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Full Length Research Paper

Genetic diversity of grape germplasm as revealed by microsatellite (SSR) markers

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In this work, cluster analysis and principal component analysis (PCA) were used to study the genetic diversity and relationships among 49 grape germplasm accessions analyzed with 19 simple sequence repeat (SSR) primer pairs. In total, 139 polymorphic loci were detected among these accessions with an average of 7.32 polymorphic loci per SSR primer pair. The average values for the effective number of alleles, Nei's gene diversity, and Shannon's information index were 1.5605, 0.3352 and 0.5064, respectively. The cluster analysis showed that the 49 accessions could be divided into five groups and an outgroup. The results of the PCA were nearly consistent with those of unweighted pair-group method with arithmetic averages (UPGMA) clustering analysis. These results will be useful for the exploitation of grape germplasm in basic and applied research.

Key words: *Vitis vinifera* L., simple sequence repeat (SSR), genetic diversity, principal component analysis.

INTRODUCTION

Vitis vinifera L. is a precious horticultural crop worldwide and is profoundly connected with the development of human culture (This et al., 2006). The genus *Vitis* L., with approximately 60 species, contains a large number of the Vitaceae and is primarily found in Europe, North America, and East Asia (Emanuelli et al., 2013). Due to the rising demand for higher-quality grape products, including fruits, raisins, juice, wine, etc., the economic value of excellent grape varieties is consistently increasing. Over the past few decades, the planting of single species with high quality and yield has resulted in the drastic reduction of genetic diversity in both cultivated and wild grapevines (Santana et al., 2008). The narrow genetic base of cultivated varieties makes them susceptible to diseases, pests, and environmental conditions. Likewise, the genetic variation of wild *V. vinifera* species has slowly diminished due to the loss of natural habitat (Emanuelli et al., 2013). To avoid further losses of valuable genes and genotypes, it is of significant importance to take effective protection measures, which requires research into genetic relationships and the reconstruction of pedigrees (Bowers et al., 1999; Benjak et al., 2005; Santana et al.,

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Abbreviations: PCA, principal component analysis; **SSR,** simple sequence repeat; **UPGMA,** unweighted pair-group method with arithmetic averages.

2008). Cultivars with desirable traits have high potential breeding value, and those with genes of enological or organoleptic interest could be important resources to plant breeders and geneticists (Santana et al., 2008). Another crucial factor in breeding success is the phylogenetic relationships between parents. Information on the amount and distribution of genetic variation in grape germplasm collections is therefore essential for the development of conservation strategies and efficient use of *Vitis* germplasm resources (De Andrés et al., 2012).

The development of DNA-based markers has provided widely used methods for quantifying variation within germplasm, including that of grapes (Emanuelli et al., 2013). Simple sequence repeats (SSRs), also known as microsatellite makers, have been widely applied to investigate genetic diversity, distinguish populations, and determine reproductive characteristics in various organisms due to their high degree of polymorphism, reproducibility, and codominant nature (Doulati-Baneh et al., 2013). Recently, several studies have been conducted to decipher the origin, construct genetic maps, and determine the genetic structure of cultivated grapes using nuclear microsatellite analysis (Bowers et al., 1996; Scott et al., 2000; Santana et al., 2010; Doulati-Baneh et al., 2013). Santana et al. (2010) reported on the origins, genetic structure, and relationships of 421 cultivated and four (allegedly) wild grapevine samples from the Castilian Plateau of Spain based on six nuclear microsatellite loci (SSRs). Doulati-Baneh et al. (2013) examined 67 grape cultivars from Iran using SSR markers and analyzed the genetic distances and population structure in the studied germplasm.

Most previous studies have focused on *V. vinifera* L. cultivars from a single location (Agar et al., 2012), which limits the utilization of the species to some extent. In this work, we selected 49 grape germplasm accessions originating from several different countries and investigated their genetic diversity and evolutionary relationships using 19 SSR markers.

MATERIALS AND METHODS

Plant materials

A total of 49 accessions were collected and analyzed in this study. Accession names and their geographic origins are listed in Table 1. The accessions were all kindly provided by the grape germplasm repository of Yantai Changyu Pioneer Wine Company Limited. Young leaves were randomly sampled from adult trees and frozen in liquid nitrogen.

DNA extraction and SSR analysis

Total genomic DNA was extracted using the Ezup Column Plant Genomic DNA Purification Kit (Sangon, Shanghai, China) following the manufacturer's protocol. DNA concentration and purity were determined by UV-spectrophotometry at 260/280 nm, and its integrity was confirmed using 1% agarose gel electrophoresis. PCR was performed in a 25 µL total volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM of Mg^{2+}, 0.2 mM of each dNTP, 0.25 µM of each primer, and 1 unit of DNA Taq polymerase (Takara Biotech Co. Ltd., Japan) with 30 ng of DNA as templates. PCR was conducted as follows: 94°C for 5 min; 36 cycles consisting of denaturation at 94°C for 30 s, annealing at 48 to 63°C (depending on primer pair) for 30 s, and synthesis at 72°C for 1 min; and a final elongation at 72°C for 10 min. Twenty grapevine SSRs were used, and a set of 19 highly polymorphic markers were considered suitable for assessing variation among the studied samples (Table 2). The PCR products were separated on 6% (w/v) polyacrylamide gels and visualized with silver staining.

Genetic diversity analysis

The data were used for the following statistical analyses. The number of alleles per locus (N), effective number of alleles (Ne), Nei's gene diversity (H), and gene diversity (Shannon's information index = I) were calculated to estimate the genetic variation level. All of the above calculations were performed using POPGENE version 1.32 (Yeh et al., 1997). Cluster analysis was performed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-PC) version 2.1 (Rohlf, 2002). A dendrogram was constructed via the unweighted pair-group method with arithmetic averages (UPGMA), and similarity coefficients were employed to reveal the relationships among the 49 accessions. Principal component analysis (PCA) was performed by NTSYS 2.1.

RESULTS

Polymorphism of SSR markers

The genetic variation statistics for the 19 SSR markers are summarized in Table 2. A total of 139 polymorphic alleles were amplified using the 19 SSR markers, ranging from 3 (scu16vv) to 17 (VrZAG62) alleles per locus. Ne among the studied markers ranged from 1.2376 (scu16vv) to 1.8449 (VrZAG64), with an average of 1.5605. The H of the 19 SSR markers ranged from 0.1834 (scu16vv) to 0.4543 (VrZAG64), with an average of 0.3352. The values of I ranged from 0.3183 (VVMD6) to 0.6458 (VrZAG64), with an average of 0.5064.

Genetic relatedness

To analyze the genetic relationships among the tested cultivars, the similarity coefficients were calculated with NTSYS-PC 2.1 using UPGMA. 'Cabernet Gernischet' 1–8 represent eight 'Cabernet Gernischet' cultivars from eight different areas in Yantai. The similarity coefficient between 'Cabernet Gernischet 6' and the other seven 'Cabernet Gernischet' cultivars, which were shown to be the same cultivar based on their similarity coefficients (1.0000), was 0.9712. The similarity coefficients of the tested grape accessions ranged from 0.4029 to 0.9856. The SSR UPGMA dendrogram partitioned the 49 tested cultivars into five main groups and an outgroup by clustering varieties with more than 60% similarity (Figure 1). Groups A, B, C, D, and E consisted of 11, 3, 8, 13, and 12 accessions, respectively. Group A was composed

Table 1. List of grape cultivars used in this study.

Cultivar	Pedigree	Species	The introduction year	Source of collection
Chaush	Unknown	*V. vinifera* L.	1980s	Russia
Cabernet Franc	Ancient variety of France	*V. vinifera* L.	1890s	France
Malvasia Istriana	Ancient variety of Greece	*V. vinifera* L.	2000s	Italy
ВиНТА	Unknown	*V. vinifera* L.	1980s	Bulgaria
Yan Tai No: 73	Muscat hamburg × alicante bouschet	*V. vinifera* L.	----	China
Beta	Unknown	*V. vinifera* L.	1960s	America
Volga-Don	Unknown	*V. vinifera* L.	1960s	Uzbekistan
Xiongyuebai	(Muscat Hamburg× *V. Amurensis*) × Longyan	*V. vinifera* L. × *V. amurensis* Rupr.	----	China
Bacco Noir	Unknown	*V. vinifera* L. × *V .vulpina* L.	1950s	France
Gongniang No: 2	Muscat Hamburg × *V. Amurensis*	*V. vinifera* L. × *V. amurensis* Rupr.	----	China
Cabernet Gernischet 1	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 2	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 3	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 4	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 5	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 6	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 7	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Gernischet 8	Ancient variety of France	*V. vinifera* L.	1890s	France
Cabernet Sauvignon	Cabernet franc × sauvignon blanc	*V. vinifera* L.	1890s	France
Muscat Hamburg	Schiava Grossa × Muscat of Alexandria	*V. vinifera* L.	1890s	England
V.amurensis Rupr.	Ancient variety of China	*V. amurensis*	----	China
Ampelopsis brevipedunculata	Ancient variety of China	*A. brevipedunculata*	----	China
Kyoho	Campbell early × centenial	*V. vinifera* L. × *V. labrusca* L.	1960s	Japan
Ruby Seedless	Emperor × pirovan075	*V. vinifera* L.	1980s	Eurasian
Jiubai	Unknown	*V. vinifera* L.	----	China
Gamay	Pinot noir × Gouais	*V. vinifera* L.	1950s	France
Dragon Oeil	Unknown	*V. vinifera* L.	1980s	Eurasian
Muscat Ottonel	Chasselas × Muscat de Saumur	*V. vinifera* L.	2000s	France
Superior Seedless	Unknown	*V. vinifera* L.	1990s	America
Rizamat	Uncertain	*V. vinifera* L.	1960s	Russia
Saperavi	Unknown	*V. vinifera* L.	1980s	Georgia
Phoenix	Uncertain	*V. vinifera* L.	1980s	West Germany
Autumn Royal	Autumn black × g74-1	*V. vinifera* L.	1998	America

Table 1. Contd.

Purple Queen	Unknown	*V. vinifera* L.	1980s	America
Black Queen	Unknown	*V. vinifera* L.	1980s	Japan
Christmas Rose	(Hunisa × emperor × nocera) × (hunisa × emperor × italia)	*V. vinifera* L.	1980s	America
Magumi	Ancient variety of Japan	*V. vinifera* L.	1990s	Japan
Honey Juice	Unknown	*V. vinifera* L.	1980s	Euro-american hybrids
Amelia	Unknown	*V. vinifera* L.	1990s	Chile
Pinot Blanc	Mutation of Pinot noir	*V. vinifera* L.	1950s	France
Galbena Veral	Unknown	*V. vinifera* L.	1970s	Romania
Grasade Cotnali	Unknown	*V. vinifera* L.	1980s	France
Kadarka 1	Ancient variety of Hungary	*V. vinifera* L.	1980s	Bulgaria
Boulgal	Unknown	*V. vinifera* L.	1970s	Turkey
Kadarka 2	Ancient variety of Hungary	*V. vinifera* L.	1980s	Hungary
Unknown	Unknown	*V. vinifera* L.	2000s	Chile
Stary goru	Ancient variety of Japan	*V. vinifera* L.	1980s	Japan
Medoc Noir	Unknown	*V. vinifera* L.	1980s	France
Vidal Blanc	Ugni blanc × seyval blanc	*V. vinifera* L.	1940s	France

Table 2. Summary of genetic variation statistics for the 19 simple sequence repeat markers.

Primer name	5’ to 3’	T/°C	N	Ne	H	I
VMC4F3	F: AAAGCACTATGGTGGGTGTAAA R: TAACCAATACATGCATCAAGGA	52	5	1.5804	0.3491	0.5277
VVS2	F: CAGCCCGTAAATGTATCCATC R: AAATTCAAAATTCTAATTCAACTGG	50	5	1.3298	0.2367	0.3899
VVIv37	F: TTTTCTCCCTACTCTTAACTTC R: GGTAGACCTTGAAATGAAGTAA	52	5	1.3561	0.2471	0.4050
VVIv67	F: TATAACTTCTCATAGGGTTTCC R: TTGGAGTCCATCAAATTCATCT	52	5	1.8060	0.4402	0.6305
VVMD5	F: CTAGAGCTACGCCAATCCAA R: TATACCAAAAATCATATTCCTAAA	50	5	1.4599	0.2790	0.4335
VVMD6	F: ATCTCTAACCCTAAAACCAT R: CTGTGCTAAGACGAAGAAGA	50	11	1.2784	0.1902	0.3183
VVMD7	F: AGAGTTGCGGAGAACAGGAT R: CGAACCTTCACACGCTTGAT	55	8	1.6948	0.3940	0.5777
VVMD8	F: TAACAAACAAGAAGAGGAAT R: AGCACATCCACAACATAATG	48	9	1.5821	0.3573	0.5394

Table 2. Contd.

VVMD31	F: CAGTGGTTTTTCTTAAAGTTTCAAGG R: CTCTGTGAAAGAGGAAGAGACGC	55	6	1.4575	0.2804	0.4328
VVMD32	F: TATGATTTTTTAGGGGGGTGAGG R: GGAAAGATGGGATGACTCGC	56	13	1.6487	0.3852	0.5708
VrZAG21	F: TCATTCACTCACTGCATTCATCGGC R: GGGGCTACTCCAAAGTCAGTTCTTG	61	6	1.5808	0.3493	0.5279
VrZAG25	F: CTCCACTTCACATCACATGGCATGC R: CGGCCAACATTTACTCATCTCTCCC	62	5	1.6406	0.3802	0.5654
VrZAG62	F: GGTGAAATGGGCACCGAACACACGC R: CCATGTCTCTCCTCAGCTTCTCAGC	62	17	1.6851	0.3886	0.5710
VrZAG64	F: GAAAGAAACCCAACGCGGCACG R: TGCAATGTGGTCAGCCTTTGATGGG	62	8	1.8449	0.4543	0.6458
VrZAG67	F: ACCTGGCCCGACTCCTCTTGTATGC R: TCCTGCCGGCGATAACCAAGCTATG	63	7	1.8025	0.4392	0.6294
VrZAG79	F: AGATTGTGGAGGAGGGAACAAACCG R: TGCCCCCATTTTCAAACTCCCTTCC	62	7	1.7306	0.4198	0.6101
scu07vv	F: CCGAAGAGGAATATGGGTTTGAG R: CCTAACTTGAAACGAAAGGACTGC	58	4	1.3265	0.2306	0.3796
scu15vv	F: GCCTATGTGCCAGACCAAAAAC R: TTGGAAGTAGCCAGCCCAACCTTC	58	10	1.6065	0.3641	0.5463
scu16vv	F: CAAAGACAAAGAAGCCACCGAC R: ACCCTCTAAAGCACACACAGGAAC	58	3	1.2376	0.1834	0.3196

T, annealing temperature; N, number of alleles; Ne, effective number of alleles; H, Nei's gene diversity; I, Shannon's information index.

by 'Muscat Hamburg,' 'Kyoho,' 'Ruby Seedless,' 'Amilia,' 'Kadarka 2,' 'Boulgal,' 'Volga-Don,' 'Magumi,' 'Galbena Veral,' 'Grasade Cotnali,' and 'Honey Juice.' Group B contained only 3 accessions: 'Beta,' 'Purple Queen,' and 'Gongniang No. 2.' Group C contained 'Chaush,' 'Pinot Blanc,' 'Saperavi,' 'Kadarka 1,' 'Xiongyuebai,' 'Jiubai,' 'Dragon Oeil,' and 'Rizamat,' while the 'Cabernet Gernischet' cultivars from Yantai were predominantly grouped in group D, together with 'Cabernet Franc,' 'Cabernet Sauvignon,' 'Gamay,' 'Muscat Ottonel,' and 'Bacco Noir.' Group E contained the other 12 accessions, except for '*Vitis amurensis* Rupr.' and '*Ampelopsis brevipedunculata*,' which composed the outgroup.

The similarity coefficient between 'Kyoho' and 'Ruby Seedless' was the highest among all accessions. Additionally, in the UPGMA dendrogram, 'Kyoho' was very close to 'Ruby Seedless,' and both accessions were clustered with 'Muscat Hamburg.' 'Gongniang No. 2' is the offspring of 'Muscat Hamburg' and '*V. amurensis* Rupr.' However, these accessions were not in the same cluster, as can be seen in Figure 1. The similarity coefficient between 'Gongniang No. 2' and 'Muscat Hamburg' was 0.7122, while that between 'Gongniang No. 2' and '*V. amurensis* Rupr.' was only 0.6619. In group D, 'Cabernet Gernischet 6' was clustered with the other seven 'Cabernet Gernischet' cultivars. 'Cabernet Sauvignon' was close to 'Gamay,' and the two accessions were grouped together with 'Cabernet

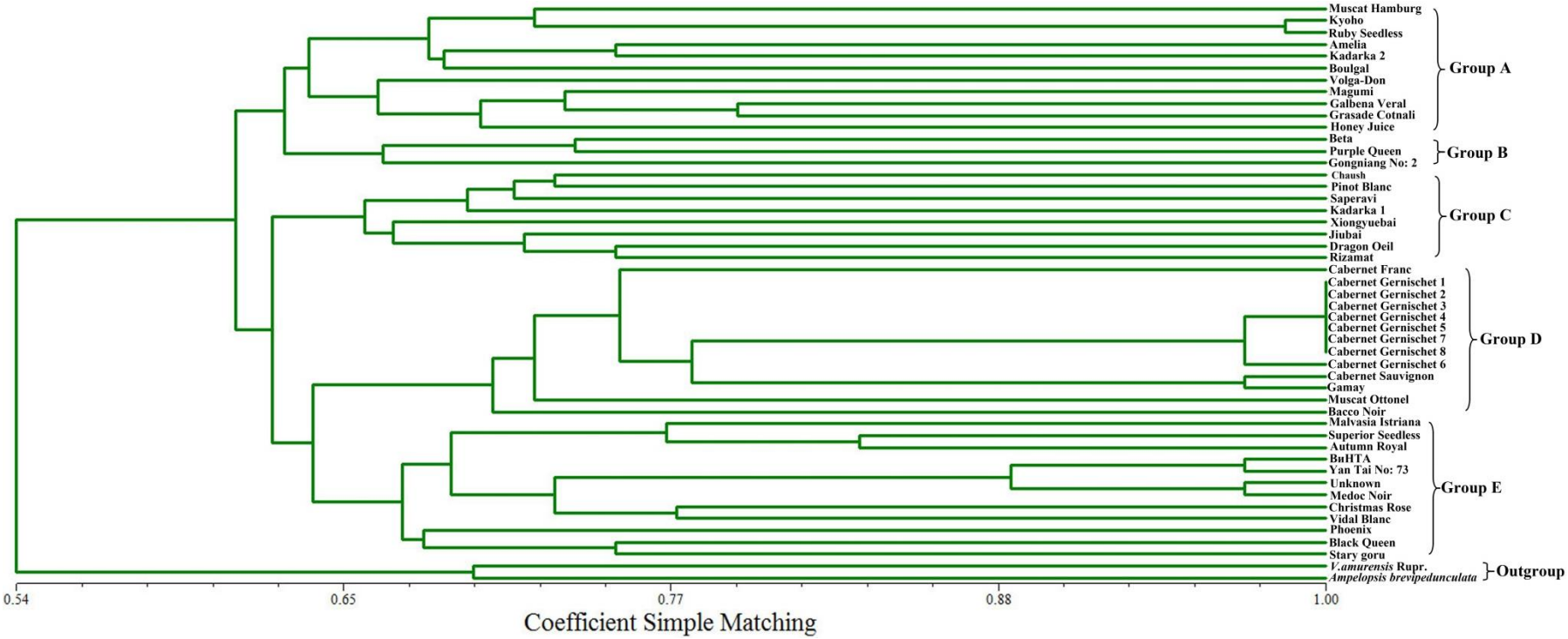

Figure 1. Unweighted pair-group method with arithmetic averages dendrogram of 49 grape germplasm accessions based on simple sequence repeat marker data

Franc.' 'ВиНТА' and 'Yan Tai No. 73' had a particularly close genetic relationship, as indicated by their similarity coefficient of 0.9712 and grouping into the same cluster. The similarity coefficient between the unknown Chilean accession and 'Medoc Noir' was also 0.9712, and a similar result can be seen in group E.

Principal component analysis

Conversely, the principal component analysis (PCA) based on the genotypic data from the SSR markers demonstrated the genetic divergence between the groups (Figures 2 and 3). Dim-1, dim-2, and dim-3 accounted for 17.33, 9.62, and 7.42% of the overall variation, respectively. The PCA results were nearly consistent with those of the UPGMA analysis, which had no difference among the 'Cabernet Gernischet' cultivars except for 'Cabernet Gernischet 6.' 'Kyoho' and 'Ruby Seedless,' the close genetic relationship which is shown in Figure 1, clearly overlapped in the PCA. However, the PCA results separated 'Saperavi' from group C. This result may have been due to dimensionality reduction.

DISCUSSION

In the present study, we selected SSR markers from these previous experiments to assess the phylogenetic relationships among 49 cultivated grapevines originating from different countries. Our results show that VrZAG64 had the highest level of genetic diversity (H = 0.4543; I = 0.6458) among all of the studied SSR markers, which suggested that VrZAG64 should have priority to be considered when estimating the genetic variation of grape cultivars. In the SSR UPGMA dendrogram, the unknown cultivar from Chile and

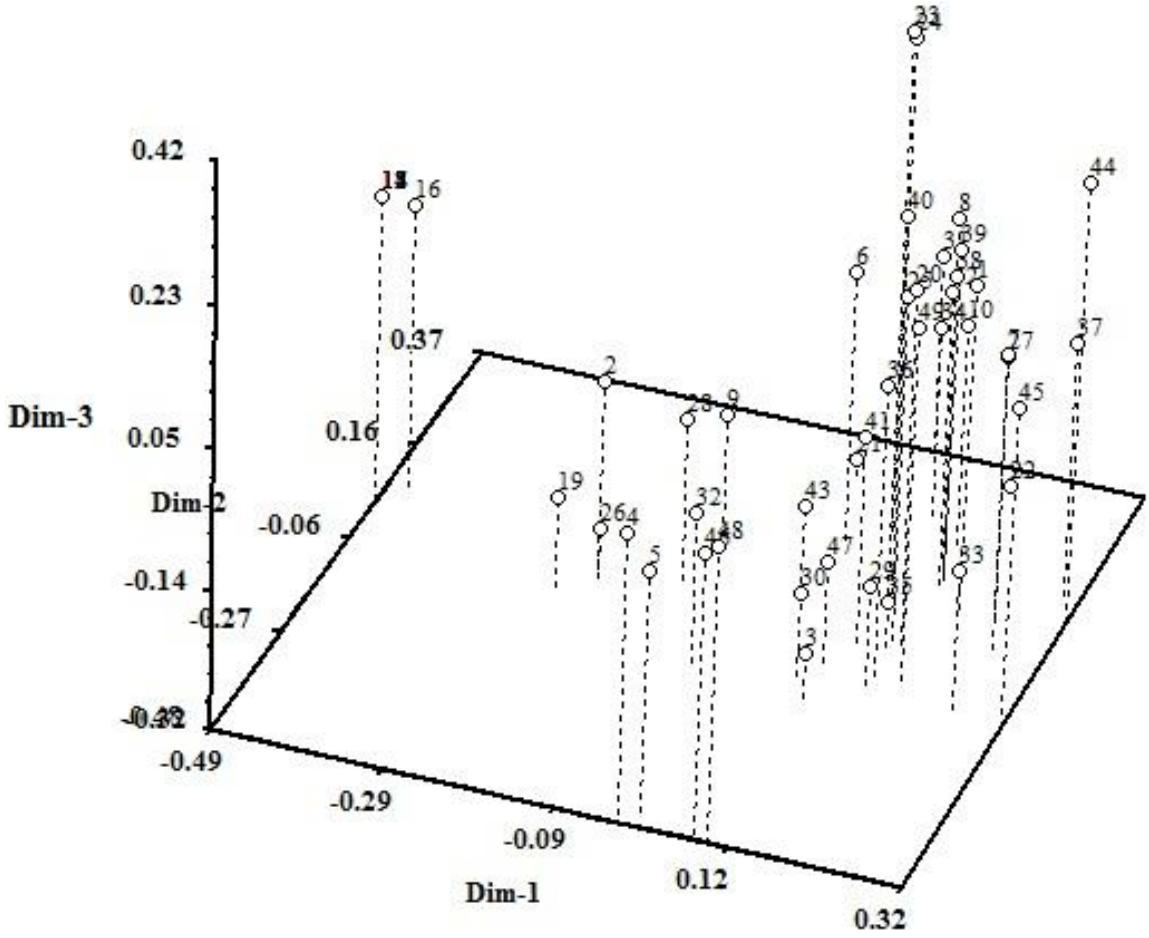

Figure 2. Principal component analysis of the simple sequence repeat markers associated with the grape germplasm accessions. The serial numbers of the accessions are shown in Table 1.

'Medoc Noir' from France (similarity coefficient = 0.9712, Figure 1) were clustered into a clade in Group E. This result implied that the unknown cultivar from Chile likely shares a common ancestry with 'Medoc Noir.' The dendrogram and similarity coefficients indicated that 'Cabernet Gernischet 6' was different from the other seven 'Cabernet Gernischet' cultivars, and the different geography and climate may be the reason why they have differences. This result also serves as a reminder that the protection of germplasm resources should be conducted to the greatest possible extent at the origins of the germplasm, as protection via relocation may potentially damage the germplasm resources. The similarity coefficient between 'Cabernet Franc' and 'Cabernet Sauvignon' was 0.7122 (Figure 1), close to the previous value found by D'Onofrio et al. (2010) using AFLP markers (0.688). These low values did not reflect the fact that 'Cabernet Sauvignon' is a cross of 'Cabernet Franc' and 'Sauvignon Blanc.' Therefore, to clarify the genetic relationship between 'Cabernet Sauvignon' and 'Cabernet Franc,' more information from the nuclear and chloroplast genomes should be considered. 'Kyoho' and 'Ruby Seedless' had a very close genetic relationship according to the cluster results and their similarity coefficient. This result is consistent with the knowledge that both accessions are offspring of the 'Emperor' cultivar. In contrast, the genetic distance between 'Muscat Hamburg' and 'Yan Tai No. 73' (Muscat Hamburg × Aicante Bouschet) is comparatively large despite their parent-offspring relationship; these cultivars were even assigned to two different groups. This result was consistent with that obtained by a previous SRAP marker study (Guo et al., 2012). A similar result also occurred between 'Muscat Hamburg,' '*V. amurensis*,' and 'Xiongyuebai' ((Muscat Hamburg × *V. amurensis*) × Longyan) (Figure 1). These observations indicated that some offspring displayed obvious heterosis, inheriting different superior qualities from their parents to obtain more desirable biological characteristics.

We also found that the similarity coefficients between cultivars from different countries were generally small, with the exception of that between 'ВиНТА' from Bulgaria

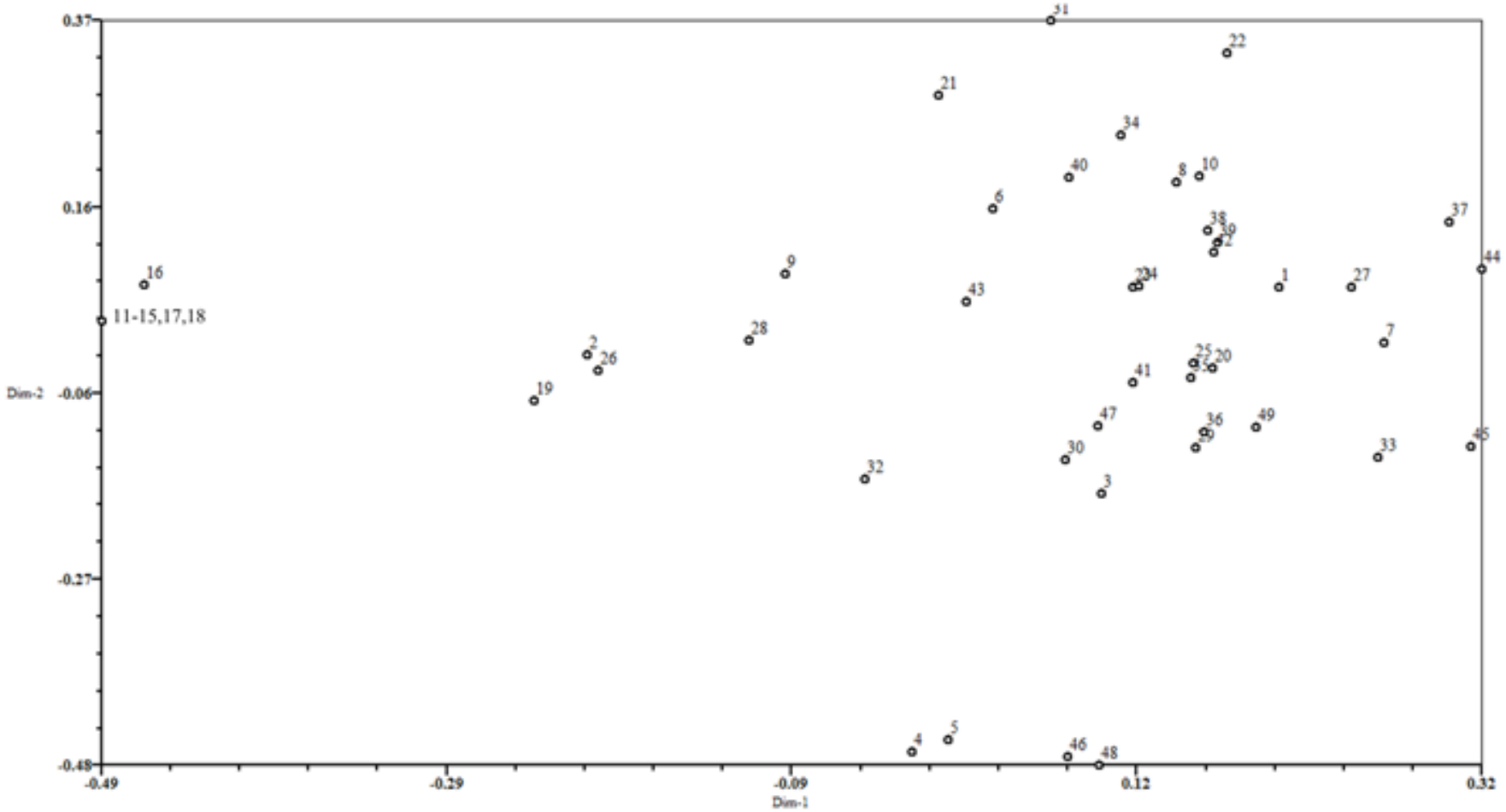

Figure 3. Simple sequence repeat markers associated with grape germplasm accessions based on principal components 1 and 2. The serial numbers of the accessions are shown in Table 1.

and 'Yan Tai No: 73' from China (0.9712, Figure 1). Given that the parents of 'ВиНТА' are not clear, 'ВиНТА' and 'Yan Tai No: 73' likely have a similar origin. In the UPGMA dendrogram, the groupings were not obviously related with the geographic origins of the cultivars (Figure 1). Cultivated populations from different countries may tend towards uniformity due to long-term adaptation to climate and human activities during the long history of cultivation for these accessions. Due to the high economic value of *V. vinifera* L., we strongly advise that core germplasm accessions of this species should be cultivated for conservation in their original regions instead of a single grape germplasm repository with a uniform

growth environment.

In conclusion, our work shows that the polymorphism of SSR molecular markers can provide important information on the inheritance and phylogenetics of grape germplasm. We identified the unknown Chilean accession using SSR markers, although we could not definitively determine its parentage. To better preserve genetic diversity, we suggest that new natural protection habitats should be established at the origins of germplasm accessions, and we recommend that the conservation and management of grape species prioritize populations with high allelic richness and heterosis (Lu et al., 2013). This work shows that assessing the genetic diversity of grape germplasm collections using SSRs is very efficient for basic and applied research. Further experiments should be performed to study grape genetic diversity. Based on the relationships among and characteristics of accessions, scientists can better protect germplasm resources and conduct breeding programs.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Characterization of a hemorrhage-inducing component present in *Bitis arietans* venom

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Better characterization of individual snake venom toxins is useful for analyzing the association of their toxic domains and relevant antigenic epitopes. Here we analyzed the *Bitis arietans* hemorrhagic-inducing toxin present in a representative venom sample. Among the 1´ to 5´ protein peaks isolated using a Sephacryl S 100 HR chromatography column, hemorrhagic activity was expressed by all according to the following intensity, P´5 > P´3 > P´2 >, P´4. The proteins were recognized and measured with antibodies present in polyvalent horse F(ab)´$_2$ anti-*B. arietans*, *Bitis* spp., *Lachesis muta, B. atrox*, *Bothrops* spp., *Crotalus* spp., and *Naja* spp. or in IgY anti-*B. arietans* and anti-*Bitis* spp. using enzyme-linked immunosorbent assays and western blotting. In addition, in an *in vitro-in vivo* assay these anti-venoms were able to block hemorrhagic-inducing activity. The evident cross-reactivity expressed by different specific anti-venoms indicates that metalloproteinases induce an immunological signature indicating the presence of similar antigenic epitopes for several snake venoms.

Key words: Snake venoms, anti-venoms, *Bitis arietans,* hemorrhage, metalloproteinases.

INTRODUCTION

In sub-Saharan Africa, snake bites are an important public health problem resulting in permanent disabilities and death (Theakston et al., 2003; Kasturiratne et al., 2008). *Bitis* spp., *Naja* spp., and *Dendroaspis* spp. include the greatest number of snake species that cause envenomation in Mozambique (Broadley, 1968; Manaçás, 1981-1982). Their venoms contain multiple and diverse toxins. *B. arietans* is a common snake found in densely populated savannah areas in sub-Saharan Africa and in the Arabian Peninsula (Navy, 1991). Victims of this snake species exhibit severe local and systemic disturbances, such as swelling, hemorrhage and necrosis (Warrell et al., 1975; Warrell, 1996; Currier et al., 2010; Calvete et al., 2006). Anti-venom supply in the African continent, however, is scarce and often unaffordable.

In 1996, we submitted a proposal to the Conselho Nacional de Ciência Tecnologia – PROÁFRICA, Ministério de Ciência e Tecnologia do Brazil Proc. No: 4800048/2006, including among the main objectives the development of procedures for producing specific anti-*Bitis* spp., anti-*Naja* spp., and anti-*Dendroaspis* spp. anti-venoms. The proposal was granted, and the anti-venoms

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were developed using whole venom as the immunogens and traditional methods of immunization (de Almeida et al. 2008; Guidolin et al. 2010).

To address the specific needs of the African region, high specificity, stable, affordable anti-venoms and poly-specific and lyophilized substitutes must be developed. To accomplish this objective, the initial step is the development of mono-specific anti-venoms. We begin this objective by developing an anti-*Bitis* spp. mono-specific anti-venom.

The metalloproteinases (SVMPs) and A-disintegrin proteinases (ADAMs) (Juárez et al., 2006; Moura-da-Silva et al., 2011; Trummal et al., 2005) comprise the M12b subfamily of zinc-dependent reprolysins. Both enzymes, along with the non-enzyme C-type lectin-like proteins, are important snake venom components (Bode et al., 1993; Stokër et al., 1995; Bjarnason and Fox, 1995). Previous studies indicate that SVMPs and ADAMs are involved in hemorrhage, edema, hypotension, inflammation and necrosis induced by snake venoms (Gutiérrez and Rucovaldo, 2000). Although Viperidae snake venoms are rich in SVMPs, these enzymes have also been observed in certain *Elapidae* and *Colubridae* venoms (Junqueira-de-Azevedo and Ho, 2002; Guo et al., 2007; Ching et al. 2006). SVMPs act by cleaving proteins on the vascular basement membrane and surrounding connective tissues as well as by altering platelet structure and function.

The introduction of toxins responsible for hemorrhage (Gutiérrez et al., 1995; Gutiérrez et al., 2005), complement system activating factors (*Tambourgi* et al., *2015*) and hypotension-inducing components (Kodama et al., 2015) present in *Bitis* sp. venom into an immunogenic mixture is considered essential to obtain high quality mono-specific anti-venom.

In this study, we purify and characterize the biological and immunological properties of a representative *B. arietans* 50-kDa protein endowed with hemorrhage-inducing activity.

MATERIALS AND METHODS

Reagents

The reagents used included: Tris buffer (Tris HCl, 25 mM; pH 7.4); Complete MMT80 (2 mL of Marcol Montanide ISA 50 in 5.0 mL of sodium chloride, 0.15 M, plus 1.0 mL of Tween 80 and 1.0 mg of lyophilized BCG); incomplete MMT80 (Marcol Montanide ISA 50 without BCG); SDS buffer (Tris, 6.25 mM, pH 6.8 plus 0.2% SDS); SDS buffer plus 1 mL of β-mercaptoethanol (SDS buffer plus 8.5 mL of glycerol and 2 mL of 1% bromophenol blue); PBS buffer (potassium chloride, 2.6 mM; monobasic potassium phosphate, 1.5 mM; sodium chloride, 76 mM; disodium phosphate, 8.2 mM; pH 7.2-7.4); AP buffer (Tris HCl, 100 mM, pH 9.5 plus sodium chloride, 100 mM, and magnesium chloride, 0.1 M); NBT solution (NBT, 50 mg; dimethylformamide in 700 µL plus 300 µL of H_2O). BCIP solution (BCIP, 50 mg; dimethylformamide in 1.0 mL of diluent); developing solution for western/dot blotting (AP buffer, 5.0 mL plus NBT solution, 33 and 16.5 µL of BCIP solution); citrate buffer, pH 5.0 (citric acid, 0.1 M, plus 0.2 M monobasic sodium phosphate); OPD solution (20 mg of OPD in 1.0 mL of citric acid); substrate buffer for ELISA (5 mL of citrate buffer plus 100 µL of OPD solution and 5 µL,). All reagents were obtained from Sigma-Aldrich (USA), except for NBT and BCIP, which were obtained from Molecular Probes (USA).

Snake venoms

African snake venoms from *B. arietans*, *B. nasicornis*, *B. rhinoceros*, *N. melanoleuca*, and *N. mossambica* were purchased from Venom Supplies Pty Ltd (59 Murray Street, Tanunda, Australia). Although African snake venoms were the focus, Brazilian snake anti-venoms from *Bothrops atrox*, *Bothrops jararaca, Lachesis muta*, and *Crotalus* spp. were also obtained to identify cross-reactions. Brazilian snake venoms were supplied by the Laboratório de Herpetologia, Instituto Butantan, São Paulo, Brazil. All venoms were collected from healthy adult snakes following standard methods, filtered through 0.45-µm membranes, assayed for protein content, and stored at -20°C.

Mice

Swiss and BALB/c isogenic mice (male and female; 18-20 g) were provided by the *Universidade Estadual de Campinas,* sp., Brazil animal facilities. The mice were used to determine the lethality (LD_{50}) of the venoms and the neutralizing potency (DE_{50}) of the anti-venoms. The animals used in this work were maintained and treated under strict ethical conditions according to International Animal Welfare Recommendations (World Health Organization, 981; Remfry, 1987). This study was approved by the *Comissão de Ética de Animais de Laboratório, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense – Darcy Ribeiro*.

Snake anti-venoms

Horse polyclonal $F(ab')_2$ anti-*B. arietans*, anti-*B. nasicornis* plus *B. rhinoceros*, and anti-*N. melanoleuca* plus *N. mossambica*, anti-*Lachesis muta*, anti-*Bothrops jararaca* and anti-*Crotalus durissus terrificus* were supplied by the Seção de Processamento de Plasmas Hiperimunes-Divisão de Desenvolvimento Tecnológico e Produção, Instituto Butantan, São Paulo, Brazil. Chicken polyclonal IgY anti-*B. arietans*, anti-*B. nasicornis plus B. rhinoceros*, and anti-*N. melanoleuca* plus *N. mossambica*, were prepared at the Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense - Darcy *Ribeiro, Campos do Goytacazes,* RJ (de Almeida et al.,2008). Horse $F(ab')_2$ anti-venoms were prepared at the Seção de Processamento de Plasmas Hiperimunes-Divisão de Desenvolvimento Tecnológico e Produção, Instituto Butantan, São Paulo, Brazil.

Quantification of proteins

The protein concentrations of the venoms, their purified fractions and the anti-venoms were assessed using the bicinchoninic acid method with a Pierce BCA Protein Assay Kit (Rockford, IL).

Determination of venom lethality (LD_{50})

The median lethal dose (LD_{50}) of each venom was determined in mice. Five serial venom dilutions were prepared in PBS. Groups of four mice were i.p. injected with each venom dilution. The control

group was injected with PBS. The death/survival ratios were determined after 48 h of observation. The LD_{50} was calculated using probit analysis (Finney, 1947; World Health Organization, 1981).

Evaluation of venom hemorrhagic activity (HD_{50})

The median hemorrhagic dose was evaluated by injecting (i.c.) 50-µL aliquots of PBS-saline, pH 7.0, containing different concentrations of crude or purified venom fractions into dorsally depilated mice anesthetized under a CO_2 atmosphere. After 48 h, the mice were again anesthetized under CO_2 atmosphere and bled.

***B. arietans* venom hemorrhagic activity**

Groups of mice were subcutaneously injected with different amounts of crude venom, with chromatograph isolated protein peaks P´1 P´2, P´3, P´5 or with 01 M NaCl (NCs) as negative control. In some experiments, *B. jararaca* venom was included as positive control. 2 h later the mice were anesthetized under CO_2 atmosphere, submitted to total bleeding, the skin dissected, and the hemorrhagic areas were exposed in the internal skin surface. The hemorrhagic areas (Figure 4A1 and 4A2, and 4 negative control NCs), were evaluated with a "SCANNER" program in Image J (Dias, 2007; Schneider et al., 2012), the images were plotted on a square millimeter chart, the plots corresponding to each hemorrhagic area were measured, the hemorrhagic square area was calculated in function of the square millimeter chart and referred as square percentages (Figure 4C). The tissue areas of each hemorrhagic area was removed, the hemoglobin was extracted and spectrophotometrically evaluated at 491 nm.

Neutralization of venom hemorrhagic activities

Samples of *B. arietans* crude venom, its chromatographically isolated protein peaks containing 4 HD_{50}, and various dilutions of anti-venoms were incubated for 30 min at 37°C. The venom samples incubated only with PBS were run in parallel. After incubation, 50 µL aliquots of the mixtures were subcutaneously injected into the mice. Each mixture was tested on a group of five mice. Two hours later, the mice were anesthetized under a CO_2 atmosphere, and the hemorrhagic areas were evaluated as described above. Neutralization activity was expressed as the minimal amount of antivenom reducing 50% of hemorrhagic areas (ED_{50}) produced by untreated antivenoms (HD_{50}).

Isolation of hemorrhagic-inducing metalloproteinases from *B. arietans* venom

Fifty milligrams of freeze-dried venom was dissolved in 5 mL of pH 5.5 acetate buffer (50 mM sodium acetate plus 150 mM NaCl), filtered through a 0.45-µm pore size membrane, and loaded on a Sephacryl S 100 HR (2.5 x 67 cm) molecular exclusion chromatography column previously equilibrated with pH 5.5 acetate buffer in a climate-controlled room. The subsequent elution was also performed with pH 5.5 acetate buffer. The samples were collected at a 60 mL/h flow rate, and their protein content was monitored by recording the absorbance at 280 nm in a spectrophotometer. The eluted fractions corresponding to peaks, P´1, P´2, P´3 and P´5 were pooled, and their protein contents were also determined as described above. The protein concentration of each fraction was adjusted to a common value before being used as antigen in western blotting and ELISA assays or as a hemorrhagic-inducing factor in the mouse skin test.

SDS-PAGE

The protein samples were separated by electrophoresis (Laemmli, 1970). Crude venoms (10 µg) or purified hemorrhagins (2 µg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved on a 12.5% polyacrylamide gel and stained with silver sulfate; molecular mass was determined using molecular markers running in parallel.

Western blotting

Samples of crude venoms (10 µg) or purified fractions (2 µg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved on a 12.5% polyacrylamide gel as described (Laemmili, 1970). Some SDS-PAGE preparations were stained with silver sulfate to be used as protein band profiles. Other SDS-PAGE preparations were electro-blotted to 0.45-µm nitrocellulose membranes (Towbin et al., 1979) to identify the protein bands recognized by anti-venoms. Membranes were incubated overnight in blocking buffer (5% non-fat milk in PBS) and then washed in PBS buffer containing 5% BSA and incubated for 2 hours with primary antibodies (horse or hen IgY anti-*B. arietans* venom antibodies) diluted to 1:5,000 in PBS containing 0.1% BSA for 1 h at room temperature on a horizontal shaker. After washing three times with PBS containing 0.05% Tween-20, the membranes were incubated with rabbit anti-horse IgG or anti-IgY conjugated to alkaline phosphatase (whole molecule) diluted 1:7,500 in PBS containing 0.1% BSA and 0.05% Tween-20. The membranes were then incubated for 1 h at room temperature on a horizontal shaker. The results were imaged after addition of the substrate.

ELISA

ELISA plates (96 wells) were coated with 1.0 µg of crude Brazilian or African snake venom in 100 µL of PBS and stored overnight at 4°C. In some assays, crotoxin or PLA_2 purified from *C. d. terrificus* or purified *B. arietans* metalloproteinase were used as the antigens. The wells were blocked for 2 hours at 37°C with 200 µL of PBS containing 5% BSA. The wells were then washed with 200 µL of PBS. For the initial antibodies, serial dilutions of horse IgG or $F(ab')_2$ preparations (1:4,000 to 2,048,000) in PBS containing 0.1% BSA were prepared, and 100 µL of each dilution was added to the individual wells. The plates were then incubated at 37°C for 1 h. The wells were then washed three times with washing buffer. As the second antibody, rabbit peroxidase-conjugated anti-horse IgG (whole molecule) (Sigma Aldrich, St. Louis, MO) diluted (1:20,000) in PBS containing 0.1% BSA and 0.05% Tween 20 (100 µL/well) was added to the plates. The plates were then incubated for 1 h at 37°C. After three washes with washing buffer, 50 µL of substrate buffer was added to each well, and the plates were incubated at room temperature for 15 min. The reaction was terminated with 50 µL of 4 N sulfuric acid per well. The absorbance was recorded at 492 nm using an ELISA plate reader (Labsystems Multiskan Ex, Thermo Fisher Scientific Inc., Walthan, MA). Horse IgG isolated from serum collected before immunization was used as a negative control. The IgG dilution giving an optical density of 0.2 was used to calculate the U-ELISA per milliliter of undiluted IgG solution. One U-ELISA is defined as the smallest dilution of antibody giving an O.D. of 0.2 under the conditions of the ELISA assay. The calculated value was then used to determine the amounts in 1.0 mL of undiluted anti-venom preparations.

Statistical analysis

The data were subjected to one-way ANOVA followed by Dunn´s

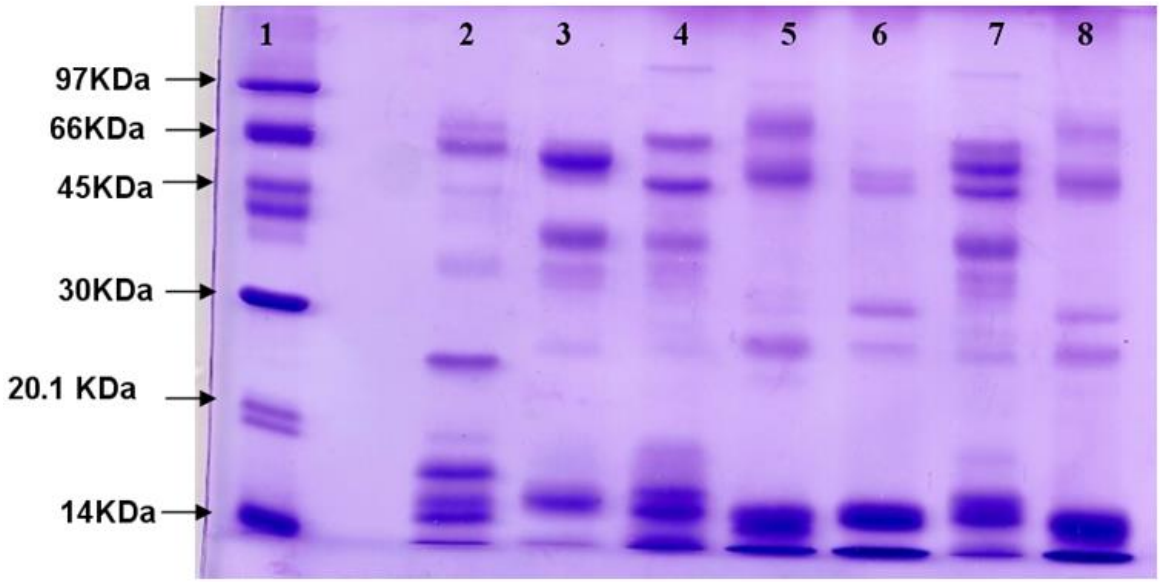

Figure 1. Polyacryalmide gel electrophoresis in SDS of snake venos.(1) Molecular mass ranging from 14 kDa to 97kDa as indicated; (2) *B. arietans* (3), *B. nasicornis* (4), *B. rhinocerus;* (5) *Bitis* spp.; *Naja melanoleuca;* (7) *N. mossambica;* (8) *Naja* spp. Samples of 15 µL containing crude venoms (30 µg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved in a 15% polyacrylamide gel. The protein bands were stained with Coomassie Blue (The SDS-PAGE was performed by David Gitirana da Rocha, Post-graduating student, LBR/UENF, Campos dos Goytacazes, RJ).

multiple comparisons test. The Kruskal-Wallis test was used to compare the hemorrhagic lesions induced by the crude venoms or the chromatographically isolated protein peaks. The Mann-Whitney method was used to compare the hemorrhagic lesions induced by the untreated or antibody-pretreated chromatographically isolated protein peaks. For all analyses, differences were considered to be significant at $P < 0.05$.

RESULTS

Venom LD_{50} values

The venom LD_{50} values were determined by i.p. injecting mice with various amounts of crude venom and calculating the death/survival ratio after 48 h. The following values were obtained: *B. arietans*, 0.96 µg/mouse; *B. nasicornis*, 123.67 µg/mouse; *B. rhinoceros*, 95.28 µg/mouse; *N. melanoleuca*, 13.41 µg/mouse; *N. mossambica*, 22.40 µg/mouse; *Bothrops atrox,* 76 µg/mouse, *Lachesis muta*, 123.4 µg/mouse, *Crotalus durissus terrificus*, 4.32 µg/mouse, and *Bothrops jararaca*, 32.6 µg/mouse. The LD_{50} values were used to determine the ED_{50}, as indicated below. The latter four venoms were included for comparison.

Visualization of the venom protein bands

Different molecular mass components ranging from 14 to 97 kDa were resolved using 15% SDS-PAGE analysis under non-reducing conditions (Figure 1): ten protein bands in *B. arietans*; (well # 2); six bands in *B. nasicornis*; (well # 3); six bands in *B. rhinoceros* (well # 4); six bands in *N. melanoleuca* (well # 6); nine bands in *N. mossambica* (well # 7); and six bands in *Naja* spp.(well # 8). The *B. arietans* venom focused on in this work exhibited five strong protein bands, one 65 kDa, one 25 kDa, one 15 kDa and two 14 kDa.

Fractionation of the *B. arietans* venom

Crude *B. arietans* venom was analyzed chromate-graphically using a sephacryl S 100 HR (2.5 cm X 67 cm) column equilibrated with 50 mM sodium acetate buffer, 150 mM NaCl at pH 5.5. The chromatography was performed at a 1 mL/min flow rate. Elution was monitored by recording the absorbance at 280 nm in a spectrophotometer. Eluted samples were successively collected, from left to right, and labeled 1 to 48. Five protein peaks (P´1, fractions 1-8; P`2, fractions 9-17; P´3, fractions 18-27; P´4, fractions 33-37; and P´5, fractions 45-48) were identified (Figure 2). After being lyophilized, the selected fractions were dissolved in 0.15 M NaCl, and their protein contents were determined. The recognition of *B. arietans* snake venom components by antibodies present in snake anti-venoms was assayed *in vitro* or by ELISA or western blotting methods. The presence of hemorrhagic-inducing factor was assayed, *in vivo*, by injecting representative aliquots into mouse skin.

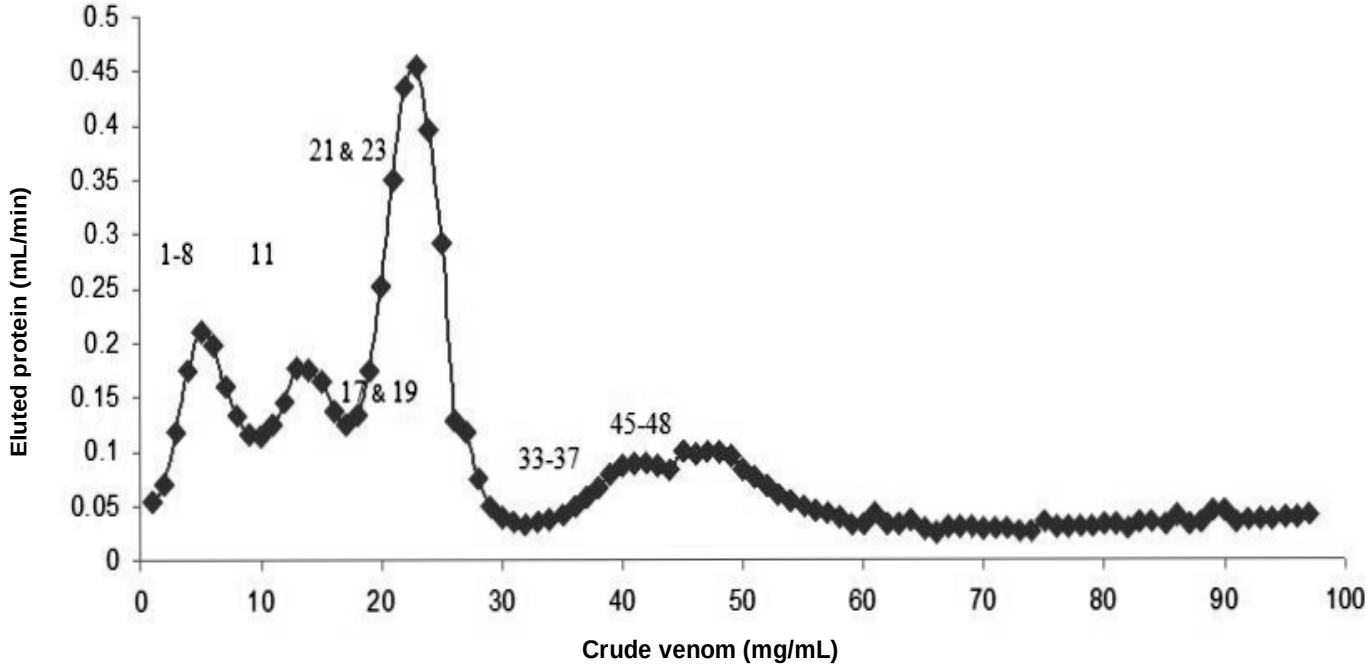

Figure 2. Isolation of hemorrhagic-inducing metalloproteinases from the *B. arietans* venom. Molecular chromatography exclusion of *B. arietans* venom. Five milliliter samples of crude venom (10 mg/mL) in 50 mM acetate-buffer, 150 mM NaCl, pH 5.5 were applied on Sephacryl S 100 HR (2.5 cm X 67 cm) column previously equilibrated with the some buffer. Chromatography was processed at 0.4 mL/min flow-rate. Elution was monitored by recording the absorbance at 280 nm in a UPC-900 Amersham Pharmacia Biotech. Elution samples were successively collected and labeled 1 to 48 from the left to right. Five eluted protein peaks (P´s) were obtained: P1´, fractions 1-8; P`2, fractions 9-17; P´3, fractions 18-27; P´4, fractions 33-37; P´5, fractions 45-48. The protein peaks were lyophilized. Before assays, the peaks were dissolved in the required buffer or solution and the protein contents were determined. The protein peaks were then submitted to, *in vitro*, ELISA and Western blotting assays by using different snake anti-venoms as the first antibodies or, *in vivo*, by injecting into mice skin to evaluated their ability to promote hemorrhage.

Characterization of the *B. arietans* protein peaks separated by sephacryl S 100 HR (2.5 cm X 67 cm) column chromatography

As indicated by polyacrylamide-gel electrophoresis (15%), the main *B. arietans* venom protein P3´ obtained from sephacryl S 100 HR chromatography was a 50-kDa protein similar to the protein present in whole venom (Figure 3A). Densitometric analysis indicated (Figure 3B) that P3´ was also prominent but with smaller-molecular weight protein contaminations. Unexpectedly, the P3´ protein was recognized by antibodies present in the different polyvalent anti-venoms.

The chromatographi-cally isolated samples were also plated in ELISA wells. The horse F(ab)´$_2$ polyvalent anti-venoms anti-*B. arietans*, anti-*Bitis* spp., anti-*Lachesis muta*, anti-*B. atrox*, anti-*Bothrops* spp., anti-*Crotalus* spp., and anti-*Naja* ssp. and the IgY anti-*B. arietans*, anti-*Bitis* spp. and anti-*Naja* spp. anti-venoms were used as primary antibodies (Figure 3C). P3´ was submitted to western blot analysis and subsequently recognized by polyvalent horse anti-venoms and stained (1 - control; 2 - anti-*Bitis arietans*; 3 - anti-*Bitis* spp.; 4 - anti-*Bothrops* spp.; 5 - anti-*Lachesis muta*; 6 - anti-*Naja* spp., 7 - anti-*Crotalus* spp.) (Figure 3D).

Hemorrhagic activity induced by crude venoms or by their isolated protein peaks

The hemorrhagic-inducing properties of *B. arietans* crude venom or of its isolated hemorrhagic-inducing protein fractions were assayed by injecting aliquots of the test samples into mice skin. After 2 h, the mice were anesthetized under a CO_2 atmosphere and bled; the skin was dissected, and the hemorrhagic areas were exposed in the internal skin surface. Figure 4A1 indicates that the hemorrhagic area developed with 5 µg (Figure 4A1a) of *B. arietans* crude venom was larger than that produced with 2 µg (Figure 4A1b) of the same venom.The amounts of hemoglobin extracted from these areas were equally proportional to injected *B. arietans* crude venom doses. Equivalent results were obtained when *B. jararaca* crude venom was used as positive control (Figure 4B). These results were also compatible when the corresponding areas were indirectly measured and expressed in percentage terms of square percentages of hemorrhagic areas: A1a = 12.8%; A1b =24.0%; $A2_1$ =11.7%; $A2_2$

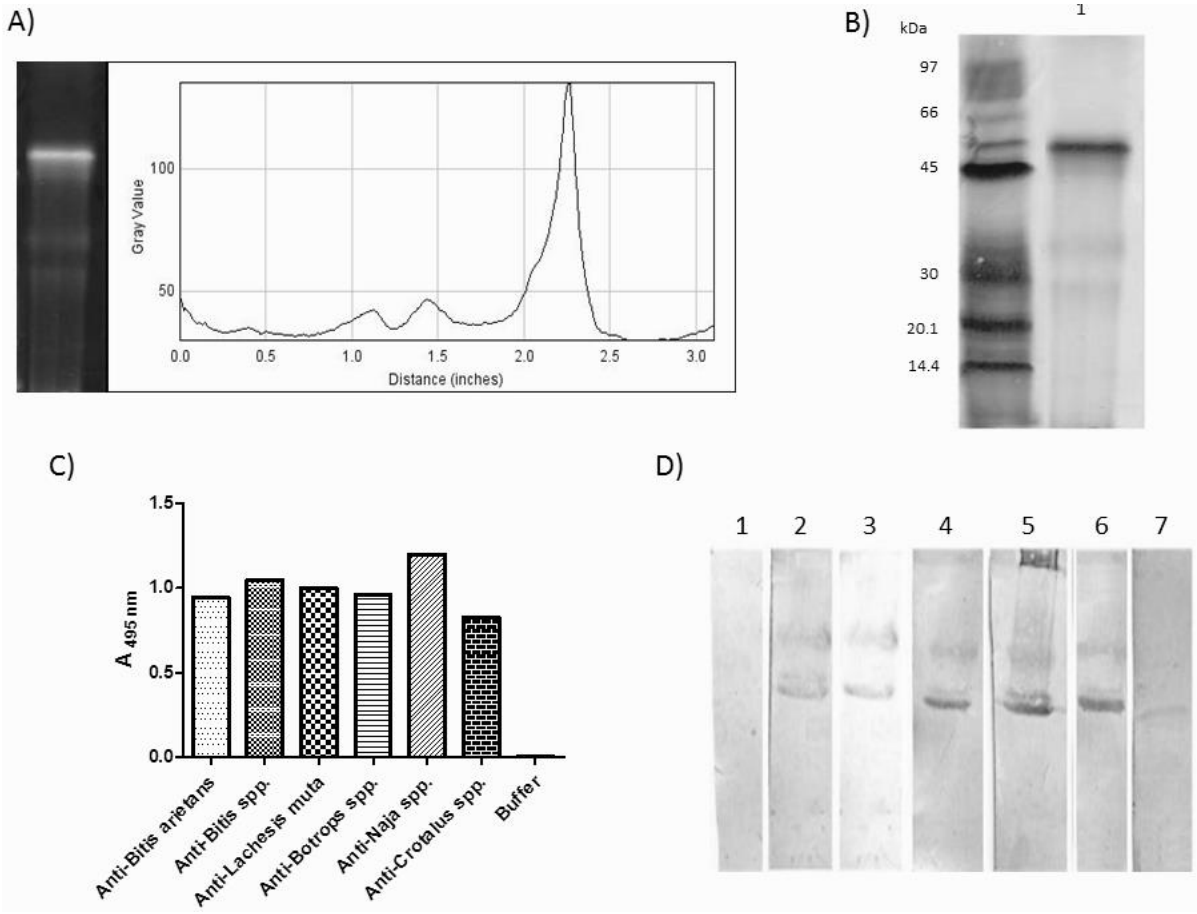

Figure 3. Characterization of the main *B. arietans* venom protein P3´ obtained from Sephacryl S 100 HR (2.5 X 67 cm) chromatography. (**A)** Polyacrilamide-gel electrophoresis (12%) of the *B. arietans* on Superose 12 HR column (10/30, ÄKTA FPLC, Pharmacia, Uppsala, Sweden), eluted protein P'3. A 50kDa band protein present in whole venom is prominent in isolated P'3. (**B)** Upon densitometry analysis the P'3 was also prominent but exhibiting smaller molecular protein contaminations. **(C)** P3´protein was recognized by antibodies present in different polyvalent anti-venoms. Samples of chromatographically isolated was plated on ELISA wells. The horse $F(ab)'_2$ polyvalent anti-venoms anti-*B. arietans*, anti-*Bitis* spp, anti-*Lachesis muta*, anti-*B. atrox*, anti-*Bothrops* spp., anti-*Crotalus* spp., and anti-*Naja* ssp, or IgY anti- *B. arietans*, anti-*Bitis* spp. and anti-*Naja* spp. were used as first antibodies. (**D)** Western blotting analysis of P3. The eluted protein peaks were submitted to polyacrylamide-gel electrophoresis (15%), electrotransferred to nitrocellulose membrane, the protein bands recognized by polyvalent horse anti-venoms and stained. 1, control; 2, anti-*Bitis arietans*; 3, anti-*Bitis* spp.; 4, anti-*Bothrops* spp.; 5, anti-*Lachess muta*; 6, anti-*Naja* spp.; 7, anti-*Crotalus* spp.

=15.6%; $A1_3$ = 18.4%; $A1_5$ = 30.3%; NCs = 0.3% (Figure 4C).

Inhibition of the isolated hemorrhagic activity by polyvalent anti-venoms

Antibodies present in horse polyclonal $F(ab')_2$ or in chicken IgY anti-*Bitis arietans*, anti-*Bitis* spp., anti-*Naja* spp., anti-*Lachesis muta*, anti-*Bothrops* spp., and anti-*Crotalus* spp. and IgY anti-*B. arietans*, anti-*Bitis* spp., and anti-*Naja* spp. were able to inhibit the *B. arietans* inducing-hemorrhagic activity present either in whole venom or in the isolated P3´ fraction (Figure 5).

DISCUSSION

The use of purified relevant snake venom toxins to immunize animals instead of crude venoms avoids anti-venom contamination with non-relevant antibodies directed to non-toxic venom components. Selecting the relevant toxins to be included in the immunogenic mixture is the first step in constructing effective mono-specific anti-venoms.

In this study, we focused on *B. arietans*; its large distribution in densely populated savannah areas in sub-Saharan Africa and in the Arabian Peninsula (Navy, 1991), associated with severity of its bite, justified its selection. Among their toxic components, we examined

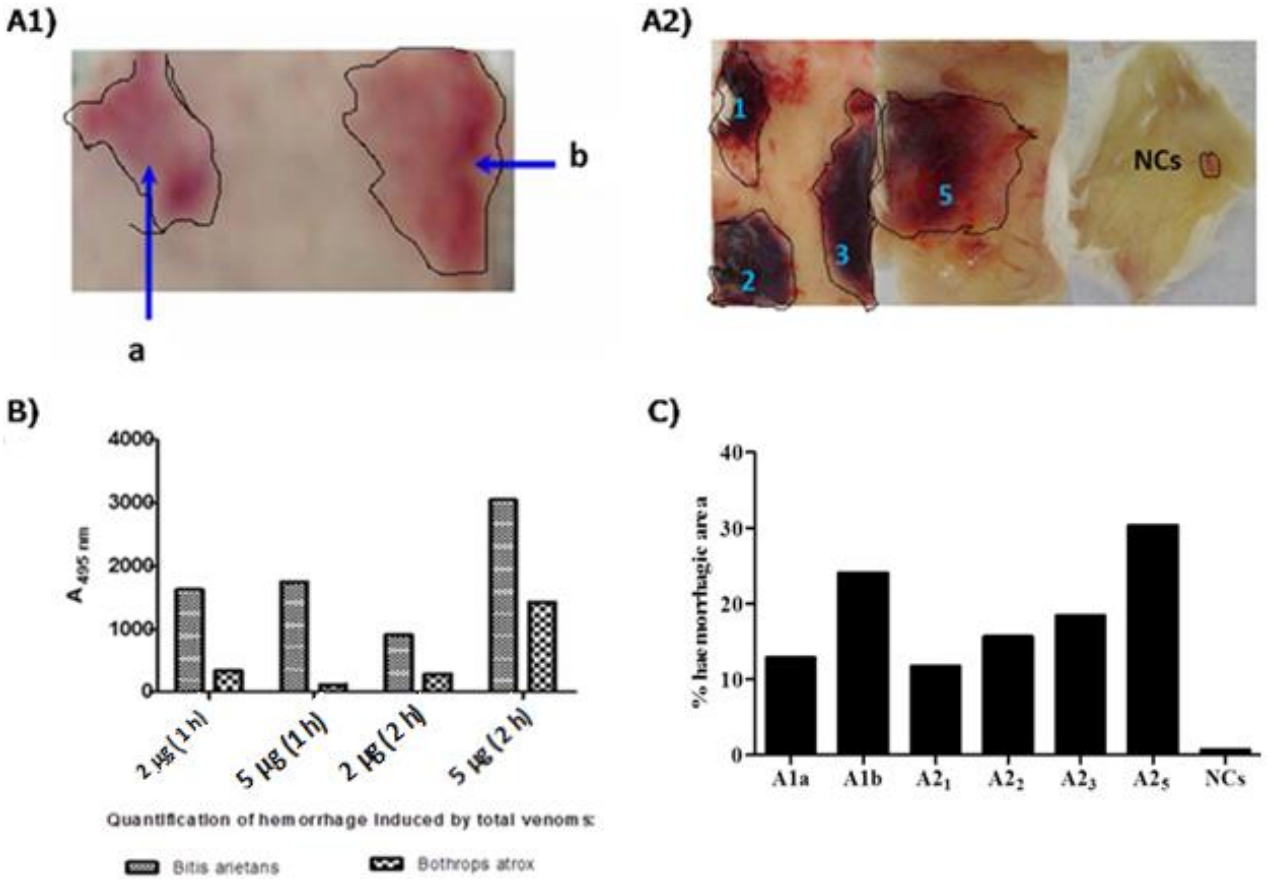

Figure 4. *B. arietans* venom hemorrhagic activity. Groups of mice were subcutaneously injected with different amounts of crude venom, with chromatograph isolated protein peaks P´1 P´2, P´3, P´5 or with 01M NaCl (NCs) as negative control. In some experiments, *B. jararaca* venom was included as positive control. Two hours later the mice were anesthetized under CO_2 atmosphere, submitted to total bleeding, the skin dissected, and the hemorrhagic areas were exposed in the internal skin surface. The hemorrhagic areas (4A1 and 4A2, and negative control NCs), were evaluated with a "SCANNER" program in Image J (Schneider, 2012), the images were plotted on a square millimeter chart, the plots corresponding to each hemorrhagic area were measured, the hemorrhagic square area was calculated in function of the square millimeter chart and referred as square percentages (**C)**. The tissue areas of each hemorrhagic area was removed, the hemoglobin was extracted and spectrophotometrically evaluated at 491 nm. (**A)** Skin fragments of hemorrhagic areas resulting from subcutaneous injections with 2 µg (A1a) or 5 µg (A1b) of *B. arietans* crude venom or with Sephacryl S 100 HR chromatography protein peaks P´1, P´ 2, ´P´3 and P´ 5 ($A2_1$, $A2_2$, $A2_3$, $A2_5$) or with 0.15 M NaCl solution (NCs**)**. (**B)** Local tissue deposited hemoglobin. (**C)** Square percentages of hemorrhagic areas: A1a = 12.8%; A1b =24.0%; $A2_1$ =11.7%; $A2_2$ =15.6%; $A1_3$ = 18.4%; $A1_5$ = 30.3%; NCs = 0.3%.

the hemorrhage-inducing factors contained in *B. arietans* venom. Data from past and recent publications have indicated that the M12b subfamily of zinc-dependent reprolysins is causative agents of local tissue lesions and systemic symptoms in *Bitis* spp. accidents (Bode et al.1993; Stokër et al., 1995; Gutiérrez and Rucovaldo, 2000). SVMPs is remarkable present in *Elapidae* and *Colubridae* venoms (Junqueira-de-Azevedo and Ho, 2002; Guo et al. 2007).

Among the *B. arietans* venom isolated fractions P´1, P´2, P´3, P´4 and P´5, P´3 were identified as having 50-kDa, P´1, P´2 and P´4 and P´5 were admittedly having lesser or higher molecular mass, respectively (Figure 2); as it was expected that SVMPs include 20-30 kDa and over 50 kDa enzymes could all mimicked venom hemorrhagic activities. In fact, the isolated *B. arietans* venom protein fractions, P'1, P'2, P'3 and P'5 exhibited, with different degree, hemorrhagic activity (Figure 4). Considering the relative molecular mass of 50 kDa for P'3, 20-30kDa for P'1 and P'2, over 50kDa for P'5, all described SVMPs were partially purified. P'3 protein fraction may exhibits the typical SVMP disintegrin domain in the presence of an MGD sequence replacing the RGD sequence found in many disintegrins (Nikai et al., 2000). The immunochemical properties of this protein were evaluated *in vitro* by standard ELISA and Western blotting methods, and the ability of this protein to induce hemorrhage was analyzed *in vivo* in mouse skin. As

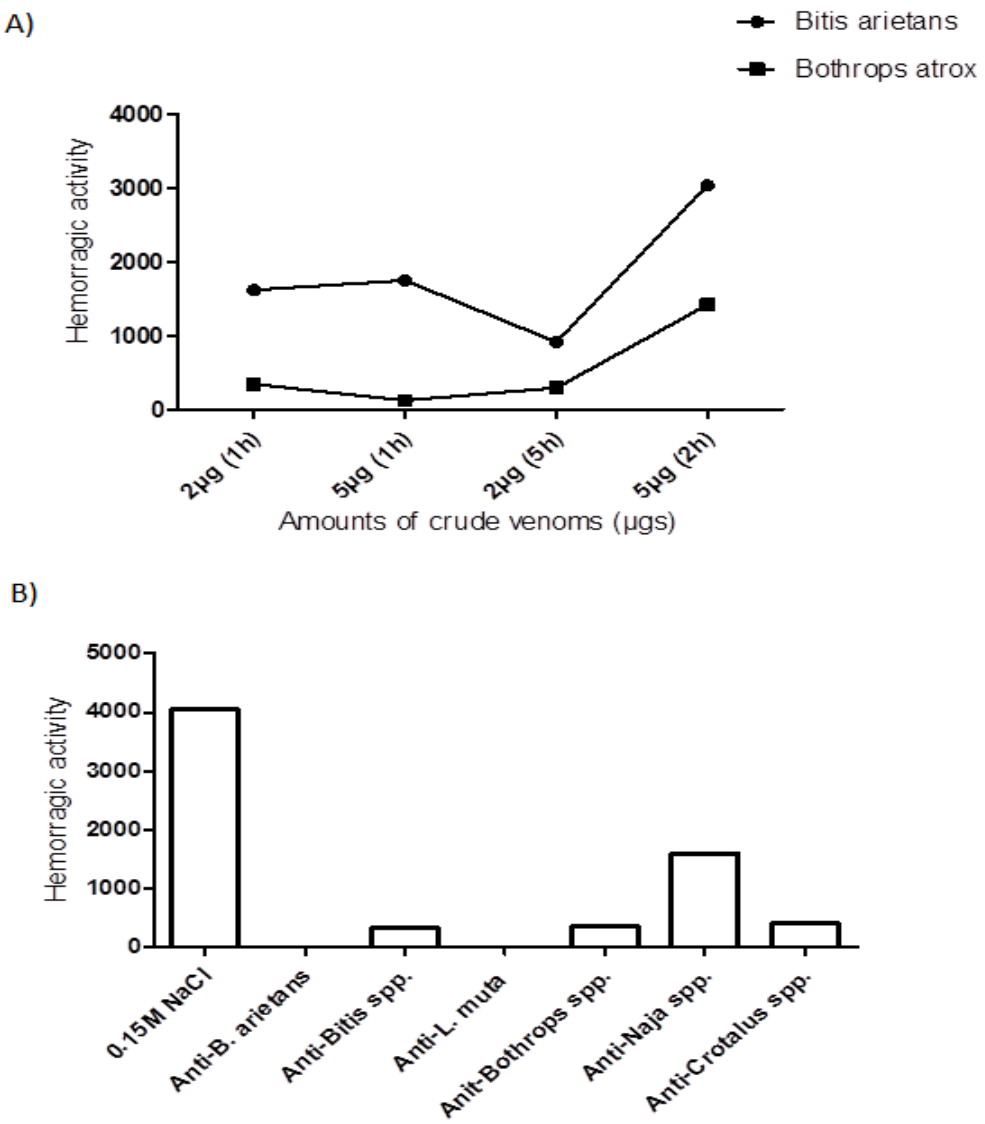

Figure 5. Antibodies developed by different crude snake venoms inhibit the hemorrhagic activities present in *B. arietans* whole venom or in their isolated fraction P3´. Mice (n=20) were subcutaneously injected with 100 µL of 0.15 M NaCl containing 20 µg of P3` eluted from on Sephacryl S 100 HR (2.5 X 67 cm) column pretreated at 37°C for 2 h with Horse $F(ab´)_2$ anti-*B. arietans*, anti-*Bitis*, anti-*Lachesis*, anti-*B.atrox,* anti-*Bothrops* anti-venoms from "Instituto Butantan" or IgY anti-*B. arietans*, anti-*Bitis* spp*., and* anti-*Naja* spp. produced in hens. The hemorrhagic areas were measured, the hemoglobin extracted and spectrophotometrically quantified at 410 nm.

expected from published data, *B. arietans* venom protein contains antigenic epitopes recognized by antibodies generated by non-Viperidae snake venoms (Junqueira-de-Azevedo and Ho, 2002; Guo et al., 2007; Ching et al., 2006). In fact, in this study, we experimentally demonstrated that the isolated 50-kDa protein was recognized by antibodies present in horse polyclonal $F(ab´)_2$ or in chicken IgY anti-*Bitis arietans*, anti-*Bitis* spp., anti-*Naja* spp., anti-*Lachesis muta*, anti-*Bothrops* spp., and anti-*Crotalus* spp. and IgY anti-*B. arietans*, anti-*Bitis* spp., and anti-*Naja* spp. (Figure 3). These antibodies were also able to inhibit the *B. arietans* hemorrhagic activity present in either *B. arietans* venom or the isolated P3´ fraction (Figure 5).

Although not tested, the 50-kDa purified protein may exhibit some biological activities similar to SVMPs, for example, fibrinolytic activity (Retzios and Markland Jr, 1998), non-hemorrhagic fibrinolytic protease activity (Willis and Tu, 1988; Kini, 2005; Kornalik and Blombäk, 1975; Yamada et al., 1996; Siigur et al., 2004; Tans and

Rosing, 2002; Brenes et al., 2010; Trumal et al., 2005; Han et al., 2010), platelet aggregation inhibition (Kamiguti et al., 1996; Laing and Moura-da_Silva, 2005; Wang et al., 2005; Zhou et al., 1996), pro-inflammatory action (Gutiérrez et al., 2005; Moura-da-Siva et al., 2007; Rucavado et al.,1995), and blood serine protease inhibitor inactivation (Kress, 1986; Krees and Catanese, 1980; Kress and Hufnagel, 1984).

The purified 50-kDa protein fraction can therefore be included in immunogenic mixtures intended to induce mono-specific anti-*Bitis* spp. venoms. This mixture has been enriched by the inclusion of purified *B. arietans* blood pressure-hypotensive factor (Kodama et al., 2015).

The immunogenic mixture will be submitted to a pre-clinical test in mice and in laying hens. Specifically, two H-2 distinct mice inbreed strains, C57BL/6 (k) and BALB/c (b), and white leghorn lineage hens (*Gallus gallus domesticus*) will be used as test animals. The immunogenic mixture containing the purified 50-kDa hemorrhagic fraction and blood pressure-hypotensive factor in various proportions will be subcutaneously (in mice) or intramuscularly (in hens) injected four times at 15-day intervals. In the primary immunization, the immunogenic mixture will be adsorbed in mesoporous silica SBA-15 as an adjuvant (Carvalho et al., 2010) prior to injection. In the three subsequent boosters, the immunizations will contain venom components without adjuvants. Blood samples will be collected before each immunization, and the sera will be used to evaluate the antibody titers and their ability to neutralize *B. arietans* venom hemorrhage, blood pressure hypotensive and lethality-inducing activities. The obtained antibodies will be evaluated in parallel assays with horse anti- *B. arietans* or *B. g. rhinoceros* plus *B. nasicornis* venoms (Guidolin et al., 2012). These anti-venoms inhibit *Bitis* spp. toxic activities (Paixão et al., 2015).

The antibody potencies for neutralizing lethality (ED_{50}), skin hemorrhage (EH_{50}), complement-inactivation (ECa_{50}), and blood-pressure hypotensive-inducing activities (EBh_{50}) will be evaluated as recommended by WHO (2010). Antibodies endowed with high specific and affinity qualities may be the first step in producing engineered mini-anti-venom antibodies as anti-snake venoms (Holliger and Hudson, 2005).

Conflict of interest

The authors declare no conflict of interest.

ACKNOWLEDEGEMENTS

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Author contributions

Tahís Louvain de Souza carried out the experiments; Fábio C. Magnoli purified the metalloproteinases; Wilmar Dias da Silva analyzed the results and wrote the paper.

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Full Length Research Paper

Germination and *in vitro* multiplication of *Helianthemum kahiricum,* a threatened plant in Tunisia arid areas

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Seeds of *Helianthemum kahiricum* have an excellent germination rate extending to about 90% within short time not more than four days after scarification (mechanical treatment) and a good protocol of disinfection. A high frequency of sprouting and shoot differentiation was observed in the primary cultures of nodal explants of *H. kahiricum* on Murashige and Skoog medium (MS) free growth regulators or with less concentration of kinetin (0.5 mg L^{-1} or 1.0 mg L^{-1} kin). *In vitro* proliferated shoot were multiplied rapidly by culture of shoot tips on MS with kinetin (0.5 mg L^{-1}) which produced the greatest multiple shoot formation. The kinetin had a positive effect on the multiplication and growth, but a concentration that exceeded 2.0 mg.L^{-1} decreased the growth. A high frequency of rooting with development of healthy roots was observed from shoots cultured on MS/8 medium hormone-free.

Key words: *Helianthemum kahiricum*, *in vitro* germination, multiplication, axillary buds.

INTRODUCTION

Helianthemum kahiricum (called R'guiga or Chaal in Tunisia) (*H. kahiricum*) is a perennial herb widely found around the Mediterranean basin (Raynaud, 1987). *H. kahiricum* is an appressed, grey-canescent perennial low shrub that reaches up to 10-25 cm long with many, branched stems. Leaves are 0.5-1.8 x 0.15-0.3 cm, oblong-lanceolate, appressed-pubescent, with strongly revolute margins, and acute to obtuse apex. Flowers are with white-villous, violaceous calyx and yellow petals equaling the sepals and are often not opening and arranged in 5-l2-flowered and 1-sided inflorescence. The fruit is an ovoid-globose and hairy capsule with ovoid-compressed, smooth, and brownish. This is a chaméphyte (Escudero et al., 2007), family of Cistaceae, arid regions and semi-arid areas (Perez- Garcia and Gonzalez- Benito, 2006). Despite its ecological and economic interests, this plant is a rare endemic flora of the western basin of the Mediterranean (Escudero et al., 2007), as a result of overgrazing (Aidoud et al., 2006). The size of their population or their range, or both, is

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Abbreviations: Zea, Zeatin (cytokinine); **IAA,** indole-3-acetic acid (auxin); **IBA,** indole-3-butyric acid (auxin); **Kin,** kinetin (cytokinine); **MS,** Murashige and Skoog medium; **NAA,** 2-naphthalene acetic acid (auxin); **2iP,** 2 isopentenyladenine (cytokinine).

Table 1. Culture medium for *Helianthemum kahiricum* seeds germination.

Concentration (mg L^{-1})	Culture medium				
	M_0	M_1	M_2	M_3	M_4
Kin	0	1	1	1	1
IBA	0	0	0.5	1	0.5
Zea	0	0	0	0	0.5

Table 2. Composition of culture medium with growth hormone (auxin and cytokinin) for the multiplication of *Helianthemum kahiricum* explants.

Concentration (mg L^{-1})	Culture medium						
	MS	C_1	C_2	C_3	C_4	C_5	C_6
Kin	0	0.5	0.5	0.5	1	2	2
IBA	0	0	1	0	0	1	0
NAA	0	0	0	1	0	0	1

restricted or is greatly diminished. The data indicates that the situation will worsen irreversibly if nothing is done to address this vulnerability. In other words, if the observed situation continues, it will anticipates the disappearance of these species more or less. Among the factors responsible, including loss or degradation of habitat, exploitation of the species, exposure to pollutants, diseases, climate change, overexploitation or other cause result in regression of range or a sustained decline in the number but the population level reaches a critical threshold. The helianthemes have a great pastoral interest. They have a very important role in the fight against desertification and stabilization of vulnerable areas (Diez et al., 2002). In addition, they are involved in the production of desert truffles (Al-Rahmah, 2001; Slama et al., 2006). Desert truffles, known locally as the "terfess" are edible and wild seasonal mushrooms hypogeal (Mandeel et al., 2007). *H. kahiricum* is considered threatened in an extremely precarious situation. The size of their population or their range, or both, is restricted or is greatly diminished. Thus, to maintain the genetic integrity of clones and conservation of this species, *in vitro* germination, cultivation microcuttings and stimulation of axillary buds are the most applied in plant micropropagation method. For the species used in this study, *in vitro* culture seems to be a very interesting alternative for preserving *H. kahiricum*. Therefore, the purpose of this work was the multiplication of the species by micropropagation highlighting the effect of the composition of the culture medium on the initiation and proliferation of plant.

MATERIALS AND METHODS

Plant material

The plant material used consists of *H. kahiricum* seed from region of Médenine (Benguerdenne: latitude 32°57'09"N, longitude 11°38'26"E, with an arid climate of an average rainfall of 150 mm/year and a sandy soil (Le Floch and Boulos, 2008). The explants which are internodes were taken from 2 months aged mother plants produced *in vitro*.

In vitro germination

Three experiments were conducted by changing the time and the type of fungicide to optimize better the germination of *H. kahiricum* seeds on MS medium (Murashige and Skoog, 1962). In all experiments, 20 seeds/replication were soaked in sodium hypochlorite followed by rinsing with distilled water. The seeds were disinfected in 70° alcohol followed by rinsing with sterile distilled water and then applying different fungicides: 1st experiment (Exp. 1): The seeds were treated with benlate (1 g L^{-1}) (10 min), followed by rinsing with distilled water; 2nd experiment (Exp. 2): The seeds were treated with mercuric chloride (1 g L^{-1}) (20 min), followed by rinsing with distilled water; 3nd experiment (Exp. 3): Without fungicide.

After the development of an adequate protocol of seeds disinfection, they were germinated in vials (20 seeds/vial). The germination test was carried out on MS (M_0) and MS modified with growth hormones (M1, M2, M3 and M4) (25 repetitions for each test) (Table 1). Incubation takes place in a growth chamber under controlled conditions with an alternating day/night (16:8 h), a relative humidity of 80 and 45%, respectively, and a temperature of 25 ± 2°C for one week.

In vitro multiplication

The vitropousses from *in vitro* germination were then stripped of their leaves, cut in microcuttings (1 or 2 cm) with 1-2 nodes. Ten explants per vial were transferred to different Culture medium: MS, MS supplemented with growth hormones (C1, C2, C3, C4, C5 and C6) (25 rehearsals for each test), and MS diluted (MS/2, MS/4, MS/6 and MS/8) (25 rehearsals for each test), without hormones (Table 2), with a view follow the morphological attributes such as, bud, the length of the main stem, number of leaves, number of nodes, root length and rooting rate. After their rooting, the vitropousses were transferred into plastic pot filled with sand to

ensure their adaptation and possible culture in an experimental plot for acclimation.

Statistical analysis

The results of analysis of variance (ANOVA) of the different parameters were obtained by the software SPSS v.11.5. Multiple comparisons of means and the setting command classes were made by Duncan's test.

RESULTS

In vitro germination

The success of *in vitro* culture depends on aseptic conditions for the cultivation of plant material used. After incubation, the best germination rate and reduced number of seeds contaminated are generated by the first test disinfection (100%) (Exp. 1). The second disinfection test (Exp. 2) yielded an average percentage of germinated seeds (32%) while the third disinfection protocol yielded no germination (0%) (Exp. 3). Seeds of *H. kahiricum* have an excellent germination rate; can reach 100% within short time not more than four days after scarification (mechanical treatment) and disinfection (Benlate). Scarification reduces germination time; it can stretch to several months because of the very rigid tegument of seeds.

Effect of growth hormone on seed germination

The seeds of *H. kahiricum* germinated on culture medium supplemented with growth hormone have present deformation (after germination) which results in vitrification and intense hydration with the formation of callus and necrotic leaves (Figure 1b, c, d, e and f).

Growth hormones have adverse effects on seed germination and thereafter are not effective for the germination of *H. kahiricum* seeds; while, the seeds on MS without growth hormones present a simple germination (Figure 2a).

Effect of growth hormone on micropropagation of *H. kahiricum*

Multiplication on MS medium without growth hormone

Shoots on MS medium are vigorous with chlorophyll leaves, normal and average percentage of vitrification. Morphological changes of vitropousses during subcultures affected neither leaves nor the appearance of the root. The leaves of plantlets contained the chlorophyll pigments, not necrotic, their size increases a subculture to another and becoming stronger. The stems reached a remarkable stiffness and strength during subcultures (Figure 1g, h and i). In *H. kahiricum*, it seems that the rooting phase is characterized by the proliferation of shoots on MS without growth hormones. Indeed, the time for obtaining vigorous shoots able to be transplanted is 8 to 12 weeks and it was during this period that shoots rooted (Figure 1j). Thereafter, the rooted vitropousses from the multiplication phase was prepared for the acclimatization stage.

Multiplication on MS medium supplemented with growth hormones

Recovery and proliferation axillary

Axillary buds subcultured on culture medium contains combinational hormones after six weeks of culturing explants bud unlike in the control medium, it takes place after two weeks (Figure 2a). MS has the highest rate of bud break (almost 90%). The effect of growth hormones is manifested by a significant reduction in the bud. The addition of the NAA causes an increase in the rate of bud compared to IBA. This can be explained by the physiological response of the explants on the interaction auxin/cytokinin, and the nature of the substance used for growth. The average shoot length appears to be strongly related to the concentration of hormones (Figure 2b). Using the results of the percentage of bud, it appears that the hormonal combinations recording bud rate reduced compared to the control. The influence of increasing the concentration of kinetin in the medium alone is shown by a significant decrease in the average length of the shoots.

Average number of nodes

The combination of auxin and cytokinin in the culture medium indicates that the increase in the kinetin medium, alone or in combination with auxin results is in a decrease in the average number of nodes relative to the control (Figure 2c). At a concentration of 0.5 mg L^{-1} Kin (C1), the average number of nodes reduced to about 20% whereas for 1.0 mg L^{-1} Kin (C4) it was in the order of 25%. In addition, 0.5 mg L^{-1} kin combined with 1.0 mg L^{-1} NAA (C3) gave a greater than 50% reduction compared to the control and the combination C2 (0.5mg L^{-1} kin + 1.0 mg L^{-1} IBA). It seems that the addition of growth regulators had a regressive effect on the average number of nodes.

Rooting

The cuttings rooted after three weeks in MS, then after a month we observed the emergence of some very harsh roots of short length on media supplemented with growth regulators. It appears that the observed formation of

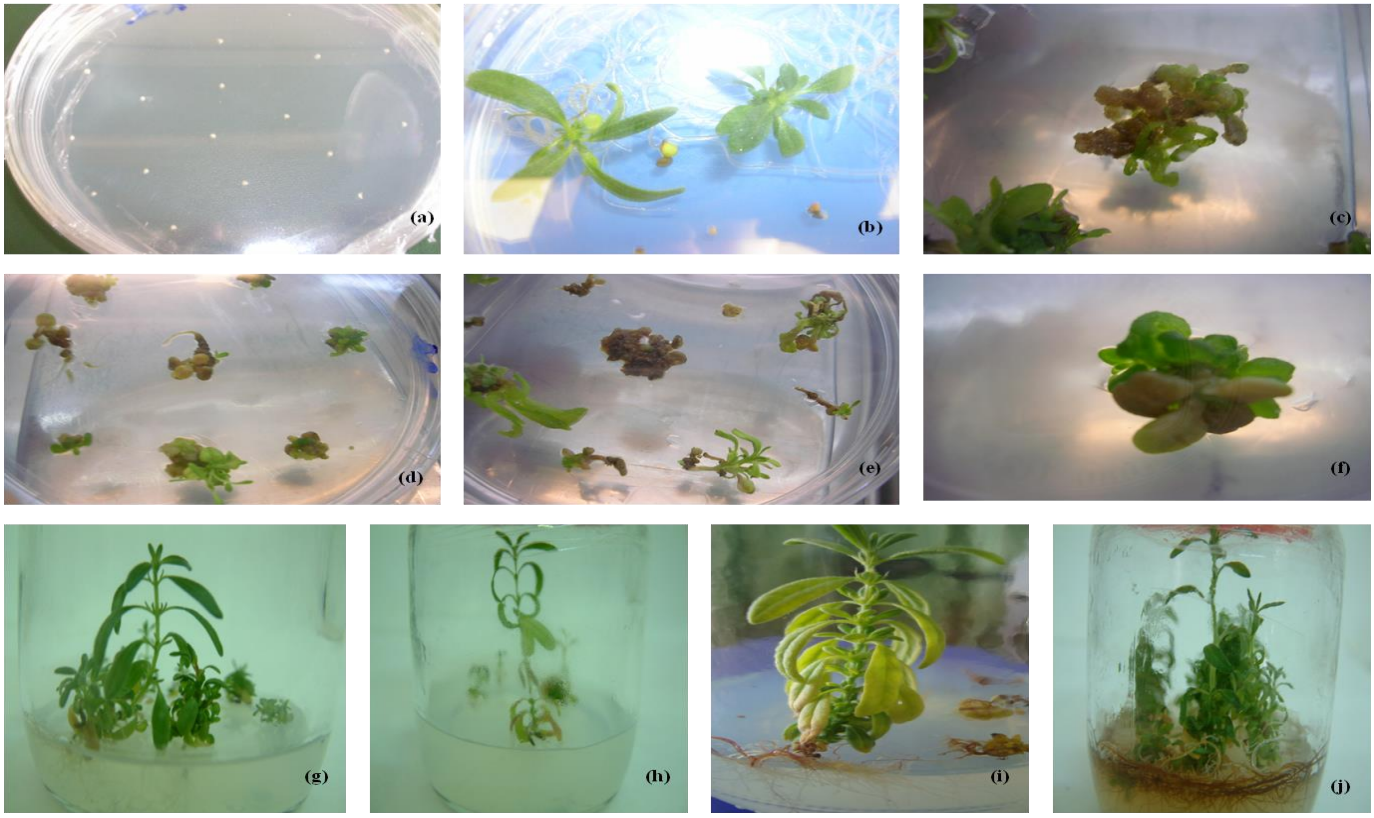

Figure 1. (a) Setting seed germination of *H. kahiricum*. **(b)** Seeds of *H. kahiricum* germinated on MS. **(c)** *H. kahiricum* seeds germinated on MS + 1 mg/l Kin + 0.5 mg/l IBA. **(d)** *H. kahiricum* seeds germinated on MS 1 mg/l Kin + 1 mg/l IBA. **(e)** *H. kahiricum* seeds germinated on MS + 1 mg/l Kin. **(f)** Seeds germinated on MS + 1 mg/l Kin + 0.5 mg/l IBA + 0.5 mg/l Zea. **(g)** Appearance of leaves during 4th subculture. **(h)** Appearance of the stems during the 4th subculture. (i) Appearance roots for 3th subculture. (j) *In vitro* plants rooting.

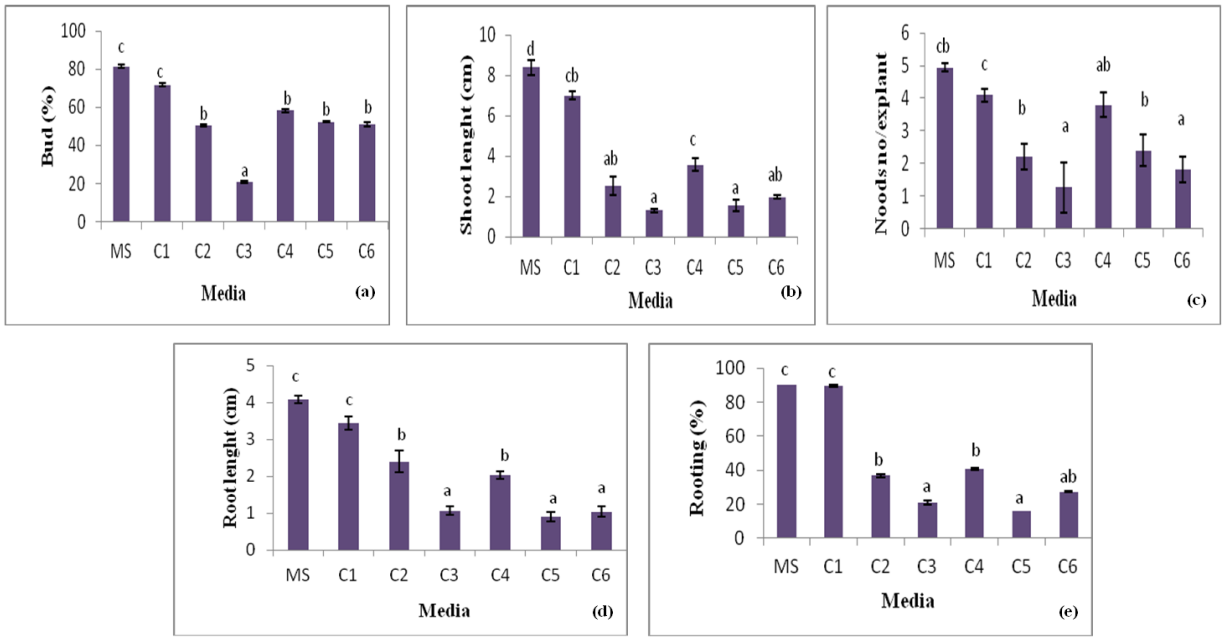

Figure 2. (a) Action of culture medium on the percentage of axillary buds of *H. kahiricum*, (b) action of media on the average length of *H. kahiricum* shoots, (c) action of media on average number of nodes, (d) effect of media on the average root length (cm) of *H. kahiricum* explants, (e) effect of media on rooting rate of *H. kahiricum* plantlets.

Table 3. Change in callus, bud break and rooting with hormone combinations of auxin/cytokinin in seedlings of two months old *Helianthemum kahiricum* from explants.

Explant	Culture medium				
	MS	C_2	C_3	C_5	C_6
Callus (%)	16±0[a]	78.4±0.78[b]	87.6±0.73[b]	91.37±0.59[c]	71.1±0.74[b]
Bud (%)	81.4±0.78[c]	50.62±1.00[b]	20.8±1.33[a]	52.4±0.88[b]	51.0±0.73[b]
Rooting (%)	90.4±0.48[d]	36.8±0.59[c]	21.1±0.71[b]	16.0±0.39[a]	27.4±0.48[ab]

Means with different letters are significantly different at threshold p<0.05 (Duncan test).

Table 4. Effect of MS dilute of the attributes morphological seedling of *Helianthemum kahiricum* aged two months from explants.

Explant	Culture medium			
	MS/2	MS/4	MS/6	MS/8
Buds (%)	90	100	100	100
Shoots length (cm)	2.24[a]	4.3[b]	5.7[c]	8.02[d]
Roots length (cm)	9.2[a]	12.4[b]	14.4[c]	15.3[d]
Leaves no/shoot	4.2[a]	6.2[b]	7.2[c]	7.7[c]
Nodes no/shoot	7[b]	6.3[a]	7.4[ab]	12.1[c]
Roots no/shoot	2.36[a]	2.44[a]	2.4[a]	3.01[b]

Means with different letters are significantly different at threshold p<0.05 (Duncan test).

callus at the base of the explants and at the location of sections inhibited rooting. Cuttings made on media containing no auxin (C1 and C4) showed highest rooting percentage. It also turns out that the addition of auxin in the medium does not improve rooting. Whatever the amount of kin in the medium, we find that IBA promoted rooting with a rate of 16% and the NAA with a rate of 21%. The rooting percentage and mean root lengths evolved in a proportional manner (Figure 2e).

Effect of ratio auxin/cytokinin on explants of *H. kahiricum*

The auxin/cytokinin ratio determines the physiological functioning of the explants, the percentage of callus is quite high compared to the control for all hormonal combinations (Table 3). However, the rate of rooting and sprouting of control are significantly higher compared to other treatments. The ratios of auxin/cytokinin about 2 (1:0.5 mg L^{-1}) promote rooting, indeed, for the combination C2 (1:0.5 mg L^{-1}) rooting percentage was 41% against a rate of 16% for the combination C5 (1:2 mg L^{-1}). The C5 (1:0.5 mg L^{-1}) promote axillary bud (62%), while C3 (1:0.5 mg L^{-1}) promoted it by 22%. However, the dilution does not influence the rate of bud, but it manifests itself on the strength of axillary shoots. There is also a strong ability of budding *in vitro H. kahiricum* associated with its proliferation of axillary explants in the presence of all medium (Table 4).

Acclimatization

The survival rate of plantlets is influenced by the nature of the substrate. The best survival rate of seedlings (63%) was recorded on a substrate perlite.

DISCUSSION

H. kahiricum is considered threatened in an extremely precarious situation. Micropropagation of this plant was an attempt conducted from nodal explants of plantlets *in vitro* to preserve/conserve this species by the micro-propagation technique which aims to safeguard bio-diversity; thereby developing an appropriate protocol for disinfecting seeds which was necessary to achieve aseptic cultures. Seed germination after strong disin-fection has the minimal contamination and a high rate of seeds germinated on MS medium without hormone. It appears that *H. kahiricum* does not require growth hormone for its germination. Hence, the study was based on regeneration by *in vitro* proliferation of axillary buds of *H. kahiricum* by the use of growth hormones in order to improve the multiplication. Microcuttings grown on MS medium without hormone presents the highest rate of budding and rooting. It seems that this plant has high potential to multiply on a hormone-free MS medium and the bud of the axillary shoots is easy without going through an induction medium. Growth substances (auxins and cytokinins)

have a depressive effect on budding, proliferation and rooting of plantlets. The acclimatization of *H. kahiricum* poses no major problems and the use of substrate consisting of sand and perlite has a high survival rate. It seems that the buds during the multiplication are actually inhibited by the effect of callus at the base of *H. kahiricum* explants. Therefore, it seems that the hormonal combination has a regressive effect on bud break, rooting and average root length; in addition, these parameters appear to be influenced by the nature of the auxin. The addition of growth hormones does not improve the rooting or the rooting period compared to the control environment. Similar results were also reported by Souayah et al. (2003) in *A. halimus* for which the root is obtained on media without growth regulators with a rooting rate improved by diluting the mineral medium; while, the work of Roy et al. (2001) on *Humulus lupulus* and Armstrong and Johnson (2001) on *Ceratopetalum gummiferum* showed that in the absence of any growth regulator, rooting could not be obtained. This may be due to the richness of this species in endogenous growth regulators. Fracaro and Echeverrigaray (2001) reported that for *Cunila galioides*, dilution of the mineral medium does not affect the induction of rooting, but negatively affects the growth of roots. However, *Pistachia vera* (Chatibi, 1999), dilution of macronutrients MS basal medium bears no significant improvement for rooting and for absence of auxin, no rooting was observed.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of temperature, light, desiccation and cold storage on germination of *Sophora tonkinensis* (Leguminosae) seeds

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Identification of the seed storage behaviour of a target species is essential prior to seed storage so that suitable storage strategies are taken. In the present experiments, germination characteristics, desiccation, and low temperature tolerance of seeds of *Sophora tonkinensis* was studied; a traditional Chinese medicine on the edge of extinction, were investigated for the first time in attempt to interpret their storage behaviour. The results indicate that the temperature optimum for seed germination ranged from 25 to 30°C, the seed germination was non-photoblastic, the seed viability declined with the decrease of moisture content by a silica gel desiccation, and a low temperature storage at -20°C for 3 months had no significant effects on the seeds desiccated to 5.4 ± 0.1% moisture content. These results suggest an intermediate seed storage behaviour. For germplasm long-term conservation purpose, a cryopreservation investigation was proposed.

Key words: Seed, desiccation tolerance, low temperature tolerance, germination, *Sophora tonkinensis*.

INTRODUCTION

The importance of long-term storage of plant genetic resources has been well recognized. *Ex situ* conservation through long-term storage of seed is, in principle, possible for a significant proportion of higher plants. Long-term seed storage, if it is feasible, is generally considered the safest, most inexpensive and most convenient method of plant genetic resources conservation (Hong and Ellis, 1996; Linington and Pritchard, 2001). However, published information on seed storage behaviour, particularly in non-crop species, is meager in the context of the task of *ex situ* biodiversity conservation (Hong and Ellis, 1996).

For seed storage purpose, that is, plant genetic/ germplasm resources conservation, identification of the seed storage behaviour of a target species is essential. Three main categories of seed storage behaviour are now recognized: orthodox, intermediate and recalcitrant (each may be further subdivided) (Roberts, 1973;

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Ellis et al., 1990).

Desiccation tolerance and low temperature tolerance are the two crucial parameter to identify seed storage behaviour. Orthodox seeds undergo maturation desiccation during development, and are shed at a relative lower moisture content, and can be desiccated further to 1-5% moisture content without damage, and can be safely stored for more than decades at a quite low moisture content. Adversely, recalcitrant seeds do not undergo maturation desiccation during development; they are shed at a relative higher moisture content and metabolic activity, and are intolerant to desiccation and sensitive to low temperature. In general, the storage lifespan of recalcitrant seeds at room temperature is only several days to weeks (Smith and Berjak, 1995). While, the storage behaviour of intermediate seeds is between orthodox and recalcitrant seeds, being desiccation-tolerant partially without the increase of storage lifespan by desiccation, being sensitive to low temperature and cannot be conserved in conventional low temperature seed bank for a long-term conservation purpose (Ellis et al., 1990). The majority of seed plants produce orthodox seeds. While, recalcitrant and intermediate seeds mainly produce from mesic plants and tropical and subtropical trees, as well as partial tropical herbs and temperate trees, such as species of Fagaceae (Song et al., 2003).

Orthodox seeds can be maintained satisfactorily *ex situ* over the long term in appropriate environments (5±1 % water content, -18 °C low temperature) (IBPGR, 1976). By contrast, the maintenance of the viability of intermediate and recalcitrant seeds storage behaviour is problematic (Hong and Ellis, 1996). In general, medium-term storage is feasible for intermediate seeds provided the storage environment is well-defined, but short-term storage is usually the best that can be achieved with recalcitrant seeds (Hong and Ellis, 1996). For recalcitrant and intermediate seed species, at present cryopreservation is the only technique available for long-term germplasm conservation (Roberts et al., 1984; Engelmann and Engels, 2002; Hor et al., 2005; Thormann et al., 2006).

Sophora tonkinensis Gagnep (Leguminosae), growing in stone hill and limestone mountain region in Guangxi, uizhou and Yunnan province in Southwest China, as well as Northern Viet Nam, is a shrub whose roots are used as traditional Chinese medicine (Chen et al., 1994; Pharmacopoeia Commission of the Ministry of Health of the People's Republic of China, 2010). Because of excessive digging, at present the wild resources of *S. tonkinensis* is on the edge of extinction (Qin et al., 2006). Therefore, it is extremely significant and urgent to conserve *S. tonkinensis* germplasm resources *ex situ*. During domestication and breeding, it is difficult to store *S. tonkinensis* seeds for a long time (Qin et al., 2011). So far, there is a few available informations on seed storage behaviour of *S. tonkinensis*.

The purpose of the present investigation is to identify the storage behaviour and germination characteristics of *S. tonkinensis* seeds, so that suitable strategies of seed storage and germination are taken for a long-term conservation of *S. tonkinensis* germplasm resources.

MATERIALS AND METHODS

Seeds

Mature *S. tonkinensis* seeds were collected from the cultivation base (23°23'N, 105°57'E; altitude, 1090 m above sea level) in Napo county, Guangxi Zhuang Autonomous Region, China, in October, 2011. The annual mean temperature is 22.1°C. Annual mean rainfall is 1115 mm. After collection, seeds were dried at 20°C and 46% relative humidity (RH) for 72 h. When the moisture content of the seeds was 17.4 ± 0.2% (wet weight basis), seeds were used for germination, desiccation and storage experiments.

Seed viability

To evaluate the potential germinability of freshly-collected seeds, viability of seeds was assessed using a tetrazolium test (Moore, 1973). Three replicates of 50 seeds each were placed on two pieces of filter paper moistened with 5 ml of distilled water, incubated at 10°C for 16 h and then longitudinally cut into halves with a scalpel. The half-seeds were incubated in a 0.1% aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride for 24 h at 30°C in the dark. Only seeds showing a strong red-stained embryo were considered viable (Meulebrouck et al., 2008) and counted.

Determination of seed moisture content

The water content of seeds was determined gravimetrically (Sershen et al., 2012). Four replicates of five seeds each were used, and the moisture content of seeds was expressed as a percentage on a wet weight basis.

Germination test

Four replicates of 50 seeds each were incubated in 9-cm-diameter Petri dishes with moist perlite (water content of perlite, 18.3 ± 0.5%) at several constant temperatures in the dark or in an alternating photoperiod (12 h light/12 h dark; PPFD, 121 μmol m^{-2} s^{-1}). Germinated seeds were counted each day, and then the cumulative germination percentages were calculated. Germination test was terminated when all seeds had germinated except rotten those or no germination was noted for five consecutive days. Radicle protrusion was used as the criterion for germination.

Desiccation and cold storage

Desiccation treatment was performed by mixing seeds with silica gel in a dryer which was placed at an ambient temperature of 20-25°C. The water loss in the seeds was monitored periodically by determining the moisture content of seeds. Seeds desiccated to about 5% moisture content were subjected to a cold storage treatment at -20°C in dark for three months.

Germination test (25°C, 12 h light/day; PPFD, 121 μmol m^{-2} s^{-1}) was executed to seeds that were desiccated to about 12 and 5% moisture content, and to those that were firstly desiccated to about 5% moisture content and followed by a three-month cold storage at -20°C.

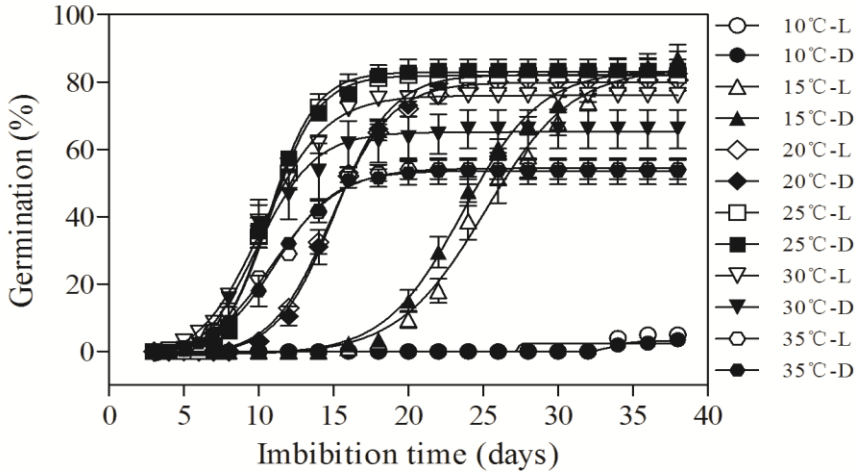

Figure 1. The time courses of germination of *Sophora tonkinensis* seeds under several combinational regimes with different constant temperatures and light. D represents dark and L represents light (12 h light/day). Values are mean ± SE of four replicates of 50 seeds each.

Data analysis

All data were analyzed with the SPSS for Windows 12.0 (SPSS, 2003). Effects of light on germination were analyzed by the independent samples *t*-test at $P = 0.05$, effects of incubation temperature on germination, germination speed and vigor of germination, and effects of desiccation and cold storage on germination were analyzed using a one-way ANOVA followed by the Student-Newman-Keuls (*S-N-K*) multiple comparisons test at $P = 0.05$. The time courses of seed germination at several constant temperature in light were performed a nonlinear regression analysis, and subsequently germination speeds were calculated by regression equations. germination speed was expressed as T_{50}, that is, the time when accumulative germination get to 50% of the final accumulative germination. To stabilize the variances, all germination data were arcsine-transformed prior to statistical analysis. All calculated values were expressed as mean ± SE (standard error).

RESULTS

Seed description and viability

The *S. tonkinensis* fruit is a legume, a pod, including one to three oval seeds. Anatomy experiments showed that a fresh mature seed included a thin black seed coat, an embryo with an axis and two expanded ivory cotyledons and no endosperm. Freshly-collected seeds were 7.7 ± 0.2 mm long, 5.9 ± 0.1 mm wide and 5.7 ± 0.1 mm thick (n = 30). The thousand seed weight (TSW) was 143.3 ± 1.0 g (n = 4). The moisture content was 17.4 ± 0.2% (n = 4). Seed viability by a TTC staining was 93.0 ± 1.3%, indicating that these seeds had a high germination potential.

Effects of light and temperature on seed germination

Light had no significant effect on seed germination at several constant temperatures ($P > 0.05$, Figure 1). In terms of final mean germination percentage, there was no significant difference between 15, 20, 25 and 30°C ($P > 0.05$), being significant higher than those at 35 and 10°C ($P < 0.05$), and the final mean germination percentage at 35°C was significant higher than those at 10°C ($P < 0.05$, Figure 1).

By a nonlinear regression analysis, mean germination speed (T_{50}) was calculated. The T_{50} at 10, 15, 20, 25, 30 and 35°C in light was 33.0 ± 0, 25.3 ± 1.0, 14.9 ± 0.1, 10.7 ± 0.2, 10.2 ± 0.4 and 11.0 ± 0.2 days, respectively. The mean germination speeds between 25, 30 and 35°C had no significant differences ($P > 0.05$), being significantly higher than those of others ($P < 0.05$, Figure 1). Vigors of germination, mean accumulative germination percentages after a 10-day incubation when the number of germinated seeds in a single day reached the maximum under most constant temperature regimes, at 10, 15, 20, 25, 30 and 35°C in 12 h/day light were 0, 0, 3.0 ± 1.3%, 34.0 ± 3.3%, 39.0 ± 5.0% and 22.0 ± 1.8%, respectively. Vigors of germination between 25 and 30°C had no significant differences ($P > 0.05$) and were significantly higher than those of others ($P < 0.05$).

Effects of desiccation and cold storage on seed germination

Initial germination of freshly-collected seeds with 17.4 ± 0.2% moisture content was 82.0 ± 4.6%. While seeds were desiccated to 8.4 ± 0.4% moisture content, the mean germination percentage significantly declined to 58.5 ± 5.6%, and 5.4 ± 0.1% moisture content, 36.0 ± 3.2% mean germination percentage ($P < 0.05$, Figure 2). A three-month cold storage at -20°C had no significant effect on seeds desiccated to 5.4 ± 0.1% moisture

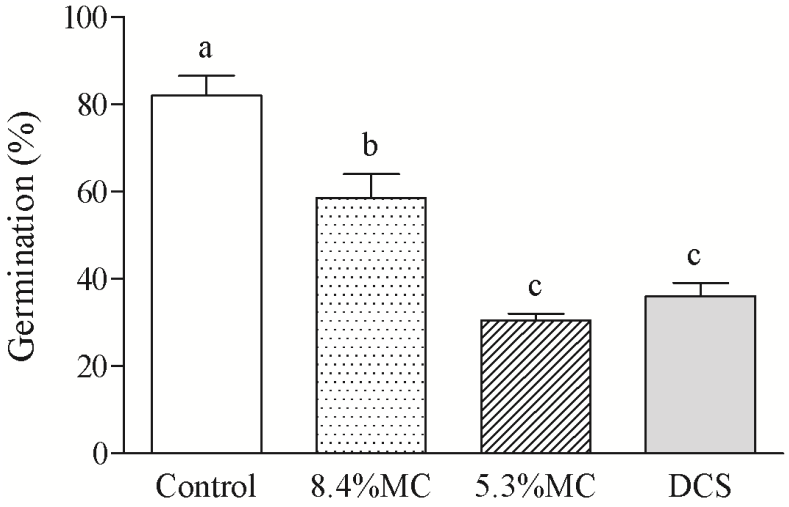

Figure 2. Effects of desiccation and cold storage on *Sophora tonkinensis* seed germination. MC, moisture content; DCS, desiccation and cold storage, that is, a desiccation by 5.4 ± 0.1% moisture content followed by a cold storage at -20°C for three months; Control represents freshly-collected seeds with 17.4 ± 0.2% moisture content. There is no significant difference between columns marked with the same lowercase letters (*S-N-K*, $P = 0.05$). Values are mean ± SE of four replicates of 50 seeds each.

content ($P > 0.05$, Figure 2).

DISCUSSION

Seeds between 30 and 13 000 g TSW may show orthodox, intermediate or recalcitrant storage behaviour. For species with recalcitrant seed storage behaviour their shedding moisture contents are distributed between 36 and 90%, for intermediate between 23 and 55%, and for orthodox between <20 and 50% (Hong and Ellis, 1996). In the present investigation, the TSW of freshly-collected *S. tonkinensis* seeds was 143.3 ± 1.0 g with 17.4±0.2% moisture content. As far as TSW and moisture content were concerned, *S. tonkinensis* seeds seemed to be orthodox.

Light is ecologically of great importance for seed germination (Bewley and Black, 1982). According to Evenari (1956), seeds are divided into three groups in terms of sensitivity of germination to white light: 1) positively photoblastic seeds whose germination is promoted by white light; 2) negatively photoblastic seeds whose germination is inhibited by white light; 3) non-photoblastic seeds whose germination is insensitive to white light. In the present investigation, white light irradiation (12 h/day) had no significant effect on seed germination under several constant temperature regimes ($P > 0.05$, Figure 1), suggesting that *S. tonkinensis* seeds might be non-photoblastic. Taking into account final mean germination percentages, mean germination speeds and vigors of germination, the temperature optimum for seed germination of *S. tonkinensis* ranged from 25 to 30°C.

Seed moisture is the most important factor in maintaining viability during storage; it is the primary control of all activities. Metabolic rates can be minimized by keeping seeds in a dry state (Bonner, 2008). Seeds that are killed by desiccation to 10-12% moisture content are likely to be recalcitrant; seeds that tolerate desiccation to about 10-12% moisture content, but whose viability is reduced when subjected to further desiccation to a lower moisture content, are likely to show intermediate seed storage behavior; seeds that tolerate desiccation (show no loss in viability) to 5% moisture content or below are likely to show orthodox seed storage behavior (Rao et al., 2006). In the present experiments, the initial germination of freshly-collected seeds was 82.0 ± 4.6% with 17.4 ± 0.2% moisture content, while desiccation treatments significantly decreased the mean germination of *S. tonkinensis* seeds, 8.4 ± 0.4% moisture content, 58.5 ± 5.6% mean germination percentage, and 5.4 ± 0.1% moisture content, 36.0 ± 3.2% mean germination percentage ($P < 0.05$, Figure 2). These results suggested that the storage behaviour of *S. tonkinensis* seeds were likely to be intermediate, according to Hong and Ellis (1996).

Low temperatures can also minimize metabolic rates of stored seeds. Meanwhile, the storage moisture content determines how low temperatures can be suitable for seed storage (Berjak and Pammenter, 2002; Bonner, 2008). In the present investigation, a cold storage at -20°C for 3 months had no significant effects on viability of seeds desiccated to 5.4 ± 0.1% moisture content ($P >$

0.05, Figure 2). According to Hong and Ellis (1996), together with desiccation tolerance, *S. tonkinensis* seeds were likely to be intermediate. So, for a purpose of long-term storage of *S. tonkinensis* seeds, an investigation of cryopreservation (liquid nitrogen conservation) is required.

According to a compendium (Hong et al., 1996), among investigated 1088 species within Leguminosae an overwhelming majority showed orthodox seed storage behaviour, and only a minority showed recalcitrant seed storage behaviour, and none showed intermediate seed storage behaviour. So far, intermediate seed storage behaviour in Leguminosae species was discovered for the first time.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effect of water deficit on Argan tree seedlings (*Argania spinosa* L. Skeels): Morphological and physiological aspect

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The Argan tree, *Argania spinosa* L., Skeels, is an endemic species in North-West Africa perfectly adapted to aridity and drought. It is in this context that we studied the physiological impact of water deficit on the Argan tree seedlings for eight weeks at a field capacity of 30%. The obtained results reveal that the stressed seedlings manifested by the strategy of the root elongation from the second week, the roots reached 31 cm compared with 15 cm of the control. However, the seedlings showed severe dehydration of 41% in leaves and 45% in roots. Besides, the content of chlorophyll pigments has relatively decreased from the second week, a slight yellowing and leaf drop was observed. The seedlings have accumulated proteins in a very significant way in leaves (from 25 to 107 mg.g^{-1} by fresh weight) and (from 23 to 90 mg.g^{-1} by fresh weight) in roots. Proline was also accumulated; the content was 4 and 2 µg.g^{-1} by fresh weight respectively in leaves and roots compared with 1.3 and 1.1 µg.g^{-1} ^{1} by fresh weight in control respectively. The accumulation of the protein and proline is higher in leaves than in roots. The content of malondialdehyde was higher in leaves than in roots. This increase is significantly related to the prolongation of the stress period from the second week. The enzymatic activity of peroxidase is in relative increase according to the duration of the water stress applied in both leaves and roots. We deduce from these results that Argan seedlings possess the characteristic of xeropyhte that tolerate aridity.

Key words: *Argania spinosa*, drought stress, proline, chlorophylls, proteins.

INTRODUCTION

The Argan tree, (*Argania spinosa* L. Skeels), is a tropical species from the Sapotaceae family which grows in North-West Africa. Its current natural area is limited in Morocco and in the Algerian southwest in Tindouf city

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(Nouaim, 2005). This species plays an important major ecological and socio-economical roles; the oil obtained from this plant is very rich in unsaturated fatty acid (Charrouf and Guillaume, 1998). The Argan tree is considered as being the least demanding tree of rainfall (Msanda et al., 2005). It is a xerophytic species which can survive at less than 120 mm of precipitation a year, as it can survive for several successive years of drought (Ferradous et al., 1996; Zahidi et al., 2013a). It is also a thermophilous species which is able to stand at very high temperatures reaching 50°C. Roots play an essential role in the survival of the Argan tree in dry climate. It has swiveling roots able to reach great depth to scoop out water (Mokhtari, 2002). Also, its physiological characteristic allows this tree to avoid water stress as its capacity of defoliation or partial closing of stomata (Faouzi, 2006; Berka and Aïd, 2009); no stomata are observed at adaxial leaf surface characterizing Argan as a hypostomatous species (Bani-Aameur and Zahidi, 2005). This tree loses its foliage only in case of a hard drought; dry conditions were spread over a longer period, trees are completely defoliated (Zahidi et al., 2013b) otherwise, the Atlantic ocean offers an important atmospheric humidity which allows the Argan tree to trap fresh air (Msanda et al., 2005). Tindouf city is an arid area where the average annual rainfall is recorded to 33.7 mm. This region has an average temperature of 22.90°C and the heat reaches its maximum in July to August until 52°C. The coincidence of the maximum heat with a period of rainfall deficiency causes a marked water stress in this region.

The Argan tree is maintained in Tindouf due to natural conditions remaining favorable in a residual ecosystem resistant but vulnerable in view of the water deficit. The nursery of Touiref Bouaâm belonging to the forest conservation of Tindouf (105 km to the north) offers some advantages in order to regenerate the Argan tree. The water deficit of the environment constitutes a constraint to circumvent in the development of the Argan tree by plantation that requires the mobilization of water resources. Under the conditions of water deficit, plants develop morpho-functional and physiological strategies to improve water absorption (Larcher, 1995). Many plants adapted to arid areas control only very little of their water loss through respiration, but they have a very major rooting able to extract water from the ground (Rivlland, 2003). The tolerance to the water constraint is a strategy that allows the plant to provide its physiological functions in spite of the degradation of its water state. It is the result of complex physical, biochemical and molecular mechanisms. The expression of various genes and the accumulation of various osmolytes coupled with an effective antioxidant system are often the principal mechanisms of tolerance to water deficit (Tardieu, 2005). Consequently, the environmental stresses including the water stress, is the appearance of an oxidative stress, that is, the accumulation of reactive oxygen species (ROS) which damages the cellular structures (Appel and Hirt, 2004). Under the optimal conditions, the leaves are equipped with enzymes such as: catalase and peroxidase and other antioxidant metabolites sufficient to face ROS.

It is for this aim that we have studied the effect of water stress on the Argan seedlings and their behavior (morphological and physiological) opposite this constraint. So, through the analysis of some stress markers such as proline or malondialdehyde that allow us to estimate the degree of damage caused by the water deficit, also the content of protein and peroxidase activity would evaluate the seedlings tolerance. We have also targeted to define the degree of the seedlings' resistance to the water deficit. Finally, *A. spinosa* needs to be reforested in the Algerian forest in order to ensure sustainable development of our forest, and this requires controlling plant growth's conditions especially at young stage in an arid environmental.

MATERIALS AND METHODS

Plant material and growth conditions

The Argan seedlings are obtained from the germination of kernels collected from Tindouf city to the north (27° 39' 15'' N latitude and 8° 9' 6'' W longitude and 450 m altitude) after soaking for 48 h in plain water (Meslem et al., 2009). The seedlings development continues under greenhouse up to one year in polyethylene bags of 15 and 8 cm of diameter containing original soil. Watering was done every week with tap water.

Water deficit experiment

Five batches of 15 seedlings (one year old each) are put under a water deficit in different durations of 2, 4, 6 and 8 weeks at 30% of field capacity (F.C). The control seedlings are watered every week. The field capacity was determined according to Côme and Corbineau (1998); we dried the soil completely in an oven. Later we weighed 1 kg that we put in a perforated cylinder on both sides, and then in a tank containing 1 L of tap water for 48 h. The water will rise surface by capillary phenomena. Finally, we measured the amount of water absorbed by the soil and this value corresponds to 100% of field capacity. At the end of the treatment, triplicate samples of leaves and roots are sorted out, washed with fresh distilled water and conserved at -80°C for further physiological analyses. The follow-up of growth was carried out throughout the water stress. The measurements of the stems' length were taken each week. However, the roots' length was measured at the end of the treatment.

Determination of relative water content (RWC)

The RWC was determined according to the method of Heller et al. (1998); the RWC was calculated as follows:

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

FW = fresh weight; TW = Turgescent weight; DW = dry weight.

Chlorophyll determination

50 mg of fresh leaves were homogenized in 2 ml of ice-cold acetone 80°, then centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant was used for the determination of chlorophyll pigments by spectrophotometer (6715 UV/Visible- JENWAY) at 645 and 663 nm. The chlorophyll (a) and (b) concentration were quantified as mg. gr^{-1} FW according to Arnon (1949) formulae:

Total chlorophyll = 20.2 (A_{645}) + 8.02 (A_{663})

Chlorophyll a = 12.7 (A_{663}) - 2.69 (A_{645})

Chlorophyll b = 22.9 (A_{645}) - 4.68 (A_{663})

Estimation of soluble total proteins

Fresh biomass was homogenized in 0.06 M phosphate-buffer (pH 7.8) with PVP (polyvinylpyrrolidone) and then centrifuged. Proteins concentration was determined as described by Bradford (1976) method; 2 ml of Bradford reagent was added to 100 µl of the supernatant or distilled water for the standard.

Determination of proline

300 mg of fresh biomass were homogenized in 10 ml of 3% sulfosalicilic acid, then centrifuged at 3000 rpm for 10 min. 2 ml of supernatant was added to 2 ml of solution contains: 1.5 g Ninhydrine, 30 ml of acetic acid and 20 ml of phosphoric acid; then heated at 80°C for 45 min. The mixture was cooled down in an ice-bath. Afterwards 5 ml of toluene was added (Monneveux and Nemmar, 1986). The proline concentration was calculated by absorbance at 520 nm using the molar extinction coefficient of 387.1 $mg.gr^{-1}$ and expressed as $\mu g.gr^{-1}$ FW according to Beer-Lambert's Law:

$$A_\lambda = -\log \frac{I}{I_0} = \varepsilon_\lambda \cdot \ell \cdot C.$$

I/I_0 is the transmittance of the solution; A is the absorbance or optical density at a wavelength λ; ε is the molar extinction coefficient; ℓ is the optical path length in the crossing solution, it corresponds to the thickness of the cuvette used and C is the molar concentration of the solution.

Estimation of lipid peroxidation (malondialdehyde content)

Lipid peroxidation was estimated by determining the malondialdehyde (MDA) contents in the leaves and roots according to the method of Rajinder et al. (1981). 100 mg of samples was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 *g* for 5 min at 4°C. Aliquot of 0.3 ml of supernatant was mixed with 1.2 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA 20%, then incubated at 95°C for 30 min and cooled down in an ice-bath. The samples were centrifuged at 10 000 × *g* for 10 min at 25°C. The supernatant absorbance was then measured at 532 nm. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the molar extinction coefficient of 155 mM^{-1} cm^{-1} expressed in µM. g^{-1} of FW according to Beer-Lambert's Law.

The assay of peroxidase

The leaves and roots were homogenized in cold (4°C) 50 mM phosphate buffer (pH 6.0) (4 ml/g of biomass). The homogenate was rapidly centrifuged at 11 000 *g* for 15 min at 4°C. The supernatant was taken as the source of peroxidase, and the determination of the activity was performed immediately (Jackson and Ricardo, 1992). Peroxidase activity was determined in triplicate by measurement of the absorbance at 470 nm of tetraguaiacol (ε = 6.65 × 103 M^{-1} cm^{-1}) according to Beer-Lambert's Law. We used 1.5 ml of 1% guaiacol and 0.4 ml of 0.1 M hydrogen peroxide in 50 mM phosphate-buffer (pH 6.0) at 35°C and the reaction was started by adding 0.1 ml of peroxidase solution (Reuveni et al., 1992; Khan and Robinson, 1994).

Analysis of data

All data were analyzed in three replications and the obtained data are evaluated statistically using Student's t-test, and least significant difference (LSD) was calculated at $P < 0.05$ and signaled faces by letters (abc), knowing that; $P > 0.05$ not significant, $P < 0.05$ significant difference (a), $P < 0.01$ very significant difference (ab) and $P < 0.001$ highly significant (abc).

RESULTS AND DISCUSSION

The follow-up of growth (length of stems and roots)

Deficit water did not influence significantly the growth of the stems. In contrast, the root length increased significantly as the duration of the water deficit was prolonged. The difference was very significant from the fourth week; 15 and 31 cm were recorded between the control and the 8-week stressed seedlings respectively (Figure 1).

Relative water content (RWC)

The relative water content in the roots and the leaves decreased gradually depending on the prolongation of the water deficit duration. From the fourth week, RWC decreased significantly from 85 to 41% in the leaves and from 88 to 45% in the roots (Table 1).

Chlorophyll content

The chlorophyll pigments; total, (a) and (b) are sensitive to water deficit. The total chlorophyll content was 15 $mg.g^{-1}$ FW in the eight weeks. The difference was very significant which is about 66 $mg.g^{-1}$ FW for the control (Table 2). Also, a slight yellowing in leaves was observed from the 6^{th} week.

Total soluble protein content

The protein content increased significantly (Figure 2). This increase was important in the first weeks in the roots. However, from the sixth week, the protein content increased more importantly in the leaves. There was a

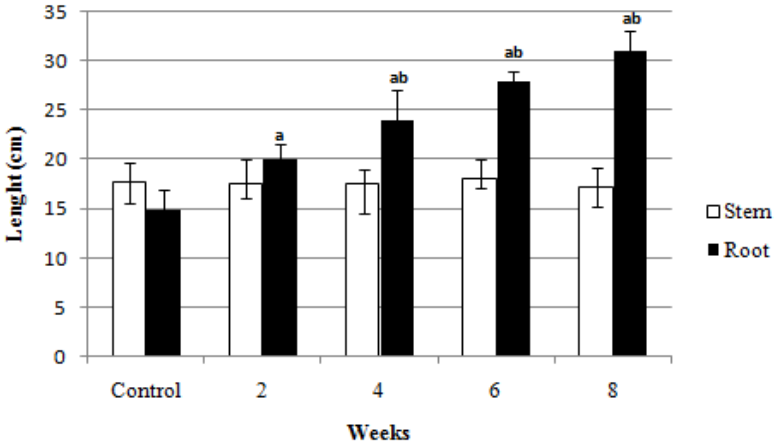

Figure 1. Effect of water deficit on the length of the 8-week stressed seedlings' stems and roots [P>0.05, not significant; P <0.05, significant difference (a), P <0.01 very significant difference (ab), and P<0.001 highly significant difference (abc].

Table 1. Effect of water stress on RWC (P>0.05 not significant, P < 0.05 significant difference (a), P <0.01 very significant difference (ab), and P<0.001highly significant difference (abc).

% RWC	Control	2 Weeks	4 Weeks	6 Weeks	8 Weeks
Leaf	85±2	83±1	70±2[a]	65±1[ab]	41±1[abc]
Root	88±1	85±2	73±1[ab]	60±1.5[abc]	45±1[abc]

Table 2. Effect of water stress on chlorophyllian pigments content [P>0.05 not significant, P <0.05 significant difference (a), P <0.01 very significant difference (ab), and P<0.001highly significant difference (abc)].

Content in mg.g^{-1} FW	Control	2 Weeks	4 Weeks	6 Weeks	8 Weeks
Chl tot	67.2 ± 0.02	66± 0.01	40±0.05[a]	27.7±0.045[abc]	15,2± 0.06[abc]
Chl (a)	61.2±0.3	54.22±0.7	50.88±1.2	38.12±0.35[abc]	30.24±0.3[abc]
Chl (b)	91.2±0.15	52.18±0.07[ab]	47.72±0.57[ab]	39.4±0,41[ab]	36.16±0.11[ab]

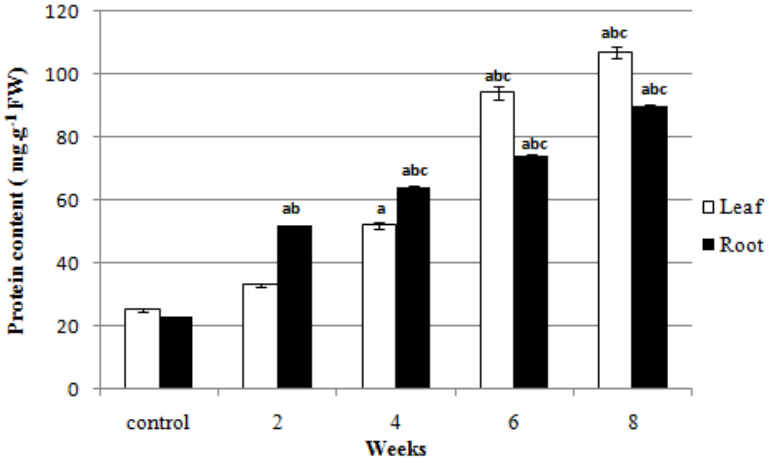

Figure 2. Effect of water deficit on the total soluble protein content [P>0.05 not significant, P <0.05 significant difference (a), P <0.01 very significant difference (ab), and P<0.001highly significant difference (abc)].

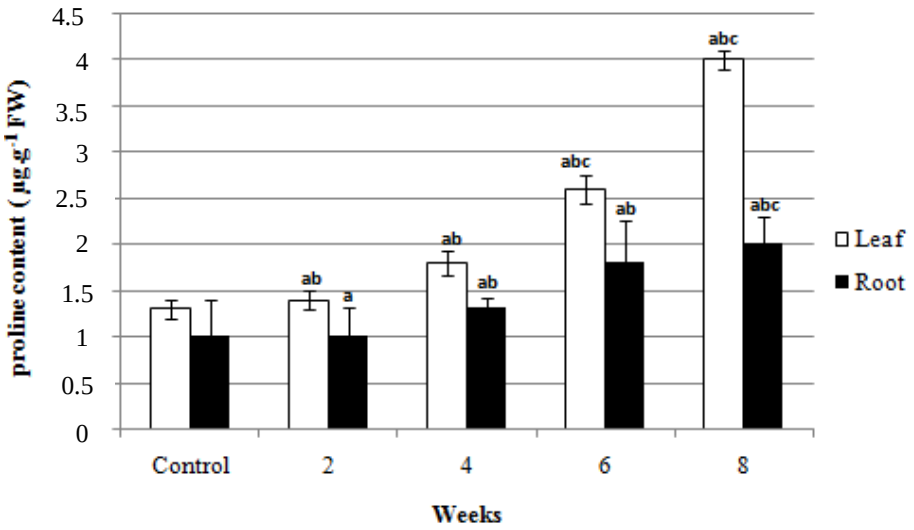

Figure 3. Effect of water deficit on proline content [P>0.05 not significant, P <0.05 significant difference (a), P <0.01 very significant difference (ab), and P<0.001highly significant difference (abc)].

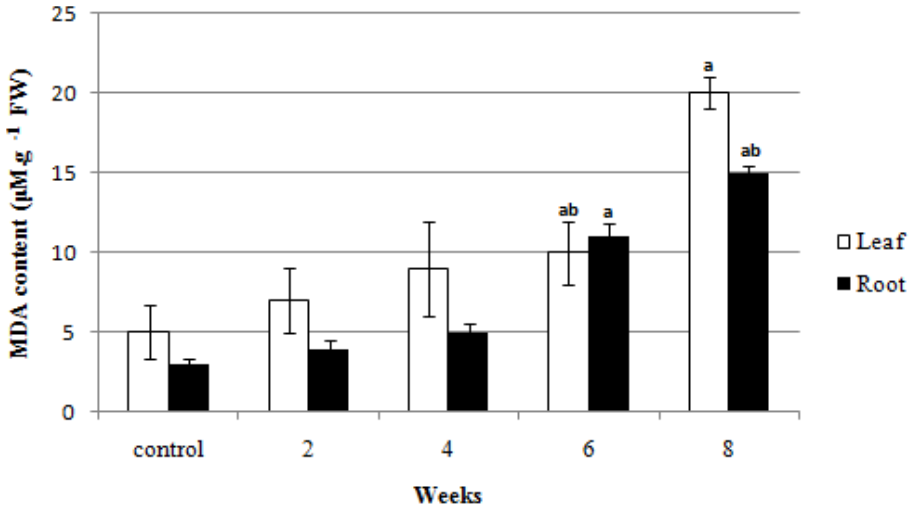

Figure 4. Effect of water deficit on the MDA content [P>0.05 not significant, P <0.05 significant difference (a), P < 0.01 very significant difference (ab), and P<0.001 highly significant difference (abc)].

positive correlation between the duration of the water deficit and the accumulation of total protein.

The proline content

Water deficit induced an accumulation of proline in leaves and roots (Figure 3); we recorded an increase from 1.3 to 4 µg.gr^{-1} FW in leaves and from 1 to 2 µg.gr^{-1} FW in roots relative to the control, respectively. This accumulation is significantly higher in leaves ($p< 0.001$) compared to roots for the eight weeks. But, in control seedlings MDA content was also higher in leaves; indeed the increase in MDA was higher in roots (x5) than in leaves (x4) with respect to controls.

The content of MDA (lipid peroxidation)

The lipid peroxidation was significantly higher in leaves than in roots compared to control; from 5 to 20 µM.g^{-1} FW and from 3 to 15 µM.g^{-1} FW (Figure 4). The amount of MDA accumulation has manifested significantly from the sixth week of stress ($p < 0.05$).

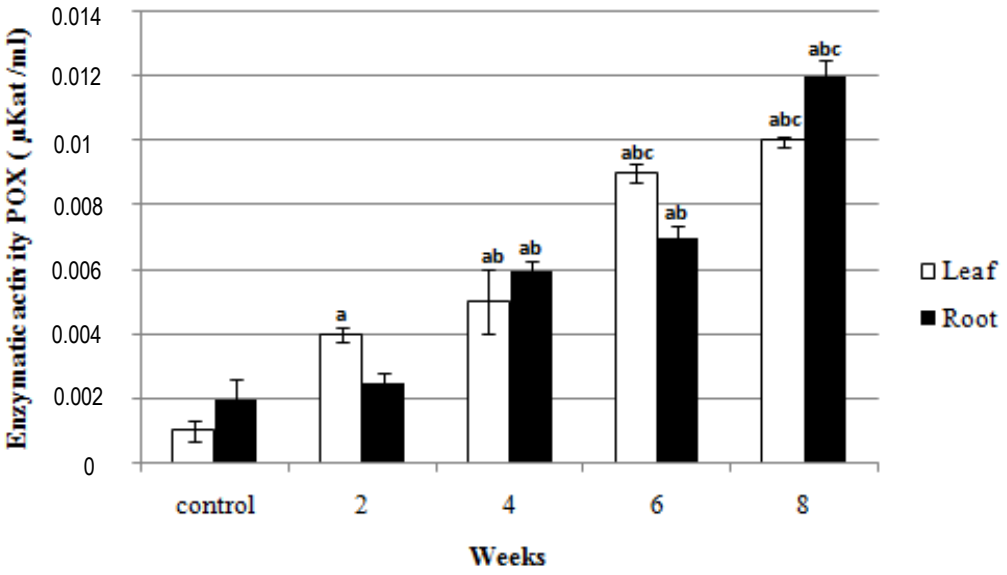

Figure 5. Effect of water deficit on the enzymatic activity of peroxidases [P > 0.05 not significant, P < 0.05 significant difference (a), P < 0.01 very significant difference (ab), and P < 0.001 highly significant difference (abc)].

Total enzymatic activity of peroxidase

The enzymatic activity of peroxidases increased progressively according to the stress duration in the leaves and the roots as well (Figure 5).

DISCUSSION

The water deficit occurs by the restriction of the availability of soil water. The degree of adaptation of the plants to the lack of water varies according to the species (Parker and Pallardy, 1985). Moreover, drought resistance is the result of complex physiological, biochemical and molecular mechanisms; at the biochemical level, there is the intervention of various osmolytes that help to maintain osmotic balance in the cell in the terms of dehydration (Wang et al., 2004). Indeed, tolerance to drought remains poorly described for the Argan tree. The mechanisms adopted by this species to withstand drought are still largely unknown (Zahidi et al., 2013a). Currently, it is proven that the Argan adult tree is drought resistant, survives in its area for many years (Emberger, 1925). We report here, responses of the Argan seedling to draught. The water deficit applied to Argan seedlings induced proteins accumulation in leaves and roots. This accumulation was from 25 to 107 $mg.gr^{-1}$ by F.W in leaves and from 23 to 90 mg^{-1} by F.W in roots after eight weeks compared to control, respectively. This accumulation of protein has been demonstrated also among abiotic stress in other species; Zerrad et al. (2008) reported an increase in protein content in wheat under water stress conditions and a stimulation of a new protein synthesis which ensures a protection of the cellular membranes (David and Grongnet, 2001).

Besides, EL-Tayeb and Ahmed (2007) reported that a tolerant broad bean to drought reveals an increase in proteins that protects the wall against damage, plus the synthesis of 13 new polypeptides. This accumulation is probably due to a protein synthesis resistance to abiotic stress, or to an activation of antioxidant enzymes. The growth of the stem during the eight weeks of stress remains indifferent compared to control. We noted also that root's elongation increased progressively as the duration of water deficit, the difference is very clear; 15 and 31cm, respectively, are recorded between the control and seedlings stress after eight weeks. These results complement those found before by Zahidi et al. (2013a) about Argan seedling, it was reported that drought stress decreased seedlings height, basal diameter, leaf number, leaf area, biomass production and water content.

Generally, any implied stress affects the growth in plants, the application of a metallic copper stress inhibits the root's elongation in radish (Sun et al., 2010) and a reduction in the amount of water in young sunflower seedlings (Jouili and El Ferjani, 2003). In spite of that, our seedlings were able to grow but at a low rate and started shedding leaves. However, Zahidi et al. (2013a) reported also that when water stress is high, leaf fall was more intense, but after lower or moderate water deficit, leaf drop was less important in humid seasons. Also, Benouf et al. (2014) reported that subjection Argan seedlings to several water stress led to reduction in the height of the stem, the number of leaves, and radial growth of biomass, and increased the length of the root portion. In seedlings of *Adenanthera pavonina*, Paliwal and Kannan

(1999) showed that water deficit causes a reduction of the stem height. In many species, root system in acquiring water has long been recognized as crucial to cope with drought conditions (Kashiwagi et al., 2006). Additionally, photosynthesis and growth (biomass production) are the primary processes to be affected by drought.

Our results agree with the finding in other species, so, in order to diminish consumption and increase absorption of water, plants in dry conditions often decrease their growth rate and biomass production, and contribute more biomass to roots (Wu et al., 2008). Our results show a remarkable reduction in the water content in roots and leaves; the RWC decreased progressively according to the duration of the water deficit. After eight weeks, we noted a decrease in the RWC from 85 to 41% in leaves and 88 to 45% in roots compared to the control. These results agree with those found by Berka and Aïd (2009) about the Argan tree subjected to several field capacity and by Fahmi et al. (2011) at different sampling arid areas. Several studies have showed the effect of the water stress on the RWC on other species; Zerrad et al. (2008) and Tahi (2008) about tomato and Kasraoui et al. (2004) about the Olive tree. The leaves of the Argan seedlings subjected to a water deficit present a gradual decrease in the content of chlorophyll pigments from the fourth week, and a yellowing of the leaves was also observed. This reflects the degradation of chlorophyll pigments which influences the photosynthetic capacity thereafter (M'Hamdi et al., 2009). It was reported that an oxidative stress causes the degradation of the total chlorophyll in Argan tree (Fahmi et al., 2011), in wheat (Tahri et al., 1998) and in sunflower (Ahmed et al., 2013). Also Majumdar et al. (1991) reported that chlorophyll is degraded following an activation of chlorophyllase. It was suggested that the existence of a probable connection between the synthesis of chlorophyll and proline, there is competition on the common precursor, which is the glutamate (Tahri et al., 1998).

Our data reveal an accumulation of proline much more in leaves than in roots. That was relative to the stress duration. It is a stress indicator that constitutes a source of nitrogen that can be used by the plant at the stress period (Stewart and Lee, 1974; Tal and Rosenthal, 1979) and a regulator of cytoplasmic pH (Pesci and Beffagna, 1984). It has even been recommended by several authors as an early screening test for the tolerance to water deficit (Singh et al., 1972). Our results show the conformity with the observations obtained by Berka and Aïd (2009) and Benouf et al. (2014) who reported that Argan seedlings overcome deficit water by osmotic adjustment resulting from a rapid and significant increase of active soluble sugars and a rapid accumulation of proline in leaves at several field capacity.

Tahri et al. (1998) and Chaib et al. (2010) reported that the PEG induced an accumulation of proline in wheat and a decrease in the activity of glutamine synthetase. The reactive oxygen species at high levels can react with unsaturated acids that cause the peroxidation of the membrane essential lipids (Scandalois, 1993). The content of MDA is often considered as an indicator of tissue damage (Ding et al., 2004). Our results demonstrate that MDA was accumulated in Argan seedlings and correlated with the stress duration. These results are consistent with the observations of Tahi (2008) who reported an accumulation of MDA in tomato and radish (Sun et al., 2010). Also, Gülen et al. (2008) reported that low temperatures induced a rise of MDA in strawberry. The peroxidase is an enzyme involved in the mechanism of detoxification. The enzymatic activity was considered as an indicator of resistance to oxidative stress (Castillo, 1987). Argan seedlings submitted under water deficit showed a high peroxidase activity in leaves and roots compared to the control. At the beginning of treatment, the enzymatic activity increased significantly in the leaves. From the second week, the phenomenon was reversed, and the activity becomes more important in roots to reach its maximum at the eighth week. Many studies show that enzymes as superoxide dismutase, ascorbate peroxidase, catalase and peroxidase especially are accumulated during the water stress (Zhang and Kirkham, 1994). The capacity of the antioxidant system is crucial to maintain the integrity of the photosynthetic system (Reddy et al., 2004). In plants, the peroxydase is known by a wide distribution of iso-enzymes, which differ by their physiological role (Maciel et al., 2007).

Also, EL-Tayeb and Ahmed (2007) have shown that a variety of tolerant broad bean to water stress had a high peroxydase activity compared to other sensitive. Thus, Gülen et al. (2008), reported the appearance of new isoforms of peroxydase in Strawberry seedlings subjected to low temperature. The detoxifying enzymes can be used as important bio-markers in response to environmental stresses (Sun et al., 2010). Indeed, the response of the Argan seedlings through the enzymatic reaction reflects its great capacity to cope with water deficit.

Finally, an important factor must be looking for which is the genotype and its effect; it was be conducted by Ait Aabd et al. (2010) in order to identify promising wild Argan trees, who found that some provenances exhibited high performance yield and appeared to be the best adapted to drought conditions, contrary to others. So, it would be interesting to select tolerant genotypes. Same observations was made by Zahidi et al. (2013b) who suggested that some genotypes are resistant to dry conditions and will be useful for selecting plus trees which are essential for management and conservation practices of genetic resources in Argan forest.

Conclusion

This study demonstrates that Argan tree seedlings have

developed metabolic strategies to cope with the effect of water deficit and to minimize damage. This study has produced interesting results on the mechanisms of resistance of the Argan seedlings against a water deficit for two months, which can be useful in the applied aspect, that is, at a young stage; we can irrigate the Argan seedlings on field at least every six weeks. It would be also interesting to look further into this study by the prolongation of the duration of the stress and to follow other parameters for the successful regeneration and reforestation of this species in the Algerian forests (Tindouf) which is on the way of defacement.

Conflict of interests

The authors did not declare any conflict of interest.

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Genetic variation of 12 rice cultivars grown in Brunei Darussalam and assessment of their tolerance to saline environment

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Genetic variations of 12 different rice cultivars in Brunei Darussalam were studied using 15 different SSR markers and their salinity tolerance mechanism was also assessed. Eight SSR markers, RM 151, 187, 206, 226, 276, 310, 320 and 334, showed polymorphic alleles while the other seven were monomorphic. A total of 158 alleles were amplified for all these rice cultivars using 15 SSR markers, with an average of 10.53. The allele frequencies per locus or marker range from 0 in RM 307 to five alleles in RM 226. PIC values varied from 0.00 to 0.7521. Similarity distance varied from 0.00 to 1.00. Dendrogram showed three distinct clusters, where both *Kuaci* and *Sp1* significantly diverted from the other ten rice cultivars. *Bandul berminya*k was the most tolerant to salinity. Quantum yield for *B. berminyak* were unaffected and it showed the least reduction in growth parameters studied when expose to salinity stress. From both salinity tolerance and genetic variation investigations for these 12 cultivars, it may probably be better to intercross between *Arat* (moderately tolerant) and *Sp1* (susceptible) as both are from different clusters, showed low genetic similarity with 0.33 and different salinity tolerance level.

Key words: Genetic variability, rice cultivars, SSR markers, salinity

INTRODUCTION

Extensive efforts have been carried out in Brunei to increase rice production which includes cultivation of fast-growing inbred paddy *Laila*, increase paddy cultivation area and improvements in facilities. However,

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Abbreviations: RFLP, Restriction fragment length polymorphism; **RAPD,** random amplified polymorphic **DNA; AFLP,** amplified fragment length polymorphism; **ISSR,** inter-simple sequence repeat**; SSR,** single sequence repeats; PIC, polymorphism information content; **RM,** rice marker.

the country has yet been able to achieve the target 20% self-sufficiency. Soil salinity is one of the major constraints that cause this situation. Low rice productivity is also caused by narrow range of genetic variability and lack of sufficient genetic information about traits inherited (Mahajan et al., 2012). Standard conventional breeding techniques which involved selection of desired parent plants using morphology and physiology study (Allard, 1999; Collard and Mackill, 2008) has been replaced by molecular breeding. The conventional breeding techniques may take five to 10 years to be completed and more work on morphology and physiology are needed for studying genetic diversity among parents (Zeng et al, 2004; Collard and Mackill, 2008). Zeng et al. (2004) have reported that the morphology measurement might not reveal the actual genetic relationships among genotypes studied. Thus, both measurements might not be discriminative enough to differentiate all the genotypes studied (Behera et al., 2012).

The constraints encountered by conventional breeders on selection of parents can be solved by using molecular breeding techniques. Some molecular markers that can be used in molecular breeding include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR) and single sequence repeats (SSR). These techniques are different in terms of speed, cost and degree of polymorphism. According to Garcia et al. (2004), RAPD is a much simpler technique when compared to RFLP, as the latter requires radioactive materials. However, RAPD lack in reproducibility cause by mismatch annealing. SSR solve these limitations as its sequence present in most eukaryote genomes, informative and reproducible. According to Saini et al. (2004), SSR is more efficient for differentiating rice genotypes when compared to both AFLP and ISSR and they also stated that both AFLP and SSR are more reliable and reproducible than ISSR.

According to Shams et al. (2012), SSR markers are usually utilized to study genetic variation in different rice germplasm as they are inexpensive, simple, rapid, easily detected by PCR and importantly only require small amount of tissue samples. These markers helped to increase efficiency and precision when selecting the parents before intercrossing and this would lead to the production of new cultivar with improved characteristics (Zeng et al., 2004; Collard and Mackill, 2008). Assessing salinity tolerance level of rice cultivars is also necessary to select the parental line for breeding program as it helps to understand salinity tolerance mechanism in the cultivars. Growth and photosynthetic efficiency measurements were taken for determining salinity tolerance of the studied rice cultivar (Cha-um et al., 2007).

Cultivar that possess high tolerance and show less reduction in these parameters would be the best parent. Rice varieties adapt to saline environments differently due to the diverse genetic background and different salinity tolerance mechanisms. Improving salt tolerance can be achieved by selecting suitable parents before intercrossing based on information provided from microsatellite markers.

Different salt tolerance components can be combined into a cultivar by intercrossing parents from different microsatellite clusters with wide salt tolerance mechanisms (Zeng et al., 2004). Investigation on genetic similarity, cluster analysis and also salt tolerance ability provide useful information for plant breeders to select best parents with diverse genetic background prior to intercrossing (Kanawapee et al., 2011).

In this study, the genetic diversity of 12 different rice cultivars grown in Brunei, with different adaption to salinity stress, was assessed using 15 SSR markers. Fifteen (15) markers with high polymorphism information content (PIC) values from Temnykh et al. (2000) were selected for the study. The result obtained from this study may help breeder to increase efficiency of breeding as it provides detailed information about genetic diversity of the 12 cultivars studied, thus allowing one to select the suitable parents for crossing to produce germplasm with better traits. Besides, no extensive investigations have yet been carried out for genetic study of the rice cultivars grown in the country using microsatellites.

MATERIALS AND METHODS

Plant material

Twelve (12) different rice cultivars: *Adan, Arat, Bandul berminyak, Jongkok, Kuaci, Laila, Pusu, Pulut Keladi, Pusu Merah*, *Raden Pinang*, *Salleh*, and an unknown rice cultivar which was named *Sp1* were used in the present study.

Genomic DNA extraction

Genomic DNA was extracted from frozen leaves (0.2 g) of the rice cultivars using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co. LLC, St. Louis, Missouri) according to the manufacturer's instructions.

PCR amplification

A total of 15 microsatellites with high PIC value were chosen from Temnykh et al. (2000) and used for the study (Table 1). The polymerase chain reaction (PCR) amplification mixture was prepared in 0.5 ml Eppendorf tubes. Each reaction mixture contained 2.5 µl of 10X PCR buffer, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 5 µl of 1 µM for each primer (forward and reverse), 100 ng of DNA, 0.3 µl Taq DNA polymerase and TE buffer (pH 8.0) was added to adjust the final volume to 25 µl. The amplification programme consisted of the following cycles: 94°C for 4 min, 30 cycles of 94°C for 45 s, 39.5 to 57.2°C (standardized for each primer) for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min, following method of Nadia et al. (2014).

Table 1. List of SSR markers used in this investigation.

Primer	Size range (bp)	PIC	Tm (°C)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
RM 72	152-198	0.85	43.8	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG
RM 151	205-317	0.87	54.2	GGCTGCTCATCAGCTGCATGCG	TCGGCAGTGGTAGAGTTTGATCTGC
RM 159	238-252	0.87	57.2	GGGGCACTGGCAAGGGTGAAGG	GCTTGTGCTTCTCTCTCTCTCTCTCTC
RM 187	136-164	0.84	45.3	CCAAGGGAAAGATGCGACAATTG	GTGGACGCTTTATATTATGGG
RM 206	121-137	0.87	39.5	TAGTTTAACCAAGACTCTC	GGTTGAACCCAAATCTGCA
RM 226	264-342	0.82	49.2	AGCTAAGGTCTGGGAGAAACC	AAGTAGGATGGGGCACAAGCTC
RM 264	148-178	0.83	46.7	GTTGCGTCCTACTGCTACTTC	GATCCGTGTCGATGATTAGC
RM 276	85-153	0.84	46.7	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA
RM 287	98-118	0.83	42.6	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC
RM 307	124-176	0.83	46.7	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC
RM 310	85-120	0.83	38.5	CCAAAACATTTAAAATATCATG	GCTTGTTGGTCATTACCATTC
RM 320	153-254	0.85	46.7	CAACGTGATCGAGGATAGATC	GGATTTGCTTACCACAGCTC
RM 333	164-215	0.83	47.5	GTACGACTACGAGTGTCACCAA	GTCTTCGCGATCACTCGC
RM 334	146-197	0.83	47.3	GTTCAGTGTTCAGTGCCACC	GACTTTGATCTTTGGTGGACG
RM 335	104-155	0.84	49.2	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG

Electrophoresis of PCR products

Amplified products were separated on 2.5% agarose gel containing 0.5 µg/ml ethidium bromide using 1xTBE buffer as explained by Behera et al. (2012) at 100 V for 1 h. A volume of 10 µl amplified product was mixed with 2 µl loading dye. The gel was visualized under gel documentation (Compact CCD Image System, Major Science, USA).

Salinity treatments for rice cultivars in soil

Salinity tolerance levels for these 12 cultivars were studied by treating the plants with 0 mM salinity (control) and at 100 mM salinity level. Once every two days, 1 L of 100 mM saline solution was supplied to salinity treatment pot and 1 L of distilled water to control pot. Experiments were carried out for three (for *Laila* cultivar only) to seven months (the other 11 cultivars). Three days after the first salinity treatment, the plantlets were subjected to physiological investigations which include photosynthesis efficiency and growth measurements.

Quantum yield measurement

Quantum yield (Fv/Fm) was taken using Fluorpen FP100 (Photon Systems Instruments, Brno, Czech Republic) once a week. Measurement was taken from the same leaf at three different locations (at the leaf base, middle and near the leaf tips). Prior to the quantum yield measurement, the leaf was dark-adapted with aluminium foil for 30 min (Wankhade et al., 2013).

Growth measurements

Towards the end of the project (after 3 months for paddy *Laila* and seven months for the other 11 cultivars), fresh and dry root weight were measured. Root fresh weights were measured immediately after harvesting. Topsoil of the extracted roots were flushed away using tap water and dry weights were taken after plants were dried at 70°C until a constant weight was attained (Zeng et al., 2004). Plant height was measured from the stem base to the tip of the top most leaf (Zeng et al., 2004) at the commencement, mid project time (one and half month or four month) and final project time (three or seven months).

Salinity tolerance scores

The salinity symptoms observed in the cultivars studied were scored according to standard evaluation systems described by Lee et al. (2007) for salinity tolerance.

Data analysis

The amplified band or allele was scored manually as present (1) or absent (0) for each genotype and primer combination. Data was entered in binary matrix using Excel and was used to calculate simple matching similarity coefficient using DARWin 5.0 with 500 bootstraps (Perrier and Jacquemoud-Collet, 2006). Dendrogram was constructed using unweighted pair group with arithmetic mean (UPGMA) following method done by Tabkhkar et al. (2012) to separate the rice cultivars into clusters and from here genetic relatedness among cultivars studied was deduced. The procedure adopted by Nei and Li (1979) was used with FreeTree software (Pavlicek et al., 1999) to calculate the distance matrix. To measure alleleic diversity of SSR markers, PIC was calculated using this formula:

$$PIC=1-\Sigma(Pi\ j)^2$$

Where, Pi j is the frequency of the ith allele in the jth population for each SSR locus (Botstein et al., 1980; Mahajan et al., 2012).

For physiology experiment, Non parametric Kruskal Wallis (if data was not normally distributed) or Two-Way Anova Tukey Test (if data was normally distributed) was used to test the following hypothesis:

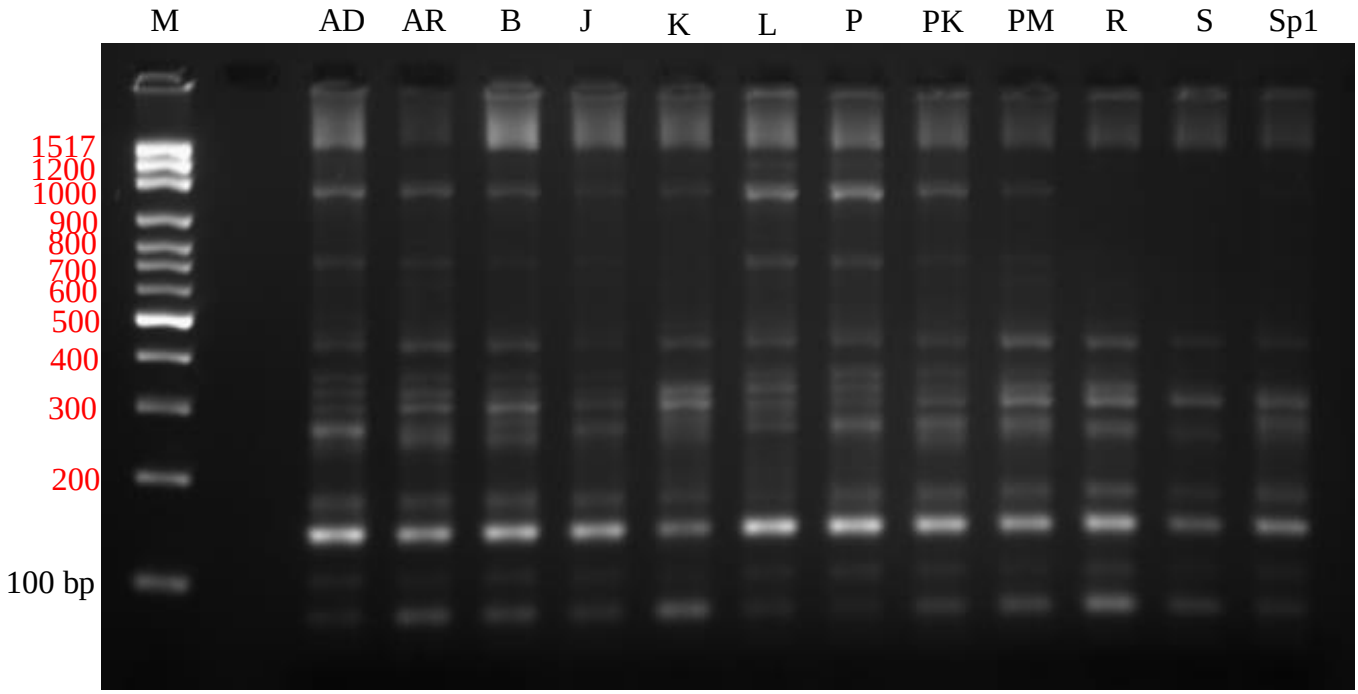

Figure 1. PCR amplification for 12 different rice cultivars (AD=*Adan*, AR=*Arat*, B=*Bandul berminya*k, J=*Jongkok*, K=*Kuaci*, L=*Laila*, P=*Pusu*, PK=*Pulut Keladi*, PM=*Pusu Merah*, R=*Raden Pinang*, S=*Salih* and *Sp1*=unnamed rice cultivar) using SSR marker RM 226 (M=marker (100 bp)).

yield for instance) of the rice cultivar; H_1: there is significant effect of salinity to parameter (quantum yield for instance) of the rice cultivar.

RESULTS

Polymorphism in SSR markers

A total of 158 alleles were amplified for all these rice cultivars using 15 SSR markers with an average of 10.53. Out of 15 markers, 8 were polymorphic while the others were monomorphic. Rice marker (RM) 151, 187, 206, 226, 276, 310, 320 and 334 resulted in polymorphic alleles while the primers, RM 72, 159, 264, 287, 333 and 335, gave monomorphic alleles. The allele frequencies per locus or marker range from 0 in RM 307 to 5 alleles in RM 226. Figure 1 shows amplified bands or alleles for 12 different rice cultivars using SSR markers RM 226. PIC values varied from 0.00 to 0.7521 (RM 226). SSR markers that produce high PIC value with 0.5 and above were obtained from RM 151, 206, 226, 310 and 320.

Genetic diversity and relationship among 12 rice cultivars studied

Both similarity distance and dendrogram were analysed using polymorphic marker RM 226. Similarity distance varied from 0.00 to 1.00 (Table 2). Genotype *Laila* showed highest similarity with *Adan* (1.00). Genotype *Pusu* and *Adan*, *Pulut Keladi* and *Bandul berminyak* and *Pusu Merah* with *Jongkok* also showed the highest similarity. Genotype *Salleh* or *Sp1* and *Kuaci* showed the least similarity (0.00). The dendrogram (Figure 2) showed 3 distinct clusters. Group 1 consists of *Sp1* while Group 2 consists of *Kuaci* and the other ten cultivars are in Group 3. Group 3 was further sub-divided into 5 groups with *Salleh* diverted from the other nine cultivars. Data shown in Figures 2 and 3 show that *Kuaci* and *Sp1* were most genetically different from the other ten rice cultivars.

Salinity tolerance

Figure 4 shows the paddy of *Arat*, *Adan* and *Bandul berminyak* grew upright and showed healthier leaves compared to the rest of the cultivars after being exposed to salinity stress for 3 months. Leaves for some of the cultivars *(Laila* and *Pusu*) died due to the high salinity stress. The other cultivars (*Jongkok, Kuaci, Pulut Keladi, Pusu Merah*, *Raden Pinang*, *Salleh* and *Sp1*) were slightly affected due to salinity stress as some of their leaves showed symptoms of dying. According to results

summarized in Table 3, *Adan* and *Arat* can be classified as moderately tolerant to salinity stress and *Bandul berminyak* was tolerant. Quantum yield of *Bandul*

Table 2. Similarity distance among 12 different rice cultivars cultivated in Brunei Darussalam investigated using RM 226 markers.

	Adan	*Arat*	*Bandul berminyak*	*Jongkok*	*Kuaci*	*Laila*	*Pusu*	*Pulut Keladi*	*Pusu Merah*	*Raden Pinang*	*Salleh*	*Sp1*
Adan												
Arat	0.75											
Bandul berminyak	0.89	0.89										
Jongkok	0.57	0.86	0.75									
Kuaci	0.67	0.67	0.57	0.40								
Laila	1.00	0.75	0.89	0.57	0.67							
Pusu	0.75	1.00	0.89	0.86	0.67	0.75						
Pulut Keladi	0.89	0.89	1.00	0.75	0.57	0.89	0.89					
Pusu Merah	0.57	0.86	0.75	1.00	0.40	0.57	0.86	0.75				
Salleh	0.33	0.67	0.57	0.80	0.00	0.33	0.67	0.57	0.8	0.8		
Sp1	0.67	0.33	0.57	0.40	0.00	0.67	0.33	0.57	0.40	0.40	0.50	

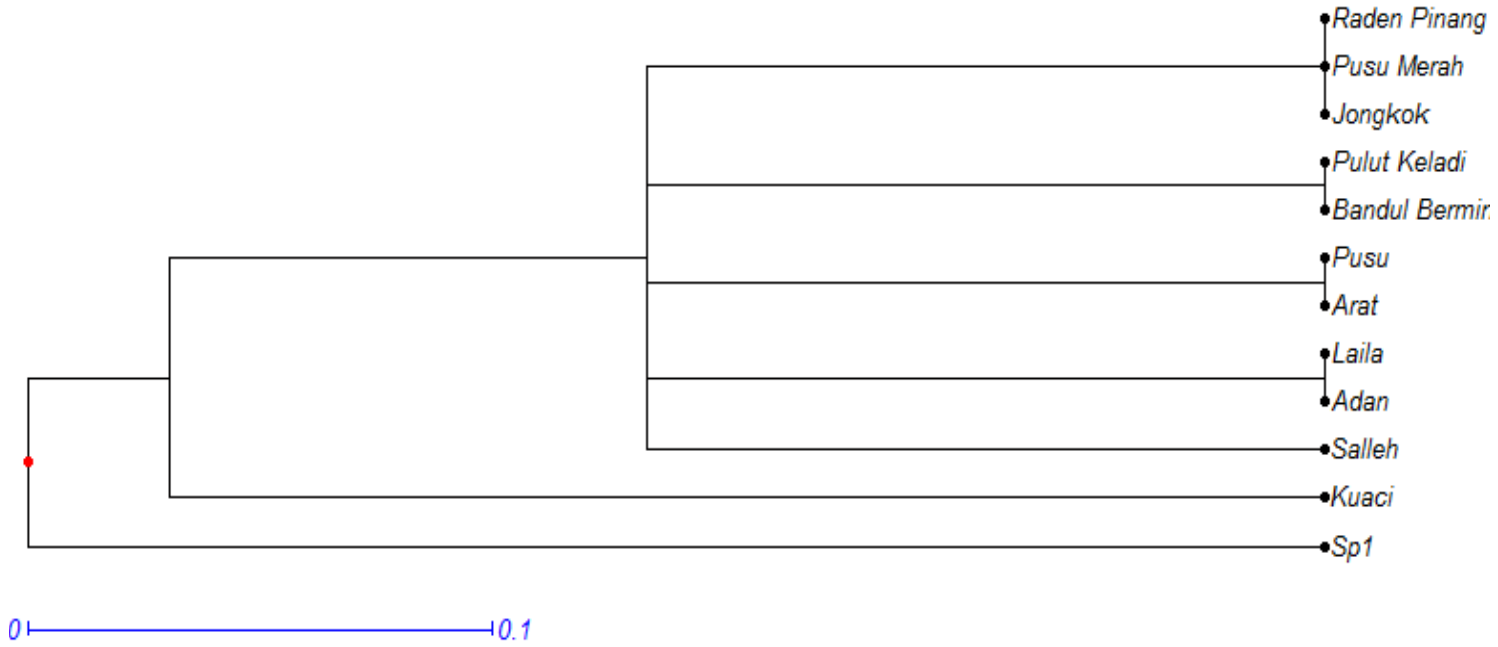

Figure 2. UPGMA based dendrogram for all rice cultivars.

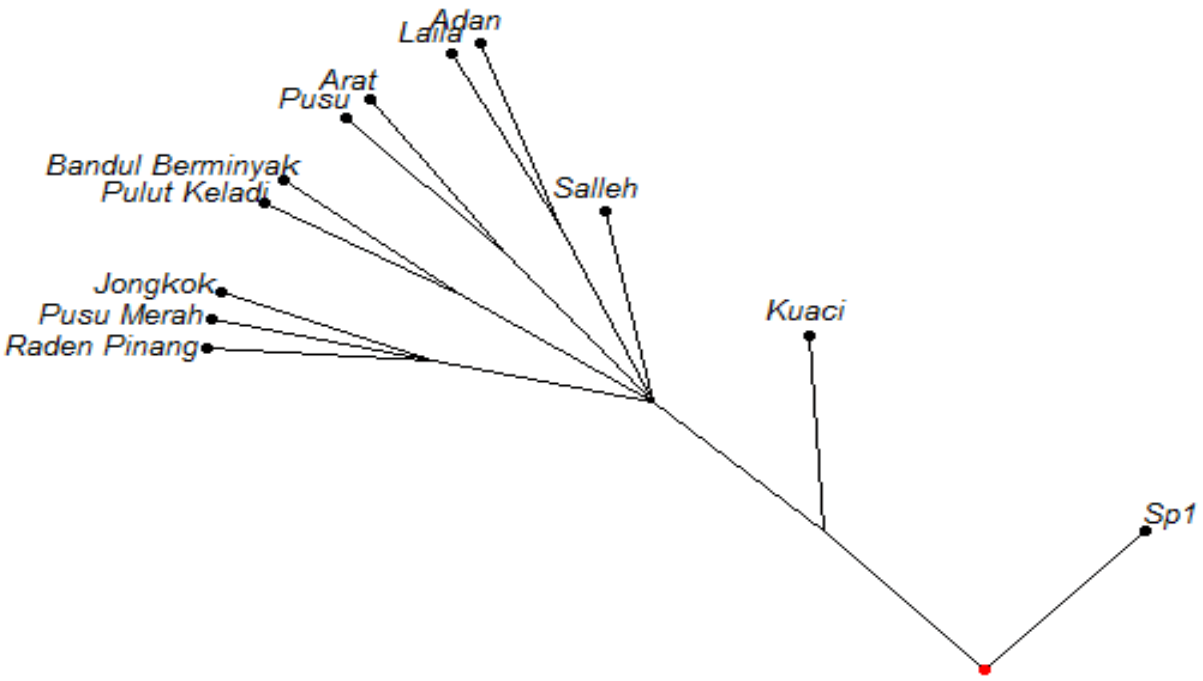

Figure 3. Tree based on neighborhood joining method showing genetic dissimilarity between all 10 rice cultivars investigated.

berminyak in control and exposed to 100 mM salinity level showed no significant different ($p>0.05$). All rice varieties showed reduction in all growth performance when exposed to salt stress, regardless whether they are tolerant or susceptible to salinity stress (Tables 4 and 5). High reduction in plant height occurred in susceptible and highly susceptible varieties except *Pusu Merah* and *Raden Pinang* while *Bandul berminyak* showed the lowest with only 2.98% (Table 4). Only *Bandul berminyak* showed less than 50% reduction in both fresh and dry root weight (Table 5). Moderately tolerant rice cultivars, *Adan* and *Arat*, showed less than 80% reduction when exposed to salinity stress. With the exception of *Raden Pinang*, all susceptible varieties showed more than 80% reduction while highly susceptible varieties showed more than 90% reduction in both fresh and dry root weights.

DISCUSSION

Polymorphism in SSR markers

Our study shows an average number of 10.53 alleles per locus, which was higher than Sarawak bario rice cultivars reported by Wong et al. (2009) and for Iranian and Malaysian rice cultivars reported by Etemad et al. (2012). These reports showed an average of 2.6 and 3.57 alleles per locus, respectively. However, our finding was lower when compared to Bangladesh rice cultivars reported by Rahman et al. (2008) with 15.6 alleles per locus. The mean allele in our study was almost comparable to Rahman et al. (2010) where they detected 11.7 alleles per SSR locus from 28 Bangladesh rice cultivars using seven markers. Etemad et al. (2012) and Shah et al. (2013) reported that the different values of average number of alleles per SSR locus among all these reports could be because of the different genotypes used and the selection of microsatellite markers. SSR markers that produce high PIC value with 0.6 and above were obtained from all loci studied except RM 334, RM 287, RM 307 and RM 151. All these markers gave monomorphic alleles, thus would not help in discriminating the genotypes studied. Wong et al. (2009) and Shah et al. (2013) explained that high PIC value of markers indicated that the number of alleles detected were also high. Their findings are in agreement with this study as the highest PIC value of RM 226 detected 5 numbers of alleles while the lowest PIC value in RM 307 gave only one allele which was monomorphic. High average PIC value of 0.59 obtained in this study might be due to high genetic diversity in all rice cultivars investigated and the fact that the markers used were chosen due to their high PIC value as reported earlier by Temnykh et al. (2000) and Behera et al. (2012). According to Sajib et al. (2012), markers with high PIC value (>0.50) could be used for genetic studies as they are greatly informative and highly polymorphic. These primers would help to differentiate the genotypes studied.

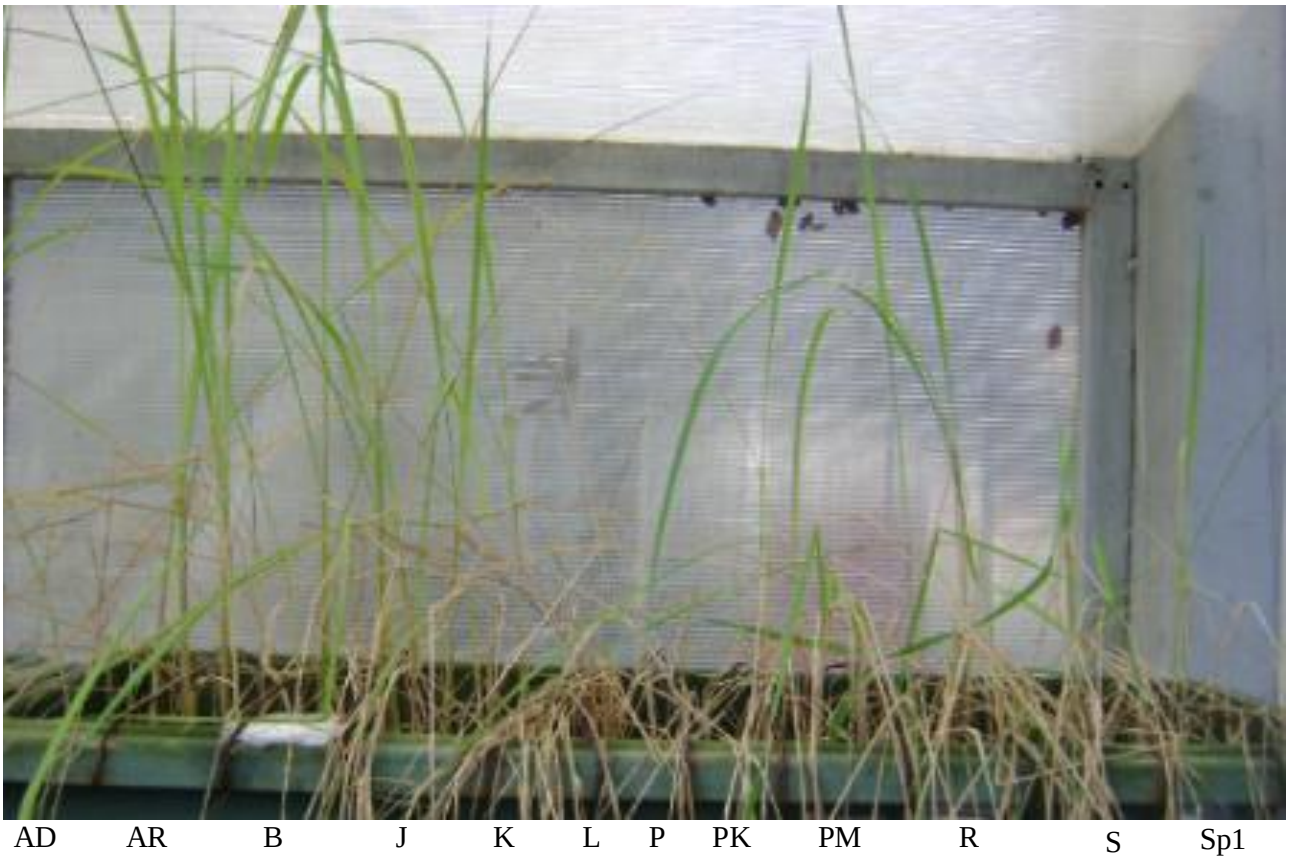

Figure 4. Salinity stress effect at 100 mM on growth of 12 rice cultivars studied.

Table 3. Salinity Tolerance status for all 12 rice cultivars studied.

Cultivar name	Score	Tolerance
Adan	5	Moderately tolerant
Arat	5	Moderately tolerant
Bandul berminyak	3	Tolerant
Jongkok	7	Susceptible
Kuaci	9	Highly susceptible
Laila	9	Highly susceptible
Pusu	9	Highly susceptible
Pulut Keladi	7	Susceptible
Pusu Merah	7	Susceptible
Raden Pinang	7	Susceptible
Salleh	7	Susceptible
Sp1	7	Susceptible

Genetic diversity and relationship among 12 rice cultivars studied

The lowest similarity distance was obtained between *Kuaci* and *Salleh* or *Sp1* while the highest was between *Laila* and *Adan, Pusu* and *Arat, Pulut Keladi* and *Bandul berminyak, Jongkok* and *Pusu Merah*. Cultivars with low genetic similarity as that for *Kuaci* and *Salleh or Sp1* can be chosen and used in breeding program to obtain higher grain quality by intercrossing as suggested by Etemad et al. (2012). Some rice cultivars showed high similarity distance with 1.00, for example, between *Pulut Keladi* and *Bandul berminyak*. According to Wong et al. (2009) such cultivars may probably originate from same source but were given different local names.

Salinity tolerance

High quantum yield for tolerant *Bandul berminyak* compared to other susceptible cultivars was in agreement to result obtained by Cha-um et al. (2007) for two cultivars exposed to 342 mM salinity stress, whereby quantum yield in salt sensitive GS No. 7032 was lower than the salt-tolerant GS No. 4371 when both were exposed to 342 mM salinity stress. This implied that the maximal quantum yield of Photosystem II of this cultivar was not affected by this stress and that this cultivar could be tolerant to salinity. Reduction in growth parameters for all 12 cultivars caused by salinity was in agreement to the

Table 4. Percentage reduction in plant height for 12 cultivars grown in salinity stress.

Rice variety		Reduction in plant height (%)
Tolerant	*Bandul berminyak*	2.98
Moderately Tolerant	*Adan*	9.05
	Arat	8.76
Susceptible	*Jongkok*	14.66
	Pulut Keladi	10.28
	Pusu Merah	8.54
	Raden Pinang	9.04
	Salleh	10.55
	Sp1	19.72
Highly susceptible	*Kuaci*	16.47
	Laila	25.28
	Pusu	22.86

Table 5. Fresh and dry root weight for 12 cultivars grown in 0 and 100 mM salinity levels with percentage reductions.

Rice variety		Reduction in root fresh weight (%)	Reduction in root dry weight (%)
Tolerant	*Bandul berminyak*	25.25	51.13
Moderately tolerant	*Adan*	72.11	74.54
	Arat	73.48	78.83
Susceptible	*Jongkok*	87.65	91.20
	Pulut Keladi	92.43	93.84
	Pusu Merah	90.04	91.04
	Raden Pinang	77.89	67.72
	Salleh	76.74	81.91
	Sp1	90.23	95.41
Highly susceptible	*Kuaci*	95.02	93.35
	Laila	97.71	97.86
	Pusu	93.69	94.77

result reported by Cha-um et al. (2007, 2009). The growth reduction is caused by decreased in photosynthesis due to salt toxicity (Cha-um et al., 2009). Photosynthetic efficiency of salt-sensitive varieties affected more than tolerant varieties. Tolerant *Bandul berminyak* shows similar pattern to salt tolerant rice variety Homjan studied by Cha-um et al. (2009), whereby both showed lower reduction in growth parameters compare to salt-sensitive varieties such as KDML105.

Plant height of all 12 cultivars showed no significant variation due to exposure to salinity stress. However, *Bandul berminyak* showed the lowest reduction in plant height, thus confirming that it is tolerant to salinity stress. Susceptible cultivars in this study showed high reduction in plant height which was similar to susceptible *japonica* variety *Daegudo* and *Guweoldo* reported by Lee et al. (2003). Reduction in plant height of susceptible varieties may have been caused by inhibition of cell expansion in leaf growth zone caused by salinity (Setter et al., 1983; Fraga et al., 2010).

In our study, highly susceptible cultivars showed the highest reduction in both fresh and dry root weights. Our results are in agreement with those reported for *Daegudo* and *Guweoldo* cultivars (Lee et al., 2003). Reduction in root weight may have been caused by the suppression in root growth cause by the production of cytokinins as a result of high saline level (Bottger et al., 1978; Hosseini et al., 2012). From this result, it can be deduced that root weight was the most affected trait by salinity stress. As stated by Lee et al. (2003) and Hosseini et al. (2012), root dry weight is a good parameter to determine salinity tolerance in these 12 cultivars. This is the most obvious

parameter affected by salinity stress, where all cultivars showed more than 50% reduction when exposed to salinity. The grouping shown in cluster and tree diagram analysis (Figure 2 and 3, respectively) does not explicitly reflect salinity tolerance levels. For example, *Pusu* and *Arat* were grouped together when the *Pusu* was regarded as highly susceptible and *Arat* was considered as moderately tolerant. This result was similar to result obtained by Kanawapee et al. (2011) whereby the moderately tolerant cultivar IR64 was grouped together with highly susceptible rice cultivar Khao Kaset and IR34. Zeng et al. (2004) had stated that it was not surprising to observe some sensitive cultivars mixing with tolerance ones in a same cluster. Cluster analysis shown by Kanawapee et al. (2011) produced from RAPD markers did show groupings based on the cultivars salinity tolerance ability. Thus, suggesting RAPD markers are more accurate in addressing cultivars affected by salinity stress. The cluster analysis produced for the 12 rice cultivars studied may have been grouped according to their location and genetic origin (Kanawapee et al., 2011).

Kanawapee et al. (2011) have recommended intercrossing cultivars from different clusters as these cultivars possess different genetic background. Out of 30 different rice cultivars investigated by Kanawapee et al. (2011), they have suggested to intercross KDML 105 with salinity tolerant SPR90 because both possess different genetic background and different physiological tolerance levels to salinity and also have different characteristics and physiology. They reported that the KDML 105-derived progeny would possess improved characteristics than both parents. Thus, SSR markers are the promising marker for rice breeder as they provide faster and precise genetic information of potential parents than the conventional breeding and also helps to produce improved rice cultivar that possess better characteristics than both parents.

Results obtained for physiological responses to salinity tolerance and genetic variation studies conducted for 12 cultivars here implies that it is desirable to intercross *Arat* (moderately tolerant) and *Sp1* (Susceptible) as both of them are from different clusters, show low genetic similarity with 0.33 and possess contrasting salinity tolerance levels. Even though *Kuaci* and *Sp1* or *Salleh* showed the lowest genetic similarity, intercrossing these cultivars may not produce an improved cultivar when compared to *Arat* and *Sp1*.

Further investigations should be carried out to assess the success of interbreeding *Arat* and by comparing the growth performance and yield between their progeny and both parents to confirm that the progeny would show better quality.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of artificially-generated wind on removing guttation and dew droplets from rice leaf surface for controlling rice blast disease

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The incidence and severity of leaf and panicle blasts were efficiently reduced in the wind-treated fields. Artificially-generated wind was performed on rice paddy field in order to measure the changes in temperature and humidity during and after the blowing process and to investigate the effects of sending wind on the formation and removal of guttation and dew droplets on rice leaves. A large electric fan was used to send wind with a depression angle of 30° at 4:00 AM for 30-40 min. During the blowing process, the humidity percentage was 100% in both treated and control paddy fields, the temperature was 0.8°C higher in the treated than the control field and the dew weight was rapidly decreased from 21 to 8 g in 20 min in the treated field. After the blowing process, the temperature started to rise at 6:28 AM (10 min earlier than the control block). It reached 29°C at 6:48 AM (30 min earlier than the control block) and remained higher than the control block till 7:20 AM (1-7°C higher). The humidity decreased to 95% at 6:30 AM (20 min earlier than the control block) and remained 95% till 6:42 AM, then rapidly decreased to reach 68% at 6:46 AM, and finally reached 60% at 7:20 AM. The humidity was 5-30% lower than that in the control block. Moreover, the dew weight increased to the initial level within 10 min and then decreased rapidly in both blocks starting from 6:20 AM. Using wind speed of 4.3 m/s or 3.2 m/s for 60 s per rotation at 4:00 AM, almost all the guttation droplets except those of less than 0.5 mm were removed from the surfaces of the 1st and 2nd uppermost leaves after two rotations (at velocity 4.3 m/s) or three rotations (at velocity 3.2 m/s) and the dew droplets were mostly removed after five rotations (at velocity 4.3 m/s) or nine rotations (at velocity 3.2 m/s). Sending wind at a speed of 3.2 m/s or faster removed guttation and dew droplets and suppressed the subsequent dew formation. Our data indicates that the removal of dew droplets on rice hills resulted in reduction of the disease development of *Pyricularia oryzea*.

Key words: Generated–wind, rice blast, microclimate, guttation, dew droplets.

INTRODUCTION

Rice blast disease, caused by *Pyricularia oryzea,* is one of the major causes of reduction of rice production in the world. The severity of rice blast disease is strongly influenced by the prevailing weather conditions that rain,

wind, temperature and sunlight. Wind is thought to reduce the number of spores attached to rice plant and to promote removal of dew droplets on the leaves. Thus, the penetration of blast into leaves might be prevented or impeded (Adachi, 1981; Hashimoto et al., 1984; Misawa and Matsuyama, 1960; Mousanejad et al., 2009; Ono and Suzuki, 1959; Suzuki, 1969). Taguchi et al. (2014) reported that outbreaks of rice blast were remarkably prevented by sending artificial wind to the paddy fields. In their study, a fan-forced wind was generated between 11:00 PM and 4:00 AM to prevent leaf surfaces from remaining moist longer than 8 h, which is sufficient time for blast fungus infection (Hashimoto et al., 1984; Yoshino, 1979). They also reported that it is necessary to adjust wind velocity to between 3 and 6 m/s to obtain sufficient blast disease control.

It is generally presumed that artificial blowing has preventive effects on rice blast because blowing causes changes in the microclimate over paddy fields, including increased temperature and reduced humidity, promoting removal of guttation/dew droplets formed on rice leaves, or wind compulsorily blows off blast spores attached to rice plants (Adachi, 1981). However, no detailed explanations have been given to how wind changes the microclimate over the paddy field and how guttation/dew droplets disappear from the leaf surface. In the present work, we studied how artificially-generated blowing influences temperature and humidity on paddy field and how it promotes the disappearance of water droplets on the uppermost rice leaves.

MATERIALS AND METHODS

Effect of artificially-generated wind on dew formation on the surface of rice leaves

The experiment was conducted in a mountainous area of Gifu prefecture suffering from serious outbreaks of rice blast for several years. Rice seedlings of “Kinuhikari” cultivar with three leaves (3 weeks after sowing) were used for transplanting. A paddy field (60 m x 30 m) was planted with rice seedlings (30 cm line interval, 18 cm hill interval and 20 plants / m^2). A large electric fan with a rotation angle of 180° (5 KW, blade diameter of 110 cm, wind volume of 4,020 m 3/min) was installed at the ridge of the paddy field (1 m away from the edge of the ridge). The fan was set on the top of 5 m pillar with a depression angle of 30°. By using this fan, rotating wind was sent to rice hills for 30 min starting from July 23 at 4:00 AM (each rotation lasts for 60s with 2 min interval). Sending wind was started from 6 weeks old rice seedlings (3 weeks after transplanting). A dew meter (Hokutow E-T11A, Japan) was installed 60 cm high from the ground level at a particular point in the paddy field where the wind speed was 3.5 m/s to study changes of the guttation/dew formation levels both during and after the blowing operation (Hashimoto et al., 1984). Additionally, the guttation/dew formation status of the control block, adjacent to the west side of the designated block, was studied.

Effect of artificially-generated wind on the temperature and humidity on paddy field

Wind was sent to the paddy field on July 19 and August 10 as described previously. A thermometer and a hygrometer were installed at a particular point where the wind speed was 3.5 m/s (80 cm high from the ground level) to measure changes in temperature and humidity on the paddy during the blowing operation and also during the subsequent time zone between 6:20 and 7:00 AM. The blowing operation lasted for 30 min starting at 4:05 AM on July 19, and for 40 min from 3:50 to 4:30 AM on August 10.

Effect of artificially-generated wind on disappearance of guttation/dew droplets from the leaf surface

For evaluation of guttation/dew droplets formation on rice leaves, the total number and location of guttation droplets of 0.2 mm or larger were recorded. Additionally, the presence or absence of microscopic dew droplets was also recorded. Guttation droplets were classified according to their size to; larger than 1.5 mm, 1.0-1.5 mm, 0.5- 1.0 mm and 0.2 - 0.5 mm. The number in each class was recorded. Since, it was difficult to count the number of dew droplets, they were evaluated collectively according to coverage area of rice leaves to: “++++” for full covering leaf surface area with dew droplets, “+++” for 2/3 of the leaf surface area covered with dew droplets “++” for 1/3 of the leaf covered with dew droplets, “+” for 1/3 or less of the leaf covered with dew droplets, and “_” for no dew droplets. For the control block, a particular position was selected in the identical paddy field to record the same measurements. The formation of guttation and dew droplets was measured at 3:50 am and at 4:50 am.

By using a large electric fan, rotating wind was sent to the paddy field for 30 min starting at 4:00 AM on July 19. Three rice hills in the zone where the wind speed was 4.3 m/s and other 2 rice hills in the zone where the wind speed was 3.2 m/s were selected to measure guttation/dew droplets formation on the unfolded first and second uppermost leaves. The measurements were performed 10 min before and after blowing process, as well as during the process. During the blowing process, the measurements were performed during the intervals of the rotation cycles. The measurements were performed 9 times (once per rotation cycle) and 5 times (every 2 rotation cycles) in the zone of wind speed 4.3 and 3.2 m/s, respectively.

RESULTS

Effect of artificially –generated wind on the dew droplets formation on the rice leaf surface

The values of the guttation/dew droplets were same in the treated and control fields just before starting the blowing operation. During the blowing process, the dew weight rapidly decreased from 21 to 8 g in 20 min. After the blowing process, the values of the dew droplets weight in the treated block were 2 g lower than the values in the control block. The weight values in both blocks decreased rapidly starting from 6:20 AM. The values of the dew droplets in the treated and control blocks were

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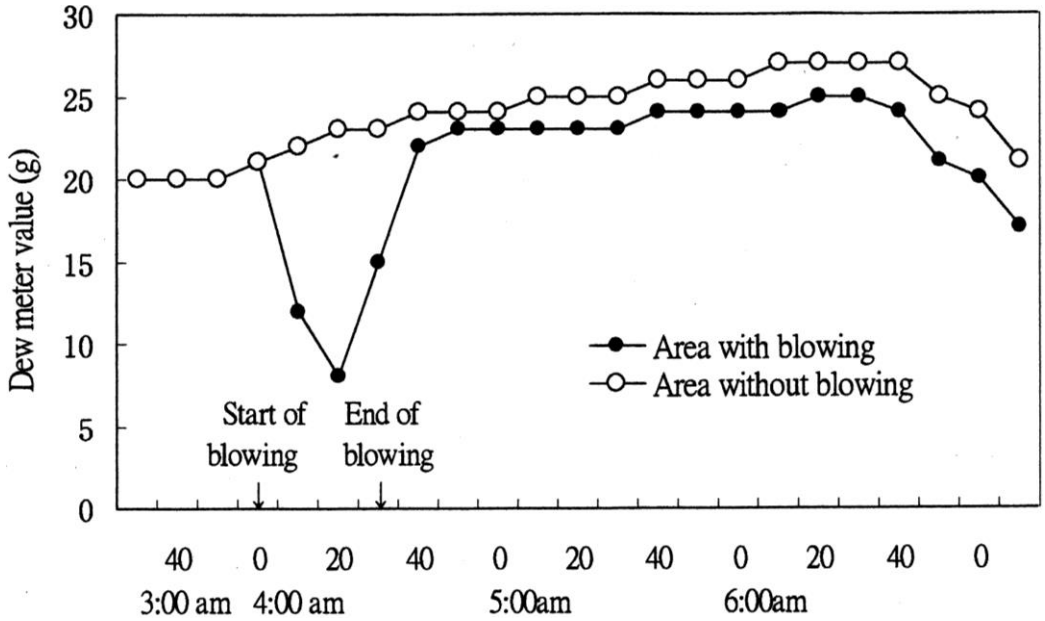

Figure 1. Dew droplets weight on the rice leaf surface in the treated and control blocks.

illustrated (Figure 1).

Effect of artificially-generated wind on the temperature and humidity on paddy field

Early during blowing process

On July 19, the temperature was 17. 2°C at 4:05 AM then increased to 17.7°C and remained at this level during the blowing process for 30 min. In the control block, the temperature was about 0.8°C lower than those in the treated block (Figure 2A). On August 10, the temperature was 19.5°C at 3.50 AM and remained almost at the same level for 40 min during the blowing process (Figure 2B). The temperature was slightly decreased after the blowing process. On the other hand, the temperature in the control block gradually decreased and was 0.9°C lower than treated block at 4:30 AM. The humidity levels in both blocks remained at the level of 100% on both July 19 and August 10 with regardless of blowing process (Figure 2A and 2B).

Later after blowing process from 6:20 to 7:20 AM

On July 19, the temperature and humidity from 6:20 to 7:20 AM were recorded in both treated and control blocks. The temperature in the control block increased gradually from 6:38 AM and reached 30°C at 7:20 AM (Figure 3A). In the treated block, the temperature started to increase at 6:28 AM (10 min earlier than the control block), and reached 29°C at 6:48 AM (30 min earlier than the control block). The temperature remained higher than the control block till 7:10 AM (1-7°C) (Figure 3A). In control block, the humidity percentage decreased gradually from 6:52 AM and reached 60% at 7:20 AM (Figure 3B). In the treated block, the humidity decreased to 95% at 6:30 AM (20 min earlier than the control block) and remained 95% till 6:42 AM, then rapidly decreased to reach 68% at 6:46 AM, which maintained for a while, and finally reached 60% at 7:20 AM. The humidity percentages in the treated block were lower than that in the control block (ranged 5- 30 %) (Figure 3B).

Although, an increase in temperature and decrease in humidity percentage were also observed in the treated block compared with the control after the blowing process on August 10, both temperature increased and humidity reduction started later after those occurred on July 19 (Figure 4). They tended to be similar changes and were consistently observed on both dates.

Artificially-generated wind effectively promoted the removal of guttation/dew droplets on the leaf surface

On July 19, the disappearance of guttation/dew droplets which formed on the first and second uppermost rice leaves was measured when the wind speed was 4.3 and 3.2 m/s in the treated block.

Using wind speed of 4.3 m/s

Just before blowing, 7 (larger than 1.5 mm in diameter), 22 (ranged from 1.0 to 1.5 mm) and many (of 0.5 -1.0

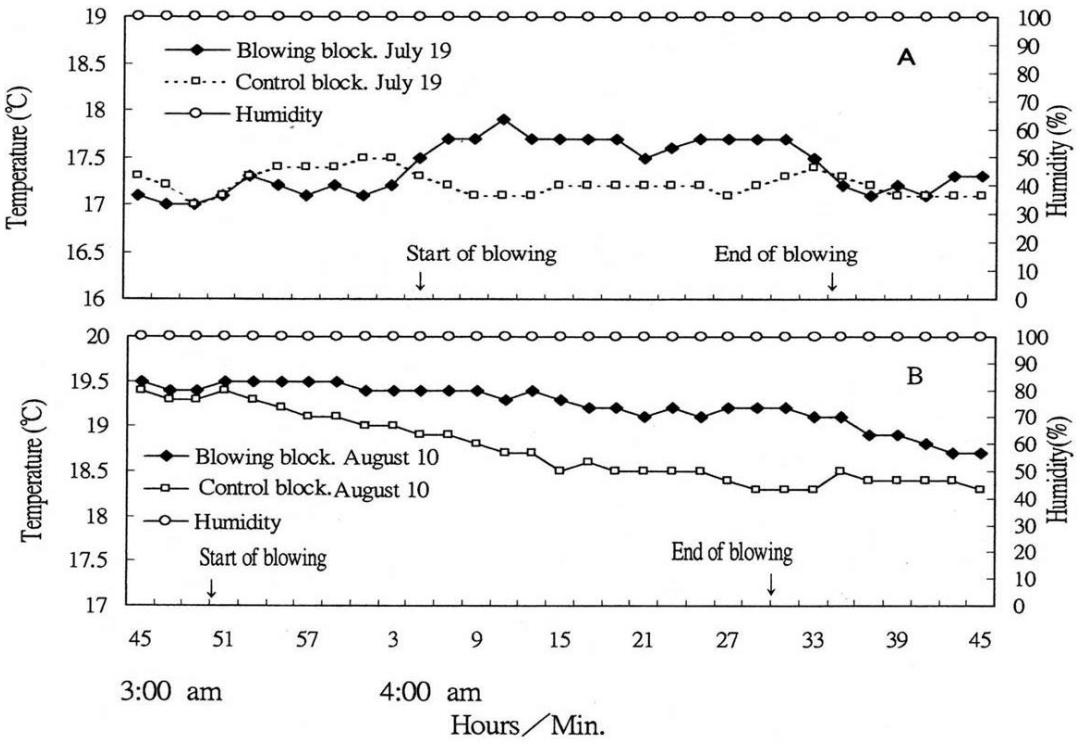

Figure 2. Temperature and humidity values in both treated and control blocks during the blowing process on July 19 (A) and August 10 (B).

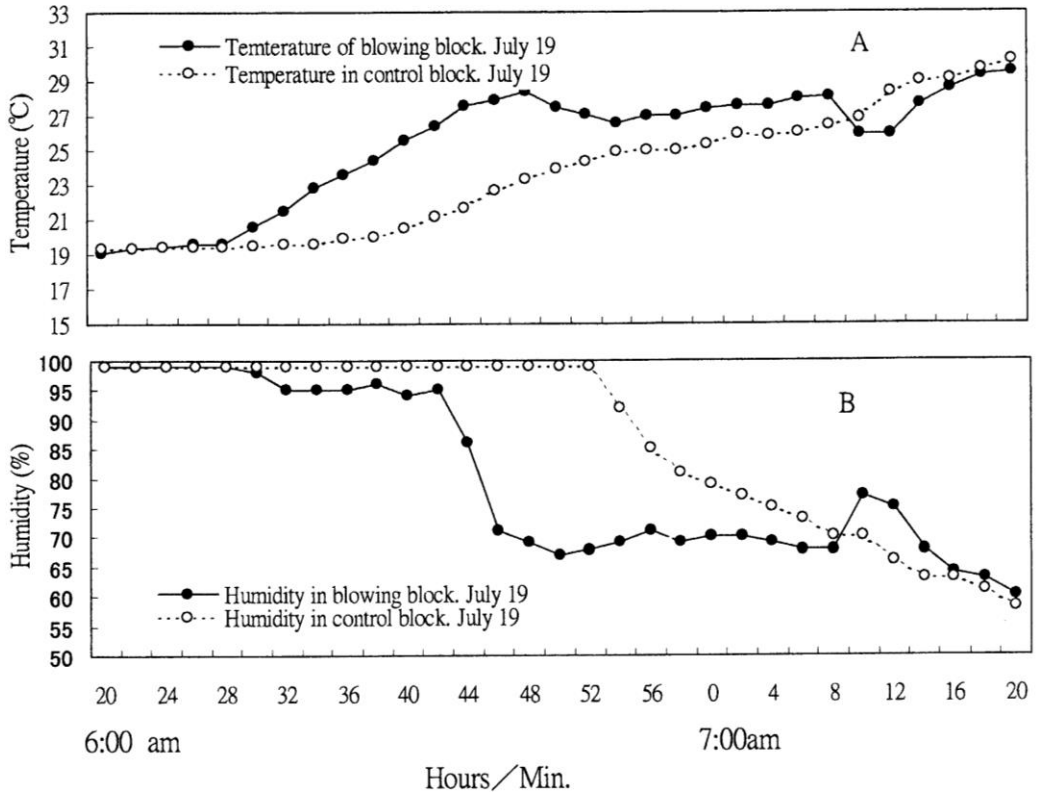

Figure 3. Temperature (A) and humidity (B) values in both treated and control blocks after the blowing process on July 19.

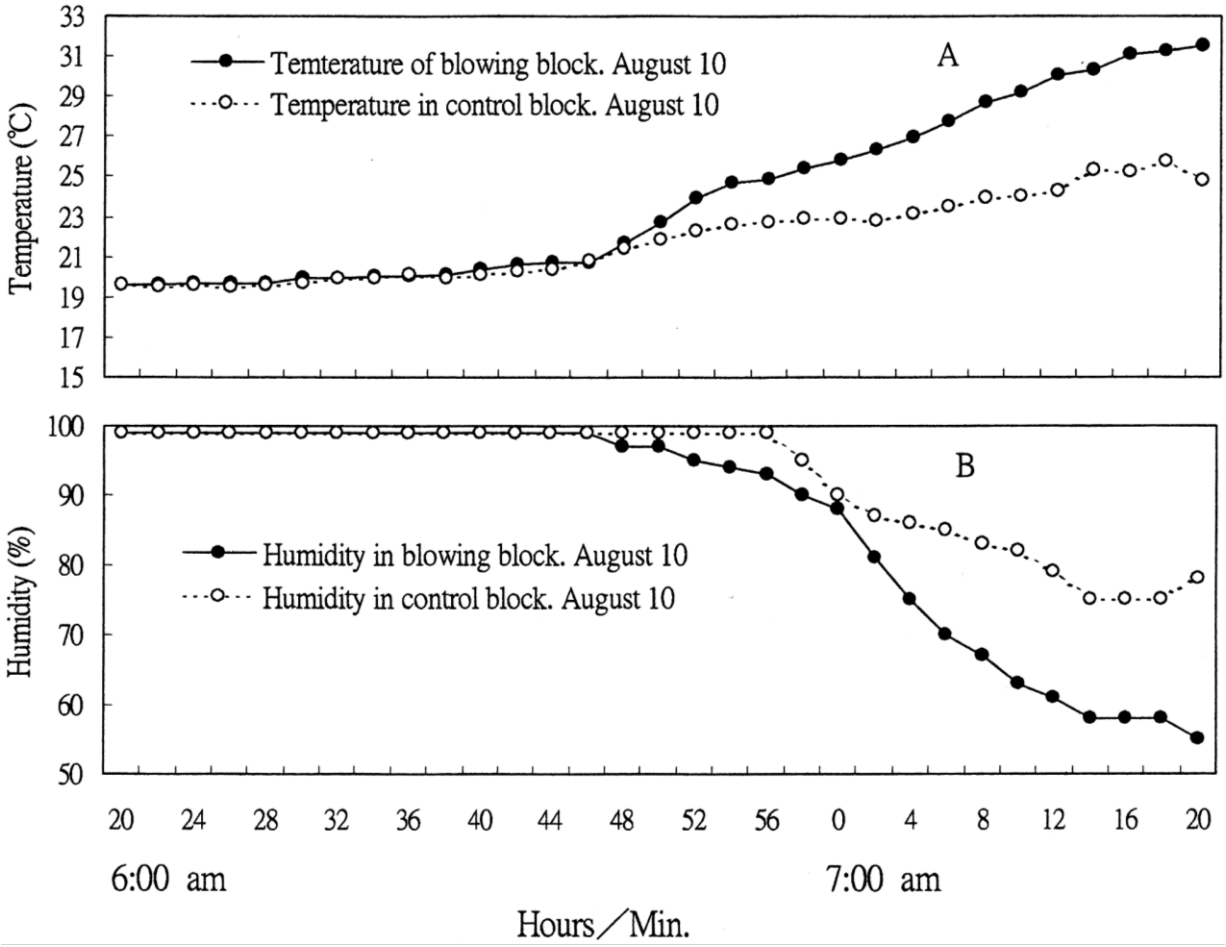

Figure 4. Temperature (A) and humidity (B) values in both treated and control blocks after the blowing process on August 10.

and 0.2 -0.5 mm in diameters) guttation droplets were observed on the first uppermost leaves as well as microscopic dew droplets that covered the entire leaf surface giving it white color. The first rotation of fan at wind speed of 4.3 m/s removed 1.5 mm or larger guttation droplets and those of 0.5-1.0 mm, leaving a small number of 1.0 -1.5 mm-sized droplets and many of those of 0.2-0.5 mm in diameter on leaf surfaces, as well as the majority of the dew droplets. The second rotation removed all guttation droplets of 0.5 mm or larger, but 12.3 of 0.2-0.5 mm-sized droplets were accumulated on the leaf edges immediately after ending of the second rotation. The dew droplets were reduced to 2/3 or less of the entire leaf surface. After the third rotation, the removal of re-accumulated guttation droplets and reduction of dew droplets were gradually succeeded. The process repeated due to consequently re-accumulation of guttation droplets on leaf edges again but no remarkable reduction was observed after the seventh rotation. Ten min after stopping the blowing, re-formed guttation droplets grew larger (1.3 droplets of 1.5 mm or larger, 15.0 droplets of 1.0 -1.5 mm). Many of re-formed guttation droplets appeared at the same locations on the leaf surface as those observed before the blowing process. However, most of the dew droplets were removed. The distribution of guttation and dew droplets before and after fan rotations is illustrated in Table 1.

Just before blowing, 7.0 (larger than 1.5 mm), 18.5 (ranged from 1.0 mm to 1.5 mm) and many guttation droplets (0.5-1.0 and 0.2-0.5 mm) as well as microscopic dew droplets covered the entire surfaces of the second uppermost leaves. The first fan rotation at wind speed of 4.3 m/s removed the majority of guttation droplets (1.5 mm or larger) and those of 1.0 -1.5 mm. However, the majority of the guttation droplets of 0.5 -1.0 mm as well as those of 0.2-0.5 mm remained on the leaf surface. Moreover, most of the dew droplets remained unremoved. The second rotation removed all of the guttation droplets of 0.5 mm or larger. But 11.0 guttation droplets of 0.2 -0.5 mm were re-formed on the leaf and dew droplets covered over 2/3 of the leaf surface. After the third rotation, removal of the re-formed guttation and decrease of dew

Table 1. Relation between appearance /disappearance of guttation /dew on unfolded uppermost leaves [a] of rice hill and blowing wind at velocity of 4.3 m/s.

Leaf position	Guttation/dew droplet diameter and classes	Number of droplets before blowing (per leaf)	Number of blowing rotations[b]									10 min after blowing
			1	2	3	4	5	6	7	8	9	
1st uppermost leaf [a]	Over 1.5 mm	7.0	0	0	0	0	0	0	0	0	0	1.3
	1.0-1.5 mm	22.0	0.3	0	0	0	0	0	0	0	0	15.0
	0.5-1.0 mm	>30[c]	0	0	0	0	0	0	0	0	0	0
	0.2-0.5 mm	>30[c]	>30	12.3	9.3	16.0	9.3	17.3	15.3	5.3	5.3	0
	Dew droplet groups[d]	++++	+++	++	++	+	+	+	+	+	+	-
2nd uppermost leaf [a]	Over 1.5 mm	7.0	0	0	0	0	0	0	0	0	0	2.0
	1.0-1.5 mm	18.5	0	0	0	0	0	0	0	0	0	16.0
	0.5-1.0 mm	>30[c]	>30	0	0	0	0	0	0	0	0	0
	0.2-0.5 mm	>30[c]	>30	11.0	3.0	3.5	8.0	17.0	19.5	9.5	0	0
	Dew droplet groups[d]	++++	++++	+++	+++	++	+	+	+	-	-	-

[a]The average leaf length of 1st uppermost leaves was 36.0 cm, and that of 2nd uppermost leaves was 38.5 cm. [b] The number of exposure processes of rice hills to the rotation wind. The blowing time in a single rotation was 60s . [c]The symbol">30" means that over 30 guttation droplets were formed on a single leaf. [d]The dew group means a colony of dew droplets . A different symbol is given to each group according to degree of the coverage over the leaf. They are "++++" for full coverage; "+++" for 2/3 coverage; "++" for 1/3 to 2/3 coverage; "+" for smaller than 1/3; and "_" for no coverage.

droplets were repeated. At the ninth rotation of blowing, no formation of guttation droplets was observed. Ten min after the blowing, the guttation droplets accumulated forming larger droplets. 2 droplets of 1.5 mm or larger and 16 of 1.0-1.5 mm were observed (Table 1).

Using wind speed of 3.2 m/s

Rice hills were examined 5 times (once every 2 rotations) using wind speed of 3.2 m/s for the disappearance of guttation/dew droplets. Just before blowing, 2.0 (of 1.5 mm or larger), 9.0 (of 1.0 -1.5 mm) and many of guttation droplets (of 0.5-1.0 mm and of 0.2-0.5 mm) were observed on the surfaces of the first uppermost leaves. After the first rotation using wind speed of 3.2 m/s, the number of guttation droplets of 1.5 or larger decreased to 0.5 and those of 0.5-1.0 mm decreased to 3.5; but the majority of the guttation droplets of 1.0-1.5 mm and those of 0.2-0.5 mm as well as most of the dew droplets remained on the leaf surface. After the third rotation, the remaining guttation droplets of 1.5 mm decreased to 0.5 and those of 0.5-1.5 mm were completely removed. Immediately after third rotation of blowing, 5.0 guttation droplets of 0.2-0.5 mm were re-formed on the leaf margins and the number of dew droplets decreased slightly. After the fifth rotation, removal of the re-formed guttation droplets and decrease of dew droplets were repeated. After the ninth rotation, a fewer dew droplets were observed. Ten minutes after the blowing, larger guttation droplets were re-formed (6.5 of 1.0-1.5 mm and 3.5 of 0.5-1.0 mm). A few dew droplets remained around the bottom of the leaves (Table 2).

Just before blowing, 7 (of 1.5 mm or larger), 18.5 (of 1.0 -1.5 mm) and many guttation droplets (0.5 -1.0 mm and 0.2-0.5 mm) as well as microscopic dew droplets covered the entire surface of the second uppermost leaves leaving them white in color. After the first rotation using wind speed of 3.2 m/s, all of the guttation droplets of 0.5 mm or larger were removed, but many of 0.2 -0.5 mm were remained on the leaf surface. Similarly, the majority of the dew droplets covered most of the leaf surface. After the third rotation, all guttation droplets of 0.5 mm or larger were removed, but 11 guttation droplets of 0.2-0.5 mm were re-formed on the margins of the leaf surface. Dew droplets covered almost 2/3 of the leaf surface. After the fifth rotation onward, removal of the re-formed guttation droplets and decrease of dew droplets were repeated. After the ninth rotation, dew droplets were reduced to 1/3 or less of the leaf surface. Ten minutes later after stopping the fan, larger guttation droplets were observed (8.5 guttation droplets of 1.0-1.5 mm and 2.5 guttation droplets of 0.5-1.0 mm) on leaf surface. Few dew droplets were observed on the bottom of second upper most leaves (Table 2).

DISCUSSION

Rice blast severity is affected by weather factors such as daily relative humidity and temperature, amount of precipitation and dew, speed direction of wind and condi-

Table 2. Relation between appearance/disapperance of guttation/dew on unfolded uppermost leaves [a] of rice hill and blowing wind at velocity of 3.2m/s.

Leaf position	Guttation/dew droplet diameter and classes	Number of droplets before blowing (per leaf)	Number of blowing rotations[b]					10 min after blowing
			1	3	5	7	9	
1st uppermost leaf [a]	Over 1.5 mm	2.0	0.5	0.5	0	0	0	0
	1.0-1.5 mm	9.0	3.5	0	0	0	0	6.5
	0.5-1.0 mm	>30[c]	>30	0	0	0	0	3.5
	0.2-0.5 mm	>30[c]	>30	0.5	3.5	0.5	5.5	0
	Dew droplet groups[d]	++++	+++	+++	+++	++	+	+
2nd uppermost leaf [a]	Over 1.5 mm	7.0	0	0	0	0	0	0
	1.0-1.5 mm	18.5	0	0	0	0	0	8.5
	0.5-1.0 mm	>30[c]	>30	0	0	0	0	2.5
	0.2-0.5 mm	>30[c]	>30	11.0	3.0	3.5	8.0	0
	Dew droplet groups[d]	++++	++++	+++	+++	++	+	+

[a]The average leaf length of 1st uppermost leaves was 37.0 cm, and that of 2nd uppermost leaves was 31.5 cm. [b] The number of exposure processes of rice hills to the rotation wind. [c]The symbol">30" means that over 30 guttation droplets were formed on a single leaf. [d] The dew group means are a colony of dew droplets . A different symbol is given to each group according to degree of the coverage over the leaf. They are "++++" for full coverage; "+++" for 2/3 coverage; "++" for 1/3 to 2/3 coverage; "+" for smaller than 1/3; and "_" for no coverage.

tion of sunshine (Adachi, 1981; Leach, 1980; Taguchi et al., 2014). Water droplets on the leaf surface are one of the important factors that are implicated in outbreaks of many fungal diseases (Burrage, 1971; El Refaei, 1977; Long, 1955; Long, 1958; Monteith and Butler, 1979; Wallin, 1963). The dew formation on the leaf surface is one of the main factors which are responsible for outbreak of rice blast (Burrage, 1971; Long, 1955; Long, 1958; Monteith and Butler, 1979; Wallin, 1963). Persistence of guttation/dew droplets for a while on the leaf surface is a requisite for contagions with rice blast (Hashimoto et al., 1984; Hemmi and Abe, 1931; Kim et al., 1975; Yoshino, 1979). In our previous study (Taguchi et al., 2014), outbreaks of rice blast (both leaf and panicle blasts) were remarkably prevented by sending artificial wind which was generated 2 times daily for 30 min each starting at 11:00 PM and 4:00 AM, respectively, to the paddy fields to prevent leaf surfaces from remaining moist longer than 8 h, which is sufficient time for blast fungus infection (Hashimoto et al., 1984; Yoshino, 1979). Our results also show that it is necessary to adjust wind velocity to between 3 and 6 m/s to obtain sufficient blast disease control. Such wind treatment was more effective than the application of chemical fungicides in controlling the outbreaks of leaf and panicle blast.

The present study investigated the influence of artificially-generated wind on removal of dew/guttation droplets from rice leaf surface based on visual observation and measurement of transitional values by dew meters and how that would effectively prevent or interfere with rice blast infection (Hashimoto et al., 1984). The correlation between guttation/dew formation and weather conditions in the natural environment have been previously reported (Arai, 1951; Crowe et al., 1978; Hsu et al., 1980; Lioyd, 1961; Lomas, 1965; Newton and Riley, 1964; Picking and Jiusto, 1978). Likely, the formation of guttation droplets is gradually increased on the tip or margins of the leaf surface until 2:00 or 3:00 AM (Barksdale and Asai, 1961). In the present study, if the wind is artificially sent at a speed of 3.2 m/s at 4:00 AM, guttation droplets on the uppermost leaves are easily dropped off after 2 or 3 rotations of fan. The small guttation droplets were re-formed again on the leaf margins during the rotation intervals. These facts suggested that such artificially-blown wind may greatly contribute to the removal of the guttation droplets.

Dew droplets form as a water film with a high surface tension on leaf surface (Beysens, 1995), which is suitable for the germination of various species of fungi (Suzuki, 1973; Wallin, 1967). In windless days, formation of dew droplets reaches the peak level between about 4:00 and 6:00 AM (Barksdale and Asai, 1961; Hashimoto et al., 1984). If the wind velocity goes beyond a particular level in the natural environment, further dew formation is prevented and the formed dew evaporates (Hashimoto et al., 1984; Hsu et al., 1980; Suzuki, 1969). In the present experiment, several rotations of artificial-generated wind at speed of 3.2 m/s or 4.3 m/s removed most of the dew droplets on the leaves, but the dew droplets re-formed again after stopping the fan. Thus, the artificial blown wind can remove the formed dew droplets on leaves, but it cannot prevent their formation because dew formation is a dynamic process in the natural environment. The humidity on the paddy was 100% in both control and treated blocks and the temperature was 0.8°C higher in the treated block than the control. Therefore, it can be presumed that the disappearance of dew/guttation droplets was due to physical removal by the artificially-

generated wind.

The disappearance time of dew begins by drying the dew rapidly to the degree that 10 g or greater volume of dew disappears in 3 h, on the condition that the weight of dew in filter paper (29 × 80 cm) increased once and was then maintained for 3 h (Hashimoto et al., 1984). They also considered that when there is a rapid decrease in dew weight due to an intermission of rainfall, etc., the dew can be regarded as being continuously maintained if there is further rainfall within 3 h. The disappearance of dew on the leaf surface due to artificial wind can be considered as an intermission of dew formation because the disappearance occurs momentary - few minutes. Furthermore, the amount of dew formed after stopping the fan is smaller than those observed in the control block. Therefore, the increase in temperature and reduction in humidity were effectively promoted after sunshine (sunrise) on the paddy field and the drying process of the rice hills gradually progressed.

Sending wind to rice nurseries for consecutive 12 h from the evening to the following morning for 15 days prevented the formation of guttation/dew droplets on the leaf surface as well as germination and penetration of conidiospores, resulting in suppression of outbreak of leaf blast (Adachi, 1981). In addition to the disappearance of guttation/dew droplets in early morning, humidity reduction and rapid increase of temperature early during sunrise could also be considered as the factors that prevent or interfere with penetration of blast fungi.

Artificially-generated wind at speed of 3.2 m/s or 4.3 m/s effectively removed the guttation/dew droplets formed on leaves. The infection rate with rice blast is correlated with the exposure time to guttation/dew droplets, thus removal of guttation/dew droplets by blowing can surely suppresses infection of blast fungi.

It can also be considered that artificial-generated wind is related to disappearance of guttation/dew droplets. Fan-generated wind speed of 3.2 m/s or 4.3 m/s at angle of 30° with a total of 9 rotations (1 min each and 2 min intervals) resulted in removing the majority of the guttation/dew droplets although a slight amount of dew remained at the bottom of leaves. On the other hand, although the guttation droplets disappeared in 20 s when wind at a speed of 3 m/s was blown in a single direction, dew droplets could not be removed even when the wind was blown continuously for 25 min (data not shown). These facts suggest that it is effective to intermittently generate wind by a large electric fan from a higher position. It can be presumed that this is because such wind can blow into the rice hills, shaking the entire rice hills. Also, rotating wind can produce frequent changes in wind speed, shaking leaves irregularly; causing the leaves to touch each other's resulting in the promoted disappearance of the guttation droplets as well as the re-formation of guttation droplets from the leaf surface. Additionally, when guttation droplets scatter, they contact the water film of dew on the leaf surface, resulting in promoted disappearance of dew. Hashimoto et al. (1984) suggested that dew flow on the leaf surface was partly contributed to washing conidiophores off of leaf surface. Although wind at velocity of 2 m/s could hardly blow spores off the lesions, wind at velocity of 4 and 5 m/s could remove them more effectively (Misawa and Matsuyama, 1960; Suzuki, 1969). This effect became more obvious in high humid conditions (Barksdale and Asai, 1961; Leach, 1980; Ono and Suzuki, 1959). These facts suggest that artificial sending wind influences the formation of conidiospores attached to the leaf surface. Thus, further study is necessary to investigate how artificial wind can influence germination of conidiospores as well as formation of appressoria and how blast fungus spores could be washed down by the flow of dew (Schrodter, 1960; Suzuki, 1973). Effect of weather on spore germination of rice blast has been studied by Mousanejad et al. (2009). They showed that the spore germination of rice blast was increased when relative humidity was increased and maximum temperature and sunny hours were decreased.

Conflict of interests

The authors did not declare any conflict of interest.

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Chemical composition and antifungal activity of essential oils of Algerian citrus

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The aim of this study was to determine the chemical composition of the essential oils of Algerian citrus. They were extracted by hydrodistillation from the leaves of citrus species (orange, Bigaradier, mandarin and lemon), using gas chromatography/mass spectrometry (GC/MS). Their chemical composition and antifungal activity against four phytopathogenic fungi (*Fusarium oxysporum f.sp., albedinis sp, Penicelium* sp.*, Alternaria* sp. and *Fusarium* sp.) were studied. The inhibiting minimal concentration (CMI) effect was also given for four oils. Ten compounds were recorded jointly among the 51 identified, of which limonene (7.18 to 36.10%), β-pinene (4.35 to 30.0%) and linalool (0.21 to 63.03%) represent the principal major compounds. These results indicate that essential oils can be employed as natural fungicides against phytopathogenic fungi.

Key words: GC/MS, essential oils , citrus, antifungal activity, phytopathogenic fungi.

INTRODUCTION

Fungal diseases are considered as the main enemies of crops. Apart from the fact that they have the potential to cause significant yield losses and deterioration of agricultural products, many of them cause a very serious risk to consumers because they produce dangerous toxins. Control methods applied involved mainly the application of chemical fungicides. In recent years, the polemic against the use of harmful chemicals on people' health and the environment has generated a debate on being exposed to the risk of having cancer and residual toxicity (Yaouba et al., 2010).

The increasing consciousness of consumers on the relationship between mode and health revolutionizes food, to justify the search for new strategies and to explore other surer solutions for replacement and biodegradable industry (Combrinck et al., 2010). For the elimination of pathogenic micro-organisms in the past years, researchers were interested in composing biologically active isolates, starting from plants with the objective of providing solutions to several challenges facing producers and agricultural distributors (Combrinck et al., 2010).

Essential oils are of a great interest because of their broad acceptance by consumers and the exploitation of their potential multipurpose utilities (Sawamura, 2000). Several researches evoked the antifungal activity of

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essential oils extracted from citrus (Mishra and Dubey, 1994; Sherma and Tripathi, 2006; Espina et al., 2011). The studies reveal that essential oils and their components have a very significant antimicrobial potential.

The studies were devoted to the determination of the chemical composition of the species of Algerian citrus (Baaliouamer, 1987). The antifungal study of the capacity of these essential oils constitutes a very promising alternative *vis-a-vis* the problems connected to the environment and the health of the consumers caused by the use of synthetic fungicides. Consequently, the objectives of this study were to determine (A) the chemical composition of essential oils of orange *(Citrus sinensis* (l) Osbeck), Bigaradier *(Citrus aurantium*), lemon *(Citrus limonum*) and mandarin *(Citrus reticulata*) by GC/SM, in order to identify the principal molecules responsible for inhibition, (b) the capacity of the antifungal activity of essential oils of the citrus against *Alternaria* sp. *(*A. sp*), Fusarium oxysporum f.* sp*. albedinis (*F. Ox*), Fusarium* sp*.* (F. sp) and *Penicelium* sp*.* (P.sp).

MATERIALS AND METHODS

Samples

Essential oils were extracted from the fresh leaves of orange, mandarin, Bigaradier and lemon from the Citrus Orchards of Chlef, Algeria in March - November 2011.

Standard cultures

Essential oils were individually examined in respect to four phytopathogenic fungi. The strain, *F. Ox was* provided by the Laboratory of the Pathology of Plants, the Institute of Agronomy, University of Chlef. The other three fungal strains, *A.* sp*., F.* sp. and *P.sp*, were from the Laboratory of Mycology of INPV Chlef. All the stocks were purified and maintained on potato dextrose agar (PDA) medium at 22 ± 2°C.

Essential oil extraction

Essential oils are obtained from the fresh leaves of orange, Bigaradier, lemon and mandarin by hydrodistillation for 3 h, using a Clevenger type apparatus. The Eos obtained are dried over anhydrous Na_2SO_4 and stored in sealed vials at 4°C until analysis

GC/MS analysis

GC/MS analysis was performed on a Hewlett Packard 6890 Series II capillary gas chromatography directly coupled to the mass spectrometer system (Agilant of the type 5973). HP-5MS non polar fused silica capillary column (50 m x 0.32 mm; 1.25 µm film thickness) was used under the following conditions: oven temperature program was from 60°C (2 min) to 260°C at 5°C/min and the final temperature was kept for 10 min; injector temperature, 250°C; carrier gas He, flow rate, 1 ml/min; the volume of injected sample was 0.1 µl in splitess mode during 0.1 min ionization energy 70 eV, in the electronic ionization (EI) mode; ion source temperature, 230°C; scan mass range of m/z 40 to 650 and interface line temperature, 280°C. The constituents of essential oils were identified based on their Kovats Index; they were calculated in relation to the retention time of a series of alkanes (C7 to C29) used as reference products, compared to those gathered by Adams (2001). The similarity of their mass spectra with those gathered in the NIST-MS library or reported in the literature was compared.

Antifungal activity

Analyzing mycelia growth

The inhibiting capacity of essential oils was examined. F. Ox, *P sp, A. sp* and *F. sp were* screwed using food poisoning technique (Grover and Moore, 1962). Necessary quantities of essential oils are separately dissolved in 0.5 ml of 5% (v/v). Tween-80 was added to various sterilized Petri dishes (9 X 1.5 cm) containing 9.5 ml of PDA medium in order to provide the concentration required from 0.001 to 1 mg/ml. The controls (without essential oil) were inoculated while following the same process. A mycelia disc (diameter of 5 mm) of pathogenic fungi, taken as periphery of the culture seven days backward, was inoculated aseptically in the center of the sets of Petri dish containing the treatments and control. Limp Petri was incubated with 22 ± 2°C during seven days. For each treatment, three repetitions were carried out. The diameter of the fungi colonies of the sets of treatment and diameter of positive control were measured. Mycelia inhibition of percentage was calculated by the average value of the diameters of colony by the following formula (Pandey et al., 1982).

Percentage of mycelia growth inhibition:

$$PI.\ (\%) = \left(\frac{dt-dT}{dt}\right) x100$$

Where, dt is the average diameter of the fungi colonies in the control and dT and average diameter of the treated fungi colonies.

Determination of dry weight

To determine the effect of essential oils on the dry weight of tested fungi in liquid medium, various concentrations of essential oils are prepared in a liquid medium of potato dextrose (PDB). This was done in test tubes (15 ml) and inoculated spores (10^7 spores/ml) of the fungi quads. Positive species tested in controls (without essential oils) are prepared at the same time. After a duration of the 15 days incubation at 22 ± 2°C, the mycelium is filtered through a Wattman filter paper No. 1, washed with distilled water and dried at 60°C for 6 h; and then at 40°C for a whole night. The filter paper containing the dry mycelium was weighed. The tests are repeated three times. Inhibition is calculated starting from the dry weight of the mycelium, by taking the control positive for the100% growth (Sharma and Tripathi, 2008).

Determination of the inhibiting minimal concentration (CMI)

The CMI of essential oils necessary for inhibition of the mycelia growth of tested fungi was determined by food poisoning technique (Grover and Moore, 1962). The inhibiting concentration minimal (CMI) of essential oils against the phytopathogenic fungi A was determined and the oil showed an absolute fungitoxicity. Various concentrations of the oil from 0.001 to 1 mg/ml as well as the control (without essential oils) were prepared by separately dissolving its necessary quantity in 0.5 ml 5% (v/v) tween-80 and then mixing it with 9.5 ml of medium PDA. Limp Petri inoculated was incubated during seven days at 22 ± 2°C. The weakest concentrations without observable growth (with the binocular magnifying glass) were defined like the inhibiting minimal concentration (CMI). For each concentration, three tests were carried out. The nature of toxicity (fungistatique/fungicide) A was given according to the study of Thomson (1989).

Production and germination of spores

The colonies of spore previously exposed to the oil (until the spores formed) of *F. ox, P.* sp.*, A.* sp. and *F.* sp. were gathered by adding 5 ml of 0.1 ml/100 ml containing sterile distilled water and Tween-80 in each Petri dish. The suspension of spore was gathered and then centrifuged. A hemocytometer was used to count the number of spores. The percentage of inhibition of the production of spore (PIs%) was calculated relative to the control. The fungi spores are obtained starting from 10 days old fungi cultures to 6 to 10 days old *F. ox, P* sp.*, A.* sp. and *F.* sp.*;* they were taken and placed on blades of glass in three specimens. The blades were incubated in an incubator at 22 ± 2°C during 48 h. For each treatment, 200 spores were examined and the germination of spores was evaluated by seeking the appearance of the tubes germinated as described by Grbic et al. (2011). The spore, whose germ tube reaches 50% of its size, is considered to be germinated. The percentage of inhibition of germination (PIg%) was calculated relative to the control as positive.

Statistical analysis

Each experiment was carried out in triples, and the average values were calculated. Statistical analysis was carried out using ANOVA and the differences between the values were given, using Duncan's test ($p < 0.05$).

RESULTS

Profile of chemical essential oils

Essential oils are extracted from the sheets of orange, Bigaradier, mandarin and lemon by hydro distillation. The yields obtained are 0.96, 1.02, 0.51 and 0.73% respectively for orange, lemon, mandarin and Bigaradier. The qualitative and quantitative analysis of the chemical profiles of essential oils is indicated in Table 1. The table includes the time of retention and percentage of the 51 components identified, accounting for 92.47 to 98.82% of all the components. The outputs obtained from the essential oils are: The results obtained proved that there are qualitative similarities between the four essential oils, although the quantities of some corresponding compounds are different. 10 componds were recorded jointly among the 51 identified, where limonene (7.18 - 36.10%), β- pinene (4.35 - 30.0%) and linalool (0.21 - 63.03%) represented the principal major compounds. The greatest volatile fraction returns of monoterpenes hydrocarbons for orange, mandarin and lemon tree are 73.64, 63.75 and 61.41%, respectively. The oxygenated monoterpenes explained approximately 71.85% of the identified components of the essential oil of Bigaradier. GC/SM of the essential oil of mandarin revealed the presence of strong concentrations in γ Terpinene (26.62%), limonene (22.52%) and β-pinene (4.35%) representing 53.49% of the total surface of the peak (Table 1). The essential oil of lemon is characterized by the prevalence of limonene (36.10%) and β-pinene (14.88%). The essential oil of orange is characterized by high concentrations in β-pinene (30%), limonene (9.37%) and the presence of two isomers sesquiterpenes (Z) and (E) β - elemene with a preponderance of the isomer (E) β - elemene (8.97%). For the essential oil of Bigaradier, linalool (63.03%) constituted the major component dominating with limonene (7.18 %) and β-pinene (5.25 %).

Antifungal activity

Inhibiting effect of essential oils on mycelia mass growth

The antifungal activity of oils essential of the Algerian citrus species with various concentrations is evaluated with respect to four phytopathogenic fungi, *F. ox, P. sp, A. sp* and *F.* sp*. in-vitro*, expressed by the inhibition percentage of the radial growth (Peak %). The results in Table 2 show the inhibiting capacity of the radial growth with a remarkable difference for the total essential oils tested. Fungi susceptibility to essential oils produces high percentages of inhibition (inhibition 100% of the mycelia growth) and has absolute toxicity. Radial growth and the biomass (Figure 1) of *F. ox, A. sp* and *F. sp* were significantly reduced in response to the various concentrations of essential oils of Bigaradier, mandarin and lemon tree, with energy of 1à 0.05 mg/ml.

Determination of inhibiting minimal concentration (CMI)

More precise data on the antifungal properties of essential oils of the Algerian citrus were obtained by determining the minimal inhibiting concentrations (CMI mg/ml) as well as the nature of the fungitoxicity (Table 3). Mandarin and lemon respectively have weakest CMI 0.01 mg/ml in respect to *F. ox* and *A. sp.* 0.05 mg/ml concentration is judged to be fungicidal as follows: Bigaradier, mandarin and lemon for *F. sp;* Bigaradier and lemon *fort F. ox* and Bigaradier *for A. sp*. The fungi species *P. sp* have been shown to be more resistant to four tested essential oils. The CMI obtained was 0.1, 1.1 and > 1.0 mg/ml respectively for mandarin, lemon, Bigaradier and orange .

Inhibiting effect of essential oils on the production and germination of spores

The results of the effects of inhibition on the production and germination of spores by essential oils are represented in Figures 2 and 3. Compared with the control, they are expressed by the inhibition percentage of the production of spores (PIs %) and the inhibition percentage of the germination of spores (PIg %). Spores production was strongly inhibited by essential oils of orange, Bigaradier, mandarin and lemon in *F. ox, P. sp* and *A. sp*. The highest inhibition percentage of the production of spores was recorded in *P. sp* with lemon (97, 85%). One note a stimulation of spores production with *F. sp* for essential oils of lemon and mandarin tree.

The capacity of citrus to inhibit spores germination was

Table 1. Chemical composition of life EOs of Algerian citrus.

Component	RT (min)	Mandarin (% area)	Lemon (% area)	Orange (% area)	Bigaradier (% area)
1-hexanol	4.68			-	-
3 hexen- 1-ol	4.71	-	-	-	0.91
α-Thujene	5.81	2.00	0.21	1.49	0.02
α -Pinene	5.99	4.36	1.28	2.04	0.38
camphene	6.37	0.10	0.13	-	0.03
β - Pinene	7.12	4.35	14.88	30.0	5.25
β - Myrcene	7.46	1.19	2.56	4.52	1.40
α Phellandrene	7.86	0.10	0.21	0.68	-
3 Carene	8.02	-	0.98	6.41	0.16
(+)-4-Carene	8.20	0.38	0.34	2.77	-
Limonene	8.71	22.52	36.10	9.37	7.18
β Ocimene	8.74	-	-	0.74	-
1,3,6-octatriene,3,7-dimethyl-	9.11	0.94	3.01	8.59	1.13
γ Terpinene	9.41	26.62	1.22	4.42	-
Cis sabinene hydrate	9.77	-	-	0.21	-
Terpinolene	10.25	1.16	0.43	2.53	-
Linalool	10.71	0.21	1.40	1.24	63.03
Z Alloocimene	11.38	0.03	0.06	0.08	-
Citronellal	12.06	-	1.21	0.90	-
Terpinen-4-ol	12.94	0.15	0.74	2.14	0.43
α Terpineol	13.34	0.10	0.79	0.07	1.00
Citronellol	14.36	-	-	1.05	-
Citral	14.70	-	6.79	-	0.46
1,6-octadien-3-ol,3,7-dimethyl	14.87	0.11	1.10		5.79
2,6-octadienal,3,7-dimethyl	15.45	-	6.79	0.22	0.15
Thymol	16.97	0.30	-	-	-
σ-Elemene	17.23	-	-	-	0.05
Cis-2,6-Dimethyl-2,6octadiene	17.53	-	-	0.45	-
Benzoic acid 2-amino- methyl	17.79	0.09	-	-	-
2,6-octadien-1-ol,3,7dimethyl	17.84	-	10.49	0.22	0.99
Copaene	18.20	-	-	-	-
(Z) *β* Elemene	18.45	-	-	0.44	-
(E) *β* - Elemene	18.71	-	-	8.97	0.08
*C*aryophylene	19.41	-	1.65	3.48	2.62
Methyl N-methylanthranilate	19.74	34.02	-	-	-
Aromadendrene	19.93	-	-	-	0.05
(E) *β* Famesene	20.20		-	0.65	0.12
α -humulene	20.29	-	0.41	1.33	0.39
β -Selinene	21.13	-	-	0.47	-
γ Elemene	21.35	-	0.10	-	0.26
α -Farnesene	21.49	-	-	0.61	-
γ -cadinene	21.80	-	-	0.56	0.01
δ -Cadinene	21.95	-	-	0.22	0.08
Nerolidol	22.95	-	-	-	0. 37
*C*aryophylene oxide	23.50	0.09	0.19	0.23	0.04
α Cadinol	25.26	-	-	-	0.04
β Sinensal	26.04	-	-	0.72	-
α Sinensal	27.27	-	-	0.30	-
β Farnesol	33.89	-	0.34	-	-
Phytol	34.36	-	-	-	0.05
Monoterpene hydrocarbons (%)		63.75	61.41	73.64	15.55
Oxygenated monoterpenes (%)		34.98	28.65	6.05	71.85
Sesquiterpene hydrocarbons (%)		-	02.16	15.95	3.66
Oxygenated sesquiterpene (%)		0.09	0.53	1.25	0.45
Other **(%)**		-	-	-	0.96
Total identified (%)		98.82	92.75	96.89	92.47

RT: Retention time , - not detected.

studied based on spores from the cultures previously exposed to essential oils. Orange, Bigaradier, mandarin and lemon exposed inhibitions of the different germinated spores. The most effective one against all the tested

Table 2. Zones of growth inhibition in Percentage Inhibition (PIc %) showing antifungal activity of Citrus EOs.

Essential oil	Dose (mg/ml)	Percentage Inhibition (PIc %)[a] (mean ± SE,n=3)			
		Fusarium sp	*Fusarium oxysporum fsp albedinis*	*Alternaria sp*	*Penicellium sp*
Orange	1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	63.92±0.3 ^{AB}
	0.8	100±00.0^{A}	76.84±7.8^{A}	100±00.0^{A}	60.62±4 ^{AB}
	0.6	100±00.0^{A}	76.59±3.5 ^{A}	100±00.0^{A}	56.91±10.0 ^{AB}
	0.4	100±00.0^{A}	65.90±6.3 ^{AB}	100±00.0^{A}	13.02±5.6 ^{C}
	0.2	88.43±0.7^{A}	50.33±8.3^{B}	73.07±4.3^{A}	N.A.
	0.1	12.35±3.3 ^{C}	19.40±5.5^{C}	23.33±2.6 ^{BC}	N.A.
	0.05	3.92±0.8 ^{D}	9.53±0.7^{C}	8.46±8.6 ^{C}	N.A.
	0.01	N.A.	2.86±0.5^{D}	5.12±1.7 ^{CD}	N.A.
	0.001	N.A.	N.A.	N.A.	N.A.
Bigaradier	1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.8	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.6	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.4	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.2	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.05	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	64.92±19.3^{AB}
	0.01	N.A.	34.11±3.0^{BC}	22.25± 2.5^{BC}	18.0±5.6^{C}
	0.001	N.A.	21.50±1.02^{BC}	13.16± 3.5 ^{C}	N.A.
Mandarin	1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.8	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	83.92±0.47^{A}
	0.6	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	84.51±0.21^{A}
	0.4	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	80.20±0.63^{A}
	0.2	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	74.51±0.62^{A}
	0.1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	N.A.
	0.05	100±00.0^{A}	100±00.0^{A}	71.87±0.13 ^{AB}	N.A.
	0.01	73.45±0.05^{A}	100±00.0^{A}	N.A.	N.A.
	0.001	N.A.	37.93±0.27 ^{BC}	N.A.	N.A.
Lemon	1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}
	0.8	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	83.14±0.20 ^{A}
	0.6	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	72,16±0.16^{AB}
	0.4	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	71.18±0.49^{AB}
	0.2	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	65.29±0.43^{AB}
	0.1	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	N.A.
	0.05	100±00.0^{A}	100±00.0^{A}	100±00.0^{A}	N.A.
	0.01	N.A.	30.56±0.17 ^{BC}	100±00.0^{A}	N.A.
	0.001	N.A.	35.25±0.10 ^{BC}	45.42±0.11^{B}	N.A.

[a]Values followed by same alphabetic letters are not significantly different according to ANOVA and Duncan's Multiple Range Test (p≤ 0.05). N.A., non-active zones of growth inhibition values are presented as mean ± standard deviation.

fungi stocks was the oil of orange, with a strong inhibition percentage of spores germination of (PIg 58.66 to 100 %). A stimulation of the germination of spores was observed in *A. sp* with lemon and mandarin tree.

DISCUSSION

The bioactivity of the oils essential extracted from the sheets of Algerian citrus in respect to four phyto-

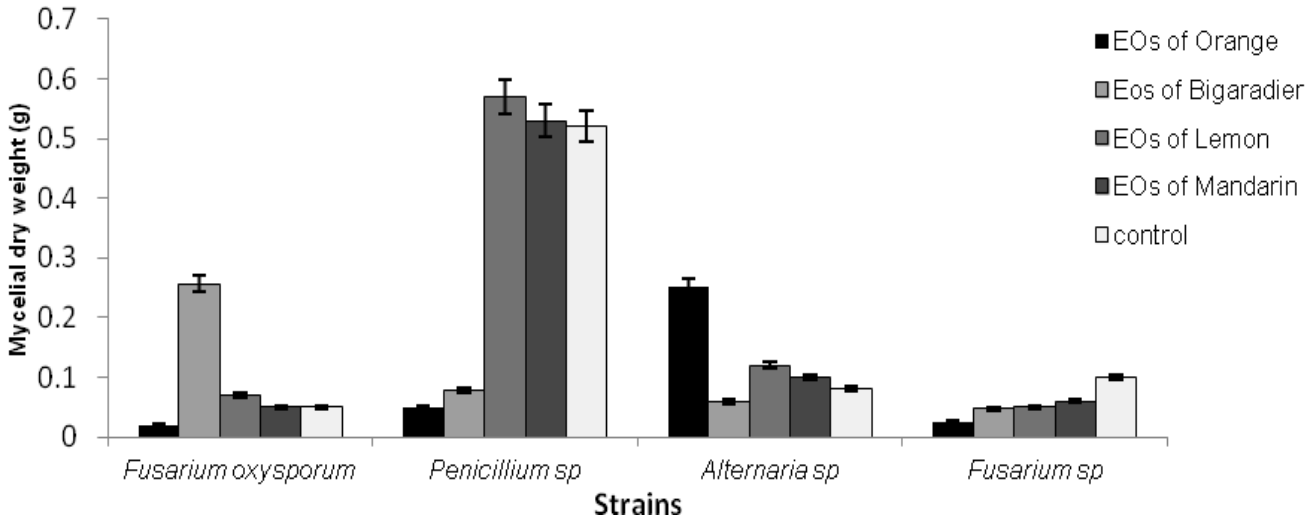

Figure 1. Effect to citrus EOs on Mycelia dry weight (g) of *fungi* after 15 day , grown in a broth of potato dextrose (PDB) and incubated at 22±2°C.

Table 3. Minimal inhibitory concentration (MIC) (mg/ml) and nature of fungitoxicity (NF) EOs of Orange, Bigaradier, Mandarin and Lemon, from (Chlef) Algeria.

Essential oil	Fungal strain							
	***Fusarium* sp.**		***Fusarium oxysporum fsp albedinis***		***Alternaria* sp.**		***Penicellium* sp.**	
	MIC (mg/m)	**NF**	**MIC (mg/ml)**	**NF**	**MIC (mg/ml)**	**NF**	**MIC (mg/ml)**	**NF**
Orange	0.4	-	1.0	+	0.4	-	>1.0	+
Bigaradier	0.05	-	0.05	-	0.05	-	0.1	-
Mandarin	0.05	-	0.01	-I	0.1	-	1	-
Lemon	0.05	-	0.05	-	0.01	-	1	+

- , Fungicidal; +, fungistatic.

pathogenic fungi was evaluated. The chemical analysis of essential oils by GC/SM indicates strong percentages in monoterpenes hydrocarbon for the whole of the oils tested except Bigaradier. Our results agree with those obtained by other authors on orange, mandarin and lemon (Baaliouamer, 1987; Espina et al., 2011; Settanni et al., 2012). The high percentage of oxygenated monoterpenes in Bigaradier was announced by Lota et al. (2001). Limonene has strong percentage in lemon tree, mandarin tree, orange and Bigaradier. Results similar to ours were given by several studies (Baaliouamer, 1987; Lota et al., 2001; Pistelli et al., 2012). The essential oil of mandarin records a strong percentage in γ Terpinene; similar results were given by Fuselli et al. (2008). With regard to the oil of orange, one noted a high percentage in β-pinene. Linalool was identified in all essential oils, but its greater quantity was announced in Bigaradier. The importance of linalool in the essential oil of Bigaradier was announced by Lota et al. (2001).

Several publications on the antifungal activity of essential oils of the citrus are reported in the literature (Viuda-Martos et al., 2008; Sharma and Tripathi, 2008). The essential oils of the Algerian citrus with proportioned concentrations demonstrated all the capacity to reduce or inhibit the growth of the fungi species tested in-*vitro.* Radial growth and biomass of *F. ox, A. sp* and *F sp* were strongly inhibited at summer in different degrees; they have a more marked activity of essential oils of Bigaradier, lemon and mandarin with very weak inhibiting minimal concentrations. Their good biological activity can be related to the presence of monoterpenes. Essential oils of the citrus were characterized by a high percentage in monoterpenes, particularly in limonene, linalool, β-pinene and γ Terpinene. Several authors allotted the antifungal activity of essential oils of citrus to the

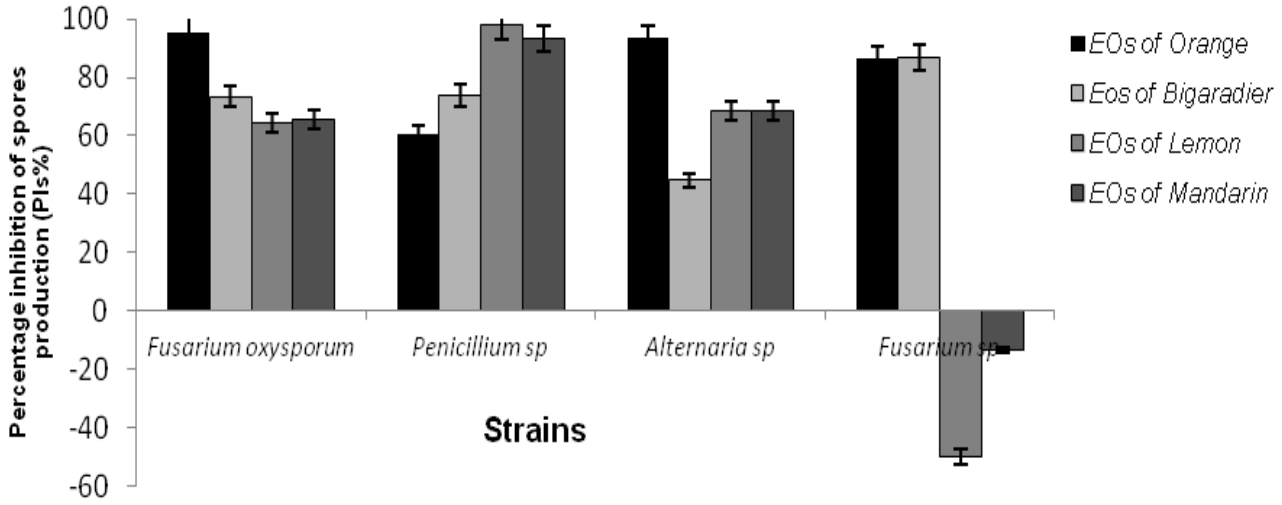

Figure 2. Percentage inhibition (compared with positive controls) of spore produced of *fungi* in colonies previously exposed to Citrus EOs. Data are means ± standard deviations (error bars).

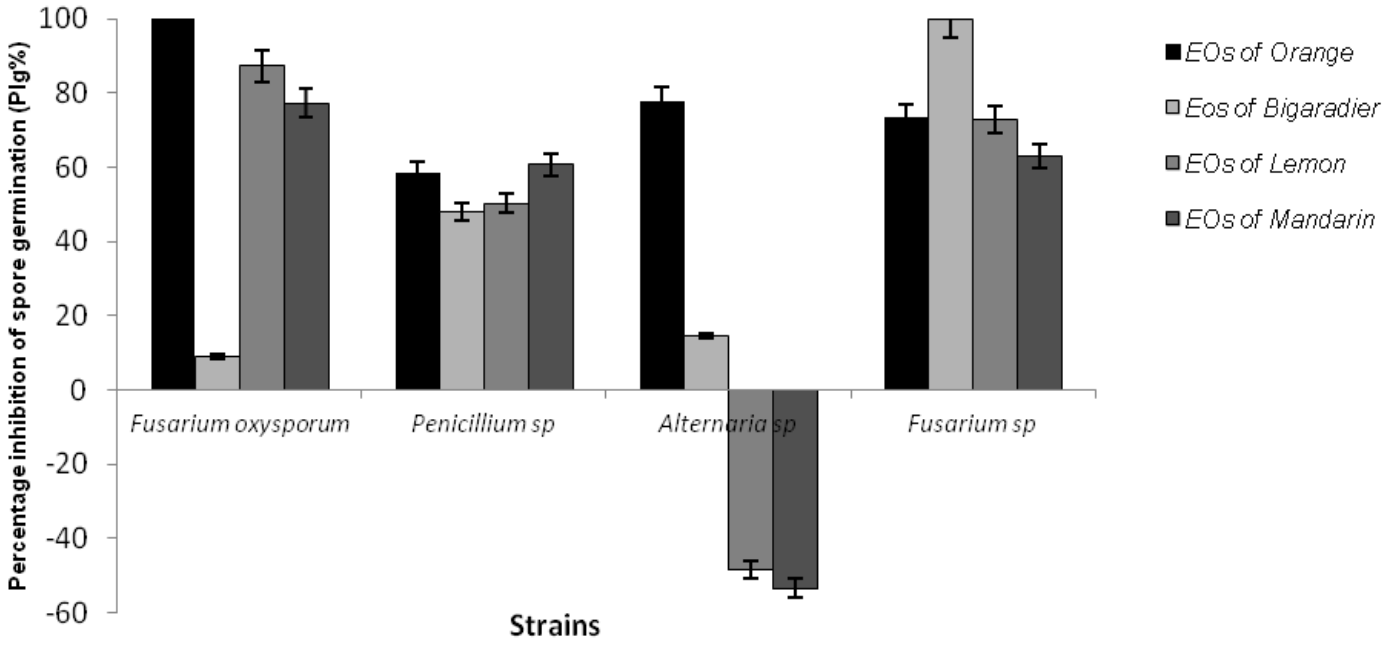

Figure 3. Percentage inhibition (compared with positive controls) of spore germination of *fungi* in colonies previously exposed to citrus Eos. Data are means ± standard deviations (error bars).

presence of monoterpenes such as linalool, limonene or central etc (Sharma and Tripathi, 2006, 2008; Viuda-Martos et al., 2008). They act on hyphas mycelia, lead to the loss of rigidity and integrity of the cellular wall of the hyphas, resulting in the death of the mycelium (Sharma and Tripathi, 2008). The capacity of citrus essential oils to inhibit the production and germination of the spores of tested fungi was evaluated *in vitro*. The tests of production and germination of spores showed the inhibiting effect of essential oils of orange, Bigaradier, mandarin and lemon on *F.* ox*, F.* sp*., P.* sp. and *A.* sp*.*

The capacity of citrus essential oils to inhibit the production and germination of spores was reported by several authors (Sharma and Tripathi, 2006, 2008; Chutia et al., 2009; Grbic et al., 2011). The best percentages of inhibition were presented by the oil of orange in respect to *F. ox, F.* sp. *and A.* sp. Sharma and Tripathi (2008) announce that the oil of orange was extremely toxic to

the germination of the spores of *Aspergillus niger* and that oil treatment led to distortions, thinning of the wall of the hyphas, reduction of the diameter of the hyphas and the absence of conidiophores. It is remarkable that the essential oil of the lemon stimulated the production of the spores of *F. sp* whereas the essential oil of the mandarin supported the germination of the spores of *A sp*. French (1985) recalled that it is extremely difficult to correlate the fungitoxic activity of simple compounds or classes of compounds. The various components of oil can act in a synergistic way while the association of several compounds can have a stimulating action on the germination of fungi spores.

The results obtained justify future research, stressing the antifungal properties of the major components of essential oils, on one hand, and the capacity of these oils to inhibit the fungi tested *in vivo,* on the other hand.

Conclusion

The data presented here shows the inhibiting powerful potential of essential oils extracted from the sheets species of Algerian citrus in respect to *F. ox, P. sp, A. sp and F. sp in vitro* and their possible integration in the programs of protection against the phytopathogenic fungi. The fungi anti capacities of essential oils of the Algerian citrus proved to be a very interesting field for applications in agro- alimentary industry.

Conflict of interest

The authors declare no conflict of interest.

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Chemical composition and anti-diabetic properties of *Jatropha curcas* leaves extract on alloxan induced diabetic wistar rats

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This study evaluates the chemical composition and anti-diabetic properties of fresh and shade dried *Jatropha curcas* aqueous leaves extracts (JCLE) on alloxan induced diabetic female wistar rats. Seven (7) kg of *J. curcas* leaves were pulverized and aqueous extracts produced. Thirty five (35) mature female rats were grouped into seven of five animals per group according to their body weights. Blood samples were collected for baseline data before inducing diabetes. Various groups of rats were fed graded doses (100, 200 and 300 mg of JCLE, respectively. Blood glucose was tested every seven days using glucometer. The animals were treated for 21 days with JCLE, blood samples were collected for liver enzyme function test, liver and pancreas tissues collected for histopathology. The results obtained were analysed statistically using analysis of variance (ANOVA) and Duncan Multiple range student test. Result shows that proximate composition of JCLE had higher protein, fat and carbohydrate concentrations in shade dried than the fresh samples. The vitamin, mineral and phytochemical compositions varied but higher in shade dried JCLE also than the fresh. Rats treated had significant ($p<0.05$) reduction in blood glucose level. Liver enzymes was higher with shade dried JCLE. The current study provides some useful insight into the anti-hyperglycemic potency of JCLE in alloxan induced diabetics and seems to repair some organs damages.

Key words: Blood glucose, *Jatropha curcus,* liver enzymes, diabetic.

INTRODUCTION

In Sub-Saharan Africa, there is a growing need to fight the scourge of diabetes among the populace. Although access to primary health care is low, diabetic patients tend to fight this disease through natures endowed plant resources. Prevalence and burden of type 2 diabetes are rising quickly and may be due to the rapid uncontrolled urbanization, environmental degradation and major changes in lifestyle of the people (Jean et al., 2010;

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Abbreviations: JCLE, Jatropha curcas aqueous leaves extracts; **AST,** Aspartate aminotransferase.

Mohan et al., 2013). This increase presents a substantial public health and socioeconomic burden in the face of scarce resources. Therefore, there is a concomitant increase in alternative but cheaper means of treating this disease condition among the rural and low economic income earners. In 2003, the International Diabetes Federation estimated that approximately 194 million people around the world had diabetes and by 2025 this figure will be expected to rise to 333 million, amounting to 6.3% of the world's population living with diabetes (Atlas, 2003). The Diabetes Association of Nigeria (2011) also reported that over 140 million people based on Nigerian Census of 2006, had an estimated six million persons with full blown diabetes mellitus. Reports from hospital records indicate the alarming increase in both prevalence and incidence of diabetes among all ethnic groups and social classes in Nigeria (DAN, 2011). Indeed, diabetes has in the last two decades become a household disease that requires urgent health care intervention.

Jatropha curcas is a drought resistant shrub or tree belonging to the family Euphorbiaceae, which is cultivated in Central and South America, South-East Asia, India and Africa (Martinez-Herrera et al., 2006). Recently, there is an increasing use of *J. curcas* in the management of diabetes and other carbohydrate metabolic related syndromes. Other ethnomedical uses of the extracts of *J. curcas* leaves include use as a remedy for cancer, as an abortifacient, antiseptic, diuretic, purgative and haemostatic (Dalziel,1995; Mishra et al., 2010). *J. curcas* leaves have been observed to be consumed by people with many ailments traditionally including diabetes mellitus in the North-east of Nigeria without scientific explanation. Thus, the main objective of this work was to assess the chemical, phytochemical and anti-diabetic properties of *J. curcas* leaves extract.

MATERIALS AND METHODS

Samples preparation

The *J. curcas* leaves were harvested, sorted by removing extraneous materials; the sample leaves were then taken to the taxonomic unit in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka for identification. The leaves were rinsed with distilled water and divided into two parts; 5 kg fresh and 5 kg was shade dried for 5 days at room temperature. The dried leaves were pulverized into powder using Warburg laboratory blender and packaged in labelled polyethylene bags for further use.

Preparation of vegetable extracts for rat studies

Two hundred grammes (200 g) each of the pulverized vegetable samples were soaked separately in 500 ml boiled distilled water and agitated intermittently for 24 h. They were filtered using fine sieve of 4.75 mm mesh (No. 4) to obtain the aqueous extracts. The extracts were stored in air tight containers at 4°C and later reconstituted in distilled water to give the required doses to be administered during the study using the formula:

$$\frac{\text{Weight of rat x conc. of extracts}}{\text{Dosage x 1000}}$$

Chemical analysis

Proximate analysis

Portions of *J. curcas* leaves were analysed for their proximate values using AOAC methods (2005).

Moisture

The moisture content of the samples was determined using the hot oven method as described by Pearson (1976). Petri dishes were thoroughly washed and dried in the oven at 100°C for 30 min and allowed to cool inside a desiccator. These various weights were determined by weighing them with a weighing balance. 2 g of finely ground sample was weighed into the dishes and placed inside the oven at 100°C for 4 h. The dishes together with the sample they contain were removed, cooled in a desiccator and weighed. They were placed back into the oven, dried for further 30 min, cooled and weighed. The drying was continued and weighed repeatedly until a constant weight was obtained. The percentage moisture content was calculated from the weight loss of the sample.

Thus:

$$\% \text{ moisture} = \frac{\text{Wt. of dish + sample – wt. of dish + sample after drying}}{\text{Wt. of sample}} \times \frac{100}{1}$$

Protein

Total nitrogen was estimated using micro kjeidahl method as described by Pearson (1976). One gram of sample was digested with concentrated sulphuric acid. The digested samples were distilled and titrated. The crude protein was calculated by multiplying the total nitrogen by the conversion factor of 6 to 25.

$$P = TN \times 6.25$$

Fat

The fat content of the samples were determined using Soxhlet extraction method as described by Pearson (1976). The fat content was determined using Soxhlet fat extraction unit/system. The Soxhlet extraction cup was washed and dried in oven and weighed in an analytical balance. One gram sample was weighed and placed in a folded filter paper inside the thimble. The thimble was immersed into an aluminium cup containing 30 ml of petroleum ether. It was further placed in the extraction unit and the extraction process began. After the extraction process the aluminium cup together with the oil it contains was sent to the oven for drying before weighing.

$$\% \text{ of wt.} = \frac{A - B}{C} \times \frac{100}{1}$$

Where, A = weight of empty cup; B = weight of cup + fat; and C = weight of sample used in grams.

Carbohydrate

Carbohydrate This was determined by difference
% carbohydrate = 100 - (% ash + protein + fat + fibre + moisture).

Ash

Ashing method by Osborne and Voogt (1973) was used. Two grams each of the samples was subjected to ashing, in a silica crucible until the food matrix was destroyed. The sample was heated gently at first at about 1500C to clay it and at 500°C in a muffle furnace to completely destroy it.

$$\% \text{ Ash} = \frac{C - B}{F} \times \frac{100}{1}$$

Where, B = weight of crucible (empty); C = weight of crucible + Ash; and F = weight in grams of sample used.

Crude fibre

The fibre content was determined using the AOAC method (1995). Three grams of sample was defatted by Soxhlet extraction or by stirring, setting and decanting with petroleum ether. The defatted sample was placed in a beaker with 200 ml of boiling H_2SO_4. It was filtered under suction, washed with distilled water and boiled again for another 30 min with 200 ml of NaOH. The digested sample was washed with 0.1 m HCl to neutralize the NaOH and severally with hot distilled water. It was washed 2 times with ethanol and three times with ether. The ash is then cooled and weighed.

$$\% \text{ fibre} = \frac{M_2 - M_3}{M0} \times 100$$

Where, M_2 = weight after drying; M_3 = weight after ignition; M_0 = weight of sample.

VITAMINS

Vitamin A (βeta carotene)

The method of AOAC (2005) was used. One gram of the sample was extracted with 50 ml of petroleum ether in triplicate. The ether extract was concentrated and evaporated to dryness. The residue was dissolved with 0.2 ml of chloroform-acetic anhydride (1:1). 2 ml of trichloroacetic acid-chloroform (1:1) was added and the absorbance taken at 620 nm every 15 s interval.

Vitamin B1 (Thiamine)

One (1) ml of the filtrate was transferred into three test tubes, 2 ml of water; 0.4 ml of 50% sodium acetate, 0.1 ml of diazotized reagent was added and shaken. 0.2 ml of 5.5% sodium carbonate was added, mixed and the absorbance taken at 540 nm against a reagent blank.

Mineral analysis

Calcium

Two grams of sample was ashed followed by classical precipitation and titration (Paul and Southgate, 1978).

Iron and zinc

The method of AOAC method (1995) was used. Two grams of sample (powdered) is weighed into a crucible and ashed in a muffle furnace at 550°C for 6 h. The ash was cooled and 6 HCl added and boiled for 10 min, while covering the crucible with a watch glass. After boiling, the sample was cooled and filtered into 100 ml volumetric flask. The crucible washed with distilled water and the washing added to ash filtrate. The ash filtrate was made to 100 ml with distilled water.

An aliquot of the filtrate was aspirated into the atomic absorption spectrophotometer (Pye Unicam) and the absorbance values corresponding to the different minerals read. Solutions of Zn and Fe were prepared and aspirated into the atomic absorption spectrophotometer and their absorbance values recorded. The percentage of element present was calculated from the absorbance values of the sample and standard solution.

Potassium

Potassium content of the samples was determined using the atomic absorption spectrometric method as described by Collins and Polkinhorne (1952). 5 g of the sample was ashed, then ash was transferred to 400 ml beaker using 100 ml distilled water. About 10 ml concentrated of HCl was added and boiled for several minutes then cooled and diluted with water to 500 ml and filtered, diluted to final conc. of solution (approximately 15 mg/L K_2O) prepared. A series of solution from the freshly prepared diluted potassium solution containing 10, 12, 14, 16, 18 and 20 mg/l K_2O was read against blank (the concentration was read between 766 to 770 nm in an atomic absorption spectrophotometry (AAS). Results were extrapolated from an already prepared calibration curve.

$K = 0.88 \times k_20$

Phosphate

The method of AOAC (2005) was used. 2 ml of sample was transferred into three test tubes and 3 ml of water added; the pH was adjusted to 7.0 with dilute ammonia and 2.5 ml of vanadate molybdate reagent added. The solution was made up with 10 ml and after 10 min the absorbance was taken at 470 nm against a blank.

Experimental design

Toxicity test

The acute toxicity and mean lethal dose (LD_{50}) of the extracts were determined in mice using the method described by Lorke (1983) (Table 1b). The experimental animals were healthy female Wistar rats weighing between 150 to 200 g. Thirty five rats were randomly divided into seven groups (1 to 7) of five rats each (Table 1a). These rats were distributed in metabolic cages and maintained under standard environmental conditions. They were fed with commercial rat chow and water *ad libitum*. Mature female Wistar rats were procured from the Faculty of Veterinary Medicine, University of Nigeria Nsukka, for this study. The test sample *J. curcas* leaves were obtained from No. 57 Ibagwa road, Nsukka, Nigeria.

Ethical consent

Ethical consent was obtained from the Ministry of Veterinary Control Enugu, Enugu state Nigeria.

Induction of diabetes

Diabetes mellitus was induced in groups' 1-6 rats by single intra-

Table 1a. Grouping of Wistar rats administered graded doses of *J. curcas*.

Group	Sample	Dose (mg/kg bwt)
1	Shade dried *J. curcas*	100
2	Shade dried *J. curcas*	200
3	Shade dried *J. curcas*	300
4	Fresh *J. curcas*	100
5	Fresh *J. curcas*	200
6	Fresh *J. curcas*	300
7	Control	

Table 1b. LD_{50} value of the aqueous extract of *J. curcas* leaves.

No. of mice	Dose (mg/kg)	No. of death of animals
3	5	0
3	50	0
3	300	0
3	2000	1
3	3000	3

LD_{50} value = 2000 mg/kg, animals used = albino mice, weight of animals = 20-25 g. No. of animals = 3, Route = oral.

peritonial injection of 150 mg/kg body weight of alloxan monohydrate (Sigma, St. Louis, USA) suspended in normal saline, after an overnight fasting on the 5th day. After 48h (7th day), diabetes was confirmed using one touch glucometer. Animals that had fasting blood glucose level ≥200 mg/dl were considered diabetic and included in the study according to Luka and Tijjani (2013).

Collection of blood samples

Blood samples were collected from the retro-bulba plexus of the medial canthus of the eye of the rat for biochemical analysis.

Blood glucose estimation

Blood glucose was estimated on day 0, 7, 14 and 21 using glucometer (One Basic, Inc.).

Assay of activities of some liver function enzymes

Serum enzymes; aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) were determined using Randox laboratory reagent kit, UK, BT 29 4QY based on method by Reitman and Frankel (1957).

Histopathology examination

At the end of the experiment, the rats were anesthetized and dissected. The liver and the pancreas tissues were extracted, rinsed in normal saline for histopathological examination after which they were fixed in 10% formalin. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5 to 6 microns. The sections were deparaffinized in xylene, taken to water and subsequently stained with Haematoxylin and Eosin (H and E) for light microscopy (Bancroft and Stevens, 1977).

Statistical analysis

Data obtained were analyzed using descriptive statistics including mean and standard deviation. Duncan new multiple range test and least significant difference (LSD) were used to separate the means. Significant differences were accepted at $p< 0.05$.

RESULTS AND DISCUSSIONS

Acute toxicity study (Table 1b) is generally carried out for the determination of LD_{50} value in experimental animals. The LD_{50} determination was done in mice by OECD guidelines 423. The aim of performing acute toxicity study was for establishing the therapeutic index of a particular treatment and to ensure the safety *in vivo*. The LD_{50} of the 50% aqueous extract of *J. curcas* was found to be 2500 mg/kg. The proximate composition of shade dried and fresh *J. curcas* leaves extracts (JCLE) as displayed in Table 2 revealed that shade dried samples contained higher percentage of protein, ash, fat and carbohydrate as compared with the fresh. However, the fresh JCLE had higher percentage of moisture content (94.12%) as compared with the shade dried leaves extract (90.00%). The lower moisture content observed in the shade dried JCLE could possibly be due to loss of moisture during the shade drying periods. Fresh vegetables are known to contain more water than either sun or shade dried vegetables. Udofia and Obizoba (2005) reported the same observation in fresh 'attama and sweet potato leaves. The protein content (1.90 and 2.01%) of fresh and dried JCLE were lower than 3.3% recorded by USDA Nutrient Database for Standard Reference (Hall, 1998). This may be because some protein fractions may still be embedded in the leave samples. Chima and Igyor (2007) also reported lower protein values for (*Pterocarpuss oyauxii*) "Oha" (2.0%) and *Gnetum africanum* "Okazi" (1.5%). Incorporating *J. curcas* vegetable in diet can furnish it with appreciable amount of protein which provides enormous benefits such as maintenance of fluid balance, formation of hormones and enzymes and contribution to immune function. Ash, carbohydrate and fat were generally lower than other authors who reported *Ipomea batata* (11.10%*)* and *Moringa oleifera* (15.09% DW) (Antia et al., 2006; Lockeett et al., 2000) and *Hibscus esculentus* (8.00% DW) reported by Akindahunsi and Salawu (2005). Ash content which is an index of mineral contents in biota was 0.69 and 0.98%, respectively, for fresh and dried JCL. Carbohydrate value for dried leaves was 6.04% lower than that observed by Akubugwo et al. (2007) in *Amaranthus hybrids* (52.18%) fresh samples, but higher than *Thuja occidentalis (*4.72%*).* As far as vegetables are concerned, some of them are rich sources while others contain traces of some the nutrients.

Table 3 shows the vitamin composition of shade dry and

Table 2. Proximate composition of shade dry and fresh *Jatropha curcas* aqueous leaves extract.

Sample	Protein (%)	Ash (%)	Fibre (%)	Fat (%)	Moisture (%)	CHO
Shade dry	2.01±0.03	0.98±0.03	-	0.97±0.04	90.00±0.33	6.04±0.30
Fresh	1.90±0.06	0.69±0.10	-	0.55±0.05	94.12±1.16	2.73±1.09

Mean ±SD of three determinations.

Table 3. Vitamin composition of shade dry and fresh *Jatropha curcas* leaves extract

Sample	Pro-vitamin A (IU)	Vitamin C (mg/100 g)	Vitamin B1 (mg/100 g)	Vitamin B2 (mg/100 g)	Vitamin B3 (mg/100 g)
Shade dry	1491.30±28.48	0.98±0.03	90.00±0.33	6.04±0.30	10.00±0.33
Fresh	1312.80±46.9	0.65±0.11	4.40±0.87	0.07±0.01	2.63±0.15

Mean ±SD of three determinations.

Table 4. Mineral composition of shade dry and fresh *Jatropha curcas* aqueous leaves extract.

Sample	Iron (mg/100 g)	Calcium (mg/100 g)	Potassium (mg/100 g)	Zinc (mg/100 g)
Shade dry	0.33±0.0	0.74±0.06	63.81±0.32	42.33±2.52
Fresh	0.60±0.02	0.49±0.01	76.45±1.77	32.00±2.00

Mean ±SD of three determinations.

Table 5. Phytochemical composition of shade dry and fresh *Jatropha curcas aaqueous* leaves extract.

Sample	Alkaloid (mg/100 g)	Tannin (mg/100 g)	Oxalate (mg/100 g)	Flavonoids (mg/100 g)
Shade dry	2.26±0.24	1.63±0.32	8.60±0.60	3.83±0.21
Fresh	0.99±0.04	0.07±0.02	4.58±0.20	2.23±0.12

Mean ±SD of three determinants.

fresh *J. curcas* leaves extract. The result shows that the vitamin composition of shade dried JCLE was higher (vitamin B190.00 mg, Vitamin B26.04 mg and Vitamin B310.00 mg) than the fresh JCLE (vitamin B1 4.40 mg, Vitamin B2 0.07 mg and Vitamin B3 2.63 mg). Pro-vitamin A and vitamin C contents were also higher in the shade dried samples than the fresh samples. This is similar to the result of other researchers who reported higher vitamin contents in dried samples than the fresh samples (USDA, 2012; Reddy and Love, 1999). Generally, vegetables are rich in vitamin, minerals, dietary fibre and protein (Otitoju et al., 2014; Humphrey et al., 1993; Mathenge, 1997). The result in this study shows that JCLE is a rich source of pro-vitamin A and vitamin B. Pro-vitamin A protects the body cells from the damaging effects of free radicals, they act as good source of vitamin A and enhance the functioning of the immune system; it also helps the reproductive system to function properly (Handelman, 2001; Young and Lowe, 2001). Table 4 shows the mineral composition of shade dried and fresh *J. curcas* leaves extracts. The fresh samples had higher iron content (0.60 mg) than the dried samples (0.33 mg). A similar trend was observed for potassium (76.45 and 63.81 mg), respectively. In shade dried *J. carcus,* calcium (0.74 and 0.49 mg) and zinc (42.33 and 32.00 mg) were higher in the dried than the fresh samples, respectively. Quantitative phytochemical compositions result (Table 5) shows that shade dried samples had higher alkaloids (2.26 mg), tannins (1.63 mg), oxalate (8.60 mg) and flavonoid (3.83 mg), respectively than the fresh samples which contained alkaloid (0.99 mg), tannins (0.07 mg), oxalate (4.58 mg) and flavonoid (2.23 mg), respectively. Oladele et al. (1995) have also reported that medicinal plants with hypoglycemic and anti-diabetic effect usually contain high concentration of alkaloids and flavonoids. This finding is in line with the result obtained from this study.

This study demonstrates hypoglycaemic effect on the fasting blood glucose level of the rats (Table 6) and had significant ($p < 0.05$) increase in the blood glucose levels after induction with 150 mg/kg alloxan. The highest percentage (70.68%) reduction of the blood glucose

Table 6. Effects of aqueous leaves extract of *J. curcas* (JCLE) on blood glucose levels in alloxan induced diabetic rats.

Group	Baseline (mg/	After induction (mg/dl)	After treatment (mg/dl)
1	87.00±6.32[a]	222.60±143.15[a]	112.33±1.53[a]
2	87.60±9.21[a]	374.0±163.21[b]	177.67±118.25[a]
3	93.40±7.79[a]	383.8±185.86[b]	112.50±6.36[a]
4	75.80±8.44[a]	313.8±198.09[b]	125.80±82.65[a]
5	67.80±4.66[a]	429.0±203.75[b]	155.25±139.09[a]
6	82.60±8.82[a]	350.4±216.47[b]	131.60±117.62[a]
7	81.60±1345[a]	75.0±7.42[a]	75.0±13.34[a]
LSD	0.002	0.056	0.80

Mean ± SD Mean values of different superscripts in the same row and significant at $p < 0.05$.

Table 7. Effect of aqueous leaves extract of *J. curcas* on aspartate amino transferase (AST) activity (IU/L) in alloxan induced diabetic rats.

Group	Baseline	After induction	After treatment
1	88.40±6.69[a]	120.80±12.85[b]	118.67±4.16[b]
2	83.0±11.27[a]	112.0±8.28[b]	105.33±9.45[b]
3	93.20±6.06[a]	108.20±7.95[b]	101.00±970[ab]
4	88.60±6.39[a]	119.40±15.47[b]	84.60±9.10[a]
5	89.0±5.96[a]	116.40±3.65[b]	89.40±3.13[a]
6	84.60±3.85[a]	109.40±9.86[b]	89.40±3.13[a]
7	95.60±4.04a	96.80±7.92 [b]	91.60±7.83a
LSD	0.077	0.014	0.000

Mean ±SD. Mean values of different superscripts in the same row and significant at $p < 0.05$.

levels was observed with the administrations of shade dried JCLE dose (300 mg) and for fresh (63.81%) with dose (200 mg) after-treatment compared with after-induction period. Similar results of hypoglycaemic effects of *J. curcas leaves* have been reported by Mishra et al. (2010) and Omale et al. (2011). Though there was significant decrease in the blood glucose level after-treatment as compared with the after-induction period, the blood glucose level was significantly ($p<0.05$) higher than the baseline blood glucose level. However, the shade dried and fresh aqueous extracts could not reverse the high blood glucose level observed in after-treatment to the initial baseline blood glucose level as reported by Mishra et al. (2010) in their work; this may be attributed to the use of the 50% ethanol extract while this study used aqueous extract for their treatments. It may also be a proof that the ethanol extract may be more effective as antidiabetic agent than the aqueous extract. Table 7 shows the mean aspartate aminotransferase (AST) level of the rats. The activity of AST after-treatment was significantly ($p < 0.05$) higher than the baseline values and the control group (group 7). Aspartate amino-transferase (AST) is an enzyme produced by the liver cells, elevated amounts of serum AST may signal toxicity which may lead to health problems. The levels of AST in the body are indicative of tissue damage and diseases and the amount released is proportional to the level of damage sustained (Evelson et al., 2005) increased with liver diseases in all species.

A number of other plants have been reported to have anti-hyperglycemic and insulin stimulatory effects (Latha and Pari, 2003; Latha and Pari, 2004; Pari and Venkateswaran, 2002). Increase in the activities of serum AST, ALT, and ALP may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (El-Demerdash and Abou, 2005), which gives an indication of the hepatotoxic effect of *J. curcas*. The levels of AST, ALT and ALP have been reported to increase in alloxan-induced diabetic rats (Nnodim et al., 2012). Both shade dried and fresh aqueous extract of *J. curcas* caused significant increase ($P<0.05$) in the level of some serum AST, ALT and ALP values after-treatment as compared with after-induction period as shown in Tables 7 to 9. However, though both the shade dried and fresh aqueous extract of *J. curcas* caused significant decrease ($p<0.05$) in the level of serum AST, ALT and ALP after-treatment when compared with after-induction, the levels of AST, ALT and ALP in the serum for the groups fed with shade dried extract were significantly ($p<0.05$) higher than the baseline values. These significantly ($p<0.05$) higher AST, ALT and ALP values obtained in after-treatment as compared with the baseline values could imply that the shade dried *J. curcas* extract had not completely revert induced diabetes in the experimental rats. Similarly, it could also imply that the hepato-protective effects of JCLE worked more in the groups treated with fresh leaves extracts than the groups treated with the shade dried leaf extract. Thus, it could be said that the fresh leaves extracts had more hepato-protective potentials than the shade dried leaves extracts.

Although, there was no significant difference in the weight of the rats following induction of diabetes and

Table 8. Effects of aqueous leaves extract of *J. curcas* on liver Alanine aminotransferase (ALT) level (IU/L) in alloxan induced diabetic rats.

Group	Baseline	After induction	After treatment
1	65.40±3.58^{a}	72.20±6.18^{a}	66.67±6.11
2	68.00±4.74^{a}	74.00±8.09 ^{a}	94.67±3.06^{b}
3	66.00±3.00^{a}	70.40±1.52^{b}	88.00±2.83^{c}
4	72.40±4.83^{a}	68.80±3.27	69.20±11.43^{a}
5	65.00±3.16^{a}	73.20±4.32^{b}	65.75±4.50^{a}
6	65.00±4.18	63.60±1.82^{a}	64.80±4.66^{a}
7	66.20±4.44	65.40±5.46^{a}	59.60±`6.43^{a}
LSD	0.08	0.015	0.00

Mean ±SD Mean values of different superscripts in the same row and significant at $p < 0.05$.

Table 9. Effects of aqueous leaves extract of *J. curcas* on liver alkaline phosphatase (ALP) in alloxan induced diabetic rats.

Group	Baseline	After induction	After treatment
1	42.40±5.77^{a}	50.60±6.54^{a}	76.0±2.65^{b}
2	47.60±4.39^{a}	51.40±5.03^{a}	62.33±5.86^{b}
3	53.0±4.90^{a}	56.60±4.04^{a}	55.50±6.36^{a}
4	48.60±6.50^{a}	48.80±6.89^{a}	44.60±4.77^{a}
5	45.80±3.49^{b}	53.80±4.49^{c}	37.50±5.26^{a}
6	39.20±2.39^{a}	63.80±3.11^{b}	42.00±3.16^{a}
7	44.0±3.16^{b}	38.80±3.56^{a}	42.20±1.79^{ab}
LSD	0.002	0.00	0.00

Mean ±SD Mean values of different superscripts in the same row are significant at $p < 0.05$.

Table 10. Effects ofaqueous leaves extract of *J. curcas* onbody weight of diabetic rats.

Group	Baseline	After induction	After treatment
1	234.20± 49.77^{a}	218.00±41.43^{a}	230.33±30.67^{a}
2	200.40±29.70^{a}	188.80±29.12^{a}	210.67±31.56^{a}
3	192.60±47.83^{a}	169.00±12.73^{a}	194.00±11.31^{a}
4	166.80±19.69^{a}	160.60±14.72^{a}	152.00±13.71^{a}
5	178.80±40.56^{a}	150.40±33.25^{a}	181.50±45.53^{a}
6	146.80±31.10^{a}	146.00±28.87^{a}	156.00±25.79^{a}
7	124.75±17.46^{b}	157.20±20.83^{b}	164.60±13.85^{b}
LSD	0.051	0.076	0.074

Mean values in the same row of different superscripts on the row are significant at $p < 0.05$.

after-treatment, untreated diabetic rats had lower body weight when compared to the normal and treated groups (Table 10). This is in line with some studies that reported significant weight reduction in untreated diabetic rats (Longe and Momoh, 2014). The histopathology result shows that the protective nature of the extract was dose dependent as there was still a clear evidence of liver cell damage in the group treated with 100 mg of shade dried leaves extract (Figure 1a). While Figure 1b showed regeneration process of the liver, a similar effect was also observed in the pancreas (Figure 1c and d). There was mild areas of vacuolations in the 200 mg treated group (Figure 1e) when compared with Figure 1f which showed normal pancreatic cell (control group).

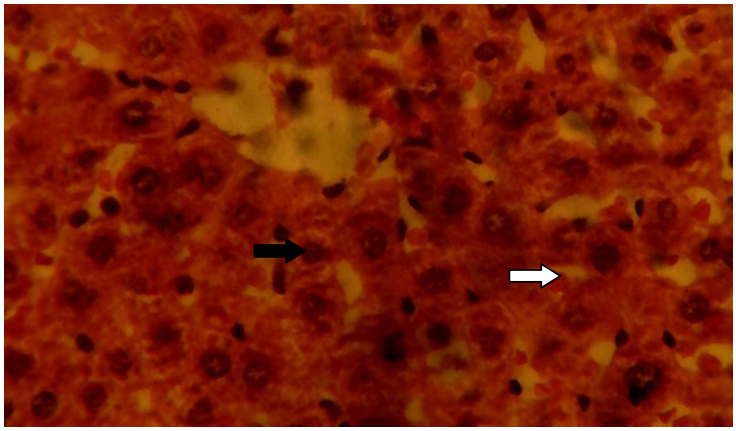

Figure 1a. Photomicrograph of liver section from group 1 (diabetic rats treated with 100 mg/kg dry leaf extract) showing mild hepatocellular degeneration (black arrow) and dilated sinusoids (white arrow). H and E x 400.

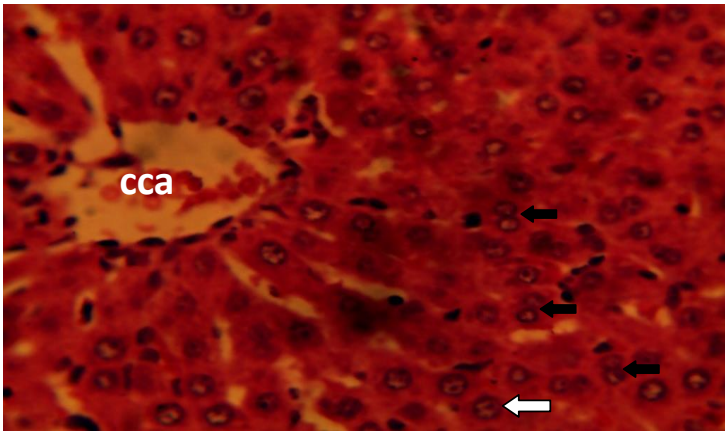

Figure 1b. Photomicrograph of liver section from group 6 (diabetic rats treated with 300 mg/kg fresh leaf extract) showing apparently normal hepatocytes (white arrows) and some binucleated hepatocytes-sign of liver regeneration (black arrows). H and E x 40.

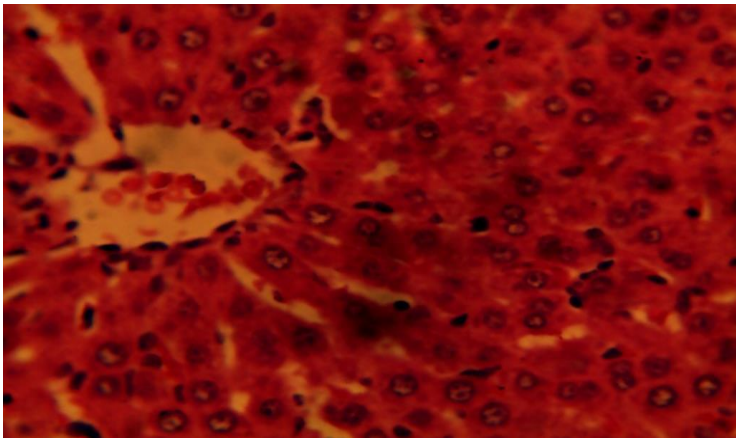

Figure 1c. Photomicrograph of liver section from group 3 (Control) showing apparently normal hepatocytes. H and E x 40).

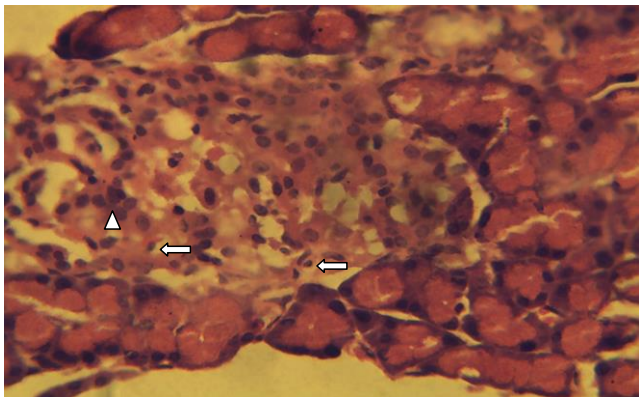

Figure 1d. Photomicrograph of sections of the pancreas from group 1 (diabetic rats treated with 100 mg/kg dry leaf extract) showing mild areas of vacuolations (arrows) and focal area of lymphocytic infiltration (arrowhead). H and E x 400.

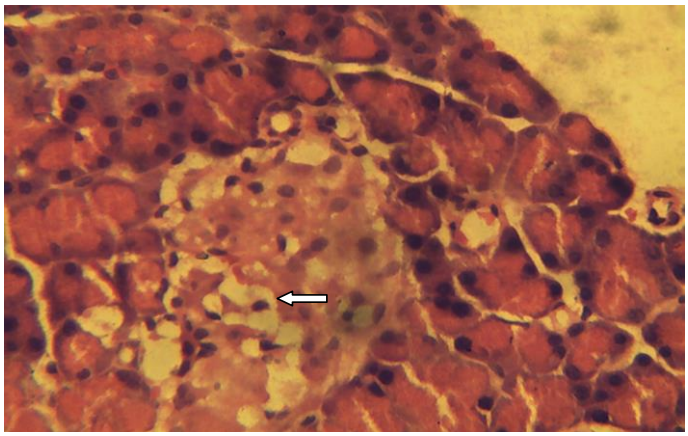

Figure 1e. Photomicrograph of sections of the pancreas from group 2 (diabetic rats treated with 200 mg/kg dry leaf extract) showing mild areas of vacuolations (arrows). H and E x 400.

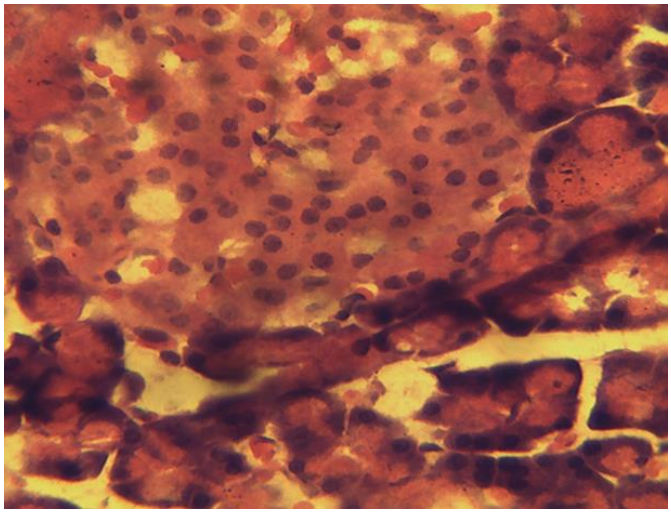

Figure 1f. Photomicrograph of pancreas section from group 5 (Control) showing apparently normal pancreatic cells. x 40.

Conclusion

The current study provides some useful insight into the antihyperglycemic potency of *J. curcas* leaves in alloxan induced diabetes. The shade dried and fresh aqueous extracts of *J. curcas* leaves used in this work showed strong anti-hyperglycemic effect as it significantly decreased the blood glucose level within the two weeks of administration. However, the shade dried and fresh aqueous extracts could not reverse the high blood glucose level observed after treatment to the initial baseline blood glucose level; this could be due to the aqueous extracts used or/and experimental duration (two weeks) at which the treatments were administered. In conclusion, *J. curcas* possess anti-diabetic property as already reported by other researchers but the aqueous plant extract may not be strong enough to revert completely diabetic condition under this experimental regime. Its mild toxicity is evident in the elevation of liver function enzyme (ALT and AST). However, some measure of processing may be required in order to reduce or eliminate its toxic potentials.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Evaluation of the antimicrobial activity of crude extracts and chromatographic fractions of *Adenanthera pavonina* Linn (Leguminosae) seeds

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Most available reports on the biological activity of *Adenanthera pavonina* (AP) are on the bark or leaves and very few are on the seeds. In particular, there are no reports on the biological effects of the chromatographic fractions of *A. pavonina* seeds hence the present study aimed to evaluate the antimicrobial activity of the crude extract and chromatographic fractions of *A. pavonina* seeds. The methanolic extract was fractionated and all of the column chromatographic fractions as well as the crude extract were evaluated against different strains of *Staphylococcus aureus*. The methanolic extract (100 mg/ml) produced zones of inhibition on PHM 002 and PHM 005 while the 50 and 100 mg/ml of the hexane extract only produced inhibition on PHM 001. On the other hand, fractions ST 10-12 F exhibited activity PHM 002 at 50 and 100 mg/ml while fractions ST-13-15F exhibited activity at all concentrations (6.25, 12.5 25, 50 and 100 mg/ ml) against *S. aureus* PHM 002 strain from the skin. The study provides some justifications for the folkloric use of AP seed powder as an antiseptic paste and warrants further studies to determine the structure of the active compound in chromatographic fraction ST 13 -15F.

Key words: *Adenanthera pavonina*, antimicrobial activity, chromatographic fractions, methanolic extract.

INTRODUCTION

Plants are valuable as sources of medicine. The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs (Rates, 2001). In the recent decades, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Vulto and Smet, 1988; Rates, 2001).

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This may be due to the fact that the conventional medicine can be inefficient (such as having side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems. Also, a large percentage of the world's population does not have access to conventional pharmacological treatment. Medicinal plants have served as valuable starting materials for drug development in both developing and developed countries. Today, more than 80% of the people living in Africa depend on medicinal plants and animal based medicines to satisfy their healthcare requirements (Yedjou et al., 2008). It has also been suggested that folk medicine and ecological awareness suggest that "natural" products are harmless. Many pharmaceutical products are from plants; though pharmaceutical industries have produced a number of new antibiotics in recent times, resistance by micro-organism remains a challenge. Action must therefore be taken to reduce this problem by controlling the use of antibiotics, developing research to better understand the mechanism of resistance and embarking on studies to develop new drugs either natural or synthetic (Ahmed et al., 1998; Hussain et al., 2011).

Adenanthera pavonina Linn (AP) is a medium to large deciduous tree of the family *Leguminosae* also called *Fabaceae*. The tree is common throughout the lowland tropics up to 300 to 400 m (Jayakumari et al., 2012). In terms of medicinal uses, it has been reported that bark and leaves are astringent, vulnerary and aphrodisiac and are used in ulcers, pharyngopathy and even the heart wood is astringent, aphrodisiac, haemostatic and is useful in dysentery, hemorrhages and gout (Vaidyaratnam and Variers, 1994). The root on the other hand is emetic in nature (Jayakumari et al., 2012). It has been reported that the plant have antiseptic and anti-inflammatory activities. The red powder is used as an antiseptic paste. Effects of *A. pavonina* include anti-inflammatory and analgesic from methanolic seed extract (Olajide et al., 2004) and ethanolic leaf extract (Mayuren and Ilavarasan, 2009); and antimicrobial from the bark extract (Ara et al., 2010). The blood pressure lowering effect of the seed extract has also been reported (Adedapo et al., 2009). The main important constituents are flavonoid compounds (Rastogi and Mehrotra, 1991). It is used as an antiseptic paste and also used to treat boils and inflammations (Chopra et al., 1956).

Most available reports on the biological activity of *A. pavonina* are on the bark or leaves and very few are on the seeds. In particular, there are no reports on the biological effects of the chromatographic fractions of *A. pavonina* seeds. Chromatography is the key to obtaining pure compounds for structure elucidation, for pharmacological testing or for development into therapeuticals. It also plays a fundamental role as an analytical technique for quality control and standardisation of phytotherapeuticals (Marston, 2007).

In this study, the chromatographic fractions of the seeds of *A. pavonina* are being explored for its antimicrobial properties with a view to determine the fraction responsible for this effect.

MATERIALS AND METHODS

Chemical and solvent

This includes: ammonia, acetone, Benedict's solution, chloroform, Dragendorff's reagents, distilled water, ethyl acetate, ferric chloride, hydrochloric acid, hexane, lead acetate, methanol, Mayer's reagent, potassium hydroxide, sodium hydroxide, sulphuric acid, toluene and Wagner's reagent.

Test organisms

Reference strains were used as suggested by Cos et al. (2006). These are different strains of *Staphylococcus aureus*; from the nose (strain 1) PHM 001, from the skin (strain 2) PHM 002, from the eye area (strain 3) PHM 003, from the armpit (strain 4) PHM 004 and the standard (strain 5) PHM 005. All microorganisms were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria.

Plant material

The seeds of *A. pavonina* were collected from the Staff School Compound in the University of Ibadan, Oyo State throughout the month of September 2009. They were authenticated at the Forestry Research Institute (FRIN), Ibadan where a voucher specimen was deposited.

Sample preparation

The seeds were air dried and powdered. The powdered seeds were extracted using cold maceration. The powdered seeds sample (1335.09 g) was put in a large flask and defatted for 24 h using 1280 ml- hexane. It was stirred occasionally and filtered. The marc was air dried and placed in a flask. The plant material was covered with 2000 ml of methanol and allowed to stand for four days after which it was filtered and the filtrate concentrated to dryness to yield 30.77 g of extract. The residue was again extracted with another 1,300 ml of methanol and the filtrate was again concentrated to dryness to yield a further 20.61 g of crude methanolic extract.

Phytochemical screening

The phytochemical analysis was performed on the ground (powered) leaf of *A. pavonina* for identification of the constituents. The constituents tested for were alkaloids, tannins, saponins, anthraquinones, cardiac glycosides and flavonoids as described by Shale et al. (1999), Moody et al. (2006) and Sawadogo et al. (2006).

Chromatographic analysis

Thin layer chromatography (TLC)

The TLC plates of dimensions 20 × 20 cm were used. The thickness of the adsorbent layer was 1 mm. Thin layer chromatography was used to monitor fractions obtained from

Table 1. TLC analysis of crude methanolic and hexane extract of *Adenanthera pavonina* seeds.

Extract	Solvent system	Colour in daylight	Colour in UV 365 nm	R_f Value
Hexane extract	SS4	Yellow	Blue	0.85
Hexane extract	SS1	Yellow	Colourless	0.69
Hexane extract	SS3	Yellow	Colourless	0.12
Hexane extract	SS2	Yellow	Yellow	0.43, 0.60
Methanolic extract	SS4	Yellow	Blue	0.64
Methanolic extract	SS1	Yellow	Blue	0.73
Methanolic extract	SS3	Yellow	Blue	0.17
Methanolic extract	SS2	Yellow	Blue	0.53, 0.67, 0.84

column chromatography fractionation of the crude methanolic extract as well as that of the crude hexane extract. The solvent systems used were: SS1-chloroform: methanol 9:1, SS2 toluene: acetone: ethyl acetate 8:2:1, SS3 chloroform: methanol 19:1, SS4 hexane: ethyl acetate 8:2, SS5 chloroform: methanol 7:3, SS6 toluene: acetone 4:1 and SS7 toluene: acetone: ethyl acetate 80:40:20. The plates used were examined under daylight and ultraviolet (UV) at 365 nm and were sprayed with Dragendorff to detect the presence of any alkaloid present (Harborne, 1984).

Column chromatographic separation

The methanolic extract was further subjected to silica gel chromatographic separation using standard procedures. The chromatographic column of length 50 cm and internal diameter 3.5 cm was rinsed with acetone and allowed to dry. It was then clamped vertically with a retort stand, and a plug of cotton wool was inserted at the bottom of the column using a clean glass rod. The column was filled with hexane, and the silica gel slurry was mixed with hexane in a beaker. The slurry was poured into the column and tapped intermittently for uniform distribution of the gel and to avoid bubbles in the column. The column was allowed to drain for some minutes to stabilize it. A portion (20.61 g) of the crude methanolic extract was subjected to chromatography in column silica gel, was eluted with solvent mixtures of increasing polarity and dissolved in methanol by warming on a water bath. The cooled methanolic extract was then introduced on top of the silica gel adsorbent in the column. The column was eluted with solvent mixtures of increasing polarity. Fractions were collected and monitored by thin layer chromatography using appropriate solvent systems. Fractions showing similar TLC patterns were pooled together (Li et al., 2012). Nutrient agar (28 g) was mixed with distilled water to make up 1 L. The mixture was heated to boiling on a hot water bath. The molten agar was poured into universal bottles and autoclaved for 30 min at 121 psi. Nutrient broth (4 g) was dissolved with distilled in a conical flask and was made up to 500 ml. The mixture was measured in 10 ml aliquots into test tubes and sterilized in an autoclave for 20 min at 121 psi. The methanolic extract and pooled chromatographic fractions were tested for antimicrobial activity using the reference organisms stated earlier. The respective organisms were sub-cultured into the nutrient broth from the stock. The overnight culture (0.2 ml) was inoculated into 10 ml of sterile distilled water in a test tube. From the diluted organism, 0.2 ml was taken and inoculated into the cooled nutrient agar and shaken for homogeneity. The sterilized agar was poured into Petri dishes and allowed to set. It was dried and bore holed with a cork borer of diameter 8 mm. Six holes were made on each plate. Different concentrations of the extracts and fractions of *A. pavonina* seeds were then introduced into the holes. The plates were allowed to stand for 30 min to achieve diffusion after which they were incubated for 24 h at 37°C (Gurib-Fakim, 2006; Jimoh et al., 2011).

RESULTS

The phytochemical screening of methanolic extract of the seeds of *A. pavonina* showed the presence of tannins, saponins, alkaloids, flavonoids and cardiac glycosides while Tables 1 to 6 show the TLC analysis of crude methanolic and hexane extract of *A. pavonina* seeds; column chromatographic separation of the crude methanolic extract of *A pavonina* seeds; TLC analysis of column chromatographic fractions of the methanolic extract of *A. pavonina* seeds using mobile phase SS5 (chloroform: methanol 7:3); TLC analysis of column chromatographic fractions of the methanolic extract of *A. pavonina* seeds using mobile phase SS7 (toluene: acetone: ethylacetate 80:40:20); TLC analysis of column chromatographic fractions of the methanolic extract of *A. pavonina* seeds using mobile phase SS6 (toluene: acetone 4:1) and anti-microbial screening of the crude methanolic extract, hexane extract and chromatographic fractions of *A. pavonina* seeds, respectively. Column chromatographic analysis of the methanolic extract of *A. pavonina* seeds resulted in 15 fractions on elution with different solvent mixtures. Similar fractions were bulked on the basis of thin layer chromatographic analysis. Fraction 15 was found to be the one with highest yield providing enough material for anti-microbial evaluations. TLC analysis of the fractions was done using three different solvent systems. Solvent system 6 (toluene: acetone 4:1) was found to be the most appropriate among the solvent systems as it gave the best resolution of the components. The most suitable solvent system for TLC analysis of the crude methanolic and hexane extracts, solvent system 2 (toluene: acetone: EtOAc 8:2:1) which found to be most appropriate gave 3 spots (Rf values: 0.53, 0.67, 0.84) for the methanolic extract and 2 spots (Rf values: 0.43, 0.60) for the hexane extract. The methanolic extract (100 mg/ml) produced zones of inhibition on PHM 002 and PHM 005 while the 50 and 100 mg/ml of the hexane extract only produced inhibition

Table 2. Column chromatographic separation of the crude methanolic extract of *Adenanthera pavonina* seeds.

Fraction collected	Solvent mixture	Volume collected (ml)	Yield (g)
ST 1F	Hex: Chloroform 70:30	25	
ST 2-4F	Hex: Chloroform 60:40	25	1.955
	Hex: Chloroform 60:40	25	
	Hex: Chloroform 60:40	25	
ST 5-7F	Hex: Chloroform 60:40	25	3.731
	Hex: Chloroform 60:40	25	
	Hex: Chloroform 60:40	25	
ST 8-9F	Hex: Chloroform 60:40	25	3.275
	Hex: Chloroform 40:60	25	
ST 10-12F	Methanol: Chloroform 10:90	25	3.451
	Methanol: Chloroform 10:90	25	
	Methanol: Chloroform 20:80	25	
ST 13-14F	EtoAC: Methanol 80:20	25	1.437
	EtoAC: Methanol 50:50	25	
ST 15F	100% Methanol	25	4.344

Table 3. Thin layer chromatography (TLC) analysis of column chromatographic fractions of the methanolic extract of *Adenanthera pavonina* seeds using mobile phase SS5 (chloroform: methanol 7:3).

Fraction	Colour in daylight	Colour in UV 365 nm	R_f Value
ST 2-4F	Colourless	Reddish-brown	
ST 5-7F	Blue	Reddish-brown	0.87
ST 8-9F	Reddish-brown	Yellow	0.80
ST 10-12F	Reddish-brown	Yellow	0.95
ST 13-14F	Reddish-brown	Yellow	0.87
ST 15F	Reddish-brown	Yellow	-

Table 4. Thin layer chromatography (TLC) analysis of column chromatographic fractions of the methanolic extract of *Adenanthera pavonina* seeds using mobile phase SS7 (toluene: acetone: ethylacetate 80:40:20).

Fraction	Colour in daylight	Colour in UV 365 nm	R_f
ST 2-4F	Reddish-brown	Yellow	0.93
ST 5-7F	Yellow	Colourless	
ST 8-9F	Reddish-brown	Colourless	
ST 10-12F	Reddish-brown	Colourless	
ST 13-14F	Yellow	Colourless	
ST 15F	Yellow	Colourless	

on PHM 001. On the other hand, only fractions ST 10-12 F and ST 13-15 F produced inhibition. The results of chromatographic analysis and antimicrobial assay are as shown in the Tables 1 to 6.

Table 5. Thin layer chromatography (TLC) analysis of column chromatographic fractions of the methanolic extract of *Adenanthera pavonina* seeds using mobile phase SS6 (Toluene: Acetone 4:1).

Fraction	Colour in daylight	Colour in UV 365nm	R_f
ST 2-4F	Yellow	Blue	0.27
ST 5-7F	Yellow	Reddish-brown	0.28
ST 8-9F	Yellow	Reddish-brown	0.15
ST 10-12F	Yellow	Reddish-brown	0.38
ST 13-14F	Yellow	Reddish-brown	0.13
ST 15F	Yellow	Reddish-brown	0.07

Table 6. Anti-microbial screening of the crude methanolic extract, hexane extract and chromatographic fractions of *Adenanthera pavonina* seeds.

Extract/fraction	Microorganism / Zone of inhibition (mm)				
Concentration mg/ml	***Staph aureus* PHM 001**	***Staph aureus* PHM 002**	***Staph aureus* PHM 003**	***Staph aureus* PHM 004**	***Staph aureus* PHM 005**
Methanolic extract					
100	-	9	-	-	14
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin	28	18	12	28	20
Hexane extract					
100	12	-	-	-	-
50	10	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin	28	13	12	24	22
ST 2-7 F					
100	-	-	-	-	-
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin	22	13	10	11	15
ST 10 -12 F					
100	-	4	-	-	-
50	-	3	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin					
ST 13 -15 F					
100	-	20	-	-	-
50	-	11	-	-	-

Table 6. Contd.

25	-	10	-	-	-
12.5	-	8	-	-	-
6.25	-	7	-	-	-
Gentamicin	22	20	5	11	10

Gentamicin 500 ug/ml – Positive control. Diameter of cork borer – 8 mm.

DISCUSSION

Phytochemical screening of *A. pavonina* seed showed the presence of tannins, alkaloids, flavonoids and cardiac glycosides. Saponins are of great pharmaceutical importance because of their relationship to compounds such as sex hormones, cortisones, diuretics, steroids, vitamin D, and cardiac glycosides. Flavonoids have influence on arachidonic acid metabolism, thus could have anti-inflammatory, anti-allergic, anti-thrombotic or vasoprotective effects. Flavonoids and tannins are phenolic compounds and plant phenolics are also a major group of compounds that acts as primary antioxidant or free radical scavengers (Ayoola et al., 2008). Tannins and saponins are also found to be effective anti-oxidants, antimicrobial and anti-carcinogenic agents (Lai et al., 2010). Flavonoids are known to target prostaglandins which are observed in the late phase of acute inflammation and pain perception (Chakraborty et al., 2004). Cardiac glycosides are steroidal glycosides which exert a slowing and strengthening effect on a failing heart. In fact cardiac glycosides are being tested for their anticancer properties (Chen et al., 2006; Frese et al., 2006; Newman et al., 2008). Thin layer chromatographic screening of *A. pavonina* seed crude extract and column chromategraphic fractions showed the presence of several alkaloid substances on spraying with Dragendorff reagent. Alkaloids are generally quite bitter and many are toxic (Takimoto and Calvo, 2008). Some alkaloids such as vinca alkaloids have been used to treat diabetes, high blood pressure, and the drugs have even been used as disinfectants. However, the vinca alkaloids are most famous for being cancer fighters (Takimoto and Calvo, 2008). The implications of all these is that this plant is of great medicinal importance.

Column chromatographic analysis of the methanolic extract of *A. pavonina* seeds resulted in 15 fractions on elution with different solvent mixtures. Similar fractions were bulked on the basis of thin layer chromatographic analysis. Fraction 15 was found to be the one with highest yield providing enough material for anti-microbial evaluations. Anti-microbial activities of the extract and components from *A. pavonina* seeds showed that only fractions ST 13 - 15 F was able to inhibit the growth of *S. aureus* strain 2 isolated from the human skin as all concentrations used. The methanolic extract was effective only at the highest concentration (100 mg/ml) against *S. aureus* strain 2 and *S. aureus* strain 5. It was ineffective at all other concentrations against other strains. The hexane extract was effective at concentrations 50 and 100 mg/ml against *S. aureus* strain 1 isolated from the nose alone, while it was inactive against all other strains of *S. aureus* at all concentrations. Fraction ST 2-7F showed no activity against any of the *S. aureus* strains, while fraction ST 10-12F was active against *S. aureus strain* 2 only at 50 and 100 mg/ml. Flavonoids, alkaloids, saponins and tannins have been reported to be active against several bacteria and fungi (Hassan et al., 2004). The presence of these in the crude extract of *A. pavonina* could be responsible for the antibacterial activity exhibited by the crude extract and components from *A. pavonina* in this study. The standard reference, gentamicin 500 µg/ml was able to inhibit all strains of *S. aureus*. Based on the results obtained from this study, there was preliminary evidence that there reside in the extract and components from *A. pavonina* seed, compounds with antibacterial activity against isolates of *S. aureus*. There is a need for further studies to determine the ED_{50}, as well as to isolate and characterize the constituents from the active fractions of this plant.

Conflict of interests

The authors did not declare any conflict of interest.

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Bioepoxidation of isosafrol catalyzed by radish and turnip peroxidases

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Peroxidases (PODs) from radish (*Raphanus sativus* L.) and turnip (*Brassica napus* L.) were extracted and precipitated with ammonium sulfate using a simple, low cost and quick method. The activities of all steps performed by the vegetable PODs were measured via guaiacol assay. The epoxidation of isosafrol, catalyzed by radish (*R. sativus L.*) and turnip (*B. napus* L.) peroxidases was conducted in 20% (v/v) aqueous ethanol solution using 30% (v/v) H_2O_2 as the terminal oxidant. High conversion (88%) and selectivity (>98%) were obtained after 48 h. The products of the reaction were analyzed by high resolution gas chromatography (GC) and mass spectrometry.

Key words: *Raphanus sativus* L, *Brassica napus* L., peroxidase, epoxidation, isosafrol.

INTRODUCTION

Peroxidases (PODs) are heme proteins involved in the oxidation of a wide variety of organic and inorganic substrates that use H_2O_2 or organic peroxides as terminal oxidants (Hamid and Rehman, 2009; Veitch, 2004). PODs can be found in multiple isoforms in several species of fruits and vegetables, and are related to changes in flavor, texture and color, during post-harvest ageing and/or the processing of vegetables and fruits (Lopes et al., 2015). In spite of being ubiquitous in nature, horseradish (*Armoracia rusticana*) is the only commercial source of these enzymes. The commercially available horseradish peroxidase (HRP) is normally used in immunoassays, diagnostic kits (Veitch, 2004) and for development of biofuel cells (Ramanavicius et al., 2015; Ramanavicius and Ramanaviciene, 2009), but it is expensive due to its elevated purification costs. Many reactions catalyzed by HRP can be found in the literature: in addition, demethylation, epoxidation, hydroxylation,

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Abbreviations: HRP, Horseradish peroxidase; **GC,** gas chromatography; **POD,** peroxidases.

polymerization of phenolic compounds (Cheng and Harper Jr, 2012; Savic et al., 2013; Gilabert et al., 2004), electro-oxidation of phenol by heterogeneous catalysis (Carvalho et al., 2007) and the oxidation of bisphenol, which is a common industrial pollutant (Hong-Mei and Nicell, 2008). Hydroxylation and epoxide production are conducted by monooxygenases or peroxidases, which have biological functions that control the transfer of one oxygen atom from the dioxygen or H_2O_2 to an organic compound (Lin et al., 2011). Several studies reported the selective oxidation of alkenes using these biocatalysts (García-Granados et al., 2004; Hirata et al., 1998, Kim et al., 2007).

Epoxides are relevant compounds in the pharmaceutical industry, as they are important synthetic intermediates (Liang et al., 2004; Choudhary et al., 2004; Lambert et al., 2005; Piovezan et al., 2005). This fact is due to the versatility of the oxirane function, which can be converted into numerous chemicals with biological activity (Archellas et al., 1997). One important example is the oxidation of isosafrol, which products epoxides, that also can be converted to aldehyde (piperonal for example), an intermediate on the route to L-Dopa (Santos et al., 2004), used in the treatment of Parkinson's disease, and to α-methyldopa, used as an anti-hypertensive agent (Gu et al., 2012).

Chemical methods to synthesize epoxides are generally based on heavy metal catalysis and/or the use of stoichiometric reagents, such as *m*-chloroperbenzoic acid (MCPBA), which generate highly polluting chlorinated byproducts (Costas et al., 2000). Some reports in the literature describe the catalytic oxidation of isosafrol in the presence of 30% H_2O_2 or other oxidants with vanadium catalysts at reflux. These conditions promote the cleavage of the double bond (C=C) to form the corresponding aldehydes and epoxides (Alvarez et al., 2006; Alvarez et al., 2007). However, this reaction was not yet reported using plant POD catalysis. The study of olefin epoxidation mediated by peroxidases under mild conditions is of great interest for the synthesis of chiral building blocks.

In this work, peroxidases from radish (*Raphanus sativus* L.) and turnip (*Brassica napus* L.) were extracted and concentrated by precipitation with ammonium sulfate using a simple, low cost and quick method. These partially purified protein fractions were used in the epoxidation of isosafrol in 20% (v/v) aqueous ethanol solution using 30% (v/v) H_2O_2 as the terminal oxidant.

MATERIALS AND METHODS

Chemicals

Isosafrol (a mixture of isomers) was obtained from "Geroma do Brasil" (PR/Brazil) and consists of isosafrol *Z* (15%) and isosafrol *E* (majority species, 85%). All solvents were purchased from Vetec (Brazil) as PA grade. All other chemicals used for the broth media were of analytical grade and purchased from Sigma–Aldrich (USA). *m*-chloro-perbenzoic acid (MCPBA) was purified according to the methodology already established in the literature. Briefly, the peracid was dissolved in ether and washed with buffer solution (410 mL 0.1 M NaOH, 250 mL 0.2 M KH_2PO_4 made up to 1 L, pH 7.5). The ether layer was dried over Na_2SO_4 and carefully evaporated under reduced pressure.

Preparation of the raw extract

Turnip and radish were obtained at a local market, and were washed, pilled, homogenized and stored in freezer in fractions of 25 g up to the experiments. Extraction of the crude enzyme was carried out according to the procedure described in the literature (Fricks et al., 2006; Fricks et al., 2010). The vegetable (25 g) was homogenized with 50 mL of 100 mM phosphate buffer $Na_2HPO_4 \cdot 2H_2O$ (pH 6.5). The extract was filtered and centrifuged at 2300 x *g* (5000 rpm) for 60 min at 4°C. The supernatant solution, which contained the enzymes, was stored at 4°C.

Precipitation and determination of proteins

21 g of $(NH_4)_2SO_4$ was slowly added to a volume of 30 mL of the raw extract, reaching up to 85% saturation. After the dissolution of the salt, the solution was placed in the freezer at -18°C for 1 h. Next, the solution was centrifuged at 2300 x *g* (5000 rpm) for 40 min at 4°C and the supernatant was discarded. The precipitate was dissolved in around 5 mL of 100 mM phosphate buffer $Na_2HPO_4 \cdot 2H_2O$, pH 6.0 and was used as a source of peroxidases. The total concentration of proteins obtained in the solutions was determined by the Bradford method, using bovine serum albumin as standard (Bradford, 1976).

Determination of peroxidase activity

The enzymatic activity of peroxidases was determined by a colorimetric method based on the change of absorbance at 470 nm due to the formation of tetraguaicol, the product of guaiacol oxidation (Fricks et al., 2006; Fricks et al., 2010). Peroxidase assay medium was composed of 2.78 mL of 100 mM phosphate buffer (pH 6.0), 0.02 mL of enzyme (previously diluted 20 x), 0.1 mL of the 100 mM guaiacol solution and 0.1 mL of 2.0 mM H_2O_2 solution at 25°C. One unit of enzyme (U) was defined as the quantity of enzyme capable of forming 1 µmol of product in a minute at 25°C and pH 6.0, $\varepsilon_{tetraguaiacol}$= 26.6 $mM^{-1}cm^{-1}$ (Hirata et al., 1998). The reaction progress was followed with a UV-Vis UV-HP8452-Diode array spectrophotometer. Control experiments were carried out in the absence of peroxidases.

Stability test of the enzyme in organic solvents

Aqueous solutions of ethanol and acetonitrile were prepared with concentrations of 20, 40 and 60% (v/v). The enzymatic samples (0.1 mL) were incubated in 25 mL of the ethanolic solution and 0.9 mL of 0.1 M guaiacol solution. At certain time intervals, aliquots (2.9 mL) were collected and added to a 2 mM solution (0.1 mL) of H_2O_2 to start the enzymatic reaction. Thus, the residual activity of the enzyme pre-incubated in the aqueous solutions of ethanol was determined. An analogous methodology has been described in the literature (Azevedo et al., 2003).

Standard oxidation reaction

10 mL of dry solvent, 0.06 or 0.08 mmol of dry *m*-chloro-perbenzoic

Table 1. Radish and turnip peroxidases activities.

Parameter	Radish		Turnip	
	Raw extract	Precipitation $(NH_4)_2SO_4$	Raw Extract	Precipitation $(NH_4)_2SO_4$
Total protein (mg)	21.6±0.55*	11.3±0.14	44.2±0.24*	15.0±0.36
Specific activity (U/mg)	13.3±0.35	20.1±0.56	41.1±2.51	36.7±1.84
Total activity (U)	96.0±2.82	76.0±3.73	605±10.08	212±5.00
Recovery of activity (%)	100.0±5.00	78.0±2.51	100±5.44	35±2.47

*mg protein/ g tissue: radish (1.72±0.12) and turnip (3.54±0.21).

acid (MCPBA) and 0.04 mmol of isosafrol were stirred in a 20 mL flask under an inert atmosphere for 48 h at room temperature. Next, the reaction medium was washed with a $NaHCO_3$ solution to eliminate excess MCPBA. The reaction products were extracted with CH_2Cl_2 and the organic phase was treated with anhydrous Na_2SO_4 and subjected to chromatographic analysis (GC).

Biotransformation by POD *R. sativus* L. and/or POD *B. napus* L.

0.04 mmol of isosafrol, 20 µL of the enzymatic solution and 0.04 mmol of 30% (v/v) H_2O_2 were added to 10 mL of 20% (v/v) ethanol solution. The reaction medium was stirred (at 120 rpm) for 48 h at 25°C. After the medium was extracted with dichloromethane and dried with anhydrous Na_2SO_4, the reaction products were analyzed by GC and GC-MS.

Methods for identification and quantification of substrate and product

Reactions were monitored by high resolution gas chromatography. An HP5890 chromatograph with an HP WCOT (25 m x 0.32 mm ID) column was used in this study. H_2 was used as a carrier gas at a flow rate of 3 mL/min (96 cm/seg), with a pressure of 20 psi. The initial temperature was 100°C and the final temperature was 250°C, with a ramp rate of 3°C/min. The injector was held at 150°C and the detector at 240°C. The injection was operated in splitless mode for 0.2 µL of the injected solution. Retention times of authentic standards and their respective retention indices were obtained from a mixture of homologous hydrocarbons and used as identification parameters. Selectivity values for each product were calculated from GC data, using the products peak area, according to the following expression:

Selectivity (%) = (area peak of the product / total area peak of the products formed) * 100

Mass spectrometry was employed to confirm the identification of the product through the use of electronic libraries and published data. The analysis was performed in a HP5973 gas chromatograph connected to a HP5972 mass spectrometer, with ionization by electronic impact at 70 eV (1 scan/min, acquisition m/z: 40-400). H_2 was used as a carrier gas, with speed of 1.0 mL/min in accordance with the conditions already described.

RESULTS AND DISCUSSION

Activity assays of radish and turnip PODs were performed based on previous experience (Lopes et al., 2015), through the reaction of a guaiacol/H_2O_2 (100 mM) system. Guaiacol was selected as a standard substrate for peroxidase activity monitoring. In recent study Kumar and co-authors showed that a plant peroxidase (*Euphorbia cotinifolia*) has maximum activity with guaiacol as reducing substrate compared with pyrogallol, dianisidine-dihydrochloride, *o*-phenelene diamine, α-aminopterin and phloroglucinol (Kumar et al., 2011). Table 1 presents the results of extraction and pre-purification of PODs from radish and turnip. The main reason for performing the precipitation of proteins from the crude extract with ammonium sulfate at 85% saturation was allowed to concentrate the vegetable peroxidases in small volumes with an easy by easily and practical method, thus reducing the volume of peroxidase solution in the epoxidation medium. 30 ml of crude extract of each plant provided 4.5 and 6.0 ml of radish and turnip precipitate, respectively. For radish POD, 78% of the enzyme was precipitated, value indicated by the recovery of the activity. However, for turnip POD, a low recovery level was observed (35%). In terms of purification, It should be noted that the precipitation of the radish raw extract with $(NH_4)_2SO_4$ was efficient, due to the increased in the specific activity (13.3 to 20.1 U/mg, purification factor 1.51) with good recovery level (78%). However, a decrease in the specific activity (41.1 to 36.7 U/mg) was observed for turnip, which indicates that part of the POD turnip activity was lost during the process.

Biochemical systems involving aqueous/organic media and mild conditions are of extreme importance due to an increased demand for environmentally friendly processes. The possibility of using peroxidases in organic solvents enhances their application in the oxidation of hydrophobic molecules. Figure 1 shows that while both extracts retain part of their original activity in aqueous ethanolic mixtures, a decrease is observed at the beginning of the exposure time. After 5 h of treatment, the activity remains constant. Higher organic solvent concentrations lead to a decrease in enzyme activity. The partially purified protein fraction of the radish extract indicated that around 50 and 15% of its initial activity is preserved after 26 h of incubation in solutions of 20 and 40% (v/v) of ethanol/water solution, respectively. The

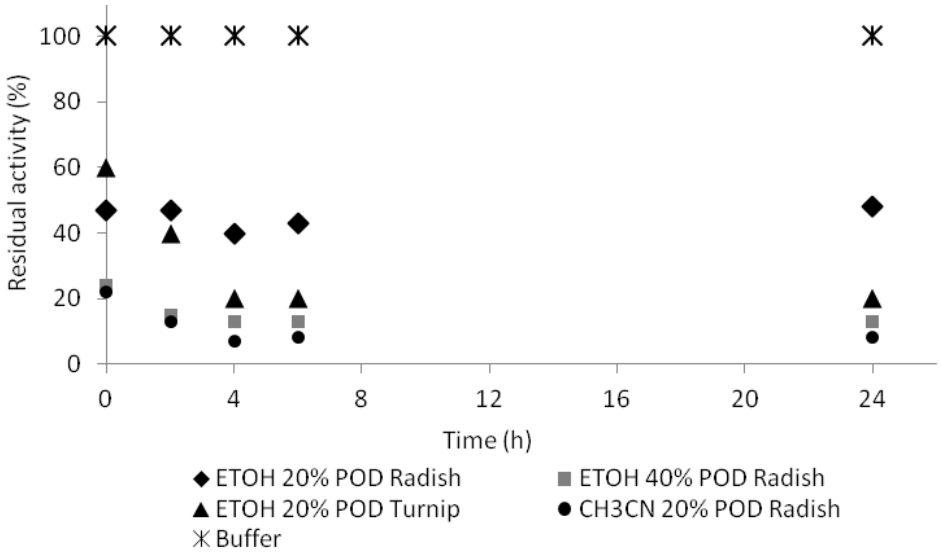

Figure 1. Residual activity of radish and turnip peroxidases in ethanol (ETOH 20 to 40% and acetonitrile (CH_3CN 20%) (v/v). In all points the deviation was less than 5%.

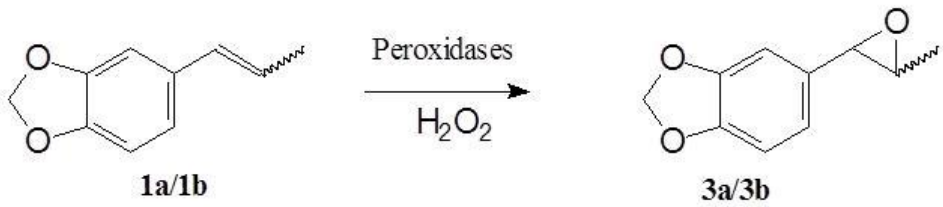

Figure 2. Microbiological oxidation of isosafrol (1a/1b) to 3a/3b.

same phenomenon was observed in the protein fraction from the turnip extract. After 26 h of incubation in 20% (v/v) ethanol/water solution, the residual activity was around 20% of the original, and virtually zero in 40% (v/v) ethanol/water (not shown in Figure 1). Radish POD extract incubated in 20% aqueous acetonitrile solution showed activities below 10% of the original activity. The results are in agreement with literature: PODs are active in organic solvents, and they have been used to catalyze the polymerization of phenolic compounds for example (Eker et al., 2009; Ryu and Dordick, 1992). In polyphenol synthesis, HRP was shown to be most stable in ethanol solutions around 20 to 40%, as higher ethanol concentrations induced a loss of activity (Ayyagari et al., 2002). Some studies in the literature indicated that HRP is more stable in polar than non polar solvents, and that sub saturated hydration levels cause a decrease in the catalytic efficiency of enzymes (Ryu and Dordick, 1992). Also, the literature shows that heme peroxidases may also have catalytic activity in non-native states (Lin and Wang, 2013).

Furthermore, large amounts of oxidant may inactivate the enzyme (Van der Velde et al., 2001). Therefore, the proportion of organic solvent, the quantity and speed of the addition of oxidant and the reaction time must be monitored to ensure enzyme activity (Azevedo et al., 2003; Santos et al., 2003; Santos et al., 2004). The epoxidation of isosafrol (1) was conducted (Figure 2) at room temperature (298 K), using the partially purified protein fractions from the extracts. Epoxidation with MCPBA was also performed to afford a direct comparison of the epoxidation with POD extracts. Blank tests showed that the substrate was not oxidized in the absence of extracts. Table 2 shows the results obtained in the experimental runs. Epoxidation with MCPBA as an oxidant gave low conversions (max. 44%) and selectivities (max. 71%) under the same experimental conditions. In addition to the epoxide, there was presence of glycol, derived from isosafrol, and piperonal, with maximum selectivities of 15 and 14%, respectively (Table 2, entry 5). According to the literature, the conventional epoxidation process utilizes acid to elicit oxygen transfer to double bonds, resulting in low yields due to side reactions such as the acid-catalyzed ring opening of oxiranes (Kim et al., 2007). In the order hand, the enzymatic epoxidation provides a mild and simple alternative, especially for the production of sensitive epoxides. The best result for the epoxidation of isosafrol

Table 2. Description of catalytic systems to oxidize Isosafrol 1 (0.04 mmol), 25°C.

#	Catalyst	Oxidant (mmol)	Solvent (10 mL)	Time (h)	Conversion (%)[a*]	Selectivity (%)			
						Epoxide	Glycol	Piperonal	By products
1	Radish -**Ia** (1,0 U)	H_2O_2 30% (0.04)	20% C_2H_5OH / H_2O	48	88	> 98	-	-	-
2	Turnip - **Ia** (1,8 U)	H_2O_2 30% (0.04)	20% C_2H_5OH / H_2O	48	7	> 98	-	-	-
3	-	MCPBA (0.06)	CH_2Cl_2	48	14	63	22	-	10
4	-	MCPBA (0.06)	CH_3CN	48	32	71	18	8	-
5	-	MCPBA (0.08)	CH_3CN	48	44	70	15	14	-

[a*]Determined by GC. Piperonal and glycol had retention times of 6.75 and 10.8 min, respectively.

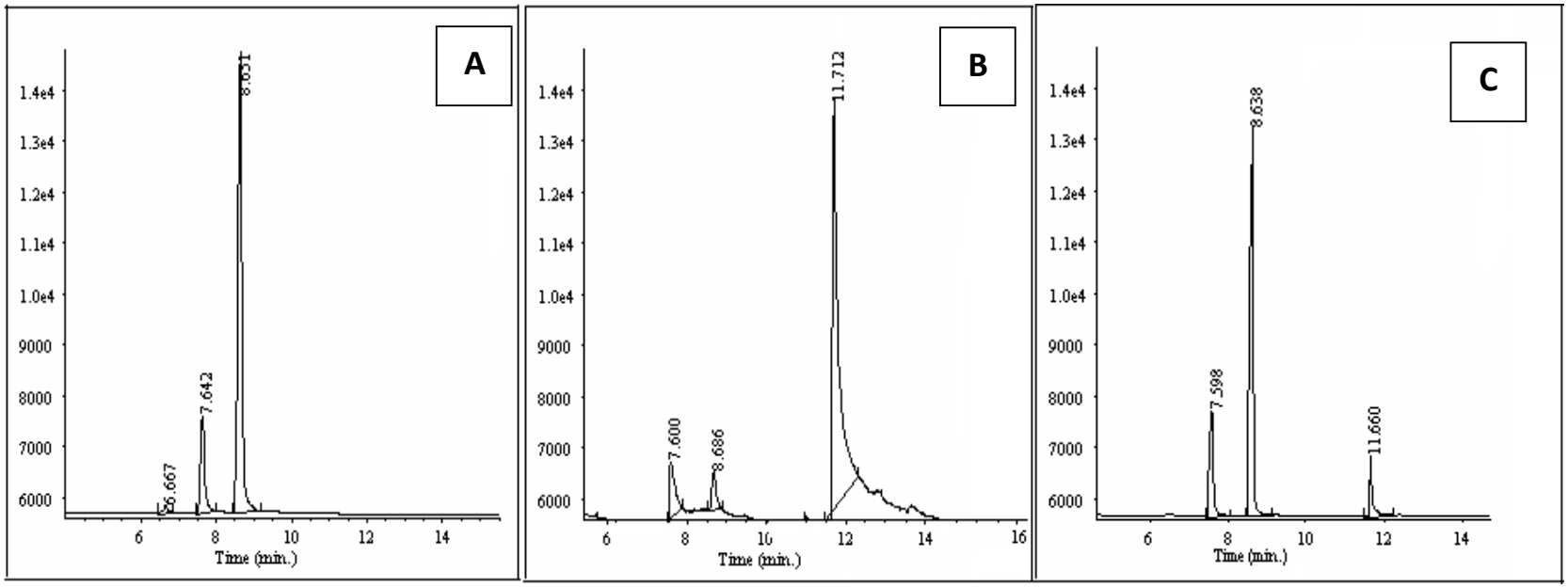

Figure 3. Chromatograms of the GC analysis of the reaction products. A) Control reaction. Retention times: Z-isosafrol (7.64 min) and E-isosafrol (8.65 min). B) Catalysis by POD *Raphanus sativus* L. (entry 1). C) Catalysis by POD *Brassica napus* (entry 2). Peak at 11.7 min is attributed to epoxide 3 (oxirane), which is validated by the mass spectrum.

was obtained with the POD extract obtained from radish as the catalyst, with the production of 3-methyl-[3´,4´-methylenedioxiphenyl]-oxirane 3 as the sole product (88% conversion and 98% selective for forming the epoxide). POD derived from turnip resulted in lower conversions of the reactant (7%), likely due to its lower stability in alcohol compared to radish POD, but with high selectivity for the epoxide (greater than 98%). Figure 3 presents the chromatograms of the GC analysis of the reaction products for the control (Figure 3A) and POD-catalyzed runs (Figure 3B,

C). Peaks derived from the isosafrol isomers are identified at 7.64 min (Z isomer) and 8.65 min (E-isomer). Control sample analysis showed only the presence of the isosafrol isomers (Figure 3A). The peak at 11.7 min is attributed to epoxide 3 (oxirane), which is validated by the mass spectrum.

The results indicate that it is possible to obtain higher conversions and selectivity with the use of plant POD as a catalyst for the epoxidation of isosafrol. In comparison, epoxidation using other plant peroxidases as catalyst show low yield. Hirata and colleagues performed the epoxidation of styrene using peroxidase from *Nicotiana tabacum*, reaching maximum yield of only 7.5% using *cis*-2-methylstyrene as substrate (Hirata et al., 1998). Our group report the oxidation of *E*- and *Z*-4-(1-propenyl)-1,2-methylenedioxybenzene (*E*- and *Z*-isosafrole) into 4-carboxaldehydro-1,2-methylene-dioxybenzene (piperonal) using different strains of *Aspergillu*s, *Cladosporiu*m, *Peacilomyces* and *Pseudomona*s. These microorganisms are able to oxidize the above compounds to piperonal, in the presence of H_2O_2, but not in its absence, indicating that this biotransformation is catalyzed by peroxidases in these microorganisms (Santos et al., 2003; Santos et al., 2004). Also, heme-monooxygenases (P-450 CIT), ω-monooxygenases and methane monooxygenases are capable of catalyzing an epoxidation reaction (Archellas and Furstoss, 1997). Some authors have also reported the oxidation of olefins using chloroperoxidase (CPO) (Allain et al., 1993). Enzymes from other sources, such as *Coprinus cinereus* peroxidase, myeloperoxidase (Tuynman et al., 2000) and chloroperoxidases (Dexter et al., 1995; Hu and Hager, 1999), are capable of catalyzing epoxidation both mildly and selectively.

Conclusion

Peroxidases from radish (*R. sativus* L.) and turnip (*B. napus* L.) were extracted and precipitated with ammonium sulfate. By this methodology only radish POD was pre-purified (purification factor 1.51). The protein fractions from the radish and turnip extracts applied in the epoxidation of isosafrol in 20% (v/v) aqueous ethanol solution using 30% (v/v) H_2O_2 as the terminal oxidant are effective catalysts to epoxidize isosafrol with high selectivity (> 98); but only with POD derived from radish, excellent chemical conversion is observed (88%).

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Extra-pulmonary oxidative stress investigations of an over-the-counter pyrethroid insecticide product in rats

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Extra-pulmonary oxidative stress investigations of exposure to aerosol, of an over-the-counter pyrethroid insecticide product in Nigeria in Wistar rats were carried out. Four groups of five animals each were used in this study, and were exposed to different concentrations of the insecticide aerosol. Malondialdehyde (MDA) and reduced glutathione (GSH) concentrations were determined in the liver, kidney and testes, while the activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) were determined in liver only. Liver, kidney and testes showed no significant difference in their weights and relative weights when compared with control, except groups II and III where significant increase in liver weight was recorded. There was no significant increase in liver, kidney and testis MDA concentration when compared with the control. There was significant decrease in testis GSH for all groups, while significant increase was seen in groups III and IV for kidney GSH and group II only for liver GSH. The activities of liver CAT, SOD, GST and GPx were reduced, but showed no significant difference when compared with the control. Our investigations therefore reveal that the compositions of the pyrethroid insecticide product may not play any role in extra-pulmonary tissue oxidative damage.

Key words: Insecticide aerosol, pyrethroid, oxidative stress, lipid peroxidation, rats.

INTRODUCTION

Eliminating mosquito vectors of the malaria parasite is one of the most effective measures against malaria. Pyrethroids are human-made forms of pyrethrins. There are two types that differ in chemical structure and symptoms of exposure. Type I pyrethroids include allethrin, tetramethrin, resmethrin, d-phenothrin, bioresmethrin and permethrin (Klaassen et al., 1996; Ray, 1991). Some examples of type II pyrethroids are cypermethrin, cyfluthrin, deltamethrin, cyphenothrin, fenvalerate, and fluvalinate (Klaassen et al., 1996; Ray, 1991). They act primarily on the nervous system by prolonging the open state of voltage-sensitive sodium channels (Soderlund et al., 2002). Since the nervous system of all the animals is quite similar, these

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compounds have the potential for non-target species (Soderlund et al., 2002).

Pyrethroid insecticide products are commonly used in the control of mosquitoes in Nigeria. The active insecticidal ingredients of pyrethroid insecticide product used in this study include cyfluthrin, imiprothrin and prallethrin. β-Cyfluthrin is a newer pyrethroid insecticide used all over the world. It is reported to be neurotoxic (Satpathy et al., 1997), hepatotoxic (Omotuyi et al., 2006) and teratogenic (Soni et al., 2011). β-Cyfluthrin toxicity is exhibited by its metabolites which in turn generate free radicals (El-Demerdash, 2007). *In vitro* study on human erythrocytes has demonstrated that cyfluthrin generates the reactive oxygen species (ROS) in cells (Sadowaska-Woda et al., 2010).

A study of imiprothrin in rats has indicated that repeated non-contiguous inhalation of an insecticide that contains imiprothrin can have immunotoxic effects in sites distal to the lungs (Emara and Draz, 2007). In human volunteers who were exposed regularly to prallethrin containing mosquito repellent, alterations in the biochemical composition of erythrocyte membranes, erythrocytic osmotic haemolysis, and plasma levels of nitrite and nitrate were found (Narendra et al., 2007).

In addition to the active insecticidal ingredients, insecticide aerosols also contain ‘‘inert’’ ingredients which are referred to as “trade secrets” by their manufacturers. Inert ingredients may not be inert in the usual sense of the word; often they are not chemically, biologically or toxicologically inert. Occupational exposure to these solvents has been associated with an increased rate of leukemia and a range of lymphoma types (McMichael, 1988; Cocco et al., 2010).

Pesticides induce oxidative stress as well as alter the defense mechanisms of detoxification and scavenging enzymes (Rasoul et al., 2012; Mossa et al., 2012; Mansour and Mossa, 2010a, 2011; Marzouk et al., 2011). Overproduction of ROS can exacerbate oxidative stress and ROS generation has been linked to a number of disease processes, such as heart disease (Giordano, 2005), diabetes (Rolo and Palmeira, 2006), liver injury (Mansour and Mossa, 2010a, 2011; Mossa, 2004; Jaeschke, 2000), cancer (Klaunig and Kamendulis, 2004) and aging (Bokov et al., 2004). It is therefore necessary to maintain the balance between ROS generation and antioxidant enzymes. This balance has been suggested to have an important role in preventing pesticides toxicity (Mansour and Mossa, 2010a, 2011; Mossa, 2004; Jaeschke, 2000).

There is a widespread misuse, incessant and inadvertent exposure to pyrethroid insecticide products in Nigeria, and there is still a dearth of information on the toxicological consequences of frequent exposure. In this study, we therefore assessed the risk and the hazard associated with exposure to aerosols of a commonly used over-the-counter pyrethroid insecticide product, using rats as an experimental animal model. We investigated if exposure to the insecticide could lead to extra-pulmonary oxidative damage by monitoring the activities of liver catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST), as well as concentrations of reduced glutathione (GSH) and malondialdehyde (MDA) in liver, kidney and testes of rats.

MATERIALS AND METHODS

Animals, test materials and reagents

Twenty albino Wistar rats with an average weight of 150 g used for this study were obtained from the animal house of the College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria. Baygon^{TM} multi-purpose pyrethroid insecticide product of Johnson Wax Nigeria Limited, Lagos (composition Cyfluthrin, 0.015%; Imiprothrin, 0.05%; Prallethrin, 0.05%; and undisclosed inert ingredients, 99.885%) was purchased from a local supermarket near the university. All other reagents and chemicals were of analytical grade, products of Sigma Chemical Co., Saint Louis, MO, USA or BDH Chemical Ltd, Poole, England.

Animal handling and insecticide aerosol exposure chamber design

The experimental animals were handled and used in accordance with the international guide for the care and use of laboratory animals (National Research Council, 1996). They were allowed to acclimatize, and kept in standard laboratory conditions under natural light-dark cycle. The experimental animals had access to diet and water *ad libitum* throughout the duration of the experiment. Four identical wooden-glass exposure chambers each with internal volume of 0.167 m^3 (0.405 x 0.800 x 0.515 m) were used. Into each chamber (containing five rats) was sprayed 0, 1, 2, or 3 ml respective volume of the pyrethroid product multi-purpose insect killer. This is equivalent to 0, 6.0, 12.0 and 18.0 mL m^{-3} of the exposure chamber, respectively. The chambers were closed for 1 h immediately after spraying. A glass top enabled observations to be made on reactions of the animals to the aerosols. These procedures were carried out once a day for 6 weeks. Effective light usage of the insecticide in Nigerian homes ranges from about 3.5 - 6.0 mL m^{-3}.

Three different doses, multiples of this light usage, were experimented within different groups of rats exposed to the insecticide aerosols thereby giving allowance for heavy aerosols spray and high level of exposure to the insecticide. The volume of the insecticide sprayed as aerosols usually depend on the users and the perceived population of insects in the house.

Experimental animals and treatments

The rats were divided randomly into four groups (I to IV) of five animals per group as follows: group I (control), not exposed to insecticide aerosols; group II, exposed to 6.0 mL of the aerosols per 1 m^3 treatment chamber volume (1 mL of the insecticide product in 0.167 m^3 exposure chamber); group III, exposed to the aerosols at 12.0 mL m^{-3}; group IV, exposed to the aerosols at 18.0 mL m^{-3}.

Sample preparation

At the end of the experimental period, the animals were sacrificed

Table 1. Liver, kidney and testis weights of control and experimental rats exposed to pyrethroid insecticide aerosols.

Group	Liver weight (g)	Kidney weight (g)	Testis weight (g)	Relative liver weight (%)	Relative kidney weight (%)	Relative testis weight (%)
I	6.51 ± 0.67	1.09 ± 0.07	2.36 ± 0.18	3.05 ± 0.24	0.51 ± 0.02	1.11 ± 0.09
II	5.15 ± 0.33[a]	1.00 ± 0.03	2.56 ± 0.08	2.67 ± 0.14	0.52 ± 0.01	1.33 ± 0.06
III	5.17 ± 0.22[a]	0.99 ± 0.05	2.23 ± 0.15	2.73 ± 0.21	0.52 ± 0.03	1.19 ± 0.10
IV	5.58 ± 0.38	1.06 ± 0.06	2.51 ± 0.06	2.77 ± 0.04	0.53 ± 0.02	1.26 ± 0.06

Each value is a mean of 5 rats ± SEM. [a]Significantly different from negative control group ($p<0.05$). Relative organ (liver/kidney/testis) weight = (organ weight/final body weight) x 100. I, Group of rats not exposed to aerosols. II, Rats in this group were exposed to 1 mL of the insecticide product aerosols in 0.167 m^3 treatment chamber (equivalent to 6.0 mL m^{-3} treatment chamber volume). III, Animals in this group were exposed to 2 mL of the aerosols (equivalent to 12.0 mL m^{-3} treatment chamber volume). IV, Rats in this group were exposed to 3 mL of the aerosols (equivalent to 18.0 mL m^{-3} treatment chamber volume).

and liver, kidney and testis were harvested. They were washed in ice-cold saline (0.9% w/v) solution, and were blotted dry before suspension in 0.1 M phosphate buffer (pH 7.4) and homogenized (1:4 w/v), followed by centrifugation at 5000 rpm for 10 min. The homogenate was kept frozen until used.

Assay for biochemical parameters

CAT activity was determined by the method of Sinha (1972), and expressed in µmole/min/mg protein. SOD was determined by the method of Misra and Fridovich (1972), and expressed in unit/mg protein, GPx was determined by the method of Paglia and Valentine (1967), expressed in µg/ml/mgprotein, and GST was determined by the method of Habig et al. (1974), expressed in µmole/min/mgprotein. MDA levels, a marker of lipid peroxidation was determined by the method of Beuge and Aust (1978) and expressed in nmol/mg, while GSH levels was determined by the method of Moron et al. (1979), and expressed in µg/ml. Protein concentration was done according to the method of Gornall et al. (1949).

Data were analyzed by one-way analysis of variance (ANOVA), followed by least significant difference (LSD) to test for significant differences among the groups of rats using Statistical Package for Social Sciences program version 17.0. Data were expressed as mean ± standard error of mean. P values less than 0.05 were considered statistically significant.

RESULTS

Liver, kidney, testis weights and relative weights of control and experimental rats exposed to pyrethroid insecticide aerosols are shown in Table 1. Exposure of rats to the insecticide aerosol did not produce any significant effect ($p > 0.05$) on liver, kidney and testis relative weights as compared to the control. Apart from 6.0 and 12.0 mL m^{-3} of the insecticide aerosol that produced significant difference ($p < 0.05$) in liver weights, there was no significant difference ($p > 0.05$) in kidney and testis weights as compared to the control.

The effects of pyrethroid insecticide aerosol on liver, kidney and testis MDA concentrations are shown in Figure 1. The obtained results showed no significant increase ($p > 0.05$) in the liver and testis MDA concentrations as compared to control, while 18.0 mL m^{-3} of the insecticide aerosol significantly increased ($p < 0.05$) the kidney MDA concentration as compared to the control.

Effects of the insecticide aerosol on liver, kidney and testis levels of GSH are represented also in Figure 2. All the concentrations of insecticide aerosol assessed (Groups II to IV) significantly reduced ($p < 0.05$) the levels of GSH in testis as compared to the control. A significant increase ($p < 0.05$) in kidney levels of GSH was recorded by 12.0 and 18.0 mL m^{-3} of the insecticide, while only 6.0 mL m^{-3} of the aerosol significantly raised ($p < 0.05$) the liver GSH level as compared to the control.

Lastly, exposure to the insecticide aerosol did not give significant reductions ($p > 0.05$) in liver GST, SOD, GPx and CAT activities as compared to the control.

DISCUSSION

Misuse of pyrethroids insecticides could cause toxicity in non-target species (Cantalamessa, 1993). Insecticides exposure constitutes a source of potent hazard especially in children and animals (Eisler, 1989; Nebeker et al., 1992; Menegaux et al., 2006). The multipurpose insect killer used, a pyrethroid insecticide product, is a popular brand for the eradication of mosquito in households in Nigeria and in some cases, it is used daily in homes. This increases the exposure of people, especially children sleeping under the aerosol.

Our investigation on the pyrethroid insecticide aerosol revealed no significant effects on liver, kidney and testes weights (Table 1), as well as their relative weights (Table 2). Measurement of MDA is mostly used to monitor lipid peroxidation and indirectly, oxidative stress *in- vitro* and *vivo* (Beltowski et al., 2000). The lipid oxidation causes disruption of the bilayer and cell integrity accompanied by leakage of cellular content from the damaged organ into the blood stream (Ologundudu et al., 2010). Our investigations in the present study revealed that the insecticide aerosol caused no significant increase ($p >$

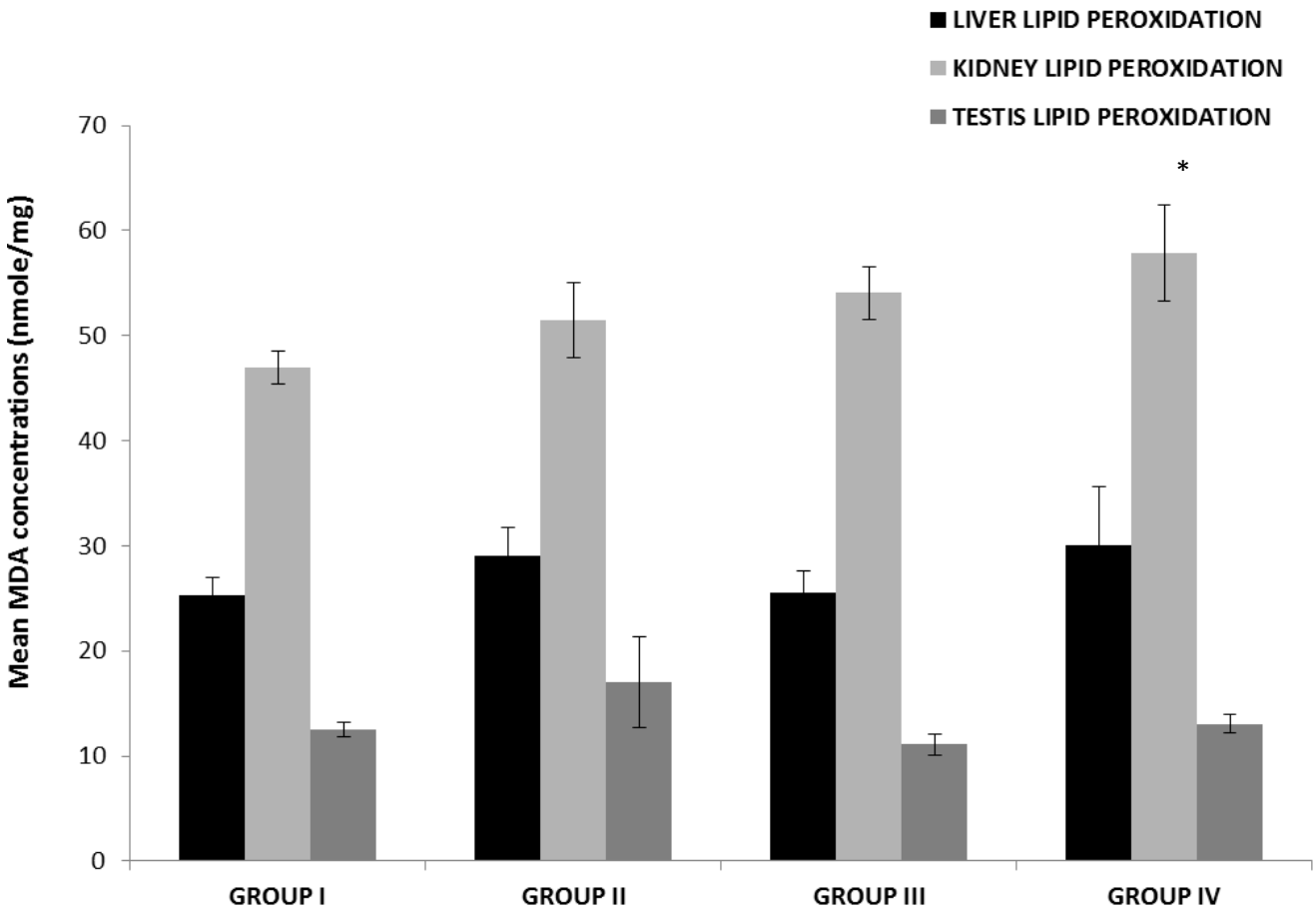

Figure 1. Effects of pyrethroid insecticide aerosols on liver, kidney and testes MDA concentrations. I, Group of rats not exposed to aerosols. II, Rats in this group were exposed to 1 mL of the insecticide product aerosols in 0.167 m^3 treatment chamber (equivalent to 6.0 mL m^{-3} treatment chamber volume). III, Animals in this group were exposed to 2 mL of the aerosols (equivalent to 12.0 mL m^{-3} treatment chamber volume). IV, Rats in this group were exposed to 3 mL of the aerosols (equivalent to 18.0 mL m^{-3} treatment chamber volume). Values are mean ± SEM; n = 5. *Significantly different from control group I ($p < 0.05$).

Table 2. Relative liver, kidney, and testis weights of control and experimental rats exposed to pyrethroid insecticide aerosols.

Group	Relative liver weight (%)	Relative kidney weight (%)	Relative testis weight (%)
I (Control)	3.05 ± 0.24	0.51 ± 0.02	1.11 ± 0.09
II (6.0 mL m^{-3} of insecticide as aerosols)	2.67 ± 0.14	0.52 ± 0.01	1.33 ± 0.06
III (12.0 mL m^{-3} of insecticide as aerosols)	2.73 ± 0.21	0.52 ± 0.03	1.19 ± 0.10
IV (18.0 mL m^{-3} of insecticide as aerosols)	2.77 ± 0.04	0.53 ± 0.02	1.26 ± 0.06

No significant difference from negative control group ($p<0.05$). Relative organ (liver/kidney/testis) weight = (organ weight/final body weight) x 100.
I, Group of rats not exposed to aerosols. II, Rats in this group were exposed to 1 mL of the insecticide product aerosols in 0.167 m^3 treatment chamber (equivalent to 6.0 mL m^{-3} treatment chamber volume). III, Animals in this group were exposed to 2 mL of the aerosols (equivalent to 12.0 mL m^{-3} treatment chamber volume). IV, Rats in this group were exposed to 3 mL of the aerosols (equivalent to 18.0 mL m^{-3} treatment chamber volume).

0.05) in the concentrations of liver and testis MDA (Figure 1), except for kidney MDA concentration in Group IV. Deltamethrin, a pyrethroid insecticide was reported to significantly increase the MDA level in testis, liver and kidney of rats (Sharma et al., 2014; Manna et al., 2005; Rehman et al., 2006). Cyfluthrin administered to rats has been reported to increase the concentration of MDA in liver and kidney of rats (Yilmaz et al., 2015).

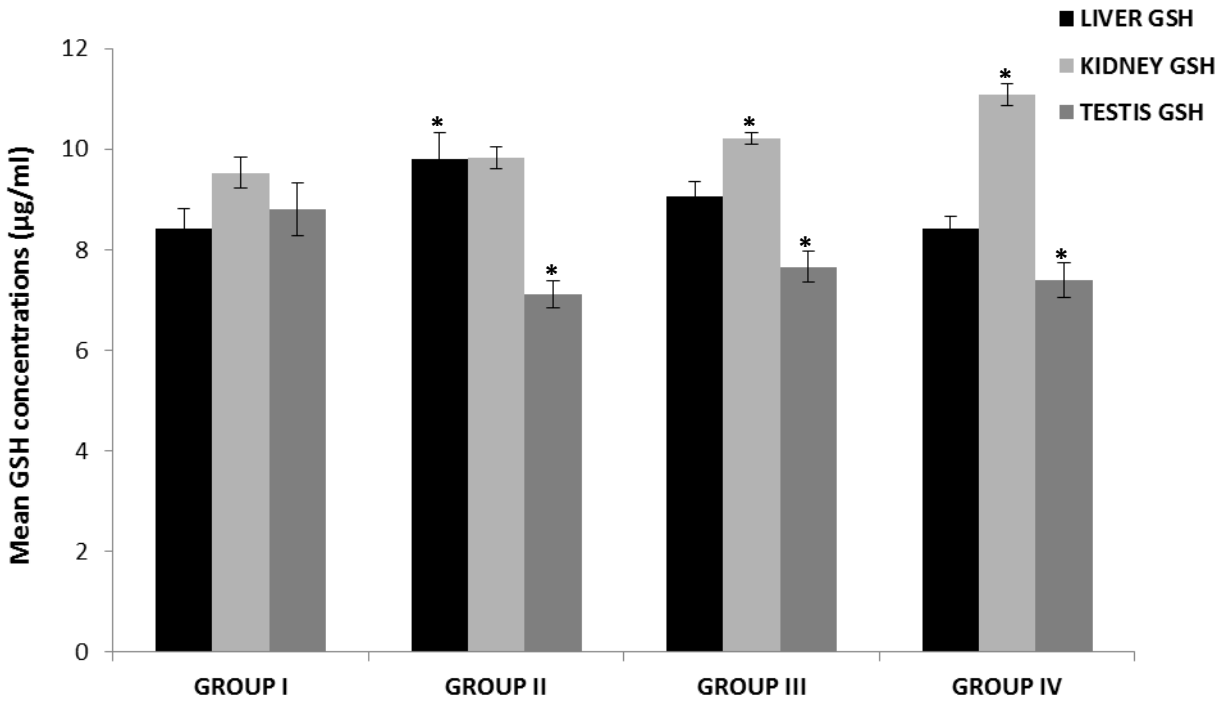

Figure 2. Effects of pyrethroid insecticide aerosols on liver, kidney and testes GSH levels. I, Group of rats not exposed to aerosols. II, Rats in this group were exposed to 1 mL of the insecticide product aerosols in 0.167 m^3 treatment chamber (equivalent to 6.0 mL m^{-3} treatment chamber volume). III, Animals in this group were exposed to 2 mL of the aerosols (equivalent to 12.0 mL m^{-3} treatment chamber volume). IV, Rats in this group were exposed to 3 mL of the aerosols (equivalent to 18.0 mL m^{-3} treatment chamber volume). Values are mean ± SEM; n = 5. *Significantly different from control group I ($p < 0.05$).

Also, in the present study, our investigations revealed that the insecticide aerosol increased significantly ($p < 0.05$) the levels of GSH in the kidney (Groups 3 and 4) of rats (Figure 2), while it significantly ($p < 0.05$) decreased the levels of GSH in testis in all the groups. The elimination of H_2O_2 is either affected by catalase or glutathione peroxidase, with the latter predominating (using reduced glutathione as substrate) in the case of testes (Zini and Schlegel, 1996; Peltola et al., 1992). This could be responsible for the decrease in testes GSH levels seen in our investigations. Incubation of testis homogenate with different concentrations of insecticide mixture for different time intervals significantly decreased the activity of antioxidant enzymes, like GST, SOD and CAT, and the level of GSH (El-Demerdash et al., 2013). Cypermethrin-induced toxicity has been reported to decrease the level of GSH in testis of rats (Huq et al., 2014). GSH participate in the elimination of ROS, acting both as non-enzymatic oxygen radical scavenger and as a substrate for various enzymes such as GPx (Ashar and Muthu, 2012). The liver is the primary site for the detoxification of xenobiotics, and it is rich in drug metabolizing enzymes. All these factors may protect the liver from the overwhelming toxic effects of the insecticide. Again, from our investigations, the non-significant decrease ($p > 0.05$) in liver GST, GPx, CAT and SOD activities (Figure 3) following exposure to the insecticide aerosol could be also due to the prompt and balanced response of these tissues to the increased and continuous generation of ROS particularly superoxide radical ($O_2^{\cdot-}$), which can be dismutated to H_2O_2 by SOD, and then to H_2O and O_2 by CAT, GPx and GST. It has been reported that 14 days exposure to beta-cyfluthrin caused a significant decrease in CAT and SOD activity as compared to the control in a dose dependent manner (Verma et al., 2013). Cyfluthrin has been reported to increase the generation of free radical and decrease SOD and CAT activity in mice (Omotuyi et al., 2006; Eraslan et al., 2007) and in cultured human erythrocytes (Sadowaska-Woda et al., 2010). Cyfluthrin administration was reported to lower the activities of CAT and GPX in liver and kidney of rats (Yilmaz et al., 2015). It was recently reported that the activities of kidney SOD and GPx, as well as activities of liver SOD, GST and CAT were significantly decreased by prallethrin administration in rats (Rafaie et al., 2014; Mossa et al., 2013). Cypermethrin-induced toxicity has been reported to decrease the activities of CAT, SOD, GST and GPx in testis of rats (Huq et al., 2014). Deltamethrin administration has been reported to significantly reduce the activities of SOD, CAT, GST and GPx, as well as levels of GSH in liver, testis and kidney of rats exposed to

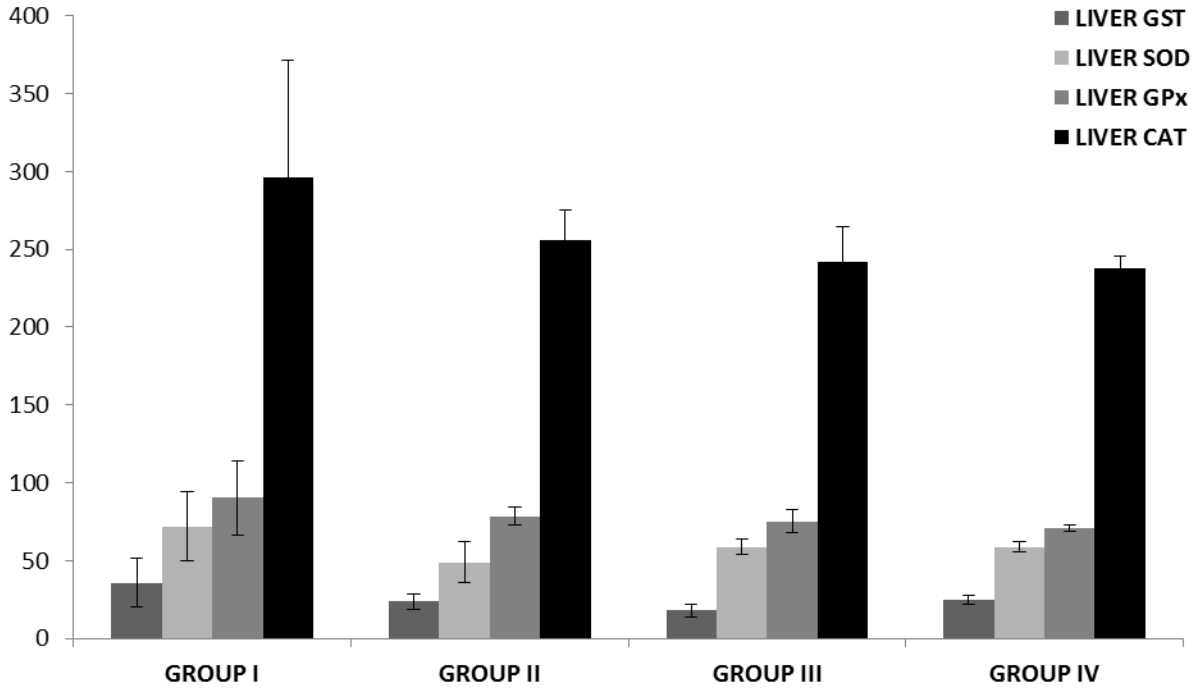

Figure 3. Effects of pyrethroid insecticide aerosols on liver GST, SOD, GPx and CAT activities. I, Group of rats not exposed to aerosols. II, Rats in this group were exposed to 1 mL of the insecticide product aerosols in 0.167 m^3 treatment chamber (equivalent to 6.0 mL m^{-3} treatment chamber volume). III, Animals in this group were exposed to 2 mL of the aerosols (equivalent to 12.0 mL m^{-3} treatment chamber volume). IV, Rats in this group were exposed to 3 mL of the aerosols (equivalent to 18.0 mL m^{-3} treatment chamber volume). Values are mean ± SEM; n = 5GST, SOD, GPx, and CAT activities are expressed as µmole/min/mgprotein, unit/mgprotein, µg/ml/mgprotein, and µmole/min/mgprotein, respectively.

it (Sharma et al., 2014; Manna et al., 2005; Rehman et al., 2006).

In conclusion, results from these investigations reveals that the concentrations of the active pyrethroid ingredients in the aerosol of this commonly used over-the-counter pyrethroid insecticide product at the dose assessed may not be associated or implicated in extra-pulmonary oxidative tissue damage.

Conflict of interests

The authors did not declare any conflict of interest.

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