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Clayton,  
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Molecular Mycology and Plant Phytopathology  
Department of Biology  
University of Isfahan  
Isfahan  
Iran

Dr. Beatrice Kilel  
P.O Box 1413  
Manassas, VA 20108  
USA

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National Cheng Kung University Medical College  
1 University road Tainan 70101,  
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Botany Department, Faculty of Science at Qena,  
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Department of Food Science & Technology,  
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Kenya

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USA

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Bioactivity of basil (Ocimum basicilum L.) on control of the spider mite (Tetranychus urticae Koch.) in peanut

Maria Isabel G. Martins¹, Antonio Euzébio G. Sant’Ana², Felipe Matheus T. Vasconcelos³, Wbyratan L. Silva², Liziane M. Lima⁴, Reginaldo Carvalho⁵*, Péricles A. Melo Filho³ and Roseane C. Santos⁴

¹Northeast Biotechnology Network RENORBIO UFRPE, Brazil.
²Chemistry Department, University Federal of Alagoas UFAL, Brazil.
³Agronomy Department, University Federal Rural of Pernambuco UFRPE, Brazil.
⁴Embrapa Algodão, Rua Osvaldo Cruz, 1143, Centenário, CEP:58428-095, Campina Grande, PB, Brazil.
⁵Federal Rural University of Pernambuco, Botany Graduate Program, 52.171-900, Dois Irmãos, Recife, Pernambuco, Brazil.

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Essential plant oils contain biopesticides that could be used to control many crop pests. Tetranychus spp. are mites that cause damage to several crops and are primarily controlled by synthetic pesticides. Literature showed that mites can be controlled with essential oils of plants containing eugenol. In this work, we evaluated the bioactivity of basil (Ocimum basicilum) accessions for peanut-spider mites control based on molecular, biochemical and agronomic assays. RNA from four basil accessions, previously chosen by divergence genetic analysis, were used to estimate the expression of eugenol synthase (EGS I) transcripts, by semiquantitative and polymerase chain reaction (qPCR) assays. Chromatography was, thereafter, performed in order to estimate the eugenol concentration. Feeding bioassays were performed using basil leaf extracts in order to estimate oviposition and mortality of spider mites females. Finally, a validation assay was carried out in greenhouse, using peanut plants previously infested with spider mites and weekly sprayed with basil water-extract. One basil accession, OVRS, revealed high phytotoxicity to spider mite females, at 15% water-extract. The mortality rate was 75% and complete inhibition of fecundity was found in BOD assays. In the greenhouse assay, the most severe damage due to mite infestations was found to plant height, number of pods and pod yield, which were reduced to 28, 53 and 52% in non-treated plants (control). Considering that basil is a short-cycle plant, with easy reproduction and management, these results represent an accessible alternative to organic control spider mites in peanut.

Key words: Basil, chromatography, eugenol, eugenol synthase, qPCR, spider mite.

INTRODUCTION

Synthetic pesticides are widely used to control pest crops due to high efficiency to plant protection. Despite this, the indiscriminate use of these products has led to several damages to mammals’ health such as neurological, respiratory and reproductive effects, and cancer and to increase pesticide resistance of insect-pests (Gill and
biopesticide activity of essential oils against crop pests have been widely reported (Isman, 2006; Lima et al., 2008; Cosimi et al., 2009; Sertkaya et al., 2010; Coitinho et al., 2011). Eugenol (4-hydroxy-3-methoxy-allyl-benzene) is a phenyl propanoid found in many species, such as cinnamon (Cinnamomum zeylanicum L.), croton (Codiaeum variegatum L.), bay leaf (Laurus nobilis L.), basil (Ocimum basilicum L.), myrrh (Commiphora myrrha Nees), nutmeg (Myristica fragmas Houtt.), pepper (Piper nigrum L.), sassafras (Ocotea odorifera Vell.), clove (Syzygium aromaticum L.), and others (Ueda-Nakanura et al., 2006; Mora et al., 2010; Wu et al., 2010; Tan et al., 2011). Clove fruits are known for containing high concentration of eugenol, between 80 and 95% (Escobar, 2011). Clove fruits are known for containing high concentration of eugenol, between 80 and 95% (Escobar, 2011). Clove fruits are known for containing high concentration of eugenol, between 80 and 95% (Escobar, 2011). Clove fruits are known for containing high concentration of eugenol, between 80 and 95% (Escobar, 2011). Clove fruits are known for containing high concentration of eugenol, between 80 and 95% (Escobar, 2011). Clove fruits are known for containing high concentration of eugenol, between 80 and 95% (Escobar, 2011).

MATERIALS AND METHODS

Germplasm and genetic diversity

Nine Brazilian basil accessions were used in this work (Table 1). In order to identify contrasting genotypes, a genetic analysis was performed based on Inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR). Seeds of each accession were seeded in pots containing soil previously fertilized (NPK, 10:10:10, ammonium sulfate, simple superphosphate and potassium chloride) and watered daily, in a greenhouse. After 30 days from emergence, young leaves were collected for DNA extraction (DNA Extraction kit Phytopure, GE Healthcare, U.S.A), following the manufacturer’s recommendations. PCR assays were performed in a 25 µL final volume containing 20 ng of each basil DNA, 1.5 µL of MgCl₂ (25 mM), 2.5 µL of 10× reaction buffer, 0.5 µL of dNTP mix (10 mM), 0.8 µL of each ISSR primer (10 mM, Table 2) and 0.3 µL of Taq DNA polymerase (Fermentas, Ontario, Canada, 5 U/µL). The program had one denaturation cycle at 94°C/5 min, followed by 30 cycles of denaturation at 94°C/30 s, annealing at 40°C/30 s and extension at 72°C/1 min. A final extension cycle was added at 72°C/5 min. The amplification products were stained with Blue Green Loading Dye (LG Biotechnology, Cotia, Brazil) and loaded in agarose gel (1%). The fragments were photographed (Bio-Imaging Systems – MiniBis Pro, Uniscience) for further genetic analysis. ISSR markers obtained from the accessions were scored for their presence ‘1’ or absence ‘0’ of bands for each primer. The binary data were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. Pair-wise similarity matrix was generated by Jaccard’s coefficient, by using NTSYS-pc (Rohlf, 1992). A dendrogram was constructed by using UPGMA method in order to identify the phenetic representation of accessions. The accuracy of clustering was evaluated by cophenetic correlation coefficient (CCC) and the significance of the groups was tested with 2,000 simulations. The GENES program (2013.0.5) was used for all analysis (Cruz, 2001).

*Corresponding author. E-mail: reginaldo.ufrpe@gmail.com.

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Expression of *EGS* I transcripts in basil accessions

Total RNA from leaves of each accession was extracted by using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany). Then, cDNA was synthesized (SuperScript III First-Strand Synthesis SuperMix for qRT-PCR, Invitrogen, Carlsbad, CA, USA) using 1 µg of each RNA. The reverse transcriptase was inactivated at 85°C for 5 min. Then, 2 U of RNase H was added to each sample and reactions were incubated at 37°C for 20 min. All procedures followed the manufacturer’s recommendations. To semiquantitative expression of *EGS I*, reactions were performed in a 25 µl final volume containing 2 µl of cDNA (1 µg), 0.04 U Taq Polimerase (Fermentas, Ontario, Canada), 0.2 mM dNTP set (10 mM), 1.5 µl MgoCl (25 mM), 1× kit buffer (10×) and 0.8 µl of each forward (ATGGAGAAATTTGATGAAAAGC) and reverse (GGCTCTTCTGTATCATGCTCTTCC) *EGS I* primers (10 mM), designed from eugenol synthase sequence (DQ372812.1), deposited on NCBI. The real time PCR reaction was made as follows: initial denaturation at 95°C/7 min, followed by 35 cycles of denaturation at 94°C/1 min, annealing at 56°C/1 min and extension at 72°C/2 min. A final extension was added at 94°C/5 min. As a constitutive control, a pair of forward (GATTTGTCATCTGACACTGGA) and reverse (GGCTCTTCTGATCATGCTCTTCC) *β-actin* primers (10 mM) was used. Both *EGS I* and *β-actin* primers were designed to amplify a fragment of 0.5 kb. The amplicons were analyzed in agarose gel (0.8%) and photodocumented.

The relative expression of *EGS I* transcripts was estimated by qRT-PCR (Eco Real-Time PCR System – Illumina, SD, USA) using Evagreen kit (Biotium Inc., Hayward, CA, USA), according to manufacturer’s instructions. The forward (GATTTGTCATCTGACACTGGA) and reverse (GGCTCTTCTGATCATGCTCTTCC) *EGS I* primers were used at 10 mM. A two-step RT-PCR procedure was performed in all experiments. First, 95°C/15 min and 40 cycles of 95°C/20 s, 60°C/20 s and 72°C/20 s were performed. Then, a curve of denaturation (melting curve) was performed after the conclusion of the amplification at 95 and 60°C/15 s, rising 2°C/min until reaching 95°C. Forward (TTGAGAAGCCGTATGAGCAAG) and reverse (ATCCTCCGATCCAGACTG) *β-actin* primers (10 mM) were used as a constitutive control. Both *EGS I* and *β-actin* primers were designed to amplify a fragment of 0.2 kb. All reactions were carried out with experimental triplicate and biological duplicate. The threshold cycle (Ct) and PCR efficiency was estimated by Real-time PCR Miner program (Zhao and Fernald, 2005). The analyses of gene expression were performed using the qBASEPlus program (Hellemans et al., 2007). The graphics, Cqs and Melt curves were automatically generated based on the normalization method with a reference gene, ΔΔCq (Livak et al., 2001). The expression pattern was estimated by relative quantification.

Chromatographic analysis of basil leaves

Fresh basil leaves from each accession were dehydrated and extracts were prepared using ethyl ether. Chromatographic analysis was performed with a flame ionization detector (Chromatograph gaseous Shimadzu 2010), equipped with RTX-1 capillary column (0.25 mm × 25 µm × 30 m). A volume of 2 µl was injected to each sample, with injector operating in splitless mode, using nitrogen as a carrier gas. The flow rate was maintained at 1 ml/min, with an initial programming of 50°C/5 min, increasing 6°C/min up to 250°C/10 min. The Kováts Index (KI) of samples was estimated by comparing with the retention time of alkane standards (C7 a C24), in the same chromatographic conditions described. The following equation was used: KI = 100n + 100 [(Rt - Rts)/(Rt0 - Rts)] where Rts is the retention time, n is the carbon number of previous alkane to Trx, and N = n+1. The quantification was performed with injection of eugenol patterns, with dilutions in hexane HPLC at 100, 200, 800 and 1500 ppb. From each pattern sample, 1.0 µl was injected and analyzed in same
Feeding bioassays with spider mite females

A *Tetranychus urticae* rearing was maintained in peanut plants (cv. BR 1), grown in greenhouse for further feeding bioassays. Spider mite females were fed on peanut leaves dipped into basil extract at different concentrations. Dehydrated basil leaves were diluted in ethanol (10 g/100 ml ethanol) and stored at RT for 24 h. Then, the extracts were filtered and concentrated on a rotary evaporator at 40°C to remove the solvent. The crude extract of each sample was diluted at 1, 5, 10 and 15%. Young peanut leaves (4 cm × 1.5 cm) were immersed for 10 s in each extract, dried on filter paper and placed in Petri plates (10 cm diameter) containing a polyethylene sponge and filter paper wetted in distilled water. Ten spider mite females were placed on each leaf. The plates were stored in BOD-growth chamber (Mod. BF2 CGFP 275, Biofoco, Brazil) at 25°C ± 1°C, relative humidity of 65 ± 10% and 12:12 h photoperiod. In control treatments, leaf discs were dipped into distilled water (control 1) and ethanol (control 2). The experimental design was completely randomized, with six treatments and four replications. Mortality rate and number of eggs were counted daily for 2 days (Siqueira et al., 2014).

Validation assay to control of peanut-spider mite in greenhouse

Seeds of peanut (cv. BR 1) were sown in pots (5 kg) containing clay loam soil previously fertilized (40 g P₂O₅ + 15 g KCl + 200 g of vegetal humus). Two plants were maintained per pot. Fifteen days after emergence, each plant was infested with 20 females of spider mites. Plants were watered daily. Ten days after infestation, plant canopies were weekly pulverized (20 ml/plant) with water-based extract of fresh basil leaves (10%), for 9 weeks, using an atomizer (1 L, mod. 78605/050, Tramontina, Brazil). The experimental design was completely randomized with six treatments and five repetitions. Control plants were sprayed with water (20 ml/plant). In the greenhouse, temperature was 35°C ± 2°C, relative air humidity 58% ± 5% and photoperiod 12:12 h during the assay.

**Statistical analysis**

For statistical analysis, we used the statistical software’s: R v2.14.1 (R Development Core Team, 2011), to verify the homogeneity of variances and normality of error; SISVAR v5.1 (Ferreira, 2007), for analysis of variance; and Scott-Knott to mean comparisons.

**RESULTS**

Genetic diversity of basil accessions

The polymorphism rate ranged from 50 to 91.6% as shown in Table 3. A rich band pattern was obtained, especially with UCB-813, UCB-824, UCB-868, UCB-834, UCB-853 and UCB-884 primers. Amplicons generated by UCB-813 and UCB-824 are shown in Figure 1. All PCR products were used to generate a similarity matrix for further clustering of accessions, which were represented by dendrogram in Figure 2. Four groups were formed by UPGMA method. Group 1 contained accessions OBA, OSJ, ODF, OCG and OPE, all of them are small green leaves, measuring from 1.8 to 3.3 cm length; groups 2 and 3 had only one accession each, OVRS and ORRS, with extra-large green and purple leaves, measuring 6.5 and 8.5 cm length, respectively; and group 4 had two accessions, OCE and OSP, both phenotypically similar for canopy and leaf traits. A panel with some morphological details of the accessions is shown in Figure 3. Based on clustering results, four accessions were chosen to represent the variability of basil genotypes and further use in molecular and biochemical assays: OVRS, OCG, ORRS and OSP.

Expression of *EGS I* transcripts by semiquantitative and qPCR assays

RNA from the four basil accessions selected by genetic analysis were used to estimate the expression of *EGS I* transcripts, based on semiquantitative and qPCR assays. *EGS I* transcripts were differentially expressed in all four basil accessions selected, as high expression in OVRS, median in OSP, and low in OCG and ORRS, based on the β-actin pattern shown in Figure 4A. These results were confirmed in qRT-PCR assays that estimated in 180× the level of *EGS I* transcript, from OVRS leaves shown in Figure 4B.

Quantification of eugenol in basil accessions by gas chromatography

Table 4A shows the standardization of eugenol by mass spectrometry based on retention time at different concentrations, and Table 4B shows eugenol concentrations from basil leaves in basil accessions, ranging from 12,635.6 ppb (OVRS) to 2,781.5 ppb (OCG), and

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**Table 3. Polymorphism for the basil accessions generated by ISSR primers.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>NB</th>
<th>NMB</th>
<th>PR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC – 813</td>
<td>22</td>
<td>7</td>
<td>68.2</td>
</tr>
<tr>
<td>UBC – 824</td>
<td>17</td>
<td>4</td>
<td>76.4</td>
</tr>
<tr>
<td>UBC – 827</td>
<td>8</td>
<td>4</td>
<td>50.0</td>
</tr>
<tr>
<td>UBC – 834</td>
<td>16</td>
<td>3</td>
<td>81.2</td>
</tr>
<tr>
<td>UBC – 853</td>
<td>14</td>
<td>4</td>
<td>71.4</td>
</tr>
<tr>
<td>UBC – 858</td>
<td>4</td>
<td>2</td>
<td>50.0</td>
</tr>
<tr>
<td>UBC – 868</td>
<td>17</td>
<td>5</td>
<td>70.6</td>
</tr>
<tr>
<td>UBC – 884</td>
<td>12</td>
<td>1</td>
<td>91.6</td>
</tr>
<tr>
<td>UBC – 892</td>
<td>10</td>
<td>1</td>
<td>90.0</td>
</tr>
</tbody>
</table>

NB - Number of bands, NMB - Number of monomorphic bands, PR - Polymorphism rate.
confirming that OVRS is different from other basil accessions selected in this study. The chromatograms of basil accessions are shown in Figure 5. No overlap peaks were observed, indicating a clear separation of the different compounds contained in the extracts analyzed. The highest peaks refer to eugenol concentrations in each sample. Only in OGC extract (Figure 5) a complex of compounds was observed, some of them with concentrations higher than eugenol.

**Bioactivity of basil extract against spider mites in BOD**

Leaf-ethanol extracts of basil accessions were used in feeding bioassays in order to evaluate the effect of eugenol in oviposition and mortality of female spider mites. It was found that the number of eggs and mortality rate of females were concentration-dependent. Data were fitted to a linear regression model shown in Figure 6. The extract obtained from OVRS leaves at 15% showed high phytotoxicity to females, with 75% of mortality rate and complete inhibition of fecundity. In previous bioassays using leaf-water extracts at 15%, the mortality rate of female spider mites were situated in 35% (OCG, OSP, ORRS) and 53% (OVRS). These results indicate that the organic components present in basil leaves were more soluble in ethanol.

**Validation of spider mites control with basil leaf extract**

Based on previous results obtained in molecular, biochemical and entomological assays, basil water-extract at 10%, from OVRS accession, was chosen to
**Figure 3.** Morphological details of basil accessions used in this work. A - OSP, B - ODF, C - OSJ, D - OCG, E - OBA, F - OCE, G - OPE, H - OVRS, I - ORRS.

**Figure 4.** Semiquantitative (A) and relative (B) expression of *EGS I* transcripts in basil accessions. M-1kb DNA ladder (Invitrogen). Graphic generated by Eco Real-Time PCR System software (Illumina) from ΔCq and Melt curve data based on the β-actin normalization method.
validate trail against spider mites in peanut plants. It was found that the characteristic symptoms of spider mites in peanut plants were seen soon after one week of infestation. Damages by mites first involved chlorotic areas on leaflets, advancing to plant’s death at the end of the life cycle, when high mite populations completely covered the canopy of plants involved in silk webbing. Figure 7 shows the performance of plants in control and treatment (basil-water extract), during three phenological phases, and pod production, at harvest. In general, the spreading of mites on basil-treated plants was slow until the seed formation period, which occurs between 60 and 65 days from planting in early genotypes. In same occasion, control plants were completely infested by mites, showing a severe reduction in photosynthetic area, at 85 days from planting. These plants showed senescence and leaf shedding, followed by wilting and plant death due to high level of infestation. In basil-
Figure 6. Number of eggs (left) and mortality rate (right) of female spider mites fed on peanut leaves dipped in leaf-ethanol extracts of basil for 48 h. A- OCG, B- ORRS, C- OVRS; D- OSP. Treatments: 1- control 1 (water), 2- control 2 (ethanol), 3 to 6-basil ethanol extract at 1, 5, 10 and 15%. The goodness of fit ($P < 0.001$) is found to each accession.

Figure 7. Performance of peanut plants infested with spider mite in greenhouse. A. Plants treated with basil-water extract at 10%; B. Plants treated with water (control).

treated plants, the spreading of mites was increased just from 80 to 85 d, but it did not commit pod production in these plants because maturation of pods was already established. The pod and seed weights, number of
Table 5. Growth and production data of peanut plants treated with basil water extract, in a greenhouse.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HMA (cm)</th>
<th>B (dae)</th>
<th>NP</th>
<th>100 PW (g)</th>
<th>100SW (g)</th>
<th>S/P</th>
<th>PL (mm)</th>
<th>PWP (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil extract</td>
<td>39^a</td>
<td>22^b</td>
<td>19^a</td>
<td>86^a</td>
<td>45^a</td>
<td>3.3^a</td>
<td>3.6^a</td>
<td>16.5^a</td>
</tr>
<tr>
<td>Control</td>
<td>28^b</td>
<td>25^a</td>
<td>10^b</td>
<td>84^a</td>
<td>45^a</td>
<td>3.4^a</td>
<td>3.4^a</td>
<td>8.5^b</td>
</tr>
<tr>
<td>Mean</td>
<td>33</td>
<td>23</td>
<td>14</td>
<td>85</td>
<td>45</td>
<td>3.3</td>
<td>3.5</td>
<td>12.3</td>
</tr>
<tr>
<td>CV (%)</td>
<td>11.5</td>
<td>12.1</td>
<td>9.1</td>
<td>8.2</td>
<td>6.5</td>
<td>8.6</td>
<td>7.2</td>
<td>9.7</td>
</tr>
</tbody>
</table>

HMA- Height of main axis at 85d, B- blooming, NP- number of pods/plant, 100PW- 100 pods weight, 100SW- 100 seeds weight, S/P- number of seeds/pod, PL- pod length, PWP- pod weight/plant. Means with same letter are not statically different by Scott-Knott test (p<0.05). Control treatment was water. CV- coefficiente of variation.

seeds/pod and pod length were not influenced by the spider mite infestation, in both control and basil-extract treatments (Table 5). The blooming was slightly delayed in control plants. The most severe damage due to mite infestations was found to plant height, number of pods and pod yield, which were reduced to 28, 53 and 52% in non-treated plants (control).

DISCUSSION

Spider mites are hosted by several commercial crops including soybean, peanuts, bean, corn, cotton and others, causing damage up to 70% at the commercial production of crops, depending on period of infestation (Haile and Higley, 2003; Oliveira and Moreira, 2009; Esteves Filho et al., 2010; Boubou et al., 2011). The spreading is fast, destroying photosynthetic area of plants, leading to dwarfism, shedding of leaves and fruits, and further losses in production. Despite the genetic improvements and tolerance of the new cultivars to spider mites, pesticides are still necessary as a control method. Therefore, the use of synthetic pesticides is still the main means of control, in spite of potential damage to humans and environment (Copping and Duke, 2007). Studies have shown that spider mites have a great ability to develop resistance to pesticides (Herron and Rophail, 2003; Khajehali et al., 2011). Considering the risks that it presents to agriculture, other strategies must be encouraged, so that the control could be more accessible and secure (Alabouvette et al., 2006). The literature offers a large number of articles reporting the biopesticide potential of extracts and essential oils from several plant species. The adoption of these products could contribute to reduction of management costs, and also environmental risks and dependency of synthetic pesticides (Mazzonetto and Vendramim, 2003; Lima et al., 2008).

The Ocimum genus is known for its aromatic and pesticide properties. The species are herbaceous or bushes, with an appreciable aroma in leaves and stems due to the components of essential oils, such as eugenol, methyl-chavicol, methyl-eugenol, saffron, geraniol, thymol, linalool, among others (Blank et al., 2005). Eugenol has been reported as a main oil component. Basil is a short cycle plant, spread by seed or branches, and commercially used in culinary, cosmetics and as insect repellents. Several articles have demonstrated the role of extract and essential oil from basil to insect and pathogen controls, most of them focusing on assays to control temperature and photoperiod using BOD growth chamber. In the present work, molecular, biochemical and agronomic approaches were adopted in order to validate the use of basil to control spider mites in peanut plants (Pasay et al., 2010). Molecular analysis focused on EGS I, a precursor enzyme of eugenol biosynthesis.

Semiquantitative and relative expressions of EGS I transcripts revealed genotype-dependence response in basil accessions evaluated. In chromatography assays, eugenol was confirmed as the main oil component, with different peaks between accessions, confirming the RT-PCR findings. Ethanol-extract from four contrasting basil accessions, identified by genetic analysis, was used in feeding bioassays using spider mite females. Extract obtained from OVRS at 15% inhibited completely the reproduction of the spider mite eggs while promoting a mortality rate at 75%. This result is promising, however, considering the ability of spider mites to acquire tolerance to pesticides (Van Leeuwen et al., 2010; Khajehali et al., 2011), a validation trial was carried out in order to certify the results in natural condition. Peanut plants were previously infested with spider mites and further sprayed weekly with water-basil (OVRS) extract at 10%, during 9 weeks. Basil extract contained the mites spreading, maintaining the pod production in satisfactory level. It is possible that the population of spider mites in peanut plants from 85d were minor, if we had used a higher concentration of basil-extract than that adopted in this work. However, as spreading of mites happened from 7th spraying, at full pod maturity phase of cv BR 1, it is suggested that basil-extract at 10% is an adequate concentration to earliness peanut cultivar (Vasconcelos et al., 2015; Santos et al., 2010). The result presented here has great potential use of basil leaf extract in mite control. The next step of the research is confirmation that eugenol is the main oil component essential through the isolation and identification of this active compound responsible for the control of insecticide.
Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviation

PCR, Polymerase chain reaction; ISSR, inter simple sequence repeat; UBC, University of British Columbia; UPGMA, unweighted pair group method to obtain an arithmetic mean; NCBI, National Center for Biotechnology Information; RT, room temperature; BOD, biochemical oxygen demand; DAE, days after emergence.

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Bioethanol production from cassava peels using different microbial inoculants

Obianwa Chibuzor1, Edak A. Uyoh1* and Godwin Igile2

1Department of Genetics and Biotechnology, University of Calabar, Cross River State, Nigeria.
2Department of Biochemistry, University of Calabar, Cross River State, Nigeria.

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The potential of bioethanol production using different microbial inoculants for the simultaneous saccharification and fermentation of cassava peels from three cassava cultivars was investigated. Peels obtained from three cassava cultivars namely TME 0505, TME 419 and TME 4779, were washed, dried in a laboratory air oven dryer at 120°C for 3 h, ground into a fine texture and sieved with 1.5 μ nylon sieve. The sieved material was cultured using the following inoculant combinations: A = Rhizopus nigricans + Saccharomyces cerevisiae; B = Aspergillus niger + Saccharomyces cerevisiae; C = Rhizopus nigricans + Aspergillus niger + Saccharomyces cerevisiae; D = Rhizopus nigricans + Spirogyra africana + Saccharomyces cerevisiae; E = Aspergillus niger + Spirogyra africana + Saccharomyces cerevisiae. These combinations have not been tested before on cassava peels. The control was inoculated with S. cerevisiae only. The cultures were distilled on the 21st day and the quantity of ethanol produced in each treatment group recorded. Results obtained showed significant differences (P<0.05) in the amount of ethanol produced and in its concentration among the five inoculants. Significant differences (P<0.05) were also obtained in ethanol yield from the three cassava varieties. Cassava peels from TME 4779 gave the highest ethanol yield of 14.46 ± 2.08 g/cm³ using R. nigricans + S. africana + S. cerevisiae. Similarly, cassava peels from TME 0505 gave the second highest ethanol yield of 13.33 ± 0.67 g/cm³ using the same combination, namely R. nigricans + S. africana + S. cerevisiae. Low ethanol yields of 4.82 ± 1.00, 6.43 ± 0.58 and 7.77 ± 0.88 g/cm³ were obtained from the cassava peels of TME 419, TME 0505 and TME 4779, respectively using S. cerevisiae alone. The yield reported in this study competes favorably with those reported from cassava peels, potato peels and millet husks using other inoculant treatments by other workers. Inoculants used in this study thus showed great potential for bioethanol production from cassava peels.

Key words: Bioethanol, cassava peels, microbial inoculants.

INTRODUCTION

The quest by many countries for energy independence as well as the widespread awareness of the need to reduce...
green-house gas emissions have heightened the search for alternative energy sources (Farrell et al., 2006). They have also served as drivers for new government initiatives to increase alternative fuel sources, principally ethanol from biological feed stocks such as cassava (*Manihot esculentum*), corn (*Zea mays*) and sweet potato (*Ipomoea batatas*). Biofuels are expected to reduce dependence on imported petroleum with associated political and economic vulnerability, reduce greenhouse gas emissions and other pollutants, and revitalize the economy by increasing demand and prices for agricultural products (Balat 2009). There is thus an increasing demand for bioethanol as alternative source of energy and Nigeria currently depends on the importation of ethanol to meet its local demand.

In Nigeria and many developing countries, there is a growing interest in the conversion of the huge biomass of organic wastes generated by the food processing sector and other human endeavors into useful products such as ethanol. A number of studies have been carried out in an attempt to optimize the yield of ethanol from cassava peel using different organisms including *Saccharomyces cerevisiae* (Adesanya et al., 2008; Marx and Nquma, 2013), *Zymomonas mobilis* and *S. cerevisiae* (Sufahri et al., 2011) *Gloeophyllum sepiarium* plus *Pleurotus ostreatus* for hydrolysis and *Z. mobilis* and *S. cerevisiae* for fermentation (Oyeleke et al., 2012; Adiotomre, 2015), *Aspergillus niger* for hydrolysis and *S. cerevisiae* for fermentation (Adetunji et al., 2015). The search is still ongoing. Odunfa and Olanbiwoninu (2012) recommended that cassava peels could be subjected to pretreatment with dilute sulphuric acid or methanolysis prior to fermentation for higher ethanol content. The present study was thus aimed at contributing to this ongoing effort by using new combinations of microorganisms (*A. niger, Rhizopus nigricans* and *Spirogyra africana*) in the combined saccharification and fermentation process to produce ethanol from the peels of cassava. To the best of the authors’ knowledge, this combination has not been tried before for this purpose.

**MATERIALS AND METHODS**

**Cassava cultivars and microorganisms**

Three cultivars of cassava identified as TME 97/ 0505, TME 419 and TME 92/4779 were obtained from National Roots Crops Research Institute (NRCRI) at Umudike, Abia State, Nigeria. The microorganisms used in this study were *A. niger, R. nigricans, S. cerevisiae* (bakers’ yeast) and the microalgae *S. africana* obtained from Mr. U. A. Offor, a Microbiologist in the Department of Microbiology, Cross Rivers State University of Science and Technology, Calabar, Nigeria.

**Preparation of broth culture of *A. niger* and *R. nigricans***

Broth cultures of *A. niger* and *R. nigricans* were prepared in 100 ml of potato dextrose broth medium using standard methods as described by Baker et al. (2001).

**Preparation of peels from cassava cultivars**

The three cassava cultivars were hand peeled using a table knife. The peels were washed under running tap to remove sand and other impurities, oven-dried at 120°C for 4 h in a laboratory air-doven dryer, milled into a powder (flour) using locally made milling machine and sieved with 1.5 µ nylon sieve. The flour was packed into sterile plastic containers, sealed and labeled accordingly.

**Simultaneous saccharification and fermentation**

Fifty grams of the sieved cassava peel flour from each of the three cultivars, was dissolved in 500 ml of distilled water in separate conical flasks. For each cultivar, this was replicated six times giving a total of 18 flasks in all. The flasks were plugged with sterile cotton wool, shaken thoroughly and autoclaved for 15 min at 120°C (Adesanya et al., 2008). The six flasks of each cultivar were inoculated with the following respectively: 1. 5 ml *A. niger + 2 g S. cerevisiae*; 2. 5 ml *R. nigricans + 2 g S. cerevisiae*; 3. 5 ml *A. niger + 5 ml R. nigricans + 2 g S. cerevisiae*; 4. 5 ml *A. niger + 1 g S. Africana + 2 g S. cerevisiae*; 5. 5 ml *R. nigricans + 1 g S. Africana + 2 g S. cerevisiae*; 6. 2 g of *S. cerevisiae*.

The mixture in each conical flask was sealed with aluminum foil and kept for twenty-one (21) days under anaerobic conditions and temperature of 28°C. Thereafter, the samples were filtered with Whatman No.4 filter paper and 30 ml of the filtrate was distilled at 78°C (standard temperature for ethanol distillation). This was done for each fermented sample.

**Determination of quantity of ethanol produced and data analysis**

The volume of the distillate collected was determined using a measuring cylinder and expressed as quantity of ethanol produced in g/cm³ by multiplying the volume of the distillate by the density of ethanol (0.8033 g/cm³) (Humphrey and Okofoagu, 2007). Means ± standard errors were obtained and subjected to analysis of variance tests. Significant tests were separated using least significant difference tests.

**Determination of ethanol concentration**

Ethanol concentration (v/v) was determined by extrapolation using the absorbance of ethanol obtained from the standard ethanol concentration curve. The standard ethanol curve was obtained according to the methods of Oyeleke and Jubril (2009).

**RESULTS AND DISCUSSION**

Analysis of variance showed a significant difference (P<0.05) in the yield (g/cm³) and the percentage concentration yield obtained amongst the inoculants and varieties of cassava. Inoculum D (*R. nigricans + S. Africana + S. cerevisiae*) consistently produced the highest volume yield in all the three cultivars while *S. cerevisae* (control) produced the least in all three cultivars.
Table 1. Ethanol yield (g/cm³) from the three cassava cultivars treated with different inoculant.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>TME 419</th>
<th>TME 0505</th>
<th>TME 4779</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.57b ± 0.67</td>
<td>10.18a ± 0.88</td>
<td>9.37b ± 0.88</td>
</tr>
<tr>
<td>B</td>
<td>8.57b ± 0.88</td>
<td>9.37a ± 1.46</td>
<td>10.71b ± 1.46</td>
</tr>
<tr>
<td>C</td>
<td>8.84b ± 1.46</td>
<td>11.00b ± 1.00</td>
<td>11.51b ± 1.20</td>
</tr>
<tr>
<td>D</td>
<td>12.59a ± 0.88</td>
<td>13.33a ± 0.67</td>
<td>14.46a ± 2.08</td>
</tr>
<tr>
<td>E</td>
<td>10.98a ± 1.45</td>
<td>13.00a ± 1.00</td>
<td>10.71b ± 1.46</td>
</tr>
<tr>
<td>Control</td>
<td>6.43b ± 0.58</td>
<td>4.82b ± 1.00</td>
<td>7.77b ± 0.88</td>
</tr>
</tbody>
</table>

A = Rhizopus nigricans + Saccharomyces cerevisiae; B = Aspergillus niger + Saccharomyces cerevisiae; C = Rhizopus nigricans + Aspergillus niger + Saccharomyces cerevisiae; D = Rhizopus nigricans + Spirogyra africana + Saccharomyces cerevisiae; E = Aspergillus niger + Spirogyra africana + Saccharomyces cerevisiae; control = Saccharomyces cerevisiae. Means followed by similar case letters in each column are not significantly different (P<0.05).

Figure 1. Percentage ethanol concentration (purity) from the three cassava cultivars treated with different inoculants. A = Rhizopus nigricans + Saccharomyces cerevisiae; B = Aspergillus niger + Saccharomyces cerevisiae; C = Rhizopus nigricans + Aspergillus niger + Saccharomyces cerevisiae; D = Rhizopus nigricans + Spirogyra africana + Saccharomyces cerevisiae; E = Aspergillus niger + Spirogyra africana + Saccharomyces cerevisiae; control = Saccharomyces cerevisiae.

as shown in Table 1. Cassava TME 4779 gave the highest concentration of 41% (v/v) when treated with inoculum E (A. niger + S. africana + S. cerevisiae) as shown in Figure 1. Fermentation results obtained on the 21st day from the three cassava varieties using five inoculants and control are shown in Table 1.

The production of bioethanol from cassava peels using different combinations of microorganisms was examined.
The microorganisms expectedly produced different amylolytic enzymes and to different levels which acted on the peels from the three cassava cultivars. The highest ethanol yield of 14.46 g/cm³ was obtained from cassava cultivar TME 4779 and a concentration of 38% (v/v) when treated with *R. nigricans* + *S. africana* + *S. cerevisiae*. This could be attributed to the presence of more carbohydrates from *Spirogyra* which is fermented to ethanol in the presence of the amylolytic microorganisms. *Spirogyra* generally is known to be autotrophic and its carbohydrate composition can also lead to increase in the release of sugars for fermentation. This result is in line with the work of Sulfahri et al. (2011) but gave a higher yield because of the presence of cassava peel substrate and good pH conditions. Sulfahri et al. (2011) obtained 9.70% of ethanol from *Spirogyra* with fermentation by *Zymomonas mobilis* and *S. cerevisiae* after 96 h (4 days). The present result is also higher than that obtained by Asif et al. (2015), who obtained 9.3 (v/v) and 8.3% (v/v) of ethanol from sugarcane molasses using *Z. mobilis* and *S. cerevisiae*, respectively. It is comparable with the report by Adiotomre (2015) of 23% ethanol from 50 g of substrate using *Gloeophyllum sepium* and *Pleurotus ostreatus* for hydrolysis of the peels and *Z. mobilis* and *S. cerevisiae* for fermentation.

Other microbial combinations such as *A. niger* + *S. cerevisiae*, as well as *R. nigricans* + *S. cerevisiae* also gave relatively high yields of 10.71 and 10.18 g/cm³, respectively. This is slightly higher than the report of Oyeleke et al. (2012), whose study gave 10.6 g/cm³ when *Z. mobilis* and *S. cerevisiae* were used to ferment cassava peels. The similarities can be ascribed to the enzyme content of *A. niger* and *R. nigricans*, both organisms are known to contain enzymes such as α-amylase, glucoamylase and cellulase necessary for the breakdown of the complex cellulose composition of cassava peels (Akpan et al., 1996). The average percentage concentration of ethanol obtained in the present study is relatively high as compared to the average yield reported from spoiled mangoes by Agulejika et al. (2005). They reported an average ethanol concentration yield of 16%. This is likely to be due to the presence of more carbohydrate content in cassava peels than in spoiled mangoes. The present report is also higher than the 8.5% given by Adetonji et al. (2015) using *A. niger* and *S. cerevisiae* on cassava peel slurry. On the other hand, the percentage concentration of ethanol obtained in the present study is much lower than reports by Oyeleke and Jubrin (2009) of 67.7 and 63.8% when *A. niger* and *Z. mobilis* were used simultaneously on guinea corn husk and millet husk, respectively. It is also lower than the 83% yield reported by Sivamani and Baskar (2015) in cassava peel using a saccharification and fermentation mixture containing glucoamylase and *Z. mobilis* with optimum conditions of 69.82 g/l substrate concentration, 24.74% (v/v) α-amylase concentration and 5.22% saccharification and fermentation mixture. Sometimes, the differences in ethanol yield may be attributed to the actual amount of carbohydrate present in the peel at the start of the experiment.

*S. cerevisiae* also known as baker’s yeast has been successfully grown on several substrates like molasses, cashew and apple juice for the production of single-cell protein and bioethanol. It is used commercially for the fermentation of glucose to ethanol and it is known for its high tolerance to ethanol, rapid fermentation rates and insensitivity to substrate concentrations (Linden and Hahn-Hagerdal, 1989). Ethanol yields as high as 65.27% have been reported from hydrolyzed pineapple peel using *S. cerevisiae* TISTER 5048 (Niwaswong et al., 2014). It has, however, been reported to be a non-amylolytic microorganism, unable to hydrolyze starch (Jamai et al., 2006). Varying concentrations of ethanol ranging from 4.82 to 7.77 g/cm³ were obtained in this study. Similarly, Ashok et al. (2014) obtained ethanol concentration of 7.95% (v/v) from sweet potato using *S. cerevisiae* MTCC-170. This shows that *S. cerevisiae* has the ability of producing ethanol from starch but at a low rate.

**Conclusion**

This study showed that the combination of *R. nigricans*, *S. africana* and *S. cerevisiae* may be the most suitable for production of ethanol from cassava peels. The study also suggests that the choice of cassava cultivar also plays a role in the optimum production of ethanol with cassava cultivar TME 4779 giving the highest yield.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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Evaluation of ethanol production from pito mash using *Zymomonas mobilis* and *Saccharomyces cerevisiae*

Ofosu-Appiah C.1*, Zakpaa H. D.1, Mak-Mensah E.1 and Bentil J. A.2

1Department of Biochemistry and Biotechnology, College of Science, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
2Department of Chemical and Biochemical Engineering, Center for Bioprocessing Engineering, Technical University of Denmark, Lyngby, Denmark.

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This study investigated the potential of pito mash (waste from sorghum brewing) as alternative and cost-effective feedstock for bioethanol production by means of fermentation using *Zymomonas mobilis* and *Saccharomyces cerevisiae* isolated from freshly tapped palm wine. Fermentation parameters such as pH, temperature and incubation period were studied. The fermentation microbes, *Z. mobilis* and *S. cerevisiae* were identified using API™ test kit and morphological characteristics, respectively. Analysis of reducing sugar residue was performed using dinitrosalicylic acid (DNS) method, while analysis of ethanol content was performed using gas chromatography. Pito mash recorded total starch content of 6.69%, reducing sugar content of 11.1 mg ml⁻¹ and cellulose content of 0.41 mg g⁻¹. Saccharification by malting increased reducing sugar content by 77.9% (19.75 mg ml⁻¹). The optimum fermentation conditions (pH, temperature and incubation period) for *Z. mobilis* and *S. cerevisiae* were 5.5, 35°C, 3 days and 6.0, 30°C, 4 day, respectively. The maximum ethanol yield of 3.03 g l⁻¹ and efficiency of 62% were obtained for *S. cerevisiae* while yield of 3.63 g l⁻¹ and efficiency of 74.2% were obtained for *Z. mobilis*. *Z. mobilis* conclusively may be better organism for ethanol production from pito mash.

**Key words:** Pito mash, agro-industrial wastes, *Zymomonas mobilis*, ethanol, reducing sugars.

**INTRODUCTION**

Energy consumption has increased steadily over the last century as the world’s population increases, and more and more countries become industrialized. The traditional source of fuel, fossil fuel is continuously being depleted irrespective of the new geographical discoveries. There have also been concerns about the pollution and various health risks associated with the use of petroleum as fuel. In view of these, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of the limited fossil fuel stock but also for safe and better environment (Chandel et al., 2007). The interest in biomass as the alternative source...
of energy is gaining momentum more and more over the last century. Production of bioethanol from biomass is one of the alternative sources of fuel that has gained a lot of attention over the past years. Ethanol produced from renewable energy source is the most promising future biofuel (Marszalek and Kaminski, 2008). To be a viable substitute for a fossil fuel, Hill et al. (2006) contended that an alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, and be producible in sufficient quantities to make a meaningful impact on energy demands, but it should also provide a net energy gain over the energy sources used to produce it. Bioethanol meets most of these criteria but the quantity of ethanol produced annually has not overtaken petroleum. The use of starchy crops such as cereals and tubers as substrates for the production of ethanol has however, been reported to be much costly and non-sustainable accounting for about 70% of ethanol production (Ramesh et al., 2004). This drawback has caused an emergence of alternative substrates such as agro-industrial wastes that are relatively abundant in the environment and serve as renewable energy resources. Some of these agro-industrial wastes are rice straw, sugarcane bagasse, corn cob, pito mash, rice husk, municipal wastes to mention a few (Ramesh et al., 2004). The problem, however, is that the technology for conversion of the lignocellulosic part of these materials to bioethanol. The choice of the best technology for the conversion of lignocellulosic to ethanol should be decided on the basis of overall economics (lowest cost), environmental (pollutants), and energy (higher efficiencies). Many investigations have been performed on the appropriate technology for the conversion of the lignocellulosic to ethanol as well as substrate with little or no lignin such as molasses (Chandel et al., 2007), but research work on the utilization of pito mash has been very limited.

Even though several microorganisms, including Clostridium species, have been considered as ethanologenic microbes, the yeast Saccharomyces cerevisiae and facultative bacterium Zymomonas mobilis are better candidates for industrial alcohol production (Castro, 2013). Traditionally, S. cerevisiae has been used for the production of ethanol; however, it has been associated with low alcohol tolerance and low productivity which for efficient ethanol production requires improvement. Z. mobilis, a gram negative bacterium possesses advantages over S. cerevisiae with respect to ethanol productivity and tolerance. Z. mobilis strain grown under anaerobic conditions can produce about 1.5 to 1.9 mol of ethanol from each mol of glucose, which is much better than ethanol produced by S. cerevisiae (Gonzalez-Sanchez et al., 2011). Zymomonas grows and ferments glucose very fast; its preference for low pH prevents contamination and grows in high glucose and ethanol concentration (He et al., 2014). The present study focuses on the potential use of pito mash as a substrate for both Z. mobilis and S. cerevisiae for bioethanol production under optimum fermentation conditions such as temperature, pH and incubation periods.

MATERIALS AND METHODS
Sample collection
The substrate for the fermentation, pito mash was obtained from local pito brewers in Kumasi in the Ashanti Region of Ghana.

Standard solid media
Five hundred milliliters of standard media was prepared by dissolving 2.5 g of yeast extract and 10 g of glucose in 500 ml conical flask containing 150 ml distilled water. Exactly 10 g of agar was then added, made up to mark and autoclaved at 121°C for 15 min.

Synthetic media
Synthetic media containing g/L: K2HPO4 1.0 g, (NH4)2SO4 1.0 g, MgSO4 0.5 and 20 g glucose was prepared in 1 L volumetric flask with 750 ml of distilled water, made up to mark and autoclaved at 121°C for 15 min.

Malt yeast peptone glucose media
Five hundred milliliters of MYPG was prepared by dissolving 1.5 g of yeast extract, 2.5 g peptone, 1.5 g malt extract, and 10 g of glucose in 500 ml conical flask containing about 150 ml distilled water. Exactly 10 g of agar (melted) was added and made up to mark and autoclaved at 121°C for 15 min. The pH of each medium was adjusted to the appropriate pH values using 1 N NaOH and 1 N HCl.

Sorghum base medium (SBM)
The SBM was prepared substituting glucose and malt extract with pito mash in the MYPG medium.

Isolation and identification of microorganisms
Z. mobilis was isolated from palm wine using the method of Cheesbrough (2003). The inoculated plates were incubated at 30°C for 3 days. Pure colonies were obtained from re-cultivation in MYPG agar. Purified isolates from fresh plates of MYPG medium were identified as Z. mobilis using API™ test kit. S. cerevisiae was serially diluted and the sediment inoculated in standard media supplemented with chloramphenicol (0.05 mg/L) (Nwachukwu, 2001) and incubated at 28°C for 24 h. Colonies suspected to be yeast were purified and morphological and fermentable sugars were determined.

Malting and mashing of sorghum grains
Sorghum cultivar grains were washed and steeped in 0.2% sodium hydroxide and water for 8 and 16 h, respectively at room
temperature. The steeped grains were allowed to germinate at room temperature for 4 days with daily sprinkling of water. The rootlets were broken and kept in oven for 24 h at 50°C. In the mashing process, a mixture of 20% malt and 80% mash was slurred and pre-heated to 45°C for 30 min after being raised to 100°C for 1 h. Complete saccharification was determined by iodine test.

Fermentation process

The procedures were adopted from Dow and McMillan (2008). Fermentation was carried out in 500 ml Erlenmeyer flasks. The fermentation lock or bubble trap consisted of rubber stopper (with hole) through which a tube was inserted. A cotton plug was inserted in the tube and the tube was connected to silicone tubing. A fermentation lock or bubble trap consisted of rubber stopper (with hole) through which a tube was inserted. A cotton plug was inserted in the tube and the tube was connected to silicone tubing. The other end was submerged in test tubes containing water. All mashers were cooled to a temperature between 27 and 30°C after liquefaction and saccharification and the pH adjusted with HCl. Saccharified mash was then inoculated with 10 ml pre-culture S. cerevisiae and Z. mobilis in separate setups. Fermentation was performed in an incubator with intermittent shaking at optimized conditions. The fermentation process was monitored by measuring the sugar and ethanol contents.

Ethanol concentration was estimated by Gas Chromatography and sugar content was measured using 3,5-dinitrosalicylic acid (DNS) method. The expected ethanol amount was calculated after fermentation stoichiometry, assuming that 1.0 g of total sugars produced 0.511 g of ethanol. The efficiency of reducing sugar conversion into ethanol by both microorganisms (%) expresses the amount of produced ethanol relative to the theoretical quantity expected based on the sugar content of the malted sorghum, and it was calculated accordingly with the following equation:

Efficiency (%) = Ethanol produced (g/l) / [TRSi – TRSi] × 100

where TRSi is the initial sugar content before fermentation and TRSi is the final sugar content after fermentation (Carter, 2014)

Optimization of pH

Ten milliliters of the mashed sorghum was placed in different test tubes and pH adjusted to ranges of 4.0, 4.5, 5.0, 5.5 and 6.0 using 1 N HCl or 1 N NaOH.

Optimization of temperature

Ten milliliters of the mashed sorghum was placed in different test tubes at varying initial temperature values of 30, 35, 40 and 45°C.

Optimization of incubation period

Ten milliliters of the mashed sorghum was placed in different test tubes and incubated at 30°C, pH 4.5 and periods of 24, 48, 72, 96 and 120 h.

Chemical analysis

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) using methods described by Negrulescu et al. (2012). Ethanol concentration was determined using a Perkin Elmer, Autosystem XL, Gas Chromatograph (USA) equipped with a flame ionization detector (FID), coupled to a Yokogawa 3021 Pen recorder.

RESULTS AND DISCUSSION

Ethanol produced from spent sorghum using S. cerevisiae and Z. mobilis separately

S. cerevisiae and Z. mobilis were employed in fermenting pito mash hydrolysate containing 19.75 g ml⁻¹ reducing sugar. In both organisms, a continuous increase in ethanol yield was accompanied with decreased reducing sugar concentration during the whole period of fermentation (Figures 1 and 2). The fermentation with Z. mobilis proceeded very rapidly and was essentially completed in three days with maximum yield of 3.63 g l⁻¹. Fermentation with S. cerevisiae required three days to complete with a yield of 3.03 g l⁻¹. In all cases, the sugar utilization was faster in Z. mobilis than in S. cerevisiae. T-test analysis showed significant difference between the amounts of sugar utilized by Z. mobilis and S. cerevisiae on each day at 95% confidence interval. This indicates that the utilization of reducing sugar on each day is dependent on the microorganism used. In both cases, the percentage yield of ethanol produced at each fermentation time examined using Z. mobilis was higher compared to that using S. cerevisiae. Bacteria are known to multiply faster than yeast thus Z. mobilis might reached the lag phase faster than S. cerevisiae and therefore utilized its substrate faster. The ethanol yield for Z. mobilis was higher than that of S. cerevisiae at all fermentation periods. S. cerevisiae is known to employ the EMP pathway to metabolize glucose producing 2 moles of ATP from 1 mole of glucose whereas Z. mobilis employing the E-D pathway produces 1 mole of ATP from 1 mole of glucose (Wang et al., 2014). Ming et al. (2014) reported that a significant amount of the carbon source is converted into biomass as a result of the E-D pathway used by this microorganism. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells’ total protein (Ming et al., 2014). Z. mobilis maintains a high level of glucose flux through the pathways to compensate for its low yield (Rutkis et al., 2013). Clark et al. (2013) reported that there is linearity between maximum cell growth) and ethanol production with S. cerevisiae strains. However, Z. mobilis perform less biomass formation and efficient production of ethanol compared to S. cerevisiae (Sootsuwan et al., 2013). The low ethanol conversion efficiency by S. cerevisiae might therefore be due to the fact that a portion of the substrate was converted to cell mass and other products. Although liquefaction and saccharification might probably kill some microorganism that might cause contamination, both organisms were able to metabolize their substrate faster.
Figure 1. Sugar utilization by *Z. mobilis* and *S. cerevisiae*.

Figure 2. Ethanol produced from pito mash using *S. cerevisiae* and *Z. mobilis* separately.

Thus competitively inhibiting the growth of other microorganisms. They can therefore be used to produce ethanol using non-sterile substrate. This could reduce energy cost involved in sterilizing the substrate. According to Tao et al. (2005) and Aggarwal et al. (2001), cheap raw material, low processing cost and high productivity are the main considerations for most ethanol production. This work therefore shows that under appropriate conditions pito mash can be used as alternative and cost-effective feed stock for the
Effect of pH on ethanol production by S. cerevisiae and Z. mobilis using pito mash as substrate.

Optimization of pH

Generally, ethanol concentration increased with increased pH in both S. cerevisiae and Z. mobilis. However, the increase was more pronounced in S. cerevisiae than Z. mobilis. For S. cerevisiae, ethanol concentration began to increase with increased pH till it reached maximum at pH of 6, and then decreased at pH 6.5 (Figure 3). In the case of Z. mobilis, fermentation took place at pH of 4 and gave higher ethanol concentration compared to ethanol concentration produced by S. cerevisiae at the same temperature. Ethanol concentration reached maximum at pH 5.5, beyond which it began to decrease. In the case of S. cerevisiae, there was significant (p<0.05) difference between the ethanol produced at all pH values. Optimum pH for ethanol was between 6.0 and 6.5 with pH of 6.0 producing the maximum ethanol volume of 0.948 mgml⁻¹ for S. cerevisiae. For Z. mobilis, there was significant difference in ethanol produced all pH values. The optimum pH was between 5.0 and 5.5 with 5.5 producing the highest ethanol of 1.85 mgml⁻¹. In all cases, ethanol produced by Z. mobilis was higher compared to S. cerevisiae. The result agrees with observation by Hwang et al. (2004) who reported that the activities of ethanol producers are slightly suppressed at pH below 4.5.

Optimization of temperature

In Z. mobilis, there was initial increase in ethanol concentration with temperature increase from 30 to 35°C; however, beyond 35°C increasing temperature became inhibitory to ethanol production (Figure 4). The decrease was more pronounced at 45°C. S. cerevisiae produced maximum amount of ethanol at 30°C and further increase in temperature (35 to 45°C) was inhibitory to its ethanol production ability. Analysis of variance indicated that for S. cerevisiae, there was significant (p<0.05) difference in the ethanol produced at each temperature. However, there was no significant (p<0.05) difference in ethanol
produced at the temperature of 35 to 45°C. The highest concentration (0.951 mg l⁻¹) was produced at temperature of 30°C for S. cerevisiae, followed by 0.849 mg l⁻¹ at 35°C. The lowest volume (0.323 mg l⁻¹) was produced at 45°C. In the case of Z. mobilis, there was significant (p<0.05) difference between ethanol produced at all temperatures. However, there was no significant (p<0.05) difference between ethanol produced at 30 to 35 and 40 to 45°C. The highest concentration of 1.951 mg l⁻¹ was produced at temperature of 35°C followed by 1.889 mg l⁻¹ at the temperature of 30°C. At all temperature values, the concentration of ethanol produced at each fermentation examined using Z. mobilis was significantly different from using S. cerevisiae. Similar observations were made by Panesar et al. (2007). It was also indicated that, decrease in the membrane phospholipids content may be responsible for the unique thermal sensitivity of Z. mobilis cells grown at higher temperature (Panesar et al., 2007).

**Optimization of fermentation period**

As shown in Table 1, maximum ethanol production of 0.855mgml⁻¹ for S. cerevisiae was observed on the fourth day of fermentation whereas maximum ethanol production of 1.269mgml⁻¹ was observed for Z. mobilis on the third day of fermentation. In both organisms, there was a sharp increase in ethanol concentration within the first two days of fermentation period (Figure 5). Generally, there was a decline of ethanol production after the optimum for both organisms (Figure 5) which could be attributed to the build-up of inhibitory toxins produced in the fermentation medium as reported previously by Zakpaa et al. (2009).

**Conclusion**

Pito mash (waste from sorghum brewing) was a suitable substrate for bioethanol production by Z. mobilis and S. cerevisiae since high yield of ethanol was produced. However, Z. mobilis (3.63 g l⁻¹) demonstrated higher biomass conversion efficiency, hence higher ethanol concentration compared to S. cerevisiae (3.03 g l⁻¹). Pito mash could therefore be used for large scale bioethanol production, hence reducing its threat to the environment.
Table 1. Effect of fermentation period on ethanol production.

<table>
<thead>
<tr>
<th>Fermentation period (days)</th>
<th>Ethanol concentration/ mg ml(^{-1})</th>
<th>Saccharomyces cerevisiae</th>
<th>Zymomonas mobilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.199 ± 0.10(^{a})</td>
<td>0.196 ± 0.030(^{a})</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.748 ± 0.04(^{b})</td>
<td>0.760 ± 0.033(^{b})</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.838 ± 0.01(^{b})</td>
<td>1.269 ± 0.063(^{b,c,d})</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.855 ± 0.007(^{b})</td>
<td>0.844 ± 0.047(^{b,e})</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.747 ± 0.06(^{b})</td>
<td>0.686 ± 0.046(^{b,f})</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in column followed by different superscript are significantly different at \(P<0.05\).

Figure 5. Effect of time duration on ethanol production by *S. cerevisiae* and *Z. mobilis* using pito mash as substrate.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES


Bioethanol production from date palm fruit waste fermentation using solar energy

Ahmed Boulal¹, Mabrouk Kihal², Cherif Khelifi¹* and Boudjemâa Benali¹

¹Unité de Recherche en Energie Renouvelables en Milieu Saharien, URERMS, Centre de Développement des Energies Renouvelables, CDER, 01000, Adrar, Algeria.
²Laboratoire de microbiologie appliquée, département de biologie, faculté sciences de la nature et de la vie, Université Oran1 Ahmed Ben Bella, 31100 Oran. Algérie.

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Every year, more than 236,807 tons, equivalent to 30% of date-palm fruits produced in Algeria, is lost during picking, storage, and commercialization processes. Gasification of this huge biomass can generate biogas such as bioethanol, biodiesel, gasoline and other useful substances. Bioethanol is becoming the main biofuel produced by chemical synthesis or anaerobic fermentation from biomass and is significant for industrial development, investment, and use. It is eco-friendly, moderately costly and cleaner than other gasses. Actually, due to modern biotechnologies, it is possible to valorise the common date-palm waste (CDPW) by bioconversion and to commercialize them in local and international markets in the form of new products with an acceptable added value such as bioethanol.

CDPW is a renewable and sustainable resource of energy that is not greatly used in industries. The date is rich in biodegradable sugars, providing bioethanol after fermentation during 72 h at 30°C in the presence of Saccharomyces cerevisiae yeast and the distillation of date’s juice obtained. In the first experience, a solar batch fermenter (SBF) of 50L capacity, and a butane gas distiller using a cocotte (cooker) of 30L capacity was designed and constructed. The bioconversion systems led to the production of 250 mL/kg of ethanol at 90° after distillation of the CDPW juice at 78°. This is in comparison to the theoretical ethanol directly produced from sugar by chemical synthesis process. The 33% efficiency that was obtained appeared satisfactory and it encouraged the great scaling development of bioethanol based on CDPW biomass and other raw materials abundant in Algeria Sahara.

Keywords: Algerian Sahara, bioethanol, dates-palms waste valorization, distillation, fermentation, solar energy, Saccharomyces cerevisiae.

INTRODUCTION

Energy produced and used are crucial elements for the development of countries and improvement on human activities (WEA, 2000). Electricity and heat generation from biomass is an efficient manner of energy conversion that is climate friendly. In Algeria, biomass potentially offers great promises with the bearing of 3,700,000 tons of oil equivalent (TOE) coming from forests and 1,330,000 TOE per year from agriculture and urban wastes (Hasni, 2006). A pre-review showed the feasibility of electricity production of 3-6MW from the discharge of Oued-Smar in Algiers (Himri et al., 2009). The removal of biogas by combustion is essential to protect the atmosphere from
the undesirable emission of unburned methane contained in biogas. The increasingly use of biogas, particularly produced from landfills, has commenced with national pilot research projects initiated by Renewable Energy Development Center (CDER, http://portail.cder.dz), studying methane recovery from cull dates and fruits processing of by-products. This operation is followed by the successful experience carried out by Renewable Energy Research Unity in Sahara Medium (URERMS) located at Adrar Province in Southern Algeria. The project is considered as a first progressive step as regard biogas energy generation in the country.

Bioenergy, including bioethanol, biodiesel, and other substances is feasible and have economically become the future solution for energetic and ecologic issues. Amongst other biogases, bioethanol has higher burning effect €2.25/kWh and lower environmental effect (Sindhu et al., 2016). According to the Renewable Fuels Association (RFA), USA, Brazil, EU and China produced 50.3%, 25.5%, 4.5%, and 2% million gallons of ethanol in 2014 respectively (Sanchez and Cardona, 2008). Certainly, the countries with agronomic based economy are therefore appropriate for bioethanol generation (Mielenz, 2001). Governments around the world have actively promoted the identification, development and commercialization of technologies for the production of alternative biofuels within the last three decades. The operation includes the production of bioethanol, which captures the attention of many researchers, consumers and investors in the hopefulness of better fuel sustainability (Oh et al., 2010). The competition between food and fuel risks the equilibrium of the equation.

Bioconversion process utilizes non-edible lignocellulose, starchy materials coming from agricultural and forestry biomass; and it is becoming the main solution to provide renewable eco-friendly and economical energy source. The characteristics and capabilities of bioethanol make it favorable to mix with gasoline (Balat et al., 2008). In addition, the need to satisfy the energy demand without environmental bearings and non-renewable fuels stock resulted from the fossil fuel, investing in research development in clean energy and sustainable development to which bioethanol is one of them (Krylova et al., 2008). Bioethanol prevents engine knocking and premature explosion due to its high octane index of 110. Moreover, higher octane index also provides wider flammability, higher heat vanishing and speed of flame (Lashinsky and Schwartz, 2006). Bioethanol has 35 to 40% lower energy content compared to gasoline, and 35% of higher oxygen content which makes the combustion cleaner and resulting in a lower emission of toxic substances (Li et al., 2008). Bioethanol helps to reduce CO₂ emission up to 80% compared to using gasoline, thus promoting a cleaner environment for the future (REN21, 2014). Chemically, bioethanol is less unsafe than gasoline and its higher flash point (13°C) gives better storage treatment ability and it is less flammable. The auto-ignition temperature is between 333 and 423°C and ethanol relatively require an elevated temperature than gasoline to be an auto-detonation; hence, ethanol vapor will be only combusted later than gasoline without a forced detonation (Shinsuke et al., 2005).

Many microorganisms (yeasts) can be used for transforming biomass into ethanol (Nigam, 2000). S. cerevisiae yeast can be used to produce ethanol of 48.8 mL/kg from pineapple cannery by-product and 120.7mL/kg from sorghum juice (Johansson et al., 2012). The augmented request of energy in homes, industries, transportation and agricultural sectors needs the rapid use of bioenergy options. Without exception, the founded biomass renewable energy is expected to progress from 50EJ/year in 2012 into more than 160EJ/year by 2050 (Kwiatkowski et al., 2006).The uncooked material and the energy request are the major cost factors in the bioethanol production (Vucurovic et al., 2012). Following the oil crisis of 1970, biofuels were perceived in many countries as a realistic solution to oil resources dependence problem. Moreover, the mixture usage with traditional fuels made it possible to consider the gain on the levels of vehicles pollutant emissions. The oil counterblows of 1986 and the too high maintenance and cost slowed down their development. To date, bioethanol is seen as the main biofuel for the future. It is subject to a significant industrial development around the world, and can be produced by chemical synthesis or fermentation (FAO Stat, 2015).

Algeria has several natural energy resources, including oil reserves of about 13.4 billion barrels, natural gas of 4502 billion m³, and coal of 65 million tons. On the other hand, the date-palm grove areas have registered a significant expansion in Algeria, estimated at 69%, growing from 101,000 ha in the year 2000 to 169,361 ha in the year 2009 with a total of 18.7 million palm trees (Nakhla in Arabic word) scattered over around 1,000 farms, producing 789,357 tons of dates every year. Approximately 50% of these palms are currently in production (Elsanhoy et al., 2012). Algeria is a country situated in the sunbelt region of the earth where the solar radiation potential is over 8 billion MWh/year. Algeria has an important area of about 2.4 million km² and a feeble population density of 15.8 inhabitants/km² where the Sahara occupies more than 84% of the whole area. Algerian climate is maritime-north and semi-arid to arid middle and south. Date-palm constitutes the principal axis of the oasis climate of the Sahara and found the basic agronomy structure of the inhabitants. These trees create the essential microclimate for all other cultivars of cereals.

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

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and vegetables, provide materials, and burn biomass for building and warming. The top ten countries producing about 90.5% of the world’s dates are Egypt, 17.2%; Saudi Arabia, 13.7%; Iran, 13%; United Arab Emirates, 9.8%; Pakistan, 9.6%; Algeria, 9%; Iraq, 7.2; Sudan, 5.4%; Oman, 3.5%; and Libya, 2% (FAO Stat., 2012). More than 236,8 tons equivalent to 30% of dates produced in Algeria are lost during picking, storage, commercialization and conditioning process, caused by the fungus, infestation by insects or simply due to their low quality, unconsumed by humans (Sofien et al., 2014). Only Adrar city region counted more than 3,000,000 of date-palm, playing an important ecological role, and offers great dates tonnage of 86,000/year. But in spite of this huge richness, these dates present a low commercial value (Figure 1a) compared to the high-class varieties of dates such as Deglet-Nour, Degla-Beida, Gars and Fergus (Figure 1b). Therefore, the CDPW are intended only for subsistence farming and animal feeds or barter exchange with foreigner countries, as in Mali, Niger, and others.

No processing industry was established in the Algerian Sahara areas to transform these huge harvests of (CDPW) into economic useful gain. Dates are generally a complete food value, rich in fermentable and biodegradable sugars, where energy reaches 2000 to 3000 Cal/kg, offering bioethanol, anaerobic fermentation, and distillation afterward. Their composition analyses showed that the Algerian Sahara dates varieties are very rich in reducing sugars, especially glucose and fructose of about 73 to 83% in dry basis (Acourene and Ammouche, 2012). Thus, making dates extract moderately suitable as feedstock for fermentation (Wei-Hao et al., 2016). For instance, we can produce ethanol after anaerobic fermentation by microorganisms such as S. cerevisiae. In addition to their sugars resource and moderately long conservation period, dates offer many other technological possibilities. They are seen as raw materials for the production of different metabolites, biopolymers, organic acids, antibiotic, amino acids, enzyme, bakery yeast, and also the butanol and hydrogen (Shahravy et al., 2012; Qureshi et al., 2012; Abd-Alla et al., 2012; Aditiya et al., 2016). The present work consists of the realization of an experimental solar batch fermenter prototype operating with the solar water heater, in order to reduce the energy consumed and to ensure anaerobic fermentation medium for the CDPW dates juice at 30%, and for producing a high quality and quantity of bioethanol at low cost after distillation.

MATERIALS AND METHODS

Raw material and microorganism

The mixture of the CDPW used in the study to produce bioethanol is composed essentially of Hchef, Kacien, and other varieties of date’s scraps of the cattle food originated from Algerian Sahara (Figure 1a). The products are dried, kept in bags and stored at room’s temperature. The microorganism S. cerevisiae used in the fermentation process of date’s juice is provided by the industrial plant of bakery yeast production, coming from Oued-Semmar in Algeria.

Ethanol production medium

1. The CDPW (Figure 2a), were washed, plunged in a water bath, rubbed carefully, and rinsed with pure water to eliminate sand, pebbles, insects and remainder plants (Figure 2b).
2. The CDPW are petted to separate seeds from coasts (Figure 2c).
3. The CDPW substrates are ground and imbibed in hot water at 90
to 95°C to facilitate sugars extraction (Figure 2d).

4. 200 g of CDPW was diluted into 800 ml of tap water, and simultaneously sulfuric acid was added and adjusted to ensure the pH between 4.3 and 4.7, for inhibiting the bacterial growth and favoring overgrowth of the yeast (Figure 2e) (Wei-Hao et al., 2016). The fermentation medium is inoculated with 1g/L of S. cerevisiae and reactivated within 60 to 90 min under an ambient temperature of 25 to 30°C in an aqueous solution in glucose with 12% V/V (Figure 2f).

5. The batch fermenter (Figure 2g) was designed and installed to operate efficiently by using solar water heater with the aim to reduce the cost of the bioethanol generation process. The fermenter was realized within the South Society of Metallic Construction (ECOMES, 2015), located at Adrar. The fermenter consisted of a tank of 50 L, built in double walls in galvanized stain steel and thermally insulated by fiberglass wool of 5 cm thickness. The heat exchanger is placed between the two walls of the tank. The lid of the tank is quite tight and contains a hole for evacuation of gasses and a second hole is
Figure 3. Sugars consumed during fermentation.

provided with a copper pipe. This was done in the middle of the tank and contained a thermostat for adjusting the substrate temperature close to 30°C. The fermenter is equipped with a manual agitator shaft and connected to a temperature data logger model Fluke 2635A with an internal memory card (Figure 2g). The batch fermenter is heated by water solar heater owned by the CDPW.

6. The water solar heater is inclined at Adrar latitude (27.88°N and 0.28°O) and oriented North-South to capture the optimum of solar energy incident during the year. The hot water is stored in a tank of 200 L and its circulation is ensured by a hydraulic pump controlled by a thermostat. Experimentation is performed during the cold season of the year, the first week of January 2015. During the fermentation process, the density sugars consumed, pH and the alcohol degree of the CDPW juice are controlled. The glucose evolution was controlled using Dubois method given in Michel-DuBois et al. (1956). Reducing sugars (RS) and total sugars (TS) are assessed by titration methods (ISO11289, 1993 and AOAC, 98, 1.12)


during the year. The hot water is stored in a tank of 200 L and its circulation is ensured by a hydraulic pump controlled by a thermostat. Experimentation is performed during the cold season of the year, the first week of January 2015. During the fermentation process, the density sugars consumed, pH and the alcohol degree of the CDPW juice are controlled. The glucose evolution was controlled using Dubois method given in Michel-DuBois et al. (1956). Reducing sugars (RS) and total sugars (TS) are assessed by titration methods (ISO11289, 1993 and AOAC, 98, 1.12)

7. The pH was measured by a digital pH-meter model Mettler Toledo methods (ISO11289, 1993 and AOAC, 98, 1.12) and the date’s juice temperature during the alcoholic fermentation was recorded using thermocouples K type connected to the data logger. After 72 h of alcoholic fermentation, the substrate juice to extract the bioethanol was used to filter. At the beginning of the distillation process (Figure 2h), the degree of alcohol is measured every 30 min, and once the process is slowed, the alcohol is recorded every one hour. The process is stopped when the degree of alcohol became very weak. The distillation temperature was kept at about 78°C.

RESULTS AND DISCUSSION

The microorganism S. cerevisiae has an optional anaerobic breathing on the alcoholic bioconversion process. In anaerobic phase, the glucose is transformed into the ethanol by fermentation effect. During the first 48 h of transformation, the process is active, especially between 24 and 48 h. However, the alcohol produced is increased throughout the last 48 h of the process and an important degradation of the sugar is noticed after 72 h (Figure 3). The total glucose rate is strongly decreased during the time from 13.8% at the beginning of the fermentation process to 3% after 72 h. The period of the fermentation process of date’s juice varied between 36 and 72 h under similar conditions. The glucose is not consumed completely due to the cessation of yeast growth caused by the accumulation of toxic substances (fatty acids), especially the octane and decane in CDPW juice (Figure 3), (Benziouches, 2011; El-Okaidi, 1987). Also, the density of the CDPW juice decreased significantly during the fermentation process from 1.07 to 1.0107 g/cm³, which was caused by the transformation of sugar into bioethanol and the loss of mass under CO2 form (Figure 4). The continuous diminution of the refractive index indicated the increase of the light speed through the date caused by the decrease of the dates density. The total solvable solids in the date’s juice were measured by a handheld refractometer for viniculture, using a refractometer, Atago model NAR-3T (°Bx), corresponding approximately to the total sugar concentration in (g/L). After distillation of the CDPW juice, a significant specific production of the bioethanol reached 250 mL/kg of dates at 90° was obtained, representing a bioconversion efficiency of 33% relatively to the theoretical sucrose transformation in the alcohol described using the following chemical synthesis equation:

\[
C_6H_{12}O_6 + S. \text{cerevisiae} \rightarrow 2C_2H_5OH + 2CO_2 + \text{Energy}
\] (2)

Where, 76 g/kg glucose produces 25 mL alcohol + x(g) of carbon dioxide if it is fermented by the yeast. Finally, the vibration sign of the biofuel produced is identified using the Infra-Red Spectra, showing different vibrations bands characterized by the
following wave numbers 2990, 3300 and 2990 cm\(^{-1}\), corresponding to the molecules group C-H, O-H and C-O respectively (Figure 5).

**Conclusion**

The current study shows that the CDPW must constitute a favorable medium for *S. cerevisiae* growth, due to its sugar content and it is becoming an attractive raw material. It is intended to produce the bioethanol by using the solar batch fermenter at relatively moderate cost, especially in Sunbelt region of Algerian Sahara, without a negative effect on human. The CDPW distilled juice produced the highest ethanol concentration of about 90°, with an acceptable productivity of 250 or 3.47 mL/kg/h, assessing a scale efficiency of 33%. Compared to the theoretical ethanol efficiency obtained from a chemical reaction using the same sugar quantity which is 50%. The present results obtained is a strong encouragement to continue Research-Development in this renewable energy field. Therefore, it is necessary to start the construction of semi-pilot and pilot fermenters and investigate new methods, microorganism, and materials by-product to improve the quantity of ethanol produced and to reduce energy consumption during the bioethanol process transformation, for decreasing the cost of the final product. The quantity of 213,000 tons/year of CDPW and 1.8 MWh/m\(^2\)/day of solar radiation appeared relatively sufficient for 40,000,000 inhabitants to develop an important biofuel industry in Algerian Sahara. Nevertheless, it is necessary to validate this purpose on bioethanol pilot installations in order to demonstrate the relevance of the proposed valorization before any transposition on an industrial scaling. Any main factor support for CDPW research and biotechnology development depend on the priority and emphasis of the national government to endorse the founding date-palms based agro-food and biofuel industry as well as local and international market to boost local economy.

**Conflict of Interests**

The authors have not declared any conflict of interests.
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REFERENCES


Full Length Research Paper

Modulation of biochemical stress initiated by toxicants in diet prepared with fish smoked with polyethylene (plastic) materials as fuel source

Department of Biochemistry, Federal University Technology Owerri, Nigeria.

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This study investigated the oxidative modulation potentials of diets supplemented with Solanum lycopersicum and Allium cepa against biochemical changes initiated in rats fed diet prepared with fish smoked with polyethylene material (FSP) and fish smoked with firewood (FSF). Thirty male Wistar albino rats were randomly grouped into six with five rats in each group. The biochemical parameters analyzed indicated significant (p < 0.05) increases in the activities of liver enzymes [alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST)] and concentration of total bilirubin and malondialdehyde (MDA) in rats maintained on diets prepared with FSF and FSP only when compared to rats maintained on rats pellets only and rats maintained on the supplemented diets. These groups of rats also presented significant (p < 0.05) fluctuations in activities of glutathione peroxidise (GPx), superoxide dismutase (SOD) and catalase (CAT) and concentrations of total protein, albumin, total cholesterol and glutathione (GSH). However, the S. lycopersicum and A. cepa treated groups showed significant restoration towards their respective normal control values. These results indicate that smoking as a food processing method (especially using polyethylene materials) generates oxidants that may induce oxidative damage. The antioxidative role of A. cepa and S. lycopersicum in the diets of treated animals emphasized their hepatoprotective potentials.

Key words: Smoked fish, polyethylene, S. lycopersicum, A. cepa, oxidants, antioxidative, toxicants.

INTRODUCTION

Fish is one of the world favourite foods and a major source of dietary protein, containing essential fatty acids and essential amino acids among others. Fish can be prepared (cooked) in many different ways. Deep-fat frying, grilling, broiling, roasting, boiling, baking, smoking, stir-frying and braising are the most common methods for fish processing. A central concern of fish processing is to prevent it from deteriorating. Roasting (smoking) enhances the flavour and taste of the fish.

However, advancing scientific knowledge have shown that cooking and processing of foods at high temperatures generates genotoxic substances such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines (HAAs), heavy metals (Sugimura, 1997;
Badry, 2010; Ujowundu et al., 2013, 2014a), among others. Grilling (broiling/roasting) meat, fish or other foods with intense heat over a direct flame results in fat dripping on the hot fire and yielding flames containing a number of PAHs (Agerstad and Skog, 2005; Ujowundu et al., 2014a) and HAAs. These chemicals adhere to the surface of the food, and the more intense the heat, the more PAHs and HAAs are present. The formation of these PAHs and HAAs on roasted foods is dependent on the distance of food from the heat source (Phillips, 2002), fat content of the fish (Knize et al., 1999), duration of roasting (Nawrot et al., 1999), temperature used (World Health Organization (WHO), 1998), whether fat is allowed to drop onto the heat source and type of fuel used (SCF, 2002; Ujowundu et al., 2014b).

Our study exposed a hazardous practice by locals that involved the use of waste polyethylene materials, plastics, cartons and other inflammable materials to generate fire, heat and smoke used to process fish and meat in some parts of Nigeria, such as Owerri in Imo state (Ujowundu et al., 2014b). The use of these materials is probably to cut corners and save cost, being readily available with little or no cost, or because these materials can generate the much needed fire/flame for fast roasting.

The by-products of polyethylene or plastic combustion are airborne particulate emission (soot) and solid residue ash (black carbonaceous colour) (Graham, 2012). Several studies have demonstrated that soot and solid residue ash possess a high potential of causing significant health and environmental concern. The soot when generated is accompanied with volatile organic compounds (VOCs), semi-VOCs smoke (particulate matter), particulate bound heavy metals, PAHs, polychlorinated dibenzofurans (PCDFs) and dioxins (Valavanidid et al., 2008). These toxicants released by these materials during combustion would probably accumulate on or absorbed by the processed foods. These foods are consumed with other food materials especially those of plant origin such as fruits, spices and vegetables. The chemical and phytochemical content of these plants are important antioxidants that ameliorate the toxic effects of the contaminants/antioxidants (Stanner et al., 2004; Poljsak, 2011).

*Solanum lycopersicum* (tomato) and *Allium cepa* (onion) are such plants God has used to bless man. Several studies have indicated the positive health effects of these plants, including anticarcinogenic, antioxidant, hepatoprotective, cardioprotective, antidiabetic and antimicrobial activities, among others (Das et al., 2005; Ciz et al., 2008; Kobori et al., 2011; Weremfo et al., 2011; Ujowundu et al., 2012, 2014b).

Consumption of tomatoes has been proposed to reduce the risk of several chronic diseases such as cardiovascular diseases and certain types of cancer, especially prostate cancer (Rao and Agarwal, 1999). Lycopene is the most prominent carotenoid in tomatoes (Karimi et al., 2005) and investigations have strengthened the hypothesis that lycopene could be a fundamental factor for the preventive effects of tomatoes and tomato products (Basu and Imrham, 2007). Although observation indicates that both tomato juice and lycopene reduced lipid peroxidation (El-Nashar and Abduljawad, 2012). Carotenoids exert antioxidants activity and lycopenes exhibits the highest overall single oxygen-quenching carotenoid, twice as that of carotene (DiMascio et al., 1989). *A. cepa* contains flavonoids and sulphuryl compounds which offers protection against cellular damage (Hodges et al., 1999). It offers direct chemoprotective roles, reduce oxidative stress and initiates production of chemical oxidative defence mechanisms by cells (Teyssier et al., 2001; Griffiths et al., 2002; Abu-El-Ezz et al., 2011). The effectiveness of *A. cepa* is adduced to the penetration of thiosulfonates and isothiocyanates in it (Block, 1985).

It is estimated that up to 2.7 million lives could potentially be saved each year if fruits and vegetables consumption are sufficiently increased (WHO, 2002). The health benefits of diet rich in vegetables have also been recognized and there are evidences that nutrient content of fruits and vegetables such as dietary fibre, folate, antioxidants, vitamins and phytochemicals are associated with low risk of cardiovascular diseases and other disorders.

Our investigation revealed that in many communities and families of developing countries, the consumption of foods roasted/smoked with hazardous materials such as polyethylene (plastic material) is common. However, the adverse health implication of this practice is scarce. This study intends to expose this and also explore the hepatoprotective activities of *S. lycopersicum* (tomato) and *A. cepa* (onion) against the adverse biochemical changes on exposure to toxic compounds present in fish smoked/roasted with polyethylene (plastic material) on male albino rats.

**MATERIALS AND METHODS**

**Plant and fish samples**

Fresh samples of *S. lycopersicum* (tomatoes) and *A. cepa* (onions) were purchased from the Relieve market, Owerri Imo State, Nigeria. These plants were identified by a plant taxonomist, Dr. F.N. Mbagwu, of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. The sample were deposited at the University Herbarium with Voucher Number IMSUH84 (*Allium sepa*) and IMSUH142 (*Solanum lycopersicum*). Each sample was sliced, oven-dried (at 45°C until 95% moisture was removed), homogenized to powder and stored in an air-tight container at room temperature prior to feed compounding. Fresh samples of mackerel fish (*Rastrelliger spp*) were bought from a Market at Obinze in Owerri-West. The fish samples were identified by Mr. C.F. Ezeafulukwe of the Department of Fisheries and Aquaculture, Federal University of Technology, Owerri (FUTO). The researchers adopted the methods used by the vendors to process the fish samples.

The fish samples were processed with smoke, heat and flame...
from firewood for 4 hours at high temperature. The smoked fish were then divided into two portions. One portion was roasted further with fire generated from plastic material (polyethylene), and the other with firewood only for 1 hour. Each portion of the smoked/roasted fish samples were homogenized and stored in an air-tight container at room temperature prior to feed compounding. The rat pellets (Poultry Growers Pellets) was purchased from Grand feed Nigeria Ltd. The processed S. lycopersicum, A. cepa, fish smoked with firewood, fish smoked with polyethylene and rat pellets were mixed together at varying combination and concentration to compound the rat’s feed as shown in Table 1. The formulated feeds were stored in well labelled, air tight containers.

Table 1. Animal groupings and feeds.

<table>
<thead>
<tr>
<th>Animal grouping</th>
<th>Feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO</td>
<td>100% rat Pellets only</td>
</tr>
<tr>
<td>PSF</td>
<td>60% Pellet + 40% Fish Smoked with Firewood (FSF)</td>
</tr>
<tr>
<td>PSP</td>
<td>60% Pellets + 40% Fish Smoked with Polyethylene materials (FSP)</td>
</tr>
<tr>
<td>PSPS</td>
<td>50% Pellets + 40% FSP + 10% S. lycopersicum</td>
</tr>
<tr>
<td>PSPA</td>
<td>50% Pellets + 40% FSP + 10% A. cepa</td>
</tr>
<tr>
<td>PSPSA</td>
<td>50% pellets + 40% FSP + 5% S. lycopersicum + 5% A. cepa</td>
</tr>
</tbody>
</table>

PO = pellets only; PSF = pellets plus fish smoked with firewood; PSP = pellets plus fish smoked with polyethylene materials; PSPS = pellets plus fish smoked with polyethylene materials plus S. lycopersicum; PSPA = pellets plus fish smoked with polyethylene materials plus A. cepa; PSPSA = pellets plus fish smoked with polyethylene materials plus S. lycopersicum plus A. cepa.

Animal groupings and treatments

Thirty (30) male Wistar Albino rats (50 to 80 g) were obtained from the animal house of the Zoology Department, University of Nigeria, Nsukka, Enugu State Nigeria. The rats were allowed free access to food (rat pellet) and water ad libitum during a one week acclimatization period at the Animal house of the Department of Biochemistry, Federal University of Technology, Owerri. The animals were kept in steel cages placed in a well-ventilated house conditions (photoperiod: 12 h light and dark cycle each) throughout the experimental period. This study adhered to the guidelines on the care and well-being of laboratory animals (NIH, 1985) and was approved by the ethical committee of the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria (FUTO/BCH/EC/2013/3). The rats were randomly distributed into six groups consisting five each as presented in Table 1. The rats were maintained on these feeds for 14 days and were allowed free access to water ad libitum.

Processing of tissues

Twenty-four hours (day 15) after the last exposure to the formulated diets, animals were anesthetized in a dichloromethane chamber. Blood was obtained by cardiac puncture and serum obtained by blood centrifugation at 1500 × g for 10 min, at 4°C. The liver from each animal was excised, weighed and stored at 4°C prior to immediate analyses. Each liver was homogenized in potassium chloride buffer (1.15 %) with ethylenediamine tetraacetic acid (EDTA) at pH 7.4 and centrifuged for 60 min. The supernatant was used to assay oxidative stress parameters.

Determination of hepatic oxidative stress parameters

Catalase (CAT) activity was determined according to the method of Aebi (1984). Briefly, 2.5 ml of phosphate buffer, 2.0 ml of H2O2 and 0.5 ml of sample was added into the test tube labelled stock. To 1.0 ml portion of the reaction aliquot from stock test tube, 2 ml of dichromate acetic acid reagent was added. The absorbance of the mixture was determined at 240 nm at a minute interval into 4 places.

Superoxide dismutase (SOD) activity was determined using the method of Xin et al. (1991). Briefly, 0.9 ml of distilled water and 0.1 ml of sample was pipetted into test tubes. Afterwards, 0.1 ml of this mixture was mixed with 0.9 ml of carbonate buffer, and 75 μl of xanthine oxidase added. The absorbance was read at 500 nm for 3 min at 20 s interval. The changing rate of absorbance was used to determine the superoxide dismutase activity. Glutathione peroxidase (GPX) activity was determined by the method of Paglia and Valentine (1967). Briefly, 3.0 ml of phosphate buffer, 0.55 ml of guaiacol, 0.03 ml of H2O2 and 0.1 ml of sample were added into test tubes and mixed. The absorbance of the mixture was taken at 436 nm for 2 min at 30 s interval.

Glutathione concentration was determined by the method of King and Wootton (1959). Briefly, into test tubes labelled test and blank 0.1 ml of sample and 0.1 ml of distilled water were added, respectively. Also, 0.9 ml distilled water and 0.02 ml of 20% sodium sulphite were added to all test tubes, mixed and stood for 2 min at 25°C. Afterwards, 0.02 ml of lithium sulphate and 0.02 ml of 20% Na2CO3 were added to all test tubes and mixed. Then, 0.2 ml phosphor – 18 – tungstic acid was added to the test tubes, shook and allowed to stand further for 4 min for maximum colour development. Finally, 2.5 ml of 2% sodium sulphite was added to test tubes and the absorbance was read at 680 nm within 10 min.

The determination of malondialdehyde (MDA) concentration was by the method of Wallin et al. (1993). Briefly, 0.1 ml of sample, 0.9 ml of distilled water, 0.5 ml of 25% trichloroacetic acid (TCA) and 0.5 ml of 17% TBA in 0.3% NaOH were pipetted into test tubes. The mixture was incubated at 95°C for 40 min and cooled in water after incubation. Afterwards, 0.1 ml of 20% sodium dodecyl sulphate was added to the mixture. The absorbance of the mixture was determined at 532 and 600 nm against a blank.

Determination of liver function parameters

Serum alanine aminotransferase (ALT) activity was determined by Reitman and Frankel (1957) method. Briefly, 0.1 ml of serum was pipetted into test tubes labelled reagent blank and sample, and 0.5 ml of the ALT reagent I was added to test tubes labelled reagent blank and sample. Also, 0.1 ml of distilled water was added into the test tubes labelled reagent blank. All test tubes were appropriately mixed and incubated at 37°C for 20 min. Afterwards, 0.5 ml of ALT reagent II was added to all test tubes, mixed and allowed to stand
at 25°C for 20 min. Later, 5.0 ml of NaOH was added to the test tubes, mixed and absorbance read against the reagent blank after 5 min and the activity determined.

The activity of aspartate aminotransferase (AST) was determined by Reitman and Frankel (1957) and Schmidt and Schmidt (1983). Briefly, test tubes were labelled reagent blank and sample and 0.1 ml of serum was added into the test tubes labelled sample, and 0.5 ml of AST reagent I was added to all test tubes. Then, 0.1 ml of distilled water was added into the test tube labelled reagent blank. All tubes were mixed and incubated at 37°C for 30 min. Afterwards, 0.5 ml of AST reagent II (containing 2, 4 – dinitrophenylhydrazine) was added to all test tubes, appropriately mixed and allowed to stand further at 25°C 20 min. Finally, 5.0 ml of NaOH was also added to the test tubes and mixed, and the absorbance of the sample was measured against the reagent blank after 5 min.

The alkaline phosphatase (ALP) activity was determined according to the Deutsche Gesellschaft für Klinische Chemie (1972) method. Briefly, 0.01 ml of the sample was added into labelled test tubes. Then 0.50 ml of ALP reagent (containing diethanolamine buffer, magnesium chloride and p-nitrophenylphosphate) was added to the tubes and mixed. The absorbance was read against water blank at 405 nm and activity (U/l) determined.

Serum total protein (TP) concentration was determined using Tietz (1995) method. Briefly, into tubes labelled reagent blank, standard sample and sample blank were added 0.02 ml distilled water, standard protein preparation and samples. Then, 1.0 ml of total protein Reagent 1 was added to all the test tubes, except in sample blank in which Reagent 2 was added. The content of these test tubes were mixed and incubated at 25°C for 30 min. The absorbance was taken at 546 nm, and the concentration determined.

Serum albumin (ALB) concentration was determined using the method of Grant et al. (1987) and Doumas et al. (1971). Briefly, 10 μl of distilled water, standard albumin preparation and samples were added to different test tubes and 300 μl of albumin reagent containing bromocresol green (BCG) was added to the tubes mixed and incubated at 25°C for 20 min. Absorbance was read at 578 nm. The determination of the plasma globulin was by the formula:

\[
\text{Plasma globulin} = \frac{\text{Total protein (TP)} - \text{Plasma albumin (ALB)}}{100} 
\]

Serum bilirubin was determined by the modified method of Jendrassik and Grof (1938), using Randox laboratory test Kit (Luton, UK). Briefly, into test tubes labelled sample blank and sample, 200 μl of bilirubin reagent I was added. Afterward 50 μl of Bilirubin reagent II was added to tube labelled sample and 1000 μl of Bilirubin reagent III added to all the test tubes. Also, 200 μl of serum was added to all the test tubes. The test tubes were mixed and incubated at 25°C for 10 min. Afterwards, 1000 μl of Bilirubin reagent IV was added to all test tubes, mixed and incubated further at 25°C for 30 min. The absorbance was read against the sample blank at 578 nm, and the concentration determined. Cholesterol was determined by Alain et al. (1974) and Melattini et al. (1978) methods for the quantitive in vitro determination of cholesterol concentration in plasma using biosystems kit (Barcelona, Spain). Briefly, test tubes were labelled blank, standard and sample. Then 10 ul of cholesterol standard solution and samples were pipetted into the test tubes labelled standard and sample, respectively. Afterwards 1.0 ml of cholesterol Reagent A (containing sodium cholate, phenol, cholesterol esterase, cholesterol oxidase, peroxidase and 4-amino antipyrine) was added to all test tubes. The test tubes were mixed and incubated at 25°C for 10 min and absorbance was taken at 500 nm.

Statistical analyses

The results were expressed as mean ± standard deviation, and the test of statistical significance was carried out using analysis of variance (ANOVA) at 95% confidence interval (P ≤ 0.05). All statistical calculations were performed with SPSS 17.0 for Windows (Ozdamar, 1991).

RESULTS AND DISCUSSION

In this study the division of the smoked fish into two portions was to achieve two things; (1) to determine the effect of fish smoked with firewood only and (ii) the effect of fish smoked with firewood and polyethylene material. The use of polyethylene (plastics) is a growing trend, in local food processing in Nigeria which adverse effects needs to be highlighted.

Food can become contaminated during thermal treatments such as drying, smoking, roasting, baking frying and grilling (Ishizaki et al., 2010). Processing of food at high temperatures generates highly lipophilic compounds (PAHs) known to be potent carcinogens (Silva et al., 2011). In this study, the fishes were roasted employing traditional roasting methods with firewood and polyethylene material as the sources of fuel, except for the fresh fish samples. The levels of PAHs in smoke depends on heat sources, temperature, flame intensity, particulate materials generated during combustion, etc (Muthumbi et al., 2003; Rey-Salgueiro et al., 2004).

Our previous study showed that roasting contributed to the contamination of the fish samples with heavy metals, and this was also dependent on the source of fuel used. This is in agreement with the research of Ersoy et al. (2005) who found that cooking methods such as frying, microwaving, baking and grilling increased the concentrations of some heavy metals (Cd, Pb and Arsenic) in fish.

The present result show that the feeding of rats with smoked fish diet (40% w/w) significantly elevated (P < 0.05) the concentration of lipid peroxidation product (malondialdehyde) in the liver after 14 days (Figure 1). Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes (Cheeseman, 1993), by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane (Bergendi et al., 1999). These latter compounds then decompose to form a wide variety of products in particular malondialdehyde (MDA) (Zeyuan et al., 1998). Lipid peroxidation in cellular membranes generates polyunsaturated fatty acids tending to reduce membrane fluidity which is essential for proper functioning of the cell (Iman, 2011). So the increase in MDA concentration (an index of lipid peroxidation) observed in the rats fed the smoked fish diets (PSF and PSP), indicates liver cell membrane damage. This is in accordance with the work of Liu et al. (2008) which recorded an increase in MDA with phenanthrene exposure. Phenanthrene is one of components observed in fish smoked with firewood, polyethylene and tyre (Ujowundu et al., 2014b). The result is also consistent with the works of Ujowundu et al. (2011), Vasanth et al.
Figure 1. Concentration of MDA in liver homogenate of rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of *S. lycopersicum* and *A. cepa*. Each bar represents the mean ± SD. Bars with different letters are significantly different (*p* < 0.05). PO = Pellets only; PSF = 60% pellet + 40% fish smoked with firewood; PSP = 60% pellets + 40% fish smoked with polyethylene materials; PSPS = 50% pellets + 40% FSP + 10% *S. lycopersicum*; PSPA = 50% pellets + 40% FSP + 10% *A. cepa*; PSPSA = 50% pellets + 40% FSP + 5% *S. lycopersicum* + 5% *A. cepa*.

(2012) and Ujowundu et al. (2012a) who observed an increase in MDA in rats after administration of diesel petroleum, anthracene and crude petroleum oil, respectively. Treatment of the rats in this study with *A. cepa* and *S. lycopersicum* supplemented diets (PSPS, PSPA and PSPSA groups) significantly reduced the MDA concentration towards the control value, indicating suppression of oxidative stress. Greater reduction in MDA concentration was presented in the PSPSA group treated with combined supplement of *A. cepa* and *S. lycopersicum*, suggesting a synergy.

We observed a significant increase in glutathione peroxidase activity in the liver of PSP rats but a non-significant increase in PSF rats when compared to normal control (PO) rats. This increase could be a response of the organ to the oxidative stress (Iman, 2011) induced by heavy metals and the metabolites of PAHs and HAAs in the smoked fish samples. Furthermore, the increase in GPx activity (Figure 2) was accompanied by a significant reduction in glutathione (GSH) concentration (Figure 3) in the liver PSP rat group. GSH is an endogenous substance that protects cells suffering from oxidative stress (Iman, 2011). Glutathione can function as an antioxidant by catalysing the reduction of *H_2*O_2* to water (Abuja and Albertini, 2001). It can react with singlet oxygen, superoxide and hydroxyl radicals (Singh et al., 2003; Hashimoto et al., 2008). Glutathione also attacks electrophilic centers and thus protects proteins, lipids and nucleic acids from the attack of electrophilic compounds which are capable of reacting with their SH groups (Hayes et al., 1991; Ahluwalia et al., 1996). The decrease in GSH concentration observed in this study corresponds to the increase in GPx activity since GSH is used in GPx pathway. The increase in GPx activity is indicative of response to the induced oxidative stress (Olagoke, 2008), due to toxic effect of toxicants, usually indicated by increase in defence enzymes (Doherty et al., 2010). Treatment with *A. cepa* and *S. lycopersicum* (groups PSPS, PSPA) resulted to non-significant decrease in the concentrations of GSH when compared to control.

The result of the present study also showed that the increase in MDA concentration in PSF and PSP was accompanied by a concomitant significant decrease in the activities SOD (Figure 4). However catalase (CAT) activity increased significantly in all the groups administered polyethylene smoked fish diets (Figure 5). SOD, CAT and GSH are crucial in the detoxification of oxy-radicals to non-reactive molecules (Van Der Oost et al., 2003). These results indicates that PAHs, heavy metals and other toxicants in the smoked fish diets, induced the production of *O_2*^- and *H_2*O_2* which are substrates of SOD and CAT (Vasanth et al., 2012). The results of this study indicate that in rats fed the
Figure 2. Activities of GPx in liver homogenate of rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of S. lycopersicum and A. cepa. Each bar represents the mean ± SD. Bars with different letters are significantly different (p < 0.05). PO = Pellets only; PSF = 60% pellet + 40% fish smoked with firewood; PSP = 60% pellets + 40% Fish smoked with polyethylene materials; PSPS = 50% pellets + 40% FSP + 10% S. lycopersicum; PSPA = 50% pellets + 40% FSP + 10% A. cepa; PSPSA = 50% pellets + 40% FSP + 5% S. lycopersicum + 5% A. cepa.

Figure 3. Concentration of GSH in liver homogenate of rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of S. lycopersicum and A. cepa. Each bar represents the mean ± SD. Bars with different letters are significantly different (p < 0.05). PO = Pellets only; PSF = 60% pellet + 40% fish smoked with firewood; PSP = 60% pellets + 40% Fish smoked with polyethylene materials; PSPS = 50% pellets + 40% FSP + 10% S. lycopersicum; PSPA = 50% pellets + 40% FSP + 10% A. cepa; PSPSA = 50% pellets + 40% FSP + 5% S. lycopersicum + 5% A. cepa.
Figure 4. Activities of SOD in liver homogenate of rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of *S. lycopersicum* and *A. cepa*. Each bar represents the mean ± SD. Bars with different letters are significantly different (p < 0.05). PO = Pellets only; PSF = 60% Pellet + 40% fish smoked with firewood; PSP = 60% Pellets + 40% fish smoked with polyethylene materials; PSPS = 50% Pellets + 40% FSP + 10% *S. lycopersicum*; PSPA = 50% pellets + 40% FSP + 10% *A. cepa*; PSPSA = 50% pellets + 40% FSP + 5% *S. lycopersicum* + 5% *A. cepa*.

Figure 5. Activities of catalase in liver homogenate of rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of *S. lycopersicum* and *A. cepa*. Each bar represents the mean ± SD. Bars with different letters are significantly different (p < 0.05). PO = Pellets only; PSF = 60% pellet + 40% fish smoked with firewood; PSP = 60% pellets + 40% fish smoked with polyethylene materials, PSPS = 50% Pellets + 40% FSP + 10% *S. lycopersicum*; PSPA = 50% pellets + 40% FSP + 10% *A. cepa*; PSPSA = 50% pellets + 40% FSP + 5% *S. lycopersicum* + 5% *A. cepa*. 
polyethylene smoked fish diets, SOD, CAT and GPX were induced. It therefore implies that a considerable amount of $O_2^-$ was produced originating abundant $H_2O_2$ that needs to be detoxified by both CAT and GPX pathways. This is in agreement with the work of Vasanth et al. (2012) who found that SOD and CAT were induced following administration of anthracene to laboratory animals. Then, since oxidative stress due to the toxicants is usually indicated by increased levels of products of oxidative damage (MDA) and subsequent increase in defence enzymes (GPX, SOD and CAT) in response to the stress (Doherty et al., 2010) or decrease due to overwhelming effect of the pollutants (Faramobi et al., 2007; Olagoke 2008; Ujowundu et al., 2011), it is then sufficient to conclude that the decrease in the activity of SOD is due to the overwhelming effect of the toxicants from the smoked fish diets where the system used the SOD to detoxify the resulting superoxide radicals.

The activities of these enzymes (CAT, SOD and GPX) were restored towards normal in the *A. cepa* and *S. lycopersicum* treated groups (PSPS, PSPA and PSPSA), suggesting the protective effect of *A. cepa* and *S. lycopersicum*. The active constituents of the plants might have caused a stabilization and repair of plasma membranes damaged by exposure to the toxicants in the roasted samples (Thabrew et al., 1987). However, the combined treatment with *A. cepa* and *S. lycopersicum* was more effective in restoring the antioxidant enzymes to normal, probably due the phenomenon/effect of synergy when compared to the individual actions of *A. cepa* and *S. lycopersicum*.

Accumulation of PAH compounds in the liver, kidney and other organs might have caused serious pathological damage due to the exposure (Vutukuru et al., 2007). When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the bloodstream making the enzymes activities in the blood to increase. Measurement of the activities of serum (plasma) marker enzymes like AST, ALT, and ALP as well as levels of serum (plasma) total bilirubin have proved a powerful tool for the assessment of liver function (Ulican et al., 2003; Porchezhan and Ansari, 2005). The significant increase in the plasma activities of ALT, AST, ALP (Figure 6) and concentration of total bilirubin (TBil) (Figure 7) observed in the rats fed with PSP is indicative of liver damage induced by the toxicants (PAHs and Heavy metals) in the smoked fish diet. The significant increase in these enzymes could either be due to their possible leakage from the cytosol across damaged plasma membrane into the general...
Figure 7. Serum Bilirubin concentration in rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of *S. lycopersicum* and *A. cepa*. Each bar represents the mean ± SD. Bars with different letters are significantly different (p < 0.05). PO = Pellets only; PSF = 60% pellet + 40% fish smoked with firewood; PSP = 60% pellets + 40% Fish smoked with polyethylene materials; PSPS = 50% pellets + 40% FSP + 10% *S. lycopersicum*; PSPA = 50% pellets + 40% FSP + 10% *A. cepa*; PSPSA = 50% pellets + 40% FSP + 5% *S. lycopersicum* + 5% *A. cepa*.

Blood circulation or increase in their synthesis as a result of the organ dysfunction (Ploa and Hewitt, 1989; Vasanth et al., 2012). It could also be due to induction of necrotic lesions in the hepatocytes of the rats (Ujowundu et al., 2011). Thus the elevated enzymes activities could be considered to be manifestation of oxidative stress (Vasanth et al., 2012) caused by the toxicants in the smoked fish diets (Ujowundu et al., 2014). These results demonstrated that both *A. cepa* (onion) and *S. lycopersicum* (tomato) exhibited hepatoprotective action against toxicants in the smoked fish diet. The reduced activities of ALT and AST and concentration of total bilirubin in groups which diets were supplemented with *A. cepa* and *S. lycopersicum* toward the normal control values indicates stabilization of plasma membrane and/or repair of damaged hepatic tissues. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew et al., 1987). The plants mediated suppression of the increased ALP activity with the concurrent reduction of raised bilirubin level which suggests the possibility of the plants constituents being able to stabilize biliary dysfunction in the rat liver in sub-acute hepatic injury by the toxicants. This agrees with the work of Weremfo et al. (2011) which investigated the hepatoprotective activity of tomato pulp against carbon tetrachloride-induced hepatic damage in rats. However, *A. cepa* significantly (P < 0.05) showed much efficacy than *S. lycopersicum* in restoring AST and ALT activities to normal, while *S. lycopersicum* was slightly more effective in reducing the levels of total bilirubin and ALP to normal. Nevertheless, the combined *A. cepa* and *S. lycopersicum* action dominated in effectiveness than the individual plants.

More so, there was significant decrease (P < 0.05) in albumin (Alb) and total protein (TP) (Figure 8) in the PSF and PSP when compared to the control (PO). Albumin specifically synthesized by the liver, is among the major antioxidant components of the plasma, and might play a major role of the total antioxidant capacity of plasma (Aycicek et al., 2005; Ujowundu et al., 2012b). The total protein, albumin and globulin level may decrease due to liver dysfunction, malnutrition and malabsorption, diarrhoea, nephrosis and acute haemolytic anaemia (Ekam et al., 2012). The decrease in the concentration of albumin and total protein may be due to compromised synthetic function of the liver induced by oxidative stress. Another factor that may be responsible for the reduction albumin, is its role in binding a host of endogenous and exogenous substances (xenobiotics) while acting as an antioxidant. This important molecules might have been used to scavenge the resulting free radicals and heavy
metals (toxicants from the roasted fish). The toxic ROS load might have exceeded the capacity of the animals' antioxidant systems, causing albumin to be used, leading to its reduction. The decrease in the concentrations of albumin and total protein in the study is corroborated with the work of Nwaogu and Onyeze (2010) and Ujowundu et al. (2012b). Higher concentrations of these proteins were observed in plants-treated groups (PSPS, PSPA and PSPSA), suggesting a hepatoprotection by A. cepa and S. lycopersicum. S. lycopersicum showed better ability than A. cepa in restoring these proteins to normal values. However the combined A. cepa and S. lycopersicum supplemented diet proved to be much more effective than the individual treatment. This suggests that the constituents of the plants (A. cepa and S. lycopersicum) could have worked synergistically.

Furthermore, the result of the study shows significant decrease (P < 0.05) in the cholesterol concentration in the PSF and PSP fed rats (Figure 9) when compared with the control. Cholesterol is primarily synthesized by the liver. Hence, the reduction in the concentration of cholesterol in the intoxicated groups may indicate a decrease in the synthetic function of the liver caused by oxidative stress (Poulos et al., 1973). Diet supplement with A. cepa and S. lycopersicum did not show much restoration potential in the rats when compared to the cholesterol concentration in the intoxicated rats.

Conclusion

From our findings, it can be concluded that roasting of fish, especially using polyethylene materials, generates a lot of toxicants. However, phytochemicals derived from plant such as A. cepa and S. lycopersicum can act as antioxidants. Because they act as free radical scavengers, singlet oxygen quenchers or metal chelators, consumption of plant products possessing antioxidant potential protects humans from the oxidative damage of reactive oxygen species (ROS).

Conflict of Interests

The authors have not declared any conflict of interests.
Figure 9. Serum Cholesterol of rats maintained on diet prepared with fish smoked with polyethylene (plastics) and the antioxidative role of S. lycopersicum and A. cepa. Each bar represents the mean ± SD. Bars with different letters are significantly different (p < 0.05). PO = Pellets only; PSF = 60% pellet + 40% fish smoked with firewood; PSP = 60% pellets + 40% Fish smoked with polyethylene materials; PSPS = 50% pellets + 40% FSP + 10% S. lycopersicum; PSPA = 50% pellets + 40% FSP + 10% A. cepa; PSPSA = 50% pellets + 40% FSP + 5% S. lycopersicum + 5% A. cepa.

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radicals and PHAs generated in particular soot emissions and
residual ash from controlled combustion of common type of plastics.


Genetic variation within and between three Vietnamese pine populations (*Pinus merkusii*) using random amplified polymorphic DNA (RAPD) markers

Ho Manh Tuong*, Nguyen Thu Giang, Chu Hoang Ha and Le Van Son

Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

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*Pinus merkusii* is an important species in Vietnam with many economic and biological contributions. The information on diversity within and between populations of a species is necessary for plantation programs, breeding and conservation strategies. Genetic diversity of three Vietnamese populations (NA, QB and QN) was analyzed using the random amplified polymorphic DNA (RAPD) markers. Nine RAPD primers produced 82 markers, 77 of which were polymorphic with 93.9% of polymorphism. The results showed higher genetic variation within populations (72%) than between populations (28%) and low Nei’s genetic differentiation index among populations (0.1867). The populations also clustered based on PCoA analysis where cluster I included NA and QB populations and Cluster II, the QN population. These results suggest that *P. merkusii* populations in Vietnam is necessary to develop the genetic resources.

Key words: DNA markers, genetic diversity, *Pinus merkusii*, random amplified polymorphic DNA (RAPD), Vietnam.

INTRODUCTION

*Pinus merkusii* is a tropical forest tree grown and planted naturally across the south Equator. This species is distributed naturally and artificially in Southeast Asia including Vietnam, Laos, Cambodia, Thailand, Malaysia, China and the Philippines (Cooling, 1968; Farjon, 2005; Razal et al., 2005; Theilade et al., 2000). It can grow from 30 to 2000 m above the sea level (Cooling, 1968; Santisuk, 1997). This species is used for the production of heavy wood, fuel, pulp, timber, resin and turpentine. Resin which is used as material for medicine, paints, printing and perfume industry is one of its most important products (Hidayat and Hansen, 2002; Razal et al., 2005; Theilade et al., 2000). *P. merkusii* is one of the principal tree in reforestation, soil erosion control and rehabilitation in Vietnam and Indonesian Islands (Hidayat and Hansen, 2002; Razal et al., 2005). This species has been extremely exploited which reduced areas of its habitats, habitat quality which led to dramatic decrease in natural

*Corresponding author. Email: tuongcns@ibt.ac.vn. Tel: +84-4-37918003. Fax: +84-4-38363144.

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areas in the past decades (Bharali et al., 2012; Kumar et al., 2000). As a result, it is listed in the International Union for Conservation of Nature as the world’s threatened forest tree species (IUCN, 2011).

The degree of genetic diversity between populations is important for re-plantation breeding and conservation strategies. Genetic variation plays the crucial role for the long-term stability (Sharma et al., 2002). The exploitation of some plant species by human have been altered for a long time (Finkeldey and Hattemer, 2007). As a consequence, many plantations of forest species show low genetic diversity. Although, most studies of *P. merkusii* were provided by isozyme analysis (Changtragoon and Finkeldey, 1995; Suwarni et al., 1999; Siregar and Hattemer, 2004; Thao et al., 2013), DNA markers such as simple sequence repeat (ISS), inter simple sequence repeat (ISSR), RAPDs have been used successfully used for analysis of pines populations (Alrababah et al., 2011; Marquardt et al., 2007; Mariette et al., 2001; Nurtjahjainingsih et al., 2007; Navascues and Emerson, 2007; Gauli et al., 2009; Thao et al., 2013; Thomas et al., 1999; Zhang et al., 2005). RAPD markers are useful tools to analyze the genetic diversity within and between plant populations (Fritsch and Rieseberg, 1996; Cruzan, 1998). Many species have been assessed using marker such as *Gentianella germanica* (Fischer and Matthies, 1998), rice (Qian et al., 2001) as well as pines (Alrababah et al., 2011; Lee et al., 2002; Xia et al., 2001; Zhang et al., 2005).

The information of genetic variation is essential for selecting, breeding and conservation strategies. This study tends to identify the level of genetic variation within and between three *P. merkusii* populations that help in breeding and conservation strategies to maintain the genetic diversity resources.

### MATERIALS AND METHODS

#### Sample collection

Young clean leaves were collected from 79 *P. merkusii* lines from three populations at three provinces of Vietnam (Table 1 and Figure 1). All samples were stored in -80°C after collecting.

#### DNA isolation

Total DNA was isolated from young leaves following Doyle and Doyle (1990) method. DNAs quality and quantity were accessed using NanoDrop Lite (Thermo scientific, USA), the DNA concentration was adjusted to 50 ng/µL for use in RAPD-PCRs.

#### PCR amplification for RAPD markers

Nine RAPD markers were selected for this study (Table 2). PCR reaction was performed in PCR vertiti@96well-Fast Thermal Cycler (Thermo Scientific, USA) with total volume of 25 µL containing: 1X polymerase chain reaction buffer, 0.25 mM dNTPs, 2 mM MgCl₂, 0.2 µM each primer F/R, 1 U Taq DNA polymerase, 100 ng DNA template and sterile ultrapure water. PCR thermal cycles consist of the following steps: 95°C for 4 min, followed by 35 cycles of denaturation at 92°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min, final extension at 72°C for 10 min and 4°C for 30 min. Amplification products were analyzed by electrophoresis on 1.5% agarose gel with TAE buffer, stained with ethidium bromide, and photographed under ultraviolet light. The bands were scored by using PyElph software (Pavel and Vasile, 2012).

#### Genetic diversity analysis

DNA fragments were scored for presence (1) or absence (0), and analyzed using GenAIEx6. The data matrix was then subjected to analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA) and the diagram for identifying the genetic diversity within and between three populations by GenAIEx software (Peakall and Smouse, 2006). Nei’s gene diversity (h), Shannon’ information index (I), mean observed number of alleles (Na); mean effective number of alleles (Ne); percentage of polymorphic loci (P) and Nei’s genetic distance between populations (Gst) were analyzed using Poppgene software version 3.5 (Yeh et al., 2000).

### RESULTS

#### Polymorphism of RAPD markers

Nine RAPD primers were able to amplify DNA fragments of 79 individuals of *P. merkusii* (Table 2). A total of 82 bands were obtained including 77 polymorphic bands with mean of 93.90%. The results also showed that the average number of markers were 9.1 for primer and the average number of polymorphic markers were 8.55 primer. The fragment sizes fluctuated between 200 and 1500 bp and the number of bands ranged from 4 (RA143) to 14 (OPE14). The PIC values of primers were high and ranged from 0.724 to 0.88 with an average of 0.82. Figure 2 showed the result of amplification for the portion...
Figure 1. The locations of the three populations of *P. merkusii* (NA, QN and QB) in Vietnam.

Table 2. Primer sequences, length and number of amplified and polymorphic bands and PIC values of nine RAPD primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Length (bp)</th>
<th>No. of amplified bands</th>
<th>No. of polymorphic bands</th>
<th>Polymorphism (%)</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA46</td>
<td>CCAGACCCCTG</td>
<td>200-1200</td>
<td>11</td>
<td>9</td>
<td>81.81</td>
<td>0.88</td>
</tr>
<tr>
<td>RA143</td>
<td>TCGGCATAG</td>
<td>500-1000</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>0.724</td>
</tr>
<tr>
<td>RA159</td>
<td>GTTCACACGG</td>
<td>250-800</td>
<td>9</td>
<td>9</td>
<td>100</td>
<td>0.848</td>
</tr>
<tr>
<td>OPB10</td>
<td>CTGCTGGGAC</td>
<td>250-1400</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>0.855</td>
</tr>
<tr>
<td>OPD20</td>
<td>ACCCGGTCAAC</td>
<td>300-1000</td>
<td>8</td>
<td>7</td>
<td>87.5</td>
<td>0.827</td>
</tr>
<tr>
<td>OPE14</td>
<td>TGGCGCTGAG</td>
<td>250-1200</td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>0.829</td>
</tr>
<tr>
<td>OPF09</td>
<td>CCAAGCTTCC</td>
<td>400-1500</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>0.781</td>
</tr>
<tr>
<td>OPG13</td>
<td>CTCTCGCACA</td>
<td>250-1500</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>0.875</td>
</tr>
<tr>
<td>OPR08</td>
<td>CCCGTGGCCT</td>
<td>250-1500</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>0.852</td>
</tr>
<tr>
<td>overall</td>
<td></td>
<td></td>
<td>82</td>
<td>77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>9.1</td>
<td>8.55</td>
<td>93.90</td>
<td>0.82</td>
</tr>
</tbody>
</table>

PIC: Polymorphic information content.

of NA and QB populations with OPF09 and RA46 primers. These results indicated that all nine RAPD primers are significant for assessing *P. merkusii* populations.
Genetic variation within populations

The genetic variation within population is shown in Table 3. The overall genetic diversity of Vietnam pine indicated that the percentage of polymorphic loci ($P$) was highest for QB (74.67%) and lowest for QN (69.33%) with mean of 72.44%. Furthermore, the NA population revealed the highest value in the number of alleles per locus with 1.627 and QN population allocated at the lowest point with 1.507. Meanwhile, the number of effective alleles per locus was the highest and lowest value at QB (1.442) and NA (1.401), respectively, with an average of 1.424.

Moreover, in QB population, the Shannon information index ($I$) and Nei’s gene diversity ($h$) reached the highest point of 0.387 and 0.258, respectively, and showed the lowest point at QN (0.359) and NA (0.238).

Genetic variation between populations

Genetic distance ranged from 0.042 (NA and QB) to 0.214 (NA and QN). The Nei’s genetic differentiation ($Gst$) between populations which is 0.1867 was also calculated. The result indicated the low genetic variation among populations. Genetic diversity in total set of population ($Ht$) and average gene diversity within

![Image](Afr. J. Biotechnol.)

Table 3. Genetic diversity within three *P. merkusii* populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>$P$ (%)</th>
<th>Na</th>
<th>Ne</th>
<th>$I$</th>
<th>$h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>26</td>
<td>73.33</td>
<td>1.627</td>
<td>1.401</td>
<td>0.360</td>
<td>0.238</td>
</tr>
<tr>
<td>QB</td>
<td>25</td>
<td>74.67</td>
<td>1.613</td>
<td>1.442</td>
<td>0.387</td>
<td>0.258</td>
</tr>
<tr>
<td>QN</td>
<td>28</td>
<td>69.33</td>
<td>1.507</td>
<td>1.430</td>
<td>0.359</td>
<td>0.242</td>
</tr>
<tr>
<td>overall</td>
<td>72.44</td>
<td>1.582</td>
<td>1.424</td>
<td>0.369</td>
<td>0.246</td>
<td></td>
</tr>
</tbody>
</table>

N: Number of samples; Na: number of alleles per locus; Ne: number of effective alleles per locus; $I$: Shannon’s information index; $h$: Nei’s gene diversity; $P$: percentage of polymorphic loci.
Table 4. Nei’s genetic distance of three P. merkusii populations.

<table>
<thead>
<tr>
<th></th>
<th>NA</th>
<th>QB</th>
<th>QN</th>
<th>Ht</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>0.000</td>
<td></td>
<td></td>
<td>0.3131</td>
<td></td>
</tr>
<tr>
<td>QB</td>
<td>0.042</td>
<td>0.000</td>
<td></td>
<td>0.2546</td>
<td></td>
</tr>
<tr>
<td>QN</td>
<td>0.214</td>
<td>0.2</td>
<td>0.000</td>
<td>0.1867</td>
<td></td>
</tr>
</tbody>
</table>

Ht: Gene diversity in total set of populations; Hs: average gene diversity within population; Gst: Nei’s genetic differentiation index among populations.

Table 5. Analysis of molecular variance (AMOVA) for three pines populations.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Est.Var.</th>
<th>Total variation (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among population</td>
<td>2</td>
<td>210.705</td>
<td>105.352</td>
<td>3.641</td>
<td>28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>76</td>
<td>727.953</td>
<td>9.578</td>
<td>9.578</td>
<td>72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>938.658</td>
<td>13.219</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df: Degree of freedom; p value: the probability of obtaining a more extreme component estimate by chance; SS: sums of square; Est.Var: estimate variation component

Figure 3. The clustering of three P. merkusii populations based on PCoA analysis. Coord.1 and coord.2 are the first and second components of PCoA analysis, respective.

The genetic diversity of three populations was established based on PCoA analysis (Figure 3). The PCoA coordinate dimensions accounted for 22.85 and 19.74% of the total variation, respectively. The population divided into two groups where cluster I included NA and QB groups and cluster II contained QN group.

DISCUSSION

The RAPD markers were successful for analyzing genetic variation in Vietnam pine populations. All the nine RAPD primers expressed high number of bands and PIC (0.82). These results illustrated that RAPD method is a powerful technique for genetic variation analysis of Vietnamese populations. Nei’s gene diversity (h) (0.246) and Shannon’ information index (I) (0.369) of this study suggested a moderate genetic diversity within three Vietnamese pine populations. The moderate genetic diversity within population may be caused by the bottlenecks during the evolutionary processes and the inbreeding after
bottlenecked of small populations (Alrabanbah et al., 2011; Zhang et al., 2005).

The Nei’s genetic differentiation index among populations (Gst) (0.1867) reflected narrow genetic variation among populations. The genetic distances among three populations were low, from 0.042 (NA and QB) to 0.214 (NA and QN). The low level of genetic distance among NA and QB populations was the characteristic of endangered species (Slatkin, 1985). Moreover, the results were caused by the genetic drift, migration, selection or isolation of gene resources (Fisher and Matthies, 1998; Sun and Wong, 2001; Gómez et al., 2010). Other causes were that the local communities selected and breeding of these plants from other locations (Gómez et al., 2010). Furthermore, the QN population was more different than NA and QB caused by the geographic distance.

Interestingly, the molecular variation within population (72%) showed higher than between populations (28%) (Table 5). This result is crucial for outcrossing woody plants (Alvarrez et al., 2001; Hamrick and Godt, 1996; Heaton et al., 1999; Steiger et al., 2002). High level of genetic within populations is significant for breeding strategies. Alvarez et al. (2001) showed that cross pollinating species always have the genetic diversity higher than self-pollinating species (Alvarez et al., 2001). In the PCOA diagram (Figure 3), the first and second coordinate accounted for 22.85 and 19.74% of the total variation. The result showed low genetic variation among the P. merkusii populations.

In conclusion, this study suggests that three P. merkusii populations need effective conservation and breeding programs to develop the genetic diversity of resources of pines in Vietnam.

Conflict of Interests

The authors certify that there is no actual or potential conflict of interest in relation to this article.

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