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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:


Short Communications

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Generation and characterization of pigment mutants of Chlamydomonas reinhardtii CC-124

Zaydan, B. K.¹, Sadvakasova, A. K.¹, Saleh, M. M.¹,² and Gaballah, M. M.²*

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The induced mutagenesis method for deriving pigment mutants of a green microalga, Chlamydomonas reinhardtii CC-124 and their pigment composition as well as ability to assess mutability of contaminated aquatic ecosystems were studied. In the present study, 14086 mutants (colonies) were obtained by exposure of the wild strain, C. reinhardtii CC-124, to 1, 2, 3, 5 min of ultraviolet (UV) irradiation. After screening, these mutants (colonies) revealed four pigmented mutants (124y-1, 124p-1, 124y-2 and 124p-2). Compared to the wild CC-124, these mutants are characterized by a decrease in chlorophyll a & b content and an increase in carotenoids. The lowest decrease in chlorophyll a was three to four folds, while the highest increase in carotenoids was two to four folds. The result of bio-test, using the resulting pigment mutant of C. reinhardtii 124y-1 showed that mutagenic activity was observed significantly in both Tekeli River and Pavlodar Oil Refinery in Kazakhstan; the waste water of the Pavlodar Oil Refinery had high-toxicity while the water of the Tekeli River had medium-toxicity.

Key words: Ultraviolet (UV) mutagenesis, Chlamydomonas reinhardtii, biotesting.

INTRODUCTION

One of the most serious ecological problems is mutagenic pollution of the natural environment. Therefore, detection of mutagenic compounds in samples taken from natural habitats is of special interest. The problem of the presence of mutagenic chemicals in natural habitats is very important because such compounds are capable of inducing serious diseases, including cancer and elicit deleterious effects on living organisms (Shigaeva et al., 1994); they are also expensive and time consuming (Wegrzyn and Czyz, 2003).

Biological assays may be an alternative to chemical analysis when mutagenic compounds are detected in the environment. Although, no currently available biological test can provide detailed and precise information on whether examined samples contain mutagens at the levels that are potentially dangerous for organisms. Therefore, it seems that the most reasonable strategy for testing environmental samples is to use a biological assay as a preliminary test to detect the presence of mutagenic compounds. Bio-testing is one of the biological methods based on native or genetically modified microorganisms as test -species have already been applied successfully to environmental toxicity, genotoxicity assessment. It depends on the easy accessibility to and/or maintenance of the organisms in the laboratory (Nendza, 2002; Allan et al., 2006). Soil unicellular green alga, Chlamydomonas reinhardtii Dang is a superb model organism for the study of a wide range of biological questions in areas such as flagellar function, photobiology and photosynthesis research (Stolbov, 1995; Pedersen et al., 2006; Schmidt et al., 2006) because of its clear genetic background. C. reinhardtii is a unique biological material that contains three genetic systems located in the nucleus, chloroplast and...
mitochondria (Merchant et al., 2007). In addition, it has rapid growth, a short breeding cycle and low-cost cultivation. The study of the consequences of the action of mutagenic substances on wild and mutant strains of interest is not only in terms of expanding our knowledge of the biological effects of factors that pollute the ecosystem, but also the emergence of opportunities receipt of test systems for genetic monitoring of the environment. Our goal in the current study was to obtain pigment mutants of green microalga, C. reinhardtii CC-124 by induced mutagenesis and to evaluate the effect of the mutability of contaminated aquatic ecosystems.

MATERIALS AND METHODS

Microalgal strain and cultivation conditions

The green soil alga, C. reinhardtii CC-124 was obtained from Kazakhstan National University, Al-Farabi, Biotechnology Department culture collection. Microalga was cultured and grown in 1000 ml conical flasks containing L2-minimal (L2m) media (Harris, 1998). It was cultured at 25±0.5°C with a fluorescent light intensity of approximately 6 W/m². Cells in the exponential growth phase were used and the initial cell density was about 1 ×10^3 cells / ml. The number of cells was determined by counting, using Goryaev’s hemocytometer under a light microscope.

UV irradiation mutagenesis of Chlamydomonas reinhardtii CC-124

According to the description of Harris (1998), microalgal cells of 4 mL in a logarithmic phase were placed in a 9 cm Petri dish, forming a thin layer covering the bottom. The dish was exposed to a UV-A lamp (5W/m²) for 1, 2, 3, 5, min, respectively. After ultraviolet (UV) irradiation, the irradiated and un-irradiated (control) cells taken from different dilutions were spread immediately on respective agar plates with L2m media; they were kept in the dark for 24 h to prevent photoreactivation, and then grown for 15 days after dividing the dishes into two groups. The first one was kept in the dark (under heterotrophic condition) and the second one was grown under constant light (under phototrophic condition). The identification of the mutants was carried out immediately after exposure and after daily dark repair of cells to prevent the increase of frequency of various kinds of mutations due to errors in DNA replication.

Growth curve and the percentage abundance of survivors

The ratio of cell survival was assessed by determining the percentage of the surviving macro colonies after irradiation exposure dose corresponded to that of the unexposed colonies of the same dilution. Survival curve was constructed by plotting the log of the surviving fraction against the time of exposure (Figure 1).

Sub-culturing of the resulting mutant cells in a liquid media to get survival sub-clones maintaining phenotypic characters

Approximately 14086 morphological surviving sub-clones were formed after UV exposure. Out of this number of colonies, 12 mutant sub-clones were selected for further breeding to study size and shape. L2m was used as a growing medium for the selection of mutant sub-clones of C. reinhardtii. Sub-clones were screened for maintaining phenotypic characters throughout series of passages. There were up to ten consecutive rounds of selections.

Analysis of pigment composition of the selected 4 mutanized sub-clones

Spectrophotometry method was used according to the study of Merchant et al. (2007). The calculation of the concentration of the pigments was determined by the optical density of pigment solutions at appropriate wavelength. UV irradiation mutagenesis of the selected four sub-clones (124y-1, 124p-1, 124y-2 and 124p-2 mutants) resulted in three new colonies characterized by different green colors pigments (dark green, light green and yellow green) to select the best one as a test organism.

Method of determining the mutagenicity of water samples by introducing a test organism in the experimental and control samples, with subsequent incubation and determination of the frequency occurrence of reverse mutations

To identify the substances that have genetic activity on cells, pigment mutants were kept in the test water and the incidence of forward and reverse mutations was calculated (counting the number of revertants). Chlorophyll b-deficient mutants were selected among the light-stable revertants by the level of fluorescence. The fluorescence level is mainly determined by chloroplasts antenna of chlorophyll a PSII. The excitation energy of PSII is a light-harvesting Chl a / b-protein complex that contains 80% of the total chl b. In this regard, the absence of chl b reduces fluorescence of the cells. The fluorescence of chl b excited wide bands of light at 469 to 640 nm. Chl florescence in the cells was observed through KS-2 filter. The absorption spectra of aqueous suspensions of cells were recorded with spectrophotometers SF-10 and SF-18. The ratios of chl a/chl b were determined by the fluorescence method.

Selection of the pigmented mutant that can be used as a test organism

Depending on UV irradiation as a mutagenic agent, we considered the percentage of revertants mutants that were induced by UV

![Figure 1](image-url)  
**Figure 1.** Effect of UV irradiation on survival of wild-type cells of Chlamydomonas reinhardtii CC-124.
irradiation as control and could be comparable with the percentage of other revertants due to contaminated water. The pigmented mutant of *C. reinhardtii* 124y-1 was selected because it is more stable its chl b is not detected and has more carotene content than the others. The maximum frequency of revertants was detected after 3 min.

**Method of determining the mutagenicity of water samples by introducing a test organism in the experimental and control samples, with subsequent incubation**

The test organism was grown in a media added with the selected water sample under testing for mutability. The assessment of water mutability was carried out by counting the number of cells revertants.

**RESULTS AND DISCUSSION**

A mutagen is anything that changes the genetic material of an organism. The most famous one is UV irradiation. Ultraviolet (UV) irradiation has a strong mutagenic agent, compared to chemical mutagenesis. UV mutagenesis offers many advantages such as less pollution, simple operation and sterile cultivation condition (Huang et al., 1993). Several successful cases of microalgae strains by using UV mutagenesis have been documented (Zhang et al., 2009; Danil'chenko et al., 2002; Deng et al., 2011). In the current study, UV mutagenesis can induce the frequency of mutation in *C. reinhardtii* CC-124. After 1 min exposure, the number of survival cells was 31% and the grown colonies did not differ from the control group in terms of their medium size and green color. Upon irradiation of the organism for 2 min, a significant reduction in the number of viable cells reached 10.5%, in addition to a heterogeneity of colonies (large, medium sizes and very fine, green, light green and dark green color). The number of grown cells after 3 min exposure to irradiation was 4.5% and the grown colonies were characterized by different sizes and dark green color. At 5-min exposure, significantly no algal growth was observed. It is clear that UV light has a lethal effect on the cells’ viability and created opportunities for optimal formation of morphological mutations due to its ability to induce highly efficient DNA damage with a survival curve of C-shape (Figures 1 and 2). This is in agreement with the reports of many researchers on the effect of UV light on algal microorganisms (Cadet et al., 1992; Danilchenko et al., 2002; Wu et al., 2005; Deng et al., 2011; IKehata and Ono, 2011).

In exposing *C. reinhardtii* CC-124 to UV radiation with 5 W/m² for 1 to 5 min, out of 130 000 cells of *C. reinhardtii* strain, 14086 morphological surviving sub clones were formed. As a result, in the mass selection without verification of the genotype in the various culture conditions, we obtained sub clones, which are characterized by changing size and color. These sub-clones are divided into six groups.

Under photoautotrophic culture conditions:

Group 1: Green color and large size (A) - 18%,
Group 2: Green color and microscopic size (B) - 32%,
Group 3: Light green color and medium-sized (C) - 33%,
Group 4: Yellow color and medium size (D) -17%.

The control group consists of colonies of green color and medium size.
Species. There was an increase of carotenoids compared to that of the wild strain. The carotenoid content in the cells of Chlamydomonas reinhardtii pigment mutants 124y-1, 124p-1, 124y-2 was 15.35, 12.19 and 23.36 µg/ml, respectively compared to the wild strain (8.12 µg/ml); that is, an increase by 2 to 4 times. Generally, under optimal light conditions, there is a certain balance between the pigment content in the algal cells which is a characteristic feature of the species. Under exposure to mutagenic agent, the balance would exchange in either direction.

UV irradiation can excite the electron shells, resulting in formation of photo-electrons; this can cause a variety of chemical reactions leading to mutations. Upon irradiation, the cells begin to synthesize carotenoids and quantity of carotenoids produced depends on the intensity of UV radiation.

Concerning UV effect on the photosynthetic pigments of plants and algae, some studies (Solovchenk and Merzlyak, 2008) revealed that the synthesis of pigments is blocked, there is retardation of cell growth as well as there is a strong trend towards increased levels of carotenoid in pigments of mutants. In confirming our data, Demmig-Adam et al. (1996) and Young and Britton (1990) reported that in response to excess of light, a rapid increase in carotenoids probably reflects the perma-

Under heterotrophic culture conditions:

Table 1. Frequency of mutations in wild-type cells CC-124 Chlamydomonas reinhardtii at various doses.

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Total number of colonies</th>
<th>Cell viability (%)</th>
<th>The number of normal colonies</th>
<th>The number of mutant colonies</th>
<th>Identification of selected colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90 000</td>
<td>92-100</td>
<td>126000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>39060</td>
<td>31</td>
<td>31248</td>
<td>7812</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>13230</td>
<td>10.5</td>
<td>8997</td>
<td>4233</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>5670</td>
<td>4.5</td>
<td>3629</td>
<td>2041</td>
<td>C</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. The content of chlorophyll and carotenoid pigment mutants in Chlamydomonas reinhardtii.

<table>
<thead>
<tr>
<th>Cipher of the strain</th>
<th>chlorophyll a content µg/10^6 cells</th>
<th>Chlorophyll b content µg/10^6 cells</th>
<th>Carotenoid content µg/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-124</td>
<td>28.73±5.72</td>
<td>13.77±2.42</td>
<td>8.12±2.42</td>
</tr>
<tr>
<td>124y-1</td>
<td>5.65±2.35</td>
<td>-</td>
<td>15.35±2.65</td>
</tr>
<tr>
<td>124p-1</td>
<td>6.32±2.38</td>
<td>4.56±3.63</td>
<td>12.19±1.48</td>
</tr>
<tr>
<td>124y-2</td>
<td>-</td>
<td>-</td>
<td>23.36±2.25</td>
</tr>
<tr>
<td>124p-2</td>
<td>6.65±3.21</td>
<td>-</td>
<td>8.69±2.30</td>
</tr>
</tbody>
</table>

Group 5: Light green color and medium size (E) - 68%,
Group 6: Yellow color and medium size (F) - 32%.

The control group consists of colonies of green color and medium size. Analysis of the output of various mutant subclones under phototrophic conditions showed that the highest percentage of subclones (33%) of the total subclones are green color and medium-sized. Under heterotrophic condition, the highest percentage of subclones (68%) are light green color and medium-sized (Table 1). For further investigation of the 12 colonies by repeated breeding, subcolonies were selected from 4 groups (3, 4, 5, 6) which have preserved the characters (yellow and light green color). They are nominated as 124y-1 and 124p-1, obtained under phototrophic conditions and 124y-2 and 124p-2, obtained under heterotrophic conditions.

Extraction of the mutant pigments was carried out on the fifth day of growth medium cultures with sodium acetate in the light; it showed (Table 2) a decrease in the content of chl a and chl b was not detected; whereas there was an increase of carotenoids compared to that of the wild strain. The carotenoid content in the cells of C. reinhardtii pigment mutants 124y-1, 124p-1, 124y-2 was 15.35, 12.19 and 23.36 µg/ml, respectively compared to the wild strain (8.12 µg/ml); that is, an increase by 2 to 4 times. Generally, under optimal light conditions, there is a certain balance between the pigment content in the algal cells which is a characteristic feature of the species. Under exposure to mutagenic agent, the balance would exchange in either direction.

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Concerning UV effect on the photosynthetic pigments of plants and algae, some studies (Solovchenk and Merzlyak, 2008) revealed that the synthesis of pigments is blocked, there is retardation of cell growth as well as there is a strong trend towards increased levels of carotenoid in pigments of mutants. In confirming our data, Demmig-Adam et al. (1996) and Young and Britton (1990) reported that in response to excess of light, a rapid increase in carotenoids probably reflects the perma-
Table 3. The study of the action of UV light on the pigment mutants of green microalga *Chlamydomonas reinhardtii*.

<table>
<thead>
<tr>
<th>The incidence of</th>
<th>Control</th>
<th>UV light (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>124y-1</td>
<td>5.5×10^4</td>
<td>4.5×10^4</td>
</tr>
<tr>
<td>124y-2</td>
<td>5.5×10^4</td>
<td>4.1×10^4</td>
</tr>
<tr>
<td>124p-1</td>
<td>5.5×10^4</td>
<td>3.9×10^4</td>
</tr>
<tr>
<td>124p-2</td>
<td>5.5×10^4</td>
<td>3.8×10^4</td>
</tr>
<tr>
<td>Total number of revertants color change</td>
<td>2.5×10^5</td>
<td>7.2×10^6</td>
</tr>
</tbody>
</table>

Figure 3. Effect of mutagenic activity of different types of polluted waters on survival pigmented mutant 124y-1.

...nently increased needs for photoprotection. Also, Kleinegris et al. (2010) stated that *Dunaliella salina* alga is bombarded with the full brunt of solar UV (ultraviolet) radiation and has evolved a novel mechanism for defending itself from its damaging effects. More than 8% of its dry body mass is β-carotene, more than any other organism that produces the compound.

In spite of some literatures reporting that response of carotenoids to UV varies, decreased carotenoids level were observed under UV (Kirchgebner et al., 2003) but they were also stimulated by UV (Xiong and Day, 2001). The decrease of chl a and b under elevated UV has also been reported by Bidigare et al. (1993), Hagen et al. (1993), Deckmyn et al. (1994) and Remias et al. (2010).

Regarding the selection of test organism for the determination of the mutagenicity of water samples, the selected mutant pigments, 124y-1, 124p-1, 124y-2 and 124p-2 were exposed to UV irradiation and the resulting 3 new types were characterized by discoloration of the colonies (dark green, light green and faint green color). The maximum frequency of mutations was observed after 3 min of UV irradiation. At the same time, there was a significant increase in the incidence of direct mutation of pigment (Table 3). Among the 3 mutants, we selected 124y-1 mutant for biotesting since it is more stable, has increased carotenoid and lacks chl b. This result is in alignment with that of Parasad et al. (1993) that the sensivity of photosynthetic pigment to UV was in order of :chl b>chl a>carotenoid. To assess the mutagenicity of water samples from Tekeli and Pavlodar Oil Refinery in Kazakhstan, the selected test organism was under subsequent incubation in the experimental and control samples to determine the occurrence frequency of reverse mutations. If the tested samples contain promutagens mutagenic chemical compound, they will induce a reverse mutation restoration of wild -type phenotype.

Consequently, samples of Tekeli River effluent were
toxic and caused an inhibition of cell growth of the mutant 124y-1. As shown in Figure 3, the cells of the test organism were about 1.5 times less than the control in the first days of the experiment. Its mutagenic activity against *C. reinhardtii* strain 124y-1 was observed, as evidenced by the lack of forward and reverse mutations. Also, samples of wastewater of Pavlodar Refinery were toxic and had mutagenic activity, induced by the appearance of the direct and reverse mutations, and shown by a slight increase in the incidence of light-stable revertants (Table 4). In the present study, wastewater samples from Tekeli River and Pavlodar Oil Refinery in Kazakhstan were evaluated for their ecotoxicological effects using 124y-1 mutant. The water of Tekeli River was of medium toxicity and wastewater of Pavlodar Refinery was of high toxicity. The current study may allow us to use UV radiation (radiation dose was 3 min) as a positive control to determine the toxicity of toxicants from contaminated ecosystems in the future. In our opinion, the system of assessment of water quality based on microalgae is promising and can be further improved by the development of new testing methods, as well as expanding the range of use of mutants.

**REFERENCES**


<table>
<thead>
<tr>
<th>The incidence of</th>
<th>Control</th>
<th>Experiment (sample No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigment mutants</td>
<td>&lt;6.5×10^-4</td>
<td>9.1×10^-5, 0.5×10^-3, 2.6×10^-3</td>
</tr>
<tr>
<td>Revertants color change</td>
<td>&lt;10^5</td>
<td>0.8×10^-5, 2.4×10^-4, 6.3×10^-4</td>
</tr>
</tbody>
</table>

Table 4. The study of genetic activity of various wastewater samples of Pavlodar Refinery.
Full Length Research Paper

Genetic variability of European honey bee, *Apis mellifera* in mid hills, plains and tarai region of India

Kumar Yogesh* and M. S. Khan

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To observe the genetic variability in European honey bee, *A. mellifera*, PCR was run separately with five primers and analysis of the banding pattern was worked out to investigate the molecular profile of honey bee genotypes collected from different locations having random amplified polymorphic DNA (RAPD) primers. All the five primer screened, amplified the product in between the range of 100 to 1300 bp and 49 scorable markers bands were generated through polymerase chain reaction (PCR), of which 38 (77.55%) were polymorphic and 11 (22.44%) were monomorphic bands identified. Based on the estimated genetic similarity matrix, the highest genetic similarity value (0.861) was noticed between the mid hill region, Jeolikote and tarai region, Pantnagar and lowest genetic similarity value (0.375) was observed between Haldwani and Rajasthan. The major gene cluster consisted of eight European honey bee, *A. mellifera* accessions from Haldwani, Almora, Do-gaun, Jeolikote, Fatehpur, Ramnagar, Aligarh, Pantnagar, while the minor gene cluster comprised single accession from Rajasthan.

Key words: Genetic variability, *Apis mellifera*, RAPD primers, DNA extraction, polymerase chain reaction (PCR).

INTRODUCTION

European honeybee (*Apis mellifera* L.) has been object of numerous studies and extensively used from different points of view like genetic, morphometrical and molecular studies (Ruttner, 1988). The need to conserve the genetic diversity of domesticated plants is well documented (Rogers, 2004). More recently, Scherf (2000) drew attention to the loss of genetic diversity in livestock worldwide, focusing on domesticated mammals and birds. Despite the widespread consensus that preserving the genetic diversity of domesticated species may prove valuable to humanity, there have been few efforts to preserve the genetic diversity of beneficial arthropod species. While several arthropod species are cultured by humans, *A. mellifera* L., the western honey bee, is the most economically important beneficial insect. *A. mellifera* is very well adapted to life in tropical conditions and have desirable characteristics for beekeeping (Delaplane and Mayer, 2000). Insects comprises the largest species composition in the entire animal kingdom and posses a vast undiscovered genetic diversity and gene pool that can be better explored using molecular marker techniques. Insect population, even within a species varies, in their behavior and morphology that attributes to their complex interaction with the environment (Dempster and McLean, 1999). In insects, DNA markers are used to provide raw information, based on which an ecologist make estimates of genetic diversity and gene flow between species (Speight et al., 2005). The greater level of polymorphism could be obtained by using DNA markers than by using protein markers (Richardson et al., 1986). Out of four species of honey bees namely, *A. mellifera*, *A. cerana*, *A. florea* and *A. dorsata* that are found in India, the former is the only one which is of great interest for commercial

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beekeeping because it can be maintained in hives. Therefore, knowledge of genetic diversity levels and population subdivisions of *A. mellifera* in India is important for understanding distribution patterns and colonization of this species. However, its conservation is also important from a biodiversity perspective, where a priority is laid on preserving the endemic races of honey bees in this region.

India is rich in biodiversity due to varied climatic and topographical conditions. It has great potential to facilitate pollination services in all of the crops. Its different states have special importance of pollinators’ services due to cash crop farming in hills, agri-export zones and limited cultivable area. Nevertheless, there are significant information missing gaps regarding various aspects of pollination and pollinators of the different states. Considering its different states, it is evident from the literature that meager information is available on the genetic diversity of insect pollinators.

**MATERIALS AND METHODS**

To observe the genetic variability of the European honeybee, *A. mellifera* samples were collected from 8 beekeepers (Table 1) at mid hills, plains and tarai region of India. Honeybee brood samples (n = 10) per colony were collected and stored in a refrigerator at -20°C and DNA was extracted for further processing. The genetic variability of European honeybee, *A. mellifera* was assessed at IPM Laboratory, Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar, U. S. Nagar.

**DNA isolation protocol**

The DNA was extracted from the honeybee (*A. mellifera*) broods. For this purpose, lyophilization of the honeybee broods was done for the easy, rapid grinding of broods and also to minimize the number of organic solvent extractions according to the Hall (1986) protocol. The following steps were used in the DNA extraction of honey bees: bee brood was lyophilized, and ground with liquid nitrogen solution with the help of mortar and pestle. The powder of bee brood collected from each location was transferred to a microcentrifuge tube using a stiff paper (for parallel preparations, the mortar and pestle are cleaned each time with a dry cloth). The powder was then suspended in 100 mM Tris-HCL, pH 8.5, 250 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulfate (SDS), 2 mg/ml RNase A (100 µl/10 mg of material) by stirring with a pipette tip and left for 10 min at 37°C. Now 0.8 ml of phenol (equilibrated with 0.1 M Tris-HCL, pH 8.0) was added to it and the tubes were shaken for 2 min for homogeneous suspension. Then, 0.4 ml of chloroform was added and the tubes were shaken for 1 min and centrifuged for 5 min at 13000 rpm (15000 x g). The upper aqueous phase was taken off, re-extracted with 1 ml of chloroform and again centrifuged. Now the supernatant aqueous phase was transferred to a sterile microcentrifuge tube and 0.6 ml of isopropanol was added, and the tube were held in a horizontal position (to maximize the area between liquids) and shaken for 1 min. DNA precipitates as a visible aggregate, and the liquid can be removed with a micropipette. Alternatively, DNA can be recovered by centrifugation for 1 min at 5000 rpm.

The DNA precipitate was rinsed twice (without centrifuge) with 70% ethanol. The tubes were spun for a few seconds, and residual liquid is removed; then the DNA is dried and re-suspended in 10 mM Tris-HCL, pH 8.0, 0.1 mM EDTA. Extracted DNA samples were store at -20°C.

**DNA purification**

2 µl RNase A was added to the eppendorf tube containing 200 µl of extracted DNA and then incubated for 3 h at 37°C in a water bath. The DNA was further extracted with equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1, v/v) and centrifuged at 10,000 rpm for 12 min at 10°C. Supernatant was taken into a fresh eppendorf tube. 0.6 m³ ice cold isopropanol and 0.1 m³ of ice cold sodium acetate (3 M) were added and the mixture was kept at -20°C for at least 2 h. The mixture was then centrifuged at 10,000 rpm at 10°C for 12 min. Supernatant was removed using a micropipette and pellet was washed with 70% ethanol and dried completely. The DNA pellet was re-dissolved in minimum amount of TE buffer.

**DNA concentration, quality and integrity determination**

The quantification of genomic DNA was done by taking the absorbance on Genesys UV spectrophotometer. The optical density was measured at 260 and 280 nm. The concentration of the DNA in the sample was related to optical density by the following formula:

<table>
<thead>
<tr>
<th>Place</th>
<th>Number of brood sample collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haldwani (tarai)</td>
<td>10 (240)*</td>
</tr>
<tr>
<td>Almora (mid hill)</td>
<td>10 (25)*</td>
</tr>
<tr>
<td>Do Gaon (mid hill)</td>
<td>10 (150)*</td>
</tr>
<tr>
<td>Jyolikot (mid hill)</td>
<td>10 (140)*</td>
</tr>
<tr>
<td>Fatehpur (plain)</td>
<td>10 (100)*</td>
</tr>
<tr>
<td>Ramnagar (tarai)</td>
<td>10 (275)*</td>
</tr>
<tr>
<td>Aligarh (plain)</td>
<td>10 (230)*</td>
</tr>
<tr>
<td>Rajasthan (plain)</td>
<td>10 (200)*</td>
</tr>
<tr>
<td>Pantnagar (tarai)</td>
<td>10 (125)*</td>
</tr>
</tbody>
</table>

*Value shown in parentheses represents the total number of honey bee colonies with the beekeeper.
Conc. of DNA (µg/ml) = \frac{OD_{260} \times 50 \times \text{Dilution factor}}{1000}

The ratio of $OD_{260/280}$ was an indication of the amount of RNA or protein contamination in the preparation. A value of 1.8 is optimum for best DNA preparation. A value of the ratio below 1.8 indicated the presence of protein in the preparation and a value above 1.8 indicated that the sample has RNA contamination.

Polymerase chain reaction (PCR)

PCR amplification was performed with primers, obtained from Eurofins Genomics India Pvt. Ltd., Bangalore. PCR conditions were standardized separately for RAPD in two steps: 1) By taking all the primers and keeping their concentration constant to find out as to which primer gave results; 2) taking template DNA concentration as 20, 40, 60 ng and primer concentration as 60, 80 and 100 ng to standardize the template DNA and primer concentration; for PCR amplifications, a total of 20 µl containing the following components were used (Table 2).

Sets of 10 oligo-nucleotide primers were employed for RAPD PCR amplification. The details of primers are given in Table 5. A master mix (minus template DNA) was prepared to reduce pipetting error. The master mix was then distributed in each tube (18.8 µl each) and finally 1.2 µl of template DNA was added in each tube. The mixture was gently mixed and centrifuged at 5000 rpm for 10 s. The PCR amplification was achieved in M. J. Research Thermocycler (PTC 200). The amplification reaction was pre-denatured at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 1.30 min and polymerization at 72°C for 1 min. The last cycle of final extension was carried out at 72°C for 2 min.

Analysis of amplification products and procedure of agarose gel electrophoresis

After the completion of PCR cycles, 15 µl of the PCR product was analyzed on 1.3% agarose gel by electrophoresis. Horizontal gel electrophoresis unit was used for fractionating RAPD markers on agarose gel. Agarose gel (1.3%) was prepared by dissolving appropriate amount of agarose in 0.5X TBE buffer. For each well, DNA loading dye and DNA samples were mixed in 1:6 ratio and loaded with a micropipette. Electrophoresis was done at 70 V for 3 h in 0.5X TBE electrophoresis buffer. The gel was then stained in ethidium bromide solution. After de-staining in de-ionized water, the gel image was viewed in U. V. transilluminator and stored in gel documentation system.

Table 2. RAPD PCR amplification.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Stock</th>
<th>Volume added (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Buffer (Invitrogen)</td>
<td>10X</td>
<td>2.0</td>
</tr>
<tr>
<td>MgCl2 (Invitrogen)</td>
<td>50 mM</td>
<td>0.8</td>
</tr>
<tr>
<td>dNTP Mix (Fermantas)</td>
<td>10 mM</td>
<td>2.0</td>
</tr>
<tr>
<td>Primer</td>
<td>10 µM</td>
<td>1.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (Invitrogen)</td>
<td>5 U/µl</td>
<td>0.3</td>
</tr>
<tr>
<td>Diluted DNA sample</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td>Nuclease free water (Promega)</td>
<td>-</td>
<td>12.2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>20.0</td>
</tr>
</tbody>
</table>

Molecular markers data analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Data were analyzed to obtain Jaccard’s similarity coefficients among the isolates by using NTSYS-pc (version 2.11s; Rohlf, 2002). The SIMQUAL program was used to calculate the Jaccard's similarity coefficients. All the gels were scored twice manually and independently. Band presence was indicated by 1 and its absence by 0. All unique bands were also scored and included in the analysis. Presence or absence of unique, shared and polymorphic bands was used to generate similarity coefficients. The similarity coefficients were then used to construct a dendrogram manually by unweighted pair group method using arithmetical averages (UPGMA). The analysis work was based on Jaccard’s similarity coefficient given as:

$$\text{Similarity coefficient} = \frac{\text{Number of polymorphic bands}}{\text{Total number of band}}$$

Based on the similarity coefficient, separate dendogram for RAPD were prepared.

RESULTS AND DISCUSSION

The results obtained from the present investigation are presented under the following subheads:

DNA fingerprinting of European honey bee, *Apis mellifera* L.

The PCR was run separately for the five primers and the amplified products were separated in agarose gel and viewed in the gel doc system and photograph was saved. The analysis of the banding pattern revealed the molecular profile of honey bee genotypes collected from different places with different RAPD primers which are given in Tables 3 and 4.

Amplification profile of RAPD primers

**Primer 1 (501)**

On agarose gel, this primer showed amplification with all
Table 3. Summary of RAPD amplified products.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of primers tested</td>
<td>72</td>
</tr>
<tr>
<td>Number of polymorphic primers</td>
<td>05</td>
</tr>
<tr>
<td>Total number of monomorphic primers</td>
<td>67</td>
</tr>
<tr>
<td>Total number of bands region amplified</td>
<td>49</td>
</tr>
<tr>
<td>Size range of amplified products (bp)</td>
<td>100 to 1300</td>
</tr>
<tr>
<td>Average number of bands per primer</td>
<td>10</td>
</tr>
<tr>
<td>Total number of unique bands identified</td>
<td>3</td>
</tr>
<tr>
<td>Total number of polymorphic bands identified</td>
<td>38</td>
</tr>
<tr>
<td>Total number of monomorphic bands identified</td>
<td>11</td>
</tr>
<tr>
<td>Percentage of all bands that were polymorphic</td>
<td>77.55%</td>
</tr>
</tbody>
</table>

Table 4. Amplified products and polymorphism obtained with RAPD primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Number of amplified products</th>
<th>Polymorphic band (numbers)</th>
<th>Monomorphic band (numbers)</th>
<th>Percent (%) polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>501</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>502</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>66.66</td>
</tr>
<tr>
<td>503</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td>81.81</td>
</tr>
<tr>
<td>504</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>505</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>66.66</td>
</tr>
</tbody>
</table>

Table 5. Base sequence of primers used in gene expression analysis.

<table>
<thead>
<tr>
<th>Oligonucleotide code</th>
<th>Base sequence (5'-3')</th>
<th>Yield (μg)</th>
<th>Yield (n/mol)</th>
<th>Volume for 100 pmol/μl</th>
<th>Temperature (°C)</th>
<th>MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>501</td>
<td>CGGTAGACG</td>
<td>473</td>
<td>154.1</td>
<td>1541</td>
<td>32.0</td>
<td>3068</td>
</tr>
<tr>
<td>502</td>
<td>CTTACGTCAC</td>
<td>400</td>
<td>134.9</td>
<td>1349</td>
<td>30.0</td>
<td>2962</td>
</tr>
<tr>
<td>503</td>
<td>CCCAACACAC</td>
<td>387</td>
<td>132.3</td>
<td>1323</td>
<td>32.0</td>
<td>2925</td>
</tr>
<tr>
<td>504</td>
<td>AAACCAGGCG</td>
<td>383</td>
<td>125.8</td>
<td>1258</td>
<td>32.0</td>
<td>3046</td>
</tr>
<tr>
<td>505</td>
<td>GGTGGGGAGG</td>
<td>469</td>
<td>149.3</td>
<td>1493</td>
<td>32.0</td>
<td>3139</td>
</tr>
</tbody>
</table>

nine samples of European honey bee, *A. mellifera* (Figure 1). This primer amplified the products in the range of 100 to 1200 bp. Loci amplified by this primer in respective genotypes were 80% polymorphic.

Primer 2 (502)

This primer amplified nine scorable RAPD loci on agarose gel with all nine genotypes, out of which six were polymorphic and three were monomorphic at 450 and 550 bp, respectively (Figure 2). Amplified product size ranged from 450 to 1200 bp. The loci amplified by this primer were 66.66% polymorphic.

Primer 3 (503)

On agarose gel, this primer revealed 11 scorable amplified RAPD loci in nine genotypes of *A. mellifera* (Figure 3). The amplified products were in the range of 100 bp to more than 1250 bp.

Out of 11 amplified products by this primer, nine loci were observed to be polymorphic while 2 loci were found monomorphic at approximately 400 and 500 bp location, respectively. Three bands at the positions 850, 1200 and 1250 bp were observed unique to genotype collected from Haldwani province.

Primer 4 (504)

This primer amplified 10 scorable RAPD loci on agarose gel with all nine genotypes, out of which nine were polymorphic and one was monomorphic at around 200 bp (Figure 4). Amplified product size ranged from 200 to 1200 bp. The loci amplified by this primer were 90%
Figure 1. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 501 on agarose gel.

Figure 2. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 502 on agarose gel.
Figure 3. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 503 on agarose gel.

Figure 4. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 504 on agarose gel.
Figure 5. RAPD profile of European honey bee, A. mellifera genotypes generated by the primer 505 on agarose gel.

Polymorphic.

**Primer 5 (505)**

On agarose gel, this primer showed amplification with all nine samples of A. mellifera (Figure 5). This primer amplified nine scorable RAPD loci in nine genotypes, out of which six were polymorphic while three bands were found monomorphic at the positions 500, 600 and 650 bp, respectively. Amplified product size ranged from 400 to 1200 bp. The loci amplified by this primer were 94.11% polymorphic. In the present study, our results show that all the five primer screened, amplified the product in between the range of 100 to 1300 bp while Hall et al. (1998) screened 700 primers, of which five were found to reveal useful distinguishable differences and the bands generated in between 0.73 to 1.75 kb. In the Africanized populations, the alleles were most frequently 105 to 108 bp in size, although some were 116 to 122 pb. This contrasts with 100 to 103 bp for most alleles in the African population, and the absence of alleles greater than 114 bp in the African population. Alleles of greater size are characteristic of European honey bee races, particularly of the Mediterranean races (Estoup et al., 1995). Raffaele et al. (2007) reported that number of alleles and expected heterozygosity per locus A107 was highly polymorphic with a total of 22 alleles detected (size range from 105 to 136 bp) and had the highest Ho values. Only five alleles were scored for locus A28 (130 to 140 bp) and loci A28 and A88 showed low heterozygosity values, in particular for the A. mellifera ligustica groups. In another study, Hunt and Page (1992) used the polymorphism and segregation of RAPD markers. The primers generated 1018 scorable marker bands (an average of 7.7 per primer), of which 409 (40%) were polymorphic, whereas in the present study, similar types of results was observed, 49 scorable markers bands generated (an average of 10 per primers), of which 38 (77.55%) were polymorphic and 11 (22.44%) were monomorphic bands identified.

Polymorphisms generated with RAPD primers result from success or failure of the primer to bind, creating the presence or absence of a particular amplified band, and from insertions or deletions that change the length of the amplified region. Most RAPD markers are of the first type and are dominant in expression, whereby a diploid with two copies of a RAPD marker cannot be distinguished from those containing one copy (Tingey et al., 1992; Williams et al., 1990). A small proportion of the screened primers were found to be useful. Thus, RAPD as a source of diagnostic genetic markers in honey bees has
not been as successful, in our hands, as RFLPs. Specific RAPD markers have been found more readily in other organisms, particularly when the characterization has been done at a higher taxonomic level, for example, as species-specific markers (Kambhampati et al., 1992) or for populations of known genetic origin, such as cultivars or strains (Guthrie et al., 1992). Sirikul et al. (2008) also reported similar type of work with *Trigona pagdeni*. The presence or absence of 51 TE-AFLP bands was scored for each individual. Eight bands (16%) were fixed. The remaining 43 bands, each of which were present in at least one individual and absent in at least one individual, were considered polymorphic. Thirty five of these bands (69%) had inferred allele frequencies in the range of 5 to 95%.

**Relationships among nine genotypes of *A. mellifera***

Using RAPD markers

Based on the estimated genetic similarity matrix (Table 6), the highest genetic similarity value (0.861) was noticed between the pair Jeolikote and Pantnagar followed by (0.842) between the pair, Jeolikote and Aligarh, (0.810) between Fatehpur and Aligarh, (0.805) between Jeolikote and Fatehpur, (0.769) between Aligarh and Pantnagar, (0.738) between Fatehpur and Pantnagar, (0.732) between Haldwani and Aligarh accession, (0.725) between Haldwani and Jeolikote, (0.7) between Haldwani and Pantnagar, (0.667) between Haldwani and Fatehpur also same between Almora and Ramnagar and also among Do-gaun and Ramnagar, (0.656) between Almora and Do-gaun accessions, (0.585) between Almora and Aligarh and (0.581) between Almora and Rajasthan. The lowest genetic similarity value (0.375) was observed between Haldwani and Rajasthan followed by (0.395) between Rajasthan and Pantnagar, and (0.45) between Do-gaun and Jeolikote. Unbiased estimates of gene diversity (Nei, 1987) were higher in the Africanized population (0.937) than the respective estimates for the Johannesburg (0.898), Forli (0.810) and Avignon (0.377) populations.

Pilar et al. (2002) reported that *A. mellifera* gene diversity varied between 0.589 ± 0.247 (Mallorca) and 0.349 ± 0.331 (Formentera). Sirikul et al. (2008) revealed significant genetic differentiation among the four populations of *Trigona pagdeni* ($\Phi_{PT} = 0.18, P = 0.01$). They detected differentiation ($\Phi_{PT} = 0.13, P = 0.001$) between samples collected north and south of the Kra (Kra) ecotone, a biogeographical zone of transition between seasonal evergreen and mixed moist deciduous forests. However, the greatest differentiation was detected between samples from the northeast and the other locations combined ($\Phi_{PT} = 0.21, P = 0.001$).

**Analysis of gene cluster formed**

The dendogram constructed from RAPD marker analysis in honey bee genotypes revealed that there were two major gene clusters formed breaking at 0.46 Jaccard’s coefficient of similarity (Figure 6). The major gene cluster consisted of eight *A. mellifera* accessions from Haldwani, Almora, Do-gaun, Jeolikote, Fatehpur, Ramnagar, Aligarh, Pantnagar, while the minor gene cluster comprised single accession from Rajasthan. The major gene cluster within accessions from Jeolikote and Pantnagar were not further separated indicating the high level of genetic similarity between the two (> 86%). The secondary gene cluster was formed within the major gene cluster around 0.5 Jaccard’s coefficient of similarity. The secondary gene cluster divided the major gene cluster into two subgroups. One of it consisted of five accessions from Haldwani, Jeolikote, Pantnagar, Fatehpur and Aligarh and the latter consisted of three accessions of honey bee from Almora, Do-gaun and Ramnagar. According to Pilar et al. (2002), in the genetic differentiation test, the honeybee populations of the Balearic Islands clustered into two groups: Gimnesias (Mallorca and Menorca) and Pitiusas (Ibiza and Formentera), which agrees with the biogeography postulated for this archipelago. As is the case with PCR reactions, the results of RAPD analyses must be carefully interpreted, especially when the nature of the amplified
A small proportion of the screened primers was found to be useful. The RAPD markers reported here are specific to groups of honey bee species and their representation in populations coincides with what had been found with RAPD markers. Nevertheless, RAPD markers can also provide an efficient assay for polymorphism which should allow rapid identification and isolation of chromosome specific DNA fragments. These RAPD markers are expected to be useful in distinguishing African and European bees and following interactions between the two. India is one, among 12- mega diversity countries and is also one of the countries whose insect fauna is very poorly known and documented.

A sound insect biodiversity knowledge base is prerequisite for effective crop cultivation, conservation, environmental assessment, ecological research management and sustainable use of biological resources. In the current millennium, India will become an economic power because of its biodiversity.

Many a time, molecular marker data help to distinguish between different species, where there is no other comprehensive way available to do so. Thus, DNA markers will be valuable in the continued elucidation of African-European honeybee interactions and population dynamics.

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REFERENCES


Full Length Research Paper

Genetic diversity study of Kenyan cassava germplasm using simple sequence repeats

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Cassava (Manihot esculenta Crantz) is an important food security crop for resource poor rural communities particularly in Africa. Little is however known about molecular diversity of Kenyan cassava germplasm. This led to a study whose objective was to identify genetic constitution of cassava accessions from different regions of Kenya using molecular tools. Seven pairs of micro satellite (SSR) primers previously developed from cassava were used to detect polymorphic 21 alleles in a sample of 69 accessions. The cluster analysis of similarity matrix obtained at 68% with SSR data showed that the 69 accessions were grouped into five marker based groups. This study proved that SSRs could be used to identify cassava accessions as well as in the assessment of level of genetic relatedness among accessions.

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

INTRODUCTION

Cassava (Manihot esculenta Crantz) belongs to the family Euphorbiaceae. Of the 98 species that belong to the genus Manihot, cassava is the only species that is widely cultivated for food production (Nassar, 2005). It is a perennial shrub grown throughout the tropical and subtropical regions of the world. It originated and was domesticated in the Neotropics. The crop is widely grown between the latitudes 30° N and 30° S, a belt that coincides with most of the developing countries of the world (Phillips, 1974). The crop was introduced into Africa in the 16th century, where it is now cultivated across an extensive area, known as the “cassava belt”. In Kenya, cassava was introduced between the 16th and 19th century by the European explorers. It was introduced along with other crops such as beans, maize and sweet potatoes. It adapted well to the environment and by the start of the 20th century, it was widely grown in the country (Suttie, 1970). For a long time, this crop has been ranked among the four major food crops in developing countries, after rice, wheat and maize (Cock, 1985).

Cassava’s adaptability to relatively marginal soils and erratic rainfall conditions, its high productivity per unit of land and labor, the certainty of a yield even under the worst conditions and the possibility of maintaining a continuous supply year round, makes this crop a basic component of the farming system in most areas of Sub-Saharan Africa (Nweke and Enette, 1999). Other advantages include flexibility in harvesting (year-round availability) and planting periods, and long period of in-ground storage after maturity. Famine therefore is rare in areas where cassava is grown, since it provides a stable base to the food production system and has the potential for bridging the food gap (Iglesias et al., 1997; Nweke and Enette, 1999; Ekwele et al., 2001).

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In Kenya, cassava is largely grown for household food security and in some areas for sale in fresh or processed form. Total output of cassava is estimated to be 864,000 tons at an average productivity of 9 tons per hectare (Kariuki et al., 2002).

Although, genetic resources have traditionally been evaluated on the basis of morphological and agronomic traits, these do not necessarily reflect food value and inherent genetic relationship among germplasm. Indeed, most morphological and physiological descriptors are greatly influenced by the environment and show continuous variation and high plasticity, with most of them only scorable at maturity. The use of reliable and standardized genetic descriptors is therefore critical in enhancing the efficiency of identification and use of high value plant germplasm to ameliorate hunger and malnutrition (Wachira, 2002). Accurate characterization and evaluation of accessions within the cassava germplasm resources in Kenya as well as assessment of the level of genetic variation in the resource is important in devising optimum management strategies for sustainable utilization and conservation of the resource.

A sustainable agricultural system requires that components of diversity be used in a way and at a rate that will not lead to a long term decline of diversity, thus maintaining its potential to meet the needs and aspirations of present and future generations. Genetic diversity is however threatened by the introduction and adoption of modern high yielding varieties (Wachira, 2002). A dramatic increase in the use of small number of highly selected accessions has led to loss of valuable genetic resources. The proportion of genetic diversity accessed by the popular varieties has often not been determined yet it is critical to the sustainable use of cassava genetic resources in Kenya. Since cassava is predominantly vegetatively propagated, over reliance on a few varieties which may also share a common ancestry may minimize the on-farm diversity and thus increase the risks posed by such co-evolving biotic factors as pests and diseases to cassava farming. Genetic diversity can most efficiently be quantified using molecular markers. The simple sequence repeat (SSR) markers are one such marker system that has been used for many genetic applications, including the assessment of genetic variability in germplasm collections and pedigree reconstruction (Fregene et al., 2003). It is envisaged that this study will help ensure a broad genetic base for such future varieties.

The objective of this study was therefore to identify genetic constitution of 69 cassava accessions from different regions of Kenya using SSR molecular tools.

**MATERIALS AND METHODS**

**Plant materials**

Sixty nine (69) cassava accessions were randomly sampled (Table 1) from the national ex-situ gene banks at Kakamega, Katumani and Kiboko. Within them, few samples from advanced IITA lines and CIAT were sampled to act as checks. All these germplasm were subjected to molecular diversity studies using SSR markers.

**DNA isolation**

Genomic DNA was isolated using two methodologies that is the 2X CTAB method as described by IAEA (2002) and as described by Dellaporta et al. (1983). For the both methods, 500 µg of young leaves was obtained from six months old plants which had been stored at two different conditions; silica gel dried and frozen (at -80°C).

**Determination of DNA concentration, purity and integrity**

The isolated DNA was purified and later the quantity and intactness (integrity) was confirmed in 1X TBE buffer alongside some uncut unmethylated lambda (λ) DNA standards (750, 500, 250, 125, 100, and 83 ng). (Sigma, UK). The gel was stained in ethidium bromide (10 µg/ml), visualised on a ultra violet transilluminator at 254 nm and photographed. The band size and staining intensity of the isolated and electrophoresed DNA samples from cassava were compared to those of the λ DNA standards to determine concentration. Inergrity of the DNA was determined by absence of smears.

**Optimization of SSR-PCRs**

PCR optimization experiments were carried out using five DNA samples by varying the concentration of the template DNA, Taq DNA polymerase, annealing temperature, number of cycles and the Mg²⁺ salt concentration.

**SSR amplifications**

Optimised SSR assays were performed using fifteen pairs of oligonucleotide primer sequences (Table 2) obtained from Operon Technologies Inc. (USA). Using the optimized PCR assay the 15 oligonucleotide pairs were screened on a sub set of 5 samples from the entire collection to reveal those that would generate unambiguous polymorphic SSR alleles. The primers which gave scoreable amplicons were then used in the analysis of all the 69 test cassava accessions. Following the initial screening, 7 SSR primers that amplified clear and reproducible SSR allele profiles were selected to study SSR variation in the samples. A negative control was also set in which sterile distilled water was used to replace template DNA. Amplification reactions were performed in a DNA thermocycler machine (Mastercycler) with a heated lid (94°C) programmed as follows; one hot start cycle of 94°C for 2 min followed by 30 cycles of 94°C for 1 min; 56°C for 1 min (DNA annealing); 72°C for 1 min and a final extension cycle of 72°C for 10 min. The samples were then maintained at 4°C.

**Gel electrophoresis**

The generated SSR amplicons by amplification were separated according to size by electrophoresis on high resolution (3%) metaphor agarose gels run in 1X TBE for 2 h at 100 V. A 100 base pair DNA ladder (Sigma, UK) was used to estimate the sizes of amplification products. Gels were stained in ethidium bromide and visualised on a UV light trans illuminator at 254 nm.
### Table 1. Sixty nine accessions of cassava sampled from the national genebanks of KARI-Katumani and KARI-Kakamega.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Accession</th>
<th>Origin</th>
<th>Accession number</th>
<th>Accession</th>
<th>Origin</th>
<th>Accession number</th>
<th>Accession</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kiringis</td>
<td>landrace</td>
<td>24</td>
<td>Matuja-2</td>
<td>Landrace</td>
<td>47</td>
<td>Muciceri-1</td>
<td>landrace</td>
</tr>
<tr>
<td>2</td>
<td>Obarodak</td>
<td>landrace</td>
<td>25</td>
<td>Nyadai</td>
<td>Landrace</td>
<td>48</td>
<td>990072-A</td>
<td>IITA</td>
</tr>
<tr>
<td>3</td>
<td>Kaleso</td>
<td>landrace</td>
<td>26</td>
<td>Tamisi</td>
<td>Landrace</td>
<td>49</td>
<td>990056</td>
<td>IITA</td>
</tr>
<tr>
<td>4</td>
<td>Kibandameno</td>
<td>landrace</td>
<td>27</td>
<td>Sabina</td>
<td>Landrace</td>
<td>50</td>
<td>990014</td>
<td>IITA</td>
</tr>
<tr>
<td>5</td>
<td>Gachaga</td>
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<td>28</td>
<td>Bwanatereka</td>
<td>Landrace</td>
<td>51</td>
<td>Kitwa</td>
<td>landrace</td>
</tr>
<tr>
<td>6</td>
<td>Marakwet</td>
<td>landrace</td>
<td>29</td>
<td>Nabwire</td>
<td>Landrace</td>
<td>52</td>
<td>Migyera-2*</td>
<td>IITA</td>
</tr>
<tr>
<td>7</td>
<td>Waite</td>
<td>landrace</td>
<td>30</td>
<td>Opondo</td>
<td>Landrace</td>
<td>53</td>
<td>SS4*</td>
<td>IITA</td>
</tr>
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<td>8</td>
<td>Nyamambakaya</td>
<td>landrace</td>
<td>31</td>
<td>Serere-1</td>
<td>CIAT</td>
<td>54</td>
<td>Muciceri-2*</td>
<td>landrace</td>
</tr>
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<td>9</td>
<td>CK-8</td>
<td>landrace</td>
<td>32</td>
<td>Bumba</td>
<td>Landrace</td>
<td>55</td>
<td>KME-1*</td>
<td>landrace</td>
</tr>
<tr>
<td>10</td>
<td>CK-9</td>
<td>landrace</td>
<td>33</td>
<td>Opongi</td>
<td>Landrace</td>
<td>56</td>
<td>MM96/4466-B*</td>
<td>IITA</td>
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<td>11</td>
<td>Sabulenyia</td>
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<td>34</td>
<td>Iuderudu</td>
<td>Landrace</td>
<td>57</td>
<td>MM96/7151*</td>
<td>IITA</td>
</tr>
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<td>12</td>
<td>Nyakatanegi-1</td>
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<td>35</td>
<td>MH95/0183-A</td>
<td>IITA</td>
<td>58</td>
<td>MM96/4884</td>
<td>IITA</td>
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<td>13</td>
<td>Adhiambolela</td>
<td>landrace</td>
<td>36</td>
<td>MM96/1871-A</td>
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<td>59</td>
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<td>IITA</td>
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<td>MM96/4466-A</td>
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<td>60</td>
<td>MM96/1871-B*</td>
<td>IITA</td>
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<td>landrace</td>
<td>38</td>
<td>MM96/7688</td>
<td>IITA</td>
<td>61</td>
<td>MM95/0183-B*</td>
<td>IITA</td>
</tr>
<tr>
<td>16</td>
<td>Nyakatanegi-2</td>
<td>landrace</td>
<td>39</td>
<td>Migyera-1</td>
<td>IITA</td>
<td>62</td>
<td>Serere*</td>
<td>CIAT</td>
</tr>
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<td>17</td>
<td>Nyakatanegi-3</td>
<td>landrace</td>
<td>40</td>
<td>Kapchelelyo</td>
<td>Landrace</td>
<td>63</td>
<td>990005</td>
<td>IITA</td>
</tr>
<tr>
<td>18</td>
<td>Agriculture</td>
<td>IITA</td>
<td>41</td>
<td>Kapchetuya</td>
<td>Landrace</td>
<td>64</td>
<td>990072*</td>
<td>IITA</td>
</tr>
<tr>
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<td>Matuja-1</td>
<td>landrace</td>
<td>42</td>
<td>KME-1</td>
<td>Landrace</td>
<td>65</td>
<td>MM96/5280</td>
<td>IITA</td>
</tr>
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<td>20</td>
<td>Mercury</td>
<td>landrace</td>
<td>43</td>
<td>Ex-Mariakani</td>
<td>Landrace</td>
<td>66</td>
<td>Arror 1</td>
<td>Wild type</td>
</tr>
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<td>21</td>
<td>Mageuna</td>
<td>landrace</td>
<td>44</td>
<td>960249</td>
<td>IITA</td>
<td>67</td>
<td>Arror 2</td>
<td>Wild type</td>
</tr>
<tr>
<td>22</td>
<td>Number 8</td>
<td>landrace</td>
<td>45</td>
<td>990183</td>
<td>IITA</td>
<td>68</td>
<td>Arror 3</td>
<td>Wild type</td>
</tr>
<tr>
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<td>Sifros</td>
<td>landrace</td>
<td>46</td>
<td>Mue</td>
<td>Landrace</td>
<td>69</td>
<td>Arror 4</td>
<td>Wild type</td>
</tr>
</tbody>
</table>

* Accessions that were used in the biochemical study.

**Scoring of SSR segments**

SSR alleles were scored from the reproducible PCRs set using different primer pairs. The size of SSR alleles was estimated from the gel photograph by comparison with 100 base pair ladder marker. Allele profiles were manually scored and compiled into a binary matrix. Positive amplification was treated as separate characters and was scored for the presence (1) or absence (0) of alleles. Only intensely stained unambiguous alleles were scored and used for statistical analysis.

**Data analysis**

The scored molecular data in a binary form (1=allele presence, 0=allele absence) was configured as an input file and analyzed with POPGENE version 1.31 (Yeh et al., 1997). Proportion of polymorphic alleles (P) was derived as:

\[
P = n_p / n_{total}
\]

Where \(n_p\) is the number of polymorphic alleles and \(n_{total}\) is the total number of amplified alleles. Single population descriptive statistics were derived. Genetic variability within the test cassava accessions was determined through derivative of average expected heterozygosity (He) of the accessions using the POPGENE software assuming Hardy-Weinberg equilibrium and no population structure. The index proposed by Nei and Li (1979) was used to calculate genetic identity (\(S_{ij}\)) between cultivars (\(i\)) and (\(j\)) as:

\[
S_{ij} = 2N_{ij} / (N_i + N_j)
\]
The average number of bands (alleles) in common between cultivars i and j are the number of alleles for cultivars i and j respectively. The similarities were used to derive genetic diversity trees by average linkage cluster analysis (POPGENE version 1.31).

**RESULTS**

The two extraction methods revealed that the method of leaf storage (silica gel and frozen at 80°C) impacted on quality of extractable DNA. Irrespective of leaf storage treatment, all DNA extracted by the CTAB methods were degraded and the isolation efficiency was not repeatable between samples. The Dellarporta method yielded high quality DNA from fresh leaf samples (refrigerated) but degraded DNA from silica gel preserved samples. For the Dellarporta protocol, the DNA extracted from silica preserved samples showed variation in DNA yields. The variations could have been attributed to the different levels of secondary metabolites in the different accessions which resulted to the variations in efficiency of extraction of DNA observed. Since the fresh leaf samples exhibited minimal variations in quantity and quality of extracted DNA, this type of tissue was chosen in subsequent DNA isolation activities.

The concentration of isolated cassava DNA was estimated by comparing the band size of 3 ul of isolated DNA with that of uncut, unmethylated lambda (λ) DNA standards (750, 500, 250, 125, 100 and 83 ng) (Figure 1). The extracted DNA ranged in concentration from 27.8 to 250 ng/µl. The isolated DNA was also high in molecular weight and was intact. Based on this, PCR optimization of cassava DNAs was carried out using diluted samples with a DNA concentration of 100, 50, 25 and 18 ng/µl. This study established the 25 ng/µl DNA sample to be optimal for PCR assays. All primers were diluted to 1 nm whereas MgCl₂ in the PCR was optimized using five serial dilutions of 2.5, 3.5, 4.0, 4.5 and 5.0 mM per PCR. The 5.0 mM MgCl₂ concentration gave best observable amplicons. After trying different types of agarose, metaphere agarose was chosen for subsequent use due to its ability to resolve alleles that differed only in a few base pairs.

The molecular size of SSR amplicons (alleles) differed with the selected primers and ranged from 230 to 310 bp with primer SSRY 13, SSRY 78 and SSRY 51, respectively. The smallest difference between the highest and lowest values of allele size was 10 bp at locus SSRY 13, and the largest difference (40 bp) was detected at locus SSRY 35. The allele sizes scored at the other remaining loci presented differences between 20 and 30 bp. A representative SSR profile of 19 cassava accessions with primer SSRY35 is presented in Figure 2. The seven SSR primers that were screened amplified a total of 39 alleles. The average number of alleles per

<table>
<thead>
<tr>
<th>SSR locus</th>
<th>Type of repeat</th>
<th>Left primer 5'-3'</th>
<th>Right primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSRY9</td>
<td>(GT)_15</td>
<td>ACAATTCTCATGAGTCATCAACT</td>
<td>CCGTTATTGTCTGTTGTCCT</td>
</tr>
<tr>
<td>SSRY13</td>
<td>(CT)_29</td>
<td>GCAAGAATTCCACCAGGAAG</td>
<td>CAATGATGTAAGATGGTACAG</td>
</tr>
<tr>
<td>SSRY35</td>
<td>(GT)<em>2(GC)(GT)</em>{11}(GA)_{19}</td>
<td>GCAGTAAAACCATTCTCCCAA</td>
<td>CTGTACAGCAAGATGCTGT</td>
</tr>
<tr>
<td>SSRY51</td>
<td>(CT)<em>{11}(CG)(CT)</em>{11}(CA)_{18}</td>
<td>AGGTTGGATGCTTGAAGGA</td>
<td>GGAATGCAGATGGTACAG</td>
</tr>
<tr>
<td>SSRY66</td>
<td>(GA)_{19}AAGA</td>
<td>ATTCAGCTTCCAATCTTTGTCA</td>
<td>CGAAATGCTTGAACAGAT</td>
</tr>
<tr>
<td>SSRY78</td>
<td>(CT)_{22}</td>
<td>TGCACACGTTCGGTTTCAT</td>
<td>ATGCCCTCCAGAGTCAAC</td>
</tr>
<tr>
<td>SSRY106</td>
<td>(CT)_{24}</td>
<td>GGAAACTGCTTGCAACAAGA</td>
<td>CAGCAAGACCATTCACTAGT</td>
</tr>
</tbody>
</table>

**Figure 1.** Lambda (λ) DNA standards (83–750 ng) and DNA samples isolated from 33 cassava accessions electrophoresed on 1% molecular grade agarose gel in TBE buffer (lane numbers represent in the same order the accessions in Table 1b).
Table 3. Number of alleles amplified by SSR primers in test cassava germplasm.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Total number of alleles</th>
<th>Number of polymorphic alleles</th>
<th>Percent polymorphism (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSRY9</td>
<td>7</td>
<td>3</td>
<td>42.8</td>
</tr>
<tr>
<td>SSRY13</td>
<td>4</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>SSRY35</td>
<td>8</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>SSRY51</td>
<td>6</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>SSRY66</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>SSRY78</td>
<td>5</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>SSRY106</td>
<td>5</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>Mean</td>
<td>5.6</td>
<td>3</td>
<td>55.4</td>
</tr>
</tbody>
</table>

The mean Nei’s gene diversity index ($H_e$) and Shannons information index (I) estimates of heterozygosity, for the 69 accessions of cassava are presented in Table 4.

Table 4. Genetic heterozygosity ($H_e$) and Shannons index (I) of 69 cassava accessions.

<table>
<thead>
<tr>
<th>Heterozygosity ($H_e$)</th>
<th>Shannons index (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36 ± 0.15</td>
<td>0.53 ± 0.19</td>
</tr>
</tbody>
</table>

primer pair ranged from 4 to 8. The number of polymorphic alleles ranged from 2 to 4. Percentage polymorphism ranged from 42.8 to 75% (Table 3). In most cases, the number of unique alleles (that is, amplified products in just one individual) was positively correlated with the total number of alleles per locus and their size differences. A binary data matrix based on 69 accessions and 21 polymorphic alleles from seven primer pairs was used for statistical analysis.

A genetic identity and distance matrix based on the proportion of shared (common) alleles (Nei and Li, 1972) was derived using Popgene version 1.31 that was used to establish the level of relatedness between the 69 accessions (Table 4). Others like Mue, a land race and 990072-B an IITA introduction were found to be the only one having the lowest genetic identity of 0.1 although there were nine pairs of accession, pairs with low genetic identity of 0.2 such as Nyamambakaya and Nyakatanegi-2. Estimation of molecular identity ranged from 0.2 between the following pairs of accessions; Nyakatanegi 3 and Nyamambakaya; Kamisi and Obarodak; Opondo and Kiringis; MM96/7688 and Nyakatanegi 3; Kapchetuya and Nyakatanegi 2; Ex-Mariakani and Kiringis; Mue and Obarodak; Mue and 990072 among others to 1.0 between accessions Agriculture and CK-9, Tamisi and Sifros, Nabwire and Sifros, Bumba and MM96/1871 among others. Pairs of accessions with an identity of 1.0 could not be distinguished by the 21 polymorphic alleles. This clearly suggests that these paired accessions could be identical genetically and possibly have only been given different names. The observation points to possible genetic redundancy of some accessions conserved in the national repository centers in Kenya. The collection held in these centres need to be rationalized to remove the genetically redundant accessions in order to contribute to generation of a truly core collection. Other accessions
which are popular in Kenya and had high genetic identity of 0.9 included Kaleso and Kibandameno, the popular landraces of the Kenyan coast; CK-8 and CK-9 together with Nabwire and Bumba which are western province land races; Kapcheleyo, Kapchetuya and Marakwet land races which are exclusively grown at Kerio valley of Kenya among others mainly found in cluster IV.

The accessions with similar names among the accessions showed varied levels of genetic identity. Varieties Serere, MH95/0183 and Migyera showed high genetic identity of 0.7. This shows that these collections with similar names could actually be the same. This was followed closely by accessions MM96/1871 and KME-1 each with 0.6 and 0.5 genetic identity respectively. Among the Kenyan land races, genetic identity ranged from 0.2 to 1 (for example Mue and Kitwa with Sifros and Tamisi respectively) while among the IITA accessions, identity ranged from 0.2 to 0.9 (for example MM96/1871 and 990072 with 990183 and 990056 respectively). The distance matrix was used to derive a dendogram using unpaired group mean linkage cluster analysis (UPGMA) (Figure 3). The dispersion of cassava accessions into various groups appeared to be random. The wild accessions collected at Kerio Valley clustered into different groups that is Arror-1 clustered into group II whereas Arror-2, Arror-3 and Arror-4 clustered in group IV. The two wild accessions, Arror 2 and 4 were very close to each other and formed a tight cluster in group IV. The three wild types of cassava found in Kenya were spread in the dendogram. This wild type cassava accessions may be the only ones to be found in Africa as has previously been reported by Halsey et al. (2008).

At a distance of 68%, the 69 accessions were clustered together into five marker based groups (Table 5). Cluster group IV had the largest number of accessions (42) which comprised two accessions originating from CIAT, 9 lines originating from IITA and 32 accessions originating from local land races. It is important to note that most land races were clustered into this group. Group I formed a distinct cluster group with accessions 990072 from IITA and landraces Nyakatane-3 and KME 1 appearing to be most distantly related from all others.

DISCUSSION

Genetic diversity in cassava has previously been studied using DNA molecular markers. Among the molecular tools that have been used include isozymes (Sarria et al., 1992), RFLPs (Angel et al., 1992), RAPDs (Tonukari et al., 1997) and SSRs (Fregene et al., 2001; Moyib et al., 2007). In most studies, low to medium genetic diversity has been observed. In the present study, also, there was generally medium genetic diversity among the land races and between the improved (introductions) accessions from IITA and Kenyan land races, as shown by the dendogram. This might be as a result of a common source of collection (IITA) from which the Kenyan cassava breeders and farmers choose their common desirable traits of cassava, such as potential for high yields and disease resistance. Because of the wide variability in biochemical quality traits, it is feasible to use some of the accessions assayed in this study as progenitors to introgress useful genes into improved cassava lines. This agrees with the study by Moyib et al. (2007) on Nigerian collections.

SSR primers have shown high levels of polymorphism in many important crops including Sorghum bicolor (Smith et al., 2000), Triticum aestivum L. (Ahmad, 2002), and Cucumis melon L. (Danin-Poleg et al., 2001). SSR primers were also polymorphic in the Kenyan cassava cultivars assessed in this study. The results of this study show that each of the seven primer pairs detected polymorphisms among the 69 cassava accessions studied. Results of this study, therefore, established a collection of these seven polymorphic SSR primers, SSRY 9, SSRY 13, SSRY 35, SSRY 51, SSRY 66, SSRY 78 and SSRY 106, that could readily be used for genotype identification and genetic diversity studies in Kenyan cultivated cassava. One of these SSR markers, SSRY 51 is located at position N on genetic linkage map of cassava (Fregene et al., 2003). A few highly polymorphic SSR markers like SSRY 66 with PIC of 75%, SSR 78 and SSR 106 all together with PIC of 60%, can be used in genetic studies of cassava. This would reduce the necessity for applying many SSR primers for the identification of cassava cultivars in Kenya and, hence, contributes to saving time and also cut the cost of research studies for genotype identification and genetic diversity studies in the species.

The genetic identity of Kenyan land races ranged from 0.2 to 1 while for IITA introductions, from 0.2 to 0.9. This shows that the Kenyan landraces are a rich source of diversity as compared to improved IITA introductions. Nonetheless, the relatively high genetic identity values for some landraces indicate close relatedness. Cassava is routinely propagated vegetatively and it is likely for two similar accessions to assigned different names. This might also stem from the fact that the Kenyan landraces were domesticated in the same ecological zones with narrow genetic base while the improved were obtained from different exotic sources that might have diverse ecological ranges. The diversity index which is the probability that two randomly selected alleles in a given accession are different, estimated by H_e, was 0.36 and the Shannon's index (I) was 0.53, indicating the average genetic diversity of cassava. Genetic diversity index (H_e) of cassava landraces and introductions was different from the Neotropics and Africa (0.46 to 0.62) but similar to that found in Guatemala as reported by Fregene et al. (2003). The reliability of estimates for genetic variation such as H_e and I and genetic distances depends more on the number of loci than on the number of individuals sampled (Fregene et al., 2003). Estimates of genetic differentiation
Figure 3. Dendogram of 69 cassava accessions based on SSR analysis of seven primers based on Nei and Li (1979) genetic distance.

ranged widely from locus to locus, underscoring the danger of assessing SSR diversity with a small set of SSR markers.

In the distribution of cassava into clusters, it is important to note that most land races were clustered into group IV. This may indicate a common ancestry for the
Table 5. Distribution of 69 cassava accessions into clusters based on SSR data.

<table>
<thead>
<tr>
<th>Cluster number</th>
<th>Number of accessions</th>
<th>Name of accessions</th>
<th>Origin</th>
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local landraces. Since IITA is a secondary centre of diversity for cassava, this helps to explain why accessions from there were found in every cluster. This is due to the movement of cassava from centre of origin to other places within the region. Land races Nyakatanegi 3 and KME 1 together with accession 990072 - A clustered together in group I and diverged from other accessions. This may be due to common ancestry or could be an indicator of duplicates. However, similarities in accessions can also arise due to convergent evolution, selection or sharing of common parentage.

Conclusion
The molecular study has also proved that SSR markers can be useful in breeding programmes of cassava allowing for the identification of new cultivars as well as assessment of genetic similarity/diversity among different genotypes. The average level of genetic diversity observed in the Kenyan landraces will benefit cassava germplasm conservation and enhancement efforts in Kenya, and contribute to the elucidation of forces that shape genetic differentiation in this asexually propagated allogamous crop.

Recommendations
The Kenyan landraces should be collected, conserved and maintained at the national cassava repository centre. Also, further introduction of IITA improved lines should be rationalized on the basis of their distance from the local landraces.

ACKNOWLEDGEMENTS
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REFERENCES

Table 5. Contd

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Group V 9

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Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX (1997). POPGENE, the user-friendly software for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.
A comparative study of the use of tiger-specific and heterologous microsatellite markers for population genetic studies of the Bengal tiger (*Panthera tigris tigris*)

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Comparison of genetic diversity indices of heterologous and species-specific microsatellite loci within a species may provide a panel of appropriate markers for genetic studies, but few studies have carried out such comparisons. We examined and compared the genetic characteristics of tiger-specific and heterologous loci in eight captive Bengal tigers. The mean polymorphic information content (PIC) value of the tiger-specific microsatellite loci (n = 15) was 0.447, and the number of alleles was from 2 to 4 per locus. In comparison, the heterologous microsatellite loci (n = 15) had a mean PIC value of 0.539, and the number of alleles per locus was three to five. Our findings indicate that the heterologous markers have a higher frequency (n = 11) of polymorphic microsatellite loci and number of alleles per locus compared with tiger-specific loci. We pooled the highly polymorphic (PIC > 0.5) tiger-specific loci (n = 5) and heterologous microsatellite loci (n = 11) except one and noted a higher mean observed heterozygosity and PIC values of 0.668 and 0.575, respectively, compared with the heterologous and tiger-specific loci taken alone. Using a locus selection criterion of PIC > 0.5, we recommend a combined panel of 16 highly polymorphic loci for genetic studies of the wild population of the Bengal tigers and suggest that either a combination of tiger-specific and heterologous microsatellite primers or heterologous primers be used in genetic studies related to the ecology, biology, socio-biology and behavior of Bengal tigers as >13 loci are needed in such studies.

**Key words:** Bengal tiger, highly polymorphic, tiger-specific, heterologous, microsatellite loci.

INTRODUCTION

The tiger (*Panthera tigris*) once had the widest geographical distribution among cat species, extending from almost 10° south of the equator (Bali and Java) to beyond 60° north (the Russian Far East) and through more than 100°

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**Abbreviations:** SSRs, Simple sequence repeats; PCR, polymerase chain reactions; PIC, polymorphic information content.
of longitude (Mazak, 1996; Nowell and Jackson, 1996). It is not surprising that the tiger exhibited considerable variation in behavior due to adaptation to diverse bioclimatic zones and the different groups of prey species across its geographical range (Pocock, 1929; Brongersma, 1935; Mazak, 1981, 1996). Three of the nine genetically identified subspecies of the tiger have become extinct (Nowell and Jackson, 1996; Luo et al., 2004).

Poaching is a major conservation threat to the species, and there is a need to curtail the global traffic in tiger parts and products (Dinerstein et al., 2007; Goodrich et al., 2008; Walston et al., 2010). Although the geographic distribution of the tiger once extended across Asia from eastern Turkey to the Sea of Okhotsk, its range has been greatly reduced in recent times due to poaching as well as human disturbances that have led to habitat loss and a reduction in the availability of wild prey species throughout its range (Dinerstein and Wikramanayake, 1993; Nowell and Jackson, 1996; Sunquist et al., 1999; Linkie et al., 2006; Sanderson et al., 2006). Now tigers survive only in small pockets spread from India to Vietnam and in Sumatra, China and the Russian Far East (Nowell and Jackson, 1996).

The Bengal tiger (P. t. tigris), the national animal of India, is listed in Schedule I of the Wildlife (Protection) Act, 1972 of India and in Appendix I of the Convention on International Trade in Endangered Species (CITES). It is one of the six extant tiger subspecies found in India, Nepal, Bhutan, Bangladesh and Myanmar (Luo et al., 2004; http://assets.panda.org/downloads/wwf_tiger_factsheet_2010_1.pdf). The reported world tiger population has declined and may be as low as 3200 individuals (http://assets.panda.org/downloads/wwf_tiger_factsheet_2010_1.pdf). The largest population (1706) is that of the Bengal tiger in India (Jhala et al., 2011).

Reliable methods of studying the population dynamics and its processes are necessary for designing long-term conservation strategies for any species in fragmented and human-dominated landscapes. Large carnivores such as the tiger are difficult to study because they are often wide ranging and have low densities (Boitani et al., 2008; Karanth, 2003) and are territorial, elusive, cryptic and nocturnal in nature (Karanth et al., 2003). It is practically very difficult to estimate numbers of tigers in terms of sex to understand the population dynamics. It is also difficult to track individual tigers to obtain the ecological, biological and genetic information needed to develop conservation strategies.

In view of the recent advances that have taken place in DNA technologies, population genetics has been widely used in research and conservation of both abundant and rare species (Schwartz et al., 2007; Goedbloed et al., 2013; Holbrook et al., 2013). Molecular methods incorporating non-invasive sampling are commonly used in monitoring the populations of carnivores (Taberlet et al., 1999; Piggott and Taylor, 2003; Waits and Paetkau, 2005; Schwartz et al., 2007; Cullingham et al., 2010; Miotto et al., 2011) and in studying their socio-biology (Langergraber et al., 2013) and behavioral genetics (Langergraber and Vigilant, 2011; Lyke et al., 2013). Among the different genetic markers used in wildlife studies are microsatellites, or simple sequence repeats (SSRs). These are regions of the genome that are made up of short repeat sequences, consisting of one to six nucleotides (Hancock, 1999; Sawaya et al., 2013), and they are widely used in studies of various organisms because of their high degree of polymorphism and their co-dominant inheritance. One of the limiting factors in the use of these markers is the development of species-specific primers (Lopes et al., 2010) to resolve genetic relationships at all levels of the population structure (Jarne and Lagoda, 1996; Hille et al., 2002). Nevertheless, attempts have been made to use these methods for the domestic cat (Felis catus) (Menotti-Raymond et al., 1999), Sumatran tiger (P. t. sumatrae) (Williamson et al., 2002), Asiatic lion (P. leo persica) (Singh et al., 2002), South China tiger (P. t. amoyensis) (Zhang et al., 2006), puma (Puma concolor) (Kurushima et al., 2006) and Bengal tiger (Bhagvatula and Singh, 2006; Sharma et al., 2008) among the Felidae.

The use of species-specific microsatellites is limited by the time and expense involved and the difficulty in isolating these short tandem repeats and their flanking regions from the genomes of the target organisms. An alternative approach to de novo development is exploitation of the available information by cross-species amplifications among a range of phylogenetically related species (Blanquer-Maumont and Crouau-Roy, 1995; Colman et al., 1996; Pepin et al., 1995; Scribner et al., 1996; Hille et al., 2002). But it is known that the level of information obtained by using heterologous primers differs among species, generally because of a decrease in the number of successfully amplified loci as the genetic distance increases (Francisco et al., 2006). Considering the information obtained by using species-specific loci, heterologous primers may not be ideal choices for estimating genetic diversity indices. Notwithstanding this, the use of heterologous primers has become quite common in genetic studies because it eliminates the need to develop new sets of primers for each species (Engel et al., 1996). Heterologous primers have been used in studies carried out on the Far Eastern leopard (P. pardus orientalis) (Uphyrkina et al., 2002), jaguar (P. onca) (Ruiz-Garcia et al., 2006), snow leopard (P. uncia) (Waits et al., 2007), clouded leopard (Neofelis nebulosa) (Witting et al., 2007), leopard (P. pardus) (Mondol et al., 2009), Siberian tiger (P. t. altaica) (Alasaad et al., 2011), cheetah (Acinonyx jubatus) (Charrau et al., 2011) and jaguarundi (Puma yagouroundi) (Holbrook et al., 2013) and provided support to plan effective conservation strategies. Janecka et al. (2008) modified primers for short amplicon originally developed for the domestic cat (Menotti-Raymond et al., 1999) and used them in genetic
studies they carried on the snow leopard.

Thus, heterologous microsatellite loci are of use in intra- and inter-population diversity analysis and can potentially be used in identifying individuals. Thus they may be used in a variety of applications, including gene mapping and analysis of family relatedness and paternity (Angers and Bernatchez, 1996; Vigilant et al., 2001; Inoue et al., 2008), whereas species-specific microsatellite loci are very useful and essential for long-term population genetic studies (Lopes et al., 2010) and for understanding genetic fitness. Therefore, in this study, we compare for the first time the genetic characteristics of tiger-specific microsatellite loci (n = 15) developed for the Bengal tiger (Sharma et al., 2008) and Sumatran tiger (Williamson et al., 2002) with those of heterologous microsatellite loci (n = 15) developed for the domestic cat (Menotti-Raymond et al., 1999, 2005) and Asiatic lion (Singh et al., 2002) in captive Bengal tigers using high quality DNA samples. Of these 30 loci, seven are different loci that have so far not been used in other studies on the Bengal tiger. We also suggest a combined panel of highly polymorphic tiger-specific and heterologous microsatellite loci for studying different genetic aspects of the wild population of the Bengal tiger.

MATERIALS AND METHODS

We obtained blood samples from eight captive Bengal tigers in Mahendra Chaudhury Zoological Park, Chhattbir, Mohali, India. Details such as individual tigers’ histories and translocation from other zoos are poorly documented in the ‘Indian National Studbook for Bengal Tigers, 2011’, and hence the geographic origin of these tigers is unknown. Genomic DNA was extracted from the blood samples using Bio Robot EZ1 (QIAGEN, Germany). We selected and amplified 15 tiger-specific loci (12 dinucleotide and three tetranucleotide repeat loci) developed for the Bengal tiger (Sharma et al., 2008) and Sumatran tiger (Williamson et al., 2002) and 15 heterologous loci (eight dinucleotide and seven tetranucleotide repeat loci) developed for the Asiatic lion (Singh et al., 2002) and domestic cat (Menotti-Raymond et al., 1999, 2005). We examined these loci for their size, range and level of polymorphism in the Bengal tiger (Table 1). Polymerase chain reactions (PCR) were carried out in an Applied Biosystems 9700 thermocycler (Applied Biosystems, Germany) in a 10 µl reaction mixture containing 1 × PCR ABI Taq gold buffer, 2.0 mM MgCl2, 0.4 mM dNTP mix, approximately 50 ng of genomic DNA, 4 pmol of forward and reverse primers and 1 U Taq Gold DNA Polymerase (Applied Biosystems). Amplification of all 30 loci was attempted for all samples using amplification conditions described in the literature. The amplified PCR products were detected on 2% agarose gel in 1 × TAE buffer. Amplified products were visualized and scored on an ABI 3130 fluorescent detection system using the GeneMapper software package (Applied Biosystems). The quality of the microsatellite data was statistically assessed for genotyping errors due to non-amplified alleles (null alleles), short-allele dominance (large-allele dropout) and mis-scoring of stutter peaks using MicroChecker 2.2.3 (Van Oosterhout et al., 2004). Statistics of the genetic diversity values were generated using GenAIEx 6 (Peakall and Smouse 2006) and GIMLET (Valiere, 2002). GENEPOP’007 (Rousset, 2008) was used to test the deviations from the Hardy-Weinberg equilibrium (HWE) and the linkage disequilibrium (LD) in the population at each locus. The polymorphic information content (PIC) of the markers was calculated from the allelic frequencies using Cervus (ver. 3.0) (Kalinowski et al., 2007). Since details of individual tigers were not available in the ‘Indian National Studbook for Bengal Tigers, 2011’, we estimated the Queller and Goodnight relatedness coefficients (Queller and Goodnight, 1989) using GenAIEx 6 (Peakall and Smouse, 2006) to ensure that the selected individuals were not related to each other. The level of relationship among individuals was established using the R-value as suggested by Blouin (2003).

RESULTS AND DISCUSSION

Different kind of genotyping errors have been reported to be involved in the use of non-invasively collected samples such as scats (Bonin et al., 2004; Pompanon et al., 2005). However, genotyping data can be validated by the use of a known individual’s DNA (Gerloff et al., 1995; Launhardt et al., 1998) and a good-quality sample that can be used as a species control DNA (Kohn et al., 1995; Paxinos et al., 1997; Wasser et al., 1997). Therefore, more studies are needed to document the genetic characteristics of microsatellite loci with a good-quality source of DNA. In view of this, we used blood samples (n = 8) as a source of high-quality DNA to minimize genotyping errors. Our data analysis using MicroChecker 2.2.3 (Van Oosterhout et al., 2004) clearly revealed the absence of null alleles, large-allele dropout (Wattier et al., 1998) and scoring errors, associated with peak stuttering (Ewen et al., 2000), in genotyping data generated using tiger-specific and heterologous microsatellite loci.

All 15 tiger-specific loci were successfully amplified in the Bengal tiger samples, and the allele size ranged from 112 to 285 bp. The average observed (H0) and expected (He) heterozygosities for the 15 polymorphic loci were 0.500 and 0.555, respectively. Five loci (PttE5, PttF4, Ptt4a, 6HDZ056 and 6HDZ210) had expected heterozygosity levels above 0.500. The number of alleles per locus in these loci ranged from 2 to 4 (average 2.933), and the mean PIC was 0.447. The effective number of alleles per locus ranged from 1.385 to 3.000 (average 2.196). Only five microsatellite loci out of 15 tiger-specific loci had PIC values higher than 0.500, which is considered to be a very informative value in population genetic analysis (Botstein et al., 1980) (Table 1).

All heterologous loci (n = 15) were amplified, and the allele size ranged from 106 to 302 bp. The average observed and expected heterozygosities for all 15 loci were 0.624 and 0.630, respectively. Fourteen loci (F41, F53, F85, F124, Fca008, Fca126, Fca304, Fca441, Fca506, Fca628, Fca740, Fca742, Ple23 and Ple57) had expected heterozygosity levels above 0.500 (Table 1). The number of alleles per locus at the polymorphic loci (n = 15) ranged from 3 to 5 (average 3.666), and the mean PIC was 0.539. The effective number of alleles per locus ranged from 1.588 to 3.447 (average 2.585). Ten microsatellite loci out of 15 heterologous loci had PIC
values greater than 0.500 and were considered to be valuable for studies related to population genetic analysis (Botstein et al., 1980) (Table 1). These observed differences in genetic diversity indices between the two types of markers may be arising from the heterologous markers (developed for the domestic cat and Asiatic lion) being phylogenetically old markers compared with the phylogenetically young markers (tiger-specific markers developed for the Bengal tiger and Sumatran tiger) as phylogenetically old markers are known to have greater genetic diversity compared with markers that are young in the evolutionary scale.

The sample size is small in the present study, which may affect the HWE analysis. However, the examined captive population was found to be in HWE at all tiger-specific and heterologous microsatellite loci except PttD6, PttB3, 6HDZ170 and F85, which showed significant ($P < 0.05$) deviations from the HWE (Table 1). The inbreeding

### Table 1. Genetic characteristics of species-specific and heterologous microsatellite loci in eight captive Bengal tigers (*Panthera tigris tigris*).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr. Asn.</th>
<th>Di-/teta</th>
<th>Size range (bp)</th>
<th>N</th>
<th>Na</th>
<th>Ne</th>
<th>H_0</th>
<th>H_E</th>
<th>PIC</th>
<th>F_IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptt10H</td>
<td>NK</td>
<td>D</td>
<td>162-164</td>
<td>8</td>
<td>2</td>
<td>1.385</td>
<td>0.375</td>
<td>0.325</td>
<td>0.258</td>
<td>-0.166</td>
</tr>
<tr>
<td>PttA2‡</td>
<td>NK</td>
<td>D</td>
<td>188-196</td>
<td>8</td>
<td>2</td>
<td>1.670</td>
<td>0.125</td>
<td>0.458</td>
<td>0.337</td>
<td>0.740</td>
</tr>
<tr>
<td>PttA4‡</td>
<td>NK</td>
<td>D</td>
<td>143-151</td>
<td>7</td>
<td>3</td>
<td>2.032</td>
<td>0.714</td>
<td>0.538</td>
<td>0.427</td>
<td>-0.363</td>
</tr>
<tr>
<td>PttC6‡</td>
<td>NK</td>
<td>D</td>
<td>174-176</td>
<td>8</td>
<td>2</td>
<td>1.800</td>
<td>0.625</td>
<td>0.458</td>
<td>0.337</td>
<td>-0.400</td>
</tr>
</tbody>
</table>
| PttE5‡     | NK        | D        | 182-190         | 8  | 4   | 2.314| 0.750| 0.650| 0.559| -0.166|†
| PttF4‡     | NK        | D        | 192-196         | 8  | 3   | 2.945| 0.625| 0.700| 0.582| 0.113|†
| PttD5‡     | NK        | T        | 201-213         | 7  | 4   | 2.510| 0.286| 0.571| 0.483| 0.520|
| PttF4‡     | NK        | D        | 283-285         | 8  | 4   | 3.000| 0.625| 0.725| 0.624| 0.146|†
| PttG4‡     | NK        | T        | 112-120         | 8  | 3   | 2.418| 0.500| 0.575| 0.447| 0.138|
| PttF1‡     | NK        | D        | 129-133         | 8  | 3   | 2.219| 0.500| 0.592| 0.456| 0.164|
| PttD6‡     | NK        | D        | 180-190         | 8  | 4   | 2.000| 0.125| 0.658| 0.483| 0.794|
| PttB3‡     | NK        | T        | 241-245         | 8  | 2   | 1.528| 0.000| 0.400| 0.305| 1.000|
| PttB2‡     | NK        | D        | 139-141         | 8  | 2   | 1.800| 0.625| 0.458| 0.337| -0.400|
| 6HDZ056‡   | NK        | D        | 172-176         | 8  | 3   | 2.656| 0.750| 0.625| 0.520| -0.191|†
| 6HDZ170‡   | NK        | D        | 216-226         | 8  | 3   | 2.656| 0.875| 0.675| 0.556| -0.376|†

### B: Heterologous microsatellite loci

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr. Asn.</th>
<th>Di-/teta</th>
<th>Size range (bp)</th>
<th>N</th>
<th>Na</th>
<th>Ne</th>
<th>H_0</th>
<th>H_E</th>
<th>PIC</th>
<th>F_IS</th>
</tr>
</thead>
</table>
| F41   | D2        | T        | 170-188         | 8  | 4   | 2.656| 0.625| 0.708| 0.616| 0.166|†
| F53   | A1        | T        | 128-152         | 8  | 4   | 2.000| 0.667| 0.500| 0.450| -0.280|†
| F85   | B1        | T        | 156-176         | 8  | 3   | 2.656| 0.375| 0.658| 0.544| 0.340|†
| F124  | E1        | T        | 258-286         | 8  | 4   | 3.306| 0.625| 0.750| 0.644| 0.102|†
| Fca008| A1        | D        | 130-134         | 8  | 3   | 1.906| 0.625| 0.542| 0.428| -0.111|
| Fca126| B1        | D        | 124-150         | 8  | 4   | 1.820| 0.625| 0.525| 0.458| -0.176|
| Fca272| A3        | D        | 112-122         | 8  | 3   | 1.588| 0.500| 0.433| 0.371| -0.142|
| Fca304| A2        | D        | 125-141         | 8  | 3   | 2.656| 0.750| 0.633| 0.511| -0.191|†
| Fca441| D3        | T        | 149-161         | 8  | 4   | 2.793| 0.875| 0.742| 0.645| -0.154|†
| Fca506| F2        | D        | 206-220         | 8  | 3   | 2.842| 0.625| 0.692| 0.575| 0.030|†
| Fca628| D2/E3     | D        | 106-110         | 8  | 3   | 2.945| 0.444| 0.660| 0.586| 0.378|†
| Fca740| C1        | T        | 290-302         | 8  | 4   | 1.820| 0.625| 0.525| 0.458| -0.176|
| Fca742| D4        | T        | 150-176         | 8  | 4   | 2.842| 0.500| 0.692| 0.592| 0.200|†
| Ple23  | NK        | D        | 152-168         | 8  | 5   | 2.793| 0.750| 0.692| 0.592| -0.154|†
| Ple57  | NK        | D        | 141-155         | 8  | 5   | 3.447| 0.750| 0.708| 0.618| -0.037|†

| Mean   | 3.666    | 2.585    | 0.624  | 0.630 | 0.539 | -0.013 |

1Locus recommended for panel of 16 microsatellite loci. T, Tetranucleotide repeat; D, dinucleotide repeat; n, number of samples; Na, number of alleles; Ne, effective number of alleles; Chr. Asn., chromosomal assignment; NK, not known; H_0, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; HW, significance of Hardy-Weinberg test based on 100 permutations ($P < 0.05$); F_IS, inbreeding coefficients. ^Sharma et al. (2008); Williamson et al. (2002); Menotti-Raymond et al. (1999, 2005); Singh et al. (2002).
coefficient ($F_{IS}$) was calculated using the procedure of Weir and Cockerham (1984), and a heterozygote excess was found at nine heterologous loci (F53, Fca008, Fca126, Fca272, Fca304, Fca441, Fca740, Ple23 and Ple57), in comparison with seven tiger-specific loci (Ptt10H, PttA4, PttC6, PttE5, PttB2, 6HDZ056 and 6HDZ0170) (Table 1). The mean $F_{IS}$ was found to be positive (0.103) with the tiger-specific loci ($n = 15$); in contrast, it was negative (-0.013) with the heterologous loci ($n = 15$) (Table 1). The observed ambiguity in the $F_{IS}$ values of the tiger-specific loci and heterologous loci may be due to the differences in the allele numbers as well the PIC values. Linkage analysis indicates no significant evidence of an LD ($P > 0.01$), demonstrating that each locus may be considered as an independent genetic marker (Rousset, 2008; Chen et al., 2005). This is an important finding because a population is likely to retain polymorphisms in the absence of an LD. The effectiveness of loci selection will be augmented if they are in a coupling LD (Wills and Miller, 1976).

Analysis of the genetic characteristics indicated a larger number of highly polymorphic loci, with a higher PIC value (>0.500) in the heterologous loci ($n = 10$) than in the tiger-specific loci ($n = 5$). It was apparent that the mean number of alleles per locus, effective number of alleles, heterozygosity and PIC values obtained using the heterologous loci were greater than those obtained with tiger-specific loci (Table 1). However, species-specific loci can be very informative genetically as they have been specifically developed for targeted species and may be quite useful in evaluating the genetic fitness because these loci are closer to the species than are the heterologous loci in the evolutionary scale.

The use of more than 13 polymorphic loci is recommended in studies related to molecular ecology for understanding population genetics, behavior, socio-biology, among others (Blouin, 2003). Similarly, Cornuet and Luikart (1996) stated that the statistical power of examining a genetic bottleneck in a wild population increases with the sample size and the number of loci. However, they also emphasize the point that it is more effective to increase the number of loci than it is to increase the sample size. Therefore, we suggest a combined panel of 16 loci, including tiger-specific loci ($n = 5$) and heterologous loci ($n = 11$), having PIC values >0.500, except F53, which was also included in the panel although it has a relatively low PIC value (0.450) because it generates a better genotyping profile that is easy to interpret and use for allele scoring. However, at the same time, the use of a greater number of loci may introduce more genotyping errors when using non-invasively collected samples of a low-quality source of DNA (Creel et al., 2003). This problem may be minimized by using the multiple-tube approach (Navidi et al., 1992; Goossens et al., 1998) and two-step multiplex PCR method without compromising with number of loci (Arandjelovic et al., 2009; Chang et al., 2012) needed to understand species biology and ecology.

Interestingly, the mean $F_{IS}$ value with the combined panel of highly polymorphic loci was close to zero (-0.004). This indicates that the population of captive tigers ($n = 8$) is in HWE. Hence, the results with the combined panel of selected loci are promising compared with the tiger-specific loci ($n = 15$) and heterologous loci ($n = 15$) alone. The combined panel yields relatively high average values of the heterozygosities ($H_0 = 0.668$ and $H_E = 0.675$). The number of alleles per locus at the polymorphic loci ($n = 16$) ranged from three to five (average 3.625), the effective number of alleles per locus ranged from 2.000 to 3.447 (average 2.781), and the mean PIC was 0.575 (Table 2). Considering the small number of individuals in our study, we support our results by examining the mean values of $H_E$ and $H_0$ (0.675 and 0.668, respectively) of the combined panel ($n = 16$), which are comparable with the reported mean values of $H_E$ (0.655 to 0.810) and $H_0$ (0.650 to 0.7624) in non-invasive genetic studies carried out on the Bengal tiger (Reddy et al., 2012; Gour et al., 2013; Sharma et al., 2013). We established the relatedness among the captive tigers ($n = 8$) using the combined panel of highly polymorphic loci and estimated the mean value of the relatedness coefficient ($R = -0.143$), which indicated that the selected

### Table 2. Comparison of genetic diversity parameters of species-specific (tiger-specific) and heterologous microsatellite loci with combined panel of highly polymorphic loci in eight captive Bengal tigers (*Panthera tigris tigris*).

<table>
<thead>
<tr>
<th>Mean values</th>
<th>Species-specific Loci ($n = 15$)</th>
<th>Heterologous loci ($n = 15$)</th>
<th>Combined panel of highly Polymorphic loci ($n = 16$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>2.933</td>
<td>3.666</td>
<td>3.625</td>
</tr>
<tr>
<td>Ne</td>
<td>2.196</td>
<td>2.585</td>
<td>2.781</td>
</tr>
<tr>
<td>$H_0$</td>
<td>0.500</td>
<td>0.624</td>
<td>0.668</td>
</tr>
<tr>
<td>$H_E$</td>
<td>0.555</td>
<td>0.630</td>
<td>0.675</td>
</tr>
<tr>
<td>PIC</td>
<td>0.447</td>
<td>0.539</td>
<td>0.575</td>
</tr>
<tr>
<td>$F_{IS}$</td>
<td>0.103</td>
<td>-0.013</td>
<td>-0.004</td>
</tr>
</tbody>
</table>

Na, Number of alleles; Ne, effective number of alleles; $H_0$, observed heterozygosity; $H_E$, expected heterozygosity; PIC, polymorphic information content; $F_{IS}$, inbreeding coefficients.
tigers were not highly related to each other, as is mostly expected in captive individuals.

Our findings clearly indicate that the combined panel of 16 loci, including Bengal tiger- and Sumatran tiger-specific microsatellite loci (n = 5) and heterologous loci (n = 11) that have been developed for the domestic cat and Asiatic lion, will be more useful for genotyping-based studies for evaluating the current range and genetic diversity and for genetic identification and characterization of various geographical populations of wild Bengal tigers from tissue, skin, hair and fecal samples (where more than 13 loci are recommended) (Blouin, 2003), compared with the use of tiger-specific or heterologous primers alone (Table 2).

REFERENCES


Full Length Research Paper

Effects of moisture barrier and initial moisture content on the storage life of some horticultural produce in evaporative coolant

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Shelf-life of five horticultural produce were studied. These include three leafy vegetables: Telfairia occidentalis, Celosia argentea and Amaranthus cruentus and two fruit vegetables: Lycopersicum esculentum and Abelmoschus esculentus. The layout plan of the experiment was a 3x2 factorial in a completely randomized design and each treatment replicated three times. The two factors examined were moisture barrier at three levels namely: thick lining, thin lining and non-lining. The other factor included initial moisture content of the produce, namely, turgid and partially wilted. Partial wilting of the produce was achieved by exposing freshly harvested materials at ambient temperature to dry for 45 min. During this period, about 20% of the moisture content was lost. Eighteen (18) vegetable baskets which work on the principle of evaporative cooling system were used. Each type of produce was stored at a time inside the vegetable basket. Some quantity of each produce were kept on the laboratory benches to serve as controls. Data recorded includes length of storage of produce, severity of disease infection, visual quality, disease incidence, ambient temperature and relative humidity of the storage baskets. The result indicates that there was a significantly higher relative humidity (P< 0.05) in the lined baskets than in the non-lined baskets. Consequently, the shelf-life of produce in lined basket was prolonged. Turgid produce had better quality retention and stored much longer than partially wilted produce. Generally, the evaporative coolant baskets provided an average temperature of 3°C lower than the ambient condition. The shelf-life of leaf vegetables T. occidentalis, C. argentea and A. cruentus was extended appreciably for 78 days compared to the controls.

Key words: Evaporative coolant, moisture barrier, shelf-life of horticultural produce.

INTRODUCTION

Post-harvest loss is the bane of agricultural production in Nigeria. Available data indicated that postharvest losses may be as high as 50% and above in Nigeria (Mbuk et al., 2011; Nwufo et al. 1990). This stemmed from the fact that fruits and vegetables are classified as highly perishables produce (Piet et al., 2011; Kay and Pallas, 1991). Once harvested, they tend to shrivel, wilt or rot at a very fast rate. This is due to their relatively high moisture content, soft texture and high respiration rate (Atanda et al., 2011).

Large quantities of fruits and vegetables may be produced during the growing season but due to lack of effective postharvest handling, much of the produce are wasted and millions of naira are spent in importing their concentrates. This then makes postharvest management of fruits and vegetables very important in any food sufficiency programme in Nigeria. Moreover, it will help to stabilize prices by carrying over produce period of high
production to period of low production (Agoda et al., 2011). Therefore, adequate storage should help to solve the problems of excess supply during the fruiting season when supply exceeds demands with the consequent low prices (Idah et al., 2007).

To achieve adequate storage, therefore, appropriate technologies should be applied. These technologies should be available to farmers adequately, affordable and easily adoptable (Ile and Bas, 2003). Evaporative coolant system (ECS) is an example of such technology in which principles the vegetable basket works (Nwufor et al., 1990). The evaporative coolant system is adaptable to environment where most of the rural farmers live. Most poor resource farmers cannot afford storage by refrigeration, irradiation and use of chemical control which may be adequate but not affordable by most farmers. ECS is a process of producing a cooling effect as a result of evaporation of liquid; electricity is not needed. It is based on the principles of adiabatic cooling of unsaturated air when in contact with water (liquid) for a sufficiently long time. The evaporative coolant is capable of reducing the temperature and producing appropriate humidity suitable for tropical fruits and vegetables (Amrat et al., 2013).

The vegetable basket has room for further modification, such as lining with polyethylene materials to enhance its effectiveness. According to Shukla et al. (2010), another method of reducing water loss from horticultural crops is placing physical barrier around the produce which also reduces air movement across the surface. Liberty et al. (2013a, b) reported that the use of very thin plastic wraps and heat - shrink films for packaging individual fruits is practiced in postharvest handling of fruits.

Another factor that influences post-harvest life of perishable produce is the initial quality of the produce before storage. Leaves attached to their parent plant function primarily to the acquisition of carbon through photosynthesis. After harvest, all these functions are not as operative as before harvest, the leaf losses its potential as energy source after harvest. With detachment from the parent plant, leaves can no longer replenish water lost through transpiration. Therefore, since water loss from the harvested leafy vegetables is inimical to the shelf life of the produce, their initial moisture content will determine their shelf life. The rate of water loss is modulated by the nature of the produce and the environmental condition in which it is stored. The objective of the study was to investigate factors that affect the effectiveness of vegetable basket in the preservation of horticultural produce.

MATERIALS AND METHODS

Five horticultural produce were used in this experiment. It was a 3x2 factorial experiment laid out in a completely randomized design and replicated three times. The two factors namely: Moisture barrier at three levels: thick lining, thin lining and non-lining. The second factor was moisture content of the produce at two levels - turgid and partially wilted. The freshly harvested vegetables were exposed to room drying for 45 min during which 20% of moisture content were lost. From preliminary test, produce exposed to drying for more than this period could not regain their turgidity after rewetting. Eighteen (18) vegetable bavets, which function on the principles of evaporative cooling, were used. The moisture barrier was made of polythene materials of two different gauges; these materials were used to line inner surfaces of the rectangular shaped vegetable baskets. The vegetables basket were made of cane or wooden frame and covered with absorbent materials such as jute materials. The baskets were wetted with tap water on the outside surfaces, two times daily. Each of the horticultural produce was stored one at a time.

Data recorded included, temperature and humidity inside the vegetable baskets and the ambient, length of storage, disease incidence and severity and visual qualities. Temperature readings were measured with the use of laboratory thermometer while humidity was measured with thermocouple. Disease severity was assessed on a five point score as suggested by Snedecor and Cochran (1967) for subjective evaluation. The grading score is as follows: 1, no disease; 2, trace; 3, slight; 4, moderate; 5, server disease. Similarly, the visual qualities were graded on a five point score as follows: 5, very fresh and no trace of colour bleaching; 4, fresh and fairly green; 3, slightly fresh and slightly bleached; 2, poorly fresh and bleached; 1, on set of deterioration. Disease incidence was determined thus:

\[
\text{Disease incidence} = \frac{\text{Number of spoilt fruits due to } x}{\text{Total number of fruit sampled}}
\]

Where, \( x \) = type of disease symptom.

RESULTS

Effect of lining and initial moisture content of produce on produce quality

Produce quality

Colour: Lining with polyethylene and initial moisture content of produce had significant effect on the quality criteria during the storage of all the horticultural produce. Table 1 shows that lining, initial moisture content of produce and; lining x initial moisture content significantly affected the colour of the three leaf vegetables: *Telfairia*, *Amaranthus* and *Celosia*.

For *Telfairia*, thick lining had the highest colour grade (5.0) which was significantly higher than thin lining and non-lining (4.3 and 4.2, respectively) (Table 1). Thin lining and non-lining did not differ with each other. In *Amaranthus*, thick and thin lining had identical and higher colour grade (3.3) than non-lining (1.8). Also, in *Celosia*, thick and thin lining had significantly higher colour grade but only thin lining was significantly higher than non-lining. Initial moisture content of *Telfairia* did not affect colour quality but in *Amaranthus* and Celosia turgid produce had significantly higher colour quality than partially wilted produce. For *Telfairia*, thick lining x turgid and partial wilting had maximum colour grade of 5.0 as well as thin lining x turgid moisture content and non-lining x partial willed. These high colour quality grades were
Table 1. Effect of lining and initial moisture content of produce on the colour of three vegetables stored in vegetable basket for 8 days.

<table>
<thead>
<tr>
<th>Lining (LN)</th>
<th>Initial moisture (IM) content of product</th>
<th>Telfairia Mean of lining</th>
<th>Amaranthus Mean of lining</th>
<th>Celosia Mean of lining</th>
<th>LSD 0.05 LN x IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick lining</td>
<td>Turgid</td>
<td>5.0</td>
<td>4.3</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Partially Wilted</td>
<td>5.0</td>
<td>2.3</td>
<td>3.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Thin lining</td>
<td>Turgid</td>
<td>5.0</td>
<td>3.7</td>
<td>3.3</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Partially Wilted</td>
<td>3.7</td>
<td>3.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Non-lining</td>
<td>Turgid</td>
<td>3.0</td>
<td>2.3</td>
<td>1.8</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>5.0</td>
<td>1.3</td>
<td>0.84</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.89</td>
</tr>
<tr>
<td>LSD 0.05 LN x IM</td>
<td></td>
<td>0.59</td>
<td>0.42</td>
<td>1.19</td>
<td>0.84</td>
</tr>
<tr>
<td>Mean on initial moisture content of produce</td>
<td>Turgid</td>
<td>4.4</td>
<td>3.4</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>4.6</td>
<td>2.2</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td></td>
<td>NS</td>
<td>0.68</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

significantly higher than thin lining x partial wilting (3.7) and non-lining x turgid moisture content (3.0).

For Amaranthus, the highest colour score was in thick lining x turgid produce (4.3), which did not differ significantly from thin lining x turgid moisture but was higher than the rest of the treatment combinations. For Celosia, the highest quality scores (5.0) (Table 1) was produced in thin lining x turgid produce, which was significantly higher than other combinations at both levels of initial moisture content. Thick lining x turgid and non-lining x turgid produce had similar colour grade (3.3 and 3.0, respectively) which were significantly higher than partially wilted produced at all lining levels, except thick lining x initial moisture with a score of 2.0 which was statistically similar to non-thinning x turgid.

On fruit vegetables (Table 2), lining significantly affected the colour quality in okra but not in tomato; thick lining produced the highest colour score of 3.8. Initial moisture content did not affect colour quality in both crops. Lining x initial moisture content significantly affected the colour moisture grade of okra but not of tomato. The highest colour grade (4.0) in okra (Table 2) occurred in thick lining x partially wilted which was not significantly different from thick lining x turgid produce (3.7). The lowest colour score of 1.7 was produced in non-lining x partially wilted moisture content in okra (Table 2).

Freshness

The result in Table 3 shows that lining and initial moisture content significantly affected the freshness of all the three leaf vegetables. Turgid moisture content consistently produced higher score on freshness quality for all three leaf vegetables than partially wilted moisture content, which consistently produced the lower scores on freshness (Table 3). Also lining x initial moisture content had significant effect on freshness of the three horticultural produce. The highest score of freshness (5.0) (Table 3) was produced in Telfairia by thick lining x turgid moisture content. This did not differ significantly with thin lining x turgid moisture content (4.7). Other combinations were statistically different from each other.
Table 3. Effect of lining and initial moisture content of produce on freshness of three vegetables stored in vegetable baskets.

<table>
<thead>
<tr>
<th>Lining (LN)</th>
<th>Initial moisture (IM) content of product</th>
<th><em>Telfaira</em></th>
<th>Mean of lining</th>
<th><em>Amaranthus</em></th>
<th>Mean of lining</th>
<th><em>Celosia</em></th>
<th>Mean of lining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick lining</td>
<td>Turgid</td>
<td>5.0</td>
<td>4.0</td>
<td>4.7</td>
<td>3.3</td>
<td>4.0</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>3.0</td>
<td>2.0</td>
<td>3.0</td>
<td>1.0</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Thin lining</td>
<td>Turgid</td>
<td>4.7</td>
<td>3.8</td>
<td>4.7</td>
<td>4.3</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>3.0</td>
<td>3.7</td>
<td>4.3</td>
<td>1.0</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Non-lining</td>
<td>Turgid</td>
<td>4.0</td>
<td>3.2</td>
<td>2.3</td>
<td>1.8</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>LSD 0.05 LN x IM</td>
<td></td>
<td>0.59</td>
<td>0.42</td>
<td>1.19</td>
<td>0.84</td>
<td>1.03</td>
<td>0.73</td>
</tr>
<tr>
<td>Mean on initial moisture content of produce</td>
<td>Turgid</td>
<td>4.6</td>
<td>3.9</td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.8</td>
<td>1.6</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td></td>
<td>0.34</td>
<td>0.22</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Effect of lining and initial moisture content of produce on freshness of two fruit vegetables stored in vegetable baskets.

<table>
<thead>
<tr>
<th>Lining (LN)</th>
<th>Initial moisture (IM) content of product</th>
<th><em>Okra</em></th>
<th>Mean of lining</th>
<th><em>Tomato</em></th>
<th>Mean of lining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick lining</td>
<td>Turgid</td>
<td>3.7</td>
<td>3.2</td>
<td>3.3</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.7</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thin lining</td>
<td>Turgid</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.0</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-lining</td>
<td>Turgid</td>
<td>2.7</td>
<td>2.3</td>
<td>1.3</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.0</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD 0.05 LN x IM</td>
<td></td>
<td>0.84</td>
<td>0.59</td>
<td>1.19</td>
<td>0.84</td>
</tr>
<tr>
<td>Mean on initial moisture content of produce</td>
<td>Turgid</td>
<td>3.0</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.2</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td></td>
<td>0.84</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

in *Telfaira*. The lowest score of freshness (2.3) was produced by non-lining x partially wilted moisture content. Lining significantly affected the freshness of *Telfaira*, thick lining produced the highest score followed by thin lining, while the least was non-lining. This trend was repeated in *Celosia for Amaranthus*, thin lining produced the highest freshness followed by thick lining and non-lining being the least.

For *Amaranthus*, the highest score on freshness quality (4.7) was produced by thick lining x turgid moisture content and thin lining x turgid moisture. The least freshness score (1.3) was produced by non-lining x partially wilted. For *Celosia*, thin lining x turgid moisture content with a score of (4.3) was statistically higher than all other combinations except thick lining x turgid moisture content (4.0). Non-lining x partially wilted (1.7), non-lining x turgid produce (2.0), thin lining x partially wilted (1.0) were statistically similar and had the lowest freshness scores. On fruit vegetables (Table 4), lining and initial moisture content significantly affected the freshness of two fruit vegetables okra and tomato.

For fruit vegetables (Table 4), thick lining produced the highest score but thin-lining and non-lining were the same in okra. For tomato, non-lining produced the highest score (2.8) followed by 2.3 score in thick lining while the least was 1.8 score produced in non-lining. For okra, the highest freshness of 3.7 score was produced by thick lining x turgid moisture content, while the lowest quality was observed in thin lining x partially wilted and non-lining x partially wilted moisture content produced significantly higher score than the other combinations. For tomato, the highest quality score of 3.3 was also produced by thick lining x turgid moisture content, followed by the score of 3.0 (Table 4) produced by non-lining x partially wilted and 2.7 score observed in non-lining x turgid moisture content. These were statistically similar and higher than the other lining x turgid moisture content which consistently produced a higher quality score than partially wilted. Generally, the freshness scores in fruit vegetables were lower than those in leaf vegetables.

**Length of time of storage**

Table 5 shows that both lining and initial moisture content
Table 5. Effect of lining and initial moisture content of produce on length of time of storage (days) of three leaf vegetables stored in vegetable basket.

<table>
<thead>
<tr>
<th>Lining (LN)</th>
<th>Initial moisture (IM) content of product</th>
<th>Telfaira</th>
<th>Mean of lining</th>
<th>Amaranthus</th>
<th>Mean of lining</th>
<th>Celosia</th>
<th>Mean of lining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick lining</td>
<td>Turgid</td>
<td>7.7</td>
<td>6.0</td>
<td>7.7</td>
<td>6.0</td>
<td>7.7</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>4.3</td>
<td></td>
<td>4.3</td>
<td></td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Thin lining</td>
<td>Turgid</td>
<td>7.0</td>
<td>5.7</td>
<td>7.0</td>
<td>5.5</td>
<td>7.3</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>4.3</td>
<td></td>
<td>4.0</td>
<td></td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Non-lining</td>
<td>Turgid</td>
<td>4.3</td>
<td>3.8</td>
<td>3.3</td>
<td>5.7</td>
<td>4.5</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>3.3</td>
<td></td>
<td>3.3</td>
<td></td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>LSD 0.05 LN x IM</td>
<td></td>
<td>1.19</td>
<td>0.84</td>
<td>1.33</td>
<td>0.94</td>
<td>0.94</td>
<td>0.66</td>
</tr>
<tr>
<td>Mean on initial moisture content of produce</td>
<td>Turgid</td>
<td>6.3</td>
<td></td>
<td>6.8</td>
<td></td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>4.0</td>
<td></td>
<td>3.9</td>
<td></td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td></td>
<td>0.68</td>
<td>0.77</td>
<td></td>
<td></td>
<td>0.54</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Effect of lining and initial moisture content of produce on length of time of storage (days) of two vegetables stored in vegetable basket.

<table>
<thead>
<tr>
<th>Lining (LN)</th>
<th>Initial moisture (IM) content of product</th>
<th>Okra</th>
<th>Mean of lining</th>
<th>Tomato</th>
<th>Mean of lining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick Lining</td>
<td>Turgid</td>
<td>3.7</td>
<td>3.5</td>
<td>5.0</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>3.3</td>
<td></td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Thin lining</td>
<td>Turgid</td>
<td>3.7</td>
<td>3.5</td>
<td>4.7</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>3.3</td>
<td></td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Non-lining</td>
<td>Turgid</td>
<td>3.0</td>
<td>2.8</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>2.7</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>LSD 0.05 LN x IM</td>
<td></td>
<td>1.19</td>
<td>NS</td>
<td>1.03</td>
<td>0.73</td>
</tr>
<tr>
<td>Mean on initial moisture content of produce</td>
<td>Turgid</td>
<td>3.4</td>
<td></td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Partially wilted</td>
<td>3.1</td>
<td></td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td></td>
<td>NS</td>
<td></td>
<td>0.59</td>
<td></td>
</tr>
</tbody>
</table>

had a highly significant effect on storage duration of the three leaf vegetables. Also lining x initial moisture content of the produce significantly influenced the length of time of storage of the three horticultural produce. For Telfairia, the longest storage time 7.7 days was produced in thick lining x turgid, followed by 7.0 days (Table 5) produced in thin lining x turgid. These two were significantly higher than the other combinations. The shortest storage time of 3.3 days (Table 5) occurred in non-lining x partially wilted. Turgid produce stored longer than the partially wilted in the Telfairia (Table 5).

For Amaranthus (Table 5) the longest time of storage (7.7 days) was produced in thick lining x turgid, and followed closely by 7.0 days in thin lining x turgid. As in Telfairia, these two were significantly higher than other lining x initial moisture treatment combinations. The shortest time of storage of 3.3 days (Table 5) was in non-lining x partially wilted. Turgid produce also has a longer-storage length than the partially wilted for Amaranthus.

In Celosia (Table 5), longest storage time (7.7 days) was in thick lining x turgid, followed by (7.3 days) in thin lining x turgid which did not differ significantly from each other. The shortest storage time of 3.7 days (Table 5) was produced in thin lining x partially wilted and it did not differ from other lining x partially wilted combinations. Thick lining consistently produced a longer storage time than thin lining and non-lining materials. On fruit vegetables (Table 6), lining had a significant effect on tomato but not in okra. Thick lining and thin lining produced similar time of storage in tomato (4.3 and 4.2 days) but longer than non-lining (3.0 days). Also turgid produce stored longer than the partially wilted in both vegetables, but was significant only in tomato. Lining x initial moisture content significantly affected the storage length in tomato but not in okra. The longest storage length of 3.7 days (Table 6) was produced in thick lining x turgid and thin lining x turgid in tomato. The least storage length was in non-lining x partially wilted in tomato storage (2.7 days). In okra the longest time of storage 5.0 days (Table 6) was produced in thick lining x turgid followed by (4.7) produced in thin lining, x turgid, the least time of storage was produced in non-lining at both levels.
of initial moisture. Turgid produce stored best at all the three lining levels.

**DISCUSSION**

Effects of lining and initial moisture content on physical qualities of produce

The result also showed that lining and initial moisture content significantly enhanced colour and freshness retention in leaf vegetables. A mean quality score of 5.00, which is very fresh with no trace of bleaching, was obtained in both colour and freshness in leaf vegetable. This result is similar to Nwufo et al. (1990), who reported that sealed leaves in polyethylene bag which was similar to the lining used in this study maintained their green fresh appearance at low temperature. Aworh (2011) observed that leaf vegetables packed with polyethylene and polypropylene recorded the lowest weight loss compared to those packed with paper materials. The result also agrees with Imonikebe (2013) who suggested that films or other materials suitable enough should be used for the lining of boxes used for the transportation of perishable produce in Australia.

The physical qualities of the fruit vegetables were considerably depressed. A mean quality colour and freshness score of 3.3 and 2.6 were obtained, respectively. This result is in disagreement with Babatola (1998) who reported that mature green tomatoes stored in the evaporative coolants system retained their colour and firmness. He obtained a quality scale of 4 indicating the fruit were very firm. The reason for the difference could be because of the difference in the age and ripeness of fruit used. Also, the high humidity favoured the growth of micro-organisms which intensified the deterioration of the ripe fruit vegetables used in this study. This is in agreement with Obetta et al. (2011) who reported that the benefits of storage humidity manipulations were not at the expense of losses due to decay by mycopathogens.

Effects of lining and initial moisture content on length of storage of produce stored in vegetable basket

The result shows that leaf produce stored in the vegetable basket lasted longer than those stored on the laboratory bench. This is in agreement with the report of Amrat et al. (2013) who reported that evaporative coolant system is appropriate for storage if many tropical leaf vegetables. This is in disagreement to Babatola (1998) who in his earlier work on shelf-life extension of green tomato fruits using evaporative coolant system reported that ECS gave the best quality followed by refrigerator and open shelf in terms of colour, firmness, weight loss and day to ripening. The disagreement could be because the fruit vegetables used in this study were already ripe, and storage in evaporative coolant could not stop further deterioration. Rather, fruit vegetables stored on laboratory benches lasted longer than those stored in the evaporative coolants. This could be as a result of lower humidity on the laboratory bench compared with higher humidity in evaporative coolants which enhances deterioration of already turgid fruit vegetables.

**Conclusion and recommendation**

Post-harvest preservation of fruits and vegetables is a sure way of achieving sustainability in global food supplies; increased production will do little to alleviate poverty and over-come distribution problem that already plague the world food supplies without adequate postharvest preservation. To ensure adequate storage and shelf-life extension of fruits and vegetable, least expensive but adequate and easily affordable technology such as vegetable basket is indispensable.

**REFERENCES**


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Full Length Research Paper

The effect of mutagens on M1 population of black gram 
(Vigna mungo L. Hepper)

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This study was carried out with black gram (urdbean) variety TNAUCo(Bg)6 to determine the effects of gamma rays (150, 200, 250, 300 and 350 Gy) and ethyl methane sulphonate (10, 15, 20, 25, 30 mM). Data collected were on seed germination and survival, pollen and seed fertility, plant height, number of primary branches, number of clusters per plant, number of pods per plant, pod length, number of seeds per plant, hundred seed weight and yield per plant in M1 generation. Thereafter, progressive reduction in germination and survival percentage, pollen and seed fertility and yield related traits were observed in the mutagenic treatments. Deleterious effects were more pronounced in higher doses, indicating almost a linear relationship. LD50 values of 41.30 and 43.50% were observed in 20 mM of ethyl methane sulphonate and 250 Gy of gamma rays, respectively. The increasing doses of gamma rays and ethyl methane sulphonate decreased in phenotypic and yield related parameters. The reduction in quantity and yield traits has been attributed to the physiological disturbance or chromosomal damage of the cells of the plant caused by the mutagens. Ethyl methane sulphonate was observed to be more effective than gamma rays as it generated more number of mutants, which later caused higher physical injury.

Key words: Vigna mungo, variety TNAUCo(Bg)6, gamma rays, ethyl methane sulphonate (EMS).

INTRODUCTION

Black gram (Vigna mungo (L.) Hepper) is a rich source of protein (20.8 to 30.5%); its total carbohydrates range from 56.5 to 63.7%. It is also a good source of phosphoric acid and calcium. It contains a wide variety of nutrients and is popular for its fermenting action and thus it is largely used in making fermented foods. It is an important pulse crop occupying a unique position in Indian agriculture. It covers an area of about 3.24 million hectares and produces 1.46 million tonnes. Its productivity is only 526 kg per ha. In Tamil Nadu, black gram covers an area of about 3.41 lakh hectares with production of 1.21 lakh tonnes and 355 kg per ha.

Jayamani et al. (2012) and Indian Institute of Pulses Research (2011) report that about 70% of the total area is in the central and southern parts of the country, and this contributes about more than 77% of the total production. But the national productivity of black gram is around 500 kg per ha due to restricted cultivation in the marginal lands, lack of genetic variability and the absence of suitable ideotypes for different cropping systems. These cause poor harvest index and susceptibility to pests and diseases (Pawar, 2001; Banu, 2005). There is paucity of research on this species compared to cereals and other legumes.

In order to improve yield and other polygenic characters, mutation breeding should be effectively utilized (Deepalakshmi and Anandakumar, 2004). Mutation induction has become an established tool in plant breeding to supplement existing germplasm and improve cultivars in certain specific traits (Kurobane et al., 1979).

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Induced mutations represent the same kind of changes that occur from natural causes (Govindan 2000). Mutagenesis has been widely used as a potent method of enhancing variability for crop improvement (Singh and Singh, 2001). Induced mutation, using physical and chemical mutagen, is a way to generate genetic variation, resulting in the creation of new varieties with better characteristic (Wongpiyasatid, 2000). Gamma rays are the most energetic form of electromagnetic radiation; their energy level is from ten to several hundred kilo electron volts and they are considered as the most penetrating compared to other radiations (Kovacs et al., 2002). Therefore, an attempt has been made to study their effects in this direction.

MATERIALS AND METHODS

Dry, healthy and uniform sized seeds of black gram variety TNAUCo(Bg)6 were treated with gamma rays at 150, 200, 250, 300 and 350 Gy doses and ethyl methane sulphonate at 10, 15, 20, 25 and 30 mM concentrations. Five hundred (500) seeds were pre-soaked for 6 h in water initially (Malarkodi, 2008). Then, the seeds were immersed for 6 h in the requisite concentration of mutagen ethyl methane sulphonate with intermittent shaking. To ensure a uniform absorption of the mutagen, the volume of mutagen solution was maintained at 10 times proportion to that of the seed volume. The whole treatment was carried out at a room temperature of 28±1°C for 4 h after washing in running water and untreated seeds were used as control. The treated seeds of gamma rays, ethyl methane sulphonate and control seeds were immediately sown in the field in a randomized block design (RBD) with three replications. Each treatment consists of three rows of 5 m length, in which 50 seeds per row were sown with 10 x 30 cm distance between plants and rows, respectively. Data were recorded on 11 quantitative characters and further statistically analyzed. Mean values for the 11 quantitative traits in different treatments and percentage over control are presented.

RESULTS AND DISCUSSION

Germination percentage was significantly reduced in all the gamma ray and ethyl methane sulphonate treatments presented in Table 1. The 50% reduction of germination was recorded at 250 Gy of gamma rays (43.50%) and 20 mM of ethyl methane sulphonate (41.30%). It indicated that germination percentage was reduced under the influence of mutagenic treatment with increasing doses per concentrations. Similar results were reported in red gram by Jayanthi (1986), in winged bean by Veeresh et al. (1995) and in blackgram by Thilagavathi and Mullainathan (2011). The significant survival reduction was observed in the higher dose / concentration of gamma rays 350 Gy (19.99) and ethyl methane sulphphonate (30 mM, 6.58). This might have been due to the effect of mutagens on meristematic tissues of the seed. Morphological variations, especially leaf abnormalities are the indicators of effective mutagen treatment. In different treatments, morphological variations like trifoliolate, tetrafoliolate, pentafoliolate, hexafoliata and fused leaves were observed in the present investigation. Plant height was also found to be significantly reduced in higher doses of physical and chemical mutagenic treatments. The maximum plant height reduction was observed in 350 Gy of gamma rays (3.00 cm) and 30 mM (2.00 cm) (Figure 1). Pollen fertility and seed fertility percentage among all the mutagenic treatments showed gradual decrease with respect to the increase in concentrations. In the current findings, the increase in pollen sterility as a consequence of mutagenesis is in accordance with the findings of Ignacimuthu and Babu (1989) on wild and cultivated Urd and mungbeans. In most cases, meiotic abnormalities are responsible for pollen sterility (Mathusamy and Jayabal, 2002) in cotton and chickpea (Khan and Wani, 2005). In addition to chromosomal aberrations, some genetic and physiological changes might have caused pollen sterility. The number of primary branches per plant was also significantly affected by 350 Gy of gamma rays (1.08) and 30 mM of ethyl methane sulphonate (1.13) treatments (Figure 1). Number of pods per plant and pod length also reduced in increasing doses in Table 2. The number of cluster per plant was also significantly affected in 350 Gy of gamma rays (3.00) and 30 mM of ethyl methane sulphonate (2.00) treatments (Figure 1). Similar results were reported in the quantitative parameters such as number of primary branches per plant, number of cluster per plant, number of pods per plant, pod length, number of seeds per pod and plant yield per ha; they all decreased in gamma rays and ethyl methane sulphonate treatment than in control in M1 generation of Vigna mungo (Thilagavathi and Mullainathan, 2011) and Vigna unguiculata (Mensah and Akomeah, 1992; Rizwana Banu, 2005).

However, plant height at 60th day, number of seed per plant and 100 seed weight per plant in different treatments indicated a significant reduction in the higher doses of physical and chemical treatment. Percentage reduction in seed weight was maximum (5.00 g) in 150 Gy of gamma rays and 10 mM of ethyl methane sulphonate treatment (4.30 g). A maximum seed yield of 10.00 g per plant was observed in control. There was significant reduction in pollen fertility, seed fertility, hundred seed weight and seed yield was non-significant in all the treatments. In the present study, reduction in seed germination and germination percentage was concentration/dose dependent and linear. Reduction of germination in mutagenic treatments is due to delay or inhibition of physiological and biological processes necessary for seed germination; they include enzyme activity (Kurobane et al., 2002). The treatments showing maximum variation in quantitative traits may show stable gene mutations in subsequent generations.

Conclusion

All the quantitative and yield traits were proportionately
Table 1. Impact of gamma ray and ethyl methane sulphonate treatment on growth characters in black gram.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination percentage</th>
<th>Survival percentage</th>
<th>Plant height at 30th Day</th>
<th>Pollen fertility percentage</th>
<th>Seed fertility percentage</th>
<th>Number of primary branches per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gamma ray (Gy)</strong></td>
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Table 2. Impact of gamma rays and ethyl methane sulphonate treatment on pod and yield characters in black gram.

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<th>Number of cluster per plant</th>
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<th>Hundred seed weight (g)</th>
<th>Seed yield per plant g</th>
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Figure 1. Comparison effect of gamma rays and EMS mutagenesis on (A) germination percentage, (B) Survival percentage (C) plant height, (D) pollen fertility (E) Seed fertility percentage (F) Number of primary branches per plant (G) Number of pods per plant (H) pod length (I) Number of cluster per plant (J) Number of seed per pod (K) Hundred seed weight, (L) seed yield per plant.
Figure 1. Contd.

The decrease in quantitative characters has been attributed to the physiological disturbance or chromosomal damage of the cells of the plant caused by the mutagens. Gamma rays belong to ionizing radiation and interact with atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the irradiation.
level. Chemical mutagens usually cause point mutation, but the loss of a chromosome segment or deletion can also occur.

Studies show that the most important parameters for inducing physical and chemical mutagen growth and yield characters were reduced based on dose per concentration and duration of treatment. In the present study, it was observed that the gamma ray irradiation and ethyl methane sulphonate affect Urdbean. We observed morphological changes such as stunted plants, reduction of the plant height and yield parameters.

REFERENCES


In vitro cytotoxic study for partially purified L-asparaginase from fresh leaves, unripe and ripe fruits of *Withania somnifera* plant

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¹Biotechnology Research Center, Al-Nahrain University, Iraq.
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This work aimed to study the cytotoxic effect of L-asparaginase isolated from local *Withania somnifera* plant on lymphocyte leukemia cells. To achieve this goal, L-asparaginase was purified from *W. somnifera* fruits by two purification steps, ion-exchange chromatography using DEAE-cellulose and gel filtration chromatography using Sephadex G-150, and the study utilized an *in vitro* evaluation for the cytotoxic effect of the partially purified L-asparaginase with concentrations ranging (12.5 to 100 μg/ml) in a two fold serial dilutions on some cell suspension culture including, acute lymphocyte leukemia and chronic lymphocyte leukemia culture at different concentrations (12.5 to 100 μg/ml) and different exposure time of treatment (24, 48 and 72 h). This two purification steps raised the specific activity from 1.73 U/mg in crude extract to 2.29 U/mg after ion-exchange and 10.5 U/mg after gel filtration; the purification fold was 1.32 after ion-exchange and 6.06 after gel filtration, the enzyme recovery was 56% after two purification steps and the results, pointed that acute lymphocyte leukemia culture showed highest sensitivity toward the cytotoxic effect (62.3±0.9%) of the partial purified L-asparaginase (100 μg/ml) than other culture after 48 h in a dose dependent manner, and highest cytotoxic inhibitory effect (73.2±1.6%) after 48 h of exposure on chronic lymphocyte leukemia culture, while healthy lymphocyte culture showed novel behavior. The lowest concentration of cell treatment gave the most significant (P<0.01) inhibitory effect. The conclusion is that there is enough evidence to support the claim that L-asparaginase from *W. somnifera* may be considered chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma.

Key words: Acute lymphocyte leukemia (ALL), chronic lymphocyte leukemia (CLL), L-asparaginase, cytotoxic assay.

INTRODUCTION

Leukemia is a cancer originating in any of hematopoietic cell that tends to proliferate as single cells within bone marrow and often circulate in the blood stream. Lymphocytic leukemia’s are derived from B or T cell precursors. Four types of leukemia are classified, Chronic Lymphocytic Leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML). The development of a malignant cell clone is due to the dysregulation of the balance between cell proliferation and the programmed cell death apoptosis (Conter et al., 2005). L-asparaginase is an enzyme that destroys asparagines external to the cell.

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Normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. The enzyme converts asparagine in the blood into aspartic acid by a deamination reaction; the leukemia cells are thus deprived of their supply of asparagine and will die (Grossman et al., 2004). Leukaemic cells lacking the mammalian asparagine-synthetase enzyme depend on exogenous sources of asparagine for protein synthesis and survival. Theoretically, the deamination of serum asparagines selectively kills leukaemic cells, leaving normal cells, which have the ability to synthesize asparagine intracellularly, unaffected (Broome, 1968). L-Asparaginase is known as a chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma, which is used mainly in the treatment of children (Borek and Jaskolski, 2001).

The current study was aimed at the in vitro cytotoxic study of the partial purified L-asparaginase on different types of suspension cell lines at different concentrations and exposure times.

MATERIALS AND METHODS

Sample collection of plant

The fresh leaves, unripe and ripe fruits of W. somnifera plant collected from the garden of plants in Baghdad University were included in this study. The plant parts were cleaned from the dust and other particles and stored in the freezer until use.

Extraction of L-asparaginase from plant tissues

After cleaning the plant tissues with distilled water, the plant tissues (leaves, unripe fruits and ripe fruits) were homogenized and approximately 3 g from each sample were ground with 2 m² of potassium phosphate buffer 0.1 M (pH 8.6) in a pestle and mortar, left on magnetic stirrer for 10 min, the extract was filtered to rid the cell debris, centrifuged at 12000 rpm for 10 min and the supernatant was taken to determine the L-asparaginase activity and protein concentration as explained in Ren et al. (2010) and Bradford (1976).

Extraction of L-asparaginase using liquid nitrogen

The plant tissues (leaves, unripe fruits and ripe fruits) were homogenized in liquid nitrogen, as the same as aforementioned.

Determination of L-asparaginase activity

0.5 ml of enzyme crude extract, 0.5 ml of 50 mM asparagine and 1 ml potassium phosphate buffer (0.02 M and pH 8.6) were mixed well, the mixture was incubated in water bath at 37°C for 15 min, after the incubation, 1 ml of 1.5 M trichloroacetic acid was added to the mixture to stop the reaction, then centrifuged at 12000 rpm for 10 min and the supernatant was collected (Ren et al., 2010). After that, the supernatant was transported to clear test tubes to determine the concentration of ammonia liberated from the enzyme action by the method of direct nesslerization, the concentration of ammonia was estimated for each sample by mixing 4 ml of distilled water with 0.5 ml of sample to be estimated and 0.5 ml of Nessler's reagent, the mixing was shaken well, incubated at 37°C for 15 min, and the absorbance was measured at wave length (450 nm). The blank was prepared by mixing 4.5 ml distilled water with 0.5 ml Nessler's reagent.

Purification of L-asparaginase

Preparation of ion exchange column (DEAE-cellulose)

The DEAE-cellulose column was prepared according to the method suggested by Whitaker (1972), the resin was packaged gently in glass column, the dimensions of resin was 1 x 24 cm, and the equilibration was achieved by the same potassium phosphate buffer at flow rate approximately 30 ml/h to next day.

Separation through ion exchange resin (DEAE-cellulose)

10 ml from enzyme crude extract was loaded onto ion exchange column, the separated fractions was collected at flow rate of 30 ml/h approximately (2 ml for each fraction), the wash was achieved by using potassium phosphate buffer, the same buffer used in equilibration, and the elution was achieved by the same buffer used in equilibrium with graduated concentrations of potassium chloride (0.1, 0.05, 0.02 and 0.01 M) respectively; the flow rate of elution was 30 ml/h too, the protein concentration of the fractions was measured at wave length 280 nm and enzymatic activity was estimated for fractions as in Ren et al. (2010) and the fractions which give higher activity was collected, lyophilized (freeze dried), and stored in the freezer until use.

Gel filtration chromatography

Preparation of sephadex G-150 column

Preparation of gel was achieved as recommended by the supply company (Sigma, USA). 5 g from gel sephadex G-150 was suspended in 1 L Tris-HCl buffer (0.1 M) and pH 8.6, then the suspension was left in water bath at 90°C for 5 h to ensure the swelling of gel beads with gentle agitation from time to time, after that, the gel was transferred to graduated cylinder, left to stagnate for 20 min, then the supernatant was removed, the gel was resuspended in 600 ml of Tris-HCl buffer, then the gel was degassed by using vacuum, then, packaged gently in glass column with dimensions (1 x 28) cm. The column was equilibrated using same buffer used in gel suspension at flow rate of 20 ml/h approximately until the next day.

Separation through sephadex G-150 column

The lyophilized extract produced from ion exchange step was suspended in 5 ml Tris-HCl buffer, the suspension was added gently on the surface of gel, the elution was done by using the same buffer (Tris-HCl) at flow rate of 20 ml/h (2 ml for each fraction), the protein concentrations for fractions was measured at wave length of 280 nm, the enzyme activity was estimated according to method mentioned in Ren et al. (2010), then the fractions with higher enzyme activity was collected, lyophilized and stored in freeze for other steps.

Preparation of L-asparaginase dilutions

Pure L-asparaginase stock solution was prepared by dissolving 5 m with 1 ml PBS, then filtered through millipore 0.22 μm filter and stored.
at -20°C until used. Serial dilutions were made starting from concentration 100, 50, 25 and 12.5 µg/ml. The dilutions were done in a sterile laminar cabinet using a sterile PBS and sterile stoppard tubes.

Sample collection of blood

5 ml of blood was collected by vein puncture from 10 (ALL and CLL) cases for each, and 5 healthy persons (control) who were admitted to the National Center of Hematology Diseases / Al Mustanisery University. The patients were diagnosed by the consultant medical staff at the centre.

Isolation of lymphocytes

Preparation of solutions and media were done according to the methods described by Marlise (1997) and Bottran and Vetvicka (2001). The lymphocytes were isolated from the peripheral heparinized whole blood as follows: 3 ml of blood was centrifuged at 1000 rpm for 15 min, buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5 ml RPMI 1640 (cell suspension). 5 ml of the diluted cell suspension was layered on 3 ml of ficoll-isopaque separation fluid, and the tubes were centrifuged at 2000 rpm for 30 min in a cooled centrifuge at 4°C. After centrifugation, the mononuclear cells were visible as cloudy band between the RPMI1640 and lymphoprep layers. The band was collected in a 10 ml test tube and the cells were suspended in 5 ml RPMI 1640. The tube was centrifuged at 2000 rpm for 5 min, the supernatant was discarded and the cells were resuspended in 5 ml RPMI 1640 (repeated twice). The suspension was centrifuged at 1000 rpm for 10 min, and the supernatant was discarded. The precipitated cells were resuspended in 1 ml RPMI media Boyum (1968) and viable count were performed (Porakishili et al., 2004), the numbers of lymphocytes were counted by improved neuber chamber and the cells concentration was adjusted to 1 × 10⁶ cell/ml. The isolated cells were grown in a flask containing 10 ml RPMI 1640 medium supplemented with bovine serum albumin (BSA) and incubated at 37°C for 48 h in CO₂ incubator (Boyum, 1968).

3 (dimethylthiazol-2-yl)-2,5- (diphenyltetrazoliumbromide) MTT

A 50 mg MTT dye was dissolved in 100 ml distilled water. The solution was filtered through 0.22 µm millipore filter to remove any blue formazan product and stored in a sterile, dark screw-capped bottles at 4°C.

Cytotoxicity assay

It is also called a cell growth inhibition assay. The in vitro method was used to investigate the effect of pure L-asparaginase on two types of leukemia culture (ALL- acute lymphocyte leukemia; CLL- chronic lymphocyte leukemia) at different concentrations (100, 50, 25 and 12.5 µg/ml) and exposure times (24, 48 and 72 h) (Masters, 2000). Using a microtitration plate (96 wells) and cell culture technique, 4 × 10⁵ cells/ml were exposed to serial dilutions of L-asparaginase in the concentrations range mentioned earlier. At the end of the exposure times, 10 µl of MTT solution was added to all wells and incubated for 4 h at 37°C. During this period, formazan crystals were formed at the bottom of each well. The spent medium was pipetted out along with suspension of culture and 100 µl of acid isopropanol (100 µl of 0.04N HCl in anhydrous isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals and incubated in shaker at room temperature for 30 min. The plates of different cell culture at the end of the assay were examined by ELISA reader at 540 nm transmitting wave length (Skehan et al., 1990; Mosmann, 1983). The inhibition was calculated according to the following equation:

\[
\text{% inhibition} = \frac{100 - \frac{\text{Correct sample}}{\text{Correct solvent}} \times 100}{\text{Correct sample}}
\]

Correct sample = Growth cell GC – negative control, correct solvent = solvent control (SC) – negative control, RPMI 1640 + PBS (negative control), RPMI 1640 + cell [cell growth control (GC)], RPMI 1640 + cell + PBS [solvent control (SC)] (Bottran and Vetvicka, 2001).

Statistical analysis

The statistical analysis is a very important final step in the research to analyse and evaluate the obtained results. Medical statistics of this study was conducted via computer based statistical program which was: 1) SPSS for windows computer package (programmer 11.5; 2) Microsoft excel 2003.

The statistical analysis system (SAS, 2004) was applied for all results to show effect of different concentration and other factors in studied parameters. The least significant difference (LSD) test and Duncan test are the comparative between means in this study. It is a well used test for the medical statistics; P value <0.01 is considered a significant correlation.

RESULTS AND DISCUSSION

Cytotoxic effect growth inhibitory assays of the partial purified L-asparaginase

L-asparaginase has been purified from W. somnifera fruits by two purification steps, ion-exchange chromatography using DEAE-cellulose and gel filtration chromatography using sephadex G-150; using these two steps, the specific activity was raised from 1.73 U/mg in crude extract to 2.29 U/mg after ion-exchange and 10.5 U/mg after gel filtration; the purification fold was 1.32 after ion-exchange and 6.06 after gel filtration; the enzyme recovery was 56% after using the two purification steps. Three cell lines were studied (ALL, CLL, Healthy) at three times of exposure (24, 48 and 72 h), using a two fold dilutions to get concentration from 100 to 12.5 µg /ml of partial purified L-asparaginase. The cytotoxic effect on acute lymphocyte leukemia culture (Table 1) revealed that the high concentrations gave a significantly (P<0.01) high inhibition rate of cells while being low gradually with low concentrations. After 48 h of exposure, a high cytotoxic inhibitory effect (5%) of the extract was started at the concentration (100 µg/ml). The extracted partial purified L-asparaginase was very sensitive and liable through many environmental changes even with suitable protection which led to the best results at 48 h of exposure than after 72 h of cells treatments. This might explain the more significant inhibition rate at different concentration for 48 h than 72 h of exposure.

Table 1 shows the results of the significant effect at P < 0.01 level after 48 h of exposure. The highest concentration
Table 1. The cytotoxic effect of extract pure L-asparaginase on three types of suspension leukemia culture (at 24, 48 and 72 h) of exposure.

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<td>51.1 ± 2.7b</td>
<td>-17.5 ± 1.1c</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>6.8 ± 0.5d</td>
<td>48.4 ± 0.6c</td>
<td>-39.6 ± 0.5d</td>
</tr>
<tr>
<td>Healthy</td>
<td>100</td>
<td>2.6 ± 0.7a</td>
<td>2.9 ± 0.5a</td>
<td>2.9 ± 0.5a</td>
</tr>
<tr>
<td></td>
<td>50</td>
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<td>2.3 ± 0.2a</td>
<td>-10.1 ± 0.09b</td>
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<td>25</td>
<td>2.2 ± 0.04a</td>
<td>2.7 ± 0.3a</td>
<td>-13.3 ± 0.2b</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1.8 ± 0.6a</td>
<td>2.2 ± 0.4a</td>
<td>-16.9 ± 0.3b</td>
</tr>
</tbody>
</table>

Mean having different small letters (a, b and c) at the same column are significantly different at P < 0.01.

(100 µg/ml) of L-asparaginase, showed the highest cytotoxic inhibitory effect (62.3 ± 0.9%); the inhibitory effect decreased (33.6 ± 0.6, 20.1 ± 0.2%) after 24 and 72 h of exposure respectively on acute lymphocyte leukemia culture and highest cytotoxic inhibitory effect (73.2 ± 1.6%) after 48 h of exposure on chronic lymphocyte leukemia culture. The L-asparaginase was an antiproliferative effect without a significant inhibitory effect (2.6 ± 0.7, 2.5 ± 0.3, 2.2 ± 0.04 and 1.8 ± 0.6%) at concentrations (100, 50, 25 and 12.5 µg/ml) respectively; after 24 h of exposure, healthy lymphocyte culture and the cytotoxic inhibitory effect decrease to reach -16.9 ± 0.3% after 72 h of exposure for 12.5 µg/ml concentration then reached (2.9 ± 0.5, 2.3 ± 0.2, 2.7 ± 0.3 and 2.2 ± 0.4%) respectively for all concentration in descending manner respectively for 48 h of exposure. Among the three types of cell suspension (ALL, CLL and Healthy) at all concentration and different intervals of exposure (24, 48 and 72 h), the extracted purified L-asparaginase had the best efficiency in inhibiting CLL culture within (100 µg/ml) 48 h which was about 73.2 ± 1.6%.

Oza et al. (2009) showed the enzyme was purified and characterized from W. somnifera, a popular medicinal plant in South East Asia and Southern Europe and a traditionally used Indian medicinal plant (Oza et al., 2009). L-asparaginase (E.C. 3.5.1.1) is used as a therapeutic agent in the treatment of acute childhood lymphoblastic leukemia (Oza et al., 2011). L-asparaginase is a known chemotherapeutic agents against cancer, such as acute lymphoblastic leukemia and lymphosarcoma. Several recent reviews are available concerning the use of L-asparaginase in cancer therapy (Muller and Boos, 1998; Oza et al., 2011). Leukaemic cells lacking the mammalian asparagine-synthetase enzyme depend on exogenous sources of asparagine for protein synthesis and survival. Theoretically, the deamination of serum asparagines selectively kills leukaemic cells, leaving normal cells, which have the ability to synthesise asparagine intracellularly and unaffected (Broome, 1968).

Asparaginase is an enzyme which produces its anticancer effects by “breaking down” asparagine, a substance normally found in the body that is involved in biological processes essential for cells to maintain life. Healthy cells are able to create asparagine for themselves; however, cancer cells are not able to create asparagine. Therefore, the depletion of asparagine by asparaginase kills cancer cells, while healthy cells are not as affected (Muller and Boos, 1998; Chumchalova and Smarda, 2003).

Conclusions

L-asparaginase purified from W. somnifera fruits may be considered as a chemotherapeutic agent against acute lymphoblastic leukemia and chronic lymphoblastic leukemia culture.

REFERENCES


Boyum A (1968). Separation of lymphocyte from blood and bone
Function of \textit{VtPGIP} in pathogenic fungus resistance of \textit{Vitis thunbergii}

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In plants, polygalacturonase inhibitor proteins (PGIPs) are very important to inactivate polygalacturonases secreted by pathogens. \textit{Vitis thunbergii} Sieb. et Zucc. polygalacturonase inhibitor proteins (\textit{VtPGIP}) was first isolated from the wild grape \textit{Vitis thunbergii} Sieb. et Zucc., which exhibits high resistance to disease. \textit{VtPGIP} is sublocalized in the plant cell wall, and this location is consistent with the function of PGIPs in the first line of host defense. The promoter of \textit{VtPGIP} contains salicylic acid (SA), abscisic acid (ABA), and fungus infection response elements. Results from real-time quantitative reverse transcriptase (RT)-PCR analysis showed that \textit{VtPGIP} expression was induced by SA, ABA, and fungi. The results indicated that \textit{VtPGIP} may have important functions in defense-related responses of \textit{V. thunbergii} against pathogenic fungi.

Key words: PGIP, disease resistance, pathogenic fungi, \textit{Vitis thunbergii}.

INTRODUCTION

Epiphytes cause significant losses as destructive pathogens of many fruits and vegetables worldwide; such organisms particularly affect the post-harvest industry. The main methods used to control fungal diseases include field and equipment sanitation, crop rotation, soil fumigation (Wheeler et al., 1994), and fungicide application; however, fungicides are deleterious to the environment. Other strategies are more promising and have been shown to decrease the incidence of fungal diseases by enhancing natural plant defense capabilities (Kessmann et al., 1994). A very important aspect is to determine potential molecules that exhibit defense functions in plants, that is, the natural defense system, and the molecules that respond to induction.

Fungal disease is caused by the secretion of hydrolytic enzymes to pectin substrates (Fish, 2005). Pathogens can release several types of enzymes, such as exo-polygalacturonases and endopolygalacturonases (endo-PGs), to breach this barrier and function with pectin methyl and acetyl esterases (Prade et al., 1999) to degrade pectin. Fungal endo-PGs, the first enzymes secreted by fungal plant pathogens, have important functions during the early stages of plant pathogenesis (English et al., 1971) to separate and macerate host tissues, thereby facilitating pathogen penetration and colonization of plant tissues. Subsequently, the products of this degradation process are used as a nutrient source for fungal growth (Karr et al., 1970).

Polygalacturonase-inhibiting proteins (PGIPs) are basic proteins present in the cell walls of most plants; PGIPs

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Abbreviations: SA, Salicylic acid; ABA, abscisic acid; PGIP, polygalacturonase inhibitor protein; PG, polygalacturonase. \textit{VtPGIP}, \textit{Vitis thunbergii} Sieb. et Zucc. PGIP.
are specific, saturable, reversible, and high-affinity ‘receptor’ endo-PGs of fungi (Cervone et al., 1987, 1990, 1989). PGIPs can directly impede pathogen invasion by inactivating polygalacturonases (PGs) (Hagedus et al., 2008). The inhibition of PG activities by PGIPs has been proposed to prolong the accumulation of oligogalacturonic acids and improve defense response (Federici et al., 2008). For instance, PGIPs of Pyrus communis (Tamura et al., 2004) and Gossypium hirsutum (James et al., 2001) inhibit fungal PG activity. However, field trials with the pear PGIP-expressing tomato lines provide contradicting results of the functions of PGIP in resistance. Hence, PGIPs from different plants vary in terms of inhibitory activity; PGIPs from a single plant also inhibit PGs from different fungi or different PGs from the same fungus with various strengths (Desiderio et al., 1997). For instance, a PG from Aspergillus niger is inhibited by PvPGIP1 and PvPGIP2 (Leckie et al., 1999) but not by PvPGIP3 and PvPGIP4 (D’Ovidio et al., 2004). Fusarium moniliforme PG is inhibited only by PvPGIP2 (Leckie et al., 1999). Hence, plants have evolved PGIPs with different recognition capabilities to counteract numerous PGs secreted by pathogenic fungi. Arabidopsis thaliana possesses two PGIP genes, which function in response to Botrytis cinerea infection; however, Arabidopsis PGIPs cannot inhibit PGs produced by F. moniliforme and A. niger; by comparison, Arabidopsis PGIPs can inhibit PGs produced by Colletotrichum gloeosporioides, Stenocarpella maydis, and B. cinerea (Ferrari et al., 2003).

Vitis thunbergii Sieb. et Zucc. (V. thunbergii) is native to China and grown throughout the central and southern parts of China as well as in Korea and Japan. V. thunbergii has a strong resistance to Coniothyrium diploidiella, Glomerella cirtula, and other disease-causing pathogens. In addition, the plant hormones salicylic acid (SA) and abscisic acid (ABA) have been implicated in various plant responses (Rao et al., 2000; Borsani et al., 2001; Turner et al., 2002; Xiong et al., 2002). The cloned VtPGIP promoter sequence contains SA and ABA-related elements components and allows the study of the expression and regulation of VtPGIP by the corresponding SA or ABA treatment. In this study, VtPGIP was characterized to determine whether or not the PGIP gene in V. thunbergii is responsible for disease resistance. We also described VtPGIP expression in response to applied SA, ABA, and fungal infection. VtPGIP may have important functions in the disease resistance of V. thunbergii.

MATERIALS AND METHODS

Plant materials and growth conditions

V. thunbergii plantlets were grown at 25°C under a 16 h/8 h (day/night) photoperiod by tissue culture. The plantlets were cultivated in the medium of 3/4 MS supplemented with 0.35 mg L⁻¹ indole-3-butyric acid.

Plant treatments

The leaves of seven-week-old intact tissue culture plantlets were selected to examine the effect of exposure to different defense response activators (signaling molecules). SA (Sigma, St. Louis) and ABA (Sigma, St. Louis) were dissolved in water, and the aerial parts of the plants were sprayed with 5 mM SA (Ferrari et al., 2003) or 50 mM ABA (Yuasa et al., 2007). The plants of the same age and treated with distilled water were used as control plants. VtPGIP expression on the leaves of tissue culture plantlets was analyzed at 0, 2, 6, 16, 24, 48, and 72 h after ABA and SA treatments. After harvest, the leaves were immediately frozen in liquid nitrogen and stored at -70°C for subsequent RNA extraction.

The leaves of V. thunbergii tissue culture plantlets were inoculated with pathogenic fungi (C. diploidiella, Erysiphe cichoracearum, G. cirtula, and B. cinerea). The plantlets of the same age and treated with water were used as control plants. After 3 d, the leaves treated with pathogenic fungi and water were harvested, immediately frozen in liquid nitrogen, and stored at -70°C for subsequent RNA extraction.

Isolating the VtPGIP gene

Genomic plant DNA was isolated using the CTAB extraction method described in a previous study (Chang et al., 1993). Total RNA was extracted by scaling down the experimental conditions previously described (Chang et al., 1993). Plant leaves (200 mg) were ground in liquid nitrogen to a fine powder by using mortar and pestle. Approximately, 900 μl of extraction buffer was added and the mixture was mixed thoroughly. The mixture was extracted with 900 μl of chloroform/isooamyl alcohol (24:1 v/v). The upper phase was transferred to a fresh tube, and the hydroxybenzene/chloroform/isooamyl alcohol (25: 24:1 v/v/v) were added. The mixture was vortexed vigorously and centrifuged at 12,000 rpm for 10 min at 4°C. The clear supernatant was carefully transferred to a clean tube and extracted again with the chloroform/isooamyl alcohol (24:1 v/v). The RNA was precipitated with 10 M LiCl for 8 h at -20°C, washed with 70% cold ethanol, air dried for 20 min, and resuspended RNA precipitate in 30 μl DEPC-treated ddiH₂O. CDNA was synthesized using the ReverTra Ace-a-TM kit (ToYoBo, Osaka, Japan) according to the manufacturer's instructions.

PCR was carried out in a 25 μl reaction volume containing 10× buffer, MgCl₂, dNTPs, primers, 50 to 100 ng of template DNA or cDNA, and 1 unit of Taq DNA polymerase using the following PCR profile: 4 min at 94°C; 38 cycles of 1 min at 94°C; 2 min at 54°C; 2 min at 72°C; final extension step of 72°C for 10 min. The gene primers [Pgfp F: 5’-ATGGAGACTTCAAAGCTTTTTCC-3’ (forward) and Pgip R: 5’-TCACTGTGCGACTCGGAGTGGG-3’ (reverse)] were used to design primers for sequencing of V. vinifera (Pinot noir, PN40024). The obtained amino acid sequence was aligned with related genes by using BioXM, and the alignment was analyzed using PROSITE (http://ca.expasy.org/prosite/). The three-dimensional structure of the VtPGIP protein and molecular modeling were analyzed using Swiss-Pdb Viewer 3.7.

Cloning of the VtPGIP promoter

The primers [F1: 5′-TGGGGAGGCTCTACCAGGA-3′ (forward) and Q1: 5′-GGACGAGTGGCGAGTAAAGTAGGAGG-3′ (reverse)] were designed based on the sequencing of Vitis (Pinot noir, PN40024). The promoter of the PGIP gene sequence was cloned by PCR. PCR was performed using genomic DNA extracted from V. thunbergii leaves with the primers p-pgip-F1/Q1. A 36-cycle PCR was conducted (one cycle was performed under the following
Figure 1. A phylogenetic tree of PGIPs from plants. In addition to the predicted proteins soybean, pear, and other PGIP used for phylogenetic analysis include the PePGIP(AAWS57430), PmPGIP(AAF79181), EgPGIP(AAR15145), PpPGIP(AAP92913), MaPGIP(ABA26937), GmPGIP(CAI99394), VvPGIP(AAM74142), VtPGIP(ABU82741), ChPGIP(BAB85785), CnPGIP(BAB85784), CjPGIP(BAA28745), CuPGIP(BAA31841), CnPGIP(BAA31842), CaPGIP(ACB30360), MpPGIP(AFC95832), PpPGIP(AEO36938), PsPGIP(ACY41032), PfPGIP(ACY41031), HaPGIP(ABW89527), VcPGIP(BAA43471), SpPGIP(AAK43459), RpPGIP(AAC43455), NhPGIP(AAK43462). They were clustered using Clustal W2 and generated a plasmid that enabled the expression of VtPGIP protein, we found that some PGIPs from plants (Figure 1). Using sequence comparison data, we found that the species of the same genus were classified into the same group except Eucalyptus grandis, Pyrus communis, and Malus pumila because the PGIPs of these plants may have different evolutionary scenarios.

Subcellular localization of the VtPGIP protein

To determine the subcellular distribution of the VtPGIP protein, we generated a plasmid that enabled the expression of VtPGIP fused to EGFP. This plasmid was used as control and was transformed into onion epidermal cells. The entire coding region of the VtPGIP gene was cloned into the plasmid of Pyk2784-EGFP (enhanced green fluorescent protein), generating the p3SS-VtPGIP-EGFP plasmid, which was designed to express the in-frame fusion protein of VtPGIP-EGFP. Onion epidermal cells were subsequently transformed with p3SS: VtPGIP-EGFP or the control plasmid p3SS: EGFP according to the particle bombardment method by using a particle delivery system (Bio-Rad Biolistic PDS-1000/He; Bio-Rad, Hercules, CA). The transformed onion epidermal cells were incubated at 25°C in the dark for 12 h, and VtPGIP-EGFP expression was then examined under a fluorescent microscope (Axio Imager A1; Carl Zeiss, Germany).

VtPGIP gene was induced by SA, ABA, and fungi

PCR was performed using 0.5 μg of cDNA to 1 μg of cDNA in a 20 μl reaction volume containing 10 μl of SYBR Green Master Mix (including Taq polymerase, dNTPs, MgCl2, 10× buffer, SYBRgreen I; ToYoBo, Osaka, Japan), and two primers, using the following PCR profile: 95°C for 2 min; and 40 cycles of 94°C for 20 s, 59°C for 20 s, and 72°C for 20 s on a 7300 Real-time PCR System (Applied Biosystems). The following primers were used:

Target gene primer:
S1: 5'-TGTCCAGTTTGATCTTTGAG-3'
S2: 5'-CAAGCCACCTAGTGGTGAAGTAG-3'

Housekeeping gene primer:
actin1: 5'-TACAATTCCCATCATGAAGTGTGATG-3'
actin2: 5'-TTGAAGGCACCTCGTCAACAAATG-3'.

The PCR efficiency of each target mRNA was obtained; the absence of primer-dimer formation, which could interfere with the specific amplification, was checked in no template control sample. Each time point was determined as an average of the data obtained from triplicate trials. Relative gene expression was calculated using the following equation:

Relative expression = [E_{target} - CP_{target (control – sample)}]/[E_{reference} - CP_{reference (control – sample)}].

The relative levels of VtPGIP used to control PGIP mRNA were analyzed using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The threshold cycle (Ct) value represents the PCR cycle in which the copy number passes the fixed threshold and can be first detected.

RESULTS

Analysis of the VtPGIP gene

A PGIP gene (EU037367) with a length of 1002 bp was isolated from V. thunbergii VtPGIP cDNA sequence was compared with the DNA sequence and showed no introns (unpublished). The amino acid sequences of some PGIPs were also compared and the results revealed sequence similarities as indicated by a phylogenetic tree of PGIPs from plants (Figure 1). Using sequence comparison data, we found that the species of the same genus were classified into the same group except Eucalyptus grandis, Pyrus communis, and Malus pumila because the PGIPs of these plants may have different evolutionary scenarios.

Similar to the majority of PGIPs, VtPGIP showed unique characteristics and the encoded products were composed of 333 amino acid residues (Figure 2). The biological site was analyzed and a signal peptide of 27 amino acid residues of the VtPGIP (Figure 3) was identified using Signal P and eight cysteine residues at conditions: 1 min at 94°C; 2 min at 58°C; and 2 min at 72°C with a final extension of 10 min at 72°C. The products were cloned into a pMD19-T vector (TaKaRa), and the two clones were sequenced.

The promoter of the VtPGIP gene was analyzed using promoter prediction software programs (Promoter Scan, Neural Network and CpG Island) and transcription factor prediction software programs (TF Search, Match 1.0-public and Plantcare).
conserved positions (Figure 2). VtPGIP was typical of extracellular leucine-rich repeat (LRR) proteins; each protein with 10 repeats exhibits variations in the xxLxx-NxL/NxL/NxL motif (De Matteo et al., 2006). In the LRR regions is considered as the main site of PG interaction (De Matteo et al., 2006). The computational and mutational analyses identified the residues within and near this region that can account for the specific PG-PGIP interactions (Figure 2).

Considering the structure of PGIP from Phaseolus vulgaris (De Matteo et al., 2006), we found that the N-terminal regions of the VtPGIP contained disulphide bridges and the C-terminal regions contain disulphide bonds (Figure 4). The N-terminal regions of the VtPGIP consist of an α-helix residue and a short β-strand that forms H-bonds with the residues of sheet B1. VtPGIP structure, which contains seven α-helix motifs, were analyzed using Swiss-Pdb Viewer 3.7. In the VtPGIP structure, the C-terminal region comprises the last two 3α-helices, the last strand of sheet B2, and a short loop (Figure 4).

**Analysis of the VtPGIP promoter**

The promoter sequence of VtPGIP, with a length of 1650 bp (Figure 5), was obtained (JF832390) and the promoter sequence was analyzed (Figure 5 and Table 1a,b). The promoter of the VtPGIP gene contained cis-acting elements involved in the responses to ABA (ABRE), SA (TCA-element), and fungal elicitor responsive element (Box-W1). The promoter also contains transcription factor binding sites, such as Nkx2-5 and c-Rel.

**Analysis of the subcellular distribution of the VtPGIP protein**

The control proteins were uniformly distributed in the cells (Figure 6A, 6B, and 6C). The VtPGIP-EGFP fusion proteins were predominately located in the plant cell wall (Figure 6D, 6E, and 6F). The signal peptide sequence of VtPGIP was analyzed online and the result indicated that the sequences should have accordant distribution located in the plant cell wall.

**Effect of the treatments on VtPGIP gene expression**

Studies on plant defense signaling have revealed that plants adopt a network of signal transduction pathways via different kinds of signaling molecules. Gene products were verified after the respective PCR products were sequenced. VtPGIP expression was analyzed in the treated
leaves and then compared with that in the untreated control leaves. The result confirmed that VtPGIP expression was induced after ABA and SA treatment (Figures 7A and 7B). The highest transcription level was found at 2 h after SA treatment compared with the control group (0 h), and the highest VtPGIP expression was increased 57.68 times compared with that of the control group (Figure 7B). For the ABA treatment, the highest VtPGIP expression was found at 6 h, and this expression was 10 times greater than that of the control group (Figure 7A). The results confirmed that the VtPGIP gene was strongly induced by ABA treatment and consisted of the promoter sequence containing cis-acting elements involved in the responses to ABA and SA.

The relative VtPGIP expressions from the leaves inoculated with four different pathogens were monitored. The results showed that VtPGIP expression from the leaves infected with fungi was higher than that in the control group (Figure 8). After the plants were infected with B. cinerea, VtPGIP expression increased 100 times higher than that of the control group. Similarly, after C. diplodiella vaccination, VtPGIP expression relative to that of the control group was also approximately 100 times higher. After E. cichoracearum and G. cigulata were inoculated, VtPGIP expression increased approximately 10 times compared with that of the control group. These results suggested that VtPGIP was involved in plant disease resistance.

**DISCUSSION**

The structural data and the close homology with other PGIPs confirmed that the sequence was PGIP. VtPGIP was previously isolated and showed a high degree of similarity to PGIP genes from other plants. For the amino acid sequence, the species of the same genus were classified into the same group, but E. grandis, P. communis, and M. pumila may indicate that PGIPs have various evolutionary histories. In all of the PGIPs studied, the LRR domain is conserved, which reveals that protein-protein interactions are involved in immune functions and
The consensus LRR domain of the VtPGIP showed similarities to the LRR regions of other PGIPs and PGIP-like plant proteins. The three-dimensional structures of VtPGIP were also analyzed (Figure 4). In the structural view, VtPGIP showed numerous sites similar to *Phaseolus vulgaris* PGIP (*PvPGIP*); hence, it may also contain PG-binding sites. The sequences of the amino acid residue of VtPGIP were analyzed and four cysteine residues were identified at the flanks of the LRR regions within the N- and C-terminal domains; this result is consistent with that of the PGIP of bean (De Matteo et al., 2006). The results suggested that the type of N-terminal extension affects substrate specificity and may determine the functional diversity of these enzymes. The N- and C-terminal cysteine-rich regions then form disulphide bridges, which are considered to be important in maintaining and providing additional stability of the secondary and tertiary structures. In the functional view, glycosylation possibly provides higher stability and increased protease resistance. This characteristic has been observed in many fungal PGs and may be crucial for their enzymatic activity. The six N-linked glycosylation sites were found in the LRR domains in VtPGIP (Figure 2). The positions and numbers of the N-linked glycosylation sites were not highly conserved among plant PGIPs (Mattei et al., 2001).

The subcellular localization of the transiently expressed PGIP protein was determined. The result showed that VtPGIP was localized in the plant cell wall, and this finding is consistent with that in previous studies on the localization of many other PGIPs (De Lorenzo et al., 2001). The plant cell wall provides structural stability (Shanmugam, 2005) and functions as the first barrier to counteract pathogens; PGs are also the first enzymes to be secreted when pathogens encounter plant cell walls (De Lorenzo and Ferrari, 2002). PGIPs are located in the plant cell wall and limit fungal invasion by counteracting the action of PGs.

Plants may have evolved mechanisms to respond to pathogens and other stress-related molecules (Ramonell et al., 2002). PGIPs may have an important function in the overall resistance to biotic and abiotic stresses (Li et al., 2003). The characteristics of *PGIP* promoter sequence was analyzed using promoter prediction software and transcription factor prediction software because the regulation of gene expression only covers a small part of the non-coding region; experimental studies are often difficult to conduct without the specific software. The promoter sequences were analyzed online to detect the presence of putative *cis*-acting regulatory elements in the promoter regions of *VtPGIP* (Table 1a,b). Several elements, including ABRE, Box-4, G-box, W1-box, HSE, TC-rich repeats, and TCA-element, were detected. *VtPGIP* expression was regulated by ABA, SA, and fungi. Such hormones and pathogens functioned as positive regulators of *VtPGIP* expression in the present experiment. In addition, several elements containing a binding site for Nkx2-5 and c-Rel transcription factors were

**Figure 4.** Ribbon representation of the crystal structure of the VtPGIP. As with the ribbon representation of the crystal structure of PGIP2 from *Phaseolus vulgaris* (De Matteo et al., 2006), the figure showed that the VtPGIP had a right-handed superhelical fold typical of LRR proteins. The fold of the VtPGIP consists of a central LRR domain flanked by the N- and C-terminal cysteine-rich regions and, also like the crystal structure of PGIP2 from *Phaseolus vulgaris*, the secondary structure of the LRR motif showed seven α-helices located on the convex side of the VtPGIP protein. Meanwhile, the majority of the LRR proteins had two B sheets connected with the helices on the convex side by loops or β-turns were analysed by Swiss-Pdb Viewer 3.7, parallel to the B2 sheet. B2 is distorted because of the twisted shape of the molecule and the variable length of the β-strands (Hegedus et al., 2008). From this figure, it showed the VtPGIP had twenty-two B-sheets. Sheets B1 and B2 are xLx and xxLxLxx regions, respectively, and the helix is the xLx/sGx region.

recognition of non-self molecules in plants (Jones, 2001). The VtPGIP protein may inhibit PG activity because the sequence ‘function-sites’ of LRR did not change during evolutionary development.

The three-dimensional structure of the bean PGIP has been investigated (Penninck et al., 1996), and results showed that the two extended B sheets in the LRR region are highly conserved; these B sheets are also involved in the interactions with pathogen PGs (De Matteo et al., 2003).
Figure 5. The promoter sequence analysed by Plantcare, it showed that it had ABRE (cis-acting element involved in the abscisic acid responsiveness), Box-4, G-box, W1-box (fungal elicitor responsive element), HSE, TC-rich repeats and other transcription factor-binding sites on the positive strand. A TCA-element (cis-acting element involved in salicylic acid responsiveness) was on the negative strand. The expression of PGIP might be correlated with abscisic acid, salicylic acid, light, the fungal elicitor, heat stress and defence and stress responsiveness.

An accumulation of VtPGIP transcripts was observed in the seven-week-old tissue culture seedlings, and VtPGIP expression was induced by SA and ABA (Figures 7A and
Table 1a. The structure of the promoter according to promoter prediction software.

<table>
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<tr>
<th>The PGIP gene promoter</th>
<th>Promoter prediction software</th>
<th>Neural network promoter prediction</th>
<th>CpG Island prediction</th>
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<td>Promoter scan</td>
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<td>Promoter region predicted on reverse strand from 1550 to 1300</td>
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<tr>
<td></td>
<td>Promoter Score: 53.33 (Promoter Cut-off point=53.000000)</td>
<td>Start End Score 1593 1643 0.93</td>
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<tr>
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<td>TATA found at 1346, Est.TSS = 1314</td>
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The structure of the promoter was analysed according to three different online software programs. Bioinformatics software was used to analyse the promoter. Some transcription factors and their positions were obtained in the TF Search, but the positions of unknown or new transcription factors could not be analysed; The promoter of the *VtPGIP* gene contain cis-acting elements involved in the responses to: abscisic acid (ABRE), salicylic acid (TCA-element) and fungal elicitor responsive element (Box-W1) and it contain some transcription factor binding sites, such as Nkx2-5;c-Rel.

Table 1b. Structure of the promoter according to transcription factor prediction software.

<table>
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<th>The PGIP gene promoter</th>
<th>Transcription factor prediction software</th>
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<td><a href="http://bioinformatics.psb.ugent.be/webtools/plantcare/html/">http://bioinformatics.psb.ugent.be/webtools/plantcare/html/</a> (Fig.5)</td>
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The structure of the promoter was analysed according to three different online software programs. Bioinformatics software was used to analyse the promoter. Some transcription factors and their positions were obtained in the TF Search, but the positions of unknown or new transcription factors could not be analysed; The promoter of the *VtPGIP* gene contain cis-acting elements involved in the responses to: abscisic acid (ABRE), salicylic acid (TCA-element) and fungal elicitor responsive element (Box-W1), and it contain some transcription factor binding sites, such as Nkx2-5;c-Rel.

VtPGIP expression in the untreated control leaves was analyzed and positively confirmed the relative expression of *VtPGIP* after ABA and SA treatment. On the basis of signaling molecules, plant defense genes were activated and regulated by different signal transduction pathways. SA, a product of the phenylpropanoid pathway, is involved in the expression of localized hypersensitivity reactions and systemic acquired resistance (Raskin, 1992). Studies have shown that SA has a critical function in the defense signaling pathway. In many plant species, SA levels increase as PR gene expression and disease resistance are activated (Johnson et al., 2003). The phytohormone ABA is involved in plant abiotic stress response and regulation of various biotic stress responses. Enhanced resistance against necrotrophic pathogens was demonstrated;
Figure 6. Subcellular localization of VtPGIP in onion epidermal cells. The onion epidermal cells were transformed with p35S:VtPGIP-EGFP(D-F). The expression and subcellular distribution of the proteins were examined under a fluorescent microscope (C, F) and a light microscope (B, E) and then merged (A, D). The data shown were representative of three independent experiments. EGFP: Enhanced green fluorescent proteins. It was found that this protein was localized in the plant cell wall.

Figure 7. Time course of the changes in mRNA levels of PGIP gene in leaves after ABA (A) and SA (B) treatments. The leaves were harvested 0, 2, 6, 16, 24, 48, and 72 h after the ABA (A) and SA (B) treatments. Analysis was performed by real-time RT-PCR (see Materials and methods). The results of this study suggested that the PGIP gene may be involved in SA and ABA-regulated defense responses, as the expression of VtPGIP strongly increased after treatment. They showed the maximum expression level of induction of VtPGIP was observed at 6 h and 2 h by ABA and SA treatments, respectively. Then expression levels of the VtPGIP transcripts decreased. After a longer period of time, it would return to basal levels of expression.

this procedure was based on primed callose accumulation controlled by an ABA-dependent defense pathway (Ton and Mauch-Mani, 2004). Plants secrete different defense proteins to protect themselves from pathogen invasion. These kinds of defense proteins are also induced by various signal molecules, such as SA and ABA. Thus, many important families of defense proteins are expressed and regulated by different signal molecules via different transduction pathways. Mechanical wounding or damage possibly occurs in the infection site where defense-related genes may be activated against pathogens (Cheong et al., 2002). Microarray studies have demonstrated that mechanical wounding and insect feeding account for distinct and overlapping sets of gene activation (Reymond et al., 2000). PGIPs are regulated during development and after wounding and pathogen infection or treatments with elicitors, SA, and cold temperature (De Lorenzo et al., 2001; Ferrari et al., 2003; Li et al., 2003).

PGIPs are also induced in many plant tissues under various environmental conditions, and PGIPs have been isolated in many plants. Other signaling molecules or defense response activators may induce PGIP gene expression (Ferrari et al., 2003). In the present study, VtPGIP gene expression was regulated by SA and ABA signal transduction pathways.
The importance of PGIPs in plant defense has been elucidated by a series of studies. For instance, the overexpression of the PGIP gene in Arabidopsis reduces symptoms and colonization by B. cinerea (Ferrari et al., 2003). In the present study, the VtPGIP expression level from pathogen-infected leaves (C. diplodiella, E. cichoracearum, G. cigulata, and B. cinerea) was higher than that of the control leaves. This phenomenon is more evident in B. cinerea and C. diplodiella than in other species.

In conclusion, PGIP has an important function in plant disease resistance. The PGIP gene expression levels were enhanced in response to applied SA and ABA. This result may be used as a basis to increase plant resistance to pathogen as induced by SA or ABA treatment in V. thunbergii. Hence, appropriate plant hormones should be applied in resistant engineering of other plants. PGIP expression in treated plants is possibly upregulated compared with untreated plants. As a result, plants may exhibit greater resistance to pathogens after treatments with appropriate plant hormones.

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Study on the extraction of dioscin by the ultrasonic-assisted ethanol

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With Dioscorea zingiberensis as row materials, and with the yield of diosgenin as assessment criteria, the effect on extraction yield of dioscin of frequency of ultrasonic, the period of ultrasonic and solid-liquid ratio (D. zingiberensis : alcohol) was studied via orthogonal test. A new and unique method to accomplish this was by utilizing the technology of ultrasonic assisted ethanol extraction. The optimal processing parameters of this method were confirmed. The method was compared with solvent extraction process for the effect on extraction yield of dioscin. It was shown that the technology of ultrasonic assisted ethanol extraction which can significantly increase the extraction yield and extraction efficiency of dioscin. The ultrasonic did not destroy D. zingiberensis cell structure, but decreased the boundary layer thickness between D. zingiberensis (solid phase) and alcohol (medium), and accelerated cells inside and outside the material exchange. International rectifier (IR) further demonstrated that ultrasonic merely increased extraction yield of dioscin instead of destroying the cell structure.

Key words: D. zingiberensis, ultrasonic waves, extraction, diosgenin.

INTRODUCTION

Dioscin, a steroid compound that is formed when diosgenin and glucides connect through β-1,3 glycosidic bond can be found mainly in the root of Dioscorea zingiberensis C.H. Wright, Dioscorea nipponica Makino, Dioscorea panthaica prain et Burkil and D. nipponica Makinovar rosthani prain et Burk (Yang et al., 2003). The hydrolysate of dioscin-diosgenin is a very important fundamental material to make steroidal hormonal drugs. Steroidal hormone has effective pharmacological capabilities such as resisting infection, hypersusceptibility, viruses and shock. Thus, it is a drug that can be used to cure rheumatism, cardiovascular disease, lymphocytic leukemia, cellularity encephalitis, and dermatosis; it is also an important anit-tumor drug and an important drug (Evans, 2002; Hu and Yao, 2002) to salvage patients at critical stages.

A traditional way of producing diosgenin was through direct acid hydrolysis. That is to say that the D. zingiberensis was pestled firstly, then hydrolyzed with acid, filtered, dried, and lastly extracted with petroleum ether. There are two biggest disadvantages of this process. One is usage of large amount of acid, which leads to serious pollution. Another is the process of acid hydrolysis; the starch in the D. zingiberensis is converted into reducing sugar, whereas it is hard to recycle, thus results in resource waste and increased the difficulty
of treatment of the acid liquor (Link, 2006). To reduce the quality of acid in the process of producing diosgenine, Xiang et al. (2008) adopted mechanical separation technology; separated starch and cellulose in D. zingiberensis, hydrolyzed turbid liquid (which contains mainly dioscin), effectively reduced the amount of acid used, but the whole process had excessive water use and pollution problem still was not solved. Chen et al. (2007) adopted alcohol fermentation, hoping to convert starch in the D. zingiberensis into alcohol, but because of dioscin bactriostasis, it resulted in low alcoholic strength in the fermentation liquid (6.5 v/v).

On the other hand, steroids of the diosgenine might be broken in the process of alcohol fermentation, which results in low purity of the diosgenine. Ultrasonic assisted ethanol extraction was used in this paper, to extract dioscin from D. zingiberensis (Which contains 2 to 3% of diosgenin); hydrolyzed dioscin with acid, washed residues, then dried and diosgenin was gotten from it. Yeast was added into D. zingiberensis which was extracted for further fermentation. Also, this method reduced the amount of acid which is used in the process of hydrolysis to get diosgenin from dioscin as well as reduced the pollution of this process. This method made full use of starch resource in the D. zingiberensis.

### MATERIALS AND METHODS

*D. zingiberensis* was provided by An Kang Institute of *D. zingiberensis*, and appraised by Professor Hu Zhenghai from Northwest University. Petroleum ether (boiling point 60-90°C), hydrochloric acid, and ethanol were purchased from Xi'an Chemical Reagent Company. A multi-frequency sonochemo reactor (SC-III) from Jiu Zhou Mechanical and Engineering Research Center, XT5 Microscope Melting Point Inspect from Shanghai Laboratory Instrument Works Co., Ltd.; FTIR Spectrum (VECTOR-22) from the German BRUKER Company; Soxhlet Extractor from Chongqing Beibang Glass Instrument Factory and Scanning Electron Microscope (CS3400), from Beijing Elaborate Technology Development Ltd were used for the study.

To simplify the experiment, dioscin was replaced by solid matter in the filter liquid in this paper; it is not accurate, but it can reflect *D. zingiberensis* nature to some extent. Generally speaking, the more content of solid matter in the extract liquid, the more amount of extraction of the dioscin. 60-mesh *D. zingiberensis* of 100 g was collected, treated with ultrasonic assisted ethanol to get the filter liquor, 1 ml of it was collected into the tube, then dried under 105°C for 2 h to get its quality. The quality was calculated according to the formula:

The amount of the solid matter in the extract liquor = the volume of the extract liquor (ml) × the quality of the 1 ml filter liquor (g/ml);

The content of the solid matter in the extract liquor = the solid matter in the extract liquor (g) / the content of the *D. zingiberensis* (100).

The filter liquor (100 *D. zingiberensis*) was vacuum concentrated which was treated with ultrasonic extraction to a paste (about 10 g), 30 ml 1.5 mol/L sulfuric acid was added, then hydrolyzed under 108°C for 4 h, the residue was washed to neutral after filtering, dried, extracted with petroleum ether, crystallized to get the diosgenin, and dried until constant weight. The yield of the diosgenin was calculated according to formula α:

\[ \text{Yield of the diosgenin} = \frac{\text{content of the diosgenin (g)}}{\text{content of the } D. \text{ zingiberensis} \times 100\%} \]

The hydrolysis product of the dioscin is diosgenin, the extract yield of the dioscin and the yield of the diosgenin had linear correlation. For convenience, the two concepts may probably change with each other in this paper.

### RESULTS AND DISCUSSION

#### The optimization of the conditions of the ultrasonic assisted ethanol extraction

On the basis of single factor experiments, the orthogonal experiment was applied to optimize the extraction. 9 shares of the 60-mesh *D. zingiberensis* (100 g) was collected, then volume fraction of 65, 75, and 85 v/v alcohol, was added respectively, according to the solid-liquid ratio of 1:8, 1:10, 1:12. Under the frequency of the ultrasonic of 14.52, 25.80, and 35.74 KHz, ultrasonic extraction, time were respectively 20, 30 and 40 min. Lastly, it was filtered to get filtrate, the filtrate was vacuum dried until paste was obtained, hydrolyzed for 4 h under 108°C after adding 30 ml 1.5 mol/L sulfuric acid, filtered and the residue was washed until neutral, which was dried, extracted with petroleum ether and crystallized to get diosgenin which was dried to constant weight. Every experiment was repeated for 3 times, and average yield of the diosgenin was calculated. The experimental arrangement is shown in Table 1, and the experimental results are shown in Table 2. Influencing factors of the yield of diosgenin are alcohol volume fraction, ultrasonic extraction time, solid-liquid ratio, and frequency of the ultrasonic. The optimal extraction condition was solid-liquid ratio of 1:10, alcohol volume fraction of 65%, extraction time of 30 min, and frequency of the ultrasonic of 25.80 KHz. Dioscin was extracted under this condition,
Table 2. The result of the orthogonal experiment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Solid-liquid ratio</th>
<th>Extraction time (min)</th>
<th>Frequency (k)</th>
<th>Volume fraction of ethanol (%)</th>
<th>Yield of diosgenin (%)</th>
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<td>20</td>
<td>14.52</td>
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<td>1.267</td>
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<td>1.142</td>
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<td>25.80</td>
<td>85</td>
<td>1.204</td>
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<td>0.43</td>
<td>0.15</td>
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Table 3. The effect of extraction methods on extraction yield of dioscin and diosgenin yield.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>The content of the solid matter/%</th>
<th>The yield of the diosgenin/%</th>
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<tr>
<td></td>
<td>1th</td>
<td>2th</td>
</tr>
<tr>
<td>Ultrasonic-assisted extraction</td>
<td>12.255</td>
<td>3.428</td>
</tr>
<tr>
<td>Ethanol extraction</td>
<td>9.474</td>
<td>0.37</td>
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<tr>
<td>Acid hydrolysis</td>
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Hydrolyzed and dried to get the diosgenin, then the experiment was repeated for three times; the mean yield of the diosgenin was 2.081%.

Comparison of the method of ultrasonic assisted ethanol extraction with that of solvent extraction

The effect of extraction methods on extraction yield of dioscin and diosgenin yield

60-mesh *D. zingiberensis* (100 g) was collected, volume fraction of 65% alcohol 1000 ml was added, which was extracted at 30 min at the frequency of 25.80 KHz, and the content of the solid matter and diosgenin yield was calculated. The first, second and third extraction of the residue over diosgenin, was collected; each experiment was repeated for three times. 60-mesh *D. zingiberensis* (100 g) was collected, 1.5 mol/L 300 ml sulfuric acid was added, hydrolyzed at 4 h at 108°C, filtered, the residue was washed until neutral, was dried, and extracted with petroleum ether to get diosgenin.

The results are shown in Table 3. Table 3 shows that it is clear that the method of ultrasonic assisted ethanol extraction can obtain a higher extraction yield of dioscin and diosgenin yield than that of the solvent extraction. Extraction yield of dioscin with ultrasonic assisted ethanol extraction for 30 min was 2.781% higher than that with solvent extraction for 30 days. Thus, ultrasonic assisted ethanol extraction not only increases the extraction yield of the dioscin and diosgenin yield but also increase the extract efficiency of the dioscin.

The diosgenin yield with ultrasonic assisted ethanol extraction (3.1345%) was 0.7122% higher than that of solvent extraction (2.4223%), and was 0.358% higher than that of direct acid hydrolyze (2.7765%).

The effect of extract methods on morphological structure of *D. zingiberensis*

*D. zingiberensis* was collected which had been extracted before, then extracted for 3 times with ultrasonic assisted ethanol extraction and solvent extraction, respectively which was vacuum dried for 24 h at 50°C. Morphology structure was analysed with SEM and the results are shown in Figure 1. Figure 1 shows that the cytoarchitecture of the *D. zingiberensis* before and after the ultrasonic extraction did not change much, and the cells...
Figure 1. The effect of extract methods on morphological structure of *D. zingiberensis*. 1, Ultrasonic assists ethanol extraction; 2, solvent extraction; 3, *D. zingiberensis* without any treatment.

were integrate, and the edges were trim, which means that the ultrasonic does not break the cytoarchitecture during the extraction process, and this result does not agree with the findings from the former experiments (Hromadkova et al., 2002). Therefore, it can be conferred that the principle for ultrasonic to assist the solvent extraction is that the ultrasonic field through the ultrasonic oscillation, ultrasonic cavitation and cavitation will effectively intensifies the liquid to perfuse outside the membrane, within the pores and a quickens surface diffusion, and all of these will bring a reduction of the boundary layer between the cells and the solvent, so that the velocity of the medium is increased.

**Properties identify of diosgenin**

The collected diosgenin which was obtained from the method of ultrasonic assisted ethanol extraction (10 g), was recrystallized, and the measured melting point was 203 to 207°C. 99% crystalline purity of diosgenin melting was 204 to 207°C. The collected diosgenin was obtained from recrystallization (0.2 g), the right amount of potassium bromide was added, ground, and then measured with IR; the results are shown in Figure 2. Figure 2 shows that the sample in 1237, 1050 cm⁻¹ (C₃OH and Δ₁₃, 978, 917, 896 and 860 cm⁻¹ (25 spironoalkyl) all made an appearance, and this is the same (Zhang and Wu, 2007) as that of the diosgenin. All of the above proves that the diosgenin obtained when ethanol is used as the assistance had a comparatively higher purity.

**Conclusion**

The technology of ultrasonic assisted ethanol extraction can significantly increase the extraction yield and extraction efficiency of dioscin. The ultrasonic did not destroy *D. zingiberensis* cell structure, but could decrease the boundary layer thickness between *D. zingiberensis* (solid phase) and alcohol (medium), and could accelerate cells inside and outside the material exchange. Dioscin, which is the hydrolyzate of *D. zingiberensis* not only is the main raw material of three synthetic hormones, but also is the essential treatment drugs of cardiovascular and cerebrovascular diseases. *D. zingiberensis* contains only 2 to 3% dioscin. In the extraction process, though a number of polar impurities (starch and protein) were extracted, the extraction volume
Figure 2. The IR of diosgenin.

(solid matter) was also less than 10%, used acid hydrolysis and acid consumption was also 10% of direct acid hydrolysis. To a certain extent, this method reduced pollution and achieves a clean production of *D. zingiberensis*.

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Light microscopic detection of *Plasmodium falciparum* *in vitro* through *Pf* histidine rich protein 2 (HRP 2) gold conjugate labeling: Rapid diagnosis of cerebral malaria in humans

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*Plasmodium falciparum (Pf)* has been found to be the deadliest of all the known species of the parasite capable of infecting humans; this is because it is capable of causing severe cerebral tissue damage. This study was carried out to demonstrate the parasite in the host blood *in vitro* through immunogold labeling using antibodies against *Plasmodium falciparum* histidine rich protein 2 (HRP 2); a major metabolite released during the cause of the parasite infection and feeding in the erythrocyte. 12 known Pf positive samples were obtained from across the six geopolitical zones of Nigeria and were further characterized by Geimsa thick and thin film for parasite identification parasite count expressed as parasites/µl of blood. An average of 400 parasites/µl of blood was obtained in each of the samples used for this study. Pf-HRP 2 antibody was conjugated to freshly prepared colloidal gold of particle size 40 nm. The conjugation process was blocked with bovine serum albumin (BSA) and the conjugate itself preserved by 1% glycerol and 0.01% sodium azide. The parasite count was titrated against the Pf-HRP 2 gold conjugate and was analyzed under the light microscope with a fluorescent filter. Reactivity and specificity of Pf-HRP 2 gold conjugate was found to be highly specific and gave direct identification of the erythrocytes infected with the parasite. A good contrast was also obtained between uninfected erythrocytes, parasite and the infected erythrocytes.

**Key words:** *Plasmodium falciparum*, malaria, fluorescent microscope, HRP 2, antibody, gold conjugates.

**INTRODUCTION**

Malaria is a dominant infection in the tropics, especially in Africa, South East Asia and the Amazon of South America, this so because these regions provide suitable climate conditions for the vector to survive and reproduce.

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**Abbreviation:** Pf-HRP 2, *Plasmodium falciparum* histidine rich protein 2.
(the vector being female anopheles mosquito) (Fukuda et al., 2009). The parasite in the host system binds to the endothelium of cerebral vessels in the brain; the binding process is called cyto-adherence and it involves knob formation (Mayer et al., 2012). Accumulation of infected erythrocyte in these blood vesseles thus prevents exchange of nutrients in the capillary bed, leading to a state of ischemia or anoxia (Hora et al., 2009; Nacer et al., 2011). The tissues of the brain are supplied in segmented tissue blocks of about 1 mm³, such that a tissue block will correspond to the region solely supplied by a terminal artery. Terminal arteries are end arteries that do not branch or anastomose; therefore shortage of blood supply cannot be compensated for eventually causing avascular necrosis primary to cerebral symptoms and hemorrhage (Willie et al., 2012).

In view of this, rapid diagnosis of falciparum infection is important. To detect plasmodium falciparum and other species of the parasite, several proteins have been identified as suitable indicators of the presence of such parasite in the blood stream (Hansen et al., 2012). Examples of such proteins include plasmodium aldolase, plasmodium lactate dehydrogenase and plasmodium histidine rich protein 2 (HRP 2) (Waisberg et al., 2012). Histidine rich protein 2 has been described as being the most effective in detecting falciparum infection (Grobusch et al., 2003). A major challenge of rapid diagnostics using in vitro kits is that the HRP 2 remains in the blood stream after the parasite has cleared post treatment with an anti-malaria molecule, thus giving false results (Grobusch et al., 2003). The World health organization (WHO) recommends microscopy as the standard for diagnosis of malaria and identification of parasite because of the various limitations of the rapid diagnostic tests (RDTs) (prozoning effects, false positive and cross reactions) (Cojoc et al., 2012). In this technique we have brought together in the same context the principle of microscopy and the biological techniques of in vitro test kits as a fast method in which Pf infected parasites can be quickly identified. This method utilizes the microscopic properties of nanoparticles (colloidal gold) and its ability to serve as a fluorescent agent and a tool for microscopy.

MATERIALS AND METHODS

Preparation of colloidal gold and gold conjugate

1% sodium citrate solution was made by dissolving 1 g of sodium citrate (Sigma) in 100 ml of deionised water (Millipore, France) the water was tested for salts and have a pH of 6.0. 2000 ml of the water was placed in a conical flask and 50 ml of 1% gold (99% pure from Sigma) chloride was added. A stirrer magnet covered with a tephlon was inserted into the solution, the hot plate was switched on such that the temperature was set at 70°C, a conical flask, 250 ml capacity was inverted to cover the opening of the larger conical flask in order to prevent loss of water by vaporization. The solution was allowed to boil and the stop watch was started, until the colour changes from red to purple (approximately 1 min). 9.6 ml of 1% gold chloride was added and the colour again changed from red to purple, 2 ml 0.7% gold chloride was then added as the colour was observed to have changed from purple to pink. The solution was allowed to cool by constantly running water on the cone region of the conical flask. The colloidal gold was analyzed using the spectrophotometer in the spectrum region, and the peak absorbance recorded was 528 nm. The particle size as analyzed under the fluorescent microscope was approximately 40 ± 1 nm, modifying the methods of Han et al. (2012).

Conjugation of gold with Pf-HRP 2 antibody (monoclonal)

The Tris HCl was poured into a beaker and the antibody was added at a concentration of 5 mg/L, the colloidal gold, twice the volume of the buffer, was the added and the solution was blocked with 1.5% bovine serum albumin (BSA, Sigma), added as 10% solution, 0.05% sodium azide (Sigma) was added to prevent microbial infection and 1% glycerol was then added to preserve the conjugate. The solution was centrifuged at 10000 rpm and 4°C, the supernatant was collected and discarded while the final conjugate particle size and concentration was determined, using the spectrophotometer. The colloidal gold and gold conjugate samples were profiled in a hollow slide under the fluorescent microscope (Han et al., 2012; Tsai et al., 2012).

Incubation of gold conjugates and blood samples

100 µl of the gold conjugate was obtained in an Appendendorf and mixed with 10 µl of the sample. The samples were mixed by gently shaking the Appendendorf tube, and was incubated at 37°C temperature for 15 min, 0.05% of phytoheamagglutinin (PHA) was added to partially lyse the red blood cell (RBC). The mixture was then smeared on a hollow slide and viewed under the fluorescent microscope and compared with a normal smear of Pf-HRP 2 conjugate. The gold conjugate was also used in soaking a conjugate pad and assembled into a rapid diagnostic kit on a filter in place (otherwise quantum dots) in the light microscope while the curves of the spheres can give fluorescence with a filter in place (green fluorescence filters were used in Zeiss Primo Star light microscope, magnification ×1000) (Figures 1A and B). Arrow heads in Figure 2A shows the symmetrical attachment of the tail of the Y shaped antibody leaving the epitope regions (the free ends of the Y) directed at right angles from the surface of the spheres. In this study, approximately four antibody projections were observed per colloidal gold in focus. Arrow heads in B shows the Y shaped slender free ends of the antibodies (magnification ×1000). In Figure 3, colloidal gold (c), parasite (p) and labeled parasite (f) were embedded in the erythrocyte. F1 represents an advanced stage of parasite infection while F2 represents the late stage of parasite infection.
Figure 1. 40 nm colloidal gold under light microscope with fluorescence filters, this is to demonstrate in the first instance that colloidal gold can be viewed without enhancement under the light microscope (B), and with fluorescence filters in the same microscope in (A) (magnification ×1000).

Figure 2. Demonstration of colloidal gold conjugated with Pf-HRP 2 using the fluorescence filters (magnification ×1000).

Figure 3. Pf infected erythrocytes shown under fluorescent filter in light microscopy (magnification ×1000). C, Colloidal gold; F, labeled parasite.
Figure 4. Demonstration of the specificity of Pf-HRP 2 to the antibody and the parasite in the infected erythrocyte (magnification ×1000). c, Colloidal gold; p, parasite; e, erythrocyte.

Figure 5. Schematic illustration of the multiplanar appearance of Pf-HRP 2 gold conjugate (Courtesy: Trinitron Biotech Limited, Nigeria).

Free end containing the epitope of the Pf-HRP 2

 Regions blocked by Bovine serum albumin

Colloidal gold

and multiplication stage of the infection (magnification ×1000). Arrow heads in Figure 4A shows the projecting free limbs of the antibodies, colloidal gold (c), parasite (p) and erythrocyte (e). Figure 4B shows parasite localized in infected erythrocytes at different stages of the infection (magnification ×1000).

In living tissues, colloidal gold can be used to study molecular traffic and transport system. When labeled with appropriate antibodies, they localize in specific regions of the cell (Nolan et al., 2012). Thus, this technique was designed to localize the colloidal gold on the surface of erythrocytes and in the cytoplasm of cells housing the parasite in the blood and also free parasites in the blood. Considering the electrical properties of the surface of colloidal gold as being positively charged, and the localization of chains of negatively charged amino acids in the tail of the Y shaped antibodies (also Pf-HRP 2), an electrostatic force of attraction can occur between colloidal gold particles and the antibodies (Gao et al., 2012).

A major advantage of this technique is that it leaves the reactive part of the antibody unbounded such that they remain as free edges of the multiplanar gold conjugate with two epitope per one Y shaped antibody and possessing four of such planar structures.

The planar structure as described in Figure 5 gives the colloidal gold conjugate stability and shows that aggregation has not occurred during the preparation of the gold conjugate. This technique has eliminated the false positive observed post treatment; when the specimen is incubated with the gold conjugate, it will show that the protein is present but the parasite is absent, thus breaking the tie for a false positive malaria test post treatment. It will also aid quick counting of parasite in the blood, as it tags both parasite and parasite infected cells, helping in diagnosis and treatment.

This feature is also of advantage over the RDTs, as the colour formation (chromoaegregation of colloidal gold conjugates) will most likely give a uniform coloration, from 750 parasites/µl till 3000 parasites/µl of blood. In this study, it is observed that the parasites are specifically labeled and can be counted on the slide to give a definitive count, by locating bounded conjugates. Since this technique utilizes antibody, it will be of advantage in identifying parasites microscopically; antibody-antigen
reactions are specific thus giving the advantage of specifically identifying Plasmodium falciparum even at lower magnifications. This technique can be employed for a wide range of blood infections including determination of the extent of spread of cancer cells. It can be used to target infection sites for live studies in tissues and to treat cancers, as the conjugated colloidal gold can be irradiated to explode tropically within tumor cells thus destroying the cancers.

REFERENCES


Full Length Research Paper

Structure of vasa deferentia and spermatophores in Parapenaeopsis stylifera (H. Milne Edwards) (Decapoda: Penaeidae)

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The structure of vasa deferentia and spermatophores has been described in Parapenaeopsis stylifera. The male reproductive system consists of two symmetrical halves; each half bears testis, vas deferens and an ejaculatory duct. Each testis comprised of two to three short, broad and milky white lobes; vas deferens is divisible into proximal, medial and distal parts. The proximal vas deferens (PVD) is a convoluted mass made up of an extremely long and thin tube having elongated rod like spermatozoa. The median vas deferens (MVD) is broad, straight, somewhat flattened and bears many membranous folds internally and contained complete spermatophores; the distal vas deferens (DVD) is straight and cylindrical tube. The ejaculatory duct is a simple dilation with a tubular basal part tapered posteriorly for extrusion of spermatophores. The spermatophores are minute, spindle shaped bodies present in large numbers in each ejaculatory duct; the size varied from 0.148 to 0.161 mm; each spermatophore bears six to eight rows of regularly arranged spermatozoa. Histological studies reveal no internal partitioning of either vas deferens or ejaculatory duct.

Key words: Parapenaeopsis stylifera, vasa deferentia, spermatophore.

INTRODUCTION

Parapenaeopsis stylifera is purely a littoral species. Its distribution is in Indo-west Pacific (Holthuis, 1980). It supports a major fishery in Pakistan and India. From Pakistani waters, 25 species and seven genera of family Penaeidae have been recorded (Kazmi, 2003), among which only 12 species have commercial significance (Majid, 1988); namely, Marsupenaeus japonicus, Penaeus monodon, P. semisulcatus, Fenneropenaeus indicus, F. merguiensis, F. penicillatus, Metapenaeus affinis, M. monoceros, M. stebbingi, Parapenaeopsis hardwickii, P. sculpitlis and P. stylifera. The studies on the male reproductive organs and structure and formation of spermatophores have been undertaken in many penaeid genera of commercial importance like Fenneropenaeus (Penaeus) (King, 1948; Tirmizi, 1958; Subrahmanym, 1965; Tuma, 1967; Tirmizi and Khan, 1970; Huq, 1981; Chen, 1986; Sultana, 1986; Champion, 1987); Penaeus (Motoh, 1981), Melicertus (Penaeus) (Malek and Bawab, 1974a, 1974b), Litopenaeus (Penaeus) and Farfantepenaeus (Penaeus) (Leung and Lawrence, 1987; Rao et al., 1990; Bauer and Cash, 1991; Chow et al., 1991), Trachypenaeus similes (Raymond et al., 1993), Sicyonia disdorsalis (Jeri, 1998) and Aristeus antennatus (Demestre and Fortuno, 1992), Astacus leptodactylus (Erkan et al., 2009; Mirheydari et al., 2012), red claw crayfish Cherax quadricarinatus

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(Lo’pez-Greco et al., 2007). Some studies on the male reproductive organs of *P. stylifera* were undertaken by Shaikhmahmud and Tembe (1958), Subrahmanyam (1963) and Sultana et al. (1994); whereas, Tirmizi (1968) presented the structure and developmental stages of genitalia. Besides this, certain studies were also done in crabs such as giant hermit crab *Petrochirus diogenes* (Raquel and Fernando, 2012).

In penaeid shrimps, the male reproductive system typically consists of paired testes, vasa deferentia and ejaculatory ducts. Each testis is comprised of several lobes of variable shapes (TL); The vas deferens is a long tube of variable diameter and length in different species, originating from the main axis of the testis and extends ventro laterally towards the base of fifth pereiopod; it is differentiated into the following parts: i) proximal vas deferens (PVD), ii) medial vas deferens (MVD), iii) and distal vas deferens (DVD), terminating into ejaculatory duct (ED); the ejaculatory duct is located on the basis of fifth pereiopod and opens to the exterior through the genital opening on the arthrodial membrane of fifth pereiopod. The penaeid shrimp vasa deferentia are more complex than most other decapods and unusual in having a large dilated ampoule termed as ejaculatory duct. The vas deferens and ejaculatory duct both play important roles in the formation of spermatophores (Malek and Bawab, 1974a, 1974b; Chow et al., 1991; Bauer and Min, 1993, Bauer and Cash, 1991). The morphological variations in different parts of vas deferens are associated mostly to the type of spermatophores. *P. stylifera* have large number of spermatophores suspended in spermatic fluid (Shaikhmahmud and Tembe, 1958; Tirmizi, 1958; Sultana et al., 1994), whereas, in other penaeid genera such as giant hermit crab *Petrochirus diogenes*, only one pair of complete spermatophores was found (King, 1948; Subrahmanyam, 1965; Tuma, 1967; Tirmizi and Khan, 1970; Huq, 1981; Motoh, 1981; Chen, 1986; Sultana, 1985; Champion, 1987, Malek and Bawab, 1974).

In all these species, spermatophores, consists of a single sperm sac attached to some non spermatic accessory structures; that is for this reason, vas deferens and ED are divided partially or completely into two equal or unequal halves for separate transportation of spermatic and non spermatic materials. The study has therefore been conducted mainly on morphological variation of vas deferens and ejaculatory duct in relation to numerous spermatophores of *P. stylifera*, suspended in fluid and devoid of any accessory structure. The histology was done to reveal the presence of any internal partitioning of vas deferens and ejaculatory duct, if exists.

**MATERIALS AND METHODS**

The fresh samples were collected from commercial fish landing sites along Sindh Coast. The shrimps were identified using key by Tirmizi (1970), the collected samples were transported to the laboratory in an insulated box containing ice. In the laboratory, shrimps were stored at -40°C in deep freezer. The dissection were followed on the next day and samples were proceeded for histological studies; samples were fixed in 10% formalin, dehydrated by isopropanol, embedded in paraffin and 5 to 7 µm sections were obtained using rotary microtome. Standard H&E staining protocol was followed. The morphological and histological variations were studied under microscope fitted with a digital camera (Nikon SMZ800 and Nikkon trinocular Eclipis S5i). Spermatophores were obtained by pressing the ejaculatory duct. A sum of 30 specimens was dissected for morphological studies; total size range was 6.0 to 7.5 cm while the size range for carapace was 0.5 to 1.0 cm.

**RESULTS**

**Morphology**

The male reproductive system (Figures 1A and 2A) consists of two halves; each half is comprised of testis, vas deferens and ejaculatory duct (Figures 1A, 2A and 2D). The testes are milky white and un-pigmented (Figure 2C) located dorsal to the hepatopancreas under the carapace and comprises of two to three (mostly three) short, broad and flattened lobes on each side; commonly three lobes were found. The opening of testicular lobes is not visible and has to be traced down after displacing the testicular lobes (Figure 2C).

The vas deferens originates from the main axis of the testis and differentiated into four distinct parts: (Figure 2A), i) proximal vas deferens (PVD); (Figure 2B), ii) medial vas deferens (MVD), iii) distal vas deferens (DVD) and, iv) an ejaculatory duct (ED) (Figures 1A and 2A). The PVD is a convoluted mass made up of an extremely long, thin and greatly convoluted tube it roughly resembles the testicular lobes in appearance but the tubules are much broader than testis; the convoluted mass of PVD can easily be differentiated from the testicular lobes. The PVD contains elongated rod like spermatozoa in large numbers. The posterior part of PVD is continued into MVD (Figure 2B).

The MVD is broad, straight, somewhat flattened, curved upward and then bent down to form a curved portion (Figure 2B). It bears many folds internally; the folds can be seen clearly when empty (Figure 2B); whereas, it appears to be reflected over itself when filled with the spermatophores.

A small notch is found at the junction where MVD transforms into the DVD. No partitioning or transportation of non-spermatic mass was seen through PVD, whereas, it was tightly packed with small spindle shape spermatophores of variable sizes in mature specimen. The DVD (Figures 1A and 2D) is almost equal to MVD in diameter, though rounded in shape and not flattened and bears no fold as found in MVD.

The ED is the dilatation of DVD with a tubular enlargement, which opens to the exterior terminally (Figure 2D) with no septum or partition inside. Large numbers of spermatophores were extruded by pressing an ejaculatory duct. Figures 1D and 2E shows the ex-
Formation of spermatophores

From the testes, spermatozoa transferred to the anterior part of PVD which is the actual site where the sperms are arranged into rows and outer layer of the spermatophore is formed; the posterior part contained spindle shape spermatophores. The spermatophores inside the MVD are found into a more or less compact form but the spindle gets its perfect shape, when the spermatophores reached to the ED, the spermatozoa are arranged in perfectly regular rows in spermatophores present in ED.

Histology

The transverse sections of testicular lobes of *P. stylifera* reveal that it has a very thin transparent outer membrane and connective tissue septa dividing the testis into lobules. The larger cells are germ cells while smaller cells are glandular. In larger cells, the cell membrane is not very clear; these cells are termed as nutritive cells by King (1948). The nutritive cells were found at the peripheral portion of the tubules. No septum or partitioning of lumen or thphlosole was found. The most anterior part of PVD contains small irregular cells; whereas the posterior part bears the spermatozoa which are of variable sizes and shape. The MVD is lined with epithelial cells that may be glandular in nature and secretes some fluids to facilitate the transfer of spermatophores. No internal partition or septum is found in the lumen. The ejaculatory duct is lined with thick layer of muscle fibers. The lumen is oblong with many complete spermatophores floating in the seminal fluid (Figure 3A and 3B).

DISCUSSION

The basic division of vas deferens in *P. stylifera* has followed the same pattern found in other species of penaeid shrimps. On the basis of gross morphology, it is divisible into same four parts namely, PVD, DVD, MVD and ED though the shape and structure of different parts of vas deferens greatly varied (Sultana et al., 1994). In *P. stylifera*, the PVD is a convoluted and an extremely long and thin tube; the MVD is broad, straight, somewhat flattened with many internal membranous folds; the DVD is straight and cylindrical tube. The ED is a simple dilation with a tubular basal part tapered posteriorly for extrusion of spermatophores. Whereas, in species of genera *Fenneropenaeus*, *Litopenaeus*, *Penaeus* and *Melicertus*, PVD is small, straight and somewhat conical, MVD is broad and inverted u-shaped, DVD is thin and ED is a large muscular, 2-chambered, pear shaped or conical structure. In penaeid shrimps, the shape and the structure of vas deferens were found to be associated mainly with the shape of spermatophores. Among species of genera *Fenneropenaeus*, *Marsupenaeus*,

truded spermatophores which are minute and spindle shaped size varied from 0.148 to 0.161 mm; six to eight rows of fine striations were found on each spermatophore (Figure 2F), which are actually the sperms arranged in rows.
Figure 2. A) Male reproductive system of *Parapenaeopsis stylifera*; B) Median vas deferens, folds visible; C) corresponding magnification of testicular lobes, proximal, median and distal vas deferens; D) MVD filled with spermatic material; ED, ejaculatory duct; E) Spermatophores; F) corresponding magnification of spermatophore showing striations. DVD, distal vas deferens; ED, ejaculatory duct; MVD, Median vas deferens; PVD, proximal vasa deferens; SP, spermatophore; T, testicular lobes; TB, tubules.

*Litopenaeus, Penaeus* and *Melicertus*, one large complete spermatophore is found from each ED, which is associated with a non spermatic accessory structure called as wing. In open thelycum penaeids (for example, *Liopenaeus* spp.), the spermatophores are more complex and bears many accessory structures to cling the sper-
matophore on thelycum (Perez Farfante, 1969, 1975; Malek and Bawab, 1974); whereas, in closed thelycum penaeids genera, Fenneropenaeus and Penaeus, a large membranous wing is found.

In either type, the entire vas deferens was divided by a complete or partial internal septum into two ducts to separately process and transport the spermatic and non-spermatic materials. The partitioning was complete in PVD; whereas in DVD and ejaculatory duct, septum was partial (Malek and Bawab, 1974a, 1974b, Champion, 1987; Sultana et al., 1994). In such species, the cellular structure revealed through histological sections was also complex and contained glandular cells which usually formed a thick lining and or typhlosole in both of the ducts. In contrary to this, in P. stylifera, several thousand tiny spindle shaped spermatophores can be extruded form a single ED; further no accessory structure is associated with the spermatophore; hence, no internal longitudinal partitioning of vas deferens and ejaculatory duct was found. The long convoluted PVD and MVD with extensive folds may contribute to the compaction and arrangement of many small spermatozoa in regular rows into the spermatophores, which are extruded through an elongated tubular opening at the end of ejaculatory duct. The tubular enlargement of ejaculatory duct may also be considered as a functional adaptation to extrude a fluid containing spermatophores.

The structures of vas deferens and spermatophore both have a close homology of structures found in Trachypenaeus (Rimapenaeus) similis (Bauer and Min, 1993) than species of other penaeid genera for having convoluted PVD, an undivided vas deferens and numerous spermatophores suspended in spermatic fluid, though no spermatic plug was found.

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UPCOMING CONFERENCES

5th International Conference on Biotechnology and Food Science (ICBFS 2014), Erzurum, Turkey, 24 Apr 2014

7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014
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