African Journal of Biotechnology

Volume 13 Number 35, 27 August, 2014
ISSN 1684-5315

Academic Journals

ABOUT AJB

The **African Journal of Biotechnology (AJB)** (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajb@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajb@academicjournals.org.

Editorial Board

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the African Journal of Biotechnology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

African Journal of Biotechnology

Table of Contents: Volume 13 Number 35, 27 August, 2014

ARTICLES

Table of Contents: Volume 13 Number 35, 27 August, 2014

academicJournals

Vol. 13(35), pp. 3553-3559, 27 August, 2014
DOI: 10.5897/AJB2014.13852
Article Number: B50687447051
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Micropropagation of *Caralluma stalagmifera* var. *longipetala:* A rare succulent medicinal plant from Karnataka, India

Ramachandra Naik M.[1], Subbiah Karuppusamy[2]* and Y. L. Krishnamurthy[1]

[1]Department of P. G. Studies and Research in Applied Botany, Kuvempu University, Shankaraghatta, Karnataka 577451, India.
[2]Department of Botany, Centre for Botanical Research, The Madura College (Autonomous), Madurai – 625 011, Tamilnadu, India.

Received 11 April, 2014; Accepted 28 July, 2014

An efficient *in vitro* protocol has been developed for the multiplication of shoots and conservation of a rare succulent medicinal plant *Caralluma stalagmifera* var. *longipetala* growing wildly in Karnataka State. Proliferation of multiple shoots was achieved on Murashige and Skoog's (MS) medium supplemented with various concentrations of 6-benzyladenine (BA), Kinetin (Kn), indole acetic acid (IAA), α- naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) alone or with various combinations from the nodal explants. The nodal explants cultured on medium containing BAP (2.0 mg/L) along with 0.5 mg/L Kn and 0.3 mg/L IAA produced the highest number of shoot sprouting (2.60±0.16) and maximum shoot length (3.96±0.20). The considerable frequency of callus induction and embryogenesis was noticed both in 1.0 mg/L NAA and 0.5-2 mg/L, 2, 4-D. The calli transferred to shoot induction medium containing the combination of hormones BAP (1.0 mg/L) plus IAA (0.2 mg/L) and NAA (0.1 mg/L) successfully regenerated *in vitro* shootlets. The *in vitro* rooting was achieved from both direct shoots regenerated from nodal explants and callus derived shootlets with NAA (0.2 mg/L). The *in vitro* rooted plantlets were successfully acclimatized (75%) in the greenhouse and gradually transferred to open field conditions.

Key word: Micro propagation, medicinal plant, *Caralluma stalagmifera* var. *longipetala.*

INTRODUCTION

Caralluma stalagmifera var. *longipetala* Karupp. & Pullaiah was originally described from Tamilnadu state (Karuppusamy and Pullaiah, 2007) and later it was recognized that its distribution was extended in Karnataka State of Southern India. The plant is a xerophytic succulent leafless medicinal herb belonging to Apocynaceae. The succulent stem of the plant is used to cure many ailments and have noted antiobesity properties (Karuppusamy et al., 2013). The herb is a rich source of flavonoidal glycosides and alkaloids (Kunert

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

et al., 2009) with significant anti-inflammatory, anti-oxidant and antimicrobial properties (Madhuri et al., 2011). It is also reported to have significant anti-arthritic activity in kaolin induced rats (Reddy et al., 1996). The chemical principles isolated from *C. stalagmifera* are steroidal glycosides, stalagmosides, carumbellosides and lasiathosides (Kunert et al., 2009).

Natural population of this plant species are declining day by day because of increase demand in the pharmaceutical market coupled with over-exploitation and habitat destruction. There are no formulated agronomic or cultivation techniques for these endemic succulent medicinal species until now. For the conservation of these important medicinal plants, several other wild *Caralluma* species have already been developed via *in vitro* multiplication protocol by various authors (Aruna et al., 2012; Ugraiah et al., 2011; Sreelatha et al., 2009). So far there is only one report available on the micropropagation of *C. stalagmifera* from *in vitro* grown seedling explants (Sreelatha and Pullaiah, 2010). Micropropagation of other related *Caralluma* species include on *Caralluma sarkariae* (Sreelatha et al., 2009), *Caralluma bhupenderiana* (Ugraiah et al., 2011) and *Caralluma adscendens* var. *attenuata* (Aruna et al., 2012). The purpose of the study was to develop a rapid *in vitro* shoot multiplication and callus regeneration protocol from plant materials collected from natural populations growing in wild.

MATERIALS AND METHODS

Plant material, surface sterilization and inoculation

Succulent plants of *C. stalagmifera var. longipetala* were collected from Muddapura of Chitradurga District in Karnataka and plants were maintained in pots containing mother soil under polyhouse condition in the Kuvempu University campus, Shivamogga (Figure 1A and B). The tender shoot segments with six to eight inter nodes were collected from the potted plants and washed with running tap water for 15 min to remove the soil particles and other dust particles. The internodes were cut into small pieces and rinsed with 1% (v/v) Tween 20 (Merck, Bangalore, India) for 5 min. They were further rinsed in distilled water three times and taken into the laminar air flow chamber where they were rinsed with sterile double distilled water. The explants were immersed in 30% ethanol for 3 min and again washed with sterilized double distilled water. It was followed by Mercuric chloride ($HgCl_2$ 0.1% (v/v)) treatment for 2 min. After sterilization, the explants were thoroughly rinsed with several changes of sterile double distilled water. The explants were trimmed into pieces of about (0.6 mm to 10 mm) and then inoculated into culture media. Murashige and Skoog's media (HiMedia, Mumbai, India) media was fortified with 3% (w/v) sucrose and 0.8% of agar for solidification. The pH of media was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved.

Shoot initiation and multiplication

For the induction of shoots, nodal explants were cultured on MS medium amended with various plant growth regulators like 6-benzyladenine (BA), Kinetin (Kn), and α- naphthalene acetic acid (NAA) (HiMedia, Mumbai, India) at different concentrations (0.5, 1.0, 2.0, 3.0 and 5.0 mg/L). Cultures were subcultured on to the fresh medium with every 30 days period of intervals. The *in vitro* response was measured in the frequency of shoot multiplication, the number of shoots per explants and the shoot lengths at the end of six week old cultures.

Callus induction and multiplication

Internodal segments of *C. stalagmifera* var. *longipetala* were cultured on MS medium fortified with auxins like 2, 4-dichlorophenoxyacetic acid (2, 4 D) and α- naphthalene acetic acid (NAA) in different concentrations (0.5 to 2.0 mg/L) alone. Regenerative and embryogenic calli were transferred to fresh MS medium supplemented with different concentration of BAP (0.5 to 2.5 mg/L), IAA (0.2 mg/L) and NAA (0.1 and 0.3 mg/L) alone or in combinations for the regeneration of shoots.

Root initiation and multiplication

The regenerated *in vitro* shoots (4 to 5 cm height) were separated and callus induced shoots with 2 to 3 cm height were isolated and transferred for root induction on to half-strength MS medium containing different concentrations of NAA, IAA and IBA (Indole-3-butyric acid). The cultures were maintained under 16 h photoperiod for one month until the micro shoots initiated the roots. *In vitro* rooting response were measured with number of roots and mean length of roots (Ugraiah et al., 2011).

Acclimatization and transplantation of plantlets

In vitro rooted plantlets were removed from culture tubes with at least two roots of 2 to 4 cm length. They were washed carefully with tap water to remove traces of agar and then transferred to the pots containing different potting mixtures namely: cocopeat (HiMedia, Mumbai, India), cocopeat + sand + soil (1:2:1) and cocopeat + sand (1:1). The planted pots were covered with transparent polythene to maintain humidity until the development of new rudimentary leaves and sprouting new roots (Aruna et al., 2012). After a month they were removed and the plants were maintained in the lab temperature conditions for 15 more days. After two months of hardening, the plants were transferred to new pots containing humus soil, kept in polyhouse for one month. During the first 15 days of acclimatization neither watering nor any fertilizers was provided to plants. The hardened plants were planted on nursery bed with frequent watering in natural condition.

Statistical analysis

The experiments were randomized and repeated three times. Each treatment consisted of 15 replicates. Data were statistically analyzed by analysis of variance (ANOVA) and mean readings were compared by Tukey's test at 0.05% probability level.

RESULTS AND DISCUSSION

Effect of cytokinin on shoot regeneration

The success of micropropagation depends on the selection of suitable explants, media composition, types of growth regulators, their concentrations and combinations

Figure 1. *In vitro* propagation of *C. stalagmifera* var. *longipetala*. **A)** Inflorescence of *C. stalagmifera* var. *longipetala*. **B)** Shoot induction from MS medium containing BA 2.0 mg/L. **C)** Rooting of regenerated shoots containing 0.2 mg/L NAA. **D)** Rootlet plant. **E)** Callus induction from medium containing 1.5 mg/l 2,4-D. **F)** Callus to shoot induction. **G)** Multiple shoots Containing BA 0.5 mg/L Kn+ 0.3 mg/l IAA. **H)** Shoot and callus induction from medium containing 0.5 Kn and NAA 0.5 mg/L. **I)** Coco peat planted pots. **J)** Polythin covered pots. **K)** Plants growing in forest soil under playhouse.

with culture conditions. The effect of cytokinins and its concentration on bud breaking from nodal explants cultured on MS basal medium is given in Table 1. The MS basal medium fortified with BAP 2.0 mg/L was found to have the best shoot sprouting, number and length of shoots without basal callus formation from nodal explants. The shoot buds sprouted on Kn containing medium showed only limited growth even if they were maintained for longer period of subculture. MS medium is the most efficient medium for shoot proliferation of *Caralluma* spp. such as *Caralluma adscendens*, *Caralluma bhubenderiana* (Ugraiah et al., 2011) and *Caralluma lasiantha* (Aruna et al., 2012). Nodal explants selected for shoot proliferation gave positive

Table 1. Effect of various concentrations of BAP and Kn on shoot formation in mature nodal explants of *C. stalagmifera* var. *longipetala* cultured on MS medium with sucrose (3%)

Hormone	Concentration (mg/L)	Shoot sprouting frequency (%)	Mean shoot number per explants ± SE	Mean length of shoots (cm) ± SE
BAP	0.5	32.5	1.35±0.09^{b}	1.68±0.15^{bc}
	1.0	40	1.40±0.11^{b}	2.25±0.15^{b}
	1.5	57.5	1.75±0.12^{b}	2.56±0.18^{b}
	2.0	80	2.25±0.17^{a}	3.11±0.20^{a}
	2.5	65	2.05±0.15^{a}	2.77±0.22^{b}
Kn	1.0	12	1.20±0.09^{c}	1.42±0.10^{c}
	1.5	32.5	1.45±0.11^{b}	2.02±0.11^{b}
	2.0	40.5	1.40±0.12^{b}	2.50±0.12^{b}
	2.5	55	1.80±0.13^{b}	2.70±0.23^{b}
	3.0	47.5	1.70±0.12^{b}	2.62±0.15^{b}

Means ± SE, n = 30. Means followed by the same letter in a column are not significantly different by the Tukey's test at 0.05% probability level.

morphogenic response with 80% of bud break and emergence of bud within a week on MS medium supplemented with BAP 2.0 mg/L and with 3% of sucrose; the highest number of shoots (2.25 shoots/explants) with mean length (3.11 cm), whereas from the Kn containing medium nodal explants obtained 1.80 shoots/explants with mean length of 2.7 cm in Kn 3.0 mg/L fortified cultures. There are several examples in literature showing the nodal explants are most effective in succulent Asclepiads (Sreelatha et al., 2009). The response of nodal explants treatment results are presented in Table 1. Out of these treatments, MS medium fortified with BAP 2.0 mg/L had a better shoot sprouting frequency (80). High concentration of BAP above 3.0 mg/L resulted in reduced shoot sprouting and frequency of response (Personal communication). Kn containing cultures produced less than 2 shoots/explants (Table 1).

Effect of cytokinins with auxin on shoot multiplication

C. stalagmifera var. *longipetala* nodal explants were cultured on MS medium supplemented with various concentrations of cytokinins (BAP 1.5 to 2 mg/L, Kn 0.2 to 1.5 mg/L) and auxins (NAA 0.2 to 1 mg/L, IAA 0.3 to 0.6 mg/L) (Table 2). The effect of BAP on multiple shoots proliferation has been demonstrated in Asclepiads (Aruna et al., 2009, 2012). Combination of 1.5 mg/L BAP + 0.6 mg/L Kn produced highest number of shoot sprouting frequency of about 76%, the mean shoot/explants was 2.05±0.17 and mean shoot length was 3.07±0.17 cm. Similarly the combination of BAP 1.5 mg/L +NAA 0.3 mg/L produced sprouting frequency of about 53.3% (Table 2). Mean shoot number per explants was about 1.40±0.11 and the mean shoot length is 2.12±0.14 cm. Basal callus formed in all the shoots were healthy (Figure 1B).

The combination of BAP (2.0 mg/L) + Kn (0.5 mg/L) + IAA (0.3 mg/L) yielded maximum shoot regeneration frequency of 90%. Number of shoots per explant was 2.60±0.16 and 3.96±0.20 cm. The mean shoot length followed by other combination of hormones such as BAP (2.0 mg/L) + Kn (1.0 Mg/L) + IAA (0.3 mg/L) showed regeneration frequency of about 86% and the number of shoots per explants was 2.10±0.18 and 3.32±0.17. The mean shoot length, of other combination of cytokinins BAP with auxins showed that the medium resulted in moderate callus formation (Table 2). The combination of BAP (2 mg/L) + NAA (0.5 mg/L) showed lower frequency of shoot sprouts (53.3%) with basal callus (Figure 1G).

Callus induction and somatic embryogenesis

The intermodal explants cultured on various concentration of 2,4-D (0.5, 1.0, 1.5, 2.0) and NAA (0.5, 1.0, 1.5, 2.0) become swollen and generally dedifferentiated and developed friable callus after two weeks of culture. Among the different auxins tested 2,4-D at 1.5 mg/L favored the best response of callus production (90%) (Table 3). NAA supplemented cultures showed good callus production but there was no further shoot regeneration or somatic embryogenesis when other auxins in different combinations were added (Table 3). The calli subcultured onto media containing a combination of 2,4-D (1.5 mg/L) with BAP (1.0 mg/L), IAA (0.2 mg/L) and NAA (0.1 mg/L) produced maximum number of globular embryos on the surface within four weeks (Figure 1E). In *Asclepias,* studies demonstrated the need of 2, 4-D (2 mg/L) and BA (0.1 mg/L) for callus induction (Vyapari et al., 1993). Other Asclepiad members like *Tylophora indica* and *Hemidesmus indicus* produced

Table 2. Effect of different combination of BAP, Kn, NAA, and IAA on shoot regeneration of mature nodal explant of *C. stalagmifera* var. *longipetala*

BAP	Kn	NAA	IAA	Shoot sprouting frequency (%)	Mean shoot number per explants ± SE	Mean length of shoots (cm) ± SE	Formation Basal Callus (CP)
1.5	0.2			66.6	1.75±0.13^{b}	2.80±0.15^{b}	-
1.5	0.4			73.3	2.15±0.13^{a}	2.98±0.20^{b}	-
1.5	0.6			76	2.05±0.17^{a}	3.07±0.17^{a}	-
1.5	0.8			70	1.95±0.15^{bc}	2.82±0.15^{b}	-
1.5		0.3		53.3	1.40±0.11^{c}	2.12±0.14^{b}	+
1.5		0.5		50	1.55±0.11^{c}	2.34±0.13^{b}	+
1.5		1.0		43.3	1.40±0.11^{c}	1.94±0.09^{bc}	++
	0.5	0.5		40	1.45±0.11^{c}	1.39±0.10^{c}	++
	0.3	0.5		46.6	1.50±0.11^{c}	2.52±0.13^{b}	++
	0.5	1.0		36.6	1.35±0.13	1.40±0.10^{c}	++
2.0	1.0			83.3	2.05±0.17^{a}	3.22±0.24^{a}	-
2.0	1.5			73.3	2.05±0.16^{a}	3.11±0.09^{a}	-
2.0		0.2		70	1.85±0.11^{b}	2.84±0.13^{b}	-
2.0		0.4		66.6	1.80±0.12^{b}	2.72±0.19^{b}	+
2.0		0.5		53.3	1.50±0.10^{c}	2.45±0.17^{b}	++
2.0	0.5	0.5		70	1.85±0.13^{b}	3.01±0.11^{a}	+
2.0	1.0	0.5		66.6	1.60±0.10	2.88±0.14^{b}	+
2.0	0.5		0.3	90	2.60±0.16^{a}	3.96±0.20^{a}	-
2.0	1.0		0.3	86	2.10±0.18^{a}	3.32±0.17^{a}	-
2.0	0.5		0.6	70	1.85±0.12^{b}	2.87±0.17^{b}	-

Means ± SE, n = 30. Means followed by the same letter in a column are not significantly different by the Tukey's test at 0.05% probability level; NR – no response.

Table 3. Effect of different combination of 2.4.D, BAP, NAA, and IAA on callus induction of mature thin explant of *C. stalagmifera* var. *longipetala*

Growth regulator (mg/L)		Callus frequency (%)	Basal callusing	Callus nature	Embryos treatment with BAP+IAA+NAA (mg/l) (subculture)	Number of shoot sprouting (%)
2.4.D	0.5	75	++	Crystal white	0.5.0+0.2+0.1	2.53±0.17
	1.0	85	+++	Greenish white	1.0+0.2+0.1	3.47±0.24
	1.5	90	+++	Greenish white	1.5+0.2+0.1	2.67±0.22
	2.0	80	++	white	2.0+0.2+0.1	CP
NAA	0.5	60	+	white	1.0+0.2+0.3	CP
	1.0	70	++	Crystal white	1.5+0.2+0.3	CP
	1.5	55	+	Crystal white	2.0+0.2+0.3	CP
	2.0	35	+	Cream white	2.5+0.2+0.3	CP

CP-Callus production.

callus on 2, 4-D and BAP (Thomas and Philip, 2005; Sarasan et al., 1994). The somatic embryos were induced within two weeks of subculture with media containing NAA and 2, 4-D at different concentrations (Stephan and Jayabalan, 2001; Inamdar et al., 1990).

Rooting of *in vitro* regenerated shoots

In *C. stalagmifera* var. *longipetala*, half strength MS medium supplemented with auxins such as IAA, IBA and NAA at different concentrations showed varied effect on rooting (Table 4). The experiment was aimed at the induction of rooting in basal portion of *C. stalagmifera*var. *longipetala* microshoots; NAA was found more effective than the IAA and IBA hormones. The NAA showed positive response of rooting in the present study, which is similar to the observations on other Asclepiads such as *Decalepis arayalpathra* (Sudha et al., 2005) and *Euphorbia tirucalli*

Table 4. Rooting response of *in vitro* regenerated shoots of *C. stalagmifera*var. *longipetala*in half strength MS media containing NAA, IAA and IBA in various concentrations with sucrose (1%) after one month.

NAA	IAA	IBA	Percentage of root response	Mean number of roots per shoot ± SE	Mean length of roots (cm) ± SE	Degree callusing (CP)
0.1			80	3.75±0.35^{c}	4.17±0.29^{b}	
0.2			90	6.10±0.56^{a}	5.90±0.47^{a}	
0.3			85	4.80±0.50^{ab}	4.40±0.40^{b}	
0.4			50	2.60±0.36^{d}	2.82±0.24^{d}	++
0.5			CP	-	-	+++
	0.2		75	3.80±0.25^{c}	3.82±0.38^{cd}	
	0.3		80	4.05±0.34^{ab}	3.25±0.33^{d}	
	0.4		60	2.80±0.19^{d}	2.50±0.22^{d}	
	0.5		40	2.10±0.22^{d}	1.28±0.13^{d}	+
	1.0		CP	-	-	+++
		0.2	65	3.05±0.33^{cd}	2.48±0.19^{d}	
		0.4	55	2.75±0.37^{d}	1.75±0.13^{d}	
		0.6	35	2.10±0.18^{d}	0.99±0.10^{e}	+
		0.8	CP	-	-	++
		1.0	CP	-	-	+++

(Uchida et al., 2004). The concentrations of 0.2 mg/L NAA induced on an average 6.10±0.56 root/shoot respectively and 5.90±0.47 root lengths (Table 4, Figure 1D). Other concentrations like NAA at 0.1 0.3 and 0.4, mg/L gradually decreased root numbers and root length and also NAA 0.5 mg/L induced the basal callus.

0.3 mg/L of IAA induced 4.05±0.34 root / shoot (80%) and 3.25± 0.33 root lengths followed by other growth hormones; IAA (0.2, 0.4, and 0.5 mg/L). The concentration of 0.2 mg/L IBA induced 3.05±0.33 root / shoot (65%) and 2.48±0.19 root lengths respectively (Table 4). Effective response of IBA in rooting has been reported for medicinal plants like *Ceropegia bulbosa* (Britto et al., 2003,) *Ceropegia candelabrum* (Beena et al., 2003) and *Decalepis hamiltonii* (Giridhar et al., 2005). The plantlets resumed good growth after four weeks of transplantation and acclimatization processes, out of 80% of plants transferred, 75% survived after three weeks of transfer under the green house condition.

Conflict of Interests

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

REFERENCES

Aruna V, Kiranmai C, Karuppusamy S, Pullaiah T (2012). Effect of medium, explants, cytokinins and node position on *in vitro* shoot multiplication of *Caralluma lasiantha* (Wight) N. E. Br., an endemic and medicinally important plant. Afr. J. Biotechnol. 11:15523-15528.

Beena MR, Martin KP, Kirti PB, Hariharan M (2003). Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum*. Plant Cell Tiss. Org. Cult. 72:285-289.

Britto JS, Natarajan E, Arockiasamy I (2003). *In vitro* flowering and shoot multiplication from nodal explants of *Ceropegiabulbosa* Roxb. var. *bulbosa*.Taiwania 48:106-111.

Giridhar P, Gururaj HB, Ravishankar GA (2005). *In vitro* shoot multiplication through shoot tip cultures of *Decalepishamiltonii* Wight &Arn. A threatened plant endemic to southern India. In Vitro Cell. Dev. Biol. Plant. 41:77-80.

Inamdar JA, Nataraj M, Mohan SS, Subramanian RB (1990). Somatic embryogenesis from callus cultures of *Crataevanurvala*Buch. Ham. Phytomorphol. 40:319.

Karuppusamy S, Pullaiah T (2007). Two new varieties of *Carallumastalagmifera* C.E.C. Fisch. (Asclepiadaceae) from Peninsular India. Rheedea. 17:41-45.

Karuppusamy S, Ugraiah A, Pullaiah T (2013). *Caralluma* (sensu lato) Antiobesity plants. Astral International, New Delhi, India.

Kunert O, Simic N, Ravinder E, Adavi Rao BV, Ravi Kumar B, Alex RM, Kuehnelt D, Appa Rao AVN (2009). Steroidal glycosides from two Indian *Caralluma* species, *C. stalagmifera* and *C. indica*. Hlev. Chim. Acta. 89:201-209.

Madhuri V, Amrutha VA, Murthy KSR (2011). DNA fingerprintings profile for *Caralluma* species: An approach to molecular taxonomy. Asian J. Biochem. Pharm. Res. 1:500-506.

Reddy BM, Byahatii V, Appa Rao AVN, Ramesh M. (1996). Anti-inflammatory activity of *Stapelia nobilis* and *Caralluma stalagmifera*. Fitoterapia 67:545-547.

Sarasan V, Sonia EV, Nair GM (1994). Regeneration of Indian sarsaparilla, *Hemidesmus indicus* R. Br., through organogenesis and somatic embryogenesis. Ind. J. Exp. Bio. 32:284-287.

Sreelatha RV, Pullaiah T (2010). Induction of somatic embryogenesis and plant regeneration from internodal explants of *Caralluma stalagmifera.* Bot. Res. Int. 3:17-20.

Sreelatha RV, Sandhya Rani S, Krishna reddy PV, Naveen M, Ugraiah A, Pullaiah T (2009). *Invitro* propagation of *Carallumasarkariae* Lavranos and Frandsen- An endemic and endangered medicinal plant, Indian J. Biotechnol. 8:236-239.

Stephan R, Jayabalan N (2001). Propagation of *Coriandrumsativum* L. through somatic embryogenesis.Ind. J. Exp. Biol. 39:387-389.

Sudha CG, Krishnan PN, Pushpangadan P, Seeni S (2005). *In vitro* propagation of *Decalepisarayalpathra*, a critically ethnomedicinal plant. In Vitro Cell Dev. Biol. Plant. 41:648-654.

Thomas TD, Philip B (2005). Thidiazuron-induced high frequency shoot organogenesis from leaf derived callus of a medicinal climber, *Tylophoraindica* (Burn.f.) Merr. In vitro Cell Dev. Biol. Plant. 41:124-128.

Uchida H, Nakayachi O, Otani M, Kajikawa M, Kohzu Y, Yamato KT, Fukuzawa H, Shimada T, Ohyama K (2004). Plant regeneration

from internodes explants of *Euphorbia tirucalli.*Plant Biotechnol. 21:397-399.

Ugraiah A, Sreelatha RS, Krishna Reddy PV, Rajasekhar K, Sandhya Rani S, Karuppusamy S, Pullaiah T (2011). *In vitro* shoot multiplication and conservation of *Carallumabhupenderiana*Sarkaria - an endangered medicinal plant from South India. Afr. J. Biotechnol. 10:9328-9336.

Vyapari S, Khatamian H, Albrecht ML (1993). *In vitro* regeneration in *Asclepias tuberosa*. Hort. Sci. 28:508.

academicJournals

Vol. 13(35), pp. 3547-3552, 27 August, 2014
DOI: 10.5897/AJB12.1406
Article Number: 1A6B33447050
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Assessment of genetic diversity of rice (*Oryza sativa*) cultivars using simple sequence repeat (SSR) markers

M. Umadevi*, P. Veerabadhiran and S. Manonmani

Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore - 641 003, India.

Received 2 April, 2012; Accepted 14 July, 2014

A set of 36 polymorphic simple sequence repeat (SSR) primers well distributed on all the 12 rice chromosomes have been used to assess the genetic diversity among the rice varieties. A total of 98 alleles were detected with an average of 2.78 alleles per locus across 39 genotypes. The number of alleles varied from two to four of these SSR loci. Among the primers, RM 401, RM 20A, RM 536, RM 7575 and RM 5862 produced maximum number of alleles (4). Out of the 98 alleles, 86 were polymorphic and this exhibited 87.76% polymorphism. SSR marker analysis differentiated the genotypes into two distinct groups. The polymorphism information content (PIC) value of the markers ranged between 0.28 and 0.50 with a mean value of 0.45. The highest PIC value (0.50) was observed for ten primers that is, RM 2, RM 24, RM 411, RM 104, RM 266, RM 125, RM 4674, RM 247, RM 3476 and RM 3351 while the lowest was 0.28 for RM 29. The genetic similarity coefficients for 39 genotypes obtained with SSR markers ranged from 23.8 to 78.4%.

Key words: Rice, genetic diversity, simple sequence repeat (SSR) markers.

INTRODUCTION

For the application of marker assisted selection (MAS) within a subspecies, it is important to obtain information on the genetic diversity within a rice subspecies over different genomic regions. Accurate identification keys based only on morphological and physiological parameters with clear cut features of distinctness are not always possible. Thus, other characteristics obtained with the help of new laboratory based technologies, supplement the need for precise description. Numerous genes of economic importance having quantitative nature are repeatedly transferred from one varietal background to another by plant breeders through conventional breeding methodologies. Phenotypic observations of many traits are time consuming and expensive, if such genes can be tagged with DNA markers, time and money can be saved when transferring them from one varietal background to another. The application of molecular marking techniques may be applied as the first step towards efficient conservation, maintenance and utilization of such genetic diversity of rice.

DNA based molecular markers are highly useful in this context since they are available in abundance and clearly allow the comparison of genetic material at juvenile phase avoiding any environmental influence on gene expression. Simple sequence repeats markers (microsatellites) are co-dominant, hypervariable, abundant and well distributed throughout the rice genome (Temnykh et al., 2001). The

*Corresponding author. E-mail: umadevitnau@gmail.com. Tel: 99525 11375.

application of microsatellites markers in rice include characterization of the genetic structure of the cultivated rice *O. sativa* at both the inter and intra-varietal level, genetic diversity and/or evolutionary analyses of landraces, weedy and wild rice germplasm, determination of the purity of breeding material or seed stocks, prediction of hybrid performance and heterosis and the analyses and tagging of valuable quantitative trait loci (QTL) and genes (Gao, 2005).

DNA marker technology has provided an efficient tool to facilitate plant genetic resource conservation and management. A wide variety of DNA markers such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), Insertion/Deletion markers (INDEL) etc. have been extensively used in rice for genetic diversity analysis, phylogenetic and evolutionary studies, mapping and tagging genes for quantitative traits of agronomic importance and marker assisted selection (Patel et al., 2014). Kibria et al. (2009) has screened several rice varieties for studying the genetic diversity by using SSR and RAPD markers. SSR markers can estimate genetic diversity between cultivars for example between parents of gene pool or between plants extracted from a population or between populations. Microsatellites are more powerful for the identification of within cultivar variation (Lapitan et al., 2007). Thus, in the present investigation 39 genotypes were characterized using SSR markers to study the level of diversity and to establish genetic similarities among themselves.

MATERIALS AND METHODS

A total of 39 rice genotypes were used in this study (Table 1). The plants were raised in Randomized Block Design with three replications during September 2008 at Paddy Breeding Station, Coimbatore. Total genomic DNA isolated from the genotypes was described by Porobreski et al. (1997) method with a slight modification. Mercaptoethanol (1%) was added to the extraction buffer to remove the phenolics. Approximately 1.0 g of leaf tissue was ground with liquid nitrogen and to this powder 750 µL of preheated (65°C) CTAB buffer was added. It was then incubated at 65°C in a water bath for 1 h. After bringing the tubes to room temperature, equal volume (750 µL) of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed well for 10 min to form an emulsion. It was then centrifuged at 10000 rpm for 15 min. The supernatant was transferred to a fresh tube and the chloroform: isoamyl alcohol step was repeated. The aqueous phase was transferred to a new tube and 2/3rd volume of ice cold isopropanol was added and incubated in a freezer overnight. The contents were then centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet was now saved by discarding the solution. The pellet was washed with 70% ethanol and air dried. It was finally dissolved in TE buffer (0.2 M EDTA+1M Tris) and stored at – 20°C.

A total of 50 random primers were utilized for SSR analysis, out of which 36 primers were selected on the basis of the clarity of banding patterns and polymorphism and the list of primers surveyed are listed in Table 2. The PCR reaction was conducted in a reaction volume of 15 µL containing nine ng of genomic DNA, 1.5 µL of 10X PCR buffer (including 15 mM $MgCl_2$), 0.6 µl of 10mM each of dATP, dTTP, dGTP and dCTP, 2 µL of forward and reverse SSR primer, 0.3 µl of Taq DNA polymerase (Bangalore Genei Pvt Ltd, Bangalore) and sterile water. Amplifications were performed in Bio-Rad (MyCycler thermal cycler) and Corbett PCR machine. The thermal cycler was programmed as follows. one cycle of 94°C for 1 min followed by 35 cycles of 94°C for 1 min, 55/61°C for 1 min., and 72°C for 2 min., and finally 1 cycle of 72°C for 5 min. PCR products were kept at 4°C until further use.

The PCR products were subjected to electrophoresis in 3% Agarose. Data were scaled as 1 (present) and 0 (not present) for all the alleles of each of the SSR locus. Polymorphism information content (PIC) was computed according to the method of Wilkie et al. (1997).

$$PICi = 1-\sum_{j=1}^{n} Pij^2$$

Where, Pij is the frequency of the jth allele at the ith locus summed over the number of alleles (n). A similarity coefficient matrix was calculated from binary data using Jaccards coefficient, followed by clustering from the estimated genetic distance and the phylogenetic tree topology was inferred with the clustering method of the Unweighted Pair Group Method with Arithmetic Averages (UPGMA). The cluster analysis and subsequent dendrogram construction was performed using NTSYS-PC VERSION 2.02 (Rohlf, 1999).

RESULTS AND DISCUSSION

Out of 50 SSR markers, 36 markers that were polymorphic in all the genotypes consistently were selected to analyze the variation among the genotypes (Table 2). The random primers produced a total of 98 alleles with an average of 2.78 alleles per primer across 39 genotypes. The number of alleles varied from two to four of these SSR loci. Among the primers, RM 401, RM 20A, RM 536, RM 7575 and RM 5862 produced maximum number of alleles (4) Out of the 98 alleles, 86 were polymorphic and this exhibited 87.76 per cent polymorphism. The band size ranged from 100 bp to 500 bp. Shefatur Rahman et al. (2009) who recorded 6.33 alleles per locus using a small set of three SSR markers on 34 varieties. Tabkhkar et al., (2012) reported that the overall size of amplified products ranged from 93bp in locus RM276 to 169bp in locus RM584. PIC is a quantification of the number of alleles that a marker has and the frequency of each of the alleles in the subset of germplasm tested. Since, a marker with fewer alleles has less power to distinguish several samples, and alleles present at low frequency have less power to distinguish, a higher PIC is assigned to a marker with many alleles and with alleles present at roughly equal proportions in the population (Jiang et al., 2010). The PIC (Polymorphism Information Content) ranged between 0.28 and 0.50 with a mean value of 0.45. The highest PIC value (0.50) was observed for ten primers *viz.,* RM 2, RM 24, RM 411, RM 104, RM 266, RM 125, RM 4674, RM 247, RM 3476 and RM 3351 while the lowest was 0.28 for RM 29. Our results both for number of alleles and PIC values are comparable to those reported by Thomson et al. (2007) and Lapitan et al. (2007). Patel et al., (2014) reported that among 9 SSR markers and 12 INDEL markers highest PIC value was obtained for INDEL marker R9M10 0.81.

Dendrogram resulting from cluster analysis of SSR data

Table 1. Materials used in the present study.

S/N	Genotypes	Pedigree	Source
1	IR 72081	IR 58025A/6*IR 65493-67-3-2-2-4-10-6-9-1/ IR 65493-67-3-2-2-4-10-6-9-1	IRRI, Philippines
2	IR 75596	D297A/7*IR 68897B/ IR 68897B	IRRI, Philippines
3	IR 75601	G46A/7*IR 68897B/ IR 68897B	IRRI, Philippines
4	IR 75608	KRISHNA A/7* 69627B/IR 69627B	IRRI, Philippines
5	IR 80154	IR 75603A/4* IR73329-80-2-3/ IR73329-80-2-3	IRRI, Philippines
6	IR 80559	IR 73328A/6*IR 73330-25-2-2/ IR 73330-25-2-2	IRRI, Philippines
7	CRMS 32	KALINGA/MIRAI	CRRI, Cuttack
8	APMS 6	-	APRRI, Maruteru
9	IR 62037-93-1-3-1-1	IR 48563-123-5-5-2/IRRI 104	IRRI, Philippines
10	IR 62036-222-3-3-1-2	IR 48563-123-5-5-2/IR 42068-22-3-3-1-3	IRRI, Philippines
11	IR 63881-49-2-1-3-2	IR 52280-117-1-1-3/IR 53294-65-1-1-3	IRRI, Philippines
12	IR 62124-83-3-2-1	IR 50358-102-2-3-3/IR 52280-117-1-1-3	IRRI, Philippines
13	IR 72865-94-3-3-2	IR 63868-2-3-2—2/IR 67023-30-3-3-2-2	IRRI, Philippines
14	IR 62030-83-1-3-2	IR 48525-65-2-1 / IR 52280-117-1-1-3	IRRI, Philippines
15	IR 59673-93-2-3-3	IR 48566-22-3-2-3/IR 28239-94-2-3-6-2	IRRI, Philippines
16	IR 68427-8-3-3-2	IR 58029-180-2-3-3/IR 44624-127-1-2-2-3	IRRI, Philippines
17	IR 68926-61-2	IR 34686-179-1-2-1R/IR 40750-82-2-2-3R	IRRI, Philippines
18	AD 01260	CR 1009 / JEERAGASAMBA	Aduthurai
19	MDU 5	RESELECTION FROM IR 10476 – 28-2-2 (*O. glaberrima* x pokkali)	Madurai
20	ACK 99017	INDUCED MUTANT OF WHITE PONNI AT 35 KR	Killikulam
21	AD 01259	CR 1009/JEERAGASAMBA	Aduthurai
22	TP 1021	ADT 43/JEERAGASAMBA	Thirupathisaram
23	RR 363-1	GAURAV / KALINGA III	CRRI, Cuttack
24	RR 361-3	SNEHA / GAURAV	CRRI, Cuttack
25	RR 354-1	RR 20-2-10 / RR 158-327	CRRI, Cuttack
26	RR 347-1	RR 19-2 / RR 149 – 1129	CRRI, Cuttack
27	RR 286-1	RR 165 – 1160 / RR 145 -22	CRRI, Cuttack
28	RR 348-6	CR 143-2-2/ANNADA	CRRI, Cuttack
29	RR 434-3	CR 222MW10 / BROWN GORA	CRRI, Cuttack
30	RR 433-1	IRAT 112 /CR 222 MW10	CRRI, Cuttack
31	ASD 06-2	ADT 36/BPT 5204	Ambasamudram
32	RR 166-645	C22/CR 289-1208	CRRI, Cuttack
33	ASD 06-3	ADT 36/TKM 9	Ambasamudram
34	ASD 06-4	ADT 39/BPT 5204	Ambasamudram
35	ASD 06-8	IR 50/TKM 9	Ambasamudram
36	ASD 06-7	IR 50/ASD 16	Ambasamudram
37	ASD 06-5	CO 43/ASD 16	Ambasamudram
38	ASD 06-6	CO 43/ADT 36	Ambasamudram
39	ASD 06-1	ASD 16/ADT 36	Ambasamudram

obtained from 39 rice genotypes is presented in Figure 1. The Jaccard's similarity coefficients for 39 genotypes obtained with SSR markers were calculated to establish the genetic relationships and the similarity index values ranged from 23.8 to 78.4%, this indicating the presence of wide range of genetic diversity at molecular level among thirty nine genotypes. Untung Sustano et al. (2008) reported parents of the mapping populations with 37%of similarity representing the maximum diversity, on the basis of 183 SSR markers. Lestari et al. (2009) observed that as nucleotide differences among genotypes are a major source of heritable variation, molecular markers derived from them should provide an effective measure of genotypic variation and hence phenotypic differences among varieties. Thirteen genotypes *viz.,* IR 80559 A, APMS 6 A, IR 72081 A, IR 75601 A, IR 75596 A, IR 80154 A,

Table 2. Number of alleles, their chromosomal locations and PIC value across 39 rice genotypes.

S/N	Marker	Number of alleles	Number of polymorphic bands	Percentage of poly morphism	Polymorphism information content (PIC)	Amplified fragment size range (bp)	Chromosome number	Annealing temperature (°C)
1	RM 216	3	2	66.67	0.44	100-200	10	57
2	RM 2	2	2	100.00	0.50	120-150	7	59
3	RM 174	2	2	100.00	0.40	180-200	2	66
4	RM 492	3	3	100.00	0.42	230-260	2	59
5	RM 24	3	1	33.33	0.50	150-500	1	59
6	RM 411	2	1	50.00	0.50	100-110	3	57
7	RM 132	2	1	50.00	0.49	100-150	3	65
8	RM 104	3	3	100.00	0.50	150-300	1	65
9	RM 570	3	3	100.00	0.44	250-320	3	59
10	RM 401	4	4	100.00	0.41	300-380	4	59
11	RM 584	3	2	66.67	0.46	150-200	6	59
12	RM 20 A	4	4	100.00	0.43	100-400	12	57
13	RM 147	3	3	100.00	0.39	100-210	10	66
14	RM 266	2	2	100.00	0.50	150-180	2	53
15	RM 536	4	4	100.00	0.49	150-500	11	59
16	RM 144	3	3	100.00	0.36	100-220	11	64
17	RM 7575	4	4	100.00	0.41	120-300	2	61
18	RM 17	2	2	100.00	0.49	120-180	12	59
19	RM 125	3	2	66.67	0.50	180-220	7	66
20	RM 248	2	1	50.00	0.48	100-130	7	59
21	RM 4674	3	2	66.67	0.50	180-210	5	51
22	RM 247	2	2	100.00	0.50	150-160	6	54
23	RM 214	3	2	66.67	0.46	100-200	7	55
24	RM 244	2	1	50.00	0.40	180-200	10	57
25	RM 9	3	3	100.00	0.45	100-210	1	60
26	RM 341	2	2	100.00	0.48	150-180	2	55
27	RM 589	2	2	100.00	0.48	150-180	6	55
28	RM 7653	3	2	66.67	0.45	100-140	5	57
29	RM 3476	2	1	50.00	0.50	120-180	5	51
30	RM 289	3	3	100.00	0.43	100-200	5	55
31	RM 29	2	2	100.00	0.28	200-220	2	59
32	RM 257	2	2	100.00	0.37	150-180	9	57
33	RM 440	3	3	100.00	0.44	180-250	5	55
34	RM 239	3	3	100.00	0.46	100-150	10	55
35	RM 3351	2	2	100.00	0.50	180-200	5	57

Table 2. Contd.

36	RM 5862	4	4	100.00	0.40	120-400	2	50
	Mean	2.72	2.36	85.65	0.45	-	-	-
	SD	0.70	0.93	21.74	0.05	-	-	-

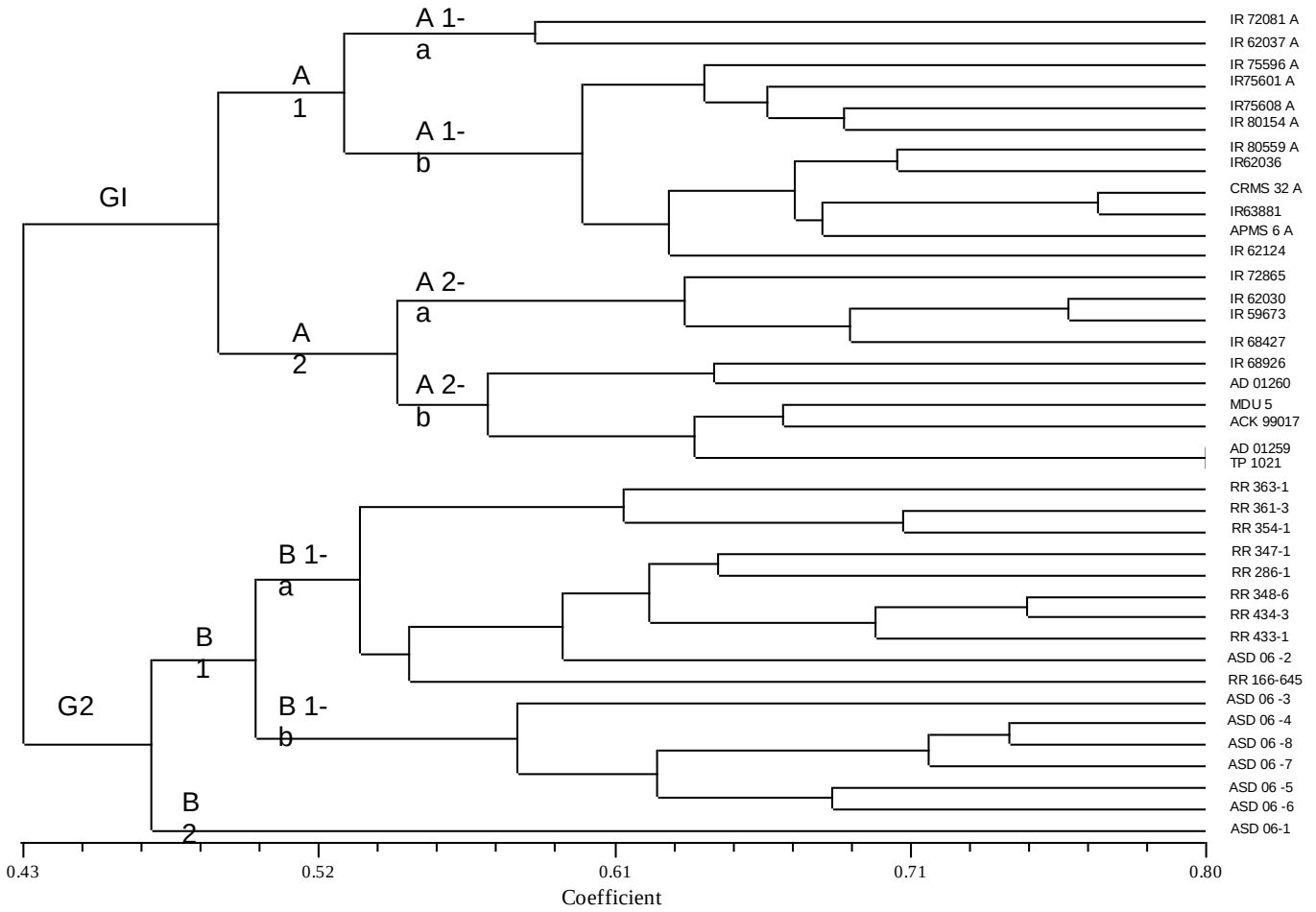


Figure 1. Dendrogram depicting genetic diversity among 39 genotypes using SSR markers.

CRMS 32 A, IR 75608 A, IR 62124-83-3-2-1, IR 62036-222-3-3-1-2, IR 63881-49-2-1-3-2, IR 72865-94-3-3-2 and IR 62030-83-1-3-2 were clustered in Group I, thirteen genotypes *viz.,* RR 354-1, RR 347-1, RR 348-6, RR 166-645, RR 433-1 RR 434-3, ASD 06-2, ASD 06-3, ASD 06-4, ASD 06-5, ASD 06-6 ASD 06-7 and ASD 06-8 were clustered in Group II, three genotypes *viz.,* RR 363-1, RR 361-1 and RR 286-1 clustered in Group II-B1a and three genotypes *viz.,* MDU 5, ACK 99017 and AD 01259 in Group I-A2b based on Jaccards similarity coefficient and these also confirmatory with D^2 values. Among the 39 genotypes, all the IR lines were clustered in cluster II (eleven) and all the RR lines (six) and ASD lines (six) were clustered in cluster I by dendrogram and also confirmed Mahalonobis D^2 statistics.]

The results indicate that geographical distribution and the source of the genotypes played major role in clustering along with the similarity and differences in their adaptation, selection criteria, selection pressure and environmental conditions. The above results provided an overview of the genetic diversity of the rice genotypes. This study provided a platform for identifying most appropriate parents for developing mapping populations to study the genetics of the biometrical and quality traits. The parents of the mapping populations were chosen keeping in mind maximization of genetic diversity estimates available from the present study. For example, parents of the mapping populations represent with >24% level of similarity representing the maximum diversity, on the basis of 50 SSR markers used in the present study. Mapping populations are currently being field evaluated and would be phenol typed for biometrical and quality related characters and genotyped with polymorphic SSR markers available from the present study for carrying out QTL mapping in the near future. The present work will be a boon for plant breeders in choosing the varieties for generating a new hybrid.

Conflict of Interests

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

REFERENCES

Gao LZ (2005). Microsatellite diversity and population genetic structure of an endangered wild rice, *Oryza officinalis* (Poaceae) from China. Mol. Ecol. 14(1): 4287-4297.

Jiang SK., Cheng H, Wang YJ, Chen WF, Zeng JX (2010). Development of a Highly Informative Microsatellite (SSR) Marker Framework for Rice (*Oryza sativa* L.) Genotyping. Agric. Sci. China 9(12):16971704.

Kibria K, Nur F, Begum SN, Islam MM, Paul SK, Rahman KS, Azam MN (2009). Molecular marker based genetic diversity analysis in aromatic rice genotypes using SSR and RAPD markers. Int. J. Sustain. Crop Prod. 4(1):23-34.

Lapitan VC, Brar DS, Abe T, Redona ED (2007). Assesment of Genetic diversity of Philippines rice cultivators carrying good quality traits using SSR markers. Breed. Sci. 57(2): 263-270.

Patel S, Ravikiran R, Chakraborty S, Macwana S, Sasidharan N, Trivedi R, Aher B (2014). Genetic diversity analysis of colored and white rice genotypes using Microsatellite (SSR) and Insertion-Deletion (INDEL) markers. Emir. J. Food Agric. 2014. 26 (6): 497-507

Porobreski S, Bailey G, Baum BR (1997). Modification of CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep. 15(1): 6-15.

Rohlf FJ (1999). NTSYS-PC Numerical taxonomy and multivariate analysis system. V.2.1, Exeter Software, Setauket, N.Y.

Shefatur Rahman M, Rezwan Molla Md, Samsul Alam Md, Rahman L (2009) DNA fingerprinting of rice (*Oryza sativa* L.) cultivars using microsatellite markers. Aust. J. Crop Sci. 3(3): 122-128.

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Cartinhour S, McCouch SR (2001). Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.). Frequency, length variation, transposons associations and genetic marker potential. Genome Res. 11(10-12): 1441-1452.

Thomson MJ, Septiningsih EM, Suwardjo F, Santosa TJ, Silitonga TS, McCouch SR (2007). Genetic diversity analysis of traditional and improved Indonesian rice (*Oryza sativa* L) germplasm using microsatillite markers. Theor. Appl. Genet. 114(6): 559-68.

Untung Susanto D, Grewal KS, Lee JH, Lee H, Aswininoor GB, Virk P (2008). Assesment of genetic diversity of some nutritionally rich corian varieties, elite lines and popular indica varieties of rice using SSR markers. Crop Improv. 35(2): 101-08.

Wilkie SE, Isaac PG, Slater RJ (1997). Genomic DNA isolation, southern blotting and hybridization. In: Plant molecular biology-a laboratory manual (ed.) Clark, M.S. Springer-Verlag, New York: 3-53.

academicJournals

Vol. 13(35), pp. 3560-3566, 27 August, 2014
DOI: 10.5897/AJB2014.13988
Article Number: 5738AD047052
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of seasonal collection on callus Induction, proliferation and somatic embryogenesis from anther cultures of *Hevea brasiliensis* Muell Arg

Wutthichai Srichuay[1,2], Soontreeya Kalawong[2,3] and Sompong Te-chato[2]*

[1]Faculty of Science and Technology, Princess of Naradhiwas University, Narathiwat, Thailand.
[2]Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand.
[3]Department of Agriculture, Faculty of Science and Technology, BansomdejchaoprayaRajabhat University, Bangkok 10600 Thailand.

Received 13 June, 2014; Accepted 4 August, 2014

Hevea brasiliensis **that is grown in Thailand and Southeast Asia is a very valuable source of natural rubber. However, few studies have been conducted to evaluate the effects of seasonal effect on plantlet regenera-tion through somatic embryogenesis from anther culture. Thus, this study discussed the effect of season on callus induction subsequent to plantlet regeneration from culturing of anther. Male flowers were aseptically opened and anthers were excised to culture on callus induction medium (CIM) which was MS supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-D, 1 mg.L^{-1} KN, 1 mg.L^{-1} NAA. Somatic embryo (SE) developed on MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA, 3 mg.L^{-1} KN and 0.05 mg.L^{-1} GA_3. This medium is so called embryo induction medium (EIM).Anther collectedfrom different season gave different result in callus induction, proliferation and somatic embryogenesis, healthy anther collected in February 15, 2013 gave the best result in callus induction (86.25%), proliferation (422 mgFW) and somatic embryogenesis (20%). Complete plantlets (15.63%)were obtained on MS medium supplemented with 5% sucrose, 0.5 mg.L^{-1} KN, 0.2 mg.L^{-1} IAA, 1 mg.L^{-1} BA, 0.3 mg.L^{-1} GA_3 and 0.2% phytagel. Seasonal collection plays influence rolein successful somatic embryogenesis in tissue culture of rubber tree.**

Key words: *Hevea brasiliensis*, rubber, antherculture, callus induction, seasonal collection, somatic embryogenesis.

INTRODUCTION

Hevea brasiliensis Muell. Arg. is one of the economic crop in Thailand and Southeast Asia as the source of natural rubber. *In vitro* propagation of *Hevea* has played a very important role in rapid multiplication of cultivars with desirable traits and production of true-to-type; healthy and disease-free plant somatic embryogenesis which appears

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

to be a promising technique that produced mass number of uniform plantlets in a short time.

Somatic embryogenesis has been reported in many species. Among those species many factors influenced plantlet regeneration through that process. *Hevea* is one species which has been studied intensively (Dijkman, 1951; Venkatachalam et al., 2013). Beyond culture media, plant growth regulators and culture environments, time of explant collection, so called seasonal effect, is also important because it affect the success of regeneration processes. Season causes variations in temperature, photoperiod and rainfall so explant had a different health. Quality of explant gave varied result. Fei et al. (2000) reported that growing condition of inflorescences had a close relation with callus proliferation.The effects of climatic changes in Southern Thailand lead to flowering of rubber tree in rainy season due to long drought period in that season. Thus, flowering of rubber tree occurred both in summer and rainy season. In case of mangosteen, low fruit-quality was greatly influenced by the high crop lost because summer rainfall induces profusionof flowers (Apiratikorn et al., 2012). Climate change resembled season change response to collectibles food and development of plant. The effect of climate change is important in determining development of pollen in Shogun citrus (Chelong and Sdoodee, 2012). The possibility of development of rubber tree flower was affected by season change.

In the past, plantlet regeneration through somatic embryogenesis in many plant species was developed by many researchers. This technique was also successfully reported in rubber tree (Jayashreeet al., 1999; Huaet al., 2010; Zhou et al., 2010; Zhou et al., 2012). However, induction of somatic embryosdepended on genotype and explant source (Fuentes et al., 2000). So far, there are still no researches reported on the effect of season on growth and development of tissue culture of rubber tree.

In this study, seasonalcollection of explant as one of external factors influencing growth and developmentof anthers of rubber tree *in vitro* are described. Those development, including, induction and proliferation of callus followed by embryogenic callus formation subsequent to plantlet regeneration are examined.

MATERIALS AND METHODS

Plant material and culture conditions

Immature male flower (1.2-1.5 mm in length) from inflorescence of *H. brasiliensis*Muell. Arg. grown around Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand was used as plant material in this experiment. Immature male flowers containing microspores at the uninucleate stage were collected and kept in a refrigerator at temperature of 4°C for 24 h. The cold-hardened explants were washed in running tap water for 20-30 min. All explants were surface sterilized in 70% (v/v) ethanol for 30 s, followed by soaking in 1.05% sodium hypochlorite solution for 20 min, then rinsed thrice with sterilized distilled water.

The anthers at length of 0.8-1.0 mm (Figure 2a) were excised from sterilized young male flowers under light microscope and cultured on MS medium. The pH of medium was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121°C for 15 min. The cultures were incubated at 26±3°C in the dark for 8 weeks orunder 14 h photoperiod with light supplied by cool-white fluorescent lamps at intensity of 12.5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Effect of seasonal collection of explants on callus induction

The inflorescences of rubber containing immature male flowers were collected at three times; 22 Jun, 2012 (Rainy season), 15 February, 2013 (Summer season) and 31 July, 2013 (late Rainy season). Anthers from right stage of pollen development were aseptically excised according to the method described above. The anthers were cultured on MS supplemented with 5% sucrose, 1 mg.L^{-1}2,4-diclorophenoxyacetic acid (2,4-D) (Fluka), 1 mg.L^{-1}kinetin (KN) (Fluka), 1 mg.L^{-1}α-naphthalene acetic acid (NAA) (Fluka), so called CIM. All cultures were maintained under the conditions as specified above. The cultures were routinely subcultured at 4 week intervals for 8 weeks to induce callus. After 8 weeks of being cultured, callus induction percentage was recorded.

Effect of seasonal collection of explants on callus proliferation

The calli induced from anthers at two different periods as mentioned earlier (22 Jun, 2012 and 15 Feb, 2013) were transferred to culture on MS supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1}6-benzyladenine (BA) (Fluka), 1 mg.L^{-1}thidiazuron (TDZ) (Sigma). The cultures were maintained under the same conditions as describe above. After 4 weeks of being cultured, fresh weight of calli was recorded and statistically compared between the two different periods.Proliferation rate of the callus was determined by the ratio of increment in fresh weight divided by initialfresh weight as the following equation:

$$\text{Proliferation rate of callus} = \frac{\text{Fresh weight at } t_t - \text{Initial fresh weight}}{\text{Initial fresh weight}}$$

Effect of TDZ and coconut water (CW) on callus proliferation

Calli derived from anther culture were transferred to MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA and 1 or 2 mg.L^{-1} TDZ or CW. The cultures were maintained under the same conditions as described above. After 4 weeks of being cultured, fresh weight of calliwas recorded and statistically compared among different types and concentrations of BA, TDZ and CW. Proliferation rate of the callus was determined by the same method as described above.

Effect of gelling agents on SE induction

Eight-week-old calli derived from anther were cultured on embryogenic callus (EC) induction medium (EIM) which was MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA, 3 mg.L^{-1} KN and 0.05 mg.L^{-1}gibberellic acid (GA_3) (Fluka). The culture medium was solidified with two different types of gelling agent, phytagel and agar.The concentrations of those two gelling agents were 0.2% for phytagel and 0.75% for agar.After 4 weeks of culture, somatic embryo (SE) induction percentage and a number of somatic embryos per callus were recorded and statistically compared. EC together with SEs were transferred to MS medium supplemented with 0.2 mg.L^{-1} IAA, 0.5 mg.L^{-1} KN, 0.3 mg.L^{-1} GA

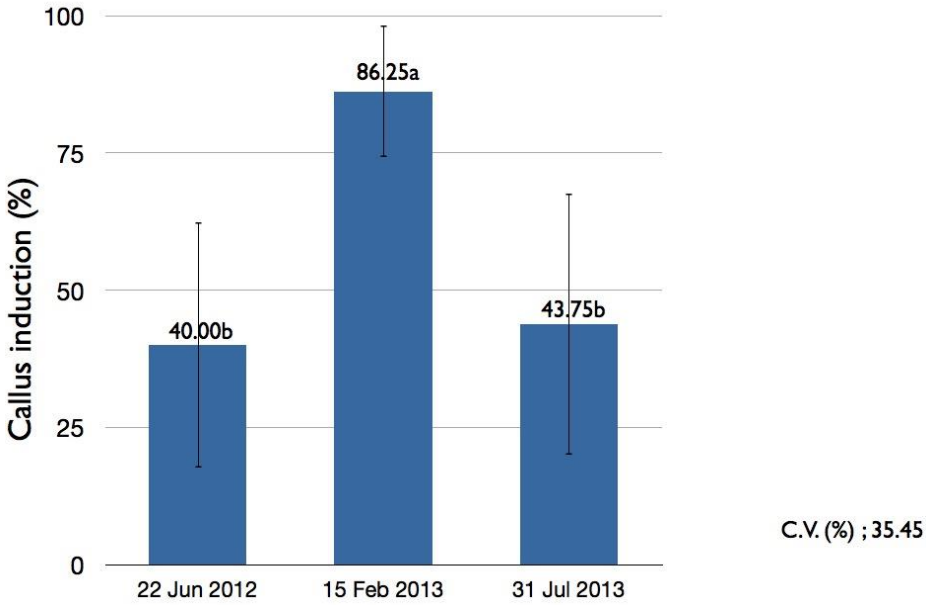


Figure 1. Effect of seasonal collection on callus induction from anther culture on MS medium supplemented with 1 mg.L^{-1} 2,4-D, 1 mg.L^{-1} KN and 1 mg.L^{-1} NAA for 4 weeks ($P < 0.01$).

and 1.0 mg.L^{-1} BAand cultured for further 4 weeks. Germination of SEs was recorded and statistically compared.

Statistical analysis

All experiments were performed in a completely randomized design (CRD). Each consisted of four replicates per treatment andten explants were performed in each replication. Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected usingleast significant difference(LSD) at the 0.01 level of probability.

RESULTS

Effect of seasonal collection of explant on callus induction

Collection period of explantsof rubber tree affect callus induction because photoperiod, temperature and rainfall affect photosynthetic rate of this plant (Sdoodee and Rongsawat, 2012). Inappropriate conditions of those environments made low productionof photosynthesis leading to the reduction growth rates of meristematic cells. Inflorescences collected in some seasonsresponded at low frequency in callus induction rate (Figure 1). Collection of inflorescence in15 February, 2012, which was the first season of flowering, showed the highest callus induction. The highest percentage callus formation was recorded to be 86.25%; significantly different ($P<0.01$) with the second season of flowering. Callus induction percentage from anther and from inflorescence collectedat second season of flowering (22 Jun, 2012 and 31 Jul, 2013) was 40.00 and 43.75%. Structures of callus induced from anther collected from difference seasons were different. Anther collected in 31 Jul, 2013 gave compact, hard and white callus (Figure 2c), whereas callus from anther collected from the first season of flower-ing was friable, watery and white in color (Figure 2b).

Effect of seasonal collection of explants on callus proliferation

Callus obtained from culturing anther collected from both seasons could proliferate on MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA, 1 mg.L^{-1} TDZ. However, callus from anthers collectedin15 Feb, 2013 gave the highest fresh weight of callus at 422 mg after being cultured for 4 weeks (Table 1). Proliferation of callus induced from anthers collected in 22 Jun, 2012 was far lower than that obtained innormal season of flowering.

Effect of TDZ and CW on callus proliferation

For proliferation of callus from anther culture, the result shows that 1 mg.L^{-1} TDZ gave the highest fresh weigh at 576 mg after being cultured for 4 weeks (Table 2). A 15% CW and 2 mg.L^{-1} TDZ containing medium reduced proliferation rate of callus. High concentration of cytokinin also resulted in low proliferation rate of callus. Among cytokinins tested, CW at concentration of 15% gave the

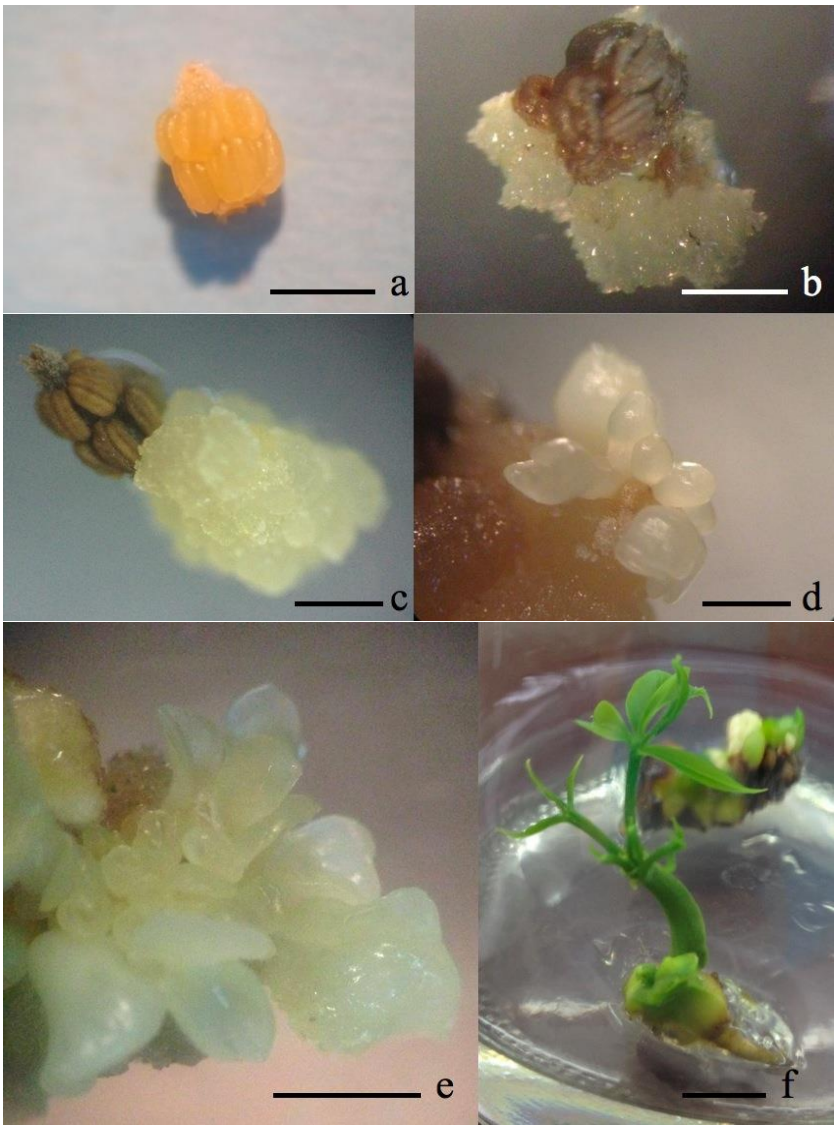


Figure 2. Development of plantlet via somatic embryogenesis from anther culture of *Heveabrasiliensis*.**a.** Anther. **b.** Friable and moist, callus induced from 15 Feb, 2013 collecting anther. **c.** Compact and hard callus induced from 31 Jul, 2013 collecting anther. **d.** SEs developed from 8-weeks-old callus on MS medium supplemented with 0.75% agar. **e.** SEs developed from 8-weeks-old callus on MS medium supplemented with 0.2% phytagel. **f.** Complete plantlet regenerated on MS medium supplemented with 5% sucrose, 0.5 $mg.L^{-1}$ KN, 0.2 $mg.L^{-1}$ IAA, 1 mg.L BA, 0.3 $mg.L^{-1}$ GA_3 and 0.2% phytagel (a, b, c, d and e bar: 1 mm; f bar : 5 mm).

Table 1. Effect of seasonal collection on callus proliferation culture on MS medium with 0.2 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ BA, 1 $mg.L^{-1}$ TDZ, and 1 $mg.L^{-1}$ $AgNO_3$ for 4 weeks.

Date	NAA/BA/TDZ	Initial fresh weight (mg)	FW callus (mg)±SD	Proliferation rate of callus
22 Jun, 2012	0.2/1/1	50	151±38[b]	2.03
15 Feb, 2013	0.2/1/1	50	422±87[a]	7.45
F-test			**	
C.V. (%)			22.69	

Mean values followed by the same letter(s) within a column are not significantly different ($P < 0.01$).

Table 2. Effect of PGRs containing MS medium with 1 mg.L^{-1} $AgNO_3$ on callus proliferation after 4 weeks of culture.

NAA	BA	TDZ	CW	FW callus (mg)±SD
0.2	1			249 ± 118^{b}
0.2	1	1		576 ± 222^{a}
0.2	1	2		393 ± 118^{ab}
0.2	1		15	227 ± 80^{b}
F-test				**
C.V.(%)				30.48

Mean values followed by the same letter(s) within a column are not significantly different (P < 0.01).

Table 3. Effect of Phytagel and Agar on SE induction from 8 weeks old callus culture on MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA, 3 mg.L^{-1} KN and 0.05 mg.L^{-1} GA_3 for 4 weeks.

Solidify type	% of SEs Induction	No. of SEs per explant
Phytagel (0.2%)	20	7.63 ± 5.57^{a}
Agar (0.75%)	15	2.17 ± 0.75^{b}
F-test		*
C.V.(%)		73.52

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

lowest proliferation rate of callus. Lata et al. (2013) reported that high concentration of TDZ decrease percentage of shoot formation in stavia. However, a low concentration of TDZ was reported to give the best result in callus proliferation in many plants (Guo et al., 2011).

Effect of phytagel and agar on SE induction

Gelling agents play important role in SE formation. In this study, phytagel increased SE induction rate (20%) from anther-derived callus leading to the highest number of SE formation at 7.63 SEs per explant (Table 3, Figure 2e). Phytagelis pure synthetic agar used at lower concentration for solidifying culture media. So, it seems that there is no impurified substances which hampered growth and development of plant tissue. Complete plantlet (Figure 2f) was obtained at 15.63% after SE was transferred to culture on 0.2% phytagel containing MS medium in the presence of 0.2 mg.L^{-1} IAA, 0.5 mg.L^{-1} KN, 0.3 mg.L^{-1} GA and 1.0 mg.L^{-1} BA for 4 weeks.

DISCUSSION

The quality, growth and development of pollens frompollen megaspore mother cells (PMC), of anther from donor plantisthe key factoraffecting callus induction and further development into plantlet. An important factor controlling quality of anther should be raised under optimum condition. In this study, we reported the relationship between the time of collection explant and the percentage of callus induction. Normal season of flowering, in February, illustrated the most suitable for callus induction in terms of growth and development. Photosynthesis is one parameter indicated quality of anther due to accumulation of photosynthates. This evident is highly impact by the suitable sunshine or radiation duration (PAR: 400-700) (Sdoodee and Rongsawat, 2012). However, long period of sunshine in a day stimulate the formation of ethylene that reduced the embryogenic callus induction in buffalograss (Feiet al., 2000). In addition, seasonal collection of explants has been reported to influence upon shoot formation in nodal culture of oak.Collecting the explants in May was the best time for the highest percentage of shoot forming explant and multiplication rate (Kartsonas and Papafotiou, 2007). A high frequency of sprouting from nodal cutting mulberry was observed in summer at 83.3% (Chitra and Padmaja, 2002). Contrary result was obtained in two pepper genotypes, Kekova cultivar gave the highest embryogenic response in summer season while 8 cultivars of Sera Demre gave a good response in winter (Ercan et al., 2006).In anther culture of flax, it also reported that collecting of explants in summer season gave the best result due to the active or meristematic activity of tissue in those explants (Krause et al., 2003).

Meristematic activity ofinitial cells is important for callus induction just after culture in culture conditions. Callus

induction from anther collecting from the first seasonal flowering gave higher proliferation rate than the second seasonal flowering (Table 1). Climate change affected the embryogenic callus growth rate due to a good quality of explants as describe above (Feiet al., 2000). Growth conditions of donor plant underappropriated photoperiod and temperature promoted a high quality of anther, like those reports in pepper (Ercan et al., 2006). Moreover, shoot was rapidly developed and proliferated from a good quality of mulberry anther (Chitra and Padmaja, 2002).

Plant growth regulators (PGRs) containing media promoted callus growth and proliferation under *in vitro* conditions. Besides types of PGR its concentration was also important in callus culture. In case of rubber tree callus, TDZ at 1 $mg.L^{-1}$ gave better result in growth rate than $2mg.L^{-1}$.Moreover, low concentration (0.2-0.6 $mg.L^{-1}$) of TDZ was reported to be the best for callus induction and combination of TDZ with NAA promoted shoot regeneration in *Astragalus cariensis* (Erisen et al., 2011).

In case of SE induction, phytagel promoted the formation ofSE fromculturing of anther of rubber tree. Zhang and Te-chato (2013) reported that a high numberof SEs from callus culture ofindica ricewas induced on phytagels up plemented medium. Phytagel solidifying culture medium was reported to increase plantlet formation in *Cleome rosea* (Simoes et al., 2010). Choudhury et al. (2008) reported that phytagel containing culture medium reduced water availability.When explants or callus could not absorb water it caused achange in osmotic potentialpromoting the development of plantlets. In case of oil palm, the increment of osmotic potentialwas reported to promote germination of somatic embryo (Hilae and Te-chato, 2005). However, increment of osmotic potential in this case was performed by adding sugar alcohol, sorbitol.

Conclusion

In rubber tree, seasonal collection of inflorescence influencedthe successful somatic embryo induction from anther culture. Callus was induced on MS medium supplemented with 5% sucrose, 1 $mg.L^{-1}$ 2,4-D, 1 $mg.L^{-1}$ KN, and 1 $mg.L^{-1}$ NAA. SEs were developed on MS medium supplemented with 3% sucrose, 0.2 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ BA, 3 $mg.L^{-1}$ KN and 0.05 $mg.L^{-1}$ GA_3. Complete plantlets were regenerated on MS medium supplemented with 5% sucrose, 0.5 $mg.L^{-1}$ KN, 0.2 $mg.L^{-1}$ IAA, 1 $mg.L^{-1}$ BA, 0.3 $mg.L^{-1}$ GA_3 and 0.2% phytagel.

Conflict of Interests

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

ACKNOWLEDGEMENTS

We would like to thank the Thailand's Office of the Higher Education Commission, the Graduate School of Prince of Songkla University and the Center of Excellence in Agricultural and Natural Resources Biotechnology, Postgraduate Education and Research Development Office, and Commission on Higher Education, Ministry of Education for financial support.

REFERENCES

Apiratikorn S, Sdoodee S, Lerslerwong L, Rongsawat S (2012). The impact of climatic variability on phonological change, yield and fruit quality of mangosten in Phatthalung province, southern Thailand.Kasetsart J (Nat Sci). 46: 1-9.

Chelong I, Sdoodee S (2012). Pollen viability, pollen germination and pollen tube growth of shogun (*Citrus reticulate* Blanco) under climate variability in southern Thailand. J. Agric. Technol. 8: 2297-2307.

Chitra DSV, Padmaja G (2002). Seasonal influence on axillary bud sprouting and micropropagation of elite of mulberry.Sci. Hortic. 92: 55-68.

Choudhury H, Kumaria S, Tandon P (2008). Induction and maturation of somatic embryos from intact megagametophyte explants in Khasi pine (*Pinuskesiya*Royle ex. Gord.).Curr. Sci. 95: 1433-1438.

Dijkman MJ (1951). Hevea thirty years of research in the far east. University of Miami. 329p.

Ercan N, Sensoy FA, Sensoy AS (2006). Influence of growing season and donor plant age on anther culture response of some pepper cultivars (*Capsicum annuum* L.). Sci. Hortic. 110: 16-20.

Erisen S, Atalay E, Yorgancilar M (2011). The effect of thidiazuron on the in vitro shoot development of endemic *Astragaluscariensis* in Turkey.Turk. J. Bot. 35: 521-526.

Fei S, Read PE, Riordan TP (2000). Improvement of embryogenic callus induction and shoot regeneration of buffalograss by silver nitrate.Plant Cell Tissue Organ.Cult. 60: 197-203.

Fuentes SRL, Calleiros MBP, Manetti-Filho J, Vieira GE (2000).The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffeacanephora*.Plant Cell Tissue Organ.Cult. 60: 5-13.

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011). Thidiazuron: a multi-dimensional plant growth regulator. Afr. J. Biotechnol. 10: 8984-9000.

Hilae A, Te-chato S (2005). Effects of carbon sources and strength of MS medium on germination of somatic embryos of oil palm (*Elaeisquineensis*Jacq.). Songklanakarin J. Sci. Technol. 27: 629-635.

Hua YW, Huang TD, Huang HS (2010).Micropropagation of self-rooting juvenile clones by secondary somatic embryogenesis in *Heveabrasiliensis*.Plant Breed. 129: 202-207.

Jayashree PK, Asokan MP, Sobha S, Ammal LS, Rekha K, Kala RG, Jayasree R, Thulaseedharan A (1999). Somatic embryogenesis and plant regeneration from immature anthers of *Heveabrasiliensis* Mull. Arg. Curr. Sci. 76: 1242-1245.

Kartsonas E, Papafotiou M (2007). Mother plant age and seasonal influence on *in vitro* propagation of *Quercuseuboica*Pap., an endemic , rare and endangered oak species of Greece. Plant Cell Tissue Organ Cult. 90: 111-116.

Krause IR, Mankowska G, Lukaszewicz M, Szopa J (2003). Regeneration of flax (*Linumusitatissimum* L.) plants from anther culture and somatic tissue with increased resistance to *Fusariumoxysporum*. Plant Cell Rep. 22: 110-116.

Lata H, Chandra S, Wang YH, Raman V, Khan IA (2013). TDZ-induced high frequency plant regeneration through direct shoot organogenesis in *Stevia rebaudiana*Bertoni: an important medicinal plant and a natural sweetener. AJPS 4: 117-128.

Sdoodee S, Rongsawat S (2012). Impact of climate change on smallholders' rubber production in Songkhla province, southern Thailand. The 2012 International and National Conference For The Sustainable Community Development of "Local Community: The Foundation of Development in the ASEAN Economic Community (AEC)" February 16-19, 2012. pp. 81-89.

Simoes C, Albarello N, Callado CH, Castro TC, Mansur E (2010). Somatic embryogenesis and plant regeneration from callus culture of *Cleome rosea*Vahl. Braz. Arch. Biol. Technol. 53: 679-686.

Venkatachalam P, Geetha N, Sangeetha P, Thulaseedharan A (2013). Natural rubber producing plants: an overview. Afr. J. Biotechnol. 12:

1297-1310.
Zhang Y, Te-chato S (2013). Improved plantlet regeneration systems in *Indica*rice (*Oryza sativa* L.) landrace *HomKra Dang Ngah*. J. Agric. Technol. 9: 1641-1654
Zhou QN, Jiang ZH, Huang TD, Li WG, Sun AH, Dai XM, Li Z (2010). Plant regeneration via somatic embryogenesis from root explants of *Heveabrasiliensis*. Afr. J.Biotechnol. 9: 8168-8173.
Zhou QN, Sun AH, Li Z, Hua YW, Jiang ZH, Huang TD, Dai XM, Huang HS (2012). Cryopreservation and plant regeneration of anther callus in Hevea by vitrification. Afr. J.Biotechnol. 11: 7212-7217.

academicJournals

Vol. 13(35), pp. 3567-3575, 27 August, 2014
DOI: 10.5897/AJB2014.13856
Article Number: BACF16E47053
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

2,4-Dichlorophenoxyacetic acid increases reserve compounds and spectaline contents in *Senna spectabilis* calli

Monaly Sado, Armando Reis Tavares and Edison Paulo Chu*

Institute of Botany, P. O. Box 68041, 04045-972, São Paulo, Brazil.

Received 15 April, 2014; Accepted 21 July, 2014

The aim of this study was to develop an *in vitro* culture system for *Senna spectabilis* and to quantify contents of storage compounds and spectaline in induced calli in relation to exogenous auxin. Explants (cotyledon, hypocotyl, epicotyl, and leaf) were cultured on MS medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). The soluble carbohydrate, starch, soluble protein and spectaline contents in the induced calli were quantified. Treatment with 0.12 mg L^{-1} of 2,4-D induced callus formation and was optimal for propagation and vegetative growth, owing to the higher concentrations of reserve compounds in the calli. Hypocotyl and epicotyl produced calli on medium containing 0.5 mg L^{-1} 2,4-D. The lowest concentrations of 2,4-D induced a higher incidence of oxidation and explants showed low viability. Spectaline accumulated at low concentrations in the different callus types and 2,4-D treatments, indicating spectaline was a constitutive compound in callus. Hypocotyl with 10.0 mg L^{-1} 2,4-D for up to five days induced cell proliferation and starch accumulation, followed by treatment with 0.12 mg L^{-1} 2,4-D to increase tissue mass and accumulation of reserve compounds enables the production of friable callus suitable for the establishment of a tissue culture system and for *in vitro* spectaline production.

Key words: Alkaloid, auxin, Fabaceae, carbohydrate, tissue culture.

INTRODUCTION

Senna spectabilis (DC.) Irwin et Barn. (syn. *Cassia spectabilis* DC., Fabaceae), popularly known as 'canafístula or cassia northeast', is a deciduous, heliophytic, selective xerophytic plant native to Central and South America. The species is found mainly in the cerrado, caatinga (savannah like) and, forests areas of small rainfall of central and northeast Brazil (Braga, 1982). In traditional medicine, extracts of this species are used as an anti-inflammatory to treat human disease, it has analgesic, laxative, anesthetic, and antimicrobial properties, exhibit inhibitory activity against superoxide generation, and show promise for treatment of Alzheimer's disease (Viegas et al., 2006).

Plant alkaloids are not only toxic to herbivores and

*Corresponding author. E-mail: chu07@hotmail.com.

microorganisms, but can also be of pharmacological importance in popular medicine, and show diverse biological activities such as reduction of blood pressure, relief of pain and spasms, stimulation of blood circulation and respiration, stabilization of mental illness, and antitumor activity (Brown and Charlwood, 1986; Wink, 2008). Plant alkaloids are also utilized as food additives, as coloring agents, for provision of a specific aroma or taste, and as preservation compounds). Because of their complexity, the chemical synthesis of many biological molecules is not yet feasible, and commercial exploitation is still dependent on agricultural production of the selected species.

Recent studies indicate that piperidine alkaloids are responsible for the pharmacological properties of *Senna* species. Such alkaloids include inhibitors of acetylcholinesterase, which have potential applications in the treatment of Alzheimer's disease (Bolzani et al., 1995, Moreira et al., 2003). *S. spectabilis* is rich in piperidine alkaloids (Viegas et al., 2006, 2013), and hence has attracted much pharmaceutical interest, which has motivated research groups to develop methodologies to yield higher quantities of alkaloids than those produced by natural sources. Pivatto et al. (2005) confirmed the presence of piperidine alkaloids in ethanol extracts from flowers and fruits of *S. spectabilis*. Melo et al. (2014) described the leishmanicidal activity of the flower crude extract and Paguigan et al. (2014) reported the anti-ulcer activity in methanol extracts of leaves. Therefore, studies on alkaloid biosynthesis, and synthesizing and accumulating of these secondary compounds by *in vitro* induced callus are relevant to explore the possible development of biotechnological production systems for piperidine alkaloids.

In vitro plant tissue culture can be initiated using organized tissues such as buds or roots, from which meristematic cells usually give rise to the same organ, or fragments of organs (for example leaf, stem, root, or petal explants) plus auxin to induce proliferation of an unorganized cell mass (callus) on an injured surface. With the proper balance of growth regulators, the callus can be propagated indefinitely or induced to differentiate into new organs or a whole plant (Loyola-Vargas and Vazquez-Flota, 2006; George et al., 2008). The transport and storage of alkaloids are correlated with specialized tissues (Wink and Roberts, 1998), and these secondary compounds are synthesized in undifferentiated plant cell cultures in very low quantities or with different chemical structures when compared to those of whole plants.

The use of *in vitro* culture techniques can accelerate cultivar development, assist in conventional breeding programs and is a potential biotechnological tool for the study of metabolism, physiology, development, plant reproduction and synthesis of biological molecules of pharmacological interest. Such techniques also enable the production of large quantities of plantlets from a small number of explants (George and Debergh, 2008).

The aim of the present study was to induce callus formation with 2,4-dichlorophenoxyacetic acid (2,4-D) from different tissues of axenic plantlets of *S. spectabilis*, quantify reserve compounds (total soluble carbohydrates, starch and soluble proteins), clarify their relationship with callus type and cell multiplication cycle, and verify the *in vitro* production of the alkaloid spectaline.

MATERIALS AND METHODS

Plantlets of *S. spectabilis* were obtained by *in vitro* seed germination from mature fruits of three specimens growing in the Institute of Botany, São Paulo, Brazil. The seeds were sterilized in concentrated sulfuric acid for 45 min followed by five washes with sterile distilled water, then germinated on complete Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3.0% (w v^{-1}) sucrose and 0.8% (w v^{-1}) agar (Bacto-agar, Difco). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. When seedlings reached 3.0 cm in length, the cotyledons, hypocotyl, epicotyl and leaves were excised with a scalpel under aseptic conditions and cultured on MS medium supplemented with 3.0% sucrose and 0, 0.12, 0.25, 0.5, 2.5, 5, 10, 20 or 40 mg L^{-1} 2,4-D in 100 mL flask containing 30 mL of medium. Each treatment comprised 20 flasks containing one explant from each tissue type. All cultures were incubated at 26±2°C with a 12-h photoperiod under white fluorescent lights (16.2 µmol m^{-2} s^{-1}). The number, type, and fresh and dry mass (lyophilized) of callus were determined after 45 days of cultivation.

The induced calli were classified as friable, nodular, and compact or oxidated combined according to the original explant type and treatment, then subdivided into three samples (0.5-2.0 g fresh mass for each sample) and stored at -20°C.

For analysis of reserve compounds, the callus samples were lyophilized, the dry mass was determined on an analytical balance, then ground using a mortar and pestle. The samples were homogenized and extracted twice with 80% (v v^{-1}) ethanol at 80°C for 5 min. After centrifugation for 10 min at 1,250 × *g*, the ethanolic supernatants (soluble carbohydrate fraction) were combined and the volume measured. Soluble proteins were extracted twice from the residue with 0.2 M phosphate buffer (pH 5.7) at room temperature for 10 min, centrifuged for 10 min at 500 × *g* at 4°C, then the supernatants were pooled and the volume measured. The soluble protein content was determined by staining with Coomassie Blue reagent and absorbance was measured with a spectrophotometer (Biospectro SP-22) at 595 nm using bovine albumin as the standard (Bradford, 1976). Starch was extracted twice from the protein residues with 52% (v v^{-1}) perchloric acid and estimated according to the method of McCready et al. (1950). The total soluble carbohydrate and starch contents were determined colorimetrically according to the phenol-sulphuric acid method (Dubois et al., 1956; McCready et al., 1950).

For preparation of the spectaline standard, fresh *S. spectabilis* flowers (500 g) were collected and lyophilized. The dried samples were homogenized in 80% ethanol at 80°C and incubated for 5 min, then cooled and the supernatant was concentrated on a rotary evaporator (Pivatto et al., 2005). Aliquot (10 mL) of the concentrated extract was lyophilized, weighed, and then 60 mL of 5% sulfuric acid was added and incubated at 4°C for 16 h. The extract was filtered and 15 mL hexane was added for lipid extraction. The aqueous acid phase was set to 9.0 using 1 N ammonium hydroxide and dichloromethane was used for liquid–liquid separation, with the crude alkaloid fraction contained in the organic phase. The ethanolic extracts from the different treatments

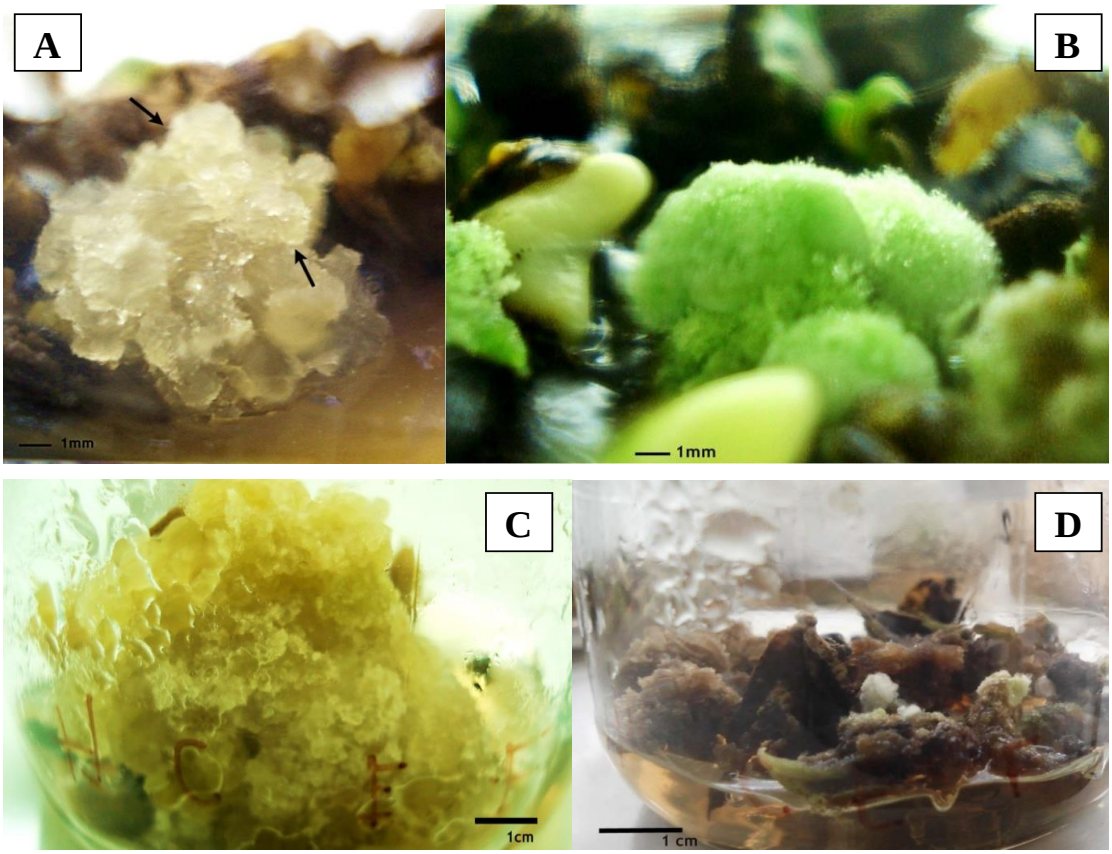


Figure 1. *Senna spectabilis* calli after cultured for 45 days on medium containing 2,4-D. **(A)** Nodular (arrows indicate callus structure). **(B)** Compact. **(C)** Friable. **(D)** Oxidized callus.

and callus types were prepared following the methodology described above, with reagents and solvents in proportional volumes. Spectaline analysis were performed with gas liquid chromatography (Silva, 2009) using a Varian Chrompack CP-3380 column and injection of 1 µL of each concentrated sample in 4.0 mg mL^{-1} ethyl acetate using the following conditions: injector 300°C; flame ionization detector 300°C; initial temperature 210°C increasing by 1°C min^{-1} to 250°C for 40 min; and 3.0 cm^3 min^{-1} helium as carrier gas.

Differences among treatments were determined by analysis of variance (ANOVA) with a post Tukey–Kramer test using Sigma Stat version 3.1 (Sigma Stat Software, Chicago, USA). Data were expressed as the mean ± standard deviation. The data were considered significant at $P < 0.05$.

RESULTS

Callus induction and growth occurred in all treatments. The calli were friable, nodular or compact (Figure 1A to C). The color of compact calli was usually green or white, not transparent and with a velvety surface, whereas the friable calli were whitish, yellowish or greyish with a spongy appearance. Nodular calli were morphologically similar (globular) to callus containing somatic embryos, suggesting a relationship between calli morphology and color.

Callus induction from hypocotyls and epicotyls was most frequent (>80%) at 0.5 mg L^{-1} 2,4-D, whereas 70% of cotyledons formed callus at 2.5, 10 and 20 mg L^{-1} 2,4-D and under 3% formed callus at 0 mg L^{-1} 2,4-D. Primary leaves showed no significant callus induction at 0 mg L^{-1} 2,4-D, whereas 80% of leaves formed callus at 2.5 mg L^{-1} 2,4-D (Table 1).

Media containing 0 and 0.25 mg L^{-1} 2,4-D did not induce nodular callus. Treatments 0.12, 0.25 and 0.5 mg L^{-1} 2,4-D had oxidized callus (Figure 1D) and cells undergoing senescence (Table 1). The fresh weight was higher ($P < 0.05$) in friable callus cultured on high concentrations of 2,4-D (Figure 2A). The dry weight was significantly higher in compact callus compared to that of friable callus ($P < 0.01$) (Figure 2B). Treatment with 0.12 mg L^{-1} 2,4-D induced compact callus with the highest dry weight compared to the other treatments. The friable callus induced by 20 and 40.0 mg L^{-1} 2,4-D showed the lowest dry weight.

Table 1. *Senna spectabilis* callus induction (%) by treatment with different concentrations of 2,4-D in the culture medium.

2,4-D (mg L^{-1})	Calli induction (%)				Type of calli (%)		
	Hypocotyl	Epycotyl	Cotiledon	Leaf	Friable	Nodular	Compact
0.0	25.0	21.9	3.1	1.6	25.0	0.0	75.0
0.12	65.8	71.0	61.8	52.6	51.1	2.0	46.9
0.25	82.0	65.3	66.7	68.1	81.8	0.0	18.2
0.5	86.7	83.3	45.0	78.3	41.9	45.2	12.9
2.5	76.6	78.1	73.4	79.7	48.8	39.5	11.6
5.0	70.0	61.7	55.0	50.0	52.6	38.6	10.5
10.0	71.7	73.3	73.3	58.3	57.9	38.6	5.3
20.0	60.0	70.0	72.5	65.0	51.5	45.5	3.0
40.0	11.7	11.7	45.0	21.7	40.0	40.0	20.0

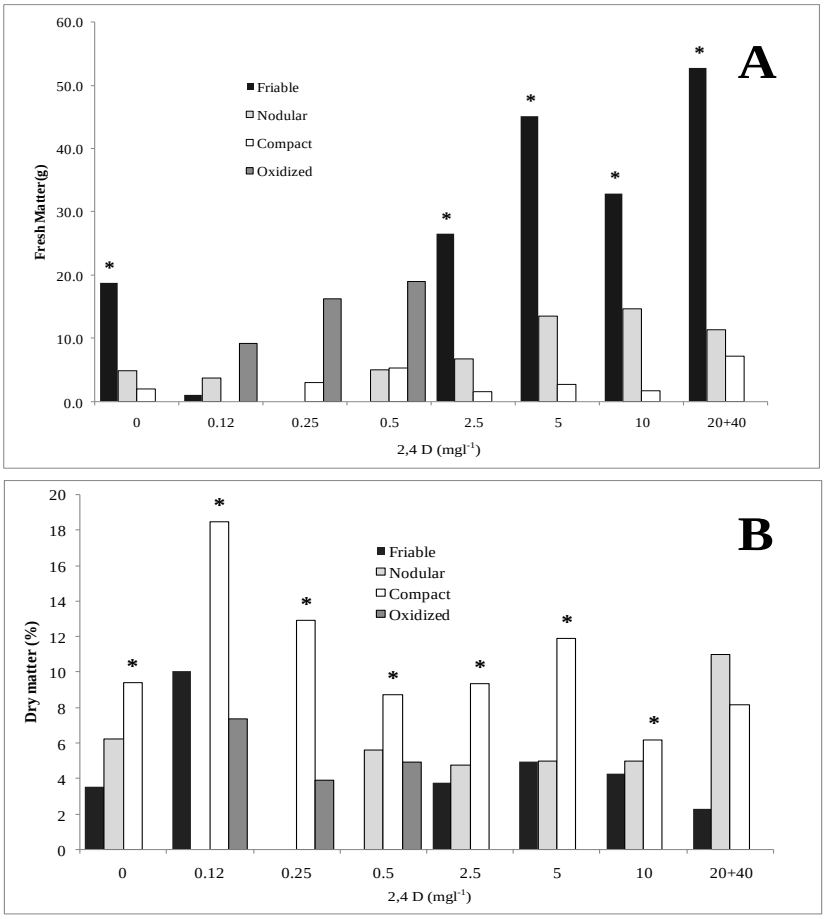


Figure 2. A) Fresh weight of *S. spectabilis* calli. *A significant difference (P < 0.05) existed between nodular, compact and oxidized calli for treatments 0, 0.5, 2.5, 5, 10 and 20+40 mg L^{-1} 2.4-D. **B)** Dry mass (%) of different calli types (friable, nodular, compact and oxidized) cultured with different 2,4-D concentrations. *A significant difference (p < 0.05) existed for friable callus at all concentrations of 2,4-D except 20+40 mg L^{-1} 2,4-D. **C)** Soluble carbohydrate content (mg g^{-1} dry matter). **D)** Soluble protein content (mg g^{-1} dry matter). **E)** Starch content (mg g^{-1} dry matter). **F)** Spectaline content (µg g^{-1} dry matter). **G)** Total content of reserve compounds (mg g^{-1} dry matter) in the different calli. Lowercase letters above bars in the same graph indicate a significant difference (C–E).

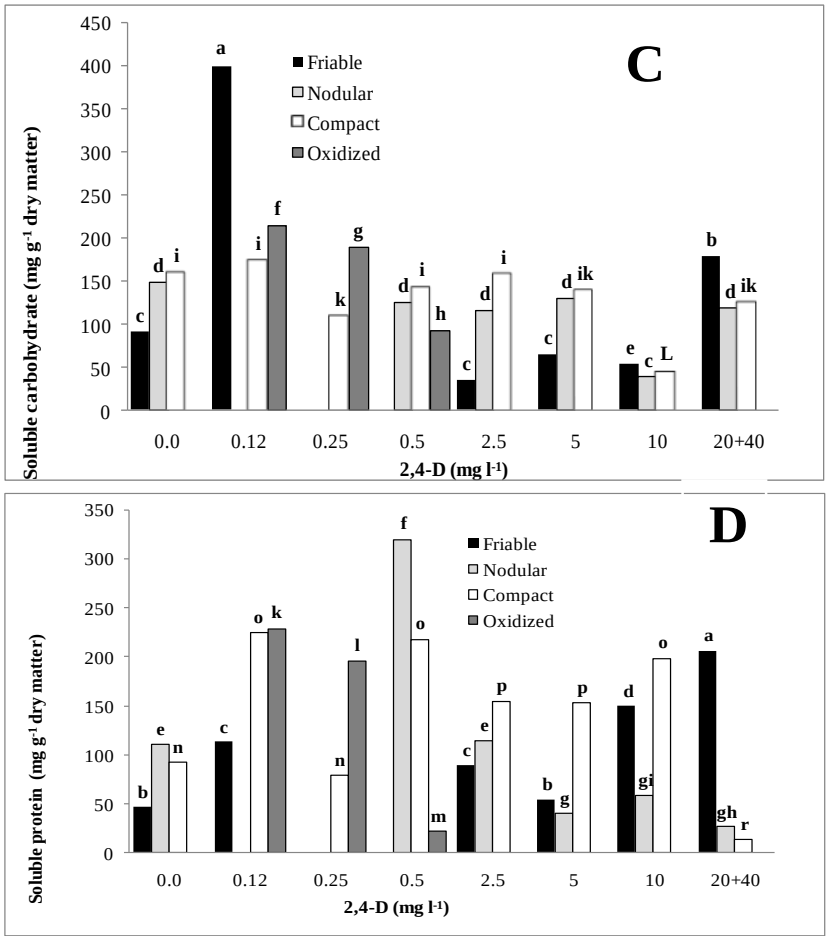


Figure 2. Contd.

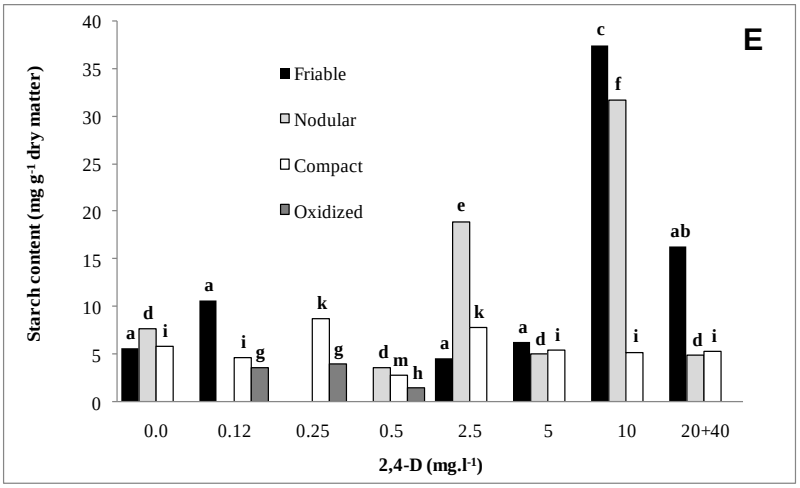
The soluble carbohydrates content was significantly higher in friable callus induced by 0.12 mg L^{-1} 2,4-D (P < 0.001) and 40.0 mg L^{-1} 2,4-D (P < 0.001) than at 0, 2.5, 5.0 and 10.0 mg L^{-1} 2,4-D (Figure 2C). Soluble carbohydrates in friable callus at 0.25 mg L^{-1} 2,4-D was lower compared to that at 0, 0.12, 0.5, 2.55 and 10.0 mg L^{-1} 2,4-D (P < 0.005). Nodular callus induced by 10.0 mg L^{-1} 2,4-D showed lower accumulation of soluble carbohydrates (P < 0.001) compared with that induced by all other 2,4-D treatments. Friable callus induced by 0.12 mg L^{-1} 2,4-D accumulated higher soluble carbohydrate contents than at all other 2,4-D concentrations (P < 0.05).

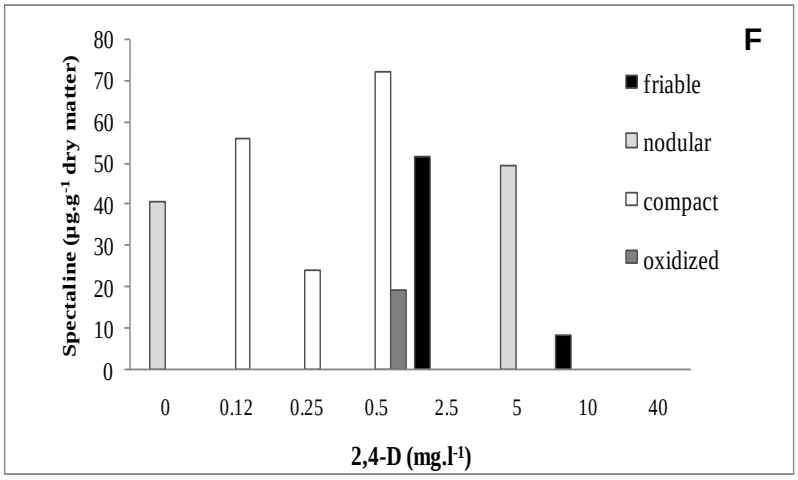
The 10.0 and 40.0 mg L^{-1} 2,4-D treatments induced friable callus with higher protein contents (P < 0.05) compared to that of the other treatments (Figure 2D). The nodular callus induced by 0.5 mg L^{-1} 2,4-D contained higher levels of soluble protein compared to that of all other treatments (P < 0.05). Soluble protein content in compact callus was significantly higher at 0.12, 0.5, 2.5, 5.0 and 10.0 mg L^{-1} 2,4-D (P < 0.01) compared to the 0.0, 0.25 and 40.0 mg L^{-1} 2,4-D treatments. Oxidized calli showed higher levels of soluble proteins in the 0.12, 0.25 and 0.5 mg L^{-1} 2,4-D treatments.

Friable and nodular callus induced by 10.0 mg L^{-1} 2,4-D contained the highest concentrations of starch (P < 0.001) compared to that of the other treatments (Figure 2E). However, the starch content of nodular callus induced at 0.25 and 2.5 mg L^{-1} 2.4-D was also significantly higher than the other treatments and callus types (P < 0.05).

Spectaline was detected in nodular callus induced by 0 and 5.0 mg L^{-1} 2,4-D (Figure 2F, Table 2), compact callus at 0.12, 0.25 and 0.5 mg L^{-1} 2,4-D, oxidized callus at 0.5 mg L^{-1} 2,4-D, and friable callus at 2.5 and 10 mg L^{-1} 2,4-D.

All types of calli induced by 0.12 mg L^{-1} 2,4-D showed higher contents of reserve compounds compared to that of the other treatments (Figure 2G). At 0.5 mg L^{-1} 2,4-D, nodular callus with large amounts of reserve compounds developed, but friable callus induced by the same treatment showed a low content of reserve compounds.





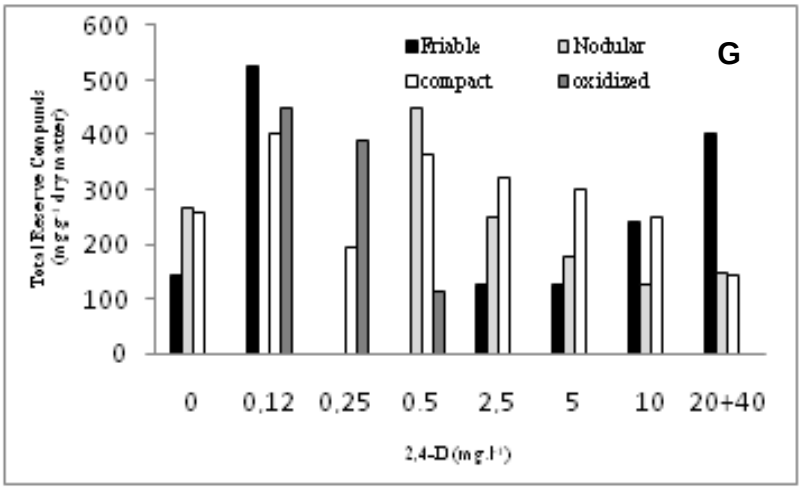


Figure 2. Contd.

Treatment with 5.0 mg L^{-1} 2,4-D induced compact callus with higher concentrations of reserve compounds compared to those of nodular and friable callus in the same treatment.

Table 2. Spectaline content (µg g^{-1} dry mass) detected by GC-FID in different callus types cultured on medium containing different 2,4-D concentrations (* not detected).

2,4-D (mg L^{-1})	Callus type			
	Friable	Nodular	Compact	Oxidized
0.0	0*	41.0	0*	0*
0.12	0*	0*	56.0	0*
0.25	0*	0*	24.0	0*
0.5	0*	0*	72.0	19.0
2.5	51.0	0*	0*	0*
5.0	0*	49.0	0*	0*
10	8.0	0*	0*	0*
20+40	0*	0*	0*	0*
Standard (flowers)	6,820.0			

DISCUSSION

The presence of spectaline in cultured calli occurred randomly, except for the 40 mg L^{-1} 2,4-D treatment, in which spectaline was not detected, showing an herbicide toxic effect in the calli growth. The accumulation of spectaline in different callus types indicates their potential for *in vitro* biosynthesis of the alkaloid. According to Shrivastava et al. (2006) and Karuppusamy (2009), the chemical composition of the culture medium, presence of growth regulators, physical components of the axenic culture, basic knowledge of the biosynthetic routes, elicitor compounds or treatments (abiotic or biotic) triggering the formation of secondary metabolites, genetic manipulation and metabolic engineering may improve the accumulation of compounds.

Trees species of Fabaceae are considered recalcitrant on tissue culture, and do not show tissue or organ induction in lower concentrations of growth regulators as recommended on protocols for cell multiplication (Gharyal and Maheshwari, 1990; Machuka et al., 2002). In the present study, all *S. spectabilis* explants have competent cells that were receptive to stimulation, induction, dedifferentiation and proliferation by auxin in the culture medium. Diverse organs from dicot tissues are easily induced, moreover, callus proliferation is more easily established from certain organs than others due some cells have the ability to perceive, translate and respond to a signal, otherwise they are not suitable targets for plant growth regulators action (Osbourne and McManus, 2005). Callus appearance is considered an important factor for successful induction of somatic embryogenesis. Pal et al. (2006), in a study of regeneration through organogenesis from hypocotyl and cotyledon fragments of *Curcubita pepo* L., considered friable and nodular callus with a milky aspect to be potentially organogenic, and discarded callus that was overly moist and spongy, too compact or brown, and the presence of green dots indicated the beginning of the somatic embryos maturation. The nodules observed on the callus surface of *S. spectabilis* could be associated with globular embryo-genic protuberances or with the globular stage of somatic embryo (Rodriguez and Wetzstein, 1998; Sharma et al., 2014).

The swelling and expansion observed in the explants has been observed on several previous studies in genus *Cassia*. Shrivastava et al. (2006) noticed swelling and increased size of explants of *Cassia senna* L. three weeks after inoculation. A similar result was observed by Agrawal and Sardar (2006) during induction of embryogenesis and organogenesis of *Cassia angustifolia* M. Vahl. Auxin stimulates acidification of the cell wall allowing extension or stretching, and during this process the cell absorbs large amounts of water (Raven et al., 2001, Silveira et al., 2004). These factors explain the amount of water absorbed by the cells, as indicated by comparison of the fresh and percentage dry mass of friable callus and low dry mass compared to that of compact callus, which suggests friable callus absorbed more water than did the compact callus.

In the present study notable concentrations of soluble proteins were present in the different callus types. In contrast, the highest soluble proteins content recorded by Silveira et al. (2004) for cell suspension cultures of *Pinus taeda* L. was 0.73 mg g^{-1} fresh weight (estimated 10% of this value for dry weight). These authors suggested that the increase in the concentration of soluble proteins might be related to mitotic activity during the exponential growth phase, when specific proteins are synthesized at the beginning of the cell cycle and which lead to biochemical and morphological changes throughout the mitotic cycle. The presence of these specific proteins in embryogenic cells might be associated with formation of proembryogenic groups (Cangahuala-Inocente et al., 2004). Total soluble protein in roots and in the photo-

synthetic aerial parts of *Phalaenopsis amabilis* were related to enzymes and their synthesis, coenzymes, nucleic acids, chlorophyll and primary plant metabolism (Ori et al., 2014). The authors suggest that these proteins are possibly not for reserve as found in potato tubers and soybean seeds. This rise expresses that the synthesis of proteins formed from the stage of degradation of carbohydrates, which is necessary for production of energy which will be used on the exponential growth phase, when the rate of cell division increases.

Reserves compounds are crucial for *in vitro* morphogenesis (Branca et al., 1994) and some studies correlated reserve consumption patterns with the development of organogenesis and also to somatic embryogenesis (Mangat et al., 1990; Martin et al., 2000). Pinto et al. (2010) pointed that starch begins to accumulate early during induction of *Eucalyptus globulus* somatic embryos with 3 mg L^{-1} α-naphthalene acetic acid, probably due to the sucrose present in the culture medium and in meristematic regions a lower abundance of starch were presented due consumption in mitotically active tissues that was reported earlier for other species (Barciela and Vieitez, 1993; Canhotoa and Cruz, 1996).

Induction of cell proliferation caused by the synthetic auxin 2,4-D also leads the cells to accumulate reserve compounds, because the cell division process has a great demand for energy and basic compounds for DNA duplication, specific protein synthesis, multiplication of organelles and production of new cell walls. Thus, treatment with 0.12 and 0.5 mg L^{-1} 2,4-D, which induced higher rates of reserve compound accumulation, is inferred to represent the optimal concentrations of auxin to induce cell proliferation in *S. spectabilis* callus. However, treatment with 10 mg L^{-1} 2,4-D represented the best option to induce friable and nodular callus with higher accumulation of starch. *Carya illinoiensis* (Wangenh.) K. Koch embryogenic clump formation is preceded by accumulation of starch grains, which are rapidly consumed during the induction of the embryogenic regions, but starch is absent in the embryo maturation stages (heart and torpedo stages) (Rodriguez and Wetzstein, 1998).

The possibility that the available sucrose, auxin and cytokinin acted as a checkpoint in the control of cell division in the plant cycle is another interesting aspect to highlight. Although the levels of reserve compounds present in the calli favored cell growth and division, the presence of high concentrations of auxin and sucrose in the culture medium was insufficient to induce differentiation of somatic embryos, buds or roots, which indicated that other factors were absent or present at inadequate concentrations to stimulate differentiation in the G2 phase checkpoint during the experiment as observed by Chu et al. (2008) for *C. echinata*.

However, data obtained in the present study could serve as the foundation for new protocols for *in vitro* propagation and differentiation of *S. spectabilis*. Our results indicate that hypocotyl fragments with an auxin shock (10 mg L^{-1} 2,4-D) for a short period (up to five days) to induce cell proliferation and starch accumulation, followed by culture with 0.12 mg L^{-1} 2,4-D to increase in tissue mass and accumulation of other reserve compounds (soluble carbohydrates and proteins), induces the production of friable callus suitable for establishment of an *in vitro* tissue culture system.

Conflict of Interests

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

REFERENCES

Agrawal V, Sardar PR (2006). *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. Biol. Plant. 50(1):118-122.

Bolzani VS, Gunatilaka AAL, Kingston DGI (1995). Bioactive piperidine alkaloids from *Cassia leptophylla*. Tetrahedron 51(21):5929-5934.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1-2):248-252.

Braga R (1982). Plantas do nordeste especialmente do Ceará. Imprensa Oficial, Fortaleza.

Brown JT, Charlwood BV (1986). Diferentiation and monoterpene biosyhthesis in plant cell cultures. In: Morris P, Scragg AH, Stafford A, Fowler MW (ed) Secondary Metabolism in Plant Cell Cultures. Press Syndicate of the University of Cambridge, Cambridge, pp. 68-74.

Cangahuala-Inocente GC, Steiner N, Santos M, Guerra MP (2004). Morphohistological analysis and histochemistry of *Feijoa sellowiana* somatic embryogenesis. Protoplasma 224(1-2):33-40.

Chu EP, Tavares AR, Pescador R, Tonello KC, Yokota ES (2008). Callus induction in *Caesalpinia echinata*, a Brazilian endangered tree. Tree For. Sci. Biotechnol. 2(1): 50-53.

Dubois M, Gille KA, Hamilton JK, Rebes PA, Smith F (1956). Colorimetric method for determinations of sugars and related substances. Anal. Chem. 28(3):350-356.

George EF, Debergh PC (2008). Micropropagation: uses and methods. In: George EF, Hall MA, Klerk GD (ed) Plant Propagation by Tissue Culture, Vol 1. The Background, 3rd ed. Springer, Dordrecht. pp. 29-64.

George EF, Hall MA, Klerk GD (2008). Plant propagation by tissue culture. Volume 1. The Background. Springer, Dordrecht.

Gharyal PK, Maheshwari SC (1990). Differentiation in explants from mature leguminous trees. Plant Cell Rep. 8(9):550-553.

Karuppusamy S (2009). A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. J. Med. Plants Res. 3(13):1222-1239.

Loyola-Vargas VM, Vazquez-Flota F. (2006). Plant cell cultures protocols. Humana Press Inc., Totowa.

Machuka J, Adesoye A, Obembe OO (2002). Regeneration and genetic transformation in cowpea. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M (ed) Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference, Ibadan. pp. 185-196.

McCready RM, Guggolz J, Owens HS (1950). Determination of starch and amylose in vegetables. Anal. Chem. 22(9): 1156-1158.

Melo GMA, Silva MCR, Guimarães TP, Pinheiro KM, Matta CBB, Queiroz AC, Pivatto M, Bolzani VS, Alexandre-Moreira MS, Viegas Jr. C (2014). Leishmanicidal activity of the crude extract, fractions and major piperidine alkaloids from the flowers of *Senna spectabilis*. Phytomedicine 21(3): 277-281.

Moreira MSA, Viegas Jr. C, Miranda ALP, Barreiro EJ, Bolzani VS(2003).

Antinociceptive profile of (-)-spectaline: a piperidine alkaloide from *Cassia leptophylla*. Planta Med. 69(9):795-799.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15(3):473-496.
Ori SS, Chu EP, Tavares AR (2014). Effects of auxins on *in vitro* reserve compounds of *Phalaenopsis amabilis* (Orchidaceae). Afr. J. Biotechnol. 13(13):1467-1475.
Osbourne DJ, McManus MT (2005). Hormones, signals and target cells in plant development. Cambridge University Press, Cambridge.
Paguigan ND, Castillo DH, Chichioco-Hernandez CL (2014). Anti-ulcer activity of leguminosae plants. Arq. Gastroenterol. 51(1):64-68.
Pal SP, Alam I, Anisuzzaman M, Sarker KK, Sharmin S, Alam MF (2006). Indirect organogenesis in summer squash (*Curcubita pepo* L.). Turk. J. Agric. For. 31:63-70.
Pinto G, Silva S, Neves L, Araújo C, Santos C (2010). Histocytological changes and reserve accumulation during somatic embryogenesis in *Eucalyptus globules*. Trees 24(4):763-769.
Pivatto M, Crotti AEM, Lopes NP, Castro-Gamboa I, Rezende A, Viegas Jr. C, Young MCM, Furlan M, Bolzani VS (2005). Electrospray ionization mass spectrometry screening of piperidine alkaloids from *Senna spectabilis* (Fabaceae) extracts: fast identification of new constituents and co-metabolites. J. Braz. Chem. Soc. 16(6):1431-1438.
Raven PH, Evert RF, Eichhorn SE (2001). Biologia Vegetal. Guanabara Koogan, Rio de Janeiro.
Rodriguez APM, Wetzstein HY (1998). A morphological and histological comparison of the initiation development of Pecan (*Carya illinoinensis*) somatic embryogenic cultures induced with naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid. Protoplasma 204(1-2):71-83.
Sharma A, Kaur R, Raina R (2014). Study of genetic fidelity of somatic embryos - derived plants of *Gentiana kurroo* royle from western himalayas - plant of great medicinal value. Br. Biotechnol. J. 4(5): 589-611.
Shrivastava N, Patel T, Srivastava A (2006). Biosynthetic potential of *in vitro* grow callus cells of *Cassia senna* L. var *senna*. Curr. Sci. 90(11):1472-1473.
Silva M (2009). Personal communication (Instituto de Botânica de São Paulo: São Paulo).
Silveira V, Floh EIS, Handro W, Guerra MP (2004). Effect of plant growth regulators on the cellular growth and levels of intracelular protein, starch and polyamines in embryogenic suspension cultures of *Pinnus taeda*. Plant Cell Tissue Org. 76(1):53-60.
Viegas Jr C, Pivatto M, Rezende A, Hamerski L, Silva DHS, Bolzani VS (2013). (–)-7-Hydroxycassine: a new 2,6-dialkylpiperidin-3-ol alkaloid and other constituents isolated from flowers and fruits of *Senna spectabilis* (Fabaceae). J. Braz. Chem. Soc. 24(2): 230-235.
Viegas Jr. C, Rezende A, Silva DHS, Castro-Gambôa L, Bolzani VS (2006). Aspectos químicos, biológicos e etnofarmacológicos do gênero *Cassia*. Quím. Nova 29(6):1279-1286.
Wink M (2008). Ecological roles of alkaloids. In: Fattorusso E, Taglialatela-Scafati O (ed) Modern alkaloids. Structure, isolation, synthesis and biology. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim. pp. 3-24.
Wink M, Roberts MF (1998). Compartmentation of alkaloids synthesis, transport, and storage. In: Roberts MF, Wink M (ed) Alkaloids: biochemistry, ecology, and medicinal applications. Plenum Press, New York. pp. 239-262.

academicJournals

Vol. 13(35), pp. 3628-3637, 27 August, 2014
DOI: 10.5897/AJB2014.13871
Article Number: 24F34A147062
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

An attempt towards standardization of the production process of dawadawan botso (a fermented condiment)

Ibrahim, A. D.[1]*, Pedavoah, M. M.[2], Bawa Saadatu M.[3] and Sani, A.[4]

[1]Department of Microbiology, Usmanu Danfodiyo University, Sokoto. Nigeria.
[2]Department of Applied Chemistry and Biochemistry, Faculty of Applied Sciences, University for Development Studies, Navrongo Campus, Navrongo, Ghana.
[3]Department of Biology, School of Science, Niger State College of Education, Minna, Nigeria.
[4]Department of Microbiology, University of Ilorin, Ilorin, Nigeria.

Received 18 April, 2014; Accepted 15 August, 2014

The production of 'dawadawan botso' a local condiment produced by traditional uncontrolled fermentation, in Nigeria and other parts of Africa is usually based on experience rather than standard measurements. This work was aimed at evaluating the effect of substrate (*Hibiscus sabdariffa* seeds) and ash leachate on the chemical composition, amino acid profile and the taste of 'dawadawan botso.' Locally prepared 'dawadawan botso' was collected from a local producer and its quality was compared to that prepared in the laboratory under optimized conditions. The ash leachate was standardized by dissolving known masses of ash in a fixed volume of water. The pH of the ash leachate was determined and then added to the substrate before the second fermentation. The pH of the unfermented, fermented *H. sabdariffa* and the 'dawadawan botso' were determined using a pH meter. Determination of the proximate composition, mineral and amino acid profile were done by AOAC method. The results were analyzed statistically using ANOVA and the post-hoc test using Duncan multiple range test (DMRT) for means that were significantly different from each other. Paired sample T- test was also done to test for the significance between groups at P<0.05 using SPSS. The results show that the volume of leachate decreased (385 ml/100 g ash to 144 ml/250 g ash) with increasing pH (13.60 to 13.70) as the mass of ash increased. Proximate analyses showed that carbohydrates were the highest followed by proteins and least was lipids. Potassium (K) and sodium (Na) were the major minerals; magnesium (Mg), phosphorus (P) and calcium (Ca) were insignificant compared to the later. The pH of the substrate decreased after second fermentation for all standards and also decreased as mass of substrate increased. The following amino acids; glutamic, aspartic and methionine were observed to decrease with increasing mass of substrate. Fermentation increased the value of total amino acids in substrate. Taste analyses indicated that monosodium glutamate-like taste was dominant, followed by bitter and sweet, respectively for all samples analyzed.

Key words: Ash, *Hibiscus sabdariffa,* "dawadawan botso", amino acid profile, proximate composition.

INTRODUCTION

Many African tribes have inherited at least one product of their tradition fermentation. These products are mostly produced from the fermentation of neglected and underutilized plants species. The process takes a long time and often it

lacks a standard protocol for its production. In Nigeria, this product includes soup condiments such as 'dawadawa' made by fermentation of soybean seeds (Popoola and Akueshi, 1984; Ogbadu and Okagbue, 1988), dawadawa-/iru by the Hausa and Yoruba people made by the fermentation of *Parkia bigloboso* seeds (Odunfa, 1981a), ogiri by Ibos made by fermentation of melon seeds (Odunfa, 1981b), ogiri-Igbo made from castor oil seeds (Odunfa, 1985), ogiri-ugu made from flutted pumpkin seeds (Barber and Achinewhu, 1992; Odibo and Umeh, 1989) owoh by the Urhobo and Tsekiri people of Niger Delta made by either African yam beans (Ogbonna et al., 2001) or cotton seeds (Sanni and Ogbonna, 1991), okpiye by the Igala and Idoma people produced from *Prosopis Africana* (Achi,1992). Dawadawan botso is a condiment produced by fermenting the seeds of *Hibiscus sabdariffa* by the rural dwellers of Zuru where it is call chwande (Ibrahim et al., 2011b). This condiment is also produced in other northern states of Nigeria such as Plateau, where the Tarok people ferment roselle seeds to make a cake to be used as "sorrel meat" or Iyu (Schippers, 2000) and Borno where the Babur/Bura ethnic groups, call it Nwanza Ntuza (Ayodele and Musa, 2008) and other African countries like Burkina Faso who called it 'Bikalga', Mali who called it 'Datou', Cameroon who called it 'Mbuja', Sudan who called it 'Furundu' and Niger who also called it Dawadawan botso (Parkouda et al., 2008; Bengaly et al., 2006).

There has been a major concern as to the required standard for the production of African food condiments as most workers are focusing on the issues of introducing starter culture or quality control. For industrial or large scale production of these condiments, there is a need for the standardization of the production process, since all industries are aiming at profit maximization. Standardizing the production process will go a long way in helping to work out the economics of its production. It was on the vein that this research work was conducted with the aim of determining the effect of substrate and ash leachate on the nutritional profile of dawadawan botso.

MATERIALS AND METHODS

Sample collection and processing

A mass of about 10 kg of the seeds of *H. sabdariffa* was purchased in Zuru market, Zuru Local Government Area of Kebbi State all in Nigeria. Locally prepared dawadawan botso was collected from a local producer in kwendo village of Zuru Local Government Area, Kebbi State.

Dried sorghum stalks were collected from harvested farms at Kwendo village in Zuru, Kebbi State Nigeria. The leaves of the dried sorghum stalks were removed and the stalks burnt to ashes. It was allowed to cool naturally and collected in bagco sack and brought to laboratory.

Testing for the effect of ash quantity on the pH and nutritional quality of dawadawan botso

The ash leachate (solution) production was standardized by weighing different amount of ash (solute) (100, 150, 200, 250 and 300 g) to fixed volume of water (solvent) (600 ml). Each of the ash leacheate collected was used to produce the dawadawan botso by adding 37.5 g/22.5 ml and 75 g/22.5 ml of the seeds of *H. sabdariffa* and ash leachate in milliliter. This was added after the first fermentation for two days and the seeds were pounded, mixed with the ash leachate and it was allowed to undergo second fermentation for one day. The second fermentation was altered by spreading the condiment on polyethene bags and dried under the sun.

Testing the effect of substrate (seed) on the pH and nutritional quality of "dawadawan botso"

The effect of substrate on the pH and nutritional quality of dawadawan botso was done by weighing 150, 250, 300 and 400 g of the seeds of *H. sabdariffa* into separate pots, washed two to three times and cooked for 8-10 h. The cooked seed were allowed in the pot for the first fermentation for two days, pounded using local mortar and pestle, and a fixed volume of ash leacheate (90 ml) was added to all seed quantities and fixed using fingers and it was allowed to undergo second fermentation for 1 day. The fermentation was altered by spreading the condiment on polyethene bags and dried under the sun.

Determination of pH

The pH was determined as done for fermented seeds of African locust beans (Ouoba et al., 2005) and *H. sabdariffa* (Parkouda et al., 2008). The pH of unfermented ground seeds, fermented seeds of *H. sabdariffa* and dawadawan botso was measured directly in a mixture prepared with 10 g of sample and 30 ml of distilled water mixed. A glass electrode pH meter was used for the measurement (CLIDA instrument PHS-25C precision pH/mV meter).

Proximate composition

Samples were analyzed in triplicate for proximate composition in accordance with the Official Methods of the Association of Official Analytical Chemists (AOAC, 1995). Ash was determined by incinerating (2 g) each of ground unfermented and fermented seeds of *H. sabdariffa* at 550°C in lenton furnaces (England) over night. Fiber was determined by drying 2 g each of ground unfermented and fermented seeds of *H. sabdariffa* overnight at 105°C in the oven (Gallenhamp Oven BS) and incinerated at 550°C for 90 min in lenton furnaces (England). Moisture content was determined by drying 2 g each of ground unfermented and fermented seeds of *H. sabdariffa* overnight at 105°C in the oven (Gallenhamp Oven BS). Crude lipid was determined by weighing a known weight of the dried sample into extraction thimble and the fat was extracted with chloroform/methanol (2:1 mixture) using soxhlet extraction apparatus as described by AOAC (2006). The extraction lasted for

*Corresponding author. E-mail: ibrahim.aliyu@udusok.edu.ng.

15 h. It was drained into an empty flask. It was placed in an oven to allow the N-hexane to evaporate in the oven (Gallenhamp Oven BS). Protein (% N * 6.25) was determined by the Micro-kjeldahl Method and the weight was determined by difference. Soluble carbohydrate was determined indirectly as the difference between crude protein and the sum of ash, protein, crude lipid and crude fiber.

Mineral content

Analysis of minerals in unfermented, locally fermented and laboratory fermented seeds of *H. sabdariffa* were done in triplicate according to methods described by Hack (2000). The investigated minerals include phosphorus, potassium, sodium, calcium and magnesium. Phosphorus was determined using spectrophometer (JENWAY 6100) at 660 γ (wavelength), potassium and sodium was determined using flame photometer (Corning 400 Essex, England), determination of calcium and magnesium was done by ethylene diamine tetra acetic acid (EDTA) titration method.

Determination of amino acid profile

The amino acid profile in the known sample was determined using methods described by Spackman et al*.* (1958). The known sample was dried to constant weight. The sequential multi-sample amino acid analyzer (TSM) was used to analyze amino acids. Four samples with the highest characteristic dawadawan botso aroma as described by the consumers were subjected to amino acid analysis. The samples were defatted by weighing a known weight of the dried sample into extraction thimble and the fat was extracted with chloroform/methanol (2:1 mixture) using soxhlet extraction apparatus as described by AOAC (2006). The extraction lasted for 15 h. Nitrogen was determined by weighing a small amount (200 mg) of ground sample, wrapped in Whatman filter paper (No. 1) and put in a Kjedhal digestion flask. Concentrated sulphuric acid (10 ml) was added. Catalyst mixture (0.5 g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio 10:5:1 was added into the flask to facilitate digestion. Four pieces of anti-bumping granules were added. The flask was then put on Kjedhal digestion apparatus for 3 h until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100 ml in standard volumetric flask. Aliquot (10 ml) of the diluted solution with 10 ml of 45% sodium hydroxide were put into the Markham distillation apparatus and distilled into 10 ml of 2% boric acid containing four drops of bromocresol green/methyl red indicator until about 70 ml of distillate was collected. The distillate was then titrated with standardized 0.01N hydrochloric acid to grey colour end point. The percentage nitrogen in the original sample was calculated using the formula:

$$\text{Percentage Nitrogen} = \frac{(a-b)\times 0.01\times 14\times V}{W\times C}\times 100$$

Where, a = titre value of the digested sample; b = titre value of blank sample; *V* = volume after dilution (100 ml); *W* = weight of dried sample (mg); *C* = aliquot of the sample used (10 ml); 14 mg = Nitrogen constant in mg; 100 = Conversion factor to percentage (Spackman et al*.,* 1958). Hydrolysis of the samples was done by weighing a known mass of the defatted sample into glass ampoule. Seven millilittres of 6 N HCl was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis for example methinonine and cystine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105°C for 22 h and the content was filtered to remove the humins. The filtrate was then evaporated to dryness at 40°C under vacuum in a rotary evaporator and the residue was dissolved with 5 ml of acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer. The hydrolysate was loaded into the TSM analyzer by loading 5 to 10 µl (5 for acidic/ neutral amino acid and 10 for basic amino acids). This was dispensed into the cartridge of the analyzer. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. The period of an analysis lasted for 76 min.

Grouping of free amino acid

This was done in accordance to the taste characteristics described by Tseng et al. (2005), amino acids were grouped as MSG-like (monosodium glutamate-like) (Asp+Glu), sweet (Ala+Gly+Ser+Thr), bitter (Arg+His+Ile+Leu+Met+Phe+Trp+Try+Val), and tasteless (Cys+Lys+Pro).

Statistical analysis

The data sets were expressed as mean ± standard deviation (n=3). Analysis of variance (ANOVA) was done using one-way analysis of variance to test for the difference in means. Post-Hoc test using Duncan multiple range test (DMRT) was carried out to test for the means that are significantly different from each other, which are presented by alphabets in superscripts. Paired sample T-test was used to test for the significance between samples at ($P< 0.05$) level of significance using the SPSS for Windows, version 15.0. (Chicago IL, USA).

RESULTS AND DISCUSSION

Ash leachate preparation was standardized and the result is presented in Table 1. To the best of our knowledge, this is one of the few work describing in details the standardization of ash leachate preparation. More ash leachate (385 and 325 ml) was harvested when low amount (100 and 150 grams) of ash is used to fix amount of water. This is probable because low ash quantity tends to hold less amount of water thereby allowing the rest to pass through the perforated container to be collected as ash leachate and at 300 g it retained the 600 ml of water used. This was the reason for not harvesting any amount of ash leachate. There was a significant difference in the pH at $P<0.05$ of ash leachate harvested from low ash concentration as compared to those obtained at higher ash concentrations.

The effect of different ash leachate preparations on the pH during the production of dawadawan botso has been conducted (Table 2). The result shows various pH values for each of the ash leachate standards after first and second fermentations. There was significant effect of the prepared ash leachate standards on pH of 'dawadawan botso' at $P<0.05$ between the pH of each of the standards before and after second fermentation. An initial pH of 11.07 to 11.70 was recorded when 100 and 250 gash leachate was used as compared to pH of 10.93 to 10.97 recorded when 150 g was used. A fall in pH was observed for all the standards after second fermentation, an indication

Table 1. Standardization of ash leachate preparation.

Quantity of ash (g)	Amount of water (H_20) (ml)	Leachate collected (ml)	pH
100	600	385 ^{e}	13.60 ± 0.000^{b}
150	600	325 ^{d}	13.63 ± 0.058^{b}
200	600	223 ^{c}	13.70 ± 0.000^{c}
250	600	144 ^{b}	13.70 ± 0.000^{c}
300	600	0 ^{a}	0^{a}

Each data point presented as mean±standard deviation (n=3).

Table 2. Effects of 1st, 2nd, and 4th standards [(100 g/600 ml), (150 g/600 ml), (250 g/600 ml)] on pH.

Quantity of seed	Effect of 1st standard (100 g/600 ml) on pH		Effect of 2nd standard (150 g/600 ml) on pH		Effect of 4th standard (250 g/600 ml) on pH	
	Before 2nd fermentation (E1)	After 2nd fermentation (E2)	Before 2nd fermentation (E3)	After 2nd fermentation (E4)	Before 2nd fermentation (E5)	After 2nd fermentation (E6)
37.5 g/22.5 ml	11.33±0.153^{b}	9.13±0.058^{b}	10.97±0.058^{a}	9.77±0.058^{b}	11.43±0.058^{a}	9.80±0.000^{b}
75 g/22.5 ml	11.07±0.058^{a}	8.43±0.153^{a}	10.93±0.058^{a}	8.67±0.116^{a}	11.70±0.000^{b}	9.27±0.058^{a}

Each data point presented as mean±standard deviation (n=3). Means along the same column with different superscript are significantly different at P<0.05.

Table 3. Effect of ash leachate on the proximate composition (%) of fermented seeds of *Hibiscus sabdariffa.*

Proximate components (%)	E1	E2	E3	E4	E5	E6
Moisture	5.33±0.29^{abc}	6.00±0.50^{c}	4.67±0.29^{a}	5.00±0.50^{ab}	5.83±0.58^{bc}	5.67±0.58^{bc}
Ash	10.17±0.76^{a}	11.17±0.58^{bc}	10.67±0.29^{abc}	10.00±0.50^{a}	10.33±0.29^{ab}	11.50±0.50^{c}
Lipid	21.33±1.76^{a}	20.67±1.61^{a}	21.67±0.76^{a}	21.33±1.76^{a}	19.67±0.76^{a}	21.00±1.32^{a}
Fibre	5.50±0.50^{bc}	4.83±0.58^{ab}	4.50±0.50^{a}	5.67±0.29^{c}	4.17±0.29^{a}	6.17±0.29^{c}
Crude protein	30.56±0.33^{c}	30.57±0.29^{c}	27.13±0.06^{b}	27.14±0.18^{b}	26.20±0.15^{a}	26.20±0.29^{a}
Carbohydrate	27.11±0.10^{a}	26.75±0.68^{a}	31.36±2.42^{bc}	30.86±1.00^{bc}	33.80±2.90^{c}	29.47±0.66^{ab}

Each data point presented as mean±SEM (n=3). Means along the same row with different superscript are significantly different at P<0.05. 100 g Ash/600 ml water, before 2nd fermentation (E1). 100 g Ash/600 ml water, After 2nd fermentation (E2). 150 g Ash/600 ml water, before 2nd fermentation (E3). 150 g Ash/600 ml water, after 2nd fermentation (E4). 250 g Ash/600 ml water, Before 2nd fermentation (E5). 250 g Ash/600 ml water, After 2nd fermentation (E6).

strongly suggesting the degradation of carbohydrates and lipid to release acid compounds. This is particularly a common scenario for many fermented condi-ment (Ouoba et al., 2003; Harper and Collin, 1992).

The effect of different ash leachate concentration on the proximate composition of 'dawadawan botso' is shown on Table 3, the least and highest lipid values were; 19.67 and 21.67% respectively. The least and highest protein values were 26.20 and 30.57% respectively. The effect of substrate concentration on the proximate composition of dawadawan botso is shown on Table 4. The least and highest lipid value (20.00 and 22.17) was recorded when 250 and 150 g were used for the fermentation respectively. The least and highest protein value were; 25.63 and 28.06 for 150 g and 300 g respectively. The highest and least carbohydrate values were; 35.93 and 31.62 for 300 g and 400 g respectively. The highest and least carbohydrate value was 26.75 and 33.80% respectively. The amount and type of the alkalinizing leachate that is added as well as the precise step during the process where it should be added has been reported to have a significant effect on the organoleptic characteristics of Bikalga (Parkouda et al., 2008). The alkalinizing leachate had effect on the proximate composition of 'dawadawan botso'. There was significant difference at P<0.05 in crude protein within the various alkalinizing leachate standards used with that from 100 g having the highest protein content (30.56 and 30.57%). This variation in crude protein could be due to the pH of the alkalinizing leachate which also has effect on the

Table 4. Effect of substrate (seeds of *H. sabdariffa*) concentration on the proximate composition of 'dawadawan botso'.

Substrate (g)/90 ml of ash leachate	Moisture (%)	Ash (%)	Lipid (%)	Fibre (%)	Crude protein (%)	Carbohydrate (%)
150	5.17±0.29^{b}	10.33±0.76^{b}	22.17±0.58^{b}	2.83±0.29^{a}	25.63±0.34^{a}	33.89±3.03^{ab}
250	6.00±0.50^{c}	8.33±0.58^{a}	20.00±0.50^{a}	3.17±0.29^{a}	27.44±0.46^{b}	35.06±0.06^{ab}
300	4.17±0.29^{a}	7.50±0.50^{a}	21.67±0.76^{b}	2.67±0.29^{a}	28.06±0.06^{c}	35.93±0.94^{b}
400	7.00±0.50^{d}	7.33±0.29^{a}	21.83±0.76^{b}	3.17±0.29^{a}	29.05±0.05^{d}	31.62±2.04^{a}

Each data point presented as mean±SEM (n=3) Means along the same row with different superscript are significantly different at P<0.05

Table 5. Effect of ash leachate on the mineral content of 'Dawadawan botso'.

Mineral content	E1	E2	E3	E4	E5	E6
Magnesium (mg/kg)	2.11±0.12^{a}	1.81±0.23^{a}	1.90±0.20^{a}	1.88±0.15^{a}	2.05±0.22^{a}	2.67±0.12^{b}
Phosphorus (mg/kg)	1.93±0.03^{c}	1.87±0.01^{ab}	1.85±0.02^{a}	1.90±0.02^{bc}	1.98±0.03^{d}	1.90±0.02^{bc}
Sodium (mg/kg)	136.67±8.04^{ab}	134.17±5.77^{a}	140.83±3.82^{ab}	135.83±7.64^{a}	141.67±3.82^{ab}	148.33±6.29^{b}
Potassium (mg/kg)	24000.00±1000.00^{b}	24000.00±1000.00^{b}	25166.67±1040.83^{b}	24166.67±1258.31^{b}	25500.0±1000.00^{b}	20666.67±1258.31^{a}
Calcium (mg/kg)	0 .54±0.08^{a}	0.63±0.13^{a}	0.50±0.13^{a}	0.59±0.08^{a}	0.50±0.25^{a}	0.47±0.03^{a}

Each data point presented as mean±SEM (n=3). Means along the same row with different superscript are significantly different at P<0.05. E1,100 g ash/600 ml water - before 2nd fermentation; E2,100 g ash/600 ml water - after 2nd fermentation; E3,150 g ash/600 ml water - before 2nd fermentation. E4, 150 g ash/600 ml water - after 2nd fermentation. E5, 250 g Ash/600 ml water- Before 2nd fermentation; E6, 250 g Ash/600 ml water- After 2nd fermentation.

fermenting organisms or their enzymes as microbial biomass also contributes to the crude protein content of fermented condiments. However, no significant difference at P<0.05 was observed in the lipid contents of the dawadawan botso. A significant difference at P<0.05 was observed in soluble carbohydrate. The constant low pH has been found to reduce protein degradation and increase non ammonia nitrogen (N) and dietary nitrogen (N) flow compared with constant high pH (Calsamiglia et al., 2002). The decrease observed in the lipid content may be due to microbial action during the fermentation process. The lipids will obviously contribute to short chain fatty acids and impart flavor on the condiment (Ibrahim et al., 2011a). Four hundred grams had the lowest soluble carbohydrate explaining that it may have supported the growth of more microbial cells which hydrolyzed the soluble carbohydrate to reducing sugars easily utilizable by the microorganisms as source of energy (Yagoub et al., 2004).

The effect of ash leachate standards on the mineral content of 'dawadawan Botso' is shown in Table 5. From the table, potassium and sodium were the major minerals which ranged from 20666.67 to 25166.67 and 134.17 to 148.33 mg/kg, respectively. In evaluating the effect of substrate concentration on the mineral content of dawadawan botso (Table 6), we also obtained similar result for potassium and sodium. The ash concentration used in the preparation of alkalinizing leachate has effect on some of mineral content of dawadawan botso. A significant difference at P<0.05 was observed in the phosphorus content of dawadawan botso with the alkalinizing leachate prepared from 250 g ash at low seed concentration having the highest value (1.98 mg/kg) compared to 100 g. Since the substrates contain different minerals apart from carbon which may serve as nutrient supplements, increasing substrate concentration has been found to increase the bioavailability of the mineral elements. No significant difference at P<0.05 between the potassium content of 250, 300 and 400 g was observed.

The effect of substrate on pH of dawadawan botso

Table 6. Effect of substrate (seeds of *H. sabdariffa*) concentration on the mineral content of 'dawadawan botso'.

Substrate (g)/90 ml of ash leachate	Magnesium (mg/kg)	Phosphorus (mg/kg)	Sodium (mg/kg)	Potassium (mg/kg)	Calcium (mg/kg)
150	1.87±0.51^{b}	2.93±0.04^{a}	162.50±5.00^{c}	23333.33±763.76^{b}	0.92±0.19^{a}
250	1.37±0.22^{b}	2.99±0.03^{ab}	140.83±3.82^{b}	15833.33±288.68^{a}	0.79±0.08^{a}
300	1.75±0.33^{b}	3.06±0.03^{b}	127.50±5.00^{a}	16333.33±763.76^{a}	0.67±0.19^{a}
400	0.54±0.14^{a}	3.04±0.04^{b}	126.67±6.29^{a}	15000.00±1500.00^{a}	1.79±0.26^{b}

Each data point presented as mean±SEM (n=3). Means along the same row with different superscript are significantly different at P<0.05.

is shown in Figure 1. The highest pH before and after fermentation (11.4 and 9.03) was recorded when 150 g of *H. sabdariffa* seeds was fermented to produce dawadawan botso. The high pH may suggest the production of more acid product during fermentation of 150 g of substrate. In another study, we detected many acidic volatile flavor compounds in dawadawan botso (Ibrahim et al., 2011a).

The ash concentration used in the preparation of alkalinizing leachate had effect on some of amino acid profile of 'dawadawan botso' (Table 7). Alkalinizing leachate prepared from 150 g ash gave the highest value for the essential amino acid valine, methionine, isoleucine, and phenylalanine as well as non-essential amino acids such as arginine, serine, glutamic acid, and tyrosine while that from 100 g gave the highest value for non- essential amino acid proline, alanine and aspartic acid, in addition to some essential amino acids such as lysine, histidine and leucine. However, these values are not significantly different at P<0.05. When amino acids are incubated together, the degradation of free amino acid by lactic acid bacteria was found to be dependent on pH (Tavaria et al., 2002).

Effect of substrate concentration on amino acid profile of dawadawan botso was conducted (Table 8). The highest value of amino acid was recorded for glutamic acid with a value of 11.24, 10.82 and 9.98 g / 100 g protein) for 150, 250 and 300 g, then aspartic acid with 10.10, 9.41 and 8.54 g/100 g protein for 150, 250 and 300 g. Two hundred and fifty grams of seeds to 90 ml of alkalinizing leachate had the highest value of essential amino acid except for methionine and threonine and the non-essential amino acid Cystine. One hundred and fifty grams seeds to 90 ml of alkalinizing leachate had the highest value of non-essential amino acid and the highest value for methionine. The decrease in the essential amino acid may be suggestive of their probable role in stimulating microbial growth and improving the fermentation of *H. sabdariffa* seeds to produce the flavour of condiment. Yimiti et al. (2004) reported that amino acid fermentation byproduct additive improved the fermentation of silage. Also, the amino acid may have been used by the fermenting organisms to stimulate their growth (Argyle and Baldwin, 1989).

The total free amino acids were evaluated (Figure 2). Fermentation increased the value of total free amino acid as compared to the unfermented seeds. Two hundred and fifty grams (250 g) had the highest value (82.97%) for total free amino acids, then 150 g/90 ml with 76.93% and finally 300 g/90 ml with 73.37%. The free amino acids groupings, based on their taste characteristics as described by Tseng et al. (2005), are shown in Figure 3. One hundred and fifty grams (150 g) had the highest value (27.74%) for monosodium glutamate free amino acid, then 300 g/90 ml with 25.24% and the least was in sweet for two classes of free amino acid (sweet and bitter). The total free amino acid and the individual free amino acid classes' result show that 250 g substrate/90 ml ash leachate had the highest value for total free amino acid and bitter free amino acids groupings based on their taste characteristics (Figures 2 and 3). This may be explained by the fact that there may be increased microbial activity in the 250 g substrate samples through proteolysis resulting to high score for total free amino acid (Ibrahim et al., 2011a). The decrease in MSG-like and sweet amino acid class may be an indication that this class have further been utilized by the fermenting organisms to produce the characteristic flavor compounds that is associated with this condiment. Similar observation had been observed for the product and other fermented product such as cheese (Tavaria et al., 2002). Despite the increase in bitter amino acid class, it was not manifested in the overall taste of the product. This is because the other classes have the potential to exhibit a masking effect of the bitter taste class, a scenario that has been reported before for doenjang soybean paste (Kim and Lee, 2003).

Conclusion

The chemical composition of 'dawadawan botso' was affected by the mass of the substrate (*H.*

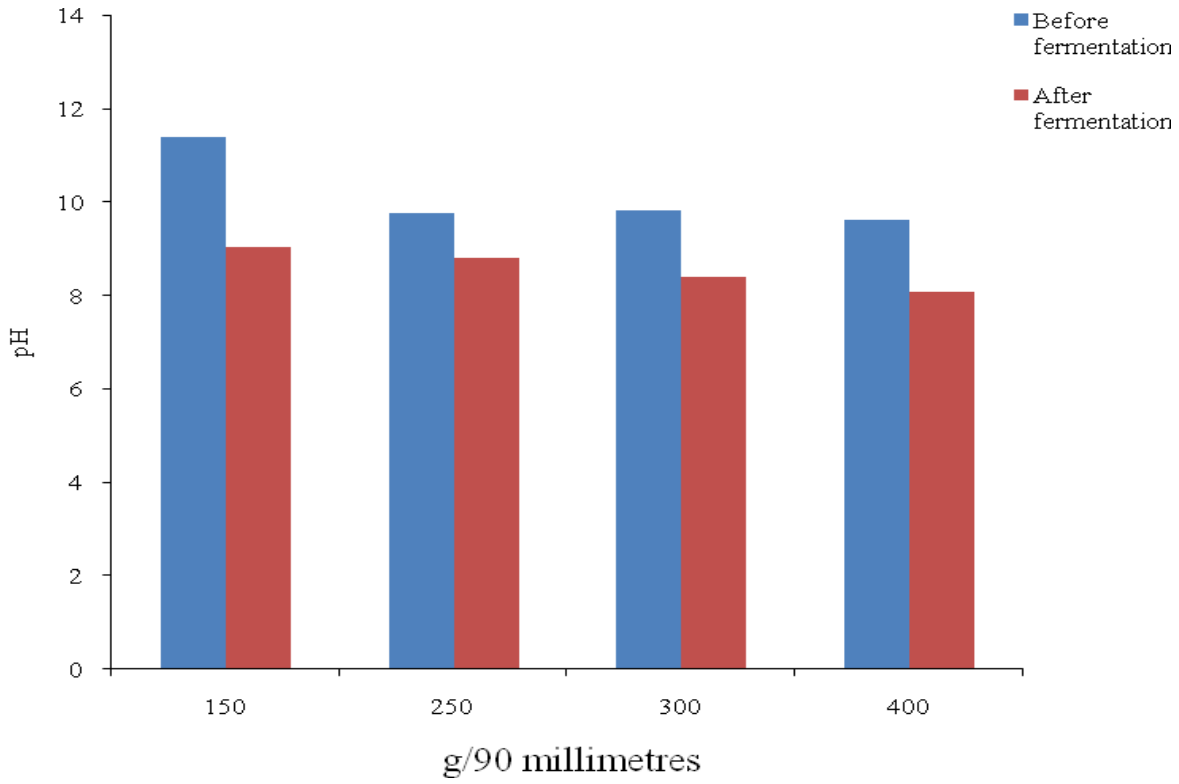


Figure 1. Effect of substrate (seeds of *H. sabdariffa*) concentration on the pH of 'Dawadawan botso'.

Table 7. Comparison of unfermented locally fermented and the effect of ash leachate size on the amino acid (g/100 g protein) profile of fermented seeds of *Hibiscus sabdariffa.*

Amino acid	A	B	E1	E4	E6
Essential amino acids (g/100 g protein)					
Lysine	2.79	3.92	4.30	3.71	3.38
Histidine	2.26	3.07	2.88	2.07	1.82
Threonine	3.39	2.16	3.29	3.39	3.21
Valine	3.95	4.30	4.56	4.87	4.47
Methionine	1.02	1.25	.83	1.44	.73
Isoleucine	3.39	4.27	4.21	5.22	4.83
Leucine	5.38	6.92	8.10	.83	5.82
Phenylalanine	4.57	5.58	5.07	6.37	4.90
Non-essential amino acids (g/100 g protein)					
Arginine	4.94	8.00	8.00	8.35	8.17
Aspartic acid	8.35	9.10	9.35	8.04	7.29
Serine	2.64	2.37	3.08	8.04	2.81
Glutamic acid	9.77	10.19	11.18	3.39	10.92
Proline	2.97	3.19	3.29	3.08	2.97
Glycine	4.96	4.52	4.89	12.29	3.79
Alanine	4.63	4.25	5.17	2.97	4.09
Cystine	1.70	1.44	1.44	4.67	1.31
Tyrosine	1.61	2.26	2.25	3.58	1.93

A = Unfermented; B = locally fermented; E1, 100 g Ash/600 ml water- Before 2nd fermentation; E4, 150 g Ash/600 ml water- After 2nd fermentation; E6, 250 g Ash/600 ml water- After 2nd fermentation.

Table 8. Comparison of unfermented seeds of *H. sabdariffa* and the effect of different seed concentration on the amino acid (g/100 g protein) profile of 'dawadawan botso' .

Amino acid	Unfermented seeds	150 g of *H. sabdariffa* seeds/90 ml ash leachate	250 g of *H. sabdariffa* seeds/90 ml ash leachate	300 g of *H. sabdariffa* seeds/90 ml ash leachate
Essential amino acids (g/100 g protein)				
Lysine	2.79	3.71 (0.92)	4.88 (2.09)	3.17 (0.38)
Histidine	2.26	2.13 (-0.13)	3.01 (0.75)	2.13 (-0.13)
Threonine	3.39	1.83 (-1.56)	2.50 (-0.89)	3.00 (-0.39)
Valine	3.95	3.89 (-0.06)	4.24 (0.29)	3.25 (-0.70)
Methionine	1.02	1.09 (0.07)	0.99 (-0.03)	1.04 (0.02)
Isoleucine	3.39	3.83 (0.44)	4.33 (0.94)	4.08 (0.69)
Leucine	5.38	6.32 (0.94)	7.36 (1.98)	6.04 (0.66)
Phenylalanine	4.57	4.73 (0.16)	5.66 (1.09)	5.24 (0.67)
Non-essential amino acids (g/100 g protein)				
Arginine	4.94	7.49 (2.55)	8.90 (3.96)	8.17 (3.23)
Aspartic acid	8.35	10.10 (1.75)	9.41 (1.06)	8.54 (0.19)
Serine	2.64	3.24 (0.60)	3.18 (0.54)	2.43 (-0.21)
Glutamic acid	9.77	11.24 (1.47)	10.82 (1.05)	9.98 (0.21)
Proline	2.97	3.61 (0.64)	3.19 (0.22)	3.40 (0.43)
Glycine	4.96	4.91 (-0.05)	5.79 (0.83)	4.38 (-0.58)
Alanine	4.63	4.79 (0.16)	5.02 (0.39)	5.41 (0.78)
Cystine	1.70	1.44 (-0.26)	1.44 (-0.26)	1.18 (-0.52)
Tyrosine	1.61	2.58 (0.97)	2.25 (0.64)	1.93 (0.32)

Values presented in parentheses are the differences between the unfermented seeds and 'dawadawan botso'.

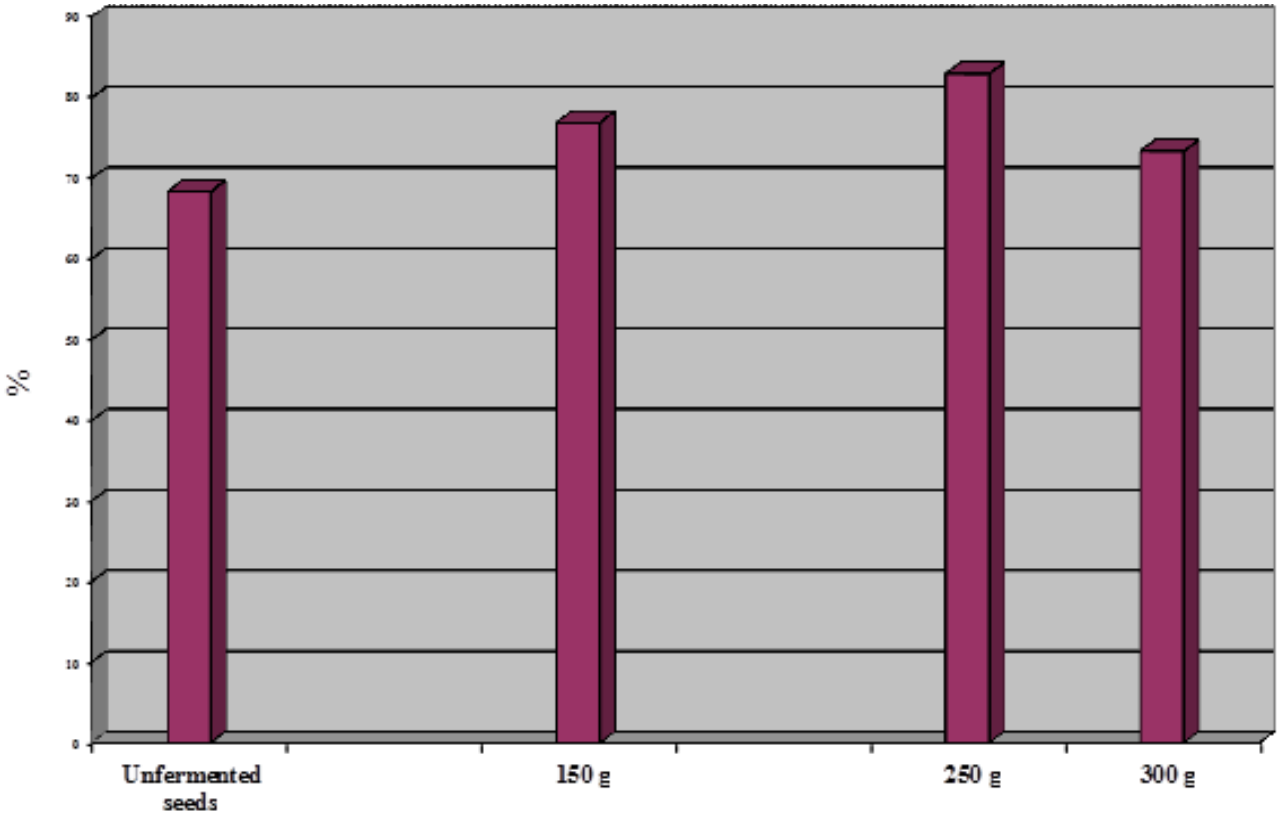


Figure 2. Content evolutions of the total free amino acid (%) in unfermented seeds and "dawadawan botso" produced from varying quantities of *H. sabdariffa* seeds per 90 mL ash leachate.

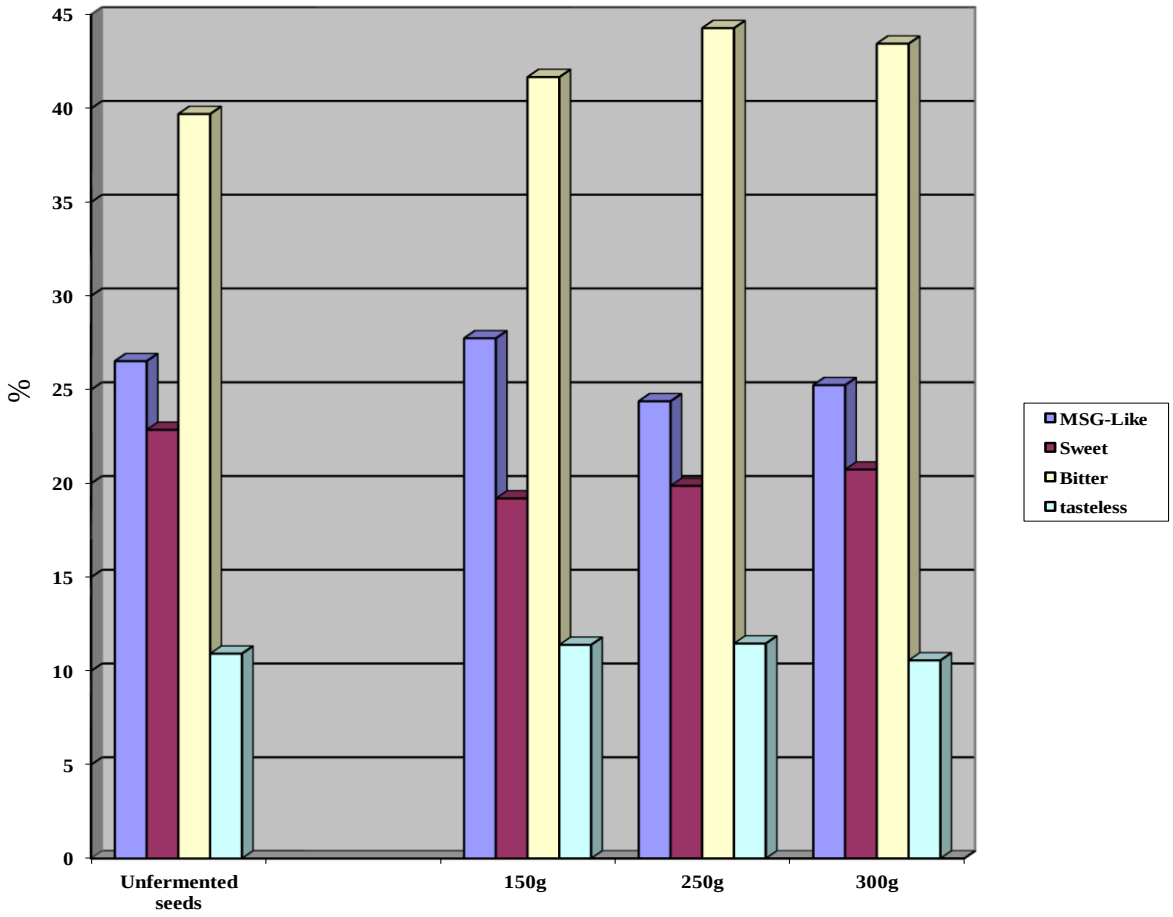


Figure 3. Content evolutions of the individual free amino acid classes (content in percent) that imparted the different taste in unfermented seeds and "dawadawan botso" produced from varying *H. sabdariffa* seeds' quantities per 90 mL ash leachate.

sabdariffa). The pH of the leachate increased with increasing mass of substrate. However, fermentation caused a reduction in pH as the mass of substrate increased. Fermentation also caused an increase in total amino acid profile in the samples.

Conflict of Interests

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

ACKNOWLEDGEMENT

Ibrahim Aliyu Dabai wish to sincerely appreciates the funding support from Nigeria–São Tomé and Príncipe Joint Development Authority through the Joint Development Authority (JDA) 2009 Nigerian Post-Graduate Scholarship.

REFERENCES

Achi OK (1992). Microorganisms associated with natural fermentation of *Prosopis africana* seeds for the production of okpiye. Plant Foods Hum. Nutr. 42:297-304.

AOAC (1995). Official Methods of analysis Association of official Analytical Chemists. Washington DC, U. S. A.

AOAC (Association of Official Analytical Chemists) (2006). Official method of Analysis of the AOAC (W. Horwitz Editor) 18 Ed. Washington, DC.

Argyle JL, Baldwin RL (1989). Effects of amino acids and peptides on rumen microbial growth yields. J. Dairy Sci. 72(8):2017-27

Ayodele PO, Musa AS (2008). Chemical and Proximate Composition of Mwanza ntuza (A Local Condiment) produced from (*Hibiscus sabdariffa*) Seeds during Production. Proceedings of the 32nd annual Conference/General Meeting of NIFST, Ogbomoso: 167-168.

Barber LA, Achinewhu SC (1992). Microbiology of Ogiri production from melon seeds (*Citrullus vulgaris*). Nig. Food J. 10:129-135.

Bengaly M, Bere A, Traore A (2006). The Chemical Composition of bikalga, a traditional fermented roselle (*Hibiscus sabdariffa L.*) Seeds Condiment. Part I. Proximate analysis and Protein quality evaluation. Electron. J. Food Plants Chem. 1:1-6.

Calsamiglia S, Ferret A, Devant M (2002). Effect of p and p fluctuations of Microbial Fermentation and Nutrients Flow from a dual-flow Continous Culture Systems. J. Dairy Sci. 85:574-579.

Hack B (2000). Analytical method of determination of mineral nutrients. In: Text on Analytical in Practice. Dolphin and John S 1st Edn. Incorp Press: New York. pp. 26-33.

Harper DB, Colins MA (1992). Leaf and Seed fermentations of Western Sudan. In: Gaden, EL., Bokanga, M., Harlander, S., Hesseltine, C. W., Steinkraus, K. H. (eds). Applications of Biotechnology in traditional fermented foods. National Academic Press. Washington DC. pp. 105-113.

Ibrahim AD, Sani A, Aliero AA, Shinkafi SA (2011a). Biocatalysis of *H.*

sabdariffa during “Dawadawan botso” production and biogeneration of Volatile Compounds. Int. J. Biol. Chem. Sci. 5(5):1922-1931.
Ibrahim AD, Sani A, Shinkafi SA (2011b). Production, microbial and physico - chemical evaluation of dawadawan botso (A condiment) produced by the fermentation of *Hibiscus sabdariffa* seeds. Int. J. Biol. Chem. Sci. 5(6):2481-2490.
Kim SH, Lee KA (2003). Evaluation of taste compounds in water soluble extract of a doenjang soybean paste). Food Chem. 83:339-342.
Odibo FJC, Umeh AI (1989). Microbiology of the fermentation of *Telferia* seeds for ogiri production. Mircen J. Appl. Microbiol. Biotechnol. 5:217-222.
Odunfa SA (1981a). A note on the microorganisms associated with the fermentation of African locust bean (*Parkia filicoidea*) during iru production. J. Plant Foods 3:245-250.
Odunfa SA (1981b). Microbiology and amino acid composition of ogiri – a food condiment from fermented melon seeds. Die Nahrung 25:811-816.
Odunfa SA (1985). Biochemical changes in fermenting African locust bean (*Parkia biglobosa*) during iru fermentation. J. Food Technol. 20:295-303.
Ogbadu CO, Okagbue RN (1988). Bacterial fermentation of soybeans for daddawa production. J. Appl. Bacteriol. 65:353-356.
Ogbonna DN, Sokari TG, Achinewhu SC (2001). Development of an Owoh-type product from African Yam beans (*Spenostylis stenocarpa*) Hoechst ex. A. Rich) Harms) by solid substrate fermentation. Plant Foods Hum. Nutr. 56:183-194.
Ouoba LII, Diawara B, Annan NT, Poll L, Jakobsen M (2005). Volatile compounds of Soumbala, A fermented African locust bean (*Parkia biglobosa*) Food condiments. J. Appl. Microbiol. 99:1413-1421.
Ouoba LII, Rechinger KB, Diawara B, Traore AS, Jakobsen M (2003). Degradation of African locust bean oil by *Bacillus subtilis* and *Bacillus pumilus* isolated from soumba, a fermented African locust bean condiment. J. Appl. Microbiol. 95:868-873
Parkouda C, Diawara B, Ouoba LII (2008). Technology and physicochemical characteristics of Bikalga, alkaline fermented seeds of *Hibiscus sabdariffa.* Afr. J. Biotechnol. 7(7):916-922.
Popoola TOS, Akueshi CO (1984). Microorganisms associated with the fermentation of soybean for the production of soybean daddawa (as condiment). Nig. Food J. 2&3:194-196.
Sanni AI, Ogbonna DN (1991). The production of owoh-A Nigerian fermented seasoning agent from cotton seed (*Gossypium hirsitium L*). Food Microbiol. 8:223-229.
Schippers RR (2000). *Hibiscus* as a vegetable. African Indigenous vegetables. An overview of the cultivated species: Natural Resources Institute/ACP-EU. Technical Centre for Agricultural and Rural Cooperation. Chatham UK. pp. 119-133.
Spackman DH, Stein EH, Moore S (1958). Automatic Recording Apparatus for Use in the Chromatography of amino acids. Anal. Chem. 30:1191.
Tavaria FK, Dahl S, Carballo FJ, Malcata FX (2002). Amino acid catabolism and generation of volatiles by lactic acid bacteria. J. Dairy Sci. 85(10):24622470.
Tseng YH, Lee YL, Li RC, Mau JL (2005). Non-volatile flavor components of *Ganoderma tsugae*. Food Chem. 90:409-415.
Yagoub AEGA, Mohamed BE, Ahmed AH, Tinay AHE (2004). Study of Furundu, a traditional Sudanese Fermented Roselle (*Hibiscus sabdariffa L*.) Seed: Effect on in vitro Protein Digestibility, Chemical Composition and Functional Properties of the total proteins. J. Agric. Food Chem. 52:6143-6150.
Yimiti W, Yahaya MS, Hiraoka H, Yamamoto Y, Inui K, Takeda M, Tsukahara A, Goto M (2004). Effects of amino acids fermentation by-product on fermentation quality and In situ rumen degradability of Italian Ryegrass (*Lolium multiflorum*) silage. Asian-Aust. J. Anim. Sci. 17(5):633-637

academicJournals

Vol. 13(35), pp. 3576-3580, 27 August, 2014
DOI: 10.5897/AJB2014.13811
Article Number: D20E17847054
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of different feed amounts on the growth performance of gilthead sea bream (*Sparusaurata*) in the Black Sea

Birol Baki

Department of Aquaculture, Faculty of Fisheries, Sinop University, 57000, Sinop-Turkey,

Received 25 March, 2014; Accepted 21 July, 2014

The aim of this study was to investigate the growth performanceof gilthead sea bream (*Sparusaurata*) fed by different feed amounts in the Black Sea. The gilthead sea bream with an average weight of 11.24±0.04 g was fed by restricted amounts of food (Group I) and *ad libitum* (Group II) for 127 days. At the end of the study, the average weight and feed conversion rates of Group I and Group II were 70.58±0.79 and 74.26±0.54 g and 1.68±0.07 and 2.19±0.15 g, respectively. Group II showed higher growth between III and VI periods where the water temperature was relatively high. The growth was limited in the periods between VII to IX under optimal temperature value. At the end of the study, growth differenceswere not significantly differed between the groups (p>0.05). While the final weight, relative growth rate, specific growth rate, feed efficiency and survival were not significantly different (p>0.05), there was a significant difference in feed conversion rate between the groups (p<0.05). At the end of the study, it could be concluded that water temperature is the most important factor in the growth rate of Gilthead Sea Bream in the Black Sea.

Keywords: Black sea, Gilthead sea bream (*Sparusaurata*), feeding, growth, feed efficiency, temperature.

INTRODUCTION

European finfish mariculture spans a broad latitude range, from the Mediterranean basin in the South, to the North Atlantic areas bordering the Arctic Circle. Marine fish cultivation is dominated by three species in this region: salmon (*Salmosalar*), European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparusaurata*). While farmed salmon production was 1,570,327 ton,the global production of farmed European sea bass and gilthead seabream were approximately 163,610 tonnes in 2011 (Anonymous, 2012). Although gilthead sea bream is scarcely found in the Black Sea (Banarescu, 1964; Svetovidov, 1964), it is very common in the other seas surrounding Turkey. Annual gilthead sea bream production in Turkey was 34 tonnes in 1986 and reached to 32,187 tonnes in 2011 (Anonymous, 2012).

The gilthead sea bream is the most common cultured fish species of the Mediterranean area and its production increased during the last decades. In the areas where

*Corresponding author. E-mail: bbaki@sinop.edu.tr. Tel: +903682876265. Fax: +903682876255.

Table 1. The recommended feed amount according to water temperature (%)

Weight (g)	Temperature (°C)			
	13-17	17-22	22-25	25-28
10-35	1.8	2.0	2.2	2.0
35-100	1.4	1.6	1.8	1.6

Table 2. Proximate composition (%) and energy content (Kcal g^{-1}) of the feeds according to the feed company.

Composition	Diet A (1-1.5 mm)	Diet B (3 mm)
Crude protein (%)	55	45
Crude fat (%)	16	20
NFE	9	13
Ash (%)	9.1	10
Cellulose	1.7	1.8
Moisture (%)	8.5	8.5
Vitamin A (IU kg^{-1})	16000	12500
Vitamin D3 (IU kg^{-1})	5000	3000
Vitamin E (mg kg^{-1})	600	300
Vitamin K3 (mg kg^{-1})	80	50
Vitamin C (mg kg^{-1})	1000	500
Gross energy (Kcal g^{-1})	5000	4800
Digestible Energy (Kcal g^{-1})	4300	4100
Metabolic Energy (Kcal g^{-1})	4000	3700

water temperature drops to 9°C, this species suffers from somedisease (Tortet al., 1998). Due to the decrease in the food intake at low water temperature levels, it is emphasized that the given amount of food should be determined in a sensitive manner (Temelli et al.,1991a).

Despite the many studies on feeding requirements, fresh and the pelleted food intake (Company et al., 1990; Goldan et al., 1997; Guinea and Fernandez, 1997), using feed stimulants (Chatzifotis et al., 2009) and alternative raw materials in food of juvenile gilthead sea bream (Emre et al., 2008; Emre et al., 2013), studies on the feed amount and rates of this species are very scarce.

The aim of the present study was to evaluatethe effects of different feed amounts on growth, feed efficiency and survival rate of juvenile gilthead sea bream in the Black Sea conditions.

MATERIALS AND METHODS

Fish stock, rearing condition and experimental design

Origin and maintenance of fish stock

Young gilthead sea bream were obtained from a commercial farm based in the Aegean Sea. The study was conducted between 24 June and 5 November for 127 days. The average weight of fish was 11.24±0.04 g and was transported to the Marine Fish Facilities of Aquaculture and Fisheries Faculty of Sinop University. Rectangular polypropylene tanks with a water volume of 55 L were used for the study. Each tank received running sea water (17-18‰) at 3 L/min.

Diet and experimental design

The study was designed astwo groups withthree replicates. A total of 120 fish (20 individuals for each tank) were used. Fish were weighted every 2 weeks throughout the study period(a total of 9 periods) of 127 days in order to adjust feed ration. Temperature was measured twice a day. The other water quality parameters (dissolved O_2, pH and salinity) were measured once a day during the study.

The feed obtained from a commercial fish feed company (Çamlı Yem Besicilik, İzmir-Turkey) was used for feeding the fish.Two feeding regimeswere used. The first group (Group I) was fed by restricted amounts of food according to the recommended table specification (based on the live weight percentage depending on water temperature) supplied by the company (Table 1). Known quantity of feed was prepared prior the study and the second group (Group II) was fed with the same feed to satiation.After fish cut the taking feed, the remaining feed was weighted to determine the feed consumption in Group II.

The specification of feed was given in Table 2. Since the mouth opening changed during the study, diet A was given until the IV. Period and diet B was given after that time.

Both groups were fed twice a day (in the morning and in the evening). Parameters were calculated according to the formulae given by Steffens (1989), Yiğit et al. (2002) and Yıldırım et al. (2009):

Table 3. The growth performance, food amount, feed conversion rate and survival rates of the experimental groups.

Parameter	Group I (% wet weight)	Group II (Satiation)
Initial total length (cm)	9.11±0.26[a]	9.14±0.23[a]
Initial weight (g)	11.22 ± 0.04[a]	11.25 ± 0.03[a]
Initial condition factor	1.49±0.13[a]	1.47±0.11[a]
Final total length (cm)	16.15±0.13[a]	16.21±0.06[a]
Final weight (g)	70.58 ± 0.79[a]	74.26 ± 0.54[a]
Final condition factor	1.68±0.05[a]	1.74±0.02[a]
Weight gain (fish/g)	59.36	63.01
Total weight gain (g)	1143.8	1266.6
Total feed intake (g)	1922	2127
Feed conversion rate	1.68±0.07[a]	2.19±0.15[b]
Survival (%)	95.00±1[a]	90.00±2[a]

[a,b]Means±SD followed by the same letter, within a row, are significantly different ($p<0.05$).

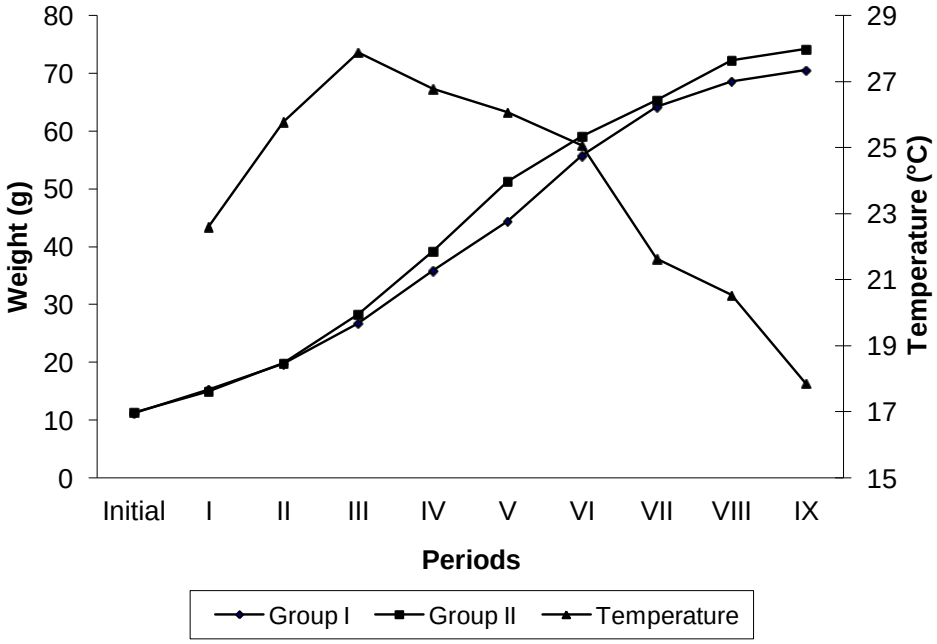


Figure 1. The mean wet weights and temperature during the study.

Condition factor; $W = aL^b$

Weight gain (fish/g): Final weight (g)-Initial weight (g); Feed conversion rate (%): FCR= Total feed intake (g) / wet weight gain (g); Survival (%)= Initial fish number/ Final fish number.

Statistical analysis

Statistical analysis included one-way analysis of variance (ANOVA) and Tukey's multiple significant difference tests using the software program (IBM SPSS 21). Survival rates data were transformed to arc-sin prior to statistical test.Differences were regarded as significant at $p<0.05$ level.

RESULTS AND DISCUSSION

At the end of the study period, except feed conversion rate, no statistically significant differenceswerefound between the two groupsfor severalgrowth performance parameters ($p>0.05$) (Table 3). A decrease in live weight percentage (%) was determined in both feed amount in accordance with the decreasing water temperature (Figure 1). While the highest feeding rate was determined in period IV at the average water temperature of 26.8±1.1°C, the lowest feeding rate was observed in period IX at the average water temperature of 17.9±0.9°C.

The best growing water temperature for gilthead sea bream was reported as 22-24°C (Benli and Uçal, 1990; Alpbaz, 2005). In this research, relative growth rates at the water temperature between 17-29°C were 23.26% in Group I and 24.20% in Group II. However, reduced growth rate was observed by decreasing temperature. Some studies showed that while growth rate was high in spring and summer months, it was low in fall and winter months and the fish got lost weight (Gjerdem and Gunnes, 1978; Okumuş et al.,1997). Şahin et al. (1999) stated thatgilthead sea bream loses weight because of low water temperature after November in Black sea.

Temelliet al. (1991b) reported that gilthead sea bream reached to 27g from 15.38 g and to144.5 g from 110.6 g by feeding 1.4-1.7 and 1-2% of body weight, respectively at 20-26°C after 3months. In the present study, total weight gains were 59.36 and 63.01 g in Groups I and II, respectively (Table 3).

The condition factor values of gilthead sea bream were 1.39-1.84 in the Aegean Sea at 14-18°C water temperature (Temelli et al., 1991b). Şahin et al. (1999) reported that the condition factor values changed between 1.4 and 1.8 in gilthead sea bream in winter months in the East Black Sea.At the end of this research, condition factors were 1.68 and 1.74 in group I and Group II, respectively which werein accordance withthe previously mentioned researches.

The specific growth rate of gilthead sea bream was 0.3 in ponds in the Aegean Sea (Gordin et al., 1987), 0.6 in net cages in the Mediterranean Sea (Bermüdez et al., 1989), 0.6 in net cages in the Aegean Sea (Alpbaz et al., 1991), 0.4 in tank media in the East Black Sea (Şahin et al., 1997),and between 1.1 and 1.3 in cages in the Black Sea (Çiftçi, 1997). It were 1.45 (Emre et al., 2008), 2.12 (Chatzifotis et al., 2009) and between 2.42 to 2.44 in tank media in the Mediterranean Sea (Emre et al., 2013), respectively. In this research, specific growth rates (1.36±0.01 in Group I and 1.40±0.02 in Group II) were higher than thatof the mentioned studies. The differences could be result from the food specifications, the fish size, the cultivation conditions and the seasonal differences.

Feed conversion ratewas 3 in cagesin the Mediterranean Sea (Bermüdez et al., 1989), between 2.3 and 2.4 in cages in the Aegean Sea (Tekin, 1996), between 1.1 and 2.2 in tanks in the Aegean Sea(Gençand Tekelioğlu, 1997), between 1 and 1.8at water temperature of 14.18°C cages in the Aegean Sea (Temelli et al., 1991b), between 1.1 and 1.48 in tanks recirculation water system (Kissil et al., 1997), between 2.1 and 2.2 in tanks in the East Black Sea (Şahin et al., 1999). It was 2.09 (Emre et al., 2008), 1.1 (Chatzifotis et al., 2009) and between 2.05 to 2.08 in tank media in the Mediterranean Sea (Emre et al., 2013), respectively. In our research, the feed conversion rates were 1.68±0.07 in Group I and 2.19±0.15 in Group II.

The growth performance of gilthead sea bream have been tried to be evaluated in the present study with two different feed amount. Group II showed higher growth between III and VIperiods where the water temperature was relatively higher (25.1-27.9°C). The growth was limited in the periods betweenVII-IX where the water temperature was under the optimal value.At the end of the study, growth difference was not significantly different between the groups ($p>0.05$).

While the feed conversion rate was high in Group II at optimum water temperature, same growth values were obtained with restricted feeding under optimum water temperature value. This situation showed that the feed given under optimum water temperature was not converted into the desired weight gain. Ultimately the feed amount rises, and this condition increased the production costs.

Therefore, feeding to satiation by taking into consideration temperature values or feeding regime according to the table values provide both reducing feed costs in production and release of less waste into water.

Conclusions

The present results reveal that temperature significantly affect the growth and feed utilization of gilthead sea bream in Black Sea ambient conditions. It can be concluded that gilthead sea bream juveniles can show optimum growth rate between June and November until the water temperature decrease to the below 17°C in Black Sea.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The support of the University of OndokuzMayıs Research Grants (Project Number: S.079) is gratefully acknowledged. Special thanks to the trout farm-Akbalik Co., Bafra, Samsun, Turkey for supplying experimental fish and feed for the study.

REFERENCES

Alpbaz AG (2005). Aquaculture.Alp Yayınları, İzmir, Turkey. ISBN:975-970-561-3. p. 225.

Alpbaz AG, Özden O, Korkut AY, Güner Y (1991). The breeding of gilthead sea bream (*Sparusaurata*L.) in SÜYO Urla.Ege University Fishery Symposium at the Education in 10. Years. pp. 553-557.

Anonymous (2012). Food and Agriculture Organization of the United Nations. Fisheries Statistics. Fishstat Plus Program (v. 2.32, by Yury Shatz).

Banarescu P (1964). Pisces-Osteichthyes. Fauna Rep. Pop. Romîne. Vol. 13. Ed. Acad. Rep. Pop. Romîne, Bucurest. p. 959.

Benli HA, Uçal O (1990).Rearing Techniques of Marine Organisms. Ministry of Agriculture, Forestry and Rural Affairs, Research Institute of Aquatic Products, Publ.No.3 Series A, Bodrum, Turkey. p. 60.

Bermudez L, Garcia-Garcia B, Gomez O, Rosique MJ, Faraco F (1989). First results of the ongrowing in cages of *Sparusaurata, Puntazzopuntazzo*and*Lithognatusmormyrus*in the Mar Menor (Murcia, S. E. Spain). European Aquaculture Society, Special

Publication Ostende. 10:27-28.
Chatzifotis S, Arias MV, Papadakis IE (2009). Evaluation of feed stimulants in diets for seabream (*Sparusaurata*).Israeli J. Aquac.Bamid. 61(4):315-321.
Çiftçi Y (1997). Gilthead sea bream (*Sparusaurata*L. 1758) rearing in Eastern Black Sea. M.Sc. Thesis, Technical UnivKaradeniz, Inst. Sci., Trabzon Turkey.
Company R, Calduch-Giner JA, Kaushik S, Perez-Sanchez J (1990). Growth performance and adiposy in gilthead sea bream (*Sparusaurata*): risks and benefits of high energy diets. Aquaculture 171:279-292.
Emre Y, Ergün S, Kurtoğlu A, Güroy, Güroy D (2013). Effects of ulva meal on growth performance of gilthead sea bream (*Sparusaurata*) at different levels of dietary lipid.Turk. J. Fish. Aquat. Sci. 13: 841-846.
Emre Y, Sevgili H, Sanli M (2008). Partial replacement of fishmeal with hazelnut meal indiets for juvenile gilthead sea bream (*Sparusaurata*). Israeli J. of Aquac.Bamid. 60 (3):198-204.
Genç MA, Tekelioğlu K (1997). The effects of different stocking densities on growth of gilthead sea bream. Mediterranean Fisheries Congress, 9-11 April 1997, İzmir, Turkey. pp. 743-749.
Gjerdem T, Gunnes K (1978). Comparison of growth rate in Atlantic salmon, Pink salmon, Arctic char, Sea trout and Rainbow trout under Norwegian farming conditions. Aquaculture 13:135-141.
Goldan O, Popper D, Karplus I (1997). Management of size variation in juvenile gilthead seabream (*Sparusaurata*), I Particle size and frequency of feeding dry and live food.Aquaculture 152: 181– 190.
Gordin H, Krom M, Neori A, Popper D, Porter C, Shpigel M (1987). Intensive integrated seawater fish ponds: fish growth and water quality. German-Israeli Cooperation in Aquac.Res. 3. Status Seminer, Tiberias. p. 45-64.
Guinea J, Fernandez F (1997). Effect of feeding frequency, feeding level and temperature on energy metabolism in *Sparusaurata.* Aquaculture 148:125-142.
Kissil WG, Lupatsch I, Higgs AD, Hardy RW (1997). Preliminary evaluation of rapeseed protein concentrate as an alternative to fish meal in diets for gilthead seabream (*Sparusaurata*). Israeli J. Aquac. 49 (3):135-143.
Okumuş İ, Şahin T, Küçük E, Akbulut B, Başçınar N (1997). Growth performance of sea bass juveniles (*Dicentrarchuslabrax*) on Eastern Turkish Black Sea coast.Mediterranean Fisheries Congress, 9-11 April 1997, İzmir, Turkey. pp. 277-282.
Şahin T, Akbulut B, Aksungur M (1999). Investigations on weight loss occuring in winter season and growth of gilthead sea bream (*Sparusaurata*) reared in the Black Sea. Turk. J. Mar. Sci. 5 (2):79-86.
Şahin T, Çiftçi Y, Erteken A, Güneş E (1997). Growth performance of gilt-head sea bream (*Sparusaurata*L., 1758) in the eastern Black Sea conditions.EU J. Fish Aqua. Sci. 14 (1-2):113-118.
Steffens SH (1989).Principles of Fish Nutrition. ElliesHorward.p. 384.
Svetovidov AN (1964). Handbook of the fauna of the USSR, fishes of the Black Sea. Izdatel'stvoNauka, Moscow.p. 550.
Tekin M (1996). Comparative researchs on the breeding of gilthead sea bream (*Sparusaurata*, L. 1758) and sea bass (*Dicentrarchuslabrax*) until market size.PhD Thesis, EgeUniv Fisheries Fac.
Temelli B, Korkut AY, Fırat A, Fırat K (1991a). The research on the using of different rates of mixed feeds in feeding of gilthead sea bream (*Sparusaurata*L.). EU J. Fish.Aqua. Sci. 8(31-32):102-115.
Temelli B, Özden O, Korkut AY (1991b). The feeding of gilthead sea bream (*Sparusaurata*, L. 1758) and sea bass (*Dicentrarchuslabrax*) in different groups.Ege University Fishery Symposium at the Education in 10. Years, 1-8.
Tort L, Rotllant J, Rovira L (1998). Immunological suppression in gilthead sea bream *Sparusaurata*of the North-West Mediterranean at low temperatures.Comp.Biochem. Physiol. 120A:175-179.
Yiğit M, Yardim O, Koshio S (2002). The protein sparing effects of high lipid levels in diets for rainbow trout (*Oncorhynchusmykiss,* W. 1792) with special reference to reduction of total nitrogen excretion. Isr. J.Aquacult. 54:79-88.
Yıldırım Ö, Ergün S, Yaman S, Türker A (2009). Effects of two seaweeds(*Ulvalactuca*and*Enteremorphalinza*) as a feed addition indiets on growth performance, feed utilization and body composition of rainbow trout (*Oncorhynchusmykiss*). Kafkas Univ. Vet. Fak. Derg. 15(3):455-460.

academicJournals

Vol. 13(35), pp. 3581-3590, 27 August, 2014
DOI: 10.5897/AJB2014.13762
Article Number: 953278247055
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Thidiazuron-induced *in vitro* bud organogenesis of the date palm (*Phoenix dactylifera* L.) CV. Hillawi

Ahmed Madi Waheed Al-Mayahi

Department of Plant Tissue Culture, Date Palm Research Centre, University of Basra, Basra, Iraq.

Received 27 February, 2014; Accepted 3 July, 2014

The objective of the present was to enhance the frequency of plant regeneration in date palm (*Phoenix dactylifera* L.) cv. Hillawi. Explants were incubated on Murashige and Skoog (MS) medium supplemented with 1 mgl^{-1} 6-benzyladenine (BA) and different concentrations (0.1 to 2.0 mgl^{-1}) of thidiazuran (TDZ), or free of BA and TDZ (control treatment). The results indicate that the Maximum response (66.67%) was observed on medium supplemented with 1.0 mgl^{-1} BA and 0.5 mgl^{-1} TDZ, producing an average of 4.2 and 18.2 buds per culture after 16 and 24 week from culture, respectively. TDZ at concentrations higher than 0.5 mgl^{-1} resulted in suppressed buds formation, where a decrease in the number of buds was noticed when the concentration of TDZ was increased from 0.5 to 2 mgl^{-1}. Regarding the activity of antioxidant enzyme peroxidase during budding of date palm cv. Hillawi, (The chemical analyses of peroxidase compounds were spectrophotometrically performed) the applied concentration of 0.5 mgl^{-1} TDZ with 1 mgl^{-1} BA enhanced peroxidase activity, where peroxidase activity was associated with increased number of buds formation. Histological studies revealed that adventitious buds were formed directly from epidermal cells without callus formation, and adventitious buds were developed from meristematic cells in shoot tip tissues. Shoots were elongated on 0.5 mgl^{-1} GA3+ 0.1 mgl^{-1} NAA MS media and rooted on MS media supplemented with 0.2 mgl^{-1} of α-naphthaleneacetic acid (NAA). Rooted shoots were successfully acclimatized and established in a mixture of peat moss and perlite (2:1) with 80% success.

Key words: Date palm, thidiazuran (TDZ), adventitious buds, peroxidase (POD), histology.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) the dioecious, monocotyledon species belonging to the family Arecaceae is a multipurpose tree having food, medicinal and ornamental importance. With the present uncertainty in the world food supply and the expected increase in demand, the date palm could be a good source of food of high nutriational value (Khan and Bi, 2012). It has long been one of the most important fruit crops in the arid regions of the Arabian Peninsula, North Africa, and the Middle East (Chao, 2007). Iraq was one of the top ten date producers

*Corresponding author. E-mail: hng_1988@yahoo.com.

producers in the world between 1991 and 2001, where it contributed a 7.5% of the total world dates production (FAO, 2011). The vegetative multiplication of date palm is traditionally achieved by offshoots. This offshoot propagation has limitations such as slow propagation rate, transmission of disease-causing pathogens and insects and production of offshoots in a limited number for a certain period in the lifetime of a young palm tree (Gueye et al., 2009). Date palm readily grows from seeds but half of the seedlings may turn out to be males and high proportion of inferior quality segregates (Al-Khalifah and Shanavaskhan, 2012; Mohammad, 2013). Furthermore, seedlings take 6 to 10 years to fruit, so male and female trees are not identifiable until flowering (Othmani et al., 2009). Hence, *in vitro* propagation is the only available alternative to produce disease free, uniform and good quality planting material to establish large scale cultivation within a short period of time (Zaid and De- Wet, 2002). *In vitro* production is applied through two main protocol, one of them is the somatic embryogenesis and the second is via meristim apexes or buds in the axil bottom of the leaves (Bekheet et al., 2001; Eke et al., 2005). Micropropagation through direct organogenesis lacking callus phase, has the advantage of producing highly identical plants to the mother plants in their vegetative characteristics (Khan and Bi, 2012). Shoot tips are most appropriate explant used for date palm *in vitro* multiplication (Al-Mayahi, 2013). Thidiazuron (TDZ) is a cytokinin-like substance that has often been used for shoot regeneration in recent years (Erisen et al., 2011). Also, it is a powerful regulator of *in vitro* plant regeneration and subsequent growth (Murthy et al., 1998). There are many reports showing that the application of thidiazuron (TDZ; N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) results in a better shoot regeneration capacity in comparison with other cytokinins (Srikandarajah et al., 2001; Zhang et al., 2001; Thomas, 2003; Husain et al., 2007). TDZ is used as a plant growth regulator to stimulate high rate of axillary shoot proliferation in many woody plant species, and its releases the lateral bud dormancy and stimulates shoot formation in wide variety of plant species (Malik and Saxena, 1992; Anandan et al., 2011). In date palm, N-phenyl N'-1,2,3-thidiazol-5-ylurea (TDZ) is used in the stimulation of direct somatic embryo regeneration from shoot tip explants (Sidky and Zaid, 2011). The plant cells possess highly efficient defence systems for elimination of the harmful effect of oxidative stress. Guaiacol- peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6) and ascorbate-peroxidase (EC 1.11.1.11) are among enzymes expressing antioxidative functions, where peroxidase (EC 1.11.1.7) is considered among enzymes expressing antioxidative functions (Kapchina-Toteva and Yakimova, 1997). There are still limited data concerning plant regeneration using thidiazuron (TDZ), and the peroxidase activity for date palm is through direct budding. The overall objectives of this study were to determine the optimal concentration of TDZ for adventitious buds regeneration from shoot tip and investigate the relationship between TDZ and peroxidase through budding in date palm tissues cultured cv. Hillawi *in vitro*. Cultivar Hillawi is a good soft date with good quality and famous in Iraq. It early ripens early and yields about 94.3 kg/palm and it is best for raw eating at rutab stage (Vij et al., 2005). But this cultivar is suffers from some problems; the ageing of the adult plants as well as study the histological events related to direct regeneration from shoot tips.

MATERIALS AND METHODS

Plant material

The experiments were conducted in the Laboratory of Plant Tissue Culture at the Palms and Dates Research Centre, University of Basra. The success of tissue culture largely relies on the selection of suitable explants for use as the starting material for the experiment. The selected offshoots cv. Hillawi were 3 to 4 years old.

Cleaning of explants

Cleaning of the explants was done according to Junaid and Khan (2009). In short, the offshoots were washed with tap water to remove the attached soil and other debris. The outer large leaves and fibers were carefully removed with a sharp knife until the shoot tip zone was exposed.

Sterilization efficient

The explants were kept in 1% sodium hypochlorite solution mixed with one drop/100 ml of Tween-20 for 20 min followed by 4 to 5 rinses in distilled water. It is recommended to give sterilization for 10 min with fresh sodium hypochlorite solution. Then, the explants were immersed in 0.1% mercuric chloride solution for 5 min, followed by 4 to 5 washes in distilled water. Sterilized explants were kept in a cold sterilized solution of ascorbic and citric acid (150 mgl^{-1}) to avoid browning (Al-Khalifah and Shanavaskhan, 2012).

Establishment of initial cultures

In this study, shoot tips were used for culture initiation (Figure 1). The shoot tip terminal, about 1 to 1.5 cm long, was sectioned longitudinally into four sections. Several media were tested. Murashige and Skoog (MS) (1962) basal medium supplemented with 1.0 mgl^{-1} NAA, 1.0 mgl^{-1} Naphthoxy acetic acid (NOA), 1.0 Indol butyric acid (IBA), 1.0 mgl^{-1} kinetin (K), 100 mgl^{-1} glutamine, 5 mgl^{-1} thiamine HCl, 1 mgl^{-1} biotin, 30 gl-1 sucrose, 2.0 gl^{-1} activated charcoal, and solidified with agar at 5.0 mgl^{-1} were used. All the media were adjusted to pH 5.8 with 0.1 N NaOH or HCl, before the addition of agar. Media were dispensed into culture jars. All jars with media were autoclaved at 121°C and 1.04 kg/cm^2 for 15 min. Cultures were kept under complete darkness at 27±2°C which provide the cultivation on initiation medium that enhanced the percentage of explant survival which was 60%, where explants started exhibiting signs expansion after 3 to 4 weeks, as well as reduced browning. Similar reports are given by (Al-Maarri and Al-Ghamdi, 1997; Al-Mayahi, 2014a).

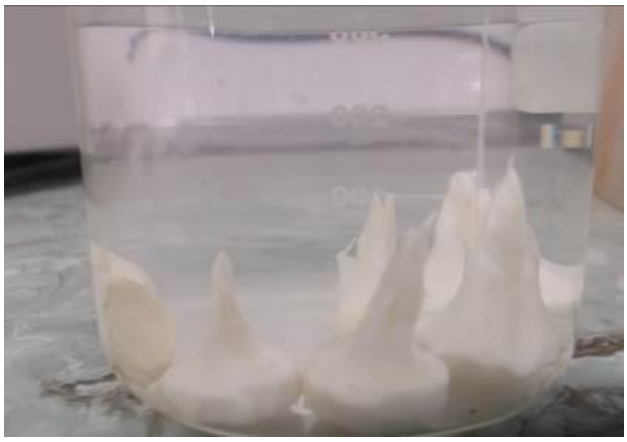


Figure 1. Apical buds used in this study.

Figure 3B. Rooted plantlets ready for transplanting to plastic pots.

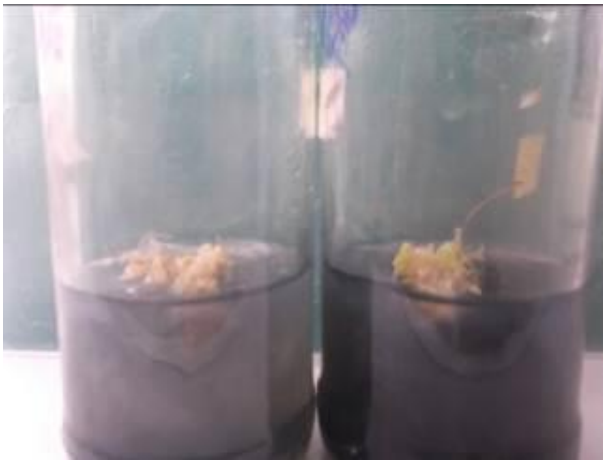


Figure 2. Subcultured buds on to fresh media.

Figure 3A. Shoots formed on the elongation medium.

Buds initiation and multiplication

The developing explants were shifted on initiation and multiplication media (MS) containing 1.0 mgl^{-1} BA and Thidiazuron TDZ at different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}), or used as free of 6-benzylaminopurine (BA) and TDZ (control treatment). All the cultures were incubated in a culture room maintained at 27 ± 2°C under 16/8 h, and light/dark with 55 to 60% relative humidity (RH). Every treatment in the experiment was replicated 6 times. Buds formed on this medium were divided and subcultured on to fresh media every 6 weeks until obtaining enough number from buds to complement this study (Figure 2). The data was recorded as follows: The percentage of response of cultures on direct bud formation after 16 week; number of direct buds/explant after 16 and 24 weeks.

Shoot elongation, rooting and acclimatization of date palm plantlets

Developed buds were isolated and transferred to the elongation medium supplemented with 0.5 mgl^{-1} Gibberellic Acid GA3+ 0.1 mgl^{-1} α-Naphthalene acetic acid (NAA). Well-developed shoots (5 cm long) (Figure 3A) were separated from each other, and transferred individually to rooting medium supplemented with NAA (0.2 mg^{-1}) (Al-Maarri and Al-Ghamdi, 1997). The rooted plants were gently removed from the vessels, washed initially to remove adhered agar and traces of the medium to avoid contamination (Figure 3B). Then, the plantlets were washed with distilled water and treated with fungicide (Benlet 500 mg^{-1}) for 20 min and transferred to plastic pots containing autoclaved a mixture of peat moss and perlite (2:1) (AL-Mayahi, 2014b). The plants were covered with glass bottles to maintain humidity (Figure 3C). The plants were initially irrigated with quarter-strength inorganic salts of MS medium for 2 week followed by tap water. Potted plantlets were grown in culture room (25±2°C, 55±5% RH, under 16 h of photoperiod with a light intensity of 40 µmol m^{-2} s^{-1}) for 45 to 60 days. The glass bottles were gradually removed upon emergence of new leaves and acclimatized plantlets were transferred to the greenhouse.

Estimation of peroxidase at a budding stage

To extract the enzyme, 1.0 g of homogenized buds tissues were ground in 20 ml. cold distilled water in a mortar at 0°C. The extract was obtained by filtering off the debris with a clean cloth and centrifuging at 3000 rpm for 15 min in a refrigerated centrifuge. The

Figure 3C. Coverage of plant by glass bottle.

Table 1. Effect of different concentrations of TDZ in combination with 1.0 BA on percentage of cultures responding and number of buds for date palm cv. Hillawi.

Treatment (mgl^{-1})	Percentage of explants responding (After 16 weeks)	Average number of Buds / explants (S.E)*	
		After 16 weeks	After 24 weeks
0	0.0±0.0^{e}	0.0±0.0^{e}**	0.0±0.0^{e}
0.1 TDZ 1 BA+	33.34±2.06^{c}	2.0±0.2^{c}	0.29 c± 4.0
0.5 TDZ 1 BA+	66.67±1.11^{a}	4.2±0.14^{a}	1.2a±18.2
1.0 TDZ 1 BA+	50.0±1.05^{b}	2.8 ±0.7^{b}	0.14b±7.2
1.5 TDZ 1 BA+	33.34±2.06^{c}	1.5±0.4^{cd}	0.75 d±2.6
2.0 TDZ 1 BA+	16.67 ±0.0^{d}	1.0 ±0.0^{d}	0.0 d±2.0

* ± Standard error (n = 6). ** Values followed by the same letter are not significantly different at P<0.05.

supernatants were recovered and kept in a tube in an ice bath until assayed. POD activity was assayed spectrophotometrically Model CECEIL CE-2021 at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide (Díaz et al., 2001). The reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H_2O_2, 2.66 mL of 0.1 M phosphate buffer pH 7 and 40 µL of the enzyme extract. The blank sample contained the same mixture solution without the enzyme extract.

Histological analysis

Histological examinations during bud formation were carried out using a freezing microtome. Microtome slide preparation and observation were made following the methods as described by Sarker and Awal (1999)

Experimental design and statistical analysis

Completely randomized design was used. The data was subjected to the analysis of variance and mean values were compared using revised LSD at 5% (Snedecor and Cochran, 1989).

RESULTS

Buds initiation and multiplication

The adventitious buds developed from shoot tips on MS medium supplemented with a combination of TDZ and BA after 4 to 6 months of culture without basal callusing (Table 1). The optimal response percentage of explants producing buds with the highest number of buds per explant was recorded on MS medium supplemented with 1 mgl^{-1} BA+ 0.5 mgl^{-1} TDZ. On this medium, 66.67% of the cultures responded (Figure 4A) with an average of 4.2±0.14 and 18.2±1.2 buds per explant after 16 and 24 week, respectively (Table 1, Figures 4A and B), which was statistically significant compared with the other treatments, followed by 1 mgl^{-1} BA + 1.0 mgl^{-1} TDZ (Figure 4C). The response percentage of explants producing buds and frequency of direct bud regeneration decreased significantly when the concentration of TDZ was increased over 0.5 mgl^{-1}, whereas TDZ at higher concentra-

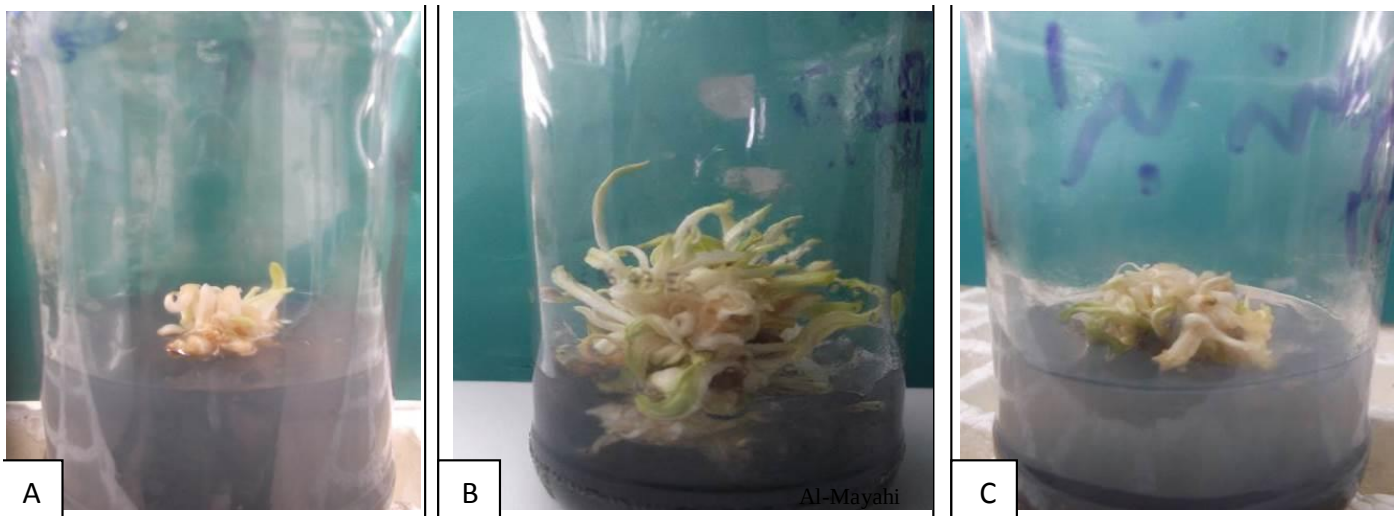


Figure 4. Bud proliferation and multiplication **A)** Bud induction from shoot tip on MS media supplemented with 0.5 mgl^{-1} TDZ + 1.0 mgl^{-1} BA after 16 weeks. **B** and **C)** Bud proliferation on 0.5 mgl^{-1} TDZ + 1.0 mgl^{-1} BA and 1.0 mgl^{-1} TDZ +1.0 mgl^{-1} BA media after 24 weeks, respectively.

Table 2. Effect of different concentrations of TDZ in combination with 1.0 BA on Activity of guaiacol-peroxidase through direct budding in *in vitro* cultured date palm cv. Hillawi.

Treatment (mgl^{-1})	POD activity (U/ml)
0	14.748^{d}
0.1 TDZ 1 BA+	19.794^{c}
0.5 TDZ 1 BA+	33.159^{a}
1.0 TDZ 1 BA+	24.166^{b}
1.5 TDZ 1 BA+	21.039^{c}
2.0 TDZ 1 BA+	16.899^{d}

* Values followed by the same letter are not significantly different at P<0.05.

tions, specially 1.5 and 2.0 mgl^{-1}, resulted in suppressed buds formation. In the absence of BA and TDZ (control treatment) there was not any response for direct buds formation, implying that these compounds are critical for bud regeneration in date palm cv. Hillawi.

Peroxidase "POD" activity

On the basis of the obtained results in the present study, Table 2, illustrates a measure of the activity of guaiacol-peroxidase "POD" regarding the effect of TDZ on bud development of *in vitro* cultured date palm cv. Hillawi. Peroxidase activity in date palm buds was stimulated by TDZ with BA. Thus, cytokinins increase peroxidase activity whereas, lowest activity was observed in control buds. In buds grown on medium containing 1 mgl^{-1} BA+ 2.0 mgl^{-1} TDZ, the activity of peroxidase did not differ significantly from control buds. More increase was exhibited in buds cultured on medium supplemented with 1 mgl^{-1} BA and 0.5 mgl^{-1} TDZ, where activity of peroxidase differed significantly compared with the other treatments.

Histological origin of adventitious budding

The anatomy of buds regenerated *in vitro* by direct organogenesis from shoot tips was investigated in date palm cv. Hillawi. The repeated cultivation on bud-forming medium in the presence of cytokinin, produced tissue masses which rapidly propagated and divided, and can always produce new buds which grow or appear at the surface of the masses or inside them. Histological sections showed that the epidermal cells were the source of organogenesis. The structure exhibited large cells not uniform in size and compactness. Also, there were scattered cell clusters near the epidermis. These cells were distinguished by their fineness and compactness, which was considered as the reason of the protrusions' formation, and consequently the formation of the promeristematic tissue. Simultaneously, with the differentiation of epidermal cells and the subsequent adventitious buds formation, meristematic cells (MC) became more and more abundant due to the continuing division in the shoot tip tissue (Figure 5A). Cell differentiation became evident through appearance of large nucleus undergoing division containing dense cytoplasm. The meristematic isolates emerged from large and strongly vacuolated parenchymatous cells. There meristematic zones could develop into meristems (Figure 5B). From the MC cells, apical meristem (AM) and leaf primordia (LP) differentiated under the same conditions in culture (Figure 5C). Several meristematic regions differentiated and these were

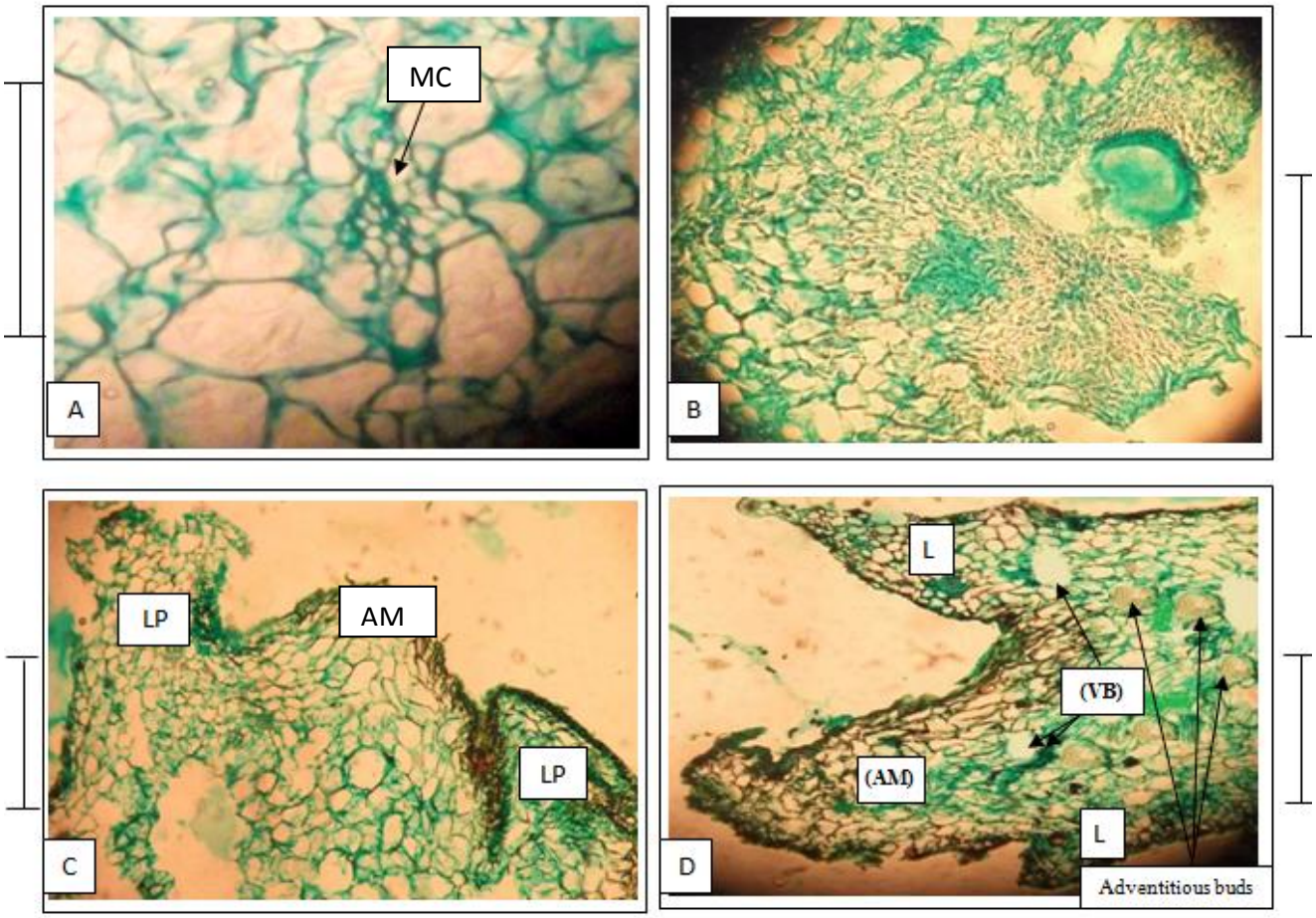


Figure 5. Transverse histological section of an adventitious bud developed from shoot tip of date palm cv. Hillawi cultured on MS + 0.5 mg/l TDZ + 1 mg/l BA. **A)** Mristematic center (MC) originating from the shoot tip, consists of very fine active cells, scale bar 100 µm. **B)** Formation of meristematic which constituted by small cells, scale bar 50 µm. **C)** Apical meristem (AM) and leaf primordia (LP), scale bar 50 µm. **D)** Adventitious buds with apical meristem (AM), leaves (L) and vascular bundles (VB), scale bar 50 µm.

responsible for the formation of adventitious buds. Continuity of cells division led to vascular bundles which can be either induced to form other independent nodules, or differentiating into a bud. It is worth mentioning the adventitious buds induction process showed that it emerged successively from basal superficial of node. Initially, small bulges raised from the epidermal cell of the node (Figure 5D). Also, the nodules developed into buds when moved to an auxin free medium. Such nodules developed into distinct buds or leaf primordium. Figure 5D shows well developed buds with apical meristem, leaves (L) and vascular bundles (VB).

Shoot elongation, rooting and acclimatization of date palm plantlets

The budding tissues formed in the second step, were transferred to the elongation medium supplemented with 0.5 mgl^{-1} GA3 + 0.1 mgl^{-1} NAA. After 8 to 10 weeks, the buds were found from shoots (Figure 6A). The regenerated shoots were transferred to rooting medium MS + 0.2 mgl-1 NAA (Figure 6B), and rooted successfully (80%) with rapid elongation, with an average of 4.4 roots per shoot and shoot of an average root length of 5.2 cm. After 6 weeks of culture (Figure 6C). Complete plants were obtained 6 to 8 weeks after the regenerated plantlets were transferred to this medium. The rooted plants were acclimatized successfully in a mixture of peat moss and perlite (2:1) with 80% after 10 weeks of transferred to plastic pots (Figure 7). All the micropropagated plants were free from external defects.

DISCUSSION

Direct regeneration is the useful means of production of plantlets with a lower risk of genetic instability than by other routes (Khan and Bi, 2012). The composition of induction media is important for adventitious buds

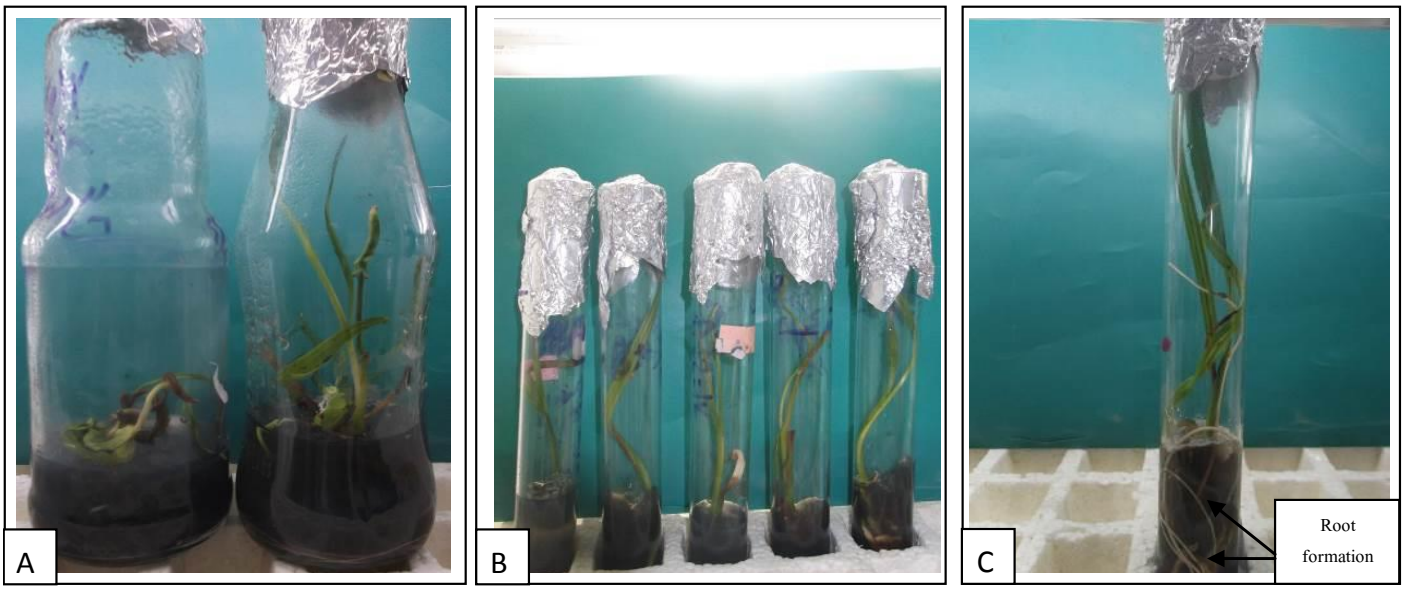


Figure 6. A) elongation of shoots on MS media supplemented with 0.5 mgl-1 GA3+ 0.1 mgl-1 NAA. **B)** Shoots on rooting medium supplemented with 0.2 mgl^{-1} NAA. **C)** Rooting of shoots on same media after 6.

Figure 7. Date palm plantlets transplanted in plastic pots filled with mixture of peat moss and perlite 2:1 ratio (v/v).

development, the presence of cytokinin is critical for buds induction and for differentiation from explants of date palm, where adventitious buds were not observed in cytokinin-free medium (control treatment). Cytokinin (BA) and cytokinin-like compound (TDZ) break apical dominance (Tawfik and Mohamed, 2006). Thidiazuron (TDZ) has gained a considerable attention during the past decades due to its efficient role in plant cell and tissue culture. The highest number of buds (18.2 per explant after 24 weeks) was induced from shoot tip explants on MS medium with 0.5 mgl^{-1} TDZ and 1.0 mgl^{-1} BA (Table 1 and Figure 5B). Such a response may perhaps be due to the increase in the levels of endogenous cytokinins by the effect of the TDZ used, which brings about an increase in the level of naturally occurring cytokinins, and it is likely to have a common site of action with the naturally occurring cytokinins (Ruzić and Vujović, 2008). Also, Casanoval et al. (2004) determined the effects of TDZ on endogenous plant growth regulators in organogenesis as low TDZ levels. Also, the TDZ mediated alteration in the cytokinin biosynthetic pathway might be responsible for the depletion of the endogenous 2iP pool and the elevated concentrations of the other purine metabolites (Zhang et al., 2005). TDZ can highly induce synthesizing, gathering and modifying other produced cytokinins (Visser et al., 1992). It is well known that cytokinins stimulate plant cell division and participate in the release of lateral bud dormancy, in the induction of adventitious bud formation, in the growth of lateral buds and in the cell cycle control (Gaspar et al., 2003). TDZ-treated plant tissues enhanced endogenous auxin metabolism and transport (Murch and Saxena, 2001). Also, Nabila et al. (2003) found that TDZ had been useful for the production of economically important secondary metabolites in some plant species. The other possibilities include the modification in cell membranes, energy levels, nutrient uptake, or nutrient assimilation (Murthy and Saxena, 1998; Murthy et al., 1998; Guo et al., 2011).

Moreover, Abbasi et al. (2011) reported that TDZ inhibits synthesis of abscisic acid according (Li and Yang, 1998), where the frequency of induction and growth of buds was found to vary significantly depending on the concentrations of TDZ. It might be due to the low concen-

trations of TDZ inhibiting synthesis of abscisic acid was more than high concentrations. These results agree with the reports of Lincy and Sasikumar (2010) which suggested that combinations of TDZ and other plant growth regulators could be more effective than TDZ used alone. Also, these results are similar to those reported by Husain et al. (2007) and Husaini and Abdin (2007) who confirmed that the frequency of shoot regeneration ability declined markedly at higher concentrations of TDZ. Also, higher concentrations of TDZ hindered further growth and development of the regenerates (Shirani et al., 2010). Generally, explants cultured on media supplemented with low levels of TDZ were positive for adventitious buds regeneration. TDZ has been shown to stimulate buds regeneration at low concentrations and was used mainly in combination with other plant growth regulators. These results suggest that the response of explants to buds formation ensure the development of budding, necessary to modify the hormonal balance in favour of the cytokinins. Moreover, it is reported that TDZ is the best choice compared with other phytohormones for protoplast proliferation (Murthy et al., 1998; Xiao et al., 2007).

The acceleration of peroxidase activity was associated with increased number of buds under TDZ effect, and had more stability and important role in POD synthesis. The stimulation of peroxidase in the present experiments in accordance to enhancement of bud growth and development at 0.5 mgl^{-1} TDZ, supports the view that TDZ might make this enzyme active, thus controlling the level of H_2O_2 and the rate of cell division (Kapchina-Toteva and Yakimova,1997). In addition, we suggest that the activity of peroxidase could be used as a biochemical marker of development in the plant object studied. Moreover, TDZ promotes the activities of POD, which may be one reason for budding. Sharifi and Ebrahimzadeh (2010) and Mamaghani et al. (2010) reported that antioxidant enzymes play an important role in the organogenesis of 20 plants as confirmed by analysis of POD which was also, reported by Ezaki et al. (1996). This enzyme has high activity as a marker during stressful conditions. In addition, such an enhancement of peroxidase activity can be caused by stress due to changes in media composition; where the TDZ is the induction of a stress response (Murthy et al., 1998; Abbasi et al., 2011), and stress has been considered to be a stimulus to developmental switch by reprogramming gene expression and reorganizing cellular state (Fehér et al., 2003). During this process, some defense-related genes may be induced to adapt the stress conditions. The ability of the explant tissue to survive the applied stresses of the culture process seems to be an integral part of the morphogenic phenomena and some studies provide indication of the factors involved in the regulation of plant regeneration, with various active forms of peroxidase involved in growth regulation, development and organogenesis. The acceleration of enzyme activity was associated with formation of more shoots (Kapchina-Toteva et al., 2005) since plant peroxidases are involved in many functions such as growth, vegetative development, resistance to biotic and abiotic stresses (Gonzalez-Verdejo et al., 2006). The results of the present study are in agreement with the results of other studies related to using cytokinins on peroxidase activity (Synková et al., 2006), and with Wang et al. (1991) who reported that many of the TDZ-stimulated enzymes were associated with cell walls membranes and membrane fluidity was modified.

Also, this result is in accordance with earlier reportof an enhancement of peroxidase activity in response to TDZ (Todor and Iordanka, 1995). As the overall result, it seems that changes in POD activity, is an index for regeneration. TDZ is resistant to oxidases, is stable, but biologically more active at low concentrations. These properties may enhance future use in tissue culture manipulations. These findings may promote further investigations of the physiological properties and selectivity of phenylurea cytokinins. The importance of the cytokinins in releasing the process of meristems and, consequently, formation, is well-known; these formations may be caused by the cytokinins that enhance the multiplication of the DNA, and the chromosomes separation which encourages the cell division (Auge, 1984). The formation of the organs in the moncotyledonae is generally, enhanced by addition of the cytokenines (Duhoux, 1988). Also, the nodules developed into buds when moved to an auxin free medium. Moreover, organogenesis, due to the influence of growth regulators present in the medium, is the result of dedifferentiation of certain cells showing a mitotic activity. This also showed that cell division is initiated in the epidermal layers and that from a multiple-layered epidermis occasional meristematic bulges are produced. Regeneration of adventitious bud meristems formed directly on explants *in vitro* is often initiated by cell divisions beginning in the epidermal. Adventitious buds primordial is initiated as a result of organized directional growth of cells from meristematic cells. Although mitotic activity was found throughout the explant, activity was concentrated in the epidermis regions that were in close contact or adjacent to the nutrient media. Clusters of cells began to appear in the subepidermal region of the explant. Regeneration in this manner is widespread, occurring in monocotyledons (Crinum macowanii) (Slabbert et al., 1995). The initial cell divisions result in a mass of small cells forming new meristematic primordia. Continuity of division cells led to vascular bundles, where development of the vascular bundles is essential to guarantee the transport between tissues and distant organs, assuring the growth.

Conclusion

In the present study, the *in vitro* protocol for regenerating plantlets of date palm cv. Hillawi using shoot tip explants was described. Since the plantlets were developed direct-

ly without intervening of callus phase, it can be concluded that results obtained in this study, permit the development of a mass propagation protocol with a good budding rate and a high regeneration percentage. Data suggest that TDZ and BA were indispensable for *in vitro* propagation of date palm since no excisable shoots were produced on MS-0 medium (control). 0.5 mgl^{-1} TDZ with 1 mgl^{-1} BA are recommended as a component of culture media. Stimulation of number of buds was accompanied by an enhancement of guaiacol peroxidase activity. Histological studies revealed the development of meristematic regions, which later developed into buds meristems.

Conflict of Interests

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

ACKNOWLEDGEMENT

The author thanks Date Palm Research Center, Tissue Culture Laboratory, University of Basra, Iraq, for providing laboratory space and the other facilities used to conduct this work.

REFERENCES

Abbasi H, Zeb A, Xu LL, Wei YH (2011). Thidiazuron: A multi-dimensional plant growth regulator. Afr. J. Biotechnol. 10(45): 8984-9000.

Al-Khalifah NS, Shanavaskhan AE (2012). Micropropagation of Date Palms. Asia Pacific Consortium on Agricultural Biotechnology (APCOAB) and Association of Agricultural Research Institutions in the Near East and North Africa (AARINENA). p. 54.

Al-Maarri K, Al-Ghamdi AS (1997). Micropropagation of Five Date Palm Cultivars Through *in vitro* Axillary Buds Proliferation. D.U.J. for Agric. Sci. 13:49-66.

Al-Mayahi AM (2014a). *In vitro* Propagation of date palm (*Phoenix dactylifera* L.) cv. khaddrawi by direct organogenesis. Jordan J. Agric. Sci.10 (1): 155-166.

Al-Mayahi AM (2014b). Effect of copper sulphate and cobalt chloride on growth of the *in vitro* culture tissues for date palm (*phoenix dactylifera* L.) cv. Ashgar. Am. J. Agric. Biol. Sci. 9(1): 6-18.

Anandan R, Thirugnanakumar S, Sudhakar D, Balasubramanian P (2011). *In vitro* organogenesis and plantlet regeneration of (*Carica papaya* L.). J. Agric. Technol. 7(5): 1339-1348.

Auge R (1984). Physiologies the phenomena related to the achievement of in vitro cultures. In: The *in vitro* culture etses horticultural applications, Ed Technical documentation and LA Lavoisier. Paris. 152p. .

Bekheet SA, Saker MM, Taha HS, Moursy HA (2001). Plant regeneration via somatic embryogenesis in date palm (*Phoenix dactylifera* L.). Arab J. Biotechnol. 4(1) :111-118 .

Casanova E, Valdes AE, Fernandez B, Moysset L, Trillas MI (2004). Levels and immunolocalization of endogenous cytokinins in thidiazuron-induced shoot organogenesis in carnation. J. Plant Physiol. 161 (1): 95-104

Chao CT (2007). The Date Palm (*Phoenix dactylifera* L.) Overview of Biology, Uses, and Cultivation. Hortic. Sci. 42(5):1077-1082.

Díaz J, Bernal A, Pomar F, Marino F (2001). Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annuum* L.) seedings in response to copper stress and its relation to linification. Plant Sci. 161: 179- 188

Duhoux E (1988). Organogenesis and vegetative propagation in trees. In: cultures of cells, tissues and organs Plant. 586 Foundations theoretical and patiques use. Ed presses Poly Technical Romand. Lavoisier. Paris. 308p. .

Eke CR, Peter A, Omorefe A (2005). Somatic embryogenesis in date palm (*Phoenix dactylifera* L.) from apical meristem tissues from 'zebia' and 'loko' landraces. Afr. J. Biotechnol. 4 (3):244-246.

Erisen S, Atalay E, Yorgancilar M (2011). The effect of thidiazuron on the in vitro shoot development of endemic *Astragalus cariensis* in Turkey. Turk. J. Bot. 35:521-526

Ezaki B, Tsugua S, Matsumoto H (1996). Expression of moderately anionic peroxidase is induced by aluminum treatment in Tobacco cells: possible involvement of peroxidase isozymes in aluminium ion stress. Physiol. Plant 96:21–28.

Fao (2011). FAOSTAT-agriculture. http://faostat.fao.org/site/567/default.aspx#ancor

Fehér A, Pasternak TP, Dudits D (2003). Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Org. Cult. 74: 201-228.

Gaspar TH, Kevers C, Faivre-Rampant O, Crevecoeur M, Penel CL, Greppin H, Dommes J (2003). Changing concepts in plant hormone action. *In vitro* Cell. Dev. Biol. Plant 39: 85-106.

Gonzalez-Verdejo CI, Barandiaran X, Moreno MT, Di Pietro A (2006). A peroxidase gene expressed during early developmental stages of the parasitic plant *Orobanche ramosa*. J. Exp. Bot. 57:185-192.

Gueye B, Morcillo F, Collin M, Gargani D, Overvoorde P, Bertossi FA, Taranbargar TJ, Sane D, Tregear J.W, Borgell A, Verdeil JL (2009). Acquisition of callogenic capacity in date palm leaf tissues in response to 2,4-D treatment. Plant Cell Tissue Org. Cult. 99:35-45.

Junaid A, Khan SA (2009). *In vitro* micropropagation of 'khalas' date palm (*Phoenix dactylifera* L.) an important fruit plant. J. Fruit Ornam. Plant Res.17(1):15-27 .

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011). Review Thidiazuron: A multi-dimensional plant growth regulator. Afr. J. Biotechnol.10(45): 8984-9000.

Husain MK, Anis M, Shahzad A (2007). *In vitro* propagation of Indian Kino (*Pterocarpus marsupium* Roxb.) using thidiazuron. *In vitro* Cell. Dev. Biol. Plant 43: 59–64.

Husaini AM, Abdin MZ (2007). Interactive effect of light, temperature and TDZ on the regeneration potential of leaf discs of Fragaria x ananassa Duch. *In vtro* Cell. Dev. Biol. Plant 43:576–584.

Kapchina-Toteva V, Yakimova E (1997). Effect of purine and phenyl urea cytokinins on peroxidase activity in relation to apical dominance of *in vitro* cultivated Rosa hybrida L. Bulg. J. Plant. Phyiol. 23(1–2):40–48.

Kapchina-Toteva VM, Iakimova, ET,Chavdarov IP (2005). Effect of cytokinins on *in vitro* cultured exacum affine balif. Proceedings of the Balkan Scientific Conference Of Biology In Plovdiv (Bulgaria) From 19th – 12st of May (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV): 714–722.

Khan S, BI T (2012). Direct shoot regeneration system for date palm (*Phoenix dactylifera* L.) cv. Dhakkias as means of micropropagation. Pak. J. Bot. 44(6): 1965-1971.

Lincy A, Sasikumar B (2010). Enhanced adventitious shoot regeneration from aerial stem explants of ginger using TDZ and its histological studies. Turk. J. Bot. 34: 21-29

Malik KA, Saxena PK (1992). Regeneration in *Phaseolus vulgaris* L.: High frequency induction of direct shoot formation in intact seedlings by N6- benzylaminopurine and Thidiazuron. Planta 186:384-389.

Mamaghani MS, Shojaei TR, Matinizadeh M, Forootan M (2010). Microsatellite loci and peroxidase alleles correlation in somaclonal variation of Eucalyptus micro theca F. Muell. Afr. J. Biotechnol. 9 (29): 4521-4545

Mohammad (2013). Electrophoretic analysis of protein patterns in date palm "Khlas" cultivar leaflets among different location of Al-Ahsa. Amer. J. Agric. Biol. Sci. 8 (4): 343-349.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–97.

Murch SJ, Saxena PK (2001). Molecular fate of thidiazuron and its effects on auxin transport in hypocotyls tissues of Pelargonium x hortorum Bailey. Plant Growth Regul. 35(3): 269-275

Murthy BN, Murch SJ, Saxena PK (1995). Thidiazuron induced somatic embryogenesis in intact seedling of peanut (*Arachis hypogaea* L.) Endogenous growth regulator level and significance of cotyledons.

Phys. Plant. 94: 268-276.
Murthy BNS, Saxena, PK (1998). Somatic embryogenesis and plant regeneration of Neem (Azadirachta indica A. Juss). Plant Cell Rep. 17: 469-475.
Nabila SK, Fawzia MJ, Naser AA, Rida AS (2003). Growth and rosmarinic acid accumulation in callus, cell suspension, and root cultures of wild Salvia fruticosa. Plant Cell Tissue Org. Cult. 73:117-121.
Othmani A, Bayoudh C, Drira N, Trifi M (2009). In vitro cloning of date palm(Phoenix dactylifera L.) cv. Deglet Bey by using embryogenic suspension and temporary immersion bioreactor (TIB). Biotechnol. Biotechnol. Equip. 23:1181-1188.
Ruzic Dj, Vujovic TI (2008). The effects of cytokinin types and their concentration on in vitro multiplication of sweet cherry cv. Lapins (Prunus avium L.). Hort. Sci. (Prague), 35(1): 12–21.
Sarker RH, Awal ST (1999). In vitro morphogenesis in chickpea (Cicer arietinum L.). Plant Tissue Cult. 9(2):141-150
Sharifi G, Ebrahimzadeh H (2010). Changes of antioxidant enzyme activity and isoenzyme profiles during in vitro shoot formation in saffron (Crocus sativus L.). Acta Biol. Hung 61: 73-89.
Shirani S, Mahdavi F, Maziah M (2010). Morphological abnormality among regenerated shoots of banana and plantain (Musa spp.) after in vitro multiplication with TDZ and BAP from excised shoot-tips. Afr. J. Biotechnol. 8(21): 5755-5761.
Slabbert MM, de Bruyn MH, Ferreira DI (1995). Adventitious in vitro plantlet formation from immature floral stems of Crinum macowanii. Plant Cell Tissue Org. Cult. 43:51-57
Sidky RA, Zaid ZE (2011). Direct production of somatic embryos and plant regeneration using TDZ and CPPU of date palm (Phoenix dactylifera L.). Int. J. Acad. Res. 3:792-796
Snedecor GM, Cochran WG (1989). Statistical Methods. 1st Edn., Iowa State University, Ames, ISBN-10: 0813815614, p. 503.
Sriskandarajah S, Frello S, Serek M (2001). Induction of adventitious shoots in vitro in Campanula carpatica. Plant Cell. Tissue Org. Cult. 67: 295–298.
Synková H, Semorádová Š, Schnablová R, Witters E,Hušák E.and Valcke R (2006). Cytokinin induced activity of antioxidant enzymes in transgenic Pssu-ipt tobacco during plant ontogeny. Biol. Plant 50:31–41.
Tawfik AA, Mohamed MF (2006). In vitro cloning of two cumin landrace lines via shoot-tip culture. J. Hortic. Sci. Biotechnol. 81:264-268
Thomas TD (2003). Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonay explants of mulberry. Biol. Plant. 46: 529–533.
Todor G, Iordanka I (1995). Effect of cytokinin-active phenylurea derivatives on shoot multiplication, peroxidase and superoxide dismutase activities of in vitro cultured carnation. Bulg. J. Plant Physiol. 21(1):73-83 .
Vij VK, Thatai SK, Monga PK (2005). Evaluation of date palm cultivars and irrigated rogations of Punjab. International Conference on mango and date palm: Culture and export 20th to 23nd June 2005.
Visser C, Qureshi JA, Gill R, Saxena PK (1992). Morphoregulatory role of thidiazuron, substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in Geranium hypocotyl cultures. Plant Physiol. 99: 1704-1707.
Wang SY, Jiao HJ, Faust M (1991). Changes in metabolic enzyme activities during TDZ-induced bud break of apple. Hortic. Sci. 26: 171- 173.
Xiao W, Huang X, Huang X, Chen Y, Dai X, Zhao J (2007). Plant regeneration from protoplasts of Musa acuminata cv. Mas (AA) via somatic embryogenesis. Plant Cell Tissue Org. Cult. 90(2): 191- 200
Zaid A, De-Wet PF (2002). In: Date Palm propagation chapter1-5,p.18-138
Zhang CL, Chen DF, Elliott MC, Slater A (2001).Thidiazuron-induced organogenesis and somatic embryogenesis in sugar beet (Beta vulgaris L.). *Invitro* Cell. Dev. Biol. Plant. 37: 305–310
Zhang CG, Li W, Mao YF, Zhao DL, Dong W, Guo GQ (2005). Endogenous Hormonal levels in *scutellaria baicalensis* calli induced by thidiazuron. Russ. J. Plant Physiol.52 (3):345-351.

academicJournals

Vol. 13(35), pp. 3591-3596, 27 August, 2014
DOI: 10.5897/AJB2013.12853
Article Number: 4C3798247056
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Response of okra [*Abelmoschus esculentus* (L.) Moench] to water stress in the soil

Rasmata NANA, Mahamadou SAWADOGO, Zoumbiessé TAMINI

Department of Biology and Plant Physiology, University of Ouagadougou

Received 27 May, 2013; Accepted 21 July, 2014

Water stress effects induced by stopping watering at vegetative (S1) and flowering (S2) stages were studied on five varieties of okra [*Abelmoschus esculantus* Moench (L.)]. Parameters such as relative water content, membrane permeability, chlorophyll content and yield of capsules varieties were evaluated. Our results show that water deficiency reduces relative water content of okra leaves. This reduction is more pronounced when water lack occurs at flowering stage of plants and, leakage of electrolytes across cell membranes is increasing. In addition, soil water deficit increased total chlorophyll content of leaves, which is much higher in stressed plants at vegetative stage. At the vegetative stage as to flowering, water deficit also reduces yield of capsules of varieties UAE 1, UAE 40 and UAE 45. This reduction is much important when plants were stressed at flowering.

Key words: Okra, water deficit, physiology, performance.

INTRODUCTION

Okra has long been considered as a marginal crop. It produces very nutritional and dietary capsules. In addition, it has long been used in traditional cooking and particularly in rural areas but nowadays okra is used in dietary habits of urban dwellers (Sawadogo et al., 2009). As tomato and onion, young capsules of okra is used in many dishes because of their binding power and also because they compete in quantity and especially as a more balanced diet. Its mineral, protein and vitamins A and C contents are significant. According to AADI (American Agency of International Development) (2006), okra is very popular in the USA and in European Union countries, EU market is about 637.837 tons, of which Kenya is the main exporter with 37% of the European market with 23 600 tons during 2000. In addition, okra is nowadays a very profitable vegetable for poor communities, especially for women, due to its strong sales potential on rural and urban markets. Indeed, in Burkina Faso, the turnover of the sale of okra from 2000 to 2004 was estimated at 276 125 364 FCFA (DGPSA, 2009). As a medicinal herb, prospective and epidemiological studies have shown that okra capsules contain antioxidants and okra consumption reduces the risk of cardiovascular disease and some cancers (Anonymous, 2009). The richness of okra mucilage content justifies the use of its sauce as diet food for gastric disorders. In fact, the raw consumption of fresh capsules soothe heartburn esophagus.

However, cultivation of okra faces several constraints, and the main one is the lack of rainfall which limits

*Corresponding author. E-mail: nanaras2001@yahoo.fr.

severely potential production of different varieties. Indeed, in Burkina Faso, national production decreased from 1.600 tons in 1989 to 726 tons in 1998, representing a decrease of approximately 60%. Faced with this situation, research resistant and / or tolerance varieties to water deficit are a promising prospect.

The goal of this work was to focus on physiological parameters of resistance and to evaluate agronomic performance of okra varieties under water deficit.

MATERIALS AND METHODS

Plant materials and culture conditions

Seeds of five (5) varieties of okra (UAE1, UAE19, UAE22 UAE40 and UAE45) were sown in pots filled with a capacity of 15 L a substrate of sandy-loam texture (13.7% clay, 15.7% silt, 70.6% sand). Two weeks after planting, a singling was performed leaving a plant in each pot. The experiment was conducted in a small greenhouse located at the University of Ouagadougou.

Experiment and application of water stress

The approach in this study was to evaluate the response of okra varieties to water interruption during vegetative stage, and also, their response in a situation of water shortage in the flowering stage. We used a factorial "split-plot" completely randomized device with three conditions of water regime, each with three replicates. Alternatives of water supply are: (i) Regular watering every 48 h until the end of the cycle, the control (T); (ii) stop watering at vegetative stage (S1) of for plants 32 days old during two weeks; (iii) stop watering at flowering stage (50% flowering) during 9 days (S2).

Relative water content (RWC) of the leaves

Measurement of relative turgidity has been determined according to the method described by Barrs (1968). The fourth leaf from apex, cut to the base of blade was weighed immediately for fresh weight (FW). Saturation is obtained by placing the whole leaf in a vial containing 200 ml of distilled water and left in the dark at a temperature of 4°C for 24 h. The weight at full turgidity (WFT) is then determined. The dry weight is obtained by drying the leaves in the oven set at 80°C for 24 h. Relative water content (RWC) is calculated using the formula below.

$$\mathrm{RWC}\,(\%) = \frac{FX - DW}{WFT - DW} \times 100$$

DW is the dry weight.

Permeability of cell membranes

In order to estimate damage of cell membranes under water restriction, the permeability of cell membranes was evaluated through the efflux of electrolytes by method of Clement (2003). Thus, five discs were taken from each leaf (third from the apex) and placed in test tubes containing 10 ml of distilled water. The whole is immersed in a water bath at 45°C for 2 h and conductivity (C1) of solution is measured. Samples are then placed in a boiling water bath for 20 min and then cooled to room temperature. Conductivity of solution is measured again (C2). The percentage of electrolyte's leakage (EL) is calculated by the formula.

$$\mathrm{EL} = \frac{C_1 \times 100}{C_2}$$

Leaf chlorophyll content

Chlorophyll content was determined by the method of Mckiney (1941). Indeed, 100 mg of fresh leaves are crushed in a mortar and chlorophyll extracted is recovered with acetone 80%. After filtration of extract, optical density (OD) is read with a spectrophotometer, respectively 663 and 645 nm. Chlorophyll concentrations are calculated using following formulas.

$$\mathrm{Chl\ a} = 12 \times OD_{663} - 2{,}67 \times OD_{645}$$

$$\mathrm{Chl\ b} = 22{,}5 \times OD_{645} - 4{,}68 \times OD_{663}$$

$$\mathrm{Chl\ a} = 12 \times OD_{663} - 2{,}67 \times OD_{645}$$

$$\mathrm{Chl\ b} = 22{,}5 \times OD_{645} - 4{,}68 \times OD_{663}$$

Total chlorophyll is obtained by the sum of chlorophyll a and b.

Measurement of yield

Capsules yield was evaluated after progressive harvest in each three days. Capsules were cut out and dried in natural conditions. Dry weight was determined by weighing with scales whose detail was 0.01 g.

Statistical analysis

Data are presented as an average of three repetitions ± standard error or the standard deviation. Results were analyzed using the analysis of variance (ANOVA) to estimate the significance of effect on the threshold $P < 0.05$ compared to the least significant difference test with Student Newman Keuls. The logical XLSTAT version 7.1 was used for data analysis.

RESULTS

Relative water content of leaves

At vegetative flowering stages, water deficit has reduced relative water content of plants leaves compared to controls (Figures 1A and B). This reduction is relatively higher in stressed plants flowering stage (Figure 1B). Comparisons of means by Newman and Keuls test at $P < 0.05$ indicate an insignificant varietal effect and water treatment at the vegetative stage ($P = 0.64$ and 0.06, respectively). But, at flowering, water treatment has highly significant different ($P < 0.0001$).

Interactions "varieties * water treatment" have shown that at vegetative stage, no significant interaction was found both in the control plants than in those under stress (Table 1).

Instead, at flowering, “varieties * treatment interactions”

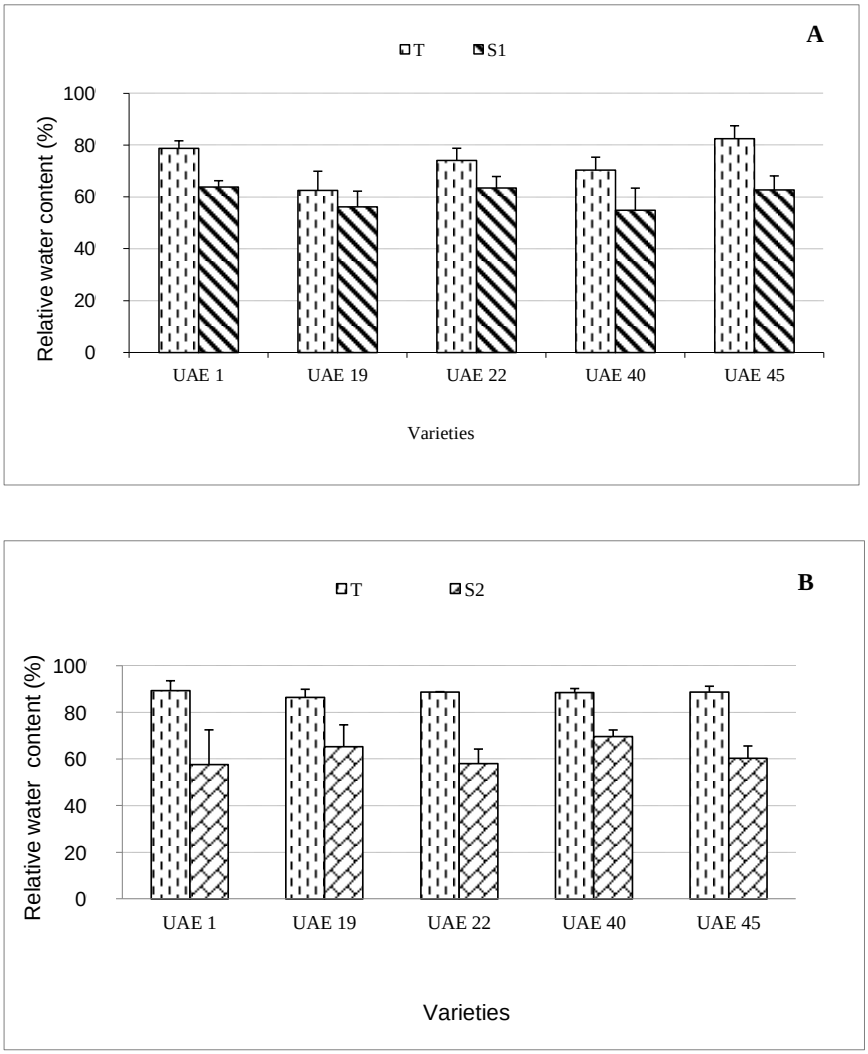


Figure 1. Relative water content of leaves of okra varieties. T = control plants; T1 = stressed plants at the vegetative stage; T2 = stressed plants at flowering.

Table 1. Interaction "varieties * water treatments" on the relative water content.

Varieties	Vegetative stage		Flowering stage	
	T	S1	T	S2
UAE 1	0.213^{a}	0.361^{a}	0.893^{a}	0.675^{c}
UAE 19	0.374^{a}	0.439^{a}	0.904^{a}	0.752^{abc}
UAE 22	0.259^{a}	0.366^{a}	0.887^{ab}	0.689^{c}
UAE 40	0.297^{a}	0.351^{a}	0.885^{ab}	0.896^{abc}
UAE 45	0.174^{a}	0.373^{a}	0.887^{ab}	0.702^{bc}

T= control; S1= water stress at vegetative stage; S2= water stress at flowering In the same water treatment; averages followed by the same letter are not significantly different and belong to the same statistics.

have significant water and were allowed to distinguish two statistics groups in the control: the first group is represented by varieties UAE 1 and UAE 19 and the second group is represented by varieties UAE 22, UAE 40 and UAE 45. In stressed plants, three statistics groups were distinguished: the first is formed by varieties UAE

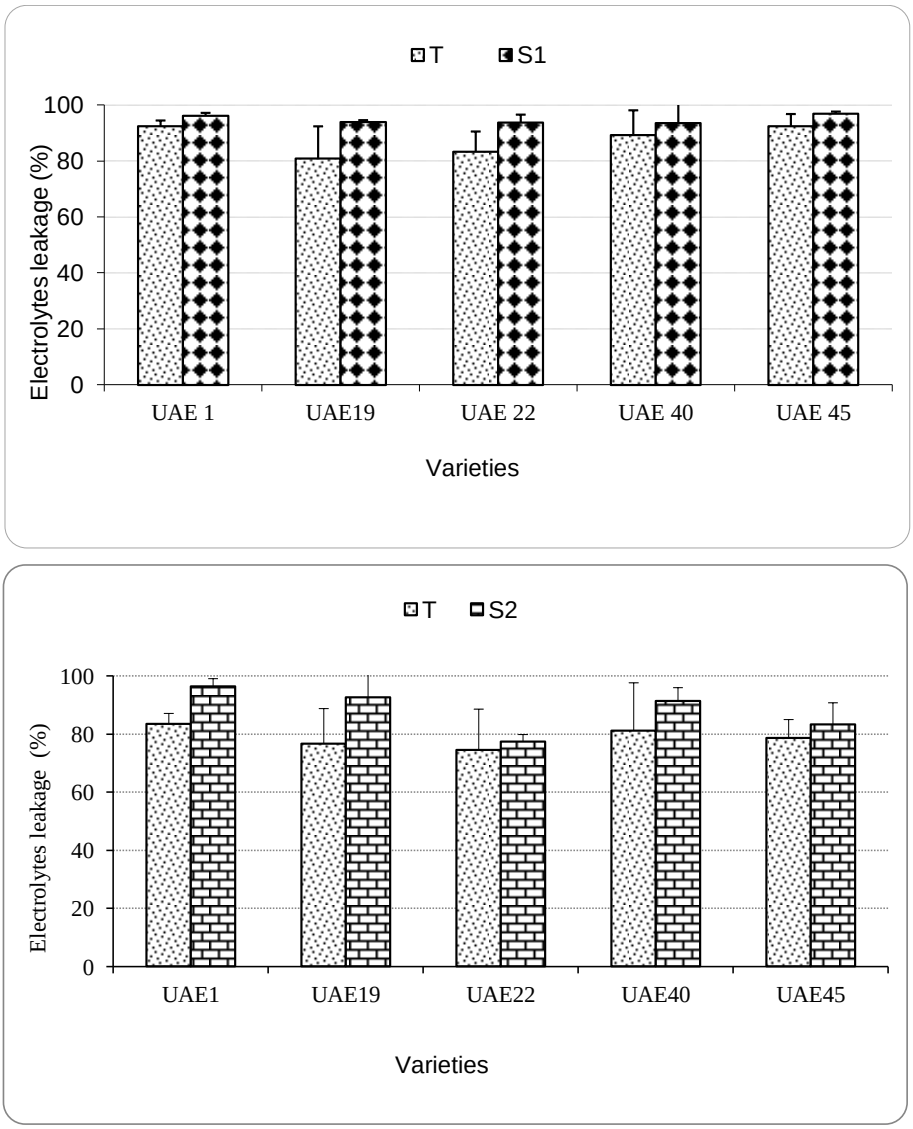


Figure 2. Percentage of electrolytes leakage in the cell membranes. T= control; S1= water stress at vegetative stage; S2 = water stress at flowering.

19 and UAE 40; the second group is represented by the variety UAE 45 and the third group is composed of varieties UAE1 and UAE 22.

Permeability of cells membranes

Permeability of cells membranes evaluated through electrolytes leakage showed an increase in plants stressed at vegetative stage and in those stressed at flowering stage compared to controls (Figures 2A and B). This increase is relatively higher in stressed plants at flowering (Figure 2B). Also, as a result of water stress at flowering stage, varieties UAE 22 and UAE 45 record a small increase in electrolytes leakage compared to other varieties.

The analysis test of variance showed a significant difference (P = 0.016) between varieties under the influence of water deficit at flowering. Regarding water treatments, differences are significant. Indeed, in the vegetative stage, there was a significant difference between control plants (T) and those under stress (S1) with an associated probability (P = 0.003). Moreover, at flowering, the difference between controls (T) and stressed (S2) is significant (P = 0.011).

Chlorophyll content

Chlorophylls *a*, *b* and total content (Table 2) shows that in control plants, chlorophyll *b* (chl *b*) is significantly lower

Table 2. Chlorophyll a, b and total of varieties according to the water treatment.

Parameter	Chlorophyll	UAE 1	UAE 19	UAE 22	UAE 40	UAE 45
T	Chl a	28.31±2.82a	25.57±1.64a	27.34± 2.62a	29.28±2.69a	27.07±1.73a
	Chl b	19.14±1.84a	13.60±1.60bc	8.65±1.66c	16.43±1.43ab	10.98±1.67bc
	Total Chl	47.44±2.47a	39.17±2.06a	35.99±2.12a	45.70±2.85a	38.06±2.38a
S1	Chl a	23.02 ± 2.07a	23.00±2.37a	23.32±2.27a	17.33±2.73a	23.51± .14a
	Chl b	42.31±2.48a	30.81±2.02a	44.00±2.79a	34.66±2.15a	28.62±1.59a
	Total Chl	65.33±3.56a	53.81±2.72a	61.34±3.24a	60.26±3.17a	52.14±3.13a
S2	Chl a	22.51±1.54a	22.70±1.99a	23.70±1.45a	24.64±1.95a	22.55±1.91a
	Chl b	30.33±2.26a	35.76±2.06a	35.48±2.13a	46.54±2.30a	26.09±185a
	Total Chl	52.83±3.94a	58.47±3.98a	59.19±3.11a	71.18±3.88a	48.64±2.50a

T= control, S1= stress at the vegetative stage, S2= stress at flowering stage. For the same water treatment, averages the same row followed by the same letter are not significantly different.

than that of chlorophyll *a* (chl *a*) in all varieties. However, under water stress effect at vegetative stage (S1) as flowering stage (S2), chlorophyll *b* content increased compared to that of controls and is much higher than that of chlorophyll *a*. Moreover, the increase in chlorophyll *b* in stressed plants is accompanied by an increase in *total* chlorophyll.

The analysis of variance shows a highly significant water treatment effect (P = 0.001) and a significant difference between varieties for chlorophyll *b* content. However, for chlorophyll *a* and *total* chlorophyll contents, varietal difference was not significant (P = 0.55).

Capsules yield

Water stress at vegetative stage and that to flowering caused a reduction of capsules yield of varieties UAE 1, UAE 40 and 45. Indeed, capsules yield decrease were respectively 35.5 and 50%, in a variety UAE 1, 14.35 and 50.11% for the variety UAE 40, 16.75 and 50% for variety UAE 45. The reduction is much greater when the water deficit occurs at flowering. Added to this, the reduction of capsules weight of plants stressed. However, varieties UAE 19 and UAE 22 showed a relative increase in the number of capsules per plant, but the average weight of these capsules is lower than that of control plants.

DISCUSSION

Whatever the stage of the development cycle, our results show a decrease in relative water content of leaves of all varieties under the effect of water stress. This decline is consistent with that already found by other authors (Mefti et al., 2000; Hamidou, 2006; Zerrad et al., 2006). However, Abdou Razakou et al. (2013) found under water-stressed the highest percentage of relative water content in some varieties of cowpea.

Electrolytes leakage increase in stressed plants corroborates results of Sawadogo et al. (2006), Hamidou (2006), and Mimoun et al. (2007). This increase can be explained by rupture of the membrane delayed, altering selective power of membranes and therefore an increased perme-ability of cellular membranes. According to Stocker (1961), the water shock causes changes or a destruction of chloroplast membranes and release into the cytoplasm of certain enzymes in cellular organelles. Relatively larger increase in stressed plants at flowering stage is con-firmed with the results of Bensalem and Vieira Da Silva (1991) that showed that the percentage of membrane damage increases with age of the plant. In addition, the increase of total chlorophyll observed in stressed plants is related to the increase of chlorophyll *b*. This is probably an adaptation of leaves to water shortages, to better protect photosystems (PSI and PSII) to ensure the photo-synthetic activity on the one hand and on the other hand, increase probability of survival and maintenance of plants production. Deeplata and Rao (2013) state the levels of okra primary metabolites as chlorophyll are strongly affected by genetic and environmental factors.

The decrease of fruit yield of varieties UAE 1, UAE 40 and UAE 45 under the effect of water stress at vegetative stage as flowering stage explains by delayed formation of reproductive organs in plants stressed at vegetative stage and a fall flowers buds and flowers seen in those under stress during flowering. These results confirm those found by Konyeha and Alatise (2013) which showed that yield and water use efficiency of okra's crop under high irrigation was highest. Son et al. (2011) in the same idea, showed a reduction in number and weight of capsules in sesame in imposition of water stress. The sharp decline in the yield of capsules in plants stressed at flowering reflects a strong sensitivity to soil moisture deficit during the development phase. Heavy water consumption of plants during flowering period was highlighted by Karam et al. (2002) (Table 3).

However, the increase of capsules number of varieties UAE 19 and UAE 22 under stress effect is a paradox. Gunawardhana and De Silva (2011) found results that indicated that okra has high yield when it grown under high temperature environment with no water stress. Our

Table 3. Yield of capsules and its weight of varieties.

Parameter	UAE 1			UAE 19			UAE 22			UAE 40			UAE 45		
	T	S1	S2	T	S1	S2	T	S1	S2	T	S1	S2	T	S1	S2
N Cap	4.00± 1.53	2.50± 1.00	2.00± 0.71	2.00± 0.58	3.33± 0.58	3.33± 1.53	3.00± 0.58	5.00± 1.73	3.67± 1.53	4.67± 1.53	4.00± 1.00	2.33± 1.53	3.33± 0.58	3.33± 1.00	2.00± 0.33
DW Cap^{-1}	2.55± 0.55	2.41± 0.69	1.07± 0.06	3.04± 0.68	1.38± 0.24	1.94± 0.37	2.06± 0.51	1.16± 0.27	1.63± 0.59	2.68± 0.70	2.31± 0.29	2.14± 0.32	2.13± 0.12	1.86± 0.81	1.88± 0.36

N Cap = number of capsules; DW Cap^{-1} = dry weight of capsules.

results can be explained by tolerance mechanisms that allow these varieties to counteract effects of water shortage.

Sorrells et al. (2000) have shown that increased production in drought conditions depends on the tolerance mechanisms that ensure cellular hydration to allow a favorable water status. Also, variety UAE 22 was identified by Sawadogo et al. (2006) as water stress tolerant because its performance of capsules is slightly reduced under water deficit effect. We had previously found that variety UAE 22 is the most productive under short intermittent water stress than others varieties of okra (Nana et al., 2009).

Conclusion

The ability of okra to tolerate the lack of water depends on strategies to reduce relative water content of leaves and to increase total chlorophyll content. It emerge from permeability of cells membranes, an increase of electrolytes leakage in plants stressed at vegetative stage and in those stressed at flowering stage. However, water deficit led to a reduction in the yield of capsules and weight for varieties UAE 1, UEA 40, UAE 45. Of the two stages of development, flowering stage is most vulnerable to water shortages in okra.

Conflict of Interests

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

REFERENCES

AADI (Agence Américain pour le Développement international), 2006. Activité de renforcement de commercialisation agricole en Guinée : la filière gombo (okra) en Guinée. PCE-I-00-99-00003-00, Task Order No. 29, 32p.

Abdou Razakou IBY, Mensah B, Addam Kiari S, Akromah R (2013). Using morpho-physiological parameters to evaluate cowpea varieties for drought tolerance. Int. J. Agric. Sci. Res. 2(5): 153-162.

Anonymous (2009). Richesses méconnues des fruits exotiques tropicaux. 22p.

Barrs H (1968). Determination of water deficit in plant tissues. In: Water Deficit and Plant. Growth, Koslowski, T. (éd.). Academy Press, New York. pp. 235-368.

Bensalem M, Vieira Da Silva JP (1991). Polymorphisme variétal de résistance à la sécheresse chez les céréales à paille. Cas du blé. *L'amélioration de l'adaptation des plantes aux milieux arides*. Ed.AUPELF-UREF. John Libbey. pp. 25-34.

Clément A (2003). *Dosages de différents constituants de la plante et de marqueurs biochimiques de stress*. Cours de formation ECOBIO, CERAAS, 16 p.

Deeplata S, Rao DV (2013). Study of metabolites of okra (*Ablemoschus esculentus)* after infection of pest. Int. J. Pharm. Sci. Rev. Res. 21 (2):347-350.

Direction Générale de Planification et de la Statistique Agricole (DGPSA) (2009). Campagne agricole 2005-2006.

Gunawardhana MDM, De Silva CS (2011). Impact of temperature and water stress on growth yield and related biochemical parameters of okra. Trop. Agric. Res. 23 (1): 77- 83.

Hamidou F (2006). *Paramètres physiologiques, biochimiques et agronomiques pertinents pour l'amélioration de l'adaptation du niébé* (Vigna unguiculata (L.) Walp.) *au déficit hydrique*. Thèse de doctorat de l'Univ. Ouagadougou, 168p.

Karam F, Breidy J, Rouphael J, Lahoud R (2002). Stress hydrique, comportement physiologique et rendement du maïs hybride (cv. Manuel) au Liban. Cahiers Agric. 11 (4): 285-291.

Konyeha S, Alatise MO (2013). Yield and water use of okra (*Abelmoschus esculentus* L. Moench) under water management strategies in akure, South-Western city of Nigeria. Int. J. Emer. Technol. Adv. Eng. 3 (9): 8-12.

Mefti M, Abdelguerfi A, Chebouti A (2000). Etude de la tolérance à la sécheresse chez quelques populations de Medicago truncatula L.Gaerth. Inst. Nat. Agron. 173-176.

Nana R, Zombré G, Tamini Z, Sawadogo M (2009). Effet du régime hydrique sur les rendements du gombo en culture de contre-saison. Sci. Nat. 6 (2): 107 – 116.

Sawadogo M, Balma D, Nana R, Sumda RMT (2009). Diversité agromorphologique et commercialisation du gombo (*Abelmoschus esculentus* L.) à Ouagadougou et ses environs. Int. J. Biol. Chem. Sci. **3** (2):326-336.

Sawadogo M, Zombre G, Balma D (2006). Expression de différents écotypes de gombo (*Abelmoschus esculentus* L.) au déficit hydrique intervenant pendant la boutonnisation et la floraison. Biotechnol. Agon. Soc. Environ. 10 (1): 43-54.

Son D, Compaoré E, Bonkoungou S, Sangaré S (2011). Effet du stress hydrique sur la croissance et la production du sésame (*Sesamum indicum*). Appl. Biosci. 37: 2460-2467.

Sorrells M.E., Diab A., Nachit M. Comparative genetics of drought tolerance. In: Royo C. (ed.), Nachit M. (ed.), Di Fonzo N. (ed.), Araus J.L. (ed.). Durum wheat improvement in the Mediterranean region: New challenges. Zaragoza: CIHEAM, 2000. p. 191-201 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 40).

Stocker O (1961). *Les effets morphologiques et physiologiques du manque d'eau sur les plantes. Recherches sur la zone aride (XV - Echanges hydriques des plantes en milieu aride ou semi-arid*e). Ed. UNESCO, 69 p.

Zerrad W, Hillali S, Mataoui BS, El Antri S, Hmyene A (2006). Etude comparative des mécanismes biochimiques et moléculaires de résistance au stress hydrique de deux variétés de blé dur. Cong. Int. Biochem. 371-376.

academicJournals

Vol. 13(35), pp. 3597-3606, 27 August, 2014
DOI: 10.5897/AJB2013.13000
Article Number: CEB8A6747057
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Henna wood as an adsorptive material for bentazon

Brahmi Mounaouer[2]*, Ahmed Wali[1], Olfa Fourti[2] and Abdennaceur Hassen[2]

[1]Laboratory "Water, Energy and Environment" (LR3E), code: AD-10-02, Department of Materials, National Engineering School of Sfax, University of Sfax, B.P.W.3038, Sfax, Tunisia.
[2]Water Research and Technology Center, Borj Cédria Science and Technology Park, P.O. Box 273, Soliman 8020, Tunisia.

Received 4 July, 2013; Accepted 14 July, 2014

In this study, the efficiency of activated carbon produced from Henna wood was studied to remove herbicide from aqueous solutions by adsorption. The parameters that affect the adsorption such as contact time, activated carbon dosage, initial concentration of adsorbate, stirring rate, temperature, and pH on bentazon adsorption were studied. The use of Henna wood as a raw material to produce activated carbon by physical activation was investigated. The activated carbons produced were characterized by nitrogen adsorption isotherms and point of zero charge properties. Brunauer, Emmett and Teller (BET) surface area of the activated carbon was determined. The removal of herbicide from aqueous solutions by adsorption on activated carbon produced was studied. The results of the present investigation showed that activated carbons prepared from Henna wood have good adsorption capacity for the removal of bentazon from aqueous solution. The Langmuir model provides the best correlation of the experimental equilibrium data. Adsorption isotherm according to BET classification was of Type I. The adsorption isotherms of bentazon revealed that adsorption increased as the concentration increases up to a saturation point. Enthalpy, entropy and free enthalpy adsorption have a negative value indicating that the adsorption of bentazon on the Henna wood activated was feasible, spontaneous and exothermic at 20 to 40°C.

Key words: Activated carbon, adsorption, thermodynamic parameters, Henna wood, Bentazon.

INTRODUCTION

In areas where intensive monoculture is practiced, pesticide use has been the standard method for pest control. Unfortunately, the use of pesticides can also result in environmental problems, such as disruption of predator-prey relationships and loss of biodiversity (Zhang et al., 2010). Additionally, the slow degradation of pesticides in the environment can

*Corresponding author. E-mail: brahmounaouer@yahoo.fr. Tel: +216 79412199. Fax: +216 79412802.

Abbreviations: b, Enthalpy of adsorption (L/mol); **BET,** Brunauer, Emmett and Teller; **Ci,** initial concentrations; **Cf,** final concentrations; **EPA,** environmental protection agency; **FTIR,** Fourier transfer infra-red; **HPLC,** high performance liquid chromatography; **GAC,** granular activated carbon; **ΔG,** free energy change; **ΔH,** enthalpy change; **k1,** pseudo 1st order rate constant; **k2,** pseudo 2nd order rate constant; **LWAC,** activated carbon prepared from *Lawsonia inermis* wood; **Q,** Langmuir adsorption capacity (mmol g^{-1}); **qe,** the adsorption capacity (molg^{-1}); **qt,** the amount of adsorbate adsorbed at time t (mol g^{-1}); **rpm,** rounds per minute; **ΔS,** entropy change; **SEM,** scanning electron microscopy; **V,** volume of sample (mL); **µg,** micro gram; **µL,** micro-liter.

lead to environmental contamination of water, soil, air, several kinds of crops, and Indirectly, of humans (Navalon et al., 2002). Pesticides are artificially synthesized, toxic pesticides in the environment can lead to environmental contamination of water, soil, air, several types of crops, bioaccumulative agents. The on-growing and uncontrolled use of pesticides to fight pests and improve agricultural production constitutes a risk for water quality (Zhu et al., 2006). Thus, pesticides have been detected by monitoring surface and underground waters. According to the European Union Directives and Regulations for drinking water hygiene, the maximum allowed concentration of total pesticides is 0.5 µg dm^{-3} (Zhang et al., 2010). Different types of pesticides can be found in water. The most frequently found pesticides are derivatives of urea, pyridazinone, phenoxy acetic acid, tryazin and the group of chlorinated pesticides (Arcury et al., 2001).

Pesticides can be eliminated from water in different ways, most frequently by adsorption on granular activated carbon (GAC) and/or by ozonization (Arcury et al., 2002). When GAC is saturated, it is usually regenerated and reused. In the majority of cases, spent GAC is thermally regenerated either on-site or transported to a thermal regeneration facility. During regeneration, the contaminants are transformed into less toxic byproducts and the sorption capacity of the carbon is re-established; thus, increasing the useful life of the GAC and the costs of water treatment are reduced (Cabrera and Leckie, 2009).

The adsorption process is being widely used by many researchers for the removal of inorganic and organic pollutants from contaminated streams. Commercially available activated carbon has been frequently employed for thousands of years in many adsorption processes for removal of impurities from liquids and gases (Chowdhury et al., 2011). It contains lot of graphite like microcrystalline unit linked together, similar to that of carbon black (Do, 1996). The effectiveness of activated carbon as an adsorbent is attributed to its unique properties including highly developed internal surface area between 500 and 2000 m^2/g, favorable pore size and high degree of surface reactivity due to presence of surface functional groups, especially oxygen groups (Ismadji and Bhatia, 2001).Their structure is complex and heterogeneous due to presence of micropores, mesopores and macro pores of different size and shape. Despite its extensive use in synthetic waste water treatment, commercial activated carbon remains an expensive material. Therefore, in recent years, the need for economical methods for elimination of pesticides from contaminated water has necessitated research interest towards the production of activated carbon from inexpensive agro based waste material (Choudhari et al., 2013). However, the adsorption property of activated carbon is highly influenced by its preparation conditions. The preparation variables of temperature, time and impregnation ratio will significantly change its surface area, pore size distribution and surface functional groups. Therefore, it is a challenge to produce specific types of activated carbon which are suitable for certain applications.

Bentazon (3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide), known by the trade name Basagran, is a selective post-emergence herbicide used to control many broadleaf weeds and sedges primarily by contact action in most gramineous and many large seeded leguminous crops such as alfalfa, beans, corn, peanuts, peas, asparagus, cereals, peppers, peppermint, rice and sorghum (Pourata et al., 2009). It is also used on two terrestrial non-food crops: ornamental lawns and turf. It has little effect on germinating seeds, and is not used pre-emergence (irrelevant) (Garrido et al., 1998).

In soil, Bentazon has an average soil half-life of 20 days and is often undetectable after 42 days. Soil microbes are responsible for rapid degradation of bentazon (Sims et al., 2009). In water, the soil organic carbon sorption coefficient, Koc, is 34 mL/g, indicating a weak sorption to soil particles, and the solubility is 500 mg/L. Due to rapid microbial degradation and photodegradation, bentazon has a low leaching potential despite having a low K_{oc} and a high solubility. In surface water, bentazon photodegrades with a half-life of 63 h (Pappiernik et al., 2007).

Generally, the environmental fate of herbicides depends on the chemical transformations, degradation and transport. Transformation determines which herbicides are degraded in the environment, and how many pesticides and their metabolites (degradation products) are present in the environment, where and for how long (Fontecha et al., 2008). Indeed, it is necessary to remove them from wastewater. Activated carbon is the most widely used adsorbent because of its extended surface area, microporous structure, and high adsorption capacity related to its great degree of surface reactivity. Many reports have described the successful use of the activated carbon as an adsorbent for the purification of water (Pintar, 2005; Ania and Beguin, 2007).

Lawsonia inermis wood is a shrub or small tree frequently cultivated in dry tropical and subtropical zones, including North Africa, India, Sri Lanka and the Middle East (Chung et al., 2002). Several studies have been carried out using activated carbon as an adsorbent by two different activation methods, physical and chemical (Garrido et al., 1998). Physical activation implies the pyrolysis of the precursor followed by the activation with gases, such as carbon dioxide, air, steam or a mixture of them. Chemical activation consists of the pyrolysis at relatively low temperature under the presence of an activating agent.

The objective of this work was to study the removal of bentazon from aqueous solutions using activated carbon prepared from *L. inermis* wood (LWAC). The adsorbent was prepared and characterized by surface and pore structural characteristics, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR) analyses. The effects of contact time, initial concentration of bentazon, activated carbon dosage, stirring rate,

temperature and initial solution pH were studied. The removal capacity of LWAC was studied by fitting the adsorption data to kinetic and isotherm models. Desorption studies of the used LWAC were also undertaken.

In this study, activated carbon was produced by physical activation of Henna wood and characterized, regarding its textural and surface chemical properties. The thermodynamic parameters such as enthalpy, entropy and free enthalpy adsorption were also considered from the adsorption isotherms measurements.

MATERIALS AND METHODS

Adsorbent preparation

The stems of *L. inermis* were collected from the field in Gabes (Tunisia). Its geographical coordinates are 33° 53' 0" north and 10° 7' 0" east. These materials were firstly carboned with distilled water to remove impurity such as sand and leaves and soluble and colored components, dried at 110°C for 12 h, crushed in a steel blender, and sieved to obtain a particle size in the range of 1-2 mm. Bentazon obtained from BASF Aktiengesellschaft, 67056 Ludwigshafen, Germany, was used as adsorbate. Distilled water was used in adsorption experiments.

Active carbon preparation

Activated carbons were prepared from Henna wood by carbonization under nitrogen flow and activation under water vapor. Carbonization was carried out in a vertical stainless-steel reactor (length 170 mm, interior diameter 22 mm) which was inserted into a cylindrical electric furnace Nabertherm (LT 5/11HA, Model 976 Gemini R, Lilienthal, Germany). The dried wood was placed into the reactor and heated from room temperature to 400°C at a constant heating rate of 10°C/min under nitrogen flow, then held at 400°C for 1 h. The samples were left to cool down after the carbonization. Activation was carried out in the same furnace. The charcoal obtained was then physically activated at 850°C for 2 h under a nitrogen flow (100 cm^3/min) saturated in steam after passing through the water saturator heated at 80°C. The tenor steam was fixed at 0.395 kg H_2O /kg N_2. After activation, the sample was cooled to ambient temperature under N_2 flow rate. The produced activated carbon was then dried at 105°C overnight, ground and sifted to obtain a powder with a particle size smaller than 45 µm, and finally kept in hermetic bottle for subsequent uses.

Active carbon characterization

The surface area of the Henna wood was calculated from the BET equation (Brunauer–Emmett–Teller) within the 0.01-0.15 relative pressure range. Nitrogen adsorption/desorption isotherms were measured at 77 K on an automatic adsorption instrument (Quantachrome Instruments, Model Nova1000e series, USA) in relative pressure ranging from 10^{-6} to 0.999. Prior to the measure-ment, all the samples were crushed and powdered to shorten the time required for reaching equilibrium in the isotherm study and degassed at 250°C under nitrogen flow for 16 h.

The nitrogen adsorption-desorption isotherms were used to determine the following parameters: specific surface area SBET (according to the BET equation), total pore volume V_{tot} (calculated from the nitrogen uptake at relative pressure of 0.99 by assuming that the pores are then filled with liquid adsorbate (N_2)), total micropore volume V_{micro}, according to simplified equations (Nickolov and Mehandjiev, 2000), total mesopore volume V_{mes} (determined by subtracting the micropore volume from the total pore volume) and external surface area were calculated by using the t-plot method of Lippens and de Boer (Lippens and de Boer, 1965). The surface morphology of the samples was examined using a scanning electron microscope (Philips XL30 microscope model, Chungmoon City, Cheju Island).

Fourier transform infrared spectroscopy (FTIR)

The surface functional groups of the prepared activated carbon were detected by Fourier Transform Infrared spectroscope using Shimadzu FTIR 8400S. A spectrum was recorded in the mid-IR range from 4000 to 400 cm^{-1} with a resolution of 1 cm^{-1}.

Point zero charge measurements (pHpzc)

Batch equilibrium technique was applied to determine the pH at the zero point of charge. By using the pH drift method (Faria et al., 2004), the pH at the potential zero charge (pHpzc) of various activated carbons was measured. The pH of a solution of 0.1 M NaCl was adjusted between 2-12 by using 0.01 mol L^{-1} NaOH. Activated carbon sample (0.2000 and 0.6000 g) was added into 100 mL of NaCl solution in 250 mL flask. The flasks were sealed, to eliminate any contact with air and then left at ambient temperature. The final pH was recorded after the pH had stabilized (typically after 24 h). The point at which initial pH and final pH were the same value was determined by using the graph of final pH versus initial pH. This was taken as the pHpzc of the activated carbon.

Adsorption studies

Batch equilibrium studies

Adsorption tests were conducted in batch mode using different concentrations of bentazon varying from 8 to 84 mg/L. However, 100 ml of bentazon solutions were put in Erlenmeyer flasks (250 mL) and equal mass of 50 mg of the prepared activated carbon was added to each flask and kept in an isothermal shaker of 200 rpm at 20, 25, 30, and 35°C, respectively for 2 h to reach equilibrium (Omri et al., 2012). The pH of the maximum adsorption of these herbicides in solutions by the activated carbon prepared was estimated at 3.3 values. Aqueous samples were taken from the herbicide solution and the concentrations were evaluated. All samples were filtered prior to the analysis to minimize the interference caused by fine carbon particles.

The concentrations of pesticides in supernatant before and after adsorption were determined by spectrophotometry at a wavelength of 224 nm, using a Shimadzu UV-VIS spectrophotometer 1650. A previously established linear Beer-Lambert relationship was used in the concentration analysis. The equilibrium adsorption capacity Q_e (mg/g) at different concentrations was calculated according to Equation 1:

$$Q_e = \frac{(C_i - C_e)\ V}{m} \quad (1)$$

Where, C_i and C_e (mg/L) are the liquid-phase concentrations of bentazon at initial and equilibrium, respectively. V is the volume of the solution (L) and m is the mass of dry adsorbent used (g).

Adsorption isotherms

Successful application of the adsorption technique demands studies based on various adsorption isotherm models (Colak et al., 2009)

because adsorption isotherm models clearly depict the relationship of amount adsorbed by a unit weight of adsorbent with the concentration of adsorbent remaining in the medium at equilibrium. Thus following Freundlich and Langmuir isothermal models were applied to the experimental data.

The Freundlich model is based on the assumption that adsorption occurs on a heterogeneous adsorption surface having unequally available sites with different energies of adsorption (Colak et al., 2009) and is given by the relation:

$$Q_e = K_F C_e^{\frac{1}{n}} \quad (2)$$

Where, K_F is roughly indicator of the adsorption capacity and 1/n is the adsorption intensity. In general, as the K_F value increases the adsorption capacity of the adsorbent increases. The magnitude of the exponent 1/n marks a favorable adsorption and values of $n > 1$ indicate a favorable condition of adsorption (Malik, 2003). Equation 2 can be rearranged to linear form to give Equation 3:

$$\ln Q_e = \ln K_F + \frac{1}{n} \ln C_e \quad (3)$$

Where Q_e is the amount adsorbed (mol/g), and C_e is the equilibrium concentration of the adsorbate (mol/L). K_F and n, the Freundlich constants, are related to adsorption capacity and adsorption intensity, respectively. The slope (1/n) and intercept (K) of a log-log plot of Q_e versus C_e are determined.

The Langmuir isotherm assumes that the surface of any adsorbent material contains a fixed number of active sites and saturation of these active sites stops the adsorption of the adsorbate (Langmuir, 1918). This indicates that the adsorption occurs until a monolayer of adsorption is completed and after completion of adsorption, no more interaction between the adsorbent and adsorbate molecules takes place (Li et al., 2009). It was adapted to model isotherms and to calculate the bentazon adsorption capacities of the produced carbons. The Langmuir isotherm used for adsorption of bentazon from the aqueous solution is given by the following Equation 4:

$$Q_e = \frac{Q_m K_L C_e}{1 + K_L C_e} \quad (4)$$

Where, Q_e is the maximum amount of adsorption and corresponds to the complete monolayer coverage on the surface (mg/g), C_e is the adsorbate equilibrium concentration (mg/g) and K_L is the Langmuir constant (L/mg). Q_m represents a practical limiting adsorption capacity when the surface is fully covered with adsorbate molecules and aids in the comparison of adsorption performance. Equation (4) can be rearranged to linear form to give Equation 5:

$$\frac{C_e}{Q_e} = \frac{C_e}{Q_m} + \frac{1}{K_L Q_m} \quad (5)$$

Where, constants K_L and Qm relates to the energy of adsorption and adsorption capacity and their values are obtained from the slope and intercept of the plot of Ce/Qe versus Ce for different temperature. The linear nature of the plot shows that the adsorption follows the Langmuir isotherm. The value of K_L, which is a measure of heat of adsorption is utilized to calculate dimensionless separation parameter RL (Weber and Chakraborti, 1974). Indeed, Weber and Chakarborti (1974) expressed the essential characteristics and the feasibility of the Langmuir isotherm in terms of a dimensionless constant separation factor RL, which is defined as:

$$R_L = \frac{1}{1 + K_L C_0} \quad (6)$$

Where, K_L is the Langmuir constant and C_0 is the initial concentration of herbicide. According to McKay et al. (1982), RL values between 0 and 1 indicate favorable adsorption.

Calculation of thermodynamic parameters

The thermodynamic equilibrium constant (b) is obtained by calculating the apparent equilibrium constant (b') at different initial concentrations of bentazon and extrapolating to zero (Bhattacharya et al., 2006):

$$b' = \frac{Ca}{Ce} \quad (7)$$

Where, Ca is the concentration of bentazon on the adsorbent at equilibrium in mg/L and Ce is the equilibrium concentration of bentazon in solution in mg/L.

According to the biosorbent dependency on the structure and surface functional groups, temperature has an impact on the adsorption capacity to a certain extent. It is well known that a temperature change alters the adsorption equilibrium in a specific way determined by the exothermic or endothermic nature of a process. The Gibbs free energy change ($\Delta G_{ad}{}^0$), enthalpy ($\Delta H_{ad}{}^0$) and entropy change ($\Delta S_{ad}{}^0$) are very important thermodynamic parameters of adsorption that can confirm the feasibility, spontaneity and heat change for the biosorption process. Parameters like Gibb's free energy ($\Delta G_{ad}{}^0$), change in entropy ($\Delta S_{ad}{}^0$), change in enthalpy ($\Delta H_{ad}{}^0$), etc. have been calculated from the relations (Amin, 2009):

$$\Delta G_{ad}{}^0 = -RT \ln b, \quad (8)$$

$$\Delta H_{ad}{}^0 = -R(T_2 T_1 / T_2 - T_1) \times \ln(b2/b1), \quad (9)$$

$$\Delta S_{ad}{}^0 = \Delta H_{ad}{}^0 - \Delta G_{ad}{}^0 / T, \quad (10)$$

Where, R is a universal gas constant, b, b1 and b2 are the Langmuir constants at 20, 25, 30 and 35°C, respectively, obtained from slopes and intercepts of Langmuir isotherms.

Integral enthalpy and entropy have been used to explain the bentazon adsorption. Generally, the entropy evolution is similar to the enthalpy. Integral enthalpy is needed to control integral entropy associated with the adsorption process. The negative values of free enthalpy indicate that adsorption is spontaneous.

RESULTS AND DISCUSSION

Characteristics of *L. inermis* wood-based activated carbon

Specific surface area and pore structure of LWAC

Identifying the pore structure of adsorbent, which is commonly determined by the adsorption of inert gases, is

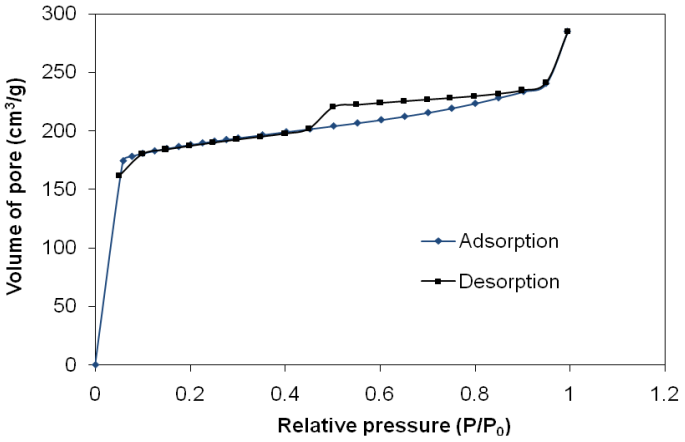


Figure 1. Adsorption-desorption isotherm of the activated carbon.

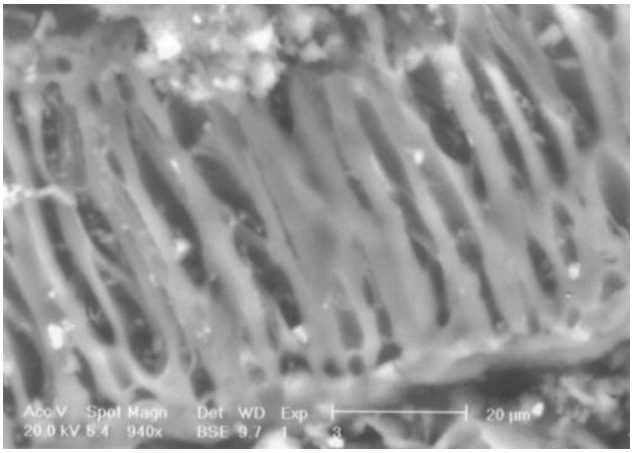


Figure 2. SEM image of Henna wood.

Table 1. Physical and chemical properties of the prepared activated carbon.

Parameter	Values
Specific surface area, S_{BET} (m^2/g)	584
Surface area micropore, S_{micro} (m^2/g)	483
External surface area, S_{ext} (m^2/g)	100
Micropore volume, V_{micro} (cm^3/g)	0.245
Total pore volume, V_T (cm^3/g)	0.441
Point zero charge (pH_{PZC})	11.32

an essential procedure before designing the adsorption process (Saritha et al., 2007). Figure 1 shows the adsorption-desorption isotherm of N_2 at 77 K. Adsorption data were obtained over the relative pressure, P/P_0, range from 10^{-7} to 1. Table 1 presents the physical pro-perties of Henna wood. The surface area of Henna wood was found to be 584 m^2/g, which was much higher than conventional activated carbons, that is, activated carbons from olive-tree wood reconsidered (474 m^2/g) (Rajashekara et al., 2007), activated carbon from sawdust of Algarroba (549 m^2/g) (Ahmad et al., 2008) and wood coal-based activated carbon (331 m^2/g) (Maldonado et al., 2006).

The graph of the adsorption isotherm in Figure 2, is a Type I isotherm and this indicates that the activated carbon is microporous. Initial step region of the isotherm is abruptly followed by a plateau indicating that the adsorption has virtually stopped because multilayer of adsorbate cannot be formed due to close proximity of the pore wall. It can be seen from Table 1 that most pores of Henna wood are in the micropore range, the micropore volume (Vmicro) occupies 55% of the total pore volume.

Surface morphology of LWAC

The SEM micrographs of LWAC sample are given in Figure 2 indicating that the porosity of the material was produced by attack of the reagent (H_2O) during activation. The increase in the steam tenor supports the following gasification process under high temperature (Ali and Gupta, 2006):

$$HO + C \rightarrow H + CO \quad (11)$$

After undergoing carbonization and activation process, the volatile matter content decreased significantly whereas the fixed carbon content increased in Henna wood. This was due to the pyrolytic effect where most of the organic substances have been degraded and discharged as gas and liquid tars leaving a material with high carbon purity (Ren et al., 2011). Halim et al. (2010) found that increasing the carbonization temperature decreased the yield progressively due to release of volatile products as a result of intensifying dehydration and elimination reaction.

Function groups of LWAC

The FTIR spectrum of Henna wood is illustrated in Figure 3. A wide absorption band at 3200-3600 cm^{-1} with a maximum at about 3411 cm^{-1} is assigned to O-H stretching vibrations of hydrogen bonded hydroxyl groups (Wang et al., 2007). Aliphatic C-H stretching vibration is found as a very weak peak at 2884 cm^{-1} while asymmetric vibration of CH_2 group appears at 2915 cm^{-1}. The bands located at about 1609 and 1414 cm^{-1} were attributed to carbonyl (e.g. ketone) and carboxylate ion COO-) groups, respectively. The shoulder at 1080 cm^{-1} can be ascribed to C-OH stretching of phenolic groups (Mourão et al., 2011).

Effect of adsorbent dosage and initial bentazon concentration

The adsorbent dosage is an important parameter because this parameter determines the capacity of

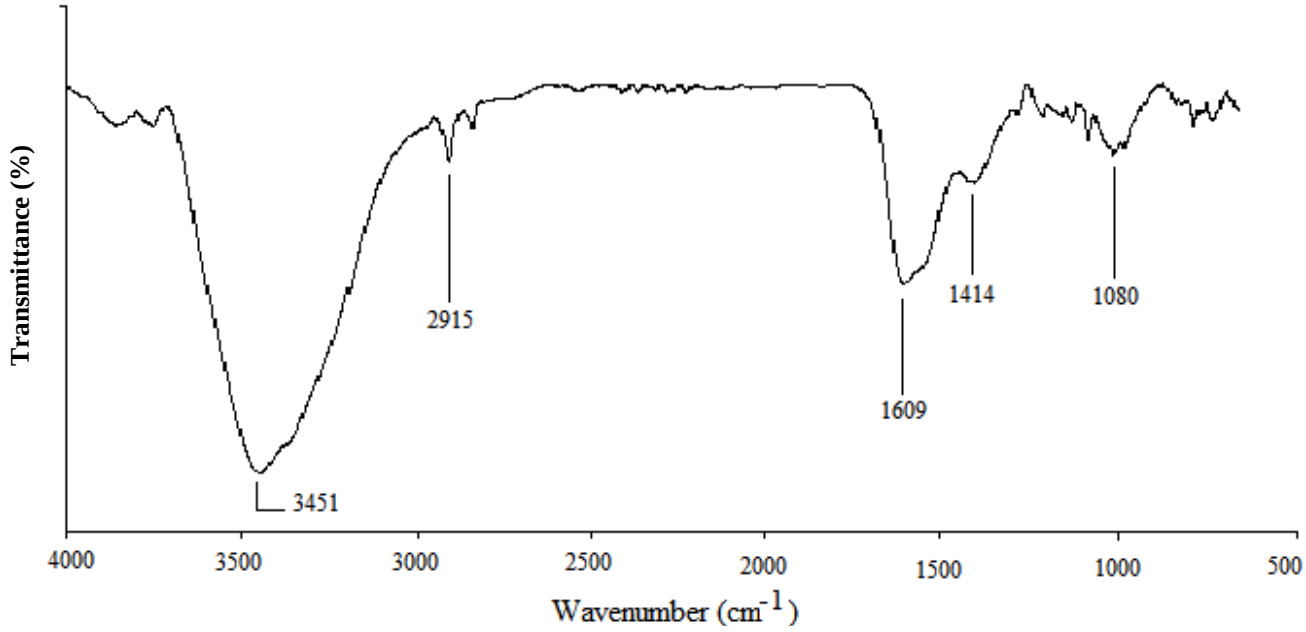


Figure 3. FTIR spectrum of the Henna wood.

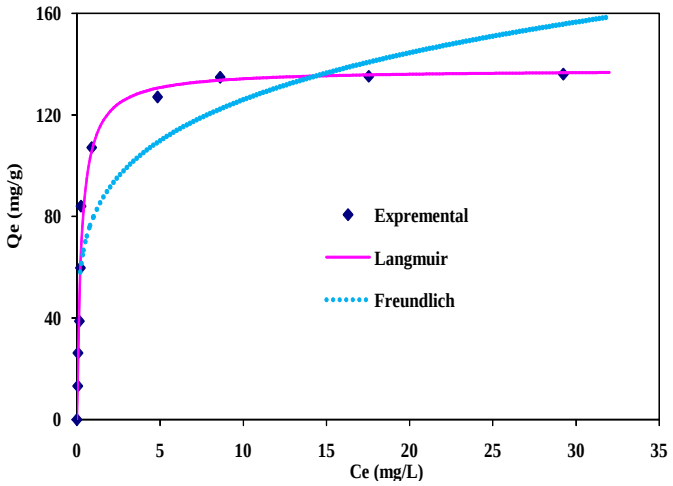


Figure 4. Adsorption isotherm of Basagron at 20°C fitted with Langmuir and Freundlich models. Q_e is the maximum amount of adsorption; C_e is the adsorbate equilibrium concentration (mg/L).

adsorbent for a given bentazon concentration and also determines sorbent-sorbate equilibrium of the system. From Figure 4, the removal of bentazon depends on the initial concentration of the adsorbate and the adsorbent dosage. For example, the percentage of removal of bentazon increased for the concentrations 22 and 78 mg/L, respectively, from 54 and 16.6% at 10 mg to 91 and 75% at 50 mg of adsorbent dose. The increased removal at high dosages is expected, because of the increased adsorbent surface area and availability of more adsorption sites (Chung et al., 2002). The optimum dosage was found to be 50 mg.

The percentage removal of the bentazon was found to decrease with the increase in initial bentazon concentration at constant amount of adsorbent of about 50 mg. This indicates that there exist reductions in immediate solute adsorption, owing to the lack of available active sites required for the high initial concentration of bentazon. Similar results have been reported in literature (Al-Sehaibani, 2000; Dweck, 2002).

The percentage removal of the bentazon was found to decrease with the increase in initial bentazon concentration at constant amount of adsorbent of about 50 mg. This indicates that there exist reductions in immediate solute adsorption, owing to the lack of available active sites required for the high initial concentration of bentazon. Similar results have been reported in literature (Al-Sehaibani, 2000; Dweck, 2002

Liquid phase adsorption

In the same focus, Ayranci and Hoda (2004) reported a Q_m value of 151 mg/g for adsorption of activated carbon-cloth. Bentazon isotherms were of type I according to the *International Union of Pure and applied Chemistry* (IUPAC) classification. This classification is in accordance with the characteristics of adsorbent.

However, activated carbon used in the present study has a high specific surface area (S_{BET} equal to 585 m^2/g) and a microporous structure that explain the registered isotherms adsorption of Type I.

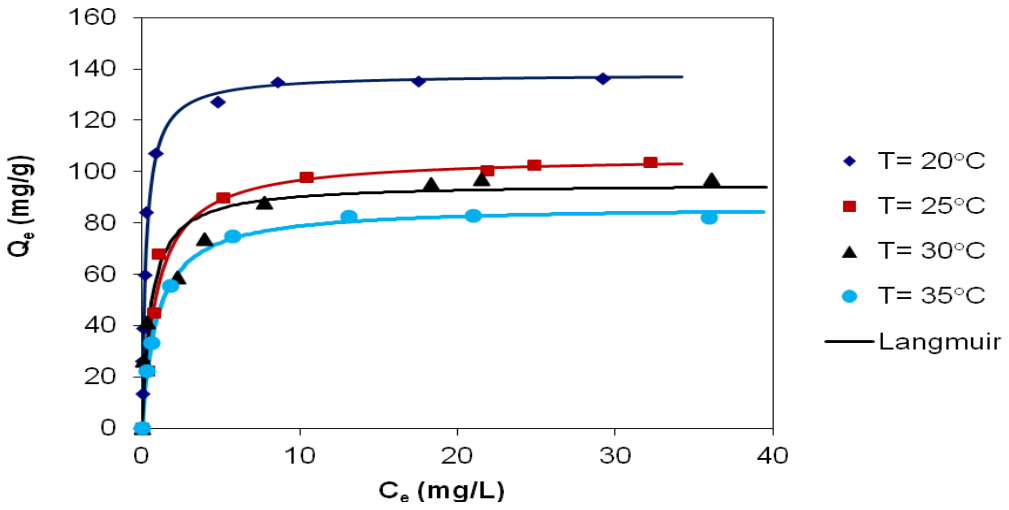


Figure 5. Adsorption isotherms of activated carbon at different temperatures fitted with Langmuir Model. Q_e is the maximum amount of adsorption; C_e is the adsorbate equilibrium concentration (mg/L).

Table 2. Langmuir constants for the adsorption of Basagran on the prepared activated carbon.

Temperature (°C)	Langmuir constants		
	Q_m (mg/g)	K_L (L/mg)	R^2
20	138.44	3.128	0.99
25	106.05	2.456	0.98
30	95.32	1.766	0.94
35	86.51	1.022	0.99

Q_m, Adsorption capacity (mg/g); K_L, Langmuir constant (L/mg); R^2, correlation coefficient.

Thermodynamic features

Isotherms of adsorption

The removal of the herbicide with activated carbon was studied at different concentrations and according to variable temperatures of 20, 25, 30 and 35°C to determine the adsorption isotherms and the thermodynamic parameters. Figure 5 shows that the amount of adsorbed bentazon on activated carbon decreased as the temperature increased. For example, Q_m decreased from 136.7 to 84.4 mg/g by increasing temperatures of the solution from 20 to 35°C, respectively. This analysis appeared important to optimize the design of an adsorption.

However, Table 2 shows the results of calculated isotherm constants at different temperatures. The Langmuir isotherm models was found to be the best fit of the experimental data over all the concentration range as indicated by the high values of correlation coefficients (R^2= 0.99).

The adsorption isotherms found was of a Type I according to BET classification (Brunauer et al., 1938). The Affinity of activated carbon to bentazon may be consented to the high specific area of activated carbon, to the bentazon mobility in liquid phase as well as to the characteristic of activated carbon and the interaction forces (Vander Waals) between adsorbent and adsorbate. The isotherms results revealed the temperature dependence on the adsorptive behavior as shown in Figure 5. This result could be explained by the exothermic nature of the adsorption reaction. Langmuir constant showed its close dependence on the surrounding adsorption temperature, if the temperature increases the Langmuir constant decreases (Table 3).

Enthalpy and entropy of adsorption

The variation of the equilibrium concentration according to the temperatures of enthalpy adsorption (ΔH_{ad}), or differential enthalpy is an indicator of the state of water adsorbed by the solid material (Tsami, 1991). The experimental data were calculated using Clausius-Claperyon equation (Kaymak-Ertekin and Gelik, 2004; Simal et al., 2007).

The experimental sorption isotherm in the form $\ln(C_e)$ versus 1/T is shown in Figure 6. The ΔH_{ad} was determined

Table 3. Thermodynamic features of the Basagran adsorption on the prepared activated carbon.

Q (mg/g)	- ΔH_{ad} (kJ/mol)	- ΔS (J/mol.K)	- ΔG (kJ/mol)
20	83.3	259.9	7.12
30	86.96	276.5	5.9
40	91.76	296.08	4.97
50	98.38	321.34	4.19
60	108.2	357.4	3.43
70	124.69	416.06	2.73
80	162.03	545.9	2.01

Q, Adsorbed quantity (mg/g); ΔH_{ad}, adsorption enthalpy (kJ/mol); ΔS_{ad}, adsorption entropy (J/mol.K); ΔG_{ad}, free enthalpy (kJ/mol).

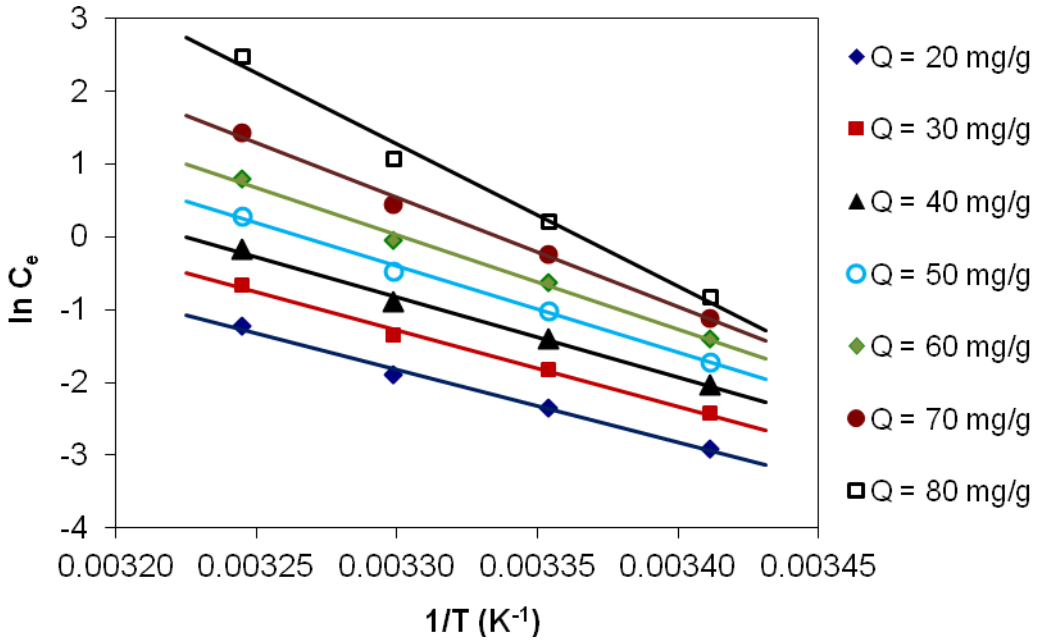


Figure 6. Variation of the equilibrium concentration according to the temperatures. Q, Adsorbed quantity (mg/g); T, temperature; C_e, adsorbate equilibrium concentration (mg/L).

from the slop ($\Delta H_{ad}/R$). This procedure is based on the assumption that ΔH_{ad} is invariant with temperature (Tsami, 1991). The enthalpy of adsorption increased according to the adsorption capacity of the activated carbon (Table 3). Similar results for silica gel have been reported in literature (Malik, 2003). It has a negative value indicating that the process is exothermic in nature which is consistent with the results obtained earlier where the bentazon uptakes decrease with the increase in solution temperature. The values, variation of the equilibrium concentration according to the temperatures, of sorption are presented in Table 3. The values show a spontaneous and favorable adsorption.

The values of adsorption entropy are shown in Table 3. The negative value of entropy reflects decreasing randomness at the solid/solution interface with some structural changes in the adsorbate and adsorbent during the adsorption process.

The feasibility of the process is shown by negative values of free energy (Table 3). This parameters had a negative value (- ΔG (kJ/mol) and Q (mg/g) varying to 7.12 (kJ/mol) from 2.01(kJ/mol) and 20 (mg/g) to 80(mg/g) respectively), which explain why the phenomenon of adsorption of bentazon by activated carbon is spontaneous nature of the adsorption processes at the range of temperature study. The decrease in the value of the free energy with increase in the temperature suggests that adsorption is favored more at the lower temperature. The exothermic nature of the process is further confirmed by the negative value of enthalpy. The negative value of

Table 4. Separation Factor RL

RL value	Type of isotherm	RL (obtained)
RL > 1	Unfavorable	-
RL = 1	Linear	-
0< RL < 1	Favorable	0.472
RL = 0	Irreversible	-

Table 5. Macro- and micro-pore diffusion rate constants.

Adsorbate concentration (mg/L)	Rate constants, Intraparticle rate parameter (mg/g·min $^{0.5}$)		
	K_1	K_2	K_3
50	2.07	0.83	0.011

entropy change indicates decrease in the dye concentration at the solid-solution interface.

The negative ΔG values determine that the reaction rate is increasing with a decrease in temperature. However, an increase of the adsorbed quantity is accompanied by a decrease in the free enthalpy that could be explained by the adsorption tendency towards the thermodynamic equilibrium.

According to McKay et al. (1982) to express the essential characteristics and the feasibility of the Langmuir isotherm in terms of a dimensionless constant separation factor RL, the data obtained represent a favorable adsorption in the case of adsorption of bentazon (RL = 0.472). The shape of isotherm is given by the value of RL as given in Table 4.

Intraparticle and pore diffusion plots for adsorption of bentazon

Besides adsorption at the outer surface, there is also possibility of intraparticle diffusion from the outer surface into the pores of the material. The adsorption mechanism of a sorbate onto the adsorbent follows three steps viz. film diffusion, pore diffusion and intraparticle transport (Pant and Singh, 2004). Though there is a high possibility for pore diffusion to be the rate-limiting step in a batch process, the adsorption rate parameter which controls the batch process for most of the contact time is the intra-particle diffusion (Allen et al., 1989). Thus, to evaluate the rate controlling step a plot was drawn between amount of bentazon adsorbed on activated carbon (time)$^{1/2}$ (graph not shown). The general shape of this curve that has not been advanced at this stage, showed three slopes. The first part of the curve is attributed to mass transfer effects (slope K1) taking place with boundary layer diffusion, while the final linear parts indicate intraparticle diffusion (slope K2 and K3). The diffusion rate parameters K1, K2 and K3 as obtained as shown in Table 5. The values for K2 and K3 indicate that the pores are micro-pores and the intraparticle diffusional resistance is due to micro-pores only (Singh et al., 2008). The diffusion rate parameters indicate that the intraparticle diffusion controls the sorption rate; which is the slowest step in adsorption.

Conclusions

The results of the present investigation showed that activated carbons prepared from Henna wood have good adsorption capacity for the removal of bentazon from aqueous solution. The adsorption characteristic has been examined with the variations in the parameters of pH, contact time, activated carbon dosage, stirring rate, initial bentazon concentration and temperature. The experimental data were analyzed using Langmuir and Freundlich isotherm models. The Langmuir model provides the best correlation of the experimental equilibrium data. Adsorption isotherm according to BET classification was of Type I.

The adsorption isotherms of bentazon are determined at different temperatures using Langmuir models. These isotherms revealed that adsorption increased as the concentration increases up to a saturation point.

Enthalpy, entropy and free enthalpy adsorption have a negative value indicating that process adsorption is exothermic, with the increase of the order degree and the spontaneous nature of the adsorption. Thermodynamic parameters suggested that the adsorption of bentazon on the Henna wood activated was feasible, spontaneous and exothermic in nature.

Conflict of Interests

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

REFERENCES

Ahmad AL, Tan LS, Shukor SRA (2008). Dimethoate and atrazine retention from aqueous solution by nanofiltration membranes. J. Hazard. Mater. 151:71-77.

Ali I, Gupta VK (2006). Advances in water treatment by adsorption technology. Nat. Protoc. 1: 2661-2667.

Allen Sj, Mckay G, Khader K (1989). Intraparticle diffusion of basic dye during adsorption onto Sphagnum Peat. J. Environ. Pollut. 50:9-50.

Al-Sehaibani H (2000). Evaluation of extraction of Henna leaves as environmentally friendly corrosion inhibitor for metals. Materialwiss. Werkstofftech. 31:1060-1063.

Amin NK (2009). Removal of direct blue-106 dye from aqueous solution using new activated carbons developed from pomegranate peel: adsorption equilibrium and kinetics. J. Hazard. Mater. 165:52–62.

Ania CO, Beguin F (2007). Mechanism of adsorption and electosorption of bentazone on activated carbon cloth in aqueous solutions. Water. Res. 41:3372-3380.

Arcury TA, Quandt SA, Cravey AJ, Elmore RC, Russell GB (2001). "Farmworker reports of pesticide safety and sanitation in the work environment. Am. J. Ind. Med. 39:487-498.

Arcury TA, Quandt SA, Russell GB (2002). "Pesticide safety among farmworkers: Perceived risk and perceived control as factors reflecting environmental justice. Environ. Health. Perspect. 110:233-

240.
Ayranci E, Hoda N (2004). Adsorption of bentazon and propanil from aqueous solutions at the high area activated carbon-cloth. Chem. 57:755-762.
Bhattacharya AK, Mandal SN, Das SK (2006). Adsorption of Zn (II) from aqueous solution by using different adsorbents. Chem. Engr J. 123:43-51.
Brunauer S, Emmett PH, Teller E (1938). Adsorption of gases in multimolecular layers. J. Am. Chem. Soc. 60:309-319.
Cabrera NL, Leckie JO (2009). Pesticide risk communication, risk perception, and self-protective behaviors among farmworkers in California's Salinas Valley. Hispanic. J. Behav. Sci. 31:258-272.
Choudhari D, Sharma D, Phadnis A (2013). Heavy metal remediation of wastewaters with agrowastes . Eur. Chem. Bull. 2:880-886.
Chowdhury ZZ, Zain SM, Khan RA, Ahmad AA, Islamand MS, Arami-Niya A (2011). Application of central composite design for preparation of Kenaf fiber based activated carbon for adsorption of manganese (II) ion . Int. J. Phys. Sci. 6:1-12.
Chung WH, Chang YC, Yang LJ, Hung SI, WR, Wong JY, Lin et al (2002). Clinicopathologic features of skin reactions to temporary tattoos and analysis of possible causes. Arch. Dermatol. 138:88-92.
Colak F, Atar N, Olgun A (2009). Biosorption of acidic dyes from aqueous solution by *Paenibacillus macerans*: Kinetic, thermodynamic and equilibrium studies. Chem. Eng. J. 150:122-130.
Do DD (1996). A Model for Surface Diffusion of Ethane and Propane in Activated Carbon. Chem. Eng. Sci. 51:4145-4158.
Dweck AC (2002). Natural ingredients for colouring and styling. Int. J. Cosmet. Sci. 24:287-302.
Faria PCC, Orfao JJM, Pereira MFR (2004). Adsorption of anionic and cationic dyes on activated carbons with different chemistries. Water. Res. 38:2043-2052.
Fontecha-Camara MA, Lopez-Ramo MV, Pastrana-Martinez LM (2008). Kinetics of diuron and amitrole adsorption from aqueous solution on activated carbons. J. Hazard. Mater. 156:472-477.
Garrido EM, Lima JLC, Delerue-Matos CM, Brett AMO (1998). Electrochemical oxidation of bentazon at a glassy carbon electrode application to the determination of a commercial herbicide. Talanta. 46:1131-1135.
Halim AA, Aziz HA, Johari MAM, Ariffin KS (2010). Comparison study of ammonia and COD adsorption on zeolite, activated carbon and composite materials in landfill leachate treatment. Desalination. 262:31-35.
Ismadji S, Bhatia SK (2001). Characterization of activated carbons using liquid phase adsorption. Carbon. 39:1237-1250.
Kaymak-Ertekin F, Gelik A (2004). Sorption isotherms and isosteric heat of sorption for grapes. Lebensm. Wiss. Technik. 37:429-438.
Langmuir L (1918). The adsorption of gases on plane surface of glass, mica and platinuim. Am. Chem. Soc. 40:1361.
Li X , Xu Q , Han G , Zhu W, Chen Z, He X, Tian X (2009). Equilibrium and kinetic studies of copper (II) removal by three species of dead fungal biomasses. J. Hazard. Mater. 165:469-474.
Maldonado MI, Malato S, Perez-Estrada LA, Gernjak W, Oller I, Domenech X, Ali S, Hussain T, Nawaz R (2006). Optimization of alkaline extraction of natural dye from Henna leaves and it's dyeing on cotton by exhaust method. J. Clean. Prod. 17:61-66.
Malik PK (2003). Use of activated carbons prepared from sawdust and rice-husk for adsorption of acid dyes a cause study of Acid yellow. 36. Dyes. Pigm. J. 56:239-249.
McKay G, Blair HS, Gardener JR (1982). Adsorption of dyes on chitin. I. Equilibrium studies, J. Appl. Polym. Sci. 27:3043-3057.
Mourão PAM, Laginhas C, Custódio F, Nabais JMV, Carrott PJM, Ribeiro Carrott, MML (2011). Influence of oxidation process on the adsorption capacity of activated carbons from lignocellulosic precursors. Fuel. Process. Technol. 92:241-246.
Navalon A, Prielo A, Araujo L, Vilchez JL (2002). Determination of oxadiazon residues by headspace solid-phase micro-extraction and gas chromatography-mass spectrometry. J. Chromatogr. A. 946:239-245.
Nickolov R, Mehandjiev D (2000). The simplified equation for micropore size distribution in adsorbents with different texture and chemical nature. In: Proceeding of 9th International Symposium of Catalysis, Varna, pp. 193-198.
Omri A, Wali A, Benzina M (2012). Adsorption of bentazon on activated carbon prepared from Lawsonia inermis wood: Equilibrium, kinetic and thermodynamic studies. Arabian. J. Chem.
Pant KK, Singh TS (2004). Equilibrium, kinetics, and thermodynamics, study for adsorption of As (III) ions on activated alumina. Sep. Purif. Technol. 36:139-147.
Pappiernik S, Yates S, Koskinen W, Barber B (2007). Processses affecting the dissipation of the herbicide Isoxaflutole and its diketonitrile metabolite in agricultural soils under field conditions. J. Agric. Food. Chem. 55:8630-8639.
Pintar A (2005). Catalytic processes for the purification of drinking water and industrial effluents. Catal. Today. 77:451-465.
Pourata R, Khataee AR, Aber S, Daneshvar N (2009). Removal of the herbicide bentazon from contaminated water in the presence of synthesized nanocrystalline TiO powders under irradiation of UV-C light. Desalination. 249:301-307.
Rajashekara MHM, Monomania HK (2007). Aerobic degradation of technical hexachlorocyclohexane by a defined microbial consortium. J. Hazard. Mater. 149:18-25.
Ren L, Zhang J, Li Y, Zhang C (2011). Preparation and evaluation of cattail fiber-based activated carbon for 2, 4-dichlorophenol and 2, 4, 6-trichlorophenol removal. Chem. Eng. J. 168:553-561.
Saritha P, Aparna C, Himabindu V, Anjaneyulu Y (2007). Comparison of various advanced oxidation processes for the degradation of 4-chloro-2 nitrophenol. J. Hazard. Mater. 149:609-614.
Simal S, Castell-Palou A, Rossellet C (2007). Water desorption thermodynamic properties of principal. J. Food. Eng. 80: 1293-1301.
Singh S, Lokesh KV, Sambi SS, Sharma SK (2008). Adsorption Behaviour of Ni (II) from Water onto Zeolite X: Kinetics and
Equilibrium Studies, Proceedings of the World Congress on Engineering and Computer Science 2008 WCECS 2008, October 22-24, San Francisco, USA, ISBN: 978-988-98671-0-2.
Tsami C (1991). Net isosteric heat of sorption in dried fruits. J. Food. Eng. 14:325-327.
Wang SL, Tzou YM, Lu YH, Sheng G (2007). Removal of 3-chlorophenol from water using rice-straw-based carbon. J. Hazard. Mater. 147:313-318.
Weber TW, Chakraborti RK (1974). Pore and solid diffusion models for fixed bed adsorbents. J. Aiche. 20:228-238.
Zhang Wei-P, Yang Yong-Q, LI M-xuan, et al. (2010). Abdominal Ultrasonic Manifestations of 284 Occupational Organophosphorus Pesticide Contacts. Practical Preventive medicine. ISSN 1006-3110. 17:4pp.
Zhu JL, Hjollund NH, Andersen AM, Olsen J (2006). Occupational exposure to pesticides and pregnancy outcomes in gardeners and farmers: a study within the Danish National Birth Cohort. J. Occup. Environ. Med. 4:347-352.

academicJournals

Vol. 13(35), pp. 3607-3616, 27 August, 2014
DOI: 10.5897/AJB2014.13775
Article Number: FE8293B47058
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Physico-chemical and biotic factors influencing microalgal seed culture propagation for inoculation of a large scale raceway pond

Mutanda, T.[1]*, Ranjith Kumar R.[2] and Bux, F.[2]

[1]Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Private Bag X54001, Durban 4000, South Africa.
[2]Institute for Water and Wastewater Technology, Durban University of Technology, Durban 4001, South Africa.

Received 5 March, 2014; Accepted 15 August, 2014

The growth of *Chlorella vulgaris* in open pond aquatic conditions poses serious challenges due to the interplay of both physico-chemical and biotic factors. We report here the monitoring of physico-chemical and biotic parameters affecting the propagation of *C. vulgaris* seed culture for inoculation of a large scale raceway pond (300 000 L capacity) in South Africa. The *C. vulgaris*strain used for this purpose was isolated from a wastewater maturation pond and characterized for its potential for biomass and lipid production. The isolate was grown aseptically in 4 × 25 L aspirator bottles in BG-11 medium under ambient laboratory conditions and the culture was supplied with filtered air and exposed to 200 µmol photons per m^2 per second using Gro-Lux agricultural fluorescent lights. The culture was transferred to a 500 L capacity portable pool under open conditions. This pond was used to further inoculate 3 more portable ponds. Physico-chemical and biotic growth parameters were monitored on a daily basis in the three ponds. The over reliance on fossil fuels will have a major impact on power supply in the near future if renewable sources of energy are not developed at a fast pace. The developed inoculum was subsequently used to inoculate an open raceway pond for large scale biomass production for biodiesel production.

Key words: Biodiesel, biomass, inoculum, lipid, raceway pond, seed culture.

INTRODUCTION

The depletion of fossil based fuels is becoming a reality as the global demand for energy fuels outpaces supply. Global energy demand is drastically increasing due to the growing world population and the improvement of the quality of human life (Razzak et al., 2013). Hence it is desirable to search for alternative, sustainable and renewable fuels such as biodiesel generated from microalgal lipids (Singh et al., 2014; Hamawand et al., 2014; Oncel, 2013; Pittmanet al., 2011). The success of this technology depends on selecting a suitable microalgal strain as well as adopting a cost effective cultivation strategy (Singh et al., 2014; Chinnasamyet al.,

*Corresponding author. E-mail: mutanda@ukzn.ac.za. Tel: +27 31 360 8226.

2010).

Successful large scale microalgal cultivation systems rely heavily on the quality, vigour and physiological properties of the seed culture, cultivation system as well as techno-economic considerations (Jonker and Faaij, 2013). In addition, the optimum combination of technical innovations in systems and processes, coupled with economic feasibility in the practical implementation and integrated scale-up for commercial production and marketing is also essential for successful development of algae based biofuels (Gendy and El-Temtamy, 2013). It is imperative to develop a seed culture propagation strategy that will result in the development of robust and high quality inoculum for seeding a large scale open raceway pond. Raceway ponds are preferred for microalgae cultivation due to a number of advantages as compared to photobioreactors (Razzak et al., 2013; Mutanda et al., 2011a; Harun et al., 2010; Chisti, 2007; Grobbelaar, 2009; Pulz and Gross, 2004).

Preliminary microalgal cultivation studies demonstrate that there are 4 fundamental steps for up scaling seed culture to be adequate to inoculate a large commercial raceway pond (Grobbelaar, 2009). The four important steps to full realisation of a high quality seed culture are: (1) bioprospecting for hyper lipid producing microalgal strain; (2) strain selection, isolation and purification using conventional and advanced methods; (3) up scaling of the seed culture under laboratory conditions in aspirator bottles and (4) seed culture propagation in a pond under open conditions (Grobbelaar, 2009; Mutanda et al., 2011a). The outdoor ponds are operated as a series of batch reactors with inoculation volumes ranging between 20 and 25% of the volumes of the next size cultures. Ambient CO_2 is adequate as a carbon source for microalgal growth but however, pure CO_2 gas is supplied on demand following an increase in pH above pH 9.5 (Bechet et al., 2013; Grobbelaar, 2009).

The production process is a stepwise increase in volume, always starting from pure laboratory grown cultures (Grobbelaar, 2009). This is done to minimise contaminating microalgae from taking over the target culture and to ensure that only the target strain is propagated to be the dominant population for inoculation of the large scale raceway pond. Finally, the entire contents of the small seed culture ponds are transferred to the next size production ponds as inoculum and eventually to the large scale raceway pond. However, it is crucial to transfer seed culture that is at the exponential growth phase to the same media previously used for seed culture proliferation so as to minimise culture shock and to prevent a long lag phase when the seed culture is transferred to the large scale raceway pond.

The commercial cultivation of microalgae and cyanobacteria on an industrial scale began with the culture of *Chlorella* in Japan in the 1960s followed by the cultivation of *Spirulina* in Mexico, the USA and China in the 1970s (Radmann et al., 2007). *Chlorella*, *Spirulina* and *Dunaliella* are commonly cultivated since they can be easily grown in highly selective media and can be cultivated in open raceway ponds and remain relatively free from contamination (Radmann et al., 2007). The main beneficial factor of the open raceway pond technology is its possible low cost microalgae cultivation system with a wide array of process designs ranging from single, multichannel and cascading systems.

Other biotic factors that feed on microalgae such as invertebrates, bacteria and viruses play havoc with microalgal cultivation under open conditions. Protozoa can devour a pond of microalgae in a matter of minutes therefore it is crucial to closely monitor these contaminants. Due to unpredictable population dynamics of the raceway pond due to seasonal variability, it is crucial to monitor any succession tendencies in the ponds by non-target microalgae. The important factors affecting microalgal growth are light intensity, temperature, nutrients, pH and salinity. These factors must be optimal for maximal microalgal growth and productivity. Therefore the aim of this study was to investigate the effect of physico-chemical and biotic factors on seed culture propagation in open ponds.

MATERIALS AND METHODS

Materials and reagents

Portable pools (500 L), fish pumps, tubings, lights and all accessories used for aerating the pools were bought from local suppliers. Aspirator bottles (Schott Duran, 25 L), oven and rotavapor were purchased from Lasec, South Africa. The Spectroquant ® Pharo 300UV / VIS 190 -1100 nm spectrophotometer was purchased from Merck, South Africa. The Hach pocket colorimeter (for the determination of free and total chlorine) and powder pillows were obtained from Universal Water Solutions, South Africa. The light intensity meter and the YSI probe were purchased from Campbell Scientific Africa and Monitoring and Control Laboratories Pty Ltd respectively. All the reagents used for media preparation were of reagent grade unless otherwise stated.

Microalgal strain isolation and purification

An extensive bioprospecting exercise for hyper-lipid producing microalgae was carried out in aquatic habitats in Kwa-Zulu Natal Province, South Africa. Subsequently, a robust high lipid producing *C. vulgaris* was isolated from a wastewater maturation pond at Kingsburgh wastewater treatment plant in Durban, South Africa. The microalgal strain was purified to monoculture using standard conventional protocols and identified using molecular tools to determine phylogenetic affiliations as previously reported in our laboratory (Bhola et al., 2011). Sequence results from BLAST searches into the GenBank databases confirmed that the obtained sequences were homologous to ribosomal genes of *C. vulgaris* with 99% similarity and the sequences obtained were deposited to GenBank with the accession number HM046832 (Bhola et al., 2011). The purified isolate was kept at 4°C in suspension and routinely subcultured until needed for further research.

Growth conditions and media composition

The microalgal cultures were grown and maintained in BG-11

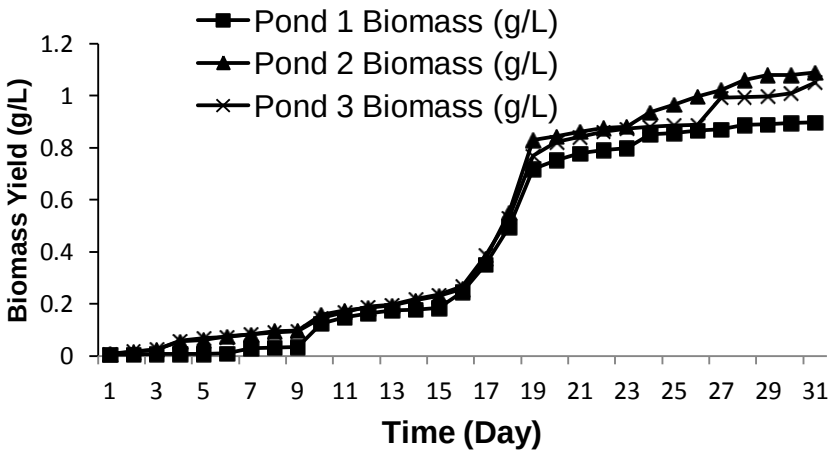


Figure 1. Graph showing biomass propagation with time in the 3 ponds.

mediumas previously described (Stanier et al., 1971;Mutanda et al., 2011b). A drop of tetracycline (0.5 µl/L) was added to the growth medium to prevent any bacterial contamination in the micro-algalcultures. The pH was adjusted to 7.4 and trace metals were added separately after autoclaving. The BG-11 medium was used for the entire seed culture preparation. The 4 ×25 L aspirator bottles were inoculated with pure seed culture (10% v/v, 1.2×10^7 cells/ml) and incubated under ambient laboratory conditions exposed to Sylvania Grow-lux 18 W lights. The seed culture growth was monitored daily and routinely checked microscopically for any contamination. Evidence of microbial contamination was determined by conventional light microscopy and in addition, bacterial contamination was investigated by spreading a diluted microalgal suspension onto solidified nutrient medium containing 1% glucose, 1% peptone and 1% yeast extract. Light microscopy was used for microalgal cell counts as described in the following section.

Inoculation of the open ponds

BG-11media composition was prepared as previously reported with the exception that it was not sterilized since it was used under open conditions. A total of 500 L of media was prepared for each portable pond with a depth of 30 cm to allow maximum sunlight penetration. The three portable pools were of equal size and had a maximum capacity of 1 000L. Each portable pool was inoculated with 10% v/v (1.2×10^7 cells/ml) of inoculum and aeration was achieved by means of fish pumps and bubbling filtered air (1 ml/min) dispersed through 10 aquarium air stones placed equidistant from each other below the microalgal suspension. Mixing of the culture was done manually by means of swirling the suspension using a hand held pump three times a day. The air bubbling through air stones also improved mixing. Evaporation rates were monitored daily by observing the deviation from the 500 L mark on each portable pool and then compensated by topping up with tap water to the 500L mark.

Monitoring of growth parameters

Physico-chemical parameters were measured using a calibrated YSI probe. The variables were monitored daily at 1100 h. The following parameters were measured: pH, temperature, salinity, evaporation rates, conductivity, dissolved O_2, total dissolved solids and oxidative reduction potential (ORP). A light intensity meter was used to measure light intensity. Samples (500 ml) were collectedfrom the ponds for laboratory analysis of microbial contaminationand determination of biomass yield.

Free and total chlorine determination

Free and total chlorine was determined using the Pocket Colorimeter (Hach) and the *N, N*-diethyl-*p*-phenylenediamine (DPD) free chlorine reagent as recommended by the manufacturer.

Determination of biomass

The microalgal cells were harvested daily from the portable pools by the centrifugation method. A seed culture sample (500 ml) was centrifuged at 4 000 RPM (20 mins, 4°C) and the microalgal biomass was placed in a preweighed watch glass. The biomass was dried in an oven at 60°C for 12 h. The watch glass with the biomass was weighed and the net mass of the microalgal cells was determined by subtracting the final weight from the weight of the watch glass. The weight of the biomass was expressed in g/L. The cell density was measured indirectly using spectrophotometry and directly by light microscopy by performing manual cell counts using a counting chamber (haemocytometer) with Neubauer rulings as instructed by the manufacturer.

RESULTS

Biomass production

The biomass was monitored in the three ponds for 30 days and the data generated show typical growth curves (Figure 1). There was a prolonged lag phase in the three ponds and this is attributed to the open nature of the growth conditions and it took a long time for the pure monoculture to adjust to the open conditions as compared to the controlled laboratory conditions. The exponential growth phase started on the 9^{th} day of growth up to the 19^{th} where the stationary growth phase sets in. These findings clearly suggest that the inoculumn in the

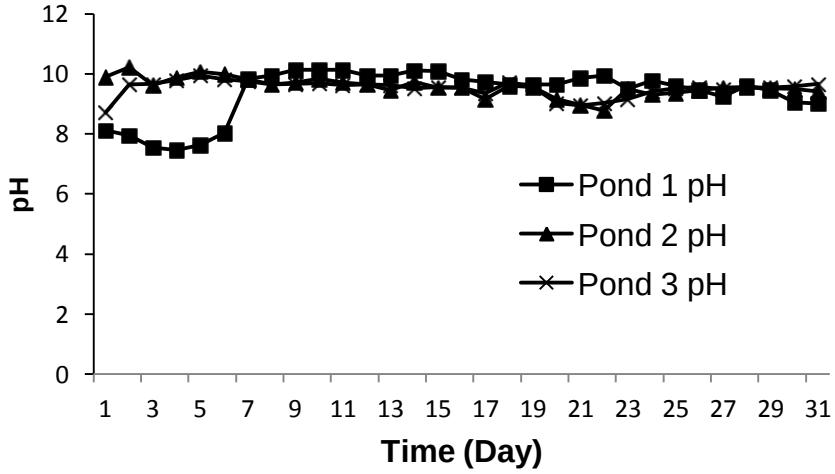


Figure 2. Graph showing pH monitoring in the three ponds.

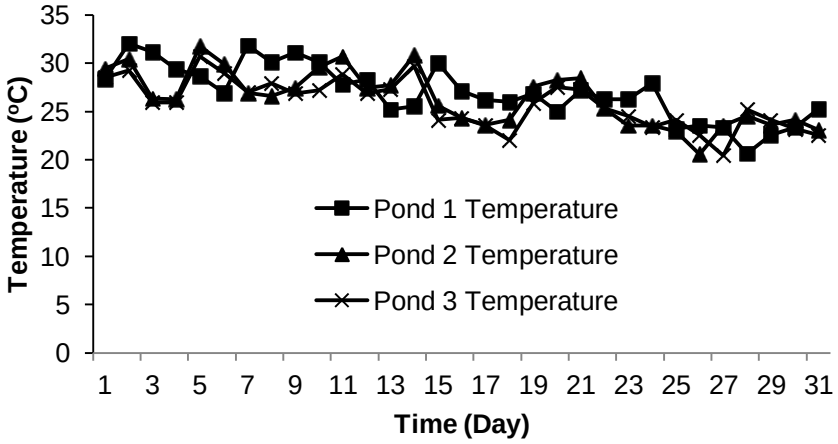


Figure 3. Graph showing temperature fluctuations in the 3 ponds.

ponds can be harvested at this prime time before the cells lose their vigour.

Effect of pH on *Chlorellavulgaris* growth

The pH values in the 3 ponds for the entire growth period were in the desirable range (Figure 2) therefore it was not necessary to supplement the ponds with additional CO_2. The pH ranged from 7.55 to 10.14, 8.78 to 10.08 and 8.72 to 9.95 in ponds 1, 2 and 3 respectively. Carbon dioxide has a strong influence on pH fluctuations in the growth ponds and the data generated demonstrate that the utilisation of CO_2 for photosynthesis results in the formation of carbonates in the medium that pushes up the pH into the alkaline range.

Effect of temperature on *Chlorellavulgaris*growth

It was found that temperature ranged from 22.56 to 32.04, 20.56 to 31.76 and 20.45 to 30.75°C in pond 1, 2 and 3, respectively (Figure 3). To validate these findings, the seasonal temperature variation at this site is suitable for microalgal cultivation throughout the year from the weather data reported by the South African Weather Services. Temperature fluctuations in the three ponds followed a similar trend since the ponds were in proximity to each other and also located at the same experimental site.

Effect of Light intensity on *C. vulgaris*growth

In the growth period under this study, the photoperiod was 8 h light and 12 h darkness. The light intensities ranged from 18.92 to 306.7, 20.21 to 462.9 and 10.31 to 315.9 $\mu molm^{-2}s^{-1}$ in pond 1, 2 and 3, respectively (Figure 4). At the initial growth stages, the light intensity was fairly high in all the experimental ponds and there was a gradual decline with time.

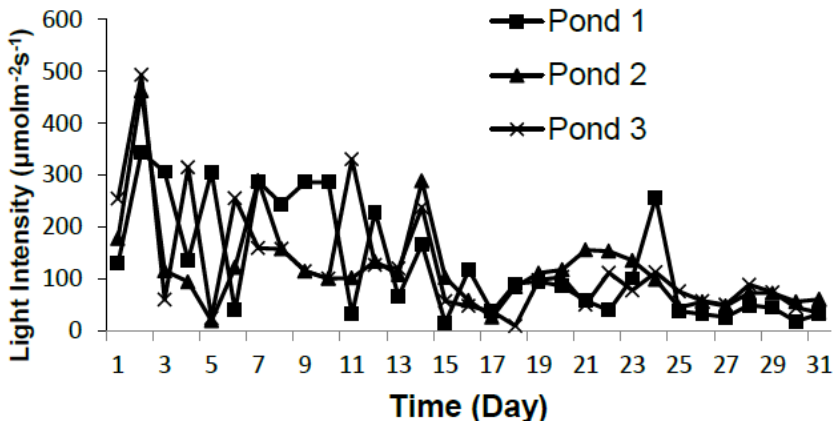


Figure 4. Graph showing light intensity in the 3 ponds.

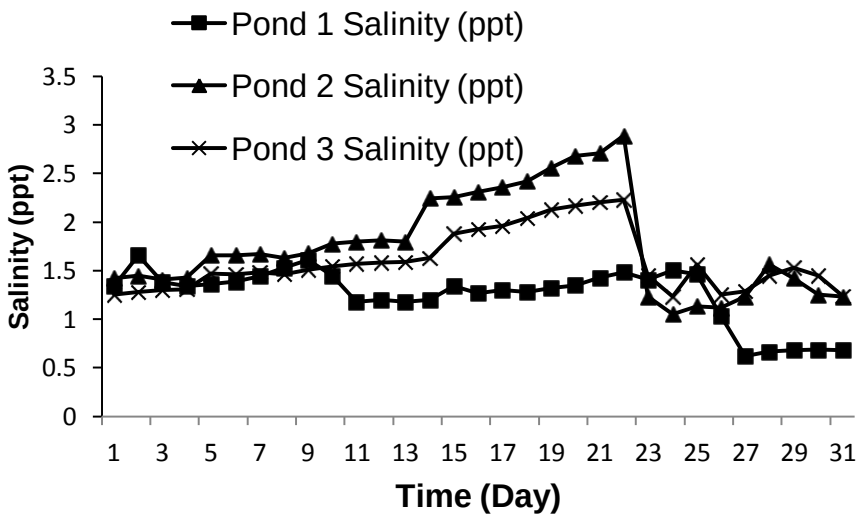


Figure 5. Graph showing salinity in the 3 ponds.

Effect of salinity on *C. vulgaris* growth

There was a slight increase in salinity in pond 1 from the initial 1.34 to 1.66 ppt on day 1 of incubation and this is possibly due to salt residues on the seed culture (Figure 5). In both pond 2 and 3, there was a gradual increase in salinity up to 2.89 and 2.23 ppt, respectively on day 22 and this is mainly attributed to slightly higher evaporation rates in the ponds (Figure 5). On this day, additional water (120 L) was added to the ponds with a corresponding decline in salinity levels. On the final day of incubation that is, day 30, the salinity levels were 0.68 ppt, 1.23 ppt and 1.24 ppt in pond 1, 2 and 3, respectively.

Effect of dissolved oxygen on *Chlorella vulgaris* growth

Data generated in our experiments show intermittent fluctuations in the concentration of dissolved oxygen with time in the three ponds. Initial DO_2 concentrations were 8.05, 19.1 and 13.45 mg/L for pond 1, 2 and 3, respectively (Figure 6). The final DO_2 concentrations on day 30 were 17.42, 15.78 and 17.13 mg/L for pond 1, 2 and 3, respectively (Figure 6).

Effect of total dissolved solids (TDS) on *Chlorella vulgaris* growth

There was a variation in TDS levels with time in the 3 ponds and a dramatic decrease in TDS (0.815 g/L) was observed in pond 2 on day 17 (Figure 7). In pond one, TDS decreased from the initial 1.394 to 0.889 g/L after 30 days of incubation. In pond 3 there was a steady increase in TDS from 1.592 to 3.116 g/L after 28 days of incubation and thereafter the TDS concentration dropped to 2.184 after 30 days of incubation (Figure 7).

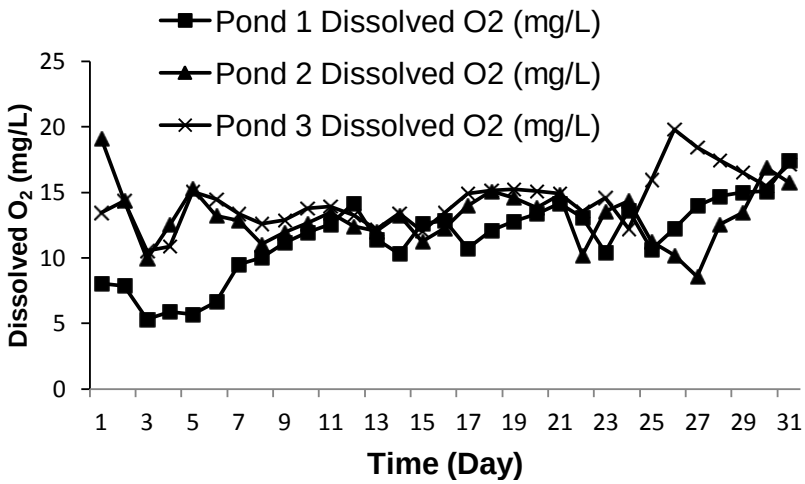


Figure 6. Graph showing dissolved oxygen in the 3 ponds.

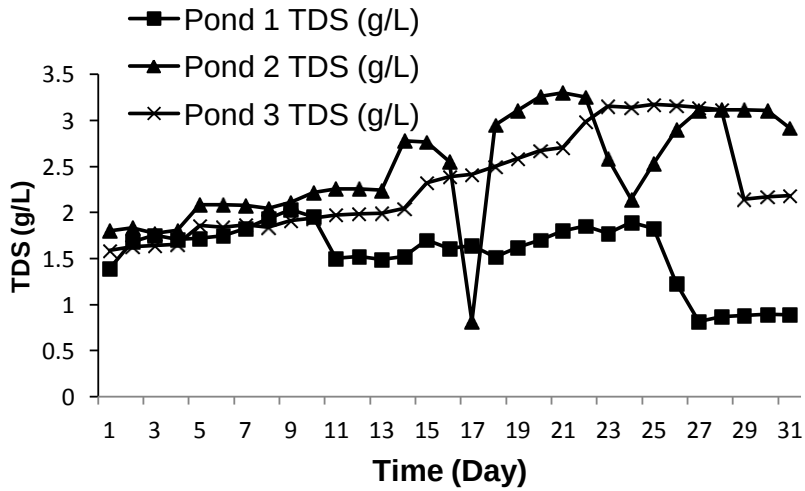


Figure 7. Graph showing total dissolved solids (TDS) in the 3 ponds.

Effect of conductivity on *Chlorella vulgaris*growth

There was a dramatic decrease in conductivity (1.214 ms/cm) in pond 2 on day 17 in response to a decrease in TDS (Figures 7 and 8). There was a steady decline in conductivity in pond 1 from the initial 2.774 to 1.374 ms/cm after 30 days of incubation (Figure 8). There was a slight increase in conductivity in pond 2 from the initial 3 to 3.125 ms/cm after 30 days of incubation. There was a steady increase in conductivity in pond 3 from the initial 2.615 to 3.415 ms/cm after 30 days of incubation.

Effect of oxidation reduction potential (ORP) on *Chlorella vulgaris* growth

The results obtained in the three ponds indicate a fluctuation in the ORP with time (Figure 9). In pond 1, the initial and final ORP was -32 and -77.6 mv, respectively. The same trend was observed in pond 2 where the initial and final ORP was -39.7 and -94.5 mv, respectively. The initial and final ORP in pond 3 was -2.6 and -74.6 mv, respectively.

Effect of biotic factors on *C.vulgaris*growth

On the last day of cultivation, microscopic analysis revealed the presence of *Scenedesmus* sp. in all the 3 ponds under investigation (Figure 10). In any microalgal seed preparation, it is desirable to closely monitor non-target microbial agents in the ponds to avert collapse of the system due to contamination by the undesirable microalgal strains.

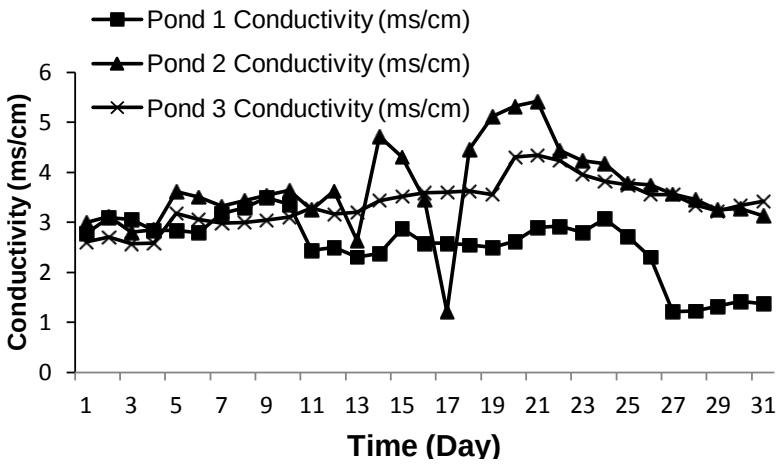


Figure 8. Graph showing conductivity in the ponds.

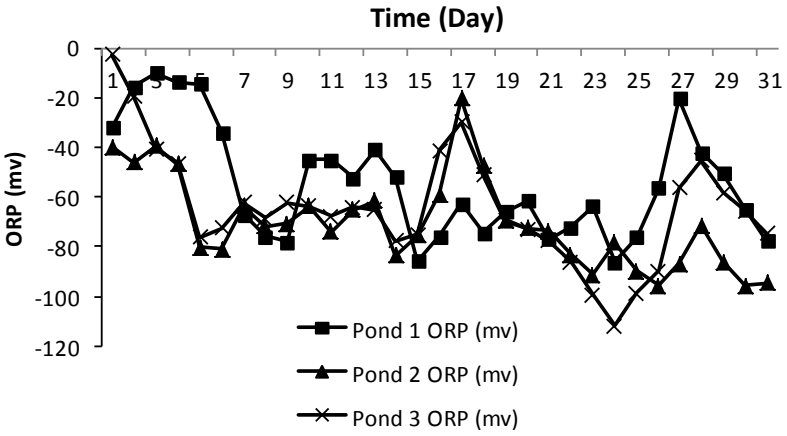


Figure 9. Graph showing oxidation reduction potential in the 3 ponds.

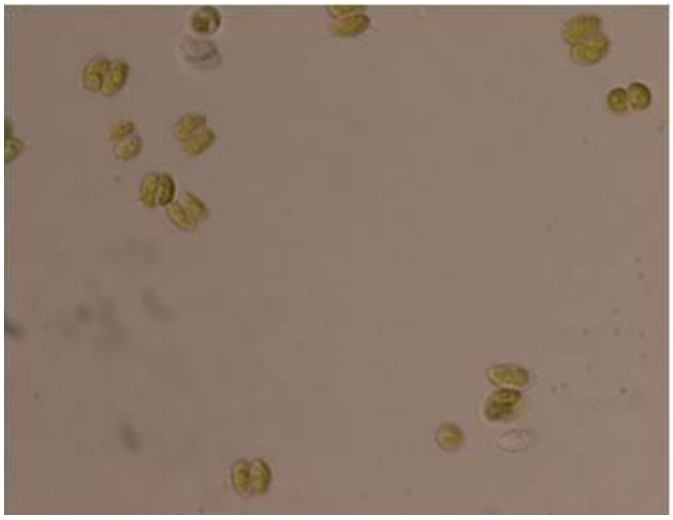


Figure 10. Light microscope slide of a wet mount of the culture in pond 1 showing the presence of *Senedesmus*sp.in the pond (x1000).

Effect of free chlorine on C. vulgaris

The free chlorine in the 3 ponds was determined and was found to be 0.05 mg/L on the first day of microalgal growth in the 3 ponds. However on the last day of growth, there was a slight decrease in free chlorine to 0.03mg/L in the 3 ponds.

DISCUSSION

Similar work done by Grobbelaar(2009) also support the assumption that continued growth of microalgal cells in batch culture could negatively impact on their viability and resuscitation and rejuvenation of these cells can lead to failure when transferred to large scale raceway pond. It was observed that settling of the cells was a major stumbling block and mechanical mixing of the suspension was only achieved by physically mixing 3 times a day using a hand held pump. The maximum dry weights achieved were 0.898, 1.09 and 1.05 g/L for pond 1, 2 and 3, respectively (Figure 1). Free chlorine is reported to be a micronutrient for microalgal growth therefore initial free chlorine in all the 3 ponds was determined and was found to be 0.05 mg/L.

pH has a major effect on microalgal growth since it controls all metabolic and physiological functions of the cell as well as influencing biomass regulation (Mayo, 1997). The pH concentration range for the existence of biological life is quite narrow (typically 6-9) and an indication of extreme pH is known to damage biological processes in biological treatment units (Akpor and Muchie, 2011). Therefore it is imperative to closely monitor pH in the ponds. The pH levels above 9 are desirable because some contaminants such as protozoans and rotifers are inhibited under these conditions and are therefore completely eliminated from the ponds but however, invertebrates can easily survive and thrive above pH 9. The presence of these contaminating microorganisms can lead to disastrous consequences since they are known grazers and can therefore devour the target microalgal cells in a short space of time. There was a gradual decrease in pH levels in pond 1 for the first three days of growth (Figure 2) and this is attributed to accumulation of dissolved CO_2 as the microalgal cells adjust to the new conditions in the open pond growth system. It is interesting to hypothesise that pH is strongly regulated by both CO_2 concentration and photosynthetic rates in the pond. Using higher concentration of CO_2 may result in decreasing the pH since unutilized CO_2 will be converted to H_2CO_3 and on the other hand, if there is not enough CO_2 gas supply, microalgae will utilize carbonate to maintain its growth (Widjaja et al., 2009). In a similar study, the addition of either or both, CO_2 and combined nitrogen (as KNO_3 or NH_4Cl), did not result in any increase in microalgal biomass productivity (Fontes et al., 1987). Optimal values for pH and temperature were 8·2 - 8·4 and 30 - 35°C, respectively (Fontes et al., 1987). However, the data generated was only for day time conditions and it will be interesting to monitor pH levels at night where photosynthesis does not take place.

Temperature is one of the most crucial factors affecting biomass accumulation and lipid production by microalgal cells since it is pivotal in all enzymological reactions and physiological functions of the cells. It is well documented that sub-tropical freshwater microalgae require temperatures in the range of 25 to 30°C for optimal growth (Grobbelaar, 2007).In this study, the temperature fluctuated in the 25 to 30°C range in agreement with data from other researchers (Mayo, 1997). Ambient temperature in this range is known to influence the biomass composition, nutrient requirements, nature of metabolism, and the metabolic reaction rates because microalgae do not have the ability to regulate their internal temperature (Carvalho et al., 2009; Mayo, 1997).

Microalgae require illumination conditions to economically achieve maximum photosynthetic rates and considering the operating conditions, light intensity (or illuminance) is one of the several parameters influencing the growth of photosynthetic organisms such as micro-algae (Zhao et al., 2013; Mata et al., 2012). Light intensity, quality and duration of exposure are the main driving forces for higher photosynthetic rates and subsequent high biomass and lipid productivities. It was reported that light intensity of around 300 µmol/m^2/s is ideal for optimal microalgal growth although too much light intensities can cause photo-oxidation that is harmful to *C. vulgaris* though intermittent light fluctuations enhance microalgal productivity (Grobbelaar, 1989). In addition, previous studies have reported that varying illumination intensities in outdoor conditions are likely to inhibit microalgae growth because of the shortage in light energy, for example, very low light intensities during rainy days or the photoinhibition caused by excessive irradiance, or very high light intensities at noon times during summer (Ugwu et al., 2007). The gradual decrease in light intensity is explained by the biomass accumulation in the suspension which retarded light penetration into the media. Furthermore, the presence of clouds and rainy conditions on some days prompted the ponds to be covered by a plastic sheet hence lowering light intensity in the microalgal suspension. From our findings (Figure 4), it is generally accepted that light intensities at this site are ideal for *C.vulgaris* cultivation and it is feasible to grow this culture in a large scale raceway pond.

The additional expenditure of metabolic energy under stress conditions is required for maintaining ion homeostasis and electrochemical gradients, for the biosynthesis of organic compounds which play an important role in protection and osmoregulation, and for supporting the maintenance of cellular structure (Alyabyev et al., 2007). In addition, salinity controls the osmotic potential of the suspension therefore has a strong influence on the water

relations of the microalgal cells. For small scale laboratory shake flask experiments, it is recommended to wash the cells with ammonium formate and deionised water to remove the salt residues (Chinnasamy et al., 2010). The salinity levels recorded for the three ponds are ideal for *C.vulgaris*growth. High salinity is reported to increase lipid production by microalgae but however, too high salinity levels above the threshold are detrimental to *C.vulgaris* and can lead to microalgal growth inhibition (Ho et al., 2010).

Environmental conditions such as temperature, salinity and atmospheric pressure greatly affect oxygen solubility in water. The dissolved oxygen is an important parameter for microalgal respiration and therefore energy production. The subsequent biomass and lipid yield is affected by the dissolved oxygen in the medium. The dissolved O_2 saturation in freshwater under atmospheric pressure at 20°C is 9.1 mg/L. The elevated dissolved oxygen concentrations on the final day of cultivation are explained by continuous and cumulative oxygen evolution due to microalgal photosynthesis, therefore exceeding the DO_2 concentration standard for freshwater. Elevated levels of dissolved oxygen are not desirable in the culture because it is well-known that dissolved oxygen is lethal to microalgal cells (Suali and Sarbatly, 2013).

Total dissolved solids (TDS) is a measure of the combined content of all inorganic and organic substances contained in the aqueous suspension in molecular, ionized or micro-granular suspended form. Salinity also comprises some of the ions that constitute TDS. The most common chemical constituents of TDS are calcium, phosphates, nitrates, sodium, potassium and chloride ions. These ions are readily found in BG-11 medium used in this investigation. The findings (Figure 7) demonstrate that TDS is a function of both salinity and evaporation rates. Microalgae cultivated in raceway pond remove TDS as they use the organic and inorganic ions for their primary production (MoheimaniandBorowitzka, 2006; Park et al., 2011). However, the TDS contributed by dust and clay particles cannot be eliminated by the microalgal cells. The presence of debris in the ponds could potentially lead to bacterial contamination and all the leaves and grass components that fell in the ponds were routinely removed from the ponds to alleviate this.

Conductivity is a measure of a material's ability to conduct an electrical current and due to the presence of electrolytes in BG-11 medium, conductivity is an important parameter to measure so as to establish general utilisation of the inorganic materials in the medium by the microalgal cells. Conductivity is closely associated with TDS and salinity and this is manifested by the findings obtained in this investigation (Figure 8). The phenomenon observed is explained by the bioavailability of chemical species in the BG-11 medium whose uptake by the microalgal cells led to a decrease in conductivity in the aqueous microalgal suspension.

ORP is a tendency of a chemical species to acquire electrons and thereby become reduced. The more positive the potential, the greater the species' affinity for electrons and tendency to be reduced. The more negative values for the ORP (Figure 9) are explained by the evolution of O_2 due to photosynthesis of the *C. vulgaris* cells and this indicate the vigour and robustness of the strain used with incubation time.

It has been reported that biotic factors that may impact negatively on algal growth include pathogenic bacteria and predatory zooplankton and also that the other micro-organisms may outcompete the target microalgal strain for essential nutrients (Pittman et al., 2011). Microscopic analysis of the cells was routinely done to check for contamination in the ponds. It is documented that under open cultivation system, *Chlorella* and *Scenedesmus*sp. usually coexist and are the predominant strains of the phytoplanktonic communities (Pittman et al., 2011).

In conclusion, this investigation clearly demonstrated that for successful seed culture preparation, there is need to closely monitor physico-chemical and biotic factors in the cultivation ponds. Under optimal conditions, these factors can lead to high growth rates of the target microalgal strains. However under the open system, it is very difficult to control the environmental factors and as a rule of thumb, population dynamics of the microalgae and any contaminants must be routinely monitored microscopically.

Conflict of Interests

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

ACKNOWLEDGEMENTS

This material is based upon work supported by the National Research Foundation (South Africa) and Claude Leon Foundation (South Africa). In addition, the authors would also like to thank Durban University of Technology and EThekwini Municipality for funding this project.

REFERENCES

Akpor OB, Muchie M (2011). Environmental and public health implications of wastewater quality. Afr. J. Biotechnol. 10(13): 2379-2387.

Alyabyev AJ, Loseva NL, Gordon LK, Andreyeva IN, Rachimova GG, Tribunskih VI, Ponomareva AA, Kemp RB (2007). The effect of changes in salinity on the energy yielding processes of *Chlorella vulgaris* and *Dunaliella maritime* cells. Thermochim. Acta. 458: 65-70.

Bechet Q, Shilton A, Guieysse, B (2013). Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation.Biotechnol. Adv. 31(8):1648-1663.

Bhola V, Ramesh D, Kumari-Santosh S, Karthikeyan S, Elumalai S, Bux F (2011). Effects of parameters affecting biomass yield and thermal behavior of *Chlorella vulgaris*. J. Biosci. Bioeng. 111: 377-382.

Carvalho AP, Monteiro CM, Malcata FX (2009). Simultaneous effect of irradiance and temperature on biochemica composition of the micro-

alga *Pavlovalutheri*.J. Appl. Phycol. 21: 543-552.
Chinnasamy S, Bhatnagar A, Hunt RW, Das KC (2010). Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications.Bioresour. Technol. 101:3097-3105.
Chisti Y (2007). Biodiesel from microalgae.Biotechnol. Adv. 25:294-306.
Fontes AG, Vargas MA, Moreno J, Guerrero MG, Losada M (1987).Factors affecting the production of biomass by a nitrogen-fixing blue-green alga in outdoor culture.Biomass 13:33-43.
Gendy TS, El-Temtamy SA (2013). Commercialization potential aspects of microalgae for biofuel production: An overview. Egypt. J Petrol. 22:43-51.
Grobbelaar JU (1989). Do light/dark cycles of medium frequency enhance phytoplankton productivity. J. Appl. Phycol.1: 333-340.
Grobbelaar JU (2007). Photosynthetic characteristics of *Spirulina platensis* grown in commercial-scale open outdoor raceway ponds: what do the organisms tell us? J. Appl. Phycol. 19: 591-598.
Grobbelaar JU (2009). From laboratory to commercial production: a case study of a *Spirulina* (*Arthrospira*) facility in Musina, South Africa. J. Appl. Phycol. 21:523-527.
Harun R, Singh M, Forde GM, Danquah MK (2010). Bioprocess engineering of microalgae to produce a variety of consumer products. Renew. Sustain. Energy Rev. 14:1037-1047.
Ho SH, Chen WM, Chang JS (2010). *Scenedesmusobliquus* CNW-N as a potential candidate for CO_2 mitigation and biodiesel production.Bioresour. Technol. 101:8725-8730.
Jonker JGG, Faaij APC (2013). Techno-economic assessment of micro-algae as feedstock for renewable bio-energy production. Appl. Energy 102: 461-475.
Mata TM, Melo AC, Simões M, Caetano NS (2012). Parametric study of a brewery effluent treatment by microalgae *Scenedesmusobliquus*.Bioresour. Technol. 107:151-158.
Mayo AW (1997). Effects of Temperature and pH on the kinetic growth of unialga*Chlorella vulgaris* cultures containing bacteria. Water Environ. Res. 69: 64-72.
Moheimani NR, Borowitzka MA (2006). The long-term culture of the coccolithophore*Pleurochrysiscarterae*(Haptophyta) in outdoor raceway ponds. J. Appl. Phycol. 18: 703-712.
Mutanda T, Karthikeyan S, Bux F (2011b). The utilization of post-chlorinated municipal domestic wastewater for biomass and lipid production by *Chlorella* spp. under batch conditions. Appl. Biochem. Biotechnol.164: 1126-1138.
Mutanda T, Ramesh D, Karthikeyan S, Kumari S, Anandraj A Bux F (2011a). Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. Bioresour. Technol. 102: 57-70.
Oncel SS (2013).Microalgae for a macroenergy world.Renew. Sustain. Energy Rev. 26:241-264.
Park JBK, Craggs RJ, Shilton AN (2011). Wastewater treatment high rate algal ponds for biofuel production.Bioresour. Technol. 102:35-42.
Pittman JK, Dean AP, Osundeko O (2011). The potential of sustainable algal biofuel production using wastewater resources.Bioresour. Technol. 102:17-25.
Pulz O, Gross W (2004). Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol.65: 635-648.
Radmann EM, Reinehr CO, Costa JAV (2007).Optimization of the repeated batch cultivation of microalga *Spirulinaplatensis* in open raceway ponds. Aquaculture 265:118-126.
Razzak S.A., Hossain MM, Lucky RA, Bassi AS, Lasa H (2013). Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing- A review. Renew. Sustain. Energy Rev. 27:22-653.
Singh B, Guldhe A, Rawat R, Bux F (2014). Towards a sustainable approach for development of biodiesel from plant and microalgae. Renew. Sustain. Energy Rev. 29:216-245.
Stanier RY, Kunisawa R, Mandel M, Cohen-Bazier G (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol.Rev. 35: 171-205.
Suali E, Sarbatly R (2012). Conversion of microalgae to biofuel.Renew. Sustain. Energy Rev.16:4316-4342.
Ugwu CU, Aoyagi H, Uchiyama H (2007). Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica 45: 309-311.
Widjaja A, Chien CC, Ju YH (2009). Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J. Taiwan Inst. Chem. E. 40:13-20.
Zhao Y, Wang J, Zhang H, Yan C, Zhang Y (2013). Effects of various LED light wavelengths and intensities on microalgae-based simultaneous biogas upgrading and digestate nutrient reduction process. Bioresour. Technol. 136: 461-468.

academicJournals
Vol. 13(35), pp. 3617-3627, 27 August, 2014
DOI: 10.5897/AJB2014.13907
Article Number: B5808AB47059
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antimicrobial activities, toxinogenic potential and sensitivity to antibiotics of *Bacillus* strains isolated from Mbuja, an *Hibiscus sabdariffa* fermented seeds from Cameroon

Bouba-Adji Mohammadou[1]*, Gwenaelle Le Blay[3,4], Carl Moses Mbofung[2] and Georges Barbier[3]

[1]Department of Food Engineering and Quality Control, University Institute of Technology, P. O. Box 454 Ngaoundere, Cameroon.
[2]Department of Food Science and Nutrition, National School of Agro-Industrial Sciences, P. O. Box 455 Ngaoundere, Cameroon.
[3]Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA 3882), Université de Brest, Université Européenne de Bretagne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.
[4]Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Université de Brest, Université Européenne de Bretagne, IUEM-UMR 6197 CNRS-IFREMER, Place Nicolas Copernic, F-29280 Plouzané, France.

Received 10 May, 2014; Accepted 4 July, 2014

This study was carried out to screen for antimicrobial activities against common pathogenic and food spoilage bacteria, yeasts and molds, toxinogenic potential and sensitivity to antibiotics of 26 *Bacillus* strains (11 *Bacillus subtilis,* 5 *Bacillus megaterium,* 4 *Bacillus amyloliquefaciens,* 4 *Bacillus pumilus* and 2 *Bacillus thuringiensis*) isolated from different samples of *Hibiscus sabdariffa* fermented seeds (Mbuja). Antimicrobial assays were realised according to the cross-streak and the overlay methods. The main toxin and bacteriocin encoding genes were screened by PCR using specific primers and antibiotic resistance was assessed by the disc diffusion method. *Bacillus* species showed variable ability to inhibit bacterial and/or fungal species. The most antibacterial strains were *B. amyloliquefaciens* (S1 and S5) and *B. subtilis* (S12) whereas the most antifungal ones were *B. megaterium* S8 and S9. Subtilin and subtilosin A genes were detected in seven strains of *B. subtilis* but they were not associated with antimicrobial activities. All strains, except *B. thuringiensis,* did not contain toxin encoding genes and were sensitive to most tested antibiotics. This suggests that antimicrobial strains could be used in starter cultures for a controlled fermentation to produce *Mbuja* in order to better control the fermentation process of Mbuja and to increase consumer's safety.

Key words: *Bacillus*, antimicrobial activities, toxins, antibiotic resistance.

INTRODUCTION

The traditional condiment produced in Cameroon by fermenting *Hibiscus sabdariffa* seeds also known as

*Corresponding author. E-mail: malbach1@yahoo.fr. Tél: + 237 77 80 31 37.

Mbuja is commonly used in the diet of people from the Sahelian region, especially for those living in rural areas with low incomes. The condiment is mostly appreciated for its nutritional value and organoleptic properties and it is used to flavour soups and sauces (Mohamadou et al., 2009). Earlier studies revealed that Mbuja could contribute to consumers' health through its anti-oxidants and phenolic compounds (Mohamadou et al., 2007). *Bacillus* spp. mainly *Bacillus subtilis* and related genera are responsible for the fermentation (Mohamadou et al., 2013).

Like for many other traditional condiments, the production of Mbuja relies on spontaneous and uncontrolled fermentation that could impact the quality and safety of the products on one hand, and on the consumers' health on the other hand. Recent studies brought evidence that safety of fermented products could be significantly improved by selecting starter cultures with protective effects against most common pathogenic and spoilage bacteria, yeasts and molds (N'dir et al., 1994; Ouoba et al., 2007). Some *Bacillus* species produce antibiotics and antimicrobial compounds such as bacteriocins including subtilin and subtilosin synthesized by *B. subtilis* (Klein et al., 1992; Stein et al., 2004; Abriouel et al., 2011). On the other hand, *Bacillus cereus* and related species (*Bacillus thuringiensis* and *Bacillus anthracis*) are well-known food poisoning bacteria which produce either emetic heat-stable toxin or diarrheal enterotoxins (HBL, NHE and BcET) (Matarante et al., 2004). Another trait of *Bacillus cereus* and related species toxicity is their ability to secrete phospholipases, cell-lysing enzymes, including sphingomyelinase (sph) and phosphatidylinositol- and phosphatidylcholine-specific phospholipase (piplc) (Matarante et al., 2004). Furthermore, some studies reported that virulence factors were also present in other *Bacillus* species than *Bacillus cereus* (Kramer and Gilbert, 1989; Phelps and McKillip, 2002).

The aim of this study was to investigate the potential food preservation and contribution to food safety as criteria for selection of starter cultures to be used in controlled fermentation of *H. sabdariffa* seeds. Hence, the work intended to assess antimicrobial activity, toxinogenic potential and sensitivity to antibiotics of *Bacillus* strains isolated from Mbuja.

MATERIALS AND METHODS

Microorganisms

Eleven strains of *B. subtilis* (S2, S7, S12, S15, S16, S17, S18, S19, S20, S21 and SY), five strains of *Bacillus megaterium* (S3, S8, S9, S11 and S14), four strains of *Bacillus amyloliquefaciens* (S1, S5, S13 and SX), four strains of *Bacillus pumilus* (S4, S6, S22 and S23) and two strains of *B. thuringiensis* (S10 and SAc) maintained at the "Laboratoire Universitaire de Biodiversité et Ecologie Microbienne" (LUBEM) culture collection were investigated. These strains were previously isolated from different productions of Mbuja (fermented *H. sabdariffa* seeds) (Mohamadou et al., 2009) and identified with molecular and phenotypic methods (Mohamadou et al., 2013). Their antimicrobial activity was investigated against 6 indicators or pathogenic bacteria (*Bacillus cereus* ATCC 6464, *Listeria innocua* HPB13 [used as indicator for *L. monocytogenes*], *Pseudomonas aeruginosa* PAO1, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Escherichia coli* and *Staphylococcus aureus*). The last three pathogenic bacteria were clinical isolates obtained from the "CHRU" (Regional University University Hospital) of Brest. Five indicators of yeasts (*Rhodotorula mucilaginosa* UBOCC-A-202007; *Debaryomyces hansenii* CLIB197; *Saccharomyces cerevisiae* CLIB227; *Kluyveromyces marxianus* CLIB282; *Candida parapsilosis* CLIB214) and four molds (*Mucor plumbeus* CBS129.41; *Aspergillus niger* UBOCC-A-101073; *Aspergillus flavus* UBOCC-A-10826; and *Fusarium oxysporum* UBOCC-A-108079) commonly encountered in food spoilage in developing countries (Njongmeta et al., 2004; Djouldé et al., 2007; Yaouba et al., 2010) were also tested. Fungi were supplied by the Culture Collection of the University of Brest (UBOCC, Plouzané, France, http://www.univ-brest.fr/ubocc) and by the CLIB Yeast Culture Collection (CLIB, Thivernal-Grignon, France).

Preparation of *Bacillus* inocula

Bacillus strains cryopreserved in culture medium containing glycerol at 30% (v/v) were cultured on nutrient agar (NA) (AES Chemunex, Bruz, France) plates and incubated for 24 h at 37°C. The strains were then subcultured for 18 h at 37°C in 10 mL of trypton soy broth (TSB) (AES Chemunex, Bruz, France). These cultures were used for antimicrobial activities screening.

Preparation of fungal inocula

Molds' inocula were prepared by growing the molds on potato dextrose agar (PDA, AES Chemunex) slants at 25°C for 7 to 10 days until sporulation. The spores were then collected by vigorously shaking the slants with sterile peptone water (0.1%, w/v). Yeast cell inocula were prepared from cultures grown in M2Lev agar (20 g/L malt extract, 3 g/L yeast extract, and 15 g/L agar). Spores and yeasts concentrations were determined by microscopic Malassez cell counts and adjusted to 10^6 cells or spores/mL with sterile peptone water (0.1%).

Inhibition of indicators bacteria by cross-streak assay

The screening of inhibitory activity of *Bacillus* strains was realized according to the cross-streak assay described by Pugsley and Oudega (1987) with some modifications. Each *Bacillus* strain was picked from TSB and a single streak of this culture was applied both on a NA agar plate and on a glucose agar with bromocresol purple (BCP) plate using a 10 µL sterile loop. Cross-streak assay on BCP was aimed at verifying that inhibitory activity of *Bacillus* strains was not due to acid production. After incubating the plates for 18 h at 37°C, the densely overgrown streak was inactivated by applying chloroform (anhydrous, ≥ 99%, Fluka, Sigma-Aldrich, Saint-Quentin Fallavier, France) using a Pasteur-pipette to form a thin film covering the whole streak of bacteria. The Petri dish was left closed for 10 min to inactivate all living cells before they were opened for 10 min (under a hood) to evaporate the toxic chloroform. A streak of the tested pathogenic bacteria was then applied perpendicularly to the chloroform-inactivated *Bacillus* strain (Figure 1) to determine the inhibition activity. Standardized suspensions in 0.85% NaCl (Mc Farland Standard 1) of tested pathogen was applied with a 10 µL sterile loop as indicated by Zihler et al. (2009).

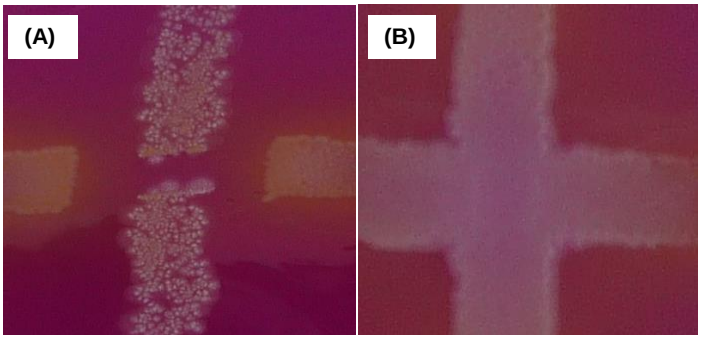


Figure 1. *In vitro* activity of *Bacillus* strains by Cross-streak with strong inhibition (A) and no inhibition on BCP agar (B).

Antifungal activity screening

The antifungal assay was performed by the overlay method described by Magnusson and Schnürer (2001). The method was realized using NA plates on which *Bacillus* strains were inoculated as a spot and incubated at 30°C. The plates were then overlaid with 10 mL of M2Lev soft agar (0.8% agar) containing 10^6 yeast cells or fungal spores per mL. The plates were examined qualitatively for clear zones of inhibition around the bacterial spots.

Total bacterial DNA extraction

The 26 *Bacillus* strains were grown for 18 h at 30°C on TSB (AES Chemunex). Tubes were centrifuged 10 min at 6000 *g* and the pellets were suspended in 0.5 mL of sterile physiological water (0.85% NaCl). DNA was extracted from suspended pellet and purified using the FastDNA® SPIN Kit (QBIOgene; MP Biomedicals, Solon, Ohio, USA) as recommended by the manufacturer's SPIN™ protocol. Pure DNA samples were frozen at -20°C until use.

PCR detection of genes encoding the bacteriocins subtilin and subtilosin

PCR were performed to screen for the presence of subtilin and subtilosin genes, two bacteriocins frequently encountered in *Bacillus* species (Abriouel et al., 2011). All the *Bacillus* strains were tested. Primers designed by Sutyak et al. (2008) were used for targeting the subtilin gene (*spaS*), whereas for subtilosin A (*sboA*), primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) on alignment of known sequence encoding the *sboA* gene from *B. subtilis* subsp. *spizizenii* (acc. n°BSUW23_18455) (Table 1). One microliter (95-105 ng) of genomic DNA from each *Bacillus* strains or *B. subtilis* LMG 8197 used as positive control were added to 24 µl of a mix PCR consisting of each primer (1 µM), dNTP (0.2 µM), $MgCl_2$ (1.5 µM), buffer (5 µL of 5× buffer), water (10 µL) and 0.5 µL of GoTaq polymerase (Promega, Charbonnières, France) to make a final volume of 25 µL. PCR was carried out using a PTC-100 programmable thermal controller (MJ Research, Waltham, Massachussetts, USA) according to the following conditions as described by Sutyak et al. (2008): denaturation for 30 s at 94°C, annealing for 30 s at 55°C (*spaS*) or 50°C (*sboA*) and elongation for 1 min at 65°C for a total of 30 cycles. PCR products were electrophoresed on agarose gel (1.5% w/v), stained with ethidium bromide and visualized under UV.

PCR detection of genes encoding a larvicidal protein (Cry1) in *Bacillus thuringiensis* strains

The presence of *Cry1* genes commonly encountered in *B. thuringiensis* and known to encode for proteins active against many insect orders including *Lepidoptera, Diptera, Coleoptera*, *Hymenoptera, Homoptera, Mallophaga*, and *Acari* (Cinar et al., 2007) was also investigated in the two *B. thuringiensis* strains (S10 and SAc) with primers (CJI-1 and CJI-2) designed by Céron et al. (1995) (Table 1). PCR mixtures were prepared using 1 µl of each primer (25 µM), 0.5 µl of dNTP (10 µM), 1.5 µl of $MgCl_2$ (25 µM), 5µl of 5× Green Buffer GoTaq® Flexi Buffer, 0.5 µl of GoTaq® DNA Polymerase (Promega), and 14.5 µl of water to make a final volume of 24 µL. Amplification was performed using 1 µl (about 100 ng) of genomic DNA from *Bacillus thuringiensis* strains and *B. subtilis* LMG 8197 (negative control) and 24 µl of PCR mixture in a PTC-100 programmable thermal controller (MJ Research) according to the following conditions defined by Bobrowski et al. (2001): initial denaturation at 95°C for 2 min followed by 30 cycles consisting of a denaturation step of 95°C for 1 min; annealing step of 52°C for 1 min, and an extension step of 72°C for 1 min and a final extension of 72°C for 5 min. PCR products were analyzed by electrophoresis, stained with ethidium bromide and visualized under UV.

PCR screening of genes encoding *B. cereus* enterotoxins and virulence factors

The genes encoding the major *B. cereus* enterotoxins and virulence factors were investigated by PCR in the 26 *Bacillus* strains. Parts of the following genes encoding hemolysin (*hbl-D/A,* 623 bp), non hemolytic enterotoxin (nheB, 769 bp), *B. cereus* enterotoxin T (*bceT,* 428 bp) and enterotoxin FM (*entFM,* 1269 bp) were screened. Virulence factors were also investigated with the targeting of genes coding for two phospholipases associated with cell lysis, sphingomyelinase (*sph* gene, 558 bp) and phosphatidylinositol-specific phospholipase C (*piplc* gene, 569 bp). Reference studies and used primers (Sigma-Aldrich) are listed in Table 1. PCR mixture of 25 µl contained 20 to 35 ng of genomic DNA (*Bacillus* strains and *Bacillus cereus* LMG 6923 used as positive control), 1 µM of each primer, 0.5 µM dNTP, 1.5 µM of $MgCl_2$, GoTaq® DNA Polymerase, 5× Green Buffer GoTaq® Flexi Buffer and distilled water. Amplification was realized according to Matarante et al. (2004) and consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 25 s, 55°C for 45 s, and 72°C for 2 min and a final extension at 72°C for 5min. PCR was performed with a PTC-100 programmable thermal controller (MJ Research). PCR products were analyzed on 1.5% (w/v) agarose gel stained with ethidium bromide and visualized by UV.

Hemolytic activity of *Bacillus* strains

Hemolytic activity was determined for all strains on blood agar plates (AES Chemunex) containing 5% of sheep blood. *B. subtilis* LMG 8197 and *Bacillus cereus* LMG 6923 were used respectively as negative and positive controls. Five microliters of cells grown overnight in TSB were inoculated as spots on blood agar plates which were incubated at 30°C for 24 h. Positive strains produced clear zone of hemolysis around the colonies.

Antibiotic susceptibility of *Bacillus* strains

The antibiotic susceptibility screening of the *Bacillus* strains was carried out by the disc diffusion method (Biomérieux, Marcy l'Etoile, France) as indicated by the producer. A single colony of *Bacillus* strains grown on NA plate was diluted in sterile 0.85% NaCl

Table 1. Primers used for PCR detection of genes encoding toxins, virulence factors, antimicrobial peptides and insecticidal proteins.

Target gene	Primer name	Primer sequence (5' - 3')	Amplicon size (bp)	Reference
Hbl-D/A	hblD-f	GGAGCGGTCGTTATTGTTGT	623	Matarante et al. (2004)
	hblA-r	GCCGTATCTCCATTGTTCGT		
nheB	nheB 1500S	CTATCAGCACTTATGGCAG	769	Granum et al. (1999)
	nheB 2269A	ACTCCTAGCGGTGTTCC		
bceT	ETF	TTACATTACCAGGACGTGCTT	428	Agata et al. (1995)
	ETR	TGTTTGTGATTGTAATTCAGG		
entFM	EntA	ATGAAAAAAGTAATTTGCAGG	1269	Asano et al. (1997)
	EntA	TTAGTATGCTTTTGTGTAACC		
Sph	Ph1	CGTGCCGATTTAATTGGGGC	558	Hisieh et al. (1999)
	Ph2	CAATGTTTTAAACATGGATGCG		
Piplc	PC105	CGCTATCAATGGACCATGG	569	Damgaard et al. (1996)
	PC106	GGACTATTCCATGCTGTACC		
Subtilin	spaSFwd	CAAAGTTCGATGATTTCGATTTGGATGT	125	Klein et al. (1992)
	spaSRev	GCAGTTACAAGTTAGTGTTTGAAGGAA		
Subtilosin	sboAf	ACAAAGGTTGTGCAACATGC	132	This study
	sboAr	TCCCCATAGACCGAATAGACC		
Cry1	CJI-1	TGTAGAAGAGGAAGTCTATCCA	280	Céron et al. (1995)
	CJI-2	TATCGGTTTCTGGGAAGTA		

Table 2. Interpretive criteria for MIC (mg/L) and MID (mm) by disc diffusion method.

Antibiotic	Minimal Inhibition Concentration (mg / L)		Minimal Inhibition Diameter (mm)	
	Sensitive	Resistant	Sensitive	Resistant
	≤	>	≥	<
Erythromycin	1	4	22	17
Vancomycin	4	8	17	X
Tetracyclin	4	8	19	17
Streptomycin	8	16	15	13
Kanamycin	8	16	17	15
Ampicillin	2	8	21	16
Trimethoprim-Sulfamethoxazol	2/38	8/152	16	10
Chloramphenicol	8	16	23	19

solution to obtain an Optical Density (OD) of Mc Farland Standard 0.5 (BioMérieux). Discs were purchased from BioMérieux and the antibiotics concentrations used were: erythromycin, 15 µg; vancomycin, 30 µg; tetracyclin, 30 µg; streptomycin, 10 µg; kanamycin, 30 µg; ampicillin, 10 µg; trimethoprim sulfamethoxazol, 1.25 µg + 23.75 µg and chloramphenicol, 30 µg. The bacterial suspension was applied to the surface of Mueller Hinton agar plates (AES Chemunex) using a sterile swab to obtain a homogenous bacterial film. The plates were then left to dry for 10 min before the disc were applied in the center of the plate. The plates were incubated at 37°C for 48 h. For tests strains susceptible to antibiotics, a clear area was observed around the disc. The diameters of inhibition areas were measured and interpreted according to the recommendations of the Antibiogram Committee of SFM, the French Society for Microbiology (2009) (Table 2).

RESULTS AND DISCUSSION

Antimicrobial activities of the *Bacillus* strains

The ability of the 26 *Bacillus* strains to inhibit indicators and pathogenic bacteria, yeasts and molds was investigated and reported in Tables 3 and 4. The antibacterial behaviour of *Bacillus* strains strongly varied between *Bacillus* strains and targets. *Bacillus* strains were both effective against Gram-positive and Gram-negative bacteria. *Bacillus cereus* ATCC6464 was inhibited by 14 strains, *Listeria innocua* HPB13 by 10 and *S. aureus* by 2 strains only. *E. coli* was the most sensitive

Table 3. Antimicrobial activity of the 26 *Bacillus* strains obtained with the cross-streak assay against 6 indicator bacteria.

Bacteria		Antibacterial activity						
Identitification	**Strain**	***Bacillus cereus* ATCC 6464**	***Listeria innocua* HPB13**	***Staphylococcus aureus***	***Pseudomonas aeruginosa* PAO1**	***Escherichia coli***	***Salmonella* Typhimurium**	**Inhibited species**
B. amyloliquefaciens	S1	+	+	+	-	+	-	4
	S5	++	++	-	-	++	-	3
	S13	+	++	-	-	+	-	3
	SX	+	++	-	-	+	-	3
B. thuringiensis	S10	+	-	-	-	-	-	1
	SAc	-	-	-	-	+	-	1
B. megaterium	S3	-	-	-	-	-	-	0
	S8	-	-	-	-	-	-	0
	S9	-	-	-	-	-	-	0
	S11	-	-	-	-	-	-	0
	S14	-	-	-	-	-	-	0
B. pumilus	S4	-	-	-	-	+	-	1
	S6	-	+	-	-	-	-	1
	S22	-	-	-	-	-	-	0
	S23	+	+	-	-	-	-	2
B. subtilis	S2	-	-	-	-	-	-	0
	S7	+	-	-	-	-	-	1
	S12	+	+	+	+	-	-	4
	S15	+	-	-	-	-	-	1
	S16	+	++	-	-	+	-	3
	S17	+	-	-	-	-	-	1
	S18	-	-	-	-	-	-	0
	S19	+	-	-	-	+	-	2
	S20	+	-	-	-	-	-	1
	S21	-	+	-	-	-	-	1
	SY	++	+	-	-	+	-	3

- , no inhibition; +, zone of inhibition between 6 and 8 mm; ++, zone of inhibition higher than 8 mm.

Gram-negative bacteria to *Bacillus* strains with nine inhibitions whereas *P. aeruginosa* PAO1 was inhibited with one strain only and *S. typhimurium* was not inhibited. The broadest spectrum of inhibition was shown by *B. amyloliquefaciens* S1 and *B. subtilis* S12 (with 4 sensitive targets) while the strongest effects were observed with *B. amyloliquefaciens* S5. *Bacillus cereus* showed weak inhibition ability and no *B. megaterium* was able to inhibit the tested bacteria.

As for bacteria, the ability of tested *Bacillus* spp. to inhibit fungal targets varied with *Bacillus* strains and tested fungi. However, the antifungal *Bacillus* species (*B. megaterium, B. thuringiensis* and *B. pumilus*) differed from the antibacterial ones (*B. amyloliquefaciens* and *B. subtilis*) (Table 4). Both molds and yeasts were inhibited. The most sensitive molds were *Fusarium oxysporum* UBOCC-A-108079 (sensitive to 9 *Bacillus* isolates), followed by *Aspergillus flavus* UBOCC-A-10826 and *Mucor plumbeus* CBS129.41 (3 *Bacillus*) while *Debaromyces hansenii* CLIB197 and *Kluyveromyces*

Table 4. Antimicrobial activity of the 26 *Bacillus* strains obtained with the spot test assay against 9 indicator fungi.

Bacteria	Code	Antifungal activity									
		Yeast					Mold				
		Rhodotorula mucilaginosa UBOCC-A-202007	*Debaryomyces hansenii* CLIB197	*Saccharomyces cerevisiae* CLIB 227	*Kluyveromyces marxianus* CLIB282	*Candida parapsilosis* CLIB214	*Mucor plumbeus* CBS129.41	*Aspergillus niger* UBOCC-A-101073	*Aspergillus flavus* UBOCC-A-10826	*Fusarium oxysporum* UBOCC-A-108079	Inhibited species
B. amyloliquefaciens	S1	-	-	-	-	-	-	-	-	-	0
	S5	-	-	-	-	-	-	-	+++	+	2
	S13	-	-	-	-	-	-	-	-	-	0
	SX	-	-	-	-	-	-	-	-	-	0
B. thuringiensis	S10	-	-	-	-	-	-	-	+	+	2
	SAc	-	-	-	+	+	+	+	-	+	5
B. megaterium	S3	++	-	+++	-	-	-	-	-	-	2
	S8	+	+++	+++	+++	++	-	-	-	+++	6
	S9	-	+++	+++	+++	+	-	-	-	+++	5
	S11	-	-	-	-	-	+	-	-	-	1
	S14	-	-	-	-	-	-	-	-	-	0
B. pumilus	S4	-	-	-	-	-	-	-	-	+	1
	S6	-	-	-	-	-	-	-	-	+	1
	S22	-	-	-	-	-	-	-	-	+++	1
	S23	-	-	-	+++	-	+++	-	+	+++	4
B. subtilis	S2	-	-	-	-	-	-	-	-	-	0
	S7	-	-	-	-	-	-	-	-	-	0
	S12	-	+	-	-	-	-	-	-	-	1
	S15	-	+	-	-	-	-	+	-	-	2
	S16	-	-	-	-	-	-	-	-	-	0
	S17	-	-	-	-	-	-	-	-	-	0
	S18	-	-	-	-	-	-	-	-	-	0
	S19	-	-	-	-	-	-	-	-	-	0
	S20	-	-	-	-	-	-	-	-	-	0
	S21	-	-	-	-	-	-	-	-	-	0
	SY	-	-	-	-	-	-	-	-	-	0

- , no inhibition; +: inhibition diameter between 4 and 6 mm; ++: inhibition diameter between 8 and 12 mm; +++: inhibition diameter higher than 12 mm.

marxianus CLIB282 were the most sensitive yeasts (inhibited with 4 *Bacillus* strains) followed by *Candida parapsilosis* CLIB214 and *Saccharomyces cerevisiae* CLIB227 (3 isolates). *Aspergillus niger* UBOCC-A-10826 and *Rhodotorula mucilaginosa* UBOCC-A-202007 were the most resistant fungi (inhibited by only 2 *Bacillus*). *B. megaterium* S8 (inactive against bacteria) showed the broadest activity spectrum and the strongest activity against six fungi (the 5 tested yeasts plus *Fusarium oxysporum* UBOCC-A-108079) followed by *B. megaterium* S9 (strong inhibition against 4 yeasts and *Fusarium oxysporum* UBOCC-A-108079). *B. thuringiensis*

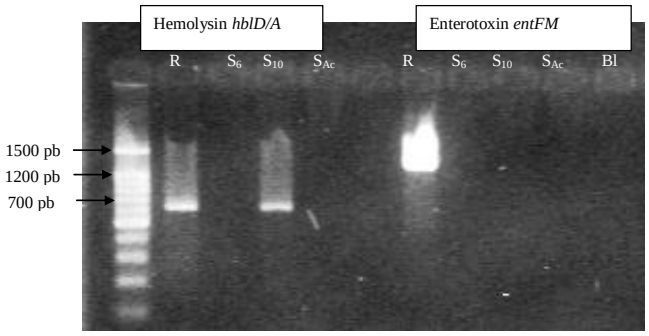


Figure 2. PCR gel of hemolysin *hbl D/A* (623 pb) and Enterotoxin FM *entFM* (1269 pb) genes. R is the reference strain of *Bacillus cereus* LMG 6923 used as a positive control, and B1 is the reference strain of *Bacillus subtilis* LMG 8197 used as a negative control.

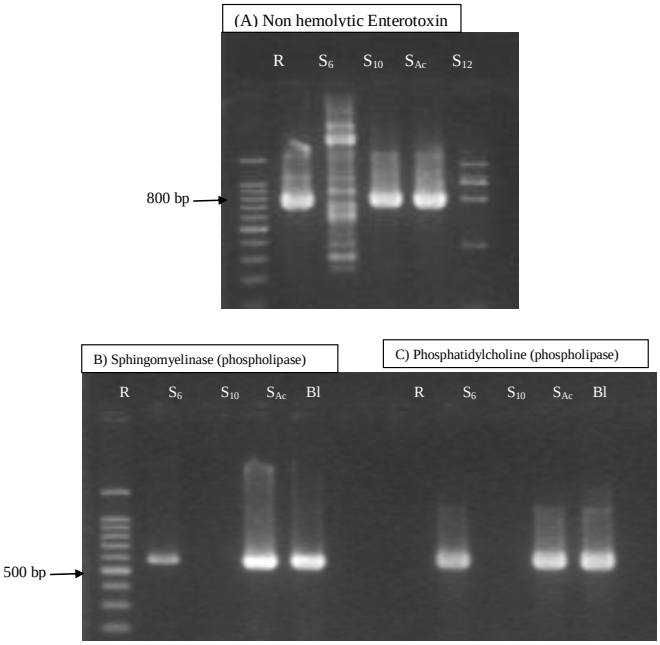


Figure 3. PCR gel of A) non hemolytic enterotoxin *nheB* gene (769pb), B) sphingomyelinase *sph* gene (558 bp) and C) phosphatidylinositol-specific phospholipase C *piplc* gene (569 bp). R is the reference strain of *Bacillus cereus* LMG 6923 used as a positive control, B1 is the reference strain of *Bacillus subtilis* LMG 8197 used as a negative control

showed a moderate but broad activity against 5 fungi while *B. pumilus* S23 showed lower antifungal ability (4 fungi inhibited). At the whole, *B. amyloliquefaciens* and *B. subtilis* isolates exhibited very
weak ability to inhibit both yeasts and molds.

PCR screening for bacteriocins and larvicidal proteins genes

The 26 *Bacillus* strains were assayed for the presence of two bacteriocins (subtilin and subtilosin) genes generally present in *B. subtilis* and related species. The PCR investigation revealed the presence of *spaS* (subtilin gene) in *B. subtilis* S2 only. Subtilosin A gene (*sboA*) was detected in *B. subtilis* S2, S7, S15, S17, S18, S19 and S20. Seven strains out of 11 *B. subtilis* contained either *spaS* or *sboA*. The screening of these bacteriocins encoding genes was negative for all other *Bacillus* species. The *Cry1* gene, encoding for a protein (Cry1) active against many insects was not detected in any of the 26 tested *Bacillus* strains including *B. thuringiensis* S10 and SAc.

PCR screening for toxins encoding genes

The presence of virulence genes was assessed for the 26 *Bacillus* strains by PCR screening (Figures 2 and 3). The presence of some genes of the enterotoxins responsible for diarrheal poisoning was confirmed for two strains identified as *B. thuringiensis.* Indeed, non hemolytic enterotoxin (*nhE*) genes were amplified by the specific primers in *B. thuringiensis* S10 and SAc while hemolysin gene (*hbl-D/A*) was detected only in SAc. However, *Bacillus cereus* enterotoxin (BcET) and enterotoxin FM genes were not amplified in all strains including the two *B. thuringiensis.* Two *Bacillus cereus* enzymes encoding genes were also screened: sphingomyelinase (*Sph*) and Phosphatidylinositol-specific phospholipase C (Piplc). These haemolytic and cell membrane hydrolytic enzymes genes were amplified in the 2 *B. thuringiensis* S10 and SAc. No other *Bacillus* species contained *Sph* and *Piplc* genes.

Hemolytic activity

Hemolytic activity was tested for the 26 *Bacillus* strains. Only the two *B. thuringiensis* strains (S10 and SAc) exhibited haemolytic activity on 5% sheep blood agar plates.

Antibiotics susceptibility

Relative susceptibilities to eight antibiotics were determined for 26 *Bacillus* strains (Table 5). The 26 strains were susceptible to erythromycin, vancomycin and streptomycin. Cases of resistance to antibiotics by at least one species were obtained for the six other antibiotics tested. Indeed, 18% of *B. subtilis* strains (strains S21 and SY) were resistant to tetracyclin; 25% of *B. pumilus* (S22) to kanamycin; 100% of *B. thuringiensis* (S10 and SAc) to ampicillin; 25% of *B. pumilus* (S22) to trimethoprim sulfamethoxazol; 25% of *B. amyloliquefaciens* (SX), 50% of *B. thuringiensis* (SAc), 40% of *B. megaterium* (S8 and S11) and 18% of *B. subtilis* (S19 and SY) were resistant to chloramphenicol.

Table 5. Antibiotic susceptibility (percentage) of the 26 *Bacillus* strains against nine tested antibiotics.

Bacteria	Number of strains	Antibiotics							
		E	VA	TE	S	K	A	SXT	C
B. amyloliquefaciens	4	100	100	100	100	100	100	100	**75**
B. thuringiensis	2	100	100	100	100	100	**0**	100	**50**
B. megaterium	5	100	100	100	100	100	100	100	**60**
B. pumilus	4	100	100	100	100	**75**	100	**75**	100
B. subtilis	11	100	100	**82**	100	100	100	100	**82**

E: Erythromycin; S: streptomycin; SXT: trimethoprim sulfamethoxazol; VA: vancomycin; K: kanamycin; TE: tetracyclin; A: ampicillin; C: chloramphenicol.

The most important antibiotic resistances for all species were observed in chloramphenicol. The most susceptible species were *B. megaterium* with resistance (40%) to only one antibiotic (chloramphenicol).

This study was carried out to investigate the potential food preservation and contribution to food safety as criteria for selection of starter cultures to be used in controlled fermentation of *Hibiscus sabdariffa* seeds. Both bacteria and fungi (yeasts and molds) are important food spoilage or pathogens in different food systems in developing countries. Some microorganisms evaluated in this study are regularly cited in food toxiinfections cases in Cameroon. Therefore, there is a need for affordable and safe methods to inhibit bacterial and fungal growth in fermented foods. This work documents the control of food spoilage and pathogenic bacteria and fungi by 26 *Bacillus* strains belonging to 5 species and their potential use as starters with a protective potential for a controlled and safe fermentation process. The strains showed different antibacterial and antifungal profiles between and within identified species. *Bacillus* strains active against bacteria were not effective against fungi.

With regard to antibacterial activities, *B. subtilis* and related species (*B. amyloliquefaciens*) induced different degree of inhibition depending on the tested species, independently of their Gram staining. Differences in antibacterial properties observed between closely related species were also observed in their phenotypic characteristics and could be explained by their genetic diversity (Mohamadou et al., 2013). The most antibacterial species were *B. amyloliquefaciens* followed by *B. subtilis* whereas *B. megaterium* induced no inhibition of bacteria and *B. pumilus* only two. *Bacillus* strains mainly inhibited, in a decreasing order, *B. cereus, L. innocua* (used as a model for *L. monocytogenes*), *S. aureus* and *E. coli,* which are among the most common foodborne pathogenic bacteria in Cameroon (Njongmeta et al., 2004; Djouldé et al., 2007). The inactivation tests showed that *B. amyloliquefaciens* S1 and S5 and *B. subtilis* S12 exhibited the broadest activity against the most common pathogens studied. *B. amyloliquefaciens* S1 and S5 inhibited Gram positive (*B. cereus* ATCC6464, *L. innocua* HPB13 and *S. aureus*) and Gram negative (*E. coli* and *P. aeruginosa* PAO1) bacteria. However, that none of the 26 strains was able to inhibit *Salmonella* Typhimurium, a leading and endemic cause of bloodstream infection in sub-Saharan Africa (Morpeth et al., 2009) is disappointing.

The pathways of antibacterial activities were partially screened. The cross-streak test on BCP indicated that the *Bacillus* species did not inhibit bacteria indicators through acid production, because the medium did not turn yellow as a sign of acidification. PCR screening of *spaS* and *sboA* genes showed that seven *B. subtilis* strains were susceptible to produce known bacteriocins: subtilin and/or subtilosin A. The production of these 2 antibacterial peptides is well documented and has been reported for *B. subtilis* and other related species like *B. amyloliquefaciens* (Klein et al., 1992; Stein et al., 2004; Sutyak et al., 2008). Subtilin, a cationic pentacyclic antimicrobial peptide is a lantibiotic that shows antimicrobial activity against a broad spectrum of Gram-positive bacteria (Abriouel et al., 2011). Subtilosin A shows a bactericidal activity against Gram-positive and Gram-negative bacteria (Shelburne et al., 2007; Sutyak et al., 2008). However, none of the most antibacterial strains (S1, S5 and S12) appears equipped to produce neither subtilin nor subtilosin A. These results suggest that *B. subtilis* and *B. amyloliquefaciens* strains isolated from Mbuja probably produced other antibacterial molecules active against Gram-positive and Gram-negative bacteria or they possess divergent sequences.

Molds and yeasts may also be pathogenic (through their toxins, like aflatoxins) and important food spoilage organisms in Cameroon (Mbiapo et al., 1989; Djouldé et al., 2007). *Bacillus* isolates were able to inhibit *in vitro* fungal growth. Both molds and yeasts were sensitive to representatives of the studied species. The most important antifungal activities were reported for *B. megaterium* strains while *B. subtilis* proved to be very weak fungi inhibitors. The strain *B. megaterium* S8 showed the broadest antifungal spectrum but inhibited mostly yeasts. In contrast, *B. amyloliquefaciens* S5 inactivated only two fungi, *A. flavus* and *F. oxysporum,* but they are among the most common food spoilage and poisoning fungi encountered in developing countries

(Yaouba et al., 2010).

As said earlier, none of the *Bacillus* strains produced sufficient organic acid to inhibit studied indicators. In addition, most fungi are weakly sensitive to organic acid. That *B. amyloliquefaciens* inhibits fungi is not surprising since Yoshida et al. (2001) earlier reported antibacterial and antifungal activity of this species through bacteriocin-like inhibitory substances (BLIS). But to our knowledge, the available literature has not yet reported the antifungal activity of *B. megaterium,* mostly known as a producer of broad spectrum bacteriocin active against food spoilage bacteria (Khalil et al., 2009). This study describes for the first time strains of *B. megaterium* active against fungi but not against bacteria.

Although no history of food intoxication by Mbuja was recorded (Mohamadou et al., 2009), the presence of at least one member of the *B. cereus* group (a well known poisoning organism) and the rare but possible production of toxin by non-cereus strains (From et al., 2005) led us to investigate the toxinogenic potential of the 26 isolates. Virulence factors and toxin encoding genes usually present in *B. cereus* were not detected in 24 strains of *B. subtilis, B. amyloliquefaciens, B. megaterium* and *B. pumilus.* However, positive PCR results were obtained for *hbL*, *nhE*, *spH* and *Piplc* genes but not for *bceT* and *entFM* genes on the two *B. thuringiensis* strains tested. These interesting results confirm the very low risk of food-borne disease due to other *Bacillus* species than *B. cereus* closest relatives and support their use as starter in a controlled fermentation process to produce a safe Mbuja. The present results go in the same line as those obtained by Matarante et al. (2004) who reported the absence of *B. cereus* toxin encoding genes in *B. subtilis* and *B. pumilus* isolated in industrial and artisanal cured sausages in Italy. When haemolytic activity was tested, only the two *B. thuringiensis* produced halos indicating a strong haemolytic power common in *B. cereus* and close related species.

This study suggests that *B. thuringiensis* strains S10 and SAc should be excluded in starter formulation to produce a controlled Mbuja. Nevertheless, another interesting use of *B. thuringiensis* is their potential to protect food crops from insects, pending on their ability to produce Cry1 proteins. These proteins are insecticidal on certain insects but not toxic to other insect, plants and animal. Due to its selective and specific action, the Cry1 protein has been use as efficient biological insecticide and an alternative to chemical insecticide (Bobrowski et al., 2001). *B. thuringiensis* tested in the present work were analysed for the presence of *Cry1* gene. No amplification product could be detected by PCR for these 2 strains. Recently, Bozlagan et al. (2010) investigated the presence of *Cry1* gene in 60 *B. thuringiensis* strains from agricultural fields and their bioactivity against larvae. These authors showed that only 17 isolates carried the *Cry1* gene, indicating that some *B. thuringiensis* may not have this gene. However, the absence of *Cry1* gene is not indicative of the total absence of insecticidal activity (Bobrowski et al., 2001).

Despite the absence of toxin genes in most of the species (except for *B. thuringiensis*), the interest in antibiotic resistance of the *Bacillus* strains could be justified by their possible side effects on certain Mbuja consumers. Indeed the immune-compromised consumers, in the context of general outbreak of HIV/AIDS in developing countries, may face increased risks of opportunistic infections. In addition possible antibiotic resistance gene transfer between *Bacillus* spp. and the intestinal microbiota on one hand and between *Bacillus* spp. and pathogenic bacteria on the other hand must be considered. All the strains displayed diversity in their susceptibility and resistance to the 8 antibiotics tested. The most effective antibiotics were erythromycin, vancomycin and streptomycin. Most species were resistant to chloramphenicol. Resistance to antibiotics was strain-dependent. Hence, *B. subtilis,* the main fermenting species isolated in Mbuja, exhibited 18% to 64% resistance to tetracyclin and chloramphenicol while all *B. thuringiensis* were resistant to ampicillin and chloramphenicol. Similar diverse susceptibilities were reported for these species and their close relatives by other studies in foods, environmental and clinical samples (Aslim et al., 2002; Schlegelova et al., 2003; Dautle et al., 2004; Luna et al., 2007; Adewumi et al., 2009; Chaves et al., 2011). At the whole, most of the strains were sensitive to different classes of antibiotics.

The most active *Bacillus* spp. against pathogenic and food spoilage micro-organisms were either antibacterial or antifungal. They were totally exempted with toxins genes and were sensitive to different antibiotics. A previous paper (Mohamadou et al., 2007) stated that *B. amyloliquefaciens* (S1 and S5) were highly amylolytic and proteolytic while *B. subtilis* S12 and *B. megaterium* S8 and S9 were highly to moderately proteolytic. These traits are important technological properties in fermenting proteinaceous *Hibiscus sabdariffa* seeds to produce Mbuja. These strains could therefore present a double significant advantage on the nutritional value and safety of Mbuja.

This work was carried out to contribute in selecting starter cultures for controlled production of Mbuja by assessing safety and potential risk of *Bacillus* strains. In the development of the starter culture, strains with important antimicrobial activities and sensitive to a maximum of antibiotics should be encouraged while toxinogenic and antibiotic-resistant strains should be discouraged. In this respect, this study raised the interest of five strains. *B. subtilis* S12 and *B. amyloliquefaciens* S1 were not toxinogenic, active against most pathogenic bacteria tested and were sensitive to all antibiotics. *B. megaterium* S9 was also not toxinogenic, sensitive to all antibiotics and active against 5 fungi. *B. amyloliquefaciens* S5 with strong activity against bacteria and two important pathogenic molds should also be

considered. Fortunately, these strains showed good technological properties. It will be suitable to undertake *Hibiscus sabdariffa* seeds fermentations based on the combination of these species to test their technological as well as their protective properties against a wider panel of pathogenic strains within Mbuja.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The authors wish to acknowledge the Université de Bretagne Occidentale for having founded this work and Dr Jérome Mounier (UBO, LUBEM, Plouzane, France) for providing us the CLIB strains.

REFERENCES

Abriouel H, Franz CM, Ben Omar N, Gálvez A (2011). Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol. Rev. 35(1):201232.

Adewumi GA, Quadri RA, Oguntoyinbo FA (2009). Antibiotic sensitivity pattern of *Bacillus* species isolated from solid substrate fermentation of cassava for *gari* production. Afr. J. Microbiol. Res. 3(11):840-843.

Agata N, Ohta M, Arakawa Y, Mori M (1995). The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. Microbiol. 141:983-988.

Asano SI, Nukumizu Y, Bando H, Hzuka T, Yamamoto T (1997). Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis.* Appl. Env. Microbiol. 63:1054-1057.

Aslim B, Saglam N, Beyatli Y (2002). Determination of some properties of *Bacillus* isolated from soil. Türk. J. Biol. 26:41-48.

Bobrowski VL, Pasquali G, Bodanese-Zanettini MH, Fiuza LM (2001). Detection of *cry1* genes in *Bacillus thuringiensis* isolates from south of Brazil and activity against *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Braz. J. Microbiol. 32:105-109.

Bozlagan I, Ayvaz A, Öztürk F, Acik L, Akbulut M, Yilmaz S (2010). Detection of the *cry1* gene in *Bacillus thuringiensis* isolates from agricultural fields and their bioactivity against two stored product moth larvae. Türk. J. Agric. Forest. 34:145-154.

Céron J, Ortiz A, Quintero R, Güereca L, Bravo A (1995). Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. Appl. Env. Microbiol. 6(11):3826-3831.

Chaves JQ, Pires ES, Vivoni AM (2011). Genetic diversity, antimicrobial resistance and toxinogenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades. Int. J. Food Microbiol. 147:12-16.

Cinar C, Apaydin O, Yenidunya AF, Harsa S, Gunes H (2007). Isolation and characterization of *Bacillus thuringiensis* strains from olive-related habitats in Turkey. J. Appl. Microbiol. 104(2):515-525.

Damgaard PH, Jacobson CS, Sorensen J (1996). Development and application of a primer set for specific detection of *Bacillus thuringiensis* and *Bacillus cereus* in soil using magnetic capture hybridization and PCR amplification. Syst. Appl. Microbiol. 19:436-441.

Dautle MP, Ulrich RI, Hughes TA (2004). *In vitro* sensitivity and resistance of 100 clinical bacterial isolates purified from microbial biofilms associated with silicone gastronomy tubes removed from pediatric patients. J. Appl. Res. 4(1):50-59.

Djouldé DR, Essia Ngang JJ, Etoa FX (2007). Nutritive Value, Toxicological and Hygienic Quality of Some Cassava Based Products Consumed in Cameroon. Pak. J. Nutr. 6(4):404-408.

From C, Pukall R, Schumann P, Hormazabal V, Granum PE (2005). Toxin-Producing Ability among *Bacillus* spp. outside the *Bacillus cereus* Group. Appl. Env. Microbiol. 71(3):1178-1183.

Granum PE, O'Sullivan, Lund T (1999). The sequence of the non-hemolytic enterotoxin operon from *Bacillus cereus.* FEMS Microbiol. Lett. 177:225-229.

Hisieh YM, Sheu SJ, Chen YL, Tsen HY (1999). Enterotoxinogenic profiles and polymerase chain reaction detection of *Bacillus cereus* group cells and *B. cereus* from food-borne outbreaks. J. Appl. Microbiol. 87:481-490.

Khalil R, Elbahloul Y, Djadouni F, Omar S (2009). Isolation and partial characterization of a bacteriocin produced by a newly isolated *Bacillus megaterium* 19 strain. Pak. J. Nutr. 8: 242-250.

Klein C, Kaletta C, Schnell N, Entian KD (1992). Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Env. Microbiol. 58(1):132-142.

Kramer JM, Gilbert RJ (1989). *Bacillus cereus* and other *Bacillus* species. In MP Doyle (Ed.), *Foodborne bacteria pathogens* New York: Marcel Dekker. pp 21-70.

Luna VA, King DS, Gulledge J, Cannons AC, Amuso PT, Cattani J (2007). Susceptibility of *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods. J. Antimicrobial Chem. 60:555-567.

Magnusson J, Schnürer J (2001). *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl. Env. Microbiol. 5(1):1-5.

Matarante A, Baruzzi F, Cocconcelli PS, Morea M (2004). Genotyping and toxinogenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages. Appl. Env. Microbiol. 7(9):5168-5176.

Mbiapo FD, Tchana A, Moundipa PF (1989). Les aflatoxines dans les céréales et les aliments prêts à la consommation au Cameroun. In J.L. Libbey (Ed.), *Céréales en régions chaudes* Paris : AUPELF-UREF. pp 157-163.

Mohamadou BA, Mbofung CMF, Barbier G (2013). Genotypic and phenotypic diversity among *Bacillus* species isolated from Mbuja, a Cameroonian traditional fermented condiment. Afr. J. Biotechnol. 12(12):1335-1343.

Mohamadou BA, Mbofung CMF, Thouvenot D (2007). Functional potential of a product from traditional biotechnology: antioxidant and probiotic potential of Mbuja, produced by fermentation of *Hibiscus sabdariffa* seeds in Cameroon. J. Food Technol. 5(2):164-168.

Mohamadou BA, Mbofung CMF, Thouvenot D (2009). Microbiological and organoleptic profiles of Mbuja, a condiment produced in Cameroon by fermenting *Hibiscus sabdariffa* seeds. J. Food Technol. 7(3):84-91.

Morpeth SC, Ramadhani HO, Crump JA (2009). Invasive non-Typhi *Salmonella* disease in Africa. Clinical Infect. Dis. 49 (4):606-611.

N'dir B, Hbid CL, Cornelius C, Roblain D, Jacques P, Vanhentenryck F, Diop M, Thonard P (1994). Antifungal properties of sporeforming microflora from Nététu. Cahiers Agric. 3:23-30.

Njongmeta NL, Ejoh RA, Djouldé R, Mbofung CM, Etoa FX (2004). Microbiological and safety evaluation of street vended meat and meat product in the Ngaoundere metropolis (Cameroon). Microbiol. Hyg. Alim. 16(47):43-48.

Ouoba LII, Diawara B, Jespersen L, Jakobsen M (2007). Antimicrobial activity of *Bacillus subtilis* and *Bacillus pumilus* during the fermentation of African locust bean (*Parkia biglobosa*) for Soumbala production. J. Appl.Microbiol. 1(2):963-970.

Phelps RJ, McKillip JL (2002). Enterotoxin production in natural isolates of Bacillaceae outside the *Bacillus cereus* group. Appl. Env. Microbiol. 68:3147-3151.

Pugsley AP, Oudega B (1987). Methods for Studying Colicins and their Plasmids. In KD Hardy (Ed), Plasmids: A Practical Approach Oxford: IRL Press. pp 105-161.

Rozen S, Skaletsky HJ (2000). Primer 3 on the www for general users and for biologist programmers. In S Krawetz, S Misener (Eds.), Bioinformatic Methods and Protocols Totowa: Humana Press. pp 365-386.

Schlegelova J, Brychta J, Klimova E, Napravnikova E, Babak V (2003). The prevalence and resistance to antimicrobial agents of *Bacillus cereus* isolates from foodstuffs. Vet. Med. 48(11):331-338.

Shelburne CE, An FY, Dholpe V, Ramamoorthy A, Lopatin DE, Lantz

MS (2007). The spectrum of antimicrobial activity of the 18 bacteriocin subtilosin A. J. Antimicrobial Chem. 59:297-300.
Stein T, Düsterhus S, Stroh A, Entian KD (2004). Subtilosin production by two *Bacillus subtilis* subspecies and variance of the *sbo-alb* cluster. Appl. Env. Microbiol. 70:2349-2353.
Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML (2008). Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. J. Appl. Microbiol. 104:1067-1074.
Yaouba A, Tatsadjieu NL, Jazet Dongmo PM, Etoa FX, Mbofung CM (2010). Antifungal properties of essential oils and some constituents to reduce foodborne pathogen. J. Yeast Fungal Res. 1(1):001-008.
Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A (2001). Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology. 91:181-187.
Zihler A, Le Blay G, de Wouters T, Lacroix C, Braegger CP, Lehner A, Tischler P, Rattei T, Hächler H, Stephan R (2009). *In vitro* inhibition activity of different bacteriocin-producing *Escherichia coli* against *Salmonella* strains isolated from clinical cases. Lett. Appl. Microbiol. 49:31-38.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals