

The background of the cover features three vertical test tubes filled with a vibrant green liquid, likely a microbial culture. The tubes are set against a dark, slightly blurred background, and the overall image has rounded corners.

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León Irapuato,
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Isfahan
Iran*

Dr. Beatrice Kilel

*P.O Box 1413
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USA*

Prof. H. Sunny Sun

*Institute of Molecular Medicine
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1 University road Tainan 70101,
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Ago-Iwoye.
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*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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*DoD Biotechnology High Performance Computing
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Review

X-Chromosome short tandem repeat, advantages and typing technology review

Imad Hadi Hameed^{1*}, Muhanned Abdulhasan Kareem² and Rafid H.H³

¹Department of Molecular Biology, Babylon University, Hilla City, Iraq.

²Babylon University, Centre of Environmental Research, Iraq.

³Ministry of Health of Iraq, Iraq.

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Microsatellites of the X-chromosome have been increasingly studied in recent years as a useful tool in forensic analysis. This review describes some details of X-chromosomal short tandem repeat (STR) analysis. Among them are: microsatellites, amplification using polymerase chain reaction (PCR) of STRs, PCR product evaluation, PCR product purification, separation and detection, data analysis of STR by Identity Software, locus information and allele frequencies for X-chromosomal STR in different populations (DXS101, DXS7423, DXS8377, DXS6789, DXS6807 genetic loci) and advantages of X-chromosomal microsatellites. In forensic casework and DNA databases, polymorphism in STR is important as a forensic genetic marker.

Key words: Short tandem repeat (STR) loci, forensic, interpretation.

INTRODUCTION

The human X chromosome (ChrX) has so far played a minor role in forensic and in population studies, but the application of ChrX markers in these fields is now accomplishing detailed attention and new doors are opening in population and forensic research (Szibor et al., 2003).

Microsatellites are DNA regions with repeat units that are 2 to 7 bp in length or most generally short tandem repeats (STRs) or simple sequence repeats (SSRs) (Ellegren et al., 2003). The classification of the DNA sequences is determined by the length of the core repeat unit and the number of adjacent repeat units. It may contain several hundred to thousands (Butler, 2012) of these. Tandem repeats occur in the form of iterations of repeat units of almost anything from a single base pair to thousands of

base pairs. Mono-, di-, tri- and tetranucleotide repeats are the main types of microsatellite, but repeats of five (penta-) or six (hexa-) nucleotides are usually classified as microsatellites as well.

DNA can be used to study human evolution. Besides, information from DNA typing is important for medico-legal matters with polymorphisms leading to more biological studies (Walkinshaw et al., 1996). Since the STR markers are important for human identification purposes (Rui et al., 2009) the number of repeats can be highly variable among individuals and can be used for identification purposes. There are three types of repeat patterns for STRs. Two or more adjacent simple repeats are considered as compound repeat. Units of similar length are called

*Corresponding author. E-mail: imad_dna@yahoo.com. Tel: 006-017-3642869.

simple repeats (Budowle, 1995; Butler et al., 2009).

Chromosome X short tandem repeats (X-STRs) analysis has recently attracted attention of the forensic community because of its usefulness in complex kinship testing. It is worth-while including X-STRs with autosomal markers for the cases when father/daughter relationships are to be tested. Analysis of ChrX short tandem repeat markers (STRs) can successfully embrace the answer that unravels the challenge presented in particular cases of kinship analysis, when the offspring is female (Szibor et al., 2003). Since fathers transmit the same X chromosome to all their daughters, they are particularly useful in deficiency paternity cases when the child is a female, in maternity testing, and in paternity cases involving blood relatives (Desmarais et al., 1998; Tun et al., 1999; Zarrabeitia et al., 2000; Szibor et al., 2003; Jia et al., 2004; Lee et al., 2004; Imad et al., 2014a, b). Forensic X-STR markers, as per size of amplicons are just like autosomal and Y chromosomal STRs (Drabek et al., 2004; Shin et al., 2005; Gomes et al., 2007; Hill et al., 2008; Diegoli and Coble, 2011).

AMPLIFICATION USING POLYMERASE CHAIN REACTION OF SHORT TANDEM REPEATS

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

Polymerase chain reaction product evaluation

For DNA amplification product evaluation in order to confirm whether the mtDNA PCR product is amplified, we

will need to examine the products of the amplification. To do this, a process called gel electrophoresis is applied. Electric current forces the migration of DNA fragments through a special gel material in this process. Since it is negatively charged, the DNA will move to the positive electrode in the electric field. The electric forces cause the longer portions of the DNA to move slower than the shorter ones (John et al., 2006; Imad et al., 2014c, d).

Polymerase chain reaction product purification

Using specialized binding buffers, EZ-10 spin column purification kits uses a silica gel membrane that selectively absorbs up to 10 µg of DNA fragments. Washed away nucleotides, oligos (<40-mer), enzymes, mineral oil and other impurities do not bind to the membrane. The DNA fragments can then be extracted from the column in small volumes. In downstream applications it can be re-used without further processing.

Separation and detection

After PCR there must be a process of separation and detection of the amplified products. A number of ways can be used to carry out the typing. Among them are:

1. Polyacrylamide gel electrophoresis (PAGE) followed by silver staining or if the primers are fluorescently tagged, detection by fluorescent gel scanners and,
2. Capillary Electrophoresis (CE) with laser induced fluorescence. Because it is automated, this method has become popular. No gel is used and samples can be inserted mechanically.

The resolution of the higher molecular weight loci is usually better than in the PAGE methods since the DNA traverses the entire length of the capillary, the resolution. There are several other components that impact DNA separations within CE systems other than the width of the sample injection zone. Among these are the polymer used for enabling the separation, the capillary, the electrophoresis buffer, and the field strength (John et al., 2004). The object of the exercise is to introduce a different dye onto the 5'-(nonreactive) end of each primer or set of primers (Giusti and Adriano, 1993). The properties of these dyes are quite unusual. Although fluoresce in different regions of the spectra they are all excited by a single argon-ion laser tuned to 488 nm. To determine which dye is present, based on the emission of each fragment as it passes the detector window, a multi wavelength analyzer, such as a charged coupled device (CCD) camera, can then be used. The advantage of this method is it allows the analysis of fragments of DNA that overlap in size as long as they are labeled with different colors, which fluoresce at different wavelengths.

The STR fragments in the sample are amplified using primers with fluorescent tags in the most commonly used

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

DATA ANALYSIS OF STR BY IDENTITY SOFTWARE

GeneScan software

Migration of ssDNA fragments ranging from 20 to 1200 base pairs is sigmoidal and not linear over the entire fragment size range, and carries out three main roles, which are: specifies peaks based on threshold values determined by the user; it differentiates the peaks according to dye color based on a matrix file and it determines the sizes the STR allele peaks based on an internal size standard. It is labeled with a different colored dye that is run for different samples. Because of the different conformations of small and large DNA fragments during migration it is difficult to acquire accurate size calls for data over 800 bps. There is a limited linear range when separating fragments ranging in size from 20-80, 80-900 and over 1000 bps because of the differences in size and shape. It is a normal practice to use different sizing algorithms for size calling. The local Southern are for large fragments and cubic spline and third order least squares are for middle fragments. These methods are all local and do not affect the results beyond the last fragment. Current sizing algorithms limit the analysis of large fragment data.

Genotyper software program

Genotyper software program converts the sized peaks into genotype calls that is (ABI PRISM Genotyper 2.5 Software User’s Manual, P/N 904648, Foster City, CA 2001) takes GeneScan data by comparison of allele sizes in an allelic ladder to the sample alleles to designate its

number. In order to make the allele calls from the allelic ladders the manufacturer of a particular STR kit normally provides Genotyper macros. These macros can be made to eliminate stutter peaks (Walsh et al., 1996) that may affect sample interpretation.

GeneMapper

The GeneMapper Software includes an Autoanalysis feature that can eliminate most of the tasks leading up to the analysis of a microsatellite project. A collection of user-defined settings (including an analysis method, size standard, and panel) that determine the sizing and genotyping algorithms is used by the GeneMapper® Software to analyze all sample files in a project.

GeneMarker (®) HID software program

A software tool for the analysis of forensic STR data and as a resource for analysis of custom STR multiplexes is GeneMarker(®) HID. It is easy to be studied and applied. It has features that may curtail user fatigue. STR data from both single-source and mixture profiles were analyzed and compared to profiles interpreted with another software package, to prove its superiority. It shows a 100% concordance for a total of 1898 STR profiles representing 28,470 loci and more than 42,000 alleles. Data obtained from a custom STR multiplex, with simplified and rapid implementation was successfully analyzed using GeneMarker HID. Finally, a time scale study is used to study the impact of the user-friendly design features of the software. It is found that using these software laboratories can save the time required for data analysis by at least 25% by using this software.

LOCUS INFORMATION AND ALLELE FREQUENCIES FOR X-CHROMOSOMAL STR IN DIFFERENT POPULATION

Allele frequencies of the population samples are shown in Tables 1 to 5, respectively. Detailed data are available by the authors.

DXS101 genetic loci

DXS101 genetic loci are highly polymorphic nucleotide. It possesses over 20 different alleles ranging from 12 to 32 repeats in length with repeat sequence - (CTT)_x-(ATT)_y- A number of same-size, different-sequence alleles have been identified through sequence analysis. Allele frequencies for the DXS101 genetic loci of ten population samples were found in this review (Jeanett et al., 2001; Edelmann et al., 2004; Coletti et al., 2006; Zarrabeitia et al., 2006; Martins et al., 2008; Yasutaka and Kiyoshi., 2010;

Table 1. Allele frequencies for the DXS101 genetic loci of ten population samples.

Allele	Ireland ^[1]	Italia ^[2]	German ^[1]	German ^[3]	Spain ^[4]	Peru ^[1]	Brazil ^[5]	Ethiopia ^[1]	Pakistan ^[6]	Japan ^[7]
12	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.008	0.000	0.000
15	0.052	0.053	0.044	0.044	0.000	0.017	0.016	0.008	0.000	0.000
16	0.000	0.000	0.005	0.005	0.000	0.000	0.008	0.024	0.000	0.000
17	0.000	0.019	0.002	0.002	0.011	0.000	0.008	0.008	0.000	0.000
18	0.073	0.178	0.084	0.085	0.046	0.008	0.055	0.098	0.000	0.000
19	0.052	0.046	0.047	0.047	0.032	0.017	0.055	0.024	0.000	0.000
20	0.021	0.033	0.012	0.012	0.000	0.025	0.031	0.024	0.000	0.000
21	0.062	0.026	0.032	0.032	0.032	0.000	0.055	0.049	0.022	0.008
22	0.010	0.033	0.022	0.022	0.096	0.008	0.023	0.073	0.033	0.041
23	0.083	0.046	0.066	0.066	0.222	0.068	0.070	0.065	0.280	0.131
24	0.115	0.177	0.213	0.212	0.170	0.305	0.289	0.122	0.264	0.305
25	0.208	0.132	0.156	0.156	0.149	0.203	0.133	0.114	0.203	0.198
26	0.146	0.092	0.114	0.114	0.117	0.220	0.156	0.171	0.071	0.179
27	0.062	9.098	0.079	0.079	0.053	0.085	0.047	0.089	0.066	0.087
28	0.062	0.033	0.070	0.070	0.021	0.025	0.039	0.081	0.044	0.033
29	0.042	0.026	0.027	0.027	0.000	0.008	0.016	0.032	0.016	0.014
30	0.010	0.007	0.024	0.024	0.000	0.008	0.000	0.008	0.000	0.003
31	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
32	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000

[1] (Edelmann et al., 2004); [2] (Coletti et al., 2006); [3] (Jeanett et al., 2001); [4] (Zarrabeitia et al., 2006); [5] (Martins et al., 2008); [6] (Muhammad et al., 2012); [7] (Yasutaka and Kiyoshi., 2010).

Table 2. Allele frequencies for the DXS7424 genetic loci of seven population samples.

Allele	Ireland ^[1]	German ^[2]	German ^[1]	Peru ^[1]	Brazil ^[3]	Ethiopia ^[1]	Japan ^[4]
8	0.000	0.001	0.000	0.000	0.000	0.000	0.000
10	0.000	0.004	0.003	0.017	0.008	0.024	0.000
11	0.010	0.006	0.004	0.000	0.000	0.032	0.000
12	0.051	0.039	0.035	0.008	0.023	0.041	0.025
13	0.031	0.072	0.069	0.042	0.094	0.024	0.029
14	0.143	0.219	0.220	0.229	0.164	0.268	0.168
15	0.388	0.303	0.291	0.297	0.281	0.252	0.288
16	0.255	0.224	0.240	0.297	0.313	0.293	0.445
17	0.082	0.098	0.104	0.085	0.102	0.049	0.037
18	0.041	0.020	0.021	0.017	0.008	0.016	0.011
19	0.000	0.013	0.011	0.008	0.000	0.000	0.000
20	0.000	0.001	0.000	0.000	0.008	0.000	0.000

[1] (Edelmann et al., 2004); [2] (Jeanett et al., 2001); [3] (Martins et al., 2008); [4] (Yasutaka and Kiyoshi, 2010).

Muhammad et al., 2012) (Table 1).

review (Jeanett et al., 2001; Edelmann et al., 2004; Martins et al., 2008; Yasutaka and Kiyoshi, 2010) (Table 2).

DXS7423 genetic loci

DXS7423 possesses over 12 different alleles ranging from 8 to 20 repeats in length with repeat sequence – (TCCA) x –. Allele frequencies for the DXS7423 genetic loci of seven population samples were found in this

DXS8377 genetic loci

A DXS8377 genetic locus is a highly polymorphic nucleotide. It possesses over 24 different alleles ranging from 37 to 60 repeats in length with repeat sequence

Table 3. Allele frequencies for the DXS8377 genetic loci of eight population samples.

Allele	Ireland ^[1]	Italia ^[2]	German ^[3]	German ^[1]	Spain ^[4]	Peru ^[1]	Ethiopia ^[1]	Japan ^[5]
37	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.000
38	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000
39	0.000	0.000	0.007	0.024	0.000	0.000	0.000	0.000
40	0.000	0.007	0.010	0.031	0.011	0.000	0.000	0.000
41	0.033	0.019	0.024	0.044	0.021	0.000	0.008	0.001
42	0.022	0.019	0.031	0.043	0.043	0.000	0.016	0.009
43	0.088	0.053	0.044	0.050	0.053	0.097	0.032	0.031
44	0.055	0.086	0.043	0.069	0.064	0.193	0.081	0.043
45	0.022	0.039	0.050	0.092	0.000	0.150	0.057	0.058
46	0.055	0.079	0.069	0.110	0.096	0.000	0.081	0.105
47	0.176	0.106	0.092	0.113	0.074	0.161	0.073	0.135
48	0.143	0.118	0.110	0.127	0.096	0.086	0.106	0.108
49	0.132	0.118	0.110	0.082	0.096	0.140	0.163	0.158
50	0.077	0.099	0.127	0.063	0.096	0.032	0.073	0.103
51	0.044	0.099	0.082	0.060	0.085	0.054	0.073	0.085
52	0.033	0.046	0.063	0.040	0.064	0.032	0.106	0.55
53	0.022	0.053	0.060	0.014	0.096	0.021	0.073	0.043
54	0.055	0.013	0.040	0.014	0.032	0.021	0.032	0.024
55	0.022	0.026	0.014	0.005	0.021	0.000	0.008	0.240
56	0.022	0.019	0.014	0.001	0.021	0.000	0.000	0.010
57	0.000	0.000	0.005	0.001	0.032	0.011	0.008	0.004
58	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.003
59	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
60	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000

[1] (Edelmanna et al., 2004); [2] (Coletti et al., 2006); [3] (Jeanett et al., 2001); [4] (Zarrabeitia et al., 2006); [5] (Yasutaka and Kiyoshi., 2010).

(GAA)_x–(GAG–GAA)_y–(GAA)₂–GAG–(GAA)₆–GAC. A number of same-size, different-sequence alleles have been identified through sequence analysis. Allele frequencies for the DXS8377 genetic loci of eight population samples were found in this review (Jeanett et al., 2001; Edelmanna et al., 2004; Coletti et al., 2006; Zarrabeitia et al., 2006; Yasutaka and Kiyoshi, 2010) (Table 3).

DXS6789 genetic loci

DXS6789 possesses over 13 different alleles ranging from 13 to 25 repeats in length with repeat sequence – TATC–(TATG)_x–(TATC)_y. Allele frequencies for the DXS6789 genetic loci of nine population samples were found in this review (Jeanett et al., 2001; Edelmanna et al., 2004; Lv, et al., 2004; Coletti et al., 2006; Yasutaka and Kiyoshi, 2010; Muhammad et al., 2012) (Table 4).

DXS6807 genetic loci

DXS6807 possesses over 8 different alleles ranging from 10 to 17 repeats in length with repeat sequence -

(GATA)_{N7}–(GATA)₂–GAC–(GATA)_x–TGT–. Allele frequencies for the DXS6789 genetic loci of seven population samples were found in this review (Jeanett et al., 2001; Edelmanna et al., 2004; Coletti et al., 2006; Yasutaka and Kiyoshi, 2010) (Table 5).

ADVANTGES OF X-CHROMOSOMAL MICROSETTE-LITE

The major advantage of X-chromosomal (ChrX) STRs arises in deficiency paternity cases, that is, when a putative father is not available and DNA from paternal relatives has to be analyzed instead (Szibor et al., 2000). Female individuals fathered by the same man share their paternal ChrX. Males inherit their only ChrX from their mother. Hence, in cases in which the putative grandmother is available for genotyping, the possible ChrX alleles of the putative father can be determined (Ellegren, 2000; Szibor et al., 2003). ChrX marker typing is highly effective in mother–son kinship and in father–daughter testing. However, linkage and possible linkage disequilibrium between the ChrX markers used have to be taken into consideration (Ellegren, 2000).

Table 4. Allele frequencies for the DXS6789 genetic loci of nine population samples.

Allele	Ireland ^[1]	Italia ^[2]	German ^[3]	German ^[1]	Peru ^[1]	Ethiopia ^[1]	Pakistan ^[4]	China ^[5]	Japan ^[6]
13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
14	0.000	0.000	0.002	0.000	0.000	0.000	0.022	0.006	0.008
15	0.070	0.059	0.036	0.036	0.025	0.163	0.192	0.190	0.159
16	0.010	0.013	0.014	0.014	0.161	0.041	0.077	0.308	0.313
17	0.010	0.000	0.001	0.001	0.000	0.008	0.027	0.045	0.017
18	0.000	0.000	0.002	0.002	0.017	0.016	0.000	0.002	0.002
19	0.000	0.046	0.030	0.029	0.051	0.016	0.022	0.033	0.024
20	0.310	0.336	0.374	0.379	0.483	0.317	0.385	0.198	0.174
21	0.340	0.289	0.286	0.286	0.136	0.211	0.170	0.165	0.179
22	0.190	0.145	0.174	0.173	0.085	0.146	0.055	0.045	0.085
23	0.070	0.086	0.064	0.063	0.025	0.065	0.044	0.004	0.036
24	0.000	0.026	0.016	0.060	0.017	0.016	0.005	0.000	0.003
25	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000

[1] (Edelmann et al., 2004); [2] (Coletti et al., 2006); [3] (Jeanett et al., 2001); [4] (Muhammad et al., 2012); [5] (Lv, et al., 2004); [6] (Yasutaka and Kiyoshi., 2010).

Table 5. Allele frequencies for the DXS6807 genetic loci of seven population samples.

Allele	Ireland ^[1]	Italia ^[2]	German ^[3]	German ^[1]	Peru ^[1]	Ethiopia ^[1]	Japan ^[4]
10	0.000	0.000	0.000	0.000	0.000	0.016	0.000
11	0.571	0.553	0.492	0.492	0.364	0.658	0.370
12	0.010	0.033	0.021	0.021	0.034	0.041	0.008
13	0.010	0.006	0.011	0.110	0.220	0.049	0.032
14	0.265	0.237	0.235	0.235	0.254	0.146	0.367
15	0.133	0.145	0.202	0.202	0.127	0.041	0.193
16	0.010	0.019	0.029	0.029	0.000	0.032	0.029
17	0.000	0.007	0.010	0.010	0.000	0.016	0.002

[1] (Edelmann et al., 2004); [2] (Coletti et al., 2006); [3] (Jeanett et al., 2001); [4] (Yasutaka and Kiyoshi., 2010).

Conflict of Interests

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

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Review

Bayoud disease of date palm in Algeria: History, epidemiology and integrated disease management

Ibrahim Elkhail Benzohra*, Mohamed Megateli and Rafik Berdja

Scientific and Technical Research Center in the Dry Areas (CRSTRA), Campus Universitaire B.P. 1682 RP, 07000 Biskra, Algeria.

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The bayoud is transmitted by the fungus *Fusarium oxysporum f. sp. albedinis*, which causes drying and rapid die back. To date, the disease has destroyed more than 12 million date palms in Morocco, or two-thirds of the producers of the best dates trees in this country, and three million of palm trees in Algeria with the threat of the spread of this disease to oasis southeast of Algeria. The research on this disease is very few in Algeria, in this case, this work had objective to study the growth and spread of the disease bayoud on the Algerian palm. is based on the observation of symptoms on palms, it showed that all regions with palm in Bechar (Saoura) and in Adrar (Touat), affected with some palm of Ghardaïa. Prophylactic measures are taken to protect and preserve our date resources (including 'Deglet Nour') in free palm (Zibans and Oued Righ in southeast of Algeria) by improving irrigation methods and the use of free releases, and determination date palm cultivars resistant to the fungus as Takarboucht in oasis of Ghardaïa and Adrar, Hmira and Hartan for Bechar's date palms.

Key words: Date palm, bayoud disease, saoura, resistance, IPM.

INTRODUCTION

The name Bayoud comes from the Arabic word, "abiadh", meaning white which refers to the whitening of the fronds of diseased palms. This disease was first reported in 1870 in Zagora-Morocco. By 1940, it had already affected several date plantations and after one century, the disease has practically affected all Moroccan palm groves, as well as those of the western and central Algerian Sahara (Killian and Maire, 1930; Toutain, 1965 ; Toutain 1972; Toutain and Louvet, 1972). Bayoud disease causes considerable damage that can sometimes take on spectacular proportions when the disease presents its violent epidemic aspect. Bayoud has

destroyed in one century more than ten million palms in Morocco and three million in Algeria (Figure 1). Bayoud destroyed the world's most renowned cultivars that are susceptible to the disease and particularly those which produce high quality and quantity fruit (Medjool, Deglet Nour, BouFegouss or Fegouss). The result is an influx of farmers who have abandoned their land and moved to large urban centres (Toutain and Louvet, 1974). The continued spread of bayoud highlights the problem threatening the important plantations of Deglet Nour and Ghars in Oued Righ, Zibans in Algeria and even in Tunisia, which is presently free of the disease, but has 70

*Corresponding author. E-mail : ibrahimelkhalil@live.fr. Tel/Fax : 033 74 18 15.

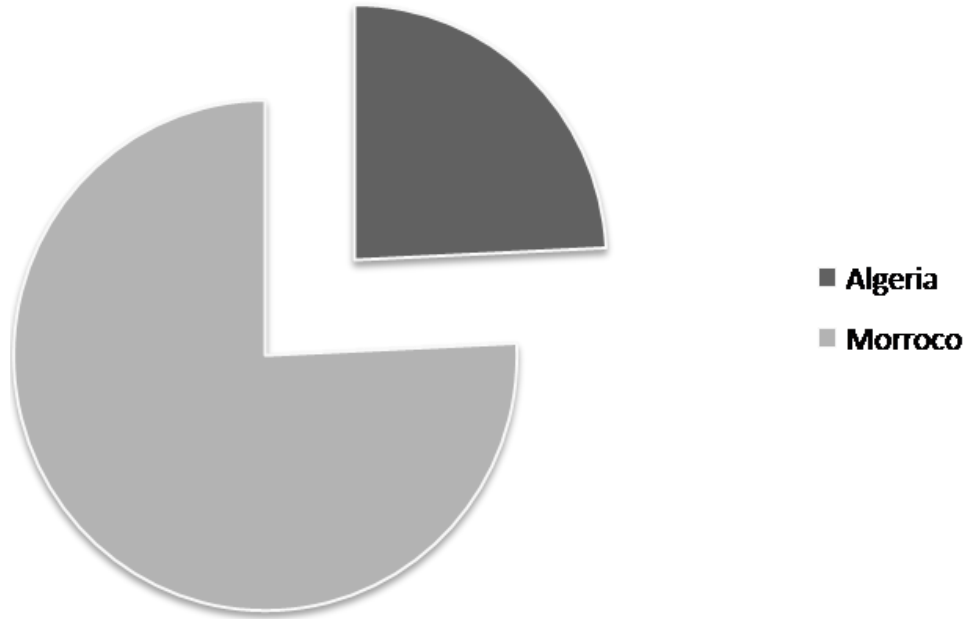


Figure 1. Date palms losses by Bayoud disease in the period of 1950 to 2000 (Benzohra et al., 2013).

to 80% of the date palm areas under cultivars are susceptible to this disease (Meloncon, 1947 ; Dubost, 1972). The disease continues to advance relentlessly to the east, despite prophylactic measures and regular attempts at eradication undertaken in Algeria (Djerbi et al., 1985). It is evident therefore, that Bayoud constitutes a plague to Saharan agriculture and at the present expansion rate, it will certainly pose serious problems of human, social and economic nature to other date-producing areas of the world.

DISEASE SYMPTOMS

The bayoud disease attacks mature and young palms alike, as well as offshoots at their base (Saaidi, 1979). The first symptom of the disease appears on a palm leaf of the middle crown (Figure 2). This leaf takes on a leaden hue (ash grey colour) and then withers, from bottom to top, in a very particular way: some pinnae or spines situated on one side of the frond wither progressively from the base upward to the apex. After one side has been affected, the whitening begins on the other side, progressing this time in the opposite direction from the top of the frond to the base. A brown stain appears lengthwise on the dorsal side of the rachis and advances from the base to the tip of the frond, corresponding to the passage of the mycelium in the vascular bundles of the rachis. Afterwards, the frond exhibits a characteristic arch, resembling a wet feather and hangs down along the trunk. This whitening and dying process of the pinnae may take from a few days to

several weeks. The same succession of symptoms then begins to appear on adjacent leaves. The disease advances ineluctably and the palm dies when the terminal bud is affected. The palm can die at any time from several weeks to several months after the appearance of the first symptoms (Figure 3). The rapid evolution of the symptoms depends mainly on planting conditions and on cultivar date palm. A small number of disease infected roots, reddish in colour, are revealed when an affected palm is uprooted. The spots are large and numerous towards the base of the stipe. As they advance towards the upper parts of the palm, the coloured conducting fascicles separate and their complicated path inside the healthy tissues can be followed. Palm fronds manifesting external symptoms exhibit a reddish brown colour when cut, showing highly coloured conducting fascicles. There is, therefore, a continuity of vascular symptoms that exist from the roots of the palm to the tips of the palm fronds. The observation of symptoms is necessary to recognise the bayoud, but to identify this disease with certainty, samples of affected fronds must be analysed by a specialised laboratory.

PATHOGEN

The causal organism responsible for bayoud is a microscopic fungus which belongs to the mycoflora of the soil and is named *Fusarium oxysporum forma specialis albedinis* (Killian and Maire, 1930; Malencon, 1934). The first precise descriptions of the fungus were done by



Figure 2. Bayoud symptoms appear on one or more leaves of the middle crown. (Béchar) (Photo : Benzohra-CRSTRA-2014-01).



Figure 3. The palm dies when the terminal bud is affected (Béchar) (Photo : Benzohra-CRSTRA-2014-02).

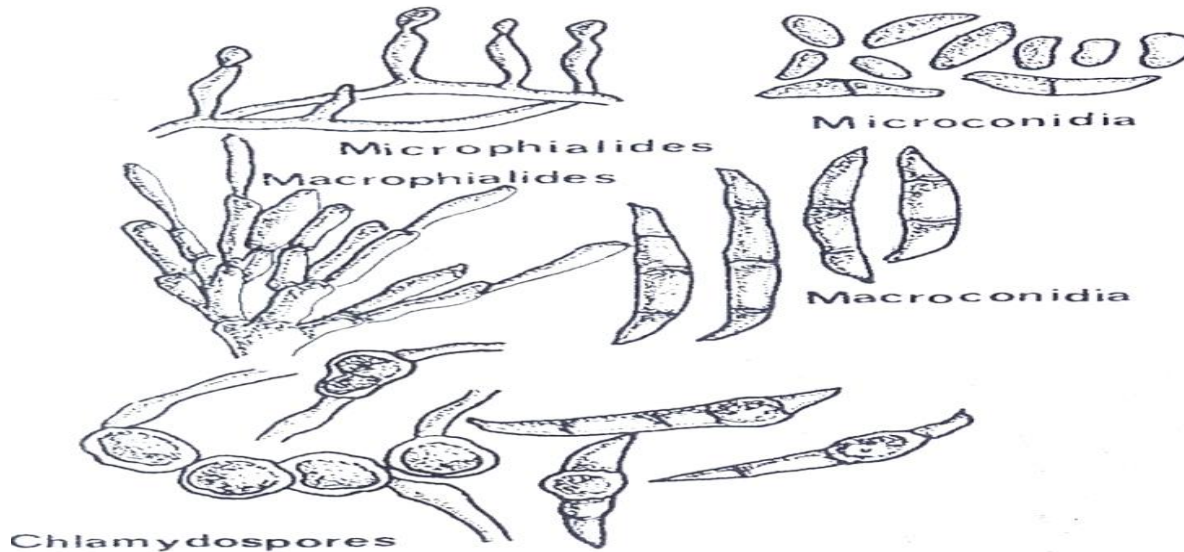


Figure 4. Conidiogenesis and conservation forms of *Fusarium oxysporum* f. sp. *albedinis*.

Malençon (Malencon, 1950a; b), Perea-Leroy (Perea-Leroy, 1958) and more recently completed by the works of Bulit et al. (1967) and Louvet et al. (1970). The causal organism responsible for Bayoud is a microscopic fungus which belongs to the mycoflora of the soil. It belongs to the group Fungi Imperfecti, the Order Moniliales, the Family *Tuberculariaceae* and, at the present time, is named *F. oxysporum forma specialis albedinis* (Malencon) Snyder and Hans.

Macroscopic characteristics

The isolation, the characterisation, and the maintenance of the *F. oxysporum* f. sp. *albedinis* culture has been the subject of numerous studies (Snyder and Hansen, 1972). Although, it is easy to obtain *F. oxysporum* f. sp. *a/bedinis* cultures from rachis fragments of palm leaves which exhibit vascular symptoms, it is very difficult to preserve the original type of colony (which is still called the wild type). Indeed, the wild form shows greatly unstable cultural characteristics under normal conditions and rapidly produces mutants. In culture, *F. oxysporum* f. sp. *albedinis* forms a fine, clear and curly mycelium in which small orange-pink sporodochia are produced (Figure 4). Blue to black sclerotia sometimes are born in the medium. They are either dispersed in the mycelium or sometimes form groups.

Microscopic characteristics

F. oxysporum f. sp. *albedinis* occurs as a septate hyaline mycelium. It is fine and uniform in young cultures, exhibiting, however, in older cultures hypertrophic cells

that occur in chains; they are round, greatly resembling chlamydospores, but without thickening of the wall. Asexual multiplication occurs by microphialides swollen at the base and pointed at the tip, arising perpendicularly from the mycelium. At the apical tip of these phialides, microconidia (microphialospores) form continuously and endogenetically, each one being pushed by the next and they adhere together in the form of small moist heads. Great numbers of hyaline microconidia, varying in form and dimensions, can be found in the same culture (3 to 15 x 3 to 5 μ m). In young cultures they are globulous, while in the older cultures they are more elongated. Microconidia are often unicellular, sometimes bicellular but rarely have two septa. Macrophialides, larger than microphialides, group together to form sporodochia and more rarely *pionnotes* (Figure 4). In culture, *F. oxysporum* f. sp. *albedinis* also produces a few macroconidia with pediform base and short, pointed tips; most have three septa, though some have four or five and measure 20 to 30 x 3 to 5 μ m (Figure 3). In older cultures, or in an agar medium covered over with earth, *F. oxysporum* f. sp. *albedinis* forms chlamydospores that are uniform and globulous, with a smooth thick wall varying from 6 to 20 μ (Baysal et al., 2010). They may be either intercalary or terminal and are isolated or grouped in two or four in short chains. They are formed either on the mycelium or from macroconidia (Figure 4). Sclerotia are dark blue-black, measure about 1 mm in diameter and occur rarely (Sedra and Djerbi, 1985).

Physiologic characteristics

The physiology of *F. oxysporum* f. sp. *albedinis* has been little studied. Malencon (1947) determined the optimum

temperature range for growth. Growth begins at 7°C, remains slow until 12°C, becomes more rapid between 21 and 27.5°C and stops at 37°C (Shabani and Kumar, 2013; Shabani et al., 2014). Louvet and Bult (1978) reported that the capacity of the fungus to use complex carbon sources and to develop in the presence of high concentrations of carbonic gas or penta-chloro-nitro-benzene. Bounaga (1975) found that the best mycelium growth occurs at 28°C. The marked preference of *F. oxysporum* f. sp. *albedinis* for pectin, mannose, xylose and cellulose was also noted and in addition to that organic nitrogenous sources are better metabolised than mineral nitrogen. The influence of sodium chloride was also studied and it was observed that there is no lessening of growth up to a concentration of 40 g/l. This observation is similar to Toutain and Louvet (1972) who found that Bayoud advances. Normally in salty fields, Dubost et al. (1970) also studied the pectinolytic and cellulolytic enzymes of the fungus. These authors showed the important difference between the enzymatic activities of different isolates of *F. oxysporum* f. sp. *albedinis*, but they did not establish a correlation between this activity and the pathogenic power of the fungus.

BIOLOGY AND EPIDEMIOLOGY

F. oxysporum f. sp. *albedinis* is preserved in the form of chlamydo-spores in the dead tissues of infected palm, especially in the roots which have been killed by the disease and in the soil. A contamination occurs regularly from palm to palm and more rapidly as the amount of irrigation increases. The appearance of the disease in locations far from the original infected area is caused primarily by the transport of infected offshoots or palm fragments harbouring the fungus. Many plants are often grown as intercrops in palm groves, notably lucerne (*Medicago sativa* L.; alfalfa), henna (*Lawsonia inermis* L.) and vegetables (Djerbi et al., 1985). These plants can harbour the bayoud organism without manifesting any symptoms (symptomless carriers) (Bengyella et al., 2012).

Survival, infection and disease cycle

Like all vascular organisms of telluric origin, *F. oxysporum* f. sp. *albedinis* is preserved in the form of chlamydo-spores in the dead tissues of infected palm trees, especially in the roots of trees killed by it. With subsequent disintegration of such tissues, the chlamydo-spores may be released into the soil where they remain in a dormant state. The fungus is found at a depth varying from 5 to 30 cm, and sometimes deeper; it can be preserved for a long period of time, even when the palm trees have long since died (eight years or more). If nutrients reach the chlamydo-spores in sufficient quantity, the spore germinates and invades a root, entering the

vascular tissues as a parasite (de la Perrière and Benkhalifa, 1991). Once the pathogen is inside the vascular element, it grows rapidly and the mycelium advances up the root and into the stem (Ghaemi et al., 2011; Laurence et al., 2012). The mycelium produces microconidia in the vessels and these are carried upwards by the water stream. When they flow up the vessel is impeded by a cross wall, the microconidia germinate, the germ tubes penetrate the wall and then microconidia formation is resumed on the other side of the wall. These new microconidia are in turn carried along to the next transverse wall in the same manner as that of the fusariose in the banana (Sedra and Djerbi, 1986). This process thus continues upward, internally through the tree to its terminal bud, leading to the death of the date palm. During the course of its upward progression, *F. oxysporum* f. sp. *albedinis* breaks out of the xylem and colonizes the surrounding parenchyma tissues of the tree by an inter- and intra-cellular mycelium which gives the tree the reddish brown colour that is characteristic of Bayoud. After the death of the date palm, the mycelium continues to develop in the parenchyma of the tree and forms numerous chlamydo-spores in the sclerenchyma cells (Sutherland et al., 2012). These constitute very favourable conditions for the survival of the fungus in the soil (Louvet, 1977; Dubost and Kada, 1974; Dubost and Kada, 1975).

Spread of Bayoud in palm groves

Little research has been done on the speed of Bayoud's advancement in a plantation. The best information comes from the experimental palm grove of Nebch at Zagora, Morocco (Toutain, 1970) and from the palm grove of In Salah in Algeria (Kada and Dubost, 1975) (Figures 5 and 6). Starting from a few affected trees (primary centres) Bayoud expands many foci; this is particularly visible in homogeneous plantations. Indeed, the contamination occurs regularly from tree to tree and more rapidly as the intensity of irrigation increases. In this case, the disease takes on an epidemic aspect; the number of affected trees increases rapidly and the life span of the diseased tree decreases. Thus, at Zagora, on a plot of land containing 125 palm trees of the susceptible Bou Feggous variety. This primary infection, began in 1956, has destroyed the whole plantation over a period of 14 years at the average rate of 6% per year. The intensive cultivation of palm groves, therefore, fosters the expansion of Bayoud. A high salt content of both soils and water (5 g/l) does not prevent or slow down the spread of Bayoud (de la Perrière and Benkhalifa, 1991). Periods of drought, accompanied by lack of water, result in a regression of the disease which then becomes dormant. On the other hand, as soon as irrigation becomes significant and frequent, the disease reappears more violently and becomes gravely epidemic, particularly when there is a predominance of susceptible

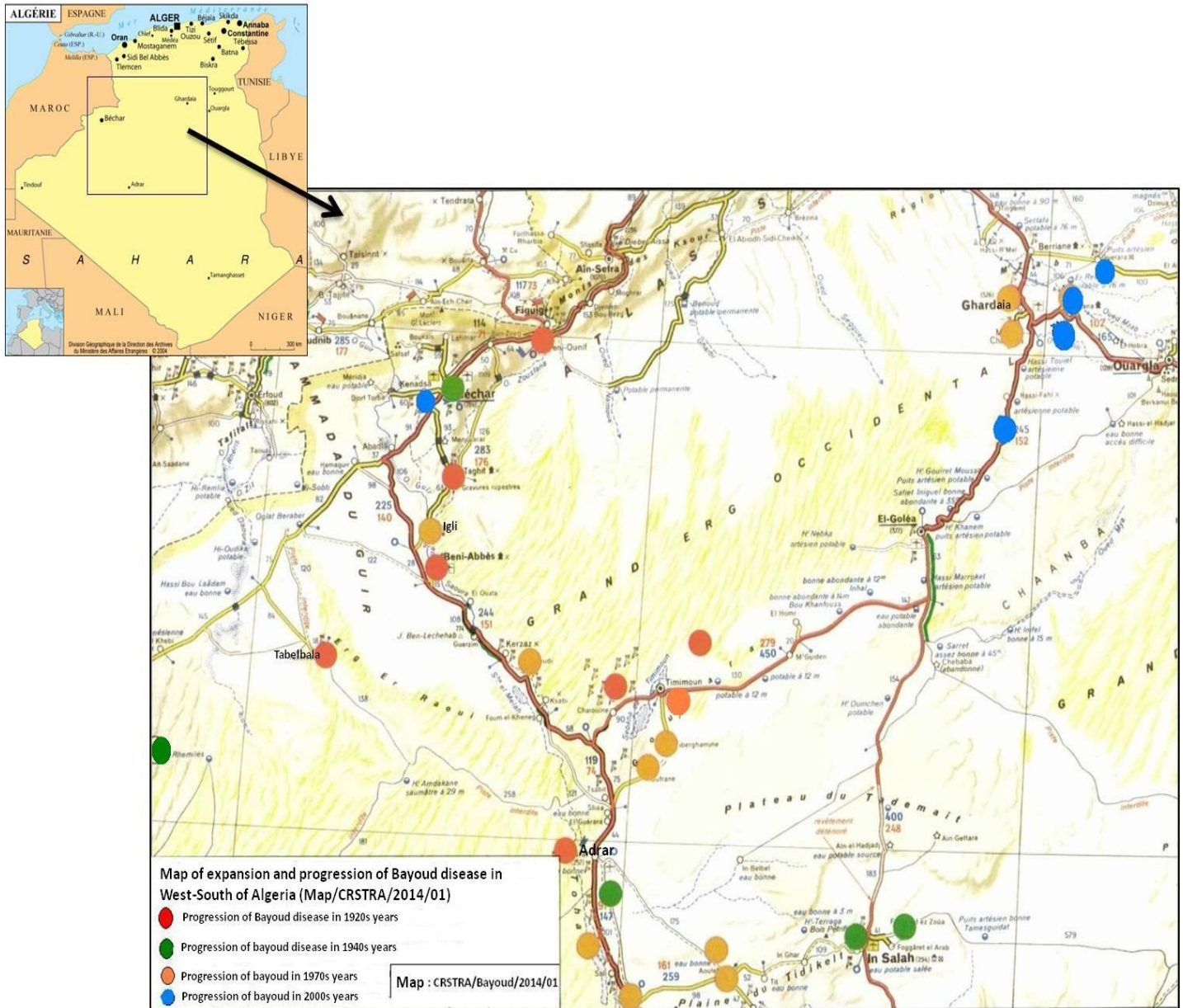


Figure 5. Progression of bayoud disease in West-South oasis of Algeria.

varieties. It thus seems that alternating dry and wet periods are favourable to an explosive expansion of the disease. This phenomenon has also been observed for other *Fusarium* diseases (Djerbi, 1970). Furthermore, according to Perea-Leroy (1958), certain intercrops such as lucerne, alfalfa *M. sativa*, henna (*L. inermis* L.) vegetables ... etc, foster strong attacks of Bayoud. The author thinks that this effect is due to the influence of copious irrigation, indispensable to these crops during the hot season. A study by Laville and Lassois (1963) shows that the direction of the advancement of the disease in the Ain Salah palm grove is related to the dominant

winds, irrigation and the salinity gradient. On the other hand, to date no work has been done to demonstrate the possible influence of nematodes on the spread of Bayoud. The appearance of foci that are rather far from the original focus is essentially related to the transport of infected offshoots or palm tree fragments harbouring the fungus. The consequences of the spread of Bayoud are particularly visible in Morocco. After the disappearance of a great number of the best varieties, presently in the Moroccan palm groves more than 50, of the trees are of seedling origin called Khalts, Sairs or Deguels (In arabic). These Sairs are often of bad quality, meaning of

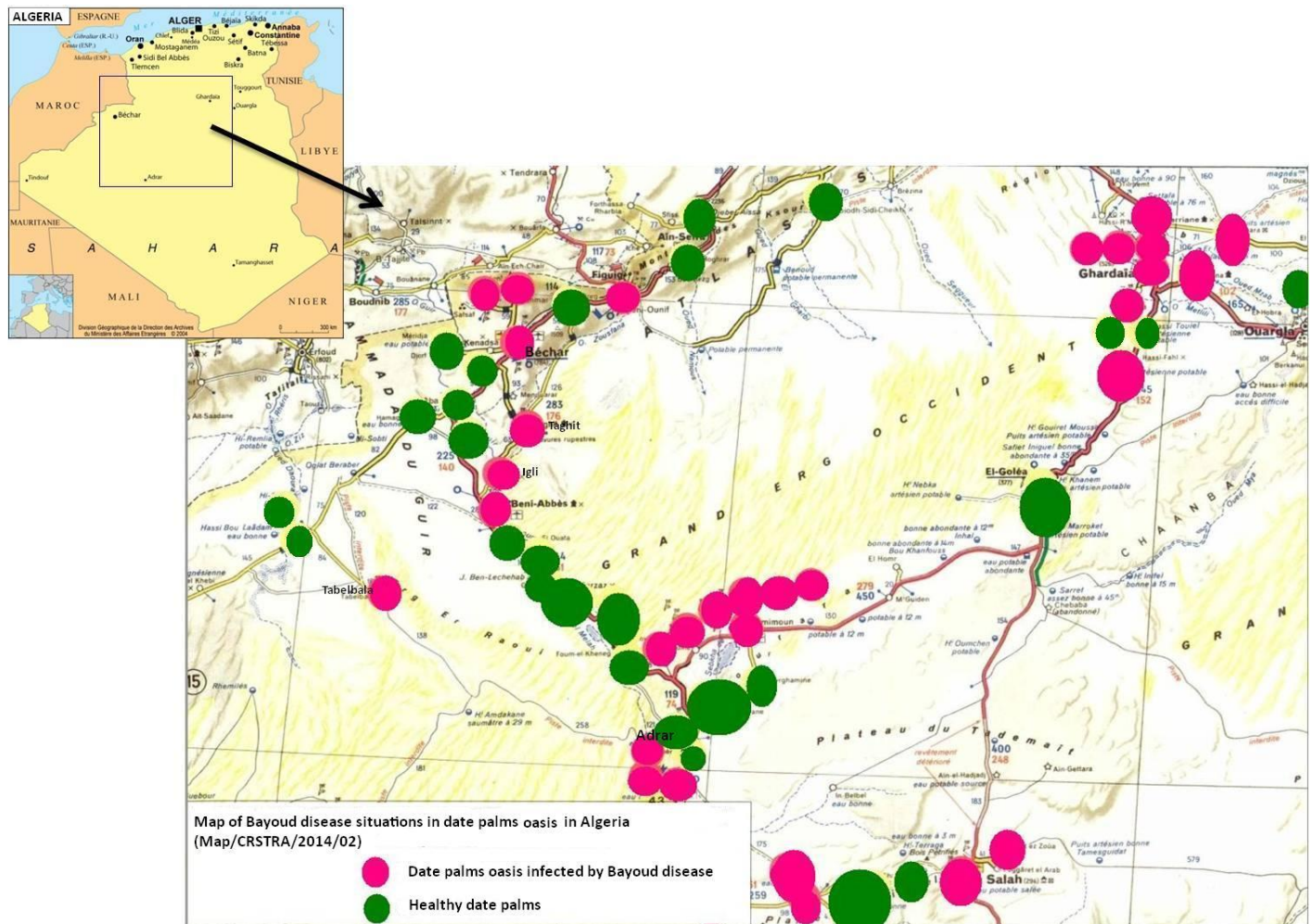


Figure 6. Map of Bayoud disease situation in date palms oasis in Algeria.

low commercial value.

HOST PLANTS

Many plants are often grown among date palms in the groves, notably alfalfa, henna, etc. To date, *F. oxysporum* f. sp. *albedinis* has only been isolated from henna (*Lawsonia inermis*); indeed, the plant harbours the Bayoud organism without manifesting any symptoms. More recently in France, Mercier and Louvet (1973) also isolated the causal organism of Bayoud on *Phoenix canariensis*, another species of palm trees, which manifests the same symptoms as the vascular fusariosis of the date palm trees.

CONTROL OF BAYOUD DISEASE

Chemical control

Soil treatment of this type of disease is destined, *a priori*,

to fail and should therefore be avoided. Chemical control can, however, be feasible in the event of the discovery of primary sources of infection in a healthy area. In this case eradication techniques should be used: palms are uprooted and incinerated on the spot. The soil is then treated with methyl bromide or chloropicrin and the area closed off with replanting prohibited until further notice.

Cultural control

Since the factors that favour high yield in date palms (irrigation, fertilisation, etc.) are the same that favour the growth of the fungus, cultural techniques are not advised. However, a significant reduction in the amount of irrigation can retard the advance of infection, that is, stopping irrigation between the months of May and October, during the hot season in the northern hemisphere (Pereau-LeRoy, 1958; Dubost and Rellon, 1974). Since the contamination occurs mainly by root contact, disease-free palms can be isolated by digging a

trench of 2 m deep around them. Water should be provided by a trough bridging the rest of the grove to this isolated plot. Under these conditions these palms can be protected for more than 10 years (Djerbi, 1983).

Prophylactic measures

The essential task is to prevent the movement of contaminated plant material from an infected palm grove to a healthy one. This material has been previously mentioned, consists mainly of offshoots, palm fragments, manure and infected soil, and artifacts made from these materials. Legislation preventing the conveyance of contaminated vegetative material from one country to another, or from one region to another, has been passed by various countries such as Algeria, Egypt, Iraq, Libya, Mauritania, Saudi Arabia, Tunisia and USA.

Genetic control

The only productive means of controlling bayoud disease lies in continued research into resistant varieties. Many resistant cultivars have already been obtained in Morocco from three sources: selection of bayoud-resistant varieties from those already existing (local and introduced), selection of high-quality, resistant clones from the natural population of the date palm, and creation of resistant and high quality varieties through a hybridisation programme (Djerbi et al., 1986). In addition, the present success of date palm propagation by *in vitro* culture will make it possible to rehabilitate the Moroccan and Algerian palm groves that have been destroyed by bayoud. It will also be possible to reconstitute the palm groves presently threatened by Bayoud and create new date-growing areas with the help of high quality, resistant varieties.

CONCLUSION AND PERSPECTIVES

The palm tree plays an important role in the ecology, economy and sociology of the Saharan environment; this tree is, in fact, irreplaceable in irrigable desert lands. Because of the considerable damage that Bayoud has caused in Moroccan, and Algerian palm groves and the threat that it constitutes to other countries, the disease has become the greatest enemy to date-growing regions of the world. As previously mentioned, the biological characteristics of *F. oxysporum* f. sp. *albedinis* and of its host make any type of successful chemical control unlikely. Furthermore, the projected prophylactic measures will never control Bayoud; they will only temporarily retard the spread of this disease. Genetic control remains the only solution to this problem. The first solution, leading to short-term (within 5 years) results. Consists of studying the date palm population existing in contaminated zones in order to select resistant varieties of good quality. This

genetic potential will be complemented by the introduction of the world's best varieties which will then be tested for Bayoud resistance. There remains, however, the problem of propagation of selected cultivars. Indeed, the release of a resistant cultivar and its planting on a mass scale demands in turn the availability of large numbers of offshoots. It is obvious that the natural production of offshoots by palm trees is very slow and insufficient to fulfil the demand for offshoots in all areas damaged by Bayoud disease. The improvement of the technique of rooting light-weight offshoots in greenhouses equipped with "mist systems" will help to accelerate, at least slightly, the technique of vegetative propagation (Saadi, 1979). Nevertheless, this course of action does not solve the need for large number of offshoots.

The solution to the problem lies in the perfection of a method of date palm tissue culture. Several laboratories throughout the world are working on this subject and they have obtained encouraging results. Those of the laboratories of Indio (USA) and Angers (France) are particularly interesting. Indeed, these authors have succeeded in obtaining young date palms from inflorescences or from meristematic tissues. Once this method is perfected, infinite numbers of high-quality, Bayoud resistant date palms will be obtained from these clones. Thus, not only will it be possible to rehabilitate the Moroccan and Algerian palm groves that have been destroyed by Bayoud, but it will also be possible to reconstitute the palm groves presently threatened by Bayoud, as well as to create new date-growing areas with the help of high-quality, resistant varieties.

In conclusion, bayoud disease is an epiphytic disease for which there is no known cure at present. Only preventive measures could protect healthy date plantations from this disease. Therefore, the following measures are imperative:

- i) Forbid the introduction of offshoots and all other plant material (palm fragments, artifacts made from date material, manure and infected soil) originating from bayoud infected countries or regions.
- ii) Forbid the import of seeds and unprocessed products of symptomless carriers such as Alfalfa (Lucerne) and Henna from bayoud-infected countries or regions.
- iii) Adopt legislation preventing the conveyance of the above plant material.
- iv) Immediately report cases where symptoms similar to the ones caused by the bayoud appear.
- v) Information on bayoud and other major diseases and pests is necessary for the success of all above actions and must be available to all date growers.

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Full Length Research Paper

Real time PCR mediated determination of the spontaneous occurrence of *Sorghum bicolor* alleles in wild sorghum populations

Titus O. Magomere^{1*}, Eliud K. Ngugi¹, Solomon I. Shibairo², Eunice Mutitu¹ and Silas D. Obukosia³

¹Plant Science and Crop Protection Department, University of Nairobi, P.O. Box 29053-00625, Nairobi, Kenya.

²Kibabii University College, Masinde Muliro University of Science and Technology, P.O Box 1699-50200 Bungoma, Kenya.

³Africa Harvest Biotechnology Foundation International, P.O. Box 642, 00621-Village Market, Nairobi, Kenya.

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The study evaluates the utility of Real Time PCR (RT-PCR) in quantitative and qualitative analysis of alleles in sorghum populations and the spontaneous occurrence of *Sorghum bicolor* alleles in wild populations of sorghum. Leaf and seed material from wild sorghum accessions were sampled in Homabay, Siaya and Busia counties to represent Western Kenya sorghum producing regions. A second sampling was done on S₂ populations of *S. bicolor*, *Sorghum halepense* and *Sorghum sudanense* maintained in the greenhouse. Crop loci were evaluated in all materials using a LightCycler® 2.0 system. Real Time PCR was effective in qualitative and quantitative determination of crop alleles in both crop and weedy backgrounds of *S. sudanense*, *S. halepense* and *S. verticilliflorum*. Crossing point values ranged between 19.7 from 30 ng template to 35.9 from 0.015 pg of template on locus SB1764. Melting peaks analysis ranged between 83.29 to 88°C on locus SB1764 and between 86.01 to 80.88°C on locus SB3420 effectively differentiating the 4 species. RealTime-PCR was successful in quantitative and qualitative analysis of specific crop alleles from loci SB1764 and SB3420 from seed and leaf DNA. Spontaneous occurrence of crop and rare alleles in wild sorghum populations growing in sympatry with crop cultivars showed the presence of crop and rare alleles in wild sorghum populations. Means of wild populations from lower midland₁ (LM₁), LM₂, LM₃ and LM₄ AEZs were not significantly different. It is therefore vital to test *S. bicolor* seeds and other plant materials in transit, at entry points and populations of growing plants for foreign genes including transgenes using RT-PCR.

Key words: Real time polymerase chain reaction (PCR), *Sorghum bicolor*, *Sorghum halepense*, *Sorghum sudanense*.

INTRODUCTION

Populations of crop and weedy sorghums have been shown to coexist in sympatry in most sorghum producing regions (Doggett, 1988). The members of this genus have shown great potential for gene flow through interspecific

hybridization. This situation has led to the existence of crop alleles in wild sorghum, attributed to ancient and recent hybridizations (Morrell et al., 2005; Mutegi et al., 2009). These crop alleles in weedy populations have

contributed to the wide variability maintained in populations of sorghum through disruptive selection process (Doggett and Majisu, 1968). The existence of domesticated sorghum alleles in wild sorghums can easily be followed by the use of PCR applications. However, conventional PCR has several technical limitations that hinder its maximum potential. The deficiencies in traditional PCR are associated with the speed, sensitivity, reliability and inability to adequately quantify target DNA (Gachon et al., 2004; Arya et al., 2005). This has led to the development of more quantitative and precise methods of analyzing target DNA sequences such as RT-PCR. The method uses instrumentation and fluorescent dyes that permit online monitoring of PCR amplification in real time (Heid et al., 1996; Wittwer et al., 1997).

Real Time-PCR offers rapidity, sensitivity, and specificity due to its ability to amplify targeted sequences from small amounts of starting DNA (Gachon et al., 2004; Bustin, 2000; Arya et al., 2005). The technique has been improved relative to conventional PCR with regard to its ability to quantify with precise calculation the starting amount of template and estimate DNA quantity during amplification (Hernandez et al., 2003; Gachon et al., 2004). In addition, RT-PCR has the capacity to differentiate the amplified strands, by applying the melting temperature (TM) approach. This is an important application in the genotyping of genetic material.

In plant studies RT-PCR has the potential of identifying genetic variability and the subsequent introgression of genes into a cultivar of interest (Gachon et al., 2004). Real Time-PCR has been applied in the absolute and relative qualitative determination of the presence of foreign DNA in plant material (seeds, leaves, roots) (Gachon et al., 2004). Foreign DNA routinely assayed through RT-PCR in plant populations include; pathogens such as bacteria, fungi and viruses (Deepak et al., 2007; Sankaran et al., 2010). The technique has the potential of detecting polyploidy events, subsequent chromosomal inversions and induced or spontaneous mutations in populations. Recently, transgenic events have also been detected through RT-PCR (Gachon et al., 2004; Deepak et al., 2007).

Transgene copy number has previously been assayed in transgenic material using duplex real time PCR (Ingham et al., 2001). German et al. (2003) showed the efficiency of the technique in differentiating hemizygous and homozygous transgenic plants with single copy inserts. In addition, MON 810 in transgenic maize was evaluated using a specific RT-PCR detection system that was shown to be a specific and accurate Taqman based technique (Hernandez et al., 2003; Pla et al., 2006). Qualitative and quantitative determination of transgene events has been reported in canola (Yang et al., 2006). Simplex and duplex RT-PCR was successfully applied in

quantification of GMO events in maize and soybean (Alary et al., 2002). Multiplex RT-PCR has also been developed for simultaneous detection of *Cry/A(b)* and *pat* genes of transgenic maize insect resistant events namely, 176, MON810, BT11 and T25 of transgenic maize (Permingeat et al., 2002). In addition RT-PCR technique has been useful in quantification of the L1781 ACCASE inhibitor resistance allele in DNA from leaf and seed pools of weedy *Lolium* populations (Kaundun et al., 2006). The technique can serve as a quick substitute to Southern blots for analysis of copy numbers of alleles or loci under investigation. RT-PCR can therefore be useful in tracking down unintended introgression of crop alleles into weedy backgrounds. This is necessary in the Eastern Africa region due to the presense of several wild and weedy sorghum species in the crop sorghum production zones. The current study evaluated spontaneous hybridization in conspecific sorghum species using RT-PCR.

MATERIALS AND METHODS

Experimental materials were obtained from two sources; first, leaf samples were obtained from S₂ populations of *S. bicolor*, *S. halepense* and *S. sudanense* grown in the greenhouse. Secondly, wild sorghums in three counties of Kenya namely Homabay, Siaya and Busia representing Western Kenya sorghum producing regions around Lake Victoria were sampled. Sampling units within the counties were clustered based on the mapped agro-ecological zones (AEZs). Sorghum growing farms were randomly sampled and a total of 175 samples were collected representing AEZs in the three counties (Table 1). The samples were collected after seed set in farms that had both weedy and crop sorghums. Weedy sorghums growing on road reserves and uncultivated land in the clusters were also sampled. The seeds and first two leaves next to the panicle were collected labelled and kept on ice and transported to the laboratory located at the College of Agriculture and Veterinary Sciences (CAVS) (-1° 14' 59.72", +36° 44' 30.79") of the University of Nairobi.

Sample preparation and DNA extraction

Young leaves were collected from 2 to 4 week old plants in the greenhouse, while old leaves were obtained from green sections of physiologically mature plants in the field. Thirty mature seeds were randomly sampled per panicle, dried and bulked. Genomic DNA from leaf and seed samples was extracted through two methods. First, genomic DNA was extracted from young leaves by using a modified CTAB extraction procedure (Doyle and Doyle, 1990; Barnaud et al., 2008). Total nucleic acid was extracted from the seeds, by a modified CTAB based protocol (Delobel et al., 2007). Agarose gel electrophoresis for genomic DNA extracted from young leaf, old leaf and seed tissues was done before RT-PCR to establish the quantity and quality of the DNA. The RT-PCR products were separated and validated using a 4% UltraPure™ Agarose gel from Invitrogen™. Densitometric analysis of agarose gels was used to determine the original concentration of genomic DNA.

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

Table 1. Agro-ecological zone characteristics in Homabay, Siaya and Busia counties of Kenya where wild –weedy sorghums were collected.

Agro-ecological Zone	Altitude (m)	Ann. Mean Temp (°C)	Ann. Mean Rainfall (mm)	Number of samples
Busia (+0° 28' 45.07", +34° 7' 11.39")				
Lower Midlands (LM)				
LM1	1200-1440	22.2-21.0	1650-2000	18
LM2	1200-1350	22.3-21.4	1420-1800	13
LM3	1140-1500	22.7-21.0	1100-1450	16
LM4	1135-1200	22.7-22.3	900-1100	14
Homabay (-0° 36' 15.25", +34° 29' 57.86")				
Upper Midlands (UM)				
UM1	1500-1570	21.0-20.5	1600-1900	11
Lower Midlands (LM)				
LM1	1300-1500	21.8-20.9	1500-1900	9
LM2	1200-1350	22.3-21.5	1400-1600	8
LM3	1140-1250	22.7-22.0	1020-1390	12
LM4	1135-1200	22.7-22.3	890-1020	21
LM5	1135-1180	22.7-22.4	-	
Siaya (+0° 2' 18.43", +34° 12' 19.88")				
Lower Midlands (LM)				
LM1	1300-1550	21.7-20.4	1600-1800	11
LM2	1300-1550	21.7-20.4	1300-1600	12
LM3	1140-1550	22.7-20.4	900-1500	11
LM4	1140-1450	22.7-20.9	800-1300	19
LM5	1135-1300	22.7-21.7	700-900	

Farm management hand book of Kenya (2005, 2009).

Table 2. Primer sequences developed for Real Time PCR analysis from the *Sorghum bicolor* physical map that exhibit polymorphism on the number and sizes of alleles present

Marker name	Chromosome	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
SB3258	5	ATTGTTGTCCTCCCCTCCACC	AGTACCTGAACCAGGCGTCGCT
SB1764	3	CTTGTGCTTGCTTGCCACCATATTC	GTCGATGAGGAGCTTCATGCTCAG
SB3008	5	ATTTTGGTCCAGTTGCTCTTGCAT	CGTACGTGAGCCGAGGATTGTTCTA
SB3420	6	GAGCCAGCATGCATGATAATTGTT	CACAAAGGCATGACAGTCAATCAA
SB5058	9	GAGAATTGGAAGAAAGCCTCGGTT	CAGAGCTCCTAAACGGTCTCCTAAA
SB5458	10	AATGTGGTGGTGTGTGTCTCCAT	TCTACTGCTATCATCGCCTCCACC
SBI000X	10	CTAGAGGATTGCTGGAAGCG	CTGCTCTGCTTGCTCGTTGAG

Primers were designed to give a 300 bp fragment in *S. bicolor*, with a melting temperature of between 60 and 62 degrees centigrade.

Primer design

Primers were designed based on the polymorphic SSR sequences from crop sorghum physical map genomic clones on five linkage groups chromosomes found in Phytozome databases (<http://www.phytozome.net/sorghum>). To ensure the specificity of the primers prior to synthesis, e-PCR was performed for all primer combinations. Annealing sites were also confirmed by using BLASTn procedure against the NCBI GenBank database. The primers selected for the study are shown in Table 2. The polymorphism in the primers were designed on presence of bands

in *S. bicolor* and absence of the same in the other members of the sorghum genus.

Real time pcr assay

The RT-PCR was performed on a LightCycler® 2.0 carousel based system in 20 µL final volume. Crop specific primer pairs for loci SB1764, SB3008, SB3420, SB5058 and SBI000X were used in the assay. The PCR reaction was optimized to obtain high end point fluorescence and low cycle threshold or crossing point (Cp) values

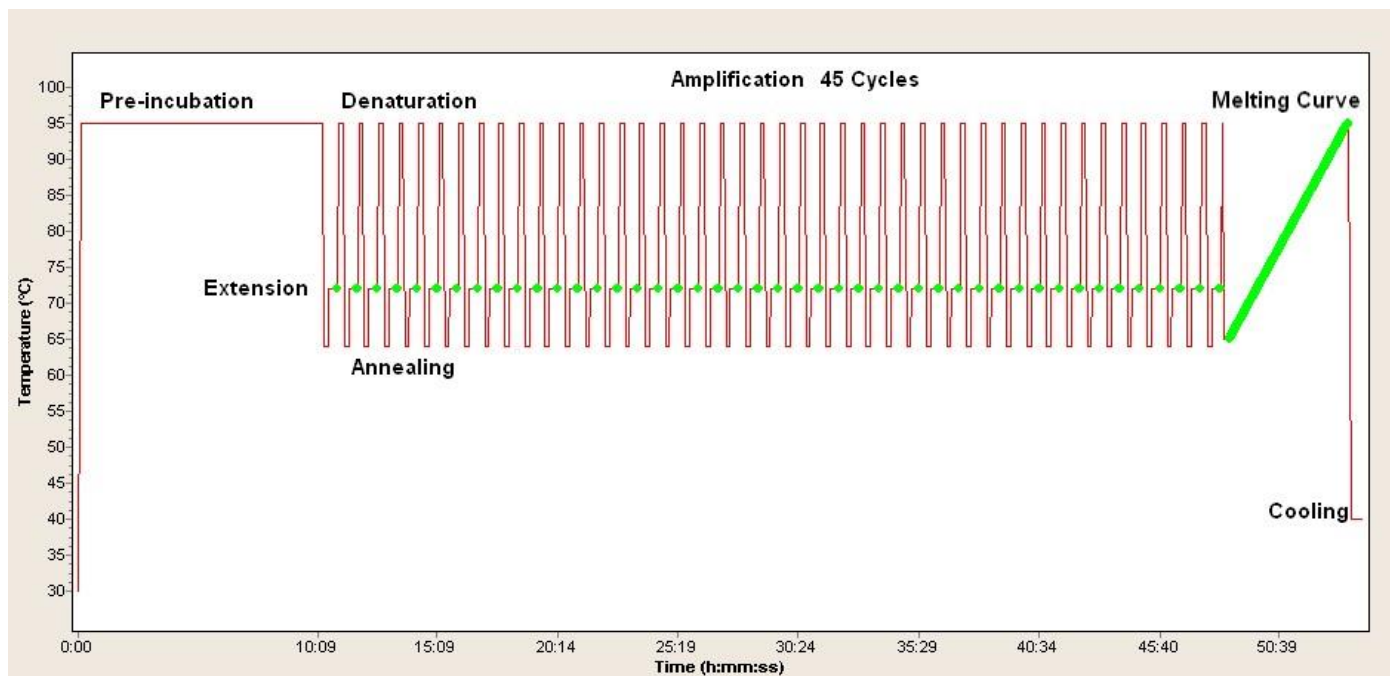


Figure 1. Reaction conditions interface on the LightCycler® 2.0 thermocycler detailing the temperatures and time for each of the stages and steps.

by varying the concentrations of the target primers, template concentrations and reaction conditions. PCR reactions were done in 20 μL of final reaction consisting of 1.5 mM MgCl_2 , 1 μM of primer mix, 1X master mix in LightCycler® 2.0 capillaries. Reaction mixtures were prepared from a LightCycler® Faststart DNA master SYBR green 1 Kit containing 62 μL of reaction mix. Vial 1a was thawed away from light and 10 μL of the enzyme from vial 1b was added. This was mixed gently by pipetting up and down to avoid degrading the enzyme. The Master Mix was shielded from light with aluminium foil and stored in a pre-cooled LightCycler® centrifuge adapters cooling block. A PCR mix for ten samples was prepared with 169.6 μL molecular grade water, 4.4 μL 25 mM MgCl_2 , 1 μL each of primer 1 and primer 1 and 22 μL of the Master Mix.

LightCycler® capillaries were placed in the numbered adapters and 18 μL of the PCR mix pipetted into each of the ten capillaries. 2 μL of the DNA sample was added to the PCR mix and centrifuged at 700 $\times g$ for 5 s. The capillaries were then placed in the numbered slots on the carousel for the reaction. The LightCycler® was programmed to amplify the target DNA for qualitative analysis, quantitative analysis and melting curve analysis through three stages. A pre-incubation stage was set at 95°C for 10 min. The second stage involved quantification through three steps; denaturation was done at 95°C for 10 s followed by annealing at 64°C for 10 s and extension at 72°C for 20 s. A single reading was acquired after this stage through each of the 45 cycles. The third stage was set for a continuous reading at the melting stage 95°C after annealing for 15 s at 65°C. The temperature transition rate/slope/ ramp rate was maintained at 20°C per sec except during melting when it was maintained at 0.1°C per sec. After melting, the reaction was cooled to 40°C for 30 s (Figure 1).

Data analysis

Fluorescence detected at 530nm was monitored at annealing and data were analyzed using the LightCycler® Software 4.x. Crossing

points (CP) were compared and utilized to evaluate the original template concentration. The presence or absence of amplification and the determination of DNA concentration approaches were useful in explaining the presence of crop alleles in wild sorghums. Fluorescence was measured at 530 nm from a SYBR Green background giving a standard amplification curve in the exponential phase, linear and plateau phase. The original template concentration was quantified during the geometric phase by analysis of the threshold cycles or crossing points (CP) cycles values. Melting curve analysis was vital for the determination of the similarity or differences of the RT-PCR products as well as the origin of alleles in the population. Melting peak analysis was also applied in genotyping the populations of wild sorghum from the three counties from Western Kenya. End point analysis was done to estimate the final amount of amplified DNA by the fluorescence emitted and the density of the band on PCR gel electrophoresis. Discriminant analysis was done in GENSTAT 14 (VSN, 2012) to evaluate the differences of populations from different AEZs in the sampled counties. The Mahalanobis (D-Squared) (Mahalanobis, 1930; GENSTAT 14, VSN 2012) test was performed to evaluate differences between subpopulations (groups) based on the crossing point and melting peak values for the wild sorghums from the sampled counties.

RESULTS

The utility of real time PCR in tracking alleles in sorghum populations

Real time PCR amplified fragments from sorghum accessions of crop and weedy backgrounds for polymorphic *S. bicolor* loci (Figure 2). Qualitative detection was done for locus SB3420 in the nine samples, where all samples showed the presence of the allele except in

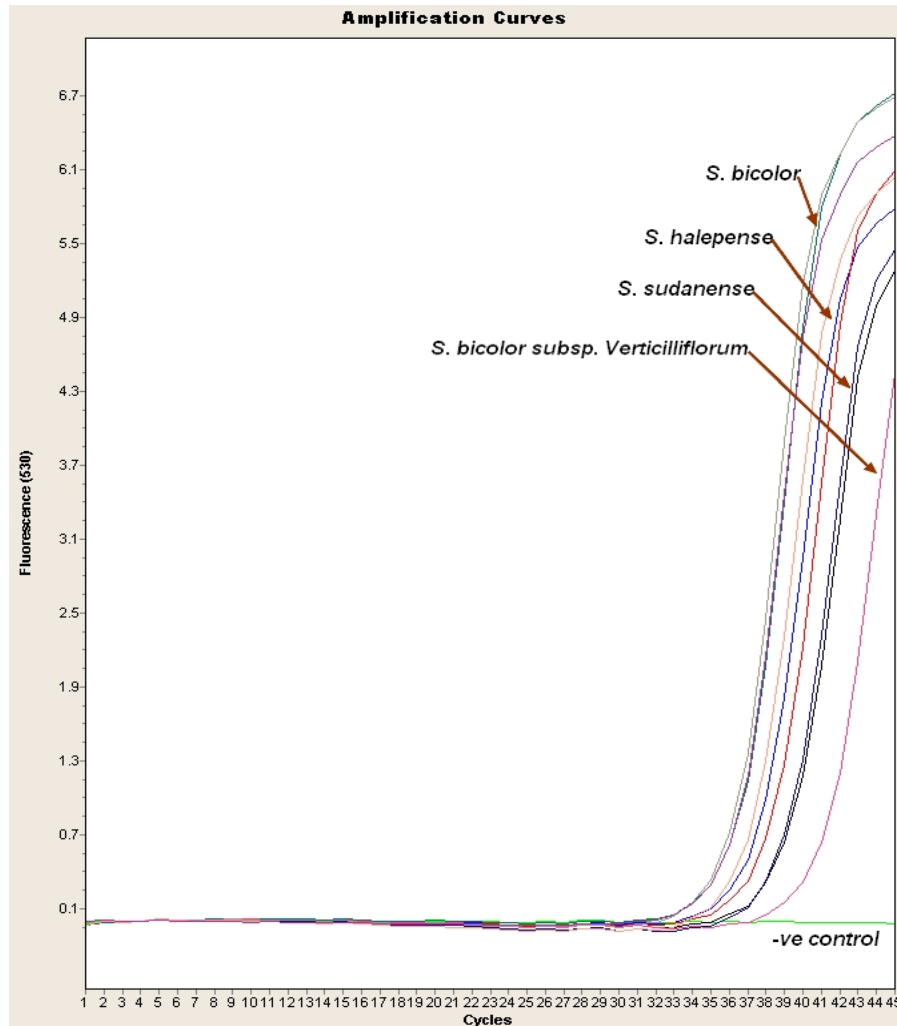


Figure 2. Real Time PCR amplification of locus SB3420 in nine sorghum samples and one negative control containing water.

the case where water replaced the sample to form the negative control (Figure 2). Using loci SB3420, amplification curves were obtained in RT-PCR experiments from templates that were known to possess the primer annealing sequences. In the reaction, *S. bicolor* samples had CP values ranging from 28 to 29 cycles, *S. halepense* had 31 to 32 cycles, *S. sudanense* had 33 to 34 cycles and *S. verticilliflorum* had 34 cycles. Samples from *S. bicolor* had higher template concentration than *S. halepense* and *S. sudanense* (Figure 2) and therefore the emitted fluorescence exceeded the standardized threshold before other species showing had higher CP values.

Quantitative detection of crop loci SB1764 in *S. bicolor* template dilutions

Sensitivity of the RT-PCR was evaluated on *S. bicolor*

templates of different concentrations (Figures 3 a, b). Serially diluted templates in T. E buffer showed differences on the CP values. Locus SB1764 positioned on chromosome 3 was amplified in all *S. bicolor* templates except in the negative control that had water. Differences in CP values among template dilutions (DNA:T.E. buffer) of 1:4, 1:5, 1:6 and 1:7 were less than those exhibited among serial dilutions of $\times 100$, $\times 10^{-1}$, $\times 10^{-2}$, $\times 10^{-3}$, $\times 10^{-4}$ and $\times 10^{-5}$ (Figure 3a, 3b, Table 3). In the reactions the 1:4 template dilutions showed CP values ranging between 19.7 and 22.58 cycles; 1:5 *S. bicolor* template dilutions had CP values of 23.10 to 24.89 cycles. 1:6 template dilutions had CP values of 24.4 to 25.53 cycles. 1:20 template dilution had CP value of 27.56 cycles. Crossing point values varied with DNA concentrations in serial template dilutions. A ten times ($\times 10$) dilution corresponding to 15 ng determined through densitometry showed a CP value of 26.11 cycles; $\times 10^{-1}$ dilution corresponding to 1.5 ng showed a CP value of 30.55

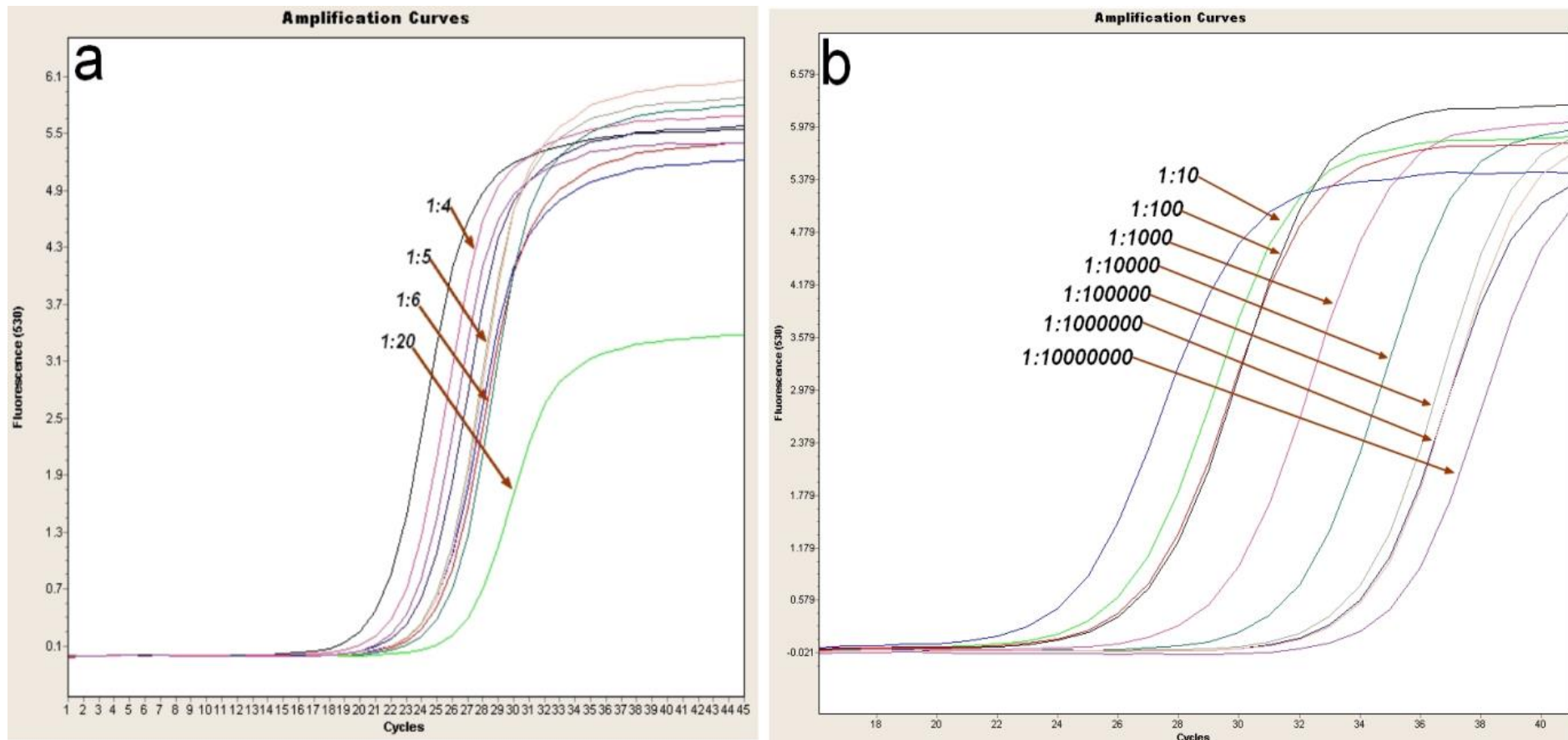


Figure 3. (a) Sensitivity of the Real Time PCR in amplifying crop specific loci in *S. bicolor*. Templates used were three samples of *S. bicolor* diluted at 1:4, 1:5, 1:6, 1:20- in TE. Primers targeting locus SB1764 were used. **(b)** Templates used were serial diluted (x100, x10-1, x10-2, x10-3, x10-4 and x10-5) in TE. Primers targeting locus SB1764 were used.

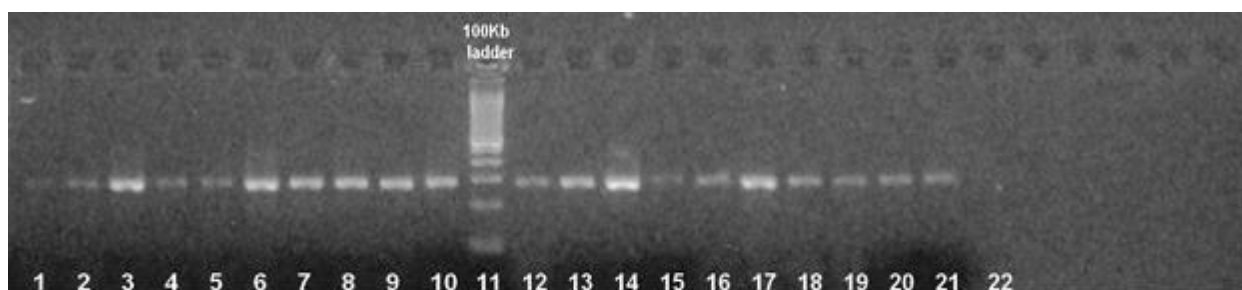
cycles; template dilution of $\times 10^{-2}$ (0.15 ng) had a CP value of 32.70 cycles; while that of $\times 10^{-3}$ (0.015 ng) gave a CP value of 32.53 cycles (Figure 3b, Table 3). Further dilution of the template showed a higher CP value, this was seen when template dilution of $\times 10^{-4}$ (1.5 pg) gave a CP value of 32.87 cycles (Figure 3b and Table

3). A dilution of $\times 10^{-5}$ corresponding to 0.15 pg gave a CP value of 33.84 cycles while a dilution of $\times 10^{-6}$ corresponding to 0.015 pg of DNA had a CP value of 35.90 cycles (Figure 3b and Table 3). *S. bicolor* template concentration was regressed to the CP values to develop a standard curve; $Y = -0.4295X + 32.95$ with an R^2 of 0.929.

End point analysis was performed to determine the fragment sizes amplified on 4% agarose gels. A 300 bp band was recovered from *S. bicolor* sample dilutions ranging from 30 ng to 0.015 pg, except the negative control (Figure 4) Denser bands were seen on agarose gel electrophoresis from samples that had higher starting template

Table 3. Precision of Real Time PCR in detection of sorghum template dilutions

Dilution from ~150ng template	Locus SB1764
	Cp values (Cycles)
1:4 (37.5 ng)	19.7-22.58
1:5 (30 ng)	23.10-24.89
1:6 (25 ng)	24.4-25.53
1:20 (7.5 ng)	27.56
1:10 (15 ng)	26.11
1:100 (1.5 ng)	30.55
1:1000 (0.15 ng)	32.70
1:10000 (0.015 ng)	32.53
1:100000 (1.5 pg)	32.87
1:1000000 (0.15 pg)	33.84
1:10000000 (0.015 pg)	35.90

**Figure 4.** End point validation of amplification, of locus SB1764, showing the expected 300bp fragment from *S. bicolor* accessions using the Real time PCR - Light cycler® 2.0 (Roche). The bands corresponding to concentrated templates (lanes 3, 6, 7, 8, 9, 13, 14 and 17) are denser. Water replaced DNA in lane 22.**Table 4.** Sensitivity of Real Time PCR in detection and characterization of sorghum accessions (*S. bicolor*, *S. halepense*, *S. sudanense* and *S. bicolor ssp. verticilliflorum*).

Loci species	SB1764		SB3420		SB3258	
	CP	TM (°C)	CP	TM (°C)	CP	TM (°C)
<i>S. bicolor</i>	32.96	88.00	30.63	86.01	21.45	81.43
<i>S. sudanense</i>	35.96	84.09	35.42	80.96	20.25	82.24
<i>S. halepense</i>	31.21	84.65	31.96	80.49	22.88	81.56
<i>S. verticilliflorum</i>	32.51	83.92	30.84	80.88	-	-

SB, *S. bicolor*; SS, *S. sudanense*; SV, *S. verticilliflorum*; SH, *S. halepense*; CP, crossing point/threshold cycle; TM, melting temperature (°C).

concentrations (above 1.5 ng) than those that were serially diluted. This validated the PCR amplification and confirmed the effect of template concentration to the product of PCR amplification (Figure 4).

Amplification and melting curve analysis for *Sorghum bicolor*, *Sorghum halepense*, *Sorghum sudanense* and *Sorghum bicolor ssp. verticilliflorum*

S. bicolor, *S. halepense*, *S. sudanense* and *S. verticilliflorum*

samples were evaluated using the RT-PCR technique (Table 4). Three loci SB1764, SB3420 and SB3258 were targeted in the templates and CP values from qualitative evaluation were compared. Analysis of locus SB1764 in *S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum* gave CP of 32.96, 35.96, 31.21 and 32.51 cycles respectively (Table 4). Almost similar CP values were obtained with locus SB3420 in *S. bicolor* (30.63 cycles), *S. sudanense* (35.42 cycles), *S. halepense* (31.96 cycles) and *S. verticilliflorum* (30.84 cycles). Amplification of the SB3258 locus had lower CP

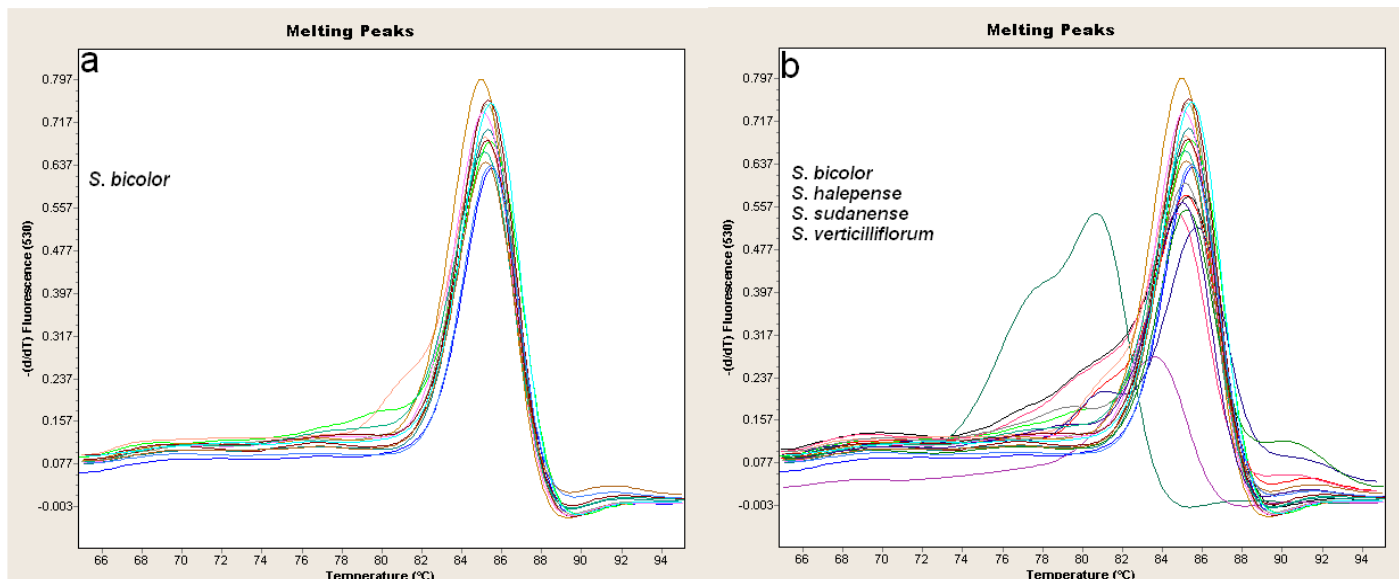


Figure 5. Melting curve analysis for *S. bicolor* templates amplified with primers targeting locus SB3420. A peak was seen at 86°C (a). For weedy sorghum accession templates, variation in melting peaks from 76 to 88°C were seen (b).

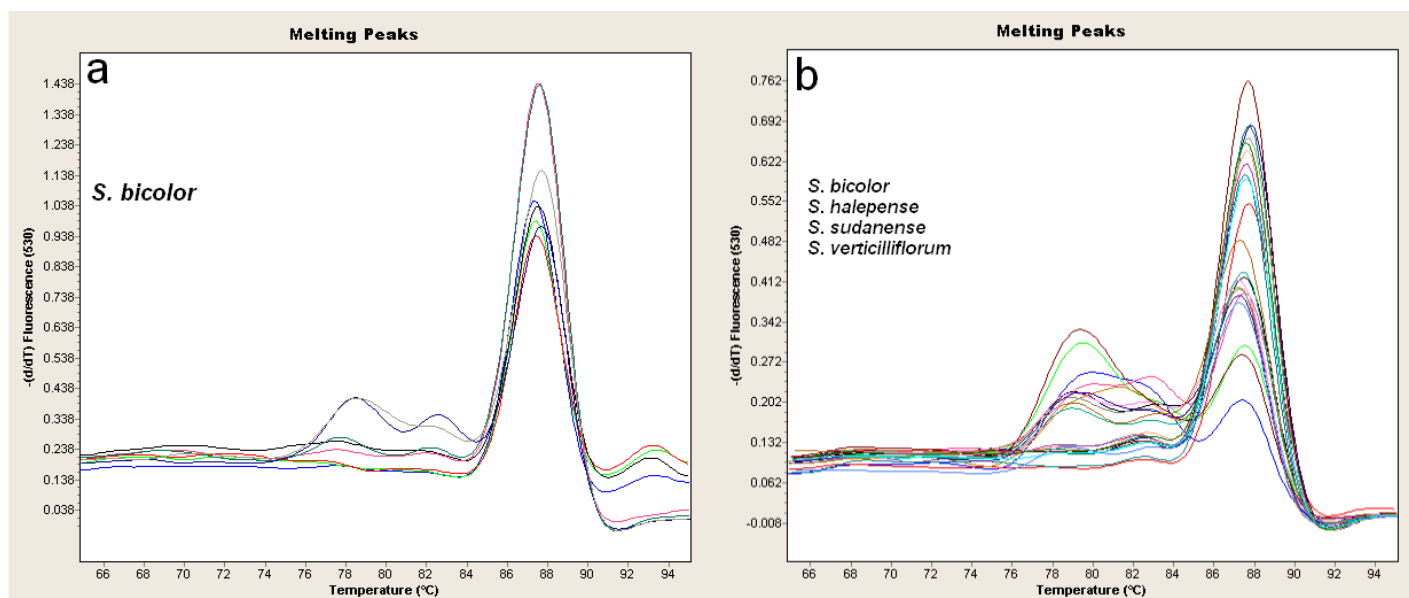


Figure 6. Melting curve analysis for *S. bicolor* templates amplified with primers targeting locus SB1764, showing peak at 88°C (a). For weedy sorghum accession templates, variation in melting peaks from 76 to 88°C were seen (b).

values of 21.45 cycles in *S. bicolor*, 20.25 cycles in *S. sudanense* and 22.88 cycles in *S. halepense* (Table 4, Figure 5). Absolute differences in CP values were attributed to the starting concentration of the template since varying yields were possible for DNA extracted from different samples.

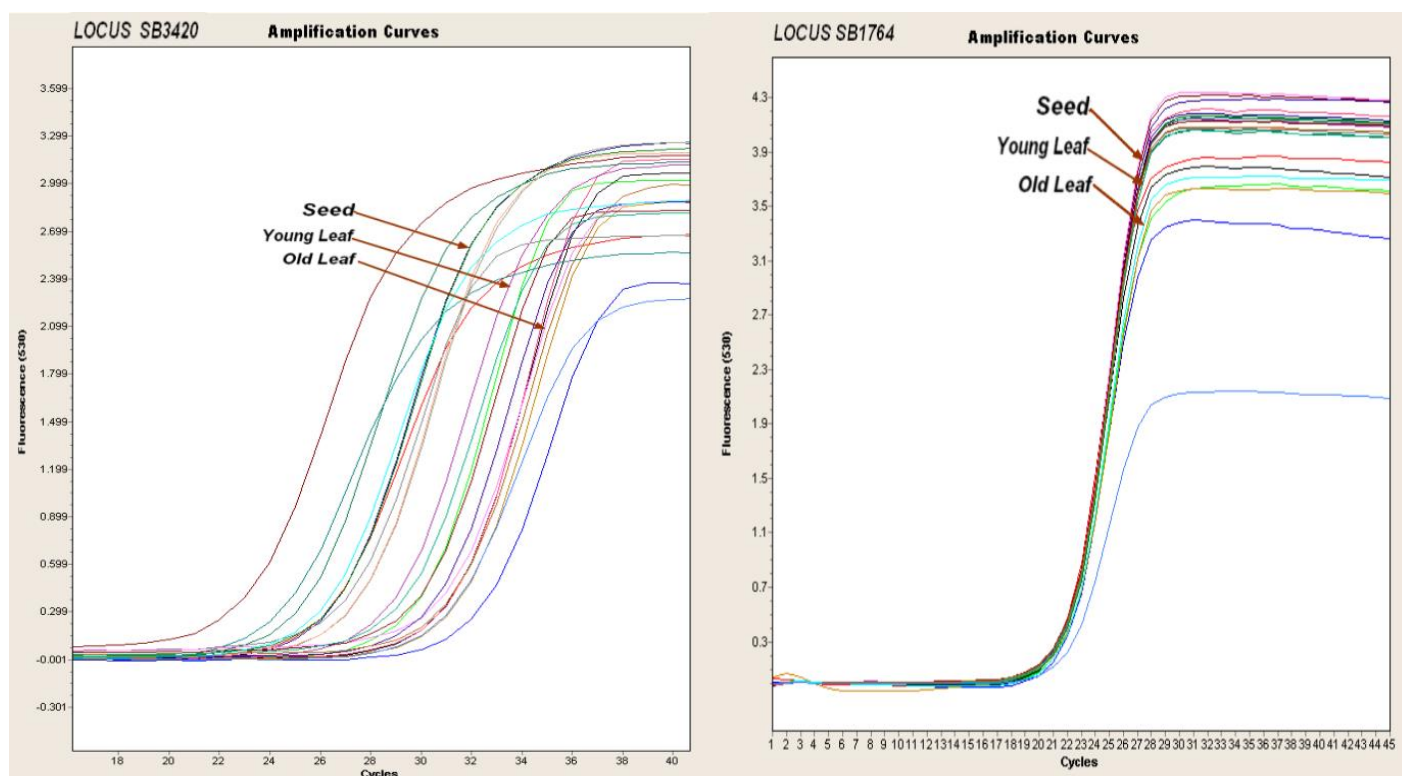
The melting curve analysis showed differences in the three loci of the sorghum accessions (Figure 5, Figure 6

and Table 4). *S. bicolor* had a peak at 88°C for locus SB1764 (Figure 6), 86.01°C for locus SB3420 (Figure 5) and 81.43°C for locus SB3258 (Table 4). *S. sudanense* showed a peak at 84.09°C for locus SB1764, 80.96°C for locus SB3420 and 82.24°C for locus SB3258. *S. halepense* had a peak at 84.65°C for SB1764, 80.49°C for SB3420 and 81.56°C for SB3258. *S. verticilliflorum* had a peak at 83.92°C for locus SB1764 and 80.88°C for

Table 5. Real Time PCR in qualitative determination of crop alleles (SB3420 and SB1764) from different plant tissues (young leaf, seed, old leaf).

Tissue	SB3420					SB1764				
	Species									
	SB	SS	SV	SH	SB	SS	SV	SH		
	CP	TM (°C)	CP	CP	CP	CP	TM (°C)	CP	CP	CP
Young leaf	23.46	85.34	25.74	24.65	21.29	20.51	87.91	20.70	20.81	20.88
Seed	23.62	85.40	24.84	21.73	25.96	20.88	87.57	20.93	20.85	20.84
Old leaf	23.12	85.32	22.13	20.60	23.76	20.81	87.58	20.86	20.91	20.84

SB, *S. bicolor*; SS, *S. sudanense*; SV, *S. verticilliflorum*; SH, *S. halepense*; CP, crossing point/threshold cycle; TM, melting temperature (°C).

**Figure 7.** Real Time PCR amplification curves of crop specific loci (SB3420 and SB1764) from different plant tissues (leaf, seed and old leaf) of twenty sorghum samples.

locus SB3420 (Figure 5, 6 and Table 4).

Analysis of real time PCR efficiency in the qualitative determination of crop alleles (SB3420 and SB1764) from different plant tissues (young leaf, old leaf and seed) of sorghum species

Using RT-PCR, templates from young leaf, old leaf and dry seed were successfully amplified from loci SB1764 and SB3420. Qualitative determination of *S. sudanense*, *S. halepense* and *S. verticilliflorum* tissues yield the expected CP and TM values (Table 8).

Using SB1764 on *S. bicolor* accessions, CP values of 20.51, 20.88 and 20.81 were obtained from young leaf derived template; seed derived template and old leaf derived template respectively (Table 5). The melting temperature peaks were in the expected range from *S. bicolor* of 87.91°C, 87.57°C and 87.58°C from young leaf, seed and old leaf respectively. In *S. sudanense*, CP values of 20.70 cycles, 20.93 cycles and 20.86 cycles were obtained from young leaf seed and old leaf respectively on locus SB1764 (Figure 7).

Amplification of locus SB1764 from *S. verticilliflorum* yield CP values of 20.81 cycles, 20.85 cycles and 20.91 cycles respectively from young leaves, seed and

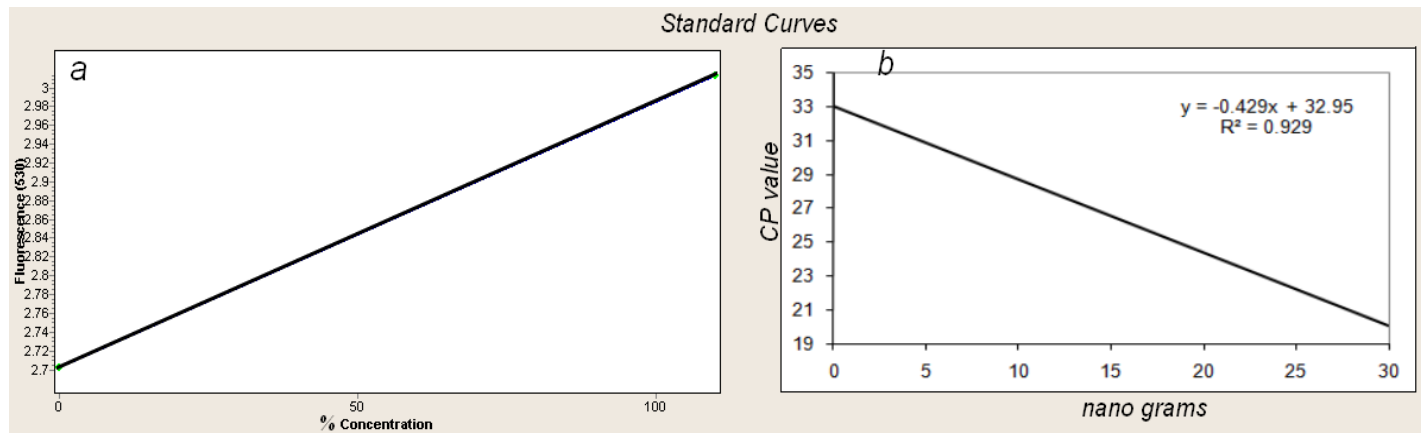


Figure 8. Standard curves showing increase in fluorescence (530 nm) with increase in concentration (a); and increase in CP values (b) with decrease in template concentration in nanograms

Table 6. Real Time PCR in quantitative determination of crop alleles (SB3420 and SB1764) from different plant tissues (young leaf, seed, old leaf).

Tissue	SB3420								SB1764							
	Species															
	SB		SS		SV		SH		SB		SS		SV		SH	
	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng
Young leaf	23.5	22.1	25.7	16.8	24.7	19.3	21.3	27.1	20.5	29.0	20.7	28.5	20.8	28.3	20.9	28.1
Seed	23.6	21.7	24.8	18.9	21.7	26.1	26.0	16.3	20.9	28.1	20.9	28.0	20.9	28.2	20.8	28.2
Old leaf	23.1	22.9	22.1	25.2	20.6	28.8	23.8	21.4	20.8	28.3	20.9	28.1	20.9	28.0	20.8	28.2

SB, *S. bicolor*; SS, *S. sudanense*; SV, *S. verticilliflorum*; SH, *S. halepense*; CP, crossing point/ threshold cycle; ng, nano grams.

old leaves (Figure 7 and Table 5). The expected melting temperatures were obtained confirming species identity. In *S. halepense*, locus SB1764 was amplified from young leaf tissues, seed and old leaf tissues to yield CP values of 20.88 cycles, 20.84 cycles and 20.84 cycles respectively (Figure 7 and Table 5). Seed DNA extraction had higher yield than young leaf and old leaf respectively, showing how best RT-PCR would be suited in evaluation of the two loci from different tissues using the stated protocols.

Locus SB3420 was amplified from *S. bicolor* accessions yielding CP values of 23.46 cycles, 23.62 cycles and 23.12 cycles from young leaf tissues, seed and old leaf tissues respectively (Table 5). The expected melting temperature peaks of 85.34, 85.4 and 85.32°C were obtained from young leaf tissues, seed and old leaf tissues respectively. In *S. sudanense*, locus SB3420 yield CP values of 25.74 cycles, 24.84 cycles and 22.13 cycles from young leaves, seed and old leaves respectively. The locus was also amplified in *S. verticilliflorum* to yield CP values of 24.65, 21.73 and 20.60 cycles from young leaf tissues, seed and old leaf tissues respectively. In *S. halepense* the locus was amplified from the young leaf, seed and old leaf tissues to

obtain CP values of 21.29, 25.96 and 23.76 cycles respectively (Table 5).

Analysis of Real Time PCR efficiency in quantitative determination of crop alleles (SB3420 and SB1764) from different plant tissues of *Sorghum bicolor*, *Sorghum sudanense*, *Sorghum verticilliflorum* and *Sorghum halepense*

The CP values obtained were applied in quantifying the concentration of the original template in *S. bicolor*, *S. sudanense*, *S. verticilliflorum* and *S. halepense* samples. A standard curve demonstrated the increase in fluorescence at 530nm with the increase in the concentration of DNA (Figure 8a). A regression, standard curve of $Y = -0.4295X + 32.95$ with an R^2 of 0.929 was applied to determine the amount of starting template in nanograms (Figure 8b). The results revealed that young leaf tissues had starting template concentrations of between 16.8ng to 27.1 ng based on the data (Table 6) from locus SB3420, seed tissues had concentration of 18.9 ng to 26.1 ng while old leaf had concentrations of between 20.6 ng to 28.8 ng (Table 6). Locus SB1764,

Table 7. Sensitivity of Real Time PCR in detection and characterization of sorghum wild and weedy species sampled from crop sorghum growing AEZs in Western Kenya.

AEZ	SB3420			SB1764			SB3258		
	CP	TM1°C	TM2	CP	TM1°C	TM2	CP	TM1°C	TM2
LM ₁	22.77	82.70	85.32	27.98	87.59	-	20.64	82.20	-
LM ₁	22.28	85.58	-	24.85	87.75	-	21.48	82.11	-
LM ₂	24.69	81.53	85.63	28.56	80.75	87.64	24.59	81.90	-
LM ₂	23.46	81.50	85.34	24.95	87.84	-	23.56	82.33	-
LM ₂	22.11	81.86	85.58	29.84	87.63	-	27.20	81.72	-
LM ₂	20.86	81.44	84.97	29.91	82.31	87.74	24.99	-	-
LM ₂	24.84	85.07	-	30.10	81.59	87.38	22.88	81.56	-
LM ₃	25.67	85.61	-	30.79	81.54	87.61	25.66	81.38	-
LM ₃	25.74	77.71	81.06	24.02	87.84	-	23.64	-	-
LM ₃	21.29	81.10	85.74	25.28	87.95	-	22.92	81.58	-
LM ₃	24.90	81.07	85.35	26.22	87.84	-	21.28	82.18	-
LM ₃	24.00	81.81	85.34	26.18	87.76	-	22.28	81.75	-
LM ₃	22.92	83.53	-	27.62	87.72	-	20.25	82.24	86.97
LM ₃	23.62	85.40	-	23.01	87.65	-	21.68	81.55	-
LM ₃	25.96	85.42	-	21.90	87.79	-	20.95	81.93	-
LM ₄	21.73	80.58	85.28	25.70	87.42	-	21.45	81.43	-
LM ₄	23.76	85.23	-	29.73	87.40	-	20.58	82.31	-
LM ₄	20.60	85.28	90.54	25.45	87.73	-	20.86	82.45	-
LM ₄	22.13	81.11	85.13	29.16	81.11	87.38	20.89	82.60	-
LM ₄	24.00	85.38	-	28.62	80.48	87.45	22.49	81.74	-
LM ₄	23.07	85.28	-	29.90	87.46	-	20.51	82.42	87.33
S.B.	23.12	85.52	-	29.62	87.43	-	20.65	81.47	86.11

LM, Lower midland; S.B., *S. bicolor* +ve control; CP, crossing point; TM, melting peak.

showed concentrations of between 28.1 ng to 29.0 ng in young leaf tissues, 28.0 ng to 28.2 ng in seed and 28.0ng to 28.3ng in old leaf tissue samples. On locus SB1764 starting concentration was determined to be between 28.0 ng to 29.0 ng in all species and tissues. *S. bicolor* had 29.0 ng from young leaf as compared to 28.3 ng in *S. verticilliflorum* and 28.1ng in *S. halepense* (Table 6). Quantitative evaluation determined the starting concentration of template thus gave an indication to the copy number and amount of target DNA in the sample.

Analysis of the spontaneous occurrence of *S. bicolor* alleles in wild populations of sorghum collected from the three counties of Kenya using real time PRC

To analyse the spontaneous occurrence of *S. bicolor* alleles using RT-PCR, loci SB3420, SB1764 and SB3258 were determined in wild populations of sorghum and the melting point analysis for the loci from wild populations showed presense of alleles of both crop and wild origin (Table 7). In addition, analysis of wild sorghums populations from different AEZs showed their distribution in Western Kenya and the contribution of rare alleles from

S. bicolor in these populations (Table 3, 4, 5). The diversity in the populations resulting from evolutionary factors such as polyploidy, mutation and interspecific hybridization could have been important in the different AEZs.

Determination of crop alleles (SB3420, SB1764 and SB3258) from wild populations of sorghum

Presence of crop loci in the wild and weedy sorghum species was evaluated using RT-PCR. Three crop loci SB3420, SB1764 and SB3258 amplified differentially in the weedy sorghum (Figure 9, Table 7) collected from Homabay, Siaya and Busia counties—and from four AEZs (LM₁, LM₂, LM₃ and LM₄). *S. bicolor* (BTX623) were used as a positive control while water was used instead of DNA template to form the negative control. Locus SB3420 in sorghum materials from LM₁ were amplified to yield CP values of 22.77 cycles and 22.28 cycles (Table 7). Weedy sorghums from LM₂ AEZs yield CP values ranging from 20.86 to 24.84 cycles (Table 7). Similar amplifications were demonstrated in materials from LM₃ which gave CP values ranging from 21.29 cycles to 25.96 cycles. Locus SB3420 was amplified from weedy sorghums

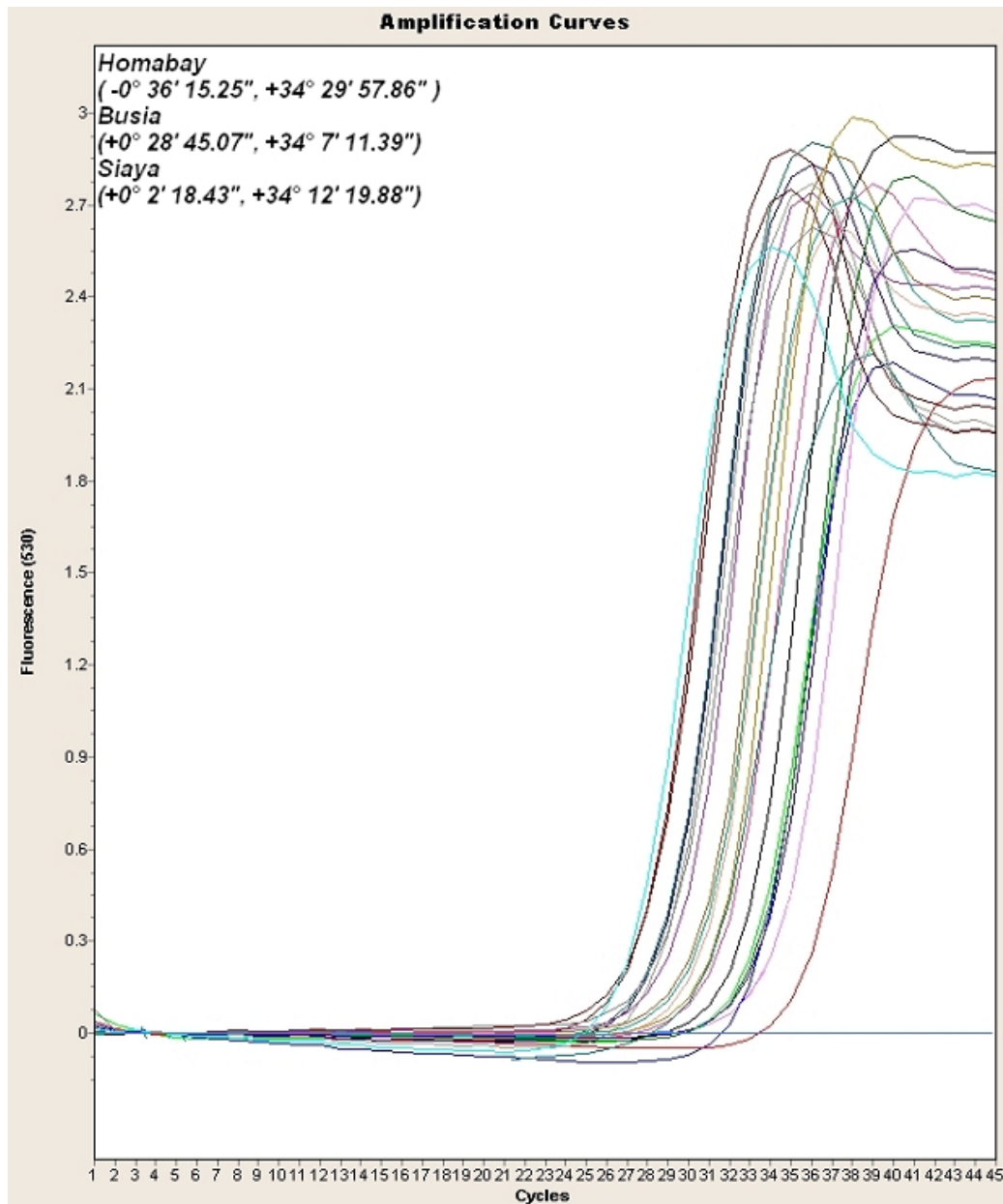


Figure 9. Real Time PCR amplification curves of crop specific locus (SB3258) found in the wild populations of sorghum collected from three counties.

collected from LM₄ zones and CP values ranged from 20.06 to 24.00 cycles (Table 7).

Locus SB1764 was amplified in the weedy sorghums from LM₁ giving CP values of 24.85 to 27.98 cycles, 24.95 to 30.10 cycles in LM₂, 21.90 to 30.79 cycles in LM₃ and 25.45 to 29.90 cycles in LM₄ (Table 7). Locus SB3258 from LM₁ had CP values of 20.64 to 21.48 cycles, sorghum from LM₂, had CP values ranging from 22.88 to 27.20 cycles while those from LM₃ exhibited CP values ranging from 20.25 cycles to 25.66 cycles. Samples from LM₄ showed CP values ranging from 20.51 to 22.49 cycles (Table 7).

Melting point analysis for loci SB3420, SB1764 and SB3258 from wild populations of sorghum

Melting peak analysis showed variations among the weedy sorghum species from the four AEZS; LM₁, LM₂, LM₃ and LM₄ (Figure 10 and Table 7). Real Time-PCR amplification of locus SB3420 gave TM peaks of between 82.70 to 85.58°C from samples in LM₁, between 81.44 and 85.63°C in LM₂, between 77.71 and 85.74°C in LM₃ and peaks between 80.58 to 90.54°C in LM₄. *S. bicolor* had a melting peak of 85.52°C (Table 7).

Locus SB1764 also had important variations among the

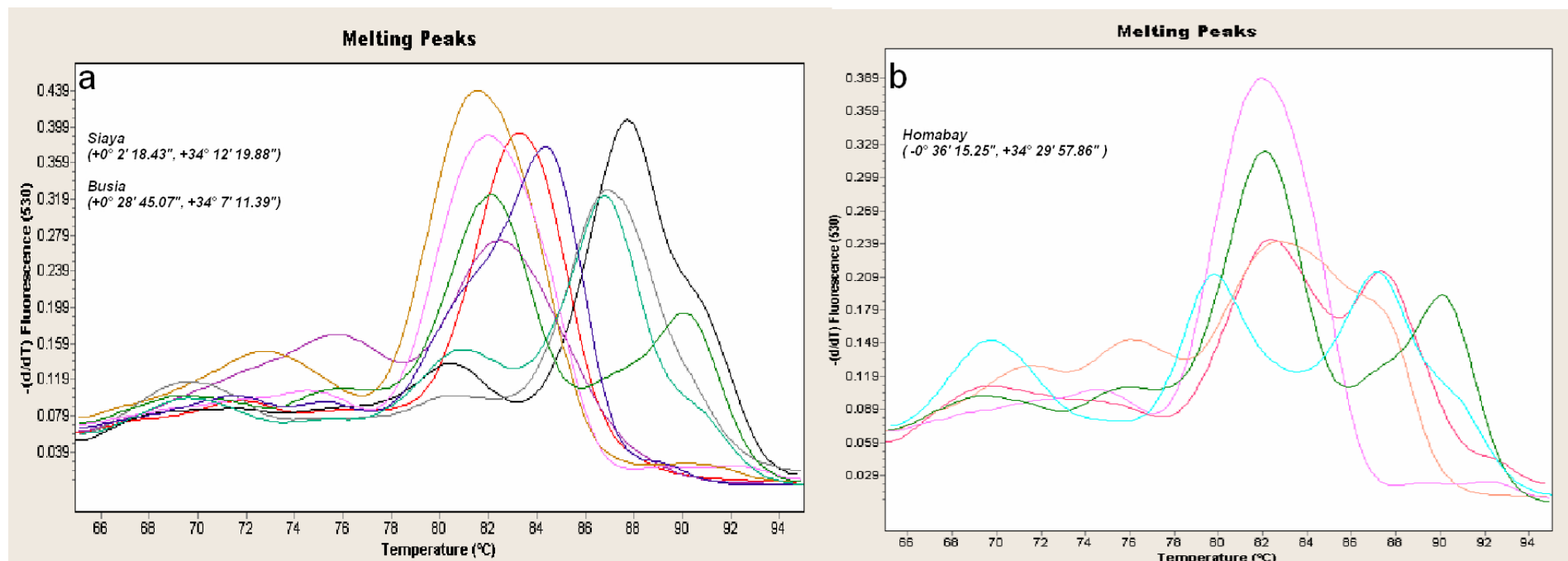


Figure 10. Melting curve analysis for weedy sorghum accession, showing variation in melting peaks from 69 to 92°C on locus SB3258 representing Siaya, Busia (a) and Homabay (b).

samples from different AEZS. Melting peaks of 87.59 and 87.75°C were obtained from LM₁ and in LM₂ peaks ranged between 80.75 to 87.84°C. Similar variability was also recorded in LM₃ where melting peaks ranged between 81.54 and 87.61°C. In LM₄ the weedy sorghums had melting peaks ranging between 80.48 to 87.73°C. *S. bicolor* showed a peak at 87.43°C (Table 7). Locus SB3258 was amplified in all the AEZS to give melting peaks between 81 and 88°C in all samples. *S. bicolor* had peaks at 81.47 and 86.11°C. LM₁ showed peaks ranging between 82.11 to 82.20°C; LM₂ had peaks between 81.56 to 82.33°C. In addition, LM₃ showed peaks between 81.38 to 86.97°C. LM₄ zone species

were analysed to yield peaks between 81.43 to 87.33°C (Table 7, Figure 10).

Inter-population analysis for wild sorghums populations in Sorghum growing AEZs in Western Kenya

Discriminant analysis for the weedy sorghums from LM₁, LM₂, LM₃ and LM₄ AEZs showed distinct population differences (Figure 11). Means of the four populations calculated from CP values and melting peaks were not significantly different. Their 95% confidence intervals showed significant overlaps. Sorghum populations from LM₁, and

LM₂ showed wider variation as compared with those from LM₃ and LM₄.

Low similarities were seen between LM₂ and LM₁ populations (1.3055) on Mahalanobis (D²-) intergroup distance test (Table 8). Midway similarities were recorded between LM₃ and LM₂ (0.5304); LM₄ and LM₂ (0.6104) and *S. bicolor* and LM₂ (0.5099). High similarities were seen between LM₃ and LM₁ (0.193); LM₄ and LM₁ (0.1824); *S. bicolor* and LM₁ (0.2769); *S. bicolor* and LM₃ populations (0.1263). High similarities were seen when LM₄ and LM₃ populations were compared giving a D² value of 0.0865 and when LM₄ populations were compared with *S. bicolor* a D² value of 0.0364 was obtained (Table 8).

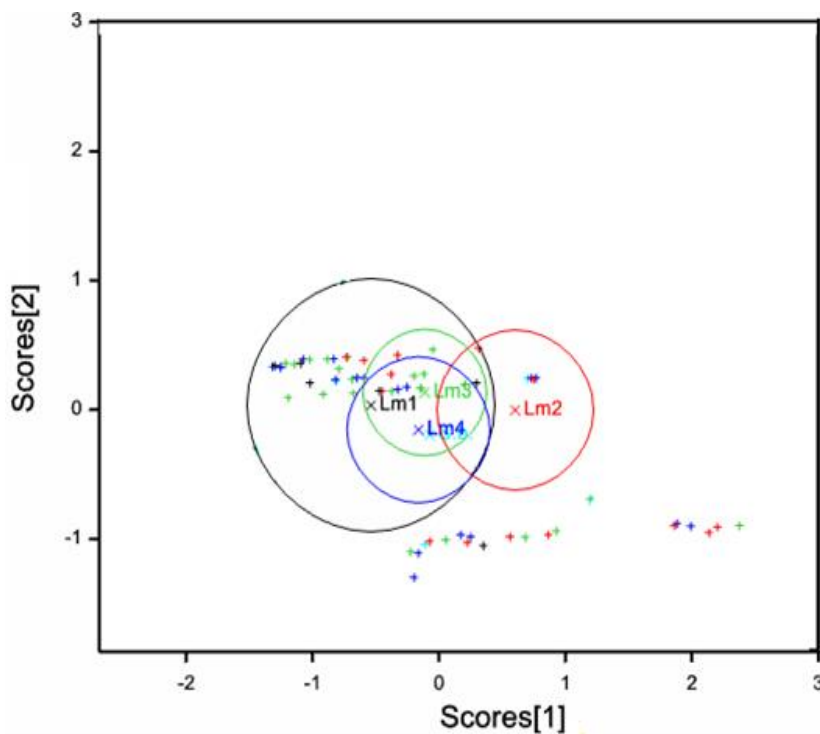


Figure 11. Discriminant analysis for weedy sorghums from LM₁, LM₂, LM₃ and LM₄ AEZs and a control *S. bicolor* (Sb) population based on crossing point and melting peak values. Circles are 95% confidence intervals around population means (N=50).

Table 8. Mahalanobis (D-squared) intergroup distances calculated from crossing point and melting peak values among weedy sorghums from LM₁, LM₂, LM₃ and LM₄ AEZs and a control *S. bicolor* (Sb).

AEZ	LM ₁	LM ₂	LM ₃	LM ₄	S.b.
LM ₁	0				
LM ₂	1.3055	0			
LM ₃	0.193	0.5304	0		
LM ₄	0.1824	0.6104	0.0865	0	
S.b.	0.2769	0.5099	0.1263	0.0364	0

Analysis of rare alleles from *S. bicolor* in wild accessions using Real Time PCR

Rare crop alleles from locus SB5458 in sorghum wild populations were evaluated using RT-PCR. Out of 22 samples the positive control (*S. bicolor*) and two samples from the weedy relatives were amplified to give the expected amplification curves (Figure 12a). Samples that did not have the alleles from the locus showed no amplification and were similar to the negative control. CP values were within the expected range with *S. bicolor* showing a threshold at cycle 23 while the other two samples exhibiting CP values at cycle 24 and cycle 33 respectively (Figure 12a). Melting curve analysis showed similar peaks for all the three samples amplified at 71°C. However the *S. bicolor* peak was three fold to six fold

longer than the other two amplifications from the wild populations (Figure 12b). The negative control and the rest of the wild sorghum samples that did not amplify did not show melting peaks.

DISCUSSION

DNA extracted from young leaf, seed and old leaf was successfully amplified on loci SB1764 and SB3420 using RT-PCR in crop and weedy species. Young leaf and seed DNA samples showed relatively lower CP value ranging from 20.51 cycles to 20.88 cycles as compared to DNA from old leaf that had CP value ranging from 20.81 cycles to 20.91 cycles on locus SB1764 (Figure 7). This was attributed to the low DNA yields from older leaves due to accumulation of compounds that limit DNA isolation. Differences among concentrations (ng) of starting template of *S. bicolor* (2N=20), *S. sudanense* (2N=20), *S. verticilliflorum* (2N=20) and *S. halepense* (4N=40) as determined on loci SB1764 and SB3420 (Table 6) may be influenced by the size of PCR fragment (bp), number of alleles per loci, the ploidy status of the species and the origin of homeologous chromosomes. For instance loci SB3420 yield 580bp in *S. verticilliflorum* and 300bp in *S. bicolor* explaining the higher concentration of 28.8ng in *S. verticilliflorum* as compared to 22.9ng in *S. bicolor*. Young leaves and seeds tissues

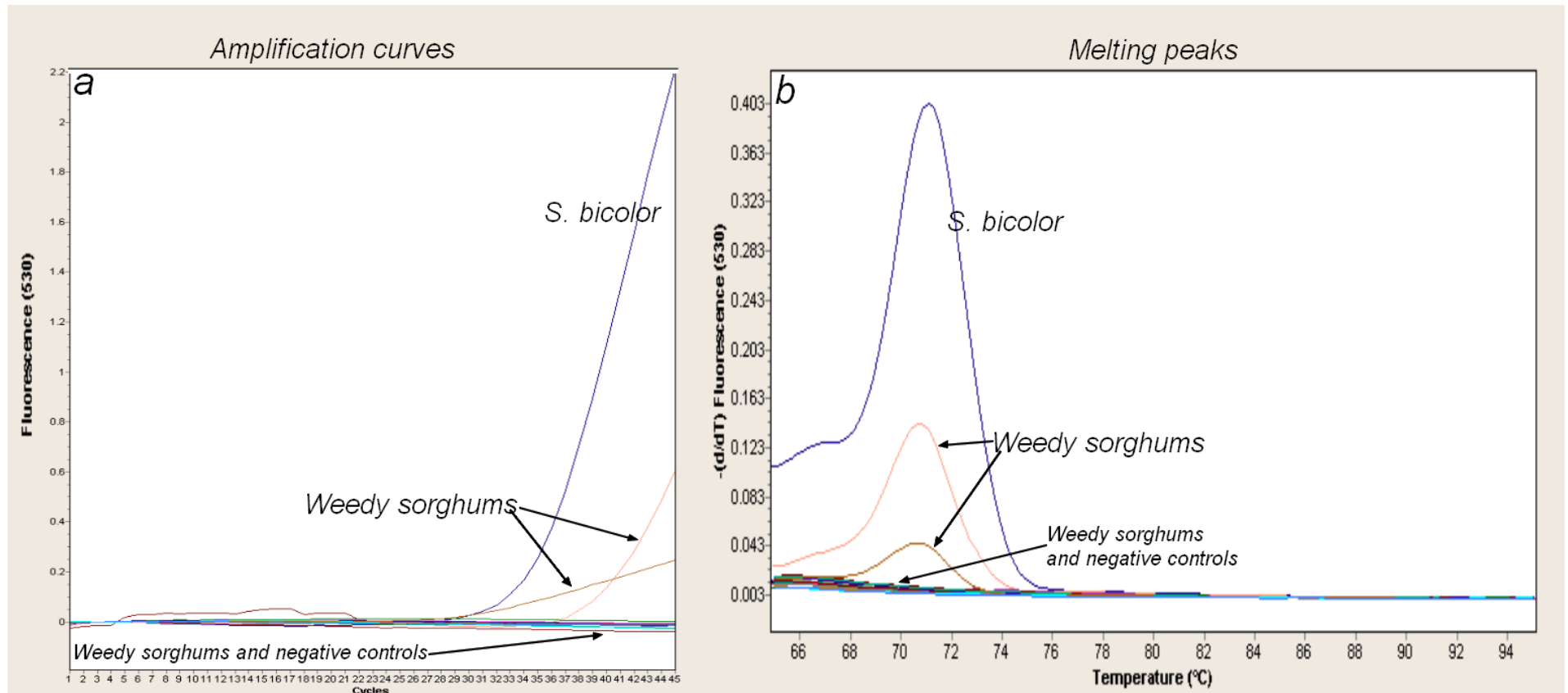


Figure 12. Real Time PCR amplification curves (a) and melting peak analysis (b) of rare crop specific loci (SB5458) from wild and weedy sorghum species showing a melting peak at 71 $^{\circ}\text{C}$.

have previously been used as source of template DNA in RealTime-PCR studies of transgenes in maize and soybean (Hernández et al., 2004, Huang and Pan, 2005). The melting peak analysis demonstrated the allelic specificity of PCR products showing a peak of 87.57 to 87.91 $^{\circ}\text{C}$ on locus SB1764 and 85.32 to 85.40 $^{\circ}\text{C}$ on locus SB3420 in the different tissues. Similar melting peaks per locus irrespective of the tissue sampled from a given species were due to base similarity

of the amplified DNA fragments, which should always occur if the tissues are from the same samples.

RealTime-PCR amplifications were obtained on loci that possessed the specific SSR primer annealing sequences in both crop and weedy accessions. Locus SB3420 was amplified in *S. bicolor*, *S. halepense*, *S. sudanense* and *S. verticilliflorum* and thus RT-PCR was effective in qualitative determination of crop alleles in both

crop and weedy backgrounds for this locus (Figure 2). The loci had different alleles in the wild species that were differentiated through melting analysis. Real Time-PCR also determined absolute and relative quantification of alleles successfully in all the species by comparing the CP values. Templates with lower dilutions in all species had higher CP values than those with higher dilutions (Figure 3a, b). However variations on CP values were observed within and among the species,

For instance in Table 4, *S. halepense* had the least CP value of (31.21 cycles) in locus SB1764 as compared to *S. verticilliflorum* (32.51 cycles), *S. bicolor* (32.96 cycles) and *S. sudanense* (35.96 cycles). This could be due to the polyploidy nature of *S. halepense*, where the homeologous allele has maintained the features of the allopolyploidy segment (Tang and Liang, 1988). Therefore, providing more templates with the primer annealing sites and thus lower CP values. However in locus SB3258 *S. halepense* had a CP value of 22.88 cycles which was higher than *S. bicolor* (21.45 cycles) and *S. sudanense* (20.25 cycles). This locus could be within the allopolyploid segment that may possess different primer annealing sites leading to less amplification and higher CP values. These variations could also be attributed to the original concentration of the template DNA and the length of the PCR product. For instance, the low CP values from *S. verticilliflorum* (30.84 cycles) in locus SB3420 could have been due to higher starting concentrations, slightly different PCR fragment or differences in specificity of primer annealing sites. Previous studies have applied the RT-PCR technique in determination and quantification of alleles in maize and sorghum (Hernandez et al., 2003; Hernandez et al., 2004; Bedell et al., 2005; Pla et al., 2006; Yang et al., 2005).

The melting point analysis procedure was valuable in the determination and discrimination of SSR alleles from different species (Figure 5; Figure 6; Table 4). Evaluation Locus SB1764, SB3420 and SB3258 in *S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum* showed useful and consistent variations in the melting peaks. However, it was difficult to characterize SSR alleles within a species using melting peaks analysis as there was apparent limited variation within the species as compared to the variations among species on the loci studied. These conserved loci would be important while designing effective tools to measure gene flow. Melting temperature ranged between 83.29 to 88°C on locus SB1764 and between 86.01 to 80.88°C on locus SB3420 in the four species. Melting temperature analysis was able to determine the alleles from different species due to the differences in the base composition of the PCR products. Fragments with higher concentration of bases G/C had higher melting temperatures than those with higher A/T concentrations. In addition, alleles with differences in the PCR products melted at higher temperatures. The melting characteristics resulting from conservation of alleles in mutating and evolving species through concerted evolution (Paterson et al., 2009) could have enhanced the ability of the RT-PCR in differentiating sorghum accessions.

The capacity of the RT-PCR to differentiate PCR products and identify primer dimer artifacts by melting analysis has been previously demonstrated (Ririe et al., 1997). Melting point analysis of RT-PCR fragments has also been demonstrated in sorghum *tb2* and *kafarin* genes (Bedell et al., 2005). Hernandez et al. (2004)

applied melting point analysis in evaluating specific quantitative detection of transgenic maize event GA21. Melting analysis has been previously applied in the analysis of products of multiplex RealTime-PCR especially while amplifying distinct loci with specific alleles (Gachon et al., 2004; Hernandez et al., 2003; Huang and Pan, 2005).

Natural occurrence of alleles of crop and wild origin were demonstrated in the wild sorghum populations growing in sympatry with crop cultivars. Crossing point (due to polyploidy in *S. halepense*) and melting point analysis of SSR loci (SB3420, SB1764, SB3258 and SB5458), showed the presence of crop and rare alleles in wild sorghum populations. CP values ranging from 20.25 cycles to 30.79 cycles were observed on wild sorghums from LM₁, LM₂, LM₃ and LM₄ AEZs. The ten cycle difference on the CP values from the AEZs could be due to the polyploidy nature of material of *S. halepense* origin and the differences in original template concentration having an exponential effect on the differences. The difference may also be due to the quality and quantity of the extracted DNA. Melting peaks ranging from 69°C to 92°C were seen on locus SB3258. This demonstrated the presence of alleles from *S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum* with peaks at 88.00, 84.09, 84.65 and 83.92°C. The overlaps in melting point analysis among the species could be attributed to interspecific hybridization. Other uncharacterized alleles were also seen in the wild populations. Rare sorghum alleles were amplified in some members of the wild accessions collected from Western Kenya (Figure 12) showing the sensitivity of the technique and its ability to track exotic alleles in wild sorghum populations. This concurs with previous results by Pla et al. (2006) in which RT-PCR was applied in identifying isolated long distance gene flow from transgenic maize Mon810 into conventional cultivars in the field. Wild sorghum populations from the different AEZs from Western Kenya were differentiated using discriminant analysis and Mahalanobis (D-squared) intergroup distances. Populations from the different AEZs did not show significant differences (Figure 11) which could be due to seed distribution patterns that transcend AEZs. Crop sorghum seed is sometimes contaminated with common weed seed from the sorghum family, such that sharing these seed leads to proliferation of the weedy species. Previous studies by Mutegi et al. (2011) demonstrated that farmers maintained some weedy species in sorghum fields. This would lead to seed mixing and sharing of weedy seeds. Similarities in selective weeding practices also tend to increase the frequency of certain weeds across the AEZs. In addition interspecific hybridization may have lead to the similarities of weedy species across AEZs. However, the intergroup distances showed higher similarities among AEZs around the lake (LM₄ and LM₃), that diminished to show wider differences towards AEZs away from the lake. This could be attributed to lake transport, such that communities around the lake share

more seed amongst themselves than they could with those further away. In addition the conditions around the lake may favour the proliferation of certain weedy species (Farm management hand book of Kenya, 2009).

CONCLUSIONS AND RECOMMENDATIONS

RealTime-PCR was useful in qualitative and quantitative determination of crop alleles in weedy sorghum. Melting analysis discriminated the species based on the allelic differentiation on specific loci. RealTime-PCR of DNA from young leaves, old leaves and seeds was effective in evaluating the presence of specific crop loci in weedy sorghums. Melting peak analysis of SSR loci SB3420, SB1764, SB3258 and SB5458 showed the presence of crop alleles in the wild sorghums. The study shows the potential for use of RealTime-PCR in evaluation of seeds and other plant materials in transit at entry points and on populations of growing plants in fields of cultivated and weedy sorghums for exotic genes including transgenes. In addition, the technique can be applied to analyze the number of copies of specific exotic genes in the target or non-target populations. Expression and transcription of such genes in the target or non target plants can also be quantified using RT-PCR.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Polyamines and WOX genes in the recalcitrance to plant conversion of somatic embryos of Habanero pepper (*Capsicum chinense* Jacq.)

Raúl Enrique Valle-Gough¹, Susana Alejandra Avilés-Viñas¹, Stephanie López-Erosa, Adriana Canto-Flick¹, Eunice Gómez-Uc¹, Luis Alfonso Sáenz-Carbonell², Neftalí Ochoa-Alejo³ and Nancy Santana-Buzzy^{1*}

¹Centro de Investigación Científica de Yucatán. Unidad de Bioquímica y Biología Molecular de Plantas. Calle 43 # 130, Chuburná de Hidalgo. 97200. Mérida, Yucatán, México.

²Centro de Investigación Científica de Yucatán. Unidad de Biotecnología. Calle 43 # 130, Chuburná de Hidalgo. 97200. Mérida, Yucatán, México.

³Departamento de Ingeniería Genética de Plantas. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. (CINVESTAV-Irapuato). Km 9.6 Libramiento Norte Carretera Irapuato-León, Gto, México.

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In order to determine the role of polyamines in the formation and development of the somatic embryos of *Capsicum chinense*, the effect of different concentrations (0, 0.01, 0.1, and 1.0 mM) of Putrescine, Spermidine and Spermine on the efficiency and morphology of the embryos was evaluated. The results show that none of the three polyamines evaluated had a significant effect on the number of embryos formed, except Spermidine (1 mM), which caused a significant reduction in their numbers, in comparison with the control treatment. However, the most noteworthy result was observed in the treatment containing 0.1 mM of Spermine. The embryos developed in this treatment showed harmonic apex-radicle development, pale-green coloration and the formation of two tiny cotyledonary leaves. Real-time PCR was used to analyze the differential expression of the *WUS*, *WOX1* and *WOX3* genes in somatic embryos treated with Spermine and untreated, including the zygotic embryo. The transcript levels of the genes analyzed were found to differ significantly between both types of embryos (somatic and zygotic), with the zygotic embryos presenting a higher level of transcripts; however, compared to the untreated somatic embryos, the somatic embryos treated with Spermine showed an increase in the transcript levels of the three genes analyzed (*WUS*, *WOX1* and *WOX3*); the *WOX1* gene in particular presented an accumulation pattern similar to that of the zygotic embryo of the species.

Key words: Somatic embryos, zygotic embryos, polyamines, transcript patterns and morphology.

INTRODUCTION

Somatic embryogenesis (SE) is defined as a process in which a bipolar structure develops from a non-zygotic cell without vascular connection with the origin tissue (Merkle et al., 1995). As somatic embryos (SEs) are formed

without any fertilization event they are genetically identical to the parent tissue and are therefore clones. Irrespective of the mode of production, the anatomical and physiological features of SEs are highly comparable

to zygotic embryos (ZEs) (Bandyopadhyay and Hamill, 2000; Deo et al., 2011; Karami et al., 2009; Karami and Saidi, 2010; Kumar-Sharma and Millam, 2004; Kumar-Sharma et al., 2008). The morphogenetic potential of *Capsicum* sp. has been reported earlier, but the process of regeneration is slow and generally produces low frequencies of shoot regeneration, thus it has been found to be highly recalcitrant (Heidmann et al., 2011; Kothari et al., 2010; Us-Camas et al., 2014). Development of a specific body plan during embryogenesis requires the coordination of cell fates according to their positions along the embryo axes (Breuninger et al., 2008). The shoot apical meristem (SAM) and cotyledons of higher plants are established during embryogenesis in the apex (Jurgens et al., 1995). The SAM is formed in the apex between cotyledons in dicotyledonous plants and generates stems, leaves, and floral organs in a set pattern (Angenent et al., 2005; Ueda and Laux, 2012). Thus, SAM formation during embryogenesis is a critical step to start subsequent vegetative and reproductive development (Aida et al., 1999; Mayer et al., 1998; Taiz and Zeiger, 2002; Takeda and Aida, 2010). However, fully functioning apical shoot meristems and dicotyledonous somatic embryos are rarely produced in the *Capsicum* sp. (Steinitz et al., 2003).

Defective somatic embryos have been reported in embryogenic cultures of different species (Halperin, 1966; Merkle et al., 1995; Schiavone and Cooke, 1987). Three categories of malformations have been described for somatic embryos of different species: fused embryos (Carraway and Merkle, 1997; Rodriguez and Wetzstein, 1994; Stipp et al., 2001); altered cotyledon morphology (Carraway and Merkle, 1997; Jayasankar et al., 2002); and lack of a shoot (abnormal apical meristem histodifferentiation) (Chengalrayan et al., 2001; Jayasankar et al., 2002; Stipp et al., 2001). High frequencies of these abnormalities and the most critical defect, the absence of a shoot in the regenerants have been reported in the *Capsicum* sp. (Lopez-Puc et al., 2006; Steinitz et al., 2003). The absence of a functional shoot and the embryo incapacity to convert into plants is how the recalcitrance in *Capsicum* sp. manifests (Aboshama, 2011; Heidmann et al., 2011; Kothari et al., 2010; Us-Camas et al., 2014).

Polyamines (PAs) are natural occurring compounds in eukaryotic cells and are essential for several processes such as growth and differentiation (Kevers et al., 2000; Kumar et al., 2007; Silveira et al., 2006; Steiner et al., 2007; Takeda et al., 2002; Yadav and Rajam, 1998). In plants, polyamines have been implicated in stress response

response, fertilization, senescence, organogenesis and somatic embryogenesis (Kakkar et al., 2000; Slocum, 1991; Takahashi and Kakehi, 2010). The continual synthesis of PAs is an essential process during the SE for the differentiation and development of the embryos (Kakkar et al., 2000). Previous studies have shown that high concentrations of PAs are found in embryos after auxin removal, also the use of PAs biosynthesis inhibitors inhibits the SE process and the addition of PAs precursors increases the number of somatic embryos (Bais and Ravishankar, 2002). Other studies have stated that the differential distribution of PAs in cell lines are related to the embryogenic capacity, where lines with high levels of PAs showed an increase in the embryo formation (Kakkar et al., 2000; Takahashi and Kakehi, 2010; Yadav and Rajam, 1998). It has been suggested that the role of the PAs in SE is due to its polycationic nature, these compounds associates to molecules such as membranes and nucleic acids, exerting a protective role against free radicals and regulating the gene transcription (Ahmadi et al., 2014; Bais and Ravishankar, 2002; Kakkar et al., 2000; Karami and Saidi, 2010; Takahashi and Kakehi, 2010).

Extensive reprogramming of gene expression accompanies the transition from somatic cells into embryogenic competent cells in response to inductive signals. Extensive effort has been focused on the identification of "master" genes required for this, although it is now apparent that the induction of the embryogenic pathway is not governed by a single gene, but is under the control of an intricated genetic network. Recent studies have revealed that the transcript accumulation of WUSCHEL-Homeobox (*WOX*) genes determines apical and basal dominions in different species (Gambino et al., 2011; Haecker et al., 2004; Lin et al., 2013; Palovaara et al., 2010 a,b; Vandenbussche et al., 2009; Van der Graaff et al., 2009; Zhang et al., 2011). One of the early expressed genes is WUSCHEL (*WUS*) which maintains a reservoir of undifferentiated cells in the SAM and its abnormal expression produces plants with discontinual growth and a compromised SAM identity (Gambino et al., 2011; Haecker et al., 2004; Ji et al., 2010; Stuurman et al., 2002; Su et al., 2009). Other genes of this family such as *WOX1* and *WOX3* are involved in the cotyledon determination; *WOX1* is expressed in the central part of the cotyledon primordia meanwhile *WOX3* is expressed earlier in the peripheral area of the cotyledon (Hacker et al., 2004; Vandenbussche et al., 2009). Mutants of *WOX1* and *WOX3* shows similar phenotypes like: dwarf

*Corresponding author. E-mail: buzzy@cicy.mx.

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Abbreviations: SE, Somatic embryos; ZEs, zygotic embryos; SAM, shoot apical meristem; WUS, WUSCHEL; WOX, WUSCHEL-Homeobox; PAs, polyamines; Put, spermidine; Spd, spermdine; Spm, spermine; MS, Murashige and Skoog basal medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA₃, gibberellic acid.

plants, reduced apical meristems and abnormal leaf morphology (Lin et al., 2013; Nakata et al., 2012; Vandebussche et al., 2009; Zhang et al., 2011)

The aim of this study was to evaluate the effect of polyamines on the efficiency and morphology of the somatic embryogenesis in *Capsicum chinense*, and characterize the most frequent deformations in the somatic embryos of this species. The differential expression of *WUS*, *WOX1* and *WOX3* genes was analyzed through real-time PCR in zygotic embryos and in somatic embryos (with and without PAs) in the early and advanced stages of their development.

MATERIALS AND METHODS

Plant material

Orange Habanero pepper seeds (*C. chinense*) var. Mayan Ba'alche (2367-CHL-021-080110/C) were surface sterilized following the protocol reported by Santana-Buzzy et al. (2005). To induce seed germination, MS (Murashige and Skoog, 1962) medium supplemented with sucrose (3% w/v), Gelrite (0.22% w/v) and 1.14 μM GA₃ was used. The pH of the medium was adjusted to 5.8 prior to sterilization, which was performed in an autoclave at 121°C and 1.5 atm for 15 min. Once the seeds were placed on the germination medium, the plates were incubated in constant darkness for 14 days at 25 ± 2°C. The plantlets were then transferred to a growth chamber under a 16 h photoperiod with light intensity of 40 to 50 mol m⁻² s⁻¹ at 25 ± 2°C, for 7 days.

Induction of somatic embryogenesis

The protocol reported by Avilés-Viñas et al. (2013) was used to induce and obtain somatic embryos. Hypocotyl segments (0.5 cm) measuring in length from aseptic plantlets were transferred onto MS medium containing 9.05 μM 2,4-D and then incubated at 25 ± 2°C, under constant light for 28 days. A total of 10 hypocotyls were transferred to flasks containing 50 ml MS medium supplemented with 4.5 μM 2,4-D and also various levels of putrescine (Put, 0, 0.01, 0.1 and 1 mM), spermidine (Spd, 0, 0.01, 0.1 and 1 mM) or spermine (Spm, 0, 0.01, 0.1 and 1 mM) for 15 days. The embryos formed were then subcultured in the same differentiation medium, without PAs, where they remained for 30 days, until completing their development. All the liquid cultures were kept in constant agitation (100 rpm), under continuous light at 25 ± 2°C. Only the treatments that showed SEs with a morphological improvement or increased efficiency were used for further analysis. Three flasks were used for each treatment and the efficiency was evaluated by taking three samples of 10 ml from each flask containing the embryo culture. The embryos were counted with the aid of a stereoscopic microscope (Nikon SMZ800). The data were submitted to variance analysis (ANOVA) and for the comparison of means; the Tukey test ($P \leq 0.05$) was used with the SPSS 16.0.0 software.

Histological analysis

Samples of embryos were fixed in FAA (37% formaldehyde: acetic acid: 96% ethanol, v/v/v) taken to a final volume of 100 ml with distilled water, from 24 to 48 h at 10°C. The procedures of dehydration, embedment and section were performed according to the method of Berlin and Miksche (1976). The sections were transferred on glass slides, deparaffinated with Ultraclear (Baker) and stained with tholuidine blue (0.05%) for 5 min.

Scanning electron microscopy

For the preparation of samples, somatic embryos in cotyledonary stages and mature zygotic embryos were selected. Samples of both types were submerged in a solution of glutaraldehyde (25%) and a buffer of cacodylate (3%, 0.1 M, pH = 7.2), followed by three rinses with same buffer, for 30 min each one. Dehydration was achieved by gradual exposure of the samples to different solutions of ethanol (50, 70 and 90% v/v), 1 h for each solution. The dehydrated explants were then submerged in absolute alcohol for 1 h and dried in a Sandri-795 Tousimis drier, substituting the ethanol with liquid CO₂ and finally with gaseous CO₂. Finally, the samples were metalized with a gold layer 21 nm thick using a Denton Vacuum Desk II metalizer. The metalized samples were observed using a JSM-6360LV JEOL scanning electron microscope.

RNA extraction and cDNA synthesis

Samples (1 g) were taken from: immature zygotic embryos (IZE), mature zygotic embryos (MZE), induced hypocotyls (IH), somatic embryos in early stages of development: globular-heart stages (GH), and somatic embryos in advanced stages: torpedo-cotyledonary stages (TC), in both cases, treated and untreated with 1 mM Spm. Extraction of total RNA was performed following the protocol reported by Chomczynsky and Sacchi (1987). The samples were treated with DNase (Ambion) and the concentration was quantified in Nanodrop (Thermo Scientific). The cDNA synthesis was carried out using the Oligo-dT primer (Invitrogen) with the SuperScriptIII enzyme (Invitrogen), following the manufacturer's instructions. RNA integrity was observed in agarose gel (1.5%) stained with ethidium bromide.

Primer design and PCR conditions

WUS (NM_001247086.2), *WOX1* (XM_004223575.1) and *WOX3* (XM_004251238.1) primers were designed from homolog sequences of *Solanum lycopersicum*, available at the NCBI. The sequences were aligned using the MEGA v 6.0. program and the primers were designed with the Primer3plus program (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>). The constitutive genes previously reported in *C. annuum* (Wan et al., 2011): Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ubiquitin 3 (UBI-3) and beta-Tubulin (β -Tub) were considered to be used as normalization genes after the analysis of gene stability in the geNorm (<http://medgen.ugent.be/~jvdesomp/genorm/>) software. Relative expression was processed with the method of Ct ($2^{-\Delta\Delta Ct}$) (Livak and Schmitgen, 2001), in a StepOne thermal cycler (Applied Biosystems). The real-time PCR reaction mixture comprised the following components at a final volume of 20 μl containing: 10 μl of SYBR-GREEN Master Mix (Applied Biosystems), 0.12 μM of each primer and 4 μl of cDNA. Reaction conditions were 94°C (3 min); 40 cycles of 94°C (15 s), 55 to 65°C (30 s) varying the alignment temperature for each primer (Table 1). The PCR was performed in six replicates per sample using three biological samples. The results were submitted to ANOVA and the means were compared with the Tukey test ($P \leq 0.05$) using the SPSS 16.0.0 software.

RESULTS AND DISCUSSION

Effect of polyamines on the efficiency and developmental morphology of embryogeny

The addition of Put and Spm to the culture media had no significant effect on the number of embryos formed in

Table 1. List of primers used for real time PCR.

Gene	Primer sequence	Tm	Fragment
<i>WUS</i>	F:5'-AGGTCTCCAAGTCTGAACA-3' R:5'-CACGAGCTTTATGGTTCTGAAA-3'	60°C	110 bp
<i>WOX1</i>	F:5'-TGCAACAAAGAAGGAGCCTACTAG-3' R:5'-TTCCATAGTTCTTGGTGTGATGGAC-3'	65°C	195 bp
<i>WOX3</i>	F:5'-AACAAAGGTGGAGTCCAACCC-3' R:5'-CCCTCAATTTTCCCCTATCC-3'	60°C	192 bp
<i>GAPDH</i>	F:5'-ATGATGATGTGAAAGCAGCG-3' R:5'-TTTCAACTGGTGGCTGCTAC-3'	55°C	276 bp
<i>UBI-3</i>	F:5'-GAGGGTGAGTGAGCAGTTC-3' R:5'-CTTCATCGTCATCTGCTGTC-3'	55°C	167 bp
β - <i>TUB</i>	F:5'-TGTCCATCTGCTCTCTGTTG-3' R:5'-CACCCCAAGCACAATAAGAC-3'	55°C	204 bp

the treatments evaluated (Figure 1A to C), while the addition of Spd causes a marked decrease in the efficiency (somatic embryos per litre) along with the increase of the Spd concentration, with significant differences when compared to the control treatment (Figure 1B). However, the addition of 0.1 mM Spm improved the embryo morphology. In the Figure 2A we observe germinated somatic embryos treated with 0.1 mM Spm; different stages of development were observed before germination (Figure 2B and C). Under these culture conditions (0.1 mM Spm) it was observed, for the first time, a harmonic apical-radical development and the emission of two tiny pale green cotyledonary leaves (Figure 2C and D), although these embryos did not subsequently evolve into plants due to a deformed shoot apex. It is likely that this change in coloration of the embryo could be attributed to the fact that PAs have been detected in vacuoles, mitochondria and chloroplasts (Slocum, 1991). Borrell et al. (1995) also detected these compounds in spinach thylakoid membranes, associated with light-reception complexes of the photosystem II. They concluded that the PAs maintain photosynthetic activity, thus preventing senescence due to osmotic stress.

Independently of the applied treatment, the embryos always emerged through the epidermis of the explant (Figure 3A, B and C) originating in the perivascular zone of the hypocotyl. The first embryos (globular-stage) were visible approximately 12-15 days after induction culture (Figure 3C and D), followed by heart, torpedo and cotyledonary embryo stages (Figure 3E, F and G) through an elongated shape and showing both poles clearly: shoot and root, with no apparent morphological differences observed among the treatments during the first stages of development (Figure 3D and E). Shoot meristem differentiation usually begins at the late globular stage and structurally organized meristems are visible at the heart or cotyledonary stage of somatic embryo development (Taiz and Zeiger, 2002). However, the evolution from

torpedo stage to cotyledonary stage failed in the majority of the embryos developed (Figure 3F and G), indicating a deformed shoot apex, incapacitated for subsequent germination.

The most frequent deformations in the shoot meristem were manifested either by the absence of cotyledons (Figure 4A and B) and deformed cotyledons (Figure 4D and 4E). Embryos with exposed shoot apical area were also observed (Figure 4C and F). All of these abnormalities incapacitated the somatic embryos to germinate and subsequently convert into plants. Our results corroborate those reported by Steinitz et al. (2003), who attributed this incapacity of *C. annuum* embryos for plant conversion due to the absence of a shoot, resulting in failure for the establishment of a normal functioning shoot meristem. The radical apex of the SEs always formed normally (Figure 4A, 4B, 4D and 4E) and radicle emission occurred spontaneously in most of the embryos formed. In contrast with the SEs, it was possible to observe the formation of well-defined cotyledons in the ZEs (Figure 5A and 5D), showing both meristems, apical and radical (Figure 5B, C and E) and the formation of provascular bundles along the apical-basal axis (Figure 5B).

The addition of PAs favored embryogenic responses in different species (Ahmadi et al., 2014; Kevers et al., 2000; Paul et al., 2009; Steiner et al., 2007; Yadav and Rajam, 1998). In *Hevea brasiliensis*, the addition of PAs increased the amount of embryogenic callus and the number of embryos per gram of callus using 50 μ M Spd (El-Hadrami et al., 1989a, b) and also in *Brassica napus* the addition of 2.5 μ M Put for 48 h increased three-fold the embryo formation and 5.56 μ M Put increased four-fold the embryo conversion (Ahmadi et al., 2014). PAs have also been found to reduce the responses of embryogenic cultures. Silveira et al. (2006) observed a reduction in the growth of *Araucaria angustifolia* suspension cultures when Spd and Spm (1.0 mM) were added to the culture media. Working on *Coffea canephora* and

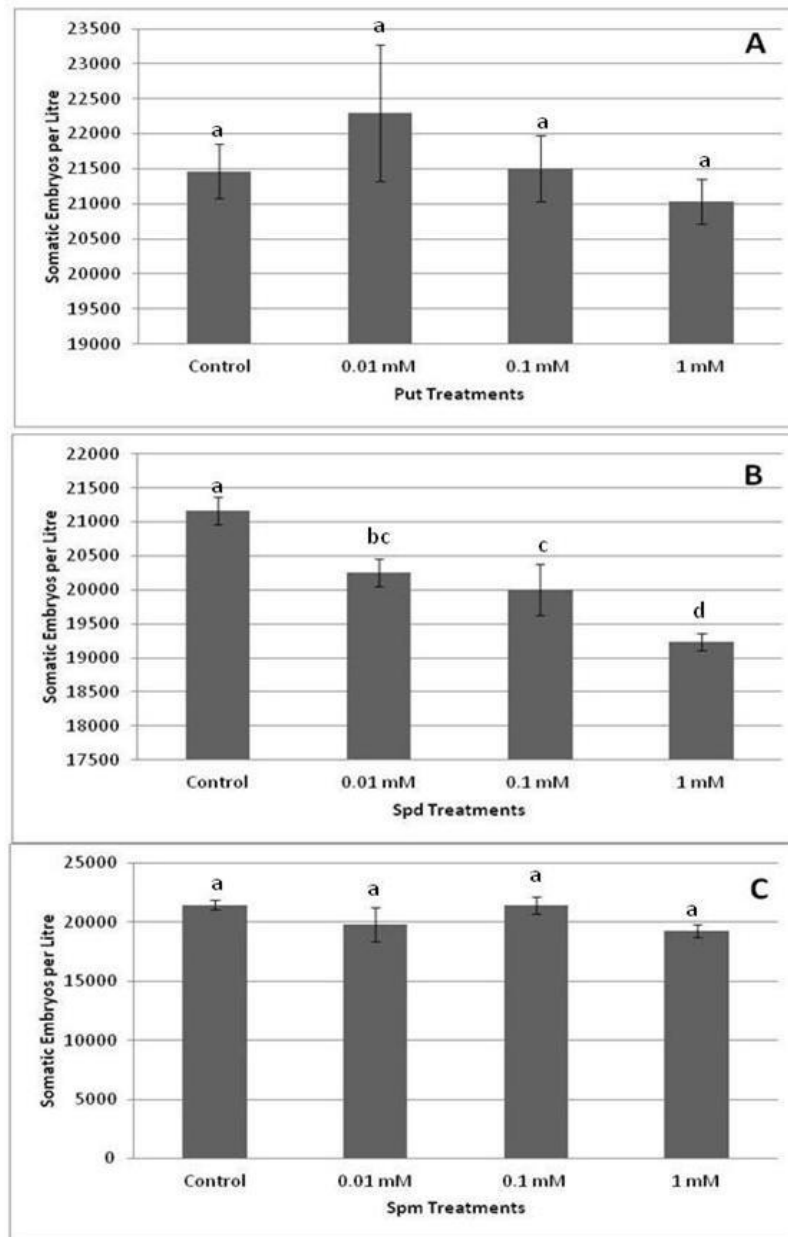


Figure 1. Effect of polyamines on somatic embryogenesis efficiency of *Capsicum chinense*. **A)** Effect of Put. **B)** Effect of Spd. **C)** Effect of Spm. Control: Liquid MS + 4.5 μ M 2,4-D. Samples taken after 30 days of culture differentiation. Mean \pm E.S. (n = 3, p < 0.05). Different letters reveals mean differences.

Coffea arabica, Calheiros et al. (1994) noted that among exogenously applied PAs, Spd significantly reduced the number of SEs in both species.

Similar results were also reported in *Solanum melongena* where concentrations of 0.5 mM Spd and Spm caused a decrease in the number of embryos formed (Yadav and Rajam, 1998). In *B. napus* microspores increased concentrations up to 23 to 56 μ M; Put reduced the embryo formation and increased the callus formation (Ahmadi et al., 2014). Spm is capable of

capturing free radicals and interacting with DNA to protect it from the action of these compounds. It has also been reported that, in *Arabidopsis thaliana*, high concentrations of Spd and Spm are able to regulate the S-adenosylmethionine decarboxylase (SAMDC) (Kakkar et al., 2000; Takashi and Kakehi, 2010).

In *Picea glauca*, increased levels of PAs during the initial developmental stages of somatic embryogenesis resulted in an increase in SAM synthetase, coinciding with high levels of stress related proteins (Lippert et al.,

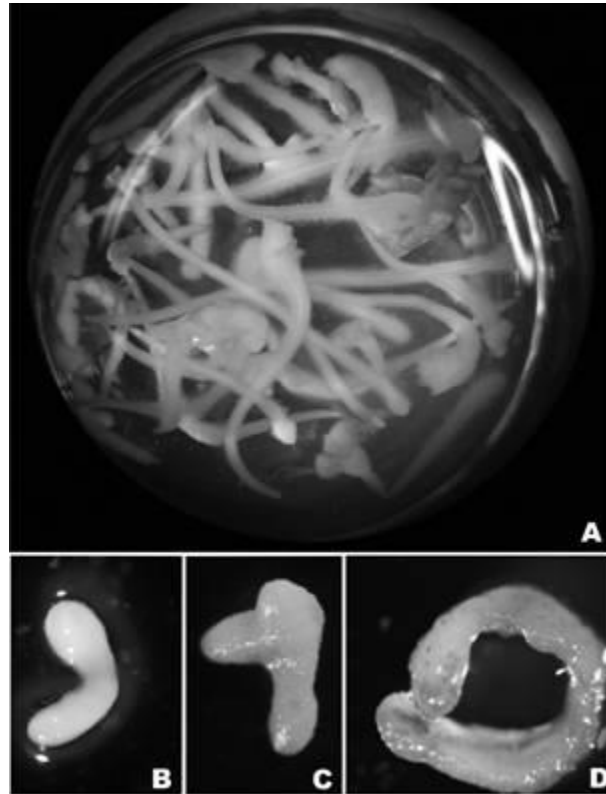


Figure 2. Effect of Spm on the morphology of *Capsicum chinense* somatic embryos. **A)** Somatic embryos with 0.1 mM Spm. **B)** Somatic embryo at torpedo stage. **C)** Somatic embryo at cotyledonary stage. **D)** Germinated somatic embryo.

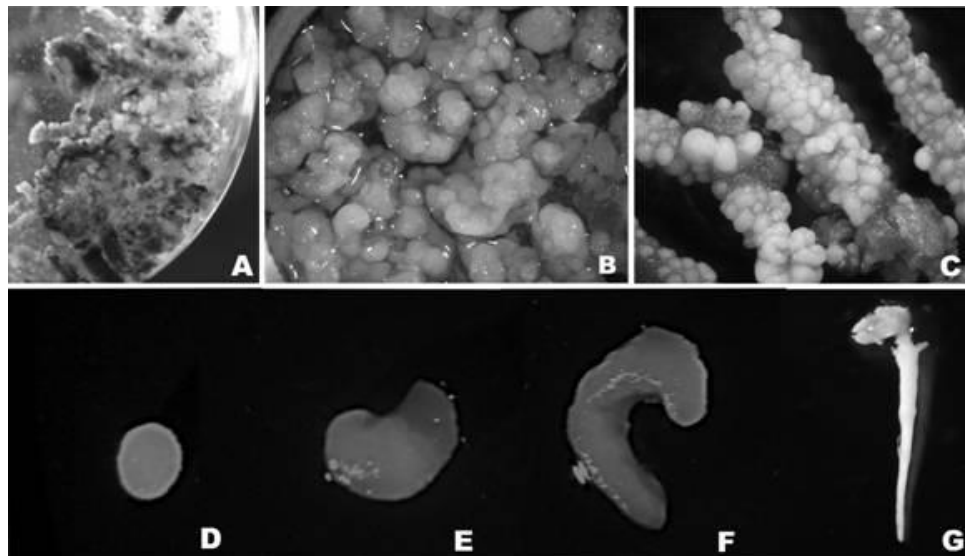


Figure 3. Microphotography of somatic embryos in *Capsicum chinense*. **A)** Hypocotyls with somatic embryos in liquid medium after 14 days of culture (MS + 4.5 μ M 2,4-D). **B)** Somatic embryos clusters formed in liquid medium after 28 days of culture (MS + 4.5 μ M 2,4-D), **C)** Hypocotyls after with 14 days of culture initiation (MS + 9.0 μ M 2,4-D). **D)** Globular somatic embryos. **E)** Heart-shaped somatic embryo. **F)** Torpedo somatic embryos and **G)** Cotyledonary somatic embryos.

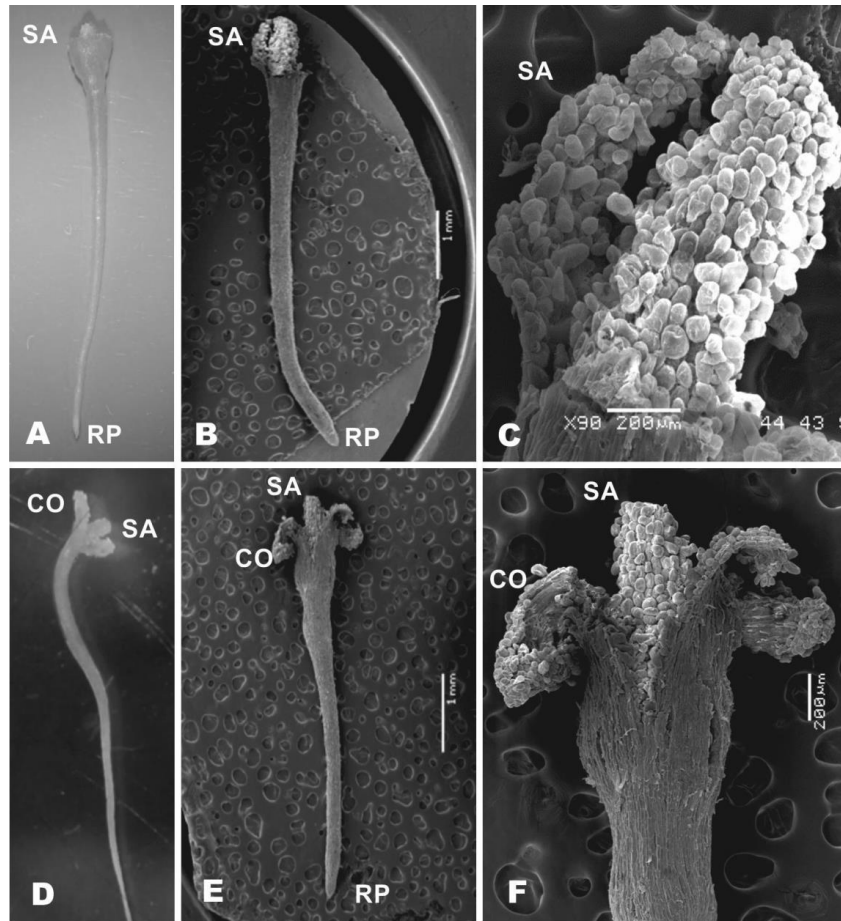


Figure 4. Characterization of deformed somatic embryos in *Capsicum chinense*. Microphotography (A, D). Scanning electron micrographs (B, C, E, F). A) Somatic embryo with absence of cotyledons. B) Somatic embryo without cotyledons. C) Close-up of the apical shoot from embryos without cotyledons with an exposed shoot area. D) Somatic embryos with deformed cotyledons. E) Somatic embryo with deformed cotyledons. F) Close-up to the deformed cotyledons and exposed shoot apex. SA, Shoot Apex; RP, Root Pole; CO, cotyledons.

2005). This allow us to infer that these compounds play a protective role during the embryogenesis due to the stress produced during this process (Karami and Saidi, 2010; Takahashi and Kakehi, 2010). This concurs with observations made by Regla-Márques (2011; Unpublished data), who detected significantly higher contents of PAs in SEs, in comparison with ZEs of *C. chinense*.

WUS transcript levels in somatic and zygotic embryos of *C. chinense*

WUS transcripts were detected in the IH, increasing significantly in the GH and diminishing drastically (up to seven-fold) in the TC (Figure 6A). A similar pattern was observed in the ZEs, showing a higher level of WUS transcripts in the IZE which also diminished significantly

in the MZE; however, this decrease was considerably lower than that observed in embryos at the TC. When SEs were treated with 0.1 mM Spm, the WUS transcript levels in GH+Spm were significantly lower to those observed in TC+Spm (Figure 6B). This behavior differed when compared to ZEs and untreated SEs, where the WUS transcript levels were significantly lower in GH+Spm compared to GH with a significant increase in TC+Spm when compared to TC. The presence of WUS has been detected in the early stages of embryogenesis, and is seen to diminish as the embryo develops (Gambino et al., 2011; Su et al., 2009), this behavior was observed in the ZEs and SEs of *C. chinense*. When the SEs were treated with Spm the WUS transcript levels showed an opposite behavior to previous reports. Alterations in the genes responsible for maintaining the shoot meristem have been observed in Arabidopsis mutants with defective SAM, deformed cotyledons and

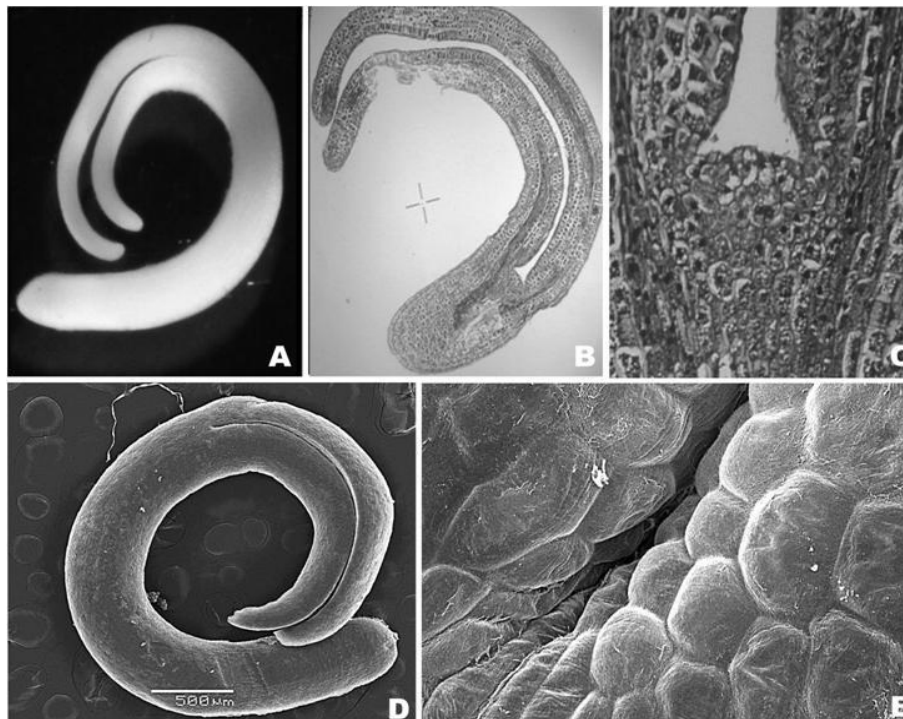


Figure 5. Ultrastructural and histological analysis of zygotic embryos in *Capsicum chinense*. **A)** Microphotography of a mature zygotic embryo. **B)** Histology of a complete mature zygotic embryo. **C)** Histology of a shoot apical meristem from a mature zygotic embryo. Scanning electron microscopy in zygotic embryos. **D)** Mature zygotic embryo. **E)** Close-up of the shoot apex in mature zygotic embryo.

lack of shoot development (Budziszewski et al., 2001; Mayer et al., 1998). Other authors have reported *wus* mutants in which interrupted growth and lack of apical dominance have been observed (Kieffer et al., 2006; Laux et al., 1996; Stuurmann et al., 2002). In *ham* (Hairy Apical Meristem) mutants of *Petunia hybrid*, a similar behavior in the expression patterns of WUS was observed in early developmental stages when compared to wild-type. However, when the plant development advances, the WUS expression reduced sharply and a disorganized shoot meristem became more evident (Stuurmann et al., 2002). This behavior of WUS was also observed in our study in which the absence of the shoot meristem or the presence of a deformed shoot meristem is also observed. A number of reports have associated these deformations in the shoot meristem with alterations in auxin transport during embryonic development (Palovaara et al., 2010a; 2010b; Vanneste and Friml, 2009), WUS can interact with auxin responsive genes (Busch et al., 2010; Kieffer et al., 2006) and is found to be involved in the establishment in the auxin transport by its association with PIN1 (Su et al., 2009). The morphological improvement with 0.1 mM Spm in the SEs could be associated with a four-fold increase in the WUS transcript levels in TC+Spm, maybe due to an improved auxin transport in these stages of development.

WOX1 transcript accumulation in somatic and zygotic embryos of *C. chinense*

During somatic embryogenesis, WOX1 transcript levels were detected in every stage of the embryos formed, including the IH phase, with a significant increase in GH decreasing significantly in TC (Figure 7A). The WOX1 transcript levels in ZEs were significantly higher in MZE when compared to IZE (Figure 7A); the behavior of the SEs was different to those observed in ZEs showing an opposite behavior between these embryos. When the SEs were treated with 0.1 mM Spm, mean differences were detected when compared with the untreated SEs, where a significant decrease was observed in GH+Spm when compared to GH (Figure 7B). The TC+Spm showed a significant increase in the WOX1 transcripts when compared to TC (Figure 7B). In general, the Spm treated SEs showed a different behavior from the SEs without Spm, in this aspect the 0.1 mM Spm treated SEs behaved similar to the ZEs, where the WOX1 transcript levels IZE were significantly lower when compared to MZE (Figure 7A); this same pattern were observed in Spm treated embryos were the WOX1 transcript levels were lower in GH+Spm when compared to TC+Spm (Figure 7B).

According to reports on other species, WOX1 transcript

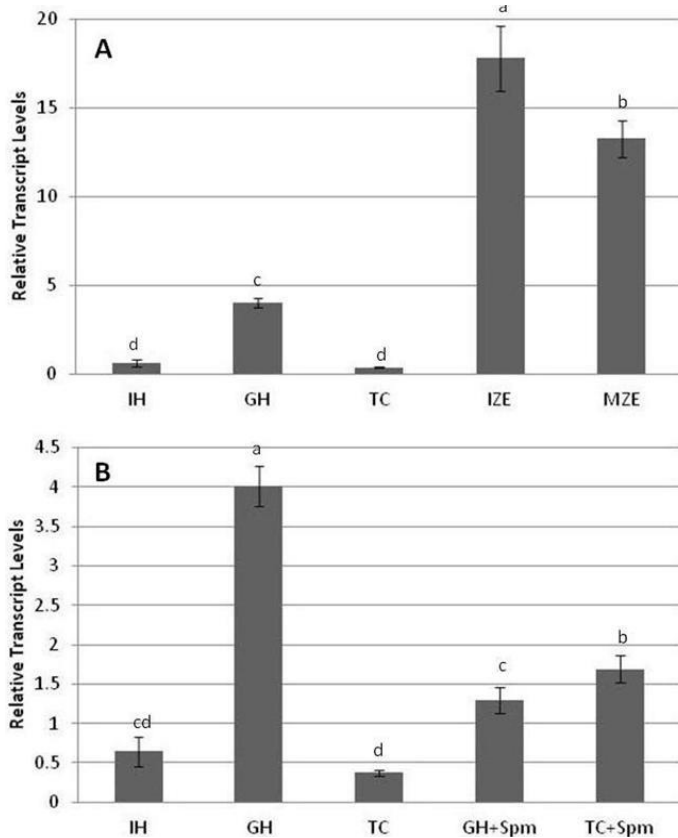


Figure 6. Quantification of WUS transcripts levels in *Capsicum chinense*. A) WUS transcript levels in zygotic and somatic embryos. B) Effect of Spm on WUS transcripts levels in somatic embryos of *C. chinense*. The PCR signals were normalized to β -TUB. Values are mean \pm S.E. (n =6, p < 0.05). Different letters indicate significant statistical differences. IH, hypocotyls in induction; GH, SEs at globular and heart stages; TC, SEs at torpedo and cotyledonary stages; IZE, immature zygotic embryos; MZE, mature zygotic embryos GH+Spm, SEs at globular and heart stages treated with Spermine and TC+Spm, SEs at torpedo and cotyledonary stages treated with Spermine.

patterns show greater presence of transcripts in the more advanced stages of embryonic development (Gambino et al., 2011; Haecker et al., 2004; Lin et al., 2013). Previous reports have demonstrated that over-expression and under-expression of WOX1 can cause alterations in plant phenotype due to the fact that this gene is associated with the organization of cellular division in leaves, organs and carpels (Lin et al., 2013; Vandebussche et al., 2009; Zhang et al., 2011). In *Nicotiana tabacum wox1*, a significant reduction in the WOX1 transcripts levels was observed, fully complemented plants showed a major increase in WOX1 transcripts similar to the wild-type (Lin et al., 2013). Moreover, the addition of PAs, particularly Spm, is capable of reverting the mutant phenotype, most likely because WOX1 is associated with the modulation of SAMDC1, a key enzyme in the biosynthesis of PAs (Ge et al., 2006; Zhang et al., 2011). The addition of Spm

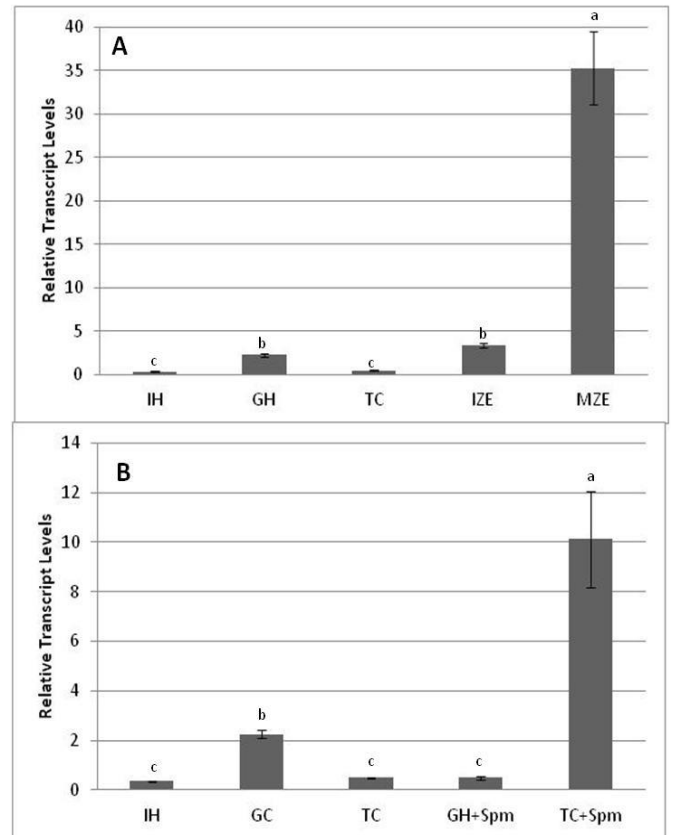


Figure 7. Quantification of WOX1 transcripts levels in *C. chinense*. A) WOX1 transcripts levels in zygotic and somatic embryos. B) Effect of Spm on WOX1 transcripts levels in somatic embryos of *C. chinense*. The PCR signals were normalized to β -TUB transcripts. Mean \pm S.E. (n =6, p < 0.05). Different letters indicate significant statistical differences. IH, hypocotyls in induction; GH, SEs at globular and heart stages; TC, SEs at torpedo and cotyledonary stages; IZE, immature zygotic embryos; MZE, mature zygotic embryos GH+Spm, SEs at globular and heart stages treated with Spermine and TC+Spm, SEs at torpedo and cotyledonary stages treated with Spermine.

increased the WOX1 transcript levels in TC+Spm in *C. chinense*; this suggests that the morphological improvement in the SEs of this species could be attributed to the role of WOX1 in the PAs biosynthetic pathway and its possible interactions with WUS and other member of the WOX family like WOX3.

Accumulation of WOX3 transcripts in somatic and zygotic embryos of *C. chinense*

The quantification of WOX3 transcripts showed no significant differences in the different developmental stages of the SEs (Figure 8A), whereas, with the ZEs, transcript levels of this gene (WOX3) was significantly higher in the MZE (Figure 8A). With the addition of 0.1 mM Spm, the quantity of transcripts increased significantly,

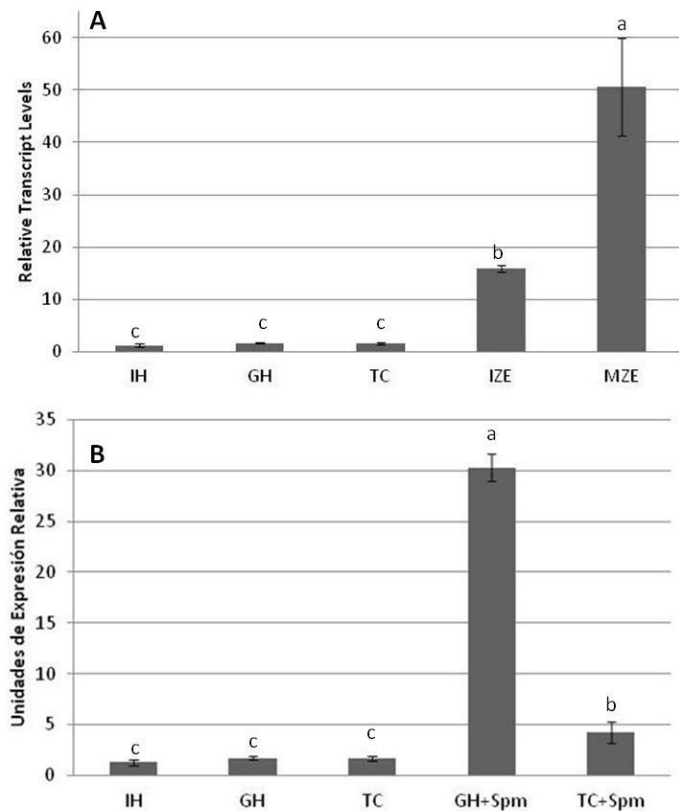


Figure 8. Quantification of WOX3 transcripts levels in *C. chinense*. A) WOX3 transcripts levels in zygotic and somatic embryos. B) Effect of Spm on WOX3 transcripts levels in somatic embryos of *C. chinense*. The PCR signals were normalized to β -TUB transcripts. Mean \pm S.E. (n =6, p < 0.05). Different letters indicate significant statistical differences. IH, hypocotyls in induction; GH, SEs at globular and heart stages; TC, SEs at torpedo and cotyledonary stages; IZE, immature zygotic embryos; MZE, mature zygotic embryos GH+Spm, SEs at globular and heart stages treated with Spermine and TC+Spm, SEs at torpedo and cotyledonary stages treated with Spermine.

in comparison with the untreated SEs (Figure 8B), but with a different accumulation pattern to those observed in the ZEs where the GH+Spm showed greater transcript levels than the TC+Spm (Figure 8B).

Our results differ from reports on the behavior of WOX3 transcript accumulation for other species, in which the transcripts have been observed to accumulate in advanced stages of embryonic development and to diminish significantly in the phases prior to germination (Haecker et al., 2004; Gambino et al., 2011). There is evidence to support the argument that WOX3 malfunction can be related to malformations of the aerial part, as this gene is associated with early leaf formation (Nakata et al., 2012; Lin et al., 2013; Shimizu et al., 2009; Vandenbussche et al., 2009). It is likely that, with the addition of Spm, WOX3 could be acting as a marker prior to organ formation. The significant increase in WOX3 transcripts in the GH+Spm could be attributed to the high

similarity existing between the proteins, WOX1 and WOX3, and thus WOX3 could be substituted by WOX1 under these culture conditions. A number of reports to this effect indicate that, due to the fact that the mutant phenotypes *wox1* and *wox3* are similar, these genes can act redundantly in embryonic development (Lin et al., 2013; Vandenbussche et al., 2009).

Shoot architecture in higher plants is highly dependent on the activity of the SAM, which maintains pluripotent stem cells at its tip (Steeves and Sussex, 1989). During the development of postembryonic shoots, the SAMs continuously produce organs in a regular pattern, for example, stems, leaves and flowers. Therefore, the subtle variations in primordia initiated by the SAM account for all of the remarkable differences and diversity in shoot architecture that we observe in nature. Recent analyses of *A. thaliana* mutants have identified several key genes that play essential roles in the specific function of SAMs (Geier et al., 2008; Kinoshita et al., 2010; Laux et al., 1996; Lohmann et al., 2001; Lenhard et al., 2002; Lenhard and Laux, 2003).

Studies on the biological function of plant Homeodomain proteins, designated WOX proteins, have revealed that they are transcription factors and are involved in the regulation of various developmental processes (Haecker et al., 2004; Lohmann et al., 2001; Qu and Zhu, 2006; Van der Graaff et al., 2009; Zhang et al., 2011). The WOX genes were all involved in maintaining a balance between cell division and differentiation (Gallois et al., 2004). WUS is an early gene expressed in the apical embryo region, while WOX1 is expressed in the central zone of the cotyledon and WOX3 in the peripheral zone (Haecker et al., 2004).

It has been observed that altered patterns of these genes have shown deformations in the apical region and can affect the function of other genes (Laux et al., 1996; Lin et al., 2013; Shimizu et al., 2009; Stuurman et al., 2002; Su et al., 2009; Zhang et al., 2011). This could suggest that the deformations observed could be the result of an anomalous accumulation of WUS, WOX1 and WOX3 transcripts during somatic embryogenesis.

In our study, the alterations detected in the levels of WUS, WOX1 and WOX3 transcripts in SEs of *C. chinense*, which are affected by the apical meristem syndrome (Steinitz et al., 2003), suggests that their incapacity to germinate and convert to plants is of a genetic nature. However, the addition of 0.1 mM Spm to the culture medium improved the embryo morphology of these deformed embryos, facilitating embryos with an adequately harmonious development of both radical and apical zones, a change in coloration from creamy white to pale green and the emission of two tiny, albeit very rudimentary, cotyledonary leaves. From the analysis of transcript levels in these embryos after Spm treatments, it was possible to observe a significant increase in WOX1 in TC+Spm (Figure 7B). The effect of polyamines has been documented previously (Galston and Shawney, 1990;

Ge et al., 2006; El-Hadrani et al., 1989a 1989b; Kevers et al., 2000; Slocum, 1991; Walden et al., 1997; Yadav and Rajam, 1998) and in the case of Spm, it has been found to be capable of modulating the activity of SAMDC1 (Zhang et al., 2011) and transcription through its association with nucleic acids (Galston and Shawney, 1990; Lippert et al., 1995; Walden et al., 1997), which would be reflected in a higher level of WUS, WOX1 and WOX3 transcripts. Even though the phenotype of the somatic embryos was improved with the addition of Spm, their inability to convert to plants persisted. SAMDC is involved in the final steps of the plant polyamine biosynthetic pathway and is essential for plant shoot architecture (Ge et al., 2006; Friedman et al., 1982; 1985; Smith et al., 1985; Rastogi and Sawhney, 1990; Thu-Hang et al., 2002).

Our results coincide with those proposed by Zhang et al. (2011) who have suggested that WOX1 plays an important role in meristem development, possibly via modulation of SAMDC1 activity and polyamine homeostasis. However, the inability of *Capsicum* embryos to convert to plants is clear evidence of the complexity of this phenomenon in which numerous genes and/or other factors do not allow the germination to follow a normal pattern during its development, perhaps because the shoot meristem is not functionally capacitated for this physiological process.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Copper-induced changes in growth and antioxidative mechanisms of tea plant (*Camellia sinensis* (L.) O. Kuntze)

Sarmishta Dey¹, P. B. Mazumder^{1*} and S. B. Paul²¹Plant Biotechnology Laboratory, Department of Biotechnology, Assam University Silchar, Assam, 788011, India.²Department of Chemistry, Assam University, Silchar, Assam, 788011, India.

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Tea is the oldest, most popular, non-alcoholic caffeine containing beverage in the world. Tea plants are prone to the attack of many diseases which can be controlled by the treatment of pesticides. These pesticides contain heavy metal which prolonged accumulation can lead to the damage of crop yield both in quantity and quality. In the present study, we observed the effects of high concentration of Cu stress on physiological and biochemical parameters. The tea cultivars (S₃A₃ and TS-491) were collected from the Rosekandi Tea Estate, Silchar, Assam. The accumulation of Cu in the different parts of the tea plants had a positive correlation with the Cu stress. The accumulation of Cu was higher in roots than in leaves and also the new stems. The results show gradual decrease in the photosynthetic activity with the increase in the concentration of the Cu stress in both the cultivars. The activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) increased with the increase in the Cu concentration but remarkably in different manner in both the cultivars. The activities of antioxidants ascorbate peroxidase (APX), CAT, SOD and POD in cultivar S₃A₃ increased up to 500 µM and at 600 µM showed a low rate of increase whereas TS-491 was tolerant up to 400 µM. The responses in the oxidative stress were characterized by an accumulation of malondialdehyde (MDA). Phenol content has also positive correlation with increase in the concentration in both the cultivars up to 500 µM. Finally it was concluded that Cu is tolerant to the cultivars of tea plant to some extent (specifically at lower concentration), but at higher concentration (beyond 400 µM) of Cu with exposure time, tea plant had a strong inhibition of growth by damaging the normal metabolism.

Key words: Tea plants, copper stress, physiological characters, lipid peroxidation, reactive oxygen species.

INTRODUCTION

Camellia sinensis (L.) O. Kuntze is an economically important crop for production of tea leaves. Tea is the oldest, most popular, non-alcoholic caffeine containing these are toxic to plants when they are present in soil or the growth media is above the permissible level (Xu and Shi, 2000). Sometimes, heavy metals like Cu, Cd, As, Zn,

beverage in the world. Copper (Cu), cadmium (Cd), nickel (Ni), manganese (Mn) and zinc (Zn) are the essential micronutrients required for the plant life but concentration (Ross 1994, Prasad and Strzalka 2002). Due to rapid industrialization and urbanization, there is an elevated

emission of toxic heavy metals which enters the biosphere and affects the growth and development of plants (Nriagu and Pacyna, 1988; Kabata-Pendias, 2001). Pesticides and fertilizers are the other major sources of heavy metal which are directly taken up by plants. As Cu is an essential element, it participates in number of physiological processes, and is an essential cofactor for many metalloproteins, but due to excess amount of copper present in cells, causes problems by inhibiting the plant growth which impairs important cellular processes like photosynthetic electron transport (Demirevska-kepova et al., 2004). Since copper is both an essential cofactor and a toxic element, involving a complex network of metal trafficking pathways that must prevent accumulation of the metal in the freely reactive form (metal detoxification pathways) and ensure proper delivery of this element to target metalloproteins is necessary. Cu as fungicides and pesticides are very effective in the control of the diseases in tea plants and are widely used (Gallagher et al., 2001; Singh, 2005), but higher concentration can cause adverse effect in plants by lowering the chlorophyll content, delay in flowering, reduction in the number and quality of shoots which leads to fall down in the quality of tea (Setia, Kaur, and Setia, 1989). The accumulation of Cu at the higher concentration through direct contact or from the food chain is harmful to human beings who consume tea. Heavy metals such as iron, copper, zinc, nickel, manganese, lead and cadmium can cause oxidative stress, producing enzymatic and non-enzymatic antioxidative reaction responses and lipid peroxidation in plants. The exposure of Cu can cause toxicity to the plants which is due to oxidative damage to the biological macromolecule by redox cycling, depletion of glutathione and alteration of homeostatis (Stohs and Bagchi, 1995). Due to the accumulation of Cu in excessive manner leads to the production of reactive oxygen species (ROS) which can damage lipids, nucleic acids, proteins, amino acids, carbohydrates and other complex molecules produced from all these in cells (Pietrini et al., 2003). To overcome such stress, plants evolved much effective mechanism to detoxify the ROS (Dat et al., 2000). A group of effective antioxidants are present in plants to maintain the antioxidant potential in the cells, which detoxify the production of reactive oxygen species. Many researches showed that amounts of antioxidant enzymes like superoxide dismutase (SOD) and peroxidase (POD) increase in plants when highly exposed to heavy metals

(Acar et al., 2001). Peroxidase plays a crucial role in physiological events which are related to diminishing growth of plants by lignification, cross-connection of cell wall polysaccharides, oxidation of indole-3-acetic acid (IAA), cell elongation and phenol oxidation (Mocquot et al., 1996). Rate of ROS formation and efficiency, and capacity of detoxification mechanisms in plants determine the level of damage of cells under stress conditions. Hence in this study, we observed the effects of Cu stress at different concentrations on some physiological indicators such as growth (shoot and root length), rate of photosynthesis, enzymatic activity of scavengers of ROS such as SOD, peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), malondialdehyde (MDA) on two cultivars of tea plants viz. clonal tea plants, S₃A₃ and the tea plants produced from seeds, TS-491.

MATERIALS AND METHODS

Experimental condition

Three months old plants of the two cultivars viz. S₃A₃ (clonal propagated) and TS-491 (developed from seeds) were collected from the Rosekandy Tea Estate, Assam, India. The plants were then transferred to Hoagland solution as nutrient media and allowed to get stable for seven days. The plants were treated with CuSO₄ at different concentrations of 50, 200, 300, 400, 500 and 600 µM in the nutrient solution (Hoagland and Amon, 1938). The control plants were left as untreated, and allowed to grow only in Hoagland solution. The top four leaves were collected for measuring the

various enzymatic activities after 2nd, 4th and 7th days of the treatment.

Morphological and growth analysis

The roots, stem and leaves of the two cultivars S₃A₃ and TS-491 were collected and analyzed for the morphological characters like growth of the different parts of the plants, appearance of new leaves, new stems etc.

Determination of chlorophyll content

The chlorophyll content of the matured leaves of both treated and control plants of both cultivars was determined following the protocol of Hegedus et al. (2001). 0.1 g leaf extract was dissolved in 80% acetone and optical density was determined using UV-VIS spectrophotometer (Thermo Fisher) at 645 and 663 nm, respectively. The values of optical density was assayed and

*Corresponding author. E-mail: pbmazumder65@gmail.com.

Abbreviations: ROS, Oxygen species; **POD**, peroxidase; **APX**, ascorbate peroxidase; **CAT**, catalase; **SOD**, superoxide dismutase; **PVP**, polyvinylpyrrolidone; **NBT**, nitrobluetetrazolium; **MDA**, malondialdehyde

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expressed as $\mu\text{g g}^{-1}$ fresh weight.

Assay of enzyme activity

For determining the enzyme activities, 0.5 g leaves were dipped

liquid nitrogen and then homogenized in a chilled mortar and pestle in 5 ml 50 mM cold phosphate buffer of pH 7.8 containing 2% polyvinylpyrrolidone (PVP). The filtrate homogenate was centrifuged at 13000 g for 20 min at 4°C and the supernatant were used for determining the enzyme activities. The antioxidant ability of leaves was determined by measuring the POD activity at absorbance of 470 nm due to the oxidation of guaiacol (Wu and von Tiedemann, 2002). SOD activity was measured by the inhibition of nitrobluetetrazolium (NBT) reduction (Krivosheeva et al., 1996). CAT activity was measured with the help of the absorbance at 240 nm (Pinhero et al., 1997).

Determination of lipid peroxidation and phenolic content:

The 0.5 g fresh weight of leaves was homogenized in a pre-chilled mortar pestle in 5 ml of 50 mM cold Na-phosphate buffer (pH 7.8), with 0.1 mM EDTA and 1% (w/v) PVP. After centrifugation at 13,000 g for 30 min at 4°C, supernatant was used for further analysis. The level of peroxidation was determined in terms of 2-thiobarbituric acid (TBA) (Liu et al., 1996). To determine the total phenolic components, the 0.5 g of leaves were extracted in ethanol and then the content was estimated with the changes in absorbance at 520 nm (Mahadevan and Sridhar, 1996).

Statistical analysis

All the experiments were done in triplicate and the mean were taken. Statistical analysis of mean values and standard deviations (SD) were performed for all the data. The significant difference was set between treatments at $p < 0.01$ or $p < 0.05$.

RESULTS

Effect of high concentration of copper on tea morphological characters

Tea plants showed different degrees of symptoms in different concentrations of copper stress. With the increase in the concentration of copper, the roots suffered mostly which is followed by leaves and then new stems (data was not given). The leaves first developed some brown spots, yellow patches, become dry and then fell off. At the highest concentration of 500 and 600 μM , less number of new leaves appeared, and most of the leaves became withered in both the cultivars. The number of new stems was very less and gradually the growth rate decreased. The fresh and dry weights of roots and leaves were analyzed and the results reflected that growth rate of the copper treated plants with respect to the control was less. The dry weight of the organs of the treated plants decreased with the increase in the concentration of the copper in both cultivars with respect

to exposure days. In the highest concentration (600 μM), the inhibition was the strongest with the smallest growth rate of roots, leaves and stem compared to tea plants treated with lower concentrations.

Effect of Cu on chlorophyll content

Chlorophyll is an important pigment which plays a vital role in the process of photosynthesis. Table 1 shows that there was a significant negative correlation between the concentration of Cu and the chlorophyll contents in the tea plants. With the exposure of high concentration of copper, the total chlorophyll content of both the cultivars decreased. The level of decrease of chlorophyll content is high in TS-491 than the cultivar S_3A_3 . From Table 1A, we can conclude that at higher concentration of 400, 500 and 600 μM , there was a gradual decrease in the chlorophyll content in cultivar S_3A_3 whereas from Table 1B, we observed the decrease was more than two fold in cultivar TS-491 compared to S_3A_3 . In S_3A_3 , at control, the total chlorophyll content was $90.12 \mu\text{g g}^{-1}$ fresh weight on 2nd day and chlorophyll content decreased gradually as the Cu concentration increased; whereas, maximum decrease in chlorophyll content was found to be started from 400 μM onwards where the content has degraded and ended up at $42.13 \mu\text{g g}^{-1}$ fresh weight on 7th day at 600 μM Cu concentration. Whereas in TS-491, the chlorophyll content was $94.12 \mu\text{g g}^{-1}$ fresh weight on 2nd day at control, and from 300 μM concentration onwards, the content degraded more than two folds and ended up at $18.09 \mu\text{g g}^{-1}$ fresh weight on 7th day at 600 μM Cu concentration. The observation indicates that compared to cultivar S_3A_3 , cultivar, TS-491 was more sensitive to copper stress.

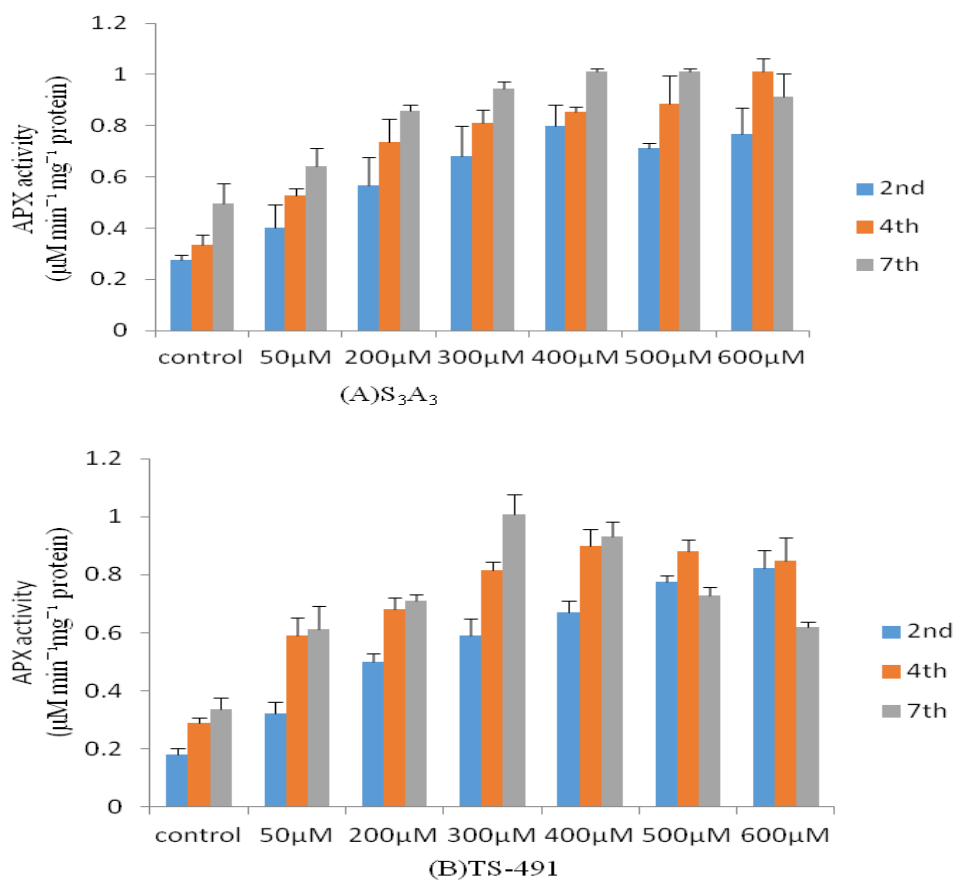
Effect of high concentration of Cu on antioxidative enzymes

Plants are exposed to abrupt stress daily and seasonal changes in the environment, and they have a wide spectrum of developmental responses and biochemical adaptations to stress condition. During normal metabolic activities and due to the consequences of environmental condition, O_2 is capable of giving rise to reactive oxygen species (Mittler et al., 2004). ROS include the superoxide radical, hydroxyl radical and hydrogen peroxide which are toxic to plants (Dismukes et al., 2001, Vellosillo et al., 2010). The activities of APX, CAT, SOD and POD gradually decreased with increase in the concentration of Cu.

It was observed that with respect to control, there was a significant increase in the APX activity in S_3A_3 cultivar up to the 4th day of 600 μM concentration, but there was a slight decrease in the APX activity on the 7th day of 600 μM (Figure 1). On the other hand, there was only a

Table 1. Effects of different concentration of Cu on total chlorophyll contents of cultivar (A) S3A3 (B) TS-491.

Treatment (μM)	2 nd day	4 th day	7 th day
	$\mu\text{g g}^{-1}$ fresh weight		
(A) S₃A₃			
control	90.12 \pm 0.52	88.92 \pm 0.78	86.58 \pm 0.93
50	86.25 \pm 0.38	85.65 \pm 0.67	82.29 \pm 1.4
200	83.31 \pm 0.26	82.62 \pm 0.81	80.19 \pm 1.36
300	81.02 \pm 0.29	79.93 \pm 0.51	75.14 \pm 0.86
400	71.92 \pm 0.42	68.23 \pm 0.86	63.89 \pm 0.99
500	65.23 \pm 0.36	62.18 \pm 0.91	56.12 \pm 0.69
600	54.23 \pm 0.39	50.19 \pm 0.57	42.13 \pm 0.86
(B) TS491			
control	94.14 \pm 1.08	92.01 \pm 1.46	89.03 \pm 1.49
50	90.13 \pm 1.09	87.32 \pm 1.26	84.28 \pm 0.99
200	83.93 \pm 0.92	80.02 \pm 1.21	78.21 \pm 1.22
300	73.32 \pm 1.03	70.89 \pm 1.22	67.12 \pm 1.36
400	61.07 \pm 0.99	52.87 \pm 0.87	49.89 \pm 1.32
500	49.27 \pm 1.29	36.87 \pm 1.27	32.13 \pm 1.36
600	36.23 \pm 1.28	23.9 \pm 0.99	18.09 \pm 1.21

**Figure 1.** Effect of increasing concentrations of Cu on APX activity in leaves of two cultivars of tea S₃A₃ and TS-491.

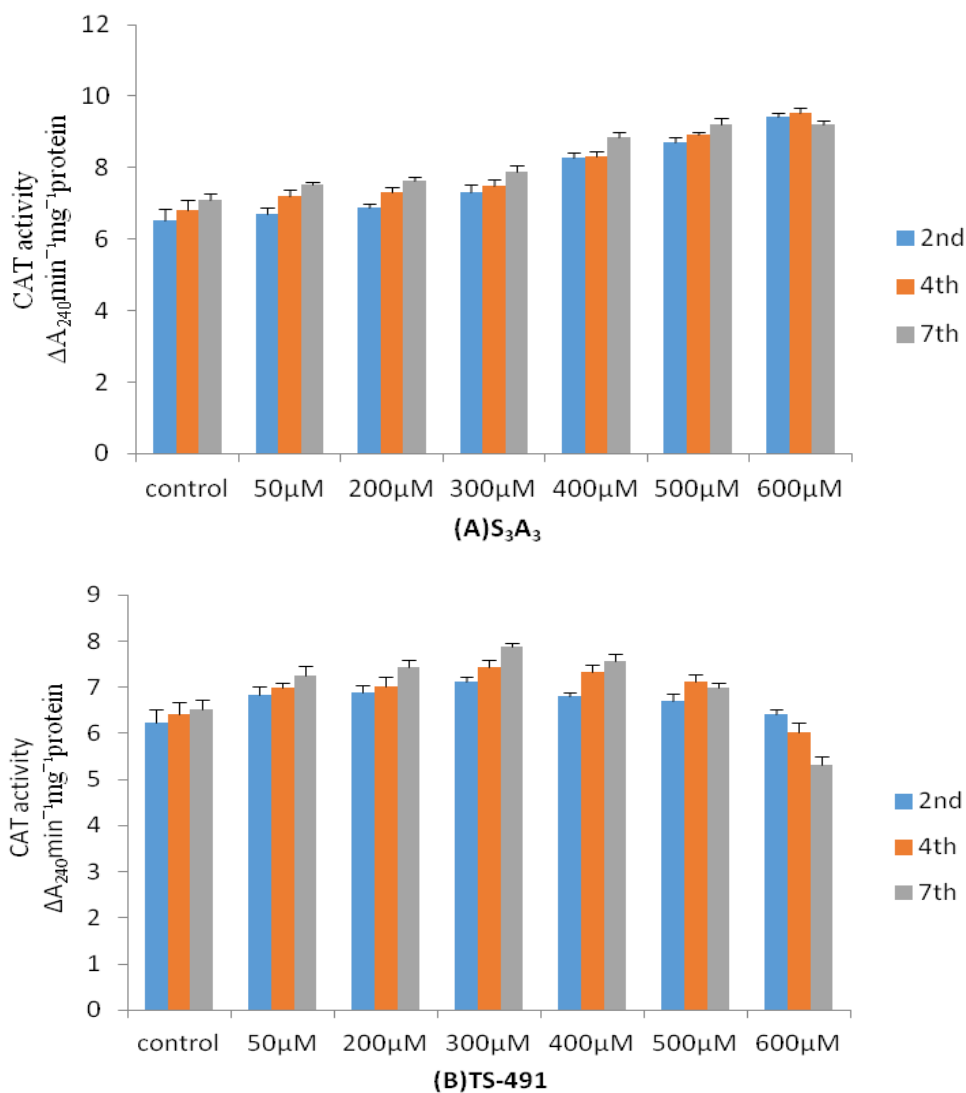


Figure 2. Effect of increasing concentrations of Cu on CAT activity in leaves of two cultivars of tea S₃A₃ and TS-491.

marginal increase in the APX activity with the exposure of Cu up to 200 μM in TS491 cultivar; but further increase in the concentration of Cu resulted in a significant increase (more than two fold) in the activity specially from the 2nd day of 300 μM and continued up to 4th day of 400 μM compared to the lower concentration, finally the APX activity started to decline from 7th day of 500 μM in TS-491. Catalase has double function, as a primary enzymatic mechanism it generally decomposes the toxic H₂O₂ generated during oxygen metabolism by the aerobic organisms and it also catalyses the oxidation of H donors with the consumption of one mole of peroxide (Havir and Mchale, 1987). From Figure 2A and B, it was observed that there was a significant increase in the CAT activity occurs in both the cultivars. S₃A₃ is found to be more

tolerant compare to the cultivar TS-491. The increase in the CAT activity of S₃A₃ cultivar continued even at higher concentration of Cu up to 4th day of 600 μM; but, there was slight decrease in the activity on the 7th day of 600 μM. whereas in TS-491 the decrease in the CAT activity was observed from 400 μM itself. The same trend of result has been found in the activity of SOD in both the cultivars. From Figure 3A, it can be observed that in the cultivar S₃A₃ the activity showed a positive correlation with the increasing concentration. There was increase in the SOD activity by one fold with the subsequent increase in the concentration with respect to the exposure days. Whereas in cultivar TS-491, the increase in the activity was up to 400 μM concentration and with further increase in the concentration the activity was

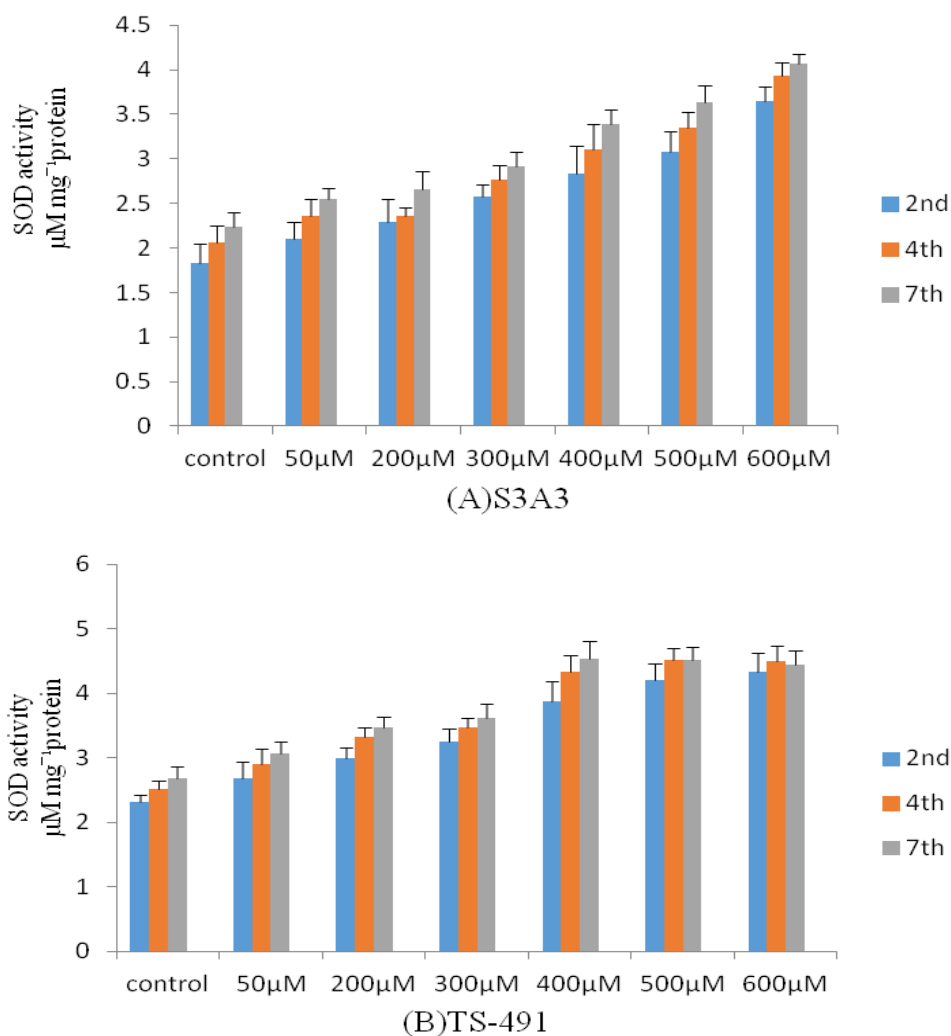


Figure 3. Effect of increasing concentrations of Cu on SOD activity in leaves of two cultivars of tea S_3A_3 and TS-491.

declined (Figure 3B). In cultivar S_3A_3 , the POD activity has increased with the exposure of higher concentration of Cu (600 μM) (Figure 4A). In cultivar TS-491, the increase in the activity was more than two fold up to 400 μM concentration of Cu, but the level of increase in the activity lowered at 500 μM whereas at 600 μM there was total decline in the activity of POD from 7th day onwards. POD plays an important role in plant respiratory metabolism and physiological resistance. From the result, it can be observed that S_3A_3 cultivar is more tolerant to higher concentration of Cu compared to TS-491. The adverse action of biotic and abiotic stress affects the active oxygen metabolism of plants.

Effect of phenolic contents and lipid peroxidation

Phenols are aromatic compounds with hydroxyl groups,

which offer resistance to diseases and pests in plants. From Figure 5A and B, it can be observed that the cultivar S_3A_3 has a significant increase in the total phenolic compound up to 500 μM and at 600 μM ; the level of increasing in the activity has slowed down slightly. On the other hand in TS491, there was increase in phenolic content up to 4th day of 500 μM and then there was a decline in the activity from 7th day of 500 μM . MDA is the final product of lipid peroxidation and its content reflect stress tolerance of plants (Liu et al., 2001; Sugiyama, 1994). It can be observed that with the increase in the concentration of Cu, the MDA content of tea cultivars increased and showed a significant positive correlation with Cu concentration. The level of increase in the peroxidation activity was less in S_3A_3 compared to TS-491 (Figure 6A and B). This shows that high concentration of Cu can lead to lipid peroxidation which

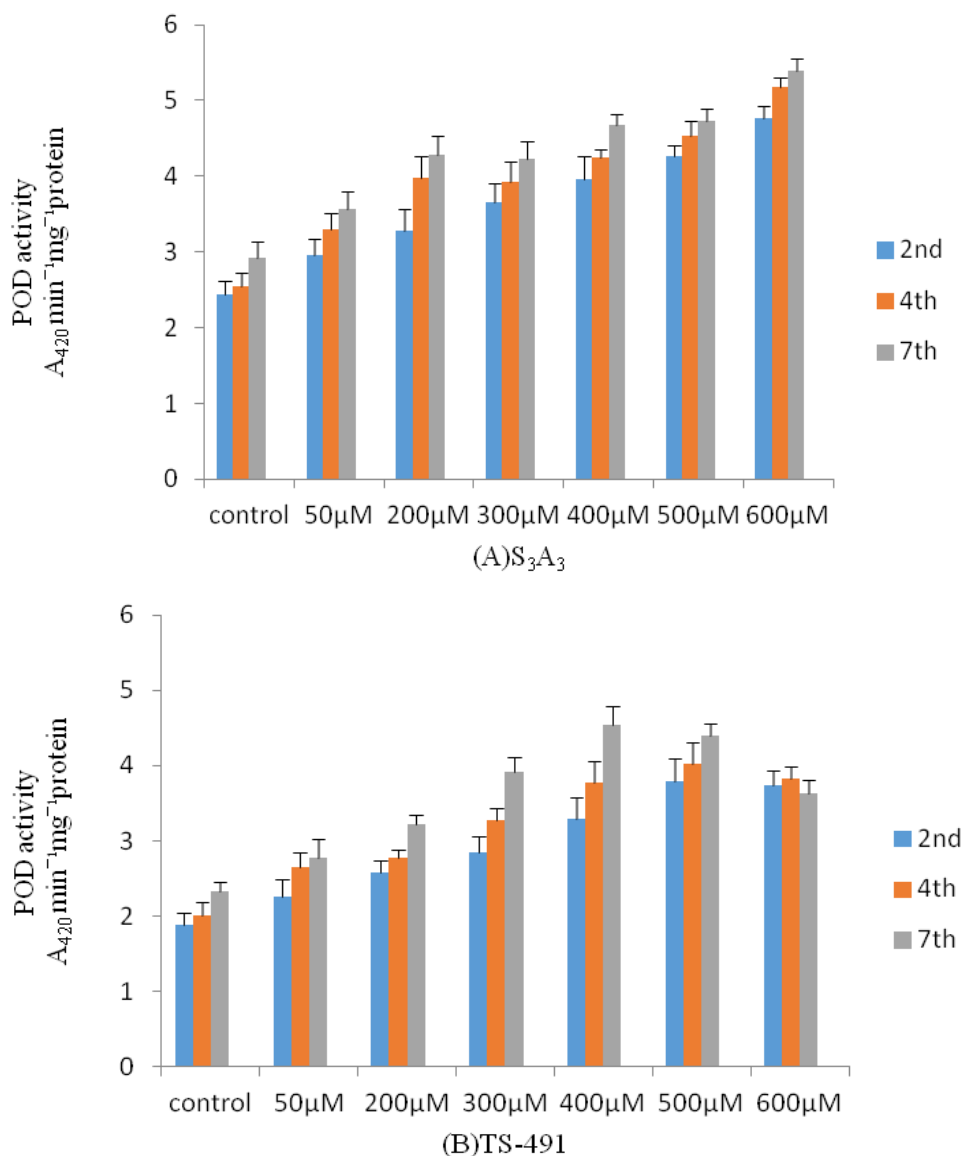


Figure 4. Effect of increasing concentrations of Cu on POD activity in leaves of two cultivars of tea S₃A₃ and TS-491.

causes damage to the balance of ROS scavenging activities of tea plants.

DISCUSSION

Heavy metals play a vital role in the physiological process of plants. In trace amounts, several ions are required for metabolism, growth and development which are present in soil or as in growth media. Number of proteins and enzymes are found to contain heavy metals which make them essential for the growth and development of plants and also helps in maintaining the optimum metabolism;

but when the amount exceeds, it creates problem in the plants by leading to cellular damage (Avery, 2001; Schutzendubel and Polle, 2002; Gaetke and Chow, 2003). Though Cu is an essential component for both the photosynthetic and respiratory electron chains, but excess of Cu can cause changes in permeability in membrane, chromatin structure, synthesis of protein, enzyme activity, photosynthesis and respiratory processes through its phytotoxic effect and also can causes lipid peroxidation and lead to activation of senescence (Baryla et al., 2000). Cu in Free State binds irreversibly to SH group which are involved in the catalytic action of enzymes (Van Assche and Clisjters,

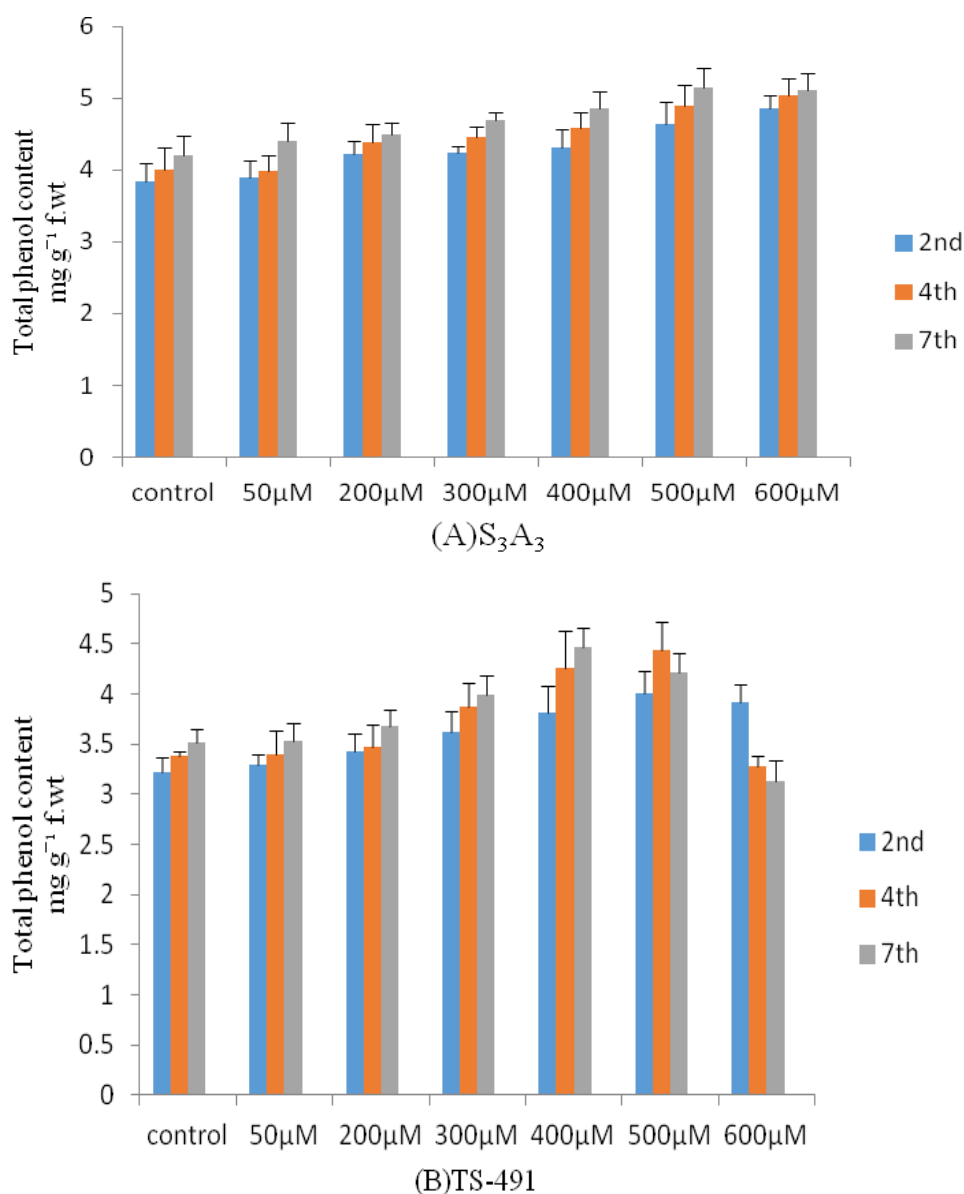


Figure 5. Effect of increasing concentrations of Cu on total phenol content in leaves of two cultivars of tea S₃A₃ and TS-491.

1990). In the present investigation, we observed that the chlorophyll content decrease with increase in the concentration of Cu. Other investigators also reported that with increase in the concentration of heavy metals, the activities of photosynthetic enzymes degrade which results in the reduction of chlorophyll content (Thapar et al., 2008). The content of chlorophyll has a close relation with photosynthesis (Liu et al., 2001), Cu ion is inserted directly to the reaction center especially PSII which due to the high irradiance causes direct damage to the reaction center (Kupper et al., 1996, 1998, 2002). Cu can change the pigment and protein composition of photo-

synthetic membranes and interferes with the biosynthesis of photosynthetic activity. Declination in the chlorophyll content is the primary bio indicator of toxicity of Cu (De Vos et al., 1992). The same result of decrease in chlorophyll content was observed in tea plants when exposed to Cr stress (Tang et al., 2011). Many other authors also observed the same trend of decreasing of chlorophyll content with increase in Cu concentration (Rama Devi and Prasad, 1998; Mohanpuria et al., 2007; Saha et al., 2011). The present study indicates that the excess of Cu concentration decrease the chlorophyll content which in turn can cause inhibition to photosystem

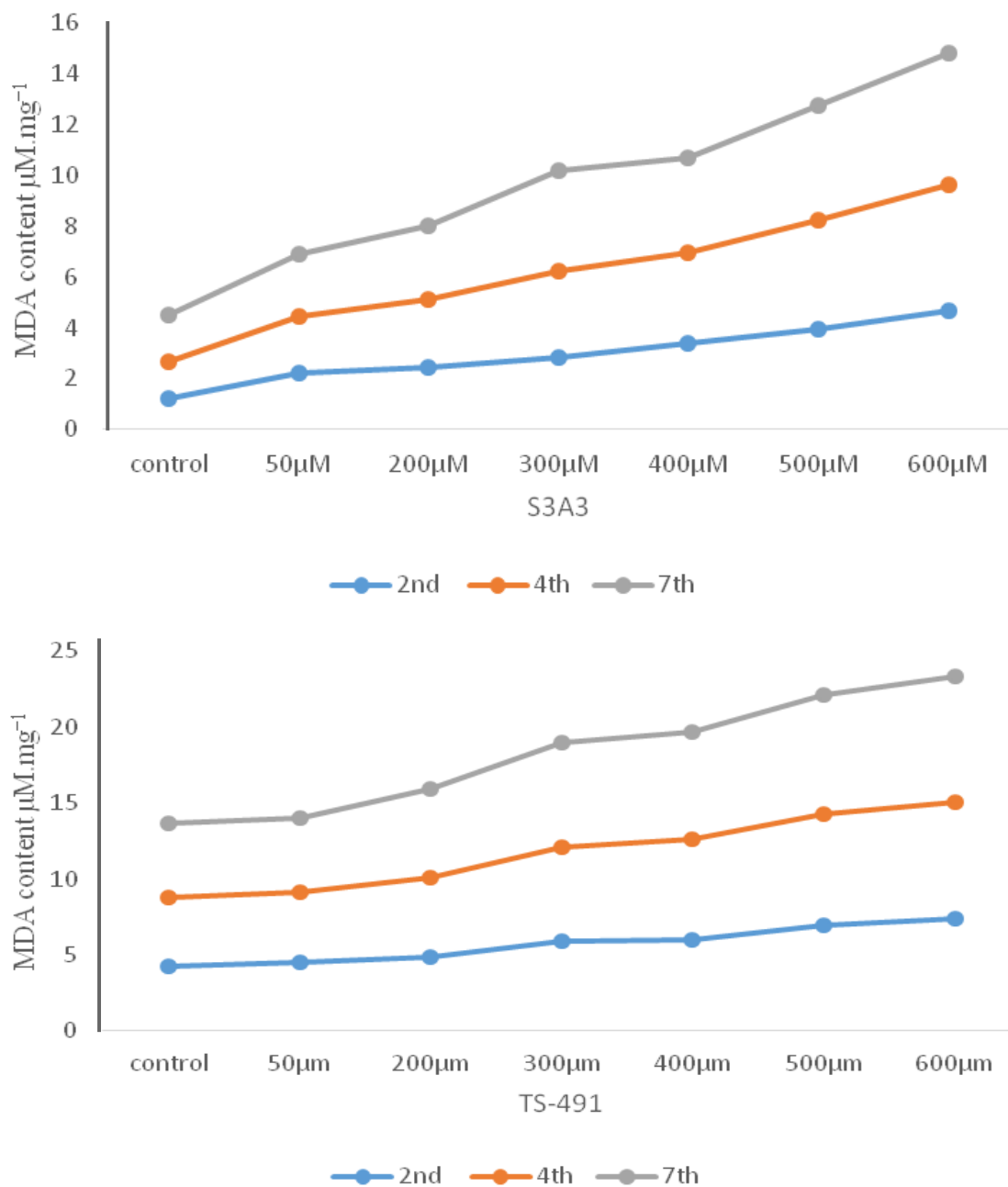


Figure 6. Effect of increasing concentrations of Cu on MDA content in leaves of two cultivars of tea S₃A₃ and TS-491

(Saha et al., 2012).

The different parts of the plants come in contact through the absorption of roots in the solution. In this study, we found that with the increase in the concentration of Cu, the level of it also increased in the different parts of the plants. The roots found to accumulate highest level of Cu as they are in direct contact with the Cu stress present in the nutrient media. The deposition of Cu in organ are in the following order

roots> leaves> stems. The accumulating ability of root was stronger than other parts of the tea plants (Tang et al. 2011). In our study both the cultivars showed different symptoms due to different concentration of Cu and in the later phases of the experiment, the growth of the tea plants were inhibited. Tang et al., 2008 reported the same results when they grow tea plant in nutrient solution incorporated with Cu stress. Cu toxicity was found to affect the growth of *Alyssum montanum* (Ouzounidou,

1994), Singh and Tewari, 2003 also observed similar results in *Brassica juncea* when treated with Cu.

Many oxidative stresses also get generated due to the accumulation of Cu in higher concentration and this as a whole cause the inhibition in the photosynthetic reactions which has been observed by many authors (Rocchetta and Kupper, 2009). The decreased concentration of the chloroplastic pigments may be the result of reduced synthesis and/or enhanced oxidative degradation of these pigments by the enhancement of oxidative stress (Romero-Puertas et al., 2002). Due to the emergence of reactive oxygen species, there is occurrence of oxidative stress. The ROS are superoxide radicals, hydroxyl radicals, singlet oxygen and H₂O₂, these damage the cells by degrading the nucleic acids (Pietrini et al., 2003). Increase in the APX activities in tea suggests that the anti-oxidative mechanism induced by Cu was invaded in detoxification of H₂O₂. Many other authors also observed the increase in APX activities due to accumulation of stress. Accumulation of Cu showed increase in APX activity in *Phaseolus vulgaris* (Gupta et al., 1999) and *Camellia sinensis* (Saha et al., 2011).

Copper was inhibitory to CAT but relatively less effective in producing oxidative damage probably due to the fairly enhanced levels of carotenoids, Cu/Zn SOD, POD and glutathione reductase (GR). CAT is also an important enzyme which catalyses H₂O₂ by breaking down it directly to form H₂O and O₂. Initially there was increase in CAT activity in cultivar TS-491 but from 4th day, there was decline in the activity. The increase in SOD and decrease in CAT activity in response to excess supply of heavy metals has been widely reported (Chaoui et al., 1997; Pandey and Sharma, 2002; Cho and Seo, 2005; Lombardi and Sebastiani, 2005). The decrease in CAT activity results in the inactivation of its reaction with superoxide ions which leads to weaken the effective detoxification of H₂O₂ (Kono and Fridovich, 1982). Cho and Seo (2005) reported that oxidative stress in response to Cd toxicity is due to H₂O₂ accumulation. Cu toxicity causes oxidative stress by generating oxygen species ROS which as a result cause lipid peroxidation. The oxidative damage in the heavy metal-stressed tea plants could be due to accumulation of H₂O₂ as a consequence of enhanced activity of total SOD and inhibition of CAT. The significant increase in CAT activity at lower concentration with the Cu exposure has also been reported in tea plant (Saha et al., 2011). However, the decrease or unaffected CAT activity due to Cu was reported in oat leaf (Luna et al., 1994), tomato seedlings (Mazhoudi et al., 1997); whereas, significant increase in CAT activity was also reported in *Prunus cerasifera* plantlets due to Cu stress (Lombardi and Sebastiani, 2005). Tang et al., 2011, also observed reduced CAT activity in tea plants due to increase in the Cr concentration.

In the present investigation, we observed increase in SOD activity in both the cultivars with the increase in the

concentration of Cu. SOD is one of the stress resistant enzymes which can catalyze O₂⁻ radicals to H₂O₂ and O₂. The decrease or increase in the level of these metals causes adverse effect in the activity. The same result has been observed by other authors (Saha et al., 2011; Wang et al., 2004; Hartley-Whitaker et al., 2001). SOD is an important antioxidant enzyme that catalyzes a disproportionate amount of superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂) (Bowler et al., 1992). Excess supply of heavy metals led to increased accumulation of ascorbate, which is associated with increased activities of APX and GR. The heavy metal-induced oxidative stress also triggers the ascorbate-glutathione cycle for detoxification of hydrogen peroxide which could be a common strategy for counteracting the over-production of the ROS (Foyer and Noctor, 2005).

Peroxidase (POD) includes a group of specific enzymes such as NAD-peroxidase, NADP-peroxidase, fatty acid peroxidase etc. POD catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydro-quinone etc. POD catalyses H₂O₂ dependent oxidation of substrates which takes part in improving the mechanical protection in plants (Dong et al., 2006). The present study observes the increase in the POD activity in both the cultivars. In TS-491, increase in the activity was only up to 500 µM concentration whereas in S₃A₃ the increase was up to 600 µM. The elevation in the activity of POD shows that it catalyses the H₂O₂ into H₂O and prevent the accumulation of H₂O₂ and O₂; which may be due to ionic micro environment or tissue specific gene expression in plants. The increase in POD helps in reduction of harmful affect caused by the free radicals to structure and function of the membrane (Sun et al., 2006; Pauls and Thompson 1984; Vetanovetz and Peterson, 1990). Saha et al., 2011 also reported the increase in the activity of POD with the increase in the Cu concentration up to 400 µM.

The present study shows the resemblance with the result of Cu stress on tea plant where they showed significant increase in the phenol content below 400 µM concentration (Saha et al., 2011). Phenolic compounds act as reducing agents, hydrogen donor and singlet oxygen quenchers which as a whole make it as an important antioxidant (Rice-Evans et al., 1997). The significant increase in the activity shows that the compound helps in the binding of the heavy metal Cu. The hydroxyls groups of phenol help in chelation by binding heavy metal (Jung et al., 2003). It was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993; Gülçin, 2005).

When the plants are grown under some stress involved in the environment, then excess of free radicals accumulate in the cells which results in the lipid peroxidation

consequently resulting to increase in the malondialdehyde (MDA) production as last product of this activity (Chaoui et al., 1997). Increased in MDA content is an indicator of physiological stress and aging (Quariti et al., 1997). Many authors also reported that exposure of heavy metals causes increase in MDA levels (Asada 1992; Gille and Singler 1995; Ozounidou 1994). In our study, we observed the increase in MDA content in both the cultivars with the increase in Cu concentration which shows similarity with the findings of Cu stress with other authors (Saha et al., 2011, Rama Devi and Prasad, 1998; Mohanpuria et al., 2007). The difference among the cultivars in response to Cu has also been reported by many authors (Ciscato et al., 1997)

Conclusion

In conclusion, our data demonstrates that among the two cultivars, TS-491 was more sensitive to the higher concentration of Cu compared to S₃A₃. The higher concentration beyond 400 µM of Cu became toxic to the tea plant. It leads to the decline in the growth by retarding many biological functions. The accumulation of Cu was higher in roots leading to the production of ROS, which are followed by lipid peroxidation and many more antioxidant enzymes. The cooperation and the interaction between the antioxidant enzymes detoxify the ROS.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

A two-stage decentralised system combining high rate activated sludge (HRAS) with alternating charcoal filters (ACF) for treating small community sewage to reusable standards for agriculture

Irene Nansubuga^{1,3}, Francis Meerburg¹, Noble Banadda², Korneel Rabaey¹ and Willy Verstraete^{1*}

¹LabMET, Faculty of Biosciences engineering, University of Ghent, Coupure Links 653, 9000 Ghent, Belgium.

²Department of Agricultural and Bio-Systems Engineering, Makerere University, P.O. 7062, Kampala, Uganda.

³National Water and Sewerage corporation, Plot 39, Jinja Road, P.O. Box 7053, Kampala, Uganda.

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Water scarcity increasingly drives wastewater recovery. Campaigns towards re-use of wastewater are not very common in Africa among other factors, due to a lack of efficient and cost-effective technology to treat wastewater to re-usable standards. In this study, two treatment systems, a high rate activated sludge (HRAS) system and alternating charcoal filters (ACF) are combined and used to treat wastewater to standards fit for reuse in agriculture. The charcoal can upon saturation be dried and used as fuel. Two different ACF lines were used in parallel after the HRAS: ACF1 with a residence time of 2.5 h and ACF2 with residence time of 5 h. Results show no significant difference ($\alpha = 0.05$) in the performance of the two filter lines, hence ACF1 with a higher flow rate was considered as optimal. The HRAS effectively removed up to 65% of total suspended solids (TSS) and 59% of chemical oxygen demand (COD), while ACF1 removed up to 70% TSS and 58% COD. The combined treatment system of HRAS and ACF1 effectively decreased TSS and COD on average by 89 and 83%, respectively. Total ammonium nitrogen (TAN) and total phosphates (TP) were largely retained in the effluent with average removal percentages of 19.5 and 27.5%, respectively, encouraging reuse for plant growth.

Key words: A-stage, sustainable wastewater treatment, resource recovery, developing countries, water reuse, nutrient management, agriculture.

INTRODUCTION

Humans depend on water for nearly all aspects of life. The diverse utilization of water coupled with population

explosion across many places in the world has made it a scarce resource. Moreover, the discharge of untreated or

*Corresponding author. E-mail: Willy.Verstraete@UGent.be. Tel: +32 9/264 59 76. Fax: +32 9/264 62 48.

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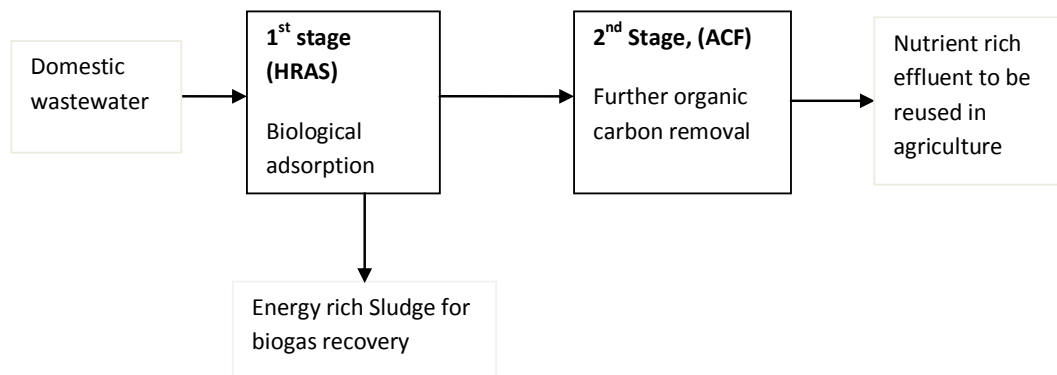


Figure 1. Representation of the combined processes treatment with use of high rate activated sludge (HRAS) system and the alternating charcoal filter (ACF).

inadequately treated wastewater leads to deterioration in the quality of fresh water sources and continues to deepen the water scarcity. Re-use of wastewater for some purposes such as agriculture is an indispensable part of integrated water management and would decrease water scarcity. This requires a change in perceptions as well as availability of simple, low cost and effective technologies. The treated wastewater should be sufficiently disinfected but not void of its nutrient content, so as to increase crop yields. In Uganda, reuse of wastewater is not widely reported; however, informal irrigation occurs in several parts of the country. For instance farmers in the Murchison Bay, which receives Kampala city's highest flow of wastewater effluent, are seen to cultivate a variety of crops. The main concern for reuse of wastewater is the health of both the farmers and the crop consumers. Unfortunately, some of the treatment methods used in developing countries may not attain sufficient disinfection, which limits reuse options (Nikiema et al., 2013) and may pose public health risks if improperly applied. Centralised systems common in the developing world are effective but very expensive and are not suitable for low density rural areas (Netter et al., 1993). These systems can cost up to € 40 per capita per year considering both capital and operational expenditure (Zessner et al., 2010). On the other hand, on-site systems are cheaper but have a number of limitations with regard to wastewater re-use. Also, some like pit latrines are known to increasingly pollute ground water sources (Katukiza et al., 2013, Nyenje et al., 2013). Therefore, efficacious and cost effective technology to boost wastewater reuse and recycling needs development for the developing world.

Verstraete and Vlaeminck (2011) proposed a new approach for optimal resource recovery, as opposed to the conventional wastewater management. In this approach which they label as the M & M treatment system, the wastewater is separated as near as possible to the source into two distinct streams: the major line (up to 90% of the flow) and the minor line (about 10% of the

flow). The major water stream is treated to reusable standards while the minor concentrated stream can undergo additional treatment to recover energy and nutrients. Small-scale decentralised systems designed for a small number of households could provide a cost-effective method for that purpose. Such systems should focus on optimising the pre-concentration methods and further treatment of the two separate streams, to maximize resource recovery. Methods of solids pre-concentration may include the biological adsorption in a high-rate activated sludge stage (HRAS), also referred to as the A-stage of the A/B Verfahren system (Böhnke, 1977). This activated sludge process operates at high sludge loading rates (2 to 10 g bCOD gVSS⁻¹ d⁻¹) and low sludge retention times (hours to days), while a short hydraulic retention time of under 30 min selects for rapid incorporation of organic matter into sludge without extensive oxidation (Bohnke, 1977). Moreover, the 'young' A-stage sludge is easily digestible by anaerobic digestion (De Vrieze et al., 2013) to recover energy. The effluent from the A-stage can be further treated to achieve reusable standards by methods such as trickling filters or sand filters. For the developing world, it is important to explore locally available materials and simple technologies in order to achieve cost effective and sustainable systems. Charcoal is such a material and it is ubiquitously available in Uganda. The use of charcoal for wastewater treatment has been widely studied (Abe et al., 1993; Samkutty and Gough, 2002; Scholz and Xu, 2002; Ochieng et al., 2004; Sirianuntapiboon et al., 2007; Nkwonta et al., 2010; Ahamad and Jawed, 2011). Its performance compared well with other media like gravel, sand rocks and zeolite, however, attaining its continued use is still a challenge.

For this reason, this study proposes and investigates the concept of the state-of-the-art of low cost small scale wastewater treatment plant which also allows for wastewater reuse. It combines two wastewater treatment systems (Figure 1). The first stage is a HRAS system similar to the A-stage, to achieve pre-concentration and

Table 1. Average operating parameters of the high rate activated sludge (HRAS), the alternating charcoal filter 1 (ACF1) with a retention time of 2.5 h and the alternating charcoal filters 2 (ACF2) with a retention time of 5 h and the removal efficiency of the HRAS combined with each of the alternating filter options.

Parameter	Raw wastewater	HRAS reactor	HRAS effluent	ACF1 effluent	ACF2 effluent	HRAS+ACF1 Average total removal (%)	HRAS+ACF2 Average total removal (%)
TSS (mg/mL)	0.322±0.163	2.174±0.932	0.102±0.049	0.032±0.022	0.026±0.019	0.089± 0.007	0.091±0.006
COD total (mg/mL)	0.613±0.244		0.233±0.106	0.093±0.045	0.091±0.047	0.083±0.008	0.084±0.008
COD soluble (mg/mL)	0.128±0.057		0.111±0.061	0.073±0.030	0.068±0.030	0.046±0.024	0.048±0.024
TAN (mg/mL)	0.036±0.011		0.033±0.010	0.030±0.009	0.029±0.009	0.019±0.016	0.020±0.010
P _{total} (mg/mL)	0.026±0.013		0.022±0.010	0.019±0.009	0.019±0.008	0.027±0.015	0.028±0.014
pH	7.2±0.2	7.4±0.2	7.5±0.2	7.6±0.1	7.6±0.1		
Temperature		21.9±0.7					
DO (mg/mL)		3.7±1.6					

major organics removal, and the second stage is filtration of the liquid fraction with use of alternating charcoal filters. The wastewater is treated to meet reusable standards for agriculture. The sludge from the process is to be used for biogas recovery in a subsequent study. Upon saturation the charcoal is replaced which allows for continuity of the system, the charcoal could then be dried and finally used as fuel, which originally was its primary use. This system is suitable for small communities.

MATERIALS AND METHODS

Sample collection

Raw domestic wastewater was collected from Bugolobi Sewage treatment plant (STP) in Kampala (Uganda) every two to three days for four months (June 2013 to October 2013). The Bugolobi STP managed by National Water and Sewerage Corporation (NWSC), is the largest sewage treatment plant in Uganda. It employs physical and biological treatment by use of screens, detritus basin, primary settling tanks, trickling filters and secondary clarifiers in that order. The plant has an average inflow of 12,000 m³ per day mainly via the centralised sewerage pipe network. However, about 300 m³ of the inflow is received via cesspool trucks that deliver septage from septic tanks and pit latrines around Kampala City and its outskirts. The cesspool dumping usually accounts for a sudden change in the influent wastewater quality. In this study, the wastewater was collected after the screens and grit chamber and stored in a 200 L container which continuously fed the HRAS experiment. Selected parameters of the raw wastewater characteristics and outflow of the HRAS stage were determined and are shown in Table 1. The values indicate that the Bugolobi STP wastewater is generally of high strength (for a comparison Metcalf and Eddy, 1991). The maximum values of total suspended solids (TSS), total phosphates (TP), total ammonium nitrogen (TAN) and chemical oxygen demand (COD) of the Bugolobi STP wastewater sampled at different times were 0.794, 0.066, 0.061 and 0.116 mg mL⁻¹, respectively. The faecal coliform (FC) colony forming units (CFU) in the influent ranged from 3.13x10² to 2.01x10⁶ CFU mL⁻¹. The wastewater characteristics are known to vary depending on the weather conditions. The variation can also be attributed to the small daily volumes (300 m³ day⁻¹) of high strength septage received by the

plant throughout the day. The reactor sludge was obtained by autonomous growth during an acclimation period of 10 days of reactor operation. The charcoal used in the study was bought from the open market, crushed into pieces ranging from 0.5 to 1.5 cm. It was then washed to remove the dust before packing it in plastic columns in the Laboratory. The porosity and dry bulk density of the packed charcoal after crushing were determined.

Experimental set-up

High-rate activated sludge (HRAS) experiment

A HRAS experiment was set up at laboratory scale as shown in Figure 2. It consisted of a continuous stirred tank reactor (CSTR) unit which was continuously aerated, a settling unit and a sludge return device. The CSTR unit had a volume of 4 L and an average hydraulic retention time (HRT) which begun at 0.5 h but was increased and maintained at 1 ± 0.3 h after 10 days. The average sludge retention time (SRT) of the CSTR was 1.5 ± 0.3 days and it was loaded at an average sludge loading rate of 2.2 g bCOD/g SS per day. Two electrical aerators (Aquatic AP1, Interpet, United Kingdom) were used to supply oxygen into the CSTR which achieved an average concentration of dissolved oxygen (DO) of 3.7 ± 1.6 x10⁻³ mg/mL. A mechanical stirrer (RW16 basic, IKA Labortechnik, Germany) was used to stir the CSTR unit. The settling unit had an effective volume of 8 L and an initial HRT of 1 h, which was increased and maintained at 2 ± 0.4 h after 10 days. The sludge from the settling unit was returned to the CSTR using a pump (Leroy Somer Varmeca, Belgium). The Recycle ratio ($Q_{\text{return}}/Q_{\text{influent}}$) of the CSTR was 1 and 2 L of sludge was wasted manually every day. The wasted sludge was kept in a 5 L container at 4°C where it settled further before the clear water was poured off and the settled sludge was used in another study. Selected parameters of the influent and effluent of the HRAS experiment were measured on the samples collected three times a week.

The alternating charcoal filter (ACF)

The effluent from the HRAS was fed into the ACF for further treatment as shown in Figure 3. It was fed into two separate ACF lines, each with three charcoal filter columns placed in series. The filter columns were 25 ± 3 cm long and had a volume of 1 L of charcoal. The charcoal particles in the filters ranged between 0.5 to

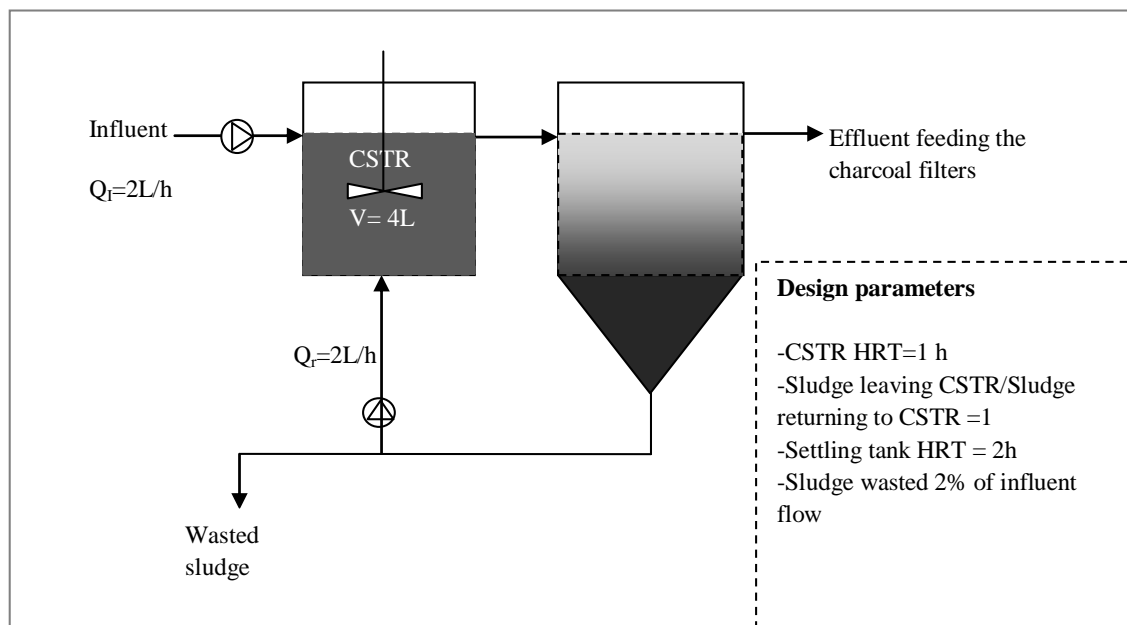


Figure 2. Schematic representation of the high-rate activated sludge (HRAS) set-up consisting of a completely mixed reactor (CSTR) in series with a settler.

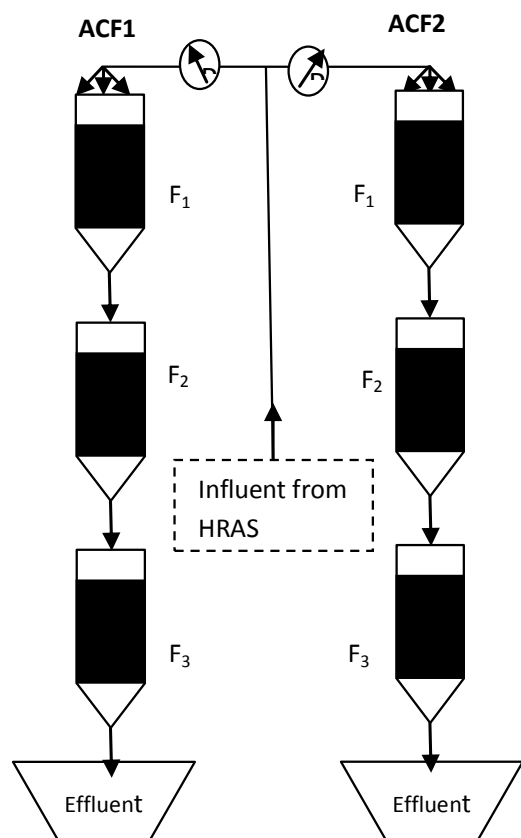


Figure 3. Schematic representation of the setup of the alternating charcoal filter 1 (ACF1) with a retention time of 2.5 h and the alternating charcoal filters 2 (ACF2) with a retention time of 5 h.

1.5 cm. The packed filters had porosity of 48% and dry bulk density of 0.3 g cm^{-3} . The residence time in the filter lines differed with filter line 1 (ACF1) having a residence time of 2.5 h, while filter line 2 (ACF2) had a residence time of 5 h. After every 30 days, the top filter column 1 (F_1) was emptied and refilled with fresh charcoal and moved to the last position in the series while filter column 2 (F_2) and filter column 3 (F_3) went a position up in the series to become F_1 and F_2 , respectively. This means that all filters were replaced every 90 days and this continued for the rest of the experimental period. Wastewater samples were taken from the effluent of the last filter columns thrice a week; and chemical oxygen demand (COD), TSS, total ammonium nitrogen (TAN), Total phosphorus (TP), and CFU were measured.

Analytical methods

The influent and effluent samples of the HRAS and the ACF were measured for organic matter, total nitrogen and phosphorous. Total phosphorus (TP), chemical oxygen demand (COD) and total ammonium nitrogen (TAN) were analyzed using Hach DR 5000 Spectrometer as described in the standard methods (APHA, 2005). The pH was measured with a pH meter (Teledo, USA) while volatile Solids (VS) and total solids (TS) were analysed according to standard methods (APHA, 2005). The FC Bacteria were determined using the Colilert-18 protocol (Idexx Laboratories, 2012) and dissolved oxygen (DO) was determined with use of a DO meter (Hach, UK). The Kruskal-Wallis non-parametric test was used to verify if there was a significant difference between the measured influent and effluent parameters of the HRAS and the ACF.

RESULTS

Performance of the HRAS reactor

In the HRAS reactor, the wastewater had an average pH

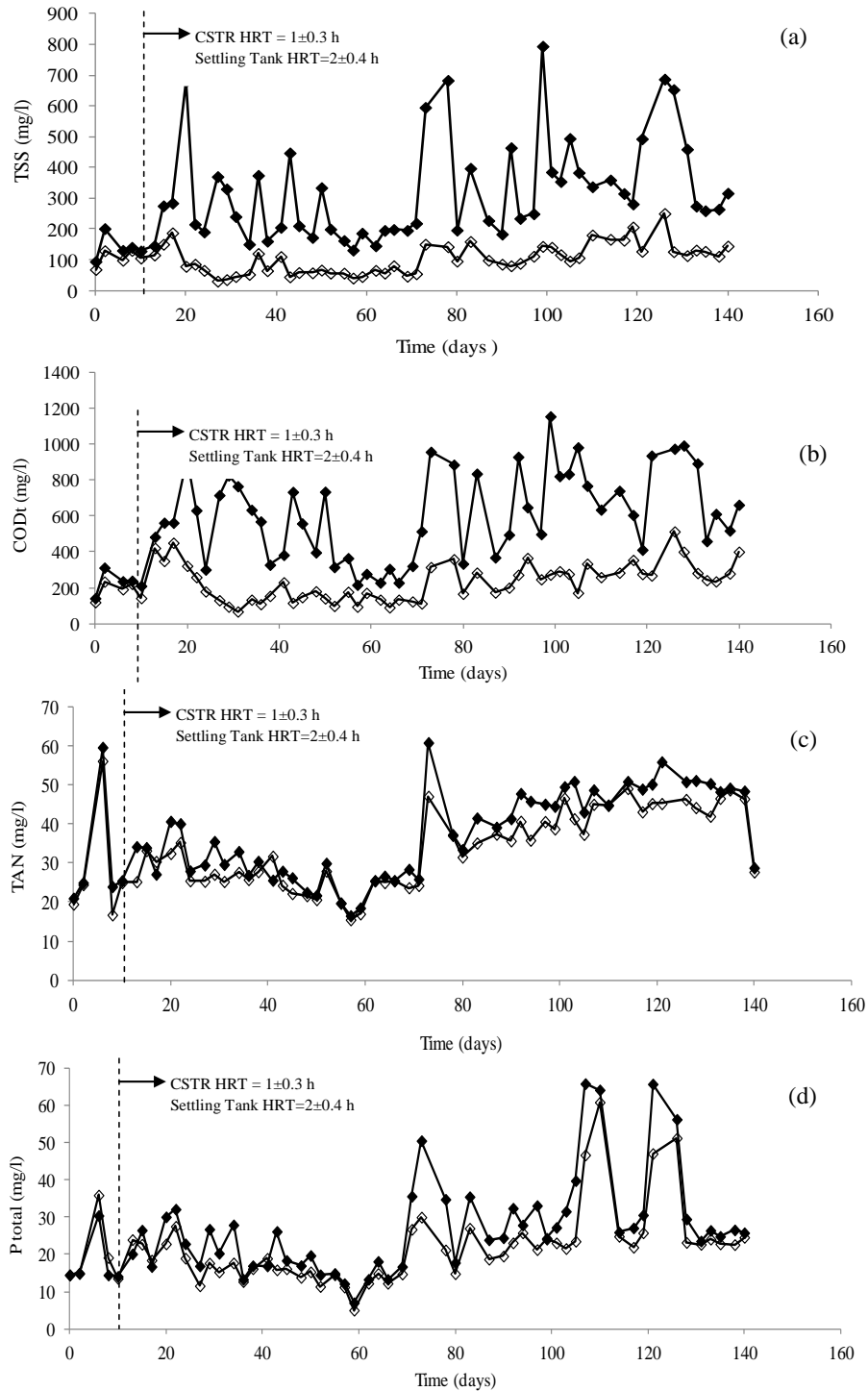


Figure 4. Influent (◆) and effluent (◇) concentrations of (a) the total suspended solids (TSS), (b) the total chemical oxygen demand (COD_t), (c) the total Ammonium nitrogen (TAN) and (d) the total phosphorous (P_{total}), in the high rate activated sludge system during the entire study period.

of 7.4 ± 0.2 , dissolved oxygen of $3.7 \pm 1.6 \times 10^{-3} \text{ mg mL}^{-1}$ and temperature of $21.9 \pm 0.7^\circ\text{C}$ (Table 1). Figure 4 shows the performance of the HRAS over the entire 140

days of the experimental run. To evaluate the performance of the HRAS, consideration is only given to the period after day 10 when the HRT in the CSTR and the

sedimentation tank were maintained at 1 ± 0.3 and 2 ± 0.4 h, respectively. Regardless of the variation observed in the influent TSS concentration (0.131 to 0.794 mg mL⁻¹), the effluent concentrations were less variable ranging between 0.030 to 0.250 mg mL⁻¹. This corresponded to an average TSS removal of 65%. The average influent COD was 0.613 ± 0.244 mg mL⁻¹ of which about 21% was soluble while the average effluent concentration was 0.233 ± 0.104 mg mL⁻¹ of which about 48% was soluble COD. This led to an average removal efficiency of 59% for total COD and 15% for soluble COD. The HRAS slightly eliminated TAN and TP with an average removal efficiency of 11 and 17%, respectively.

Performance of the ACF reactor

The effluent of the HRAS was fed to two ACF reactors for further treatment. ACF1 had a residence time of 2.5 h while ACF2 had a residence time of 5 h. Figure 5 shows the performance of the two filter lines over the entire 140 days of the experiment. For consistency, the period after day 10 was considered for evaluation of the performance of the filters. The average TSS concentration of the effluent from ACF1 and ACF2 were 0.032 ± 0.022 and 0.026 ± 0.019 mg mL⁻¹, respectively. This corresponds to an average removal efficiency of 70% for ACF1 and 76% for ACF2. The concentration of total COD of the effluent from ACF1 was on average 0.093 ± 0.045 mg mL⁻¹ of which 78% was soluble COD, for ACF2 the average total COD was 0.091 ± 0.047 mg L⁻¹ of which 74% was soluble. This corresponds to a total COD removal efficiency of 58 and 60%, observed for ACF1 and ACF2, respectively, while for soluble COD, it was 27 and 30%, respectively. Like in the HRAS reactor, the removal of TAN and TP was low in both filter lines. The average removal of TAN was 11 and 13% in ACF1 and ACF2, respectively, while the average TP removal was 12% in ACF1 and 13% in ACF2. Statistical analysis showed that there was no significant difference ($\alpha = 0.05$) in the performance between ACF1 and ACF2 in removal of all the above considered parameters.

Overall performance of the combined treatment system

In general, the combination of the HRAS and ACF registered high COD and TSS removal efficiencies (Table 1). The overall average TSS removal was $89\% \pm 7$ and $91\% \pm 6$ when the HRAS was combined with ACF1 and ACF2, respectively. The same combinations attained average total COD removals of $83\% \pm 8$ and $84\% \pm 8$ and average soluble COD removal of $46\% \pm 24$ and $48\% \pm 24$, respectively. The overall removal of TP and TAN was generally lower compared to TSS and COD: the combination of HRAS with ACF1 obtained an average

TAN removal of $19\% \pm 16$ while with ACF2 it was $20\% \pm 10$. TP removal was $27\% \pm 15$ and $28\% \pm 14$ for the HRAS combination with ACF1 and ACF2, respectively. There was no significant difference ($\alpha=0.05$) in the performance of the two filters. CFU counts were monitored from day 34 up to the end of the experiment. The HRAS influent CFU counts varied widely from 3.13×10^2 to 2.01×10^6 CFU mL⁻¹. During the experimental study period, the HRAS system achieved on average 1 log decrease of CFU and a further 2 log decrease was achieved by the ACF treatment system.

DISCUSSION

High rate activated sludge (HRAS) system

Bohnke et al. (1997) proposed that the HRT of HRAS should be 30 min or less. However, at that HRT which was used in the first 10 days of the experiment, the performance of our HRAS unit was insufficient, with COD and TSS removals going below 40 and 45%, respectively, hence the HRT was increased to 1 h. The HRAS reactor thereafter effectively removed TSS and total COD by an average of 65 and 59%, respectively. The results in this study are similar to those observed in other studies (Zamalloa et al., 2013; Bohnke, 1977). Apart from biological uptake and degradation, removal in the HRAS systems is partially due to physico-chemical processes which include adsorption and bio-flocculation (Bohnke et al., 1997, 1998).

The contribution of physico-chemical processes on the overall removal is a result of the short SRT and high sludge loading rate of HRAS processes, which alter the kinetics of substrate removal (Larrea et al., 2002; Makinia et al., 2006). The adsorption of particulate substrates may act as a buffer against fluctuations in organic loads (Bunch and Griffin, 1987), which ensures that the effluent sent to the second stage had a more stable composition for optimal filter performance (Bohnke et al., 1997). TP and TAN were removed to a lower extent in comparison to TSS and COD. TAN and TP removal is generally known to be low in HRAS and other high rate activated sludge processes. To ensure sufficient removal of these compounds, additional treatment is typically incorporated after such systems.

Zamalloa et al. (2013) applied a flocculant in the HRAS to decrease phosphates while Bohnke et al. (1997) ensured TAN and TP removal in a second activated sludge stage at low sludge loading rates. For this study however, since the final effluent from the treatment system is proposed for reuse in agriculture, there would be no need for removal of TP and TAN. The sludge generated in the HRAS is known to be highly degradable (Hernandez Leal et al., 2010; De Vrieze et al., 2013) and will be anaerobically digested for energy recovery in a subsequent study.

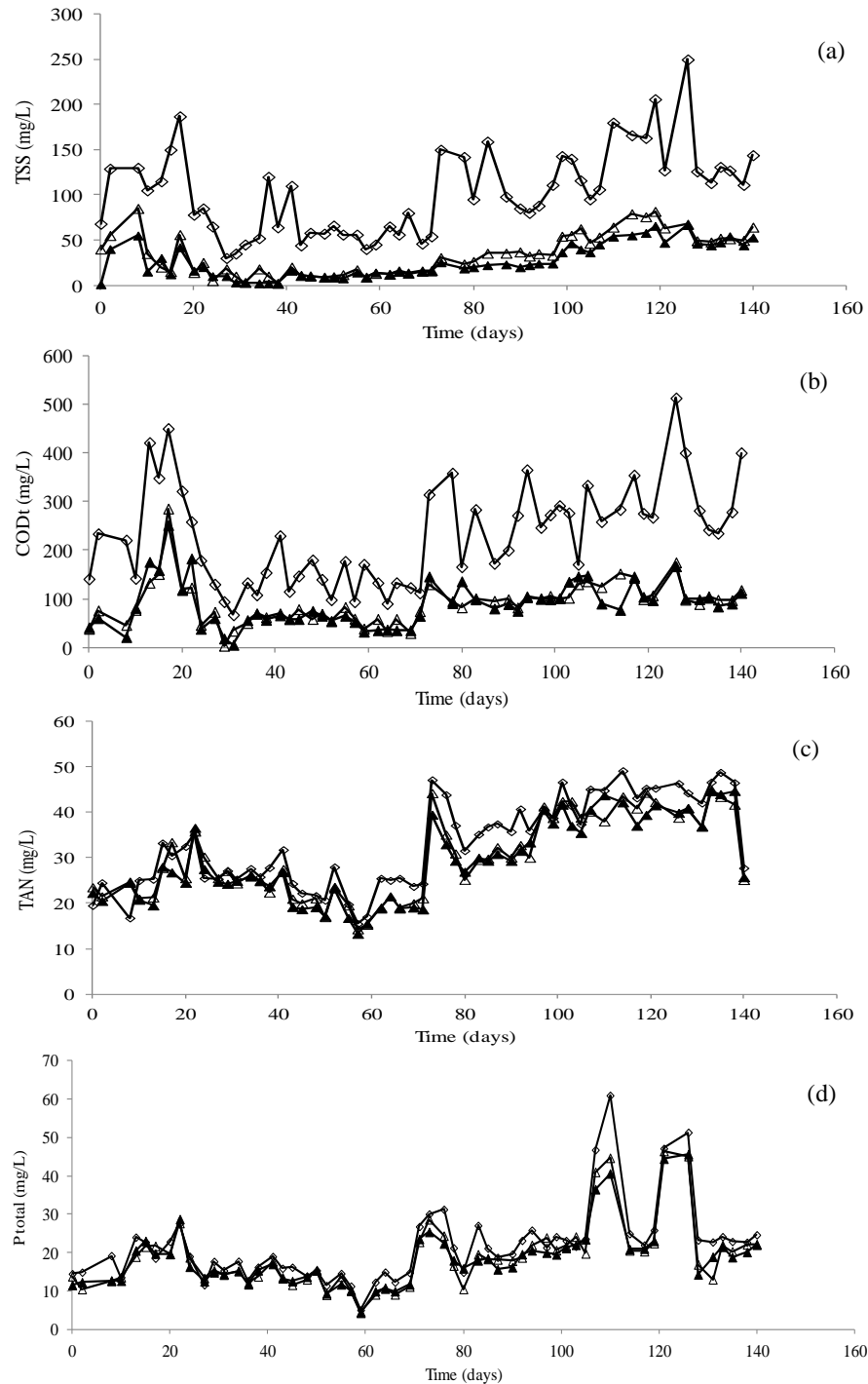


Figure 5. Concentrations of (a) the total suspended solids (TSS), (b) the total chemical oxygen demand (COD_t), (c) the total Ammonium nitrogen (TAN) and (d) the total phosphorous (P_{total}) in the Influent (◇), ACF1 Effluent (△) and ACF2 Effluent (▲) during the entire study period.

Alternating charcoal filters (ACF) system

The charcoal filters benefited from the HRAS stage which had an effective treatment and produced a more uniform effluent (TSS and COD did not vary as much as they did

in the influent). The two filters had similar performance in which they effectively removed TSS and total COD by an average of 73 and 59%, respectively. Similar to the HRAS, a limited removal was observed for TAN and TP, so the final effluent still contained sufficient nutrients for

plant growth. Removal mechanisms of pollutants by the charcoal filter are similar to those in other filters. These include physical filtration, sedimentation, adsorption and biological degradation due to biofilm development. When compared to other filter materials like gravel and rocks however, charcoal has a number of essential properties such as a high number of many micro pores on the surface, high porosity and a high specific surface area of 200 to 300 m²/g (Darmstadt et al., 2000). The higher specific surface area and porosity in charcoal enhances sedimentation and other filtration processes in charcoal filters (Ochieng and Otieno, 2006) and the micro-pores provide good conditions for micro-organisms to attach. Also, like granulated carbon, charcoal is a good adsorbent and has been widely used as such in wastewater and water treatment (Abe et al., 1993; Khalfaoui et al., 1995, Kamal and Mohammad, 2012). Due to its adsorbent properties, charcoal can accumulate sufficient organic matter and nutrients for biomass to grow. It is believed that in the first few days before biofilm growth, adsorption is responsible for most of the COD removal. All these processes contribute to the high efficiency of TSS and COD removal observed throughout the filter's operation. In addition, the small-sized charcoal particles used in this study are cheap, light and easily available at charcoal making stores as waste, and hence offers a cost-effective filter medium for application in the developing world. Actually, the cost for regular replacement of the charcoal are quite reasonable, they are only of the order of 9% of the total cost capita⁻¹ year⁻¹. Unlike other media however, charcoal is not easy to clean in case of clogging, which would potentially limit its application for prolonged operation times. Therefore, it is proposed in this study that the charcoal filters be used in series and be moved up the chain as the first filter is replaced every month. As demonstrated in this study, such an alternating use of charcoal filters ensures consistently high removal efficiency for both TSS and COD. Interestingly, the spent charcoal can be sun dried and subsequently used for fuel. Thus, the charcoal can be used in a coherent sustainable way.

Overall performance

Overall, the combination of the HRAS with each of the filters showed an effective system for the removal of TSS and COD. It produced an effluent whose average values of TSS and COD met the National effluent standard as required by the National Environment Management Authority (NEMA). NEMA is the regulatory body of effluent discharge in Uganda and its standards require both the TSS and COD of the effluent to be below 0.1 mg mL⁻¹. The combination of the HRAS and the ACF also showed that it could on average achieve a 3 log decrease of CFU mL⁻¹ from the influent. The removal efficiency of CFU is at least 60% in an activated sludge process or

biofilm process (Farrell et al., 1990). The treatment system in this study performed as well as expected achieving 99.9% (3 log decrease) of CFU for the combined systems of the HRAS and the ACF. In porous media systems, pathogen removal is partially achieved by straining and sorption, which are largely determined by the filter pore sizes, hydraulic loading and clogging (Stevik et al., 2004). Straining would be predominant with small pore sizes (when bacteria sizes are bigger than the pore sizes), low hydraulic loading and where clogging has occurred, otherwise adsorption would take over. With the charcoal particle sizes up to 1.5 cm it is clear that adsorption was the most important mechanism of pathogen removal at the beginning of the experiment. However, with time, clogging brought about straining as the other pathogen removal mechanism. Also, the continued running of experiment allowed accumulation of macro-organisms which contribute to pathogen removal through predation. With the influent ranging from 3.13 × 10² to 2.01 × 10⁶ FC mL⁻¹, it was possible to achieve the NEMA effluent standard of 10² CFU mL⁻¹ for more than half of the samples (53%). Given that on average, a 2 log decrease of CFU can be achieved by the ACF system alone which consists of three filter columns, it would be possible to increase percentage of compliance by increasing the number of filter columns in the ACF system. Further studies could aim at optimising the system with regard to additional filters required to achieve 100% compliance of the CFU effluent to NEMA standards. Furthermore, with the effluent proposed to be reused in agriculture, it should also meet the standards for reuse. The World Health Organisation (WHO) guidelines require at least a 6 log decrease of pathogens from the wastewater source considering a level of contamination of 10⁶ CFU mL⁻¹ in the untreated wastewater (WHO, 2006). On the other hand, designing a plant to achieve a log decrease of 6 or more, only to eliminate pathogen contamination would be too expensive. It would include additional processes like chemical coagulation, flocculation and disinfection, which would generally preclude its application in many developing countries. It is therefore important that wastewater reuse strategies for pathogen removal are not just based on wastewater treatment alone. Instead, a multiple control approach should be adopted to effectively eliminate or inactivate the various microorganisms spread through different routes. WHO (2006) proposes different control measures such as cooking and washing of foods before consumption, that can be combined to achieve a total log decrease sufficient to eliminate risk of pathogen infection.

Preliminary estimation of costs

The preliminary cost estimates of the HRAS/ACF treatment system serving a small farming community of

Table 2. Capital and operational cost estimation of HRAS/ACF system. Assuming a small agricultural community of 10 houses, with 5 inhabitants producing 100 L of wastewater IE⁻¹day⁻¹.

Capital costs	€
HRAS CSTR ^a	60
HRAS Settler ^b	110
Charcoal filter ^c	114
Filter material ^{cd}	5
HRAS/ACF Instrumentation ^e	100
Total Capital cost	389
	7.8 € Capita ⁻¹
Operational costs	€/m ³ /d
ACF material ^{df}	0.012
Electricity costs ^g	0.003
Labour costs ^h	0.093
Total operational cost	0.1
	3.6 € Capita ⁻¹ year ⁻¹
Annualised overall cost for the treatment system ⁱ	4.9 € Capita ⁻¹ year ⁻¹

^aWastewater flow rate plus recycle of 0.4 m³h⁻¹, requires a durable plastic water tank of 0.5 m³, volume price according to a local plastic water tank manufacturer is 60 €. ^bFor a HRT of 2 h, the settling tank volume required is at least 0.8 m³. Use a durable plastic water tank of 1 m³ volume, local manufacture's price is 110 €. ^cFor a flow rate of 0.2 m³h⁻¹ (no recycle), total charcoal volume required is 0.5 m³ (0.2 m³ per filter). Use 3 plastic tanks of 0.25 m³ of a local price of € 38 each. ^dA bag of charcoal (0.33 m³) costs between € 10 - 20 depending on the season. However, a bag of the small pieces (< 2 cm) arising from the charcoal making process is wasted or sold at 3 €. ^eHRAS/ACF instrumentation (pump, aerator and pipe work) is estimated at 100 €. ^fMaterial in only one filter is replaced monthly. ^gBased on a consumption of 0.018 kWh/d/m³ wastewater treated. Installed power of 6 W/m³ reactor is assumed (10 m hydraulic head, for a flow rate of 5 m³/d and a pump efficiency of 60%) and 3 h pumping at an electricity cost of 0.09 €/kWh. ^hCheap unskilled labour is required to monitor pump operation time and change material. ⁱA life span of 10 years was considered and a real interest rate of 10%.

10 houses, each with 5 inhabitants is shown in Table 2. The costs are based on the lab-scale reactor operational conditions and use of locally available but durable material in Uganda. These estimations indicate that the system can treat wastewater at an overall (capital and operational) annualised cost of 5 € capita⁻¹ year⁻¹. This estimate excludes the sludge line treatment. If it is included, it could be possible to recover an additional value from electricity generated estimated at 1 € capita⁻¹ year⁻¹ for sludge with at least 3 to 5 kg DW/m³ (Verstraete and Vlaeminck, 2011) through anaerobic digestion. The overall (capital and operational) cost of the HRAS/ACF system is less than a third the overall cost of a small scale (10,000 to 50,000 IE) conventional activated sludge system (CAS), which is estimated at about 18 to 24 € capita⁻¹ year⁻¹ (Zessner et al., 2010), excluding sludge treatment. It was also less than half the cost of the waste stabilisation pond (WSP) and the horizontal subsurface flow constructed wetlands (HSSF-CW) which can cost about 13 and 14 € capita⁻¹ year⁻¹, respectively, in East Africa (Mburu et al., 2013). Apart from the already mentioned added value that could arise from anaerobic

digestion of the sludge, the proposed system offers the community other benefits which include fuel that can be derived from the sun dried used charcoal. Furthermore, a nutrient rich effluent would go a long way to boost crop productivity for farmers.

Conclusions

The results in this study have shown that a combination of the HRAS and the ACF can effectively remove TSS and COD from domestic wastewater to meet the NEMA discharge standards. The treatment system achieved the NEMA effluent standard for CFU for more than half of the samples. However, it would be possible to attain higher CFU removal if more filter columns are added in the ACF system. Further research is proposed to optimise the system in order to achieve 100% compliance to the CFU standard. TAN and TP were largely retained in the effluent, allowing nutrient reuse by crops. The proposed treatment system has an estimated cost which is less than half the cost of other systems such as, the small-

scale CAS, WSP and HSSF-CW. It further offers a nutrient-rich effluent which will advance the re-use of wastewater for agriculture through generation of higher crop yields and profits. The novel design is therefore suggested for further development as a technology for wastewater treatment and reuse to benefit small agricultural communities. In order to effectively eliminate microorganisms and reduce pathogen transmission, it is recommended that the effluent be reused in an agricultural setting with a multi-barrier approach for example where food will be washed and or cooked before consumption.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Characterization of indole acetic acid endophyte producers in autochthonous *Lemna gibba* plants from Xochimilco Lake

Orlando Ortega-Acosta¹, Aida Verónica Rodríguez-Tovar², Eugenia López-López³ and Angélica Rodríguez-Dorantes^{1*}

¹Laboratorio de Fisiología Vegetal, Departamento de Botánica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D.F. 11340, México.

²Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D.F. 11340, México.

³Laboratorio de Bioconservación y Manejo, Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D.F. 11340.

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Xochimilco's lacustrine zone is a network of channels that, along with the chinampas, conform a unique ecosystem which has served as source of aquatic resources. Duckweeds are small free-floating monocotyledon aquatic plants classified as macrophytes that serve as nutrient pumps and reduce eutrophication effects. Recently, there are number of new studies related to the aquatic plant-microbial interaction focused on the direct functional analysis that investigate plant microbe interactions at full biological hierarchy. The aim of this study was to compare the auxin *in vitro* production efficiency of the endophyte phyto-bacteria isolated from *Lemna gibba* L. plants collected nearby the Xochimilco aquatic agrosystem. There were 17 isolates obtained from the *L. gibba* plants collected in the dry season and 14 isolates for the rainy season. The environmental conditions and seasonal characteristics determined the number and identity of the isolated endophyte phyto-bacteria in *L. gibba* plants according to the several apparent differences in the water quality. This work contributes to the knowledge of the phyto-bacteria diversity in aquatic plants, particularly in Lemnaceae species; here the majority of the isolates have been characterized as higher indole acetic acid producers, recommended as candidates for their use as biofertilizers.

Key words: Plant growth-promoting bacteria, biofertilizers, *Lemna gibba*, Xochimilco.

INTRODUCTION

The Xochimilco's lake is located at the southern part of Mexico City basin and comprises a unique ecosystem

which has served as source of aquatic resources, while its waters have been used for irrigation. The important of

*Corresponding author. E-mail: rodorantes@yahoo.com.mx.

field cultures are named chinampas, artificial islands made by interweaving twigs and branches of trees filled with sediment from the lake's bottom (Quiroz et al., 1982; Juárez-Figueroa et al., 2003). López-López et al. (2006) and Gagné et al. (2002) mention that this lake had been used as a water supply by humans since the beginning of 1900, but began being negatively influenced by the municipal effluent that have been discharged into it from Mexico City waste waters.

Duckweeds are one of the main groups considered as small (1 to 15 cm) free-floating macrophytes abundant in the Xochimilco's lake; that serve as nutrient pumps, reducing the eutrophication effects and providing oxygen from their photosynthetic activity; these plants are often seen growing on still nutrient rich fresh and slightly brackish waters (Hasan and Chakrabarti, 2009). Recently, there are number of new studies related to the aquatic plant-microbial interaction, particularly regarding to the duckweeds *Spirodela polyrrhiza* (Rahman et al., 2007), *Lemna minor* (Hou et al., 2007; Uysal and Taner, 2009), and *L. gibba*; focusing on the direct functional analysis that investigate plant-microbe interactions at full biological hierarchy, starting with the genomic, transcriptomic, and proteomic analysis of plant-associated bacteria (Farinati et al., 2009; Stout and Nüsslein, 2010). There were reports of some studies of *Lemna* and its associated bacteria that included microscopic observations and enumeration of bacteria on plant surfaces as well as several culture-dependent studies (Landolt, 1986); examples of this kind of studies are the works of Stout and Nüsslein (2005) whose compared the bacterial communities associated with the roots of *L. minor* plants; Yamaga et al. (2010) reported the response of phenol-degrading bacteria in the rhizosphere of *Lemna aoukikusa* to understand their beneficial symbiotic interactions. Sharma et al. (2013) mention that certain compounds produced by bacteria promote plant growth and Rajkumar and Freitas (2008) suggested that indole-3-acetic acid (IAA) increase plant biomass, this effect is produced by some plant-beneficial bacteria due to their bacterial production of plant hormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins (Idris et al., 2007). Yamaga et al. (2010) and Suzuki et al. (2014) reported the characterization of plant growth promoting bacteria associated with aquatic plants; the authors isolated the phyto-bacteria *Acinetobacter calcoaceticus* P23 isolated from *L. aoukikusa*, and reported that this bacteria is a plant growth promoting bacteria associated with *L. minor*, *S. polyrrhiza*, and *Wolffia arrhiza*. Their works demonstrated that this strain promotes the growth of *L. aoukikusa* to a 2-fold increase in growth rate (Yamaga et al., 2010). The authors reported that the bacteria colonized the plant surfaces and increase the chlorophyll content of *L. minor* and suggest that this isolated P23 has the potential to be a good biofertilizer. Idris et al. (2004) reported the results of a miniaturized biotest that analyzes the phytostimulatory effects by the

isolated phyto-bacteria *Bacillus amyloliquefaciens* FZB42 on clones of *L. minor*; the authors showed that this Gram positive bacteria was able to produce significant amounts of IAA and its production increase after the addition of Trp (Idris et al., 2007). Koga et al. (1991) reported that the stimulation of IAA synthesis by tryptophan was described previously for gram-negative plant associated bacteria and Patten and Glick (2002) also employed a mutant of the gram-negative plant-beneficial bacterium *Pseudomonas putida*, with low IAA production that were added to *L. minor* fronds showing that plant-growth-promoting effects decreased. These authors demonstrated a close correlation of plant growth promotion and auxin production like the response did by *B. amyloliquefaciens* FZB42. The aim of this study was to compare the auxin *in vitro* production efficiency of the endophyte phyto-bacteria isolated from *L. gibba* plants collected nearby the Xochimilco aquatic agrosystem.

MATERIALS AND METHODS

Isolation and selection of endophyte phyto-bacteria from *L. gibba* plants

Three distinguish zones were selected in the Lake of Xochimilco, interconnected by a system of water channels according to their land use and environmental conditions (Lopez-López et al., 2006), namely: A) Chinampa zone ("CH", an agricultural zone adjacent to the water channels), B) Tourist zone ("T", a located zone of markets and boat rides on trajineras in water channels) and C) Urban zone ("U", where domestic waters are discharged into the water channels). Plants of *L. gibba* L. were collected from each water channel zone in two seasons: dry season [May, 2013 (MA)] and rainy season [August, 2013 (AG), in each selected channel there were established three sites along them, taken in each site samples of *L. gibba* plants (100 g) with a phytoplankton net (60 cm x 25 µ). The samples were deposited in Ziploc bags and transported in cold (4°C) to the laboratory. The endophyte phyto-bacteria were isolated according to Yamaga et al. (2010) suggestions; 10 g of *L. gibba* plant biomass was collected. Plants were surface sterilized with 10% sodium hypochlorite for few seconds, rinsed several times with sterile distilled water and finally deposited in sterile mortar and pestle to homogenize them with 10 mL of sterile distilled water. The plants homogenate was transferred to sterile bottles containing 90 mL of sterile distilled water and the plant suspension from each sample were analyzed by appropriate dilutions (10^{-1} and 10^{-2}); 0.2 mL was taken from each sample and placed on plates containing Nutrient Agar (NA) medium. The plates were incubated at 28°C in the dark for 24 h and the endophyte phyto-bacteria were selected and isolated according to their different colony morphology and maintained and preserved on NA medium plates, for their conventional bacterial test. The phyto-bacteria isolates were identified by the determination of gene 16S rRNA sequences. Colony PCR was performed from live cell cultured on NA medium plates. Cells were harvested after 24 h and processed for DNA isolation using the Allers and Linchen procedure (2000). Using the purified genomic DNA, the molecular target gene 16S rRNA was amplified using universal primer set fD1 and rD1 designed by Weisburg et al. (1991). Aliquots of PCR reaction products were electrophoresed in 1% agarose gel and then stained with ethidium bromide. These PCR products were purified and sequenced by the Unidad de Biotecnología y Prototipos de FES-Iztacala (UNAM). The sequences were then compared to similar sequences in the databases using Basic Logical Alignment Search Tool analysis

(BLAST) at NCBI).

Measurement of the IAA production by the endophyte phyto-bacteria of *L. gibba*

The selected endophyte phyto-bacteria isolated from *L. gibba* plants were analyzed by their induction and efficiency of Indole Acetic Acid (IAA) production with the addition of the amino acid Tryptophan, according to the methods of Sheng and Xia (2006) and Zaidi et al. (2006), employing Salkowski reagent (Bric et al., 1991). Auxin production by the selected isolated strains was analyzed in the presence and absence of L-Tryptophan and determined by colorimetry (Melo et al., 2011). The assays were done taking 4.9 mL of sterile nutritive broth media, added to culture tubes (10 x 15 cm) without (control) and supplemented with L-Trp at final concentrations of 1, 2 and 5 mg/L. The culture tubes were inoculated with 0.2 mL of each rhizobacteria inoculum of 5×10^7 cells/mL in sterile distilled water. The culture tubes were incubated at 28°C for 120 h. After the incubation, the cultures were centrifuged at 3,500 rpm, at 25°C for 45 min to discard the bacteria pellets and to recover the supernatant where the auxins were excreted; 2 mL of each supernatants were mixed with 2 mL of Salkowski's coloring reagent and the development of a pink color indicates IAA production and was quantified reading its absorbance at 535 nm and the concentration was estimated by a standard IAA curve. The assays were performed by triplicate.

Statistical analysis

Data were analysed by one-way ANOVA analysis of variance and the mean differences were compared applying a Tukey-Kramer post-test, using the statistics program Graph Pad InStat Ver. 3.10. A numerical comparative analysis of the IAA production with and without the addition of L-Trp of the phyto-bacteria isolated from each zone was done; a distance matrix built using the conventional standard distance coefficient and a phenogram was resolved using the unweighted pair group method of arithmetic averages (UPGMA) method, and finally a correlation coefficient of Pearson was obtained using the version 2.11T Numerical Taxonomy and Multivariate Analysis System (NTSyS-PC) software.

RESULTS AND DISCUSSION

Isolated endophyte phyto-bacteria from *L. gibba* plants

Table 1 lists the isolated endophyte phyto-bacteria from *L. gibba* plants for each collected zone and season, and were identified based on its 16S rDNA sequence homology analysis. There were 17 isolates from the *L. gibba* plants collected in the dry season: six isolates from plants collected in urban zone, three isolates from plants collected in tourist zone and eight isolates from plants collected in chinampa zone.

For the rainy season, there were 14 isolates: nine isolates from plants collected in tourist zone, one isolate from plants collected in urban zone and four isolates from plants collected in chinampa zone. The environmental conditions and seasonal characteristics determined the number and identity of the isolated endophyte phyto-bacteria in *L. gibba* plants with several apparent differences

in the water quality among the selected three zones. The environmental behavior as indicated by López-López et al. (2006), may be the result of a high nutrient enrichment in all three areas and the presence of microalgal blooms in the urban and tourist zones; the apparent differences in the studied zones also seems to be the result of the local wastewater inputs, run off and leached from the different zones of Lake Xochimilco studied which are a consequence of the land use and the level of man-made disturbance in adjacent land areas.

Another reason of the diversity and distribution of endophyte phyto-bacteria of *L. gibba* plants, could be the relative phosphate concentrations in the channels of the selected zones; as Martínez-Cruz et al. (2006) mention, the drier months (April and May) presented high phosphate concentrations by the effect of water evaporation and in June to early November (rainy season), the phosphate content diminished due to the dilution of it by rain falls.

Richardson (1985) believed that emergent macrophytes were capable of higher phosphate absorption, due to rhizosphere activity and Martínez-Cruz et al. (2006) believe that chemical precipitation of reactive phosphate can also occur and thus prevent higher total phosphate removal. Martin and Gerald (1994) found that phosphorus absorption by plants occurs slowly and only for soluble phosphate compounds. Lalke-Porczyk and Donderski (2003) mention the number of epiphytic bacteria and heterotrophic epiphytic bacteria displayed a distinct seasonal variability; Niewolak (1974) and Olah (1974) explain the summer maximum of the number of bacteria on certain plants by citing the increased amount of organic substances secreted by the plants and the increase in the temperature of the water. On the other hand, the fall in the total number of bacteria observed in summer in macrophytes may be caused due to the excretion of antibacterial substances by plants or by algae inhabiting them, or due to the excessive exposure of plant surfaces to sunlight (Lalke-Porczyk and Donderski, 2003).

Efficiency of IAA produced by the selected endophyte phyto-bacteria from *L. gibba* plants

The results of the evaluation of IAA production by the isolates are presented in Figures 1 and 2. The isolated endophyte phyto-bacteria were screened for their ability to produce the auxin IAA, without and with different concentrations of tryptophan (0, 1, 2 and 5 mg/L) as inducer and precursor of it.

The IAA production of the isolates, considered as basal production without the addition of the amino acid was less in the endophyte phyto-bacteria of *L. gibba* plants collected in dry season (between 0.64 to 43.4 µg/mL), compared to the isolates from *L. gibba* plants collected in rainy season (between 24.45 to 53.98 µg/mL); apart from one of the isolates of *L. gibba*

Table 1. Auxin production by the endophyte phyto bacteria isolated from *Lemna gibba* plants collected from two seasons.

Dry Season					Rainy season				
Identified endophyte phyto bacteria	Zone	Gram behavior	Identity (%)	IAA producer	Identified endophyte phyto bacteria	Zone	Gram behavior	Identity (%)	IAA Producer
<i>Bacillus subtilis</i> U-MA-1	A	Bacilli Gram +	98	L	<i>Stenotrophomonas</i> spp. TU-AG-1	B	Bacilli gram -	100	H
<i>Bacillus</i> spp. U-MA-2	A	Bacilli Gram +	96	L	<i>Pseudomonas</i> spp. TU-AG-3	B	Bacilli gram -	100	H
<i>Bacillus</i> spp. U-MA-3	A	Bacilli Gram +	97	H	<i>Serratia</i> spp. TU-AG-5	B	Bacilli gram -	99	H
<i>Bacillus pumilus</i> U-MA-4	A	Bacilli Gram +	96	H	<i>Serratia</i> spp. TU-AG-6	B	Bacilli gram -	99	H
<i>Bacillus stratosphericus</i> U-MA-5	A	Bacilli Gram +	96	H	<i>Serratia</i> spp. TU-AG-8	B	Bacilli gram -	98	H
<i>Bacillus stratosphericus</i> U-MA-6	A	Bacilli Gram +	96	H	<i>Stenotrophomonas</i> spp. TU-AG-9	B	Bacilli gram -	97	H
<i>Enterobacter</i> spp. TU-MA-7	B	Cocci Gram +	96	H	<i>Serratia</i> spp. TU-AG-10	B	Bacilli gram -	99	H
<i>Paenibacillus</i> spp. TU-MA-9	B	Bacilli Gram +	9	L	<i>Serratia</i> spp. TU-AG-11	B	Bacilli gram -	100	H
<i>Bacillus simplex</i> TU-MA-10	B	Bacilli Gram -	95	L	<i>Exiguobacterium</i> spp. TU-AG-12	B	Bacilli gram -	97	H
<i>Achromobacter</i> spp. CH-MA-13	C	Bacilli Gram +	95	L	<i>Stenotrophomonas</i> spp. U-AG-17	A	Bacilli gram -	100	H
<i>Deinococcus</i> spp. CH-MA-15	C	Bacilli Gram -	92	L	<i>Serratia</i> spp. CH-AG-19	C	Bacilli gram -	85	H
<i>Enterococcus faecium</i> CH-MA-16	C	Bacilli Gram -	95	L	<i>Serratia</i> spp. CH-AG-20	C	Bacilli gram +	97	H
<i>Achromobacter</i> spp. CH-MA-17	C	Bacilli Gram -	97	H	<i>Serratia</i> spp. CH-AG-21	C	Bacilli gram -	99	H
<i>Pseudomonas</i> spp. CH-MA-19	C	Bacilli Gram -	96	H	<i>Serratia</i> spp. CH-AG-22	C	Bacilli gram -	95	H
<i>Bacillus pumilus</i> CH-MA-21	C	Cocci Gram +	96	L					
<i>Rahnella aquatilis</i> CH-MA-23	C	Bacilli Gram -	95	H					
<i>Enterobacter</i> spp. CH-MA-24	C	Bacilli Gram -	94	H					

A, Urban zone; B) Tourist zone; C, Chinampa zone.

plants collected in dry season from the chinampa zone *Enterobacter* spp. CH-MA-24, with 76.84 µg/mL of IAA produced. In general, the increase of IAA production by the isolates as the concentration of Trp increased, was present only in three isolates of *L. gibba* plants collected in dry season and in six isolated plants collected in rainy season; again this response were more evident in the isolates from the plants collected from this season with an increase of IAA production in average from 40 to 55 µg/mL. According to the classification of Khalid et al. (2004) for the *in vitro* production of IAA by bacteria; categorized in three

principal groups: lower producers (L= 1 to 10 µg/mL IAA), medium producers (M= 11 to 20 µg/mL IAA) and higher producers (H= 21 to 30 µg/mL IAA), the isolated endophyte phyto bacteria based on their IAA basal production could be classified as Table 1 show.

Figure 3, shows the phenogram with the associated groups according to the selection of the higher IAA producers without and with the addition of Trp; this figure two groups forming at first: Group I made only by *Enterobacter* spp. strain CH-MA-24 with the highest production of IAA and the rest of the phyto bacteria comprise the

Group II: with Group IIa organized by the selected phyto bacteria with a good IAA production with no distinction amongst their origin and season of collected *L. gibba* plants and the other endophyte phyto bacteria forming Group IIb classified as relative medium and lower IAA producers. Among the principal genera identified, the endophyte phyto bacteria isolated from the *L. gibba* plants collected in dry season, there were mainly eight isolates that belong to the *Bacillus* genera, six of them from plants collected in urban zone, one from the plants collected in tourist zone and one from the plants collected in chinampa zone and

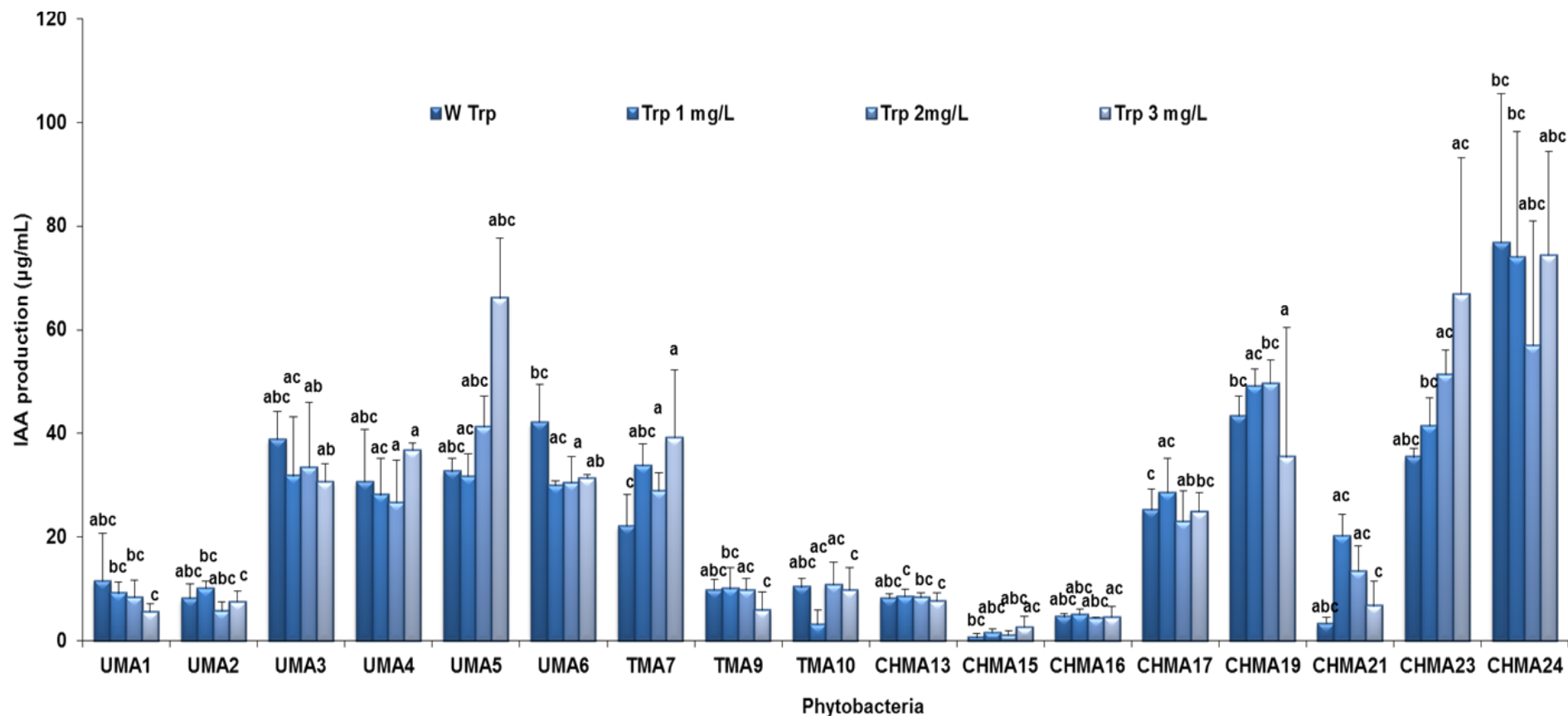


Figure 1. IAA production of the endophyte phyto-bacteria of *Lemma gibba* plants collected in rainy season, where, WTrp is without Tryptophan and Trp is concentration: 1, 2, 3 mg/L. Values are mean values + SD. from three replicates for IAA production. The different lower-case letters shows the significant difference between experiments ($p < 0.001$).

diverse genera like: *Enterobacter*, *Achromobacter*, *Paenibacillus*, *Deinococcus*, *Enterococcus*, *Rahnella* and *Pseudomonas*. Nine of the fourteen identified endophyte phyto-bacteria isolated from the *L. gibba* plants collected in the rainy season, belong to the *Stenotrophomonas* genus and the rest of the isolates to the genus: *Exiguobacterium*

and *Pseudomonas*. It is important to mention that all the isolates of *L. gibba* plants collected from the chinampa zone in the rainy season were *Serratia* bacteria with a diverse morphology. The isolated genera from *L. gibba* plants were new, compared with the findings by Stout et al. (2010) and Stout and Nüsslein (2005); these authors

reported members of the genera *Flavobacterium* isolated from Environmental Protection Agency (EPA) *L. minor* plants and the response of one plant growth promoting bacteria that plays a role in the elongation of root zones improving heavy metal phytoremediation.

Regarding the ecological context, this work

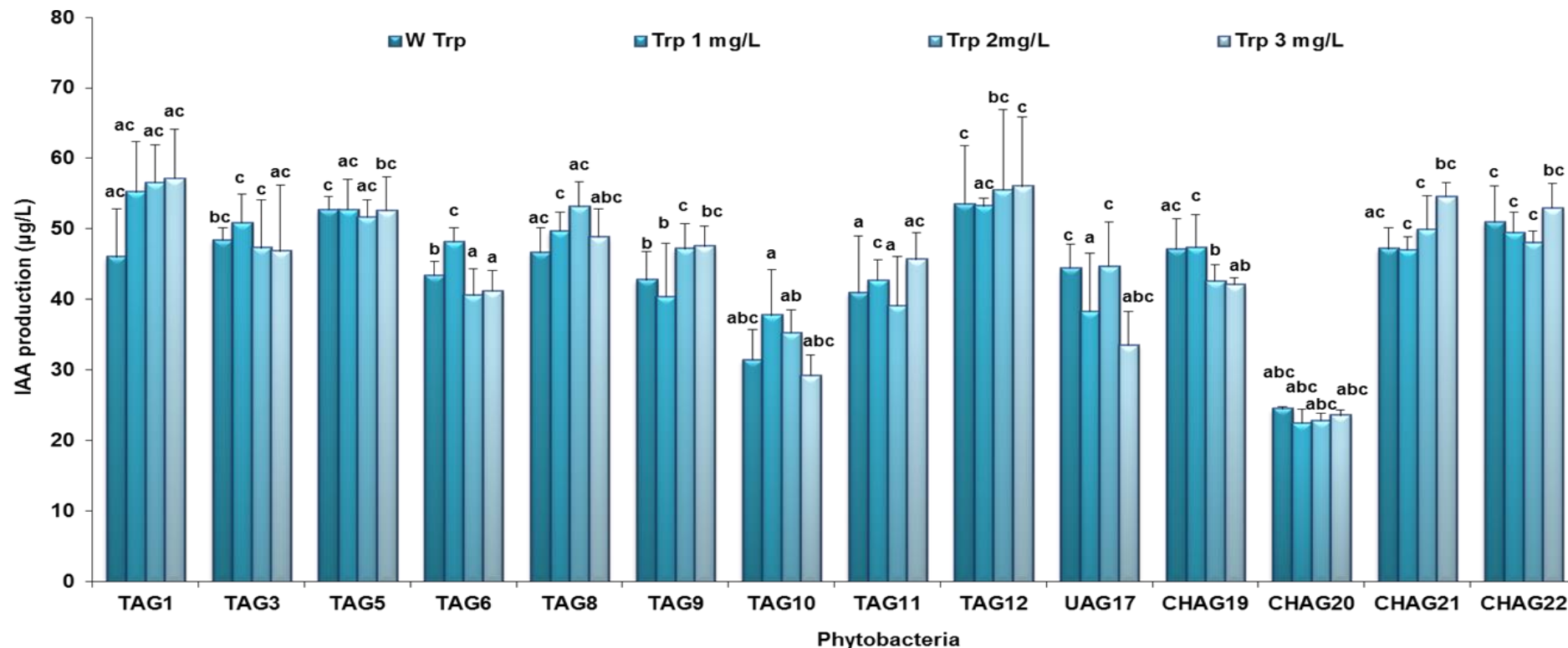


Figure 2. IAA production of the endophyte phytobacteria of *Lemna gibba* plants collected in rainy season, where, WTrp is without Tryptophan and Trp is concentration: 1, 2, 3 mg/L. Mean values + SD. from three replicates for IAA production. The different lower-case letters shows the significant difference between experiments ($p < 0.001$).

agrees with the commentaries of Idris et al. (2004) regarding the nature of the IAA production obtained by the isolates identified in the plants of *L. gibba* collected in the channels of the Xochimilco Lake, because the IAA production was induced with the addition of Trp to the cultures. The authors' mention that, it is possible that the presence of tryptophan-like compounds liberated in plant exudates could stimulate the IAA synthesis in the plant growth promoting bacteria

that colonize the structures of plant surface. The authors particularly mention that they reisolated *B. amyloliquefaciens* directly from *L. minor* plants, indicating that this bacterium lives in the vicinity of the plant's surface and could uptake the excreted plant compounds. Kamilova et al. (2006) mention that one of these compounds may be the amino acid. Thus, they suggested that the production of IAA stimulated in *B. amyloliquefaciens* FZB42 by the presence of Trp leads a promotion of plant

growth giving close relationship between it and *L. minor* plants.

Conclusions

This work contributes to the knowledge of the phytobacteria potential in aquatic plants, particularly in Lemnaceae species; here the majority of the isolates have been characterized as

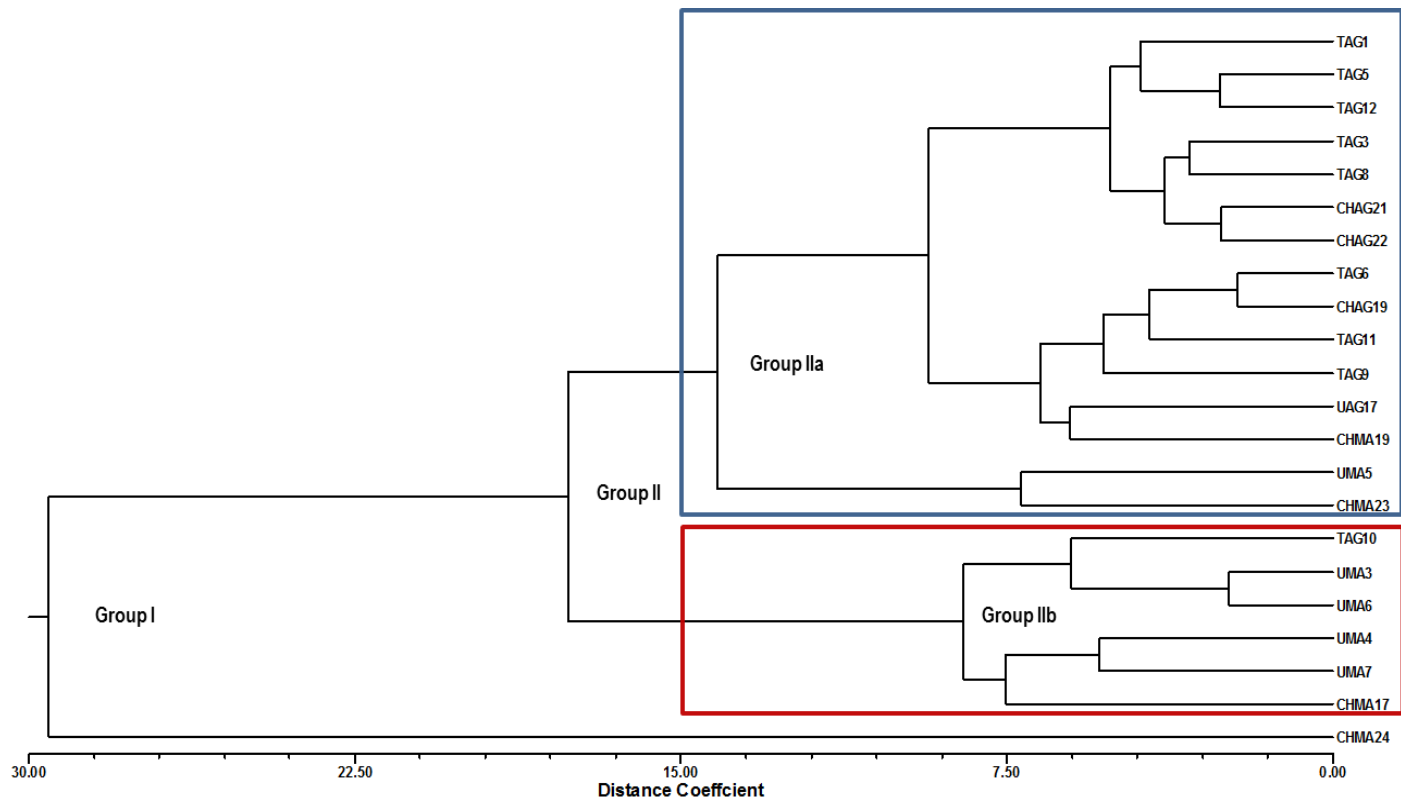


Figure 3. Phenogram of the isolated endophyte phyto-bacteria from *Lemna gibba* related to their IAA production ($r=0.85$).

higher IAA producers; recommended to candidates for their use as biofertilizers. The authors suggest further works with these isolates that involves plant-microbe systems as bioassays to demonstrate the ecological role that these endophyte phyto-bacteria have in association with *L. gibba* plants, for the conservation of aquatic ecosystems.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of gamma radiation on enzymatic production of lignolytic complex by filamentous fungi

Nelânia Maria de Queiroz Baptista^{1,2}, Evelyne Gomes Solidônio^{1,2}, Flávia Virgínia Ferreira de Arruda¹, Erik Jonne Vieira de Melo¹, José Robson Neves Cavalcanti Filho¹, Maira Judith de Azevedo Callou¹, Rita de Cássia Mendonça de Miranda^{3*}, Waldeciro Colaço² and Norma Buarque de Gusmão¹

¹Departamento de Antibióticos, Universidade Federal de Pernambuco, Recife/PE, Brasil.

²Pós Graduação em Tecnologias Energéticas e Nucleares, Universidade Federal de Pernambuco, Recife/PE, Brasil.

³Faculdade do Nordeste da Bahia, FANEB, Brasil.

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This work aimed to study the effect of gamma radiation on the production of enzymes by filamentous fungi present in the seawater used for thermoelectric Termope S / A, in the vicinity of Port of Suape, Pernambuco. The isolated microorganisms were screened for their ability to produce enzymes. Subsequently, the fungi were irradiated at 3 doses (500, 1000 and 1500 Gy), using 4 inoculation techniques: lyophilisation, scraping of the spores, glass beads and agarose block. A fermentation assay for enzyme production was made in simple batch using a bioreactor New Brunswick, model Bioflo 110. The best enzyme produced was lignin peroxidase (676 U/L) by *Aspergillus awamori* in 500 Gy dose. Manganese peroxidase enzyme (1720 U / L), produced by three species of fungi (*Penicillium* sp.), was the highest in 500 Gy dose. *Aspergillus terreus* produced laccase enzyme (980 U/L) better in 500 Gy dose. In the assay of the fermentation bioreactor, the best results were found in BH-amid corn steep liquor, where 650 U/L of laccase was produced at the baseline and also in the medium containing corn steep liquor, where 620 U/L laccase was produced during 72 h. This is better than Sabouraud. The use of gamma radiation increased the production of enzymes by filamentous fungi compared to tests without radiation. Statistical analysis shows there are no significant differences between the inoculation techniques, and the best dose of radiation tested was 500 Gy. In trial bioreactor containing Bushnell Haas + corn steep, *Penicillium* sp. produced larger amounts of phenoloxidases enzymes compared to Sabouraud medium. Irradiation in a dose of 500 Gy stimulates *A. awamori*, *A. terreus* and *Penicillium* sp. to produce phenoloxidases enzymes.

Key words: *Aspergillus*, *penicillium*, corn steep, radiation.

INTRODUCTION

Marine biotechnology is considered as an area of great interest because of its high potential and contribution to the construction of an eco-sustainable society. Aquatic environment is not yet fully explored and its resources

can play an important role in many industrial activities. Different research priorities can be identified in the field of marine biotechnology to show the vision of evolution and perspectives for the coming years (Melamed et al., 2002;

Adams, 2006; AS, 2009). The fungi present in marine ecosystems are associated with different bodies. However, these microbial groups are still not fully relatively studied in terms of their ecological functions and evolutionary origin as sources of useful metabolites for medicine, agriculture or industry (Osterhage et al., 2002; Klemke et al., 2004). Those considered as marine fungi are of biotechnological interest, since they produce metabolites such as enzymes. Microorganisms are a major producing sources of enzymes used in industrial society. Attractive and cost effective production of these metabolites is considered source and may be grown in large quantities and in a relatively short time (Zimmer et al., 2009). Enzymes are biocatalysts used in industry, and can be used in molecular biology, biomedical applications (Demain and Adrio, 2008), for the development of analytical methods for product manufacturing and technological treatment of wastes (Chirumamilla et al., 2001). The enzymes may be of different organisms such as animals (glands), plant (seeds, fruits, exudates) and microorganism cultures. The latter make use of a total culture, extracting the enzyme from the culture medium (Coelho and Amaral, 2013). There is a growing recognition that enzymes may be used in many bioremediation processes such as the treatment of pollutants. The potential application of lignolytic enzymes has been the subject of extensive academic and industrial interest due to their ability to degrade a variety of toxic and recalcitrant pollutants.

The literature stresses the largest families of enzymes produced by fungi lignolíticos: -MnP manganese peroxidase (EC: 1.11.1), laccase - Lac (EC: 1.10.3.2) and lignin peroxidase - Lipstick (including EC 1.11.1.14), and the two most important processes in lignin degradation with a wide application in industries. Laccase is a copper containing enzyme active site iron while lignin peroxidase contains a prosthetic group. Lignin peroxidase is a heme protein having a high oxidation potential to oxidize phenolic and non-phenolic substrates. Laccase is an oxidase which catalyses the reduction of H₂ and O₂ to oxidize aromatic amines (D'Souza et al., 2006). As these enzymes do not have substrate specificity, they are employed in degradation, with application in chemical, food, agriculture, paper, textiles, cosmetics industries and in bioremediation treatments (Bonugli-Santos et al., 2010; Gomes et al., 2009; Sette et al., 2008; Pearce, 1997). Many microorganisms have been investigated in relation to their ability to produce enzymes, among which filamentous fungi are of great biotechnological interest. An important factor to be taken into account is that most fungi producing enzymes of this class are higher fungi of

the phylum basidiomycote that are very difficult to cultivate in the laboratory. Among the fungi called lower, we can highlight the anamorphic of ascomycote and basidiomycote and zigomycote that has been reported in the literature as promising for enzyme production since coloados under ideal conditions (Miranda et al., 2013). One alternative for improving the production of these enzymes by other groups of fungi is induced mutation by radiation, one widely used technique that contains antibiotics and drugs derived from microorganisms. On changes in the molecules caused by ionizing radiation, directly or indirectly, studies have been developed in different fields of biology, including: creation of new plant varieties with improved characteristics; increased and improvement in food production through plant metabolism, animal and control or elimination of insects, fungi and / or bacteria (Okuno et al., 1982; Cardoso, 2006).

The biotechnological application of irradiation positively increases production of enzymes. This study is on the effect of gamma radiation on the enzymatic production of filamentous fungi isolated from water of Port Complex of Suape, Pernambuco.

MATERIALS AND METHODS

Microorganisms

In this work, the fungi, *Penicillium* sp., *Aspergillus terreus* and *Aspergillus awamori* used were obtained from seawater. 60 l of water (8 ° 24 '22.1" S 34 ° 57' 57.2 " W) was collected from three random points: two points on the dike to capture sea water of Termope SA and a point on the river Ipojuca (next watershed in the collection point), the Suape Port Complex - Pernambuco.

Isolation of microorganisms

The seawater samples were processed through the manifold on nitrocellulose filter membranes with a porosity of 0.22 microns. After filtering these membranes with any condensate, they were transferred to Petri dishes with agar Sabouraud medium - BSA (10 g peptone, 40 g glucose and 15 g agar in 1 L of distilled water). The medium was prepared for use and after preparation, it was taken to autoclave. The whole procedure was performed in a laminar flow chamber. The Petri dishes were incubated at 30°C for up to 15 days and new isolations were performed every 15 days.

Preparation of samples for irradiation

In order to test the best technique of fungus inoculation, samples were prepared according to four different methods. With the previously fungi grown in Petri dishes, lyophilization (Figueiredo, 2001), scraping of spores (Carvalho et al., 2012), glass beads

*Corresponding author. E-mail: ritamend30@gmail.com.

(Droce et al., 2013) and agar block (Kali et al., 2014) were performed.

Irradiator with cobalt source

In carrying out the irradiation, the fungus grown was placed individually in Petri dishes or flasks penicillin, wrapped in plastic wrap. The samples were subjected to radiation doses of 500, 1000, 1500 Gray (Gy) at a rate of 3,532 kGy / h in March 2014. In irradiating with cobalt-60, an MDS Nordion Gammacell 220 Excel source has also been prepared as a sample for this control procedure.

Enzyme assays

Inoculation of irradiated fungi

After irradiation the samples were inoculated, according to the technique used in each preparation. In the freeze drying techniques, scraping the spores from agar block, fungi were directly transferred to Erlenmeyer flask (500 ml) containing 200 ml of Sabouraud broth and incubated under stirring of 150 rpm for 72 h. For the glass bead technique, 5 ml of sterile distilled water was added to a Petri dish and the material was homogenized. After this process, an aliquot of 2 ml of spores was removed and transferred to Erlenmeyer flasks (500 mL). It was kept in the same conditions described above.

Assessment of enzyme activity

The mycelium of fungi grown on Sabouraud broth was filtered with liquid metabolite in sterile membrane of 0.22 micrometre (Merck Milipore®). The experimental procedure consisted of two stages: the first analysis was performed with samples of fungi in the control group (zero radiation) and the second was done with the samples after irradiation (500, 1,000 and 1,500 Gy). For each fungus and processing assays were performed in triplicate. To determine the enzymatic activity, the absorbance and appropriate length for each test wave was measured in a Thermo Scientific® spectrophotometer.

Activity of lignin peroxidase (LiP)

The LiP activity was determined by oxidation of veratryl alcohol according to the method of Gill and Arora (2001). The mixture is composed of 1 ml of buffer 125 mM sodium tartrate (pH 3.0), 500 µl 10 mM veratryl alcohol, 500 µl 2 mM hydrogen peroxide and 500 µl of enzyme extract. With the addition of the hydrogen peroxide reaction, the reading was done at 310 nm. One unit of each enzyme was defined as 1.0 micromol of product formed per minute under the assay conditions.

Activity of manganese peroxidase (MnP)

To quantify the manganese peroxidase (Bonugli-Santos et al., 2010), oxidation of phenol red (0.01% v / v) plus 500 µL of the enzyme extract, sodium lactate (0.25 M), bovine albumin (0.5% w / v) MnSO₄ (2 mM) and H₂O₂ in citrate phosphate buffer (20 mM, pH 4.5) was measured. The reading was done at 610 nm.

Activity of laccase (Lac)

The activity of the laccase was determined using 2,2-azino-bis-ethylbenzothiazoline (ABTS) as described by Gill and Arora (2001). In the mixture, 0.1 ml of sodium acetate buffer 0.1 M (pH 5.0) and 0.8 mL of a solution of ABTS were used at 0.03% (w / v), and 0.1 ml of the enzyme extract was the absorbance at 450 nm.

Statistical analysis

To determine if there was no statistically significant difference between enzyme production after radiation and the various inoculation techniques used, the student t test with significance level of p < 0.05 was used, with the help of statistic 8.0 software.

Assays for enzyme production by fermentation bioreactor

For the fungus with the highest enzyme production, an assay on biological reactor, brand Bioflo 110 (5 L) was carried out in tubes, containing two types of middle Sabourad (SAB) and liquid Bushnell Haas -BH compound 1 g KH₂PO₄, 1 g of K₂HPO₄, 1 g of NH₄NO₃, 0.2 g MgSO₄·7H₂O, 0.05 g FeCl₃, and 0.02 g CaCl₂ and 2H₂O (Atlas, 1995) milhocina- plus 40 mL/l. 3 L of SAB 250 blocks of agar (Ø 6 mm) was placed per liter, with microbial growth in irradiated best dose of the test vial, obtained in the enzymatic assay. In the second trial, 3 L Bioreactor middle BH (modified) was used, 250 blocks of agar (Ø 6mm) per liter, with irradiated microbial growth obtained in the enzymatic assay. The experiment was conducted at the temperature of 30°C, agitation of 150 rpm and pH 5.6. The trials were held for 72, 24 h where each was taken in a 10 ml aliquot of the sample for the evaluation of enzyme activity.

RESULTS AND DISCUSSION

Isolation of microorganisms

From the 140 strains of filamentous fungi isolated from water, 23 strains belong to the genus *Aspergillus* (16.43%), 20 strains, *Penicillium* (14,29) and the other 97 (69.28%), fungal species. The three selected specimens were *Penicillium* sp., *A. terreus* and *A. awamori*, having historical enzyme activity. Several authors agree with the results obtained in this work. Rajesh and Rai (2013) reported the high enzyme productivity of *Ventilago madraspatana* Gaertn fungus after being isolated from soil and indoor plants.

Enzyme assays

Assessment of enzyme activity

Activity of lignin peroxidase (LiP): The three fungi tested behaved differently in the production of lignin peroxidase enzyme under the three methods of inoculation employed (Figure 1). The best method of inoculation, scraping spores (ER) was used for *Penicillium*

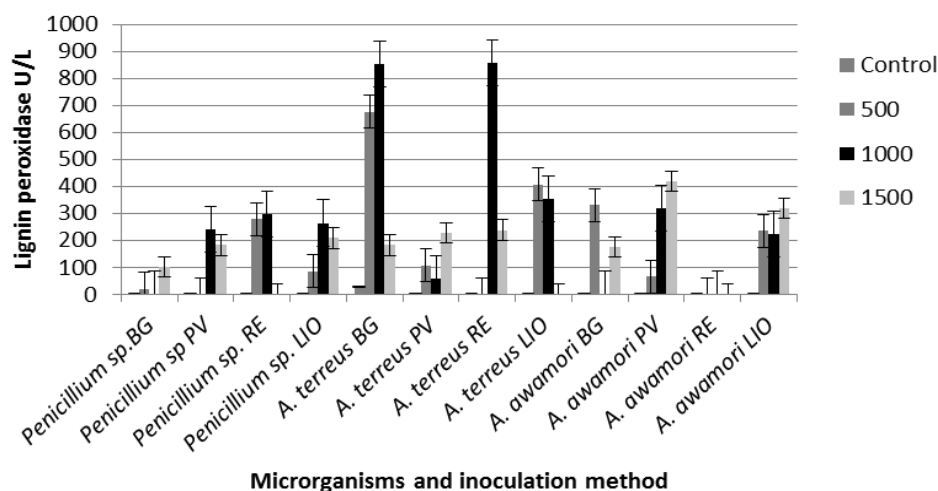


Figure 1. Values of enzyme assay for lignin peroxidase.

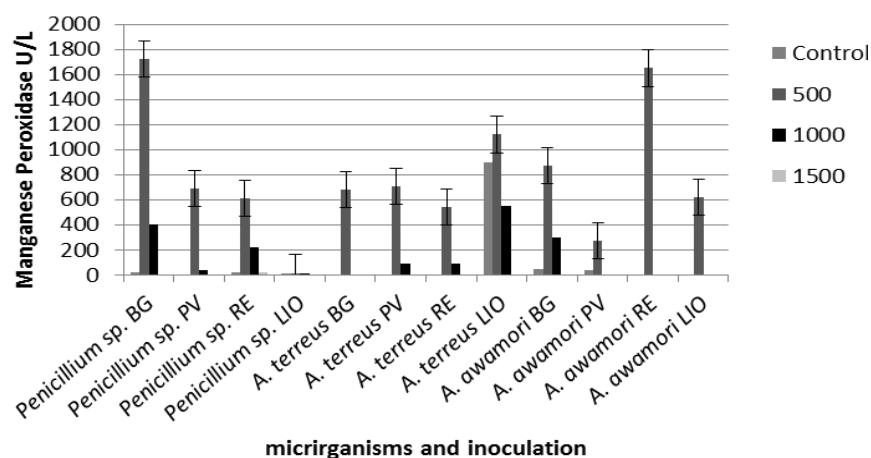


Figure 2. Values of the enzymatic assay for manganese peroxidase (MnP).

sp., where there was a production of 298 U/l enzyme at a dose of 1000 Gy. Although all the methods of inoculation favored the enzymatic production after irradiation of *Penicillium* sp. in all dose levels, there was a small production compared to other fungi studied. When the irradiated fungus was *A. terreus*, Block Agar (BG) and Scraping Spores (RE) methods had similar efficiency. The same yielded 852 to 858 U/L at 1000 Gy. Different from *Penicillium* sp., *A. terreus* had a higher significant production under two methodologies and in a dose of radiation. *A. awamori* had similar behavior with *Penicillium* sp.; they had similar enzyme production under the three irradiation methodologies (agar block, glass bead and lyophilization) in the three tested doses. The authors reported that genera of filamentous fungi have intermediate resistance to ionizing radiation, mainly *Aspergillus* and *Penicillium* genera (Rowley et al., 1978).

Filamentous fungi of the genus *Aspergillus* stand out as excellent producers of secondary metabolites of industrial and environmental interest, since they have a high rate of growth and a large thermotolerance, which favors studies of selection and production of high value-added bio-products (Berka et al., 1992; Ward et al., 2005; Lotfy et al., 2007; Mata-Gomez et al., 2009; Samson and Varga, 2009; Dhillon et al., 2012; Goswami et al., 2012; Singh and Mukhopadhyay, 2012; Chavan and Deshpande, 2013; Gopinath et al., 2013; Maldonato et al., 2014).

Activity of manganese peroxidase (MnP): When manganese peroxidase enzyme was studied, it was observed that all three fungi tested were producers (Figure 2). When the fungus *Penicillium* sp., was irradiated, agar block (BG) method of inoculation was the most effective, leading to the production of 1720U/L

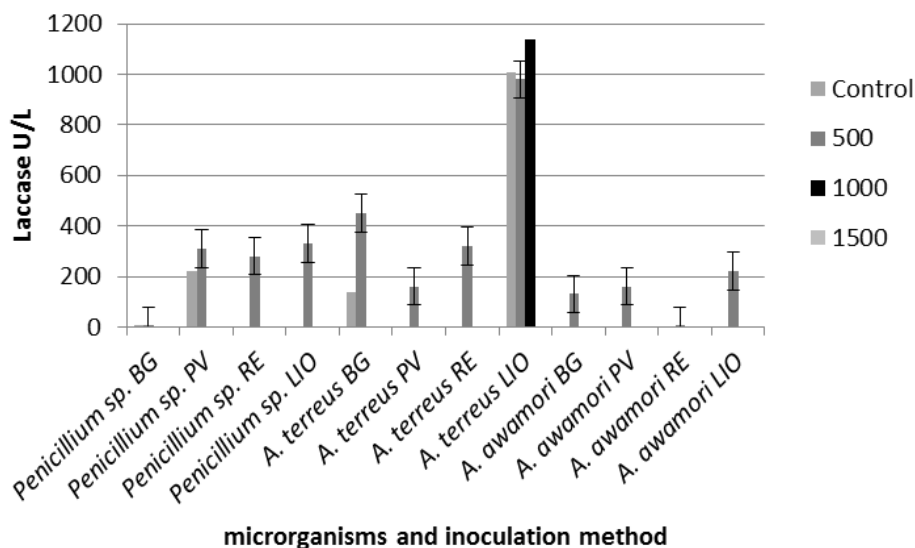


Figure 3. Values for laccase enzyme assay.

enzyme at 500 Gy. Although the fungus *Penicillium sp.* has produced manganese peroxidase with all methods of inoculation and in all doses of radiation, the values were insignificant compared to 500 Gy dose under block agar. When *A. terreus* was subjected to radiation, it was observed that freeze drying method was most effective in producing enzyme in all doses. *Penicillium sp.* at 500 Gy was able to induce the production of 1120 U / L manganese peroxidase in the fungus *A. terreus*. The fungus, *A. awamori* showed that the best method of inoculation was scraping the spores, which led to the enzymatic production of 1650 U/L at 500 Gy dose. It can be observed at this step that 500 Gy dosages proved the most efficient for the three fungi tested, regardless of the inoculation method used. The growth capacity is lost at low doses; however, the culture age may considerably influence the radio sensitivity and therefore, experiments are conducted by different authors to determine the sensitivity to radiation in yeast and filamentous fungi. Yeasts are generally more resistant filamentous fungi (Diehl, 1995).

Treichel et al. (2010) describe that the genus *Aspergillus* excels due to its high biotechnological potential to produce high value-added bio-products, especially microbial enzymes. Colla et al. (2012) reported that the production of microbial enzymes by filamentous fungi is especially prized biotechnologically. This facilitates their recovery in the middle of production, and describes the genus *Aspergillus* as a good producer.

Activity of laccase (Lac): The production of laccase enzyme by fungi in three different doses of radiation is seen in Figure 3. The production of laccase enzyme by

Penicillium sp. at 500 Gy was 330 U / L, but there was no production at 1000 and 1500 Gy. It was observed that for all inoculation methods described above and in all the doses, enzyme was produced by the fungus. *A. awamori* produced 220 U / L at a dose of 500 Gy, but there were no results at 1000 and 1500 Gy doses. It was observed that enzyme produced by this fungus was small in all the methodologies. *A. terreus* produced 980 U / L enzyme at 500 Gy dose and 1140 U / L at 1000 Gy; these were the highest enzyme production, but there was no production at 1500 Gy. It was observed that the fungus at 500 and 1000 Gy doses was efficient when freeze-drying (IOL) method was used. Since there are few studies on using fungi for the production of irradiated metabolites, the closest microorganisms reported are lichens which when subjected to gamma or ultraviolet radiation tend to produce larger amounts of phenols; therefore, they act as sunscreens and foto inducibles or protect the interior of the stem while preserving their physiology (Seaward, 1977). The same thing may have occurred with the micro-organism under study.

In *Cladonia salzmännii* found in soil leachates, increased production of acid barbático (FAB) occurred when they were irradiated at 5 Gy (152,9 mg bar / stem mg) and 10 Gy (86,8 mg bar / mg stem). Here, higher concentrations of these compounds were detected. The concentration of BAR in samples subjected to 60 and 100 Gy doses, respectively, was not higher than that of the control. It can be suggested that, doses from 60 Gy negatively influence biosynthesis liquênicos compounds analyzed, inhibiting, with increasing doses of gamma radiation, the production of BAR (Melo, 2011). These results are similar to that of this work, where 500 Gy dose

applied gave better results, and 1500 Gy dose negatively influenced enzyme production. The production of atranorina (ATR) by *Cladonia verticillaris* not exposed to gamma radiation was 0.024 mg.mL^{-1} . Later, the TR range of exposure of the substance was approximately 27 min. The output 10 for samples irradiated at 1,000 and 10,000 Gy was 0.15, 0.06 and 0.20 mg.mL^{-1} , respectively (Silva, 2011). In this experiment, the higher dose of gamma radiation was the one with higher production. Another isolated from *Cladonia verticillaris* production of ATR for the samples irradiated at 10, 1000 and 10,000 Gy was 0.07, 0.03 and 0.08 mg.mL^{-1} , respectively. Thus, there is a greater production with an irradiated sample at 10,000 Gy. This change is probably due to changes in the chemical composition of the substance blocked in the metabolic pathways by radiation (Silva et al., 2010).

The data concerning the enzymatic activity of fungi tested in this work are still insufficient. That is why it is important to continue and deepen the studies, as well as to implement other complementary techniques. However, the present study demonstrates that there is an improvement in enzyme production in filamentous fungi, especially at 500 Gy, which allows further development of this metabolite.

Statistical analyses

According to the T test, there were statistically significant differences in enzyme production at 500 Gy dose. Comparing the control treatment with radiation dose of 500 Gy, lignin was $p = 0.007832$ and manganese was $P = 0.000155$. To control at 1000 and 1500 Gy, significant differences of $p = 0.001881$ and $p = 0.000627$, respectively were observed for lignin. Comparing the doses from 500 to 1000 MN showed only a difference of $p = 0.000032$; when comparing the laccase of 500 with 1500, we obtained $p = 0.000067$ and $P = 0.000001$, respectively for manganese. The comparison between 1000 and 1500 revealed only a difference for manganese peroxidase with $p = 0.008845$.

According to the results obtained in this work it can be inferred that the three proposed dosages improved the enzymes produced by the selected fungus, according to the proposed objective. It is of utmost importance for industries which use strains to produce metabolites of industrial interest to make the process cheap. There was an improvement in the production of three enzymes of industrial interest when the yeast was subjected to low doses of radiation.

The results indicate that the different techniques in which the inoculation was subjected to fungi caused no abnormalities (statistically significant differences at the level of $p = 0.05$), and any of such techniques can be repeated for the reproduction of enzyme. The best technique for reproduction is agar (BG) block, for it is

easy and economically important for use in scientific research and industrial production criteria.

Assays for enzyme production by fermentation bioreactor

In an attempt to reduce the environmental impacts generated by agro-industrial activities, government agencies and industries are seeking new environmental policy. And research shows that waste still contains a lot of organic matter and other by-products, which can be used as a source for the generation of other products, such as animal feed, nutrients for micro-organisms in various processes (Pelizer et al., 2007). The corn steep liquor is a byproduct of processing corn. Corn steep liquor contains a lot of nitrogen, amino acids and other nutrients, and is used primarily as a food supplement for manufacturing feed for poultry and ruminants. Some studies are being developed for fermentation processes that accrue as a source of nutrients for micro-organisms (Amartey and Leung, 2000). Based on the results obtained from the enzyme production and the statistical analysis, one bioreactor test of this process was done on two types of media. *Penicillium* sp was irradiated at 500 Gy and led to the production of the best enzymes- laccase and manganese peroxidase. For the analysis of lignin in the middle SAB enzyme Bushnell Haas + corn steep liquor, the highest values obtained were 108 and 560 U/L (Figure 4).

Recently, soil fungi have been examined for their ability to degrade hydrocarbons and producing ligninolytic enzymes (Silva et al., 2009). Lignin peroxidase has been used to mineralize a range of recalcitrant aromatic compounds such as hydrocarbons and dyes (Wesenberg et al., 2003). Clement et al. (2001) studied the degradation of hydrocarbons by thirteen deuteromycete ligninolytic fungi and found that the degradation degree varies with the ligninolytic enzymes. Moreira (2006) reported in his work that *Psilocybe castanella* produced a high ligninolytic activity, probably in response to the concentration of the organic pollutant hexachlorobenzene. Anastasi et al. (2009) reported a lipase production of 19 U/L by Basidiomycetes. Compared to that produced in this work, a better result was obtained. Eight fungal strains used in bioremediation were isolated from agricultural soils cultivated with rice. The major enzyme activities detected were related to the production of lignin peroxidase. The maximum detected level was 6079 U.L^{-1} (strain P11SA4F), followed by $3,332 \text{ U.L}^{-1}$ (strain P11SA4F). None of the tested fungi can be compared to LiP production ($18,851 \text{ U.L}^{-1}$) by *Ganoderma* sp strain GAS13.4, used as the control (Silva et al., 2004). The fungus *Penicillium commune* produced 2,500 U/L lignin (Baptista et al., 2011). Lignin peroxidase was produced thus: *Paecilomyces* sp. produced $94 \text{ U/L} \pm 9$

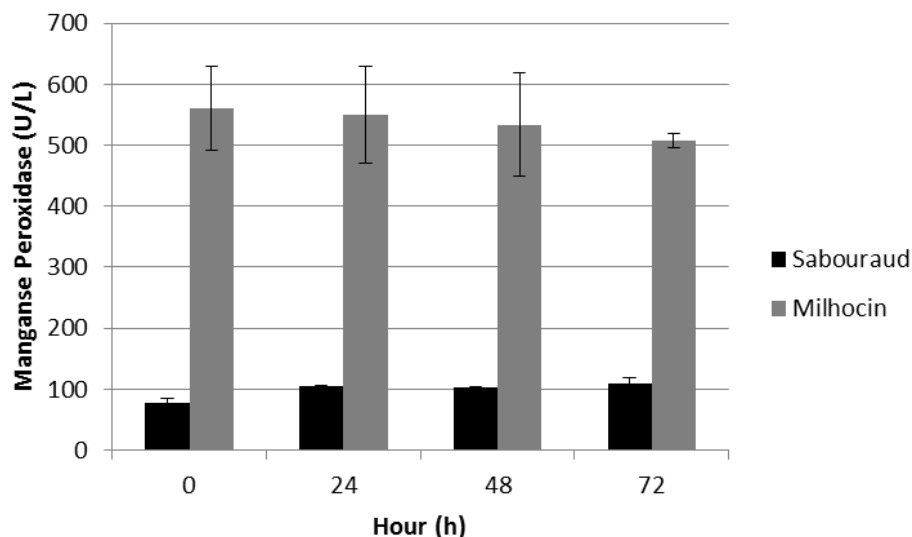


Figure 4. Test bioreactor for lignin.

lignin peroxidase; *Penicillium* sp., 100 U/L \pm 22; *Aspergillus* sp., 100 U/L \pm 14; and *Penicillium* sp., 144 U/L \pm 13 (Maciel, 2010). Gomes et al. (2009), in decolourisation of stains, obtained 9 U/mL of lignin peroxidase after five weeks of incubation. Anastasi et al. (2009), in his study, observed a LiP production of around 19 U / L by Basidiomycetes. Thus, the results obtained for *Penicillium* sp., and *Aspergillus* sp. proved to be superior to that of the literature. Of all the tested fungi grown in pure cultures that produce lignin peroxidase, *P. commune* had higher enzyme production, reaching 2,515 U/L (Arruda, 2011). The maximum activity of LiP by Basidiomycetes found was 20 mmol/L at 24 h incubation in the work cited. The LiP activity detected was 3.58 U/L, greater than the activity detected by Zhao et al., (1996) and Arora et al., (2002), where they found only 0.173 U/g and 1 U/ml, respectively, from other white rot fungi of the wood.

For manganese enzyme (Figure 5), from the initial period to the middle in SAB medium containing corn steep liquor, good results were obtained, close to 600 U/L for 24 h period. This was almost the same for both media production, but we observed a slightly better outcome in the SAB; at the middle in SAB, there was a low level of enzyme production (40 U/L) for 48 h; the medium containing corn steep liquor had a great result of 550 U/L. For 72 h, enzyme (4 U/L) was produced only in corn steep liquor media. The results were directly proportional to the increase in hours. Thus, the starting time (72 h) stimulated an increased enzyme production of 300 U/L in the bioreactor milhocin medium; for lignin peroxidase and manganese, best result found in the medium containing corn steep liquor was 555 U/L.

Regina et al. (2009) obtained maximum values of MnP

activity around 1400 U/L for *Lentinus edodes* grown in liquid-based infusion of cassava bagasse and dextrose medium. However, the same authors also observed the influence of the substrate on the expression of the enzyme; with the infusion of crushed cane sugar and dextrose, the maximum value was obtained at 400 U/L. Betini (2006) demonstrated that the fungus, *Aspergillus niger*, xylanase produced a concentration higher than 30% when cultured in a medium containing wheat bran as only carbon source compared to medium that also contains corn cobs. This demonstrates that supplementing with cob meal appears to satisfactorily answer the enzyme production by the fungi; although Kadowaki et al. (1997), in their studies, obtained maximum production of xylanase by *A. tamaritii* when it was grown in medium supplemented with a high concentration of solid waste from *Zea mays* (corn cob). *Gloeophyllum byrsina* and *Coriolopsis striatum* are excellent producers of MnP and produced 67.1 U/ml (21 days bagasse) and 590.3 U/ml (28 days in rice straw), respectively compared to those of Nuske et al., (2002), where *Nematoloma frowardii* produced 1.5 U/ml after 11 days of fermentation in wood chips as a substrate. *Phanerochaete chrysosporium* fungus was widely studied for its ability to produce ligninases (Fujian et al., 2001). The highlight in the production of manganese peroxidase was: 60.0 U/L \pm 8 produced by *Penicillium* sp, about 56 U / L \pm 6 generated by *Curvularia lunata* and 51 U/L \pm 4 by *Paecilomyces* sp. (Maciel et al., 2010). Similar results were obtained by Gomes et al. (2009), who decolorized dyes using rice as a substrate and obtained 0.6 U/mL of peroxidase-Mn. However, better results were observed by Anastasi et al. (2009) in their degradation tests using basidiomycetes that produced about 124 U/L MnP.

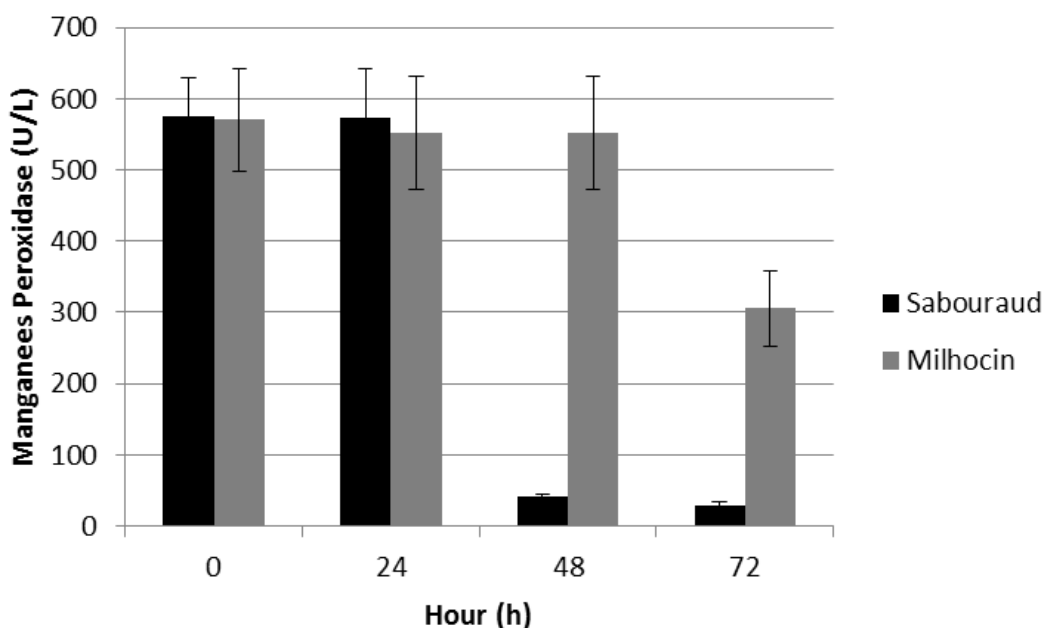


Figure 5. Test bioreactor for manganese peroxidase.

For the enzymatic activity of laccase (Figure 6) at the initial period, sample with the greater amount of corn steep liquor produced 650 U/L enzyme than at the middle in SAB where 460 U/L enzyme was produced for 24 h; at the middle, enzyme production (300 U/L) decreased in SAB and in the medium containing corn steep liquor (559 U/L enzyme) for 48 h. The medium containing corn steep liquor presents a slight increase over the 24 h period, with 620 U/L enzymatic production. For 72 h, in the medium containing the milhocin, there was enzymatic production of 610 U/L. The enzyme laccase showed the best results compared to the other enzymes produced in this work.

It is possible to observe that the condition where supplementation of corn steep liquor was found has the best enzyme production. One of several factors that can influence the growth of a microorganism is the nature of the culture medium. The difference in composition of media is a factor that could change the metabolites produced. *Penicillium* sp. (290 U/L \pm 28) and *C. lunata* (210 U/L \pm 17) stand out statistically in the production of laccase enzyme compared to other fungi (Maciel et al., 2010). For Quarantino et al. (2008), laccase production by *Panus trigrinus* ranged from 0.024 to 2.04 U/mL, confirming the results obtained in this work. Amid Sabouraud broth plus diesel oil, glucose was used as control and diesel oil as inducer of enzyme activity, where laccase activities range from 4.35 to 4.62 U/L, highlighting the highest production for *Cunninghamella echinulata* and fungi *Penicillium commune* with 4.62 U/L for both (Baptista et al., 2011). The biological functions of

laccase in micro-organisms are still not very clear. In fungi, there are reports about its involvement in rapid cell growth, sporulation (Gianfreda et al., 1999) and degradation of lignin (Eggert et al., 1996). The largest production of laccase was in Sabouraud liquid medium by the fungus *Penicillium commune*, reaching 1,947 U/L (Baptista et al., 2011)

According to Rothschild et al. (2002), the activity of lipase and laccase has been reported in some white rot fungi. Using diesel oil as substrate resulted in the highest value for *C. echinulata*, 2,594 U/L. The results obtained are comparable or higher than those found by Narkhede and Vidhale (2005) who observed the production of polyphenol oxidases by *C. lunata* isolated from industrial effluent. *Paecilomyces* species have been reported in literature as being able to degrade substrates, lignolíticos, with production of polyphenol (Kluczek-Turpeinem et al., 2003). According to Kluczek-Turpeinem et al. (2007), the secretion of lignin degrading enzymes is a key step for the metabolism of carbon per *Paecilomyces* spp. representing a significant potential for detecting the expression levels of these enzymes.

Conclusion

The use of gamma radiation increases enzyme production by filamentous fungi. These results obtained in the research can prove to be useful in areas such as biotechnology, and thus add further information to the topic that is still pioneering.

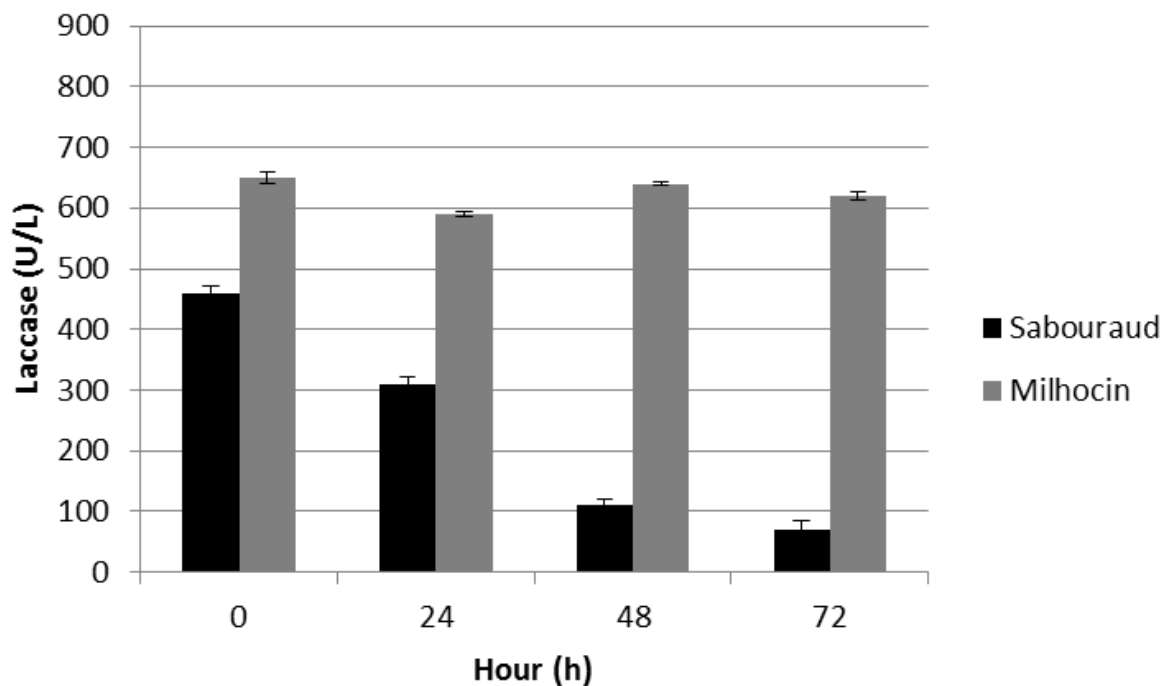


Figure 6. Test bioreactor for laccase.

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Full Length Research Paper

Production of α -amylase by solid state fermentation by *Rhizopus oryzae*

Osanía Emerenciano Ferreira¹, Nayara Abrão Montijo¹, Eduardo da Silva Martins² and Marcia Justino Rossini Mutton^{1*}

¹Technology Department- Postgraduate Program Agropecuaria Microbiology, FCAV/UNESP, Jaboticabal -SP, Brazil; UEMG University of Minas Gerais - Brazil.

²UEMG – Universidade do Estado de Minas Gerais, Brasil.

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A thermotolerant strain of *Rhizopus oryzae* was grown in three agro-industrial by-products: brewers' rice, corn grits and wheat bran. Different substrates, cultivation time, moisture content, additional nitrogen sources, pH and temperature of incubation were evaluated aiming to optimize growing conditions. The highest enzymatic activity was observed after 24 h of cultivation using wheat bran as substrate with the following salt solutions: NH_4NO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{SO}_4$ 0.1% at temperature of 35°C. It was observed that changes in the pH range 4.0-6.0 did not significantly affect α -amylase activity. The optimum operation conditions were 75°C and pH 4.5. The enzymes remained stable at 75°C in the absence of substrate for 25 min.

Key words: Starch hydrolysis, fermentation parameters, amylolytic enzyme.

INTRODUCTION

Amylases are a group of enzymes commonly used in the food, textile, pharmaceutical and detergent industries and in the sugar-energy sector. Several microorganisms have been investigated for potential production of this enzyme. However, only a few strains of fungi and bacteria meet the criteria for production of this commercial enzyme (Souza and Magalhães, 2010).

Submerged fermentation is commonly used for amylase production, but solid-state fermentation (SSF) is emerging as a promising technology with the use of several agro-industrial by-products as substrates (Balkan and Figen, 2010). Most substrates used in SSF are agro-

industrial by-products such as soy bran, rice bran, cassava bagasse, wheat bran and sugarcane bagasse among others (Bhargav, 2008). These by-products usually have several substances of high nutritional value, essential for the cultivation of microorganisms, especially filamentous fungi, which have the ability to convert these materials into other biomolecules or materials that can be used in different processes (Couto and Sanromán, 2006).

In addition to the type of substrate, enzyme production by microorganisms is affected by several factors, such as fermentation time, and by physicochemical factors such as moisture, pH, temperature and nitrogen sources

*Corresponding author. E-mail. marcia.mutton@gmail.com. Tel/Fax: +55 16 3209-2675.

(Pandey et al., 2000). This study aimed to evaluate the type of substrate, cultivation time, nutrient supplementation, moisture, pH, and temperature, parameters that can affect the activity of α -amylase using a *Rhizopus oryzae* strain.

MATERIALS AND METHODS

Media, cultivation of microorganism and enzyme production

Some agro-industrial by-products of rice, corn and wheat were used as substrates for the strain cultivation because, in addition to their high availability and low cost, they have great potential for production of amylases. All substrates were obtained from pet food and supply stores in the municipality of Frutal, MG, Brazil and were sieved through a 9-mesh sieve. Sugarcane bagasse (10%, total amount of the culture medium) was used to support the growth of the amyolytic strain.

The fungus was grown in 500 mL-Erlenmeyer flasks containing Sabouraud agar medium supplemented with 1% starch, and it was incubated at 35°C for 48 h. In order to obtain mycelial suspension, 100 mL of distilled sterile water were added to each vial containing the fungus. The direct spore count was performed by optical microscopy using a Neubauer chamber.

Cultivation of *R. oryzae* in the agro-industrial by-products was performed on 250 mL Erlenmeyer flasks containing 5 g of each substrate, which were autoclaved for 40 min at 120°C. Initially, the fermentation media were inoculated with the mycelial suspension in saline solution composed of 1% (p v⁻¹) of (NH₄)₂SO₄, MgSO₄·7H₂O and (NH₄NO₃), with 10⁵ mL⁻¹ spores and incubated for 168 h. The initial moisture content was 70%; samples were taken every 24 h to determine enzyme activity.

To obtain the enzyme extract, 40 mL of distilled water were added to each flask; the flasks were shaken on a horizontal shaker at 100 rpm for 30 min; the extract was filtered in a funnel lined with gauze, and centrifuged at 1800 g for 20 min; the supernatant containing the crude enzyme extract was obtained.

Two additional nitrogen sources were evaluated: an inorganic source, 1% (p v⁻¹) of (NH₄)₂SO₄, MgSO₄·7H₂O and (NH₄NO₃), and an organic source composed of 1% (p v⁻¹) soy bran; both were sterilized at 121°C for 30 min. Distilled water was used as the control. After defining the best nutrient supplemental source (or the additional nitrogen source), three moisture content values (50, 60 and 70%) were evaluated. Enzymatic activity was evaluated every 24 h of cultivation.

The incubation temperatures of 30, 35, 40, and 45°C and the pH values of 4.0, 4.5, 5.0, 5.5 and 6.0 were evaluated under optimum conditions of substrate, supplemental nutrient source, moisture, and cultivation time.

Enzyme activity measurements

The α -amylase activity was determined by measuring the decrease in iodine-binding capacity, between starch and iodine, of a starch solution treated with a crude enzyme solution, according to the method described by Fuwa (1954). One enzyme unit (U) was defined as the amount of enzyme needed to hydrolyze 10 mg of starch in 10 min under the assay conditions.

Enzyme characterization

To determine the optimum pH of enzymatic activity, the crude extract was incubated at the pH range 3.0-10.5 and 60°C using the

following buffer systems: acetate (pH 3.0-5.5), citrate/NaOH (pH 5.5-7.0), Tris-HCl (pH 7.0-8.5) and glycine/NaOH (pH 8.5-10.5).

To determine the optimum temperature of enzyme activity, the enzyme extract was incubated at the previously determined optimum pH and at temperatures ranging from 30 to 90°C with variation range of 5°C. The enzymatic activity was measured using the dextrinizing method. The stability of the enzyme was assessed by incubating the crude enzyme solution in screw-cap tubes at the previously determined pH and temperature values until it exhibited no activity. Samples were taken every 2 min, and residual activity was measured by the dextrinizing method.

Statistical analysis

The data obtained were analyzed using the Microcal Origin 6.1 software. Analysis of variance and comparison of means by the Tukey test at 5% probability were also conducted. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Under the initial conditions evaluated, the highest α -amylase activity in *R. oryzae* was achieved after 24 h of fermentation with the production of 6.350 U mL⁻¹ on wheat bran (Figure 1). On the brewers' rice substrate, the highest activities were achieved after 120 h of fermentation, with values of 2.906 and 2.963 U mL⁻¹, respectively; on corn grits, it was achieved after 120 h of fermentation and exhibited lower enzymatic activity (1.835 U mL⁻¹) (Figure 1).

The supplementation with the salts suspension increased the synthesis of α -amylase by *R. oryzae* on wheat bran up to 96 h of fermentation. However, there was a decrease in the enzyme activity when corn grits and rice bran were used as substrates (Table 1).

The absence of salts increased the enzyme activity (5.295 U mL⁻¹) in the substrate containing rice after 48 h of fermentation; the highest activity value was achieved after 72 h of fermentation (6.100 U mL⁻¹). The lowest enzyme activity was achieved using the corn based substrate (Table 1), regardless of supplementation with salts.

There was a significant reduction in enzyme activity by the fungus when soy bran suspension (1%) was used in the rice and corn based substrates. There was a significant reduction in enzyme activity by the fungus when soy bran suspension (1%) was used in the rice and corn based substrates. These results similar to those obtained by Celestino et al. (2014), who found that the use of supplemental organic solution *Aspergillus oryzae* was less effective in the production of α -amylase (Table 1). In the period of time at which there was greater activity (24 h) on wheat bran, the absence of supplementation delayed the synthesis of α -amylase.

According to Bakir et al. (2001), *R. oryzae* showed higher affinity for inorganic nitrogen sources for xylanase production. This behavior was also observed in the present study for the synthesis of α -amylase when wheat bran was supplemented with soy bran and inorganic salt solution. The time for higher production of enzyme was

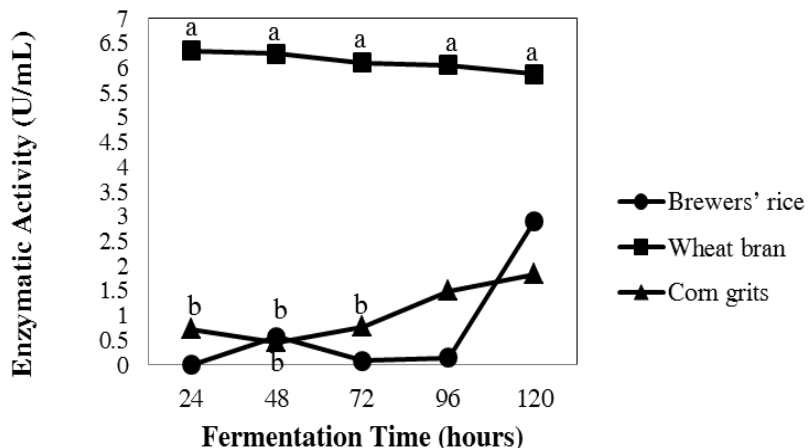


Figure 1. Activity of α -amylase in *R. oryzae* in culture medium containing brewers' rice, wheat bran and corn grits as substrates. Means followed by the same lower case letters in the times for each additional nitrogen source do not differ statistically between themselves by the Tukey test at 5% probability.

Table 1. Influence of nutrient supplementation on the enzymatic activity of *Rhizopus oryzae*.

Time (h)	Control (H ₂ O)	Soy bran (1%)	NH ₄ NO ₃ / MgSO ₄ .7H ₂ O and (NH ₄) ₂ SO ₄ 1%
Rice			
24	2.428 ^c	0.404 ^c	0.001 ^c
48	5.295 ^b	0.500 ^c	0.569 ^c
72	6.099 ^b	1.709 ^{bc}	0.089 ^c
96	5.928 ^b	1.131 ^{bc}	0.147 ^c
120	5.609 ^b	0.977 ^c	2.906 ^b
Wheat			
24	3.425 ^c	5.289 ^b	6.349 ^a
48	5.988 ^b	4.369 ^b	6.290 ^a
72	5.545 ^b	5.356 ^{ab}	6.107 ^a
96	5.760 ^b	5.206 ^{ab}	6.055 ^a
120	4.874 ^b	5.148 ^b	5.880 ^b
Corn			
24	1.482 ^c	0.673 ^c	0.731 ^c
48	1.567 ^c	1.173 ^c	0.465 ^{bc}
72	1.041 ^c	0.434 ^c	0.771 ^{bc}
96	1.723 ^c	0.574 ^c	1.502 ^{bc}
120	1.3292 ^c	1.006 ^c	1.835 ^b

CV: Time: 0.710 Substrate: 0.5430; additional nutrient source = 1.214; MSD=21.30. Means followed by the same lower case letters in the rows for each additional nitrogen source do not differ statistically between themselves by the Tukey test at 5% probability. CV = Coefficient of Variation; MSD = Minimum significant difference.

reduced from 72 to 24 h, respectively (Table 1).

In the culture medium containing wheat bran as substrate, the moisture contents did not significantly

affect the synthesis of α -amylase by *R. oryzae*. This was the best substrate for enzyme production under the three moisture conditions evaluated (Figure 1).

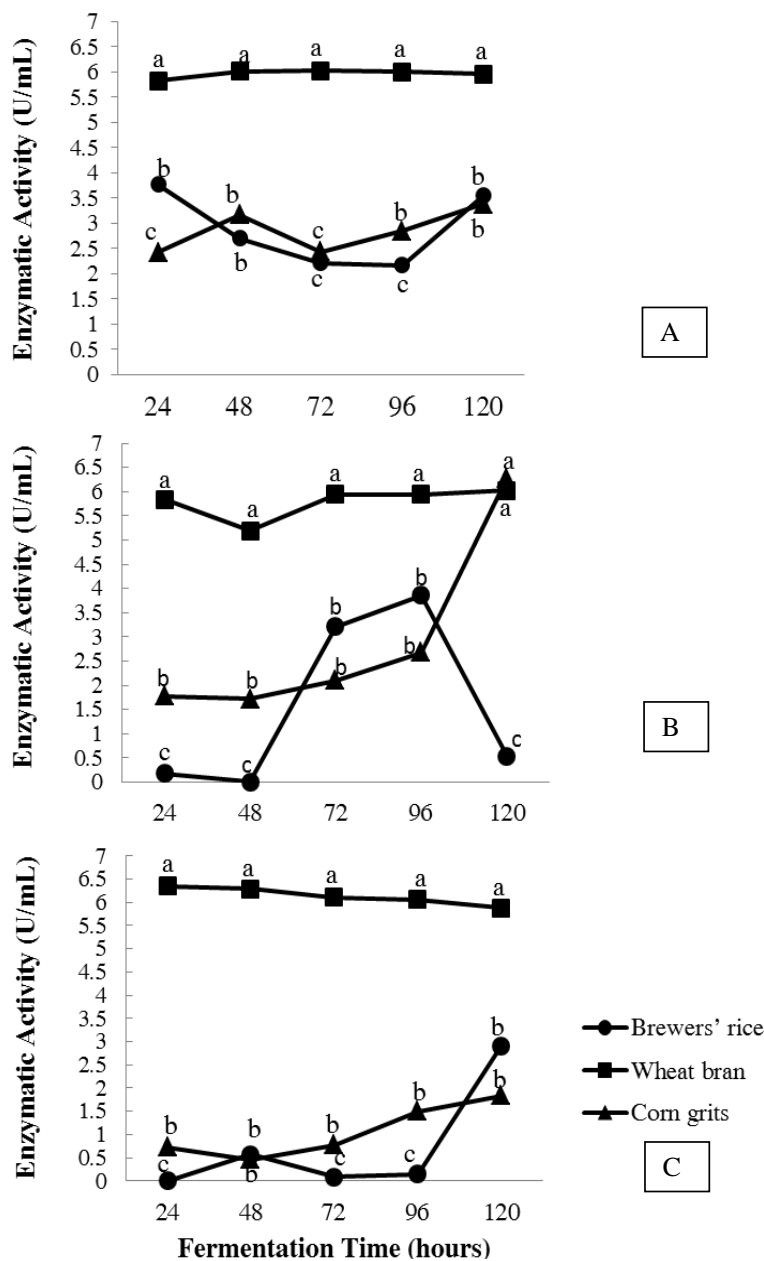


Figure 2. Influence of the moisture content on the activity of α -amylase in *R. oryzae*: A) 50% moisture, B) 60% moisture, and C) 70% moisture in culture medium containing brewers' rice, wheat bran, and corn grits as substrates. Means followed by the same lower case letters in the times for each additional nitrogen source do not differ statistically between themselves by the Tukey test at 5% probability.

As for the substrate containing brewers' rice, the reduced moisture favored α -amylase activity and there was significant difference between the moisture contents of 50 and 60%; for moisture content of 50%, the highest values were achieved after 24 and 48 h (3.769 U mL⁻¹ and 2.812 U mL⁻¹, respectively) and 120 h (3.542 U mL⁻¹) of fermentation, and for moisture content of 60%, the highest values were observed after 72 h (3.205 U mL⁻¹)

and 96 h (3,861 U mL⁻¹) of fermentation (Figure 2).

Moisture content of 60% was favored with the use of the substrate containing corn grits, since after 120 h of fermentation, the highest activity (6.279 U mL⁻¹) was achieved, which is similar to that obtained with the substrate containing wheat bran (Figure 2). The results obtained for brewers' rice and corn corroborate those found by Ramachandran et al. (2004), who used *A. niger*

Table 2. Enzyme activity of *R. oryzae* at different conditions of temperature and pH in wheat bran during 24 h of fermentation.

pH	Temperature (°C)			
	30	35	40	45
4.0	5.941 ^a	6.057 ^a	5.553 ^{ab}	5.104 ^b
4.5	5.940 ^a	6.067 ^a	5.484 ^{ab}	5.271 ^b
5.0	6.013 ^a	5.953 ^a	5.474 ^a	5.515 ^a
5.5	5.962 ^a	5.986 ^a	5.445 ^a	5.518 ^a
6.0	6.053 ^a	5.897 ^a	5.481 ^a	5.488 ^a
CV	6.64	5.26	4.79	5.33
MSD	0.7898	0.6283	0.577	5.72

Means followed by the same lower case letters in the rows for each additional temperature do not differ statistically between themselves by the Tukey test at 5% probability. CV = Coefficient of Variation; MSD = minimum significant difference.

and reported that the best moisture content to obtain amylase was 64%. Tunga and Tunga (2003) who used *A. oryzae* strains in solid state fermentation of sugarcane bagasse and reported the need for higher moisture content for amylase production, achieving highest production of this enzyme after 72 h of fermentation with approximately 80% moisture.

Guandalini (2007), studying *Metarhizium anisopliae* and using starch waste as a substrate for amylase production, obtained results similar to those found in the present study in brewers' rice and corn grit substrates, in which the highest values of enzyme activity decreased with increasing moisture.

After determining the best substrate and the parameters: cultivation time (24 h), nutrient supplementation with inorganic salt solution, and initial substrate moisture content of 70%, the evaluation of the optimum temperature of incubation of the fungus and the initial pH of the best substrate (wheat bran) was performed. There was no significant difference in the parameters pH and temperature, except for the temperature of 45°C, at pH 4.0 and 4.5 (Table 2).

Studying thermotolerant *R. oryzae* strains, Kitprechanich et al. (2008), found that the fungus had the ability to withstand and grow up to 45°C, and the highest mycelium growth occurred at 34°C. Huang et al. (2005) investigated the impact of temperature on the synthesis of lactic acid in *R. oryzae* and *Rhizopus arrhizus* using potato starch waste wastewater as substrate; the authors reported an increase in the hydrolysis of the substrate starch at 40°C. Peixoto et al. (2003) studied a *Rhizopus microsporus* strain and observed that it was highly tolerant to high temperatures, and that there was a 4-fold increase in the amylase synthesis at 50°C.

The pH of the culture medium is one of the most important factors to be controlled; pH changes during the growth of a microorganisms directly affects the synthesis and stability of excretory-secretory products in the culture media. Liao et al. (2007) investigated the formation of

pellet in *R. oryzae* and observed that there were no significant differences on pellet formation at pH of 3.0-7.0. The strain studied in the present study exhibited similar behavior when evaluated at the pH range between 4.0 and 6.0 without affecting the α -amylase synthesis. This behavior indicates that this strain is probably not very sensitive to changes in pH; a fact that has already been highlighted by other authors in other species of filamentous fungi, such as *Aspergillus niger* and *Penicillium chrysogenum* (Galbraith and Smith, 1969) and *Syncephalastrum racemosum* (Freitas et al. 2014)..

Analyzing the graph of enzyme activity in terms of changes in pH (Table 2, Figure 3), it can be said that there is more than one type of enzyme in the enzyme extract since there is more than one enzyme activity peak; the first peak is between pH 4.5- 6.0 and the second, with lower values, one is near pH 8.5.

The profile of the enzyme produced by *R. oryzae* is desirable since according to the literature, the optimum pH of activity of most enzymes is between 4.0-5.5. The results of this study highlight the abilities of this strain to synthesize active enzymes at alkaline pH. It is worth mentioning that there are few enzymes with this pH profile, and that they have become highly sought after.

Souza and Magalhães (2010), in a literature review on α -amylase, found that of 18 species of fungi investigated only three displayed activity at pH between 7.0 and 9.0; all others were most active at pH between 4.75 and 6.0. Michelin et al. (2010) reported that the highest α -amylase activity produced by *Paecilomyces variotti* occurred at acidic pH.

The α -amylase from *R. oryzae* was found to be thermophilic since it exhibited highest activity at 75°C (Figure 3), which is a desirable result considering that, according to the literature data, most fungi strains exhibit a range of α -amylase activity within a much narrower range of temperature (50-60°C). Michelin et al. (2010) reported that the optimal temperature of an α -amylase produced by *Paecilomyces variotti* was 60°C. Souza and Magalhães (2010) in a literature review on the application

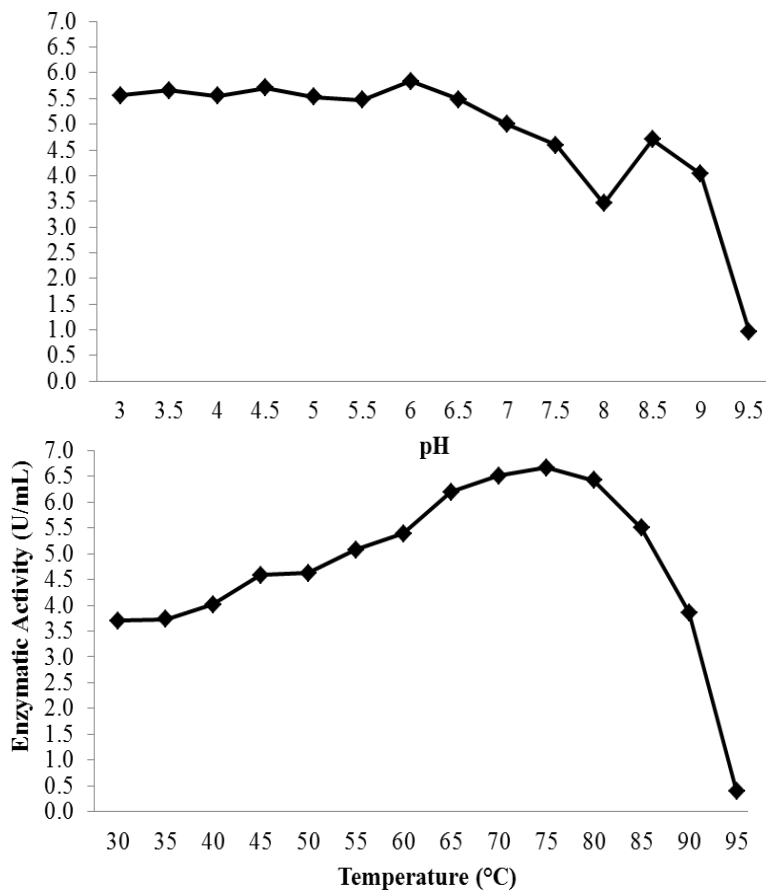


Figure 3. Physicochemical characterization of crude enzymatic extract of *R. oryzae*; evaluation of effects of pH and temperature changes on the enzyme activity.

of α -amylase in the industry found that among 10 species of yeasts and fungi filaments investigated, none exhibited optimum temperature of enzyme activity higher than 75°C.

In another literature review on α -amylase, Freitas et al. (2014) also found that among the fungal species investigated, none showed stability above 70°C. Haki et al. (2003), considering industrial development and the application of thermostable enzymes, reported that the majority of α -amylases of fungal origin are thermostable between 50 and 60°C.

The half-life of the fungal α -amylase was approximately 50% in 25 min at 70°C although the literature reports much higher α -amylase thermal stability values. Michellin et al. (2010), studying *Paecilomyces variotti*, found that this enzyme was stable for 60 min at 55°C; this characteristics allow its use in industrial processes in which no residues of the enzyme is desired, for example in the sugar production from sugarcane.

Conclusion

The *R. oryzae* strain was proven to be effective for α -

amylase production in the substrates studied, showing the feasibility of using agro-industrial by-products as substrates for the production of this enzyme, with optimal operation conditions at 75°C, pH 4.5, and stability at 75°C in the absence of substrate for 25 min.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Acute oral toxicity and cytotoxicological evaluation of the ethanol extract of *Samanea tubulosa* pods in Swiss mice

Paulo Alex Bezerra Sales¹, Lucas Antonio Duarte Nicolau², Janylla Mirck Guerra de Oliveira¹, Marina Rebeca Soares Carneiro de Sousa¹, Mariana Helena Chaves³, Fernando Aécio de Amorim Carvalho², Enoque Pereira Costa Sobrinho Junior² and Amilton Paulo Raposo Costa^{1*}

¹Department of Veterinary Morpho physiology, Federal University of Piauí, School of Agrarians Sciences, Teresina, Piauí, Brazil.

²Medicinal Plants Research Center, Federal University of Piauí, Teresina, Piauí, Brazil.

³Department of Chemistry, Federal University of Piauí, Teresina, Piauí, Brazil.

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Acute toxicity and cytotoxicity of ethanol extract of *Samanea tubulosa* (EEST) pods were evaluated in Swiss mice. Acute toxicity studies were conducted based on OECD guidelines 420, where the limit test dose was 5000 mg/kg. Observation was made and recorded systemically for 1, 2, 4 and 24 h after the administration of dose for skin changes, morbidity, aggression and sensitivity of the behavior of the animals. For the cytotoxicity, 3-[4,5-dimethylthiazol-zil] -2,5-diphenyltetrazolium (MTT) test and hemolysis were performed with concentrations of 6.25 to 800 µg/ml. No significant variation ($p < 0.05$) in the body and organ weights between the control and the treated group was observed after 14 days of treatment. Pathologically, neither gross abnormalities nor histopathological changes were observed. No mortality was recorded in 14 days. Moreover, both cytotoxic tests made no significant alterations to be able to display the evidence of the effect of cytotoxic. Therefore, we suggest that EEST use is safe in a systemic and cellular level.

Key words: *Samanea tubulosa*, toxicity, 3-[4,5-dimethylthiazol-zil] -2,5-diphenyltetrazolium (MTT), hemolysis, natural product.

INTRODUCTION

Medicinal plants have biological activities that are beneficial to humans because they contain certain compounds

that have useful properties. However, the potential toxicity of their bioactive substances has not been well

*Corresponding author. E-mail: amilfox@uol.com.br.

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established (Rosidah et al., 2009). Long-term use without any evidence of risk may indicate that a medicine is harmless. However, the absence of any reported or documented side-effects is not an absolute declaration of the safety of herbal medicines (Who, 2000). Detailed toxicological evaluation has to be performed using suitable experimental animals to supply guidelines for selecting a 'safe' dose for human and animal use. Tests to assess toxicity are performed to classify and label substances appropriately according to their lethality or toxicity potential as prescribed by law (Valadares, 2006). They are conducted to know the conditions in which the chemicals have toxic effects, the nature of these effects and the safe levels of being exposed to them (Júnior and Borges, 2012). Among the medicinal plants, *Samanea* genus, that has been studied a little, has a promising biological potential for having rare therapeutic applicability in scientific research. Botanical studies highlight the existence of some species such as *Samanea saman*, *Samanea inopinata* and *Samanea tubulosa*, representatives of this genus. There are few morphological and anatomic differences between *S. saman*, *S. innopinada* and *S. tubulosa*. The basic difference between *S. tubulosa* and *S. inopinata* is observed in the fruiting; while *S. saman* is different from *S. inopinata* and *S. tubulosa* in structures, like leaflets and bark texture (Zapater et al., 2011; Durr, 2001). *S. tubulosa* is known in Piauí, Alagoas, Maranhão, Paraíba, Pernambuco and Sergipe as "bordão-de-velho"; in Bahia as samaneiro and sete cascas, Ceará. This species belongs to the family Leguminosae (Mimosoideae); it is a large tree that can reach 4 to 18 feet and 25 to 45 cm diameter. The fruit pods are sessile and indehiscent; and 10 to 18 cm long, with 20 to 30 seeds. It has wide applicability in animal and human feed; its fermented fruit can be used for ethanol production and apiculture with flowers. Its pods are edible and the pulp is sweet (Lorenzi and Souza, 1995; Carvalho, 2007).

This study was conducted at the Federal University of Piauí. Ethanol extract of *S. tubulosa* administered on female Wistar rats was toxic to the fetus, causing malformations, fetal resorption and pronounced decrease in birth weight (Sales et al., 2011). Due to these results, we aimed to evaluate the toxicity *in vitro* and *in vivo* of ethanol extract (EEST) pods of *S. tubulosa*.

MATERIALS AND METHODS

Preparation of the extract

The ethanolic extract of the pods was prepared from dry pods in an oven at 45°C. It was subsequently ground in a mill and subjected to electric trituration with 99.5% ethanol for seven days at room temperature; and then protected from light. The extract was concentrated in a rotary evaporator at 50°C, packed in amber glass bottles and kept in the refrigerator and subsequently lyophilized.

The pods of *S. tubulosa* were collected from Teresina-PI, Center of Agricultural Sciences, Federal University of Piauí (UFPI). They were identified in the Herbarium Graziela Barroso- UFPI, where a voucher specimen was deposited under TEPB number – 27.261.

Animals

Female mice (*Mus musculus*, Swiss variety; 25 to 35 g) were used. They were reared in Maintenance of Animals Intended for Experimentation Department of Veterinary Morphophysiology - CCA/UFPI. The animals were housed in standard cages and kept at 24 ± 1°C and 12 h light dark cycle with water and food (FRI-LAB Rats - Fri-Ribe) *ad libitum*. Experimental protocols used are in accordance with the International standards and were approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEE/UFPI) protocol number 025/14.

Cytotoxicity assay *in vitro* of EEST

The macrophages used in this study were obtained from the peritoneal cavity of Swiss mice of four to five weeks. The removal of macrophages was performed in a laminar flow, in which the animals after euthanasia were kept in a supine position in a plate. 8 mL of phosphate buffered saline (PBS – NaCl 145 mM, Na₂HPO₄ 9 mM, Na₂HPO₄ 1 mM, pH 7,4) was administered to them and sterilized at 4°C in the abdominal cavity. After it was performed, the abdominal region was massaged gently, and aspiration of PBS injected using a sterile syringe attached to a needle was held. The material obtained was transferred to a conical polystyrene tube with a capacity of 50 ml, and centrifuged at 1000 rpm for 10 min. Shortly after, it was washed three times with PBS at 4°C. To investigate cell viability, it was counted in a Neubauer chamber diluted in Trypan blue dye.

We evaluated the cytotoxicity of ethanol extract of *S. tubulosa* using the test of bromide 3-[4,5-dimethylthiazol-zil] -2,5-diphenyltetrazolium (MTT) (Sigma-Aldrich, St. Louis, EUA). To a 96 well plate, 100 µl of supplemented RPMI 1640 and 2 x 10⁵ macrophages were added. For cell adhesion, these cells were incubated at 37°C and 5% CO₂ for 4 h. After that time, supplemented RPMI media used for the removal of cells that did not adhere were washed twice. 100 µl of supplemented RPMI 1640 with diluted EEST (800 to 6.25 µg/ml) was added later. They were then incubated for 48 h at the end of the incubation. 10 µl of supplemented RPMI 1640 with EEST was added to MTT diluted in PBS (5 mg/ml). The samples were incubated for 4 h in an oven at 37 with 5% CO₂, and then the supernatant was discarded. Then, 100 µl DMSO was added to all the wells. Then, the plate was placed under stirring for about 30 min in an agitator Kline (AK model 0506) at room temperature to complete dissolution of the formazan. Finally, the reading was performed at 550 nm in a Biotek reader (model ELx800) plate. The results were expressed in percentage and mean cytotoxic concentration (CC₅₀); the control group was taken as 100% (Nogueira et al., 2007). The negative control was given supplemented RPMI 1640 media 0.2% DMSO.

Acute toxicity of EEST

This procedure followed the Acute Oral Toxicity protocol recommended by OECD 425 (OECD, 2001). The animals were divided into five groups, with six animals each. They were treated orally with a single dose of the compound dissolved in distilled water and increasing doses of 2000, 3000, 4000 and 5000 mg/kg in

Table 1. Number of deaths of Swiss (female) mice after acute treatment of ethanol extract of *Samanea tubulosa* (EEST) at doses of 2000, 3000, 4000 and 5000 mg/kg orally.

Treatment (mg/kg)	Number of animals/group	Deaths of animals after 72 h	Deaths of animals after 14 days
Vehicle	6	0	0
EEST 2000	6	0	0
EEST 3000	6	0	0
EEST 4000	6	0	1
EEST 5000	6	0	0

Vehicle group received distilled water at 0.1 ml/10 g body weight. There was no difference between the groups (one-way ANOVA).

10 ml/kg volume. The control group received distilled water as a single dose, orally, in 10 ml/kg volume. All animals were observed after treatment, every 30 min during the first 4 h and daily until day 14. The parameters evaluated were: death, alertness, sedation, ptosis, dyspnea, urination, diarrhea, convulsions, spontaneous motor activity, postural reflex, piloerection, response to touch, among others. The total number of deaths in each group was quantified by the end of the period. The animals were then anesthetized by anesthetic combination (50 mg/kg ketamine + 5 mg/kg xylazine) for blood collection and then euthanized by overdose of the same anesthetic combination for the removal of organs.

Cytotoxicity in human erythrocytes

For evaluation of the hemolytic activity, human red blood cells type O⁺ blood was collected in anticoagulant (EDTA). After collection, the erythrocytes were diluted in 80 μ l PBS, adjusting the concentration of blood to 5% of erythrocytes. Then, EEST was added (800 to 6.25 mg / mL), and diluted in a volume of 20 μ l PBS. After this procedure, it was incubated for 1 h at 37°C and the reaction was stopped by adding 200 μ L of PBS. Then, the suspensions were centrifuged at 1000 g for 10 min at room temperature. The supernatant was subjected to spectrophotometry at a wavelength of 550 nm to quantify the hemolytic activity. The 0 (negative control) and 100% hemolysis (positive control) was determined by replacing the sample solution tested with an equal volume of PBS and sterile Milli-Q water, respectively. The results were expressed in percentage and average concentration in hemolytic (CH50), considering the positive control as 100% hemolysis (Löfgren et al., 2008).

Statistical analysis

All assays were performed in triplicate and in three independent experiments. Analysis of variance (ANOVA) followed by a post-hoc Newman-Keuls and Tukey test was performed, taking a *P* value of < 0.05 as the minimum level required for statistical significance.

RESULTS AND DISCUSSION

Tests assessing acute systemic toxicity are used to classify and label substances appropriately according to their lethality or toxicity potential as prescribed by law. Besides the lethality, other parameters are investigated in

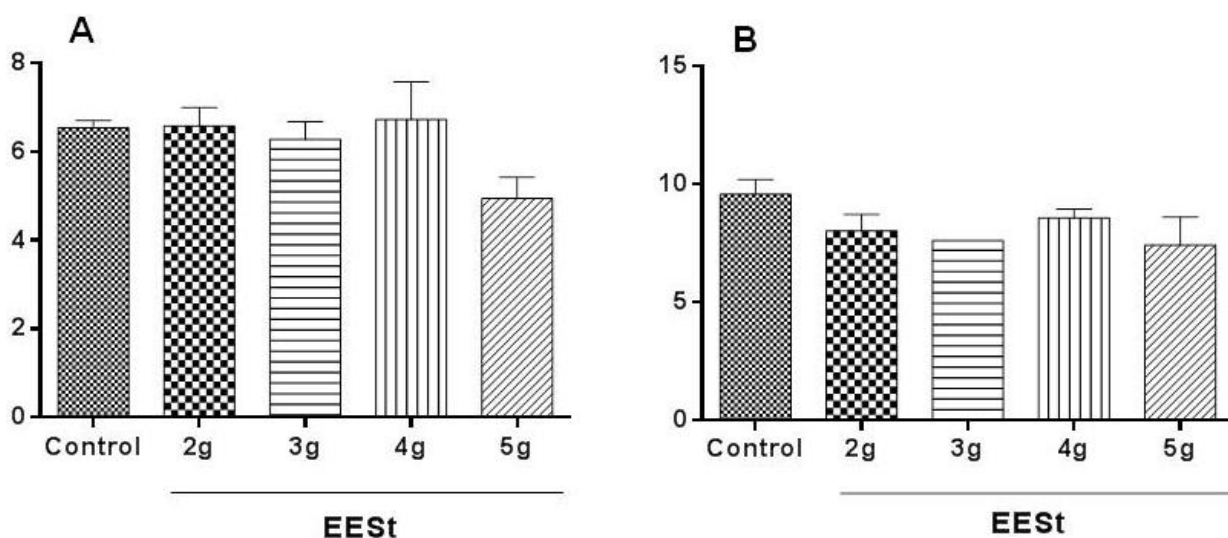
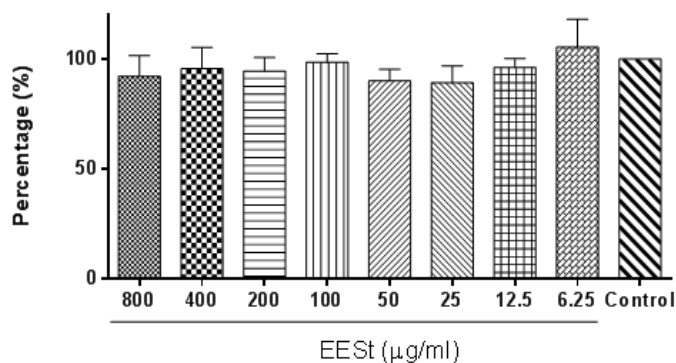
studies of acute systemic toxicity to identify toxicity potential in specific organs, identify toxicokinetics and dose-response relationship (Valadares, 2006). Toxicology studies are designed to evaluate the erroneous idea that herbal products are not toxic or do not have adverse effects because they are natural, and that the popular use of medicinal plants serves as validation for the effectiveness of such drugs (Simões et al., 2004). In the experimental model recommended by OECD 425 (OECD, 2001), acute treatment with distilled water and ethanol extract of *S. tubulosa* (EEST) at doses of 2000, 3000, 5000 mg/kg did not cause death in animals. Evidence shows that only one death was in the group treated with 4000 mg/kg (Table 1). The calculation of LD₅₀ could not be performed because no toxicity was observed in the tested concentrations. This shows that it is safe as the administration route used and the doses studied. This supports the use of EEST in several experimental trials. Macroscopic analysis of liver, adrenal, ovaries, kidneys, heart, uterus, spleen and lung of Swiss mice treated orally with EEST at the doses recommended in this study showed no changes in their color and morphology. This indicates that the extract was not able to produce structural changes in these organs. This corroborates the data in Table 2 that there was no significant difference between the tested groups regarding the relative organ weights. Several observations were made when administering the EEST, such as alertness, sedation, dyspnea, urination, diarrhea, convulsions, voluntary motor activity (ptosis) and automatic (postural reflexes), piloerection, response to tactile stimuli, the presence of death, which were not observed in any change between the groups tested. Figure 1 shows that the EEST tested in different doses (2, 3, 4 and 5 g) did not differ significantly regarding the consumption of food and water between the groups.

Another very important test in our study was the *in vitro* cytotoxicity (MTT/Hemolysis), where it is in a biological system that materials have direct contact with cell cultures in order to analyze their direct effect on cells (Christina et al., 2009). Although the results of the tests that assess cytotoxicity *in vitro* may not have a direct

Table 2. Relative Mass (mg/10g body weight) of organs of Swiss mice acutely treated orally with ethanol extract of *Samanea tubulosa* (EEST) at doses of 2000, 3000, 4000, 5000 mg/kg.

Group	Liver	Adrenal	Ovaries	Kidneys	Heart	Uterus	Spleen	Lung
Vehicle	550±38	4.1±0.4	9.2±3	167±27	48±2	28±5	90±21	94±8
EEST 2000	544±36	3.7±0.3	8.1±0.5	131±3	43±1	49±6	56±3	87±4
EEST 3000	522±18	2.7±0.4	9.9±4.9	145±13	45±2	24±6	77±21	131±37
EEST 4000	518±23	2.7±0.5	6.7±0.5	141±8	46±2	30±2	67±6	96±6
EEST 5000	632±50	3.4±0.4	7.9±1.7	149±7	47±2	34±11	76±9	102±7

Values represent mean ± S.E.M. of the number of animals used in the experiment (n = 5-6). There was no statistical significant difference between groups (One-way ANOVA followed by Tukey test).

**Figure 1.** Consumption of ration (A) and water (B). Data represent the mean ± standard error of experiments carried out during all 14 days of experiment.**Figure 2.** Cytotoxicity of ethanol extract of *Samanea tubulosa* (EEST) on the viability of murine peritoneal macrophages. Peritoneal macrophages were seeded at 1×10^5 /well in 96-well microplates and incubated for 48 h in the presence of EEST at concentrations of 6.25 to 800 µg/ml. Viability was determined with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), and the optical density was determined at 540 nm. Data represent the mean cell density ± standard error of 3 experiments carried out in triplicate.

correlation *in vivo*, it is safe to say that if a material induces demonstrably a cytotoxic reaction in tests involving cell culture, it is very likely to develop toxicity when applied to living tissue (Osorio et al., 1998). Figure 2 shows that in our assay at different concentrations (from 6.25 to 800 µg/ml), the extract did not degenerate the cell membrane of macrophages, so there is no cytotoxicity in *S. tubulosa*. This is because it was not possible to calculate the value of CC50.

Tests performed with erythrocytes allow the evaluation of the potential of a drug to cause damage to the cell plasma membrane. This could be either by forming pores or rupturing totally, leading to cell damage or changes in membrane permeability (Costa-Lotufu et al., 2002). This model is used for a preliminary study of the effects of toxic substances; a possible indicator of damage to cells *in vivo* (Aparicio et al., 2005). The results of the hemolysis test with EEST (Figure 3) corroborate with previous results and the tested concentrations (6.25 to 800 µg/ml). EEST showed low toxicity with erythrocytes,

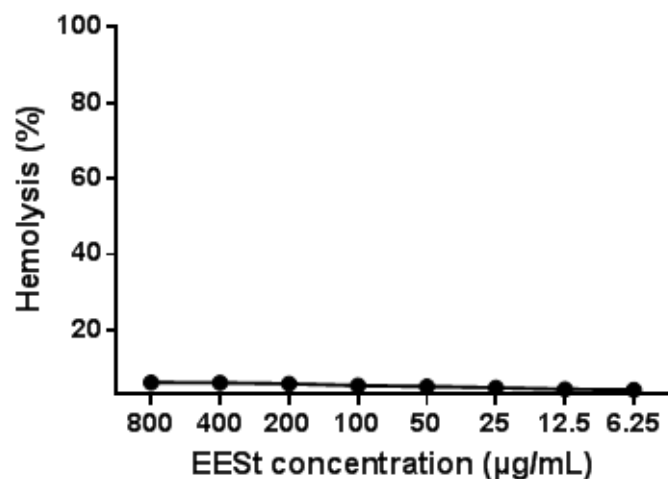


Figure 3. Hemolytic activity of ethanol extract of *Samanea tubulosa* (EEST) in a 4% suspension of human O+ red blood cells after 1 h of incubation.

producing no significant effect.

The results obtained through the various toxicological studies demonstrated that EEST has no acute oral toxicity at the doses studied, as there was no significant effect on the study of the cytotoxicity in macrophages and erythrocytes. Thus, we can suggest that its use is safe in systemic and cellular level of Swiss mice and human erythrocytes.

Conflicts of interest

The authors declare no conflict of interest.

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Full Length Research Paper

Evaluating the antioxidant and anticancer property of *Ficus carica* fruits

Jasmine R*, Manikandan K and Karthikeyan

P.G. & Research Department of Biotechnology, Bishop Heber College, Tiruchirappalli-17, Tamil Nadu, India.

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The present study was designed to investigate the antioxidant and anticancer properties of *Ficus carica* (FC). The dried coarse powder of *F. carica* fruits was exhaustively extracted with ethanol and the resulting crude ethanolic extract (FCE) was assayed for antioxidant and anticancer activities, since both are inter-related. The antioxidant activity of FCE was determined by reductive potential in reducing ferric ions and anticancer activity was determined using breast cancer cell lines (MCF7). The extract showed strong antioxidant and anti-cancer activities, which suggest the use of the plant for therapeutic purposes, supporting the traditional claims.

Key words: Ficus, MCF7, antioxidant, breast cancer.

INTRODUCTION

One of the largest causes of mortality worldwide is cancer. So far, abnormalities in about 350 genes have been demonstrated in human cancers (Futreal et al., 2004; Broadhead et al., 2010) and epidemiologically, cancer is responsible for one in eight of worldwide deaths (Wolf and Davidovici, 2010). Increasing interest and research on herbal medicine have revealed its importance in treating many diseases including cancer. Epidemiologic studies have suggested that some antioxidant agents as well dietary constituents with antioxidant properties may be acting as naturally occurring cancer prevention agents. Therefore, there is an urgent need to develop alternative therapeutic measures against this deadly disease. Many components from dietary or medicinal plants have been identified that they possess substantial chemopreventive properties. India has unique plant varieties yet to be studied for anticancer

components. Breast cancer is the second most common cancer amongst women, comprising 21.4% of female cancers. Antioxidants also have known to play a vital role in preventing cancers. Several phytochemicals which possess antioxidant potential are known to prevent cancers and the anticancer effects may be associated with antioxidant properties due to its polyphenolic components (Gheldof et al., 2003; Liu et al., 2000; Laandrault et al., 2001; Siddhuraju and Becker, 2003; Zuo et al., 2002). Among the several plants reported to possess medicinal properties, *Ficus* constituted one of the largest genera of medicinal plants with about 750 species of woody plants, trees, and shrubs primarily occurring in subtropical and tropical regions throughout the world. The genus is remarkable for the large variation in the habits of its species. In India, the most important species of *Ficus* are *Ficus bengalensis*, *Ficus carica*,

*Corresponding author. E-mail: jasmine_selvakumar@yahoo.com. Tel: 9500423739.

Ficus racemosa and *Ficus elastica*. *F. carica* is commonly referred as "fig". Various parts of the plant like bark, leaves, tender shoots, fruits, seeds, and latex are medicinally important. The fig is a very nourishing food and used in industrial products. It is rich in vitamins, mineral elements, water, and fats. Figs are one of the highest plant sources of calcium and fiber.

According to USDA data for the Mission variety, dried figs are richest in fiber, copper, manganese, magnesium, potassium, calcium and vitamin K relative to human needs. They have smaller amounts of many other nutrients. Figs have a laxative effect and contain many antioxidants inhibiting the oxidative mechanisms that may lead to degenerative illnesses (du Toit, 2001). They are good source of flavonoids and polyphenols and some bioactive compounds such as arabinose, β -amyryns, β -carotenes, glycosides, β -setosterols and xanthotoxol. The dried figs produced a significant increase in plasma antioxidant capacity and also used in various disorders such as gastrointestinal respiratory, inflammatory, cardiovascular disorders, ulcerative diseases, and cancers. In traditional medicine, the roots are used in treatment of leucoderma and ringworms and its fruits which are sweet, have antipyretic, purgative, aphrodisiac properties and have shown to be useful in inflammations and paralysis. *F. carica* has been reported to possess antioxidant activity by previous study (Solomon et al., 2006). The plant has been reported to possess antiviral, antibacterial, hypoglycemic, hypotriglyceridaemic and anthelmintic effects. There are no reports on the *F. carica* crude ethanolic extract (FCE) possessing both antioxidant and anticancer activity. Hence, this study was aimed to investigate the antioxidant and anticancer activity of *F. carica* fruits. The present review correlates antioxidant activity of *Ficus* species with its anticancer activity.

MATERIALS AND METHODS

Collection of plant materials

F. carica fruits were purchased from local supermarket at Tiruchirappalli District, Tamil Nadu, India during the month of March and authenticated by the botany department of our college and the voucher specimen was deposited.

Preparation of plant extract

The fruits chosen for the study were been washed, macerated and lyophilised. The collected fruits were chopped into small pieces, sun dried for about five days and grinder into coarse powder with a mechanical grinder and stored in an air tight container. Then 25 g of powder was taken mixed with 100 ml of ethanol for five days at room temperature $25 \pm 2^\circ\text{C}$ with occasional stirring. After 5 days the ethanol extract was filtered with Whatman No. 1 filter paper. The extract was concentrated under reduced pressure below 50°C through rotary vaccum evaporator. The concentrated extract was collected in a Petri dish and allowed to dry for complete evaporation of ethanol. The whole process was repeated three times and finally,

collected the blackish green color, concentrated plant extract. It was stored at 4°C for future use. About 500 g of fruits yielded 37 g powder. The procedure was repeated to collect the needed quantity.

Cell line and culture

Breast cancer (MCF-7) cell line was obtained from NCCS, Pune, India. The cells were maintained in minimal essential media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g/ml}$) in a humidified atmosphere of 50 $\mu\text{g/ml}$ CO_2 at 37°C .

Reagents

MEM, Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were needed for the study. All the chemicals and reagents were obtained from Sigma Aldrich Mumbai, India.

In vitro assay for anticancer activity (MTT assay)

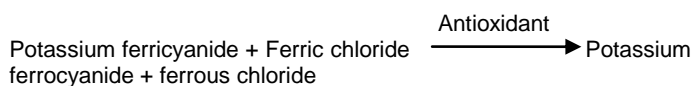
The anticancer activity of FCE on MCF-7 was determined by the MTT assay (Mosmann, 1983). Cells ($1 \times 10^5/\text{well}$) were plated in 1 ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 48 h of incubation, the cells reached the confluence; then the cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 24, 48 and 72 h at 37°C . After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200 $\mu\text{l/well}$ (5 mg/ml) of 0.5% MTT solution was added. After 4 h incubation, 0.04 M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined. The absorbance at 570 nm was measured with a UV- spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of MCF-7 was expressed as the % cell viability, using the following formula:

$$\% \text{ cell viability} = (\text{O.D at 570 of treated cells} / \text{O.D at 570 of control cells}) \times 100\%.$$

Reducing power assay

Principle

The reducing power of ethanol extract of *Ficus* fruits was determined by the reducing antioxidant power method, and was conducted according to Pellegrini et al. (2003). Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.



Chemicals required

Potassium ferricyanide (1% w/v), phosphate buffer (0.2 M, pH 6.6), trichloro acetic acid (10%), ferric chloride (0.1%) and ascorbic acid (1%).

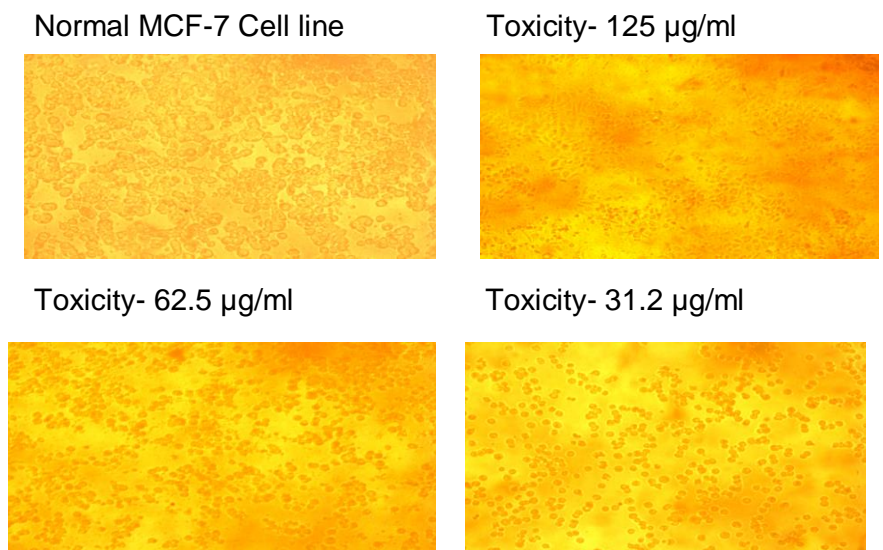


Plate 1. Anticancer effect of sample (24 h) on MCF-7 cell line.

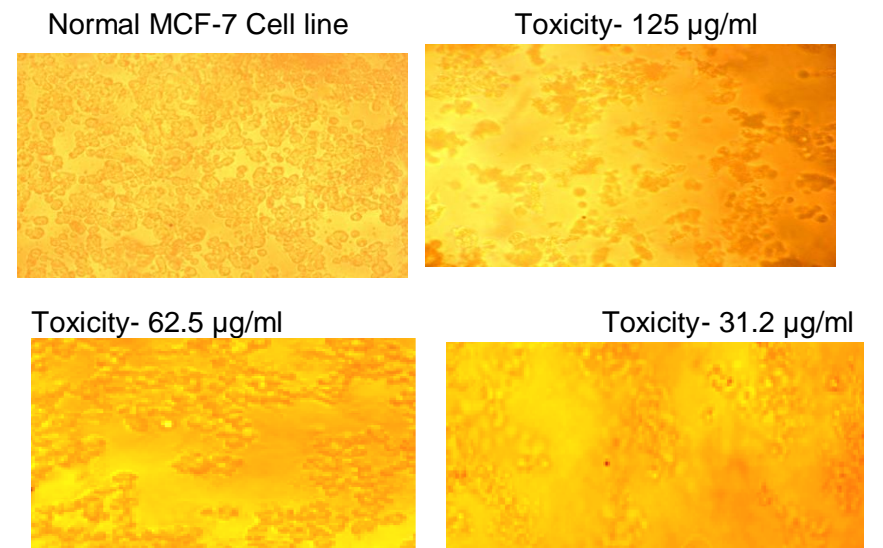


Plate 2. Anticancer effect of sample (48 h) on MCF-7 cell line.

Phosphate buffer preparation

Dibasic sodium phosphate (18.75 ml of 0.2 M) is mixed with 31.25 ml monobasic sodium phosphate and diluted to 100 ml with water.

Procedure

Various concentrations of the plant extracts in 1.0 ml of deionized water were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml) and incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5

ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard; increased absorbance of the reaction mixture indicates increase in reducing power.

RESULTS AND DISCUSSION

The anticancer activities of *F. carica* ethanol extracts at 24, 48 and 72 h have been shown in Tables 2 to 5 and Plates 1 to 3. The results show a dose and time dependent decrease in viability. The percentage of cell death at

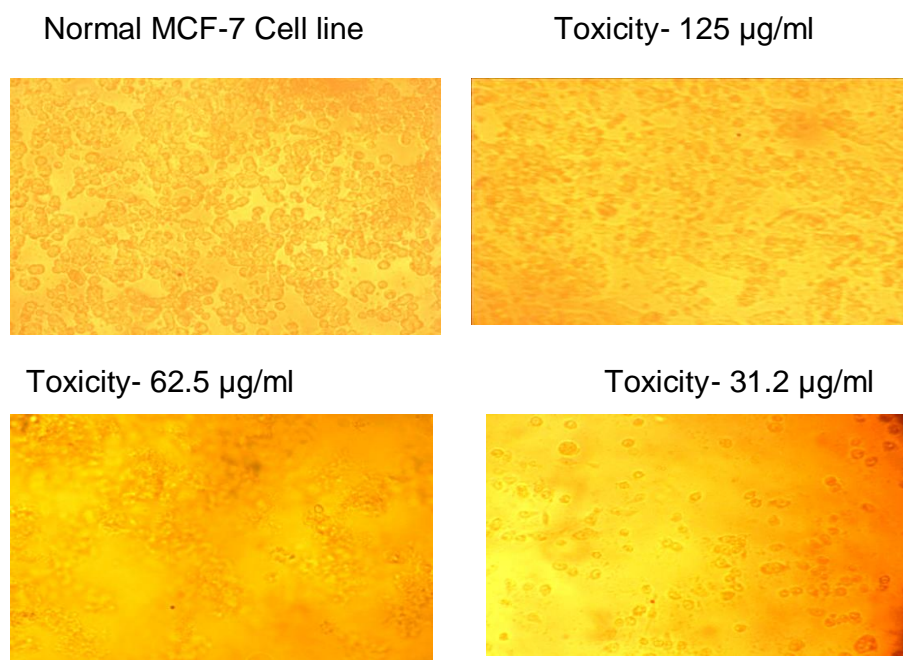


Plate 3. Anticancer effect of sample (72 h) on MCF-7 cell line.

Table 1. Shows that the volume of ascorbic acid and extract taken for the presence study, where A test is absorbance of test solution. A blank is absorbance of blank. The antioxidant activity of the *Ficus carica* extract was compared with the standard ascorbic acid.

Ascorbic acid concentration (mg/ml)	Absorbance at 700 nm
0.2	0.40
0.4	0.58
0.6	0.85
0.8	1.00
1.0	1.2

Table 2. Antioxidant activity of *Ficus carica* ethanol extract.

Ascorbic acid concentration (mg/ml)	Absorbance at 700 nm
0.2	0.09
0.4	0.10
0.6	0.11
0.8	0.79
1.0	0.81

1000 µg/ml varied in time and was 85.5% at 24 h, 89% at 48 h and 90.5% at 72 h, while at dose of 500 µg/ml, the response was also time dependent as the 76% inhibition at 24 h increased to 80.5 and 82.5% at 48 and 72 h, respectively (Figures 2 to 4). This was also supported by the antioxidant activity as depicted in Table 1 and Figure

1. Similar findings were already reported by Jeune et al. (2005). The ability of plant extracts to significantly decrease the cell viability in a time and dose dependent manner had also been observed by other researchers (Svejda et al., 2010). There has been growing interest in the beneficial health effects of consuming fruits and

Table 3. Anticancer effect of sample (24 h) on MCF-7 cell line.

Concentration ($\mu\text{g/ml}$)	Absorbance (O.D)	Cell viability (%)
1000	0.09	14.28
500	0.15	23.80
250	0.22	34.92
125	0.27	42.85
62.5	0.32	50.79
31.2	0.37	58.73
15.6	0.44	69.84
7.8	0.53	84.12
Cell control	0.63	100

Table 4. Anticancer effect of sample (48 h) on MCF-7 cell line.

Concentration ($\mu\text{g/ml}$)	Absorbance (O.D)	Cell viability (%)
1000	0.07	11.11
500	0.12	19.04
250	0.18	28.57
125	0.24	38.09
62.5	0.29	46.03
31.2	0.31	49.20
15.6	0.38	60.31
7.8	0.46	73.01
Cell control	0.63	100

Table 5. Anticancer effect of sample (72 h) on MCF-7 cell line

Concentration ($\mu\text{g/ml}$)	Absorbance (O.D)	Cell viability (%)
1000	0.06	9.52
500	0.11	17.46
250	0.18	28.57
125	0.23	36.50
62.5	0.28	44.44
31.2	0.33	52.38
15.6	0.40	63.49
7.8	0.43	68.25
Cell control	0.63	100

vegetables. Mainly, the presence of phenolic antioxidants is believed to have the protective mechanisms (Sun, 1990). Phenols have always known to play a vital role in alleviating cancers and also possess antioxidant activity (Rice-Evans et al., 1996; Son and Lewis, 2002).

In the present study, the ethanol extracts of *F. carica* exhibited reducing activity and anti-cancer activity. The results indicate that the extent of antioxidant activity of the extract is in accordance with the anti-cancer activity. The most common cause of hereditary breast cancer is

an inherited mutation in the *BRCA1* and *BRCA2* genes. In normal cells, these genes help prevent cancer by making proteins that keep the cells from growing abnormally. Although, in some families with *BRCA1* mutations, the lifetime risk of breast cancer is as high as 80%, on average, this risk seems to be in the range of 55 to 65%. For *BRCA2* mutations, the risk is lower, approximately 45%. Breast cancers linked to these mutations occur more often in younger women and more often affect both breasts than cancers not linked to these

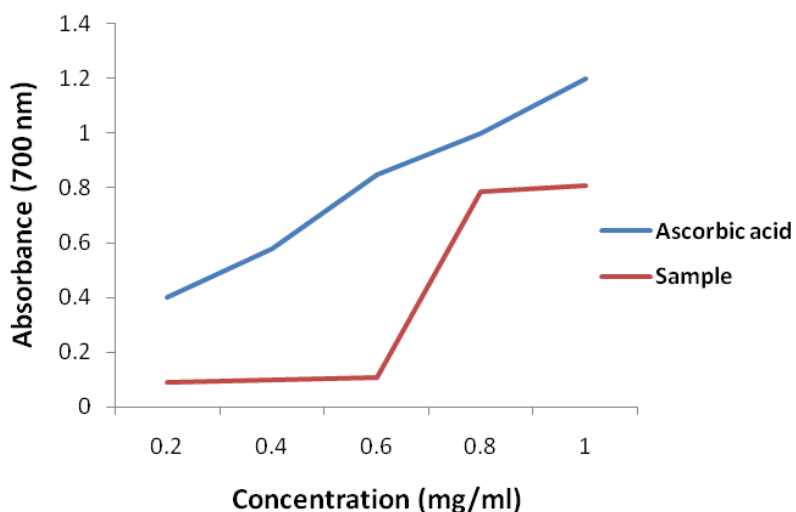


Figure 1. Antioxidant activity of the standard and sample.

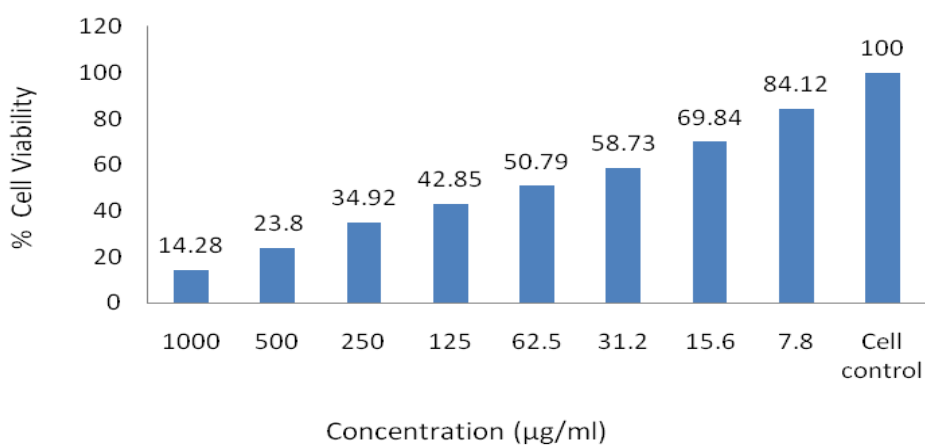


Figure 2. The effect of *Ficus carica* ethanol extracts against breast cancer (MCF-7) cell line at 24 h.

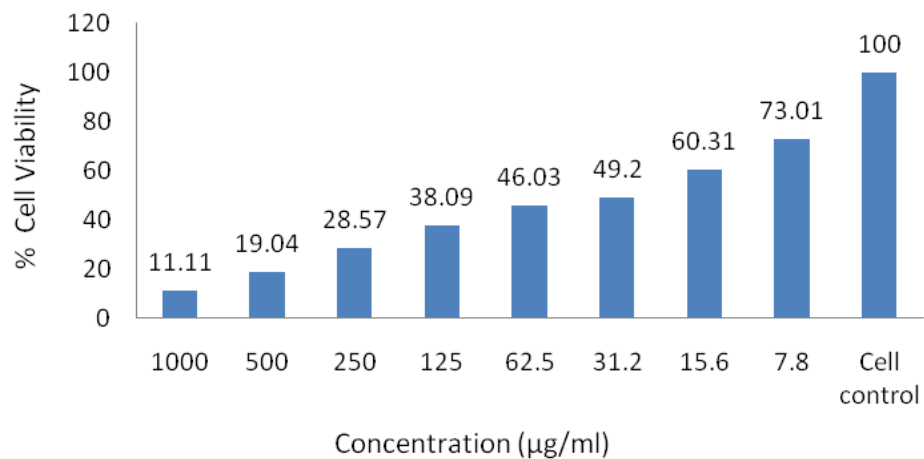


Figure 3. The effect of *Ficus carica* ethanol extracts against breast cancer (MCF-7) cell line at 48 h.

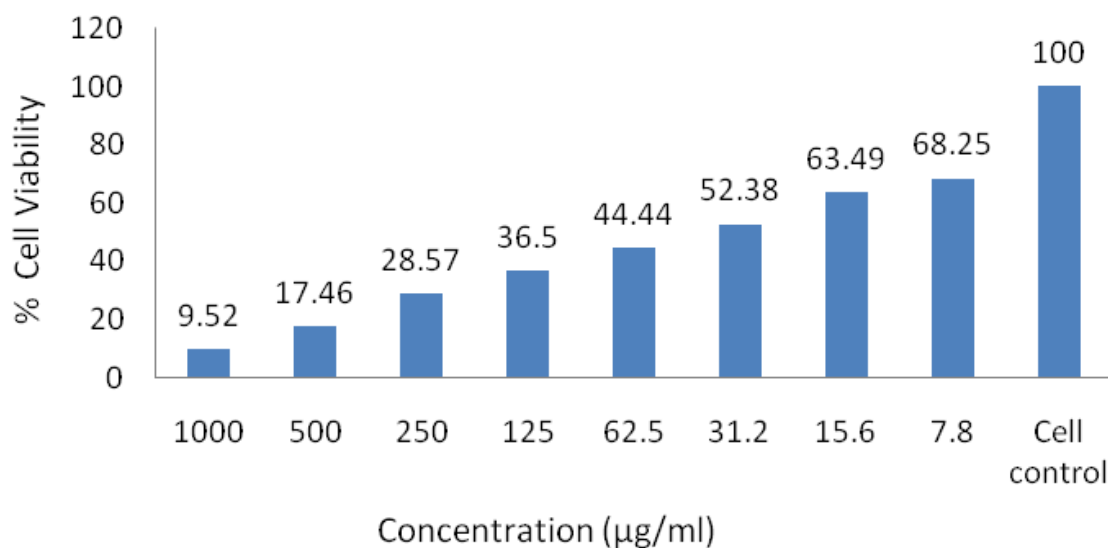


Figure 4. The effect of *Ficus carica* ethanol extracts against breast cancer (MCF-7) cell line at 72 h.

mutations. Women with these inherited mutations also have an increased risk for developing other cancers, particularly ovarian cancer. It has been reported that ethanolic extracts are normally used for anticancer screening because it is believed that the polar compounds were mostly claimed for anticancer properties. As mentioned formerly, the ethanolic extracts of FC showed significant cytotoxic activities towards MCF-7 cells. The anti-cancer activity was also supported by the anti-oxidant activity displayed by FCE (Chiang et al., 2005).

The present results, together with previous studies, suggest that *Ficus* extract has great potential as an anticarcinogenic and antioxidant agent. In general, the beneficial effect of plant products such as *Ficus* may be attributable to one or more phytochemicals including antioxidants, phenolic compounds and other substances. Understanding the modes of action of these compounds should provide useful information for their possible applications in cancer prevention and perhaps in cancer therapy (Taraphdar et al., 2001; David, 2004). Further work to identify the active principle and its role in signaling the key apoptotic enzymes is under progress.

This study has provided insight towards the possibility of using plants effectively as a possible alternative treatment for cancer treatments, supporting the traditional use of this plant. Further fractionation and spectral analysis are under progress to identify an effective and safer drug for anti-oxidant and anti-cancer activities. Experimental results reveal that the plant extract possesses significant anticancer activity which may be due to its cytotoxicity and antioxidant properties. Further research is going on to find out the active principle(s) of FCE for its anti-cancer activity.

Conflict of interests

The authors have not declared any conflict of interests.

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