

African Journal of Biotechnology

Volume 15 Number 5, 3 February 2016

ISSN 1684-5315



*Academic
Journals*

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ARTICLES

- Current status of Colletotrichum capsici strains, causal agents of Brown blotch disease of cowpea in Burkina Faso** 96
Thio I. G., Zida E. P., Sawadogo M. and Sérémé P.
- In vitro seed germination and seedling development of the orchid Coelogyne stricta (D. Don) Schltr.** 105
Gaurav Parmar and Bijaya Pant
- Adsorption of copper, zinc and lead on biosurfactant produced from cassava wastewater** 110
Larissa Kummer, Natássia Jersak Cosmann, Glaucia Maria Pastore, Ana Paula Resende Simiqueli, Vander de Freitas Melo and Simone Damasceno Gomes
- Investigating the antioxidant properties and rutin content of Sea buckthorn (Hippophae rhamnoides L.) leaves and branches** 118
Aliye Aras Perk, F. Duygu Ceylan, Onur Yanar, Kadir Boztas and Esra Capanoglu

Full Length Research Paper

Current status of *Colletotrichum capsici* strains, causal agents of Brown blotch disease of cowpea in Burkina Faso

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Received 18 September, 2015; Accepted 13 January, 2016

Brown blotch disease, caused by *Colletotrichum capsici*, is an important disease of cowpea with a significant yield losses ranging from 42 to 100% in West Africa. In this study, a specific polymerase chain reaction (PCR) primer set CC1F1/CcapR was used to characterize and to study the phylogenetic relationship of thirty eight strains of *Colletotrichum* species. This primer set is capable of amplifying only *C. capsici* from different fungal structures and provide a powerful tool for *C. capsici* detection in brown blotch disease in cowpea. Phylogenetic analysis from neighbor-joining (NJ) showed a high genetic variability in the rDNA-ITS region of the isolates. The isolates formed four groups or clusters on the basis of specific fragment analysis. Groups I, II, and III consist of strains containing specific region length of twenty one nucleotides and were considered as variant 1 of *C. capsici*. Group IV was a heterogeneous and consists of variants 1, 2, 3, and 4 of *C. capsici*.

Key words: Cultivars, internal transcribed spacer (ITS) sequence, *Colletotrichum* species.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of main grain legume crops grown in sub-Saharan Africa in terms of cultivated areas, production, and consumption (Singh et al., 2002; Ajeigbe, 2006). In Burkina Faso, cowpea is a staple food crop, which is grown essentially for human food and nutrition. Cowpea production is subjected to abiotic stresses, such as drought, heat, and poor soil fertility and to various biotic agents including soil borne

and seed borne, fungal pathogens, which cause diseases leading to a significant yield losses and reduction in grain density (Katile et al., 2010). Of the diseases, Brown blotch caused by *Colletotrichum capsici/truncatum* has been cited to be of great importance not only in Burkina Faso, but also in other parts of the world. In a worldwide perspective, *C. capsici* pathogen causes a disease commonly known as anthracnose on a wide range of

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plants, including legumes, vegetables, and small fruits (Séréme, 1999; Banerjee et al., 2007; Torres-Calzada et al., 2011). Recently, strains of *C. capsici* and *Colletotrichum dematium* f. *truncatum* have been described as the same species (Hyde et al., 2009) and are now considered to be synonym of *Colletotrichum truncatum* (Damm et al., 2009). The genus *Colletotrichum* includes several species, such as *Colletotrichum graminicola*, *C. capsici*, *C. truncatum*, *Colletotrichum gloeosporioides*, *C. dematium*, *Colletotrichum destructivum*, and *Colletotrichum coccodes*, which may occur as endophytes, saprobes or pathogens (Hyde et al., 2009). *Colletotrichum* is a cosmopolitan fungus and includes a number of economically important plant pathogenic fungi, occurring predominantly in tropical and subtropical regions on a wide range of crops (Sharma et al., 2014a)

Working on brown blotch disease of cowpea in Nigeria, Emechebe (1986) identified 8 races of *C. capsici*. Four of these races were reported to be specific to habitats in Guinea and Sudan Savannah ecologies. The four other races were reported to be common in rain forest zones. The spread of the pathogen can be facilitated by high humidity and heavy rains during the growing season. The identification of *Colletotrichum* species has been reported to be difficult due to morphological similarities of the pathogen races (Hyde et al., 2009). In Burkina Faso, Séréme (1999) reported 12 pathogenic groups of *Colletotrichum* spp., including all *C. capsici* which are associated with brown blotch disease. The existence of these pathogen races in Burkina Faso create confusion especially when quick, accurate and proper pathogen identification is needed.

Traditionally, identification and classification of *Colletotrichum* spp. Have been based on the morphological characters, pathogenicity tests, and biochemical approaches (N'Guetia et al., 2013; Saxena et al., 2014; Chai et al., 2014; Enyiukwu et al., 2014). In most cases, there were no clear differences in conidial or appressorial size and shape among these species.

Thus molecular characterization is essential for completing the morphological description of *Colletotrichum* spp. (Sharma et al., 2014a, b; Enyiukwu et al., 2014). The rDNA region of *Colletotrichum* spp. has been investigated for species identification and phylogenetic relationships (Cannon et al., 2012). The internal transcribed spacer (ITS) markers have also been successfully used for detection of many species including plants, fungi and bacteria (Cros et al., 1993; Shivaprakash et al., 2011). Some ITS markers notably *C.capR/C.capF* and *CC1F1/CC2R2* have been used for *C. capsici* species detection and phylogenetic analysis (Banerjee et al., 2007; Chandra et al., 2009; Torres-Calzada et al., 2011).

In this paper, species specific polymerase chain reaction (PCR) primer pair *CC1F1/CcapR* were used to characterize and study the phylogenetic relationship of different strains of *Colletotrichum* spp. responsible of

brown blotch disease in cowpea in Burkina Faso.

MATERIALS AND METHODS

Collection of *Colletotrichum* isolates

Colletotrichum isolates were obtained from infected cowpea tissues (leaf, stem and pod) from farmer's fields in seven different sites, situated in three agro-ecological zones of Burkina Faso, namely Saria, Kamboinse, and Kouare (North Soudanian zone), Farako Ba and Gaoua (Soudanian zone), and Pobe Mengao and Bani (Sahelian zone) during the period from October to November, 2013. The collected tissues materials were surface sterilized in 70% (v/v) ethanol for 1 min followed by immersion in sodium hypochlorite (NaOCl) 1% (v/v) for 5 min and three successive rinses in sterilized distilled water. The samples were left to dry under the laminar air cabinet for 1 h. Then small pieces (approximately 5 × 5 mm) from the margins of infected tissues were transferred to Petri dishes containing three layers of wet blotter papers. Petri dishes containing samples were incubated at 28°C under 12 h/12 h light: dark for 7 to 9 days.

Identification of *Colletotrichum* spp.

Colletotrichum spp. (complex *C. capsici/truncatum*) were identified based on morphological characters of the acervuli and conidia produced on the infected tissues on the blotter papers under the stereomicroscope and compound microscopes based on the identification key established by Marthur and Kongsdal (2003). Then, using a sterile loop, pure isolates were further placed onto potato dextrose agar (PDA) containing streptomycin (0.3 µg/L of PDA). The plates were incubated at 24°C for 7 days under ultraviolet (UV) light of alternating 12 h light and darkness to obtain pure culture.

Purification of *Colletotrichum* single spore isolates

To obtain fresh single spore pure culture of *Colletotrichum* isolates, distilled water (100 ml) was added to PDA and 200 µl of the suspension obtained were spread on new PDA plates and incubated for 24 to 48 h as previously described. Then three to five single cells from each isolate were again transferred onto new PDA. After seven days of growth, single spore pure cultures were stored at 20°C until used.

Molecular characterization

DNA extraction

The Flinders Technology Associates (FTA) plant cards were used for fungal DNA extraction. The fungi suspension of 100 µl containing approximately 10³ to 10⁶ spores/ml was applied as a single spot on the paper, and the paper was air-dried for 1 to 2 h at room temperature. The spotted FTA plant cards were stored at room temperature until use. Using a Harris Micro Punch instrument, discs of 2-mm in diameter were taken from spots made on the FTA plant card. The sample discs were each placed in separate Eppendorf tubes. The discs from each FTA plant card were washed and air-dried for 1 h at room temperature following the manufacturer's instruction (www.whatman.com).

ITS amplification and sequencing

PCR detection assay was conducted using the genus *Colletotrichum*

specific primer CC1F1 5'ACCTAACTGTTGCTTCGGCG-3' (Chandra et al., 2009) and the species *C. capsici* specific primer CcapR 5'-CCCAATGCGAGACGAAATG3' (Torres-Calzada et al., 2011), which gave a specific band size of 425 bp. The PCR mixture of 25 µl contained 1 FTA disk, 5 µl of Buffer (Flexer) 5X, 0.5 µl of dNTP (10 nM), 18.42 µl of distilled H₂O, 0.5 µl of each primer (5 µM), and 0.08 µl of DNA Go Taq (Promega). The PCR was performed using a thermocycler Master Cycler Gradient at 95°C for 5 min for initial denaturation, followed by 25 cycles of denaturation at 94°C for 30s, primer annealing at 62°C for 30s and extension at 72°C for 2 min. The final extension was set at 72°C for 5 min. Eight microliters of the PCR products was analyzed on 1.5% agarose gel stained with 5 µl of ethidium bromide. After approximately 45 to 60 min of running at 70 to 80 mV, the gel was visualized. The PCR positive products were then sent to Beckman Coulter Genomics service (USA) for sequencing.

Molecular identification and phylogenetic analysis

BioEdit software was used for the Clustal W Multiple sequence alignment, and BLAST for species identification in the NCBI GenBank. Due to complications in distinguishing *C. capsici*, *C. truncatum* and *C. dematium* (Hyde et al., 2009), specific fragment of *C. capsici* was targeted on the BioEdit multiple sequence alignment software and was compared with that in the NCBI database. The phylogenetic tree was produced using DARwin 6.0.4 software.

RESULTS

The isolates of *Colletotrichum* spp. which were identified from the lesions of cowpea tissues were identified as *C. capsici* based on size and shape of conidia. The isolates of *C. capsici* showed differences in morphological characteristics such as colony color (brown, orange, black or grey or white), conidial shape and size (Figure 1). Conidia of fungal isolates were typically ellipsoidal and hyaline, and produced acervuli. Our results indicated that mycelium of *C. capsici* from cowpea varied and could be aerial, compact or cottony (Figure 1). The size of conidia also varied within the isolates (Table 1).

The present study characterized thirty eight isolates of *Colletotrichum* spp. using molecular approaches; specific region in the International Transcribed Spacer regions of *C. capsici* was targeted and was used to determine phylogenetic relationships between these fungal strains.

The ITS sequence analysis of the 38 isolates of *Colletotrichum* spp. showed that all the strains were *C. capsici* (Table 2). Among the strains, 92% contained the specific sequence of *C. capsici* (Torres-Calzada et al., 2011) and were designated as variant 1. In total, four variants of *C. capsici* were identified on the basis of the sequence analysis of species (Table 3).

The four variants of *C. capsici* identified (Figure 2) in this study are in alignment with four physiological races identified by Emechebe (1986) which are specific to Guinea and Sudan Savanna habitats. The species *capsici* variant 1 represented the most, with 92% of the strains, and was distributed over all agro-ecological zones of the country. But variants 2, 3 and 4 seemed to be specific to

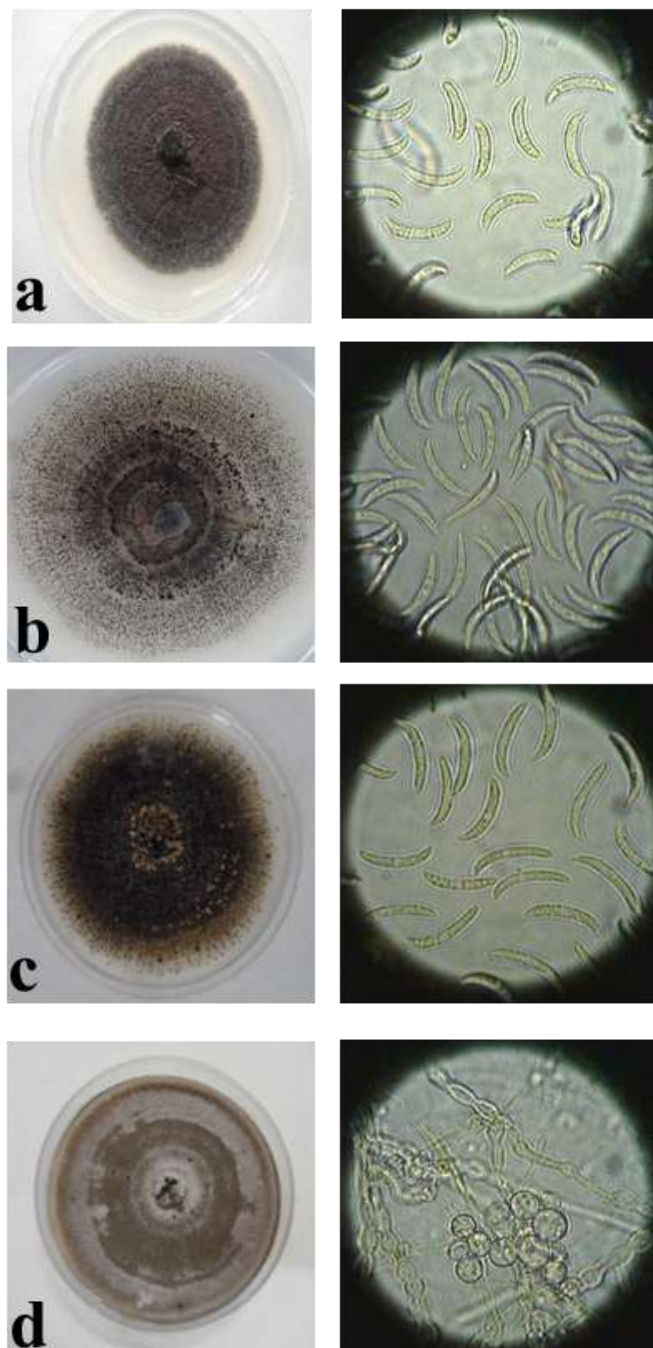


Figure 1. Different morphological aspects and close view of conidia of four *C. capsici* isolates in PDA culture; a: 03-KO-1 (var. 1); b: 35-GA-5 (var. 2); c: 42-GA-7 (var. 3); d: 20-KB-5 (var. 4).

the Sudanian zone (Gaoua).

Based on the phylogenetic relationship of the 38 clones, 4 clusters at a distance coefficient of 0.1 (Figure 3) were formed. The major cluster I had sixteen isolates with 65% similarity. Three of the identified variants of *C. capsici* belonged to the heterogeneous cluster IV.

Table 1. Comparatives morphological characters of eight *C. capsici* isolates, 10 days on PDA.

Isolate	Location	Organ	Conidia length (µm)	Morphological characters		
				Colony colour	Acervulus	Mycelia form
03-KO-1	Kouare	Pod	20	Brown	Abundant	Compact
20-KB-5	Kamboinse	Stem	-	Brown-white	Absent	Cottony
35-GA-5	Gaoua	Pod	28	Orange	Abundant	Cottony
42-GA-7	Gaoua	Pod	23	Orange	Abundant	Aerial
71-FA-6	Farako-Ba	Pod	22	Brown	Abundant	Compact
74-BA-1	Bani	Pod	21	Brown	Abundant	Aerial
77-PM-1	Pobe	Pod	21	Black	Abundant	Compact
96-SA-2	Saria	Stem	20	Orange	Abundant	Cottony

Table 2. Molecular identification of *Colletotrichum* spp. single spores isolates from cowpea in Burkina Faso.

<i>Colletotrichum</i> spp. isolate	Organ	Location	NCBI identification	GenBank accession number	Specific sequence analysis identification	Variants
001-KO-1	Pod	Kouare	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
003-KO-1	Pod	Kouare	<i>C. capsici</i>	KM213014 (99% id.)	<i>C. capsici</i>	Var. 1
005-KB-1	Pod	Kamboinse	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
006-KB-1	Pod	Kamboinse	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
013-KB-3	Stem	Kamboinse	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
016-KB-3	Stem	Kamboinse	<i>C. capsici</i>	KM213014 (99% id.)	<i>C. capsici</i>	Var. 1
020-KB-5	Stem	Kamboinse	<i>C. capsici</i>	HM197759 (85% id.)	<i>C. capsici</i>	Var. 2
024-GA-2	Stem	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
025-GA-3	Pod	Gaoua	<i>C. capsici</i>	KM213014 (99% id.)	<i>C. capsici</i>	Var. 1
026-GA-3	Pod	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
027-GA-3	Pod	Gaoua	<i>C. capsici</i>	J185787 (99% id.)	<i>C. capsici</i>	Var. 1
028-GA-3	Pod	Gaoua	<i>Colletotrichum</i> sp.	HQ130691 (99% id.)	<i>C. capsici</i>	Var. 1
033-GA-4	Pod	Gaoua	<i>C. capsici</i>	KM213014 (99% id.)	<i>C. capsici</i>	Var. 1
035-GA-5	Pod	Gaoua	<i>C. capsici</i>	HM197759 (92 % id.)	<i>C. capsici</i>	Var. 3
036-GA-5	Pod	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
042-GA-7	Pod	Gaoua	<i>C. capsici</i>	HM191710 (99 % id.)	<i>C. capsici</i>	Var. 4
043-GA-7	Pod	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
045-GA-8	Pod	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
047-GA-9	Stem	Gaoua	<i>C. truncatum</i>	JQ936245 (99% id.)	<i>C. capsici</i>	Var. 1
051-GA-10	Stem	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
053-GA-10	Stem	Gaoua	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
058-FA-2	Leaf	Farako-Ba	<i>C. capsici</i>	KM213014 (99% id.)	<i>C. capsici</i>	Var. 1

Table 2. Contd.

059-FA-3	Leaf	Farako-Ba	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
060-FA-3	Leaf	Farako-Ba	<i>C. capsici</i>	HQ271451 (99% id.)	<i>C. capsici</i>	Var. 1
062-FA-4	Stem	Farako-Ba	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
064-FA-4	Stem	Farako-Ba	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
071-FA-6	Pod	Farako-Ba	<i>C. capsici</i>	KM213014 (98% id.)	<i>C. capsici</i>	Var. 1
072-FA-6	Pod	Farako-Ba	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
074-BA-1	Pod	Bani	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
077-PM-1	Pod	Pobe-Mengao	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
082-PM-3	Stem	Pobe-Mengao	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
083-PM-3	Stem	Pobe-Mengao	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
085-PM-3	Stem	Pobe-Mengao	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
089-PM-5	Stem	Pobe-Mengao	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
090-PM-5	Stem	Pobe-Mengao	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
091-PM-5	Stem	Pobe-Mengao	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1
092-SA-1	Stem	Saria	<i>C. capsici</i>	HM197759 (99 % id.)	<i>C. capsici</i>	Var. 1
096-SA-2	Stem	Saria	<i>C. capsici</i>	HQ271457 (99% id.)	<i>C. capsici</i>	Var. 1

Table 3. rDNA sequence comparison of the specific fragment from the internal transcribed spacer (ITS) region of the 38 *Colletotrichum capsici* species isolated from cowpea plants.

Variant of <i>C. capsici</i>	Sequence variability in the specific region	Seq. identity	GenBank	Representative clone
Variant 1 (<i>C. capR</i> specific fragment)	-AACATTTTCGTCTCGCATTGG(T)G	92 to 99%		045-GA-8
Variant 2	-ACCATTTTCGCCCTCGGATTGGG	92%		035-GA-5
Variant 3	-AACATTCCGCTT-GGATTGGG	88%		042-GA-7
Variant 4	-TACTTTCGCCCTCGGATTGGG	85%		020-KB-5

Variants 2, 3, and 4 had 71% similarity with variant 1 in cluster IV. The sequences similarities of the 38 strains of *C. capsici* were consigned in Table 4.

DISCUSSION

In this study, thirty eight *Colletotrichum* spp.

isolates were characterized using molecular approaches. The present findings have revealed that there exist high level of variation between the rDNA-ITS region of *Colletotrichum* spp. in Burkina Faso.

High number of clades obtained on the dendrogram shows a strong intraspecific differentiation within the *C. capsici* species. All the four clusters were representative of the three

agroecological zones and the distribution of the isolates was not depending on the geographic area indicating a high genetic diversity at all locations. Kumar et al. (2010) observed the same results; working with *C. falcatum* from sugarcane in India. Such variations could be due to similar climatic conditions, which might favor the coexistence of the isolates.

The analysis of sequence identity between *C.*

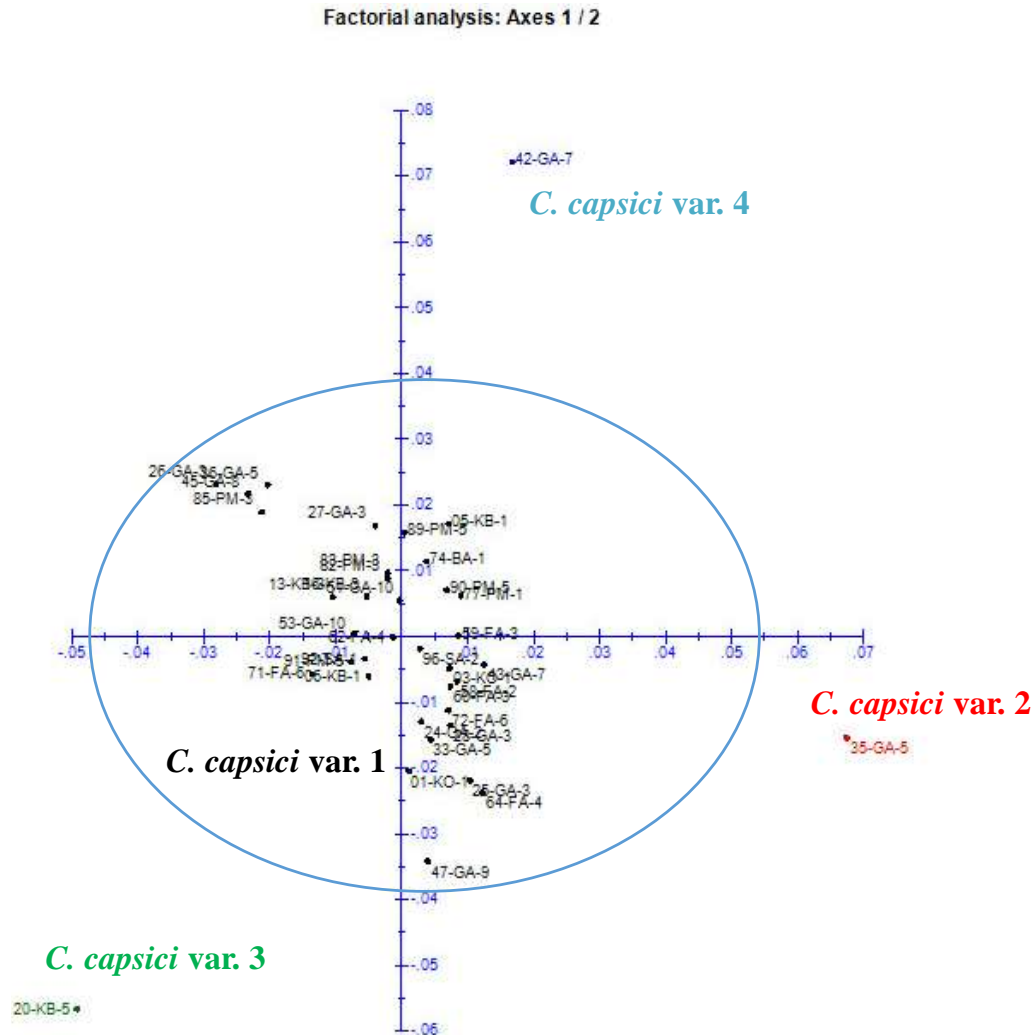


Figure 2. Distribution of *Colletotrichum capsici* variant.

Capsici strains ranged from 0.773 to 0.974 (Table 3). This similarity indicates a high penalty for closely related sequences. The highest degree of sequence identity was observed between strains 96-SA-2 and 91-PM-5 (0.974), 92-SA-1 and 77-PM-6 (0.961), and the lowest degree (0.773) was observed between strains 20-KB-5 (variant 3) and 28-GA-3 (variant 1). *C. capsici* isolates from Saria (North sudanian) were very close to those from Pobe Mengao (Sahelian).

Working with Anthracnose disease on chilli fruits, using Randomly Amplified Polymorphic DNA (RAPD), Kumar et al. (2015) demonstrated genetic variability among isolates of *C. capsici* from different locations in India. Contrary to our study, they found a low value of similarity coefficient. The difference in similarity coefficients between this study and Kumar et al. (2015) could be due to the fact that ITS sequences cannot be used to distinguish closely related species.

The concept of a species in the genus *Colletotrichum* is not well defined due to insufficient variation in classical

descriptive criteria and difficulties in dealing with pathogens of similar morphology, but different host specificity (Sutton, 1992). The molecular study of *C. capsici* strains provides criteria that permit differentiation of strains or species with similar conidial morphology. The examination of highly conserved rDNA, along with other criteria represents a useful approach of addressing taxonomic uncertainties (Sherriff et al., 1994; Bailey et al., 1996; Sexena et al., 2014). The phylogenetic relationship of the strains of *C. capsici* showed that the variants 2, 3 and 4 may be evolved from variant 1 from a mutation of two or three nucleotides in the specific fragment region.

In a previous report, *C. capsici* strains from cowpea in Burkina Faso were different based on both biomorphological and biochemical characters (Sereme, 1999).

In the current study, the use-fulness of rDNA-ITS markers was demonstrated to distinguish and establish the phylogenetic relationship of different strains of *C. capsici* isolated from cowpea plants in Burkina Faso. This

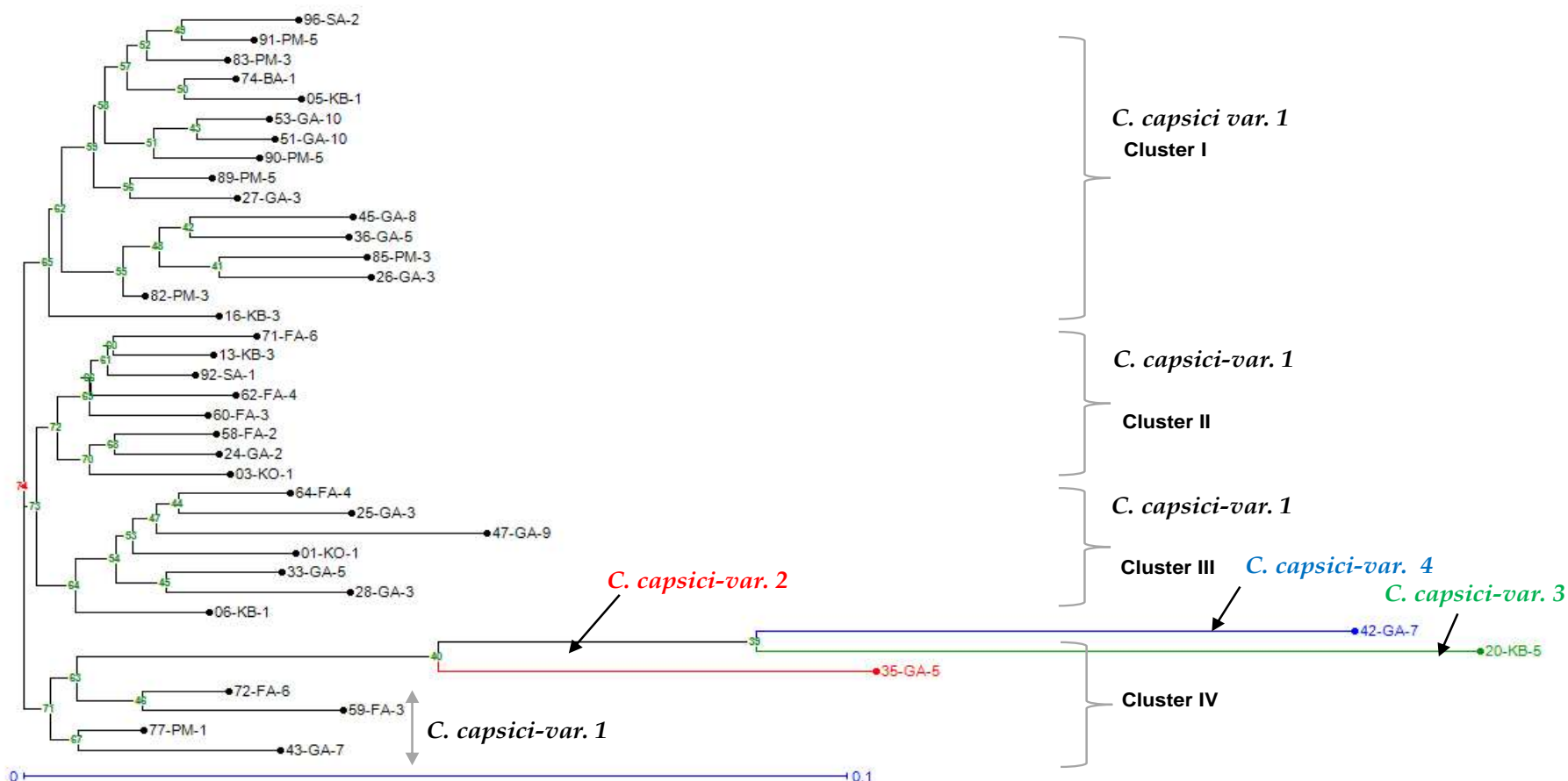


Figure 3: Phylogenetic relationship of 38 strains of *C. capsici* based on NJ/UnWeighted method. The distance coefficient = 0.1

research provides a quick and accurate tool for specific PCR detection of *C. capsici*, causative agents of brown blotch disease in cowpea. In this study, the ITS markers were recognized to be phylomarkers for intra and extra-specific relationship within species population as per Lei et al. (2012), and for validation of species status as per the study by Dabert (2006). Thus, findings

were presented, which will help future researchers to avoid confusion when distinguishing the *C. capsici* from other *Colletotrichum* spp. infecting cowpea.

Conflict of Interests

The authors have not declared any conflict of

interests.

ACKNOWLEDGEMENTS

This research was supported financially by different donors, the West Africa Agricultural Productivity Program (WAAPP), the International

Table 4. Sequence identity/similarity Matrix of *Colletotrichum capsici* strains from cowpea in Burkina Faso.

Seq->	01-KO	03-KO	05-KB	06-KB	13-KB	16-KB	20-KB	24-GA	25-GA	26-GA	27-GA	28-GA	33-GA	35-GA	36-GA	42-GA	43-GA	45-GA...
01-KO-1	ID																	
03-KO-1	0.946	ID																
05-KB-1	0.914	0.917	ID															
06-KB-1	0.943	0.948	0.926	ID														
13-KB-3	0.932	0.948	0.914	0.964	ID													
16-KB-3	0.957	0.929	0.919	0.957	0.941	ID												
20-KB-5	0.780	0.778	0.758	0.803	0.785	0.797	ID											
24-GA-2	0.933	0.928	0.906	0.944	0.933	0.939	0.778	ID										
25-GA-3	0.944	0.934	0.924	0.951	0.941	0.953	0.774	0.919	ID									
26-GA-3	0.923	0.907	0.913	0.930	0.920	0.935	0.779	0.915	0.910	ID								
27-GA-3	0.925	0.916	0.933	0.946	0.936	0.941	0.780	0.927	0.930	0.950	ID							
28-GA-3	0.920	0.913	0.906	0.946	0.925	0.932	0.773	0.914	0.928	0.918	0.929	ID						
33-GA-5	0.951	0.937	0.910	0.960	0.943	0.957	0.786	0.931	0.949	0.937	0.941	0.955	ID					
35-GA-5	0.867	0.846	0.842	0.871	0.855	0.887	0.816	0.854	0.868	0.861	0.872	0.855	0.874	ID				
36-GA-5	0.930	0.909	0.910	0.939	0.939	0.948	0.787	0.919	0.933	0.960	0.941	0.916	0.932	0.872	ID			
42-GA-7	0.792	0.792	0.779	0.810	0.792	0.800	0.833	0.791	0.782	0.795	0.793	0.783	0.795	0.821	0.786	ID		
43-GA-7	0.906	0.906	0.909	0.924	0.924	0.921	0.770	0.944	0.907	0.906	0.916	0.907	0.913	0.850	0.906	0.786	ID	
45-GA-8	0.928	0.916	0.915	0.941	0.932	0.944	0.790	0.917	0.926	0.948	0.948	0.916	0.932	0.865	0.960	0.783	0.906	ID

Table 4. Contd.

Seq->	47-GA	51-GA	53-GA	58-FA	59-FA	60-FA	62-FA	64-FA	71-FA	72-FA	74-BA	77-PM	82-PM	83-PM	85-PM	89-PM	90-PM	91-PM	92-SA	96-SA
47-GA-9	ID																			
51-GA-10	0.907	ID																		
53-GA-10	0.902	0.978	ID																	
58-FA-2	0.903	0.921	0.915	ID																
59-FA-3	0.873	0.895	0.893	0.917	ID															
60-FA-3	0.905	0.928	0.933	0.931	0.898	ID														
62-FA-4	0.907	0.926	0.935	0.938	0.902	0.962	ID													
64-FA-4	0.939	0.935	0.939	0.939	0.911	0.941	0.941	ID												
71-FA-6	0.903	0.932	0.930	0.944	0.900	0.949	0.949	0.939	ID											
72-FA-6	0.885	0.905	0.896	0.927	0.927	0.890	0.897	0.919	0.897	ID										
74-BA-1	0.888	0.942	0.937	0.932	0.936	0.911	0.913	0.926	0.915	0.928	ID									
77-PM-1	0.906	0.943	0.939	0.953	0.915	0.951	0.958	0.948	0.953	0.923	0.937	ID								
82-PM-3	0.886	0.920	0.918	0.914	0.895	0.909	0.912	0.921	0.918	0.922	0.914	0.934	ID							

Table 4. Contd.

83-PM-3	0.898	0.955	0.948	0.916	0.906	0.912	0.914	0.928	0.918	0.920	0.960	0.938	0.936	ID						
85-PM-3	0.905	0.923	0.923	0.905	0.902	0.903	0.905	0.916	0.923	0.892	0.910	0.927	0.927	0.925	ID					
89-PM-5	0.905	0.960	0.957	0.923	0.913	0.914	0.921	0.944	0.930	0.916	0.953	0.950	0.934	0.966	0.934	ID				
90-PM-5	0.903	0.974	0.967	0.915	0.896	0.931	0.928	0.933	0.941	0.899	0.944	0.946	0.920	0.951	0.934	0.962	ID			
91-PM-5	0.900	0.957	0.957	0.918	0.911	0.921	0.918	0.937	0.925	0.909	0.955	0.938	0.931	0.969	0.927	0.966	0.955	ID		
92-SA-1	0.909	0.939	0.939	0.946	0.907	0.967	0.967	0.946	0.967	0.905	0.924	0.969	0.920	0.923	0.920	0.930	0.941	0.932	ID	
96-SA-2	0.902	0.962	0.957	0.930	0.906	0.926	0.926	0.939	0.932	0.907	0.942	0.948	0.925	0.962	0.927	0.960	0.960	0.974	0.934	ID

Mixte Laboratory (LMI, INERA-IRD) Patho-bios, and the Kirkhouse Trust Foundation. They thank Dr. Christophe Brugidou and Martine Bangratz for technical support and helpful discussion.

REFERENCES

- Ajeigbe A (2006). Gestion de *Striga gesnerioides* et *Alectra vogelii* en culture de niébé. In: FAO, Atelier sous régional sur la gestion intégrée durable en agriculture des espèces du genre *Striga*., IITA, Kano, Nigeria.
- Bailey JA, Nash C, O'Connell RJ, TeBeest DO (1996). Molecular taxonomy of *Colletotrichum* species causing anthracnose on the *Malvaceae*. *Phytopathology* 86:1076-1083.
- Banerjee AK, Arora M, Murty USN (2007). How far is ITS2 reliable as a phylogenetic marker for the mosquito general. *Electronic J. Biol.* 3(3):61-68.
- Cannon PF, Damm U, Johnston PR, Weir BS (2012). *Colletotrichum*- current status and future directions. *Stud. Mycol.* 73:181-213.
- Chandra NC, Udaya Shankar AC, Niranjana SR, Prakash HS, Mortensen CN (2009). Anthracnose disease of chilli pepper. *Technical Bulletin.* 15 p.
- Cros J, Lashermes PH, Marmey PH, Harmon S, Charrier A (1993). Molecular analysis of genetic diversity and phylogenetic relationship in coffee. ASIC, 15e Colloque, Montpellier 7 p.
- Dabert M (2006). DNA markers in the phylogenetic of the Acari. *Biol. Lett.* 43(2):97-107.
- Damm U, Woudenberg JHC, Cannon PF, Crous PW (2009). *Colletotrichum* species with curved conidia from herbaceous hosts. *Fungal Divers* 39:45-87.
- Emechibe AM (1986). Cowpea pathology. In Grain Legume Improvement Program Annual Report 1985. IITA, Ibadan, Nigeria. pp. 69-100.
- Enyiukwu DN, Awurum AN, Ononju CC, Nwaneri JA (2014). Biology and management strategies of cowpea anthracnose disease caused by *Colletotrichum* species. *Greener J. Biochem. Biotechnol.* 1(2):052-065.
- Hyde KD, Cai L, McLenzie EHC, Yang YL, Zhang JZ, Prihastuti H (2009). *Colletotrichum*: a catalogue of confusion. *Fungal Divers* 39:1-17.
- Katile SO, Perumal R, Rooney WL, Prom LK, Magill CW (2010). Expression of pathogenesis-related protein PR-10 in sorghum floral tissues in response to inoculation with *Fusarium thapsinum* and *Curvularia lunata*. *Mol. Pathol.* 11: 93-103.
- Kumar N, Jhang TS, Satyavir, Sharma TR (2010). Molecular and Pathological Characterization of *Colletotrichum falcatum* Infecting Subtropical Indian Sugarcane. *J. Phytopathol.* 159(4):260-267.
- Kumar S, Singh V, Singh M (2015). Assessment of genetic diversity in *Colletotrichum capsici* using Randomly Amplified Polymorphic DNA. *Indian J. Appl. Res.* 5:29-39.
- Lei R, Rowley TW, Zhu L, Bailey CA, Engberg SH, Jr EEL, Lu G (2012). Phylomarker- A tool for mining phylogenetic markers of mouse genome comparison: Application of the muse lemur (genus *Microcebus*). *Phylogeny. Evol. Bioinform.* 8:423-435.
- Marthur SB, Kongstal O (2003). Common Laboratory Seed Health Testing Methods for Detecting Fungi. Published by the International Seed Testing Association (ISTA), 425 p.
- Saxena A, Raghuvanshi R, Singh HB (2014). Molecular, phenotypic and pathogenic variability in *Colletotrichum* isolates of subtropical region in north-eastern India, causing fruit rot of chillies. *J. Appl. Microbiol.* 117:1422-1434.
- Sereme P (1999). *La maladie des taches brunes du niébé (Vigna unguiculata) au Burkina Faso: connaissance des agents pathogènes impliqués et développement de méthodes de lutte.* Thèse de Doctorat d'Etat ès-Sciences Naturelles, Université de Cocody, UFR Biosciences, Côte d'Ivoire 159 p.
- Sharma, K, Goss EM, van Bruggen AHC (2014a). Isolation and identification of *Colletotrichum cordylinicola* causing anthracnose on Cordyline (*Cordyline fruticosa*) in Florida, USA. *HortScience* 49:911-916.
- Sharma K, Merritt JL, Palmateer A, Goss E, Smith M, Schubert T, Johnson RS, van Bruggen AHC (2014b). Isolation, characterization and management of *Colletotrichum* spp. causing anthracnose on lucky bamboo (*Dracaena sandariana*). *Hort. Sci.* 49(4):453-459.
- Sherriff C, Whelan MJ, Arnold GM, Lafay J-F, Brygoo Y, Bailey JA (1994). Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. *Exp. Mycol.* 18:121-138
- Shivaprakash MR, Appanavar SB, Dhaliwal M, Gupta A, Gupta S, Gupta A, Chakrabarti A (2011). *Colletotrichum trunctum*: an Unusual Pathogen causing Mycotic Keratitis and Endophthalmitis. *J. Clin. Microbiol.* 49:2894-2898.
- Singh BB, Ehlers JD, Sharma B, Freire Filho FR (2002). Recent progress in cowpea breeding. In: Challenges and opportunities for enhancing sustainable cowpea production. Fatokun, S. A. Tarawali, B. B. Singh, P. M. Kormawa and M. Tamò (eds), IITA, Ibandan, Nigeria, pp. 22-40.
- Sutton, BC (1992). The Genus *Glomerella* and its Anamorph *Colletotrichum*. In *Colletotrichum: Biology, pathology and control.* Edited by J.A. Bailey and M.J. Jeger. CAB International, Wallingford, UK. pp. 1-26.
- Torres-Calzada C, Tapia-Tussell R, Quijano-Ramayo A, Martin-Mex R, Rojas-Brito D (2011). A species-specific polymerase chain reaction assay for rapid and sensitive detection of *Colletotrichum capsici*. *Mol. Biotechnol.* 49:48-55.

Full Length Research Paper

***In vitro* seed germination and seedling development of the orchid *Coelogyne stricta* (D. Don) Schltr.**

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Received 19 July, 2015; Accepted 29 December, 2015

***Coelogyne stricta* (D. Don) Schltr., an orchid of high ornamental and medicinal values, is native to Nepal at 1400 to 2000 m elevations. *In vitro* seed germination and seedling development was carried out on 0.8% (w/v) agar solidified Murashige and Skoog (MS) medium, supplemented with various combinations of α -naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP). MS medium supplemented with 1 mg/L BAP and 1 mg/L NAA was found to be the best condition for the development. The germination started after 7 weeks of culture and complete seedlings were obtained after 23 weeks of culture on the medium supplemented with 1 mg/L BAP and 1 mg/L NAA suggesting the usefulness of both hormones in root induction. In the hormone, free MS medium germination started after 5 weeks, but root initials were not developed even after 32 weeks of culture.**

Key words: *Coelogyne stricta*, *in vitro*, Murashige and Skoog (MS), 6-benzylaminopurine (BAP), α -naphthalene acetic acid (NAA).

INTRODUCTION

Nepal, situated in the lap of Himalaya, harbors 451 species of orchids from 107 genera (Rajbhandari, 2015). Orchids as a whole are cited under Appendix II of CITES except *Paphiopedilum insigne* and *Paphiopedilum venustum* in Nepal. They are important aesthetically, medicinally and also regarded as ecological indicator (Joshi et al., 2009). Due to their varied shape, size, colourful-long lasting flowers, shining green leaves and variously shaped pseudobulbs, they are very popular around the world.

A total of 90 species of orchids of Nepal have medicinal value (Pant and Raskoti, 2013). *Coelogyne* represented

by 14 plant species (Rajbhandari, 2015), is one of them. It is also the most threatened orchids in Nepal due to its over collection from nature.

Coelogyne stricta (D. Don) Schltr., a native orchid of Nepal, is commonly known as 'The Rigid *Coelogyne* Pseudobulbs'. It is an epiphyte on tree trunks or lithophytes on mossy rocks at elevations of 1400 to 2000 m in Nepal (Raskoti, 2009; Rajbhandari, 2015). It has high aesthetic value (Figure 1), so it is often used as an ornamental plant in many gardens, nurseries, hotels, etc. Its medicinal value is due to paste of its pseudobulb which is applied to the forehead against headache and

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Figure 1. A flower of *Coelogyne stricta*.



Figure 2. An immature capsule.

fever (Baral and Kurmi, 2006). Owing to its high demand in the national and international markets, over collection from its natural habitat and slow growth rate in nature, this species is restricted only to narrow pocket areas in the nature.

Orchid seeds lack functional endosperm, so the germination of the seeds requires an aid of suitable fungus. The germination rate of orchid seeds in nature is only 2 to 5% (Rao, 1977); even if they do so, the seeds take a long time to germinate and any disturbance in the habitat may destroy the whole population. The seedlings take 12 years to grow to maturity (Basker and Narmatha Bai, 2006). Vegetative propagation of this orchid through division of clumps of rhizomes, bulbs or by the rooting of off-shoots is slow; so often, that it is difficult to obtain the desired number of plants. These difficulties in natural germination and slow vegetative propagation may drive this species to extinction. *In vitro* propagation of orchids through seeds can produce large number of orchids in reasonable short time.

Hence, the present study was undertaken to develop an efficient protocol for *in vitro* propagation of *C. stricta* through seeds and ultimately assist in its conservation.

MATERIALS AND METHODS

Eight weeks old immature capsule of *C. stricta* (Figure 2) collected from the orchid house of National Botanical Garden, Godawari, Kathmandu was used in this research.

The capsule was sterilized by washing under running tap water besides 2 to 3 drops of tween 20 solution (Qualigens Fine Chemicals Pvt. Ltd.) for 50 min until the water became totally clear and transparent. The capsule was then rinsed in 70% ethyl alcohol for 2 min and in 1% solution of sodium hypochlorite for 10 min. Finally, it was rinsed with sterile water five times.

Murashige and Skoog (MS) medium was used alone and in different combinations of 6-benzylaminopurine (BAP) and α -

naphthalene acetic acid (NAA) (Table 1). The medium was supplemented with 3% sucrose. The pH of the medium was adjusted to 5.8 before autoclaving and solidified with 0.8% (w/v) agar. The medium was autoclaved at 15 psi for 15 min.

The sterilized capsule was then dissected longitudinally into two halves (Figure 3) using sterile surgical blade inside pre-sterilized laminar air flow cabinet. The seeds were then inoculated on the surface of MS medium alone and in different combinations of BAP and NAA using sterile forceps. The cultures were incubated at $25 \pm 2^\circ\text{C}$ under photoperiod of 16/8 h light/dark cycle.

RESULTS AND DISCUSSION

Immature capsule was selected for this research as it shows better germination response and saves time (Pant, 2006). The most effective germination response for *C. stricta* with complete development of roots and shoots was found to be on MS medium supplemented with BAP (1 mg/L) and NAA (1 mg/L). The quantity and nature of growth regulators have significant effect on the germination of orchid seeds (Arditti, 1979).

The most appropriate medium was selected on the basis of time taken for germination of seeds and their growth and development. Initiation of seed germination was observed after five weeks of culture in five different hormonal combinations of the medium (Table 1). This was supported by the findings of Reddy et al. (1992), who studied the seed germination and seedling growth in four different species of orchids (*Cymbidium aloifolium*, *Dendrobium crepidatum*, *Epidendrum radicans* and *Spathoglottis plicata*) and found the seed germination after 5 weeks. It was also supported by similar findings of Hoshi et al. (1994) on the seed germination of four species of *Cypridium* and Pradhan and Pant (2009) on *Dendrobium densiflorum*.

Protocorms were obtained after 8 weeks of culture in

Table 1. Effect of growth hormones supplemented to MS medium on seed germination and seedling growth of *C. stricta* (D. Don) Schltr.

Medium	Growth hormones	Concentration of hormones (mg/L)	Observation taken in weeks			
			Initiation of germination	Protocorm formation	1st shoot formation	1st root formation
MS	-	-	5	8	13	-
MS	BAP	0.5	5	9	16	-
MS	BAP	1	8	14	19	-
MS	BAP	1.5	8	14	19	-
MS	BAP	2	7	13	21	-
MS	NAA	0.5	5	8	12	25
MS	BAP+NAA	0.5+0.5	5	8	11	24
MS	BAP+NAA	1+0.5	6	9	14	29
MS	BAP+NAA	1.5+0.5	5	9	14	25
MS	BAP+NAA	2+0.5	6	9	13	25
MS	NAA	1	8	10	13	-
MS	BAP+NAA	0.5+1	7	9	12	26
MS	BAP+NAA	1+1	7	9	13	23
MS	BAP+NAA	1.5+1	7	10	13	24
MS	BAP+NAA	2+1	7	10	13	25

Culture conditions: 25± 2°C, 32 weeks, 16 h photoperiod and 6 replicates were used in each combination.

**Figure 3.** Capsule cut longitudinally into two halves.**Figure 4.** Clumped protocorms on MS medium supplemented with 0.5 mg/L BAP after 10 weeks of culture.

three different hormonal combinations of the medium (Table 1). Similar findings were also reported by Basker and Narmatha Bai (2010) in the seed germination of *Eria bambusifolia* which took 7 weeks for protocorms formation, Pant et al. (2011) on *Phaius tancarvilleae* which took 9 weeks and Gogoi et al. (2012) on *Cymbidium eburneum* which also took 9 weeks. Clumped protocorms were observed in this case on MS medium supplemented with 0.5 mg/L BAP after 10 weeks of culture (Figure 4). The first shoot initial was obtained after 11 weeks of culture on MS medium supplemented

with BAP (0.5 mg/L) and NAA (0.5 mg/L) while it was observed after 13 weeks of culture on hormone-free MS medium (Figure 5). This was supported by the findings of Pant et al. (2011) on *P. tancarvilleae* which took 12 weeks for first shoot formation. Dense shoot formation was common under various hormonal combination of the medium (Figure 7) except on MS medium supplemented with 0.5 mg/L BAP (Figure 6). The first root initial was obtained after 23 weeks of culture on MS

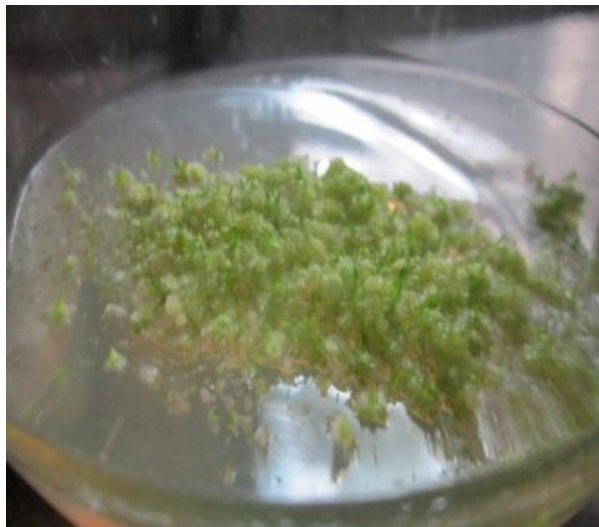


Figure 5. Initiation of shoots on hormone-free MS medium after 13 weeks of culture.

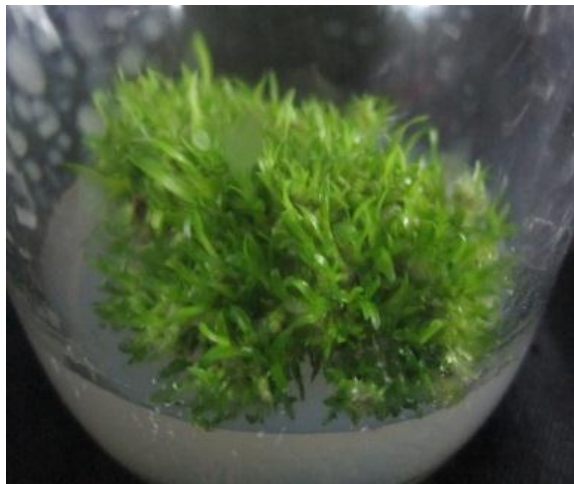


Figure 7. Shoots on MS medium supplemented with 1 mg/L BAP and 1 mg/L NAA after 20 weeks of culture.



Figure 6. Formation of shoots on MS medium supplemented with 0.5 mg/L BAP after 20 weeks of culture.



Figure 8. Initiation of root on MS medium supplemented with 0.5 mg/L NAA after 25 weeks of culture.

medium supplemented with BAP (1 mg/L) and NAA (1 mg/L) while on MS medium supplemented with 0.5 mg/L NAA it was observed after 25 weeks of culture (Figure 8). Pradhan and Pant (2009) in the seed germination of *D. densiflorum* found 19 weeks needed for the first root formation.

Complete plantlet of *C. stricta* was obtained after 23 weeks of culture. This was supported by the findings of Pant et al. (2011) on *P. tancarvilleae* which took 24 weeks to develop into complete plantlets and Paudel et al. (2012) on *Esmeralda clarkei* which took 25 weeks. MS medium supplemented with BAP (1 mg/L) and NAA (1 mg/L) was found to be the best for seed germination of *C.*

stricta. This was supported by the findings of Pant and Swar (2011) in the study of seed germination of *Cymbidium iridioides* who found MS medium supplemented with BAP (1 mg/L) and NAA (1 mg/L) to be best for the protocorms formation and seedlings growth.

Phytohormone NAA was found to be essential for root initiation as the medium combination lacking NAA did not develop root even after 32 weeks of culture (Table 1). It may be due to genetic constitution of explants and the endogenous growth regulators present in them. According to Yam et al. (1989), the nutritional requirements of germinating orchid seeds vary with their physiological state and this may be species specific. This also revealed that the addition of root hormone NAA might be essential in the nutrient medium for the

successful root growth and development. The nutrient requirement of orchid seeds in terms of quantity as well as form may vary at different stages of development for various species (Ernst, 1974; Arditti and Ernst, 1984).

Conclusion

The phytohormones BAP and NAA are both necessary for *in vitro* seed germination and seedlings growth of *C. stricta*. MS medium supplemented with BAP (1 mg/L) and NAA (1 mg/L) was found to be the best for this purpose.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to express sincere gratitude to Prof. Dr. Pramod K. Jha, Head, Central Department of Botany, Tribhuvan University and Dr. Sushim Ranjan Baral, former Chief, National Herbarium and Plant Laboratories, Godawari for providing necessary laboratory facilities for the research. We would also like to thank National Botanical Garden, Godawari for providing the orchid capsule.

Abbreviations

MS, Murashige and Skoog medium; **BAP**, 6-benzylaminopurine; **NAA**, α -naphthaleneacetic acid.

REFERENCES

- Arditti J, Ernst R (1984). Physiology of orchid seed germination. In: Orchid Biology: Reviews and Perspectives, Arditti J. (ed.), Cornell University Press, New York. 3:177-222.
- Arditti J (1979). Aspects of the physiology of orchids. Adv. Bot. Res. 7:421-655.
- Baral SR, Kurmi PP (2006). A Compendium of Medicinal Plants of Nepal. Mass Printing Press, Kathmandu, Nepal. P 534.
- Basker S, Narmatha Bai V (2006). Micropropagation of *Coelogyne stricta* (D. Don) Schltr. Via pseudobulb segment cultures. Trop. Subtrop. Agroecosyst. 6:31-35.
- Basker S, Narmatha Bai V (2010). *In vitro* propagation of an epiphytic and rare orchid *Eria bambusifolia* Lindl. Res. Biotechnol. 1:15-20.
- Ernst R (1974). The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. Am. Orchid Soc. Bull. 43:35-38.
- Gogoi K, Kumaria S, Tandon P (2012). *Ex situ* conservation of *Cymbidium eburneum* Lindl.: a threatened and vulnerable orchid, by asymbiotic seed germination. 3 Biotech. 2(4):337-343.
- Hoshi Y, Kondo K, Hamatani S (1994). *In vitro* seed germination of four Asiatic taxa and notes on the nodal micropropagation of American *Cypripedium montanum*. Landline 9:93-97.
- Joshi GC, Tewari LM, Lohani N, Upreti K, Jalal JS, Tewari G (2009). Diversity of orchids in Uttarakhand and their conservation strategy with special reference to their medicinal importance. Rep. Opin. 1(3):47-52.
- Pant B (2006). Biotechnology in orchid conservation. Proceeding of Natural Resource Management, Nepal Biological Society (NBS).
- Pant B, Raskoti BB (2013). Medicinal Orchids of Nepal. Himalayan Map House (P.) Ltd, Kathmandu, Nepal. P 104.
- Pant B, Shrestha S, Pradhan S (2011). *In vitro* seed germination and seedling development of *Phaius tancarvilleae* (L'Her.) Blume. Sci. World 9(9):50-52.
- Pant B, Swar S (2011). Micropropagation of *Cymbidium iridioides*. Nepal J. Sci. Technol. 12:91-96.
- Paudel M, Pradhan S, Pant B (2012). *In vitro* Seed Germination and Seedling Development of *Esmeralda clarkei* Rchb.f. (Orchidaceae). Plant Tissue Cult. Biotechnol. 22(2):107-111.
- Pradhan S, Pant B (2009). *In vitro* seed germination in *Cymbidium elegans* Lindl. and *Dendrobium densiflorum* Lindl. ex Wall. (Orchidaceae). Bot. Orient. J. Plant Sci. 6:100-102.
- Rajbhandari KR (2015). A Handbook of the Orchids of Nepal. Department of Plant Resources, Thapathali, Kathmandu, Nepal. P 168.
- Rao AN (1977). Tissue culture in the orchid industry. In: Applied and Fundamental Aspects of Plant Cell Tissue. Organ Cult., Reinert J. and Bajaj Y.P.S. (eds.), Springer Verlag, Berlin. pp. 44-69.
- Raskoti BB (2009). The Orchids of Nepal. Published by Bhakta Bahadur Raskoti and Rita Ale, Kathmandu, Nepal. P 252.
- Reddy PV, Nanjan K, Shanmugavelu KG (1992). *In vitro* studies in tropical orchids: seed germination and seedling growth. J. Orchid Soc. India 6(1, 2):75-78.
- Yam TW, Arditti J, Weatherhead MA (1989). The use of darkening agents in seed germination and tissue culture media for orchids: a review. J. Orchid Soc. India 3:35-39.

Full Length Research Paper

Adsorption of copper, zinc and lead on biosurfactant produced from cassava wastewater

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Received 18 September, 2015; Accepted 1 February, 2016

The remediation of soils contaminated by hydrophobic compounds, especially petroleum derivatives, using surfactants has been fairly studied. The use of these compounds in heavy metal contaminated areas is incipient and their mechanism of action has not been totally elucidated yet. The biosurfactants are compounds with surfactant characteristics produced by living organisms and have several environmental advantages, like greater biodegradability and production from renewable sources. The aim of this study was to evaluate the adsorption potential of the metals copper, zinc and lead onto the biosurfactant structure and to relate it to the Langmuir and Freundlich physicochemical models of adsorption. The biosurfactant produced presented an emulsification index (EI) around 60%, which was stable even after 72 h of emulsion formation. The analysis of FTIR and HPLC confirmed that the biosurfactant produced is composed mainly of surfactin, found in concentration of 28%. The biosurfactant was capable of adsorbing the metals in its structure. In the adsorption process of Pb^{2+} , the biosurfactant was considered as an interesting adsorbent, but this behavior did not adjust satisfactorily to Langmuir and Freundlich models. Thus, the biosurfactant has the potential to be utilized in remediation processes of soils and treatment of effluents contaminated by heavy metals.

Key words: *Bacillus subtilis*, trace elements, heavy metals, remediation of contaminated areas, agro industrial residue, surfactin.

INTRODUCTION

The surfactants are an important class of chemicals widely utilized in several industrial sectors, as

pharmaceutical, cosmetic, fine chemical, food and environmental applications, and are usually synthesized

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from petroleum derivatives (Silva et al., 2010). However, the growth of environmental concern between the consumers allied with new legislations of environmental control, lead to the pursuit of natural surfactants as an alternative to the available products. Biosurfactants are bacteria, fungi and yeast metabolic byproducts that show surfactant properties (Barros et al., 2007; Das et al., 2009; Marin et al., 2015; Cortés-Camargo et al., 2016). The application of surfactants produced biotechnologically is restricted to certain areas, since the production process is still not economically competitive as compared to surfactants derived from petroleum (Henkel et al., 2012). Biosurfactant production in industrial scale is usually limited by the high cost of culture media, associated with inefficient downstream methods and to relatively low incomes of the product (Henkel et al., 2012). The use of agroindustrial wastes as substrates for microbial production has been suggested as an alternative to make the process commercially feasible (Colla and Costa, 2003; Barros et al., 2007; Bezerra et al., 2012; Henkel et al., 2012) and enables, as well, the utilization of million tons of environmentally dangerous residues in biotechnological processes (Cortés-Camargo et al., 2016).

Different industrial wastes were studied for biotechnological purposes, such as: cooking oil residues (Haba et al., 2000), effluents of olive oil production (Mercade et al., 1993), molasses and whey (Joshi et al., 2008), okara - solid residue from the production of soy milk (Slivinski et al., 2012), vine-trimming wastes (Cortés-Camargo et al., 2016) and liquor from sisal (*Agave sisalana*) pulp hydrolysis (Marin et al., 2015).

World cassava production is around 263 million tons (Mt) per year with Africa accounting for about 57% of the supply in 2012. Asia is second with 30.7%, while Latin America and the Caribbean are third with 12.2%. Nigeria is the largest producer of cassava in the world with more than 54 Mt at the end of 2012 and The Democratic Republic of Congo is the second largest producer of cassava in Africa after Nigeria in 2012 (FAOSTAT, 2014). A positive characteristic is that cassava crop is very resilient and can be cultivated in a wide variety of ecological zones, making it ideal for poor farmers to cultivate (Okudoh et al., 2014).

The industrial processing of the cassava generates residues that require different treatments for their disposition that are conditioned to the technological and economic level of each company. The liquid residue, cassava wastewater, is generated during the process of shredded mass pressing for flour production or during cassava starch extraction and purification (Suman et al., 2011). The high concentration of remaining carbohydrates and several important micronutrients can cause a serious problem of environmental pollution if the effluent is not properly treated before disposition (Damasceno et al., 2003). On the other hand, this is also an interesting characteristic in a culture medium, since there might be no need for supplementation to biosurfactant production

(Barros et al., 2007; Bezerra et al., 2012).

The application of surfactants in order to remediate soils contaminated with hydrocarbons and/or heavy metals has been widely studied and the biosurfactants are an attractive option because of their biodegradability and lower toxicity (Song et al., 2008; Wan et al., 2011). The efficiency of these compounds for the efficient removal of metals depends on several physicochemical mechanisms of interaction and environmental conditions that have still not been totally elucidated (Franzetti et al., 2009; Singh and Turner, 2009). Among the basic factors involved, the types of surfactant, soil and contaminant, as well the complex interaction between them may be cited (Juwarkar et al., 2007; Asçi et al., 2010; Torres et al., 2012). According to Ramamurthy et al. (2008), anionic surfactants have the largest effects on metals mobilization. Burrows et al. (2010) reported the surfactant acidity as a determining factor for its larger or smaller interaction with the metals, which is stronger for slightly acidic carboxylates than for sulphates or sulphonates. Although these interactions should be due in great part to electrostatic effects, they are also, in some cases, followed by an increase in entropy as a result of the release of water from hydrated cations.

Stalikas (2002) discussed that the interaction of metal species with surfactants occurs through electrostatic interactions with the surfactant polar head, or by hydrophobic interactions of the metal chelates with the micelles hydrophobic chain, which could be a strategy to, for example, promote the removal of metallic ions from an effluent (Costa, 2011).

Rhamnolipid-type biosurfactant interaction with metals was reported by Wang and Mulligan (2009), whose results suggested that the interaction of biosurfactant with soils and/or residues containing heavy metals, may occur due to several factors, such as interfacial tension reduction and solubilization of metals by the formation of micelles or complexes. Das et al. (2009) tested a biosurfactant produced by *Bacillus circulans* in different critical micellar concentrations (CMC) and concluded that as the concentration of the surfactant increases, the metal removal (Cd and Pb) of the environment (liquid effluent) also increased, reaching a complete removal with a concentration corresponding to 5xCMC.

Thus, this study aimed to evaluate the adsorption capacity of heavy metals (Cu^{2+} , Zn^{2+} and Pb^{2+}) into the biosurfactant obtained using cassava wastewater, liquid waste from cassava flour production, in order to assess its use as a possible alternative in decontamination of effluents and soils contaminated with metals.

MATERIALS AND METHODS

Biosurfactant production

The biosurfactant was produced by *Bacillus subtilis* LB5a isolates pertaining to the culture collection of the Bioflavors Laboratory of

DCA/FEA/Unicamp (Brazil). The liquid wastewater from the cassava flour, the cassava water, was previously treated by heating until boiling point, then chilled and centrifuged to be used as culture medium. The fermentation process was carried out in a pilot fermenter (Pilot New Brunswick 8000 MP 80), at 30°C, 4 vvm (air volume per volume of culture medium per minute) and agitation of 150 rpm (Barros et al., 2008a).

The foam produced during the fermentation process was continuously collected and collapsed (Barros et al., 2007). For biosurfactant extraction, the collapsed foam was centrifuged at 12.700 G for 10 min to get cell-free solution, then had its pH adjusted to 2.0 using 6N HCl and was kept at 4°C overnight for decantation. After, the liquid was centrifuged at 12.700 G per 20 min, the precipitate was collected, neutralized with 3 M NaOH, and then dried at 50°C 50²+C (Makkar and Cameotra, 1997, 1999; Barros et al., 2007). This material, named raw biosurfactant, was then crushed in mortar and stored for later analysis.

Emulsification index

Screw cap test tubes containing 6 mL of hydrocarbon and 4 mL of a 48 mg.L⁻¹ solution of raw biosurfactant were vortexed for 2 min and then left to rest at room temperature. The hydrophobic compounds utilized were sunflower oil, soybean oil and gasoline. The emulsification index was determined after 24, 48 and 72 h (EI_{24h}, EI_{48h} and EI_{72h}, respectively) by the difference between the height of the emulsion layer (EL) and the mixture's total height (TH), as shown in Equation 1 (Cooper and Goldenberg, 1987):

$$EI (\%) = (EL/TH) \times 100 \quad 1$$

Biosurfactant characterization by infrared spectroscopy

The biosurfactant obtained was characterized by Fourier transform mid-infrared spectroscopy (FT-MIR), using the Shimadzu IR Prestige 21 infrared spectrophotometer with detector DTGS. A quantity of 2 to 5 mg of the samples were diluted in 100 mg of KBr and analyzed by the transmission technique.

The organic attributions for the observed spectroscopic bands were done by the comparative method with the data available in Silverstein and Webster (2007) and other papers in the area to confirm the associations, which were presented during the discussion of the obtained results.

Biosurfactant characterization by high performance liquid chromatography

The biosurfactant was analyzed by high performance liquid chromatography (HPLC) utilizing a Shimadzu liquid chromatographer, model LC 20 AT, with a Kromasil C18 column. The mobile phase used was acetonitrile and trifluoroacetic acid (3,8 mmol.L⁻¹) in ratio of 80:20.

To confirm the surfactant type, a co-chromatography with surfactin standard from Sigma- Aldrich was performed. Both the biosurfactant and the standard sample were diluted in acetonitrile: methanol (1:1, v:v) (2 mg.mL⁻¹) and 20 µL were used for the injection. This injection methodology was based on Slivinski et al. (2012).

Adsorption Isotherms for the ions Cu²⁺, Zn²⁺ and Pb²⁺

A volume of 20 mL of several solutions containing metallic ions in different concentrations were brought into contact with 50 mg of

adsorbent (biosurfactant) at 25°C, agitation of 200 rpm and pH ~ 6.3. The pH of the metallic solutions was adjusted using NaOH (0.1 mol.L⁻¹) and was not corrected during the experiments of adsorption. In order to prevent an increase in competition of the different species for the adsorption spots, a pH buffer was not used. The metallic solutions were prepared from commercial analytical standards of 1,000 mg.L⁻¹ and then diluted to concentrations between 50 and 750 mg.L⁻¹ for Zn²⁺ and from 20 to 200 mg.L⁻¹ for both Cu²⁺ and Pb²⁺. All the tests were performed in triplicate.

After 24 h of shaking to reach the balance, the samples were centrifuged at 3,500 G for 10 min and then filtered through qualitative filter paper. The metal concentration in the supernatants was measured by atomic adsorption spectroscopy (AAS) in a Shimadzu spectrophotometer model AA 6030. Isotherms models of Langmuir and Freündlich were applied to the obtained data.

The quantity of adsorbed metal (q) was the difference between the initial concentration of metal in each solution and the concentration in the supernatant after contact with the adsorbent (C_e). The data collected experimentally were adjusted to the models of Langmuir and Freündlich whose equations are presented below. The isotherm of Langmuir is given by the Equation 2 where: q_e = quantity of adsorbed metal per mass adsorbent unity (mg.kg⁻¹); K_L = Langmuir constant related to the connection energy of the metal to the adsorbent (L.mg⁻¹); C_e = metal concentration in the balance solution (mg.L⁻¹).

$$\frac{C_e}{q_e} = \frac{1}{K_L b} + \frac{C_e}{b} \quad 2$$

The Freündlich equation is presented in Equation 3, where q_e = amount of metals adsorbed per unit mass of the adsorbent (mg.kg⁻¹); K_F = Freündlich adsorption constant (L.mg⁻¹); n = Freündlich constant related to surface heterogeneity (dimensionless); C_e = metal concentration in the balance solution (mg.L⁻¹)

$$q_e = K_F C_e^{\frac{1}{n}} \quad 3$$

RESULTS AND DISCUSSION

Emulsification index (EI)

The EI_{24h} for the biosurfactant solution with the three types of oil evaluated, sunflower oil, soybean oil and gasoline, were about 60% and it was observed that the emulsions formed were stable for at least 72 h (Table 1). Bezerra et al. (2012) who produced ramnolipid from cassava water with *Pseudomonas aeruginosa* obtained the emulsification index of the biosurfactant solution with kerosene ranging from 50 to 68% for the first 24 h. A lot of them are maintained stable for 72 h or more. Batista et al. (2006) that worked with surfactin, got values of 50% of the EI for the commercial kerosene. Barros et al. (2008b) analyzed the surfactin obtained from *B. subtilis* Lb5a and produced under the same conditions as described in the present study with 15 different types of hydrophobic compounds. The emulsions with sunflower

Table 1. Emulsification index (EI) of different emulsions of hydrocarbons with the biosurfactant produced by *Bacillus subtilis* LB5a.

Hydrophobic compound	EI (%)		
	24h	48h	72h
Soybean oil	59.4	59.4	59.4
Sunflower oil	59.7	59.7	59.7
Gasoline	60.3	60.3	60.3

oil, soybean oil and gasoline were stable for at least 72 h and values of EI_{24h} were close to 70%. This difference is probably due to the level of purification of the surfactin used, as the biosurfactant used by Barros et al. (2008b) had gone through an additional process of extraction with solvents. Besides, these same authors verified the relation between the sizes of the fatty acids carbonic chains in the tested oils and the EI_{24h} of the emulsions with surfactin and could not identify any relation between the type and the size of the chains. The stability of the emulsions found in the present study and also reported in other papers might indicate that, once the metal is adsorbed into the biosurfactant, the possibility of desorption of the metal to the environment is reduced.

Biosurfactant characterization

The results have confirmed that the biosurfactant obtained in this work is really surfactin, once the bands are very similar to the standard of surfactin (Sigma-Aldrich) (Figure 1). The Fourier transform infrared spectroscopy (FTIR) is a methodology that detects similarities or differences in the chemical structures of compounds (Franca et al., 2010). According to Silverstein and Webster (2007), functional groups and structures can be discriminated by their molecular vibrations. This technique can be used for the identification of the biosurfactants because most of them have one radical carbonyl, ester binding or carboxylic acid, which absorb energy in the infrared region of the electromagnetic spectrum (Gartshore et al., 2000).

The absorption spectrum in the infrared zone of the biosurfactant was compared with the spectrum of surfactin standard from Sigma Aldrich (Figure 1), as well as to the data described by Teixeira et al. (2009) and Oliveira et al. (2013) studies that obtained surfactin using different carbon sources. Oliveira et al. (2013) used clarified cashew juice and Teixeira et al. (2009), artificial mineral nutrient broth.

It is possible to observe an absorption in 3.282 cm^{-1} , characteristic of axial strain of the connection N-H present in peptides; absorption in 2.952 cm^{-1} corresponds to the asymmetric stretching of the connection C-H of grouping CH_3 (fatty acids); band in 2.922 cm^{-1}

correspondent to the asymmetric stretching of the connection C-H of grouping CH_2 ; band of axial strain C=O in 1.720 cm^{-1} of carboxylic acid. This band was observed in all works used as comparison and also in all of them, this strain is discrete (Teixeira et al., 2009; Oliveira et al., 2013). There are two absorptions, one in 1.647 cm^{-1} , that feature the amide I group (C=O; C=N), and other in 1.535 cm^{-1} that feature the amide II. Oliveira et al. (2013) also obtained a very similar spectrum, assigning such bands likewise to the amide groups. In the zone of 1.230 to 1.200 cm^{-1} , the vibration C=O in aromatic ring or CH_2 strain in alkyl carboxyl is possible. The strain in 1.056 cm^{-1} can represent the C-O stretch of polysaccharides.

The area obtained from the integration of the 5 peaks of the homologous by HPLC was applied in the regression equation calculated through the calibration curve constructed using different concentrations of surfactin standard from Sigma-Aldrich ($R^2 = 0.9904$) to estimate surfactin concentration in the raw biosurfactant. The elution profile in HPLC of the homologous lipopeptide produced presented retention times equivalent to the ones obtained for the standard surfactin. Doing the comparison of the chromatogram obtained for the biosurfactant produced with chromatogram of the standard Sigma-Aldrich sample, as well the results of FTIR and the literature results, it is possible to identify the surfactant produced as surfactin.

Five peaks have been identified: at 6, 14, 18, 25 and 27 min. The integration of the 5 peak areas showed that the concentration of surfactin in the raw biosurfactant was 28%. Since after acid precipitation the biosurfactant did not suffer any other downstream process, a low level of purity was expected. Oliveira et al. (2013) evaluated the biosurfactant production by *B. subtilis* using cashew juice and obtained 11.17% of surfactin in the semi-purified biosurfactant. Franca et al. (2010) produced biosurfactant from *Pseudomonas aeruginosa*. EQ 109 from glycerol and obtained low production yields, and after the purification process, the aforementioned authors obtained about 6.57% of rhamnolipid in biosurfactant.

The choice to avoid further steps of purification was made considering the intended use of this biosurfactant. It was reported that the extraction by solvents, and resulting increase in costs and generation of chemical residues, was not necessary for the process of remediation of soils or effluents.

Adsorption of metals onto the biosurfactant

In Figure 2, there are experimental data, as well as the obtained isotherms by the models of Langmuir and Freundlich for adsorption of Zn, Cu^{2+} and Pb^{2+} by the biosurfactant. Resulting parameters of the isotherms are listed in Table 2.

The high determination coefficients (R^2), obtained from

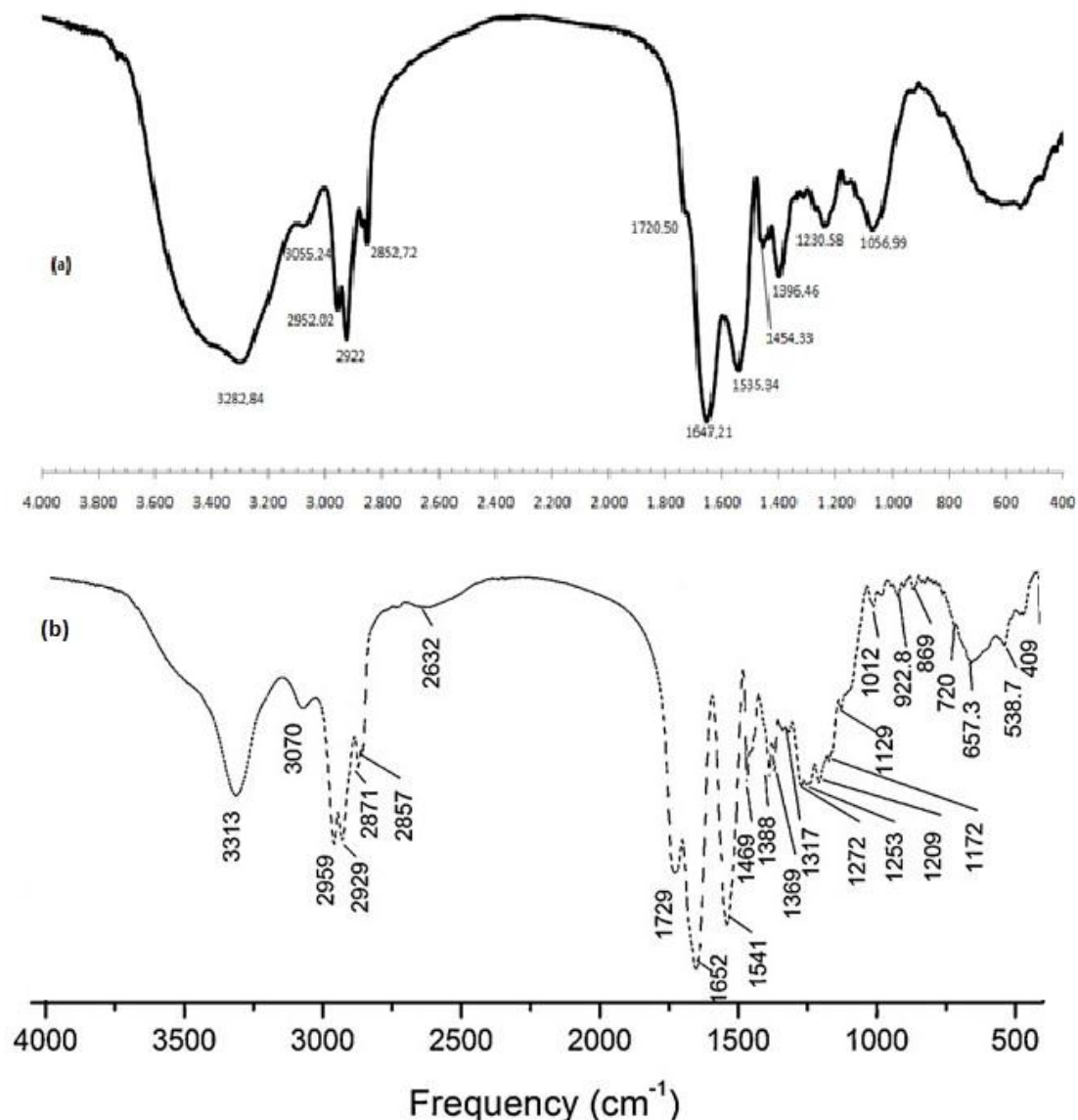


Figure 1. Infrared spectrum of the biosurfactant obtained by the bioprocess (a) and surfactin standard Sigma-Aldrich (b).

Table 2. Langmuir and Freundlich parameters for adsorption of Zn^{2+} , Cu^{2+} and Pb^{2+} into the biosurfactant.

Parameter	Langmuir			Freundlich		
	$K_L (L mg^{-1})$	$B (mg g^{-1})$	R^2	$K_F (L mg^{-1})$	$1/n$	R^2
Zinc	0.25	5.00	0.9981	100.79	1.21	0.8710
Copper	0.11	10.00	0.9879	303.53	1.04	0.8230
Lead	0.05	3.33	0.5355	32.02	2.08	0.8125

K_L - Langmuir isotherm constant; b - maximum monolayer coverage capacity; R^2 - coefficient of determination; K_F - Freundlich isotherm constant.

the linearized equations of Langmuir and Freundlich (Table 2), indicate that both adsorption models are

significantly able to demonstrate the adsorption of Zn^{2+} and Cu^{2+} into the studied biosurfactant.

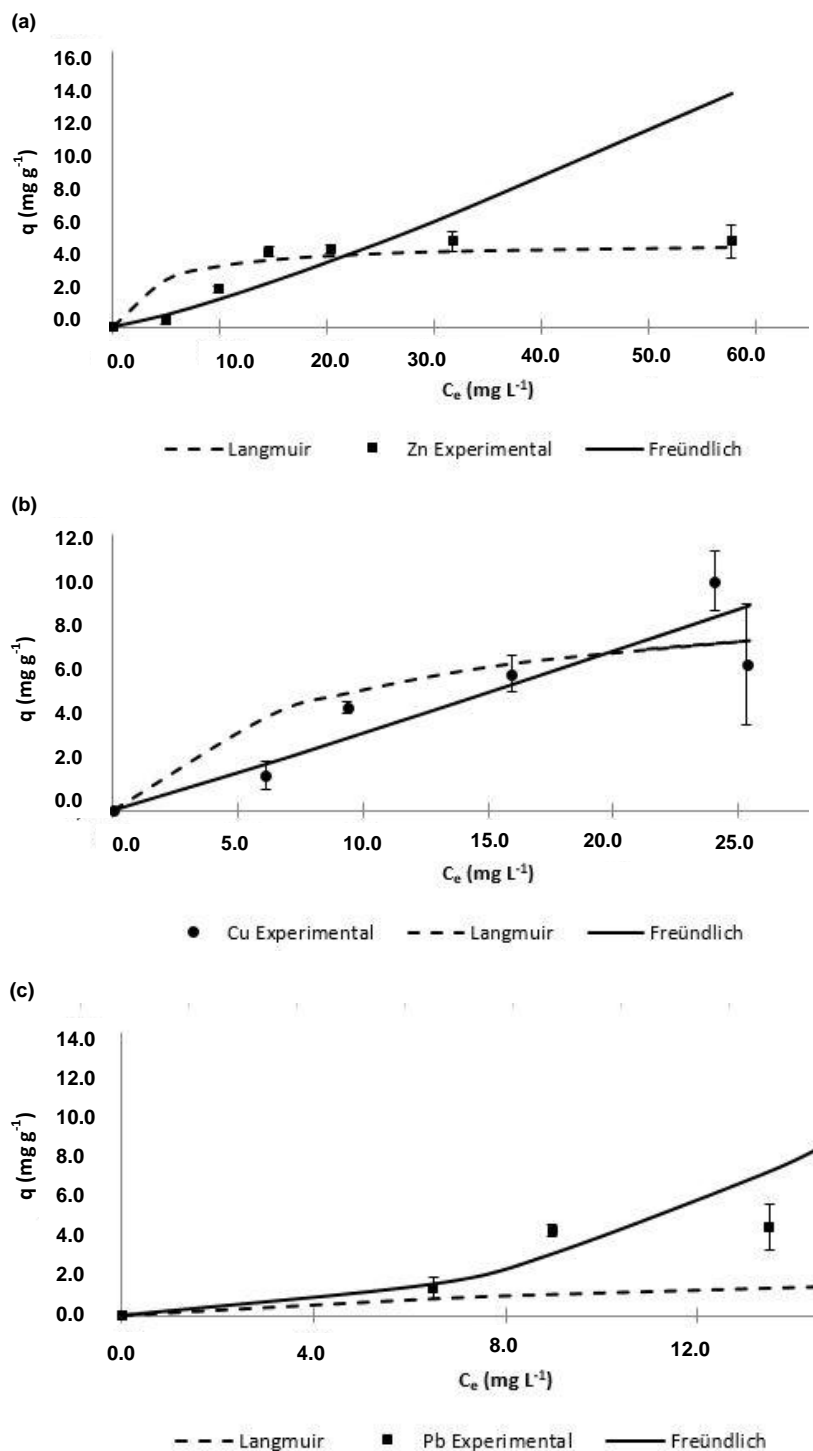


Figure 2. Isotherms obtained from the adsorption data obtained experimentally for Zn²⁺ (a) Cu²⁺ (b) and Pb²⁺ (c) in the equilibrium solution.

Langmuir model represented better the behavior of adsorption of Zn²⁺ into the biosurfactant, and Freundlich model overestimated the experimental values (Figure 2).

Based on the b values obtained by the Langmuir

isotherm, that estimates the maximum capacity of adsorption (MCA), it is possible to affirm that the biosurfactant has high affinity for the studied metals. The value for Cu²⁺ was the highest, 10 mg.g⁻¹, followed by Zn²⁺ (5 mg.g⁻¹). For Pb²⁺, the curves showed that the

system did not adjust well to the Langmuir model in the studied concentration range, which makes the MCA value not valid. Freundlich model was the most appropriate to describe the adsorption of this element.

The experimental values were important to confirm that the biosurfactant produced from cassava wastewater have shown high capacity to adsorb metals, independent whether an adjustment to the evaluated physicochemical models has been observed. According to Zeraik and Nitschke (2010) and Seydlová and Svobodová (2008), the surfactin is characterized as an anionic biosurfactant is a result of the negative charges in its polar group, which is due to the presence of functional groups as amide and carboxylic acids.

Therefore, this capacity to adsorb metals on the cation forms occurs because of the anionic nature of the surfactin, which allows the interaction between the negatives charges of the biosurfactant and the positives charges of the metallic ions. According to Piron and Domard (1997), who studied the uranium adsorption into different types of chitosans, the adsorption capacity increases with the average degree of acetylation (DA). The DA measures the percentage of amino groups available in the chitosan chain and is one of the main parameters that affect the adsorption properties.

Colla et al. (2012), in a study that compared the Cd removal in liquid containing biosurfactant and different species of filamentous fungi, concluded that in those liquids containing biosurfactant, the metal removal was better. The association between *Aspergillus* fungi and biosurfactant promoted 100% removal of the Cd existence in the solution. The authors concluded that the presence of biosurfactants in liquids improves the metal removal efficiency.

The difference between the adsorption potential for the three metals studied is due to the properties of each one of them. According to Vieira et al. (2011), concentration, pH and ionic strength of the solution, beyond the effect of competitors ions, widely influences the adsorption properties, besides contributing to the protonation of the amino groups from the biopolymers. Thus, the knowledge of the behavior of these metallic species in solution, may contribute to the understanding of the adsorption mechanism (ionic change, electrostatic action, chelation, etc).

According to Miller (1995), the biosurfactant presents potential to increase the efficiency of metal removal in soils because they facilitate sorption processes, mass transferences and resistance to transport in aqueous phase. The addition of biosurfactants may promote the desorption of heavy metals of solid phases through the complexation of free forms of metals in solution or by the accumulation of the biosurfactants in the liquid-solid interface, caused by the reduction of interfacial activities, which promotes a direct contact between the biosurfactants and the metals. Moreover, Valdman et al. (2005) demonstrated that an exopolysaccharide

extracted from the microorganism *Serratia* sp., with biosurfactant activities, presented a high efficiency in the Cd removal from liquids. In these experiments 50 mg.L⁻¹ of Cd²⁺ and 0.1 g.L⁻¹ of the previously purified biosurfactant were used, achieving a Cd removal of 170 mg.g⁻¹ of biosurfactant.

Conclusions

The biosurfactant produced presents emulsifier properties and stability, and the emulsification index was about 60% even after 72 h. The biosurfactant produced consists fundamentally of surfactin with 28% of purity.

The biosurfactant produced from cassava wastewater has shown capability to adsorb Cu²⁺, Zn²⁺ and Pb²⁺ into its structure. The maximum capacity of adsorption of Cu²⁺ and Zn²⁺ was estimated at 10 and 5 mg.g⁻¹, respectively.

In the adsorption process of Pb²⁺, the biosurfactant presented as a good adsorbent, however the satisfactory adjustment has not been observed for the Langmuir model. The Freundlich model presented better adjustment.

Further studies are recommended to verify the behavior of the biosurfactant in liquid effluents and soils containing heavy metals.

Conflicts of interest

The authors declare that there is no conflict of interest.

REFERENCES

- Asçi Y, Nurbas M, Açıkel SY (2010). Investigation of sorption/desorption equilibria of heavy metal ions on/from quartz using rhamnolipid biosurfactant. *J. Environ. Manage.* 91:724-731.
- Barros FFC, Ponezi AN, Pastore GM (2008a). Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. *J. Ind. Microbiol. Biotechnol.* 35:1071-1078.
- Barros FFC, Quadros CP, Maróstica Jr MR, Pastore GM (2007). Surfactina: propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. *Quim. Nova* 30:409-414.
- Barros FFC, Quadros CP, Pastore GM (2008b). Propriedades emulsificantes e estabilidade do biosurfactante produzido por *Bacillus subtilis* em manipueira. *Cienc. Tecnol. Aliment.* 28:979-985.
- Batista SB, Munteer AH, Amorim FR, Tótola MR (2006). Isolation and Characterization of Biosurfactant/Bioemulsifier-Producing Bacteria from Petroleum Contaminated Sites. *Bioresour. Technol.* 97:868-875.
- Bezerra MS, Holanda VCD, Amorim JA, Macedo GR, Santos ES (2012). Produção de biotensioativo utilizando *Pseudomonas aeruginosa* (P.A.) e resíduo agroindustrial (manipueira) como substrato. *Holos* 1:14-27.
- Burrows HD, Chimamkpam TO, Encarnação T, Fonseca SM, Pereira RFP, Ramos ML, Valente AJM (2010). Trivalent metal ion binding to surfactants and polyelectrolytes: a review. *J. Surface Sci. Technol.* 26:197-212.
- Colla LM, Costa JAV (2003). Obtenção e aplicação de biosurfactantes. *Vetor* 13:85-103.
- Colla LM, Hemkemeier M, Gil ASL (2012). Biossorção de cádmio e produção de biosurfactantes por fungos filamentosos em fermentação submersa. *Revista CIATEC – UPF* 4:1-10.
- Cooper DG, Goldenberg BG (1987). Surface-active agents from

- two *Bacillus* species. *Appl. Environ. Microbiol.* 53:224-229.
- Cortés-Camargo S, Pérez-Rodríguez N, Oliveira RPS, Huerta BEB, Domínguez JM (2016). Production of biosurfactants from vine-trimming shoots using the halotolerant strain *Bacillus tequilensis* ZSB10. *Ind. Crops Prod.* 79:258-266.
- Costa PBA (2011). Síntese e propriedades de surfactantes di-aniónicos: interação com íons trivalentes. MsC Dissertation, University of Coimbra, Portugal.
- Damasceno S, Cereda MP, Pastore GM, Oliveira JG (2003). Production of volatile compounds by *Geotrichum fragrans* using cassava wastewater as substrate. *Process Biochem.* 39:411-414.
- Das P, Mukherjee S, Sen R (2009). Biosurfactant of marine origin exhibiting heavy metal remediation properties. *Bioresour. Technol.* 100:4887-4890.
- FAOSTAT (2014). Cassava production and trade, Food and Agricultural Organization of the United Nations. Rome, Italy.
- Franca FP, dos Santos FPB, Lopes LMA (2010). Optimization of Biosurfactant Production from Glycerol by *Pseudomonas Aeruginosa* EQ 109 Using Factorial Design 2³. *J. Chem. Chem. Eng.* 4:1-12.
- Franzetti A, Caredda P, Ruggeri C, La Colla P, Tamburini E, Papacchini M, Bestetti G (2009). Potential applications of surface active compounds by *Gordonia sp.* strain BS29 in soil remediation technologies. *Chemosphere* 75:801-807.
- Gartshore J, Lim YC, Cooper DG (2000). Quantitative Analysis of Biosurfactants Using Fourier Transform Infrared (FT-IR) Spectroscopy. *Biotechnol. Lett.* 22:169-172.
- Haba E, Espuny MJ, Busquets M, Manresa A (2000). Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. *J. Appl. Microbiol.* 88:379-87.
- Henkel M, Müller MM, Kügler JH, Lovaglio RB, Contiero J, Syltatk C, Hausmann R (2012). Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. *Process Biochem.* 47:1207-1219.
- Joshi S, Bharucha C, Jha S, Yadav S, Nerurkar A, Desai AJ (2008). Biosurfactant production using molasses and whey under thermophilic conditions. *Bioresour. Technol.* 99:195-199.
- Juwarkar AA, Nair A, Dubey KV, Singh SK, Devotta S (2007). Biosurfactant technology for remediation of cadmium and lead contaminated soils. *Chemosphere* 68:1996-2002.
- Makkar RS, Cameotra SS (1999). Structural characterization of a biosurfactant produced by *Bacillus subtilis* at 45°C. *J. Surfactants Deterg.* 2:367-372.
- Makkar RS, Cameotra SS (1997). Biosurfactant production by a thermophilic *Bacillus subtilis* strain. *J. Ind. Microbiol. Biotechnol.* 18:37-42.
- Marin CP, Kaschuk JJ, Frollini E, Nitschke M (2015). Potential use of the liquor from sisal pulp hydrolysis as substrate for surfactin production. *Ind. Crop Prod.* 66:239-245.
- Mercade ME, Manresa MA, Robert M, Espuny MJ, Deandres C, Guinea J (1993). Olive oil mill effluent (OOME)-new substrate for biosurfactant production. *Bioresour. Technol.* 43:1-6.
- Miller RM (1995). Biosurfactant-facilitated remediation of metal-contaminated soils. *Environ. Health Perspect.* 103:59-62.
- Okudoh V, Trois C, Workneh T, Schmidt S (2014). The potential of cassava biomass and applicable technologies for sustainable biogas production in South Africa: A review. *Renew. Sustain. Energy Rev.* 39:1035-1052.
- Oliveira DWF, França IWL, Félix AKN, Martins JLL, Giro MEG, Melo VMM, Gonçalves LRB (2013). Kinetic study of biosurfactant production by *Bacillus subtilis* LAM1005 grown in clarified cashew apple juice. *Colloids Surf. B Biointerfaces* 101:34-43.
- Piron E, Domard A (1997). Interaction between chitosan and uranyl ions Part 1. Role of physicochemical parameters. *Int. J. Biol. Macromol.* 21:327-335.
- Ramamurthy AS, Vo D, Li XJ, Qu J (2008). Surfactant-enhanced removal of Cu (II) and Zn (II) from a contaminated sandy soil. *Water Air Soil Pollut.* 190:197-207.
- Seydlová G, Svobodová J (2008). Review of surfactin chemical properties and the potential biomedical applications. *Cent. Eur. J. Med.* 3:123-133.
- Silva SNRL, Farias CBB, Rufino RD, Luna JM, Sarubbo LA (2010). Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. *Colloids Surf B Biointerfaces* 79:174-183.
- Silverstein RM, Webster FX (2007). Spectrometric identification of organic compounds. 7ed. (Rio de Janeiro: LTC).
- Singh A, Turner A (2009). Surfactant-induced mobilisation of trace metals from estuarine sediment: implications for contaminant bioaccessibility and remediation. *Environ. Pollut.* 157:646-653.
- Slivinski CT, Mallmann E, Araújo JM, Mitchell DA, Krieger N (2012). Production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation using a medium based on okara with sugarcane bagasse as a bulking agent. *Process Biochem.* 47:1848-1855.
- Song S, Zhu L, Zhou W (2008). Simultaneous removal of phenanthrene and cadmium from contaminated soils by saponin, a plant-derived biosurfactant. *Environ. Pollut.* 156:1368-1370.
- Stalikas CD (2002). Micelle-mediated extraction as a tool for separation and pre concentration in metal analysis. *TRAC Trends Anal. Chem.* 21:343-355.
- Suman PA, Urbano LH, Leonel M, Mischán MM (2011). Efeitos de parâmetros de fermentação na produção de etanol a partir de resíduo líquido da industrialização da mandioca (manipueira). *Acta Sci. Technol.* 33:379-384.
- Teixeira ML, Cladera-Olivera F, Dos Santos J, Brandelli A (2009). Purification and characterization of a peptide from *Bacillus licheniformis* showing dual antimicrobial and emulsifying activities. *Food Res. Int.* 42:63-68.
- Torres LG, Lopez RB, Beltran M (2012). Removal of As, Cd, Cu, Ni, Pb, and Zn from a highly contaminated industrial soil using surfactant enhanced soil washing. *Phys. Chem. Earth* 37-39:30-36.
- Valdman E, Leite SG, Caldas SM (2005). Effect of biosurfactant concentration on cadmium biosorption by *Serratia sp* isolated from tropical soil. Paper presented at the 13th International Conference on Heavy Metals in The Environment, Rio de Janeiro, Brazil.
- Vieira RS, Oliveira MLM, Guibal E, Rodrigues-Castellanã E, Beppu MM (2011). Copper, mercury and chromium adsorption on natural and crosslinked chitosan films: An XPS investigation of mechanism. *Colloids. Surf. A Physicochem. Eng. Asp.* A374:108-114.
- Wan J, Chai L, Lu X, Lin Y, Zhang S (2011). Remediation of hexachlorobenzene contaminated soils by rhamnolipid enhanced soil washing coupled with activated carbon selective adsorption. *J. Hazard. Mater.* 189:458-464.
- Wang S, Mulligan CN (2009). Rhamnolipid biosurfactant-enhanced soil flushing for the removal of arsenic and heavy metals from mine tailings. *Process Biochem.* 44:296-301.
- Zeraik AE, Nitschke M (2010). Biosurfactants as agents to reduce adhesion of pathogenic bacteria to polystyrene surfaces: effect of temperature and hydrophobicity. *Curr. Microbiol.* 61:554-559.

Full Length Research Paper

Investigating the antioxidant properties and rutin content of Sea buckthorn (*Hippophae rhamnoides* L.) leaves and branches

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Received 29 April, 2015; Accepted 10 December, 2015

The present study focused on the antioxidant properties and rutin content of leaves and branches of *Hippophae rhamnoides* L. (Sea buckthorn) in Turkey. Dried leaves (leaf tea), processed (PB) and unprocessed branches (UB) of Sea buckthorn (SBT) were extracted with ethanol and prepared in forms of aqueous extract (AE). All samples were analyzed for their contents of rutin, total phenolics (TPC), total flavonoids (TFC) and total antioxidant capacity by using DPPH and CUPRAC methods. TPC of leaves from ethanolic extracts (EE) were significantly higher than UB and PB extracts. The DPPH scavenging activity of extracts ranged from 41.93 ± 3.57 and 132.43 ± 6.57 mg trolox equivalent (TE)/g, and the antioxidant capacity measured with CUPRAC method were in between 129.4 ± 18.1 and 538.5 ± 34.8 mg TE/g. Both EE and AE of leaf samples had significantly higher rutin content compared to the UB and PB samples. Taking the high antioxidant and rutin content of leaves into account and with respect to their positive health effects, consumption of SBT as a herbal tea should be investigated.

Key words: Sea buckthorn, *Hippophae rhamnoides*, leaves, antioxidant, phenolic profile, rutin content.

INTRODUCTION

Sea buckthorn (SBT) (*Hippophaë rhamnoides* L.) is a species of flowering plant in the family Elaeagnaceae, deciduous shrub with good adaptability to various climate conditions and extensive genetic variability with numerous greenish-yellow flowers and bright orange, globular, ellipsoid fruit. It is native to Europe and Asia and has been domesticated in several countries (India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania, France, etc.) (Khan et al., 2010; Yogendra Kumar et al., 2011; Pop et al., 2013). In

Turkey, SBT is widely distributed throughout North and East Anatolia and known locally as 'Yalancı igde, Cıdırgan, Dijirgan, Cıcılık' or 'Sincan Çalısı' (Aras-Tayhan, 1997). All parts of the plant are considered to be good source of large number of bioactive substances like vitamin, carotenoids, and flavonoids, organic acids, sterols and some essential amino acids. For instance, berries and seeds of SBT are well known for their antioxidative properties, attributed to hydrophilic and lipophilic compounds including ascorbic acid, flavonoids,

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Table 1. Some characteristics of the sampling sites.

Sampling site	Latitude/altitude longitude	Annual precipitation (Mm)	Annual temperature (°C)	Climate type	Sampling dates
Sivas-Sincan stream	39°54'N- 2000 m 37°59'E	794 -	2.8 -	Humid -	June 2013 August 2014

proanthocyanidins and carotenoids. The leaves of SBT are also considered for their antioxidant potential correlated to flavonoids and phenolic acids derivatives, and they have been used in some countries to make extracts, tea, animal feed, pharmaceuticals and cosmetics (Michel et al., 2012; Wani et al., 2013).

Flavonoids are the widest group of secondary metabolites involved in many biological functions in plants. They are classified into flavonols, flavones, flavanones, catechins, anthocyanidins and chalcones (Gupta et al., 2011). Rutin is one of the bioactive flavonoid compound, which is present in substantial amounts in various plants (Attanassova and Bagdassarian, 2009). Grapes and buckwheat are the most important rutin containing foods between fruits, vegetables and grain crops (Kreft et al., 2006). Rutin, a naturally occurring flavonol consisting of aglycone quercetin and a rutoside moiety in position 3 of the C ring, is found in many food substances. It has been reported to exhibit beneficial effects against several types of liver diseases (Pan et al., 2014). Within the group of flavonoids, many studies have been conducted on rutin, since this flavonoid is of great therapeutic importance. Rutin causes an increase in pancreatic lipase with consequent reduction in triacylglyceride levels in rats. It has also been described in events associated with the immune system, as seen with other flavonoids. Rutin has been shown to inhibit neoplasia induced by immunosuppression with azoxymethanol and to reduce oxidative stress in leukocytes in rheumatoid arthritis (Marcarini et al., 2011). *H. rhamnoides* is generally reported to be rich in a wide range of biologically active substances. Due to the antioxidant properties of sea buckthorn leaves the number of studies investigating their potential utilization has grown in recent years, but there are limited number of studies on SBT branches in the literature and therefore, this study was focused mainly on leaves and branches of SBT. The aim of the present study is to investigate the antioxidant properties and rutin content of sea buckthorn leaves, processed branches and unprocessed branches.

MATERIALS AND METHODS

Plant material

Wild Sea buckthorn (*H. rhamnoides* L.) were harvested from

Central Anatolia, particularly Sivas region (Table 1). The leaves and branches were separated from each other and ventilated. In order to inhibit the activation of oxidation enzyme, leaves and branches were subjected to shocking procedure. For this purpose plant materials were shocked with 90 to 100°C vapor in rotary evaporator. Output materials of the shocking unit were cooled with dry air. Cooled materials were subjected to folding procedure for 25 to 30 min. After folding unit, leaves and branches were transferred into the drying chamber and dried at 110°C. At the end of this treatment dried leaf tea and processed branch were obtained. These materials were cut into small pieces and stored in the dark at room temperature before use (up to 1 week).

Dried leaves, processed (PB) and unprocessed branches (UB) of *H. rhamnoides* were extracted with ethanol as described below, also prepared in forms of infusion of leaf tea and decoction technique was used for PB and UB. All samples were analyzed for their contents of total phenolics and total flavonoids as well as their total antioxidant capacity by using two different methods including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Copper Reducing Antioxidant Capacity (CUPRAC). The samples were also analyzed by using HPLC to identify their phenolic profiles.

Extraction procedure

Ethanolic extraction

Ethanolic extractions were carried out for dried leaves, PB and UB samples as described previously by Maheshwari et al. (2011) with slight modifications. 2.0 ± 0.01 g of each sample was extracted with 15 ml of 70% aqueous-ethanol in a cooled ultrasonic bath (Azakli, Turkey) for 15 min. After 24 h incubation, the treated samples were centrifuged (Hettich Zentrifugen Universal 32R, UK) for 10 min at 4000 rpm at 4°C and the supernatant were collected. Then 15 ml of 70% aqueous-ethanol was added to the pellet and this extraction procedure was repeated two more times. All four supernatants were combined and adjusted to a final volume of 50 ml. Prepared extracts were stored at -20°C until analysis.

Aqueous extraction

To improve the efficiency of extractions, different methods were used for aqueous extraction of leaves, UB and PB. Dried leaf tea samples were prepared in forms of infusions. For the preparation of infusions, 2 ± 0.01 g of dried leaves were weighed and dissolved in 200 ml of pure water at 90°C. After waiting for 30 min, the leaves were removed and the infusion was filtered, and further analyzed. Because of the woody structure of UB and PB, similar technique to the preparation of wood for microscopic examination was applied. 2 ± 0.01 g of UB and PB were weighed and 75 ml of water added. It was heated until boiling. At the boiling point 25 ml of pure water was added and was reboiled. This procedure repeated four more times until final volume of 200 ml. Branches were removed and extract was filtered and further analyzed (Aras-Tayhan, 1997).

Determination of total phenolic content (TP)

The TP of extracts was determined using Folin-Ciocalteu reagent according to the method modified from Velioglu et al. (2006) using gallic acid as a standard. A mixture of 100 μ l of the extract, 900 μ l of distilled water and 1.5 ml of 0.2 N Folin-Ciocalteu reagents were prepared and allowed to react for 5 min. Then 1.2 ml of 7.5% Na_2CO_3 solutions added into the reaction mixture. After incubation for 90 min at room temperature, the absorbance was measured at 765 nm using Optima SP-3000 nano spectrophotometer. The TP of extracts was expressed as mg of gallic acid equivalent (GAE) per g sample.

Determination of total flavonoid content (TF)

The TF was measured colorimetrically as described by Kim et al. (2003). Mixture of 0.25 ml of each extract, 1.25 ml of distilled water and 75 μ l of 5% NaNO_2 were prepared and allowed to react for 6 min. Then 150 μ l $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was added and mixed. After 5 min 0.5 ml of 1 M NaOH was added. The total volume was adjusted to 2.5 ml with distilled water. Absorbance of the mixture was measured at 510 nm versus prepared blank. The TF of extracts was determined by a rutin standard curve and expressed as milligram of rutin equivalent (RE) per gram sample.

Determination of total antioxidant capacity (TAC)

Antioxidant capacity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals. Therefore, in this study the TAC was estimated by two different assays. The DPPH and CUPRAC assays were performed according to Kumaran and Karunakaran (2006) and Apak et al. (2005) respectively. Trolox was used as a standard and results were expressed in terms of milligram of Trolox equivalent (TE) per gram sample.

DPPH-radical scavenging activity assay

The free radical scavenging activity of sea buckthorn leaves, UB and PB aqueous and ethanolic extracts on DPPH radical were determined according to the method introduced by Kumaran and Karunakaran (2006) and Rai et al. (2006). 0.1 mM of DPPH was prepared by dissolving 3.943 mg DPPH with 100 ml ethanol. 100 μ l of each extract was mixed with 2 ml ethanolic solution of DPPH (0.1 mM). Extracts were substituted by methanol and distilled water blanks. Decolourisation of purple free radical DPPH solution was measured at 517 nm after 30 min incubation in the dark and at room temperature. A trolox calibration curve was done between 0.01 and 0.2 mg/ml. Results were expressed in mg of trolox equivalents/g of sample (mg TE/g).

Copper reducing antioxidant capacity (CUPRAC) assay

CUPRAC assay was carried out according to Apak et al. (2005) which is based on the absorbance measurement of Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction of chain-breaking antioxidants with the CUPRAC reagent, Cu(II)-Nc, where absorbance is recorded at the maximal light-absorption wavelength of 450 nm. 10^{-2} mM of CuCl_2 solution was prepared by dissolving 0.4262 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water, and diluted to 250 mL. Ammonium acetate buffer at pH =7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in distilled water and diluted to 250 mL. Neocuproine (Nc) solution was prepared daily by dissolving 0.078 g Nc in 96% ethanol, and diluted to 50 mL with ethanol. 100 μ l of

extract was mixed with 1 ml of CuCl_2 solution, 1 ml of Nc solution, 1 ml of ammonium acetate buffer and 1 ml of distilled water. After 30 min absorbances were measured at 450 nm against reagent blank. Results were expressed in milligram of TE per gram of sample.

HPLC analysis of rutin and other flavonols

Rutin and major flavonols were determined following the method of Capanoglu et al. (2008). Extracts were filtered through a 0.45 μ m membrane filter and analyzed using a Waters 2695 HPLC system with a PDA (Waters 2996) detector. A Supelcosil LC-18 (25 cm \times 4.60 mm, 5 μ m column Sigma-Aldrich, Steinheim, Germany) was used. The mobile phase consisted of solvent A, Milli-Q water with 0.1% (v/v) Trifluoroacetic acid (TFA) and solvent B, acetonitrile with 0.1% (v/v) TFA. A linear gradient was used as follows: At 0 min, 95% solvent A and 5% solvent B; at 45 min, 65% solvent A and 35% solvent B; at 47 min, 25% solvent A and 75% solvent B; and at 54 min returning to initial conditions. The flow rate was 1 ml/min. Detection was done at 360 nm. Identification was based on the retention times and characteristic UV spectra. Quantification was done using external standards (rutin, quercetin-3-O-galactoside, kaempferol) as well as taking the information from the literature into account.

Statistical analysis

All the experiments were replicated three times and the data were represented as mean \pm SD. For multiple comparisons, data were subjected to statistical analysis using SPSS software (version 16.0 for Windows, SPSS Inc.) for the analysis of variance (ANOVA). Duncan's new multiple range test was used to analyze differences between treatments ($p < 0.05$).

RESULTS

Total phenolic content (TPC) and total flavonoid content (TFC)

The TPC of SBT leaves, unprocessed branches (UB) and processed branches (PB) were reported in the range of 25.8 \pm 2.9 to 75.9 \pm 6.5 mg GAE/g (Table 2). The TPC of leaves from ethanolic extracts (EE) were significantly higher than UB and PB extracts ($p < 0.05$). It was observed that TPC of PB extracts were higher than UB extracts. When considering PB and UB extracts with respect to the differences between aqueous and ethanolic extraction methods, the TPC of aqueous extracts (AE) were substantially higher than ethanolic extracts (Figure 1). This circumstance was interpreted, as decoction was a more efficient extraction technique vis-à-vis ethanolic extraction.

The TFC of SBT leaves, UB and PB were indicated in the range of 21.2 \pm 3.8 to 74.0 \pm 3.0 mg RE/g. In contrast with TPC results, TFC of all EE was higher than the AE content. The highest level of flavonoids was found in EE of leaves (74.0 \pm 3.0 mg/g) followed by UB (64.6 \pm 4.5 mg/g) and PB (59.4 \pm 6.8 mg /g). But there is no significant difference between EE of UB and PB statistically ($p > 0.05$).

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), Free radical scavenging activity (DPPH) and Copper reducing antioxidant capacity (CUPRAC) of SBT ethanolic and aqueous extraction.

Variables	Sample	Total phenolics (mg GAE/g)	Total flavonoids (mg RE/g)	DPPH (mg TE/g)	CUPRAC (mg TE/g)
Leaf	Ethanolic extraction	75.9± 6.5 ^a	74.0 ± 3.0 ^a	89.6 ± 2.0 ^c	538.5 ± 34.8 ^a
	Aqueous extraction	66.9± 5.9 ^a	21.2 ± 3.8 ^e	132.4± 6.6 ^a	293.5 ± 26.7 ^b
Processed Branch	Ethanolic extraction	43.1± 5.7 ^c	59.4 ± 6.8 ^b	83.3 ± 9.8 ^c	291.3 ± 17.2 ^b
	Aqueous extraction	53.8± 8.9 ^b	45.2 ± 0.6 ^c	113.1± 6.1 ^b	235.5 ± 34.1 ^c
Unprocessed Branch	Ethanolic extraction	25.8± 2.9 ^d	64.6 ± 4.5 ^b	41.9 ± 3.6 ^e	129.4 ± 18.1 ^e
	Aqueous extraction	43.2± 3.6 ^c	30.5 ± 3.4 ^d	71.1 ± 7.9 ^d	181.7 ± 27.6 ^d

*Data represent average values ± standard deviation of three independent samples. Different letters in the columns represent statistically significant differences ($p < 0.05$).

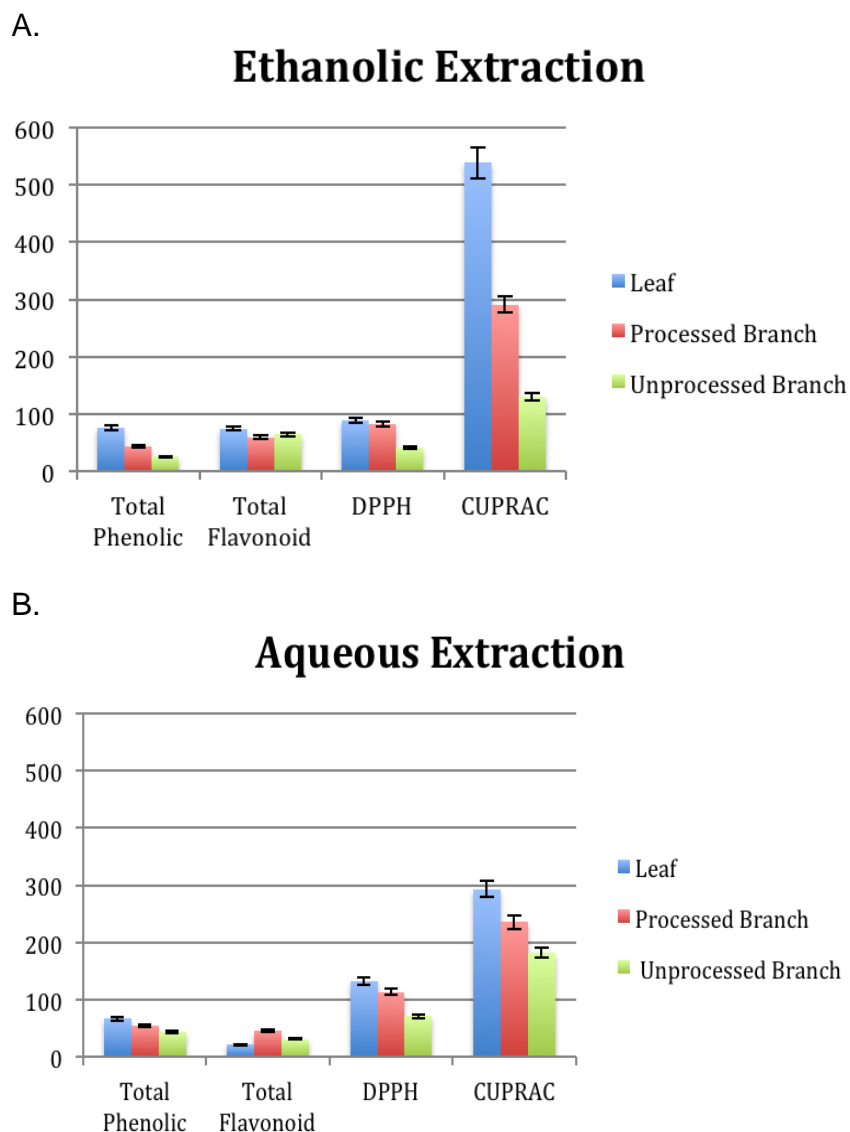
**Figure 1.** Total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activity (DPPH) and copper reducing antioxidant capacity (CUPRAC) of both ethanolic (A) and aqueous (B) extracts.

Table 3. Flavonol profile of ethanolic and aqueous extracts of SBT.

Compound ($\mu\text{g/g}$)	Leaf		Processed branch		Unprocessed branch	
	Ethanolic	Aqueous extraction	Ethanolic	Aqueous extraction	Ethanolic	Aqueous extraction
Rutin	8377 \pm 96 ^a	6939 \pm 46 ^b	1229 \pm 98 ^d	1433 \pm 47 ^c	757 \pm 13 ^e	418 \pm 108 ^f
Quercetin-3-O-galactoside	703 \pm 13 ^a	586 \pm 37 ^b	164 \pm 23 ^d	231 \pm 30 ^c	152 \pm 7 ^d	85 \pm 31 ^e
Kaempferol	129 \pm 2 ^a	65 \pm 1 ^b	Nd*	Nd	Nd	Nd

*Data represent average quantities \pm standard deviation (determined by HPLC) of three independent samples. Different letters in the rows represent statistically significant differences ($p < 0.05$). **Nd: not detected.

Antioxidant activity

To investigate the antioxidant activities of SBT leaves UB and PB two different *in vitro* assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Copper Reducing Antioxidant Capacity (CUPRAC) were used. The DPPH scavenging activity of extracts ranged from 41.9 ± 3.6 and 132.4 ± 6.6 mg TE/g. EE of leaf (89.6 ± 2.1 mg TE/g) had significantly higher antioxidant properties than PB (83.3 ± 9.8 mg TE/g) and UB (41.9 ± 3.6 mg TE/g). But statistically there is no significant difference between EE of leaves and PB ($p > 0.05$). This correlation was also valid for AE. When compared to extraction methods, higher scavenging activity was observed in AE of leaves, UB and PB (Figure 1). The CUPRAC values of extracts ranged from 129.4 ± 18.1 to 538.5 ± 34.8 mg TE/g. Higher antioxidant capacity was observed in EE of leaf, PB and AE of UB.

HPLC analysis

The results as shown in Table 3 indicated that the extracts are rich in rutin, Quercetin-3-O-galactoside and kaempferol.

DISCUSSION

In the present study, relatively higher phenolic compounds were observed from EE in comparison to AE of SBT leaves (Figure 1). These results were in accordance with previous studies (Upadhyay et al., 2010; Yogendra Kumar et al., 2013). Leaves were found to contain maximum TPC followed by processed branches and unprocessed branches.

In a study performed by Michel et al. (2012), different SBT extracts were evaluated for their antioxidant activity. The DPPH scavenging activity of extracts ranged from 174.8 and 528.6 mg TE/g.

In another study, Yogendra Kumar et al. (2011), evaluated antioxidant activity of the leaf extracts of SBT using DPPH and FRAP assays. The trolox equivalent

antioxidant capacity (TEAC) values reported for the extracts by using FRAP assay were in the range of 2.03 to 182.13 mg/g, while the values for the DPPH assay were 6.97 to 282.75 mg/g. In addition, according to the researchers the higher antioxidant activity exhibited by the sub critical water extracts (SWE) over the other Soxhlet and decoction extraction methods clearly demonstrated the relative advantage of SWE for obtaining formulations with high antioxidant compounds.

The profile of lipophilic antioxidants (LA) was also studied in SBT leaves, harvested in summer (June) and autumn (October) from plants of both sexes (female and male) by Górnas et al. (2014). According to this study, results indicated a greater significance of plant sex rather than harvest time; however, autumn samples of both plant sexes had a slightly higher antioxidant activity than summer samples. But an opposite phenomenon was observed in the case of isolated LA fraction from SBT leaves extracts. The higher antioxidant activity of the isolated LA fraction from 80% ethanol extracts of SBT leaves harvested in autumn, compared with the summer samples, were explained by an increase in the content of lipophilic compounds during leaf development in plants of both sexes.

According to the results, there are differences between DPPH and CUPRAC assays; higher values were obtained by the CUPRAC method. This might be related with the diversity of the reaction conditions including the required reaction time, and the wavelength at which the measurements are performed (Antolovich et al., 2002; Capanoglu et al., 2010). It is known from the literature that the chromogens of CUPRAC method have a good solubility in solvent systems (both aqueous and organic) (Apak et al., 2007), which can be the reason of high TE values. The differences are basically as a result of the non-standardized assay techniques with different radicals that is generated, time of reaction or mechanism. Especially, with regard to the antioxidant capacity tests, it will be beneficial to apply different test procedures for a full evaluation of antioxidant activity (Antolovich et al., 2002; Capanoglu et al., 2010). Moreover, in order to investigate these metabolites in detail it is recommended to use more comprehensive methods such as LC-MS.

Several authors have determined the profile of major phenolic compounds of SBT (Zu et al., 2006; Arimboor et al., 2008; Upadhyay et al., 2010; Arimboor and Arumughan, 2012; Pop et al., 2013, etc). In these studies, compounds belonging to groups of phenolic acids, flavonols, and flavones were identified. In the group of phenolic acids, gallic acid (GA) was the dominant phenolic acid for SBT leaves. Bittova et al. (2014) monitored the HPLC profiles of polyphenolic compounds in different SBT plant parts during annual growth cycle and estimation of their antioxidant potential. Their results showed that catechin, epicatechin and gallic acid were the most abundant analytes in annual green shoots and leaves, and their content varied significantly during the studied period. Besides, according to the result of Pop et al. (2013) leaves had higher levels of flavonol glycosides than berries, at average 1118 mg/100 g dry weight. On the other hand, isorhamnetin were the predominant flavonoid for berries, but rutin, quercetin-3-glucoside and kaempferol were found to be predominant in leaves.

This study also focused on the analysis of flavonols, particularly rutin, from sea buckthorn leaves and branches. In the literature, there is limited information on the content of rutin in SBT. According to our results, rutin content changed between 418 ± 108 and 8377 ± 96 $\mu\text{g/g}$. Rutin content is critical since it was reported to have positive health effects against several types of liver diseases and it has great therapeutic importance. It also causes an increase in pancreatic lipase with consequent reduction in triacylglyceride levels in rats. It has also been described in events associated with the immune system, as seen with other flavonoids (Marcarini et al., 2011). The highest rutin amounts were determined in EE of leaf (8377 ± 96 $\mu\text{g/g}$). Both EE and AE of leaf had significantly higher rutin content compared to processed branches and branches. On the other hand rutin content of all samples were significantly different ($p < 0.005$).

The quercetin-3-O-galactoside amounts of SBT leaves, processed branches and branches were reported between the range of 85 ± 31 and 703 ± 13 $\mu\text{g/g}$. The highest quercetin-3-O-galactoside amounts were determined in EE of leaf too (703 ± 13 $\mu\text{g/g}$). But there is no significant difference between EE of UB and PB ($p > 0.005$). But kaempferol was only observed in EE (129 ± 2 $\mu\text{g/g}$) and AE (65 ± 1 $\mu\text{g/g}$) leaf extracts.

HPLC results indicated that parallel to the spectrophotometric analysis results, infusion resulted with lower flavonol content. Ethanolic extraction presented the highest concentration of flavonoids except processed branches. Martins et al. (2014), evaluated and compared the antioxidant and antibacterial activities, and phenolic compounds of the infusion, decoction and hydroalcoholic extract of oregano. According to their results, both preparations, mostly decoction, gave higher antioxidant activity than the hydroalcoholic extract. The antioxidant properties seem to be related to phenolic compounds,

mainly flavonoids, since decoction presented the highest concentration of flavonoids and total phenolic compounds, followed by infusion and hydroalcoholic extract, respectively.

Conclusion

According to the results of the current study, leaves, UB and PB of SBT acquire high amounts of phenolics, flavonoids and also present some degree of antioxidant activities. Taking the high antioxidant and rutin content of leaves into account and with respect to their positive health effects, consumption of SBT in forms of an herbal tea should be investigated. Wide variations in the flavonoid profile and antioxidant activity of samples were observed which is probably related with the effect of type and variety of leaves and branches and other related factors as well as processing conditions including temperature, relative humidity, etc. Further studies should be performed in order to establish bioactive properties *in vivo* and *in vitro*.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study was financially supported by Istanbul University, Scientific Research Project (BAP) Unit, Project no. 35978.45891 and Ministry of Science, Industry and Technology, Techno-initiative Project no. 0239.TGDS.2013.

REFERENCES

- Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K. (2002). Methods for testing antioxidant activity. *Analyst* 127:183-198.
- Apak R, Guclu K, Demirata B, Ozyurek M, Celik SE, Bektasoglu B, Berker KI, Ozyurt D (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules* 12:1496-1547.
- Apak R, Guclu K, Ozyurek M, Karademir SE, Altun M. (2005). Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: the CUPRAC method. *Free Radic. Res.* 39:949-961.
- Aras -Tayhan A (1997). Türkiye'nin *Hippophae rhamnoides* L. Taksonu Üzerinde Ksilolojik ve Palinolojik Araştırmalar. PhD Thesis (Unpublished).
- Arimboor R, Arumughan C (2012). HPLC-DAD-MS/MS profiling of antioxidant flavonoid glycosides in sea buckthorn (*Hippophae rhamnoides* L.) seeds. *Int. J. Food Sci. Nutr.* 63:730-738.
- Arimboor, R, Kumar, KS, Arumughan C (2008). Simultaneous estimation of phenolic acids in sea buckthorn (*Hippophae rhamnoides*) using RP-HPLC with DAD. *J. Pharm. Biomed. Anal.* 47(1):31-38.
- Attanassova M, Bagdassarian V (2009). Rutin content in plant products. *J. Univ. Chem. Technol. Metall.* 44:201-203.
- Bittova M, Krejzová E, Roblová V, Kubáň P, Kubáň V (2014). Monitoring

- of HPLC profiles of selected polyphenolic compounds in sea buckthorn (*Hippophaë rhamnoides* L.) plant parts during annual growth cycle and estimation of their antioxidant potential. *Cent. Eur. J. Chem.* 12:1152-1161.
- Capanoglu E, Beekwilder J, Boyacioglu D, De Vos R, Hall RH (2010). The Effect of Industrial Food Processing on Potentially Health-beneficial Tomato Antioxidants. *Crit. Rev. Food Sci. Nutr.* 50:919-930.
- Capanoglu E, Beekwilder J, Boyacioglu D, Hall RH, de Vos R (2008). Changes in antioxidant and metabolite profiles during production of tomato paste. *J. Agric. Food Chem.* 56:964-973.
- Górnas P, Sne E, Siger A, Seglina D (2014). Sea buckthorn (*Hippophaë rhamnoides* L.) leaves as valuable source of lipophilic antioxidants: The effect of harvest time, sex, drying and extraction methods. *Ind. Crops Prod.* 60:1-7.
- Gupta N, Sharma SK, Rana JC, Chauhan RS (2011). Expression of flavonoid biosynthesis genes vis-à-vis rutin content variation in different growth stages of *Fagopyrum* species. *J. Plant Physiol.* 168:2117-2123.
- Khan BA, Akhtar N, Mahmood T (2010). A comprehensive review of a Magic Plant, *Hippophaë rhamnoides*. *Pharmacogn. J.* 2:65-68.
- Kim D, Jeong SW, Lee CY (2003). Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.* 81:321-326.
- Kreft I, Fabjan N, Yasumoto K (2006). Rutin content in buckwheat (*Fagopyrum esculentum* Moench) food materials and products. *Food Chem.* 98:508-512.
- Kumaran A, Karunakaran RJ. (2006). Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. *Food Chem.* 97:109-114.
- Maheshwari DT, Yogendra Kumar MS, Verma SK, Singh VK, Singh SN. (2011). Antioxidant and hepatoprotective activities of phenolic rich fraction of Sea buckthorn (*Hippophaë rhamnoides* L.) leaves. *Food Chem. Toxicol.* 49:2422-2428.
- Marcarini JC, Tsuboy MSF, Luiz RC, Ribeiro LR, Hoffmann-Campo CB, Mantovani MS. (2011). Investigation of cytotoxic, apoptosis-inducing, genotoxic and protective effects of the flavonoid rutin in HTC hepatic cells. *Exp. Toxicol. Pathol.* 63:459-465.
- Martins N, Barros L, Santos-Buelga C, Henriques M, Silva S, Ferreira ICFR (2014). Decoction, infusion and hydroalcoholic extract of *Origanum vulgare* L.: Different performances regarding bioactivity and phenolic compounds. *Food Chem.* 158:73-80.
- Michel T, Destandau E, le Floch G, Lucchesi ME, Elfakir C. (2012). Antimicrobial, antioxidant and phytochemical investigations of sea buckthorn (*Hippophaë rhamnoides* L.) leaf, stem, root and seed. *Food Chem.* 131:754-760.
- Pan PH, Lin SY, Wang YY, Chen WY, Chuang YH, Wu CC, Chen CH (2014). Protective effects of rutin on liver injury induced by biliary obstruction in rats. *Free Radic. Biol. Med.* 73:106-116.
- Pop RM, Socaciu C, Pintea A, Buzoianu AD, Sanders MG, Gruppen H, Vincken JP (2013). UHPLC/PDA-ESI/MS Analysis of the Main Berry and Leaf Flavonol Glycosides from Different Carpathian *Hippophaë rhamnoides* L. Varieties. *Phytochem. Anal.* 24:484-492.
- Rai S, Wahile A, Mukherjee K, Saha BP, Mukherjee PK (2006). Antioxidant activity of *Nelumbo nucifera* (sacred lotus) seeds. *J. Ethnopharmacol.* 104:322-327.
- Upadhyay NK, Yogendra Kumar MS, Gupta A (2010). Antioxidant, cytoprotective and antibacterial effects of sea buckthorn (*Hippophaë rhamnoides* L.) leaves. *Food Chem. Toxicol.* 48:3443-3448.
- Velioglu S, Turkmen N, Sari F (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chem.* 99:835-841.
- Wani TA, Wani SM, Shah AG, Masoodi FA (2013). Optimizing conditions for antioxidant extraction from Sea Buckthorn leaf (*Hippophaë rhamnoides* L.) as herbal tea using response surface methodology (RSM). *Int. Food Res. J.* 20:1677-1681.
- Yogendra Kumar MS, Dutta R, Prasad D, Misra K (2011). Subcritical water extraction of antioxidant compounds from Sea buckthorn (*Hippophaë rhamnoides*) leaves for the comparative evaluation of antioxidant activity. *Food Chem.* 127:1309-1316.
- Yogendra Kumar MS, Tirpude RJ, Maheshwari DT, Bansal A, Misra K. (2013). Antioxidant and antimicrobial properties of phenolic rich fraction of Sea buckthorn (*Hippophaë rhamnoides* L.) leaves *in vitro*. *Food Chem.* 141:3443-3450.
- Zu Y, Li C, Fu Y, Zhao C (2006). Simultaneous determination of catechin, rutin, quercetin kaempferol and isorhamnetin in the extract of sea buckthorn (*Hippophaë rhamnoides* L.) leaves by RP-HPLC with DAD. *J. Pharm. Biomed. Anal.* 41:714-719.

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