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About the Cover: A view of Roselle fruits (*Hibiscus sabdariffa* L.). For further reading on nutritional and functional properties of Roselle seed protein hydrolysates, refer article # 3 on page 409.

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FOOD SCIENCE AND NUTRITION

Investigating the antioxidant and anticytotoxic activities of propolis collected from five regions of Indonesia and their abilities to induce apoptosis

A. E. Z. Hasan[1,2,3], D. Mangunwidjaja[1,2], T. C. Sunarti[1,2], O. Suparno[1,2*] and A. Setiyono[4]

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Abstract

Propolis is a resinous substance collected by stingless bee or honey bee from various plant sources. The substance is known to contain beneficial properties for human. The geographical origin of propolis determines its biological properties. In this study, propolis were collected from five regions of Indonesia with the objective of determining the yield, their total flavonoid content, their capacity to induce apoptosis, and their toxicity to the Michigan Cancer Foundation-7 (MCF-7) cell line. The inhibition of antioxidant 1,1-diphenyl-2-picrylhydrazyl (DPPH), the induction of apoptosis to Saccharomyces cerevisiae and the anticytotoxic ability were determined. Propolis from Pekanbaru region had higher yield than other regions with value of 19.97%; propolis from Kendal had higher quantity with value of 46.60%, total flavonoid content; propolis from Pandeglang was higher in DPPH oxidation capacity with value of 68.94 $\mu g.ml^{-1}$; propolis from Kendal, expressed petite cell induction in *S. cerevisiae* cells with value of 81.44%, and the anticytotoxic to MCF-7 breast cancer cell line were best observed in propolis from Makassar region with a value of 47.71% life cells. All of the propolis extracted from the stingless bee hive *Trigona* spp from five regions in Indonesia contained flavonoids.

Key words: Propolis, *Trigona* sp, flavonoids, anticancer, MCF-7, MTT-assay

Introduction

Propolis is a resinous hive product collected by honeybees or stingless bees and is used to make a nest as well as for defense. Propolis (in Greek) means defense of the city or the bee hives. It thus implies a product involved in the defense of the bee community (Salatino et al., 2005). Honey bees belonging to the *Trigona* sp do not have a sting for defense which likely causes them to produce relatively more propolis than stinging bees. Their are capable of producing a chemical for defense (Caron 1988).

One of the functions of propolis is to protect the beehive from bacterial contamination and insect attack. Propolis has been used in folk medicine since ancient times and recent studies have been conducted which reveal an advantage of propolis as antibacterial, antifungal, antiviral, antiinflammation, local anesthesia, hepatopro-tective, immuno-stimulant, antiparasitic, and antitumor functions (Yousef and Salama, 2009; Fearnley 2005; Woo, 2004). More than 180 active compounds in propolis are known (Kasahara et al., 2004; Khismatullina, 2005), among which the flavonoids, aromatic acids, terpenoids, phenylpropanoids, and fatty acids (Lustosa et al., 2008). For the last 40 years, many studies have focused on the chemical composition, biological activity, pharma-cological, and therapeutical uses of propolis (Khismatullina,2005).

Nunes et al. (2013) stated that the composition of the propolis depends on the season, the vegetation, and the area of collection. Fernandes-Silva et al. (2013) also stated that the chemical composition of propolis depends on the plant or plants from which the resin is collected and, consequently, on the geographic location of the hive. According to Franchi et al. (2012), the

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difference in the quality of propolis could be seen from the location and color of a beehive. However, the composition of propolis is more complex and unpredictable than previously assumed (Teixiera et al., 2005). Nowadays, most of the propolis used for commercial production to improve health and prevent illnesses are from Europe and America (especially Canada and Brazil), and very little from Asia.

The objective of this study was to compare the properties of the *Trigona* propolis extracted from five regions of Indonesia by determining the total flavonoid content, antioxidant activity, apoptosis induction, and the anticytotoxic activity to MCF-7 breast cancer cells line.

Materials and Methods

Materials and Equipment

The materials used in this study include: *Trigona* bee hives from Pandeglang, Kendal, Banjarmasin, Makassar, and Pekanbaru, 70% ethanol as solvent, Roswell Park Memorial Institue (RPMI) media, MCF-7 cancer cell line from LAPTIAB The Agency for The Assessment and Applicantion of Techno-logy, *S. cerevisiae*, DPPH, and $AlCl_3$. Equipment used in this study were an autoclave, an incubator with 5% CO_2, microwave processor, a laminar air flow, UV-Vis Spectrophotometer, and an orbital shaker.

Methods

Propolis Extraction

Propolis was extracted by using a modification (in time) of the method reported by Trusheva et al. (2006) and Jang et al. (2009). Extraction was conducted by using a combined method of maceration in 70% ethanol and microwave heating (Kriss Microwave Oven, 2450 MHz and 800 Watts). Aliquots of 2.0 g of crude propolis were obtained from each regions and combined with 18 ml of 70% ethanol. Each sample was mixed for 18 h at 200 rpm using an orbital shaker. After that, the samples were heated with microwave oven for 10 minutes. The extract was filtered and the filtrate was evaporated in a water bath at temperature of 50±2°C for three hours or until the water and ethanol evaporated.

Total flavonoid determination (Chang et al., 2002)

Total flavonoid content was determined by the colorimetric method using aluminium chloride. Aliquots of 2.0 ml of the extract solution, or standard solutions of quercetin (60, 50, 40, 20, 15, 10, and 5 $\mu g.ml^{-1}$), then were added to 100 µl of 10% $AlCl_3$, 0.1 ml of 1 M Na-Acetate and 2.8 ml of distilled water. The mixtures produced a yellow solution which was , then shaken vigorously until homogeneous and then left for 30 mins. The absorption was measured at a wave length of 415 nm. Total quercetin expressed as total flavonoid equal weight (g) of each dried sample weight (100 g).

DPPH Antioxidant Assay

The antioxidant activity assay was carried out according to Chang et al. (2002) and Chang et al. (2007). The propolis extract was redissolved in 70% ethanol and prepared at various concen-trations of 500, 250, 125, 62.5, 31.25, 15.5, and 7.75 $\mu g.ml^{-1}$. These were then mixed with 125 µmoles DPPH. The mixtures were the shaken vigorously and left to stand at room temperature for 30 mins in the dark. The absorbance at 515 nm of the reaction solutions was measured. The percentage of DPPH decolorization of the sample was cal-culated according to the following equation:

$$\text{DPPH radical scavenging (\%)} = (1 - A/A_o) \times 100$$

where A_o is the absorbance of the mixture without a sample and A is the absorbance of the mixture with sample after 30 mins. The IC_{50} of antioxidant activity was calculated as the concentrations of sample that inhibited by 50% the scavenging activity of DPPH radicals under these conditions.

Cell Apoptosis Test

Cell apoptosis assays were carried out according to Laun et al. (2001). YEPD agar plates were added to the propolis extract (50 $\mu g.ml^{-1}$) and poured with 200 µl of *S. cerevisiae* culture and then incubated at room temperature for 24 h. The growth of *S. cerevisiae* was measured by direct colony counting.

MCF-7 Cancer Cell Line Anticytotoxic Assays

Anticytotoxic activity assays were performed on cultured MCF-7 cancer cell line using the methylene blue test method that has been reported by Lin and Hwang (1991). The extract was dissolved in a viscous solvent of DMSO to make a 10% (v/v) substock solution, and then diluted in the RPMI 1640 medium to make a 1% (v/v) substock solution. Dissolution samples stratification was conducted to obtain a final concentration of test solutions of 100 $\mu g.ml^{-1}$. Then 20 µl of the test solution was added to the well plate which contained 100 µl cancer cell line and incubated for 24 h at 37°C in an incubator with 5% CO_2. The number of living cells was calculated using an ELISA (enzyme linked immunosorbent assay) reader, i.e. a serological test

that is commonly used in various immunology laboratories at a wavelength of 515 nm.

Analysis of biologically active compounds in Propolis

The methods for analysis of biologically active compounds reported by Harborne (1987) were employed for conducting the tests.

a) Alkaloids. A total of 100 mg of propolis extract was put into the test tube and combined with two drops of ammonia and 5 ml of chloroform and then filtered. The filtrate was added to 1 ml of 2.0 M H_2SO_4, and the acid fraction was taken then added by Dragendorf, Meyer and Wagner reagents. The presence of alkaloid was charac-terized by the formation of a red precipitate with Dragendorf rea-gent, white precipitate on Meyer reagent, and a brown precipitate on Wagner reagent.

b) Triterpenoids and Steroid. A total of 100 mg of extract was put into the test tube, then added by 5 ml of hot ethanol, and filtered. The filtrate obtained was evaporated, and then combined with 1 ml of diethyl ether. After mixing by vortex, then added by 1 ml of concentrated H_2SO_4 and 1 ml CH_3COOH. Red or purple color indicates the presence of triterpenoids and green or blue showed the presence of steroid.

c) Flavonoids. A total of 100 mg of extract was put into the test tube, then combined with 5 ml distilled water, and filtered. The filtrate obtained was combined with Mg powder, 1 ml of concentrated HCl, and 1 ml of amyl alcohol. The mixture then was shaken to allow the separation. Color (yellow-red) that forms at the interface of amyl alcohol showed the presence of the flavonoids.

d) Tannins. A total of 100 mg of extract was put into the test tube, then combined with 5 ml distilled water, and then filtered. The filtrate obtained was combined with 3 drops of 1% $FeCl_3$. The formation of a blue or greenish black color indicated tannin.

e) Saponins. A total of 100 mg of extract was put into the test tube, then combined with 5 ml distilled water and filtered. The filtrate obtained was vigourously shaken and left for 10 minutes. The formation of stable foam showed the presece of saponin compounds.

Results and Discussion

Propolis Extraction

Propolis is produced from beehives *Trigona* spp extracted using microwave treatment (Microwave-assisted extraction, MAE), which can increase the contact between solvent and sample (Jang et al., 2009). It is hoped that the desired compounds can be extracted properly. However, before the extraction by using a microwave, stinglessbees hive was macerated in ethanol. The 70% ethanol was capable to extract the flavonoid as the important compounds in propolis. The 70% ethanol is semipolar solvent which can extract all active components with different polarity in the propolis (Cunha et al., 2004; Hasan et al.,2006) and the optimum conditions for extracting flavonoid for *Alpinia pricei* rhizome (Hseu et al., 2011).

The chemical composition of propolis as well as colors and odor was strongly influenced by the source materials and the bee hives age. Filtrate colors of propolis produced from five different regions were light-yellow, yellow-black to brown and black. The compositions of each propolis were different from nest to nest, location to location, season to season and because bees take resin from the plants around the nest, then the composition of propolis vary depending on the locations or regions. Color, indicates the level of lightness and chromaticity of an object (Suparno et al., 2007). The chemical ingredient of a material or the existence of oxidized products (Suparno et al., 2009), can be used to predict yield of propolis in a propolis extract. Propolis extract having a darker color indicate a higher extractable yield compared with a brighter color. A higher flavonoid content is indicated by darker color (Woo, 2004). The yields of propolis extract obtained from five regions in Indonesia were different. Table 1 shows that the yield of propolis from Pekanbaru was significantly different from that from Makassar, but for other locations yields were almost the same. According to Paviani et al. (2011), the difference in the origin of propolis and solvent polarity resulted in differences in yield and lead to differences in the types and amounts of flavonoids.

Total Flavonoid

Trigona Spp. propolis produces large quantities of flavonoids compared to other types of bees. The higher concentration of flavonoid was indicated the color of the solution when reacted with $AlCl_3$. A high concentration was shown by color changes from yellow to the darker color and indicated by higher absorbance value which means that the extract contains high levels of flavonoid (Surendra et al., 2012; Woo, 2004).

Table 1. Yield, total flavonoid, antioxidant activity, and anticytotoxic activity of the propolis extracted from five regions in Indonesia.

Character	Regions				
	Makassar	Pekanbaru	Kendal	Pandeglang	Banjarmasin
Yield, %	1.85±0.51[c]	19.97±2.19[a]	7.28±1.59[b]	11.05±3.20[b]	8.38±0.70[b]
Total Flavonoid, μg. ml^{-1}	38.78±1.62[b]	16.90±0.537[e]	46.60±0.78[a]	30.62±1.50[c]	24.60±0.73[d]
Antioxidant Activity (IC_{50}), μg.ml^{-1}	1125.56±133[b]	308.88±12[c]	144.06±52.53[d]	68.935±5.63[e]	4162.61±845.9[a]
Anticytotoxic Activity at 100 μg.ml^{-1}, % Live Cell	47.71±9.31[c]	76.35±1.48[a]	50.26±2.70[c]	70.64±1.21[b]	75.79±1.33[a]

Remark: Result (number) followed by the same superscript letter on the same row shows that the results were not significant at α=0.05.

Table 2. Phytochemicals comprizing the propolis extracted from five regions in Indonesia.

No.	Class of Compounds	Regions				
		Makassar	Pekanbaru	Kendal	Pandeglang	Banjarmasin
1.	Alkaloids	-	-	-	-	-
2.	Flavonoids	+	+	+	+	+
3.	Saponins	-	+	+	-	-
4.	Tannins	-	+	+	+	-
5.	Steroids	-	-	-	-	-
6.	Triterpenoids	-	-	-	-	-

Remarks: + = positive results, - = negative results

Table 1 shows that propolis from Kendal contained the highest flavonoid content, and this was followed by propolis from Makassar, Pandeglang, Banjarmasin and Pekanbaru. Syamsuddin et al. (2010) reported that propolis originated from three regions of Java Island, each containing different types and values of flavonoids. Similarly, Daugsch et al. (2008) and Paviani et al. (2011) reported that for propolis from Brazil the differences in the quantities and types of flavonoids depended on bee-hive and solvent polarity. Antioxidant activities derived from plant sources are often associated with the content of flavonoids (Table 2). Flavonoids as antioxidants can capture free radicals capture by providing a hydrogen atom to the radical (Ratnam et al., 2006). The relationship between total flavonoid extract and antioxidant activity can be seen from the results of the antioxidant test. The flavonoid from propolis have antioxidant capacities that are much stronger than those of vitamins C and E (Prior and Cao, 2000).

Antioxidant Activity

The antioxidant test was conducted to determine the antioxidant potential of propolis extract, using the DPPH scavenging method. DPPH usage to scavenge radicals has some advantages, i.e. it is easy to use, it has high sensitivity, and it enables the analysis of large numbers of samples in a short time.

The parameters used for this test is IC_{50}, i.e. the concentration of extract required to capture the DPPH free radical by 50%. IC_{50} values were obtained from an equation which relates the concentration of the extract to the percent age of captured radicals. A smaller IC_{50} value of the extract means higher capturing activity of the DPPH radical, and therefore, increased activity as an antioxidant. Potential propolis extracts as an with high antioxidant activity were the propolis extracts from the Pandeglang with an IC_{50} value of 68.935 μg.ml^{-1}. That propolis extracts originated from Pekanbaru, Makassar, and Banjarmasin were not active as antioxidants is shown by their IC_{50} values wich were more than 150 μg.ml^{-1}. The extract originating from Kendal was moderately active antioxidant, as having an IC_{50} value was less than 150 μg.ml^{-1}.

The difference in the antioxidant activity is mainly due to the flavonoid content of propolis extracts or other compounds, which are potential antioxidants. The composition of propolis was influenced by the type and age of the bee-hive and existing vegetation around the hive of *Trigona* spp (Table 2). Total flavonoid content shows that the highest value was from Kendal, but its antioxidant properties was lower. This is in accordance with the

statement of Bankova et al. (2000) that the active ingredient in natural propolis is variable, depending on the resin plant origin, climate, and the time collecting resin plant by the bees. This difference is caused by the influence of different types of flavonoids (Jang et al., 2009). Antioxidant activity has a good correlation with flavonoid content (quercetine, apigenine, and kaempferol) and caffeic acid concentration (Coneac et al., 2008). The presence of techtochrysine (Lee et al., 2003) or propoline (Chen et al., 2004) increases enzymes that contributing in the antioxidant activity.

S.cerevisiae Apoptosis Induction

Apoptosis, or programmed cell death, is a normal development in the health of multicellular organisms. Cell death is a response to various stimuli and during apoptosis; the organisms are in a self-regulated and controlled state. This makes apoptosis distinct from another forms of cell death called necrosis, which is uncontrolled cell death caused by the lysis of cells, inflammatory responses and, potentially, to serious health problems (Granot, 2003). After receiving specific signals which instruct cells to undergo apoptosis. Typically a number of changes occur in the cell. Proteins known as *caspase* are normally activated. Bhatia-Kissova and Camougrand (2010) stated that the apoptosis mechanism in yeast caused by the addition of rifampicin or lactate originated in the mitochondria to form the mitochondria of *caspase* 1 (Yca1). This is the case also with the presence of propolis in this study. Many chemicals cause apoptosis in *S. cerevisiae* such as glucose, acetic acid and propolis (Sukhanova et al., 2011). The process of apoptosis occurring in *S. cerevisiae* has been described by Lotti et al. (2011). Cytochrome *c,* but not *endonuclease G* (Nuc1p), was involved in the propolis-mediated cell death in *S. cerevisiae* (de Castro et al., 2011).

This study shows that the induction of apoptosis on *S. cerevisiae* cell caused by propolis originated from Pekanbaru, Banjarmasin, Pandeglang, Makassar, and Kendal gave apoptosis induction potential of 50.94, 65.08, 67.75, 71.09 and 81.43%, respectively (Figure 1).

This difference was caused by the type of flavonoids contained in the propolis (Jang et al. 2009). In the research reported by Umthong et al. (2011), Thailand *Trigona* propolis contained compounds with antiproliferative activity in-vitro on cancer, but not on normal cell lines. The differences of type and number of flavonoids distinguished the mechanism of propolis in apoptosized cancer cell.

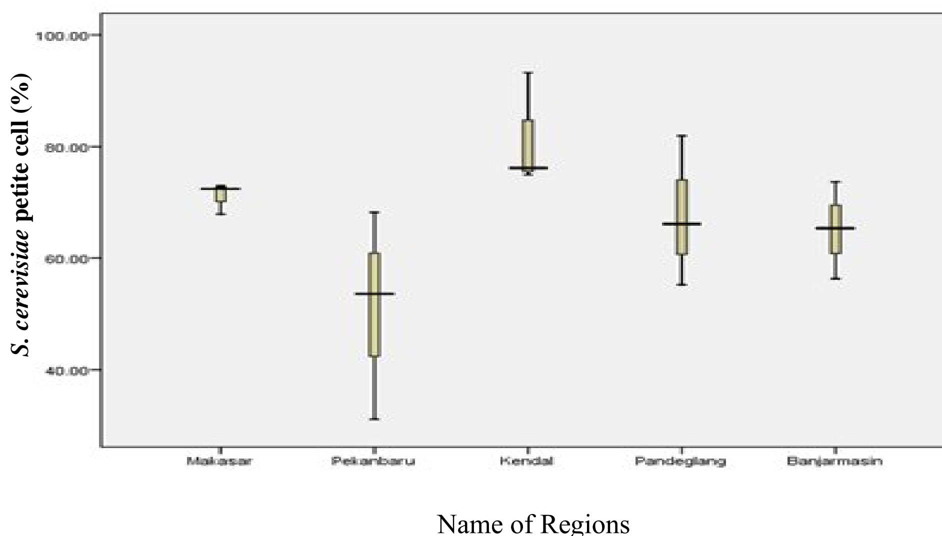

Figure 1. The persentage of *S. cerevisiae* petite cell with 50 $\mu g.ml^{-1}$ propolis from five regions in Indonesia.

MCF-7 Cancer Cell Line Anticytotoxic

The results shows that propolis originating from Pekanbaru provided an opportunity to live MCF-7 cells more than others, the opposite is the originated from Makassar that has better capacity as an anticancer agent (Table 1). This shows that the location of the beehive affected the quality of propolis produced. Data in Table 1 demonstrates the inhibition of cancer cell growth inhibition by propolis. Research conducted by Syamsuddin et al. (2010) suggests that there are differences in the results of MCF-7 cell IC50 of ethanol extract of propolis originated from Batang, Lawang and Sukabumi. The difference in the results is shown by the effect of the location of the hive on antibacterial activity has been done by Daugsch et al. (2008). Similarly, when compared between Fatoni (2009) and Hasan et al. (2011) showed differences in the antibacterial activity of propolis from different locations (Bukittinggi and Pandeglang) with different origin plant. The difference in effect between propolis origin may be due to the differences of propolis's chemical content (Zhu et al., 2011). Inhibition of cancer cells by propolis is caused by the activation of the caspase enzyme pathway and protein transcription pathways (Madeo et al., 2004).

Huang et al. (2012) reported that there were propolis components controlling cancer cell proliferation and maintaining or upregulating the function of tumor suppressor genes in normal cells. Research reported by Umthong et al. (2011) stated that *Trigona propolis* originating from Thailand contained compounds with antiproliferative activity in-vitro on cancer but not on normal cell lines. This difference is caused by the influence of type and number of flavonoids in the propolis (Jang et al., 2009), such as the chrysin and cafeic acid which inhibits the growth of cancer cells directly (Sawicka et al., 2012). In addition, different types of flavonoids would affect inhibitory mechanisms (Sawicka et al., 2012; Watanabe et al., 2011). The difference in propolin amounts, especially D, C, E, A, and B, will cause a difference in strengths of propolis as antitumor agents (Chen et al., 2004).

Conclusions

Propolis from Pekanbaru region had higher yield than other regions with value of 19.97%; propolis from Kendal had higher quantity with value of 46.60%, total flavonoid content; propolis from Pandeglang was higher in DPPH oxidation capacity with value of 68.94 $\mu g.ml^{-1}$; propolis from Kendal, expressed petite cell induction in S. cerevisiae cells with value of 81.44%, and the anticytotoxic to MCF-7 breast cancer cell line were best observed in propolis from Makassar region with a value of 47.71% life cells. All of the propolis extracted from the bee hive *Trigona* spp. from five regions in Indonesia contained flavonoids.

Therefore, propolis from Indonesia indicating may have the potency as anticancer agents and has potential in anticancer drug research.

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NUTRITION AND FOOD SCIENCE

Investigation of moisture sorption behavior of soluble sodium caseinate

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Abstract

Casein is a potential food protein, used in a number of food applications. In this work, moisture sorption isotherms of casein were determined at 10, 20 and 30°C under different relative humidity in the range of 12 to 98% using gravimetric method. The equilibrium moisture uptake data were interpreted with the help of three well-known sorption isotherm models namely, (GAB, Halsey and Oswin models) using non-linear least square method. The experimental moisture uptake data were best interpreted by GAB model. The monolayer moisture content Xm was found to vary from 54.08 x 10-3 to 10.48 x10-3 kg kg^{-1} (db) for a temperature rise from 10 to 30°C, thus indicating negative temperature dependence. The Clausius-Clapeyron equation was used to determine the isosteric heat of sorption (qst). The uptake data was also used to determine isokinetic temperature Tβ (315.9 K) and harmonic mean temperature Thm (292.8 K). The enthalpy-entropy compensation theory indicated that moisture sorption process was enthalpy driven.

Key words: GAB model, Beta casein, Isosteric heat of sorption, Oswin, Enthalpy

Introduction

Casein is a major milk protein and it accounts for almost 80% of the total protein contents of milk (Silva et al., 2005). This major protein product is used as a food additive in dairy, dietary and baby foods (Aneja et al., 2002). Because of a wide range of applications, last two decades have witnessed sincere efforts in manufacturing of food and pharmaceutical grade casein. In fact, production of dried casein powder has been recognized as a significant part of dairy industry.

It has been reported that by the year 2011 the annual casein/cheese production in India might have reached to 1.23 billion US$ by the year 2011, (Gupta, 2007). Such a large scale production of casein has imposed serious challenges on the manufacturing units for its safe and moisture–free production as well as storage, particularly in the seasons when there is enough moisture in the environment. The presence of a large number of polar groups, in casein also acts as a driving force for absorption of water vapors. In general, dehydrated protein powders have atendency to absorb moisture at moderate and high water activities thus resulting in protein insolubility and enhanced flavor deterioration. Thus, it is necessary to predict the exchange of moisture between food materials and surroundings because it affects the physicochemical properties, drying process, storage capacity and microbial safety (Rangel-Marron et al., 2011). The adsorption of moisture by foods can be defined as a process in which there is reversible and progressive combination of water with the food solids via a number of sorptive processes such as chemisorptions, physical adsorption, and multilayer condensation The term Equilibrium Moisture Content (EMC) is usually regarded as the water content of food stuff when its vapor pressure equals that of the surroundings. The quantitative relation between EMC and the water activities at any temperature is quantified in the form of moisture sorption isotherms. A correlation between EMC and corresponding water activities has been described by a number of isotherm models (Van Den Berg et al., 1981). Some of the models also consider the effect of temperature. In addition to applications in food engineering, the sorption isotherms of a food product play significant role in are product and process

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development (Delgando et al., 2002). The water activities greately influence the deteriorative mechanisms in food system and therefore for longer storage stability it is desirable to make water activity modifications. The storage and packaging problems can be better handled by evaluating thermodynamic parameters of water sorbed (Maltini, 2003; Acosta-Esquijarosa et al., 2011). In addition, these parameters are also helpful in appraising the shelf life of food product under varying product, package and environment interactions.

In spite of the fact that casein has potential applications in field of food and nutrition, its hydrophilicity and stickiness create problems during its production, drying and storage and applications. This also affects its shelf life storage capacity. However, there are a few studies that have attempted to investigate its moisture absorption behavior. In a study (Mauer et al., 2000), β-casein was isolated and its sorption isotherms were determined at +4°C and +22.5°C. The isotherms were further determined after 9 months of storage at -29°C and +22.5°C. The results indicated that storage temperature had varying effect on moisture sorption isotherms.; however, at any aw differences in moisture content were small (< 0.03g H2O/g solids at high aw). β-casein stored at -29°C had lower mo . The production of casein and its associated products from buffalo milk has been adopted as very popular small scale industry in most of the parts of India where there is huge production of milk. Therefore it is required to study the moisture absorption tendency of casein under the Indian climatic conditions so as to provide some useful basic information to the manufacturers.

With these objectives this work was undertaken to study the water vapor sorption behavior of casein at different temperatures and to make a quantitative interpretation of the data using suitable isotherm models. The sorption data has been used to determine various thermodynamic parameters also.

Experimental

Materials

The milk protein casein (in soluble form as sodium caseinate, product code No. 4-2628, mol. wt 23000) was purchased from Hi Media Chemicals, Mumbai, India. The relative constant humidity environments were created by using saturated solutions of various salts i.e. KOH, CH_3COOK, K_2CO_3, $Mg(NO_3)_2$, NaCl, KCl and K_2SO_4. These salts were purchased from Merck Chemicals, Mumbai, India and were analytical grade. The whole study was carried out with double distilled water.

Water activities (a_w) of saturated solutions of the above salts at 10, 20 and 30°C were adopted from Labuza (1984) and are given in Table 1 (Ramesh, 2003).

Table 1. The water activities (aw) of saturated salt solutions at 10, 20 and 30°C.

Salt	aw at 10°C	aw at 20°C	aw at 30°C
KOH	0.1234	0.0932	0.0738
CH_3COOK	0.2338	0.2311	0.2161
K_2CO_3	0.4314	0.4316	0.4317
$Mg(NO_3)_2$	0.5736	0.5438	0.5140
NaCl	0.7567	0.7547	0.7509
KCl	0.8677	0.8511	0.8362
K_2SO_4	0.9818	0.9759	0.9700

Study of moisture sorption isotherms

The accurately pre-weighed casein samples, in triplicate, were weighed in small crucibles of aluminium foils and then placed in the desiccators. This desiccator had various saturated salt solution to provide the required relative humidity environment. The desiccators were placed in temperature-controlled cabinets maintained at 10, 20 and 30°C (±1°C). The reason for selection of the given temperature was that the room temperature normally falls between 20 and 30^{0}C in Asia subcontinent countries like India. In addition, the preservation of casein under low temperature conditions justifies 10^{0}C as the lower temperature selected. The pre-weighed casein powder samples were placed in different desiccators till they all attained equilibrium weight which was indicated by the fact that there was no measurable weight change (±0.001g). The equilibrium moisture absorption was attained in almost twelve days. The weight gain was recorded every day. It took almost 30 s to remove, weigh, and then put back the samples into desiccators finally, the moisture content of each sample was determined by hot air oven drying method (AOAC, 1984). All measurements were made in triplicate and the average values were used in the analysis.

Sorption isotherm models

In this study, the EMC data was analyzed using four ‘two parameters models’ i.e. the Oswin, the Henderson, the Cauri, Halsey models and one ‘three parameters model’ namely the GAB model. All these models along with related parameters are given in the Table 2. The two parameter models,

viz., Halsey Henderson, Cauri and Oswin, in their linear form, were used for the determination of related constants with the help of linear regression program. However, a non-linear least square procedure was applied to determine constants for three-parameter GAB model. It is often more convenient to re-arrange GAB equation into a second degree polynomial equation.

$$\frac{a_w}{M} = \alpha a_w^2 + \beta a_w + \gamma \quad .....(1)$$

where

$$\alpha = \frac{k}{M_o}\left[\frac{1}{C-1}\right] \quad ....(2)$$

$$\beta = \frac{1}{M_o}\left[1-\frac{2}{C}\right] \quad ....(3)$$

And $$\gamma = \frac{1}{M_o Ck} \quad ...(4)$$

A non-linear regression analysis of a_w/M versus a_w gives a second order polynomial. The coefficients α, β and γ were thus obtained from this polynomial equation and substituted one by one to obtain GAB constants M_o, C and k, where M_o is the moisture content when all primary sites are occupied by single water molecules, C is the Guggenheim constant and k is the factor comparing the properties of multilayer water molecules as compared to the bulk liquid.

The mean relative deviation modulus (P) and the standard error of estimate (SE) were used as error functions to access the accuracy of fitness of different models.

Modeling isotherm models

The equilibrium moisture uptake data of Casein samples, at three temperatures, namely 10, 20 and 30°C, were fitted to various isotherm models displayed in Table II. The parameters of the GAB sorption model were calculated using non-linear regression analysis whereas other models were applied in linearized forms to evaluate associated parameters. Two criteria namely mean relative deviation modulus (P) and the standard error of estimate (SE) were used to evaluate the fitness of sorption models to the experimental data.

$$P = \frac{100}{N}\sum_{i=1}^{N}\frac{|M_{ex} - M_{pr}|}{M_{ex}} \quad ...(5)$$

$$SE = \sqrt{\frac{\sum_{i=1}^{N}(M_{ex} - M_{pr})^2}{N-n}} \quad ...(6)$$

Table 2. Sorption isotherm models used in this study.

Name of model	Equation	Reference
GAB (Guggenheim-Anderson-de Boer)	$M = \frac{M_0 Ck.a_w}{(1-ka_w)(1-ka_w+cka_w)}$	(Anderson, 1946)
Halsey (Linearized)	$a_w = \exp\left(\frac{-A1}{M^{A2}}\right)$ In M = InA$_1$+A$_2$ In (-In a$_w$)	(Halsey, 1948)
Oswin (linearized)	$M = A\left(\frac{a_w}{1-a_w}\right)^B$ In M = In A + B In $\frac{a_w}{1-a_w}$	(Oswin, 1946)
Henderson (Linearized)	Log [-In (1-a$_w$)] = n log M$_c$ + log K	(Henderson, 1952)
Caurie	$\ln\frac{1}{m} = -\ln(CM_0) + \frac{2C}{M_0}\ln\frac{1-a_w}{a_w}$	(Caurie, 1970)

Where, M_{ex} and M_{pr} were the experimental and predicted moisture content values, respectively; N and n were the number of observations and the number of constants in each model respectively. The P value of less than 10 percent indicates acceptability of the model (Arslan et al., 2006). A model is said to be best fit if SE and P exhibit lowest values for it while the regression coefficient (R^2) has highest value.

The temperature dependency of GAB parameters C and K can be expressed by Arrheniutype equation (Goula et al., 2008).

$$C = C_o \exp[\Delta H_c/RT] \quad \ldots(7)$$

And

$$K = K_o \exp\left[\frac{\Delta Hk}{RT}\right] \quad \ldots(8)$$

Here, ΔH and ΔK are functions of heat of sorption of water; and C_0 and K_0 are the adjustable constants for the temperature effect.

Monolayer moisture contents M_o, calculated using GAB isotherm model was used to calculate solid surface area S of the sample (Zareiforoush et al., 2009) using following expression.

$$s = \frac{M_0 N_A A_m}{M_{H_2o}} = 3.5\times10^3 M_o \quad \ldots(9)$$

Where, S is the solid surface area (m^2g^{-1} dry solids); M_o is the monolayer moisture content (kg water kg^{-1} dry solid); M_{H2O} is the molecular weight of water (18 $9mol^{-1}$), N_A is Avogadro number ($6.023x10^{23}$ molecules mol^{-1}), and A_m is the area of a water molecule ($1.06x^{-19}$ m^2).

The number of adsorbed monolayer was calculated using the formula

$$S=2/N \quad \ldots(10)$$

Where, S is cauries slope. In Cauris equation, C represents density of bound water whereas the product of monolayer moisture content M_0 and number of adsorbed monolayers N gives the percent bound or non-freezable water (Jayendra Kumar et al., 2005).

Isosteric heat of sorption and entropy of sorption

The net isosteric heat of sorption (q_{st}) is defined as the amount of energy by which the heat of vaporization of moisture in a product exceeds the latent heat of pure water (Labuza, 1968). The differential entropy of sorption (s_d) is proportional to the number of available sorption sites at a specific energy level. For a thermodynamic system, the net isosteric heat (q_{st}) and the differential entropy of sorption (s_d) are related as (Rosa et al., 2010):

$$\text{In } a_w = -\frac{q_{st}}{RT} + \frac{S_d}{R} \quad \ldots(11)$$

Where, q_{st}, S_d, and R are expressed as $kJmol^{-1}$, $kJmol^{-1}K^{-1}$ and kJ mol^{-1} K^{-1}, respectively.

For a given range of EMC, corresponding water activities were calculated and the linear plots, obtained between In a_w and I/T were used to calculated q_{st}, and S_d, with the help of slope and intercept respectively.

Entropy – enthalpy compensation theory

According to the compensation theory, there is linear relationship between the net isosteric heat of sorption (q_{st}) and differential entropy of sorption (S_d,) for given moisture content range (Igathinathane et al., 2007):

$$q_{st} = T_\beta S_d + \alpha \quad \ldots(12)$$

The linear regression analysis enables us to calculate isokinetic temperature T_β and constant α. The parameter T_β is obtained from the slope of linear plot between q_{st} and S_d. T_β is the isokinetic temperature with an important physical meaning as it represents the temperature at which all reactions in the series proceed at the same rate and α is a constant (Madamba et al., 1996). In order to corroborate the compensation theory, Gabas et al. (2000) proposed statistical analysis test to corroborate the compensation theory. The harmonic mean temperature (T_{hm}) was given as follows:

$$T_{hm} = \frac{n}{\Sigma_1^n (1/T)} \quad \ldots(13)$$

The compensation theory only applies $T_\beta \neq T_{hm}$. The moisture uptake process is enthalpy driven or entropy controlled as per condition that $T_\beta > T_{hm}$ or $T_\beta > T_{hm}$ (Igathinathane et al., 2007).

Results and Discussion

Moisture sorption isotherm

The moisture sorption isotherms for soluble casein (SC) at 10, 20 and 30°C are depicted in Figure 1. It is clear that the EMC increases with water activity at constant temperature. The isotherms exhibit sigmoidal shaped curves, thus indicating a Type – II isotherm (Brunauer et al., 1940), which is characteristic for most of the foods. A close look at the isotherms reveals some

interesting results. Firstly, the isotherms obtained at 10 and 20°C exhibit a S-shaped curve while the isotherm obtained at 30°C show a little different behavior with a cross-over at the water activity of 0.8. The observed findings could be explained as below:

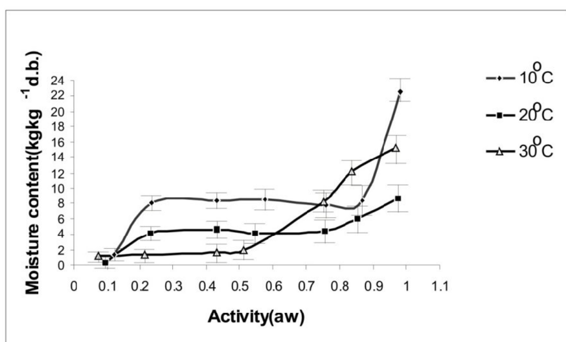

Figure 1. Moisture uptake as a function of water activity at different temperature.

It is commonly observed that moisture sorption isotherm consists of three zones. Here, in the isotherms obtained at 10 and 20°C, three zones can be noted; zone – I (aw: 0.0 to 0.2), zone – II (aw; 0.2 to 0.7) and finally zone – III (aw: 0.7 to 1.0) In the region I (termed as monolayer sorption region), the EMC increases with water activity due to the fact that casein contains a large number of polar groups along the macromolecular chains. These groups act as strong binding sites for incoming water vapor molecules. In addition, according to Falade et al. (2004), there is possibility of swelling or unfolding of protein chains thus offering new active sites for binding of water molecules. The zone – II, also termed as multilayer sorption region, consists of multilayer moisture which is under transition to natural properties of free water and is available for chemical reactions. In this zone, there is linear increase in moisture content with water activity but relatively at a slower rate as compared to the rate observed in the zone-I. In this zone, sorption takes place at less active sites. Finally, in the zone – III (usually termed as capillary condensation zone) there is sharp increase in EMC which may be attributable to diffusion of moisture into voids and capillaries. The water in this zone is in the free state. A close look at the two isotherms reveals that there is more moisture uptake at the later stages of the curves. The sharp rise in EMC is also attributable to partial dissolution of casein. Here, it is also noteworthy that for a given water activity, the EMC shows negative temperature dependence. This may simply be due to the fact that as the temperature is increased from 10 to 200C, vapor molecules gain more kinetic energy and this prevents them from binding at available sorption sites.

The isotherm behavior, exhibited at 30°C, appears to be a little different. There is gradual water absorption in linear fashion till the water activity reaches a value of nearly 0.6. However, a drastic increase in EMC is observed as the water activity of the surrounding environment exceeds 0.6. In addition, a crossover is also observed. This could probably be due to dissolution of casein at 30°C beyond the water activity of 0.6. Such as intersection or cross-over behavior has also been reported for moisture sorption by dry powder (Hassan, 2004), basundi mix (Sharma et al., 2009), gingerbread (Cervenka et al., 2008) etc. Overall, all the three curves showed continuity.

Fitting of isotherm models

The experimental data on moisture sorption was fitted to various isotherm models given in Table 2. The estimated parameters and various error functions for selected isotherm models in the water activity and temperature range studied are given in Table 3. A close look at the values of error functions P, and SE indicate that out of various isotherm models applied, the GAB isotherm model appears to fit best on the equilibrium moisture uptake data. The best quantitative interpretation of the moisture uptake data by the GAB model was almost expected because it is a semi-theoretical, multi molecular, localized homogenous adsorption model. It is also reported frequently that GAB is the most versatile sorption model available (Ayrarci et al., 2005).

The treatment of sorption data, according to GAB model, allows us to evaluate monolayer moisture content value M_o of foods. The M_o value represents the moisture content of a material when its entire surface is fully covered with a uni molecular layer of water vapor molecules (Sawhney et al., 2011). The significance of M_o values is that deterioration of foods is very small below M_o. because the reason is that water is strongly bound to the food below M_o and does not take part in any deteriorative reactions either as solvent or as one of the substrates. Therefore, at a given temperature the water activity level corresponding to M_o or less is the safest one. The values of M_o, in this work, were found to be 5.40, 3.34 and 1.04 g/100g solid at 10, 20 and 30°C

respectively. The M_o values reported for dried acid casein from buffalo skim milk are reported to be 5.60, 4.70 and 4.54 g/100g solid at 25, 35 and 45°C respectively. Similarly, the moisture contents for soy protein, as reported by Cassini et al. (2006) at 10, 20 and 30° were 7.40, 6.40 and 5.50 g/100g db respectively. The overall assessment of all these values reveals that in the temperature range of 45 to 10°C, the moisture uptake of food proteins falls in the range of 1.0 to 8.0g/100g dry basis. The observed decrease in moisture content with temperature range mentioned above may be due to reduction in number of active sites for water binding because of folding of macromolecular chains , thus causing a decrease in the number of active binding sites exposed (Ariahu et al., 2006). However this reasoning does not seem to be justified in the present study as the temperature range selected was 10 to 30°C. Also, there is another possibility that as the temperature is raised, there is increase in kinetic energy of water vapor molecules and this makes them less stable, thereby, favoring their departure from the binding sites of food material (Tunc et al., 2007). Finally, this causes the monolayer moisture content to decrease. The negative temperature dependency of EMC is well known phenomenon and reported by a large number of workers.

It has been observed that strong adsorbent – adsorbate interactions, is exothermic in nature and is favored at lower temperature. These results in an increase in parameter C when temperature is lowered (Diosady et al., 1996). This behavior is also in agreement with above equation (7) that suggests negative temperature dependency of C. A quite compatible behavior is also found in this study; increasing temperature from 10 to 30°C caused by decrease in C value from 507.9 to 66.0 From a study of 30 different foods, Iglesias et al. (2008) reported that in 74% of them, C did not show negative temperature dependence. They suggested that there could be some irreversible changes associated with increasing temperature such as enzymatic reaction, protein de-naturation. However, in this study a change of temperature from 10 to 30°C is not sufficient to cause denaturation of casein in this work. Finally, as the values obtained are >2, the isotherms could be classified as type II as already mentioned earlier. Similar observations are reported elsewhere (Farahnaky et al., 2009).

The interactions between the molecules in multi layers and the adsorbent are interpreted in terms of value of K. When K is unity, the properties of multi-layer resemble with those of bulk water, and under this situation the BET isotherm model can well interpret the sorption process. However, in this study, the values of K, displayed in Table 3, are less than unity except that obtained at 30°C.

Finally, the specific surface area enables us to determine the water binding properties of particulate materials. In order to determine surface area, the monolayer moisture content values (M_o), obtained from GAB isotherm model, were put in equation (9). The values of S were found to be 189.2, 110.5 and 35.1 m^2g^{-1} for the equilibrium sorption temperatures of 10, 20 and 30°C, respectively. It is clear that specific surface area decreases with temperature, which may be attributed to the decrease in the number of active sorption sites available (Rizvi et al., 1983).

Properties of bound water

The spoilage of food stuff is usually governed by the physical state of water absorbed by them (Van den Berg et al., 1981). Hence, it is necessary to determine various characteristics of bound water such as its density, its relation to surface area of adsorbent, number of adsorbed monolayers etc. All the parameters describing the property of sorbed water are shown in Table 4. The data reveals that density of water, percent of bound or unfreezable water, monolayer moisture content and specific surface area of sorbent decrease with increase in temperature. The observed decrease is simply attributable to the increase in the kinetic energy of water molecules, thus resulting in a decrease in the percent bound water, monolayer moisture content and density of water.

Net Isosteric heat of adsorption (qst) and differential entropy (sd)

The isosteric heat of adsorption can be determined by the use of Clausius-Clapeyron equation, provided the isotherm data is available at two or more temperatures. The isosteric heat of sorption varies with the amount of water sorbed by the substrate. In this work, the In a_w versus 1/T plots were obtaining for various moisture contents using the equilibrium moisture uptake data obtained at different temperature (shown in Figure 1). The slopes and the intercepts of linear plots, as shown in Figure 2, were used to evaluate q_{st} and S_d respectively. As the slopes obtained are linear, it may be concluded that the heat of sorption is independent of temperature for different values of EMC.

Table 3. Parameters obtained for various isotherm models.

Model	Constants	Temperature (^{0}C)		
		10	20	30
GAB	Mo	5.40	3.34	1.04
	C	507.964	477.354	66.0393
	K	0.78613	0.5368	1.02667
	SE	0.5213	0.4187	0.3217
	P	3.4813	4.6143	5.1305
	r^2	0.9736	0.9711	0.7884
Halsey	A1	0.07371	0.04212	0.0237
	A2	-0.22	-0.1849	-0.5847
	SE	1.6783	0.9917	2.3154
	P	8.6189	9.2785	11.5680
	r^2	0.774	0.8667	0.5311
Oswin	A	0.08579	0.0475	0.0282
	B	0.1901	0.1856	0.8666
	SE	3.6023	2.1765	1.7436
	P	7.3542	6.6076	5.2258
	r^2	0.4284	0.7054	0.8622
Henderson	N	0.0291	0.0175	0.0056
	K	0.491	0.183	0.091
	SE	3.6002	1.2487	1.6321
	P	10.6548	8.2416	9.3581
	r^2	0.827	0.727	0.870
Cauri	M_o	8.18	6.67	2.25
	C	0.560	0.964	0.973
	SE	2.3126	3.1763	1.9457
	P	9.5682	7.3785	8.1793
	r^2	0.884	0.843	0.822

Table 4. Properties of sorbed water of casein at different temperatures.

Temperature (^{0}C)	Monolayer moisture content (g/100g dry solid) M_0	Cauri slope S	No.of adsorbed monolayer N	Density of sorbed water C	Bound water %	Surface Area m^2g^{-1}
10	5.40	0.364	14.5	0.0926	0.973	186
20	3.34	0.189	6.8	0.0648	0.964	397
30	1.04	0.116	2.3	0.0590	0.560	63

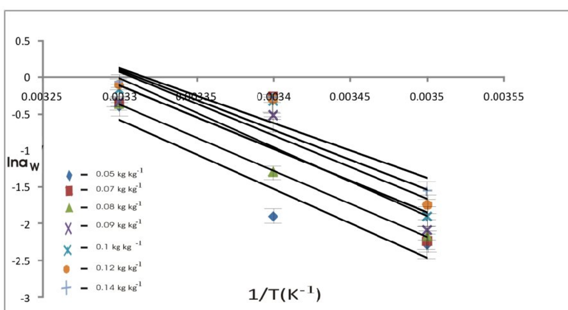

Figure 2. lna_w versus 1/T plots for evaluation of qst.

The variation in net isosteric heat of sorption q_{st} with moisture contents is well depicted in Figure 3. It is obvious that q_{st} shows a negative dependence on EMC. This decrease is attributable to the fact that when moisture content is quite low, the water vapors are adsorbed at the most active sites, thus producing highest interaction energy. This results in higher values of q_{st} at fairly low moisture contents. However, with the increase in the moisture content, the number of active sites available for water vapor sorption decreases, and this finally results in lower q_{st} values (Paulo et al., 2010). When the isosteric heat of sorption approaches towards zero, the interaction between the adsorbent and the adsorbed molecules becomes negligible i.e. these water molecules are termed as ‘free water’ which is available to microorganisms. The energy required for removal

of water during the drying process can be estimated form the heat of sorption (i.e.Q_{st}) data.

Finally, the S_d values obtained at the three experimental temperature were plotted against respective moisture content experimental temperature and the results, as shown by the net isosteric heat of sorption (q_{st}) as depicted in Figure 4. Similar types of results have also been shown elsewhere (Goula et al., 2008).

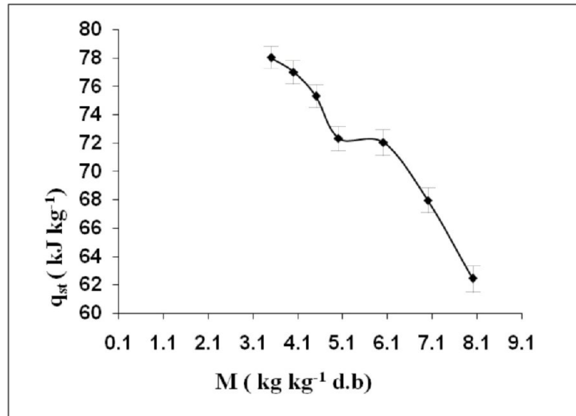

Figure 3. Variation of q_{st} with moisture content.

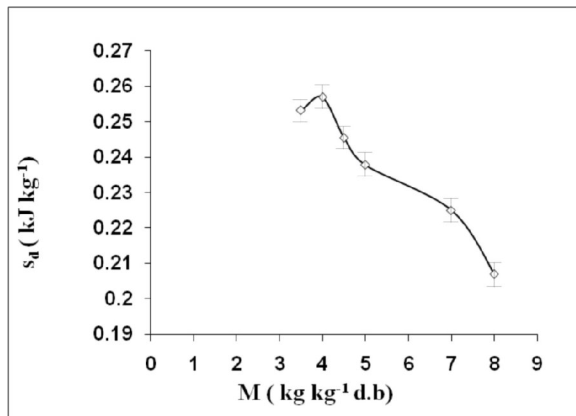

Figure 4. Variation of differential entropy absorption sd with moisture content.

Entropy – Enthalpy compensation theory

The plot of q_{st} versus S_d was observed to exhibit a linear relationship for casein as shown in Figure 5. The value of isokinetic temperature $T_\beta$and constant α were calculated from the linear regression analysis and were found to be 315.9 K and 2.893, respectively (r^2 = 0.983). According to Gabas et al. (2000), in order to apply the compensation theory, it is necessary that calculated harmonic mean temperature T_{hm} should differ significantly from T_β. In this work, the value of T_{hm}, obtained using eq. (12) was found to be 292.8 K, which is finitely different from the T_β value.

Finally, as $T_\beta > T_{hm}$, the water vapor sorption onto casein could be said to be enthalpy driven process.

Conclusions

Casein exhibited typical Type-II adsorption isotherms with sigmoidal shape. For a given water activity, the equilibrium moisture content was found to decrease with temperature. The EMC data was best interpreted in terms of GAB isothermal model. The q_{st} and S_d were calculated using Clausius – Clapeyran equation and showed negative dependence on moisture contents. The enthalpy-entropy compensation theory was successfully applied on moisture sorption data obtained for casein. The moisture uptake process was found to be enthalpy driven.

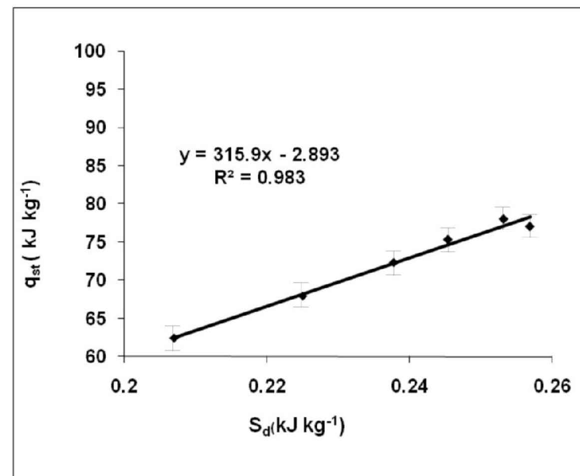

Figure 5. Sd versus q_{st} plot to test the compensation theory.

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FOOD SCIENCE AND NUTRITION

Nutritional and functional properties of Roselle (*Hibiscus sabdariffa* L.) seed protein hydrolysates

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Abstract

The nutritional and functional properties of Roselle seed protein hydrolysates (RSPH) were studied. Roselle seed protein isolates were digested (for 1.5 h and 3h) using pepsin followed by pancreatin. The maximum nitrogen solubility values of 94.17 and 90.8 were observed at pH 12 and pH 10 after 1.5 h and 3 h of hydrolysis, respectively. RSPH1.5 and RSPH3 exhibited a good foaming capacity of 310 and 300% respectively. Furthermore, RSPH1.5 showed the best foaming stability (300%) after 60 min compared to RSPH3 (144%) at the same time. For both hydrolysates, the amounts of essential amino acids exceeded those recommended by the Food and Agricultural Organization/World Health Organization (2007) for humans. The emulsifying capacity of RSPH1.5 was higher than that of RSPH3 90 and 100 mL/g respectively. RSPH1.5 possessed also the highest water holding and oil holding capacities compared to RSPH3. RSPH3 was more heat sensitive than RSPH1.5. The denaturation temperature for RSPH1.5 and RSPH3 were 95.80ºC and 85.93ºC respectively. Our results demonstrated that RSPH may be used wholly or partially to replace high-price materials like egg albumen and casein as well as a potential food ingredient.

Key words: Enzymatic hydrolysis, Functional Properties, Nutritional quality, Protein isolates, Roselle seed

Introduction

Proteins are essential constituent of any human diet, because they provide nitrogen and amino acids, which cannot be synthesized by the body (Irakoze and Zhou, 2005). In food products, protein isolates are used as additives to improve some properties, such as water-binding, foaming, gelation and emulsifying capacities, viscosity and texture. Protein isolates extracted from maize germ (Zayas and Lin, 1989), sesame seed (Lopez et al., 2003), peas (Dagorn et al., 1987), soybean and wheat proteins (Boneldi and Zayas, 1995), have been added as replacements for egg albumin to a variety of food products. To diversify its applicability in different food systems, protein isolates have been used in hydrolysed form.

Enzymatic hydrolysis of proteins has a great of potential for modifying functional properties of food proteins. In recent years, a lot of consideration has been put on the protein hydrolysates produced by enzymatic processes. Protein hydrolysates can be classified according to their degree of hydrolysis. Protein hydrolysates with different degrees of hydrolysis can be used as flavorings in soups, sauces, and meat products (Weir, 1986). Protein hydrolysates most likely have improved functional properties compared to the original proteins and are commonly used as food ingredients (Vioque et al., 2000). In medical field, protein hydrolysates are widely used as diet supplements or hypoallergenic foods (Frokjear, 1994).

Studies have indicated that functional properties of proteins can be enhanced by enzymatic hydrolysis. Mannhein and Cheryan (1992) reported that enzymatic hydrolysis improved solubility of corn gluten meal protein compared to its unmodified form. In the case of whey and casein proteins on the other hand, Van der ven et al. (2002) found that although foam formation showed improvement after enzymatic hydrolysis and that foam stability was poorer than it was before

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modification. Findings by Qi et al. (1997) suggest that pancreatin modified soy protein exhibited better emulsifying activity index than the original protein, making it a potential ingredient in the emulsified foods formulation.

Although, some nutritional and functional properties of Roselle seed protein have been studied (data not shown), there is currently no information available especially, on the nutritional and functional properties of the Roselle seed protein hydrolysates. Therefore, this study undertaken to evaluate the nutritional and functional properties of Roselle seed protein hydrolysates obtained at different hydrolysis time for the purpose to be used in food formulation systems.

Material and Methods

Materials

Seeds of *H. Sabdariffa* were obtained from Koutiala, southern region of Republic of Mali and transported to Wuxi, China. All enzymes used were of food grade. Pepsin and pancreatin were purchased from Sigma Chemical Co. (St. Louis, USA). All the other chemicals used in the experiments were from commercial sources and of analytical grade.

Preparation of defatted Roselle seed flour

Roselle seeds were cleaned by removing dust, stones, and plant debris. The seeds were milled using a laboratory scale hammer miller and the resulting powder was sieved through a 60 mesh screen until fine powder was obtained. Thereafter the powder was defatted twice with n-hexane at room temperature at a ratio of 1:10 (w/v) and stirred for 7 h for oil extraction step. The oil-free flours was desolventized and stored in desiccator at room temperature for subsequent uses.

Preparation of Roselle seed protein isolate

Roselle seed protein isolates (RSPI) were obtained from defatted flour as reported by El-Tinay et al. (1988) with some modifications. The defatted flour was dispersed in distilled water at ratio of 1:10; the pH was adjusted to 10 with 1 M NaOH and stirred for 3 h at room temperature. The suspension was then centrifuged at 4000 rpm for 20 min. The residues were re-extracted for the second time as described above. The supernatants were combined and protein was precipitated by adjusting to pH 3.5 with 1 M HCl before centrifugation at 4000 rpm for 20 min. The protein isolate (precipitate) was washed twice with distilled water then resuspended in distilled water and the pH was adjusted to 7.0 with 1 M NaOH prior to freeze-drying. The dried protein (protein isolates) was stored in desiccator at room temperature for subsequent analyses. The protein content was determined using Kjeldahl method (AOAC, 2000).

Preparation of Protein hydrolysates

To produce hydrolysates from RSPI, enzymatic hydrolysis was performed using deux enzymes (pepsin followed by pancreatin) under their optimal conditions. The RSPI samples were divided into two groups (each containing 20 g and 400 mL of water) and hydrolyzed in 500-mL reactor under temperature and pH control devices. Sample 1 was hydrolyzed by pepsin (1% at pH 2) for 0.5 h followed by pancreatin (2% at pH 7) for 1 h. Sample 2 was hydrolysed (under the same condition) by pepsin for 1 h followed by pancreatin 2 h. Conditions were constantly monitored and maintained throughout the process. Upon completion of the hydrolysis, the enzymes were deactivated by heating in a boiling water bath for 10 min. The reaction mixtures were then centrifuged at 7000 xg, 4°C for 10 min, and the supernatants were collected. The protein hydrolysates obtained were freeze-dried and stored at -20ºC for subsequent analysis. The degree of hydrolysis was determined by measuring the nitrogen content soluble in 10% trichloroacetic acid as discussed by Kim et al. (2001).

Amino acids analysis

The dried samples (100 mg) were subjected to acid hydrolysis using 5 mL of 6 M HCl under nitrogen atmosphere for 24 h at 110°C. The hydrolysate was washed into a 50 mL volumetric flask and made up to the mark with distilled water. The amino acids were subjected to RP-HPLC analysis (Agilent 1100, USA) after precolumn derivatization with o-phthaldialdehyde (OPA). Each sample (1 µL) was injected into a Zorbax 80 A C18 column (i.d. 4.6X180 mm, Agilent Technologies, Palo Alto, CA, USA) at 40ºC with detection at 338 nm. The Amino acid composition was expressed as g per 100 g protein.

Protein nutritional parameters

The nutritional parameters of both hydrolysates were calculated using their amino acid composition including:

(1) Proportion of essential amino acids (E) to the total amino acids (T) of the proteins.

(2) Amino acid score (AAS) = (mg of amino acid per g of test protein/mg of amino acid per g of FAO/WHO/UNU standard reference pattern) ×100.

(3) Predicted protein efficiency ratio (PER). The predicted PER values of Roselle protein isolates

hydrolysates were estimated by three regression equations developed by Chavan et al. (2001).

I. PER= - 0.684+0.456(Leu)-0.047(Pro)

II. PER= -0.468+0.454(Leu)-0.105(Tyr)

III.PER=-1.816+0.435(Met)+0.780(Leu)+0.211(His)-0.944(Tyr).

Nitrogen solubility (NS)

Nitrogen solubility was determined according to the procedure of Diniz and Martin (1997), with slight modification. Samples were dissolved in distilled water (10 g/L) and pH of the mixture was adjusted to 2, 4, 6, 8, 10 and 12 with either 0.5 N HCl or 0.5 N NaOH under continuous shaking (Lab-Line Environ-Shaker; Lab-Line Instrument, Inc., Melrose Park, IL, USA) at room temperature for 35 min and 25 mL aliquot was centrifuged at 2800 ×g for 35 min. A 15 mL aliquot of the supernatant was analyzed for nitrogen (N) content using the Kjeldahl method and the NS was calculated according to equation:

Nitrogen solubility (%) = [Protein content in supernatant/protein content in sample] ×100

Differential scanning calorimetry

Thermal properties of the hydrolysates were evaluated using Differential scanning calorimetry (DSC). Seventy milligram of various samples were dissolved into 1 mL of 0.05 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. The protein solutions (45 µL) were transferred and hermetically sealed in a stainless steel pan. The samples were heated by scanning from 20 to 200ºC at a rate of 10ºC/min against a reference containing 45 µL buffer without protein in a differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, Conn., USA). Thermal denaturation temperature and enthalpy (ΔH) were calculated from thermograms.

Water holding capacity

The Water Holding Capacity (WHC) of the hydrolysates was determined as described by Diniz and Martin (1997) with some modifications. Triplicate samples (0.5g) were placed in centrifuge tubes, dissolved with 10 mL of distilled water and vortexed for 30 sec. The mixture was allowed to stand at room temperature for 30 min and later centrifuged at 3000 ×g for 25 min. The supernatant was filtered using whatman Number 1 filter paper and the volume retrieved was measured. The difference between initial volumes of distilled water added to the protein sample and the flow through was determined. The results were reported as mL of water absorbed per gram of protein sample.

Oil holding capacity

Oil holding capacity (OHC) was determined as described by Chakraborty (1986). One gram of each sample (W_0) was weighed into pre-weighed 15 mL centrifuge tubes and thoroughly mixed with 10 mL (V_1) of soybean refined pure oil using Vortex mixer. Samples were allowed to stand for 30 min. The protein- oil mixture was centrifuged at 3000 ×g for 20 min. The supernatant was immediately poured into a 10 mL graduated cylinder, and the obtained volume reading was recorded (V_2). Oil holding capacity (mL of oil per g of protein) was calculated as:

$$OHC = (V_1-V_2)/W_0.$$

Emulsifying capacity

Emulsifying capacity of the samples was measured as described by Rakesh and Metz (1973) , with some modification. One gram of each freeze–dried sample was transferred into a 250 mL beaker and dissolved in 50 mL of 0.5 N NaCl and then 50 mL of soybeans pure oil was added. The homogenizer equipped with a motorized stirrer driven by a rheostat Ultra-T18 homogenizer (Shanghai, China) was immersed in the mixture and operated for 120 sec at 10 000 rpm to make an emulsion. The mixture was transferred into centrifuge tubes, kept under a water- bath at 90°C for 10 min and then centrifuged at at 3000 xg for 20 min. Emulsifying capacity was calculated using the equation :

$$EC= (V_A-V_R)/ W_S$$

Where:

V_A is the volume of oil added to form an emulsion

V_R is the volume of oil released after centrifugation

W_S is the weight of the sample.

Foaming capacity and foaming stability

Foaming capacity (FC) was determined using the method described by Makri et al. (2005). Sample concentrates of 1% were prepared in de-ionized water and adjusted to pH 7.4 with 1.0 N NaOH and 1.0 N HCl. A volume of 100 mL (V_1) of the suspension was blended for 3 min using a high–speed blender, poured into a 250 mL graduated cylinder, and the volume of foam (V_F) was immediately recorded. Foaming capacity was calculated using the following equation:

$$FC (\%) = (V_F/V_1) \times 100$$

Foam stability was determined by measuring the change in volume of the foam after 60 min.

Statistical analysis

Results were expressed as the mean values±standard deviation (S.D.) of three separate determinations. The data were averages of triplicate observations and were subjected to a one way analysis of variance (ANOVA), followed by Duncan's multiple range test. The data was subjected to correlation analysis, using SPSS software (version 16.0).

Results and Discussion

Enzymatic Hydrolysis

In quantitative work on protein hydrolysis it is necessary to have a measurement for the extent of hydrolytic degradation. It should be evident that the number of peptide bonds cleaved during the reaction is the parameter that most closely reflects the catalytic action of proteases (Alder-Nissen, 1986). The Degree of hydrolysis (DH) is generally used as a parameter for monitoring proteolysis and is the most widely used indicator for comparison among different protein hydrolysates. The results showed that the DH of RSPH1.5 was 15.82% which was lower than that observed at 3 hours hydrolysis time (21.56 %) (Tounkara et al., 2013b).

Amino acids analysis

The biological activity of protein is more related to its amino acid make up. In order to appreciate the physicochemical properties of the hydrolysates, amino acid composition analysis was carried out. The obtained amino acid test results were shown in Table 1 along with FAO/WHO/UNU (2007) recommended essential amino acid composition values. The results suggest that the hydrolysates contained a good proportion of all essential amino acids as reported by Sathivel et al. (2003). Glutamic acid was the major amino acid in both hydrolysates. In general, arginine, aspartic acid and glutamic acid were predominant in all the samples. The amino acid compositions of Roselle seed protein hydrolysates in this study were in agreement with the findings of El-Adawy and Khalil (1994), Tounkara et al. (2013a) for Roselle seed protein. On the other hand, the Roselle seed hydrolysates comply with the findings of Abu-Tarboush et al. (1997), this was the same for Roselle seed protein concentrates and Roselle seed protein isolates.

Table 1. Comparative amino acid profiles of the RSPH1.5 and RSPH3 (g/100g of protein).

Amino acids [a)]	RSPH1.5	RSPH3	FAO /WHO Child (Adult)
Essential amino acids			
Lysine	4.48	4.04	4.8(4.5)
Histidine	2.34	2.28	1.6(1.5)
Leucine	7.90	6.26	6(5.9)
Isoleucine	3.82	3.93	3(3)
Phenylalanine	5.41	4.31	
Phenylalanine+Tyrosine	8.19	6.33	4.1(3.8)
Methionine	1.79	2.26	
Methionine+Cystein	3.04	3.88	2.3(1.6)
Valine	5.11	5.61	2.9(3.9)
Threonine	3.25	3.76	2.5(2.3)
Non-essential amino acids			
Glycine	4.03	4.59	
Cysteine (Cys-S)	1.25	1.62	
Aspartic acid	10.37	11.69	
Glutamic acid	23.48	24.78	
Serine	4.43	4.48	
Arginine	10.37	11.69	
Alanine	4.45	4.74	
Tyrosine	2.78	2.02	
Proline	4.73	5.56	

[a)] Data are mean of 3 replications

Protein nutritional parameters

The quality (nutritional or nutritive value) of protein in human diet depends on the level at which it can provide the amount of essential amino acids required by the body for growth and maintenance (Zhu et al., 2006). In this study, amino acid composition was used as a basis for estimating the nutritional quality of Roselle seed protein hydrolysates. Results of the ratio of essential to total amino acids (E/T), amino acid score (AAS) and protein efficiency ratio (PER) of the hydrolysates are shown in Table 2. In all samples the ratio of essential to total amino acids was higher than 36% (the recommended value by FAO/WHO/UNU), and 1.5 hydrolysates had the highest ratio of 41.12% (Table 2). In general PER below 1.5 implies a protein of low or poor quality, while PER between 1.5 and 2.0 indicates an intermediate protein quality and then PER above 2.0 indicates protein of a high quality (Friedman, 1996). The predicted PER values of all the samples were in the range of high quality (Table 2). The AAS results showed that amino acid compositions were well balanced in both hydrolysates and their values were almost the same as those recommended by Agricultural Organization/World Health Organization for adult requirements (FAO, 2007).

Differential scanning calorimetry

Differential scanning calorimetry is a method that has been used extensively on protein denaturation in various food systems to obtain data for heat capacity, enthalpy and entropy (thermodynamic) and reaction rate and activation energy (kinetic) due to its rapidity and easiness (Ryan et al., 2008; Foh et al., 2011). The thermal properties of the two different hydrolysates were investigated to examine the impact of hydrolysis time on the peptide conformation. The knowledge on thermal properties of protein is important for strategizing food-processing and designing heat-processing (Ju et al., 2001). Because functional properties of protein meal products are greatly influenced by their conformation, therefore, differential scanning calorimetry as a technique highly sensitive to conformational changes is often applied to protein hydrolysates and related products (Goreinstein et al., 1996). Thermal properties of the RSPH1.5 and the RSPH3 were given in Table 3. The denaturation temperature and enthalpy of the RSPH1.5 and the RSPH3 were 95.80°C, 5.31 J/g and 85.93°C, 3.65 J/g, respectively. The result shows that both hydrolysates were less heat sensitive. These results means, that the RSPH could be subjected to acceptable heat treatment during processing conditions without undergoing denaturation.

Table 2. Nutritional parameters of the different Roselle (*Hibiscus sabdariffa* L.) protein seed hydrolysates.

Parameters [a)]	RSPH1.5	RSPH3
E/T %	41.12	37.81
Estimated of PER		
I	2.69	1.91
II	2.83	2.16
III	2.95	2.63
Amino acid scores		
Leucine	131.67	104.33
Histidine	146.25	142.25
Threonine	130.00	150.4
Valine	176.20	193.45
Met + Cys	132.17	168.69
Isoleucine	127.33	131.00
Phe+Tyr	199.02	154.39
Lysine	93.33	84.16

[a)] Data are mean of 3 replications. E/T = Amount of essential amino acids (E) to total amino acids (T), PER = protein efficiency ratio.

Table 3. Thermal properties of Roselle seed protein hydrolysates.

DSC Measure [a)]	Sample	
	RSPH1.5	RSPH3
T_o	78.44	60.47
T_p	86.95	75.45
T_e	95.80	85.93
ΔH (J/g)	5.3176	3.651
Ar (Mj)	16.006	25.525

[a)] Data are mean of 3 replications. To: Start Temperature; Tp: Peak Temperature; Te: End Temperature; ΔH: Delta H; Ar: Area

Nitrogen solubility (NS)

Nitrogen solubility is used to measure protein hydro solubility. Nitrogen solubility is one of the most essential functional properties due to its impact on the other properties. As indicated in Figure 1, the nitrogen solubility of both hydrolysates was pH dependent. The curves showed that the solubility of the hydrolysates reached their minimum values of 63.85% and 73.85% at pH 4.0 for the RSPH1.5 and the RSPH3 respectively, while increased at high pH. The lowest solubility of all hydrolysates was achieved at pH 4.0 because the isoelectric point of Roselle seed protein is between 3.5 and 4, and the minimum solubility usually occurs at isoelectric point. Thus, the hydrolysis might be responsible for the

increased solubility at the isoelectric point and at other pH levels as a result of shortening of molecular chains and the increase in the amount of polar groups (Chobert et al., 1988; Nielsen, 1997; Slattery and Fitzgerald, 1998). This trend in solubility is in agreement with previous reports (Clemente et al., 1999; Aluko and Monu, 2003). The high nitrogen solubility of different hydrolysates (80 to 90%) would make them suitable for food applications (Yiqiang et al., 2000). The values of protein solubility at various pH may be helpful for predicting how protein hydrolysate would perform when used in food systems.

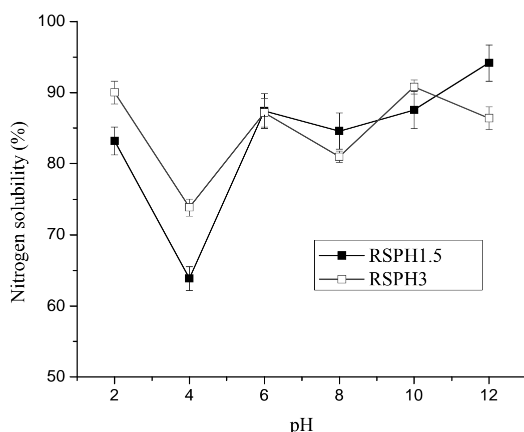

Figure 1. Nitrogen solubility of the two Roselle seed protein hydrolysates.
Values represent the means ± SD of triplicates.

Water holding capacity

Water and oil interactions with proteins are essential in food systems as they have a great impact on food organoleptical attributes. Protein conformation, amino acid composition and surface hydrophobicity/ polarity are some of the intrinsic factors that affect water holding (WHC) capacity of food proteins (Barbut, 1999). Water holding capacity is the ability of protein to absorb water and retain it against a gravitational force within its matrix. The WHC of the RSPH1.5 and the RSPH3 were 2.5 and 2 mL/g (Table 4) respectively. The result of WHC of the hydrolysates obtained from this study was comparable to those previously reported by Foh et al. (2010). Water holding capacity is a valuable factor for protein additives used in food systems. The WHC of the hydrolysates can be used to determine how those proteins can be added into food formulation systems and how they can replace animal proteins traditionally used (Zayas and Lin 1989).

Oil holding capacity

Oil holding capacity (OHC) may be defined as the ability of a substance to absorb and retain oils. It is an important property in food processing because it influences organoleptical aspects of food products. The OHC of RSPH1.5 and RSPH3 were 5.6 mL/g and 4.9 mL/g, respectively (Table 4). It is proposed that high oil absorption capacity of Roselle seed protein hydrolysates may give an advantage for their consideration in the formulation of food products such as cake, sausages, salad dressings and mayonnaise. Wasswa et al. (2007) also produced protein hydrolysates with OHC.

Emulsifying capacity

Food emulsions, the mixtures of immiscible liquids (water and oil), are usually thermodynamically unstable. In food systems such as salad dressings, their formation and stability are very important. Proteins and lipids commonly interact in food systems. The proteins' ability to stabilize emulsions is vital. Formation of emulsions occurs due to the presence of hydrophilic and hydrophobic groups of proteins. This primarily depends on diffusion of peptide chains at water-oil interfaces. Hydrolysates with smaller molecular sizes and high solubility facilitate that diffusion and improve the protein-lipid interaction. As shown in Table 4, all hydrolysates were good emulsifiers with EC of 100 mL/g and 90 mL/g for the RSPH 1.5 and RSPH3 respectively. Roselle seed protein digested using pepsin followed by pancreatin were degraded into peptides (oligopeptides and polypeptides), consequently enhancing the protein solubility, emulsion capacity and stability. Hydrolysis was effective in improving the EC of hydrolysates. Related findings have indicated that enzymatic hydrolysis of tilapia (Foh et al., 2010) and grass carp skin (Wasswa et al., 2007) produced hydrolysates with higher emulsifying capacity.

Table 4. Functional properties of RSPH.

Functional properties[a)]	RSPH1.5	RSPH3
Water holding capacity (mL/g)	2.5±0.02 [a]	2.00±0.10 [b]
Oil binding capacity (mL/g)	5.6±0.30 [a]	4.9±0.14 [b]
Emulsifying capacity (mL/g)	100±4.0 [a]	90±3.10 [b]
Foaming capacity (%)	310±3.5 [a]	300±4.0 [b]

[a)]All values are means and standard deviations of three replicates. Mean values with different superscripts along the same row were significantly different ($P < 0.05$).

Foaming capacity and foaming stability

The foam formation is equivalent to the emulsion formation. The ability of proteins to form stable foams is an important property. Increase in net charge of the protein likely increases the flexibility of the protein and weakens hydrophobic interactions. Results in Table 4 suggested that all the hydrolysates had good foaming capacities (FC) of 310% and 300% for the RSPH1.5 and RSPH3, respectively. These results corroborate with the findings of Puski (1975), Alder-Nissen (1986), and Wang et al. (2008), who reported that enzymatically-modified food proteins improved foaming properties. Moreover, the RSPH1.5 showed more stable foam compared to the RSPH 3 (300 and 144% at 60 min, respectively) (Figure 2). The FC of the hydrolysates was improved by hydrolysis. Nevertheless, it is clear that the FC may depend on the protein structure, molecular size and hydrophobicity of the hydrolysate (Martin et al., 2002), which also depend highly on the hydrolysis procedure and the parent protein from which they are obtained. The effect of hydrolysis on foam stability is dependent on the degree of hydrolysis (Althouse et al., 1995; Mutilangi et al., 1996; Slattery and Fitzgerald, 1998). The short chain molecules form a wicker interaction network, which result in to a less stable foam.

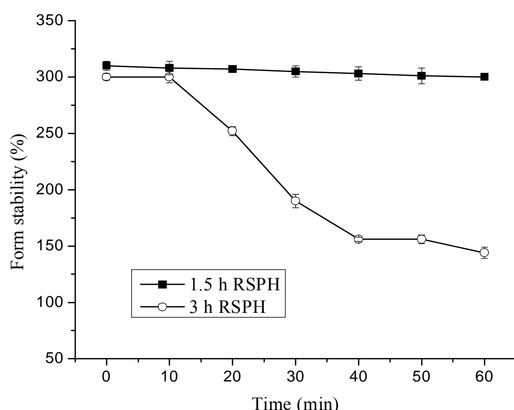

Figure 2. Foaming stability of Roselle seed protein hydrolysates.
Values represent the means ± SD of triplicates

Conclusion

The primary functionality of Roselle seed protein hydrolysates is due to its soluble peptide content. Roselle seed protein hydrolysates have high nitrogen solubility, good emulsifying capacity, WHC, OHC, foaming capacity and foam stability. The proportion of essential amino acids of both samples was higher than the recommended values by FAO/WHO. All the estimated nutritional parameters based on amino acids composition showed that RSPH has a good nutritional value. These results proposed that RSPH could be useful as whole or partial replacement of high-price protein sources such as egg albumin and casein.

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PLANT SCIENCE

Grain physical characteristics and bread-making quality of alternative cereals towards common and durum wheat

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Abstract

The present study is aimed at explaining the physical characteristics and bread-making quality of Khorasan wheat (BGR 40365, BGR 12389), einkorn (B2E0417) and emmer (B2000528). Here we will compare the aforementioned grains to both, common wheat (cv. Enola and Sadovo 1) and durum wheat (cv. Progres and Denitza). The physico-chemical characteristics (i.e thousand kernel weight, gluten content and Pelshenke value), are known to differ significantly among the wheat species, however, few studies have examined these characteristics in ancient grains. The highest thousand kernel weight and test weight were observed in BGR 40365. The highest wet gluten content was found in BGR 12389 (34.02%). One variety of einkorn (B2E0417) was determined to be 'gluten free'. Emmer (B2000528) was characterized by high content of wet gluten (30.13%). The best balance between gluten quantity and quality was observed in two varieties of Khorasan wheat (BGR12389, BGR 40365). PC-analysis was applied to group varieties according to their similarity on the basis of ten traits. Three sub-groups could be identified where the first one composed by the hexaploid wheat cultivar Sadovo 1 and cultivar Enola. The second sub-group included cultivar Progres and BGR 40365, while cultivar Denitza, BGR 12389 and B2000528 constituted the heterogeneous third sub-group.

Key words: Bread-making quality, Einkorn, Emmer, Khorasan, Physical characteristics of grain

Introduction

The consumer's interest in natural, unconventional and nutritional foods led to the development of new specialty foods based on grain blends. These components of foods are often termed 'ancient wheat' or 'primitive wheat', suggesting that the species were not the subject of modern plant breeding programs. Examples of these "ancient wheat" are einkorn (*Triticum monococcum* L.), emmer (*T. dicoccon* Schrank.) and Khorasan (syn. Oriental) wheat (*Triticum turgidum* L. *ssp. turanicum* (Jakub.) Mk.) are such neglected and underutilized wheat species, which probably survived over the centuries in subsistence farming systems in Europe, the Near East and Central Asia (Abdel-Aal et al., 1998; Grausgruber et al., 2004; Piergiovanni et al., 2009). These species are used for production of different cultural foods and breads based on religious and/or cultural; customs. Various types of breads are made in different cultures that are consumed as staple or at special festivals (Asghar, 2011). The attention towards these ancient species have also been renewed by the increasing demand for traditional products, the request for species suitable to be grown in marginal areas and the need to preserve genetic diversity (Stagnari et al., 2008).

In Bulgaria the interest in primitive wheat has increased during the past few years. Today the farmers practicing organic agriculture initiate the testing of various varieties of einkorn and emmer applicable to organic or low-input farming. A lot of practical questions originate because of difficulties with sowing norms and terms, unknown growth type, no possibilities and/or problems with threshing and de-hulling, or relatively low yield in relation to cultivated wheat. As presented by the Bulgarian Ministry of Agriculture and Foods the agricultural areas with einkorn and emmer amount 1,000 ha (www.limec-bgagro.bg). So called 'primitive wheat varieties' are cultivated in different country regions: Silistra, Vratsa, Plovdiv and village Rabevo- Eastern Rodopes.

The aim of this study was to illuminate the characteristics of Khorasan wheat, einkorn and emmer. Here we will compare the grain physical characteristics of these 'ancient wheat to the bread-

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making quality of common wheat and durum wheat.

Material and Methods

The study was conducted in the Institute of Plant Genetic Resources "Konstantin Malkov" - Sadovo, during 2013. As standards two cultivars of common winter wheat (Enola and Sadovo 1) and two cultivars of durum wheat (Progres and Denitza) are used. In parallel varieties of species einkorn (*Triticum monococcum* L.), emmer (*T. dicoccon* Schrank.) and Khorasan (syn. Oriental) wheat (*Triticum turgidum* L. *ssp. turanicum* (Jakub.) Mk.) were investigated (Table 1). The grain physical characteristics such as, thousand kernel weight, test weight and general vitreousness were determined according to the methods described in BDS ISO 520 (2003), BDS ISO 7971-2 (2000) and BDS 13378 (1976) . The moisture content of the flour samples was determined according to BDS ISO 712 (1997). Wet and dry gluten content in different flour samples was estimated by the hand washing method according to BDS 13375 (1988). The sedimentation value of whole wheat flour was determined using the method presented by Pumpyanskiy (1971). Bread making strength index and gluten weakness were defined according to BDS 13375 (1988). The whole wheat meal flour of each wheat variety was also tested for fermentation value (Pelchenke et al., 1953).

The experimental data of evaluated parameters were analyzed using the statistical program SPSS 13.0. The analysis of variance was applied to determine the following descriptive characteristics: mean value, standard error of means, standard deviation and coefficient of variation. Principal Component Analysis (PCA) for similarity was applied to wheat varieties on the basis of ten characteristics: thousand kernel weight, test weigh, vitreousness, moisture content, wet and dry gluten contents, sedimentation values, Pelshenke value, bread making strength index and gluten weakness. PCA was implemented in three components in the factor plane.

Results and Discussion

Physical characteristics of grain

The physical characteristics thousand kernel weight, test weight and vitreousness showed significant variation among different wheat species (Table 2). The thousand kernel weight ranged from 22.67 to 57 g. The highest thousand kernel weight confirmed statistically was observed in BGR 40365 (Khorasan wheat), followed by cultivar Progres (56.60 g). The other Khorasan wheat (BGR12389) and cultivar Denitza were found statistically at par with respect to their thousand kernel weight as recorded 31.67, 36 g, respectively. In comparison with the cultivars Sadovo1, Enola, Pobeda and Denitza significantly the lowest thousand kernel weights were found in the grains of covered wheat's of *Triticum monococcum* L. - B2E0417 (22.67 g) and *Triticum dicoccon* Schrank. - B2000528 (25.73 g), but both of these possessed non-significant differences among each other. The thousand kernel weight is a useful tool for the assessment of the potential milling yield. The kernel size contributes directly towards the improvement of grain yield as well as milling yield. Khan et al. (2009) indicated that the wheat varieties possessing a higher grain weight present a better potential for grinding and flour extraction. That is why the wide variation of grain weight could be used for improvement of this trait for creation of genotypes appropriate for maximum flour outputs. In our investigation BGR 40365 can be used as a donor of gene by this trait into the common winter wheat.

Table 1. List of wheat species and varieties.

No.	Genus/species	Subtaxa	Name of variety	Origin
1	*Triticum aestivum* L.	var. lutescens	Sadovo 1	Bulgaria
2	*Triticum aestivum* L.	var. erythrospermum	Enola	Bulgaria
3	*Triticum durum* Defs.	var.leucurum	Progres	Bulgaria
4	*Triticum durum* Defs.	var.alexandrinum	Denica	Bulgaria
5	*Triticum turgidum* L. ssp. turanicum (Jakubz.) Mk.	var. notabile	BGR 12389	Russia
6	*Triticum turgidum* L. *ssp. turanicum* (Jakubz.) Mk.		BGR 40365	Germany
7	*Triticum monococcum* L.	var.vulgare	B2E0417	Bulgaria
8	*Triticum dicoccon* Schrank	var.farum	B2000528	Italy

The highest test weight was recorded of BGR 40365, respectively 83 kg/hl and the lowest of durum wheat cultivar Denitza (78.10 kg/hl). The wheat cultivars Enola, Sadovo 1 and Progres are designed with very closely values (resp. 80.10; 80.30 and 80.60 kg/hl). There should be pointed that variety BGR 40365 was large seeded with well-filled grains which contain relatively more endosperm and less coats.

The grain vitreousness is important as a character for kernel grading and bread-making properties (Popov, 1965; Wang et al., 2002). The vitreousness of the evaluated varieties ranged from 62 to 99.67%. The highest value was recorded from Khorasan varieties where 99.67% for BGR 12389 and 97.67% for BGR 40365. The wheat with the lowest vitreousness 62% was eincorn (B2E041) and durum wheat cultivar Progres.

As presented the highest values of the physical grain characters were observed of Khorasan wheat (BGR 40365) in comparison with other varieties included in the study. This finding confirms the reported before by other researchers regarding hundred kernels weigh and test weigh of different wheat species (Grausgruber et al., 2005; Stagnari et al., 2008; Abdel-Haleem et al., 2012).

Chemical characteristics

Moisture content

The moisture content ranged from 7.83% to 9.47% (Table 3). The highest moisture content was determined in the common wheat varieties (Enola and Sadovo 1), 9.0% and 9.47%, respectively. The group of tetraploid wheat varieties (Progres, Denitza, BGR 12389, BGR 40365 and B2000528) showed close values of this character. The lowest moisture content was determined in the diploid wheat (B2E0417) -7.83%. The variation of moisture content in different wheat varieties might due to their different chemical components – proteins and starch as well to the shape of seeds and seed covering structures. Practically all components of the environment that influence the equilibration seed moisture could affect the water content in seeds. In another studies this effect is also confirmed taking attention both to the genetic characters of each variety and the environment including climate and agro-factors during vegetation (Mahmood, 2004; Khan et al., 2009).

Table 2. Physical characteristics of grain of different wheat species.

	Triticum aestivum L.		*Triticum durum* Defs.		*Triticum turgidum ssp. turanicum* (Jakubz.) Mk.		*Triticum monococcum* L.	*Triticum dicoccon* Schrank
	Sadovo 1	Enola	Progres	Denitza	BGR 12389	BGR 40365	B2E0417	B2000528
Vitreousness, %								
Mean	64.00	70.00	62.00	81.67	99.67	97.67	62.00	93.67
Std. Error of Mean	1.15	0.58	0.58	0.33	0.33	0.33	1.15	0.33
Std. Deviation	2.00	1.00	1.00	0.58	0.58	0.58	2.00	0.58
CV,%	3.13	1.43	1.61	0.71	0.58	0.59	3.23	0.62
Thousand kernel weight, g								
Mean	49.40	44.40	56.60	36.00	31.67	57.00	22.67	25.73
Std. Error of Mean	0.12	0.58	0.35	0.29	0.33	0.69	0.33	0.47
Std. Deviation	0.20	1.00	0.60	0.50	0.58	1.20	0.58	0.81
CV,%	0.40	2.25	1.06	1.39	1.82	2.11	2.55	3.14
Test weight, kg/hl								
Mean	80.10	80.30	80.60	78.10	81.60	83.00	-	-
Std. Error of Mean	0.17	0.10	0.35	0.06	0.40	0.58	-	-
Std. Deviation	0.30	0.17	0.60	0.10	0.69	1.00	-	-
CV,%	0.37	0.22	0.74	0.13	0.85	1.20	-	-

Table 3. Chemical characteristics of whole wheat flour of different wheat species.

	Triticum aestivum L.		*Triticum durum* Defs.		*Triticum turgidum ssp. turanicum* (Jakubz.) Mk.		*Triticum monococcum* L.	*Triticum dicoccon* Schrank
	Sadovo 1	Enola	Progres	Denitza	BGR 12389	BGR 40365	B2E0417	B2000528
mousture content, %								
Mean	9.47	9.00	8.00	8.60	7.87	8.20	7.83	8.00
Std. Error of Mean	0.03	0.06	0.06	0.06	0.03	0.06	0.03	0.06
Std. Deviation	0.06	0.10	0.10	0.10	0.06	0.10	0.06	0.10
CV,%	0.61	1.11	1.25	1.16	0.73	1.22	0.74	1.25
wet gluten content, %								
Mean	31.92	32.08	23.10	36.70	34.02	26.30	-	30.13
Std. Error of Mean	0.08	0.08	0.10	0.10	0.02	0.30	-	0.03
Std. Deviation	0.11	0.11	0.14	0.14	0.03	0.42	-	0.04
CV,%	0.35	0.35	0.61	0.39	0.08	1.61		0.14
dry gluten content, %								
Mean	10.40	10.69	8.16	12.62	11.48	9.24	-	10.32
Std. Error of Mean	0.23	0.18	0.10	0.04	0.24	0.09	-	0.18
Std. Deviation	0.40	0.32	0.18	0.07	0.41	0.16	-	0.32
CV,%	3.85	2.95	2.21	0.55	3.57	1.73	-	3.10
bread-making strength index								
Mean	55.00	78.00	40.00	67.00	76.00	71.00	-	51.00
Std. Error of Mean	1.73	0.58	2.89	1.15	2.89	1.15	-	1.73
Std. Deviation	3.00	1.00	5.00	2.00	5.00	2.00	-	3.00
CV,%	5.45	1.28	12.50	2.99	6.58	2.82	-	5.88
gluten weakness, mm								
Mean	10.51	6.03	13.03	7.98	6.50	4.50	-	12.01
Std. Error of Mean	0.20	0.09	0.04	0.07	0.23	0.12	-	0.14
Std. Deviation	0.34	0.15	0.06	0.13	0.40	0.20	-	0.24
CV,%	3.24	2.53	0.47	1.58	6.15	4.44	-	2.00
sedimentation value, cm3								
Mean	52.00	55.00	19.67	26.00	25.33	22.33	14.67	30.00
Std. Error of Mean	0.58	0.58	0.33	0.58	0.33	0.33	0.33	0.58
Std. Deviation	1.00	1.00	0.58	1.00	0.58	0.58	0.58	1.00
CV,%	1.92	1.82	2.94	3.85	2.28	2.59	3.94	3.33
Pelshenki test, min								
Mean	47.00	171.00	32.67	38.00	108.00	58.00	29.67	28.67
Std. Error of Mean	1.73	2.89	0.33	0.58	1.15	0.58	0.33	0.33
Std. Deviation	3.00	5.00	0.58	1.00	2.00	1.00	0.58	0.58
CV,%	6.38	2.92	1.77	2.63	1.85	1.72	1.95	2.01

Gluten content

As known the wet gluten is a visco-elastic proteinaceous substance obtained after washing out the starch granules from wheat flour dough (Mis, 2000; Yanchev and Ivanov, 2012). Quality of the resulting gluten is a considerable index of the wheat baking potential. Gluten quality is characterized by the degree of extensibility and the elasticity (Curic et al., 2001).

The variation of gluten content between examined varieties was in a large scale (Table 3). In our study was found out that from eincorn (B2E0417) the wet gluten is not washing out. This sample was assigned as ‘zero gluten’ from all evaluated wheat varieties. The highest wet gluten content was found in *T. durum* Defs. cv. Denitza

(36.70%) and *Triticum turgidum* L. *ssp. turanicum* (Jakub.) Mk. BGR 12389 (34.02%). In BGR 40365 was determined that the wet gluten content was relatively low but it was tight, stiff and with the minimum allocation of gluten ball (4.6 mm). Emmer (B2000528) and bread wheat cv. Sadovo 1 were characterized by high wet gluten content (resp. 30.13% and 31.92%), but with lower gluten quality indicated by gluten weakness (10.5 and 13.0 mm). Similar results were reported by Grausgruber et al. (2004). In their study was shown that emmer varieties possess high protein and wet gluten contents but low gluten quality. The best combination of grain gluten properties was observed in wheat cv. Enola and in varieties of Khorasan wheat (BGR12389, BGR 40365). The bread-making strength index of above samples was very high as an indication for good gluten quality.

Dry gluten content correlates positively with the wet gluten content (Zaidel et al., 2009; Baslar and Ertugay, 2011). In our study it varied from 8.16% for cv. Progers to 12.62% for cv. Denitza.

Sedimentation values (SV)

The highest rate of sedimentation value was described of bread wheat cultivars Sadovo 1 and Enola, respectively 52 cm^3 and 55 cm^3. Contrariwise the lowest value of SV (14.67 cm^3) was detected for einkorn (B2E0417). The tetraploid wheat in this study (Progres, Denitza, BGR 12389, BGR 40365 and B2000528) was described with sedimentation value between 19.67 to 30 cm^3 (Table 3).

As other authors indicated SV is in a positive relation to the crude protein content and wet gluten content (Yanchev and Ivanov, 2012). Our results confirm this suggestion comparing gluten content and sedimentation value of evaluated varieties.

Pelshenke test (PT)

The Pelshenke test is one of the most important tests for assessment of the gluten strength (Khan et al., 2009). The variation of this character between evaluated samples of wheat species ranged from 28.67 to 171 min (Table 3). The highest value of PT was observed for bread wheat cv. Enola (171 min), followed by Khorasan wheat BGR 12389 (108 min). The lowest value was determined of coated wheat varieties: B2000528 (*T. dicoccon* Schrank.) and B2E0417 (*T. monoccocum* L.), respectively 29.67 and 28.67 min.

Principal component analysis (PC)

PC-analysis was applied to arrange varieties by their similarity. The analysis was carried out on the basis of presented above ten traits by three components in the factor plane. The values of the three components to each of the studied parameters were calculated empirically (Table 4). The analysis showed that the first component explains 38.536% of the total variation, the second – 28.007% and the third – 20.133%. The all three components explain total **86.68**% of the variation in the experiment. The most effective by the first component were four characters: test weight, Pelshenki test, gluten weakness, and bread-making strength index. The second component correlated to thousand kernel weight and wet and dry gluten content. The third component was influenced by moisture content, vitreousness and sedimentation value. Three-dimensional scatter plot presented the distribution of varieties according to factor's scales (Figure 1). Three sub-groups could be identified where the first one composed by the hexaploid wheat cultivar Sadovo 1 and cultivar Enola. The second sub-group included cultivar Progres and BGR 40365, while cultivar Denitza, BGR 12389 and B2000528 constituted the heterogeneous third sub-group.

Table 4. Weighted factors (PC1, PC2 and PC3) of descriptive characteristics on the rotated matrix with tree factors.

Characters	Component		
	1	2	3
Moisture content, %	0.148	0,064	0.935
Vitreousness, %	0.287	0.413	-0.803
Thousand kernel weight, g	0.178	-0.929	0.231
Test weight, kg/hl	0.652	-0.461	0.276
Sedimentation value, cm3	0.184	0.205	0.876
Pelshenki test, min	0.742	0.123	0.283
Wet gluten, %	0.307	0.871	0.246
Gluten weakness, mm	-0.956	-0.098	0.149
Bread-making strenght index, or	0.922	0.371	-0.034
Dry gluten, %	0.306	0.873	0.121
% of total variance explained	38.536	28.007	20.133
Cumulative variation, %	38.536	66.543	86.676

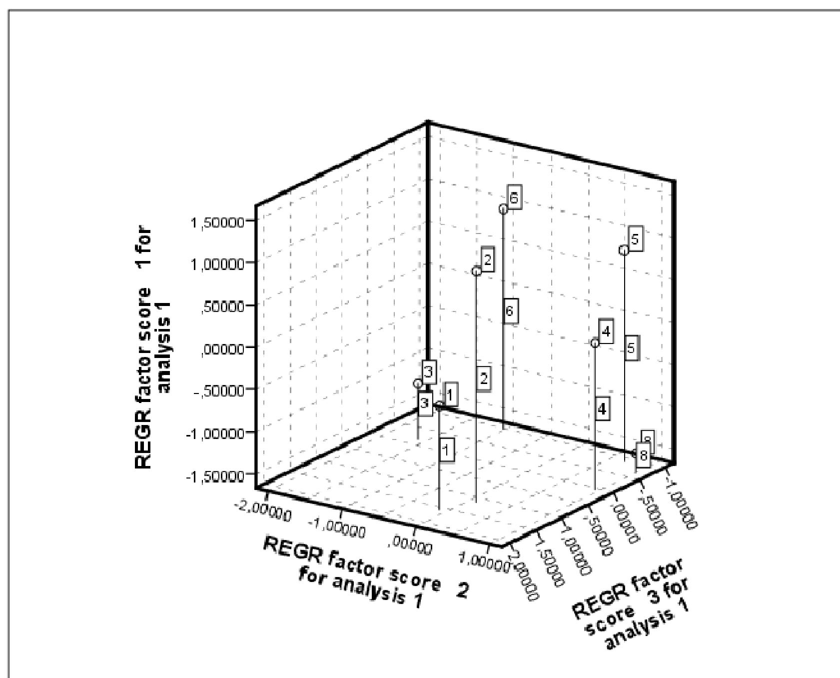

Figure 1. Three-dimensional scatter plot of the first three principal components. The varieties presented with the conditional numbers 1-8 of the graph correspond to the description in Table 1.

Conclusion

The physical characteristics of grain such as thousand kernel weight, test weight and vitreousness showed significant variation among evaluated wheat species. In this comparative study the highest physical properties of grain were described for Khorasan wheat, BGR 40365. This indicates that, Khorasan wheat has the potential to become high yield flour in grain. It is important to note that wet gluten was not washed out from einkorn, B2E0417. This means that einkorn is good selection for people requiring 'gluten free' diets. However emmer (B2000528) is characterized with high gluten content its gluten has lower quality because the gluten weakness. The most adequate balance between gluten quality and quantity traits were observed for cv. Enola (*T. aestivum* L.) and two varieties of Khorasan wheat (BGR12389, BGR 40365). So indicated three varieties possessed the highest bread-making strength index. Principal Component analysis illustrates the grouping of accessions by three components in the factor plane, where they explain total **86.68**% of the variation in the experiment. The effectiveness of evaluated characters was illustrated by their correlation to components. The most effective by the first component were four characters: test weight, Pelshenki test, gluten weakness, and bread-making strength index. The second component correlated to thousand kernel weight and gluten content. The third component was influenced by moisture content, vitreousness and sedimentation value.

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Desert truffles of the North Algerian Sahara: Diversity and bioecology

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Abstract

This study reports on the bio-ecology of desert truffles collected from the Northern Algerian Sahara. It aims focused on (i) the identification of desert truffle species with a morphometric characterization, (ii) the determination of their geographical distribution, and (iii) the description of the edaphic, climatic and geomorphological characteristics of their natural habitat. The harvest of 78 truffle fruiting bodies from seven different locations resulted in the identification of three species of the family Pezizaceae: *Terfezia arenaria* (Moris) Trappe, *Terfezia claveryi* Chatin and *Tirmania nivea* (Desf.) Trappe. These hypogeous ascomycetes live in mycorrhizal association with *Helianthemum lippii* (Cistaceae). Desert truffles grow in heterogeneous soils of sandy texture, moderately calcareous (10.19 ± 1.37%), slightly alkaline (7.87 ± 0.22), with low organic matter (0.86 ± 0.1%) and slight phosphorus contents. The development of desert truffles is closely linked with high rainfall occurring during fall and/or winter. The truffles colonize desert depressions "Dayas" and beds of Wadis, since these geomorphological zones accumulate rainwater, which promotes the growth of both truffles and its host–plant.

Key words: Algerian Sahara, Biodiversity, Bioecology, Desert truffles, Mycorrhizae, Terfeziaceae

Introduction

Desert truffles are hypogeous ascomycetes in Mediterranean countries consisting of genera such as *Terfezia*, *Delastria*, *Mattirolomyces*, *Picoa*, and *Tirmania* (Trappe, 1979; Kovács and Trappe, 2014). From their name, desert truffles include species typically distributed in regions with arid and semi-arid climates (Honrubia et al., 1992). These truffle species have developed adaptations to exploit various types of soil of various characteristics, particularly in association with plant species of the family Cistaceae, mainly the genus *Helianthemum* (Malençon, 1973; Alsheikh and Trappe, 1983; Chevalier et al., 1984; Alsheikh, 1985; Kagan-Zur et al., 1994, 1999; Khabar et al., 2001; Slama et al., 2006; Kovács et al., 2007).

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Besides their wide distribution, the order Pezizales predominates in arid and semi-arid regions of the Mediterranean basin and adjacent areas, including North Africa (Malençon, 1973; Trappe, 1979; Khabar et al., 2001; Slama et al., 2006), the Middle East (Al-Ruqaie, 2002; Mandeel and Al-Laith, 2007), Southern Europe (Janex-Favre et al., 1988; Honrubia et al., 1992; Ławrynowicz et al., 1997; Moreno et al., 2002) and also the Kalahari Desert (Díez et al., 2002; Mandeel and Al-Laith, 2007; Trappe et al., 2008a, 2014b). In the Mediterranean regions, *Terfezia* and *Tirmania* spp. form mycorrhizal associations mainly on roots of various species of the genus *Helianthemum* Miller (Dexheimer et al., 1985; Fortas and Chevalier, 1992; Gücin and Dülger, 1997). Other genera of desert truffles occur in Africa, Australia and North America (Kovács and Trappe, 2014).

In Algeria, research on desert truffles is sparse, especially in the Sahara desert. Although existing reports described a few species of the genera *Terfezia* and *Tirmania*, they performed mycorrhization tests on desert truffles with other plants in addition to *Helianthemum* spp. All documented studies mainly focus either on the steppe regions (Bessah, 1999), or approached the

Occidental Sahara of Algeria (Fortas, 1990; Tadja, 1996). Therefore the importance and benefits of these symbiotic fungi remain unknown in the Sahara Desert, which represents the most hostile ecoregion worldwide. Furthermore, the current study focuses on identification and morphological characterization of edible species of desert truffles found in Northern Algerian Sahara in relation with their distributional habitats. This pioneer study aims to provide information about species bio-ecology including the geographical distribution, habitat description, species occurrence frequency, main edaphic and climatic factors controlling their distribution and occurrence.

Materials and Methods

Study area

The study area is located in province "Wilaya" of Ghardaia and Ouargla in the Northern Sahara (Algeria), covering an area of 200,000 km², between 28°40'N to 33°40'N and 02°00'E to 08°00'E (Figure 1). The study was conducted during January to March of 2006 to 2012.

The climate is typically hot-arid. Average temperatures are high, with absolute maxima in July–August exceeding 50 °C, and minima in January ranging from 2 to 9 °C (Le Houerou, 1990). Soil surface temperature may exceed 70 °C; however, the temperature rapidly deceases with depth. Because of low cloudiness, the sunlight in the Sahara Desert is relatively strong and has a drying effect by raising the temperature (Ozenda, 2004). Practically, precipitation always occurs as rain characterized by its slight importance; torrential rains are rare. Rains are related to Sudano-Saharan and Saharan meteorological disturbances (Dubief, 1963). Such insufficient Saharan rains are associated with a significant irregularity of rainfall patterns and a considerable interannual variability, which induce more or less lengthy severe droughts (Ozenda, 2004).

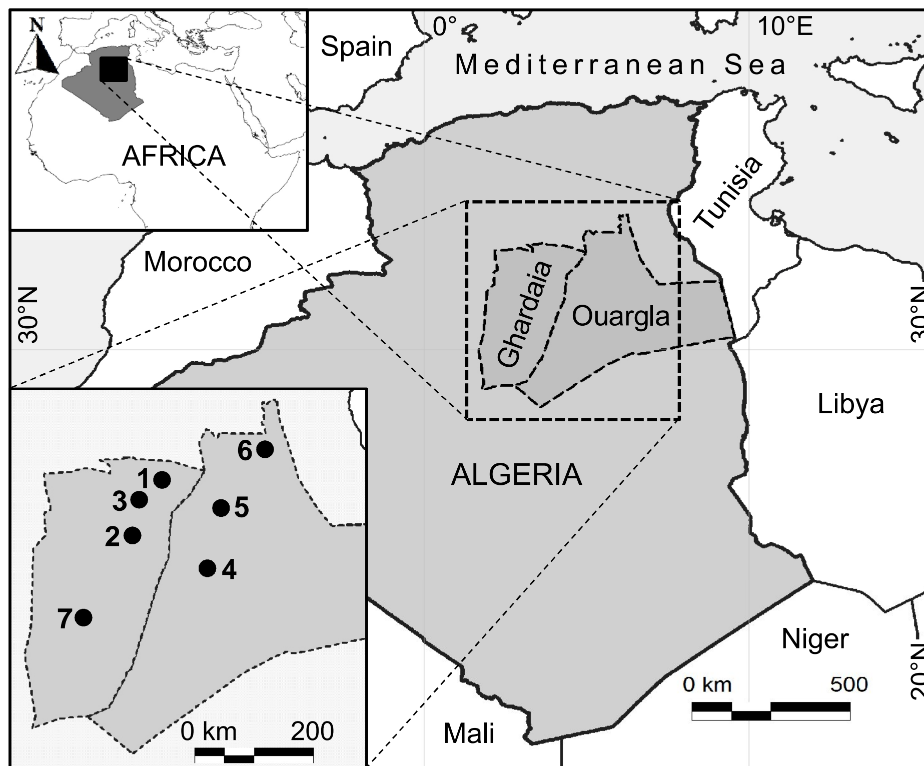

Figure 1. Location of the surveyed area in Northern Algerian Sahara. The study area includes Ghardaia and Ouargla spotted inside the dashed square. Solid dots (●) represent location of the seven sampling sites: 1: Guerrara, 2: Hassi El Fehal, 3: Noumrate, 4: Oued M'ya, 5: Oued N'ssa, 6: Stah El Merdfa, 7: Golea.

Choice of sampling sites

Seven sampling sites were chosen inside the study area for the collection and harvest of desert truffles. Site selection was based on the guidance of local inhabitants and nomads of the Sahara Desert. Seekers of desert truffles, "Truffle hunters" were also consulted. The selected sites were known to be usual producing areas of desert truffles.

Within each sampling site, a station of one hectare area (100 × 100 m) was delineated. The choice of all stations was based on the use of a purposeful sampling by choosing stations which looked particularly homogeneous and representative (Gounot, 1969).

In the vicinity of host plants in the genus *Helianthemum* (Alsheikh, 1985), the desert truffles were detected *in situ* by observing cracks and swellings in soil surfaces caused by emergence of the ascocarps (Figure 2).

Morphological study of Truffles

By use of a stereo zoom binocular microscope, morphology of macroscopic characters, in particular the shape and colour, was described for each part of the truffle, namely the ascocarp, the peridium and the gleba. To identify and characterize the species harvested, fresh samples were cut with a sharp blade, placed in 5% KOH, and stained with Melzer's reagent. Various characteristics such as ascospore shape, number per ascus, colour and orientation were observed under an optical microscope (magnification ×100) connected to a computer for species recognition. The dimensions of the asci and ascospores were measured using the Bio microscopic software “Motic Image Plus 2.0”. Truffle species were identified by available keys such as Trappe (1979) and Ferdman et al. (2005).

Soil sampling and analysis

At each truffle harvest point, a soil sample was collected at a depth of about 10–25 cm. Soil samples were dried in open air in the laboratory at ± 25°C, sieved through a 2 mm sieve and analysed to determine the physicochemical characteristics by standard methods (Baize, 2000).

The analysed soil parameters were particle size, determined by the international method “Robison’s pipette”; electrical conductivity “EC” was measured with a conductivity-meter at 25°C on a soil:water ratio of 1:5, then the concentration of salts "salinity" was calculated by this formula: soil salinity in mg/L = 640 × EC in dS/m (Baize, 2000); pH was determined with a pH-meter with glass electrode on a soil:water ratio equal to 1:5; total $CaCO_3$ was measured by Bernard calcimeter; organic matter was identified by Anne’s method (AFNOR, 1996); HCO^{3-} was determined by titration with H_2SO_4 and SO_4^{2-} by the gravimetric method after precipitation as barium chloride (AFNOR, 1996); phosphorus, Ca^{++}, Mg^{++} and K^+ were determined by an atomic absorption spectrophotometer on a soil:water extract ratio of 1:5 (Baize, 2000).

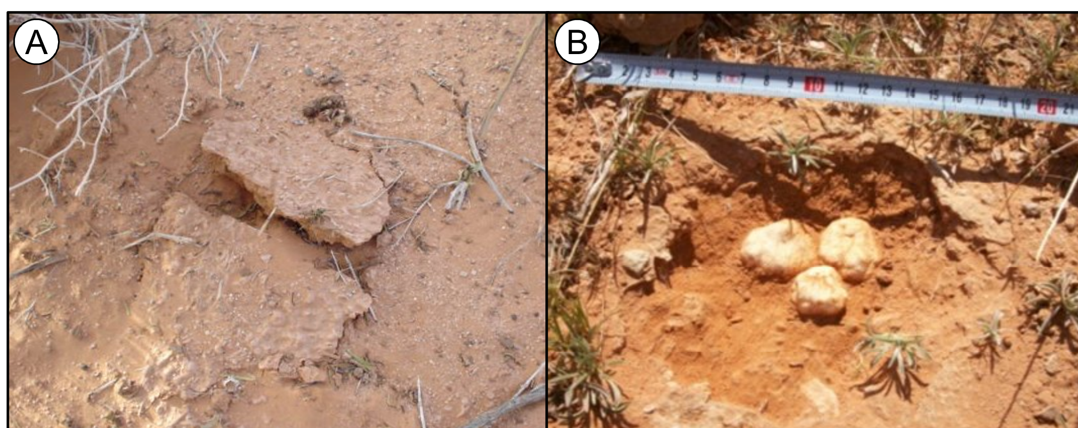

Figure 2. General view of the collection points of desert truffles (A: Swelling of the ground caused by Truffle, B: View of *Tirmania nivea* growing deep in the earth and the surrounding vegetation, including young seedlings of *Helianthemum lippii*).

Statistical analysis

A Pearson's Chi-square test (χ^2) was performed to test the null hypothesis that no association exists between the occurrence of desert truffle species and the study sites.

Means and standard deviations of soil factors were given for each site. After assessing the normal distribution and homogeneity of data by the Shapiro-Wilk normality test, all soil parameters were tested for significant differences between sampled sites using One-Way ANOVA followed by Tukey's post hoc test when the test is positive. R-commander {Rcmdr} was used as a statistical package for computations (Fox, 2005).

Results and Discussions

Identification and distribution of truffle species

Following the sampling of seven sites at the Northern Algerian Sahara during the period 2006 to 2012, a total of 78 fresh fruiting bodies of truffles were collected. The harvested desert truffles belonged to *Terfezia* or *Tirmania* (Pezizaceae) and three species: *Terfezia arenaria*, locally called in Arabic "Terfesse Lahmar", *Terfezia claveryi*, known vernacularly as "Terfesse Lakhal" and *Tirmania nivea*, commonly called "Terfesse Labyadh".

The most abundant species over all sampled sites was *Tirmania nivea* with a mean relative abundance of 70 ± 10.1%, followed by *Terfezia arenaria* whose occurrence ranged from 19 to 37% (mean = 22.9 ± 8.1%). *Terfezia claveryi* occurred r between 0 and 17% with an average of 9.9 ± 5% (Figure 3). According to Pearson's Chi-square test, no significant relationship was found between number of harvested fruiting bodies of each truffle species and the sampled sites (χ^2 = 11.01, *df* = 12, *P* = 0.528).

Desert truffles are limited to semi-arid and arid areas, especially in countries of the Mediterranean basin, including Southern Europe as Portugal, Spain, France, Italy, Hungary and Turkey (Janex-Favre et al., 1988; Honrubia et al., 1992; Moreno et al., 2002), and North Africa, extending from Morocco to Egypt (Malençon, 1973; Bokhary, 1987; Khabar et al., 2001), and the Middle East (Alsheikh and Trappe, 1983; Alsheikh, 1985; Mandeel and Al-Laith, 2007). Some species of desert truffles also occur in Botswana, Namibia, and South Africa (Marasas and Trappe, 1973; Trappe et al., 2008a, 2014a), Australia (Trappe et al., 2008b, 2010, Claridge et al., 2014), and Mexico and the USA of North America (Moreno et al. 2012, Trappe et al. 2014b).

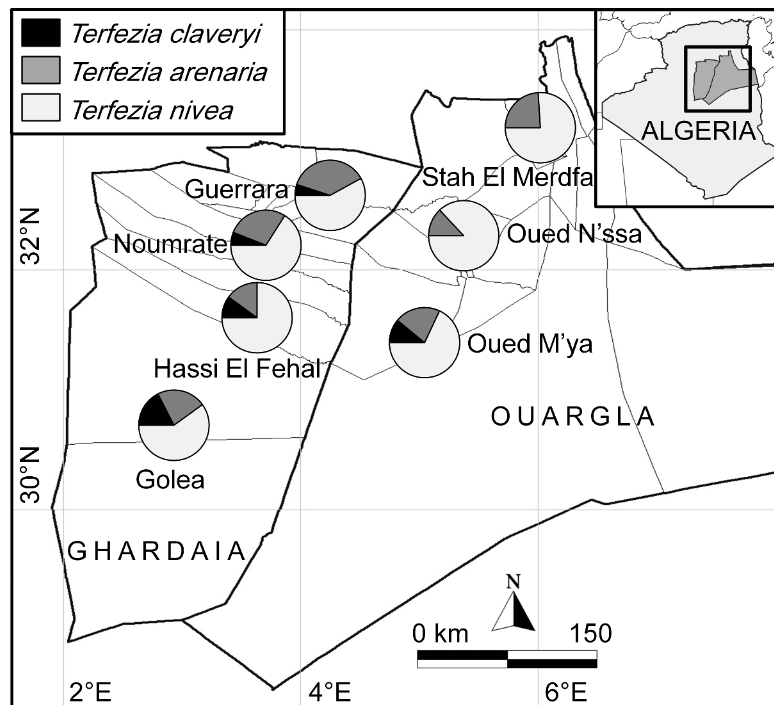

Figure 3. Distribution and density of desert truffles in the Northern Algerian Sahara. (Charts represent occurrence frequencies of the surveyed truffle species within each site).

Morphometric description of surveyed truffles

The macroscopic and microscopic characters and anatomy of the fruiting body for the identified truffle species are detailed in Figure 4.

• ***Terfezia arenaria*** — **Ascocarps**: subglobular to tuberiform, 7 – 12 cm in diameter, with peduncle at the base. The colour ranges from dark-brown to brown (Figure 4A). **Peridium**: brownish, 0.75 – 2 mm thick with a smooth, often cracked surface (Figure 4B). **Gleba**: fleshy, mottled reddish to pinkish, with visible sterile veins irregularly delineating the fertile areas. **Asci**: subglobose, sometimes ovoid, randomly arranged in fungal tissues, 62–71 × 73–83 μm in diameter. At maturity it contains six ascospores. **Ascospores**: yellowish, thin-walled hyaline, freely placed inside asci, with spherical shape and size ranging from 21–25 × 22–23 μm (Figure 4C).

• ***Terfezia claveryi*** — **Ascocarps**: measuring 5–8 cm in diameter, sub-globular sometimes pyriform, gibbous sterile surface, pale-brown to brown, tinted blackish-brown at maturity (Figure 4D). **Peridium**: orange-brown coloured, 0.8–1.2 mm thick (Figure 4E). **Gleba**: fleshy, compact, first yellowish then reddish at maturity, divided by pale veins. **Asci**: globose, 73–93 × 62–74 μm, contain eight spores. **Ascospores**: yellowish, arranged randomly in hyphae tissue at maturity, globose, 17 – 22 μm broad (Figure 4F).

• ***Tirmania nivea*** — **Ascocarps**: often subglobose or lobed pyriform, short and smooth pedicel, can reach 4–8 cm in diameter, whitish cream-coloured to white (Figure 4G). **Peridium**: 1.5–2 mm thick, yellowish milky white (Figure 4H). **Gleba**: fleshy white, solid, slightly marbled with some veins of 1.8–4.9 mm wide. **Asci**: ellipsoid to obovoid, 56–73 × 38–47 μm, amyloid, most with eight hyaline spores with short pedicel. **Ascospores**: bluish, thin-walled, freely arranged within the asci, ellipsoid, 15–18 × 11–13.5 μm (Figure 4I).

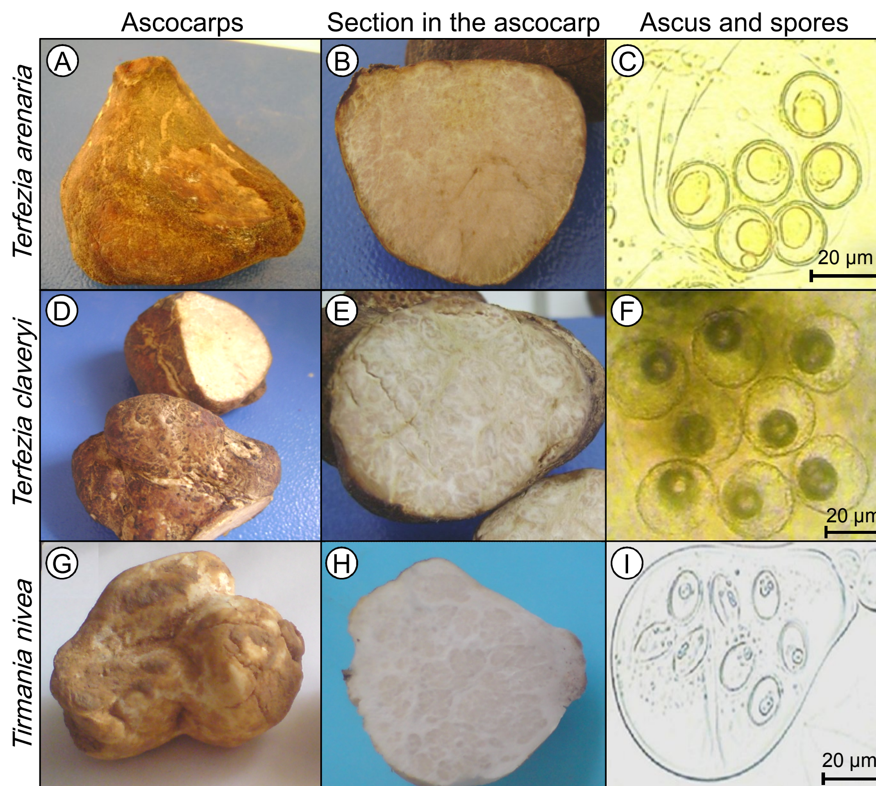

Figure 4. An overview of some morphometric and anatomical characteristics of fruiting bodies of *Terfezia arenaria* (A: Ascocarp, B: section of ascocarp, and C: ascus with and six ascospores), *Terfezia claveryi* (D: Ascocarp, E: ascocarp section, and F: ascospores) and *Tirmania nivea* (G: Ascocarp, H: cross section of ascocarp, and I: ascus with and eight ascospores) collected from the Northern Algerian Sahara Desert.

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The morphological and anatomical characteristics of *Terfezia arenaria*, *Terfezia claveryi*, and *Tirmania nivea* found in the study area are in ideal agreement with descriptions of the same truffle species of Africa (Malençon, 1973; Khabar et al., 2001; Slama et al., 2006), those of Kuwait (Alsheikh and Trappe, 1983), Saudi Arabia (Bokhary, 1987), Irak (Abd-Allah et al., 1989), Egypt (El-Kholy, 1989), Mediterranean Basin (Morte et al., 2009), and at world scale (Trappe, 1979).

Bioecology of desert truffles

Edaphic parameters

Results of the physicochemical analyses applied for soil samples extracted from different parts of the North Algerian Sahara are shown in Table 1.

Soil analyses of sites producing desert truffles showed wide heterogeneous pedological characteristics between the sites. Indeed, ANOVA tests showed a significant variation (P <0.001) in values of most edaphic factors (except K^+ and $SO_4^=$) between the sampled sites. Tukey's post hoc showed that these sites belong to different classes (2–7 groups, following soil factors) which support the finding on heterogeneous pattern of desert truffles distribution over the Northern Algerian Sahara, where truffles recorded different occurrence frequencies within each site.

Despite this inter-site differences, the grain size analysis, based on the American classification of soil textures (Eswaran et al., 2010), showed that all habitats of truffles in the Northern Sahara have soils of sandy texture and single grained structure, where sand grains have high rates of 80.6–91.7%.

Chemically, the habitats of desert truffles were characterized by soils having slightly alkaline pH (Baize, 2000) whose values varied slightly between 7.60 and 8.05. According to the classification scale of salinity of the 1:5 aqueous extract (Aubert, 1978), the sampled soils possess salinity degrees less than 100 mg/L because electrical conductivity values were slight and varied between 0.65 and 0.79 dS/m, which indicates non-saline soils. The content of organic matter ranged from 0.78 to 0.98%. These values reveal a high deficient in soil organic matter according to the scale of organic matter defined by Morond (2001). In addition, the contents of $CaCO_3$ varied from 9.09 to 12.01%, revealing that the sampled soils were moderately calcareous following the scale of total $CaCO_3$. The phosphorus content ranged between 23.42 and 25.54 ppm, indicating soils are poor in phosphorus (Baize, 2000).

Table 1. Means (± standard deviation) of physico-chemical soil analyses carried out at sites of harvesting desert truffles in the Northern Sahara of Algeria. Outcomes of One-way ANOVA test are given as F (*df* between groups, *df* within groups) and P-value. Letters show significant differences between sites according to Tukey's post hoc test (95% family-wise confidence level): Tukey (HSD) critical value = 3.027. (EC: electrical conductivity, n: number of soil samples analysed in each site).

Soil parameter	Sampled sites (n = 78)							ANOVA	
	Guerrara (n = 10)	Hassi El Fehal (n = 7)	Noumrate (n = 15)	Oued M'ya (n = 17)	Oued N'ssa (n = 11)	Stah El Merdfa (n = 10)	Golea (n = 8)	$F_{(6, 71)}$	P
Clay (%)	2.38 ± 0.18^{a}	2.07 ± 0.19^{b}	0.18 ± 0.04^{c}	1.64 ± 0.29^{d}	2.69 ± 0.25^{e}	3.19 ± 0.12^{f}	2.05 ± 0.08^{b}	316.57	<0.001
Silt (%)	13.9 ± 2.18^{a}	6.2 ± 1.38^{b}	18.03 ± 0.49^{c}	9.66 ± 6.52^{b}	16.72 ± 0.7^{ac}	15.29 ± 0.89^{ac}	6.98 ± 0.61^{b}	20.84	<0.001
Sand (%)	83.72 ± 2.25^{a}	91.73 ± 1.24^{b}	81.79 ± 0.51^{a}	88.67 ± 6.53^{b}	80.6 ± 0.78^{a}	81.53 ± 0.94^{a}	90.97 ± 0.62^{b}	19.74	<0.001
EC (dS/m)	0.66 ± 0.04^{a}	0.66 ± 0.06^{a}	0.79 ± 0.07^{a}	0.78 ± 0.07^{a}	0.67 ± 0.03^{a}	0.78 ± 0.08^{b}	0.65 ± 0.07^{a}	11.63	<0.001
pH	7.88 ± 0.12^{ab}	7.78 ± 0.39^{ac}	7.96 ± 0.07^{b}	8.05 ± 0.11^{b}	7.60 ± 0.21^{c}	7.90 ± 0.07^{ab}	7.84 ± 0.1^{bc}	10.62	<0.001
Total $CaCO_3$ (%)	10.21 ± 0.76^{ab}	11.28 ± 1.26^{ac}	11.66 ± 1.13^{c}	9.09 ± 0.54^{d}	9.19 ± 0.32^{bd}	9.09 ± 0.77^{d}	12.01 ± 0.11^{c}	27.09	<0.001
Organic matter (%)	0.83 ± 0.11^{ab}	0.83 ± 0.05^{bc}	0.85 ± 0.04^{ab}	0.84 ± 0.08^{ab}	0.78 ± 0.07^{b}	0.97 ± 0.09^{c}	0.98 ± 0.1^{ac}	6.42	<0.001
Phosphorus (ppm)	22.09 ± 1.32^{a}	23.42 ± 1.38^{ab}	21.95 ± 1.09^{a}	24.22 ± 1.05^{b}	21.83 ± 1.50^{a}	22.28 ± 0.94^{a}	25.54 ± 0.50^{c}	14.83	<0.001
Ca^{++} ($cmol^+$/kg)	6.26 ± 0.17^{a}	5.19 ± 0.49^{b}	6.26 ± 0.17^{a}	4.62 ± 0.7^{c}	5.31 ± 0.08^{b}	4 ± 0.46^{d}	5.02 ± 0.12^{bc}	42.70	<0.001
Mg^{++} ($cmol^+$/kg)	1.44 ± 0.19^{ab}	1.13 ± 0.13^{c}	1.28 ± 0.2^{ac}	1.46 ± 0.1^{b}	1.36 ± 0.06^{ab}	1.3 ± 0.1^{bc}	1.4 ± 0.12^{ab}	7.47	<0.001
K^+ ($cmol^+$/kg)	0.14 ± 0.02^{a}	0.18 ± 0.03^{a}	0.14 ± 0.02^{a}	0.18 ± 0.02^{a}	0.19 ± 0.03^{a}	0.2 ± 0.02^{a}	0.19 ± 0.02^{a}	1.04	0.405
HCO_3^- ($cmol^+$/kg)	0.21 ± 0.02^{ab}	0.21 ± 0.03^{b}	0.22 ± 0.01^{ab}	0.26 ± 0.08^{ac}	0.29 ± 0.01^{c}	0.28 ± 0.04^{c}	0.23 ± 0.02^{bc}	7.45	<0.001
$SO_4^=$ ($cmol^+$/kg)	0.44 ± 0.02^{a}	0.4 ± 0.02^{a}	0.42 ± 0.04^{a}	0.4 ± 0.09^{a}	0.44 ± 0.04^{a}	0.41 ± 0.02^{a}	0.4 ± 0.03^{a}	1.56	0.171

Due to physicochemical properties of soils in the Algerian hyper arid lands, such as the lack of cohesion between soil particles, low values of the organic matter, salinization…, ecosystems of this region are classified as degraded habitats (Halitim, 1998). Moreover, truffles were reported occurring in semiarid and arid areas of Algeria on calcareous soils that have sandy texture and very slight organic matter values (Fortas, 1990). Besides, it has been reported that *T. claveryi* and *T. nivea* occur in deserts of salty and/or gypseous soils (Singer, 1961; Halwagy and Halwagy, 1974; Alsheikh and Trappe, 1983; Bradai et al., 2013).

In general, soil properties of truffles biota in Northern Algerian Sahara are very similar to those reported in some truffle autoecological studies, whether in North Africa (Fortas, 1990; Khabar et al., 2001; Slama et al., 2006) or in the Middle East (Abd-Allah et al., 1989; Hashem and Al-Obaid, 1996; Al-Ruqaie, 2002; Mandeel and Al-Laith, 2007). However, our results are different from those observed in the Kalahari Desert, where the soil of truffle habitats had a low pH values ranging from 5.5 to 6.5; as well low total CaCO3 content (0.3 and 3.1%) (Taylor et al., 1995). This difference is probably due to multiple dissimilarities of regional landscape-type (geomorphology, hydrology and the type of habitat); climate patterns (temperature, precipitation and seasonality); soil traits (soil type and evolution); and the type of host plants (Díez et al., 2002).

Geomorphological parameters

In the Algerian Sahara, the producing areas of desert truffles occur especially in depressions (Daya) and beds of temporal Wadis. The trend towards these geomorphological zones is not random, it follows that these formations are characterized by their ability to accumulate rainwater, which promotes the development of truffles as well as its host plant species. Ozenda (2004) argues that the Wadis and Dayas (depressions) are among the most favourable biotopes for the installation of vegetation in the Saharan regions. In addition, Chehma et al. (2005) also demonstrated that the beds of Wadis are the richest and most diverse in species and plant families in the Northern Sahara habitats. Moreover, this area is well known for its richness of medicinal plants (Hadjaidji-Benseghier and Derridj, 2013).

Climatic parameters

According to our findings, the development and distribution of desert truffles in the Northern Algerian Sahara are mostly related to the existence of favourable habitats in relation to climate. Indeed, truffle development is closely related to climatic conditions, particularly rainfall, which mainly occurs during the fall and winter seasons (Figure 5). In fact, truffles thrive in warm climates provided in autumn and/or winter given quantities of rains fall then periods of drought follow. These rains, even of low-quantity, play important roles in the establishment of truffles including the transport and dispersion of fungal spores, spore germination and also the germination and development of host plant (*Helianthemum* spp.).

However, the production of truffles can be disrupted by excessive rainfall or rains poorly distributed during the year or even by prolonged periods of extreme heat or cold or even more prolonged periods of drought (Chafi, 2004). Indeed, we find that annual rainfall well distributed between October to March often give good results for harvesting desert truffles in Algeria, which takes place between January–February in the Saharan regions (arid climate) and from March to April in steppe regions, areas with semi-arid climate.

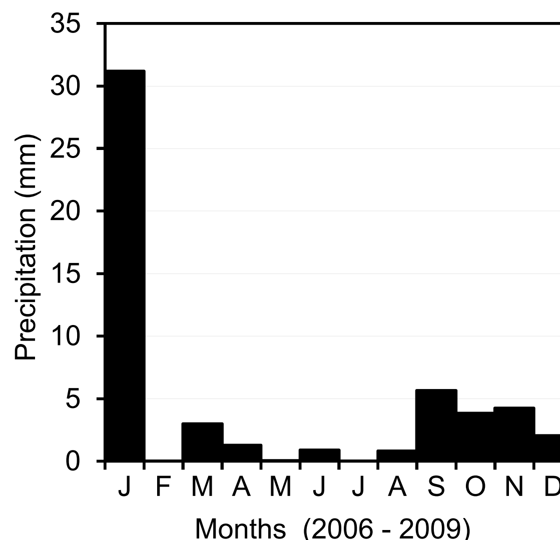

Figure 5. Variation in average monthly precipitation of Northern Sahara, Algeria during the period 2006–2009.

Based on our monitoring of desert truffles in the sites sampled throughout 2009 and for rainfall of 130.06 mm (NOM, 2010), truffle productivity was about 4.3 kg per hectare, knowing that for the same sites and during the year 2007, no production has been achieved since the rainfall did not exceed 51.05mm (NOM, 2008). The growth of fruiting bodies of truffles depends on several factors such as the periodicity of the rainy season including rainfall

amount, types and characteristics of soil, water availability and climatic conditions (Bokhary and Parvez, 1988).

Generally, areas where desert truffles grow have an annual rainfall ranging from 50 to 380 mm. During the season of harvest, production yield of truffles is greater if the rainfall varies from 70 to 120 mm in the countries of North Africa and if it fluctuates between 100 to 350 mm in South European countries. The seasonal distribution of rainfall is as important as its quantity, i.e. the rain is required, for both fungi and host–plant growth, no later than early December in North Africa and the Middle East; and at least in early October in southern Europe (Morte et al., 2009).

Symbiotic host plant

Desert truffles surveyed in the Northern Sahara of Algeria establish symbioses with plant roots of the family Cistaceae, especially with *Helianthemum lippii*. The genus *Helianthemum* is well known in literature for the establishment of associations with truffles in several regions in the world (Dexheimer et al., 1985; Fortas and Chevalier, 1992; Díez et al., 2002; Mandeel and Al-Laith, 2007).

The species *Helianthemum lippii* is a small plant, very branched, of stiff stems and partially lignified, that measures up to 30 cm of height in good rainfall conditions. The leaves are opposite, oblong, covered with very short hair, which gives them a whitish green colour. Tiny yellow flowers of five petals, sessile as leaves, are visible in clusters (Ozenda, 2004).

Within the sampled sites, desert truffles are harvested in habitats characterized by a high density of the host plant. In addition to the syncing of truffle development with fall–winter rainfalls, there is a significant synchronization in the growth of the two symbionts, where the maturity of the desert truffles, assessed by the formation of asci, usually and timely corresponds to the flowering stage of the host plant.

Conclusion

The present taxonomic and bioecological study of desert truffles, conducted for the first time in Northern Algerian Sahara, has identified three species *T. arenaria*, *T. claveryi* and *T. nivea*. These species, living in association with *H. lippii* (Cistaceae), are few in number but have remarkable adaptations to the environment that is characterized by a severe aridity and rudimentary soil traits. Indeed, from an ecopedological point of view, the harvested truffles grow in moderately calcareous sandy soils, slightly alkaline and poor in organic and mineral matter. In terms of climate, these fungi grow in a hot arid climate, as long occasional rains occur in autumn–winter, then periods of drought follow. Future work is necessary to investigate truffles of the Algerian Sahara through studies at molecular scale (DNA sequencing, phylogeny, eco-physiology, and therapeutic and biotechnological benefits.

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PLANT SCIENCE

Allelopathic activity of leaves, stalks and roots of *Cymbopogon nardus*

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Abstract

In this study, leaves, stalks and roots of *Cymbopogon nardus* were separately evaluated to determine the most active parts that contained the strong growth inhibitory activity. Each aqueous methanol extracts of *Cymbopogon nardus* were determined their allelopathic activity by using six test plant species; alfalfa (*Medicago sativa* L.), cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), barnyard grass (*Echinochloa crus-galli* L.), Italian ryegrass (*Lolium moltiflorum* Lam.) and jungle rice (*Echinochloa colonum* (L.) P. Beauv.). Four extract concentrations (0.01, 0.03, 0.1 and 0.3 g dry weight equivalent extract/mL) were used for the bioassay. The results showed that these three extracts have inhibitory activity and the percent inhibition increased concentration dependently. However, the inhibitory activity of leaf and root extracts was more effective than stalk extract at 95% level of significance. Barnyard grass, Italian ryegrass and jungle rice were the most sensitive to the leaf, stalk and root extracts, respectively. The concentrations required for 50% growth inhibition of *C. nardus* leaf, stalk and root extracts on all test plants were 0.000-0.025, 0.009-0.077 and 0.003-0.023 g dry weight equivalent extract/mL, respectively. In addition, separation of these extracts through silica gel column indicated that root extract contained the most active fractions with strong growth inhibition. The present results suggest that *C. nardus* may have allelopathic compounds and the root extracts have the greatest inhibitory activity. Studies are in progress for the isolation and identification of allelopathic compounds in aqueous methanol extracts of *C. nardus* roots for the development of natural herbicides.

Key words: *Cymbopogon nardus,* Inhibitory activity, Aqueous methanol extract, Weed control

Introduction

Weed infestation in crop field results in a reduction in quality and quantity of crop productivity. Currently, synthetic herbicides have been considered to solve weed problems and prevented crop yield loss. However, the overuse of synthetic herbicide may affect the environment, human health and the increasing of herbicide resistance weeds (Owen and Zelaya, 2005; Hager and Refsell, 2008; Bhadoria, 2011). Hence, to overcome the disadvantages of herbicide applications, efforts to utilize natural plant products or natural eco-friendly chemicals are in demand.

Plant allelochemicals are defined as natural compounds that influence on the development of neighboring plants by releasing into the environment in several ways such as leaching, volatilization, root exudation and plant decomposition (Rice, 1984). The allelochemicals can be presented in every organ of plant parts including flowers, leaves, stems, roots and seeds (Rice, 1984; Fateh et al., 2012; Grisi et al., 2012). Naderi and Bijanzadeh (2012) identified the potential of allelopathic effects of leaf, stem and root extracts of ten Iranian rice cultivars on barnyard grass, which leaf extract exhibited the strongest growth inhibitory activity followed by root and stem extracts. Numerous researchers also reported that each plant part showed significant difference effects on the growth of test plant species (Dorning and Cipollini, 2006; Fateh et al., 2012; Grisi et al., 2012; Pirzad et al., 2012; Liu et al., 2003; Tabrizi and Yarnia, 2011). It has also been reported that the compounds in roots reach more easily to the surrounding plant roots than the compounds in leaves (Wu et al., 2009).

Cymbopogon nardus (L.) Rendle is a perennial grass that widely cultivated in Southeast Asia (Shasany et al., 2000; Nakahara et al., 2003). This plant is well known as mosquito repellent and also has several pharmacological properties (Simic et al., 2008; Nurhanani and Othman, 2010; Istianto

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and Emilda, 2011; Kongkaew et al., 2011; Silva et al., 2011; Sritabutra et al., 2011). For food productivity, Lonkar et al. (2013) analyzed the chemical constituents from leaves of six varieties of *C. flexuosus* for preparation as medicinal tea. However, there have been only a few studies of allelopathy in the genus of *Cymbopogon*. Zeng and Luo (1996) reported the effects of root exudates released by *C. citratus* that affected seedling growth of radish, rice and cucumber by decreasing seed germination, root length and seedling height. Additionally, the volatile compounds of *C. citratus* significantly inhibited seedling growth of corn and barnyard grass (Li et al., 2005).

Our previous studies confirmed that whole plants of *C. nardus* have strong inhibitory activity on common agricultural weeds such as barnyard grass, Italian ryegrass, jungle rice and timothy (Suwitchayanon et al., 2013). Therefore, in the present study, *C. nardus* was divided into three parts such as leaves, stalks and roots and investigated their allelopathic activities. The aqueous methanol extracts were determined their growth inhibitory activity on monocotyledonous and dicotyledonous species to develop as alternative weed management options.

Materials and Methods

Plant Materials

The whole plants of *Cymbopogon nardus* (L.) Rendle were collected from Chiang Mai province, Thailand in July 2012. Plants were washed several times to get rid of soil particles and separated into 3 parts; leaves, stalks and roots, then dried in oven at 70°C and ground into powder. Dry powder was then vacuum sealed in a plastic bag and kept at 4°C. Dicotyledonous species such as alfalfa (*Medicago sativa* L.), cress (*Lepidum sativum* L.) and lettuce (*Lactuca sativa* L.) were chosen because of their known seedling growth behavior. Monocotyledonous species such as barnyard grass (*Echinochloa crus-galli* L.), Italian ryegrass (*Lolium moltiflorum* Lam.) and jungle rice (*Echinochloa colonum* (L.) P. Beauv.) were chosen because there are common agricultural weeds.

Extraction

Leaf, stalk and root powder (100 g) was extracted separately with 1 L of 70% (v/v) aqueous methanol for two days. The extract of each plant powder was then filtered through one layer of filter paper (No. 2; Toyo Ltd., Japan), using a vacuum pump. The residue was extracted again with 1 L of cold methanol for one day and filtrated. The two filtrates of each part were combined and evaporated to dryness with a rotary evaporator at 40°C. Each crude extract was dissolved in cold methanol and subsequently used for the next experiments.

Bioassay

The bioassay was conducted with four concentrations (0.01, 0.03, 0.1 and 0.3 g dry weight equivalent extract/mL). An aliquot of the extract was added to a sheet of filter paper (No. 2) in 28 mm Petri dish. After the solvent evaporated, the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of polyoxyethylenesorbitan monolaurate (Tween 20; Nacalai, Kyoto, Japan), a surfactant that did not cause any toxic effects. Ten seeds of alfalfa, cress, lettuce or 10 germinated seeds of barnyard grass, Italian ryegrass or jungle rice were arranged on the filter paper in Petri dishes.

For germination, barnyard grass, Italian ryegrass and jungle rice were germinated by soaking in distilled water in Petri dish (9 cm diameter) and incubated in the darkness at 25°C for 72 h.

Control seeds were sown on the filter paper moistened with the aqueous solution of Tween 20 without the extract. The shoot and root lengths of those seedlings were measured at 48 h after incubation in the darkness at 25°C. Percent inhibition of seedling growth was calculated by reference to the length of control seedlings.

The bioassay was repeated three times with 10 plants for each determination. The inhibition percentage was calculated using the equation as follow: Inhibition (%) = [1-(treatment /control)] × 100. In addition, the concentrations required for 50% inhibition (I_{50}) of the test plant species in the assay were calculated from the regression equation of the concentration response curves.

Separation of the extracts

Leaf, stalk and root powder of *C. nardus* was extracted as described above. The extract was then concentrated at 40°C in vacuo to produce an aqueous residue. The aqueous residue was adjusted to pH 7.0 with 1 M phosphate buffer, and partitioned three times against an equal volume of ethyl acetate, and separated ethyl acetate and aqueous phase. The ethyl acetate fraction was carried out by drying over anhydrous Na_2SO_4 then filtrated and evaporated to dryness. The residue was chromatographed on 60 g of silica gel (60 Merck, 70–230 mesh) and eluted stepwise with *n*-hexane that contained increasing amount of ethyl acetate (10% per step, v/v; 150 mL per step) and methanol (300 mL). The inhibitory activity was determined by using cress seedlings.

Statistical Analysis

All experiments were carried out with triple replicated and repeat twice. The statistical data processing was analyzed by SPSS version 16.0 using one-way ANOVA and general linear model/ univariate. GraphPad Prism 6 was used to analyze the concentrations required for 50% inhibition.

Results

Effect of aqueous methanol extracts of leaves, stalks and roots of *C. nardus* on shoot growth

The inhibitory activity of *C. nardus* leaf, stalk and root extracts on shoot growth of six test plant species are shown in Figure 1. Three extracts showed different percent inhibition on test plant species. Threshold of growth inhibition for *C. nardus* leaf and root extracts was 0.03 g/mL while threshold of growth inhibition for stalk extract was 0.1 g/mL.

Leaf extract at the concentration of 0.03 g/mL completely inhibited shoot growth of lettuce (100%) and inhibited shoot growth of alfalfa, cress, Italian ryegrass, barnyard grass and jungle rice by 23.61, 28.74, 29.39, 39.30 and 51.28% of control, respectively.

Root extract at the concentration of 0.03 g/mL inhibited shoot growth of lettuce, alfalfa, cress, jungle rice, barnyard grass and Italian ryegrass by 4.21, 23.91, 25.29, 28.49, 30.39 and 31.91% of control, respectively.

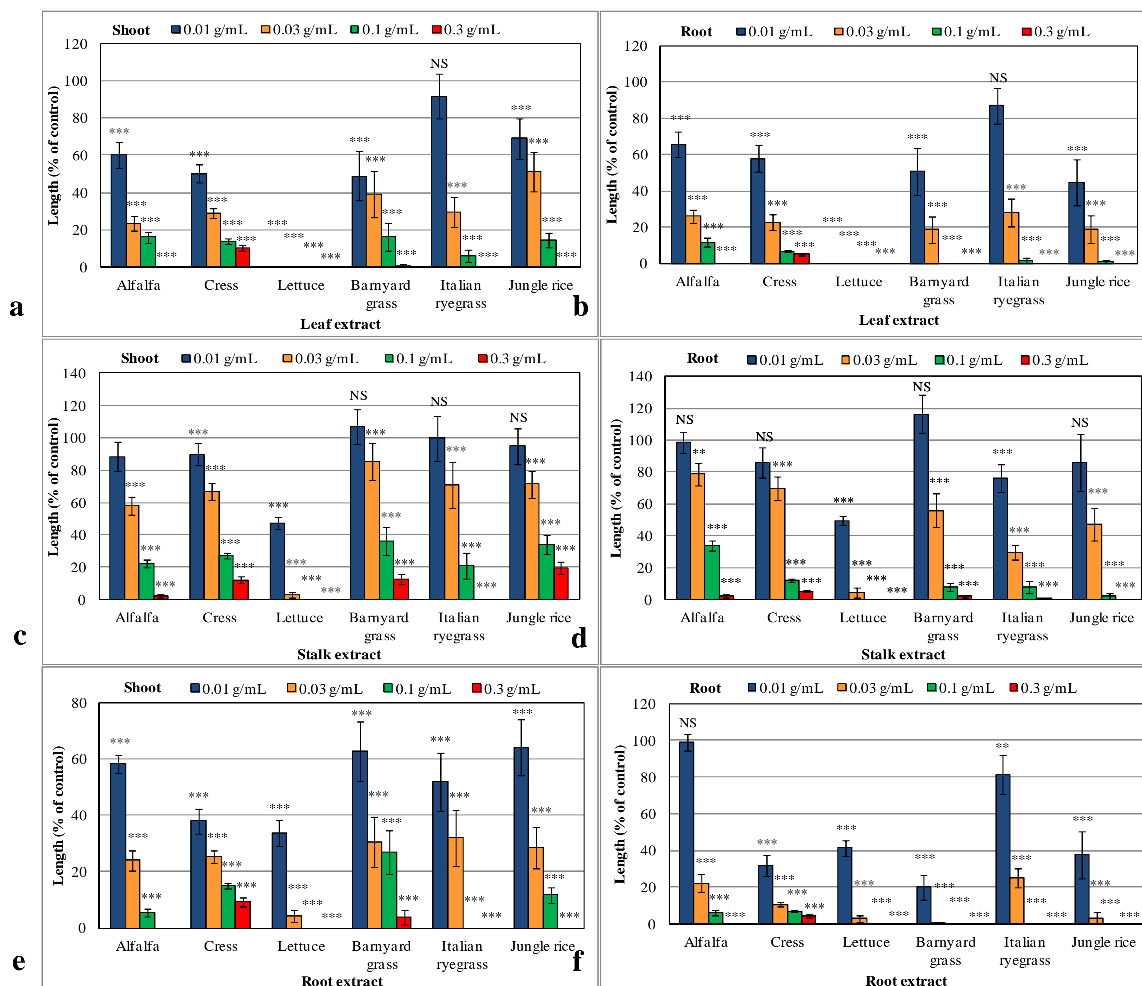

Figure 1. Effects of leaf (a, b), stalk (c, d) and root (e, f) extracts of *Cymbopogon nardus* on shoot and root growth of six test plant species. The bioassay was conducted with 0.01, 0.03, 0.1 and 0.3 g dry weight equivalent extract/mL. Means ± SE from three independent experiments with 10 seedlings for each determination are shown. *P< 0.05, **P< 0.01, ***P< 0.001 (Student's t-test).

Table 1. Effects of leaf, stalk and root extracts of *Cymbopogon nardus* on seedling growth of six test plant species.

Plant parts		Average % inhibition					
		Dicotyledonous species			Monocotyledonous species		
		Alfalfa	Cress	Lettuce	Barnyard grass	Italian ryegrass	Jungle rice
Leaf	Shoot	74.50 a	75.07 a	100.00 a	73.68 a	68.16 b	66.25 a
Stalk		57.36 b	51.44 b	87.50 c	39.85 b	52.12 c	44.98 b
Root		78.12 a	78.02 a	90.54 b	69.01 a	79.05 a	73.96 a
Leaf	Root	74.16 a	78.65 b	100.00 a	82.64 b	70.83 a	83.83 a
Stalk		46.64 c	57.01 c	86.54 b	54.57 c	71.50 a	66.31 b
Root		68.26 b	86.52 a	88.94 b	94.85 a	73.42 a	89.74 a

The bioassay was conducted with four concentrations; extracts obtained from 0.01, 0.03, 0.1 and 0.3 g dry weight of *C. nardus* per mL, and average % inhibition was calculated. Same letter in column is not significantly different at $P<0.05$.

Table 2. The concentration required for 50% inhibition on shoot and root growth of test plant species.

Plant parts	Test plant species	I_{50} (g dry weight equivalent extract/mL)	
		Shoot	Root
Leaf	Alfalfa	0.013	0.015
	Cress	0.009	0.012
	Lettuce	0.000	0.000
	Barnyard grass	0.012	0.010
	Italian ryegrass	0.022	0.021
	Jungle rice	0.025	0.009
Stalk	Alfalfa	0.039	0.066
	Cress	0.050	0.042
	Lettuce	0.010	0.010
	Barnyard grass	0.077	0.030
	Italian ryegrass	0.049	0.019
	Jungle rice	0.066	0.028
Root	Alfalfa	0.013	0.023
	Cress	0.004	0.004
	Lettuce	0.007	0.009
	Barnyard grass	0.016	0.007
	Italian ryegrass	0.012	0.019
	Jungle rice	0.015	0.008

The values were determined by a logistic regression analysis after bioassays

Stalk extract at the concentration of 0.1 g/mL completely inhibited shoot growth of lettuce seedling (100%) and inhibited shoot growth of Italian ryegrass, alfalfa, cress, jungle rice and barnyard grass by 20.76, 22.38, 26.92, 33.95 and 36.02% of control, respectively.

Comparison of the average percent inhibition of these three extracts is shown in Table 1. Leaf and root extracts have greater percent inhibition than stalk extract and demonstrated significantly different from stalk extract ($P<0.05$).

The concentration required for 50% inhibition (I_{50}) is shown in Table 2. Leaf and root extracts exhibited I_{50} at 0.00-0.025 and 0.004-0.016 g/mL, which were lower that stalk extract at 0.010-0.077 g/mL. For dicotyledonous species, all plant extracts were strongly inhibited lettuce and cress shoots. For monocotyledonous species, barnyard grass shoots were the most sensitive to leaf extract and Italian ryegrass shoots were the most sensitive to stalk and root extracts.

Effect of aqueous methanol extracts of leaves, stalks and roots of *C. nardus* on root growth

The inhibitory activity of *C. nardus* leaf, stalk and root extracts on root growth of six test plant species are shown in Figure 1. Leaf extract at the concentration of 0.03 g/mL completely inhibited root growth of lettuce (100%) and inhibited root growth of barnyard grass, jungle rice, cress, alfalfa and Italian ryegrass by 18.69, 18.80, 22.71, 26.12 and 28.18% of control, respectively.

Root extract at the concentration of 0.03 g/mL inhibited root growth of barnyard grass, lettuce, jungle rice, cress, alfalfa and Italian ryegrass by 0.59, 2.93, 3.21, 10.54, 22.21 and 25.00% of control, respectively.

In contrast with leaf and root extracts, stalk extract required the concentration of 0.1 g/mL for completely inhibited root growth of lettuce seedling (100%) and inhibited root growth of jungle rice, barnyard grass, Italian ryegrass, cress and alfalfa by 1.83, 7.57, 7.85, 12.20 and 34.03% of control, respectively.

Table 1 shows that the inhibition of plant part extracts on root growth of test plant species were also corresponding to their shoot growth (P<0.05).

Leaf and root extracts exhibited I_{50} at 0.00-0.021 and 0.004-0.023 g/mL, which were lower than stalk extract at 0.010-0.066 g/mL (Table 2). For dicotyledonous species, lettuce and cress roots were sensitive to all plant extracts than their shoot. For monocotyledonous species, jungle rice roots were the most sensitive to leaf extracts while Italian ryegrass and barnyard grass roots were the most sensitive to stalk and root extracts, respectively.

Separation of the extracts

Several active fractions separated by silica gel column were found in the root, leaf and stalk extracts (Figure 2). Six fractions eluted with 30 (F2), 40 (F3), 50 (F4), 60 (F5), 70 (F6) and 80% ethyl acetate (F7) in *n*-hexane separated from root extract inhibited shoot growth of cress seedling by 36.32, 22.11, 45.79, 28.42, 15.79 and 34.74% of control, respectively. Those fractions also inhibited root growth by 29.80, 7.45, 46.05, 18.28, 6.09 and 23.70% of control, respectively.

Three fractions eluted with 60 (F5), 70% ethyl acetate (F6) in *n*-hexane and methanol (F9) separated from leaf extract inhibited shoot growth by 33.71, 42.13 and 47.19%, respectively and inhibited root growth by 36.88, 36.88 and 35.46% of control, respectively.

In contrast, only one active fraction, which was eluted with 70% ethyl acetate in *n*-hexane (F6), was detected in stalk extract. The activity of the fraction on cress shoots and roots was 27.57 and 24.57% of control, respectively.

Discussion

Aqueous methanol extracts of *C. nardus* leaves, stalks and roots exhibited different growth inhibitory activity which were depending on test plant species such as monocotyledonous species (barnyard grass, Italian ryegrass and jungle rice) and dicotyledonous species (alfalfa, cress and lettuce), and also the extract concentrations. The inhibition increased with increasing extract concentrations (Figure 1). Islam and Noguchi (2013) also indicated that the growth restriction of cress and Italian ryegrass were more clearly observed with increasing concentration of allelopathic compounds.

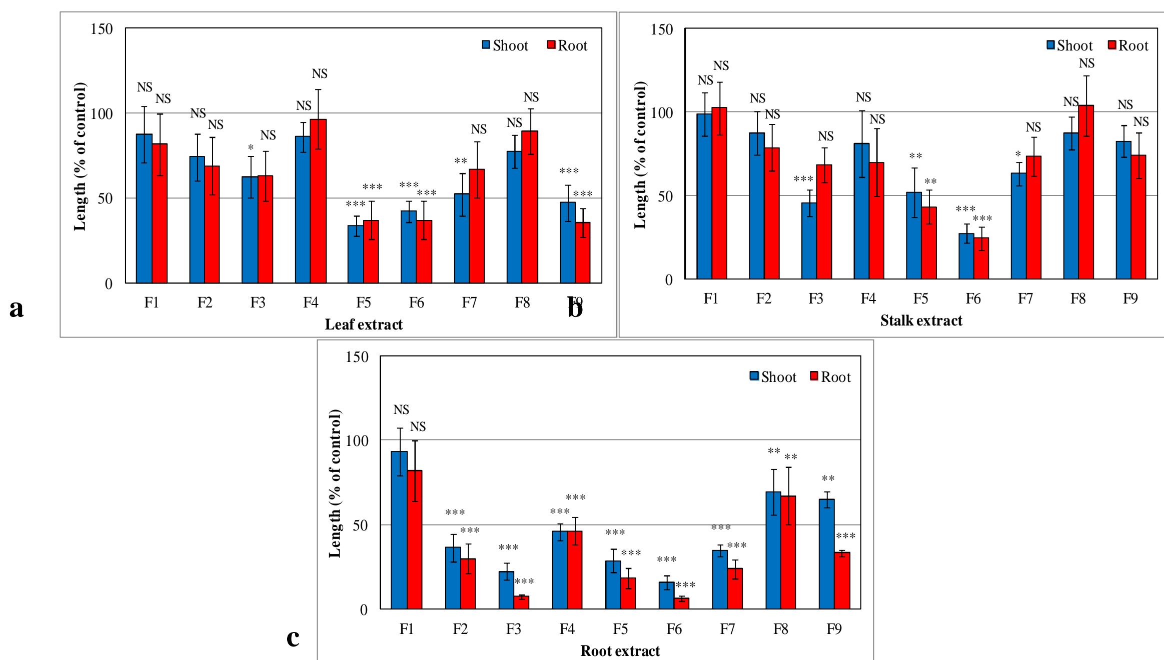

Figure 2. Effects of fractions separated by a silica gel column on seedling growth of cress. The bioassay of *Cymbopogon nardus* leaf (a), stalk (b) and root (c) extracts was conducted at the concentration of 0.3 g dry weight equivalent extract/mL. Means ± SE from three independent experiments with 10 seedlings for each determination are shown. *P< 0.05, **P< 0.01, ***P< 0.001 (Student's t-test).

The threshold concentration for growth inhibition on the test plants was lower in leaf and root extracts than stalk extracts ($P<0.05$) (Table 2). In addition, effectiveness of the extracts to test plant roots was greater than that to their shoots which correspond to the reported by many researchers (Olofsdotter et al., 2002; Pukclai et al., 2010; Zhang and Fu, 2010; Hussain and Reigosa, 2011; Esmaeili et al., 2012). It may be possible reason that roots easily contact and absorb the compound in the medium and soil (Salam and Noguchi, 2010).

The present results show that leaf and root extracts have greater inhibitory activity on test plant species than stalk extract, and also required lower concentration for 50% growth inhibition than stalk extract (Table 2). It was reported that leaves and roots were the main source of allelopathic compounds (Rice, 1984; Fateh et al., 2012). Dicotyledonous species were more sensitive to all plant extracts than monocotyledonous species (Table 1), which the inhibitory activity of the allelopathic substances was species specific and concentration dependent (Barnes and Putnam, 1987; Kruse et al., 2000).

Separation of *C. nardus* root extract through silica gel column showed the most active fractions and stronger growth inhibitory on cress seedlings than leaf and stalk extracts (Figure 2). Additionally, these three extracts had the same two active fractions, F5 and F6, however those fractions of each extract exhibited different level of growth inhibitory activity. It may be due to the differences in concentrations of allelopathic substances or variation of chemical composition between plant parts (Wu et al., 2009; Grisi et al., 2012; Sarkar et al., 2012).

Conclusions

Aqueous methanol extracts of leaves, stalks and roots of *C. nardus* exhibited the growth inhibitory activity especially on common weeds in agriculture fields such as barnyard grass, Italian ryegrass and jungle rice. Leaf and root extracts demonstrated strong growth inhibition. This study suggests that the growth inhibitory activity of each extract may be due to the allelopathic substances in *C. nardus*. The isolation and identification of allelopathic substances in aqueous methanol extracts of *C. nardus* roots are in progress with the purpose for development of natural herbicide for controlling weed control purpose.

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PLANT SCIENCE

Effect of urea and ammonium sulphate on some physiological aspects and chemical compositions of *Pennisetum glaucum* plants

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Abstract

A research experiment was carried out to study the effect of soil application of urea and/or ammonium sulphate on some physiological aspects and biochemical composition of pearl millet (*Pennisetum glaucum*) plants during the vegetative growth period. The parameters analyzed were dry weight, water content, polysaccharides, total carbohydrates, phosphorus content, nitrogenous content and proline in roots and shoots as well as photosynthetic pigments content in the leaves. The results for roots indicated significant increases in dry weight, water content and direct reducing value due to the combination of urea with ammonium sulphate at 0.5% during the first and second periods and urea with ammonium sulphate at 0.3% in the third one. Total carbohydrates significantly increased due to application of ammonium sulphate at 0.5% in the second and third samples. Organic and total phosphorus significantly increased due to application of urea at 0.3% in the first and second samples. Applying urea at 0.5% achieved the highest value in total soluble N, protein-N, total-N and proline in first sample. Meanwhile, the combination of urea and ammonium sulphate at 0.3% resulted in high increases in total- N and protein - N in the second and third samples. Likewise, application of urea with ammonium sulphate at 0.5% caused the highest significant value in total soluble - N in the second sample compared with control and other treatments in roots.

Key words: *Pennisetum glaucum*, Ammonium sulphate, Photosynthetic pigments, Urea

Introduction

Pearl millet (*Pennisetum glaucum*) is the most widely grown type of millet and one of the major field crops in Yemen. In 1999, the total cultivated area of millet is 649551 hectare and the total production was 464240 tons. The crop is principally produced for both human and animals.

Nitrogen is one of the most important nutrients for the growth and yield of several crop. Nitrogen is the most limiting nutrient for cereal crops production. Also, it is an essential component of structural amino acid, amides, nucleotides, nucleoproteins and is essential to cell division, expansion and nonstructural components of plant cells (Mengel and Kirkby, 1979). Ammonium (NH_4) and urea in soils are the two main sources of available N for plant growth. Although other nitrogen sources such as fertilizer applications have noticeable effects on growth of *Pennisetum glaucum* plants (Powell et al., 1991; Youngquist et al., 1992; Hassanein, 1996). Nouret al. (1989) found that among different nitrogen forms, urea gave the highest mean values of seedling growth parameters. Iptas and Brohi (2011) observed that the N rate had no significant effect in the first and third cutting, but in the second one dry matter yields, crude protein content and yield, increased significantly with the increase in N rate. The highest yield of 9.1 ton/ hectare was obtained with 80 kg N/hectare for the average of 2 years at the second cutting.

Allen (1984) noted that small grain absorb large amount of nitrogen early in their growth cycle, store it in leaves, and transfer it to the developing seed, which results in very efficient use of rapidly available nitrogen fertilizer. Therefore, the main objective of this study was to determine the effects of urea and ammonium sulphate applied separately or in combinations (at 0.3% and 0.5% solution), to the soil at seedling stage on some physiological aspects and chemical compositions of *Pennisetum glaucum* plants.

Materials and Methods

A pot experiment was carried out in a greenhouse at the Experimental Station, Department of Biology, Faculty of Education,

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Sana'a University, Yeman during the growing season of 2011, to study the effect of soil application of urea and/or ammonium sulphate on some physiological aspects and biochemical composition of *Pennisetum glaucum* plants, during the vegetative growth period. The grains were obtained from crops market in Sana'a city, the capital of Yeman Republic.

Nitrogen sources

Two nitrogen sources i.e. urea and ammonium sulphate were used and applied at two levels 0.2 and 0.4% solution alone or in combination. The treatments were as follows: (1) without any addition (control) (2) urea at 0.3% (3) urea at 0.4 % (4) ammonium sulphate at 0.3% (5) ammo sulphate at 0.5% (6) combination between urea and ammonium (0.20 : 0.30%) (7) combination between urea and ammonium (0.40 : 0.40%). Plastic pots of 20 cm in diameter were packed with about 3 kg mixtures of clay and sand (1: 1 v /v), Phosphorus and potassium fertilizers were added to the soil before sowing. Before planting the grains selected were surface sterilized by sooking in 0.01 M HgCh solution for 3 minutes and washed thoroughly with distilled water. Ten grains were sown at 1st of March in the season 2011 in each pot. The pots were kept in a greenhouse in which plants were subjected to normal day/ night conditions and watered for 20 days. Then, the seedlings were thinned to 5 uniform plants per pot for the subsequent study. At day 30, the pots were divided randomly into 7 groups and the treatments were added to the soil of each pot in one dose. Irrigation was conducted every 4 days in the first 2 weeks then every 7 days in the second 4 weeks. The experiment continued for period of 60 days. The treatments were arranged in a completely randomized design with 12 replicates (12 pots) for each treatment.

Sampling and collecting data

Three samples were taken from each treatment during vegetative growth period at three times 30, 40 and 50 days after sowing. At each sampling date, ten plants of each treatment were randomly taken for different measurements. The plants were cleared and separated into roots and shoots. The samples were then dried at 70°C. Fresh and dry weights of roots and shoots systems, water content and direct reducing value of roots and shoots were recorded, or calculated.

Data concerning growth and chemical compositions were subjected to statistical analysis according to Snedecor and Cochran (1980).

Chemical analysis

Fresh and dry samples of the two organs were used for the following determinations:

1) Photosynthetic pigments: The chlorophylls a, b and carotenoids were determined in the fresh leaves using the spectrophotometer method recommended by Nornai (1982).

2) Phosphorus compounds: The different phosphorus compounds were extracted and determined according to the method described by Chapman and Parker (1961).

3) Carbohydrates contents: Polysaccarides and total carbohydrates were determined using the method adapted by Doubis et al. (1956).

4) Nitrogenous compounds: Total soluble nitrogen fractions and total nitrogen were determined using the method described by Horneck and Miller (1998). The subtraction of total soluble N from total- N multiplied by 6.25 (gave the value for protein- N (A.O.A.C. 1980).

5) Proline: Free proline concentration was determined according to Bates et al. (1973).

Data concerning growth and chemical compositions were subjected to statistical analysis according to Snedecor and Cochran (1980).

Results and Discussion

The effects of applying different nitrogenous treatments on dry weight, water content and carbohydrate contents in roots of *Pennisetum glaucum* plants are presented in Table 1 for the first sample. The combination of urea and ammonium sulphate at 0.5% solution significantly increased dry weight, total reducing value, polysaccharides and total carbohydrates of *the* roots. Differences among the seven treatments in dry weight and water contents percentages were significant. Similar results were found in sample 2. The combination of urea and ammonium at 0.5% solution also resulted in the highest values of dry weight, water content and direct reducing value (DRV) in the second sample. In addition, the combination of urea and ammonium at 0.3% solution had the highest value of TRV compared with the control. The results of the sample showed that the combination of urea and ammonium at 0.3% solution significantly increased dry weight, water content % and DRV compared with the control. However, urea alone at 0.5% solution gave the highest value of polysaccharides 15.16 g/100g.

Table 1. Effect of urea and ammonium sulphate (0.3%, 0.5%) and their combination on dry weight, water content, direct reducing value (DRV), total reducing value (TRV), polysaccharides and total carbohydrates of *Pennisetum glaucum* roots.

Treatments sample		Dry weight	Water content	DRV	TRV	PoIysaccharides	Total carbohydrates
		(g)	%	(g/100g)	(g/100g)	(g/100g)	(g/100g)
		First (30 days after sowing)					
Control		0.13	1.90	10.62	14.32	12.24	26.56
Urea 0.2%		0.14	1.89	8.09	11.14	12.16	23.30
Am Sulphate 3%		0.14	1.69	9.92	11.22	11.19	22.41
Urea 0.4%		0.13	1.56	9.04	20.34	10.19	30.43
Am Sulphate 5%		0.16	2.55	9.12	21.36	12.22	33.58
Urea + Am. Sulphate 0.3%		0.22	3.20	10.16	20.33	13.24	33.47
Urea + Am. Sulphate 0.5%		0.23	3.40	9.19	22.16	13.16	35.32
LSD at	0.05	0.017	·0.321	0.272	0.092	0.188	1.76
	0.01	0.022	0.451	0.880	0.120	0.255	2.360
		Second (40 days after sowing)					
Control		0.62	3.12	9.45	15.24	13.08	28.32
Urea 0.2%		0.66	4.42	8.95	22.25	16.09	38.21
Am Sulphate 0.3%		0.52	3.13	8.85	20.15	14.09	34.24
Urea 0.4%		0.64	3.62	8.84	15.16	13.18	28.34
Am Sulphate 0.5%		0.56	7.00	8.74	22.16	16.19	38.33
Urea + Am. Sulphate 0.3%		0.64	6.59	9.96	23.22	15.18	38.30
Urea + Am. Sulphate 0.5%		0.69	7.23	10.23	20.18	15.36	35.54
LSD at	0.05	0.031	0.060	0.081	0.289	0.326	1.860
	0.01	0.042	0.810	0.120	0.401	0.436	2.621
		Third (50 days after sowing)					
Control		0.94	6.11	8.15	12.12	12.19	24.31
Urea 0.2%		1.22	5.66	8.04	14.14	11.19	25.33
Am Sulphate 3%		0.46	4.94	8.13	15.09	14.22	29.31
Urea 0.4%		1.16	6.86	8.33	20.08	15.16	35.04
Am Sulphate 5%		0.92	7.70	9.02	22.25	13.13	35.38
Urea + Am. Sulphate 0.3%		1.84	9.59	9.14	12.17	11.13	23.30
Urea + Am. Sulphate 0.5%		0.89	7.66	8.99	22.19	12.04	33.23
LSD at	0.05	0.047	0.044	0.081	0.311	0.316	2.101
	0.01	0.062	0.056	0.111	0.404	0.412	2.221
LSD at	0.05	0.030	0.104	0.070	0.280	0.328	1.811
	0.01	0.042	0.140	0.092	0.361	0.421	2.112

Am = Ammonium

The impact of applying different nitrogenous treatments on dry weight, water content and carbohydrate contents in roots are presented in Table 2 for the first sample. Urea at 0.3% solution significantly increased dry weight and water content of shoots at 30 days from germination over the control. The combination of urea and ammonium sulphate at 0.5% resulted in the best DRV TRV g, polysaccharides and total carbohydrate compared with other treatments. In addition, significant differences between urea 0.3% and 0.5% and combination of urea and ammonium sulphate solution at 0.5% in polysaccharides.

As per the second sample, urea 0.3% led to obtain the highest value of DR V 9.32 g/100g. Am. sulphate 0.3% produced the highest values of dry weight 3.46 g TRV 18.45 g/100g, polysaccharides 15.40 g/100g and total carbohydrates 33.85 g/100g. However, ammonium sulphate 0.5% resulted in the best water content 20.15%.

The treatments significantly increased dry weight over the control treatment of shoots. Ammonium sulphate at 0.5% had the highest value of dry weight, whereas, the combination of urea and ammonium sulphate at 0.5% resulted in the highest water content, DRV, and total carbohydrates. Treatment of urea at 0.5% solution produced the highest polysaccharides content. The effects of the combination of urea and ammonium sulphate added at 0.5% solution on dry weight and water content in the three samples in both root and shoots systems could be attributed to their effect on several physiological factors such as, cell division, uptake of elements, photosynthetic pigment

content, carbohydrate and protein content and cytokines content in roots. Hence, such mechanism results in increasing the growth of roots and shoots system. Similar results were obtained by Powell et al. (1991), Youngquist et al. (1992) on *Pennisetum glaucum* plants and by Nouret al. (1989) and Khedret al. (2000) on wheat and by Iptas and Borhi (2011) on *Pennisetum glaucum* plants. In addition, the superiority of urea on vegetative growth may be due to continuous and slow release of N to *Pennisetum glaucum* plants as a result of transforming of Nll, to $N0_3$ or extended NH_4 nutrition. Also, it was reported by Zhou et al. (1997) that high N inputs (270 kg N ha) increased both DM production and N uptake by corn.

Concerning carbohydrates content, the higher increment of carbohydrate fractions in roots than in shoots with the application of urea and ammonium at 0.5% alone or their combination, could be attributed to the active translocation of different photosynthesis from source organs (leaves) to the sink organs (roots) during vegetative growth. Also, NH4 assimilate into organic compounds in roots before translocation to the shoots this process requires energy and a flux of carbohydrates from the photosynthesized tissues to roots. Fentem et al. (1993a) and Marschner (1995) indicated that the high demand of carbon skeletons for ammonium in roots is reflected on sugar and carbohydrate content in roots.

Table 2. Effect of urea and ammonium sulphate (0.3%, 0.5%) and their combination on dry weight, water content, direct reducing value (DRV), total reducing value (TRV), polysaccharides and total carbohydrates of *Pennisetum glaucum* shoots.

Treatments sample		Dry weight	Water content	DRV	TRV	PoIysaccharides	Total carbohydrates
		(g)	%	(g/100g)	(g/100g)	(g/100g)	(g/100g)
				First (30 days after sowing)			
Control		0.69	4.26	7.28	14.42	12.66	26.68
Urea 0.2%		0.97	6.25	8.79	15.54	15.42	30.96
Am Sulphate 3%		0.76	5.23	7.62	13.49	13.32	26.81
Urea 0.4%		0.58	4.25	7.99	14.52	14.40	28.92
Am Sulphate 5%		0.59	4.47	8.52	17.42	13.33	30.75
Urea + Am. Sulphate 0.3%		0.59	5.22	9.33	14.33	11.54	25.87
Urea + Am. Sulphate 0.5%		0.66	4.24	9.42	18.29	14.62	32.91
LSD at	0.05	0.068	0.151	0.122	0.282	1.212	1.082
	0.01	0.101	0.212	0.214	0.432	1.721	2.271
				Second (40 days after sowing)			
Control		2.18	12.22	8.24	14.40	13.23	27.63
Urea 0.2%		2.14	14.30	9.32	15.32	14.33	29.55
Am Sulphate 0.3%		3.56	12.34	8.45	18.45	15.40	33.85
Urea 0.4%		1.89	11.50	7.63	17.38	14.22	31.60
Am Sulphate 0.5%		2.61	20.45	9.34	15.34	14.37	29.71
Urea + Am. Sulphate 0.3%		2.43	17.64	8.63	14.22	15.26	29.48
Urea + Am. Sulphate 0.5%		1.99	19.50	8.55	13.25	13.23	26.48
LSD at	0.05	0.110	0.212	1.021	1.521	1.421	2.122
	0.01	0.156	0.362	1.341	2.121	2.002	3.563
				Third (50 days after sowing)			
Control		3.22	30.24	7.28	13.87	12.26	26.13
Urea 0.2%		4.23	31.34	8.35	14.96	13.38	25.34
Am Sulphate 3%		3.52	28.40	7.22	16.28	13.28	29.46
Urea 0.4%		3.64	33.54	8.61	17.70	14.49	31.99
Am Sulphate 5%		4.22	28.95	9.23	14.46	12.50	26.96
Urea + Am. Sulphate 0.3%		4.25	30.41	8.42	13.35	13.60	26.95
Urea + Am. Sulphate 0.5%		3.95	34.33	9.24	18.22	12.30	30.52
LSD at	0.120	1.522	0.429	0.687	1.596	1.567	0.120
	0.221	2.321	0.712	0.992	2.100	2.120	0.221
LSD at	0.101	1.021	0.267	0.419	1.460	2.012	0.101
	0.132	1.212	0.399	0.594	1.607	2.212	0.132

In general, the positive effects of nitrogen fertilizer on sugar and carbohydrates content may be due to its influence on photosynthetic efficiency. Similar effects were nearly found by Khedr et al. (2000) on wheat and Abo El- Ghait (1993) who found that urea and ammonium nitrate at 80 and 120 kg N/fed significantly increased total carbohydrate content in leaves of *Strelitzia reginae*. In addition, Marschner (1995) indicated that, when the nitrogen supply is suboptimal, ammonia assimilation increases both protein content and leaf growth and correspondingly the leaf area index which correlated with an increase in net photosynthesis.

The effects of different nitrogenous treatments on photosynthetic pigments are presented in Table 3. Treatment of 0.3% urea resulted in the highest chlorophyll A and B, while ureaat 0.5% resulted in the highest value of carotenoids in the first sample. Similarly, the results of the second sample showed that application of urea at 0.3% resulted in the highest chlorophyll A. Urea 0.5% solution resulted in the best chlorophyll A in the third sample.

The results obtained in this study are in agreement with those results obtained by Abo EI-Ghait (1993) and Khedr et al. (2000) and Riedell and Kiechefer (1993) who found that the content of chlorophyll A and B significantly increased by increasing nitrogen rate. In this connection, Marschner (1995) reported that nitrogen supply causes an enhancement of protein synthesis and chloroplast formation leads to an increase in the lipid content of leaves as well as to an increase in chloroplast constituents such as chlorophyll and carotenoid.

Table 3. Effect of urea and ammonium sulphate (0.3%, 0.5%) and their combination on chlorophyll a, band carotenoids of *Pennisetum glaucum* leaves.

Treatments sample		Chlorophyll (a)	Chlorophyll (mg/g fresh wt)	Carotenoids
		First (30 days after sowing)		
Control		6.22	4.26	5.61
Urea 0.3%		7.42	5.55	6.37
Am. Sulphate 0.3%		7.32	4.43	6.27
Urea 0.5%		6.67	4.52	7.23
Am. Sulphate 0.5%		5.56	5.35	5.16
Urea + Am. Sulphate 0.3%		5.26	4.52	4.18
Urea + Am. Sulphate 0.5%		6.53	5.26	5.26
LSD at	0.05	0.168	0.101	0.163
	0.01	0.232	0.132	0.224
		Second (40 days after sowing)		
Control		5.32	4.22	4.20
Urea 0.3%		7.25	5.34	7.37
Am. Sulphate 0.3%		6.32	5.12	4.22
Urea 0.5%		6.29	4.31	3.10
Am. Sulphate 0.5%		7.22	4.24	4.38
Urea + Am. Sulphate 0.3%		5.65	5.26	5.27
Urea + Am. Sulphate 0.5%		6.24	5.62	3.22
LSD at	0.05	0.122	0.124	0.202
	0.01	0.286	0.201	0.301
		Third (50 days after sowing)		
Control		5.10	3.75	3.17
Urea 0.3%		6.22	4.26	5.22
Am. Sulphate 0.3%		5.25	3.26	4.16
Urea 0.5%		6.36	3.26	3.29
Am. Sulphate 0.5%		4.31	4.68	2.21
Urea + Am. Sulphate 0.3%		5.20	3.38	3.37
Urea + Am. Sulphate 0.5%		6.12	3.22	2.76
LSD at	0.05	0.132	0.118	0.106
	0.01	0.212	0.102	0.150
LSD at	0.05	0.167	0.101	0.151
	0.01	0.213	0.144	0.216

The effects of different nitrogenous treatments on phosphorus content are presented in Tables 4 and 5. The results showed that urea 0.3% caused the highest values of organic –P in both first and second samples. The combination of urea and ammonium sulphate at 0.5% resulted increase for all parameters in the first sample compared with other treatments. While combination of urea and ammonium sulphate at 0.3% solution produced the higher values for organic and organic parameters compared with the control in the second sample. These results are in agreement with those reported by Powell et al. (1991) and Khedr et al. (2000).

The impacts of different nitrogenous treatments on TSN, protein, total N and Proline contents of roots and shoots are presented in Tables 6 and 7, respectively. Urea at 0.5% solution caused a significant increase in total soluble N (TSN), protein- N, total - N and proline of roots for the first and second samples, while the combination of urea and ammonium sulphate at 0.3% solution resulted in the highest values of protein - N and total – N, compared with other treatments in the third sample. As per shoots, ammonium sulphate at 0.3% solution increased TSN in the first sample. The urea at 0.5% solution resulted in higher value for total N as compared to other treatments in both second and third samples.

Table 4. Effect of urea and ammonium sulphate (0.3%, 0.5%) and their combination on inorganic phosphorous, organic phosphorous and total phosphorous of *Pennisetum glaucum* roots.

Treatments Sample		Inorganic -P	Organic-P	Total-P
		(g/100g d.wt)		
		First (30 days after sowing)		
Control		0.25	0.65	0.90
Urea 0.3%		0.29	0.86	1.15
Am. Sulphate 0.3%		0.32	0.65	0.97
Urea 0.5%		0.25	0.64	0.89
Am. Sulphate 0.5%		0.25	0.63	0.88
Urea + Am. Sulphate 0.3%		0.13	0.56	0.75
Urea + Am. Sulphate 0.5%		0.18	0.64	0.82
LSD at	0.05	0.021	0.062	0.120
	0.01	0.039	0.101	0.148
		Second (40 days after sowing)		
Control		0.43	0.92	1.35
Urea 0.3%		0.65	0.98	1.63
Am. Sulphate 0.3%		0.14	0.96	1.10
Urea 0.5%		0.16	0.83	0.98
Am. Sulphate 0.5%		0.64	0.66	1.30
Urea + Am. Sulphate 0.3%		0.19	0.68	0.87
Urea + Am. Sulphate 0.5%		0.12	0.86	0.97
LSD at	0.05	0.042	0.092	0.020
	0.01	0.063	0.122	0.028
		Third (50 days after sowing)		
Control		0.24	0.79	1.31
Urea 0.3%		0.22	0.66	0.88
Am. Sulphate 0.3%		0.23	0.59	0.82
Urea 0.5%		0.16	0.72	0.88
Am. Sulphate 0.5%		0.19	0.61	0.80
Urea + Am. Sulphate 0.3%		0.19	0.75	0.95
Urea + Am. Sulphate 0.5%		0.17	0.76	0.93
LSD at	0.05	0.041	0.106	0.044
	0.01	0.052	0.146	0.062
LSD at	0.05	0.026	0.068	0.071
	0.01	0.049	0.112	0.095

Table 5. Effect of urea and ammonium sulphate (0.3%, 0.5%) and their combination on inorganic phosphorous, organic phosphorous and total phosphorous of *Pennisetum glaucum* shoots.

Treatments Sample		Inorganic -P	Organic-P	Total-P
		g/100g d.wt		
		First (30 days after sowing)		
Control		0.22	0.41	0.63
Urea 0.3%		0.15	0.42	0.57
Am. Sulphate 0.3%		0.22	0.62	0.84
Urea 0.5%		0.15	0.68	0.83
Am. Sulphate 0.5%		0.16	0.42	0.58
Urea + Am. Sulphate 0.3%		0.26	0.72	0.98
Urea + Am. Sulphate 0.5%		0.26	0.72	0.98
LSD at	0.05	0.016	0.018	0.132
	0.01	0.021	0.012	0.202
		Second (40 days after sowing)		
Control		0.24	0.56	0.80
Urea 0.3%		0.28	0.56	0.82
Am. Sulphate 0.3%		0.27	0.73	0.99
Urea 0.5%		0.19	0.26	0.45
Am. Sulphate 0.5%		0.16	0.64	0.80
Urea + Am. Sulphate 0.3%		0.34	0.94	1.28
Urea + Am. Sulphate 0.5%		0.26	0.92	1.18
LSD at	0.05	0.016	0.022	0.034
	0.01	0.022	0.035	0.056
		Third (50 days after sowing)		
Control		0.22	0.44	0.66
Urea 0.3%		0.19	0.35	0.54
Am. Sulphate 0.3%		0.24	0.56	0.80
Urea 0.5%		0.19	0.76	0.95
Am. Sulphate 0.5%		0.16	1.14	2.30
Urea + Am. Sulphate 0.3%		0.24	1.52	1.67
Urea + Am. Sulphate 0.5%		0.26	0.94	1.20
LSD at	0.05	0.020	0.046	0.052
	0.01	0.031	0.062	0.075
LSD at	0.05	0.016	0.33	0.081
	0.01	0.022	0.042	0.115

These increments in protein fractions (protein - N, total soluble Nand total- N in both roots and shoots of *Pennisetum glaucum* plants in the three samples may be attributed to the stimulatory effects of urea and ammonium sulphate on proteolytic enzymes and enhancement of biosynthesis of amino acids and protein. These effects were referred to the increments in total N content in both roots and shoots in response to increased urea and ammonium sulphate at 0.3% or 0.5% concentration. The increase in ammonia assimilation in roots into amino acids which was accompanied with a marked increase in all nitrogenous fractions content parallel to increase in high level of added urea (0.5%) or the combination of urea and ammonium sulphate (at 0.5%). Also, it was accompanied by dry weight stimulation of roots and shoots as shown in Tables 1 and 2. On the other hand, the reduction in protein N of shoots system was accompanied by increment in total soluble nitrogen and unassimilated NH4, in response to the high level of urea and ammonium sulphate at 0.5%. In addition, when urea was used as N- source it can be taken up directly by the roots and it is hydrolyzed by urease after translocation to the shoots in maize plant. Meanwhile, NH_4, is toxic to plant tissue and cannot be strode. Consequently, plants assimilate it into organic compounds in the roots (amides, amines and amino acids) before translocation to shoot (Marschner, 1995).

Marschner (1995) reported that, polyamine content and free amino acids (such as proline) were particularly high in meristematic tissues, in plants supplied with high levels of ammonium and low external pH, this increases are most likely a reflection of pH homeostasis.

These results are in agreement with those obtained by Khedr et al., 2000 and Iptas and Brohi, 2003.

Conclusion

From the results, it can be concluded that there are significant increases in dry weight, water content and direct reducing value due to the combination of urea with ammonium sulphate at 0.5% during the first and second periods, and urea with ammonium sulphate at 0.3% in the third one. Total carbohydrates significantly increased due to application of ammonium sulphate at 0.5% in the second and third samples. Likewise other studied parameters also significantly increased due to application of urea and ammonium sulphate in the first and second samples.

Table 6. Effect of urea and ammonium sulphate (0.3%, 0.5%) on TSN, protein -N, Total-N and proline of *Pennisetum glaucum* roots.

Treatments Sample		Total soluble nitrogen (TSN)	Protein-N	Total-N	Proline
		(g/100g D.WT)			
		First (30 days after sowing)			
Control		4.32	3.18	7.50	0.44
Urea 0.3%		5.22	3.80	9.16	0.66
Am. Sulphate 0.5%		4.36	4.16	8.78	0.58
Urea 0.5%		5.62	5.22	10.66	0.68
Am. Sulphate 0.5%		4.74	5.20	9.45	0.46
Urea + Am. Sulphate 0.3 %		5.26	4.60	9.82	0.34
Urea + Am. Sulphate 0.5 %		5.22	5.44	10.88	0.48
LSD at	0.05	0.108	0.261	0.583	0.079
	0.01	0.028	0.332	0.796	0.109
		Second (40 days after sowing)			
Control		5.44	6.52	11.86	0.34
Urea 0.3%		5.34	8.67	15.16	0.68
Am. Sulphate 0.5%		5.66	8.34	13.93	0.45
Urea 0.5%		5.59	6.24	13.00	0.56
Am. Sulphate 0.5%		5.66	7.25	15.12	0.44
Urea + Am. Sulphate 0.3 %		5.86	9.26	15.15	0.46
Urea + Am. Sulphate 0.5 %		6.50	8.66	15.71	0.58
LSD at	0.05	0.016	0.169	0.891	0.096
	0.01	0.029	2.39	1.310	0.210
Control		Third (50 days after sowing)			
Urea 0.3%		4.18	5.22	9.49	0.26
Am. Sulphate 0.5%		3.36	7.68	11.38	0.28
Urea 0.5%		4.45	7.63	12.08	0.26
Am. Sulphate 0.5%		3.26	8.29	11.55	0.28
Urea + Am. Sulphate 0.3 %		5.46	7.35	12.82	0.25
Urea + Am. Sulphate 0.5 %		4.45	9.96	14.41	0.24
LSD at	0.05	3.66	6.66	10.16	0.29
	0.01	0.034	0.193	0.806	0.083
LSD at	0.05	0.037	0.297	0.917	0.129
	0.01	0.032	0.197	0.732	0.099

Table 7. Effect of urea and ammonium sulphate (0.3%, 0.5%) on TSN, protein -N, Total-N and proline of *Pennisetum glaucum* shoots.

Treatments Sample		Total soluble nitrogen (TSN)	Protein-N	Total-N	Proline
		(g/100g D.WT)			
		First (30 days after sowing)			
Control		2.32	1.44	3.76	0.56
Urea 0.3%		4.29	2.26	6.55	0.28
Am. Sulphate 0.5%		5.52	3.25	8.77	0.46
Urea 0.5%		4.28	6.19	10.47	0.50
Am. Sulphate 0.5%		5.19	3.28	8.37	0.40
Urea + Am. Sulphate 0.3 %		5.25	4.53	9.78	0.52
Urea + Am. Sulphate 0.5 %		4.18	3.29	7.44	0.60
LSD at	0.05	0.217	0.366	0.511	0.083
	0.01	0.299	0.591	0.721	0.116
		Second (40 days after sowing)			
Control		3.34	2.34	5.69	0.56
Urea 0.3%		6.42	5.47	11.89	0.69
Am. Sulphate 0.5%		6.25	6.54	12.79	0.58
Urea 0.5%		5.29	8.42	13.71	0.55
Am. Sulphate 0.5%		5.56	7.39	12.85	0.79
Urea + Am. Sulphate 0.3 %		6.66	6.28	12.94	0.78
Urea + Am. Sulphate 0.5 %		5.66	7.29	12.95	0.58
LSD at	0.05	0.246	0.526	0.633	0.081
	0.01	0.364	0.761	0.901	0.112
Control		Third (50 days after sowing)			
Urea 0.3%		1.45	4.26	5.71	0.36
Am. Sulphate 0.5%		3.42	5.36	8.78	0.48
Urea 0.5%		4.26	6.42	10.68	0.39
Am. Sulphate 0.5%		6.29	5.26	11.55	0.29
Urea + Am. Sulphate 0.3 %		4.36	4.25	8.62	0.38
Urea + Am. Sulphate 0.5 %		3.25	5.37	8.72	0.49
LSD at	0.05	3.28	6.26	9.54	0.48
	0.01	0.360	0.056	0.916	0.081
LSD at	0.05	0.432	0.769	1.261	0.099
	0.01	0.239	0.430	0.667	0.082

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Short Communication

PLANT SCIENCE

Agro-ecological study of forage productivity of some annual untraditional drought-resistant fodder species for foothill regions in Central Balkan Mountains (Bulgaria)

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Abstract

Comparative testing of some annual untraditional drought-resistant cereal (foxtail millet and true millet) and legume crops (bitter vetch and chickpea) was conducted during 2011-2013 in the Research Institute of Mountain Stockbreeding and Agriculture (RIMSA) in Troyan (Bulgaria). The largest part of the forage yields had the stems in comparison with leaves and inflorescences. The chickpea had more green mass (12.82 t ha^{-1}) and dry mass (3.27 t ha^{-1}) yields than bitter vetch – respectively by 18.46% and 18.48%. Regarding the cereal crops it turned out that the true millet had more yields (12.98 t ha^{-1} green mass and 3.32 t ha^{-1} dry matter) than the foxtail millet (respectively 16.80% and 11.14%). Judging from the factual data it can be considered that the forage species studied in details are suitable for ecological fodder preparation in foothill regions of the Central Balkan Mountains in Bulgaria.

Key words: Cereal, Legume, Drought-resistance, Forage productivity, Yield structure

Introduction

During the last years of global warming plant species have mostly suffered its adverse effects. As a result the average annual temperature variation in recent years is between 11-12°C. The average annual rainfall on the territory of the country decreases to 672 mm and varies considerably per months (Ivanova and Mishev, 2012). This therefore greatly affects the growth and development of field crops (Alexandrov and Hoogenboom, 2000; Alexandrov, 2008).

According to Wilkins and Vidrich (2000) herbage fodder species suitable to grow in dry conditions should be found. Due to the climate changes some researchers focus on examining some cereal and legume pure crops so as to guarantee for their sustainable forage production (Paul et al., 2002).

Testing of different annual species is very important for animal feeding especially in the summer dry period – June to August (Posler et al., 1993; Gordon and Newmann, 1997).

The current use of annual summer monocultures and mixtures relates to the present climate changes in both regional and global aspect.

Due to the specific soil and climate conditions typical of the foothill regions in the Central Balkan Mountains in Bulgaria and the lack of studies from recent past, the suitability of some annual cereal and legume forage crops was demonstrated in series of studies over the last few years (Lingorski and Kertikov, 2005a; 2005b; Lingorski, 2011a; 2011b; 2012). Their season mixtures were also used for the production of green forage (Lingorski and Kertikov, 2006; 2009; 2010).

The purpose of this study was to establish the parameters for production of ecologic forage mass (without mineral fertilization) from some untraditional cereal (foxtail millet and true millet) and legume crops (bitter vetch and chickpea) for foothill areas of the Central Balkan Mountains in Bulgaria.

Materials and Methods

The experiment was carried out on an area situated at 384 m above sea level in the experimental field of RIMSA, Troyan during 2011–

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2013. The territory of the institute is located in the most Southern parts of the foothill region of the Central Balkan Mountains and belongs to the Northern Balkan climate region of moderate continental climate subzone. During the period of study the average monthly air temperatures during the vegetation period (from March to July) of forage species amounted to 14.7^{0}C (compared to 21-year period – 1988-2008 - 13.8^{0}C, with more than 0.9°C), and rainfall was 401.9 mm (compared to 21-year period – 404.6 mm, i.e. it was less than 2.7 mm). The soil–type of the trial area was light–gray forest (pseudopodzolic) with low native fertility and high acid reaction (Dinkova, 2009).

The soil–cultivating process included the following: ploughing (in autumn of previous year), disk harrowing and cutting (in spring of the corresponding year of sowing). The trial area was pressed by rool pressors once after sowing immediately (annually at the end of April).

The experiment was conducted in four consequent repetitions. The different cereal and legume crops were broadcast sown by hand. All investigation crop variants had by 5 m^2 harvesting area and they were the following: 1. Bitter vetch (*Vicia ervilia* (L.) Willd. – as a Standard for legumes (var. 1 and var. 2), 2. Chickpea (*Cicer arietinum* L. *ssp. euroasiaticum*), 3. Foxtail millet (*Setaria italica ssp. mocharicum* Alef.), 4. True millet (*Panicum miliaceum* L. *ssp. effusum* Al. – with branched panicle) – as a Standard 2 for cereal crops (var. 3 and var. 4).

The experimental area of each variant was harvested for fodder and seeds divided into two equal parts. The studied forage crops were grown under non-irrigated conditions in compliance with the generally accepted standard technologies in Bulgaria (Nikolova and Todorova, 1986; Moskov and Tenova, 2005)

Note: Considering the fact that most of the country's water supply zones are located in the Central Balkan Mountains, annual fertilization was not applied in this experiment.

The annual sowing of the above mentioned crops was carried out in stages, depending on their biological characteristics and climate conditions of the year. The legumes (chickpea and bitter vetch) were first sown because they can grow in colder climate conditions. Because of their thermophylic the sowing of foxtail millet and true millet was done later by permanent of soil temperature 10-12°C. Thus, in 2011 the sowing was done on March 28 for legumes and May 10 for cereals, in 2012 – on March 23 and May 9 and in 2013 - on April 12 and April 23. It can be seen that, depending on the climatic conditions of the year, the sowing dates varied as follows: for legumes – from March 23 to April 12 and for cereals – from April 23 to May 10.

The sowing of the tested forage cultures was accomplished on inter-crop distances 12 cm with values as follows: for bitter vetch - 140 kg ha^{-1} (300 germinable seeds m^{-2}); for chickpea - 100 kg ha^{-1} (50 germinable seeds m^{-2}), for foxtail millet - 15 kg ha^{-1} (500 germinable seeds m^{-2}); for true millet - 25 kg ha^{-1} (400 germinable seeds m^{-2}), and they were in consistence with the purity and germination of seeds. The care for the crops during vegetation period was limited to maintenance of the experimental areas free of weeds.

In the cereals harvesting for green mass was carried out at the beginning of ear formation phase and in legume crops at beans formation phase.

Results and Discussion

Structural elements of the forage yield

Structural elements of the forage yield are included in Table 1 for the different forage crops by years and average for the period of the study. From the table it is seen that in the production of forage the yields are determined by different morphological above-ground plant organs.

Thus, in 2011 for legumes the highest weight had stems - 50.00% for chickpea and 75.00% for bitter vetch. The smallest share (12.50%) of legume crops and inflorescences of bitter vetch had the leaves. As for the cereals, the weight of the stems was more than of leaves and inflorescences (56.25% for foxtail millet and 62.86% for true millet). Due to ear formation phase of harvesting, the inflorescences had the smallest forage proportion (12.50% for foxtail millet and 17.14% for true millet), followed by leaves – 31.25% and 20.00% respectively.

The same table shows that in 2012 the legume stems were the heaviest - 52.63% for chickpea (var. 1) and 39.74% for bitter vetch (var. 2). The leaves again weighed less - respectively 15.38 and 10.53%. The participation of inflorescences (44.89% for chickpea and 36.83% for bitter vetch) is relatively high in the formation of fresh mass yields. Considering the tested cereal crops their stems also exceeded the weight their morphological organs (leaves and inflorescences) - 45.45% and 51.72% respectively. The leaves'percentage from the yield was 36.36% (foxtail millet) and 27.59% (true millet). Due to ear formation phase the inflorescences had the smallest proportion - 18.19% and 20.69% respectively.

Table 1. Structural elements of the yields of forage (in %).

Variant (forage crop)	Leaves, %	Stems, %	Inflorescences, %
2011			
1. Bitter vetch (Standard)	12.50	75.00	12.50
2. Chickpea	12.50	50.00	37.50
3. Foxtail millet	31.25	56.25	12.50
4. True millet (Standard 2)	20.00	62.86	17.14
2012			
1. Bitter vetch (Standard)	10.53	39.74	36.83
2. Chickpea	15.38	52.63	44.89
3. Foxtail millet	36.36	45.45	18.19
4. True millet (Standard 2)	27.59	51.72	20.69
2013			
1. Bitter vetch (Standard)	26.70	60.00	13.30
2. Chickpea	41.50	55.30	3.20
3. Foxtail millet	21.10	47.40	31.50
4. True millet (Standard 2)	19.20	57.70	23.10
Average for the period 2011-2013			
1. Bitter vetch (Standard)	16.58	58.25	20.88
2. Chickpea	23.13	52.64	28.53
3. Foxtail millet	29.57	49.70	20.73
4. True millet (Standard 2)	22.26	57.43	20.31

In 2013, the stems of the legumes had biggest proportion in the yields - 55.30% for chickpea and 60.00% for bitter vetch. Inflorescences had a smaller one - 3.30% and 13.30%, recpectively. The formation of fresh mass yields reached relatively high values which were 41.50% for chickpea and 26.70% for bitter vetch. The same was valid for the stems whose exceeded that of the other morphological plant organs - 47.40% for foxtail millet and 57.70% for true millet. The inflorescences values that were respectively 31.50% and 23.10% determined the yields. The leaves had the smallest value from the yield (21.10% and 19.20%).

The observational data of this indicator for the experimental period of 2011-2013 shows that the stems (55.45% of legumes and 53.57% of cereals) had the largest proportion, followed by leaves (19.86% of legumes and 25.92% of cereals) and finally the inflorescences (24.71% and 20.52% respectively).

In our previous experiment (Lingorski and Kertikov, 2005b) with comparative testing of some annual spring legume forage crops (vetch, white lupine and bitter vetch) was to establish that the stems participation in the yields forming was from 51.23% to 55.96%. In second place forage yields were determined from the leaves.

It was found similar results in our other experiment (Lingorski and Kertikov, 2005a) but with annual spring cereal forage crops (maize, sorghum-sudan grass hybrid, sudan grass, oats and true millet). And in these cultures the stems' share in the yield formation was greater than that of the leaves or the inflorescences. Consequently, for maize the proportion was highest – 66.16%, and the lowest one for true millet – 49.05%. The foliar mass percentage was greatest in the yield from sudan grass and sorghum-sudan grass hybrid – respectively 33.65% and 31.80%. The corresponding values for inflorescences were highest for true millet and oats - 23.84% and 19.12%. This confirms the obtained results in the present experiment.

Yield of forage

The forage yield for different crops are shown in Table 2. They included the experimental period 2011-2013.

During the 1st year (2011) the green and dry mass productivity was established by dates and species as follows: on June 28 for bitter vetch (var. 1) and chickpea (var. 2), and on July 7 for foxtail millet (var. 3) and true millet (var. 4).

In 2012 the fodder harvest was done as follows: on June 20 and June 25 for bitter vetch and chickpea and on July 5 - foxtail millet and true millet.

In 2013, the harvest of green mass and dry matter was done on the following dates: on June 30 and July 2 for bitter vetch and chickpea and on June 28 and July 2 - foxtail millet and true millet.

Table 2. Green mass and dry mass yields by years and average for the 2011-2013 period (in t ha^{-1} and in %).

Variant (forage crop)	Green mass t ha^{-1}	Green mass %	Dry mass t ha^{-1}	Dry mass %
2011				
1. Bitter vetch (Standard)	15.47	100.00	4.37	100.00
2. Chickpea	18.67	120.69	4.84	110.76
3. Foxtail millet	12.27	88.46	3.43	112.83
4. True millet (Standard 2)	13.87	100.00	3.04	100.00
2012				
1. Bitter vetch (Standard)	9.20	100.00	2.05	100.00
2. Chickpea	10.27	111.63	2.77	135.12
3. Foxtail millet	7.33	84.54	1.95	74.14
4. True millet (Standard 2)	8.67	100.00	2.63	100.00
2013				
1. Bitter vetch (Standard)	7.87	100.00	1.87	100.00
2. Chickpea	9.53	121.09	2.21	118.18
3. Foxtail millet	12.80	78.05	3.47	80.51
4. True millet (Standard 2)	16.40	100.00	4.31	100.00
Average for the period 2011-2013				
1. Bitter vetch (Standard)	10.85	100.00	2.76	100.00
2. Chickpea	12.82	118.16	3.27	118.48
3. Foxtail millet	10.80	83.20	2.95	88.86
4. True millet (Standard 2)	12.98	100.00	3.32	100.00
$LSD_{0.05}$	705.71	74.03	210.25	95.28
$LSD_{0.01}$	1068.65	112.10	318.38	144.28
$LSD_{0.1}$	1716.75	180.08	511.47	231.78

It can be seen that, depending on the climatic conditions of the year, the harvesting dates varied as follows: in larger limits (12 days) for legumes – from June 20 to July 2 and for a short time (5 days) for cereals – from July 2 to July 7.

From Table 2 it can be seen that in 2011 regarding the legumes (var. 1 and 2) more fresh and dry mass (18.67 and 4.84 t ha^{-1}) was obtained from chickpea compared to bitter vetch (Standard) - by 20.69 and 10.76%. As for cereals (var. 3 and 4) a Standard 2 (true millet) exceeded only fresh mass yields of foxtail millet by 11.54%. Due to the local soil and climate conditions more dry matter was accumulated by foxtail millet during vegetation (3.43 t ha^{-1}). It exceeded the true millet by 12.83%.

The above mentioned table also shows that in 2012, more green mass and dry matter of the legumes (10.27 and 2.77 t ha^{-1}) was obtained by the chickpea compared to bitter vetch (Standard) - respectively by 11.63 and 35.12%. Regarding cereal crops the fresh and dry mass proportion of the true millet exceeded that of the foxtail millet by 15.46% and 25.86%.

In 2013 more fresh and dry mass of the legumes (var. 1 and 2) - 9.53 and 2.21 t ha^{-1} was obtained by the chickpea compared to bitter vetch (21.09% and 18.18%). As far as cereals (var. 3 and 4) are concerned true millet (Standard 2) exceeded in the production of forage the foxtail millet respectively by 21.95% for green mass and 19.49% for dry matter.

Considering the whole experimental period by years (2011-2013) it can by concluded that from the legumes the yields of chickpea (12.82 and 3.27 t ha^{-1}) were higher than these of bitter vetch - 18.16% for green mass and 18.48% for dry matter. Regarding the cereal crops the true millet had more yields (12.98 and 3.32 t ha^{-1}) than foxtail millet – in this way more forage was obtained respectively by 16.80 and 11.14%.

In our previous experiment with annual cereal crops (Lingorski and Kertikov, 2005a) highest yields were obtained from sudan grass, followed by sorghum- sudan grass hybrid and true millet. They exceeded the standard (maize) by 37.77%, 29.29% and 21.06% respectively.

In our experiment with some annual legumes (Lingorski and Kertikov, 2005b) highest dry mass yields were obtained from white lupine, followed by horse bean and vetch – more than standard (field pea) with 98.0%, 39.3% and 10.4% respectively.

Conclusions

The comparative agro-ecological study of some annual drought-resistant untraditional forage species for the conditions of foothill areas of the

Central Balkan Mountains (Troyan region) showed the following:

The largest share of the forage yields had the stems, followed by the leaves and inflorescences.

The chickpea had a more yields (12.82 t ha^{-1} green mass and 3.27 t ha^{-1} dry matter) compared to bitter vetch - in more respectively by 18.16% and 18.48%. As for the cereal crops the true millet had more yields (12.98 t ha^{-1} green mass and 3.32 t ha^{-1} dry matter) than the foxtail millet – more forage mass was obtained respectively by 16.80 and 11.14%.

From the obtained data it can be considered that the studied forage species are suitable for preparation of ecological forage in the foothill regions of the Central Balkan Mountains.

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ANIMAL SCIENCE

Effect of Methyl Testosterone (17α-MT) on the phenotype, bioindices and gonads of adult male dwarf Gourami (*Colisa lalia*)

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Abstract

The effect of different concentrations of synthetic androgen 17α- methyl-testosterone (MT) on the adult fish, *Colisa lalia* was studied in the present investigation. It was found that fishes fed with homogenous mixture of the hormone in ethyl alcohol, exhibited phenotypically, morphometric, gonadal changes and differences in GSI value. Significant differences for length, weight, body color and GSI values were observed between hormones treated and control groups. Fishes fed with hormonal doses of 10 and 15 mg/Kg feed showed significantly elevated body color compared to 5 mg/Kg feed. Length, weight and GSI values were found significantly higher in 10 mg than 5mg/Kg and control group. The highest mean length and mean weight of fish was recorded as 4.76±0.13 mm and 1.50±0.15g respectively with the hormone treatment of 10mg/Kg of feed. The present study revealed that the synthetic hormone had no significant effect on the gonad development of *C. lalia*.

Key words: *Colisa lalia,* 17α- methyl-testosterone, Ornamental fish, Synthetic androgen

Introduction

The dwarf gourami (*Colisa lalia*) also called *Trichogaster lalius* belonging to Order- Perciformes and Family- Ospheronemidae / Belontidae is inhabitant of slow moving streams, rivulets and lakes with plenty of vegetation. They generally grow to a size of 3.6-5.0 cm or to a maximum of 8.8 cm. Males can be easily distinguished from females for their colour. The dwarf gourami male is a bit bigger than the female and has turquoise and orange-red iridescent vertical bands on the entire body and on fins, mutants with total orange-red body and turquoise dorsal fin, or total turquoise body with just some red at the edges of the fins are available. The dwarf gourami female is totally silver with pale turquoise vertical stripes (www.aqua-fish.net).

Keeping colorful ornamental fishes in aquarium is one of the oldest and most popular hobbies in the world. In India, the hobby of ornamental fish keeping is nearly 70 years old. The ever-increasing demand for aquarium fishes gradually paved the avenue towards global trade of ornamental fishes. The top exporting countries include Singapore followed by Honkong, Malaysia, Thailand, Philippines, Srilanka, Taiwan, Indonesia and India. India's share in ornamental fish trade is estimated to be less than 1 % of the global trade. The largest importer of ornamental fish is the USA importing fish worth over US$ 500 million every year followed by Europe and Japan. The emerging markets are China and South Africa.

India hosts 11% of global ichthyic diversity of about 31,300 fish species, so far it possess 930 described ornamental fish species and of these approximately 250 are now available to aquarium hobbyists. Despite the huge potential offered by the rich diversity and environment, export of ornamental fish from India continues to remain negligible. Approximately 267 fish species belonging to 38 families has been identified as ornamental fishes from North East India comprising about 85% of the total aquarium fish trade of India.

Ornamental fishes are acceptable to consumers if they have striking and vibrant colours. Colouration, which is one of the most important factors deciding the market value of the ornamental

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fish, is controlled by the endocrine and nervous systems (Asimi, 2009). However, dietary sources supplemented with external hormones also play a role in enhancing the fish colour. People involved in the trade of ornamental fish are constantly exploring methods of enhancing skin coloration. 17α- methyl-testosterone was the first successful hormone used to produce functional males from female genotype in Medaka (*Oryzias latipes*) (Yamamoto, 1958). Several methods for hormone administration include dietary implementation, oral administration, injection and silastic implantation. Dietary administration of hormone is most practical and effective methodology (Yamamoto, 1953). The diet supplementation generally involves homogenous mixing of the steroid in the diet and the alcohol evaporation method is the most widely used for steroid application (Guerrero, 1975). The hormone has been applied for masculinization, inducing colour and anabolic consideration in many ornamental fishes.

Current investigation was carried out to study the effect of exogenous steroid, methyl testosterone (MT) on phenotypical and gonadal changes in adult male Dwarf Gourami, *Colisa lalia* and its response in the induction of body colour, enhancement in the length and weight without suppressing the gonad of adult fish.

Materials and Methods

Collection and acclimatization of fish species

The study was conducted at the Department of Fishery Biology and Resources Management of West Bengal University of Animal and Fishery Sciences at Chakgaria during 2011. Male adults of *Colisa lalia* ranging from the length 3.2 to 4.2 cm and weight of 0.85 to 1.44 mg were collected from Gullif Street, Kolkata, West Bengal. In the laboratory, the fishes were given a short bath treatment with 2% potassium permanganate ($KMnO_4$) solution for 3 to 5 minutes as prophylactic measures. Subsequently, they were transferred carefully to the aquarium (60 x 30 x 30) cm^3 containing iron free tap water. For acclimatization to laboratory condition, they were stocked at a density of 30 fish per aquarium in 35 L of water for 15 days before starting the experiment. Experiment was conducted with 3 replicates having 30 fish in each aquarium, in which adult *Colisa lalia* were fed with diet supplemented with 3 doses of 17 α -MT i.e. 5, 10, 15 mg/Kg diet for 90 days.

A control without hormone dosage fed with a commercially available aquarium feed was maintained. The feed was given to the stocked fishes at the rate of 3% of their body weight daily with equal rations i.e. during morning to evening hours. Left out feed and accumulated fecal matter was siphoned out daily morning in order to maintain healthy condition of fishes. The important water quality parameters were fixed and recorded i.e. water temperature (30.21± 3.1°C), pH (7.39 ±0.12) and D.O. (4.65± 0.45ppm). The biotic factors like length, weight and colour of fish as well as the GSI (Gonado Somatic Index) were measured and documented in every 15 days interval. For each sampling 10 fishes were randomly selected from 3 replicates of each treatment doses. For the colour estimation the views of ten different persons were collected secretly and separately. Gonado Somatic Index was calculated with the help of following formulae

GSI (Gonado Somatic Index) =Weight of gonad / Weight of fish x 100

Statistical Calculation

The average body weight and length of the hormone treated fish were compared to that of the control by multivariate ANOVA using time and treatment followed by Duncan's multiple range test (DMRT) between the treatments. Similarly the biological end points like GSI and treatment doses were analyzed through Pearson's correlation to determine the influence of hormones on GSI. All this statistical analysis were done using SPSS software package 17.

Preparation of hormone incorporated feed

Three different feeds containing different concentrations (5, 10 and 15 mg per Kg of feed) of MT hormone (obtained from Sigma Chemicals Ltd., USA) were used for the analysis. Each dose was dissolved separately in 100 ml of 95% ethanol and the hormone mixture was spread over the feed and air dried. Control fishes were fed with feed speeded with 10 ml of 95% ethanol / kg without any hormone. The prepared feed was kept in sealed packets and stored in the freeze (4°C). Feed were taken out from the freeze before 15 minutes of use. During use maximum pre-cautionary measures are taken.

Results

Current investigation resulted in the enhancement of body color in hormone treated groups (5, 10 and 15 mg/Kg feed) in contrast to control where no color was detected after 90 days experiment. Among the hormone treated group 10 and 15 mg/Kg feed treated fishes showed more coloration compared to 5mg/Kg treated fishes (Plate 1 to 4). The average length and weight of fishes ranged between 3.49±0.18 to 3.54±0.12 mm

and weight 0.90±0.24 to 1.08±0.19 gm respectively. The average body weight and length of the hormone treated fish were interpreted to find the probability of occurrence and represented in Table 1, 2.

Plate 1. Control group of *Colisa lalia* fishes.

Plate 2. 5 mg/Kg treated group of *Colisa lalia* fishes.

Measurement of male gonads (testis)

In the initial stages of the experiment no significant differences were found between control and hormone treated groups, but at the end of experiment control fish showed difference in average length 3.99±0.12 mm and weight 1.28±0.03g compared to hormone treated group (Figure 1, 2). The highest mean length and mean weight at the end of experiment was 4.76±0.13 mm and 1.50±0.15g in 10 mg/Kg feed respectively. GSI values also increased in MT treated fish than control fish that means the hormone was not suppressing the gonadal development (Figure 3).

Plate 3. 10 mg/Kg treated group of *Colisa lalia* fishes.

Plate 4. 15 mg/Kg treated group *Colisa lalia* fishes.

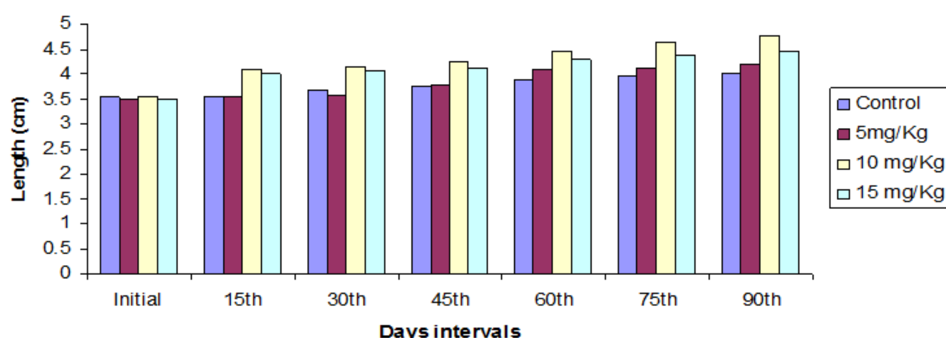

Figure 1. Fortnightly variations in length of *Colisa lalia* fishes upon treatment with exogenous steroid, methyl testosterone (MT).

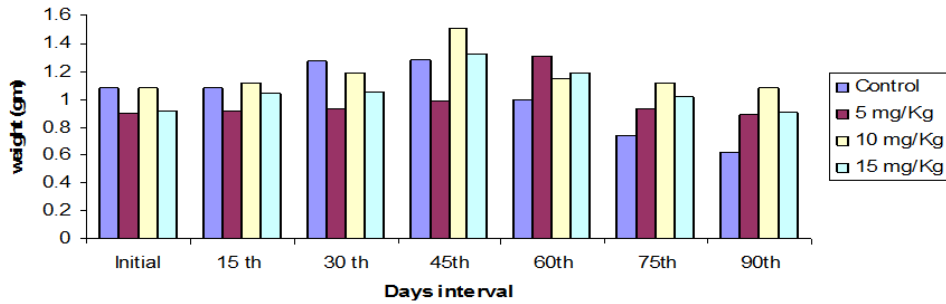

Figure 1. Fortnightly variations in weight of *Colisa lalia* fishes upon treatment with exogenous steroid, methyl testosterone (MT).

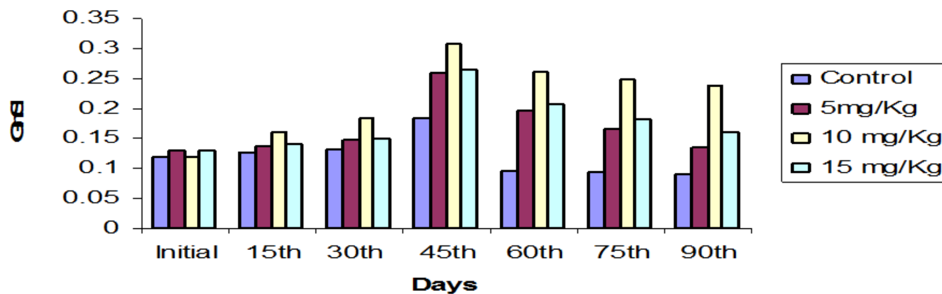

Figure 1. Fortnightly variations in Gonado-Somatic Index (GnSI) of *Colisa lalia* fishes upon treatment with exogenous steroid, methyl testosterone (MT).

Table 1. Comparison of length, weight and GSI value with time through Post-Hoc Test.

Time	Number	Length	Weight	GSI
Initial	40	3.5150^{a}	0.8979^{a}	0.1243^{a}
15th day	40	3.7975^{b}	0.9966^{b}	0.1817^{ab}
30th day	40	3.9175^{c}	1.0868^{c}	0.2587^{bc}
45th day	40	3.9400^{c}	1.1042^{c}	0.3267^{c}
60th day	40	3.9500^{c}	1.1833^{c}	0.4429^{c}
75th day	40	4.0450^{d}	1.2750^{d}	0.4592^{d}
90th day	40	4.0650^{d}	0.9511^{ab}	0.3778^{bc}

Values with a common superscript in the same column with the same letter index did not differ significantly (P>0.05).

Table 2. Post-Hoc Test comparison with treatment groups in case of Length, Weight, and Gonado-Somatic Index.

Treatment	Number	Length	Weight	GSI
Control	70	3.7314^{a}	1.0063^{a}	0.2533^{a}
5mg/Kg	70	3.8571^{b}	1.0098^{a}	0.2765^{ab}
10mg/Kg	70	4.0114^{cd}	1.1617^{b}	0.3720^{c}
15mg/Kg	70	4.0543^{bc}	1.0488^{a}	0.3617^{bc}

Values with a common superscript in the same column with the same letter index did not differ significantly (P>0.05).

Discussion

The colour changes in treated fishes were found more than that of the controlled fishes (Plate 1 to 4). The change in fish color may be due to the actions of hormone on neuropeptide system of fish. The hormone (MT) is triggering the pituitary to produce more melanophore dispersing hormone (MDH) thus increasing the level of concentration in the blood stream, which is resulting in the color variations according to hormone concentration.

In the present study length, weight and Gonado-Somatic Index showed an increasing trend (Fig-1, 2 & 3). The maximum length was attained in 10 mg/Kg hormone treated fish than other treatments and control groups which was in well accordance of the finding of Simone (1990) in channel cat fish, *Ictalurus punctatus*. At the end of experiment smallest length and weight was observed in control than other hormone treated group (Table-1&2). Lone and Matty (1980)

reported that 17 α -MT induced better growth by acting in three different ways - improved food conversion, activation of other exogenous anabolic hormones and direct effect on gene expression in muscle cells. MT has shown its impact on the growth enhancement of various fish species such as Pacific salmon, *Onchorhynchus tschawytscha* (Bride and Fagerlund, 1973), Common carp, *Cyprinus carpio* (Lone and Matty, 1980) and Nile tilapia, *Oreohromis niloticus* (Tayamen and Shelton, 1978). Androgenic activity of the gut lead to the growth enhancement in Mirror carp (Lone and Matty, 1981).

Fry of *Oreochromis niloticus* treated with MT @5-25 mg/Kg diet was found to be significantly heavier than the control (Jo et al., 1995). Best growth was observed with MT compared to control at 10-60 ppm (Hanson et al., 1983). Hormone treated group was much heavier than that of the fry feed on hormone free diet (Cleide et al., 2000). Contradictory to the present findings stated that growth depression was observed in the Gold fish, *Carassius auratus* treated with MT at higher concentration than 10 mg/Kg and growth enhancement at low concentration i.e. 1mg/Kg (Yamazaki, 1976).

GSI value in this research was higher among hormone treated adult fish compared to control group at the end of experiment. In the present study the development of gonadal materials (Primary spermatocytes, Secondary spermatocytes and Spermtozoa *i.e.* sperms) inside the follicles of the male indicated the maturation stages of gonads (Plate 5&6). It revealed that hormone had no effect on gonadal development in adult fish. The length, weight and the GSI values were increasing with respect to the experimental period (Fig-1,2&3). Contradictory to the present findings 100% sterility of gonad was reported in grass carp by using mibolerone hormone (Kavumpurath and Sampath,1990) and in common carp of same age group (Rao and Rao,1983; Basavaraja and Rao,1988; Das et al.,1990). Gonad weight and GSI value of Red swordtail, *Xiphophorous helleri* and Siamese fighting fish, *Betta splendens* decreased with the increase of hormone dose beyond the optimum dosage and it negatively reflected on the reproductive performance (James and Sampath, 2006).

Gonads sterility of Salmon was achieved at a dose of 30 mg/Kg diet (Simpson et al, 1978). Rainbow trout fry with 10 ppm MT for 8 week and 30 ppm MT for 4 week obtained sterility (Hurk and Slof, 1981). The administration of steroid hormones has been reported to cause sterility in rainbow trout (Yamazaki, 1983), grass carp (Boney et al., 1984) and carp (Basavaraja, 1984). Sterility due to suppression of gonad development in common carp was reported by Pandian and Sheela (1998). Present study indicated neither hormonal doses (5, 10 and 15 mg of MT per Kg of feed) nor duration of treatment (90 days) could result in degeneration of the testis. This may be either due the inadequate hormonal dose or lesser experimental time period in our study as in other earlier studies time period was longer and fishes been encountered at higher doses of the Methyl Testosterone (17α-MT).

Conclusion

Despite the marketability and commercialization of Gourami (*Colisa lalia*) on large scale, its captive breeding with the help of inducing agents specially colour inducer i.e. MT hormone is still a virgin field.

This study is an initiation in adult male dwarf Gourami (*Colisa lalia),* which will form the basic platform for further research and provide a strategy to study sex ratio of offspring while breeding this hormone treated males. In the present study it was revealed that hormone doses 5 mg/kg-15 mg/kg for a maximum period of 90 was not enough to depress the gonadal development. Moreover, a marked increment was observed in weight, length and Gonado-somatic Index value at 10 and 15 mg/kg MT harmone in feed. Therefore, along term study is necessary to optimize the hormonal dose and experimental time in Gourami (*Colisa lalia*). More important, survival of fish after hormonal manipulation which reduces the chance of success and reliability are the major criteria to be studied on a long way.

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ANIMAL SCIENCE

Microbiological characterization of camel and sheep meat preserved by refrigeration and lactic acid

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Abstract

The microbial growth of certain bacteria contaminating camel and sheep meat, kept under refrigeration was evaluated. The samples were collected at the Ouargla slaughterhouse. The selected compartment for monitoring was the thigh (most demanded by consumers of the region). The shelf life of the two types of meats studied was five days against the total mesophilic aerobic flora, yeasts, enterobacteria and fecal coliform contamination whose percentages were respectively 30.26%, 26.55% , 22.74% and 20.44% for camel meat; 28.91%, 28.21%, 22.87% and 20% for sheep meat. The lactic acid concentration that ensures better conservation, was 4% for sheep meat while a concentration of 2% was sufficient for camel meat. The duration of cold preservation of both meats (treated and untreated) was nine days except for yeasts whose duration was seven days.

Key words: Meat, Dromedary, Sheep, Lactic acid, Conservation

Introduction

The richness of meat in water, proteins of high biological value makes it an essential food for a balanced diet. However, these vertues make it a favourable breeding ground for most microbes (Clinquart et al., 1999). Most of the germs contaminating carcasses after different stages of slaughter (skinning and evisceration) are saprophytes. These bacteria, yeasts and molds. are germs that cause alteration or putrefaction of meat. In addition, the presence of pathogens in food is often responsible for borne illness (Cottin et al., 1985). Food preservation is conservation of its edibility, taste and nutritional properties. This requires the prevention of microbial growth and retarding the oxidation of fats which cause rancidity (Bourgeois et al., 1991). Conservation at low temperature retards the growth of microorganisms. The majority of germs such as coliforms have limited metabolic activites at temperatures below 5°C (Craplet, 1966). It is the preferred method of preserving meat, and the best currently known (Laurent, 1974).

Refrigeration is the storage of food at low positive temperatures. In general, the temperature is around 0°C to +4 °C. Refrigeration should be applied initially to fresh healthy foods. During this type of storage, water maintains liquid constitution (Bourgeois et al., 1996). Most bacteria grow rapidly in fresh non-acidic meat, fish and vegetables causing deterioration. Other forms of spores make them resistant to preservation techniques and resume their multiplication upon return to ambient conditions (Multon, 1984). The use of chemical additives to acidify the meat can preserve them in the best conditions. The addition of these agents is designed to optimize the preservati of food while conserving the organoleptic and nutritional qualities (Multon, 1984). Organic acids, such as lactic acid are widely used as condiments in food preparations. Lactic acid bacteria inhibit the growth of pathogenic microorganisms especially *E. coli* (Huxley, 1969; Houstma et al., 1986; Miller, 1994).

The present work aims to characterize the microbiological properties of meats from two species (camel and sheep) kept under refrigeration after undergoing treatment with lactic acid solutions at concentrations of 2% and 4% through daily monitoring of the proliferation of aerobic mesophilic total flora, yeasts, Enterobacteriaceae and coliforms. This is initiated to evaluate preservative effect of organic acid (lactic acid).

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Materials and Methods

Biological material

The samples used in this study were taken immediately after slaughtering at the Ouargla slaughterhouse. The samples were collected using a sterile knife, (thigh) for both types of meat (sheep and camel). In total, 20 samples of meat (coming from thighs) investigated 10 samples for each studied species (camel and sheep). Because sample types are perishable, transportation of the samples was carried out in a cooling system (isothermal cooler). In the laboratory, the meat was cut aseptically into 10g pieces, using a chisel and sterile forceps. The weighing was determined using an analytical balance. Manipulations were carried out with a maximum asepsis (Bunsen burner lit bench for 15 minutes and washed with bleach).

Series of samples (for both meat origins camel and sheep) were deceived or in a solution of lactic acid 2%, 4% or sterile distilled water. A series of samples was left untreated (control). Each sample was placed (10 g) individually in a sterile bag and stored in a refrigerator at a temperature between 0 and 4°C.

Preparation of the initial suspension and decimal dilutions

The procedure was performed according to the French standard NF V-057-2. Ten gram of meat was introduced aseptically into a sterile "Stomacher" bag containing 90 ml of diluent (Water Buffered Peptone). After grinding and homogenization of the solution, the obtained initial suspension was subsequently diluted 1/100 (10^{-2}), and 1/1000 (10^{-3}) and one ten thousandth (10^{-4}).

Microbiological characterization of camel meat

The culture medium used for the enumeration of total aerobic mesophilic flora was the *plate count agar* (PCA), according to the ISO 4833 standard. Fecal coliforms were counted on VRBL environment, according to NF V 08-017. For the Enterobacteriaceae, the selective medium used was VRBG with incubation of Petri dishes seeded depth for 24 h at 37°C. Enumeration of yeasts was performed by counting colonies on OGA (Glucose agar with oxytetracycline) medium after seeding the surface with 0.1ml of the stock solution and serial dilutions and incubation Petrie plates at 25°C for 2 to 5 days (NF V 03-454).

Statistical analyses

Statistical analyses included two stages, (i) analysis of internal variability in each species of meat (camel and sheep) and (ii) comparative analysis to determine the variability between germs sought.

-The analysis of internal variability included- the description of the mean and standard deviation for each of the germ,

-Correlations between rates for each meat contamination (Pearson correlation).

Establishing a contingency table between germs sought in each species of meat. These analyzes were performed using the software XLStat (Addinsoft©)

Results and Discussion

Effect of the nature and treatment of meat on the evolution of the mesophilic aerobic flora total enterobacteria, coliforms and yeasts

The results for the microbiological quality of meat, camel and sheep kept under refrigeration, namely the total mesophilic aerobic flora, fecal coliforms, Enterobacteriaceae and yeasts are summarized in (Figure 1).

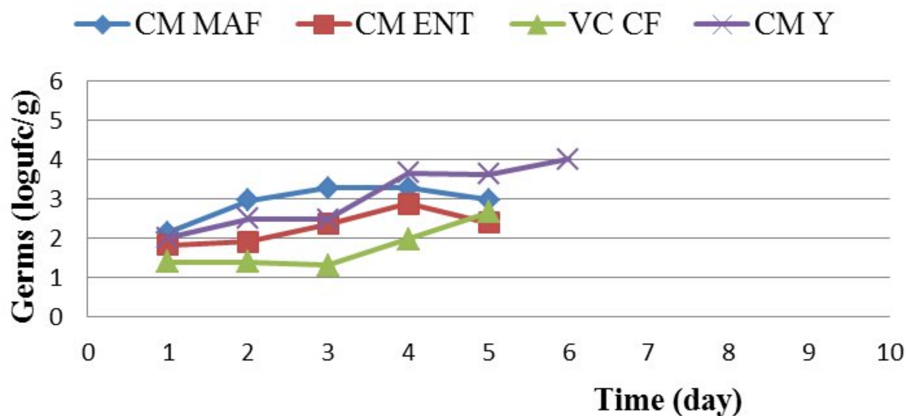

Figure 1. Changes in germs over time in the control camel meat. CM: Camel Meat, MAF: mesophilic aerobic flora total, ENT: Enterobacteriaceae, FC: fecal coliforms, Y: yeast, d: day.

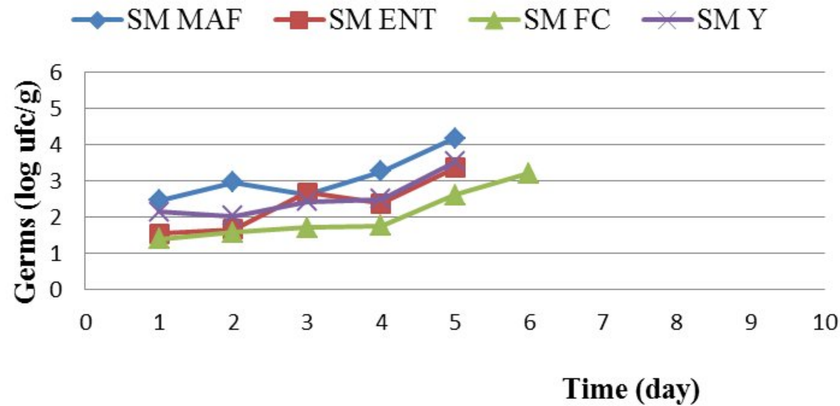

Figure 2. Changes in germs sought over time in the control sheep meat
SM: Sheep Meat, MAF: mesophilic aerobic flora total, ENT: Enterobacteriaceae, FC: fecal coliforms, Y: yeast, d: day.

Overall, the rate of contamination maximum total mesophilic aerobic flora samples of camel meat is 3.17 ± 0.62 logufc / g (Figure 1), while that of sheep meat samples is about 3.27 ± 0.57 logufc / g (Figure 2). Values of the flora that we obtained in this study are consistent with those reported by Hamad (2009). According to this author, contamination rates were of the order of 1.79 logufc/cm^2 for camel meat and 3.08 logufc/cm^2 for the sheep meat. Camel meat has a maximum rate of contamination by enterobacteria 2.88 ± 0.71 logufc / g (Figure 1), relatively lower than that of the (3.35 ± 0.05 logufc / g). Our results were within the range of values reported by Hamad, (2009) whose results were 2.60 log10ucf/cm^2 and 3.38 log10ucf/cm^2 respectively for camel and sheep meat.

Fecal coliforms samples of sheep and camel meat reached maximum contamination levels of 3.21 ± 0.21 logufc / g and 3.65 ± 0.46 logufc / g, respectively. So, as the rates for yeast was 3.52 ± 0.62 log ufc / g and 3.66 ± 0.49 log ufc / g (Figure 1 and Figure 2).

The comparison of the maximum contamination by germs counted recorded on both studied meat left out that camel meat was less contaminated than the sheep. This could be explained by the difference in skinning techniques for animal skinning, despite the fact that these meats were processed in the same slaughterhouse.

The predominant flora of contamination for both meats was constituted by the total mesophilic aerobic flora reflecting their hygienic quality. The presence of fecal coliforms was indicative of poor hygiene and in particular defects that occur during evisceration because coliforms are saprophytes of the digestive tract of man (Basel et al., 1983).

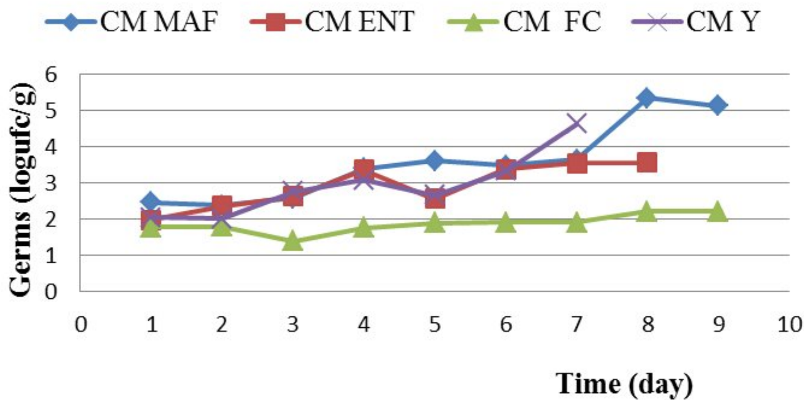

Figure 3. Changes in germs sought over time in processed camel meat by lactic acid 2%
CM: Camel Meat, MAF: mesophilic aerobic flora total, ENT: Enterobacteriaceae, FC: fecal coliforms: yeast, d: day.

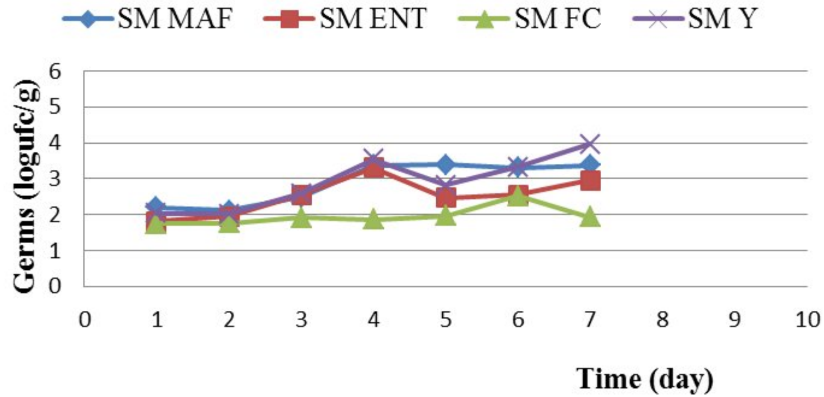

Figure 4. Changes in germs sought over time in processed sheep meat by lactic acid 2%
SM: Sheep Meat, MAF: mesophilic aerobic flora total, ENT: Enterobacteriaceae, FC: fecal coliforms,Y: yeast, d: day.

Immersion of meat before refrigeration in a solution of lactic acid 2% or 4% appeared to slow the multiplication rate of bacteria. The maximum rate of contamination of the camel meat rinsed with a solution of 2% lactic acid were respectively 5.12 ± 0.14 logufc/g, 3.54 ± 0.21 logufc/g, 2.20 ± 0.28 logufc/g and 4.63 ± 0.38 logufc/g. The duration of its conservation was seven days for yeast, eight days for Enterobacteriaceae and nine days for the total mesophilic aerobic flora and coliforms (Figure 3).

Sheep meat was pretreated with a solution of 2% lactic acid storage before refrigeration, presented maximum contamination rate, total mesophilic aerobic flora of 3.38 ± 0.21 logufc/g of Enterobacteriaceae logufc/g 3.31 ± 0.32 of fecal coliforms logufc 2.52 ± 0.66 logufc/g and 3.97 ± 0.30logufc/g of yeast (Figure 3). The shelf life of the meat was seven days for these germs (Figure 4).

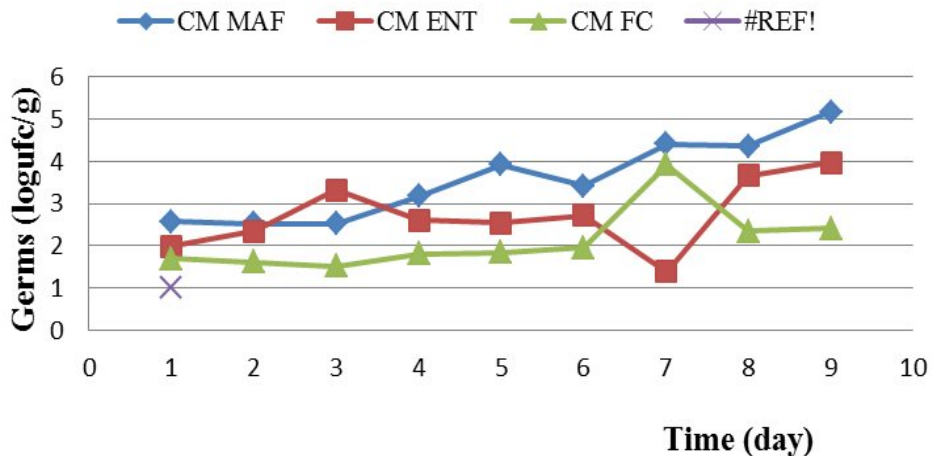

Figure 5. Changes in germs sought over time in processed camel meat by lactic acid 4%.
CM: Camel Meat, MAF: mesophilic aerobic flora total, ENT: Enterobacteriaceae, FC: fecal coliforms: yeast, d: day.

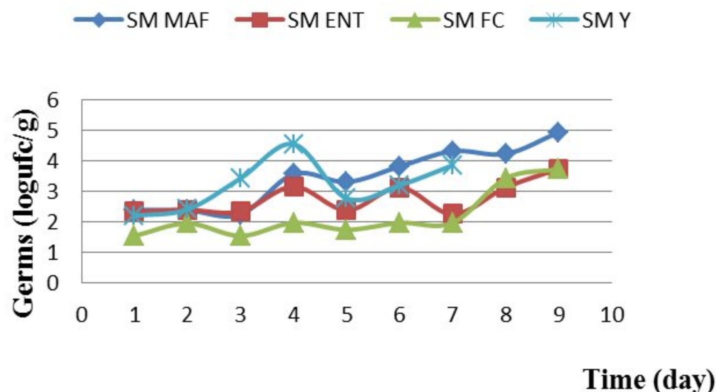

Figure 6. Changes in germs sought over time in processed sheep meat by lactic acid 4%.
SM: Sheep Meat, MAF: mesophilic aerobic flora total, ENT: Enterobacteriaceae, FC: fecal coliforms, Y: yeast, d: day.

A lactic acid concentration of 4% did not reduce the rate of contamination of camel meat by the total mesophilic aerobic, Enterobacteriaceae, coliforms and yeasts whose respective values were 5.17 ± 0.3 logufc / g, 3.98 ± 0.50 logufc / g, 3.93 ± 0.26 logufc / g and 4.65 ± 0.70 logufc/g, nor the duration of its conservation compared to the results obtained with concentration of 2%, except the shelf-life against enterobacteria which was extended to nine days (Figure 5).

Whereas in the presence of a lactic acid concentration of 4%, the maximum rate of contamination of sheep meat were of the order of, 4.94 ± 0.10 logufc/g for mesophilic aerobic flora total, 3.74 ± logufc 0.67/g for Enterobacteriaceae, 3.73 ± 0.84 logufc/g for fecal coliforms and 4.55 ± 0.27 logufc/g for yeasts (Figure 5). The shelf life of the sheep meat was nine days against the majority of organisms investigated (Figure 6).

The lower infection rates of the total flora, Enterobacteriaceae and coliforms in processed meat by lactic acid could be explained by the fact that organic acids inhibit pathogenic microorganisms. The latter cannot grow in foods at acid pH (below 4.5). The decrease in pH affects even the heat resistant spores (Bourgeois et al., 1996; Lyreal and Vierling, 1997).

The high rate of yeast in the treated meat could be explained by the selective effect exerted by organic acids on the microbial population. They inhibit pathogenic microorganisms but stimulate yeasts. Yeasts are extremely tolerant to changes of pH; they can grow at pH 4 to 6.Moreover, those germs can locally adapt their optimum pH. The pH is not a good indicator to control their development (Lyreal and Vierling, 1997).

Table 4. Correlation matrix between the rates of infection in camel and sheep meat.

Sheep meat	FAMT ST	FAMT 2%	FAMT 4%	ENT ST	ENT 2%	ENT 4%	CF ST	CF 2%	CF 4%	L ST	L 2%	L 4%
FAMTST	**1**	0,442	0,162	0,572	**0,694**	0,212	0,136	0,206	0,082	0,659	0,445	**0,683**
FAMT2%	0,442	**1**	**0,650**	0,648	**0,839**	0,323	0,716	**0,498**	0,116	0,842	0,889	0,699
FAMT4%	0,162	**0,650**	**1**	0,238	**0,536**	0,700	0,502	0,374	**0,765**	0,421	0,681	0,437
ENTST	**0,572**	0,648	0,238	**1**	0,528	0,029	0,449	0,301	- 0,012	**0,723**	0,401	0,338
ENT2%	0,694	**0,839**	**0,536**	0,528	**1**	0,281	0,335	**0,286**	0,103	0,698	0,918	0,961
ENT4%	0,212	0,323	0,700	0,029	0,281	**1**	0,368	0,376	0,791	0,301	0,261	0,324
CFST	0,136	0,716	0,502	0,449	0,335	0,368	**1**	0,887	0,103	**0,510**	0,521	0,159
CF2%	0,206	**0,498**	0,374	0,301	**0,286**	0,376	0,887	**1**	0,078	0,215	0,439	0,166
CF4%	0,082	0,116	**0,765**	0,012	0,103	0,791	0,103	0,078	**1**	0,104	**0,130**	**0,097**
LST	**0,613**	0,842	0,421	**0,723**	0,698	0,301	**0,510**	0,215	0,104	**1**	0,591	0,594
L2%	0,445	0,889	0,681	0,401	0,918	0,261	0,521	0,439	**0,130**	0,591	**1**	**0,830**
L4%	0,683	0,699	0,437	0,338	0,961	0,324	0,159	0,166	**0,097**	0,594	**0,830**	**1**

The characters in bold are significant at P <0.05.

Table 5. Mean ± standard deviation of rates of infection in camel and sheep meat.

	Camel meat	Sheep meat	Significant level P <0.05
FAMTST	3,036 ± 0,697	2,922 ± 0,463	N Sign
FAMT2%	3,073 ± 0,573	2,910 ± 0,614	N Sign
FAMT4%	3,365 ± 0,801	3,283 ± 0,849	N Sign
ENTST	2,927 ± 0,897	2,318 ± 0,749	N Sign
ENT2%	2,636 ± 0,545	2,484 ± 0,479	N Sign
ENT4%	2,730 ± 0,672	2,894 ± 0,861	N Sign
CFST	2,027 ± 0,727	2,025 ± 0,867	N Sign
CF2%	1,826 ± 0,211	1,960 ± 0,261	N Sign
CF4%	2,126 ± 0,742	2,206 ± 0,805	N Sign
LST	2,927 ± 0,776	2,852 ± 0,751	N Sign
L2%	2,927 ± 0,897	2,899 ± 0,748	N Sign
L4%	2,966 ± 0,905	3,269 ± 0,739	N Sign

Infection rates by total mesophilic aerobic flora, enterobacteria, fecal coliforms and yeasts were not significantly different in both species of meat and when these meat were tree ted by lactic acid (Table 4). The correlations were generally not significantly in the two meats (Table 5).

Conclusion

The present study regarding meat from two different species (camel and sheep) aimed to highlight the preservative effect of a physical technique (refrigeration). The presence of certain flora from the first day was indicator of contamination of the carcasses during slaughtering operations and contamination of meat during cutting operations. The daily counts of these organisms were highlighting important points: (i) the difference in sensitivity of germs to refrigeration as well as treatment with lactic acid. (ii) the preservation of meat at a temperature between 0 and 4°C to slow the multiplication of germs without being able to destroy them. (iii) the storage temperature of meat which presented an undeniable effect in prolonging the shelf life of meat. (iv) the combination of two methods of conservation for improving the shelf life of meat, whatever the species (camel and sheep), and finally which could help more families to keep their surplus product directly without freezing. (v) a concentration of 2% sufficient to increase the shelf life of camel meat to nine days for the majority of bacteria sought, while a concentration of 4% was needed to achieve this length for sheep meat.

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Alhadrami, G. A. 1991. Effect of preservatives and maturity on the nutritional value of alfalfa hay for lactating dairy cows. Ph.D. dissertation, University of Arizona, Tucson.

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