30**	-6.08*	0.33	-0.72	-52.61	-68.47**	-1.84	-11.48**	-4749.65**	-4188.65**	-3.53*	-2628.65**
SE (±)	2.03	1.51	1.50	0.18	6.86	7.97	0.53	2.33	555.89	655.26	0.91	389.98
Female entries												
EX-DAMBOA WHITE	0.48	1.77	1.57	3.35**	11.84	-5.94	3.89**	2.50	856.62	743.95	3.50*	585.95
EX-DAMBOA YELLOW	-4.89*	1.50	1.57	-1.18*	14.89*	24.58*	-1.16	-6.03*	-529.98	1389.35**	-1.40	-383.20
EX-BIU WHITE	-3.58*	-3.10*	-3.23*	1.98	-19.87*	-13.86*	-1.32	4.83*	1568.02**	-529.98	-0.87	783.59*
EX-BIU YELLOW	-1.78	-0.17	0.10	-1.18*	-10.46	-4.88	-1.41	-1.30	-1843.32**	-103.22*	-1.23	-986.35*
SE (±)	1.6600	1.3000	1.3000	0.1600	5.9400	6.9000	0.4600	2.0100	481.4100	567.4700	0.7700	337.7300

NSP, Number of stands per plot; ASI, anthesis silking interval; NCPL, number of cobs per plant; DC, dehusked cobs; DTT, days to 50% tasseling; PHT, plant height; NCPT, number of cobs per plot; HSW, 100 seed weight; DTS, days to 50% silking; EHT, ear height; WC, weight of cobs; GRY, grain yield; *Significant; **Highly significant (P< 0.05).

(2013). TZE-WDTSTRQPMC0 is the third highest general combiner after line TZE-COMP₃DTC₁ exhibiting positive significant effects in number of stands per plot, days to tasseling, plant height, ear height and 100-seed weight. The result also shows that EVDT-99WSTRQPMC0 exhibited negative significant GCA effects for number of stands per plot, ASI and 100-seed weight.

In the case of testers, EX-BIU WHITE revealed superior positive significant GCA effects for number of cobs per plot, weight of cobs and grain yield. However, negative significant GCA effects were observed for number of stands per plot, days to 50% tasseling, days to 50% silking, plant height and ear height. Therefore, EX-BIU WHITE was the highest general combiner tester. EX-DAMBOA WHITE expressed positive significant GCA effects for the ASI, number of cobs per plant and 100-seed weight. It was the second highest general

combiner tester.

The parents with a high GCA effects for traits could produce superior segregants in the F₂ as well as in later generations. The line EVDT-99WSTRC0 and tester EX-BIU WHITE had high GCA effects for most of the traits. Therefore, these parents could be utilized in a hybridization programme for selection of superior recombinants. These results are supported by Premlatha and Kalamani (2010) and Kanta et al. (2005), who have also identified good combiners and superior hybrids in maize.

Specific combining ability effects of hybrids

Estimates of specific combining ability for twelve agronomic traits in 20 hybrids in maize combined across years are presented in Table 3. EVDT-

99WSTRQPMC0 x EX-DAMBOA WHITE exhibited the highest positive and significant SCA effects for grain yield. Hybrid EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW was the only one that exhibited positive and significant SCA effects for both number of stands per plot and grain yield. Therefore, these hybrids had the highest and significant specific combining ability effects among the hybrids and can be used for further selection.

The EVDT-99WSTRC0 x EX-DAMBOA WHITE hybrid exhibited significant and negative SCA effect for both days to 50% tasseling and silking. TZE-COMP₃DTC₁ x EX-DAMBOA WHITE had the highest positive and significant SCA effects for ASI. Eight hybrids showed significant SCA effect for plant height, being EVDT-99WSTRQPMC0 x EX-BIU WHITE and EVDT-99WSTRQPMC0 x EX-BIU YELLOW those that express the highest

Table 3. Estimate of specific combining ability effect for the hybrids for 12 agronomic traits in maize combined across years.

Entry	NSP	DTT	DTS	ASI	PHT	EHT	NCPL	NCPT	WC	DC	HSW	GRY
EVDT-99WSTRC0 x EX-DAMBOA WHITE	-4.40	-8.10**	-6.98**	-2.93	-40.09**	-38.23*	-4.25	-3.17	-100.78	276.05	2.79	88.60
EVDT-99WSTRC0 x EX-DAMBOA YELLOW	2.20	1.83	0.68	1.60	-0.74	-7.08	1.01	-0.50	-982.18	-1332.68	-0.06	-642.38
EVDT-99WSTRC0 x EX-BIU WHITE	2.67	4.43	4.82	0.40	27.62*	23.03	2.09	1.37	1297.15	1253.32	1.76	524.41
EVDT-99WSTRC0 x EX-BIU YELLOW	-0.47	1.83	2.48	0.93	13.21	22.29	1.15	2.30	-214.18	-196.68	1.11	29.38
TZE-WDTSTRQPMC0 x EX-DAMBOA WHITE	-3.82	-3.02	-2.73	-3.35**	-20.92	-18.56	-4.02	-8.75*	-3099.95	-3148.95*	-4.21*	-1970.80*
TZE-WDTSTRQPMC0xEX-DAMBOA YELLOW	-0.55	-3.42	-3.40	1.85	-25.90*	-38.42*	1.11	-0.75	1078.65	1402.32	2.19	740.65
TZE-WDTSTRQPMC0 x EX-BIU WHITE	0.92	2.18	2.07	1.32	7.79	21.36	1.09	3.12	1384.65	1034.98	0.33	1049.26
TZE-WDTSTRQPMC0 x EX-BIU YELLOW	3.45	4.25	4.07	0.18	39.05*	35.62*	1.82	6.38	636.65	711.65	1.69	180.89
EVDT-99WSTRQPMC0 x EX-DAMBOA WHI	6.52	1.15	0.10	-4.68	73.00*	28.09	-2.70*	10.42*	4626.72**	3936.05**	1.54	2262.54*
EVDT-99WSTRQPMC0xEX-DAMBOAYELLOW	7.45*	2.08	0.43	-0.15	34.01**	68.90**	1.32	5.08	2635.32**	2510.65**	0.94	2018.83*
EVDT-99WSTRQPMC0 x EX-BIU WHITE	-1.75	-4.32	-3.77	1.65	-56.47**	-54.53**	0.58	-5.72	-3712.02**	-3063.35**	-0.93	-1971.96*
EVDT-99WSTRQPMC0 x EX-BIU YELLOW	-12.22**	1.08	3.23	3.18	-50.54**	-42.47*	0.80	-9.78*	-3550.02**	-3383.35**	-1.56	-2309.41*
TZE-COMP ₃ DTC ₁ x EX-DAMBOA WHITE	-0.73	12.15**	10.60**	15.65**	-3.33	32.76*	14.96**	-1.25	-2044.37**	-1902.53	8.75**	-695.59
TZE-COMP ₃ DTC ₁ x EX-DAMBOA YELLOW	-6.13	-5.58*	-5.40*	-5.48	-1.35	-23.16	-4.72**	-1.25	-902.10	-734.27	-2.02	-765.63
TZE-COMP ₃ DTC ₁ x EX-BIU WHITE	3.33	-2.32	-1.93	-5.68**	21.84	8.08	-5.43**	2.28	440.57	158.40	-5.55*	-88.54
TZE-COMP ₃ DTC ₁ x EX-BIU YELLOW	3.53	-4.25	-4.27	-4.48**	-17.16	-17.69	-4.81**	0.22	2504.90*	2478.40*	-1.18	1549.76*
BG97TZECOMPO _{3x4} x EX-DAMBOA WHITE	2.43	-2.18	-0.98	-4.68**	-8.66	-4.06	-4.00*	2.75	618.38	839.38	-3.29	315.26
BG97TZECOMPO _{3x4} x EX-DAMBOA YELLOW	-2.97	5.08*	6.68*	2.18	-6.01	-0.25	1.29	-2.58	-1829.68	-1846.02	-1.06	-1351.47
BG97TZECOMPO _{3x4} x EX-BIU WHITE	-5.17	0.17	-1.18	2.32	-0.78	2.06	1.68	-1.05	589.65	616.65	4.41*	486.83
BG97TZECOMPO _{3x4} x EX-BIU YELLOW	5.70	2.92	-4.52	0.18	15.45	2.25	1.04	0.83	621.65	389.98	-0.06	549.38
SE (±)	3.5200	-2.6100	2.6000	1.3300	11.8800	13.8000	0.9200	4.0400	962.8200	1134.9400	1.5700	675.4700

NSP = Number of stands per plot; ASI = anthesis silking interval; NCPL = number of cobs per plant; DC = dehusked cobs; DTT = days to 50% tasseling; PHT = plant height; NCPT = number of cobs per plot; HSW = 100 seed weight; DTS = days to 50% silking; EHT = ear height; WC = weight of cobs; GRY = grain yield; *Significant; **highly significant (P < 0.05).

negative and significant SCA effects for this traits. These two hybrids also expressed the highest negative and significant SCA effects for ear height. EVDT-99WSTRQPMC0 has been identified in this study as one of three best combining parents. A similar result was reported by Aminu and Izge (2013). TZE-COMP₃DTC₁ x EX-DAMBOA WHITE hybrid showed the highest positive and significant SCA effects for number of cobs per plant and 100-seed weight, while EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE had the highest positive and significant SCA effects for number of cobs per plot and dehusked cobs. In most of the hybrids that had the highest SCA

effects for drought tolerant traits such as days to 50% tasseling, days to 50% silking, plant height, ear height and ASI one of the parents was EX-DAMBOA WHITE, which is the best general combining parent, and therefore, the combination of favourable genes from the parents for the corresponding traits could have resulted in high and desirable SCA effects.

This study reveals hybrids with significant and highly desirable SCA effects for different traits such as days to 50% tasseling, days to 50% silking, plant height, ear height, ASI dehusked cob and grain yield which were found. Some of the superior hybrids were from either one of the

parents with high GCA effect or parents that are low x low general combiners. It therefore, means that the parents with either high GCA or low SCA would have a higher chance of having excellent complementary with other parents that have high GCA. Similar findings have been reported by Asif et al. (2007), Premlatha and Kalamani (2010) and Aminu and Izge (2013).

Heterosis

Estimates of heterosis for 12 agronomic traits in maize combined across years are presented in

Table 4. Heterosis of the hybrids over higher parents for twelve agronomic traits in maize combined across years.

Entry	NSP	DTT	DTS	ASI	PHT	EHT	NCPL	NCPT	WC	DC	HSW	GRY
EVDT-99WSTRC0 x EX-DAMBOA WHITE	0.53	-3.50	-2.65	-12.20	4.43	1.95	9.90	13.45	3.20	5.62	-2.46	-2.19
EVDT-99WSTRC0 x EX-DAMBOA YELLOW	-4.16	-17.06	-13.42	-1.18	-22.86	-37.87	-11.79	-19.97	-22.09	-20.03	-7.69	-23.02
EVDT-99WSTRC0 x EX-BIU WHITE	-4.59	-15.67	-12.39	7.32	-38.33	-55.52	-22.12	-31.13	-43.33	41.09	-13.85	-44.15
EVDT-99WSTRC0 x EX-BIU YELLOW	1.56	-310.76	-640	-2.38	-38.27	-46.10	-11.52	-18.35	-43.54	-41.58	-10.15	-45.20
TZE-WDTSTRQPMC0 x EX-DAMBOA WHITE	1.93	-6.15	-4.27	-1.27	0.45	18.28	11.27	5.44	-2.45	-1.01	1.92	-2.50
TZE-WDTSTRQPMC0 x EX-DAMBOA YELLOW	-1.04	-17.65	-13.42	-2.44	-30.96	-55.80	-16.98	-28.84	-47.73	-48.52	-4.49	-52.78
TZE-WDTSTRQPMC0 x EX-BIU WHITE	0.51	-9.54	-4.56	13.12	-34.96	-46.90	-20.35	-23.64	-50.56	-52.46	-14.91	-55.54
TZE-WDTSTRQPMC0 x EX-BIU YELLOW	-12.99	-13.54	-8.55	-20.99	-27.27	-52.35	-17.05	-20.80	-41.85	-40.88	-10.19	-44.39
EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE	-0.76	1.55	0.86	14.88	3.89	13.80	12.75	14.43	3.00	27.71	30.64	54.88
EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW	-9.39	-15.59	-10.14	13.18	-31.52	-48.03	11.60	10.07	4.23	39.30	20.41	44.00
EVDT-99WSTRQPMC0 x EX-BIU WHITE	-8.88	-6.83	-3.14	-17.07	-29.47	-37.43	-17.70	12.01	-43.29	-43.83	-14.29	-41.65
EVDT-99WSTRQPMC0 x EX-BIU YELLOW	-8.12	-6.21	-1.71	9.05	-27.36	-42.67	-14.22	-15.98	-47.52	-48.32	-15.29	-52.03
TZE-COMP ₃ DTC ₁ x EX-DAMBOA WHITE	-2.91	1.00	0.29	8.43	5.27	2.93	4.81	13.16	-6.1	-4.62	0.64	-5.66
TZE-COMP ₃ DTC ₁ x EX-DAMBOA YELLOW	-2.60	-15.29	-12.60	-16.28	-34.32	-57.60	-16.04	-27.18	-45.32	-44.18	-10.90	-44.73
TZE-COMP ₃ DTC ₁ x EX-BIU WHITE	-2.04	-11.29	-6.05	-3.61	-31.86	-41.78	-10.62	-22.09	-42.97	-42.99	-11.18	-49.90
TZE-COMP ₃ DTC ₁ x EX-BIU YELLOW	-2.08	-13.88	-11.56	1.18	-33.93	-53.77	4.60	7.67	-23.75	-33.22	-3.82	43.62
BG97TZECOMP _{3x4} x EX-DAMBOA WHITE	-2.19	-5.41	-4.13	6.17	-0.56	10.80	3.70	13.20	-6.29	-3.72	-6.54	-2.39
BG97TZECOMP _{3x4} x EX-DAMBOA YELLOW	-5.45	-14.71	-8.49	-19.05	-32.14	-51.41	0.93	10.25	-20.95	-10.65	-12.15	34.51
BG97TZECOMP _{3x4} x EX-BIU WHITE	-7.65	-12.54	-8.07	3.70	-35.19	-44.71	-22.12	-26.54	-46.94	-48.58	-16.77	-54.38
BG97TZECOMP _{3x4} x EX-BIU YELLOW	-2.86	-13.61	-11.05	-3.61	-23.14	-49.47	20.74	-16.32	-50.89	-51.93	-14.95	-52.58

NSP, Number of stands per plot; ASI, anthesis silking interval; NCPL, number of cobs per plant; DC, dehusked cobs; DTT, days to 50% tasseling; PHT, plant height; NCPT, number of cobs per plot; HSW, 100 seed weight; DTS, days to 50% silking; EHT, ear height; WC, weight of cobs; GRY, grain yield; *Significant; **Highly significant (P< 0.05).

in Table 4. The results reveal that hybrid TZE-WDTSTRQPMC0 x EX-DAMBOA WHITE had the highest positive heterotic effect for number of stands per plot. All the hybrids indicated negative higher parent heterosis for days to 50% tasseling and days to 50% silking, except, EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE and TZE-COMP₃DTC₁ x EX-DAMBOA WHITE.

Hybrids EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE, EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW and TZE-WDTSTRQPMC0 x EX-BIU WHITE had the highest positive higher parent

heterosis for ASI. Positive high value heterosis is actually desirable for ASI, implying that these hybrids could tolerate drought. EVDT-99WSTRC0 x EX-BIU WHITE and EVDT-99WSTRC0 x EX-BIU YELLOW had the highest negative higher parent heterosis for plant height. While, TZE-COMP₃DTC₁ x EX-DAMBOA YELLOW had the highest negative higher parent heterosis for ear height. Negative heterosis for plant height and ear height are also desirable implying that these hybrids would mature earlier and could escape drought respectively.

BG97TZECOMP_{3x4} x EX-BIU YELLOW hybrid recorded the highest exhibited positive higher parent heterosis for number of cobs per plant. With respect to number of cobs per plot, EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE exhibited the highest positive higher parent heterosis effects. Hybrids EVDT-99WSTRC0 x EX-DAMBOA WHITE and EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW recorded the highest positive higher parent heterosis for weight of cobs. EVDT-99WSTRC0 x EX-BIU WHITE exhibited the highest positive

higher parent heterosis for dehusked cobs.

Four hybrids expressed positive higher parent heterosis for 100-seed weight, being EVDT-99WSTRC0 x EX-DAMBOA WHITE, the hybrid with the highest higher parent heterosis. Hybrids EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE and EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW expressed the highest positive higher parent heterosis for grain yield. High heterotic values in grain yield have also been reported in maize by Joshi et al. (2002) and Aminu and Izge (2013).

It is noteworthy that these hybrids EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE, EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW, TZE-COMP₃DTC₁ x EX-BIU YELLOW and BG97TZE-COMP_{3x4} x EX-DAMBOA YELLOW appeared to have genes that could be introgressed to exploit heterosis for earliness and high grain yield. These results are in line with earlier independent studies of Bello and Olaoye (2009), Kumar et al. (1998), Joshi et al. (1998)

Conclusion

The present study identifies parents: EVDT-99WSTRC0, EVDT-99WSTRQPMC0, TZE-COMP₃DTC₁ and EX-BIU WHITE as the best general combiners, and hybrids EVDT-99WSTRQPMC0 x EX-DAMBOA WHITE and EVDT-99WSTRQPMC0 x EX-DAMBOA YELLOW as the best among the 20 hybrids evaluated since they have the best level of high parent heterosis in ASI, number of cobs per plot, 100 seed weight and grain yield. Desirable heterotic levels in days to tasseling, days to silking, ASI and plant height are of tremendous advantage in areas with marginal rainfall like the study area.

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Full Length Research Paper

Effect of *Dodonaea viscosa* Jacq. residues on growth and yield of mungbean (*Vigna mungo* L. Hepper)

Kamil M. M. Al-Jobori^{1*} and Sumeia Abbas Ali²¹Institute of Genetic Engineering and Biotechnology for Post Graduate Studies- Baghdad Univ.-Iraq.²College of Science University of Baghdad, Iraq.

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This study was to evaluate the effect of *Dodonaea viscosa* Jacq. residues on mungbean (*Vigna mungo* L. Hepper) local cultivar. An experiment [using randomized complete block design (RCBD) design] with three replications was conducted in 2010. The trial comprised of four treatments such as mulching, incorporation into soil and extract, along with control with mungbean crop without adding residues of *D. viscosa*. Data showed a significant increase in chlorophyll - b (Chl.b) and total chlorophyll in leaves of mungbean in mulching treatment as compared to control. Plant height, number of branches per plant, leaf area index, number of seed per pod and 100 seed weight were slightly higher for *D. viscosa* residues than for the control treatment. All *D. viscosa* residues treatments had positive effects on number of pods per plant, plant seed yield and total seed yield as compared to control. However, mulching treatment was the superior. Neither protein nor oil content in mungbean seeds were significantly affected by *D. viscosa* residues, even though there was slight increase. Carbohydrates content in the seeds were not significantly different due to *D. viscosa* residues treatments, although some decrease was observed due to slight increase in protein and oil content. The leaf tissue N, P, K, Mg or Fe concentrations were not significantly affected by *D. viscosa* residues treatments. However, the chemical analysis of field soil properties after harvesting demonstrated the increase in inorganic elements as compared with soil before sowing. Mulching gave the best results, followed by incorporation into the soil, and then spraying of extract in comparison to control.

Key words: Mungbean, *Dodonaea viscosa* residues, growth, yield, quality, mineral elements content.

INTRODUCTION

Mungbean is an important legume crop grown in rainfed and irrigated conditions. Weed infestation in mungbean crop is one of the main causes of low yield per hectare against the potential yield. Uncontrolled weeds can reduce mungbean yield by 28% (Ali, 1992) or may reduce mungbean yield as much as 50 to 90% compared with weed free conditions (Poehlman, 1991). Iraq grows various types of pulse crops. Among them broadbean,

lentil, mungbean, chickpea, field pea and cowpea are important. Among the pulse crops, mungbean has special importance in intensive crop production of the country for its short growing period. This also applies in many other countries like Bangladesh (Ahmed et al., 1978). Mungbean grain contains 51% carbohydrates, 26% protein, 10% moisture, 4% mineral and 3% vitamins (Kaul, 1982). The green plants can also be used as animal feed and

*Corresponding author. E-mail: kamilaljobori@yahoo.com.

its residues have capacity to improve soil fertility thus, increase the productivity of land. The crop is potentially useful in improving cropping pattern as it can be grown as a catch crop and inter crop due to its rapid growth and early maturing characteristics. It can also fix atmospheric nitrogen through the symbiotic relationship between the host mungbean roots and soil bacteria and thus improves soil fertility. It may play an important role to supplement protein in the cereal-based low-protein diet of the people of Bangladesh, but the acreage and production of mungbean is steadily declining (BBS, 2005).

Dodonaea viscosa Jacq. belongs to the family Sapindaceae. The center of origin of *D. viscosa* is believed to be Australia. In Iraq, *D. viscosa* widely cultivated as a hedge plant (Townsend and Guest, 1980). It is an evergreen branched shrubs or small tree reaching height about 3 to 8 m (Teffo, 2006). The allelochemicals released from *D. viscosa* Jacq contain the following components: flavonoids, glycosides, tannins, volatile oils, terpenes, saponins and phenols and absence of alkaloids and sugars in the leaf extracts while alkaloids, comarins, volatile oils, steroids and resins were not detected in bark extract (Esmaeel and Al-Jobori, 2011). Much research has been concerned with the detrimental effects of living plants or their residues upon growth of higher plants and crop yields. The allelopathic properties of plants can be exploited successfully as tool for pathogens, weed reduction and enhanced the yields in crops (Xuan et al., 2005). Recent research work identified a number of species that have chemicals suitable for promoting or suppressing the growth and yield of surrounding plants including *Lactuca sativa* (Chon et al., 2003), *Prosopis juliflora*, *Eucalyptus camaldulensis* and *Acacia nilotica* (Marwat and Khan, 2006), *Cassia angustifolia* (Hussain et al., 2007), *Brachiaria decumbens* (Elizabeth et al., 2008) and *D. viscosa* Jacq (Barkatulla et al., 2010). Incorporating allelopathy into natural and agricultural management systems may reduce the use of herbicides, insecticides, and other pesticides, reducing environment/soil pollution and diminishing autotoxicity hazards (Chon et al., 2002). Management systems that maintain crop residues on the soil surface have several attractive features, including weed control (Barkatullah et al., 2010), reduced erosion, less on-farm energy use, more available soil water (Weston, 2005), improved soil nutrient status (Akemo et al., 2000), which could help increase organic matter contents over time, and provide positive benefits for these soils (Qasem and Foy, 2001).

Hence, the present study was taken to detect the effective and sustainable treatment by using *D. viscosa* residues for the best growth, yield, and quality of mungbean and estimate nutrients contributed by *D. viscosa* residues to mungbean.

MATERIALS AND METHODS

Plant materials collection

Mature leaves, bark and stems of *Dodonaea viscosa* were collected

from gardens of Baghdad University during May and June of 2010. The collected parts were air-dried for several days under sun light and weighted using digital balance. For mulching and incorporating treatment, the dried plant parts were chopped into pieces (0.5 to 1cm length) and kept until use. The amount of residues for spray treatment were washed with distilled water and dried, and then homogenized to fine powder by grounding separately in an eclectic grinder and then kept in plastic bags at room temperature.

Preparation of extracts

The aqueous extracts were prepared from dried plant parts (bark, leaves and stems). A total of 7 200 kg were soaked in 72 L of distill water (100 g in 1000 ml) , and kept at room temperature. After 48 h, aqueous extract was filtered through the sieve (Hoque et al., 2003). Then, the suspension was filtered through eight layer of cheese cloth (Meyer et al., 2006), and kept in plastic bottle under refrigeration at 4°C until use. According to Rafiqul Hoque et al. (2003), these extracts had 100% concentration. 6 L from these extracts were sprayed on each 1 m² of the plot area after crops sowing.

Site location and species selection

Experiments were conducted in a farmer's field in AL-Shaab district, Baghdad province in the period of July to November 2010. The seeds of mungbean were obtained from local markets (local cultivar).

Treatments and experimental design

The experiment was laid out in a completely randomized block experimental design (RCBD) with three replications. Each replication comprised randomly the following treatments: i. Mulching: 600 gm (3 g per kg soil) of *D. viscosa* residues were maintained on the soil surface for each 1 m² of the plot area after sowing maize or mungbean (6 ton ha⁻¹); ii. Incorporation in soil: 600 gm (3 g per kg soil) of *D. viscosa* residues were incorporated in the soil for each 1 m² of the plot area before sowing the crops (6 ton ha⁻¹); iii. Spray with residues extracts: 6 L. of *D. viscosa* residues extracts were sprayed on each 1 m² of the plot area after crops sowing; iv. Control: plots were sown with mungbean without adding residues of *D. viscosa*.

The crop was managed according to the recommended conventional agronomical practices.

Chlorophyll extraction and quantification

Chlorophyll content of dry leaves of mungbean was measured following the method of Linchtenthaler (Zhang and Kirkham, 1996). The absorbance of the pigment was measured at 646.8, 663.2 and 470 nm for Chlorophyll - a, Chlorophyll -b and Carotenoids (Carotene + xanthophylls), respectively using the following equations:

$$\text{Chla} = 12.25A_{663.2} - 2.79A_{646.8} \quad (1)$$

$$\text{Chlb} = 21.5A_{646.8} - 5.10A_{663.2} \quad (2)$$

$$\text{Chltotal} = \text{Chla} + \text{Chlb} \quad (3)$$

$$\text{Cx} + \text{C} = (1000A_{470} - 1.82 \text{Chla} - 85.02 \text{Chlb})/198 \quad (4)$$

Determination of Inorganic elements

Leaf samples of mungbean were collected during flowering and

Table 1. Physical and chemical properties of field soil.

Parameter	Value before sowing	Value after harvesting
pH	6.4	6.2
Electrical conductivity (E.C)	3.95	2.68
Sand (%)	17	19
Clay (%)	19.5	18.5
Silt (%)	63.5	62.5
Soil texture	Silt	Silt
Organic mater (%)	1.1	1.4
N (ppm)	25	150
P (ppm)	1.2	2.0
K (ppm)	72	165
Fe (ppm)	0.02	0.04
Mg (ppm)	0.8	0.6

grain formation. The leaves were dried for seven days at 60°C, ground, and analyzed for N, P, K, Mg or Fe. Analysis was carried out in the Central Laboratory, Department of Biology, College of Science, Baghdad University.

Plant growth parameters

Ten plants from each plot were randomly selected during flowering period to record data on the morphological growth: plant height, number of branches per plant, and leaf area index (LAI).

Harvesting

Mungbean plants were harvested at maturity stages on 22 November. Ten plants from each plot were randomly selected. The data regarding various yield components parameters; pod number per plant, number of seeds per pod, 100 seed weight, and plant seed yield were recorded at maturity. Seed yield was collected from the second and third rows of each plot were sun dried properly. The weight of seeds was taken and converted to yield in ton ha⁻¹.

Chemical analysis

For mungbean seed proteins, oils or carbohydrates analyses, samples were obtained from the harvests made at maturity. Then the samples were dried at 60°C for two weeks, ground and analyzed in Post Studies Laboratories, College of Agriculture, Baghdad University.

Soil sampling and analysis

Soil samples were taken after harvest. Two samples were taken randomly from each plot, 10 to 15 cm deep. The samples were mixed, air-dried, sieved through a sieve with 2 mm openings to remove large rock and plant debris, and pulverized. The small roots and stones were picked out. Soil texture and organic matter were carried out in Department of Laboratories, Ministry of Water Resources. The electrical conductivity (Ec) , pH , inorganic nutrients N, P, K , Mg and Fe were conducted in the Central Laboratory, Department of Biology, College of Science , Baghdad University. The physical and chemical characteristics of the soil (before sowing and after harvesting) are listed in Table 1.

The mean monthly temperature, monthly rainfall and relative humidity are presented in Table 2.

Statistical analysis

The recorded data were statistically analyzed to obtain the level of significance using the MSTAT-computer package program. The means were separated following least significance deference (LSD) test.

RESULTS

The amount of rain fall during the growing season is almost non-existent (Table 2). Plants had to rely entirely on irrigation water based on the fact that the autumn growing season is characterized by non-rainfall. The measurements recorded for Chlorophylls and carotenes parameters are presented in Table 3. The data shows that Chl.b and total Chl. in leaves of mungbean were increased significantly by 16.86 and 17.79% in mulching treatment as compared to the control. The data show that carotenes were not significantly affected by *D. viscosa* residues treatments. However, carotenes tended to increase with mulching and incorporation treatments to 2.24 and 2.28 mg g⁻¹ dry weight.

The allelopathic effect of *D. viscosa* residues on growth and yield of mungbean are presented in Table 4. Plant height, number of branches per plant, and leaf area index were slightly higher at *D. viscosa* residues than at control treatment, although not significant. Maximum plant height was 69.87 cm in incorporation in soil treatment, branch per plant was 4.6, and leaf area index was 3.84 in mulching treatment. On the other hand, dry weight of mungbean increased significantly by 53.96, 52.80 or 42.47% when *D. viscosa* residues were used as mulching, incorporation in soil or extracts, respectively compared with control. Maximum dry weight of mungbean was 66.45 g recorded in mulching treatment but this was not significantly different ($P \leq 0.05$) from other

Table 2. Total monthly rainfall, mean temperature and relative humidity at the experimental site during the growth period of 2010.

Month	Monthly rainfall (mm)	Monthly mean temperature (°C)	Monthly mean relative humidity (%)
July	0.0	45.5	18.0
August	0.0	46.6	17.0
September	0.0	41.7	13.0
October	TR*	35.5	17.0
November	2.5	27.7	4.0

*RT= Less than 0.1 mm

Table 3. Chlorophylls and carotenes content in leaves of mungbean as influenced by *D. viscosa* residues.

Treatment	Chl.a (mg/g dry weight)	Chl.b (mg/g dry weight)	Total chl. (mg/g dry weight)	Carotene (mg/g dry weight)
Mulching	14.89	6.10	20.99	2.24
Incorporation in soil	14.57	5.94	20.52	2.28
Extract	12.07	5.19	16.93	1.86
control	12.60	5.22	17.82	1.93
LSD. 0.05	N.S	0.60	3.10	N.S

N.S, not significant.

D. viscosa residues treatments (Table 4). The results show that mulching produced highest Pod per plant (65.47) compared to control which gave the lowest pod per plant (39.67).

However, there was no significant difference with other *D. viscosa* residues treatments (Table 4). Numbers of seeds per pod measurements in mungbean were statistically same with the control.

D. viscosa residues treatments increased 100-seed weight of mungbean but did not reach significant level (Table 4). Seed yield per plant was significantly higher when *D. viscosa* residues used compared to control (untreated). Mulching treatment produced maximum seed yield (21.69 g) and it enhanced the plant yield by 8.8% as compared to control (Table 4). Neither protein nor oil content in mungbean seeds were significantly affected by *D. viscosa* residues, even though there was slight increase. Mulching treatment gave the best results of protein and oil (23.57 and 1.85%), respectively. Carbohydrates content in mungbean seeds was not significantly different due to *D. viscosa* residues treatments; although some decrease was observed due to slight increase in protein and oil content (Table 5). There was a tendency for carbohydrates content to increase in control treatment and reach 62.13 as compared with *D. viscosa* residues treatments.

The leaf tissue N%, P%, K%, Mg ppm or Fe ppm concentrations were not significantly affected by *D. viscosa* residues treatments (Table 6). However, there were a slight increase in N, P, K, and Mg elements, and slight decrease in Fe element.

DISCUSSION

There were several changes that took place in the life cycle of mungbean plant from germination to maturity. Each physiological and morphological characteristic may affect yield in many ways, the net effect of which depends on other characteristics, on environmental conditions, and on agronomic practices (Kuo, 1998). The different responses of chlorophyllase a and b activities to the same concentrations of allelochemical imply that chlorophyllase a and b may be two different enzymes, located in the chloroplast of higher plants (Yang et al., 2004). Using crop residues as a mulching may moderate the temperature in the top soil layer which can enhance the activity of soil microorganisms, promoting the release of nutrients, improving water infiltration, and facilitating root development and increase photosynthesis (Kladivko, 2001). Aerial parts of *D. viscosa* contain several flavonoids, diterpenoid acids, some biologically active saponins and plant acids, a novel p-coumarin acid ester, essential oils, sterols and tannins (Esmaeel and AL-Jobori, 2011; Barkatullah et al., 2010). Chou et al. (1995) provides evidence that when saponins produced by mungbean plants are added to the soil; they enhance the growth of new mungbean plants as an allelochemical plant growth regulator. That is, saponins stimulated Chl accumulation, which in turn caused stimulation of photosynthesis and finally increased total plant growth.

A different response was observed in the leaf area index (Rebetzke, 1994). Angiras et al. (1987) stated that no suppressive effect was seen on leaf establishment of

Table 4. Effect of *D. viscosa* residues on growth parameters and yield of mungbean.

Treatment	Plant height (cm)	Number of branches	Leaf area index	Plant dry weight (g)	Number of pods/plant	Number of seed/pod 100	Seed weight (g)	Plant seed yield (g)	Total seed yield (ton/ha ⁻¹)
Mulching	66.90	4.6	3.84	66.45	65.47	9.45	3.51	21.69	2.41
Incorporation in soil	69.87	4.2	3.38	65.95	53.33	9.82	3.55	18.70	2.08
Extract	66.87	4.4	3.33	61.49	56.60	9.55	3.61	19.67	2.19
Control	63.53	4.0	3.02	43.16	39.67	9.43	3.43	12.8	1.43
LCD 0.05	N.S	N.S	N.S	17.31	24.55	N.S	N.S	5.49	0.70

N.S, not significant.

Table 5. Quality parameters of mungbean seeds and maize grains as influenced by *D. viscosa* residues.

Treatment	Carbohydrate (%)	Oil (%)	Protein (%)
Mulching	59.28	1.85	23.57
Incorporation in soil	62.05	1.80	20.98
Extract	59.20	1.76	23.52
Control	62.13	1.65	19.35
LSD 0.05	N.S	N.S	N.S

Table 6. Mineral elements content in the leaves of mungbean as influenced by *D. viscosa* residues.

Treatment	Iron (ppm)	Magnesium (ppm)	Potassium (ppm)	Nitrogen (ppm)	Phosphorus (ppm)
Mulching	48.13	242.67	0.68	1.76	0.147
Incorporation in soil	49.43	243.00	0.66	1.73	0.154
Extract	49.00	231.67	0.64	1.74	0.156
Control	57.27	236.67	0.65	1.70	0.145
LSD 0.05	N.S	N.S	N.S	N.S	N.S

soybean by *Sorghum halepense* (Cheema et al., 2001), but LAI of mungbean recorded high mean compared with control means. Similar results were reported by other researchers (Aslam et al., 2004; Onuh et al., 2011). The accumulation of lower dry matter for control treatment might be

due to internal nutrient stress and high competition with weeds, which caused reduction in both cell division and cell elongation of mungbean and reduced carbohydrate synthesis and hence the growth was reduced (Asaduzzaman et al., 2008). Leather and Einhellig

(1985), considered dry weight of crop to be a better indicator of injury due to the presence of weed. In mungbean, weeding play an important role because weed crop competition commences with germination of the crop and continues till its maturity (Sultana et al., 2009).

Numbers of seeds per pod are determined during the reproductive stage of mungbean growth. So, these contradictory results can be attributed to differences in climatic conditions and genetic makeup for crop plant (Hussain et al., 2011). Seed yield per plant was significantly higher when *D. viscosa* residues used was compared to control (untreated). These results are similar to those summarized by Cheema et al. (2001). This increase in seed yield may be due to better weed management, better leaf area and more number of pods per plant (Table 4).

Similar results were recorded by Rakha (1999). In general, an increase in one component at a certain level, often leads to a decrease in another. Often the number of pods per plant declines as the number of plants per unit area increases. Similarly, the weight seed per pod decreases as the number of seeds per pod increases. This means that, for maximum yield, all these yield components should in an appropriate balance (Kuo, 1998). Allelochemical can be leached from the living plant during precipitation (rainfall, snow, dew, mist etc.) and mulching of fresh residues on the soil surface or incorporated into soil lead to suppress weed growth (Chon and Kim, 2004). Khan et al. (2001) observed that grain yield production was due to many yield-contributing traits that were positively correlated with yield. For example, seed yield was positively correlated with number of branches, and thus Reddy et al. (1991) stated that the increase in number of branches enhanced the seed yield.

Weeds compete with the main crops for nutrients and other resources and reducing the yield both qualitatively and quantitatively (Ahmed 2004; Jabeen and Ahmed, 2009). So, the residue of *D. viscosa* inhibited the growth of weeds and increase quality and quantity of the yield of mung bean. These results tend to support the observation of Meso et al. (2005) who indicate that peanut residue does not contribute significant amounts of N to succeeding crops; however, retaining residue on the soil surface provides other benefits to soils. Nitrogen uptake at control is an indication of the nitrogen released by the soil and adding fertilizer. These results suggest that the initial soil available -p and adding fertilizer were sufficiently high to adequately meet the phosphorus needs of the plants. Also these results indicated that the amount of potassium, iron and magnesium in the soil was sufficient, and additional treatment did not affect the nutrient status of the plants (Table 1). The chemical analysis of field soil properties N%, P%, K%, Mg ppm or Fe ppm after harvesting demonstrated the increase soil mineral elements as compared with soil before sowing (Table 1).

Conclusions

D. viscosa residues had a clear positive effect on the growth, yield, yield components, chlorophylls, carotenes,

protein, oil and elements content characteristics of mungbean; however, mulching gave better results in comparison to control. These agriculture crops may be cultivated with the use of *D. viscosa* residues without/or least harm. Allelopathic effect depends upon the method of application and test pieces. Results indicate that *D. viscosa* residue does not contribute significant amounts of nutrients to growing crops; however, retaining residue on the soil surface could help increase organic matter contents over time, which can provide positive benefits for the soil. Mulching gave the best results, followed by incorporation into the soil, and then spraying of extract in comparison to control.

Conflict of Interests

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

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Full Length Research Paper

***In vitro* propagation of the new disease resistant *Coffea arabica* variety, Batian**

Lubabali, A. H.^{1*}, Alakonya, A. E.², Gichuru, E. K.¹, Kahia, J. W.³ and Mayoli, R. N.¹

¹Coffee Research Foundation, P.O. Box 4-00232, Ruiru, Kenya.

²Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya.

³World Agroforestry Centre (ICRAF), Cote d' Ivoire Country Program Cocody Mermoz, 08 BP 2823, Abidjan, Côte d' Ivoire.

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Batian is a true breeding commercial coffee variety that was released in Kenya in 2010. It is resistant to coffee berry disease and coffee leaf rust which are the main coffee diseases in Kenya. Coupled with early ripening, good beverage quality and high yields, demand for planting material has surpassed supply. Conventional propagation methods do not provide enough planting materials, hence the need to develop alternative methods. The objective of this study was to develop an effective *in vitro* protocol for propagating the coffee variety, Batian. Leaf explants were harvested and cultured on Murashige and Skoog (MS) media supplemented with different concentrations of cytokinins benzyl amino purine (BAP) and thidiazuron (TDZ) separately, 100 mg/l myo-inositol 3% sucrose and gelled with 0.3% gelrite. The results show differences among cytokinins levels in induction of somatic embryos. BAP at 13.3 μ M gave the highest mean of embryos per explants, 6.06 ± 1.18 and highest percentage of embryogenic cultures of 58.33%. Development of somatic embryos was achieved on hormone free MS media with highest mean length of 0.32 ± 0.03 mm. Indole butyric acid at 9.8 μ M was best for induction of a well-developed root system with a mean length of 1.22 ± 0.09 mm. This protocol opens new prospects for massive propagation of Batian in nine months.

Key words: Batian, somatic embryo, *Coffea arabica*.

INTRODUCTION

Kenyan coffee is of highly rated beverage quality in the world and contributes significantly to the country's economic growth. One of the constraints to coffee production in Kenya is infection by diseases. Coffee berry disease (CBD) that is caused by *Colletotrichum kahawae* and coffee leaf rust (CLR), caused by *Hemileia vastatrix*

are economically important coffee diseases in Kenya (Gichuru et al., 2012). Coffee breeding programmes in the country have recently resulted into the release of the variety, Batian in 2010. This new variety is resistant to both CBD and CLR (Gichimu and Omondi, 2010). The release of Batian coincided with favourable coffee prices

*Corresponding authors. E-mail: Lubabalihudson@gmail.com or Hudson.lubabali@crf.co.ke.

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that resulted in overwhelming demand for planting materials. As a rapid multiplication method for planting materials, tissue culture offers a feasible alternative to supplement the conventional propagation methods to meet the high demand for planting materials.

Somatic embryogenesis allows for the initiation and development of embryos from somatic tissues without the involvement of sexual fusion. It is a process by which somatic cells undergo bipolar development to give rise to genetically identical whole plants in nine months. There are two types of somatic embryogenesis; direct somatic embryogenesis (DSE), where embryos originate directly from the explants and, indirect somatic embryogenesis (ISE) where embryos are derived from an embryogenic dedifferentiated callus (Carneiro, 1999). The time taken to regenerate coffee plantlets using DSE is normally shorter (nine months) as compared to the time taken for ISE (12-13 months). However, the later one is more prolific and yields much more embryos per explants. In *Coffea* sp., somatic embryogenesis was first reported by Staristky (1970) and since then, a number of protocols for somatic embryogenesis have been developed for various genotypes of coffee (Yasuda et al., 1985; Hatanaka et al., 1991, 1995; Sandra et al., 2000; Giridhar et al., 2004). This paper documents the development of an effective *in vitro* protocol for propagating the coffee variety, Batian.

MATERIALS AND METHODS

Study site and sampling

The study was carried out at the Plant Tissue Culture laboratory of the Coffee Research Foundation situated at Ruiru (Altitude 1620 m above sea level 1°06'S; 36°45'E), Kenya. Third pair of leaves were excised from mother plants grown in the greenhouse at Ruiru and placed in a beaker containing tap water and taken to the laboratory for cleaning. They were washed with tap water containing a few drops of detergent teepol® and rinsed five times. The explants were transferred to the lamina flow cabinet, immersed in 70% (v/v) ethanol for 30 s and rinsed twice with sterile distilled water. This was followed by surface sterilization using 25% of a commercial bleach (JIK)® which contains 3.85% sodium hypochlorite for 25 min.

Media preparation

Direct induction of somatic embryos was carried out using Murashige and Skoog (MS) basal salts supplemented with 30 mg/l cysteine, 100 mg/l inositol and 2% (w/v) sucrose. This medium was supplemented with various plant growth regulators (PGR). Cytokinins were evaluated separately; benzyl amino purine (BAP) at 4.4, 8.8, 13.3, 17.7, 22.0 µM and thidiazuron (TDZ) at 4.5, 9.0, 13.6, 18.2, 22.7 µM. BAP at 2.2, 4.4, 8.8, 13.3, 17.7, 22.0 µM was used for development media. On the other hand, half strength MS media supplemented with auxins indole butyric acid (IBA) at 4.9 9.8 19.7 µM and naphthalene acetic acid (NAA) at 2.5, 5.3 10.7 µM, and 100 mg/l inositol and with 2% sucrose was used for rooting. The pH was adjusted to 5.8 using 1 N NaOH or 0.1 M HCL before agar was added and media heated to dissolve the agar and

dispensed in 10 ml aliquots into culture bottles. The media was autoclaved at 1.06 kg cm⁻² and 121°C for 15 min. Inoculation was carried out in a sterile laminar airflow hood. The leaf explants were cut into sections of 1cm² and cultured in MS media to produce somatic embryos. The somatic embryos were excised and transferred to MS media containing cytokinin (BAP) for development, followed by culturing on MS media with auxins IBA and NAA singly, for root initiation.

Experimental design, data collection and analysis

The experiments were laid out in completely randomized design (CRD). For embryo induction each treatment was repeated three times with 48 explants per repetition. For development of somatic embryos, each treatment was repeated three times with 30 explants per repetition. For root induction, each treatment was repeated three times with 20 explants per repetition. Data on number of somatic embryos per explants, percentage embryogenic cultures, length of developing embryos as well as length of roots were recorded. All the data were subjected to one-way analysis of variance (ANOVA) and the significant differences between treatment means were assessed by Duncan's multiple test range (DMRT). The results are expressed as a mean ± SE. Percent (%) embryogenic cultures was calculated as total number of explants with embryos/total number of cultured explants x 100, while percentage root induction was calculated as total number of plantlets with roots/total number of cultured plantlets x 100.

RESULTS

Somatic embryos were observed from the cut edges of the leaves 30 days after culture. Several stages of somatic embryogenesis were observed in the same culture (Figure 1). These somatic embryos later developed to the cotyledonary stage after 90 days. The MS media supplemented with BAP 8.8 µM gave the highest number (6.06 ± 1.18) of embryos per explant as well as the highest percentage of embryogenic cultures (58.33%). There were no embryos regenerated when the concentration of BAP was increased from 8.8 to 22.0 µM (Table 1).

MS media supplemented with TDZ 9.0 µM gave the highest mean number (2.06 ± 0.63) of embryos per explant and the highest percentage of embryogenic cultures (33.33%). There was no response when the concentration of TDZ was increased from 9.0 to 22.7 µM (Table 2).

On development media, the somatic embryos increased in size and synthesized photosynthetic pigments (Figure 2A). These pigments facilitate photosynthetic activity, extra storage reserves like lipids, triglycerides, proteins and other hydrates which favor *in vitro* germination (Nasim et al., 2010). Somatic embryos were considered to have germinated by development of the shoot (epicotyl) and elongated with the presence of a radicle structure which is a precursor to root induction (Figure 2B). In this study, embryos cultured on embryo elongation media responded by forming shoots only



Figure 1. Formation of somatic embryos at cut edges of the leaf discs.

Table 1. Effect of BAP on regeneration of somatic embryos in Batian variety.

Concentration (μM)	Embryogenic cultures (%)	Mean no. of embryos per explant
4.4	16.67	1.93 ± 0.86^b
8.8	14.58	1.75 ± 0.79^b
13.3	58.33	6.06 ± 1.18^a
17.7	25.00	1.11 ± 0.37^b
22.0	0.00	0.0 ± 0.0^b
P value		<.0001

n= 48. Values represent means \pm SE. Means within a column followed by different letters are significantly different at P = 0.05.

Table 2. Effect of TDZ on regeneration of somatic embryos in Batian variety.

Concentration (μM)	Embryogenic cultures (%)	Mean no. of embryos per explant
4.5	0.00	0.0 ± 0.0^b
9.0	33.33	2.06 ± 0.63^a
13.6	18.75	1.00 ± 0.45^{ab}
18.2	16.67	0.82 ± 0.44^b
22.7	0.00	0.0 ± 0.0^b
P value		<0.0001

n= 48. Values represent means \pm SE. Means within a column followed by different letters are significantly different at P = 0.05.

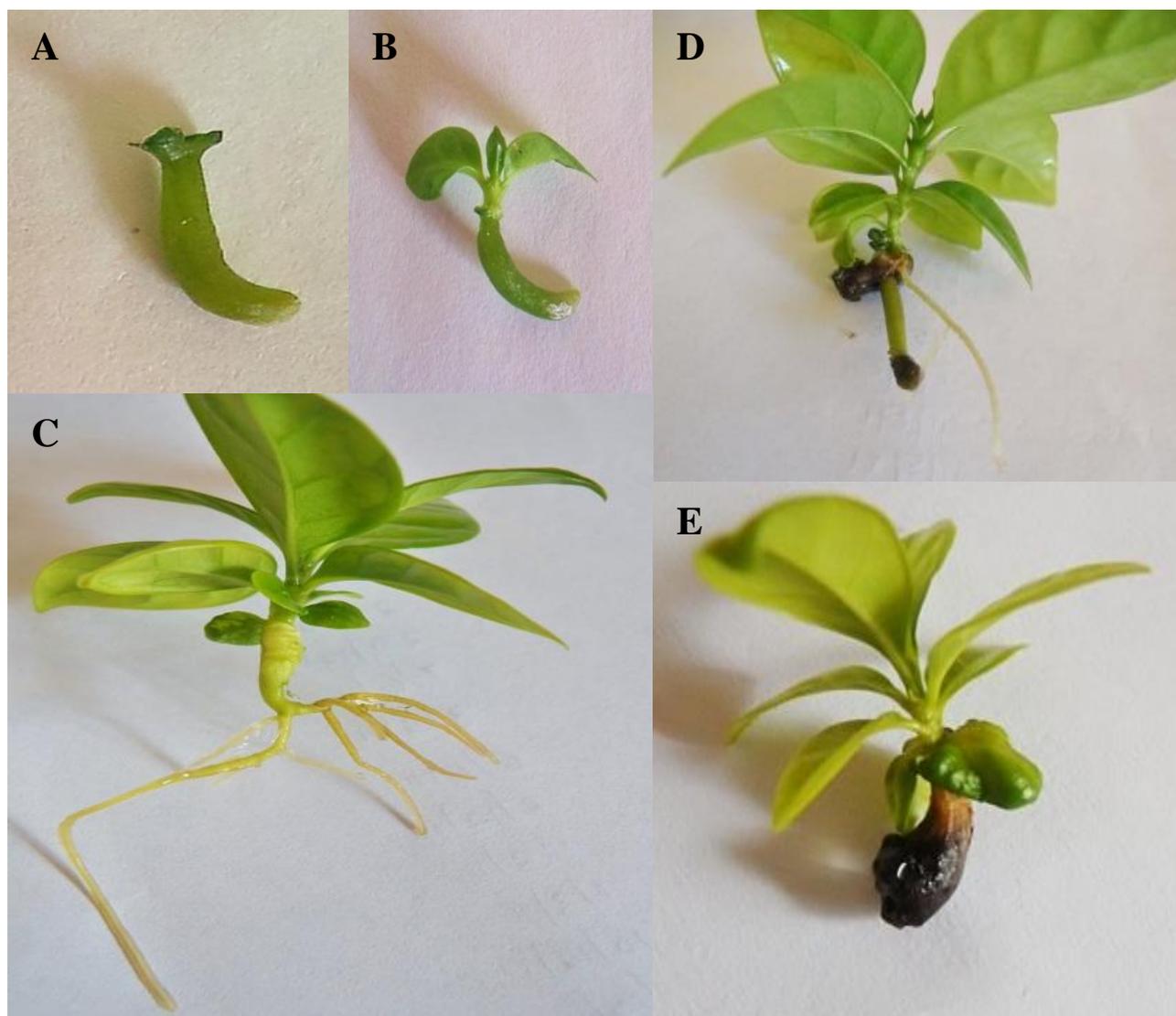


Figure 2. The different regeneration stages of somatic embryos. **A**, Maturing somatic embryo after 14 days in germination media. **B**, Matured somatic embryo ready for root induction. **C**, Rooting of plantlet on NAA 4.9 μM 8 weeks after culture in rooting media. **D**, Inhibited root elongation on NAA 10.7 μM . **E**, Desiccation of plantlet due to high levels of auxins (IBA 19.7 μM).

hence the need for a rooting step. The effects of BAP on elongation of somatic embryos showed no significant difference. However, control had the highest mean length of 0.27 ± 0.05 mm while BAP at 4.4 μM had the lowest mean absolute length (Table 3).

MS media supplemented with NAA 2.5 μM gave the highest mean root length of 1.02 ± 0.14 mm and highest percentage (66.67%) of root induction (Table 4). Low levels of auxins promoted root elongation (Figure 2C). Increasing the concentration of NAA from 5.3 to 10.7 μM resulted in inhibition of root elongation (Figure 2D).

The embryos cultured on media supplemented with 9.8 μM IBA gave the highest mean root length, 1.22 ± 0.09 mm. Increasing IBA from 9.8 to 19.7 μM resulted in

reduction in the percentage root induction as well as the mean root length. This was similar to the trend observed for somatic embryo cultured on medium supplemented with NAA where an increase in concentration resulted in low percentages of root induction (Figure 2E). On the other hand, 9.8 μM IBA gave the highest mean length and the highest percentage (68.62 %) of root induction (Table 5).

DISCUSSION

In vitro growth is highly dependent on the interaction between naturally occurring endogenous substances and

Table 3. Effect of BAP on development of somatic embryo of Batian.

Concentration (μM)	Mean shoot length (mm)
Control	0.27 ± 0.05^a
2.2	0.20 ± 0.05^a
4.4	0.15 ± 0.05^a
8.8	0.23 ± 0.05^a
17.7	0.19 ± 0.05^a
P value	<0.0001

n = 30. Values represent means \pm SE. Means within a column followed by different letters are significantly different at $P = 0.05$.

Table 4. Effects of different NAA concentrations on rooting of Batian.

Concentration (μM)	Mean root length (mm)	Root induction (%)
2.5	1.02 ± 0.14^a	66.67
5.3	0.72 ± 0.18^{ab}	53.84
10.7	0.53 ± 0.11^b	50
P value	<0.0001	

n = 20. Values represent means \pm SE. Means within a column followed by different letters are significantly different at $P = 0.05$.

Table 5. Effects of different IBA concentrations on rooting of Batian.

Concentration (μM)	Mean root length (mm)	Root induction (%)
4.9	1.05 ± 0.09^{ab}	52.38
9.8	1.22 ± 0.09^a	68.62
19.7	0.85 ± 0.09^b	36.11
P value	<0.0001	

Values represent means \pm SE. Means within a column followed by different letters are significantly different at $P = 0.05$.

the analogous synthetic growth regulators added to the medium (George, 1993). During the current study, somatic embryos were formed only at the cut edges. These results are in agreement with those of Hatanaka et al. (1991) who reported that somatic embryos were formed only at the cut edges which were in contact with the media containing growth regulators. They suggested that the cut surface (wounding) might have been sites for rapid uptake of minerals and PGR which resulted in the high percentage of embryogenic cultures. These results are also in agreement with that of Gatica et al. (2007) and Catie and Yasuda et al. (1985) who reported that culture media supplemented with low levels of BAP stimulated development of somatic embryos. During the present study, increasing the concentration of BAP resulted in the decrease of number of somatic embryos. This is contrary to the results of Yasuda et al. (1985) who observed that

media supplemented with 4 to 6 mg/l BAP were more efficient in induction of somatic embryos of F1 hybrid *Coffea arabica*. These differences could be explained by the influence of genotype and other precise conditions that may not have been reproduced in the two studies.

TDZ is chiefly used as cotton defoliant (Giridhar et al., 2004) but it also acts as a growth regulator in tissue culture systems. In peanut and geranium, the use of TDZ has effectively replaced the requirement of auxins and/or cytokinins in induction of somatic embryogenesis (Gill and Saxena, 1993). In this study, results show that TDZ at 9.0 μM produced the highest (33%) frequency of embryogenic cultures. However, these results are contrary to those of Kahia (1999) who reported 100% embryogenic cultures when coffee leaf explants of Ruiru 11 an F1 hybrid were cultured on modified MS media supplemented with 1 μM TDZ, and Gill and Saxena (1993)

who reported 100% induction of somatic on *Nicotiana tabacum* L. leaf explants using TDZ. In this study, the use of TDZ in formation of somatic embryos was achieved with low concentrations. These is in agreement with the study of Giridhar et al. (2004) who reported that low levels of TDZ stimulated direct somatic embryogenesis in *Coffea*.

The time taken to regenerate coffee plantlets using DSE is normally shorter (nine months) as compared to the time taken for ISE which is 12-13 months (Ducos et al., 2010; Etienne et al., 2010). It is a two-step procedure thus similar to high frequency somatic embryogenesis (HFSE). On the other hand, the later one is more prolific and yields much more embryos per explants. Due to the long period, the cultures stay in a PGR supplemented media, the chances of somaclonal variation in ISE are much higher. The protocol developed in this study ensures production of true to type plantlets.

Auxins stimulate root initiation by activating quiescent pericycle cells to initiate division and then expansion which facilitate lateral root emergence, although response varies with concentration (Fukaki and Tasaka, 2009). Taiz and Ziegler, (2003) reported that auxins are required for root induction; though, root growth is inhibited at higher auxin concentration. Kollmeier et al. (2000) stated that high auxin concentration inhibits root elongation. This was also reported by Riov and Yang (1989) who observed that auxins intensify the rate of ethylene biosynthesis. Subsequently, it is conceivable that high concentration of NAA and IBA induced ethylene biosynthesis which is inhibitory to root elongation. IBA proved to be better than NAA in induction of rooting system across all evaluated concentrations.

In general, the effectiveness of each PGR on the various regeneration stages of the selected *C. arabica* varieties was influenced by the concentration. During the current study, a reproducible somatic embryogenesis protocol for regenerating Batian in nine months was developed. The protocol involves culturing the leaf disc explants on half strength MS media supplemented with BAP 13.3 µM. The somatic embryos elongated on hormone free full strength MS media. The somatic embryos were rooted on half strength MS media supplemented with 19.7 µM IBA.

Conflict of Interests

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

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Full Length Research Paper

Studies on seed yield potential of some selected kenaf (*Hibiscus cannabinus* L.) genotypes

J. O. Olasoji^{1*}, O. A. Aluko¹, G. O. Agbaje², O. N. Adeniyani¹, K. O. Kareem¹ and S. O. Olanipekun¹

¹Institute of Agricultural Research and Training, Obafemi Awolowo University, P.M.B. 5029, Moor Plantation, Ibadan, Oyo State, Nigeria.

²Department of Crop Production and Protection, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

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Kenaf seed yield depends on morpho-physiological traits between varieties, and the interaction between genotype and the environment. Studies were conducted in Ibadan, Ilora and Kisi stations of the Institute of Agricultural Research and Training (IAR&T), Obafemi Awolowo University, Moor Plantation, Ibadan, Oyo State, Nigeria during 2009 and 2010 cropping seasons to determine the influence of location and genotypes on seed yield potentials in 20 kenaf genotypes. The experiment was carried out using Randomized Complete Block Design (RCBD). Result shows that, kenaf plants had tallest average capsule height in Ilora in both years, while higher numbers of capsule/plant and seed number/capsule were recorded in Kishi. There were no significant differences among all the genotypes planted irrespective of the location on seed weight/plant, 100 seed weight and seed yield/ha. Average highest capsule height was recorded in 2009 across genotypes. However, average number of capsule/plant, seed number/capsule, seed weight/plant and seed yield/ha were higher in 2010. Local kenaf had highest number of capsules (55.04) with about the least seeds per capsule (9.45) and least weight of seed per plant (3.30 g). While BS-1 had the least capsule number (18.86) and highest seeds per capsule (15.74) was recorded in SF-549. AU-75 recorded the highest seed weight/plant. The 100 seed weight ranged from 3.37 to 2.19 g. Seed yield/ha across genotypes ranged from 660 kg/ha in Local line 36 to 1454.3 kg/ha in AU-75.

Key words: Kenaf, capsule height, capsule number, seed number, seed weight/capsule, 100 seed weight and seed yield/ha.

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) is one of the most important fibre crops in the world. It has been cultivated and used as cordage crop to produce twine, rope, gunny bag and sackcloth for over six millennia (Dempsey, 1975;

Charles, 2002). New applications of kenaf have been developed such as pulping and paper making, oil absorption, potting media, board making, filtration media and animal feed (Sellers and Reichert, 1999; Cheng,

*Corresponding author. E-mail: juliusolasoji@gmail.com.

2001).

Kenaf is commercially cultivated in more than 20 countries, particularly in India, China, Thailand and Vietnam (FAO, 2003). The seeds are good source of low cholesterol vegetable oil and also for biodiesel production (Webber and Bledsoe, 1993). Kenaf has a high growth rate, reaching heights of 4-6 m in about 4-5 months and its yields of 6-10 tonnes of dry mass per acre each year, is generally 3-5 times greater than the yield for the southern pine tree (LeMahieu et al., 2003) which can take from 7-40 years to reach harvestable size. Kenaf has a wider range of adaptation to climatic conditions than other fiber crops grown for commercial use (Liu, 2003). The development of cultivars, which are adapted to a wide range of diversified environments, is the ultimate aim of plant breeders in a crop improvement program (Muhammad et al., 2003).

Genotype x Environment (GXE) interaction is an important issue to agronomists, who transfer a new variety from another environment. The adaptability of a variety over diverse environments is commonly evaluated by the degree of its interaction with different environments in which it is grown. A variety is considered to be more stable if it has a high mean yield but a low degree of fluctuation in yielding ability when planted over diverse environments (Purchase, 1997). Kenaf is now grown under different climatic conditions and a wide range of yield had been reported (Bhangoo et al., 1986). Literatures reported a seed yield in a range of 1-1.5 ton/ha in US and Mexico (Scott and Cook, 1995; Mullens, 1998). In Nigeria, seed yield reported are lower than 1 ton/ha.

However, the sustainable commercial production of kenaf depends on the availability of good seeds in enough quantity. Seed yield in kenaf is influenced by the population density or plant spacing and plant genotype (Mullens, 1998; Berti et al., 2013; Webber and Bledsoe, 2002).

Other factors like maturity ratings of cultivar, photosensitivity of varieties, latitudinal location soil fertility, cultural practices, rainfall in terms of distribution and intensity may significantly influence kenaf performance, seed quality and yield (Webber, 1996; Mullens, 1998; Webber and Bledsoe, 2002). Agbaje et al. (2011) also observed that time of planting and rainfall pattern had significant effect on higher seed weight and seed yield in 2007 as compared to 2006. The bulk of fats and oils, whether for human consumption or for industrial purpose is presently derived from plant sources.

Therefore, improvement has to be more on yield potentials of some selected plant species such as kenaf that has ability to produce unique desirable fats and oils. This study therefore seeks to determine the influence of geographical factors or elements on the seed yield. Hence, the suitability of different genotypes to these geographical locations will be determined for crop management recommendations in order to improve seed production.

MATERIALS AND METHODS

Location, experimental design, treatments and agronomic practices

The experiment was conducted in 2009 and 2010 cropping seasons at three different research stations of the Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria. The research stations are; Ibadan (Transitional rainforest belt), Kisi (Guinea savannah) and Ilora (Derived savannah). Rainfall (mm) records were supplied from IAR&T meteorological unit during the experimental period for both years (Figure 1). Twenty (20) genotypes of kenaf were sourced from the Kenaf and Jute Improvement Programme of the Institute. The experimental design was randomized complete block design (RCBD) with three replicates. Each sub-plot was (3 x 5) m² in size. Planting was done on 17th July and 11th July in 2009 and 2010, respectively. Plots were ploughed and harrowed and a pre-emergence herbicide, Pendimethalin (500 EC) was applied at the rate of 1.7 kg ha⁻¹, using a Knapsack sprayer. Manual weeding was done 4 WAP and NPK fertilizer was applied 2 days after weeding at the rate of 80:30:30 in both years. Monoforce® (Monocrotophos) was applied at the concentration of 0.68 kg ha⁻¹ active monocrotophos in 225 L of water at 5 WAP and at 50% flowering to protect plants from leaf beetle attack (*Podagrica* spp.) and pod sucking insects, respectively.

Agronomic data collection

Ten plants were tagged randomly within the inner rows at 4 WAP for the assessment of height (m), capsule number, seeds number per capsule and seed weight per capsule. The tagged plants were cut and separated from others at harvest. Harvesting of plant was done manually by cutting stems with cutlass in December, 2009 and 2010, respectively. The capsule height on the plant was determined from the above ground level to the first capsule from the base using graduated meter rule.

The number of capsules per plant was counted to obtain the mean values in each treatment. Seed number per capsule was determined by counting the number of seeds in 10 capsules and recording the average. This was also weighed to determine the seed weight per capsule.

One hundred seeds were taken randomly from the threshed seeds for weight determination using a gravimeter scale model GF-2000. The seed yield from each plot was determined after the manual threshing and converted into kilogram/hectare. The traits measured were analyzed for each year with SAS using General Linear Model (GLM) procedure and significant means were separated using Duncan's Multiple Range Test (DMRT) at 5% probability level.

RESULTS AND DISCUSSION

The mean squares due to location were highly significant ($p < 0.001$) on average height of the capsule, number of capsule/plant and seed number/ capsule (Table 1). Seasonal effect was also significant ($p < 0.001$) on average height of capsule, seed number/capsule, seed weight/plant and seed yield. Also, genotypic effect significantly ($p < 0.001$) affected number of capsule/plant and seed number per capsule. First order interaction of location x season was significant ($p < 0.05-0.001$) on all the parameters measured with the exception of 100 seed

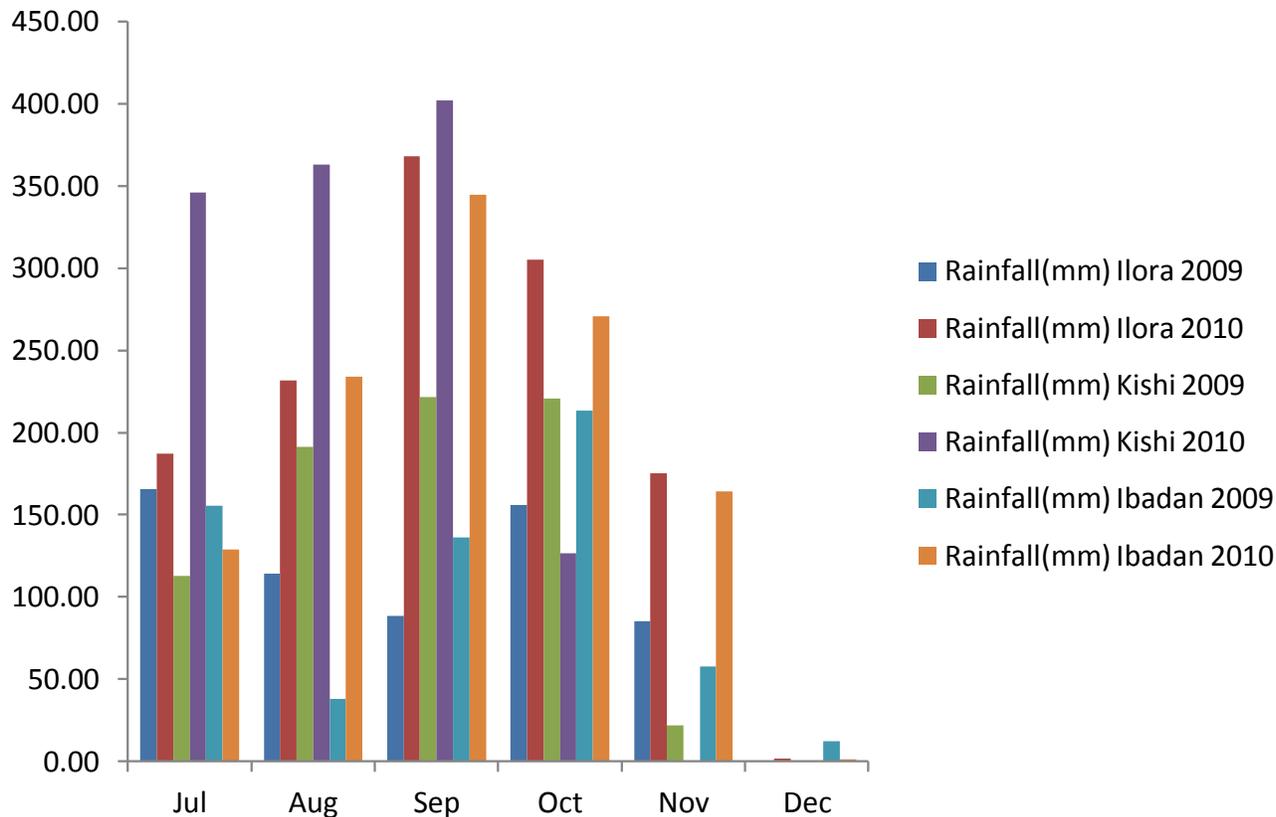


Figure 1. Rainfall distribution pattern at the experimental stations.

Table 1. Mean squares derived from combined analysis of variance for seed yield and yield components in 20 kenaf genotypes.

Source of Variation	DF	Capsule height (cm)	Capsule number/plant	Seed number /capsule	Seed weight/plant (g)	100seed weight (g)	Seed yield (Kg/ha)
Replicate	2	***	Ns	Ns	Ns	Ns	Ns
Location (L)	2	***	***	***	Ns	Ns	Ns
Year (Y)	1	***	Ns	***	***	Ns	***
Variety (V)	19	Ns	***	***	Ns	*	*
L X Y	2	***	***	***	*	Ns	*
L X V	38	*	Ns	Ns	Ns	Ns	Ns
Y X V	19	Ns	Ns	Ns	Ns	Ns	Ns
L xY xV	19	Ns	Ns	Ns	Ns	Ns	Ns

*, ** and *** Significant at $P < 0.05$, 0.01 and 0.001 respectively. Ns, Not significant.

weight. Only one character, average height of capsule was significantly different ($p < 0.001$) for the replication mean square. It means that environmental difference between locations existed in that character. Average height of the capsule ranged from 150.76 cm in Kishi to 175.25 cm in Ilora (Table 2). However, number of capsule/plant was highest in Kishi (29.78) and lowest in Ilora (20.34). In case of seed number / capsule, maximum seed number was recorded in Kishi, while

minimum was noted in Ilora with values of 15.51 and 11.20, respectively. Average seed weight / plant, 100 seed weight and seed yield / hectare were not significantly affected by location. This may be due to the fact that had been reported by Dempsey (1975) that kenaf have a wider range of adaptation to environmental factors than other fibre plants cultivated for commercial use. Despite no statistical yield differences among the locations, seed weight/plant and 100 seed weight were

Table 2. Cumulative effect of location on seed yield and yield parameters in 20 kenaf genotypes.

Location	Capsule height (cm)	Capsule number/plant	Seed number/capsule	Seed weight/plant (g)	100 seed weight (g)	Seed yield (Kg/ha)
Kisi	150.76 ^b	29.78 ^a	15.51 ^a	6.45 ^a	2.33 ^a	1290.6 ^a
Ibadan	158.00 ^b	23.03 ^b	11.51 ^b	7.06 ^a	2.60 ^a	1411.7 ^a
Ilorra	175.25 ^a	20.34 ^c	11.20 ^b	5.14 ^a	2.38 ^a	1067.3 ^a

Mean values within a column with the same letter are not significantly ($P<0.05$) different.

Table 3. Seasonal effect on seed yield and yield components in 20 kenaf genotypes.

Year	Capsule height (cm)	Capsule number/plant	Seed number/capsule	Seed weight/plant(g)	100seed weight (g)	Seed yield (Kg/ha)
2009	190.34 ^a	22.82 ^a	11.94 ^b	3.19 ^b	2.58 ^a	944.46 ^b
2010	126.87 ^b	26.71 ^a	13.65 ^a	10.19 ^a	2.46 ^a	1359.27 ^a

Mean values within a column with the same letter are not significantly ($P<0.05$) different.

Table 4. Effect of genotype on kenaf seed yield and yield components.

Variety	Capsule height (cm)	Capsule number/plant	Seed number/capsule	Seed weight/plant (g)	100seed weight (g)	Seed yield (Kg/ha)
2QC	158.91 ^{ab}	24.28 ^b	9.44 ^d	4.64 ^{ab}	2.37 ^b	928.8 ^{ab}
6QX	168.69 ^{ab}	21.56 ^b	12.72 ^{abcd}	6.24 ^{ab}	2.50 ^b	1247.9 ^{ab}
A-60-282	166.19 ^{ab}	21.67 ^b	13.09 ^{abc}	6.03 ^{ab}	2.51 ^b	1205.7 ^{ab}
A-60-284	175.0 ^a	24.39 ^b	14.71 ^{ab}	6.59 ^{ab}	2.56 ^b	1318.4 ^{ab}
AC-313	156.98 ^{ab}	19.52 ^b	13.98 ^{abc}	6.50 ^{ab}	2.42 ^b	1299.6 ^{ab}
AMC-108	162.85 ^{ab}	23.46 ^b	10.96 ^{cd}	7.24 ^a	2.47 ^b	1451.7 ^a
AU-75	171.66 ^a	25.38 ^b	13.41 ^{abc}	7.27 ^a	2.47 ^b	1454.3 ^a
BS-1	172 ^a	18.86 ^b	11.85 ^{bcd}	5.41 ^{ab}	2.31 ^b	1082.4 ^{ab}
Cuba 108	157.40 ^{ab}	22.59 ^b	14.25 ^{abc}	5.52 ^{ab}	2.56 ^b	1103.3 ^{ab}
Ex Funtua	165.85 ^{ab}	20.99 ^b	15.03 ^{ab}	5.10 ^{ab}	2.47 ^b	1019.7 ^{ab}
Ex Shika	168.41 ^{ab}	20.98 ^b	12.65 ^{abcd}	6.43 ^{ab}	2.44 ^b	1285.7 ^{ab}
G-45	165.69 ^{ab}	23.63 ^b	14.77 ^{ab}	5.79 ^{ab}	2.41 ^b	1155.2 ^{ab}
Ifeken 100	170.60 ^a	23.10 ^b	14.98 ^{ab}	7.10 ^a	2.36 ^b	1419.3 ^a
Ifeken 400	167.76 ^{ab}	23.90 ^b	14.30 ^{abc}	5.74 ^{ab}	2.19 ^b	1147.3 ^{ab}
Local Kenaf	146.63 ^b	55.04 ^a	9.45 ^d	3.30 ^b	3.07 ^{ab}	1361.6 ^a
Local line 36	167.02 ^{ab}	24.70 ^b	15.16 ^{ab}	6.81 ^a	2.28 ^b	660.0 ^b
S-72-78-10	167.20 ^{ab}	24.55 ^b	13.86 ^{abc}	6.73 ^{ab}	2.43 ^b	1345.5 ^{ab}
SF-549	158.53 ^{ab}	20.26 ^b	15.74 ^a	6.24 ^{ab}	3.67 ^a	1210.0 ^{ab}
Tainung 1	167.14 ^{ab}	22.57 ^b	14.17 ^{abc}	4.98 ^{ab}	2.35 ^b	996.7 ^{ab}
V ₂ - 400	164.45 ^{ab}	26.06 ^b	12.72 ^{abcd}	6.35 ^{ab}	2.39 ^b	1269.7 ^{ab}

Mean values within a column with the same letter(s) are not significantly ($P<0.05$) different.

better in Ibadan and this contributed to higher yield (1411.7kg). Height of the first capsule produced in 2009 was higher as compare to the height of capsule in 2010 (Table 3). The implication of this is that in 2010 capsule formation on mother plant was more as compared to 2009. Average number of capsules/plant, seed number/capsule, seed weight/plant and seed yield/plant was higher in 2010 than in 2009. Differences in yield traits and seed yield between years were due to differences in rainfall from July through December each

year. Rainfall for these months during 2009 and 2010 were 609.50, 786.40, 613.40 and 1269.40, 1237.80, 1143.40 mm in Ilorra, Kishi and Ibadan, respectively. This result corroborates the findings of Webber (1996), Mullens (1998) and Webber and Bledsoe (2003). They reported that factors like maturity ratings of cultivar, photosensitivity of varieties, latitudinal location, soil fertility, cultural practices, rainfall in terms of distribution and intensity; may significantly influence kenaf performance, seed quality and yield. Table 4 shows that

Table 5. Influence of season and location on kenaf seed yield and yield components.

Location	Capsule height (cm)	Capsule number/plant	Seed number/capsule	Seed weight/plant (g)	100 seed weight (g)	Seed yield (Kg/ha)
2009						
Kishi	161.05	23.48	12.20	3.63	2.36	925.90
Ibadan	202.72	26.70	10.53	3.00	2.59	1020.90
Ilorra	207.25	18.27	11.77	2.93	2.69	886.13
2010						
Kishi	140.47	26.07	13.82	9.28	2.30	1312.23
Ibadan	113.28	29.36	15.48	11.11	2.61	1502.57
Ilorra	142.30	22.41	12.21	7.34	2.58	1212.25
F-Test	***	***	***	*	Ns	*

*, ** and *** Significant at $P < 0.05$, 0.01 and 0.001 respectively. Ns, Not significant.

average height of capsule ranged from 146.63 cm in local kenaf to 175.0 cm in A-60-284 with no statistical difference among the genotypes used. All the genotypes used with the exception of Local Kenaf could be planted for both seed and fibre production. Of all the genotypes used, Local kenaf produced 55.04 capsules/plant while BS-1 recorded 18.86 capsules/plant. Average seed number / capsule ranged from 15.74 in SF-549 to 9.44 in 2QC. Average seed weight/plant also ranged from 7.27 g in AU-75 to 3.30 g in Local kenaf. Both 100 seed weight and seed yield per hectare were statistically different among all the genotypes used. However, SF-549 recorded the highest hundred seed weight and lowest in Ifeken 400 with values of 3.67 g and 2.19 g, respectively. Genotypes AU-75 and Ifeken 100 recorded highest yield of 1454 and 1419 kg/ha, respectively. The least yield of 660 kg was recorded by Local kenaf. Four of the genotypes used recorded yield of less than 1 ton/ha. Average capsule height produced across the 3 locations in 2009 was higher than what was recorded in 2010 (Table 5). Both Ibadan and Kishi had kenaf with capsule height of more than 2 m above ground level. In 2010, average height of capsule was lower than 1.5 m in all the three location. There were 11.03, 9.90 and 22.66% increase in capsule number/plant in 2010 in Kishi, Ibadan and Ilora over 2009, respectively. There were increase in seed number/capsule and seed weight/plant in 2010 in all the three location as compared to what was recorded in 2009. Seed yield/ha of 41.83, 47.25 and 36.79% increase in 2010 over 2009 were recorded in Kishi, Ibadan and Ilora, respectively.

Conclusion

Sixteen (16) lines with high seed yield (> 1 ton/ha) and fibre yield potential were identified for the promising performance in Ibadan. These genotypes showed promising results as dual purpose kenaf lines; seed and fibre production, since the height at which capsules set

were above 1.5 m from ground level. This will give necessary information in the development of combine harvester for kenaf for both seed and fibre production.

Conflict of Interests

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

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Full Length Research Paper

Physiological characteristics and pathogenicity of *Xanthomonas campestris* pv. *musacearum* strains collected from enset and banana in Southwest Ethiopia

Befekadu Haile¹, Girma Adugna^{1*} and Fikre Handoro²

¹Department of Horticulture and Plant Sciences, College of Agriculture and Veterinary Medicine, Jimma University, P.O. Box 307, Jimma, Ethiopia.

²Southern Agricultural Research Institute, P.O. Box 6, Hawassa, Ethiopia.

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Xanthomonas campestris pv. *musacearum* (Xcm) is a deadly bacterial pathogen causing wilt of enset and banana plants since the first record in Keffa province in Southwest Ethiopia as early as the 1960s. The disease remains a dominant constraint to enset production although its impact on banana has declined over the past four decades. The disease is ravaging banana plantations and spreading at alarming rates since its recent outbreak in other east and central African countries, including Uganda, Democratic Republic of Congo, Rwanda, Tanzania and Kenya. Enset wilt management strategies such as sanitation have been recommended although it is tedious for farmers to apply them for various reasons. The efforts to develop enset clones tolerant/resistant to Xcm strains have not been efficient for inconsistent reactions/performance of the selected materials, mainly attributed to variations in the bacterial isolates used across the studies. Thus, it is important to determine ranges of variation within the pathogen and host populations for developing resistant varieties and further breeding work. The objectives of this study were to collect and characterize Xcm strains from enset and banana plants in three major enset growing zones of Southwest Ethiopia and determine host-pathogen interactions. Nineteen (19) Xcm strains were selected from a total of 72 isolates collected from leaf petioles of enset and banana plants infected with bacterial wilt in six districts of Sheka, Keffa and Bench-Maji zones. The bacterial strains were typically creamy to yellow mucoid, circular with dome-shaped colonies. The strains were Gram-negative, KOH and catalase positive, suppressed on asparagine medium and negative for nitrate reduction; most isolates (84.2%) were insensitive to 2% NaCl while few strains (15.8%) were retarded by 1% NaCl concentration. All the strains were positive to hypersensitivity test with reaction varying from chlorosis to necrosis on tobacco leaves. Six enset and two banana strains of Xcm were pathogenic to the susceptible enset 'Yeko' and banana 'Butuza' (AAA) clones. The banana strains induced typical bacterial wilting symptoms on both hosts that ultimately led to complete death (100%). The host-pathogen interaction evidenced differences mostly among the enset clones in their resistance/tolerance and variation in aggressiveness (virulence) between the bacterial strains. The enset clones 'Nobo' and 'Gudiro' were consistently resistant while 'Yeko' was highly susceptible to the three Xcm strains, namely: Xcme-9, Xcme-10 and Xcme-19, whereas 'Chikaro' was moderately tolerant to two strains (Xcme-10 and Xcme-19) but most susceptible to strain Xcm9. The strains were less or non-aggressive to the resistant clones 'Nobo' and 'Gudiro' but most aggressive on the susceptible plants while ranges of aggressiveness were demonstrated on 'Chikaro'.

Key words: Banana, *Ensete ventricosum*, enset bacterial wilt, *Xanthomonas campestris* pv. *musacearum*, Ethiopia.

INTRODUCTION

Xanthomonas campestris pv. *musacearum* (Xcm) has been the most devastating bacterial disease of enset (*Ensete ventricosum* (Welw.) Cheesman), threatening crop production in Ethiopia since it was first officially reported in 1968 (Yirgou and Bradbury, 1968, 1974; Ashagari, 1985; Weldemichael et al., 2006, 2008). The disease is widely distributed and has caused significant losses in almost all enset growing areas of the country over the past 45 years (Ashagari, 1985; Handoro and Weldemichael, 2007; Weldemichael et al., 2008), affecting farmers' livelihood. Ashagari (1985) reported that bacterial wilt was very severe in 23 out of 29 surveyed districts sampled in central, southern and southwestern Ethiopia. In general, unlike the report by Addis et al. (2004), the disease is more serious on enset than on banana where both crops are grown together in southwest Ethiopia.

Although *X. campestris* pv. *musacearum* was confined for many years as an endemic disease in Ethiopia, widespread outbreaks of bacterial wilt incited by the same pathogen have been reported on banana plantations, inflicting great losses, in other east and central African countries. In these regions, the bacterial wilt of enset is commonly known as banana *Xanthomonas* wilt and was first reported in Uganda in 2001, Democratic Republic of Congo in 2003, Rwanda in 2004, Tanzania in 2005 and Kenya in 2006 (Biruma et al., 2007; Smith et al., 2008; Tripathi et al., 2009). However, there has been no report on wild enset in these countries.

Enset is cultivated only in Ethiopia (Zippel, 2005; Bizuayehu, 2008), being a multipurpose crop used for food, feed and fibre. The crop is said to ensure food security, especially in the face of recurrent drought and climate change (Brandt et al., 1997). Enset is supposed to have been domesticated and distributed in the higher areas of Keffa, in the southwestern part of the country (Westphal, 1975). Both contemporary techniques and classical classification systems indicate that there exists considerable diversity in the cultivated and wild enset populations of the country (Almaz et al., 2002; Birmeta et al., 2004; Bizuayehu, 2008).

To date, management of enset bacterial wilt has not been successful in Ethiopia for a number of technical and practical reasons. Although enormous extension efforts on sanitation (disinfection of farming and processing tools, roguing/eradication of infected enset plants) have been undertaken to curb the disease problem, the measures are not easy to implement by enset farmers. Enset is a giant, single-stemmed, herbaceous perennial plant (10-13 m high and >2 m in diameter) with a deep-rooted underground corm and a large pseudostem that

make it difficult to uproot and bury or burn the infected mature trees. In addition, despite considerable diversity within cultivated and wild enset (Almaz et al., 2002; Birmeta et al., 2004; Bizuayehu, 2008), developing resistant/tolerant enset clones has not been effective. Among other problems, the reactions of most clones identified as tolerant/resistant were not consistent across locations and over time (Ashagari, 1985; Handoro and Michael, 2007, 2008). This inconsistent performance of the clones was partly attributed to variations in the bacterial wilt pathogen isolates used across the studies (Michael et al., 2008). Nevertheless, the interactions of host-pathogen-environment-related factors determine disease expression. Hence, the present studies addressed physiological characteristics, pathogenicity and host-pathogen interactions of *X. campestris* pv. *musacearum* strains collected from infected enset and banana plants in southwest Ethiopia.

MATERIALS AND METHODS

Collection of *X. campestris* pv. *musacearum*

During September - November 2011, a large number of tissue pieces (25 cm long) were randomly collected from leaf petioles of enset and banana plants with active bacterial wilt symptoms in farmers' fields, after checking for the presence of bacterial cells in the dissected sections. The sample areas were represented by 54 farmers' fields (2 plants per field) in six major enset-growing districts of three zones in southwest Ethiopia, namely Sheka, Keffa and Bench-Maji varying in altitude from 1450 to 2450 m.a.s.l. The bacterial cells were easily isolated and then purified on yeast peptone sucrose agar (YPSA) (5 g yeast extract, 10 g peptone, 20 g sucrose, 15 g agar per litre of distilled water) after incubating at 28°C for 48 to 72 h (Quimio, 1992; Schaad et al., 2001). The field-collected isolates were maintained as a stock culture on the same medium at 4°C and then used for further study. The cultural and physiological characterizations of the bacterial isolates were conducted in the plant pathology laboratory and the hypersensitivity and pathogenicity tests were undertaken in the greenhouse at College of Agriculture and Veterinary Medicine, Jimma University.

Characterization of *X. campestris* pv. *musacearum*

Gram reaction

The standard Gram-staining procedure (Schaad et al., 2001) and KOH solubility test (Fahy and Hayward, 1983) were conducted to determine Gram positive and negative bacterial isolates.

Growth on asparagine medium

The bacterial isolates that showed Gram negative reaction with mucoid thread were grown on asparagine medium

*Corresponding author. E-mail: girma.adugna@yahoo.com.

(0.5 g asparagine, 0.1 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KNO_3 , 0.1 g CaCl_2 , 0.1 g NaCl and 15 g agar per litre of distilled water) at 28°C for 48 to 72 h (Dye et al., 1980). This diagnostic test is used to detect *Xanthomonas* because it is not able to grow on asparagine medium while other species, such as yellow *Enterobacteriaceae* and many *Pseudomonas* can. The growth of the bacteria on asparagine agar plates and broth were recorded and those isolates that were unable to grow on the medium were considered for further tests.

Growth on nutrient agar with 5% glucose

Each isolate was streaked on nutrient agar with 5% glucose (23 g nutrient agar, 5% glucose per litre of distilled water) and incubated at 28°C for 48 to 72 h. The colony growth characteristics such as form, elevation and color were scored on this medium to differentiate *X. campestris* from other *Xanthomonas* species (Bradbury, 1984).

Catalase and salt tolerance tests

A few drops of 3% hydrogen peroxide were added on the surface of the 48 h-old culture of each isolate on YPSA medium and bubble formation was recorded as positive for catalase activity (Dickey and Kelman, 1988). Salt tolerance of the strains was also tested by inoculating each isolate into nutrient broth in a test tube with varying NaCl concentrations (1 to 5%) (Hayward, 1964). The nutrient broth without salt (0%) was included as a negative control and the presence or absence of bacterial growth was examined after 12 to 14 days incubation at 25°C.

Nitrate reduction

The ability of the isolates to reduce nitrate to nitrite was determined in a growth medium that contained 1 g KNO_3 , 5 g peptone, 3 g yeast extract and 3 g agar per litre of distilled water in test tubes. Each isolate was inoculated by stabbing and sealing with 3 ml sterilized molten agar to avoid false positives and incubated at 28°C. The growth of each bacterial isolate and bubble formation beneath the upper agar layer was observed and recorded as a positive result for nitrate reduction after three, five and seven days inoculation (Dickey and Kelman, 1988).

Hypersensitivity test

Forty-hour-old cultures of 19 selected bacterial strains (out of 72 collected isolates) were suspended in sterilized distilled water and adjusted to 0.3 O.D. at 460 nm (equivalent to about 10^8 CFU/ml bacterial cell concentration) using a spectrophotometer. An aliquot of 2 ml bacterial suspension was injected into the intercellular spaces of expanded leaves (2 leaves per plant) of one-month old tobacco plants (*Nicotiana tabacum* var. White Burley) with a sterile syringe. Similarly, treated plants with distilled sterile water were used as negative control. All the inoculated plants were then kept in a greenhouse with a daytime temperature of about 27 - 30°C for subsequent symptom development. The appearance of chlorotic to necrotic tissue around the injection point on inoculated leaves was considered as positive for the test (Quimio, 1992).

Pathogenicity test

Growing susceptible clones

One-year old suckers propagated from a susceptible enset clone,

locally known as 'Yeko', and a banana variety 'Butuza' (AAA) were planted in a mixture of sterilized soil, sand and amended with composted manure (in a 2:1:1 ratio) in 25 L plastic pots (Quimio 1992). The suckers were grown for six months in the greenhouse at 25 - 30°C daytime temperature.

Inoculum preparation and inoculation

A total of eight Xcm strains (six from enset and two from banana), that showed positive reactions in the hypersensitivity test was selected and reproduced on nutrient agar in 90 mm Petri dishes incubated at 28°C for 48 h. The bacterial cell suspension of each strain was uniformly prepared as mentioned above and aliquots of 3 ml of the suspension (1×10^8 CFU/ml concentration) were inoculated using a sterile syringe to the second innermost leaf petiole of young (six month- old) enset and banana plants. In this case, the six enset strains were inoculated only into the susceptible enset plants while infectivity of the two banana isolates were tested both on the banana variety 'Butuza' and the enset clone 'Yeko'. Seedlings of both hosts treated in the same manner with distilled sterile water were included as negative controls. This experiment was laid out in a completely randomized design with three replications; each strain was tested on three plants per replication. The progressive wilting symptoms, including number of infected leaves, number of completely dead seedlings, and number of days to first symptom appearance and days to complete death were recorded at weekly intervals for three to four months. Finally, the presence and/or absence of bacterial mass was checked in the leaf petioles and pseudostems and re-isolated from symptomatic plants.

Enset clone-*X. campestris* pv. *musacearum* strain interaction in the field

This set of experiment was conducted under field conditions using four enset clones locally identified as 'Yeko', 'Chikaro', 'Nobo' and 'Gudiro' and three bacterial strains confirmed to be pathogenic in the previous trial. The enset clones were purposely selected based on their reactions to Xcm as susceptible, intermediate and tolerant/resistant in most surveyed farmers' fields in the six major enset producing sample districts. Suckers of each enset clone were transplanted at Gecha (1960 m a.s.l.) in Anderacha district of Sheka zone and carefully grown for about eight months. The three Xcm strains Xcme-9, Xcme-10 and Xcme-19, respectively represented Keffa, Sheka and Bench-Maji zones with their respective enset agroecology described as lowland (1450 m.a.s.l.), highland (2450 m.a.s.l.) and intermediate (1790 m.a.s.l.). Fresh bacterial inoculum of each selected Xcm strain was separately prepared as mentioned above and artificially inoculated (5 ml) with each enset clone following the procedures adopted by Michael et al. (2008). A row of five enset plants per clone was inoculated with sterile water and included as a negative control.

Experimental design, data collection and analysis

Each of the tests conducted in the laboratory was repeated at least three times with three replication per test including negative and positive control. The field experiment, consisting of 12 treatments (three bacterial strains by four enset clones) arranged in a factorial treatment combinations, was laid out in a randomized complete block design with three replications (five plants/treatment). The disease data, such as number of days to first symptom appearance, number of wilting and dead plants, and number of days to complete death, were recorded at fortnight intervals for five to six months. In addition, disease severity was assessed employing a 0 - 5 scale developed by Winstead and Kelman (1952), scoring 0 for a plant

Table 1. Physiological characteristics of *Xanthomonas campestris* pv. *musacearum* (Xcm) strains isolated from leaf petioles of enset and banana plants in southwest Ethiopia (2012).

Xcm strains ¹	Gram staining	KOH test	Nutrient agar (5% glucose)	Catalase test	Nitrate reduction	NaCl concentrations (%) ²					
						0	1	2	3	4	5
Xcme-1	-	+	+	+	-	+	+	+	-	-	-
Xcme-2	-	+	+	+	-	+	+	+	-	-	-
Xcme-3	-	+	+	+	-	+	+	+	+	+	-
Xcme-4	-	+	+	+	-	+	+	-	-	-	-
Xcme-5	-	+	+	+	-	+	+	+	-	-	-
Xcme-6	-	+	+	+	-	+	+	-	-	-	-
Xcme-7	-	+	+	+	-	+	+	+	-	-	-
Xcme-8	-	+	+	+	-	+	+	+	+	+	+
Xcme-9	-	+	+	+	-	+	+	+	-	-	-
Xcme-10	-	+	+	+	-	+	+	+	-	-	-
Xcme-11	-	+	+	+	-	+	+	+	+	+	-
Xcme-12	-	+	+	+	-	+	+	+	+	-	-
Xcme-13	-	+	+	+	-	+	+	+	+	-	-
Xcme-14	-	+	+	+	-	+	+	-	-	-	-
Xcme-15	-	+	+	+	-	+	+	+	+	+	+
Xcmb-16	-	+	+	+	-	+	+	+	+	-	-
Xcmb-17	-	+	+	+	-	+	+	+	+	+	-
Xcme-18	-	+	+	+	-	+	+	+	-	-	-
Xcme-19	-	+	+	+	-	+	+	+	+	+	-

¹Xcm strains Xcme-1, Xcme-2 and Xcme-3 were collected from Gatimo, Yina and Kanga (Masha, Sheka zone); Xcme-4, Xcme-5 and Xcme-6 were collected from Gebina, Chicha and Tugiri (Andiracha, Sheka zone); Xcme-7, Xcme-8 and Xcme-9 were collected from Ermichi, Kubito and Achany (Yeki, Sheka zone); Xcme-10, Xcme-11 and Xcme-12 were collected from Yerkichity, Dirbado and Damonechity (Giesha, Keffa zone); Xcme-13, Xcme-14, Xcme-15 and Xcmb-16 were collected from Sheda, Gawaty and Dachadifa (Bita, Keffa zone); Xcmb-17, Xcme-18 and Xcme-19 were collected from Kuka, Maha and Ziagin (Shebench, Bench-Maji zone), respectively. ²The salt tolerance test was replicated and three independent tests were conducted. The letters 'e' and 'b' stand for the host enset and banana, respectively. The sign '-' and '+' indicate negative and positive responses.

without visible symptom, 1 for one leaf wilted, 2 for two to three leaves wilted, 3 for four leaves wilted, 4 for all leaves wilted and 5 for completely dead plant. Finally, the incubation period, the number of days to complete death, disease severity index and incidence were computed for statistical analyses. The disease incidence (%) was calculated as the number of dead enset plants divided by the total number of inoculated plants per plot multiplied by 100, then transformed to arc sin square root values before analysis of variance, and treatment means were compared using Tukey's test with SAS software version 9.2 (SAS Institute Inc. 2008).

RESULTS

Physiological characteristics of *X. campestris* pv. *musacearum* strains

Colony characteristics

Nineteen bacterial strains including two isolates from banana were studied (Table 1), and the colonies were circular, dome-shaped and highly mucoid with a shiny appearance on the YPSA medium. All the strains showed Gram-negative reaction in Gram staining and did not

dissolve in 3% KOH solution; instead they formed a thin strand of slime while mixing the bacterial cells and lifting with the inoculating loop.

The colonies of Xcm strains showed slight variations in colour and growth character on nutrient agar with 5% glucose medium. Most of the isolates from enset (68%) had slimy mucoid to mucoidal yellow colonies, whereas those identified from some enset clones and banana (32%) appeared creamy mucoidal to slightly yellow (Figure 1). The strains were catalase positive, producing gas bubbles when a 48-h-old colony of each strain was dissolved in a few drops of 3% hydrogen peroxide. The tested bacterial isolates failed to grow on asparagine medium and did not reduce nitrate to nitrite (Table 1).

In salt tolerance tests, 84.2% of the strains grew well on nutrient broth amended with 2% NaCl, while few strains (15.8%), including three from enset (Xcme-4, Xcme-6 and Xcme-14), were retarded by 1% NaCl, being highly sensitive to salt. On the other hand, 31.6% of the strains (Xcme-3, Xcme-8, Xcme-11, Xcme-15, Xcmb-17 and Xcme-19) demonstrated salt tolerance, being insensitive even up to 4 or 5% NaCl concentration (Table 1).

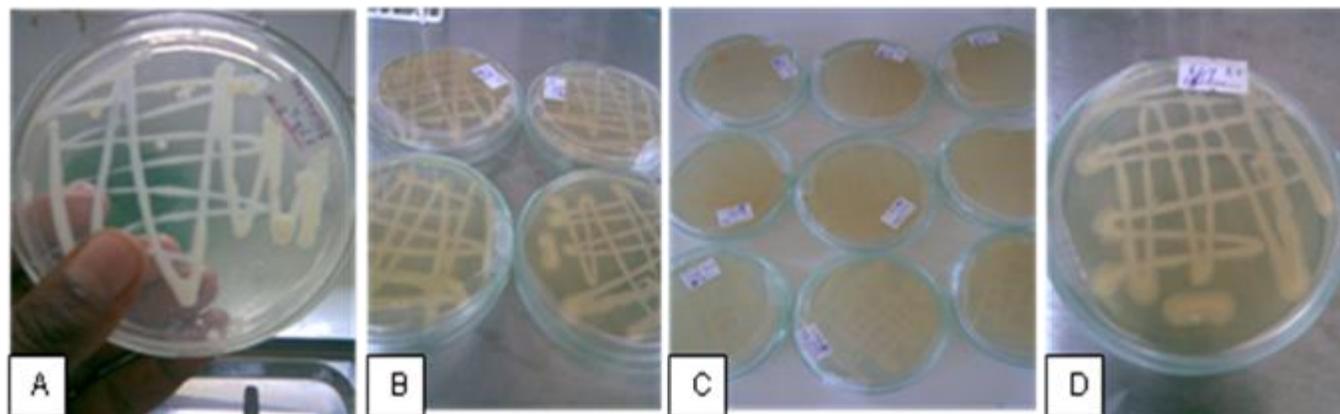


Figure 1. Colonies of *Xanthomonas campestris* pv. *musacearum* strains grown on nutrient agar with 5% glucose for 48 h at 28°C with creamy/slimy, less-mucoid (A and B) to mucoid and deep yellow (C and D) appearance.

Hypersensitive reaction of *X. campestris* pv. *musacearum* strains on tobacco leaves

All the 19 bacterial strains inoculated into the leaves of tobacco seedlings induced hypersensitive reactions ranging from small chlorotic spots to extensive necrotic areas around the injection point within 48 to 72 h. Some enset strains, such as Xcme-9, Xcme-10 and Xcme-19, showed more aggressiveness, inducing extensive necrosis, while untreated leaves appeared green.

Pathogenicity of Xcm strains on young enset and banana plants

Among Xcm strains that demonstrated an aggressively positive hypersensitive reaction, six isolated from enset and two from banana (Xcmb-16 and Xcmb-17) were found to be highly pathogenic on young seedlings of a susceptible enset clone 'Yeko' and a banana variety 'Butuza' (AAA). The number of days to first symptom appearance (incubation period) and days to complete death varied significantly ($P < 0.05$) among the bacterial strains (Table 2). The two banana strains Xcmb-16 and Xcmb-17 induced typical wilting symptoms on its host variety 'Butuza' within 30 days, as compared to significantly ($P < 0.05$) longer incubation periods ranging from 45 to 60 days on the enset clone. In addition, some enset isolates Xcme-2, Xcme-6 and Xcme-13 took about two months (50 to 60 days) to infect and initiate first wilting symptoms. All eight strains eventually resulted in complete death of inoculated plants of both hosts within 45 to 90 days (Table 2).

The inoculated enset leaves first revealed gray to light yellow chlorosis around the inoculated areas that gradually turned to yellowish brown necrosis and finally the whole leaf dried and then the petiole collapsed. These external symptoms spread progressively to the

remaining leaves, leading to complete death of the plant. Internally, groups of creamy to yellowish bacterial cells were evident in dissected petioles of symptomatic leaves and in cross-section of pseudostem of dying enset (Figure 2A - G) and banana plants (Figure 2H - J).

Interactions of enset clones with *X. campestris* pv. *musacearum* strains

There were highly significant ($P < 0.001$) differences among the selected strains, enset clones and their interactions in all disease parameters considered in the study (Table 3). The three strains Xcme-9, Xcme-10 and Xcme-19, respectively, collected from Sheka, Keffa and Bench-Maji zones of southwest Ethiopia, induced wilting symptoms on enset clone 'Yeko' within short incubation periods of 52 to 53 days that subsequently resulted in higher wilt severity and complete death of the plants (Table 3). However, the same bacterial strains did not incite disease on all inoculated plants of clone 'Nobo' 120 days after inoculation. Similarly, these strains showed infection symptoms only on the inoculated leaf of 'Gudiro' plants after about 56 to 62 days incubation (Table 3). The infection did not progress and the plants entirely remained healthy even after the trial termination. Thus, the result indicated that enset clones 'Nobo' and 'Gudiro' showed high levels of resistance to the bacterial wilt strains, while 'Yeko' was markedly susceptible.

The enset clone 'Chikaro' had differential reactions to the three bacterial strains. This clone was moderately attacked by Xcme-10 and Xcme-19 with respective disease incidences of 55 and 66% that were significantly ($P < 0.05$) different from strain Xcme-9, causing complete death (Table 3). The three Xcm strains caused the least disease severity (0 to 6.7%) and did not kill 'Nobo' and 'Gudiro' clones (0% incidence) although they showed high percentage of wilt severity index and incidences on

Table 2. Pathogenicity of *Xanthomonas campestris* pv. *musacearum* (Xcm) strains inoculated with a susceptible enset clone 'Yeko' and a banana variety 'Butuza' in the greenhouse at College of Agriculture and Veterinary Medicine, Jimma University Ethiopia (2012).

Xcm strains ¹	Host	Incubation period (days) ²	Mean number of days to complete death ³	Wilt incidence ⁴
Xcme-2	'Yeko'	50 ^{ab}	75 ^{a-d}	88.4 (100)
Xcme-6	'Yeko'	55 ^a	85 ^{ab}	88.4 (100)
Xcme-9	'Yeko'	35 ^{ab}	55 ^{de}	88.4 (100)
Xcme-10	'Yeko'	45 ^{ab}	65 ^{b-e}	88.4 (100)
Xcme-13	'Yeko'	50 ^{ab}	80 ^{a-c}	88.4 (100)
Xcmb-16	'Yeko'	45 ^{ab}	75 ^{a-d}	88.4 (100)
Xcmb-16	'Butuza'	30 ^b	45 ^e	88.4 (100)
Xcmb-17	'Butuza'	30 ^b	60 ^{c-e}	88.4 (100)
Xcmb-17	'Yeko'	60 ^a	90 ^a	88.4 (100)
Xcme-19	'Yeko'	45 ^{ab}	70 ^{a-d}	88.4 (100)
LSD (P < 0.05)		20.95	22.40	NS

¹*Xanthomonas campestris* pv. *musacearum* strains Xcme-2, Xcme-6, Xcme-9 were collected from Masha, Anderacha and Yeki districts of Sheka; Xcme-10, Xcme-13, Xcmb-16 were collected from enset in Geisha and banana at Bita districts of Keffa; Xcmb-17 and Xcme-19 were collected from banana and enset at Shebench in Bench-Maji, respectively. ²Mean number of days to first symptom appearance and ³Mean number of days to complete death of the plants after inoculation (incubation period). ⁴Disease incidence transformed to angular values before analysis and those figures in the brackets are actual mean values. Means followed with the same letter(s) in the column are not significantly different from each other according to Tukey's test. NS = non significant.

the plants of 'Yeko' (up to 100%) (Table 3). This finding also showed *X. campestris* pv. *musacearum* populations varied from non- or least to high levels of aggressiveness or virulence.

DISCUSSION

In this study, 72 isolates of *X. campestris* pv. *musacearum* were identified from symptomatic enset and banana plants sampled in six districts of three major enset growing zones namely, Sheka, Keffa and Bench-Maji in southwest Ethiopia. The bacterial strains had circular, typical mucoid growth varying from light cream to very yellow colonies on nutrient agar with 5% glucose. In physiological tests, all the 19 bacterial isolates were Gram-negative, KOH and catalase positive, did not grow on asparagine medium and failed to reduce nitrate to nitrite. In addition, they demonstrated relatively varying growth sensitivity to NaCl concentrations and 84.2% of the isolates grew well on nutrient broth amended with 2% NaCl, while few strains (15.8%) were retarded by 1% NaCl, being highly sensitive to salt. The strains induced positive hypersensitive reactions, producing chlorotic to necrotic areas on tobacco leaves.

Eight of 19 strains were pathogenic to the susceptible enset clone 'Yeko', including the two banana strains inoculated into enset and banana, exhibiting progressive bacterial wilting symptoms from yellowing of the inoculated leaves to complete death of the young plants

of the respective hosts in the greenhouse. Besides, the characteristic external symptoms, creamy to yellowish pockets of bacterial cells were commonly encountered inside the leaf petioles and pseudostems of infected plants of enset and banana that were consistent with bacterial *Xanthomonas* wilt of both hosts infected in the fields. There were some discernible differences in cultural (creamy to yellow colony), physiological (salt tolerance) and hypersensitivity (chlorotic to necrotic) responses among Xcm strains. In the pathogenicity test, although all Xcm isolates eventually killed both enset and banana plants, the two banana strains Xcmb-16 and Xcmb-17 infected its host variety 'Butuza' (AAA) within significantly (P < 0.05) shorter incubation time (30 days) and caused death with in 45 to 60 days than on enset plants. This may suggest that the banana strain is relatively more aggressive on banana than on enset. The banana cultivars such as 'Dwarf Cavendish', 'Giant Cavendish' and 'FHIA-17' with similar genome to 'Butuza' were reported to be highly susceptible to Xcm strain in Uganda (Tripathi and Tripathi, 2009).

The 19 bacterial strains detected in infected and symptomatic enset and banana trees thus conform to the phytopathogenic bacterial species *X. campestris* pv. *musacearum*. These findings are in agreement with the pioneer work of Yirgou and Bradbury (1968, 1974) who first detected and identified enset bacterial wilt on enset and banana plants in Keffa province of southwest Ethiopia. Based on physiological, biochemical and pathological characteristics of the bacterial strains, the



Figure 2. Typical characteristic symptoms of bacterial wilt caused by *Xanthomonas campestris* pv. *musacearum* on infected enset and banana plants under field conditions in southwest Ethiopia. External progressive wilting symptoms (A - C) and dead enset plant (D), pocket of bacterial cells in dissected leaf petiole (E) and yellowish bacterial cells oozing out of cross-section of leaf petioles (F) and pseudostems of enset (G). External early wilting symptom (H) and finally dead (I) and yellowish bacterial exudates in the stem cross-section (J) of banana plant.

authors proposed the epithet *X. musacearum* sp.n. as causal agent of the disease on both hosts (Yirgou and Bradbury, 1968, 1974). Later, the species was grouped as *X. campestris* pathovar and named as *X. campestris* pv. *musacearum* providing detailed classical taxonomic descriptions (Bradbury, 1986; Young et al., 1991). The results of molecular genetic analyses (Rep-PCR and RAPD) indicate that the present populations of *X. campestris* pv. *musacearum* in central and east Africa (Ethiopia, DR Congo, Rwanda, Tanzania and Uganda) are homogenous regardless of time of isolation,

geographic location and hosts (Aritua et al., 2007, 2008; Odipio et al., 2009; Lewis Ivey et al., 2010). Nevertheless, based on fatty acid methyl esters and *gyrase B* gene analyses, Aritua et al. (2008) proposed reclassification of this species as a pathovar within *X. vasicola* (*X. vasicola* pv. *musacearum*). As commented by Ivey et al. (2010), we also suggested that several research data should be generated on many collections of *X. campestris* pathovars (including the *Musacearum* strains from enset and banana) through host-pathogen interaction (cross-inoculation) and molecular analyses.

Table 3. Interaction of enset clones inoculated with *Xanthomonas campestris* pv. *musacearum* isolates in incubation period (days), disease severity index and incidence (%) and days to complete death under field conditions at Gecha (Sheka) in southwest Ethiopia (2011/2012).

Enset clones ¹	Bacterial isolates ²	Incubation period (days) ³	Disease severity index (%) ⁴	Days to complete death ⁵	Incidence ⁶
<i>Gudiro</i>	Xcme-9	61.7 ^{ab}	6.7 ^c	0.0 ^c	1.6 ^c (0.0)
	Xcme-10	55.8 ^{bc}	6.7 ^c	0.0 ^c	1.6 ^c (0.0)
	Xcme-19	62.5 ^{ab}	6.7	0.0 ^c	1.6 ^c (0.0)
<i>Nobo</i>	Xcme-9	0.0 ^e	0.0 ^d	0.0 ^c	1.6 ^c (0.0)
	Xcme-10	0.0 ^e	0.0 ^d	0.0 ^c	1.6 ^c (0.0)
	Xcme-19	0.0 ^{e2}	0.0 ^d	0.0 ^c	1.6 ^c (0.0)
<i>Chikaro</i>	Xcme-9	46.7 ^d	100 ^a	86.7 ^b	88.4 ^a (100)
	Xcme-10	67.5 ^a	68.9 ^b	110.0 ^a	66.0 ^b (83.4)
	Xcme-19	63.3 ^a	71.2 ^b	100.0 ^{ab}	54.8 ^c (66.7)
<i>Yeko</i>	Xcme-9	51.7 ^{cd}	100 ^a	86.7 ^b	88.4 ^a (100)
	Xcme-10	51.7 ^{cd}	100 ^a	88.3 ^b	88.4 ^a (100)
	Xcme-19	53.3 ^{cd}	100 ^a	90.0 ^b	88.4 ^a (100)
Coefficient of variation (cv)		9.25	2.21	12.46	13.89

¹Enset clones *Gudiro*, *Nobo*, *Chikaro* and *Yeko*; and ²*Xanthomonas campestris* pv. *musacearum* isolates Xcme-9, Xcme-10 and Xcme-19 were obtained from Sheka, Keffa and Bench-Maji zones of southwest Ethiopia. ³Incubation period indicates mean number of days between inoculation and first symptom appearance and 0.0 means no diseased plant was recorded until end of the trial (120 days after inoculation). ⁴Disease severity index (%) calculated from wilt score based on 0 - 5 scale of Winstead and Kelman (1952). ⁵Mean number of days to complete death of the plants after inoculation date. ⁶Figures in the brackets are the original/untransformed mean values of disease incidence. Means (of three replications) with in each column followed with the same letter(s) are not significantly ($P < 0.05$) different from each other according to Tukey's test.

The enset clone by Xcm isolate interaction study showed significant differences in incubation period, severity index, date to complete death and disease incidence under field conditions (Table 3). This result demonstrates that there are remarkably contrasting responses in tolerance/resistance to the bacterial wilt strains among enset clones grown in southwest Ethiopia. The two clones '*Nobo*' and '*Gudiro*' are highly resistant, with little or no infection symptoms as opposed to the partial to complete death of inoculated plants of '*Chikaro*' and '*Yeko*' clones. There were no wilting symptoms, except on the inoculated leaves of '*Gudiro*' trees treated with three isolates Xcme-9, Xcme-10 and Xcme-19, four months after inoculation. Similarly, Handoro and Michael (2007) observed very low bacterial wilt infection on '*Meziya*' enset clone artificially inoculated with three Xcm isolates originating from Sidama, Dawro and Kembata with 8.3, 5.7 and 2.6%, respectively, as compared to 75 - 100% death on '*Arkia*' clone by the same isolates under field conditions. The '*Meziya*' trees that showed slight yellowing symptoms recovered from infection and became healthy after four to six months (Handoro and Michael, 2007). Michael et al. (2008) also reported that some clones, such as '*Buacho*' and '*Wonigoro*' (Sidama collection) and '*Bazeriel*' and '*Dere*' (Gurage collection) recovered from initial infections. The *in vitro* plantlets and potted plants of *Musa balbisiana* and cv. '*Nakitembe*', inoculated with a *Xanthomonas* wilt isolate, have recovered and appeared healthy after showing initial

symptom of infections under screen house conditions (Tripathi and Tripathi, 2009).

Although many authors speculated hypersensitive type of resistance that are also common in nonhost crops like tobacco and maize, some kinds of physical and/or biochemical defense reactions are perhaps operating in the resistant hosts of enset and banana clones. Thus, further investigation on the resistance mechanisms and/or pathogenicity factors with histopathological and biochemical techniques through to genomics are worthwhile.

The present study also indicates that there is variation in pathogenicity within the bacterial populations. Among the three Xcm isolates; on '*Chikaro*' clone, Xcme-10 and Xcme-19 caused significantly lower disease severity index, incidence and longer time to complete death as compared to isolate Xcme-9. Xcme-9 was found to be highly aggressive to '*Chikaro*' and '*Yeko*' plants, while all strains were slightly or non-aggressive to '*Gudiro*' and '*Nobo*'. Handoro and Michael (2007) tested the pathogenicity of five Xcm isolates artificially inoculated into '*Meziya*' (resistant) and '*Arkia*' (susceptible) enset clones and observed variations in disease incidence. The Sidama and Dawro isolates were very aggressive (100%) followed by Kembata, Gurage and Hadiya strains with wilt infection of 75.0, 66.7, and 58.3%, respectively, on the susceptible clone '*Arkia*'. Tripathi et al. (2008) found significant variation ($P < 0.0001$) in susceptibility of eight banana cultivars but reported no variation in pathogenicity

(wilt incidence) between 16 Xcm isolates tested on the eight cultivars. However, these isolates were significantly ($P < 0.0001$) different from each other in two important parameters, incubation period for appearance of symptoms and number of days to complete wilting of plants (Tripathi et al., 2008).

In conclusion, *Xanthomonas* bacterial wilt is widespread in different enset growing regions of Ethiopia including Sheka, Keffa and Bench-Maji zones. It is found to be an important disease of enset, but with minor intensity on banana plants. The bacterial strains identified from enset and banana in these areas showed similar cultural, morphological and physiological characteristics to the species *X. campestris* pv. *musacearum* with some differences in salt tolerance and aggressiveness. The host-pathogen interaction evidenced diversity among the enset clones in their resistance/tolerance and variation in virulence between the bacterial strains. The enset clones 'Nobo' and 'Gudiro' are consistently resistant while 'Yeko' is highly susceptible to the three Xcm strains. 'Chikaro' is moderately tolerant but most susceptible to third one. The three strains are least or non-aggressive towards the resistant clones 'Nobo' and 'Gudiro' but most aggressive on the susceptible 'Yeko' with varying pathogenicity levels to 'Chikaro' ranging from moderate to high aggressiveness. Therefore, this study implies that the response of 'Nobo' and 'Gudiro' enset clones should be verified in major enset producing areas including yield and quality assessment. In addition, aggressive (virulent) Xcm strain should be used in screening and testing enset landrace collections in endeavor to identify resistant/tolerant clones for sustainable management of enset bacterial wilt.

Conflict of Interests

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

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Full Length Research Paper

Influence of temperature, pH, and ionic strength on the rheological properties of oviductus ranae hydrogels

Qing Liang, Jinsong Zhang*, Changjiang Xu, Jianpeng Dou and Shouqin Zhang

College of Biological and Agricultural Engineering, Jilin University, Changchun Jilin 130022, China.

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The main rheological features of oviductus ranae (OR) hydrogels were investigated as a function of temperature, pH and ionic strength. With respect to the steady shear measurements, a reduction in viscosity was observed as the temperature increased. The flow behavior can be well described by the Herschel-Bulkley model in the down curve at a shear rate of 300 to 0 s⁻¹, where standard error is lower than 20, and it behaved like a viscoplastic fluid. OR gels showed greater extent of thixotropy with the increase of temperature. Temperature dependence on apparent viscosity at a specified shear rate can be described by the Arrhenius model with high R^2 values. The thermostability of OR hydrogels were verified by temperature sweep test, which showed that there was no phase transition observed in the temperature range of 10 to 90°C. It was found that at pH 5 to 10 and the salt concentration of 0 to 10 g/L, the OR hydrogels showed very weakly pH-dependent and salt-dependent rheological properties and dominant elastic behavior. All data indicated that OR has great potential for application as gelling and thickening agent with multiple healthy functions.

Key words: Rheological properties, oviductus ranae hydrogels, temperature, pH, ionic strength.

INTRODUCTION

Oviductus ranae (OR), a traditional Chinese medicine and functional food, is made from dried oviduct of the female forest frog, *Rana chensinensis*. As OR contains many nutrients and functional components such as proteins, polysaccharide, fat, and physiological activators (e.g. estradiol, progesterin, and testosterone) (Liu and Liu, 2007; Zheng et al., 2008; Hu et al., 2003), it has been reported and recorded in 'Compendium of Materia Medica and Chinese Pharmacopoeias' for the functions of anti-fatigue (Chen et al., 2005), antioxidation (Li et al., 2008), regulating blood fat (Yang et al., 2011) and invigorating the kidney (Hu et al., 2003).

When OR contacts with water, the biopolymers unfold,

exposing the reactive surfaces of neighboring biopolymers molecules, which then interact to form intermolecular bonds. When sufficient bonding (hydrogen bonds, ionic linkages, hydrophobic interactions, and covalent bonds) occurs, it expands and transforms into hydrogel with three-dimensional cross-linked hydrophilic biopolymer network that can absorb large quantity of water. The reason why OR can be turned into hydrogel is attributed to its most important feature of multicomponent nature. This system is made up of two major kinds of biopolymers: protein and polysaccharide. Many proteins/polysaccharides are hydrocolloids and considerable interest has been devoted to the study of polysaccharide-protein

*Corresponding author. E-mail: jluzjs@126.com. Tel: +86 431 85562173. Fax: +86 431 85562173.

Table 1. Amino acid composition of oviductus ranae (as g amino acid/ 100 g OR).

Amino acids	% of amino acid	Amino acids	% of amino acid
Asp	4.00	Cys	ND
Thr	6.66	Leu	2.10
Ser	2.76	Tyr	1.80
Glu	3.81	Phe	1.40
Gly	1.81	His	4.86
Ala	0.75	Lys	3.35
Val	1.74	Arg	1.33
Met	0.39	Pro	3.48
Ile	2.04	H-Pro	ND

ND for not detected.

mixtures. The structural functions of these biopolymers are greatly affected by their interactions with each other and with other components in food systems (Girard et al., 2002; Andrade et al., 2010; Kerry et al., 1999; van den Berg et al., 2008; Turgeon et al., 2003; Agbenorhevi and Kontogiorgos, 2010; Ribotta et al., 2007; Mohamed and Xu, 2003; Gaaloul et al., 2009). Because of their ability to modify the rheological and functional properties of food systems, they can be used in the food industry for changing texture such as gel formation, water retention, emulsification and aroma retention. Moreover, OR is also attracting the increasing attention of pharmaceutical and functional food industry, not only because of its multiple beneficial effects on human health mentioned above, but also its thickening or gelling properties.

As compositionally and structurally complex materials, hydrogels exhibit a wide range of rheological properties under different conditions. And those properties are strongly affected by temperature, pH, and ionic strength (Tang et al., 2007; Lai et al., 2008; Romero et al., 2009; Venugopal et al., 2002; Cakir and Foegeding, 2011). To our surprise, however, there is no report concerning the rheological characteristics of OR hydrogels influenced by factors mentioned above to date. Therefore, in this work, the influences of temperature, pH, and electrolyte (NaCl and CaCl₂) conditions on rheological properties of OR were extensively investigated using both steady shear and small amplitude oscillatory measurements based on our previous corresponding study (Liang et al., 2012). Our work may give an insight into the dependence of molecular structure on specific conditions (such as temperature, pH, ionic strength of the system) and reveal the rheological properties of OR hydrogels as a new source of hydrocolloid gum.

MATERIALS AND METHODS

Proximate composition and amino acid analysis of oviductus ranae

OR was purchased from Antu Baoli health food Co., Ltd., China, and it was produced in Changbai Mountain of Jilin province in

Northeast China. The moisture (11.9±0.2%), ash (4.5±0.1%), fat (2.0±0.1%) and crude polysaccharide (13.1±0.1%) contents of OR was determined according to the method as described by AOAC (AOAC, 1990; Dubois et al., 1956). The crude protein (62.0±0.1%) content was determined by estimating its total nitrogen content by Kjeldahl method (AOAC, 1990). A factor of 5.8 was used to convert the nitrogen value to protein. Amino acid composition of OR was determined after hydrolyzation in 6N HCl/0.1% mercaptoethanol solution for 24 h at 110°C. The sample in aminoethyl cysteinyl dilution buffer was then analyzed using amino acid analyzer (S-433D, Sykam, Amtsgericht Augsburg, Germany) (Table 1).

Preparation of oviductus ranae hydrogels

The sample of dry OR was ground in porcelain mortar and then sifted through a 40 mesh sieve. Deionized water was used to mix with the powdered OR to form hydrogels. The hydrogels were prepared at 1:80 (OR: water, w/w, sample A), 1:120 (B), 1:160 (C), and 1:200 (D) concentrations. The OR powder was mixed with pHs 4, 5, 6, 7, 8, 9, 10, 11 water solutions (1:120) to get corresponding hydrogels with different pH value respectively. The pH values were adjusted by 0.1 M NaOH or HCl. The OR powder was suspended in 0, 0.05, 0.1, 1 and 10 g/L NaCl or CaCl₂ solutions to get corresponding hydrogels with different electrolyte concentration. Different ionic strengths were obtained by adjusting with 200 g/L NaCl or CaCl₂. All the above samples were allowed to equilibrate for 24 h at room temperature (20°C). In order to get rid of entrapped air bubbles and acquire homogeneous samples, gentle stirring was imposed on the hydrogel samples. Then, they were allowed to equilibrate for another 24 h at 20°C to complete hydrogels structure formation.

Rheological tests

Rheological measurements were performed on a controlled-stress rheometer (AR500, TA Instruments, Texas, USA) and equipped with an aluminum parallel plate geometry (40 mm diameter, 1 mm gap). Each kind of rheological experiment was performed at its corresponding temperature, and the temperature was controlled by a water bath connected to the Peltier system in the bottom plate. A thin layer of silicone oil was applied on the surface of the samples in order to prevent evaporation. The linear viscoelastic (LVE) region was determined for each sample through a frequency strain-sweep measurement at 1 Hz and 20°C (amplitude range is 0.01 to 100%, data not shown). Viscoelastic properties of OR hydrogel samples were determined within the linear viscoelastic region. An equilibration of 3 min was performed before each measurement.

Shear stress-shear rate data were obtained at 20°C on the samples (A, B, C or D). The instrument was programmed for set temperature and equilibration followed by two-cycle shear in which the shear rate was increased linearly from 0 to 300 s⁻¹ in 3 min (up curve) and immediately decreased from 300 to 0 s⁻¹ in the next 3 min (down curve) at 5, 25, 50, 75 or 95°C. This process was repeated two more times for each sample. Data from the descending segments of the shear cycle were used to characterize the flow of the samples and to estimate the Herschel-Bulkley (H-B) parameters by using the equation:

$$\tau = \tau_0 + K\dot{\gamma}^n \quad (1)$$

Where, τ_0 is yield stress, K is the consistency index, and n is the flow behavior index. The dimensions of K depend where, τ_0 is yield stress, K is the consistency index, and n is the flow behavior index. The dimensions of K depend upon the value of n . The physical meaning of K and n (<1) in equation is similar to the equation of power-law. With the use of the parameter τ_0 , this model provides a somewhat better fit to some experiential (Chhabra and Richardson, 2011).

The influence of temperature on the viscosity at 100 s⁻¹ for non-Newtonian fluids of each concentration could be expressed by the Arrhenius model:

$$\eta = A \exp\left(\frac{E_a}{RT}\right) \quad (2)$$

Where, A is the frequency factor or viscosity coefficient at a reference temperature (Pa.s), E_a is the activation energy (kJ/mol), R is the gas constant (kJ/mol K) and T is the absolute temperature (K). Activation energy can be determined from the slope of $\ln\eta$ versus $1/T$ plot.

The thermostability of the samples were determined according to the procedure of Tischer et al. (Tischer et al., 2006) with small changes. After the sample was equilibrated at 10°C for 2 min, the temperature of the sample was increased to 90°C and subsequently its temperature was decreased to 10°C with a ramp rate of 5°C/min for both heating and cooling. A sweep of this temperature profile is performed one more time. In the tests a constant frequency of 1 Hz and strain amplitude of 1% were used.

The viscosity and flow properties were measured for hydrogels (1:120) with different pH values (3.0 to 11.0) and salt concentrations (0 to 10 g/L) at shear rate of 50 s⁻¹ and constant temperature of 20°C. Small amplitude oscillatory tests were also performed at 20°C over the frequency range of 0.1 to 10 rad/s. The strain amplitude for the frequency sweep measurements was selected as 1%, which was in the linear viscoelastic region for all samples. The mechanical spectra were obtained through recording storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta = G''/G'$) as function of angular frequency.

Statistical analysis

The experimental results obtained from this study were fitted to different kinetic and mathematical models using TA Rheology Advantage Data Analysis software V 5.4.7 (TA Instruments, UK). The fit and estimates were calculated at a significance level of 95%. The best fit regression model could be selected on the basis of standard error (SE), which is defined as:

$$\frac{\sum(X_m - X_c)^2 / (n - 2)^{0.5}}{\text{Range}} \times 100 \quad (3)$$

Where, X_m is the measured value; X_c is the calculated value; n is the number of data points and *range* is the difference between the maximum value of X_m and the minimum value.

RESULTS AND DISCUSSION

Effect of temperature

The effect of temperature on the flow behavior is shown in Figure 1. There was a reduction in stress (viscosity) as the temperature increased. The experimental data of shear stress versus shear rate for the OR hydrogels at different concentrations and temperatures were fitted by H-B model. The consistency index (K), the flow behavior index (n) and the yield stress (τ_0) as influences of OR concentrations and temperatures were derived from the flow curves of OR hydrogels (Table 2) by fitting the descending flow curve with the H-B model ($SE < 20$). H-B model is often used to describe the flow behavior of food gels which belong to viscoplastic materials showing shear-thinning behavior at stress levels exceeding τ_0 (Wang et al., 2011). The yield stress (τ_0) represents a finite stress required to initiate flow. The τ_0 values of the samples determined using H-B model approximately show the trend with concentration and temperature: yield stress increased with increase of concentration and decreased with increase of temperature. The K values have similar trends which relates to the capacity of water binding. The thixotropy of OR hydrogels at various temperature are also presented in Figure 1. For the range of shear rates used in this study, the H-B model describes flow behavior of each sample. Appearance of a hysteresis area in the plot of shear stress versus shear rate means that all hydrogels at different temperature exhibit time-dependent behavior. And the larger the enclosed area, more severe is the time-dependent behavior of the materials. All the samples exhibit clockwise hysteresis loop, indicating that shear stress obtained from the increasing-order of shear rate (up curve) is much larger than the corresponding value of the decreasing-order of shear rate (down curve), which suggests thixotropic behavior of the hydrogels. At low shear rate of up curve, the shear stress increase quickly with increase of shear rate. So, the hydrogels exhibited extremely thixotropic behavior, which manifests as a regional peak in the ascending curve of hysteresis loop, neither the H-B nor any other typical viscous model could fit well to the ascending flow curve. Therefore, only the parameters obtained by fitting the descending flow curve were reported in the Table 2.

The clockwise loops can be interpreted as structure breakdown by the shear field (thixotropic behavior) to alter a structure or form a new structure. Some authors have determined thixotropic behavior by the coefficient of thixotropic breakdown, K_d , which is defined as the ratio of the hysteresis area to the area beneath the ascending shear curve (Dokic et al., 2010):

$$K_d = \frac{A_{up} - A_{down}}{A_{up}} \quad (4)$$

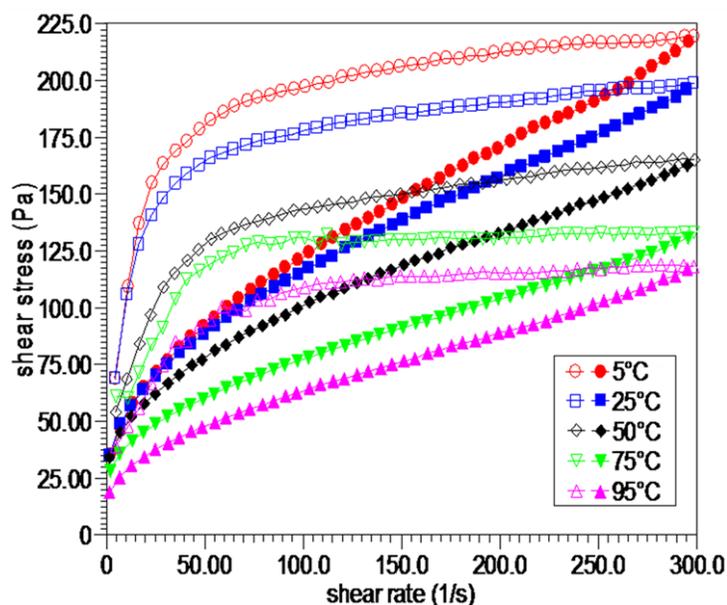


Figure 1. Flow curves of 1:120 (w/w) oviductus ranae hydrogel at various temperature. Open symbols, up curve; closed symbols, down curve.

Table 2. The Herschel-Bulkley equation parameters for oviductus ranae hydrogels at different concentrations and temperatures (downward curve).

Samples	Temperature (°C)	K_d	Down curve			
			τ_0^A (Pa)	K^B (Pa·s ⁿ)	n^C	SE
A	5	0.3052	49.78	7.610	0.6264	10.56
	25	0.2754	44.66	6.933	0.5883	8.087
	50	0.2546	42.54	6.447	0.5971	8.604
	75	0.3241	39.09	3.844	0.6529	8.091
	95	0.3681	21.37	3.332	0.6747	13.02
B	5	0.2903	31.96	5.873	0.6272	7.991
	25	0.2587	31.58	5.585	0.5908	6.375
	50	0.2194	30.73	5.050	0.5523	5.625
	75	0.2953	28.48	3.330	0.6187	8.504
	95	0.2977	18.12	3.051	0.5932	9.289
C	5	0.2796	22.86	5.595	0.5823	7.701
	25	0.2573	21.21	4.278	0.5877	7.127
	50	0.2158	19.41	3.609	0.5506	4.443
	75	0.2656	16.59	2.699	0.6300	10.62
	95	0.2782	15.29	2.023	0.6081	8.953
D	5	0.2400	16.17	4.139	0.5926	6.905
	25	0.2254	15.10	3.745	0.5743	7.269
	50	0.2019	14.14	2.707	0.5906	7.101
	75	0.2328	12.19	2.301	0.5844	5.855
	95	0.2645	10.83	1.091	0.6685	13.84

^A Yield stress, ^B Consistency coefficient, ^C Flow behavior index.

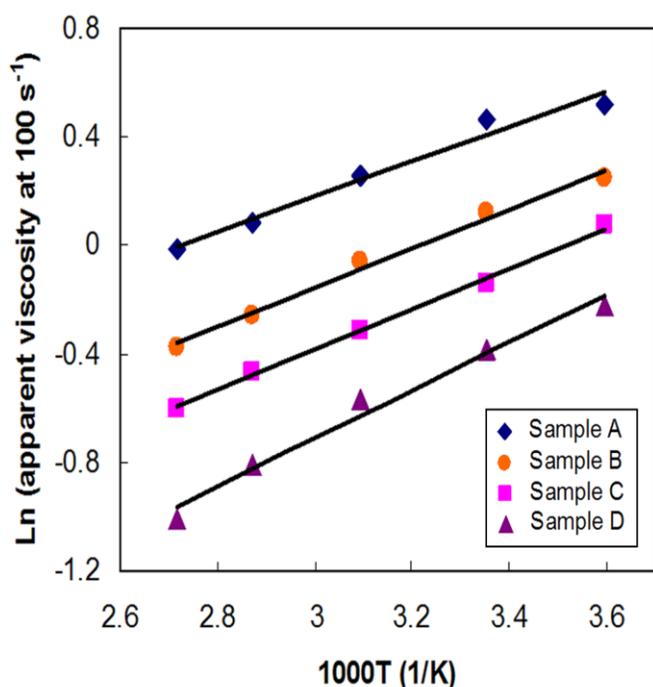


Figure 2. Arrhenius plot for the apparent viscosities at the shear rate of 100s^{-1} .

Table 3. Arrhenius model ($\eta=A\exp^{(E_a/RT)}$) for temperature dependency of apparent viscosity (at 100s^{-1}) for OR hydrogels at different concentrations.

Samples	A (Pa s)	E_a (kJ/mol)	R^2
A	0.173	5.362	0.972
B	0.163	6.014	0.992
C	0.073	6.207	0.995
D	0.035	7.316	0.979

Where, A_{up} and A_{down} are the areas under ascending and descending flow curves, respectively.

Table 2 shows the data of K_d at the different temperatures and concentrations studied. It was found that K_d increased as the concentration of the extract increased, which means an increased extent of structuring of the samples with concentration. Furthermore, K_d is greater at higher temperatures for all the concentrations studied. Since a coefficient of thixotropic breakdown is an index of the energy needed to destroy the structure of the system, it can also be explained as a result of increased intermolecular interaction as suggested previously for the greater concentration dependence of viscosity at higher temperatures (Karazhiyan et al., 2009).

An increase in temperature from 5 to 95°C did not

significantly influence the critical shear rate limiting the H-B region or the shape of the OR hydrogels, hence the H-B flow index (n) hardly changed sharply (Table 2). It must be emphasized that the shear thinning properties were still clear enough at 95°C as pointed out by the H-B flow index value of 0.59 to 0.67. The flow behavior index indicated that at higher temperature the systems were obvious pseudoplastic which was confirmed by K_d .

The effect of temperature on apparent viscosity at a specified shear rate can be described by the Arrhenius relationship (Eq. (2)), Figure 2 illustrates that the change of apparent viscosity with temperature generally obeyed the Arrhenius model. Frequency factors (A), activation energies (E_a) and coefficients of determination (R^2) are show in Table 3. Good agreement with linearity was found. The activation energy indicates the energy barrier that must be overcome before the elementary flow process can occur (Rao, 2007). The magnitude of the temperature effect varied with concentration as shown by the E_a values in Table 3. E_a decreased and constant A increased with the increase in OR concentrations. This effect is reversible and it is due to the interactions of the molecules in system which varies with the temperature. As showed in Table 3, the activation energy for the OR hydrogel decreased from 7.316 kJ/mol at 1:200 concentration to 5.362 kJ/mol at 1:80. This indicates that the higher the activation energy, the greater the effect of temperature on the viscosity. Therefore, as temperature increases, the thermal energy of the molecules varies and the intermolecular distances change.

To assess the thermostability of the OR hydrogels, a temperature swing test is an effective method in terms of physical stress because by changing the temperature in a defined manner conditions can be simulated. If the moduli G' and G'' have identical values at recurring temperatures at constant frequency and amplitude (in the linear viscoelastic range) regardless of the number of temperature sweeps performed, a sample can be classified as stable. The temperature dependences of G' and G'' of the sample A are shown in Figure 3 (the curves of other samples are similar to the sample B, so they are not shown). The curves for the storage and loss module were nearly parallel through the given temperature range and the storage modulus G' was always larger than the loss modulus G'' over the entire measuring window. If the storage modulus increases, so do the loss modulus. These experimental phenomena are beyond our expectation, because the macromolecules of proteins always denature in the environment of high temperature. If the denaturation happens, the moduli G' and G'' would change sharply. But the fact is not so which indicates that OR hydrogels are thermostable. Among many gels, there is always a phase transition from a liquid to gel or adversely with the change of temperature (Moraes et al., 2009; Chiou et al., 2006). But the sol-gel transition cannot be seen in the temperature range of 10 to 90°C which

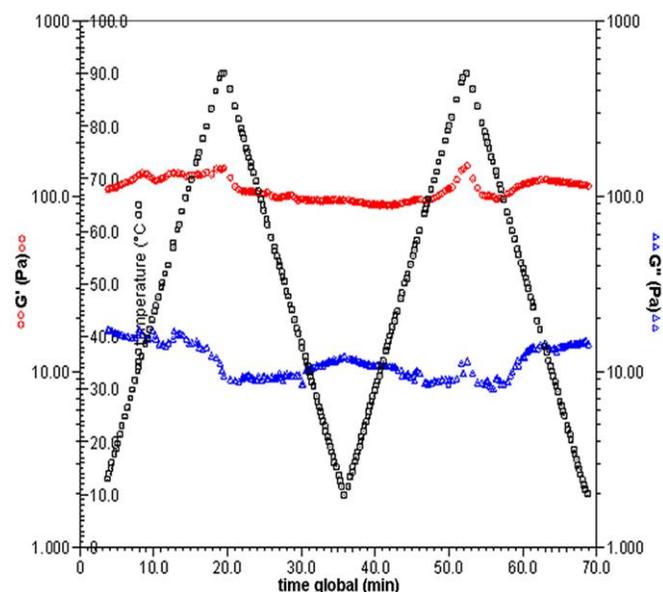


Figure 3. Dynamic swing test for 1:120 (w/w) oviductus ranae hydrogel.

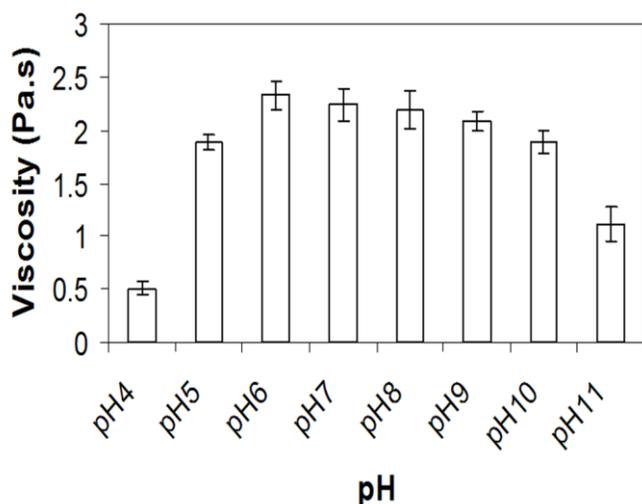


Figure 4. Influence of pH on steady flow viscosity of 1:120 (w/w) oviductus ranae hydrogels at the shear rate of 50 s^{-1} .

indicates that, for the samples of OR hydrogels, there is no obvious phase transition.

Influence of pH on the rheological properties

Changes in pH may influence the viscosity of OR. The effect of pH (4 to 11) on the steady flow viscosity (at the shear rate of 50 s^{-1}) of OR hydrogels (1:120) is displayed

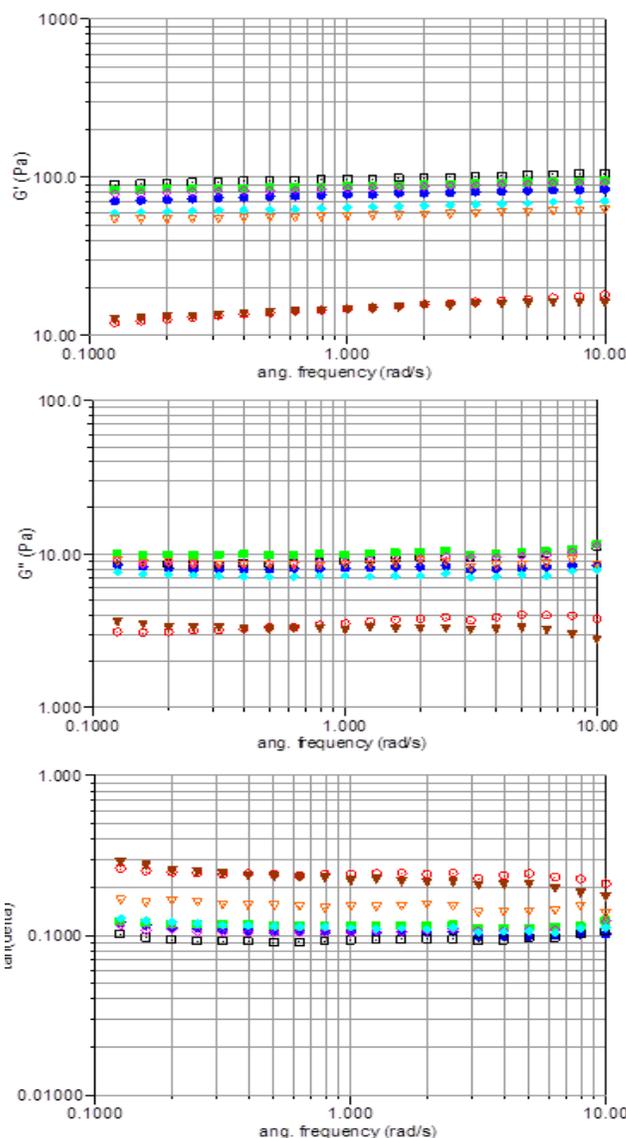


Figure 5. Dependence of storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta = G''/G'$) on frequency (f) for Oviductus Ranae hydrogels at different pH. (○) pH4; (●) pH5; (□) pH6; (■) pH7; (◇) pH8; (◆) pH9; (▼) pH10; (▽) pH11.

in Figure 4. The viscosity was found to be fairly stable over a wide range of pH (5 to 10) and decreased both at lower (pH < 4) and higher (pH > 11) pH which presented the initial and ultimate pronounced decrease of the viscosity at more acidic or alkaline conditions. The influence of pH on the viscosity of different protein-polysaccharide systems has been reported in some literatures (Totosaus et al., 2002; Bazinet et al., 2004). But the similar phenomenon with that in this study is scarce. The G' , G'' and $\tan \delta$ values for hydrogels were also dependent on pH (Figure 5). And there have been some reports on the viscoelastic properties of protein-

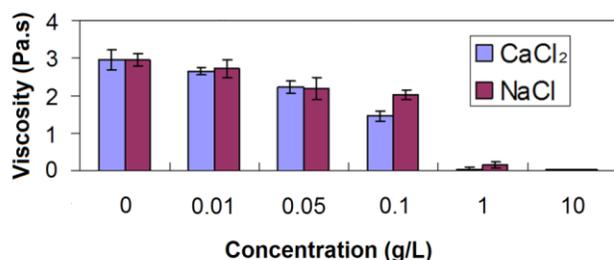


Figure 6. Influence of NaCl and CaCl₂ concentrations on steady flow viscosity of 1:120 (w/w) Oviductus Ranae hydrogels at the shear rate of 50 s⁻¹.

polysaccharide systems (Miquelim et al., 2010; Tadpichayangkoon et al., 2010; Romero et al., 2009; Ribotta et al., 2007; Bazinet et al., 2004; Mohamed and Xu, 2003; Gaaloul et al., 2009). From Figure 5, it can be seen that G' and G'' display the similar changing tendency as with pH change. The G' -frequency curve had decreased values at both acidic (pH 4) and alkaline (pH 10) values which is consistent with the influence of viscosity. Moreover, G' is more sensitive to the effect of pH than G'' and G' is always greater than G'' at pH 4 to 11, indicating that OR hydrogel shows dominant elastic behavior. At pH values below 4 (including pH 4) the storage modulus decreased sharply. This is probably due to increased net positive charge in the chains, which could inhibit junction zone formation and therefore result in declined gel rigidity. Above pH 11, the storage modulus also decreases obviously, probably due to deamination of acid amides and changes in the charge density to give a high net negatively charged biopolymer and high repulsive force of protein molecules. The increased charge density can oppose the ability of chains to make contact and form junction zones and thus decrease the gel stiffness. So, the pH has a paramount importance in controlling the electrostatically mediated interaction between proteins and polysaccharides as it can directly control the degree of ionization of the charged groups carried by these two biopolymers (Schmitt et al., 1998).

The pH-tan δ relationship also showed high points at both acidic and alkaline pH with maximum values. As tan δ values represent the G''/G' values, a low tan δ value indicates a stronger role for G' in the network formed and can represent the relative elasticity of the network. As a result, the lower tan δ values from pH 5 to 9 would suggest better networks. While the low G' , high tan δ values would suggest very little biopolymers interaction. The reduction in G' also suggests that the OR could be degraded or chemically changed at extreme pH values.

Influence of ionic strength

Considering salts' effect on the rheological properties, we

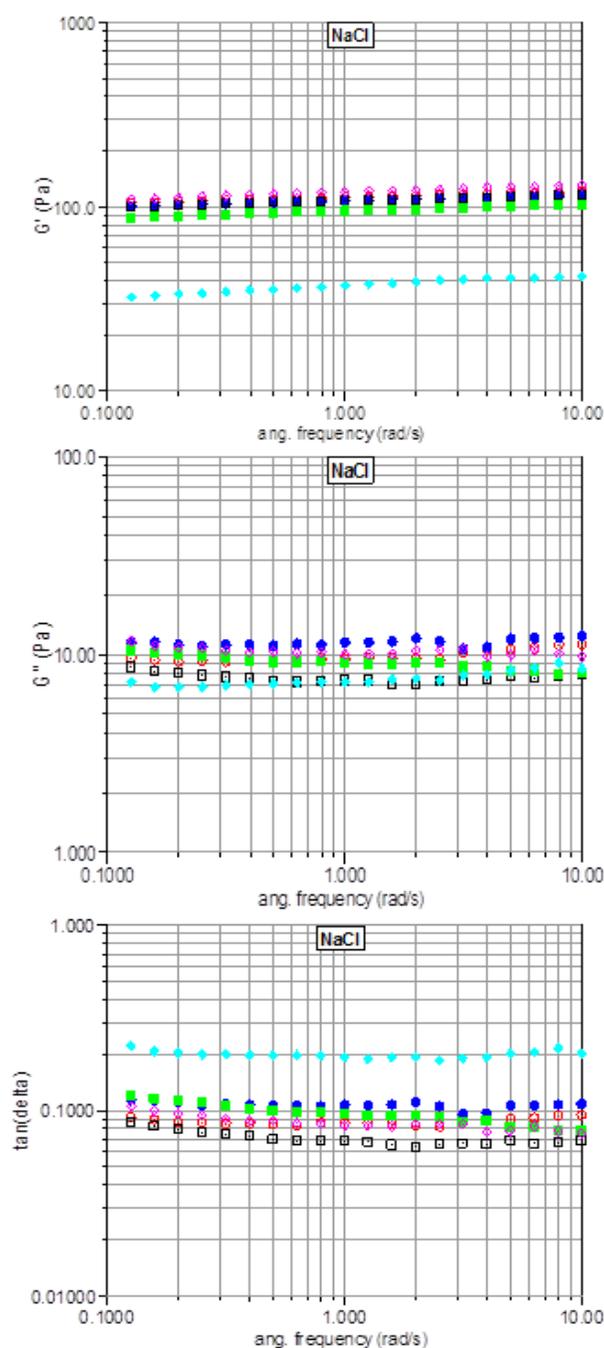


Figure 7. Dependence of storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta = G''/G'$) on frequency (f) for Oviductus Ranae hydrogels at different NaCl concentrations. (○) 0 g/L; (●) 0.01 g/L; (□) 0.05 g/L; (■) 0.1 g/L; (◇) 1 g/L; (◆) 10 g/L.

studied monovalent (that is, NaCl) and divalent (that is, CaCl₂) salt concentration on the steady flow viscosity (at the shear rate of 50 s⁻¹, Figure 6) and viscoelasticity of OR hydrogel (Figures 7 and 8). As expected, the increase

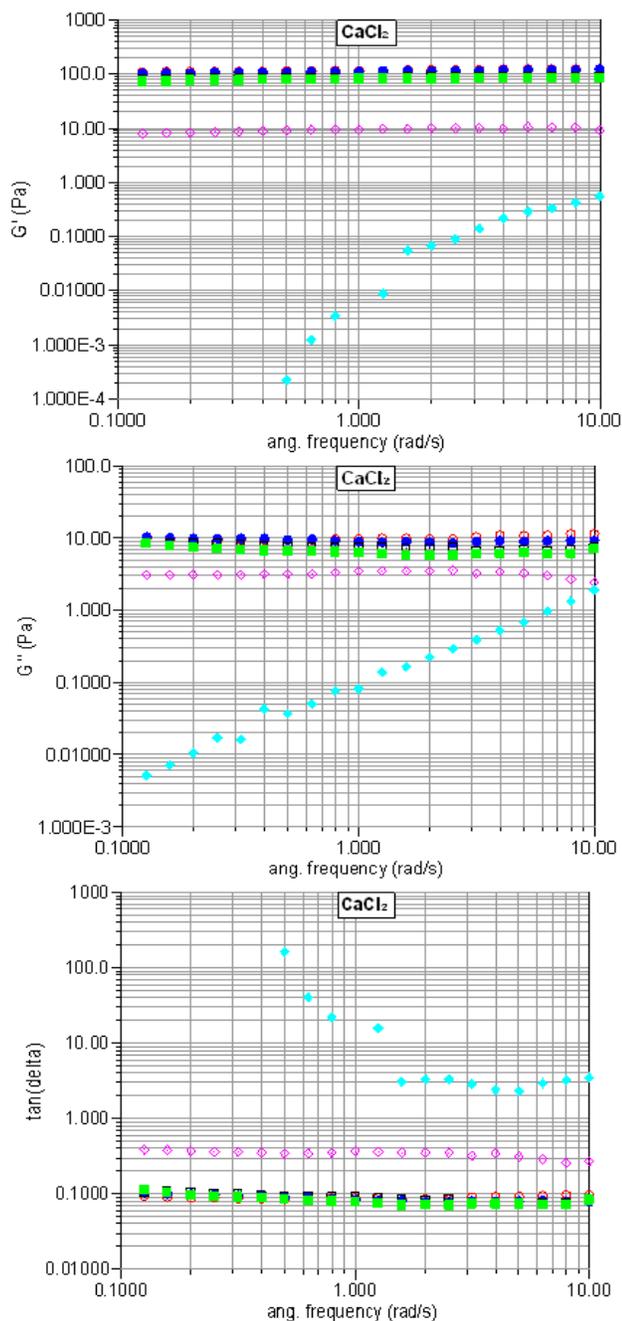


Figure 8. Dependence of storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta = G''/G'$) on frequency (f) for *Oviductus Ranae* hydrogels at different CaCl_2 concentrations. (\circ) 0 g/L; (\bullet) 0.01 g/L; (\square) 0.05 g/L; (\blacksquare) 0.1 g/L; (\diamond) 1 g/L; (\blacklozenge) 10 g/L.

of salt decreased the apparent viscosity and dynamic modulus at high concentrations, and significant ionic selectivity was observed. At salt concentration (NaCl and CaCl_2) higher than 1 g/L, it can be seen that the presence of electrolyte causes a drastic decrease of the viscosity.

And more obvious influence on viscosity can be seen from the change of CaCl_2 concentration. A decrease of viscosity in salt solutions is a common effect in protein-polysaccharide systems. The decrease arises from the screening effect of the charges of the macromolecules due to small ion-pairing. This effect reduces the number of protein molecules which is able to interact with the polysaccharide chains (Matsunami et al., 2007) and can cause the decrease of the three-dimensional network strength, resulting in the decrease of the viscosity of OR hydrogel.

The influence of ionic strength on OR hydrogel properties was also evaluated by dynamic oscillatory rheometry. At the NaCl concentration (C_{NaCl}) lower than 1 g/L or CaCl_2 concentration (C_{CaCl_2}) lower than 0.1 g/L, an invariability in G' , was observed. When higher concentrations ($C_{\text{NaCl}} > 10$ g/L and $C_{\text{CaCl}_2} > 1$ g/L) of salt were added, the storage modulus rapidly decreased. It was initially thought that the network structure would become weaker since salt addition could have led to decreased attractive interactions among biopolymers due to the salting-in effect on proteins. The effect could promote exposure of new regions of the protein surface to the solvent, enabling new electrostatic interactions to occur. It is well known that low concentrations of salt only have a small effect on coacervation of electrostatically stabilised complexes. Higher ionic strengths, on the other hand, prevent complexation due to a reduced entropic driving force from the release of counter-ions (Schmitt et al., 1998).

With the addition of electrolytes, the OR hydrogel is dominant elastic fluid ($G' > G''$, $\tan \delta < 1$) at C_{NaCl} used; but at $C_{\text{CaCl}_2} > 10$ g/L, it becomes to dominant viscous fluid ($G' < G''$, $\tan \delta > 1$), indicating that the OR could not form a gel. When divalent ions were used, suppression of coacervation occurred at lower ionic strength values than for monovalent ions. Thus, ionic strength dependence for complex formation between protein and polysaccharide might not only be related to salt concentration, but to the type of ion.

Conclusion

In this study, the influences of temperature, pH and electrolytes (NaCl and CaCl_2) on the viscosity and dynamic modulus (G' and G'') of OR were examined. With the increased of temperature from 5 to 95°C, it was found that the shear stress (apparent viscosity) of the samples decreased. H-B model was suitable for representing the flow behavior of the tested samples and it was suitable for predicting the yield stress of the samples. Though, the low apparent viscosity was observed with increasing of temperature, the samples still present great extent of thixotropy, which indicates that the inter-macromolecular interactions are still significant at high temperature.

Magnitudes of consistency index (K) and apparent viscosity ($\eta_{a, 100}$) for the OR hydrogels were influenced by temperature and their concentrations. The effect of temperature on $\eta_{a, 100}$ is described well by Arrhenius relationship with high correlations (R^2). As concentration increased, the activation energies and frequency factors decreased and increased, respectively. Temperature sweep test showed that both G' and G'' were nearly independent on temperature, which suggested that the OR hydrogels' are superior to thermostability. At pH 5 to 10 and electrolyte concentration 0 to 10 g/L, the rheological properties of OR show very weak pH and salt dependence, respectively, and dynamic measurements also suggested that elastic behavior dominates throughout the entire frequency range examined. Thus, the gel-like behavior of OR hydrogel was relatively dependent on conditions of solvent (that is, pH, ionic strength). All data indicated that OR, a hydrogel with multiple healthy functions, has unique rheological properties and could be developed as a new source of gelling and thickening agent in modern food industry.

Conflict of Interests

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

ACKNOWLEDGEMENTS

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Full Length Research Paper

Extraction, partial purification and characterization of pectinases isolated from *Aspergillus species* cultured on mango (*Mangifera indica*) peels

Daniel Ikenna UDENWOBELE^{1*}, Chukwunonso Anthony NSUDE¹, Arinze Linus EZUGWU¹, Sabinus Oscar Onyebuchi EZE¹, Chukwudi ANYAWU², Peter Nzemndu UZOEGBU³ and Ferdinand Chiemeka CHILAKA¹

¹Enzymology and Protein Chemistry Unit, Department of Biochemistry, University of Nigeria, Nsukka, Enugu, Enugu State, Nigeria.

²Department of Microbiology, University of Nigeria, Nsukka, Enugu, Enugu State, Nigeria.

³Department of Biochemistry, University of Nigeria, Nsukka, Enugu, Enugu State, Nigeria.

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Pectinase was produced from a culture of *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*. Pectinase synthesis was achieved using mango (*Mangifera indica*) pectin extract as an inducer during pectinolytic fungi isolation while submerged fermentation process was carried out using ground mango peels as the sole carbon source. Substrate fermentation was evaluated within seven days by monitoring the pectinase activity every 24 h. The highest pectinase secretion was obtained from *A. niger* and *A. fumigatus* after 92 h (day 4) of incubation, while in *A. flavus*, it was after 120 h (day 5). Crude enzyme extracts from the three organisms were partially purified by a combination of ammonium sulphate precipitation and dialysis with an approximately two-fold purification of the pectinase and a yield of 5.4, 7.66 and 5.99% for *A. niger*, *A. fumigatus* and *A. flavus*, respectively after dialysis. The specific activities of 1.62, 1.79 and 1.86 U/mg for *A. niger*, *A. fumigatus* and *A. flavus* enzymes were calculated, respectively. Pectinase from *A. niger* and *A. fumigatus* had pH and temperature optima of 5.0 and 40°C, respectively, while that from *A. flavus* had pH and temperature optima of 5.0 and 45°C. The Michealis constant, K_m and the maximum velocity, V_{max} determined from Lineweaver-Burk plots of initial velocity data at different concentrations of the mango pectin extract were 0.357 mg/ml and 35.34 U; 0.156 mg/ml and 68.0 U; and 0.261 mg/ml and 60.61 U; for the enzymes from *A. niger*, *A. fumigatus* and *A. flavus*, respectively. The results suggest that mango peels can be used for value added synthesis of pectinase, an important enzyme with numerous biotechnological applications.

Key words: Mango peels, mango pectin extract, pectinase, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, submerged fermentation, partial purification, characterization.

INTRODUCTION

In the processing and utilization of mango fruit in products such as juices, nectars concentrates, jams, jelly powders and flakes, wastes are generated in the form of

peels and kernels. According to Larrauri et al. (1996), by-products of industrial mango processing may amount to 35 to 60% of the total fruit weight. These by-products

represent a serious disposal problem and ways for a sustainable agricultural production have to be discovered. Mango kernels have been utilized as source of oil (Moharram and Moustafa, 1982), natural antioxidants (Puravankara et al., 2000), starch (Kaur et al., 2004), flour (Arogba, 2002) and feed (Ravindran and Sivakanesan, 1996). Mango peels can be utilized in the production of value added products such as biogas, pectin peel oil, dietary fiber and predominantly pectinases that can be easily harnessed. Among these products, pectin and pectinases have a wide global market (Bali, 2003). Pectins are complex and structural polysaccharides found in the primary cell wall and middle lamella of fruits and vegetables where they function as hydrating agent and cementing material of the cellulosic network (Jarvis et al., 2003; Favela-Torres et al., 2006). Pectic polysaccharides such as homogalacturonan (HGA), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) have been isolated from plant primary cell wall (Harholt et al., 2010). Pectinases are a group of enzymes that catalyze the breakdown of pectins. They are classified into protopectinases, esterases and depolymerases based on their preferred substrate, the degradation mechanism and the type of cleavage (Jayani et al., 2005). Submerged (SMF) and solid state fermentation (SSF) processes have been widely used for pectinase production by different organisms. However, microbial pectinases are produced mostly by SMF in a process that is influenced by the type and concentration of the carbon source, the culture pH, the incubation temperature and the oxygen concentration (Rashmi et al., 2008).

MATERIALS AND METHODS

3,5-Dinitrosalicylic acid (DNS) was a product of Sigma chemical company (USA). All other chemicals used in this work were of analytical grade and were products of Merck (Germany), BDH chemical limited (England), May and Baker limited (England).

Collection of mango fruits

Mature mango fruits (*Mangifera indica*) were collected from the major markets in Amechi Awkunanaw in Enugu State, Nigeria.

Ground mango peels

The fruit peels were peeled, cut into pieces and washed with 96% ethanol to disinfect the peels. The washed peels were then sun dried for seven days and ground into powder.

Extraction of pectin

Pectin was extracted using the method described by McCready (1970) and the yield determined by the equation:

$$\text{Percentage yield} = \frac{\text{mass of pectin extracted}}{\text{mass of ground mango peels}} \times 100$$

Isolation of microorganism

Three *Aspergillus* strains were isolated from soil of decaying mango fruits and vegetables located in the University of Nigeria Nsukka Campus Enugu State, Nigeria using the method described by Martin et al. (2004). The soil samples were collected in clean dry plastic containers and transported to the laboratory.

Fungal identification

Three days old pure culture of *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*, were used in preparing microscopic slides. A clump of the mycelia was dropped on the slide and a drop of lactophenol blue was added to it. Identification was carried out using 400 magnifications according to the method of Barnett and Hunter (1972). Species identification was performed by examining both macroscopic and microscopic features of a three day old pure culture. Colour, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as separation, spore shapes and so on were examined. The spores of the three *Aspergillus* strains were grown and maintained on potato dextrose agar.

Pectinase production

Pectinase was produced by submerged fermentation according to the method of Martin et al. (2004). Submerged fermentation (SmF) technique was employed using 21 250 ml Erlenmeyer flask containing 100 ml of sterile cultivation medium optimized for pectinase with 0.1% NH_4NO_3 , 0.1% $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ and 1% of ground mango peel. The flask was stoppered with aluminium foil and autoclaved at 121°C for 15 min to sterilize the culture medium. Three days old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10 mm. The culture was incubated for seven days at room temperature (30°C). The fermentation media was agitated at 250 rpm on rotary shaker. At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration through Whatman filter paper No. 1. The filtrate for each day was assayed for pectinase activity till the 7th day of fermentation. After the seven days pilot SmF studies, the day of peak pectinase activity was chosen for mass production of enzyme from the respective fungal isolates. Several (21) 250 ml Erlenmeyer flasks were used to produce 3.0 L of the enzyme. Harvesting was carried out on the respective peak days of enzyme activity. The harvested 3.0 L of the enzyme was used as crude enzyme.

Pectinase assay

Pectinase activity was evaluated by assaying for polygalacturonase

*Corresponding author. E-mail: daniel.udenwobele@unn.edu.ng. Tel: +2347031524415.

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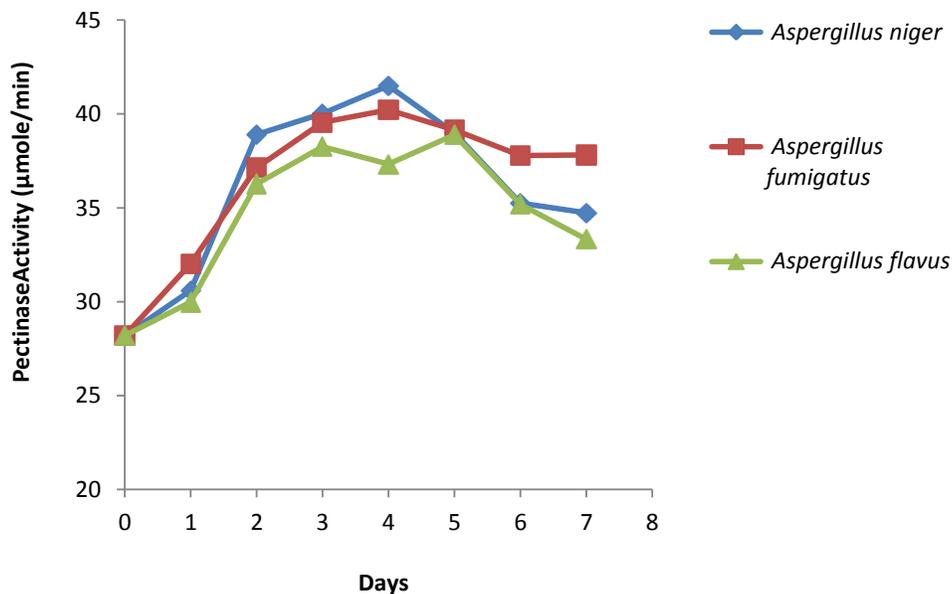


Figure 1. Determination of duration for peak pectinase production from *Aspergillus* species.

(Pg) activity of the enzyme. This was achieved by measuring the release of reducing groups from mango pectin using a modification of the 3,5-dinitrosalicylic acid (DNS) reagent assay method described by Miller (1959) as described in Wang et al. (1997) with little modifications. The reaction mixture containing 0.5 ml of 0.5% mango pectin in 0.05 M sodium acetate buffer of pH 5.0 and 0.5 ml of enzyme solution were incubated for 1 h. 1 ml of DNS reagent was added and the reaction was stopped by boiling the mixture in a boiling water bath for 10 min. The mixture volume was made up to 4 ml with 1 ml of Rochelle salt solution and 1 ml of distilled water. The reaction mixture was allowed to cool and then absorbance was read at 575 nm. The standard curve was prepared for reducing groups with galacturonic acid. One enzyme unit of pectinase is the number of reducing groups measured in terms of galacturonic acid, produced as a result of the action of 0.5 ml of enzyme extract in 1 min at 30°C.

Protein was determined by the method of Lowry et al. (1951). Specific activity is the ratio of the total activity to total protein.

Enzyme purification

The crude enzyme was brought to 80% ammonium sulphate saturation and then dialysed. Dialysis tubes preserved in 90% ethanol were rinsed several times with distilled water and then buffered till they were clean of the ethanol. 0.05 M sodium acetate buffer pH 5.0 was used for enzyme dialysis. Dialysis was carried out for 14 h with continuous stirring and buffer changed every 6 h aiming to remove low molecular weight substances and other ions that may interfere with enzyme activity. After dialysis was complete, the partially purified enzyme was stored frozen at -24°C.

Enzyme characterization

The effects of pH and temperature were determined according to the method described by Miller (1959) as described in Wang et al. (1997) with little modifications as described previously under pectinase assay. The kinetic parameters were calculated from

Lineweaver-Burk plots of initial velocity data at different concentrations of mango pectin extract (0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 mg/ml).

RESULTS

All the experiments were conducted in triplicate and the result is the mean of the data derived. Three strains of pectinolytic fungi (*A. niger*, *A. fumigatus* and *A. flavus*) were isolated from soil of decaying fruits and vegetables. Pectin was extracted from mango peel with a yield of 15.2% at pH of 2.2, temperature of 70°C and extraction time of 60 min. The three *Aspergillus* species: *A. niger*, *A. fumigatus* and *A. flavus*, grown in media containing mango peel, produced significant quantities of pectinases. Crude enzymes from *A. niger* and *A. fumigatus* had their highest activities on day 4, while *A. flavus* had its highest activity on day 5 (Figure 1). Figure 2 shows the pectinase activities of mass-produced crude extract. The polygalacturonase activities of *A. niger*, *A. fumigatus* and *A. flavus* obtained from ammonium sulphate precipitation of pectinases were 45.92, 41.69 and 51.19 µmol/min, respectively, at 80% ammonium sulphate saturation (Figure 10). Tables 1, 2 and 3 show the percentage yield and purification fold of pectinases from the three pectinolytic microorganisms after dialysis, while the specific activity of the partially purified enzyme is presented in Figure 3. The effect of pH on the activity of pectinases from the three microorganisms is shown in Figure 4. Figure 4 also shows minor pH peaks for pectinases from *A. fumigatus*, *A. flavus* and *A. niger*. The effect of temperature on pectinase activities of the three isolates is presented in Figure 5. The effect of temperature

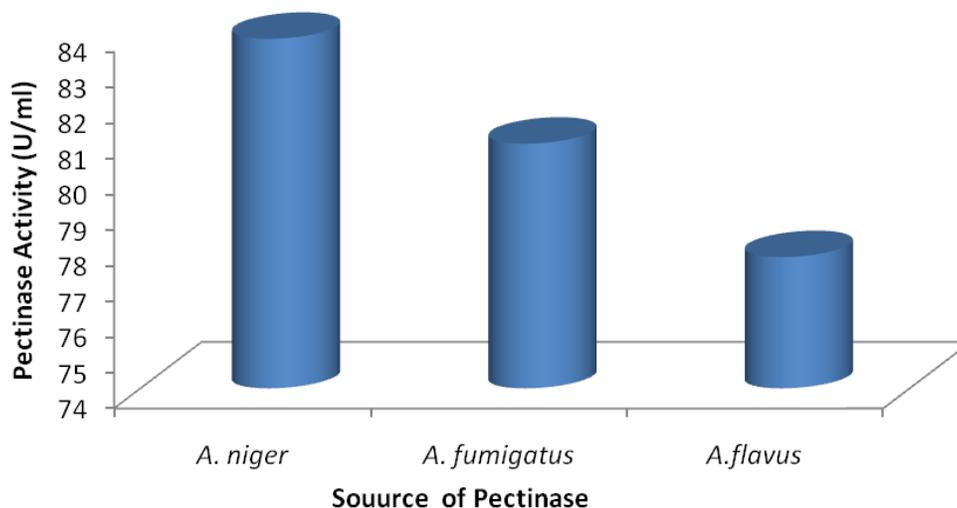


Figure 2. Pectinase activity of crude extracts from the three *Aspergillus* species.

Table 1. Purification of pectinases from *A. niger*.

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme filtrate	500	310	41900	135.16	1	100
Ammonium sulphate precipitation	20	15.6	1131.6	72.54	0.54	2.70
Dialyzed enzyme	23	10.58	2311.04	218.43	1.62	5.40

$\mu\text{mol}/\text{min} = \text{Unit (U)}$.

Table 2. Purification of pectinases from *A. fumigatus*.

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme filtrate	500	345	40430	117.19	1	100
Ammonium sulphate precipitation	20	21.4	2073.2	96.88	0.83	5.13
Dialyzed enzyme	22.7	14.76	3095.83	209.82	1.79	7.66

$\mu\text{mol}/\text{min} = \text{Unit (U)}$.

Table 3. Purification of pectinases from *A. flavus*.

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme filtrate	500	300	38840	129.47	1	100
Ammonium sulphate precipitation	20		1203.2	79.16	0.61	3.10
Dialyzed enzyme	22.5	9.65	2326.5	240.56	1.86	5.99

$\mu\text{mol}/\text{min} = \text{Unit (U)}$.

on the activity of pectinases at minor pH peaks is shown in Figure 6. Table 4 shows pectinase characterisation

from different *Aspergillus* species. Figures 7, 8 and 9 show the Lineweaver-Burk plot of initial velocity data at

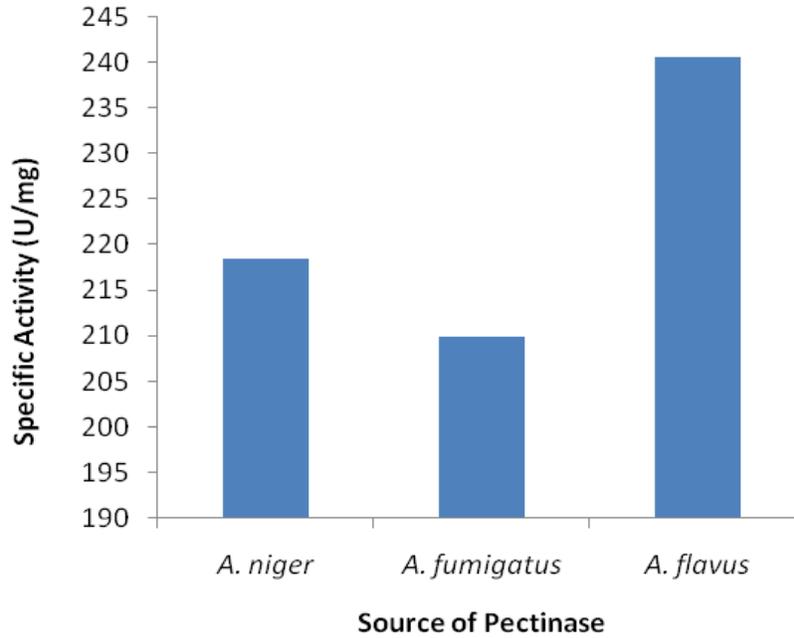


Figure 3. Specific activity of partially purified enzymes from different *Aspergillus* species.

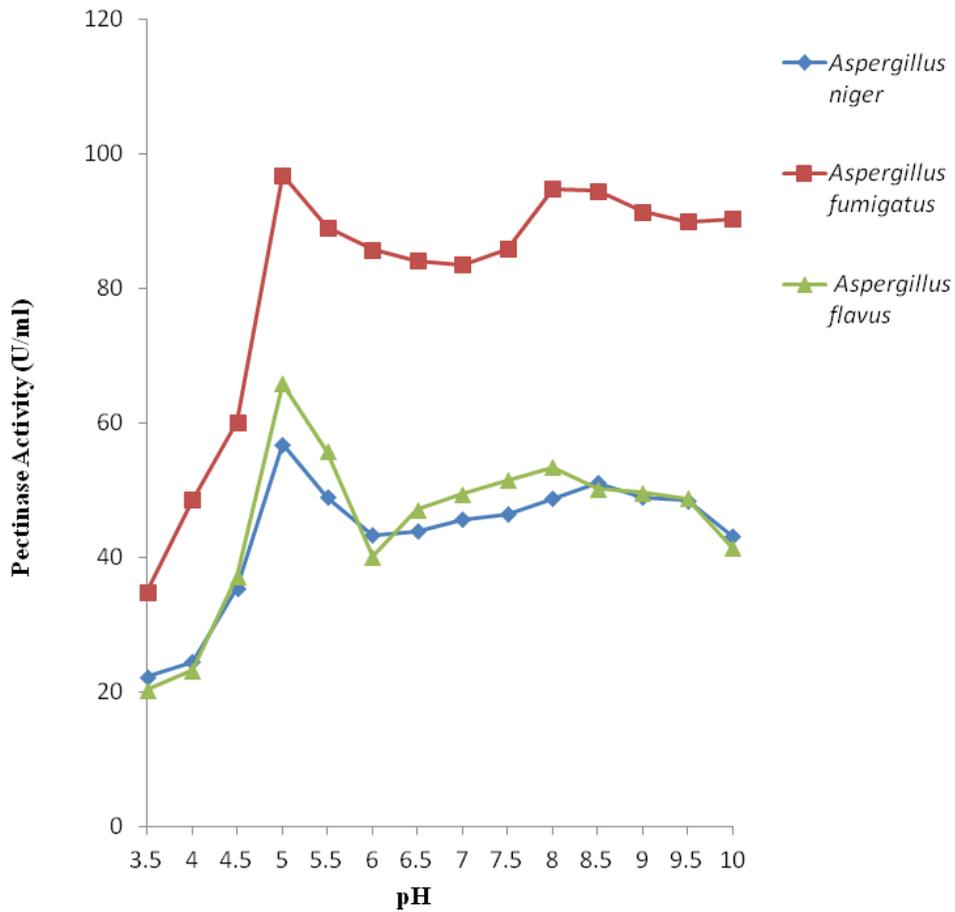


Figure 4. Effect of pH on the activity of pectinases from the three *Aspergillus* species.

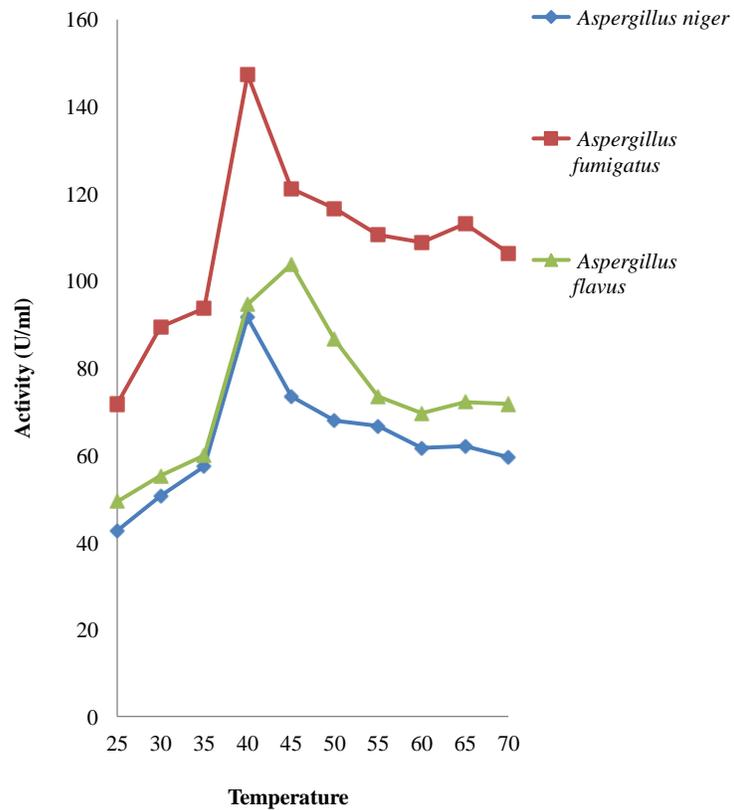


Figure 5. Effect of temperature on the activity of pectinases from the three *Aspergillus* strains.

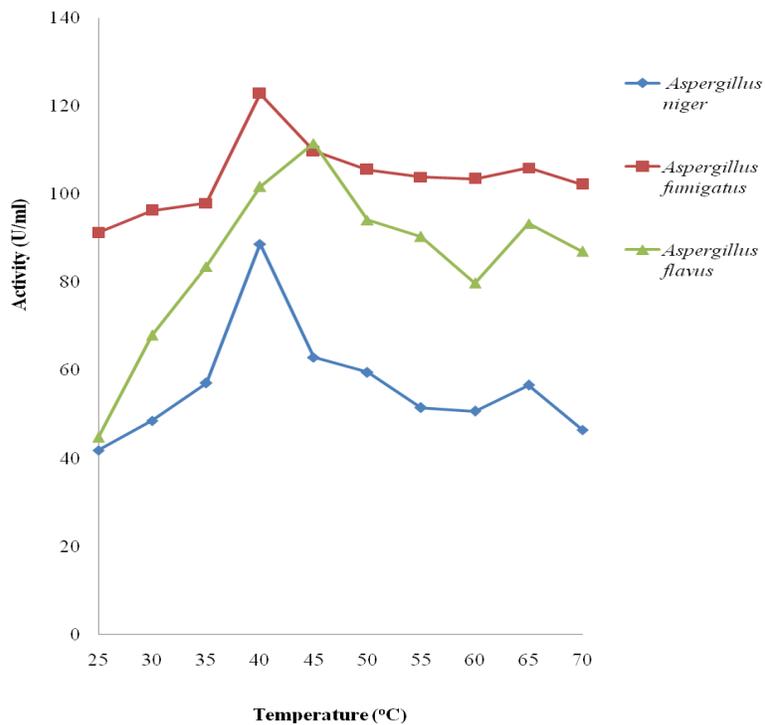


Figure 6. Effect of temperature on the activity of pectinases at minor pH peaks.

Table 4. Pectinase characterization.

Parameter	<i>A. niger</i>	<i>A. fumigatus</i>	<i>A. flavus</i>
pH	5.0	5.0	5.0
Temperature (°C)	40	40	45
V _{max} (μmol/min)	35.34	68.03	60.61
K _m (mg/ml)	0.357	0.156	0.261

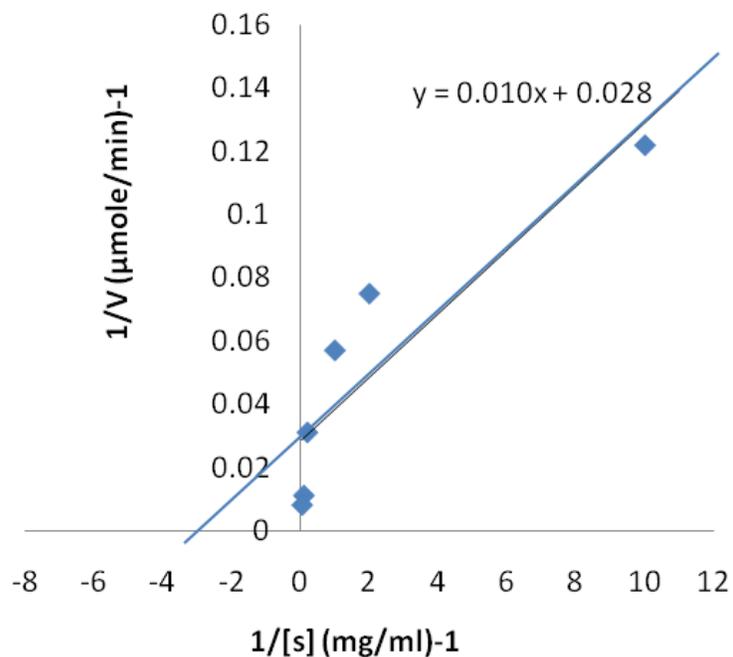


Figure 7. Lineweaver-Burk plot of pectinases from *A. niger*.

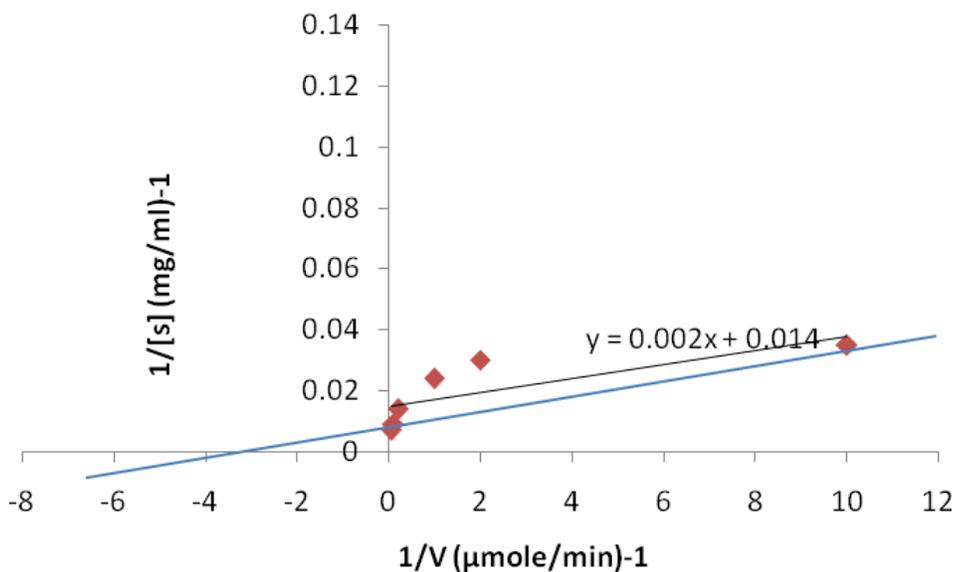


Figure 8. Lineweaver-Burk plot of pectinases from *A. fumigatus*.

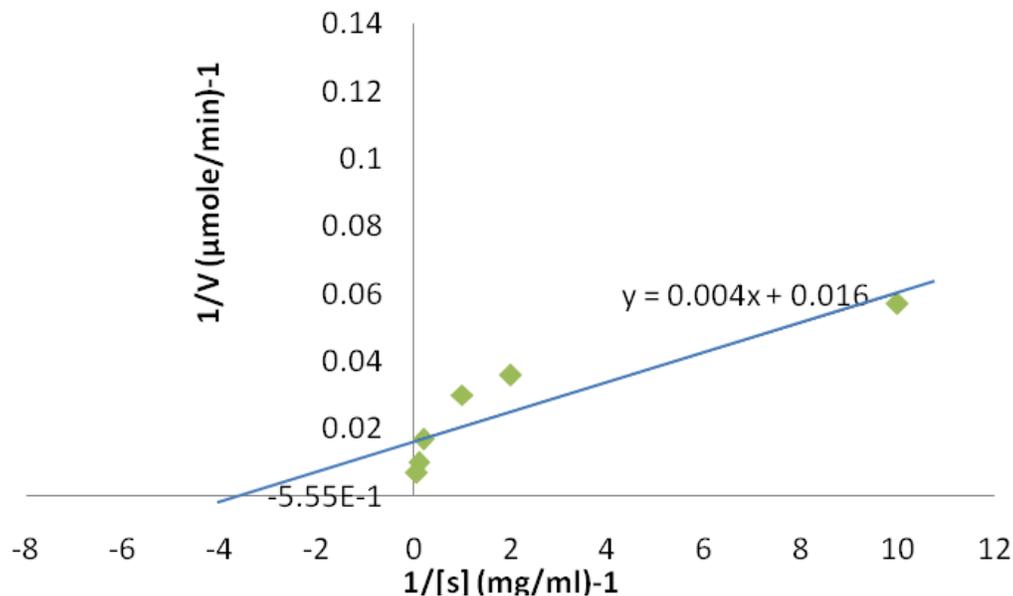


Figure 9. Lineweaver-Burk plot of pectinases from *A. flavus*.

data at different concentrations of mango pectin extract for pectinases from *A. niger*, *A. fumigatus* and *A. flavus*, respectively.

DISCUSSION

Pectin extraction

The percentage yield is comparable with the results obtained by Sharma et al. (2006), Rouse and Crandall (1976), Hussain et al. (1991) and Rehman et al. (2004). Any differences in the yields may have resulted from the differences in mango species, extraction technique, changes in pH, temperature and extraction time (Kertesz 1951; Rehman et al., 2004).

Pectinase production

The results showed that *A. niger*, *A. fumigatus* and *A. flavus* can grow well on mango peels. Pectinases production was analyzed over a period of 7 days by studying polygalacturonase (Pg) activities of extracted crude enzymes (Figure 1). Crude enzymes from *A. niger* and *A. fumigatus* had their highest activities (41.5 and 40.22 μmol/min, respectively) on day 4, while *A. flavus* had its highest activity (38.89 μmol/min) on day 5 (Figure 1). The peak days were chosen for optimal pectinase production by individual species. Pectinase activities of crude extract from *A. niger*, *A. fumigatus* and *A. flavus* were 83.80, 80.86 and 77.68 U/ml, respectively (Figure 2).

Partial purification

The polygalacturonase activities of *A. niger*, *A. fumigatus* and *A. flavus* obtained from ammonium sulphate precipitation of pectinases were 45.92, 41.69 and 51.19 μmol/min, respectively, at 80% ammonium sulphate saturation (Figure 10) which is comparable with the reports of Buga et al. (2010) and Adejuwon and Olutiola (2007) for pectinase from *A. niger* and *Lasidiopodia theobromae*, respectively. The results show that the purification fold was 1.62, 1.79 and 1.86 for *A. niger*, *A. fumigatus* and *A. flavus*, respectively (Tables 1, 2 and 3). The differences in these values might be due to differences in *Aspergillus* strains since pectinase production by filamentous fungi varies according to the strain and other genetic factors (Souza et al., 2003). Total protein decreased from crude values of 310, 345 and 300 mg to dialyzed values of 10.58, 14.76 and 9.65 mg for *A. niger*, *A. fumigatus* and *A. flavus*, respectively during the purification process (Tables 1, 2 and 3). The reduction in total protein from crude values of 310, 345 and 300 mg to dialyzed values of 10.58, 14.76 and 9.65 mg for *A. niger*, *A. fumigatus* and *A. flavus*, respectively during purification process might be due to loss of unwanted proteins. In addition, the increase in specific activities of enzymes from the three sources from crude values of 135.16, 117.19 and 129.47 U/mg to values of 218.43, 209.82 and 240.56 U/mg (Tables 1, 2 and 3) for *A. niger*, *A. fumigatus* and *A. flavus*, respectively after dialysis might result from the loss of unwanted proteins that interfere with the enzyme activity, thus leading to an increase in specific activity of the enzyme. However, increment in specific activity is a measure of purification

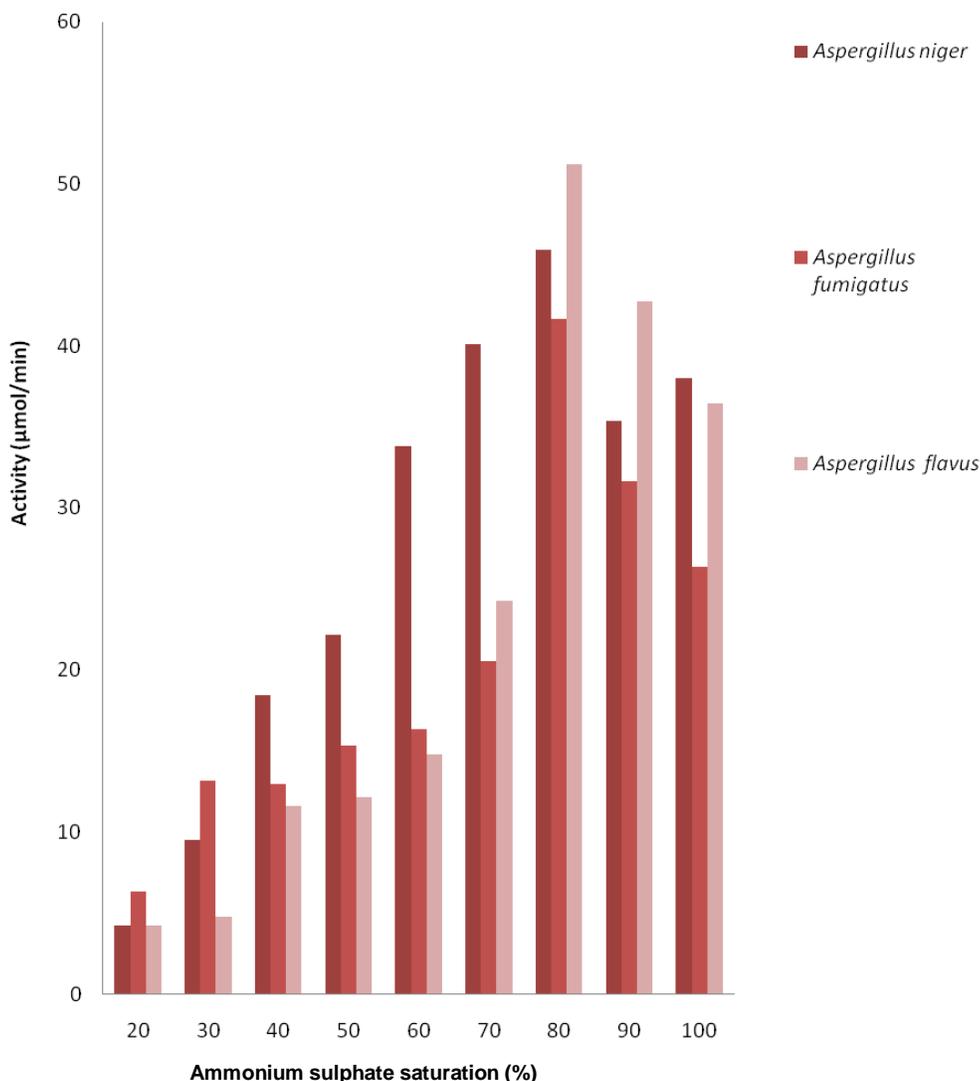


Figure 10. Ammonium sulphate precipitation profile of pectinases from the three *Aspergillus* strains.

achieved, indicating that pectinases from *A. flavus* that have the greatest enzyme specific activity, were the most purified. They were followed by pectinases produced by *A. niger* and then *A. fumigatus*, after partial purification (dialysis).

Pectinase characterization

The optimal pH of 5.0 was obtained for the pectinases from the three *Aspergillus* species (Figure 4) while an optimal temperature of 40°C was obtained for *A. niger* and *A. fumigatus* and 45°C for *A. flavus*, respectively (Figure 5). These results are in consonance with the reports of Favela-Torres et al. (2006), Niture and Pant, (2001) and Manachini et al., (1987). Figure 4 also shows minor peak activities for *A. fumigatus* and *A. flavus* at pH

8.0 and *A. niger* at pH 8.5. At pH of 8.0, pectinases from *A. fumigatus* and *A. flavus* indicated an optimal temperature of 40 and 45°C respectively, while that from *A. niger* indicated a temperature optimum of 40°C at pH of 8.5 as shown in Figure 6. The minor peaks at pH 8.0 for *A. fumigatus* and *A. flavus* and 8.5 for *A. niger* in Figure 4 may represent optimal pH for an isoform of pectinase, possibly pectin lyase. Pectin lyases have pH optima in the alkaline range 7.5-10.0 and temperature optima of 40-50°C (Jayani et al., 2005).

Kinetic studies

Kinetic parameters, V_{max} and K_m , of the enzymes were calculated from Lineweaver-Burk plot (Figures 7, 8 and 9). Pectinases from *A. niger*, *A. fumigatus* and *A. flavus*

had V_{max} and K_m values of 35.34 U and 0.367 mg/ml; 68.03 U and 0.156 mg/ml and 60.61 U and 0.261 mg/ml, respectively (Table 4). Thus, the increasing order of V_{max} for the three organisms is *A. niger*>*A. flavus*>*A. fumigatus*. K_m values less than 0.15 and up to 5.0 mg/ml (<0.15 to 5.0 mg/ml) and specific activities 8.8 to 7000 U/mg were reported for some fungal pectinases by Lucie (2000). The K_m value obtained in this study is not only comparable but also in agreement with the report by Rombouts and Pilnik (1980) that the K_m for most fungal pectinases is less than 1 mg/ml.

Conclusion

Most work on pectinase microbial production has been oriented in the direction of using pure pectin extract, especially apple pectin extract, as the sole carbon source, while in this work we used mango peels for pectinase production. Our findings provide alternative and cheaper source of substrates for microbial pectinase production. Thus, mango peel could be an attractive and promising substrate especially in submerged fermentation for the production of pectinases by *Aspergillus* species. Pectinase from *A. fumigatus* with the highest yield during the purification process, indicated highest V_{max} and the lowest K_m (highest catalytic efficiency) value at acidic pH of 5.0 and optimum temperature of 40°C. Therefore, it can be employed in industries for hydrolysis of pectic biomass to utilizable bio-products.

Conflict of Interests

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

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Full Length Research Paper

Expression and characterization of a novel spore wall protein from *Nosema bombycis*

Haihong Qiu^{1,2}, Mingqian Li², Xinyi He², Xiangkang He² and Xingmeng Lu^{2*}¹Department of Biological and Chemical Engineering, Shaoyang University, Shaoyang 422000, P. R. China.²College of Animal Sciences, Zhejiang University, Hangzhou 310058, P. R. China.

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Microsporidia are obligate intracellular, eukaryotic, spore-forming parasites. The environmentally resistant spores, which harbor a rigid cell wall, are critical for their survival outside their host cells and host-to-host transmission. The spore wall comprises two major layers: the exospore and the endospore. In *Nosema bombycis*, five spore wall proteins have been identified which contain two endospore proteins (SWP25 and SWP30) and three exospore proteins (*Nb*SWP5, SWP26 and SWP32). In the current study, we identified a novel endospore protein *Nb*SWP12 with calculated molecular mass of 25.56 kDa and pI of 6.69 using SDS-PAGE and MALDI-TOF MS technique. Followed by gene cloning and protein expression, polyclonal antibody production, indirect immunofluorescence antibody test, and immunoelectron microscopy analysis, the results indicate that this protein is localized to the endospore and has no obvious enhancement on adherence to host cells. The characterization of this novel spore wall protein from *N. bombycis* may facilitate our further investigation of the relationship between *N. bombycis* and its host, *Bombyx mori*.

Key words: Microsporidia, *Nosema bombycis*, Spore wall protein, *Nb*SWP12.

INTRODUCTION

Microsporidia, which are unicellular eukaryotes and obligate intracellular parasites, have long been recognized as pathogenic agents in sericulture, apiculture, and mammals (Wittner, 1999). Microsporidia were previously divided into primitive eukaryotes, however, more and more molecular evidences based on the recent phylogenetic analyses of rDNA sequences, conserved proteins and the complete genome sequences of microsporidia *Encephalitozoon cuniculi* have demonstrated that these organisms are phylogenetically related to the fungi with remnant mitochondrial organelles (Hirt et

al., 1999; Keeling et al., 2000; Fabienne et al., 2004; James et al., 2006; Goldberg et al., 2008; Lee et al., 2008). To date, the identified microsporidia have approximately 160 genera and 1300 described species (Corradi et al., 2008). Of these, at least 13 species are reported to infect humans (Dider et al., 2008), and five microsporidian genera have been found to infect the silkworm *Bombyx mori* (Bhat et al., 2009). *Nosema bombycis*, as the causative agent of silkworm pebrine (Naegli, 1857), ravaged the silkworm industry of Europe, especially in Italy and France during the mid-19th century

*Corresponding author. E-mail: xmlu@zju.edu.cn. Tel: +86-571-88982305. Fax: +86-571-88982305.

(Becnel and Andreadis, 1999). Even now, it is still epizootic and causing heavy economic losses in silk-producing countries such as China.

Although microsporidia have no active stages outside their host cells and can survive only by living inside cells, all microsporidia produce environmentally resistant spores with a unique extrusion apparatus that contains a coiled polar tube ending in an anchoring disc at the apical part of the spore (Vavra et al., 1999). Under appropriate conditions inside a suitable host, the polar tube is discharged through the thin anterior end of the spore thereby penetrating a new host cell and inoculating the infective sporoplasm through the hollow tube into the new host cell (Bigliardi and Sacchi, 2001). The sporoplasms released into host cells then enter a proliferative phase, undergoes the sporogony, sporonts, and the sporoblasts stages and then differentiates into mature spores protected by a thick wall. The spore wall, which consists of an electron-dense, proteinaceous outer layer (exospore), an electron-lucent inner layer (endospore) composed of chitin and protein, and a plasma membrane (Vavra et al., 1999), provides structural rigidity and protects the mature spore from the outer environment. The spore wall proteins (SWPs) of microsporidia may play a role in recognition by the host during the invasion process (Hayman et al., 2005; Southern et al., 2006), and the interactions between the SWPs and the host plasma membrane mediate the microsporidian spores being phagocytized by the host cell (Couzinet et al., 2000; Franzen et al., 2005).

Due to little sequence similarity between the known microsporidian spore wall proteins with any other eukaryotic proteins, it is very difficult to identify a new spore wall protein. To date, only a total of seven spore wall proteins have been reported in *E. cuniculi* (Bohne et al., 2000; Brosnon et al., 2005; Peuvel-Fanget et al., 2006; Southern et al., 2007; Xu et al., 2006) and *Encephalitozoon intestinalis* (Southern et al., 2007; Hayman et al., 2001), and four spore wall proteins from *N. bombycis* including SWP25, SWP30, SWP26 and SWP32 were addressed on the location and function (Wu et al., 2008; 2009; Li et al., 2009). In our previous study, we identified an exospore protein *NbSWP5* that can protect spores from phagocytic uptake by cultured insect cells (Cai et al., 2011).

In the current study, we identified another endospore protein *NbSWP12* which was found in mature spores of *N. bombycis*. Its location and function were elucidated through matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique, gene cloning and protein expression, polyclonal antibody production, indirect immunofluorescence antibody (IFA) test, and immunoelectron microscopy (IEM) analysis. These results propose reliable experimental data for the interaction of endospore protein *NbSWP12* with *N. bombycis* *in vitro* and *in vivo*, and provide foundation for further study of the mechanism of microsporidia of *N.*

bombycis.

MATERIALS AND METHODS

Production and purification of *Nosema bombycis*

N. bombycis was originally isolated from infected silkworms in Zhejiang, China. Spores were propagated and purified as previously described (Zhang et al., 2007). Briefly, spores were harvested from infected moths. The fifth molted silkworm larvae were challenged by feeding on mulberry leaves artificially polluted by *N. bombycis* spores (10^8 spores/200 larvae). The moths developed from the infected fifth larvae were dissected, homogenized, and centrifuged. Spores were purified on Percoll and centrifuged at 21,000 *g* for 90 min (Canning et al., 1999). Purified spores were stored in deionized water supplemented with antibiotics (Sigma, 100 mg/ml streptomycin, 100 U/ml penicillin) at 4°C for later use (Gatehouse and Malone, 1998).

Spore wall proteins extraction and MALDI-TOF MS analysis

Spore wall proteins of *N. bombycis* were extracted as described previously (Wu et al., 2008) with slight modifications. Briefly, 10^9 spores were disrupted in a lysis buffer (Takara, 0.1M DTT, 4% CHAPS and 0.2% SDS) adding acid-washed glass beads (Sigma, diameter: 425 - 600 μ m) with a FastPrep-24 (MP BIO). Following, proteins were incubated in an extraction buffer containing 2 M thiourea, 7 M urea, 0.1 M DTT, 4% CHAPS and 0.2% SDS for 6 h at room temperature. After centrifugation for 10 min at 20,000 *g*, the supernatant was collected and the samples were stored at -80°C for later use.

After quantification with Plus-One 2D Quant kit (Amersham), the protein samples were analyzed by standard SDS-PAGE on 12.5% polyacrylamide gels and stained with Coomassie Blue. Protein bands were then excised for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis as described previously (Cai et al., 2011). The generated data were used to search the UniprotKB/

SwissProt database using the software GPS Explorer (version 3.6, Applied Biosystems) and MASCOT (version 2.1, Matrix Science) with the following parameters: trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance set to 100 ppm, fragment tolerance set to \pm 0.3 Da, and minimum ion score confidence interval for MS/MS data set to 95%.

Recombinant protein expression of spore wall protein *NbSWP12* and polyclonal antibody production

The *NbSWP12* protein was expressed with prokaryotic expression vector pET-30a (Novagen). Based on the genomic DNA sequences of *N. bombycis* (GenBank: EF683112.1), polymerase chain reaction primers were designed using Primer 5.0 software (Premier Inc.) as forward primer: 5'-GCGGATCCATGAAAGATTTAAAAAG-3' and reverse primer: 5'-TCAAGCTTACTTAGTCCTCTCTAATGC-3'; the forward primer and reverse primers contained *Bam*H I and *Hind* III restriction sites (underlined) respectively. These primers amplified a 687-bp genomic DNA fragment corresponding to the amino acid regions 1-228 of the 228-amino acid *NbSWP12* protein.

PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 5 min, 35 amplification cycles (denaturing at 95°C for 45 s, annealing temperature at 52.6°C for 45 s, and extension at 72°C for 1 min), and a final

extension step at 72°C for 10 min. The PCR products were analyzed on 1% agarose gels with ethidium bromide staining, the primer-specific product band with the expected size was excised from the gel and recovered with QIAquick PCR purification kit (Qiagen) following the protocol of the manufacturer.

The amplified products were digested with *Bam*HI and *Hind*III and inserted into a *Bam*HI/*Hind* III -digested prokaryotic expression vector pET-30a. The resultant recombinant plasmids were transformed into *Escherichia coli* Transetta (DE3) competent cells and expression of recombinant *Nb*SWP12 was induced for growth for 6 h at 37°C in the presence of 1 mM isopropyl- β -thiogalactopyranoside (IPTG). The expressed fusion proteins were then purified with His-Bind Purification Kit (Novagen) from recombinant protein expression of spore wall protein *Nb*SWP12. Mono specific polyclonal antiserum against the purified recombinant *Nb*SWP12 was produced by immunization of native rabbit using the standard 56-day antibody production protocol. Meanwhile, a rabbit was injected with PBS, its sera were collected and stored at -20°C and used as a negative control. The animal house and experimental staff were approved by Chinese veterinary services, and experiments were conducted in accordance with ethical guidelines.

Immunoblotting analysis

N. bombycis proteins were extracted according to the procedure as previously described (Wang et al., 2007). Briefly, *N. bombycis* spores (10^9 cells) were suspended in a lysis buffer, containing 100 mM DTT, 4% CHAPS and 0.5% Triton X-100 to protect the protein from disruption, and 0.5 g acid-washed glass beads (0.425-0.600 microns, Sigma) were added in a 1.5-ml Eppendorf tube, followed by vigorously shaking for 2-3 min at maximum speed on the Fast-Prep24. The homogenate was transferred to a fresh Eppendorf tube, containing 200 μ l extraction buffer (0.2% (w/v) SDS, 4% (v/v) CHAPS, 100 mM DTT, 2M thiourea, 7 M urea), and incubated at room temperature for 6 h. After centrifugation at 3,000 g for 5 min, the supernatant was collected as protein samples and stored at -80°C for later use.

For immunoblotting analysis, protein samples were subjected to SDS-PAGE on 12.5% polyacrylamide gels. Electrophoresis, transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore) and blocking were performed under standard conditions. Anti-*Nb*SWP12 sera (1:200 dilution) was used as the primary antibody. The secondary antibody, a goat anti-rabbit IgG-IgM antiserum (Sigma) labelled with peroxidase, was detected by addition of the substrate tetrahydrochloride (DAB).

Indirect immunofluorescence assay analysis (IFAs)

For indirect immunofluorescence assay analysis, BmN cell line derived from the ovary of *B. mori* was cultured in TC-100 insect cell culture medium (Sigma) in glass bottom culture dishes (GBD-35-20, Nest Biotechnology Co.) at 27°C, supplemented with 10% fetal bovine serum (GibcoBRL Life Technologies) and 50 μ g/mL of gentamycin (Takara). Approximately 1×10^6 cells with 2 ml growth medium were allowed to grow at 27°C for 16 h. The purified *N. bombycis* spores (10^7 cells) were added into the cell culture medium and host cells were incubated for 24 h at 28°C. The host cells including some *N. bombycis* spores were fixed with 100% methanol for 10 min at 4°C, and then permeabilised with 0.5% Triton X-100 for 30 min. Incubated with polyclonal antisera or a negative serum, the slides were kept in a moist chamber for 60 min at 37°C. Bound antibodies were detected with 1:64 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma). DNA was stained with 1 mg/ml DAPI for 20 min. The spores were examined with an OLYMPUS BX50 immunofluorescence microscope (excitation wave WL of FITC-IgG: 495 nm; DAPI: 359 nm; magnification, 1000 \times).

Ultrastructural localization by immunoelectron microscopy (IEM)

N. bombycis was prepared as described above, and IEM was performed as previously described (Wu et al., 2008). Briefly, the mature spores were fixed with 3.0% (v/v) paraformaldehyde and 1.0% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, and then rinsed five times (15 min each time) in PBS buffer (pH 7.2) at 4°C. To enable samples to be imbedded and photopolymerised in K4M resin, the fixed spores were dehydrated with graded ethanols and sequentially permeabilised in K4M at -20°C. Ultrathin sections were placed on 300-mesh nickel grids coated with Formvar and carbon. In order to immunostaining, these grids were transferred into a blocking solution, obtaining 1% bovine serum albumin (BSA), 0.02% polyethyleneglycol mol. wt. 20,000 (PEG 20,000) and 0.1 M NaCl, 1% Na₂SO₄, for 30 min, and then incubated with a 1:200 dilution of the primary anti-*Nb*SWP12 polyclonal antibodies, or a negative serum or polyclonal antibodies against total proteins of *N. bombycis*, in blocking solution for 1 h at 25°C, followed by incubation with a 1:100 dilution of the gold conjugated anti-rabbit IgG (Sigma, 10 nm ID) for 1 h at 25°C. Grids were rinsed five times in PBS buffer (pH 7.2), stained with uranyl acetate and lead citrate, and then dried, examined, and photographed with a JEM-1230 transmission electron microscope (JEOL).

Spore adherence assays and infectivity assays

BmN cells were used as host cells to measure adherence of *N. bombycis* spores and infection. Adherence of *N. bombycis* spores and infection were measured as previously described (Hayman et al., 2005; Wu et al., 2009). Approximately 1×10^6 cells with 2 ml growth medium in each dish were allowed to grow at 27°C for 16 h. Recombinant proteins (0.1-10 μ g/ml), as well as the control sample (negative serum), were incubated with 5.0×10^6 *N. bombycis* spores on monolayers for 4 h at 28°C. The culture dishes were thoroughly washed with PBS to remove unbound spores. To identify host cell infection, fresh cell culture medium was added and incubated at 28°C for additional 48 h. An immunofluorescent assay was performed to treat these samples and the average numbers of spores bound to per host cell were calculated by counting microsporidia in at least 30 host cells of magnification. The results were shown as the inhibition percentage of adherent spores relative to the control samples-at least 30 host cells of 400 \times magnification. The significance of the differences between the control and experimental assays were measured using the two-tailed Student's t-test in the Statistical Package for Social Science (version 12.0, SPSS). P values of 0.001 or less were considered statistically significant. The aforementioned experiments were repeated three times with similar results.

RESULTS

Identification of a spore wall of *N. bombycis* *Nb*SWP12

The band for protein *Nb*SWP12 with calculated molecular mass of 25.56 kDa and pI of 6.69 was excised and analyzed by MALDI-TOF-MS. It matched a 228-amino acid protein (Table 1) which was correspondent with that previously named as *Nb*SWP12 under GenBank Accession number EF683112 (Wu et al., 2008). Analyses indicated that the protein possesses one predicted N-glycosylation site and 16 phosphorylation sites, but

Table 1. MALDI-TOF MS analysis of the spore wall protein *NbSWP12* of *Nosema bombycis*.

Protein ID	pI/MM (kDa)	Cov (%)	Mmobs (Da)	Mmcalc (Da)	Mmdiff (Da)	Position	Sequence
SWP12	6.49/25.56	12.72	812.3641	812.3347	-0.0294	189-194	TIEMMR
			910.3835	910.3457	-0.0378	125-131	FNEQCGR
			1167.5211	1167.4718	-0.0493	123-131	EKFNEQCGR
			1323.6539	1323.5967	-0.0572	178-188	LSELFENSQTR
			1423.6998	1423.6396	-0.0602	63-75	IYHGLSMVSSASR
			1458.7223	1458.6606	-0.0617	211-222	DLNIEFHQESVK
			1461.7697	1461.7043	-0.0654	195-208	DFIGADGLQGVLTR
			1798.7740	1798.6978	-0.0762	76-90	MNYFSDADIFEGFAR

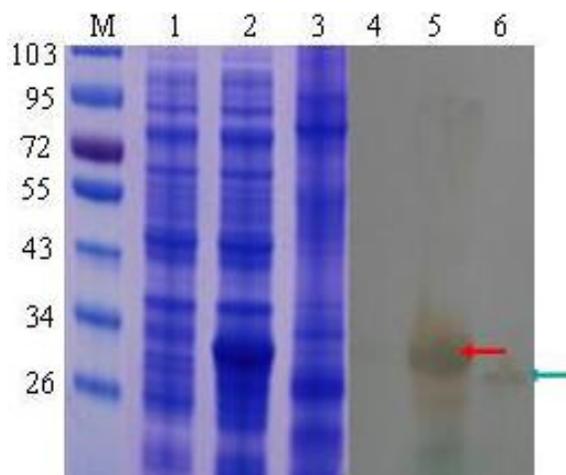


Figure 1. SDS-PAGE and western blot analysis of spore protein SWP12 of *Nosema bombycis*. *N. bombycis* spore proteins were run on a 12% SDS-PAGE gel and detected with anti-*NbSWP12* polyclonal antibody. M, molecular mass standards; lane 1 to lane 3, SDS-PAGE analysis; lane 1, crude extract from *E. coli* transetta (DE3) with pET30 vector induced by 1 mmol/L IPTG; lane 2, crude extract from *E. coli* transetta (DE3) with pGT30-SWP12 induced by 1mmol/L IPTG; lane 3, crude extract of *N. bombycis* spore proteins; lane 4 to lane 6, western blot analysis, lane 4, no band of normal rabbit sera (1:200 dilution) as negative control of the lane1; lane 5, apparent fractions (~31.95 kDa) of the anti-recombinant *NbSWP12* sera (1:200 dilution) after His-Binding affinity chromatography; lane 6, only tenuous band (~26kDa) of *N. bombycis* spore proteins (1:200 dilution) as positive control of the lane 3. The blotting bands are indicated by red arrowhead and blue arrowhead, respectively.

neither O-glycosylation sites nor transmembrane domain are predicted (<http://www.cbs.dtu.dk>). *NbSWP12* is predicted to be an extracellular (for example, cell wall) protein (<http://www.psорт.org/>) by in silico analysis, but it possesses no amino-acid signal peptides (www.cbs.dtu.dk/services/SignalP/).

Expression of *NbSWP12* fusion proteins and western blot analysis

To further characterise *NbSWP12*, we constructed the recombinant expression plasmid pET30-*NbSWP12* and transformed the recombinant plasmids into the *E. coli* transetta (DE3) strain. The heterologously expressed proteins with about 31.95 kDa were purified by affinity chromatography using a His-Bind Purification Kit (Novagen). The *NbSWP12*-specific polyclonal antibody was generated by immunising rabbit with the purified fusion protein and used in western blot analyses. A single 26-kDa band was detected from the *N. bombycis* spore protein lysates (Figure 1), which is in agreement with the size calculated from the sequence. The result clearly demonstrated that the antiserum was successfully produced in rabbit and had strong reactivity to *NbSWP12*.

Expression of *NbSWP12* in *Nosema bombycis* spores

The antiserum to *NbSWP12* was used in an immunofluorescence assay with purified mature spores of *N. bombycis* added in BmN cells (Figure 2A₁). Little to no fluorescence signal was detected in the control (Figure 2B₁). Rabbit anti-*NbSWP12* sera were shown to bind to *N. bombycis* spores (Figure 2 A₁, A₃). The fluorescence patterns were consistent with these proteins reacting specially with the spore walls, showing strong immunofluorescence in the *vitro*-infected host cells, and also in the purified mature spores, which display a bright signal and are readily recognized at 1,000× magnification. The existence of spores was confirmed by DAPI staining (Figure 2 A₂, B₂).

Location of *NbSWP12* in the *N. bombycis* parasite

IEM was employed to determine the cellular location of the *NbSWP12* protein. The mature spores were postfixed with 1% osmium tetroxide, and then were treated using immunogold. As shown in Figure 3, a number of gold particles were distributed along the endospore regions of

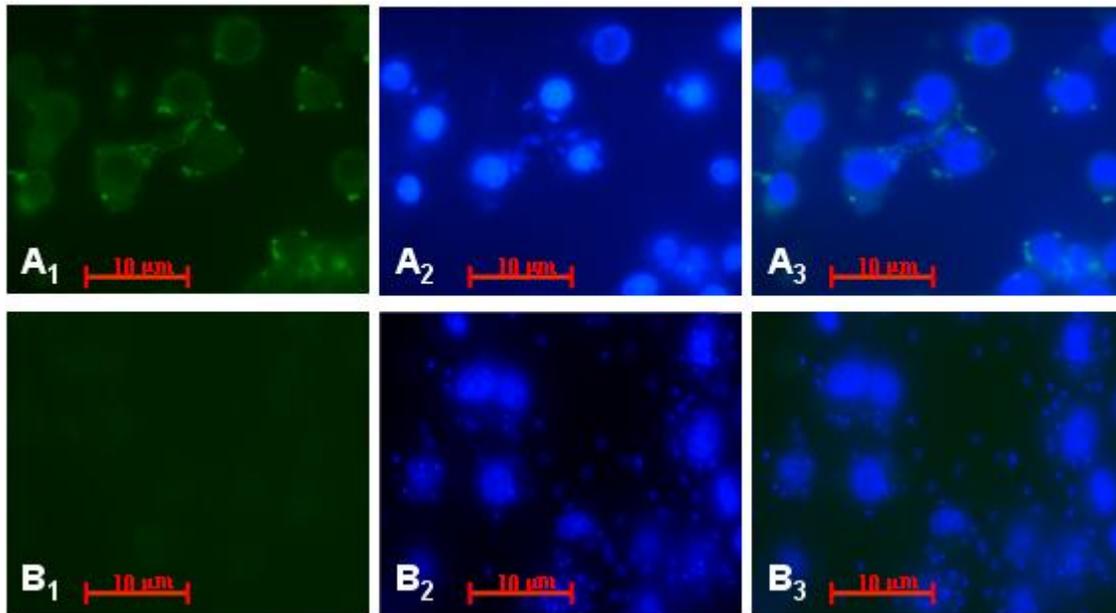


Figure 2. Indirect immunofluorescence assay with rabbit anti-*NbSWP12* showing labeling of the intracellular parasite and the purified mature spores. *N. bombycis* spores infected BmN cells and purified mature spores (A) are visualized with a fluorescence microscope, after incubation with the primary antibodies against *NbSWP12* (A₁). Image B₁ is the negative control, image A₂ and B₂ are DAPI staining. A₃ and B₃ are the merged images of A₁ and A₂, B₁ and B₂, respectively. Anti-*NbSWP12* was used at a 1:100 dilution. The secondary antibody was FITC-conjugated goat anti rabbit IgG(Sigma) used at a 1:64 dilution. All images are magnified 1000x. The scale bar represents 10 μm.

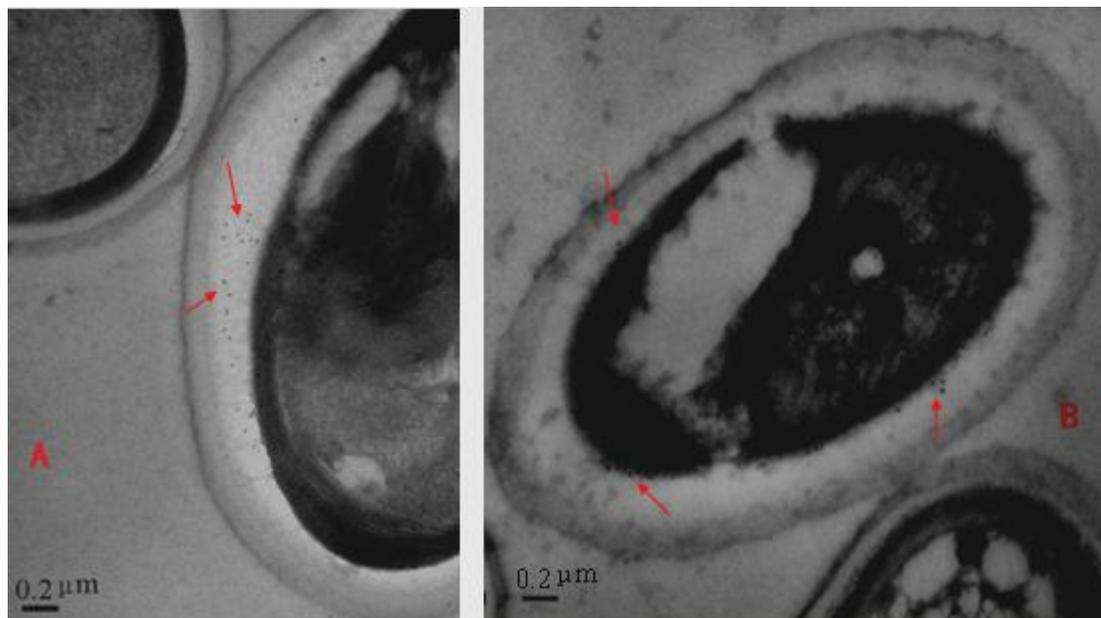


Figure 3. Immunogold electron microscopy of *N. bombycis* using the anti-*NbSWP12* polyclonal antibodies and secondary antibodies conjugated with 10nm colloidal gold (Sigma). Immunogold labeling of *NbSWP12* demonstrated the location of *NbSWP12* in the endospore of *N. bombycis*. The colloidal gold particles are marked by red arrowheads. The scale bar represents 200 nm.

the spore wall but few in the exospore regions (Figure 3). No gold particles were detected in the control sample

using control sera (data not shown). These results implied that *NbSWP12* may be located in the endospore

Table 2. Statistics of the spore adherence and host cell infection assay with exogenous recombinant *NbSWP12* protein.

Exogenous <i>NbSWP12</i> protein ($\mu\text{g/mL}$)	0	0.5	1.0	2.0	5.0
Adherence spores/host cell (average)	15.67	14.56	15.67	14.66	14.55
Percentage of infected cells (%)	25.33	24.61	25.33	24.66	24.52

region of the *N. bombycis* spore.

The effect of exogenous *NbSWP12* protein on *N. bombycis* attachment and host cell invasion

A host cell invasion experiment was performed to determine whether SWP12 functions in the spore attachment process, and the generated data are shown in Table 2. Statistical analysis shows that there were no statistically significant differences in spore adherences or host cells infection compared with that of control samples.

DISCUSSION

As a group of eukaryotic intracellular parasites, microsporidia infect almost all vertebrates and invertebrates (Didier et al., 1998). As composition of rigid spore wall, spore wall proteins play an important role in microsporidian invasion (Southern et al., 2007; Frixione et al., 1992). However, little is currently known about the components of the spore wall. Only seven spore wall proteins in *E. cuniculi* and *E. intestinalis* were identified by monoclonal or polyclonal antibodies (Bohne et al., 2000; Brosion et al., 2005; Peuvél-Fanget et al., 2006; Southern et al., 2007; Xu et al., 2006; Hayman et al., 2001). Among fourteen hypothetical spore wall proteins from *N. Bombycis* deposited in GenBank data, only five complete spore wall proteins have been identified, which contain two endospore proteins (SWP25 and SWP30) and three exospore proteins (*NbSWP5*, SWP26, and SWP32) (Wu et al., 2008; Wu et al., 2009; Li et al., 2009; Cai et al., 2011).

In the current study, based on protein technique, MALDI-TOF MS analysis, and on the GenBank data, we have identified a new spore wall protein named as *NbSWP12* in the silkworm parasite *N. Bombycis*. Sequence analysis demonstrated the protein to have a calculated molecular mass of about 25.56-kDa that is distinct from the previously reported spore wall proteins. IEM data implied *NbSWP12* was located into endospore, even so there is much work to be done in order to attain an accurate positioning of this protein. Only the function of spore wall proteins has been illuminated, can we execute some related downstream research?

Little reduction of spore adherence or host cells infection compared with that of control samples was

implied by the following spore adherence and host cell infection assays. It may be presumed that *NbSWP12*, as a supposed endospore wall protein, may not be a surface adherence ligand or not the main adherence factor, and that this protein is more likely to be involved with spore wall construction/maintenance than the infection process. Further studies on the molecular function of *NbSWP12* on parasite-host interactions are currently carried out in our laboratory, which will help us to clarify the infection mechanism of *N. Bombycis*.

In summary, studies of the spore wall proteins should facilitate our further investigation of the relationship between these ubiquitous pathogens and their hosts, *B. mori*, which is beneficial for us to control the silkworm, *B. mori* pebrine disease in sericulture.

Conflict of Interests

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

ACKNOWLEDGMENTS

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Full Length Research Paper

Ascorbic acid and mineral elements composition of powdered antimalarial (Maloff-HB) and haematinic (Haematol-B) herbal formulations from Ogbomoso, Nigeria

Adepoju Tunde Joseph OGUNKUNLE¹, Olugbenga Solomon BELLO^{2*} and Adijat Funke OGUNDOLA¹

¹Environmental Biology Unit, Department of Pure and Applied Biology, Ladoke Akintola University of Technology, P.M.B 4000, Ogbomoso, Oyo State, Nigeria.

²Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology, P.M.B 4000, Ogbomoso, Oyo State, Nigeria.

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Different types of powdered antimalarial and blood-enriching (haematinic) recipes are used in traditional health system of Southwestern Nigeria. Two of these from Ogbomoso (that is Maloff-HB and Haematol-B, respectively) were recently named following a quantitative definition of their botanical constituents. However, information on the physico-chemical and phytochemical properties as well as the residual constituents of both polyherbal formulations (PHFs) is lacking. The amount of ascorbic acid in them was therefore determined using ultraviolet (UV)-visible spectrophotometric method. Their elemental compositions (magnesium, calcium, manganese, iron, zinc, potassium, sodium and copper) were also quantified spectrophotometrically. Maloff-HB and haematol-B contain high quantities of ascorbic acid (mean values of 542.35 and 414.14 mg/100 g, respectively). This is the antioxidant that has been implicated in many redox reactions which promote good health. Both drugs are also rich in Mg (1319.04 and 2340.00 mg/100 g, respectively) and Ca (784.31 and 1011.67 mg/100 g, respectively), these values being comparable to the recommended dietary intakes. The values obtained for Fe/Cu and Fe/Zn ratios can promote bioavailability of these important mineral elements. The important role of ascorbic acid in enhancing iron absorption is discussed with the conclusion that the two drugs have the potential to meet some dietary requirements which promote healthy blood that prevents infections. The study recommends safety and efficacy evaluations of the two PHFs based on their residual constituents, *in vivo* activity and bioavailability of their beneficial constituents.

Key words: Antioxidants, ascorbic acid, herbal formulations, anti-malaria, mineral elements composition.

INTRODUCTION

World Health Organization defines herbal medicines as finished, labeled medicinal products that contain active ingredients (aerial or underground parts of plants), or

other plant material (such as juices, gums, fatty oils and essential oils) or combinations thereof, whether in the crude state or as plant preparations (WHO, 2000). The

part of plants (otherwise called medicinal herbs) or other plant material is known to contain bioactive phytochemicals, which are the basis for their therapeutic properties (Coker et al., 2008; Soetan and Ayelaagbe, 2009). Many of these herbs have also been acknowledged for their mineral elements and proximate compositions; they have nutritional qualities that could provide the users with additional nutrients (Abolaji et al., 2007). In fact, therapeutic and prophylactic properties of some medicinal plant parts have been attributed to their nutritional value rather than their phytochemical constituents (Akpinar- Bayizit et al., 2012; Pandey et al., 2012).

The Chiang Mai 1998 declaration at the first WHO/IUCN/WWF international consultation on conservation of medicinal plants recognized that medicinal plants were essential in primary health care, both in self-medication and in national health services (WHO, IUCN and WWF, 1993). In consonance with the provisions of this declaration, the medical and dental practitioners (Amendment) Decree number 78 of 1992 was promulgated by the Federal Government of Nigeria to place traditional and alternative medicines side by side with orthodox medicine (ABFR and Co, 1992). Ever since that time, there had been an overwhelming increase in the public awareness and usage of herbal medicines in Nigeria. Rukangira (2001) has also reported an increasing demand for herbal medicines in Africa with its growing population, but admitted that constraints and challenges existed in relation to conservation, science and technology, use of medicinal plants at local level, marketing and efficacy requirements. Similarly, Lau et al. (2003) and WHO (2003) have called the safety and efficacy of African herbal products to question on the basis of adulteration, substitution, contamination, mis-identification, lack of standardization, incorrect preparation and/or dosage, and inappropriate labeling and/or advertisement.

Herbal product cannot be considered scientifically valid until the drug being tested has been authenticated and characterized so that one can ensure reproducibility in the manufacturing of the product. So, for an herbal formulation to be accorded acceptability as a therapeutic agent of disease, the issue of its standardization has to be addressed first. This is an important step for establishing a consistent biological activity and a consistent chemical profile towards putting in place a quality assurance programme for production and manufacturing of herbal drugs (Choudhary and Sekhon, 2011). The practice in which botanical materials are converted into medicines where modern scientific techniques and traditional knowledge are properly integrated to ensure standardization and quality control is known as herbal

drug technology (Patra et al., 2010).

Annexure I of the guidelines published by the World Health Organization (WHO, 2000) on evaluation of traditional medicines provide, among others, quantitative list of active ingredients to accompany each herbal product for the information of the consumer. This aspect of the guidelines is being violated with impunity, especially by many traditional healers, believing that such pieces of information constitute a trade secret that must be jealously guarded. This practice has effectively hindered the development of phyto-medicine in Africa. United States Embassy in Nigeria (2011) has listed malaria fever as a major public health problem in Nigeria. This disease is said to be responsible for over 70% of outpatient hospital visitation with its attendant toll on productivity and major source of discomfort and complications in children and pregnant women (Oyibo et al., 2008). Much as in orthodox medical practice, Fabeku and Akinsulire (2008) have pointed out the care of the blood as paramount to preventive and curative health care with respect to herbalism in southwestern Nigeria.

In recognition of the aforementioned facts on Nigerian health requirements and with regard to the guidelines provided by WHO on herbal drug standardization, Ogunkunle et al. (2014) have qualitatively and quantitatively defined the botanical constituents of the commonly used antimalarial and haematinic powdered herbal formulations in Ogbomoso southwestern Nigeria which they named Maloff-HB and Haematol-B, respectively (Table 1). However, the physico-chemical and phytochemical properties as well as the residual constituents of these drugs are yet to be documented. Therefore, the objective of the present study was to elucidate the ascorbic acid and mineral elements composition of Maloff-HB and Haematol-B as a contribution to the standardization of these herbal formulations with a view to examining them for general acceptability. Even though the direct link between the essential elemental composition of herbal drugs and their curative efficacy has not been established, the results of this study will be of immense importance in defining the nutritional status of Maloff-HB and Haematol-B. In addition, since the proportions of the various 'active ingredients' in the two herbal formulations are known, the outcome of the present study may be useful in deciding and managing the dose of each of the two drugs.

MATERIALS AND METHODS

Preparation of herbal formulations

Dried herbal materials representing nine plant species for Maloff-HB and 10 species for Haematol-B were procured from traditional

*Corresponding author. E-mail: osbello06@gmail.com.

Table 1. Botanical characterization and percentage composition (wt/wt) of antimalarial (Maloff-HB) and haematinic (Haematol-B) powdered herbal formulations from Ogbomoso Nigeria*.

Plant species	Family	Part used	Parts per 100 (wt/wt)	
			Maloff-HB	Haematol-B
<i>Enantia chlorantha</i> Oliv	Annonaceae	Stem bark	30.0	-
<i>Sorghum bicolor</i> Moench.	Poaceae	Leaf sheath	-	30.0
<i>Alstonia boonei</i> De Wild	Apocynaceae	Stem bark	20.0	-
<i>Hibiscus sabradiffa</i> L.(red variety).	Malvaceae	Fruit calyx	-	20.0
<i>Calliandra haematocephala</i> Hassk	Fabaceae	Root	10.0	-
<i>Mangifera indica</i> L.	Anacardiaceae	Stem bark	10.0	5.5
<i>Theobroma cacao</i> L.	Sterculiaceae	Stem bark	-	10.0
<i>Okoubaka aubrevillei</i> Phelleg et Nomand	Santalaceae	Stem bark	8.0	-
<i>Sarcocephalus latifolius</i> (J.E.Smith) E. A.Bruce	Rubiaceae	Root bark	8.0	5.5
<i>Aristolochia ringens</i> Vahl.	Aristolochiaceae	Roots	-	7.0
<i>Parquetina nigrescens</i> (Afz.) Bullock	Periplocaceae	Root bark	6.0	-
<i>Garcinia kola</i> Heckel	Guttiferae	Seed	-	5.5
<i>Khaya senegalensis</i> (Desr.) A. Juss.	Meliaceae	Stem bark	-	5.5
<i>Uvaria chamae</i> P. Beauv.	Annonaceae	Root bark	-	5.5
<i>Zanthoxylum zanthoxyloides</i> (Lam.) Zepern & TimLer	Rutaceae	Root bark	-	5.5
<i>Cassytha filiformis</i> L.	Lauraceae	Vines	4.0	-
<i>Pterocarpus osun</i> Craib.	Papilionaceae	Stem bark	4.0	-
Total			100.0	100.0

*Adapted from Ogunkunle et al. (2014); - = not applicable.

herbal practitioners in Ogbomoso, Nigeria in March 2012 (Table 1). The plant materials were chipped, cleaned and re-dried in the sun for five days and then, milled separately by passing them several times through a fabricated milling machine head (with sterile interiors) attached to a 2 HP Lister engine. Sieving of each fraction was carried out separately using a 450 micrometer sieve. 200 g of each of the two herbal remedies were formulated by measuring out a double portion of the quantity of each of the herbal constituents in Table 1 which were mixed for about 2 min in a sterilized electricity-operated kitchen blender (Figure 1). The powdered herbal formulations were stored as drug samples in two labeled bottles for subsequent analyses.

Determination of ascorbic acid content

One gram of each sample was accurately weighed into a 25 mL conical flask, 10 mL of oxalic acid (0.05 M) solution and the sample was placed under shade for 24 h for extraction of vitamin C contents. After 24 h, the samples were filtered through a 0.45 µm filter paper. Then 2.5 mL of each sample was transferred to a separate 25 mL volumetric brown flask containing 2.5 mL of oxalic acid (0.05 M) solution. 0.5 mL of metaphosphoric acid mixed with 0.5 mL of acetic acid, 1 mL of (5%v/v) sulphuric acid solution and 2 mL ammonium molybdate solution was added to each volumetric brown flask and made up to 25 mL mark with distilled water. Each sample was then analyzed for Vitamin C at a wavelength of 760 nm and compared with the standard (Anon, 1995-1996). For the sake of accuracy and precision, analysis of each herbal drug for its ascorbic acid content was replicated three times and the data were analyzed by means of descriptive statistics (means and standard deviations). The means of ascorbic acid contents in the two herbal formulations were also compared at $\alpha = 0.05$ using independent sample t-statistic.

Determination of mineral elements composition in herbal formulations

The formulations were dried in oven at 70°C for 24 h until the dry weight was constant. The dried formulations were then ground and passed through a 0.2 mm plastic sieve. Then, 0.5 g of each sample was wet digested with an Ultra-pure nitric acid (HNO₃) (10 mL) in a polyethylene test tube using a heating block digestion unit at 120°C. The final solution was filtered into a 50 mL volumetric flask through a 45-µm filter paper and diluted to the mark with deionized water. All reagents used in this study were of analytical grade. Mineral contents of the two herbal formulations were analyzed using atomic absorption-spectrophotometer (Perkin Elmer Model 3300). In order to ensure some level of precision, the two samples were analyzed for magnesium (Mg), calcium (Ca), manganese (Mn), iron (Fe), zinc (Zn), potassium (K) sodium (Na) and copper (Cu) in triplicate and each result obtained was analyzed by means of descriptive statistics. In order to ensure some level of precision, each result obtained was analyzed by means of descriptive statistics as in the ascorbic acid content. Also, variations in the ratios of some minerals (Na/K, Ca/K, Zn/Cu, Na/Mg, Ca/Mg, Fe/Cu and Fe/Zn) were computed in replicates of three and their means and standard deviations were determined. The mean values for all the mineral contents and ratios in the two herbal drugs were then compared at $\alpha = 0.05$ using independent sample t-statistic.

RESULTS AND DISCUSSION

Antioxidant potentials of the herbal formulations

The presence of free radicals or highly reactive oxygen species has been linked to many diseased conditions in humans because they are capable of inducing oxidative

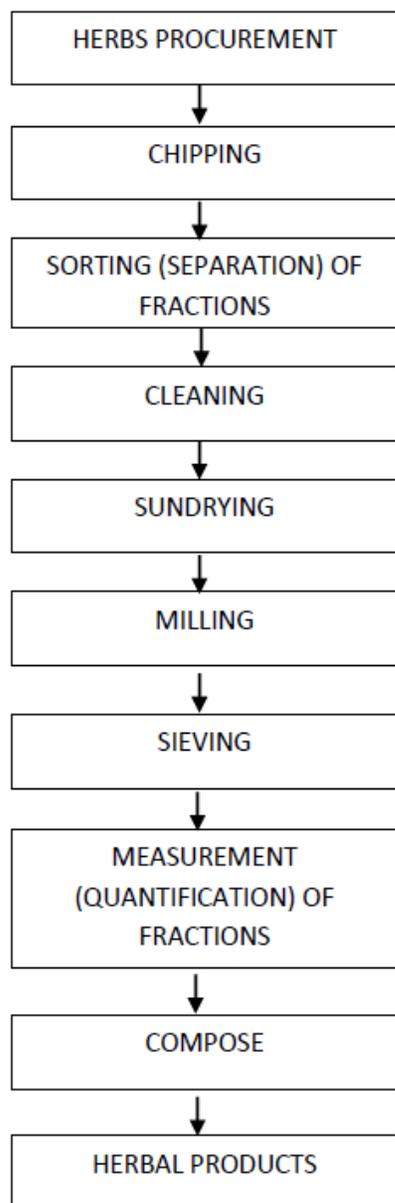


Figure 1. Flow chart of the steps followed in the manufacture of powdered antimalarial and haematinic herbal formulations used for the study (Option A of the manufacturing protocols reported in Ogunkunle et al., 2014).

damage to human body that invariably suppresses the body immune system (Lobo et al., 2010). Reduction in the risk of diseases is however ensured to some extent with the help of enzymes such as super-oxide dismutase and catalase; and through the activities of antioxidant compounds such as ascorbic acid (or vitamin C), tocopherol, phenolic acids, polyphenols, flavonoids and glutathione (Meena et al., 2012). Dietary antioxidants (in

the form of antioxidant supplements) have been in use for a long time against the damaging effects of free radicals but more attentions have continued to be focused on the use of antioxidants from natural sources as spices and herbs (Khalaf et al., 2008).

The mean values of ascorbic acid and mineral elements content of the two polyherbal formulations (PHFs) studied are shown in Table 2. In general, the standard deviation for the nine parameters studied were less than 10% of the means. The results show that the two formulations are rich in ascorbic acid, a demonstration that they might have good antioxidant activity and could be a good source of natural antioxidants. Ascorbic acid, being an important precursor of redox reactions is known to promote a healthy immune system. It is therefore an integral part of many drugs, for it enhances the quality and efficacy of the products (Joshi, 2012). The presence of high concentrations of ascorbic acid in the two PHFs (542.35 mg/100 g and 414.14 mg/100 g in Maloff-HB and Haematol-B respectively) might be responsible for their therapeutic effects and uses in the traditional system of medicine.

Mineral element composition of the herbal formulations

The herbal drugs studied are also rich in Mg and Ca (Table 2) and could be said to be potential sources of these two essential elements for proper functioning of the body. Although the direct link between the essential elements of herbal drugs and their curative capacity is yet to be established, awareness is on the increase, as regards the importance of dietary minerals in the prevention as well as in the cure of several diseases (Prasad, 1993). Minerals are also globally acknowledged to be of critical importance in human diets. Major minerals are those required in amounts greater than 100 mg per day (mpd) and they include calcium (Ca), phosphorus (P), magnesium (Mg), sulphur (S), potassium (K), chlorine (Cl) and sodium (Na). Trace minerals on the other hand are required in much smaller amounts (less than 100 mpd) and they include zinc (Zn), iron (Fe), silicon (Si), manganese (Mn), copper (Cu), fluorine (F), iodine (I) and chromium (Cr) (Imelouane et al., 2011).

Many secondary metabolites found in herbal drugs are known to make them to be medicinally potent for therapeutic purposes. In addition, it is now well established that many of the mineral elements earlier enumerated play vital roles in general well-being of humans who consume the plant material either as diet or as herbal product. These minerals are known to work in synergy with vitamins and a deficiency in some of them can lead to poor health and serious illnesses (Miller, 2012; Chandler, 2014; WebMD, 2005-2014). Using the entries in Table 3 as a reference, it can be observed that the composition of ascorbic acid (vitamin C), Ca and Mg

Table 2. Mean ascorbic acid content and mineral compositions in the powdered antimalarial (Maloff-HB) and haematinic (Haematol-B) herbal formulations from Ogbomosho, Nigeria.

Parameter	Mean (mg/100 g) \pm SD	
	Maloff-HB	Haematol-B
Ascorbic acid	542.350 ^b \pm 0.845	414.140 ^a \pm 2.498
Mg	1319.040 ^a \pm 4.401	2340.000 ^b \pm 2.205
Ca	784.310 ^a \pm 4.942	1011.670 ^b \pm 2.797
Mn	0.026 ^a \pm 0.002	0.052 ^b \pm 0.004
Fe	0.613 ^a \pm 0.004	0.977 ^b \pm 0.006
Zn	<0.001 ^a \pm 0.000	0.282 ^b \pm 0.004
K	2.459 ^a \pm 0.046	4.483 ^b \pm 0.003
Na	0.265 ^a \pm 0.008	0.608 ^b \pm 0.002
Cu	0.471 ^a \pm 0.001	0.486 ^b \pm 0.005

n, number of replicates of each parameter) = 3; mean values in a row with the same superscript are not significantly different ($P \geq 0.05$) while means in a row with different superscripts are significantly different ($P < 0.05$).

in the two PHFs (Table 2) can adequately be compared with the recommended dietary requirements for all age groups. Table 2 also indicates that Maloff-HB contained a significantly higher amount of ascorbic acid (542.35 mg/100 mg) than Haematol-B (414.14 mg/100 mg) while the amount of all the eight mineral elements studied was significantly higher in Haematol-B. The active ingredients in the two PHFs are yet to be identified, but within the context of the findings of this study, the therapeutic property of Maloff-HB as claimed by the traditional medical practitioners could be attributed mainly to its comparatively high ascorbic acid content while the blood-enriching function of Haematol-B could be due to its comparatively higher amount of mineral elements, some of which have been acknowledged as blood enhancers (Herber and Stoeppler, 1994; Miller, 2012; Chandler, 2014; WebMD, 2005-2014). Although the mean values of Mn, Fe, Zn, K, Na and Cu in Haematol-B (Table 2) did not come any close to the recommended values in Table 3, the results obtained in the present study are in fair conformity with those obtained by Lokhande et al. (2010) from the roots of *Withonia somnifera* and *Hemidesmus indicum* which are largely used as blood purifiers in India. However, the percentage of Fe, Zn, Na and K in Haematol-B are comparatively lower than those reported by Abolaji et al. (2007) in *Xylopiya aethiopica* (fruit), *Parinari polyandra* (fruit) and *Blighia sapida* (root) which are traditional herbs used by pregnant women in some parts of Southwestern Nigeria as blood purifiers and fertility enhancers.

The mineral ratios in a diet or an herb are frequently considered more important in defining its nutritional and/or possible therapeutic status than the individual mineral concentrations themselves. According to Watts (2010), these ratios, not only reveal the important balance between these elements, but also provide information regarding the many possible factors that may be represented by disruption of their relationships. A

proper interpretation of the relationships between minerals within the body can therefore provide information on the state of the health as well as enable a specific and targeted approach to therapy. On the strength of the above argument, the relationships between several elements in a medicinal herb can be said to be suggestive of synergistic or antagonistic effects, with the resultant provision of various elements to the body in bioavailable forms, and in a balanced manner with negligible harmful effects except for some environmental contaminants (Lokhande et al., 2010).

Inter-elemental correlations

Based on the foregoing, and the information provided in Table 4, it is arguable that the ratios of the mineral elements are an important factor for consideration in determining possible extent of bioavailability of the nutrients to the body. In recognition of this factor, it can be said that only Fe/Cu ratio in both Maloff-HB and Haematol-B and Fe/Zn ratio in Haematol-B appear to meet the acceptable dietary requirements (Table 4). By implication therefore, dietary iron availability appears to be guaranteed with the use of these herbal formulations even though the absolute quantities of Fe, Zn and Cu were insignificant (Table 2). The entries for Na/K, Ca/K, Na/Mg, Ca/Mg and Zn/Cu in Table 4, which fell short of the stated dietary requirements should however, not be interpreted to mean that the two herbal formulations are invalid with respect to the enumerated mineral nutrients. The fact is that, the reservoir of the various elements required in the human body at a given period is as a result of an additive process, such that an herb consumed or a meal taken can always add its piece of mineral constituents.

Bioavailability of a mineral nutrient is a condition that has a direct relationship with the quantity absorbed into the body of the consumer. The rate of mineral absorption

Table 3. Nutritional goals for age gender groups based on dietary reference intakes and dietary guidelines recommendations*.

Parameter	Source of goal	Amount of daily recommended dietary intakes													
		1-3 years		4-8 years		9-13 years		14-18 years		19-30 years		31-50 years		51 years and above	
		Child	F	M	F	M	F	M	F	M	F	M	F	M	
Ascorbic acid (mg)	RDA	15	25	25	45	45	65	75	75	90	75	90	75	90	
Calcium (mg)	RDA	700	1000	1000	1300	1300	1300	1300	1000	1000	1000	1000	1200	1200	
Iron (mg)	RDA	7	10	10	8	8	15	11	18	8	18	8	8	8	
Magnesium (mg)	RDA	80	130	130	240	240	360	410	310	400	320	420	320	420	
Phosphorous (mg)	RDA	460	500	500	1250	1250	1250	1250	700	700	700	700	700	700	
Potassium (mg)	AI	3000	3800	3800	4500	4500	4700	4700	4700	4700	4700	4700	4700	4700	
Sodium (mg)	UL	<1500	<1900	<1900	<2200	<2200	<2300	<2300	<2300	<2300	<2300	<2300	<2300	<2300	
Zinc (mg)	RDA	3	5	5	8	8	9	11	8	11	8	11	8	11	
Copper (mcg)	RDA	340	440	440	700	700	890	890	900	900	900	900	900	900	

*Source: USDA and HHS (2010); F, female; M, male; RDA, recommended dietary allowance; AI, adequate intake; UL, upper limit.

Table 4. Mean values of some mineral ratios in Maloff-Hb and Haematol-B, in comparison with recommended dietary ratios and ranges.

Mineral ratio	Mean ± SD		Ideal ratio	Acceptable range of ratio
	Maloff-HB	Haematol-B		
Na/K	0.108 ^a ± 0.005	0.135 ^b ± 0.001	[2.4:1]	[1.4 - 3.4]
Ca/K	319.013 ^b ± 4.892	225.667 ^a ± 3.861	[4.2:1]	[2.2 - 6.2]
Na/Mg	<0.001 ^a ± 0.000	<0.001 ^a ± 0.000	[4:1]	[2 - 6]
Ca/Mg	0.594 ^b ± 0.004	0.432 ^a ± 0.001	[7:1]	[3 - 11]
Zn/Cu	0.002 ^a ± 0.000	0.580 ^b ± 0.007	[8:1]	[4 - 12]
Fe/Cu	1.301 ^a ± 0.011	2.009 ^b ± 0.014	[0.9:1]	[0.2 - 1.6]
Fe/Zn	613 ^b ± 3.605	3.464 ^a ± 0.055	{< 2:1}	{0.67 - 3.33}

n, number of replicates of each computed ratio) = 3; mean values in a row with the same superscript are not significantly different (P>=0.05) while means in a row with different superscripts are significantly different (P<0.05); [] source = Watts (2010) and { } source = Solomons and Jacob (1981).

in turn, has been found to be dependent on additional factors other than the ratios of the mineral elements as earlier highlighted. A discussion of iron absorption is of particular interest in evaluating the qualities of Maloff-HB and Haematol-B with the understanding that both

drugs should have a direct bearing to blood enhancement and body immunity boosting. Iron absorption refers to the amount of dietary iron that the body obtains and uses from food or herb. Healthy adults absorb only about 20% of dietary iron (NIH, 2007) but absorption by individuals is

influenced by several factors which can be grouped into three: dietary status of the body (form and storage level of iron); amount of iron absorption enhancers; and quantity of iron absorption inhibitors (phytic acid) (NIH, 2007; Dewar, 2009).

Potential for iron absorption enhancement

Ascorbic acid has long been recognized as a versatile enhancer of dietary iron absorption in the body system (Hallberg, 1981) and, using a multiple regression analysis, this antioxidant has been reported as a biochemical predictor of iron absorption ($P = 0.0441$) along with the content of animal tissue ($P = 0.0001$) and phytic acid ($P = 0.0001$) by Reddy et al. (2000). With this background information, it can be said that the two herbal drugs studied are potential sources of essential mineral elements with the compositions that fairly agree with the recommended dietary intake of all ages (Table 3). Even though the observed quantities and or derived ratios of the elements in the drugs did not agree perfectly with the recommended values, the high amount of ascorbic acid confers on them the potential to enhance iron absorption in the body, even from other sources. In a related study, Hussain et al. (2011) reported high amounts of this iron absorption enhancer in some selected medicinal plants.

Conclusion and recommendations

Based on the results of this study, it can be concluded that Maloff-HB and Haematol-B are good reservoirs of ascorbic acid. This antioxidant has been found in many redox reactions as promoters of good health. The primary functions of these polyherbal formulations as drugs can therefore be traced to the good supply of this antioxidant. The two herbal formulations are also rich in Ca and Mg and their Fe/Cu and Fe/Zn ratios are such that can promote bioavailability of these important mineral elements. Hence, these drugs have the potential to meet some dietary requirements which will promote good quality blood that prevents infections. Further explanations of the basis for using the formulations can be sought through a study on their biochemistry. Their consumption rate should however be under some caution because there is lack of empirical information on their safety and efficacy, more so, the present study did not evaluate the drugs for their residual constituents (heavy metals, pesticides, toxins, etc). In order to confirm the beneficial effects of the two herbal drugs, it is important to carry out studies on the *in vivo* activity and bioavailability of their constituents.

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Conflict of Interests

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

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Full Length Research Paper

Antidiabetic effect of aqueous extract of *Basella alba* leaves and metformin in alloxan-induced diabetic albino rats

Bamidele, O.^{1*}, Arokoyo, D. S.¹, Akinnuga, A. M.² and Oluwarole, A. O.¹

¹Department of Physiology, Bowen University, Iwo, Osun State, Nigeria.

²Department of Physiology, Cross River University of Technology, Cross River, Nigeria.

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The present study was carried out to evaluate the anti-diabetic effect of aqueous extract of *Basella alba* leaves in alloxan-induced diabetic albino rats. Thirty (30) male albino rats weighing 100 to 150 g were used for this work. The rats were randomly distributed into five groups containing six (6) rats per group. Group I rats served as the healthy control group and were neither induced with diabetes nor given any treatment. Group II rats served as the diabetic control. They were induced with diabetes but not given any treatment throughout the experiment. Rats in group III and IV were induced with diabetes and administered *B. alba* leaf extract at different doses, 100 and 200 mg/kg, respectively. Group V rats were also induced with diabetes and treated with Metformin at a dose of 100 mg/kg body weight. The various treatments were administered orally for a period of three weeks. The mean fasting blood glucose (FBG) of the rats was determined weekly using a glucometer. The rats treated with *B. alba* at doses of 100 and 200 mg/kg had their mean FBG levels significantly lower than the diabetic control group ($p < 0.05$). Similarly, there was significant difference between rats in the group treated with Metformin and the diabetic control group ($p < 0.05$). The mean FBG levels of rats treated with 200 mg/kg of *B. alba* leaf extract was not statistically different from that recorded in rats treated with Metformin. The results show that *B. alba* leaf extract has antidiabetic effect in alloxan-induced diabetic rats, varying with the quantity ingested. Hence, consumption of *B. alba* leaf as a vegetable should be encouraged for the treatment of diabetes mellitus.

Key words: *Basella alba*, diabetes mellitus, fasting blood glucose, metformin, hypoglycemia.

INTRODUCTION

Diabetes mellitus is a major worldwide health problem involving endocrine pancreas. It is implicated in oxidative stress which induces insulin resistance in the peripheral tissue and impairs insulin secretion from pancreatic β -cells (Ceriello and Mortz, 2004; Wang et al., 2006). It is a major cause of adult blindness, kidney failure, neuro-

pathy, heart attack and strokes. It is also characterized by excessive disturbance of carbohydrates, proteins and lipid metabolism, thickening of capillary basement membrane throughout the body leading to microangiopathy, macroangiopathy and long term complications which affect eyes, kidneys, nervous system and circulatory

*Corresponding author. E-mail: femi_bayo_is@yahoo.co.uk. Tel: +2348067463528.

system (Dhasarathan et al., 2011). Due to increasing obesity and altered dietary habits in both western and developing countries, the prevalence of type 2 diabetes is growing at an exponential rate (Zimmet and Lefebvre, 1996). It has become a major menace in the last 10 years. In 2004, according to the World Health Organization (WHO) more than 150 million people worldwide suffer from diabetes. The WHO has predicted that the major burden will occur in developing countries. Life expectancy may be halved by diabetes mellitus especially in developing countries where its prevalence is increasing and adequate treatment is often unavailable (Dhasarathan et al., 2011).

Plants have been the major source of drugs for the treatment of diabetes mellitus in some countries of the world like India and China (Dhasarathan et al., 2011). The importance of antidiabetic plants in the development of economic and effective treatment for diabetes currently estimated to affect over 30 million people worldwide has been recognized by the World Health Organization (WHO Technical Report Series, 1985). Most of the anti-diabetic plants have been found to contain substance like glycosides, alkaloids, terpenoids, flavonoids and so on (Loew and Kaszkin, 2002). The ethnobotanical information reports stated that about 800 plants may possess anti-diabetic potential (Aquilara et al., 1998). Among the plants reported, *Basella alba* may be included in that it contains most of the substances possessed by the already discovered anti-diabetic plants but not yet scientifically proven to possess the potential. *B. alba* (family *Basellaceae*), is a fast growing vegetable, native to tropical Asia, probably originating from India and Indonesia and extremely heat tolerant areas (Grubben and Denton, 2004). It is grown throughout the tropics as a perennial plant and in warmer temperate regions as an annual crop. Its thick semi-succulent heart-shaped leaves have a mild flavour and mucilaginous texture. It is commonly known as Malabar, Ceylon, East-Indian, Surinam and Chinese Spinach (Facciola, 1990). It is known to be high in vitamin A, vitamin C, vitamin B9 (Folic acid), Calcium, Magnesium and several vital antioxidants. It is low in calories by volume and high in protein per calories (Duke and Ayensu, 1985).

The flowers are used as an antidote for poisons (Duke and Ayensu, 1985). It is also a safe aperient for pregnant women and its decoction has been used to alleviate labour (Duke and Ayensu, 1985). The aqueous extract of *B. alba* leaves has been reported to reduce anaemia and maintain good health (Bamidele et al., 2010). *B. alba* traditionally claimed to increase libido (Kuate and Efferth, 2010). A recent literature survey revealed that the plant extract possess analgesic, (Anandarajagopal et al., 2011) anti-inflammatory activities (Chaitanya, 2012), microbial activities (Oyewole and Kalejaiye, 2012), gastro protective action (Kumar et al., 2012) and CNS depressant activity (Anandarajagopal et al., 2011). Species of the plant called *Basella rubra* has been scientifically investigated to possess hypoglycemic activity

in streptozotocin-induced diabetes in rats (Nirmala et al., 2009). Aside this, information about the anti-diabetic effects of *B. alba* is scanty. In the light of this, this work was carried out to evaluate the anti-diabetic effect of aqueous extract of *B. alba* leaves in alloxan-induced diabetic albino rats.

MATERIALS AND METHODS

Animals

A total of 30 male Wistar albino rats with weight range of 100 to 150 g purchased from the Department of Biochemistry, Bowen University, Iwo, Osun State, Nigeria were used in this study. The animals were kept in cages under standard conditions (temperature, 25±2°C, 12 h light and 12 h dark cycle) in the animal house of the Physiology Department, Faculty of Basic medical science, Bowen University, Iwo, Osun State, Nigeria. All animals were fed with commercially formulated rat feed and water *ad libitum*.

After randomization into various groups, the rats were acclimatized for a period of 2 weeks in the environment before the initiation of the experiment. Their cages were cleaned of waste daily. All procedures involving the use of animals in this study complied with the guiding principles for research involving animals as recommended by the declaration of Helsinki and the Guiding principles in the care and use of animals (World Medical Association, 2002).

Plant materials

The fresh leaves of *B. alba* (Indian spinach) were procured from Odori market in Iwo, Osun state, Nigeria. The plant materials were identified and authenticated by the Chief Herbarium Officer of the Department of Biological Sciences, Bowen University, Iwo, Osun state, Nigeria. The leaves were washed in tap water and shade-dried after which they were reduced into fine powder by grinding. 100 g of the powdered leaves was stirred into 1000 ml of boiling distilled water. Boiling was allowed to continue for 5 min. The mixture was kept off the hot plate, for 30 min to allow it to infuse. It was then filtered using cheese cloth. The filtrate was then concentrated by evaporation to dryness using a water bath to obtain the solid mass. The extract was then dissolved in normal saline and used for the study.

Preparation of standard drug (metformin)

Five grams of metformin was obtained from the Pharmacy Unit of Bowen University clinic, Iwo, Osun state, Nigeria. The tablets were then grounded to fine powder. The powder was then dissolved in 25.70 ml of distilled water to get a stock solution of 19.20 g/dl.

Induction of diabetes mellitus

The animals were weighed and injected via intraperitoneal route; 100 mg/kg of alloxan dissolved in distilled water (Carvalho et al., 2003) using insulin syringes. Diabetes mellitus was confirmed after 72 h of alloxan injection by testing the fasting blood glucose levels in the blood obtained from the tail vein of the animals using glucometer and glucose test strip. The result of blood glucose measurement by glucometer correlates excellently well with the result obtained from standard laboratory methods (Ajala et al., 2003). The accuracy of the test result was confirmed by the use of glucose test kit.

Table 1. The fasting blood glucose levels in different experimental groups of rats (n=5).

Treatment mode	Group I (healthy control)	Group II (diabetic control)	Group III (DM + <i>B. alba</i> 100 mg/kg)	Group IV (DM + <i>B. alba</i> 200 mg/kg)	Group V (DM + metformin)
Baseline	76.2 ± 3.20	65.2 ± 5.48	60.2 ± 4.83	77.0 ± 3.54	63.5 ± 3.27
Week 0	75.7 ± 4.13	336.7 ± 4.79*	310.5 ± 5.36*	371.0 ± 7.28*	368.2 ± 1.03*
Week 1	60.0 ± 5.12	396.0 ± 5.29*	218.0 ± 4.65 ^{ax}	177.4 ± 3.76 ^{ax}	138.4 ± 1.59 ^{ax}
Week 2	69.5 ± 4.58	392.0 ± 5.22*	228.0 ± 4.40 ^{ax}	134.8 ± 2.48 ^{ax}	109.6 ± 9.42 ^a
Week 3	80.2 ± 4.41	380.2 ± 5.05*	232.8 ± 5.22 ^{ax}	127.9 ± 5.78 ^a	100.2 ± 5.51 ^a

Experimental design

Thirty (30) wistar rats were grouped into five (5) different groups containing six rats per group. Each group was kept in different cages. The grouping was done as follows: group I, healthy control (HC): they were neither induced with diabetes nor given any treatment throughout the experiment; group ii, diabetic control (DC): they were induced with diabetes but not given any form of treatment throughout the experiment; group III (DM+low dose of *B. alba*): the rats were induced with diabetes and treated with *B. alba* aqueous leaf extract at a low dose of 100 mg/kg; group IV (DM+high dose of *Basella alba*): the rats were induced with diabetes and treated with *B. alba* leaf extract at a high dose of 200 mg/kg; Group V (DM+metformin): the rats were induced with diabetes and treated with a standard drug (metformin) at a dose of 100 mg/kg.

Administration of drugs

Both metformin and *B. alba* were administered via the oral route with the aid of an oropharyngeal canular. The rats were handled appropriately to restrict movement and prevent trauma to the rats during drug administration. The drugs were administered for a period of three weeks.

Measurement of fasting blood glucose level

Baseline fasting blood sugar was recorded after the two weeks of acclimatization in all rats and after diabetes mellitus has been induced in the test groups. The fasting blood glucose (FBG) levels were measured using glucometer by obtaining blood samples from the tail vein and recorded weekly in all the groups.

Statistical analysis

The results were tabulated as mean ± Standard Error of Mean (SEM). The one way ANOVA was used to analyze the data, followed by a post-hoc test (LSD). The results are considered significant at $p < 0.05$.

RESULTS

Effect of *basella alba* leaf extract and metformin on fasting blood glucose in alloxan-induced diabetic rats

The results of this experiment are shown in Table 1. Diabetes mellitus was induced at the beginning of week 0, which represents the values after induction. Rats with

FBG level above 200 mg/dl were considered as having diabetes mellitus (Carvalho et al., 2003). The results show that normal rat chow has no effect on fasting blood glucose in non-diabetic healthy (Group I) and diabetic (Group II) control rats. There was sustained hyperglycaemia in diabetic control (group II) throughout the experiment.

The mean FBG levels in group II, III, IV and V were significantly higher than that of group I (healthy control group) ($p < 0.05$) during week 0 and week 1. There were also increases in mean FBG levels in groups II, III, and IV (392.0.0±5.22 mg/dl, 228.0±4.40 mg/dl and 134.8±2.48 mg/dl) compared to healthy control group (59.5±4.58 mg/dl) in week 2 and this is statistically significant ($p < 0.05$).

Group V showed no statistical difference when compared to the healthy control group in week 2. FBG in groups II and III (380.2±5.05 and 232.8±5.22 mg/dl) were significantly higher ($p < 0.05$) than the healthy control group (80.2±4.41 mg/dl) while there was no statistical increase in group IV and V in week 3.

As depicted in Table 1, FBG levels in Group III, IV and V were significantly decreased ($p < 0.05$) when compared to group II (diabetic control). The decreases lasted throughout the treatment period. There were no statistical differences between Group IV and V while there were statistical differences ($p < 0.05$) between group III and V from weeks 1 to 3.

DISCUSSION

The anti-diabetic effects of aqueous leaf extract of *B. alba* and Metformin on alloxan-induced diabetes mellitus was examined in this study. The healthy control group has normal fasting blood glucose level. Therefore, this showed that normal rat chow has no effect on the fasting blood glucose levels. The increases in FBG levels above 200 mg/dl in groups II, III, IV and V, which were significant when compared to healthy control group confirmed induction of diabetes mellitus (Carvalho et al., 2003) in the test rats. Diabetic rats treated with *B. alba* aqueous leaf extract significantly had lower FBG levels when compared to the diabetic control group. The anti-hyperglycaemic effect of *B. alba* was noticed at two

different levels, for two different doses. It was observed that *B. alba* leaf extract had a more pronounced effect at a high dose than when administered at a low dose. The decrease in FBG levels in diabetic rats treated with *B. alba* may be due to the regeneration of beta cells of the pancreas by the presence of anti-oxidants (Duke and Ayensu, 1985; Nirmala et al., 2009; Olajire and Azeez, 2011) in the plant which are known to scavenge the free radicals produced by oxidative damage in the disease state (Olmendilla et al., 1997; Bamidele et al., 2010; Nirmala et al., 2011). Other reasons which may account for the reduction in the FBG levels observed may possibly include: inhibition of glucose absorption, increase sensitivity of receptors to insulin and stimulation of peripheral glucose uptake. Although, the present findings suggest the presence of anti-diabetic compound(s) in the leaf extract of *B. alba*, the exact mechanism of this effect is still speculative and requires further studies for clear understanding. The reduction in the FBG levels observed in the present work had been also reported by Nirmala et al. (2009) in another species of the plant (*B. rubra*).

The result of this study shows that three weeks of treatment is not enough time for treating diabetes mellitus if *B. alba* aqueous leaf extract is administered at a low dose of 100 mg/kg but if the dose is increased as high as 200 mg/kg, three weeks may be enough to treat diabetic rats and return the FBG levels near normal level.

Metformin however, had the greatest reduction effect on FBG level. Evident drop in FBG levels were noticed in the diabetic rats treated with Metformin from the first week to the third week of treatment. The FBG levels in Metformin-treated diabetic rats nearly appeared the same with the values obtained in the healthy control rats. The reduction in FBG levels in metformin-treated diabetic rats observed was similar to the earlier reports (Stalin et al., 2012; Shareef et al., 2013). Metformin at 100 mg/kg per body weight slightly reduced FBG levels than *B. alba* at 200 mg/kg per body weight in diabetic rats. This suggested that at high dose (200 mg/kg) used in this study, *B. alba* appears to have effect similar to metformin.

In conclusion, based on the results of this current work, aqueous leaf extract of *B. alba* has anti-diabetic effect in alloxan-induced diabetic rats. At a dose of 200 mg/kg, the anti-diabetic effect of aqueous leaf extract of *B. alba* is comparable to that of metformin-treated diabetic rats. Thus, *B. alba* leaf consumption as a vegetable should be encouraged to manage or treat diabetes mellitus.

Conflict of Interests

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

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