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African Journal of Biotechnology

Full Length Research Paper

Extraction and characterisation of gelatin from the skin of striped catfish (*Pangasianodon hypophthalmus*) and studies on its colour improvement

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Functional properties of gelatin from skin of striped catfish with and without bleaching for 48 h by 5% H_2O_2 (w/v)) were studied. Gelatin from skin bleached with 5% H_2O_2 for 48 h showed the highest yield (16.18 g). Bleaching not only improved the colour of gelatin gel by increasing the L* (lightness)-value and decreasing a*(redness/greenness)-value, but also enhanced the bloom strength and the emulsifying and foaming properties of the resulting gelatin. Fourier transform infrared spectroscopic study showed higher intermolecular interactions and denaturation of gelatin from bleached skin than that of the control. These results indicated that hydrogen peroxide most likely induced the oxidation of gelatin, resulting in the formation of gelatin cross-links, hence improved functional properties.

Key words: Gelatin, catfish, color, bloom strength.

INTRODUCTION

Gelatin, the denatured form of collagen, has been extensively applied in the food industry as an ingredient to improve the elasticity, consistency and stability of foods. Its parent form, collagen, constitutes approximately 30 g/100 g of total animal protein. Skin, bones, the vascular system, tendons and the connective tissue sheaths surrounding muscle are the major sources of collagen. In general, gelatin is manufactured from the waste generated during animal slaughter and processing, that is, skin and bone (Patil et al., 2000). Generally, pig and cow skin and bones are the main sources of gelatin. Recently, outbreaks of mad cow disease (bovine spongiform encephalopathy, BSE) have caused anxiety for customers. Additionally, the gelatin obtained from pig skin and bone cannot be used in kosher and halal foods due to religious constraints (Sadowska et al., 2003), while as Sikhs and Hindus, they do not use bovine gelatin due to religious constraints (Singh et al., 2011). Furthermore, an increasing attention to health issues of consumers has also gained momentum. As a consequence, increasing interest has been paid to alternative sources of gelatin, especially from the skins and bones from fish processing by-products (Kittiphattanabawon et al., 2005). In addition, the collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews, both of whom require bovine to be religiously prepared. As a consequence, the alternative sources of collagen,

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especially from aquatic animals including freshwater and marine fish and mollusks have received increasing attention (Singh et al., 2011).

Pangasianodon hypophthalmus or Pla Sawai (in Thai), a large freshwater catfish, belongs to the order Siluriformes and is a member of Pangasidae family. It is one of the most important aquaculture species in Thailand (Froese and Pauly, 2007), especially in the northeast part of Thailand. This fish is also known as Siamese shark or sutchi catfish and is native to the Chao Phraya River in Thailand and the Mekong in Vietnam. It has become an important fish for many countries like Indonesia, Malaysia and China (Roberts and Vidthayanon, 1991). This freshwater fish normally lives in a tropical climate and prefers water with a pH of 6.5 to 7.5 and a temperature range of 22 to 26°C. Adults reach up to 130 cm (4 ft) in length and can weigh up to a maximum of 44.0 kg (97 lb) (Roberts and Vidthayanon, 1991). Its meat has been popular among the consumers worldwide. During processing and filleting, a huge amount of skin from this fish is generated as a byproduct, which can be used as a potential source for collagen extraction. The skin from this fish is thick and tough, which may be associated with the collagen cross-links, especially cross-linking caused by hydroxylysine. The information on composition and molecular properties of collagen from the skin of this species has been reported in our previous study (Singh et al., 2011). The mother substrate of the gelatin obtained from this species was a bit darker in color and hence it was apprehended that its gelatin may not draw the attention for industrial uses. Nevertheless, the pigments in skin may pose a colour problem and bleaching could be performed prior to gelatin extraction. Hydrogen peroxide is a potent oxidant that is widely used as bleaching agent in seafood processing (Kolodziejska et al., 1999; Thanonkaew et al., 2008). Kolodziejska et al. (1999) reported that soaking squid skin in 1% H_2O_2 in 0.01 M NaOH for 48 h could improve the colour of the resulting collagen. The decomposition of H_2O_2 in aqueous solution occurs by dissociation and hemolytic cleavage of O–H or O–O bonds, with the formation of highly reactive products: hydroperoxyl anion (HOO^-), hydroperoxyl (HOO^-) and hydroxyl (OH^-) radicals, which can react with many substances, including chromatophores (Perkins, 1996). Wash water containing H_2O_2 also showed a gel-enhancing effect in surimi, via induced protein oxidation (Phatcharat et al., 2006). Hence, this study was undertaken to isolate and characterize gelatin, to study the effect of H_2O_2 on the bleaching of striped catfish skin and its impact on the functional properties of resulting gelatin.

MATERIALS AND METHODS

Chemicals

Sodium dodecyl sulfate (SDS), acetic acid, hydrogen peroxide (H_2O_2), and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Methane sulfonic acid, citrate buffer, calf skin gelatin (bloom strength 120 to 150 g) and calf skin acid-soluble type I collagen were from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All chemicals for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Fish skin preparation

Whole fresh farmed striped catfish (*P. hypophthalmus*) (approximately 2 years old) weighing 1 ± 0.5 kg/fish stored on ice were procured from the local fish market of Hatyai, Thailand. Fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported within 1 h to the laboratory. Upon arrival, fish were washed using tap water and deskinned. The skin was washed with cold water (5 to 8°C) and cut into small pieces (0.5 to 0.5 cm^2). The prepared skin samples were packed in polyethylene bags and kept at 20°C until used. The storage time was not longer than 1 month.

Extraction of fish skin gelatin

Gelatin was extracted from washed giant catfish skin as described by Jongjareonrak et al. (2006). To remove non-collagenous proteins and pigments, washed skin was soaked in 0.2 mol/L NaOH with a skin to solution ratio of 1:10 (w/v) at 4 ±1°C with a continuous gentle stirring. The solution was changed every 40 min for three times. Alkaline-treated skin was then washed with tap water until neutral or faintly basic pH (pH<7.5) of wash water was obtained. To remove the fat content of the skin, it was treated with butanol with a skin to solution ratio of 1:10 with a continuous stirring at 4°C. The solution of the skin was changed every 8 h and six defatting changes were done. Butanol treated skin was washed with ten volumes of water till the water pH become basic. The prepared skin was subjected to bleaching in 2% H_2O_2 using a sample:solution ratio of 1:10 (w/v) for 48 h. Bleached samples were washed three times with 10 volumes of water. The alkali-treated skin without bleaching was used as the control. To swell, the collagenous material in the fish skin matrix, the alkaline-treated skin was soaked in 0.05 mol/L acetic acid with a skin to solution ratio of 1:10 (w/v) for 3 h at room temperature (25±1°C) with a continuous gentle stirring with change of the solution at hourly intervals. Acid-treated skin was washed as previously described. The swollen fish skin was soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45±1°C for 12 h with continuous stirring to extract the gelatin. The mixture was then filtered using two layers of cheese cloth. The resultant filtrate was freeze dried and the dry matter from freeze-dried process was ground and referred to as ‘‘gelatin powder’’.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the method of Laemmli (1970). Gelatin samples were dissolved in 5% SDS solution. The mixtures were then heated at 85°C for 1 h, followed by centrifugation at 8500 ×g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS and 20% glycerol). Samples were loaded onto polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel. After electrophoresis, gels were stained for 1 h with a mixture of 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained for 1 h with a mixture of 30% (v/v) methanol and 10% (v/v) acetic acid and destained again with the same mixture for 30 min. High molecular weight markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins.

Fourier transform infrared (FTIR) spectroscopy

FTIR analysis of freeze-dried gelatins of the bleached and non bleached skins was conducted and compared. A Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated L-alanine triglycine sulfate (DLATGS) detector was used. The horizontal attenuated total reflectance (HATR) accessory was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000 to 650 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25°C.

Bloom strength

Gelatin gel was prepared as per the British Standard 757: 1975 (BSI, 1975) with a slight modification. Gelatin was dissolved in distilled water (60°C) to obtain a final concentration of 6.67% (w/v). The solution was stirred until gelatin was solubilized completely and transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solution was incubated at the refrigerated temperature (4°C) for 18 h prior to the analysis. The bloom strength was determined according to the British Standard 757: 1975 method (BSI, 1975). The bloom strength was determined at 8 to 10°C using a texture analyzer (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (in grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded. The measurement was performed in triplicate.

Determination of colour

Gelatin gel (6.67%, w/v) was prepared as described previously. Colour of gel samples was determined using a Colorimeter (ColourFlex, HunterLab Reston, VA). CIE L* (lightness), a* (redness/ greenness) and b* (yellowness/blueness) values were measured. Color of the dry gelatin powder was also measured.

Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin were determined according to the method of Pearce and Kinsella (1978), with a slight modification. Soybean oil (2 ml) and gelatin solution (1% protein, 6 ml) were homogenised (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu). EAI and ESI were calculated by the following formulae:

$$EAI\ (m^2/g) = (2 \times 2.303 \times A \times DF)/l\phi C$$

where $A = A_{500}$, DF = dilution factor (100), l = path length of cuvette (m), ϕ = oil volume fraction, and C = protein concentration in aqueous phase (g/m^3);

$$ESI\ (min) = A_0/\Delta A \times \Delta t$$

where A_{500} = absorbance at 500 nm, $\Delta A = A_0 - A1_0$, and Δt = 10 min.

Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined, as described by Shahidi et al. (1995) with a slight modification. Gelatin solution with 1% protein concentration was transferred into 100 ml cylinders. The mixtures were homogenized for 1 min at 13,400 rpm for 1 min at room temperature. The sample was allowed to stand for 0, 30 and 60 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_T / V_o) \times 100$$

$$FS\ (\%) = (V_t / V_o) \times 100$$

where V_T is the total volume after whipping, V_0 is the original volume before whipping, and V_t is the total volume after leaving at room temperature for different times (30 and 60 min).

Statistical analysis

All data were subjected to analysis of variance and differences between means were evaluated by Duncan's multiple range test. For pair comparison, t-test was used (Steel and Torrie, 1980). SPSS statistical program (Version 10.0) (SPSS Inc., Chicago, IL) was used for the data analysis.

RESULTS AND DISCUSSION

Yield

Gelatin was extracted from the skin of striped catfish with yields as shown in Table 1 on a dry weight basis. The gelatin yield recoveries have been reported to vary among fish species, mainly due to the differences in collagen content, the compositions of the skin as well as the skin matrix. The yields of gelatin obtained from fish skin have been reported for sin croaker (14.3 g/100 g), shortfin scad (7.25 g/100 g) (Cheow et al., 2007), big eye snapper (6.5 g/100 g) and brown stripe red snapper (9.4 g/100 g) (Jongjareonrak et al., 2006), Atlantic salmon (15.3 g/100 g) and cod (11.8 g/100 g) by a 2 step extraction (Arnesen and Gildberg, 2007). As shown in Table 1, it was found that the gelatin yield was higher in bleached samples as compared to non bleached samples and gelatin yield increased with increase in defatting changes. H_2O_2 was found to break the hydrogen bond of collagen (Courts, 1961). Donnelly and McGinnis (1977) reported that tissue containing collagen was liquefied through agitation with H_2O_2 for 4 to 24 h. In the presence of sufficient H_2O_2 (5%), hydrogen bonds of collagen molecules in striped catfish skin might be broken, resulting in an increased efficiency in gelatin extraction, as evidenced by the increased yield.

SDS page pattern of gelatin

Protein patterns of gelatin from the skin of striped catfish treated with and without H_2O_2 are as shown in Figure 1a and b, respectively. In general, no differences were seen

Table 1. Yield of gelatin isolated from skin of striped cat fish *(Pangasius hypothalamus*).

Sample	Treatment	Yield (g)
Skin gelatin without bleaching	Control	5.38 ± 0.01
	2 Defatting changes	4.15 ± 0.01
	4 Defatting changes	5.98 ± 0.01
	6 Defatting changes	7.54 ± 0.01
Skin gelatin with bleaching	Control	6.66 ± 0.03
	2 Defatting changes	12.68 ± 0.04
	4 Defatting changes	14.02 ± 0.02
	6 Defatting changes	16.18 ± 0.01

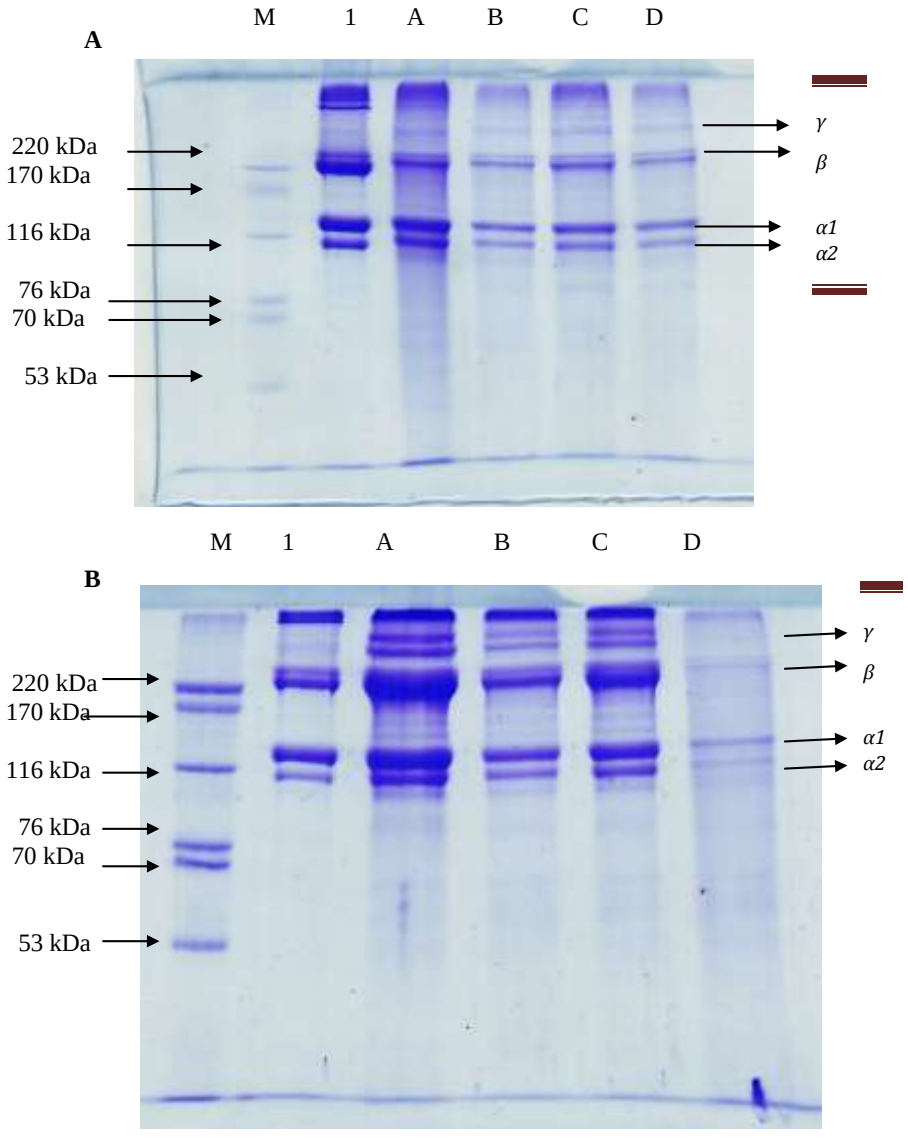

Figure 1. Protein patterns of the skin gelatin from striped catfish (A) with and (B) without H_2O_2 treatment (*Pangasius hypothalamus*). 1: type 1 calf skin collagen, A: gelatin with no defatting, B: gelatin with two defatting changes, C: gelatin with four defatting changes, D: gelatin with six defatting changes. M: Molecular weight markers.

in protein patterns of gelatin under bleached and non bleached conditions. α1- and α2-chains were found as the major components and similar to that of standard collagen type I. Gelatins extracted from both bleached and unbleached skin had smear protein bands with molecular weight equivalent to γ-chain, α-chain and less than α-chain. Proteins with smear bands were generated during extraction. Muyonga et al. (2004) reported that during conversion of collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds obtained. Without bleaching, skin matrix was denser and the conversion of collagen to gelatin was less effective. This suggested that the peroxide decomposition products, such as the hydroxyl radicals and superoxide anion radicals O_2^-, were presumed to destroy H-bond-stabilising α-chains, resulting in increased extractability. H_2O_2 might induce some fragmentation of α-chain, leading to a slightly lower MW (97 kDa). Collagen extracted from both cuttlefish and squid skin composed mainly of α-chains and low content of dimmer (β-components) and higher molecular weight aggregates (γ-components) (Gomez-Guillen et al., 2002; Nagai et al., 2001).

FTIR spectra of gelatin

FTIR spectra of gelatin extracted from skin of striped cat fish with and without bleaching in 5% H_2O_2 for 48 h are as shown in Figure 2a and b, respectively. FTIR spectroscopy has been used to study changes in the secondary structure of gelatin. Spectra of both bleached and non-bleached skin gelatin displayed major bands at 3264 cm^{-1} (amide A, representative of NH-stretching, coupled with hydrogen bonding), 1628 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^-), 1550 cm^{-1} (amide II, representative of NH bending, coupled with CN stretching) and 1240 cm^{-1} (amide III, representative of NH bending). FTIR spectra of striped catfish skin gelatin were similar to those found in other gelatins (Muyonga et al., 2004). Bleaching skin with H_2O_2 resulted in decreases in the intensity of amide A, I, II and III bands of gelatin in case of both bleached and non-bleached skin. These changes are indicative of greater disorder (Friess and Lee, 1996) in gelatin and are associated with loss of triple helix state (Muyonga et al., 2004). Muyonga et al. (2004) reported that the amide I and II peak of collagen extracted from adult Nile perch was at a higher frequency than the young fish skin collagen, due to more intermolecular cross-links in the adult fish collagen. A shift of peaks to lower wave numbers is associated with a lower molecular order (Payne and Veis, 1988). Amide I is the most useful peak for infrared analysis of the secondary structure of protein including gelatin (Surewicz and Mantsch, 1988).

Yakimets et al. (2005) reported that the absorption peak at 1633 cm^{-1} was the characteristic for the coil structure of gelatin. The change in amide I band of gelatin suggested that the use of H_2O_2 might affect the helix coil structure of gelatin. This result suggested that hydrogen peroxide might induce the changes in secondary structure and functional groups of resulting gelatin, associated with the increased intermolecular interactions and denaturation of gelatin.

Bloom strength

The effect of bleaching of cuttlefish skin with H_2O_2 on bloom strength of gelatin gels is shown in Table 2. The lowest bloom strength was observed in gels of the control gelatin when compared with bleached skin gelatins. According to Holzer (1996), the gel strength of commercial gelatin, expressed as bloom value, ranges from 100 to 300 g, but gelatin with bloom values of 250 to 260 g are the most desired. Bleaching 5% H_2O_2 for 48 h resulted in marked increases in bloom strength ($p < 0.05$). Bloom strength of gelatin gel from striped catfish skin increased with increasing defatting changes ($p < 0.05$). The highest bloom strength of gelatin was obtained from skins with six defatting changes followed by bleaching in 5% H_2O_2 for 48 h (316.63 ± 2.94 g). Bloom strength of the resulting gelatins was higher than that of the control. This result suggested that H_2O_2 might induce the oxidation of protein with the concomitant formation of carbonyl groups. These carbonyl groups might undergo Schiff base formation with the amino groups, in which the protein cross-links were most likely formed (Stadtman, 1997). Moreover, OH^- can abstract H atoms from amino acid residues to form carbon-centered radical derivatives, which can react with one another, to form C–C protein cross-linked products (Stadtman, 1997). The larger protein aggregates were mostly associated with the improved bloom strength.

Colour

L*, a* and b*-values of gelatin gels from striped cat fish skin with and without bleaching under different conditions are shown in Table 3. Gelatin gel from skin without bleaching was more pink-purple in colour, as indicated by a lower L*-value, but a higher a*-value, when compared with gelatin gel from bleached skin. Thus, soaking cuttlefish skin in 5% H_2O_2 solution could improve the colour of gelatin gel by increasing L*-value and decreasing a*-value. For fish skin, H_2O_2 treatment resulted in increases in L* and lower a*-value of the resulting gelatin ($p < 0.05$). In general, the control gel from dorsal skins had higher colour intensity than the gel, most likely due to the higher content of chromatophore. Thus, higher H_2O_2 concentration was necessary for

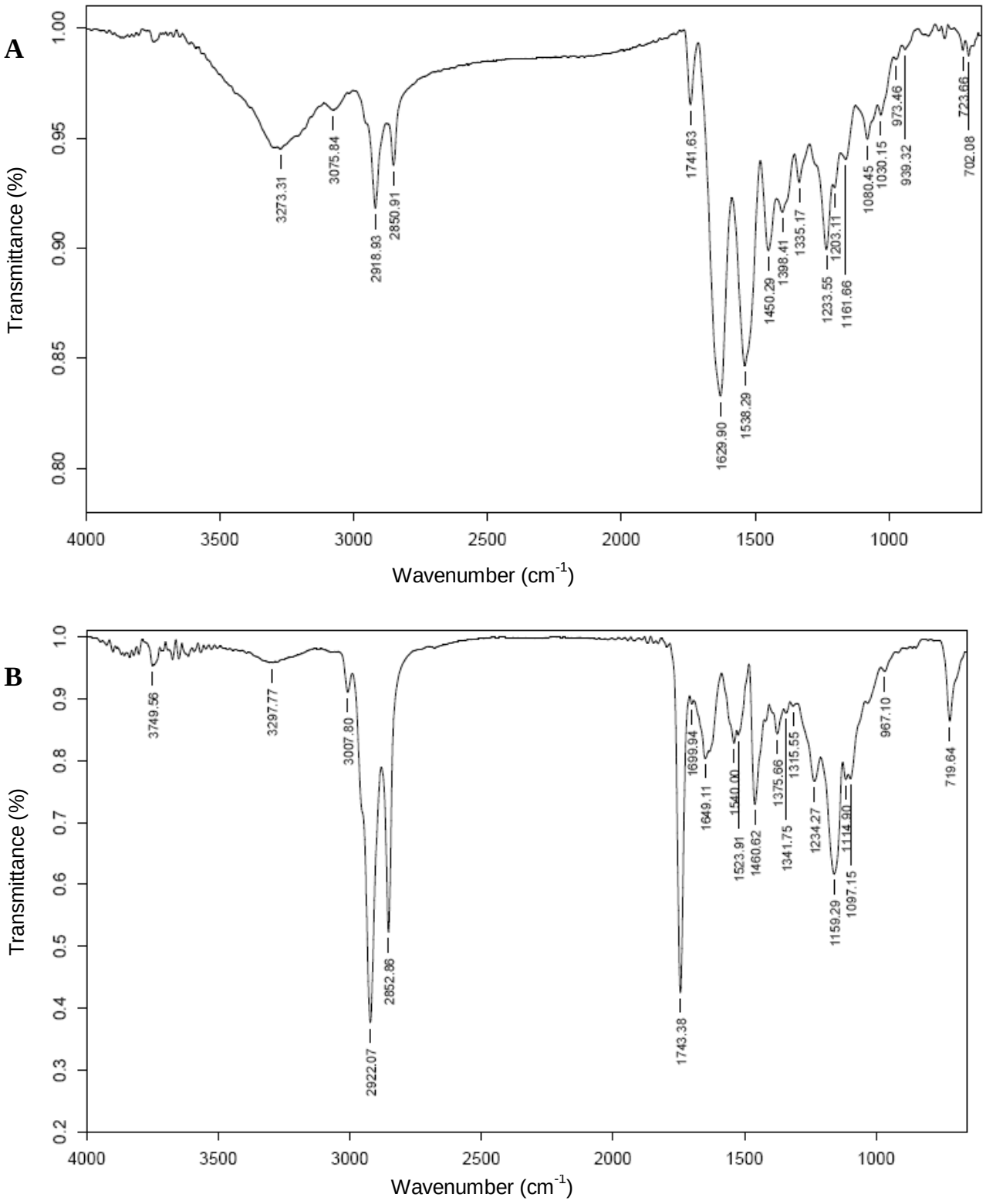

Figure 2. FTIR spectra of gelatin from skin of striped catfish (A) with no and (B) with bleaching.

improvement of colour of gelatin. The fish needs to be bleached, because the flesh could be stained by ink, viscera and colour pigments during handling and processing (Thanonkaew et al., 2008). Oxidising agents,

Table 2. Bloom strength of gelatin extracted from skin of striped cat fish *(Pangasius hypothalamus*) treated with and without H_2O_2.

Sample	Treatment	Bloom strength (g)
Skin w/o bleaching	Control	233.56 ± 6.51^{c}
	2 Defatting changes	237.33 ± 3.91^{c}
	4 Defatting changes	268.37 ± 7.1^{c}
	6 Defatting changes	292.08 ± 8.36^{c}
Skin with bleaching	Control	242.75 ± 4.3^{a}
	2 Defatting changes	294.87 ± 1.87^{a}
	4 Defatting changes	303.10 ± 2.02^{a}
	6 Defatting changes	316.63 ± 2.94^{a}

Data represent mean ± SD of four determinations. Letter in the same column with different superscripts differ significantly ($p<0.05$).

Table 3. Color of gelatin extracted from skin of striped catfish *(Pangasius hypothalamus*) with and without H_2O_2 treatment.

Sample	Treatment	L	A	B
Skin gelatin w/o bleaching	Control	62.69 ± 0.06^{a}	71 ± 0.56^{c}	5.56 ± 0.43^{d}
	2 Defatting changes	66.63 ± 0.02^{a}	-0.57 ± 0.58^{c}	6.48 ± 0.07^{d}
	4 Defatting changes	68.08 ± 0.08^{a}	-0.76 ± 0.72^{c}	7.08 ± 0.11^{d}
	6 Defatting changes	70.14 ± 0.45^{a}	0.24 ± 0.19^{c}	10.41 ± 0.05^{d}
Skin gelatin with bleaching	Control	47.25 ± 0.00^{b}	2.28 ± 0.02^{c}	17.13 ± 0.02^{d}
	2 Defatting changes	68.71 ± 0.01^{b}	-0.64 ± 0.58^{c}	14.11 ± 0.03^{d}
	4 Defatting changes	59.76 ± 0.14^{b}	-0.97 ± 0.58^{c}	14.69 ± 0.10^{d}
	6 Defatting changes	63.30 ± 0.02^{b}	-1.17 ± 0.58^{c}	11.90 ± 0.06^{d}

Data represent mean ± SD of four determinations. Letter in the same column with different superscripts differ significantly ($p<0.05$) while letter in the same row with differ superscripts differ significantly.

derived from the decomposition of hydrogen peroxide, were able to destroy the chromophore. Hydroperoxyl anion is a strong nucleophile which, during bleaching, is able to break the chemical bonds that make up the chromophore. This changes the molecule into a different substance that either does not contain a chromophore, or contains a chromophore that does not absorb visible light (Perkins, 1996). On the other hand, hydroperoxyl and hydroxyl radical (OH^{-}) generated by the decomposition of hydrogen peroxide may induce free radicals, causing the oxidation of protein, changes in protein structure and functional properties of gelatin. As a result, bleached skin contained a low content of chromophore, or still had the chromophore, which was colourless.

Emulsifying properties of gelatin

EAI and ESI of gelatin from striped catfish skin with and without bleaching are shown in Table 4. For gelatin from bleached skin, bleaching using 5% H_2O_2 for 48 h resulted in lower EAI ($p < 0.05$), compared with skin without bleaching. It was presumed that bleaching of fish skin for a long time caused aggregation of protein to a large extent. Aggregated proteins might be rigid and could not unfold rapidly at the interface and form a film around an oil droplet effectively. Emulsions containing gelatin from bleached skin was more stable than that of the control ($p < 0.05$). Larger and longer peptides could stabilise the protein film at the interface more effectively. However, proteins oxidised to a higher degree might possess a lower ability for stabilising emulsions. It was noted that a longer bleaching time and higher H_2O_2 concentration led to a lower ESI of gelatin for all samples, except for gelatin from the control ($p < 0.05$). Surh et al. (2006) found that the oil-in-water emulsion prepared with high molecular weight fish gelatin (~120 kDa) was more stable than that prepared with low molecular weight fish gelatin (~50 kDa). Thickness of an adsorbed gelatin membrane increased with increasing molecular weight. This was associated with the increased stability of emulsions to coalescence during homogenization (Lobo and Svereika,

Table 4. Emulsifying properties. Emulsifying properties of the gelatin extracted from the skin of striped catfish *(Pangasius hypothalamus*) with and without H_2O_2 treatment.

Sample	Treatment	Emulsion activity index (m^2/g)	Emulsion stability index (min)
Skin gelatin w/o bleaching	Control	16.57 ± 0.06^{a}	15.14 ± 0.05^{c}
	2 Defatting changes	15.64 ± 0.21^{a}	18.57 ± 0.55^{c}
	4 Defatting changes	16.16 ± 0.07^{a}	18.28 ± 0.96^{c}
	6 Defatting changes	16.89 ± 0.05^{a}	35.92 ± 3.29^{c}
Skin Gelatin with bleaching (48 h)	Control	11.91 ± 0.06^{b}	16.96 ± 0.16^{d}
	2 Defatting changes	11.21 ± 0.72^{b}	22.18 ± 0.23^{d}
	4 Defatting changes	13.30 ± 0.26^{b}	34.89 ± 2.29^{d}
	6 Defatting changes	11.80 ± 0.27^{b}	31.17 ± 0.76^{d}

Data represent mean ± SD of four determinations. Letter in the same column with different superscripts differ significantly ($p<0.05$) while letter in the same row with different superscripts differ significantly.

Table 5. Foaming properties. Emulsifying properties of the gelatin extracted from the skin of striped catfish *(Pangasius hypothalamus*) with and without H_2O_2 treatment.

Sample	Treatment	Foam expansion (%)	Foam stability (%)	
			30 min	60 min
Skin gelatin w/o bleaching	Control	112.5 ± 10.61^{a}	127.50 ± 10.61^{c}	122.50 ± 10.61^{da}
	2 Defatting changes	127.50 ± 3.54^{a}	112.50 ± 3.54^{c}	107.50 ± 3.54^{da}
	4 Defatting changes	136.50 ± 0.71^{a}	130.50 ± 0.71^{c}	125.50 ± 0.71^{da}
	6 Defatting changes	127.50 ± 3.54^{a}	122.50 ± 3.54^{c}	117.50 ± 3.54^{da}
Skin gelatin with bleaching (48 h)	Control	119 ± 1.41^{b}	115.00 ± 0.00^{d}	110.00 ± 0.00^{ba}
	2 Defatting changes	127.50 ± 3.54^{b}	135.00 ± 0.00^{d}	118.00 ± 0.00^{ba}
	4 Defatting changes	131.50 ± 7.78^{b}	143.50 ± 3.54^{d}	128.00 ± 4.24^{ba}
	6 Defatting changes	142.00 ± 7.07^{b}	151.00 ± 7.07^{d}	138.00 ± 7.07^{ba}

Data represent mean ± SD of four determinations. Letter in the same column with different superscripts differ significantly ($p<0.05$) while letter in the same row with different superscripts differ significantly.

2003). This possibly results from the differences in the intrinsic properties of proteins, composition and conformation of protein between gelatins from different sources (Damodaran, 1997).

Foaming properties of gelatin

FE and FS of gelatin extracted from cuttlefish skin with and without bleaching are shown in Table 5. Gelatin from unbleached skin had a slightly lower FE than gelatin extracted from bleached skin ($p < 0.05$). The foaming ability of proteins is related to their film-forming ability at the air-water interface. In general, proteins, which rapidly adsorb at the newly-created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). At both 30 and 60 min, bleaching with 5% H_2O_2 for 48 h exhibited the highest FS ($p < 0.05$). Gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via inter-bubble gas diffusion contribute to instability of foams (Yu and Damodaran, 1991). Coalescence of bubbles occurs, because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture (Damodaran, 2005). Thus, foam stability could be improved by bleaching the skin of catfish with H_2O_2 under the appropriate conditions.

Conclusion

Bleaching of striped catfish skin with 5% H_2O_2 not only improved the colour of resulting gelatin, but also enhanced the bloom strength effectively. Furthermore, bleaching could increase the yield of gelatin. Bleaching also improved emulsifying and foaming properties of the

resulting gelatin, mostly via the oxidation of the gelatin molecule.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Electrolyte ions and glutathione enzymes as stress markers in *Argania spinosa* subjected to drought stress and recovery

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Understanding the mechanisms underlying *Argania spinosa* responses to drought stress is essential for its regeneration and domestication. Toward that end, an integrative study of tolerance responses to drought stress in four *A. spinosa* ecotypes (2 contrasting coastal ecotypes (Adm and Rab) and 2 contrasting inland ecotypes (Alz and Lks)) have been conducted. Responses to soil drying and re-watering were measured at physiological and biochemical levels. Soil drying resulted in significant increase in leaf concentrations of potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) with differential responses between ecotypes. The glutathione-related enzymes: glutathione peroxidase (GP), glutathione reductase (GR) and glutathione S-transferase (GST) showed a significant increase in their enzymatic activity in *A. spinosa* plants subjected to drought stress. Additionally, a significant increase in thiol protein content in the four ecotypes was recorded, during drought stress. These antioxidant traits responded differently depending on ecotype. However, rapid and significant changes in the studied physiological and biochemical traits were observed during recovery from drought, only after four days. According to the traits having the most discriminating power, the both inland ecotypes, especially Lks ecotype, seem to be potential candidates for regeneration of argan forest and their domestication in arid and semi-arid environments.

Key words: *Argania spinosa*, drought stress, glutathione enzymes, thiol compounds, recovery.

INTRODUCTION

Drought caused by water scarcity and/or the uneven distribution of rainfall is the main abiotic factor limiting

crop productivity worldwide. Currently, drought becomes a global challenge to ensure the survival of agricultural crops and sustainable food production. It is related to almost all aspects of biology. Study of the drought stress has been one of the main directions in global plant biology (Somerville and Dangl, 2000). In the Mediterranean ecosystem, plants are subjected to severe and permanent drought stress, particularly during summer months (Nogués and Baker, 2000). Some plants as argan tree [*Argania spinosa* (L.) Skeels], endemic species to Southwestern part of Morocco, have developed a variety of strategies and mechanisms in response to changes in the environment, especially drought stress (Diaz-Barradas et al., 2010, 2013; Chakhchar et al., 2015a, b or c). Water restriction can lead to morphological, physiological, biochemical and molecular changes. Nonetheless, the drought tolerance is present in almost all plants, but its magnitude varies from one species to another and even within the same species. It causes subtle changes in physiological and biochemical processes of plants. Understanding the metabolic and physiological aspects of drought stress responses in plants is therefore of critical importance.

Drought stress often disturbs the balance of nutrients and electrolyte ions in the plants such as potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). However, high concentrations of these elements in the leaves can mitigate the negative effects of drought stress (Cakmak, 2005; Wu et al., 2013). In addition, the drought stress can alter cell homeostasis through the production of reactive oxygen species (ROS), potentially damaging agents (Parent et al., 2008). When the reduction of oxygen in the chloroplasts is incomplete, the majority of these ROS are generated and oxidative damage may occur (Mittler, 2002; Miller et al., 2010; Gill and Tuteja, 2010). In order to maintain homeostasis and prevent oxidative stress, plants have evolved a defense system included, among others enzymes, the glutathione antioxidant enzymes such as glutathione reductase (GR, EC 1.6.4.2), the glutathione S-transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GP, EC 1.11.1.9) (Noctor et al., 2002; Gill and Tuteja, 2010). Besides these enzymatic antioxidants, other non-enzymatic compounds are considered to be relevant markers of oxidative stress, such as the thiol compounds (Deneke, 2000).

Despite recent studies and our understanding of physiological and biochemical response of argan tree to drought stress (Diaz-Barradas et al., 2010, 2013; Chakhchar et al., 2015a, b or c), information regarding the adaptive mechanisms underlying the regulation of *A. spinosa* metabolism during recovery from drought is scarce. This tree has important socio-economic and ecological roles in South-West Morocco, where it grows in over 800,000 hectares, and in which it also plays a great role in the biodiversity of the forest's ecosystem (Msanda et al., 2005). *A. spinosa* is a potential very important tree species for vegetable oil, which could generate a great interest from the horticultural industry. Also, the argan trees can be exploited for firewood, timber, as a forage for cattle, especially in drought years, and as a shade tree for cereal crops, thereby, supporting the economy of the indigenous population. About 1.3 million people of local population are living in rural areas where traditional sylvo-pastoral systems are based on the argan tree (Chaussod et al., 2005).

For a better understanding of how *A. spinosa* ecotypes differ in their tolerance to drought stress and recovery, this study was planned to better characterize some drought tolerance traits through an integrative analysis of physiological and biochemical responses to drought in *A. spinosa*. Therefore, these questions were asked: (1) how physiological and biochemical mechanisms vary under drought and recovery conditions; (2) how these responses to drought and recovery conditions vary between the studied ecotypes; and (3) how could discriminateion be made between these ecotypes in terms of tolerance degree using the studied adaptive traits.

MATERIALS AND METHODS

Plant and experimental design

Sampling of seeds of *A. spinosa* was conducted in four regions of the argan tree forest in South-West Morocco. Climatic, geographical and hydrological conditions of these four regions are markedly different (Chakhchar et al., 2015a, b). Two contrasting coastal ecotypes (site: Rabia (Rab) and Admine (Adm)) and two contrasting inland ecotypes (site: Aoulouz (Alz) and Lakhssas (Lks)) were chosen for a better interpretation of the mechanisms regulating biochemical and physiological processes. The protocol of cultivation and experimental layout was the same used previously (Chakhchar et al., 2015a, b, c). Uniform young *A. spinosa* plants of similar height, aged 29 months, were selected for the experiment for each ecotype. The effect of prolonged drought stress by cessation of irrigation for 15 and 30 days followed by rehydration during 4 days were simulated. The environmental conditions in chamber during the experiment were maintained at 28 ± 1°C temperatures during day and 25 ± 1°C during night in a 16:8 photoperiod and the rate of relative humidity varied between 65 and 70%. The average maximum photosynthetically active radiation (PAR) was approximately 400 µmol $m^{-2}s^{-1}$ ensured by a combination of incandescent and fluorescent lamps.

Soil moisture content

Pots were randomized to each treatment to determine the soil moisture content. Soil samples were taken 5 cm deep without plant residues, weighed (fresh weight) and dried in an oven at 80°C for

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72 h before measuring the dry weight. Moisture content of the soil sample was expressed in percent according to the formula:

Soil moisture content (%) = [(wet weight - dry weight) / dry weight] × 100.

Physiological traits

Endogenous content of ions (Mg^{2+}, K^{+} and Ca^{2+})

Leaf material collected was carefully rinsed with deionized water and the fresh weight of each sample was determined. Then, the leaf material was calcined at 600°C for 6 h and the dry weight of each sample was measured. Each sample was ground into a fine powder and digested with concentrated nitric acid (HNO_3) overnight at 120°C. Samples were then dissolved in (1:1, v/v) HNO_3/$HClO_4$ (perchloric acid) to 220°C, resuspended in 5% (v/v) HNO_3 and analyzed for the determination of Mg^{2+}, K^{+} and Ca^{2+} content using inductively coupled argon plasma emission spectrometry. Ion contents were expressed in mmol/g DM. Three independent measurements per treatment (one repetition per plant) were opted for.

Biochemical traits

Glutathione enzymes extraction

Fresh leaves samples from control and treated plants were immediately ground to a fine powder in a mortar in the presence of liquid nitrogen. Enzymes were extracted on ice by homogenizing the powder (0.1 g for each enzyme × 5 replicates per treatment) in 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing 0.1 mM EDTA, 1% (w/v) polyvinyl pyrrolidone (PVP), 0.1 mM phenylmethanesulfonyl fluoride solution (PMSF) and 0.2% (v/v) Triton X100, 1 mM dithiothreitol and 20 mM ascorbate. There were 5 replicates per treatment (one plant per replicate).

Total soluble protein concentration for determination of the specific activities of the enzymes was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard. All spectrophotometric analyses were conducted on a Jenway (6305 UV/Vis. England) spectrophotometer.

Glutathione reductase activity (GR)

Glutathione reductase (GR; EC 1.6.4.2) was assayed by monitoring the ß-nicotinamide adenine dinucleotide 2'- phosphate (NADPH) oxidation coupled to the reduction of GSH at 340 nm using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ (Edwards et al., 1990). Reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM NADPH, 1 mM oxidized glutathione (GSSG) and 0.1 ml of enzyme extract. GR activity was expressed in nmol oxidized GSSG per min per mg of proteins. There were 5 replicates per treatment (one plant per replicate).

Glutathione-S-transferase activity (GST)

GST activity (EC 2.5.1.18) was measured in a reaction mixture containing 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5), 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 0.1 ml of enzyme extract. The reaction was initiated by the addition of 1 mM GSH and the formation of 2, 4-dinitrophenyl-S-glutathione (DNP-SG) was followed at 340 nm according the method described by Habig et al. (1974) and Habig and Jacoby (1981). GST activity was expressed in nmol GSH per min per mg of proteins using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ for the compound formed. There were 5 replicates per treatment (one plant per replicate).

Glutathione peroxidase activity (GP)

GPX activity (EC 1.11.1.9) was determined by adopting a coupled assay with GR to follow the GSH oxidation according the method described by Nagalakshmi and Prasad (2001). Reaction mixture contained 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5), 10 mM Na_2-EDTA, 1 M NaCl, 10 mM GSH, 2 mM NADPH and 2.5 mM H_2O_2. Reaction was initiated by addition of 5 µl of GR (500 units/2.8 ml) and 0.1 ml of the enzyme extract. The consumption of NADPH was measured at 340 nm for 5 min. GPX activity was expressed in nmol NADPH oxidized per min per mg of proteins using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ for NADPH. There were 5 replicates per treatment (one plant per replicate).

Thiol compounds content

Aliquots of the fine powder plant material were homogenized in 50 mM Tris-HCl (pH 8.0) containing 20 mM EDTA. Homogenates were centrifuged for 20 min at 15,000 × g and supernatants were used for thiol assays. Thiol compounds content was determined by the method described by Nagalakshmi and Prasad (2001).

For determination of total thiols content, 0.2 ml of supernatant were mixed with 0.8 ml of 0.2 M Tris-HCl (pH 8.2) and 0.05 ml of 0.01 M dithionitrobenzoic acid (DTNB, dissolved in methanol). Mixture was brought to 3 ml by addition of absolute methanol. After incubation for 15 min at room temperature, the absorbance was measured at 412 nm. Total thiols content was estimated using an extinction coefficient of 13.1 $mM^{-1}cm^{-1}$. For non-protein thiols content, 0.5 ml of supernatant was mixed with 0.8 ml of distilled water and 0.2 ml of 50% (w/v) trichloroacetic acid. After incubation for 15 min incubation under stirring, the homogenates were centrifuged at 15,000 × g for 15 min. Supernatant (0.25 ml) was mixed with 1 ml of 0.4 ml M Tris-HCl (pH 8.9) and 0.025 ml of 0.01 M DTNB. After a second incubation for 15 min, the absorbance was measured at 412 nm against a reagent blank. Thus, protein thiols content was calculated by subtracting the non-protein thiols content from the total thiols content. The results were expressed in µmol/g. There were 5 replicates per treatment (one plant per replicate).

Statistical analysis

Each data pointed the mean of five separate replicates and mean values and standard deviations were calculated. Results were examined by the three-way analysis of variance (ANOVA) in order to test the effect of ecotype, time, watering regime and their interactions in each of the physiological and biochemical study variables (traits). Means were compared using the Tukey's Post hoc test. A Pearson correlation analysis was done for some variables for each ecotype. A canonical discriminant analysis (CDA), by entering all independent variables into the equation at once, was performed on the four contrasting *A. spinosa* to determine which variables discriminated between them. Statistical analyses were conducted using SPSS version 17.

RESULTS

Soil moisture content was affected by the cessation irrigation of *A. spinosa* plants. Significant differences were recorded in the level of soil moisture content in the four contrasting ecotypes (Figure 1). After 30 days of

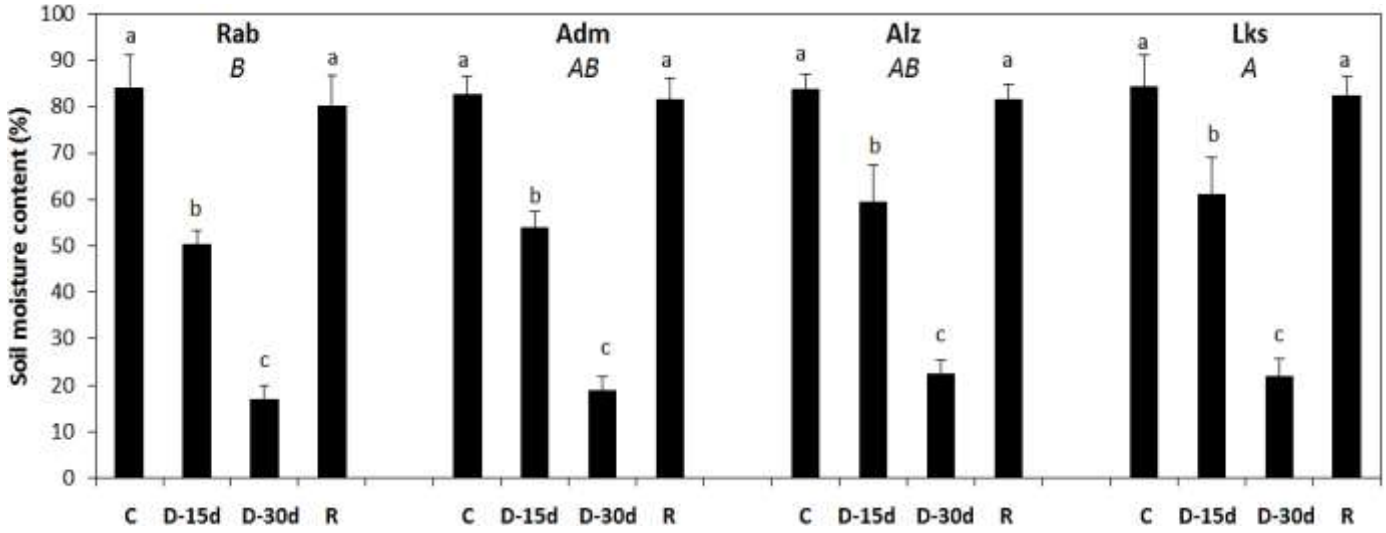

Figure 1. Soil moisture content recorded under drought stress and rehydration conditions. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

withholding watering, soil moisture content decreased very significantly compared to the control (80.0, 77.3, 73.3 and 74.1% in Rab, Adm, Alz and Lks, respectively). This physiological-edaphic trait evolved similarly in the four ecotypes during the drought and rehydration periods. Indeed, significant differences among these four ecotypes have not been registered. The rehydration of pots containing argan plants quickly restored the level of the soil moisture content only after four days (Figure 1). According to multivariate analysis of variance, the ecotype × watering regime interaction was not considered statistically significant for this trait ($P = 0.014$).

Withholding of water to *A. spinosa* plants induced a significant accumulation in leaf concentrations of inorganic ions K^+, Ca^{2+} and Mg^{2+} ($P < 0.001$) (Figure 2a, b and c). After 30 days of withholding watering, the percentage of K^+ accumulation varied between 45 and 61%, while Mg^{2+} varied between 47 and 91% in the four contrasting ecotypes. Adm and Rab ecotypes showed the highest accumulation of K^+ (61.0%) and Mg^{2+} (91.9%), respectively. However, Ca^{2+} concentration has been doubled even tripled in the leaves of stressed plants in some ecotypes compared to control plants. The highest accumulation of Ca^{2+} was noted in Alz ecotype. In addition, significant differences in the concentrations of these three inorganic ions in leaves of the control plants were recorded ($P < 0.001$), which Lks ecotype showed the greatest constitutive concentration of K^+ and Ca^{2+}. Thus, both inland ecotypes (Alz and Lks) showed the highest constitutive concentration of Mg^{2+}. During rehydration period, the leaf concentrations of K^+, Ca^{2+} and Mg^{2+} quickly decreased significantly, by referring to the levels noted under drought conditions, to achieve concentrations close to those recorded in control conditions (Figure 2a, b and c). The kinetics of recovery was different for the three inorganic ions depending on the ecotype. According to multivariate analysis of variance, the ecotype × watering regime interaction was statistically significant for all these three ions ($P < 0.001$).

The activity of glutathione enzymes GP, GR and GST was significantly increased in the leaves of plants subjected to drought stress ($P < 0.001$) (Figure 3a, b and c). In the four contrasting ecotypes studied, a significant stimulation of these enzymes depending on the stress prolongation was found. Induced activities of these enzymes glutathione revealed ecotype-dependent differences. However, ecotypic differences regarding the constitutive activity except for GST have not been recorded. After 15 days of withholding watering, the activities of these three enzymes have been shown to be higher in both inland ecotypes paralittoraux than both coastal ecotypes. Whereas after 30 days of withholding watering, GP activity increased significantly by approximately 12.8, 13.7, 20.9 and 20.3% in Rab, Adm, Alz and Lks, respectively (Figure 3a), the activity of GR increased by approximately 36.6, 33.0, 37.8 and 33.2% in Rab, Adm, Alz and Lks, respectively (Figure 3b) and the significant increase of GST activity was estimated to be 65.6, 79.6, 70.5 and 70.0% in Rab, Adm, Alz and Lks, respectively (Figure 3c). However, after 4 days of rehydration, the specific activity of these enzymes decreased significantly in the four ecotypes suggesting significant recovery kinetics of glutathione enzymes in *A. spinosa*. Using the multivariate analysis of variance, the ecotype × watering regime interaction was judged statistically significant only for the GR ($P < 0.001$) and GP

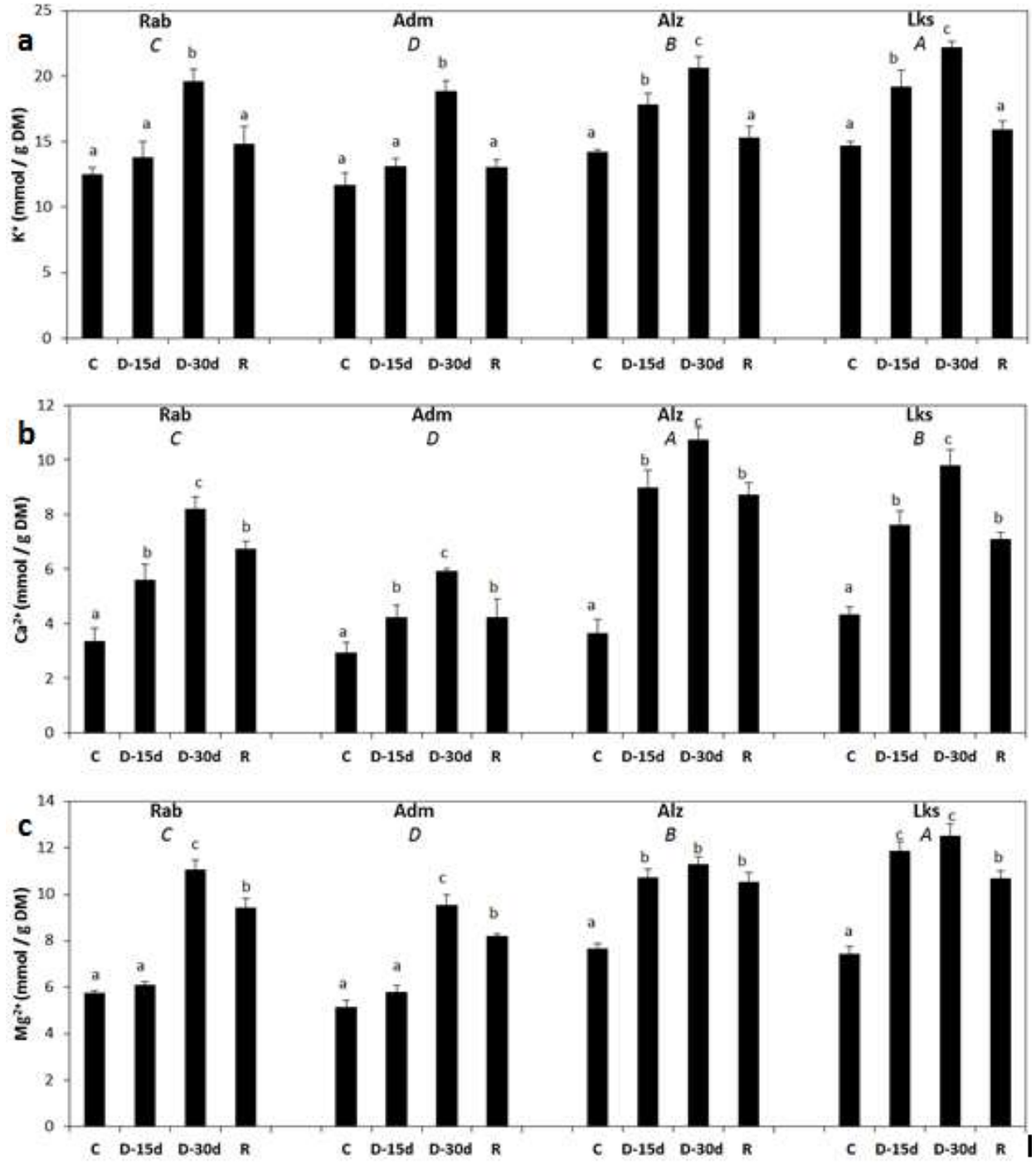

Figure 2. Effect of drought stress and rehydration conditions on leaf concentrations of K^+ (a), Ca^{2+} (b) and Mg^{2+} (c) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

(P = 0.007).

Drought stress caused significant increase in total thiols content in the leaves of argan tree plants (P < 0.001) (Figure 4). After 30 days of withholding watering, this significant increase was estimated at 31.5, 41.2, 13.5 and 18.2% in Rab, Adm, Alz and Lks, respectively. The increase of total thiols content in the four contrasting ecotypes was mainly due to the significant increase in

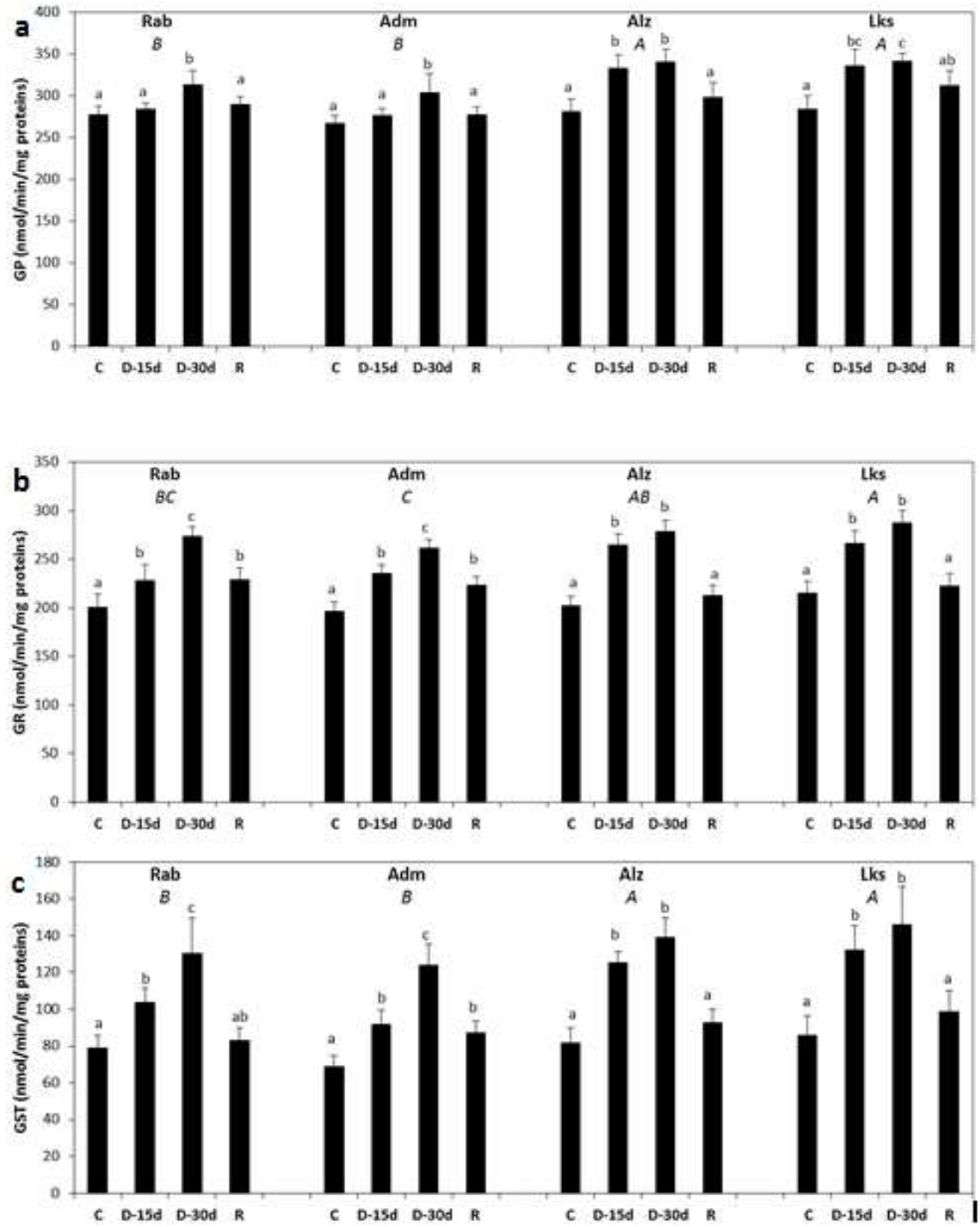

Figure 3. Effect of drought stress and rehydration conditions on enzymatic activity of GP (a), GR (b) and GST (c) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

protein thiols under drought conditions (Figure 4). The rate of these proteins is one of the relevant markers of oxidative stress. This increase recorded in stressed plants was 2 to 4 times higher compared to control plants.

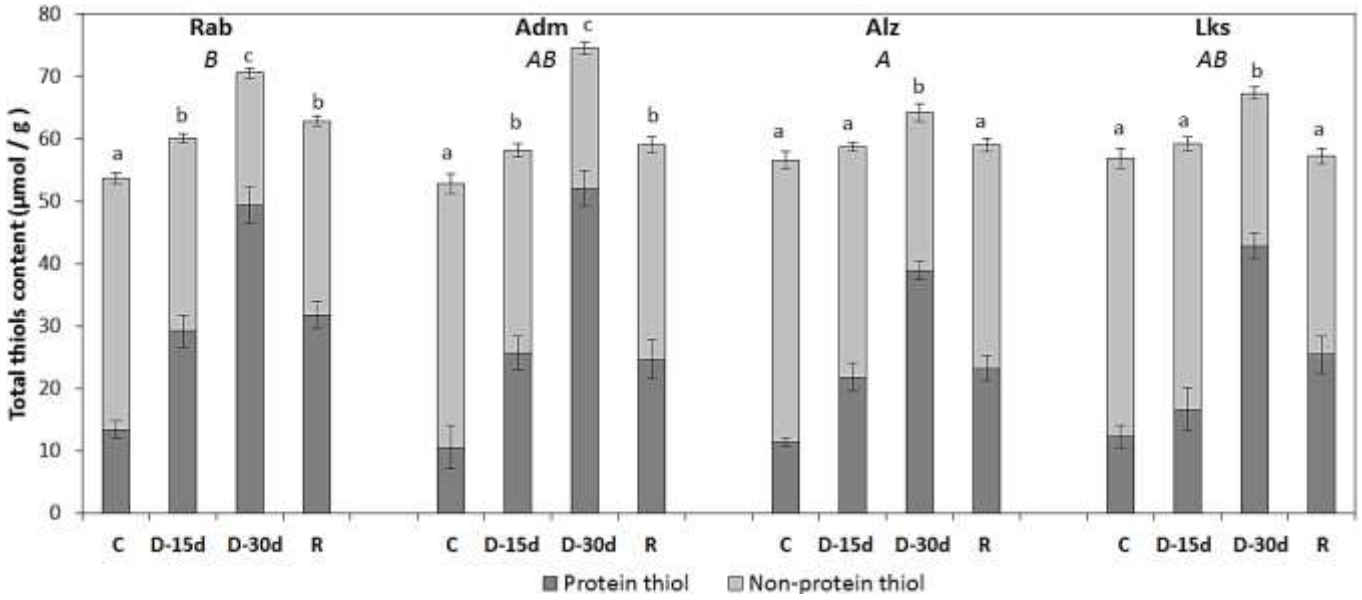

Figure 4. Effect of drought stress and rehydration conditions on thiol compounds content in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

Table 1. Statistical characteristics of the discriminant functions extracted from CDA.

Parameter	Discriminant function	Eigen value	Variance (%)	Cumulative (%)	Canonical correlation
CDA_1	1	2098.64	98.6	98.6	1.00
	2	19.49	0.9	99.5	0.98
	3	11.12	0.5	100.0	0.96
CDA_2	1	490.34	96.4	96.4	0.99
	2	16.12	3.2	99.6	0.97
	3	2.26	0.4	100.0	0.83

However, the non-protein thiols content decreased significantly during the drought stress period ($P < 0.001$). This decrease was estimated at 47.3, 46.8, 43.8 and 45.4% in Rab, Adm, Alz and Lks, respectively. Under control conditions, significant differences were noted among ecotypes especially for the non-protein thiols content ($P < 0.001$) and total thiols ($P < 0.002$). After the rehydration phase, levels converging to those noted in the control plants were recorded (Figure 4). The recovery kinetics of these thiol compounds were significant depending on the ecotype. Following the multivariate analysis of variance, ecotype × watering regime interaction revealed a statistically significant difference in these three compounds ($P < 0.001$).

Canonical discriminant analysis

The results obtained of the CDA confirmed the existence of differences in global characteristics of ecotypes (Table 1). Wilk's lambda denoted a high significance of the both selected models (CDA_1 for drought stress after 30 days (Figure 5) and CDA_2 for rehydration conditions (Figure 6)) ($P \leq 0.001$). The first two discriminant functions (DF) accounted for approximately 99.5 and 99.6% for CAD_1 and CDA_2, respectively. The null hypothesis of discriminant functions is tested using χ^2-test. The canonical correlations for the first two functions in each model were highly significant.

The canonical plot of CDA_1 (Figure 5) showed a clear separation of the four contrasting ecotypes taking into account both first functions. Based on the standardized coefficients of the canonical discriminant functions, the non-protein thiols content and the concentration of Ca^{2+} were highly weighted in the positive part of DF1 whereas the GST activity and the concentration of Mg^{2+} were strongly weighted to negative part. The non-protein thiols

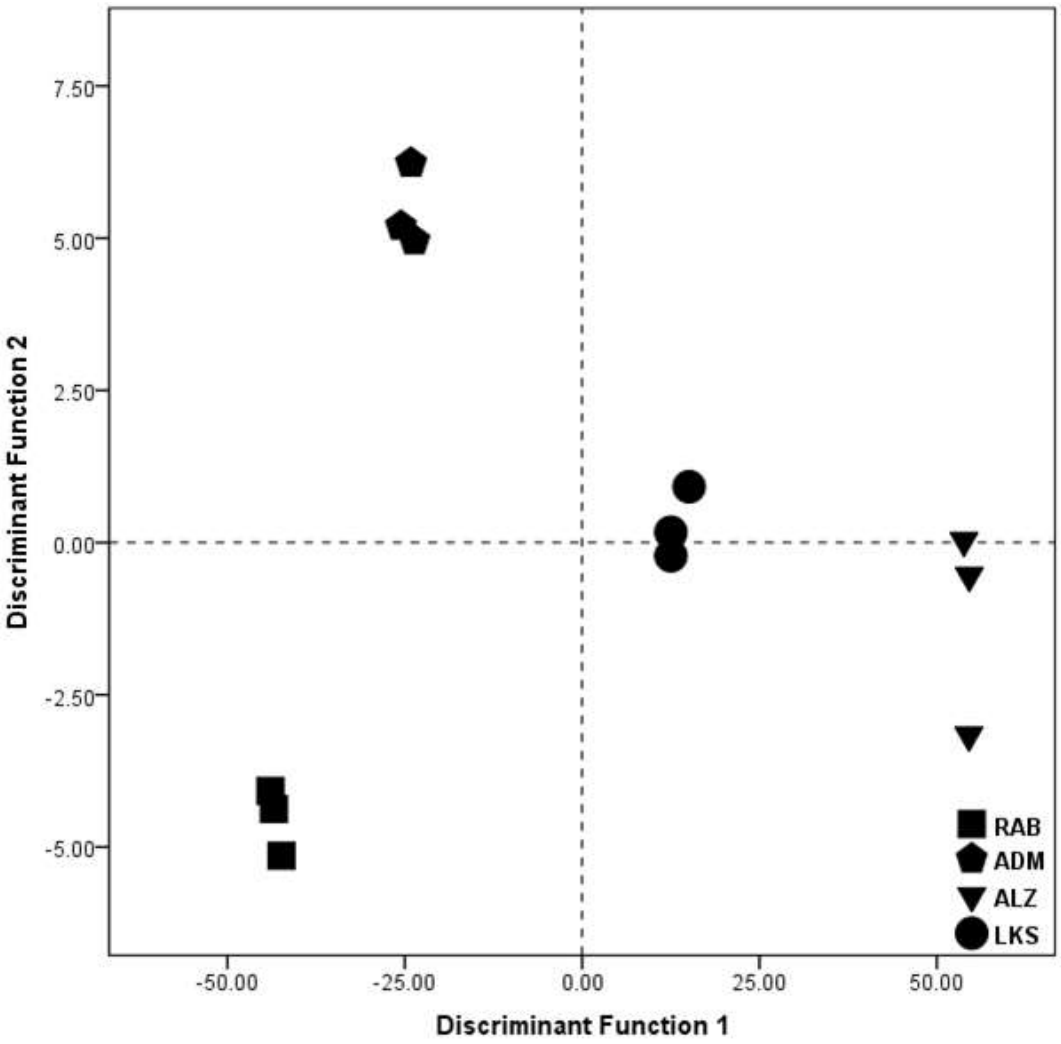

Figure 5. 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits under drought stress conditions.

content and the GR activity were highly weighted in the positive part of DF2, while the both GST and GP activities were greatly weighted in the negative part. Concerning CDA_2 (Figure 6), the concentration of Ca^{2+} and the GR activity were highly weighted in the positive part of the first DF, while the protein thiols content and the concentration of K^+ were highly weighted in negative part. The both protein thiols and non-protein thiols contents were strongly weighted in the positive part of DF2; however, both GP and GST activities were highly weighted in negative part.

The first DF in both analyses (drought stress and rehydration) contributed mostly to distinguish between coastal ecotypes (Rab and Adm) and inland ecotypes (Lks and Alz). However, the second DF clearly separated between both coastal ecotypes on the one hand and secondly between both inland ecotypes, according the CDA_1 and CDA_2, respectively.

DISCUSSION

Behavioral responses of plants to drought stress are complex and different mechanisms are adopted when they are subjected to this water constraint. Drought stress induces a number of physiological, biochemical and molecular reactions that regulate plant growth and productivity.

The results of the soil moisture content revealed a similar soil water status for all ecotypes. However, the drought prolongation significantly affected this trait. The reduction of soil moisture content is an obvious consequence of withholding watering and existence of the plant in the pot. This trait plays an important role in the hydrological and biological processes. In arid and semi-arid areas, soil water is a critical factor that affects plant growth and can thus determine plant distribution models (Engelbrecht et al., 2007). At the same time,

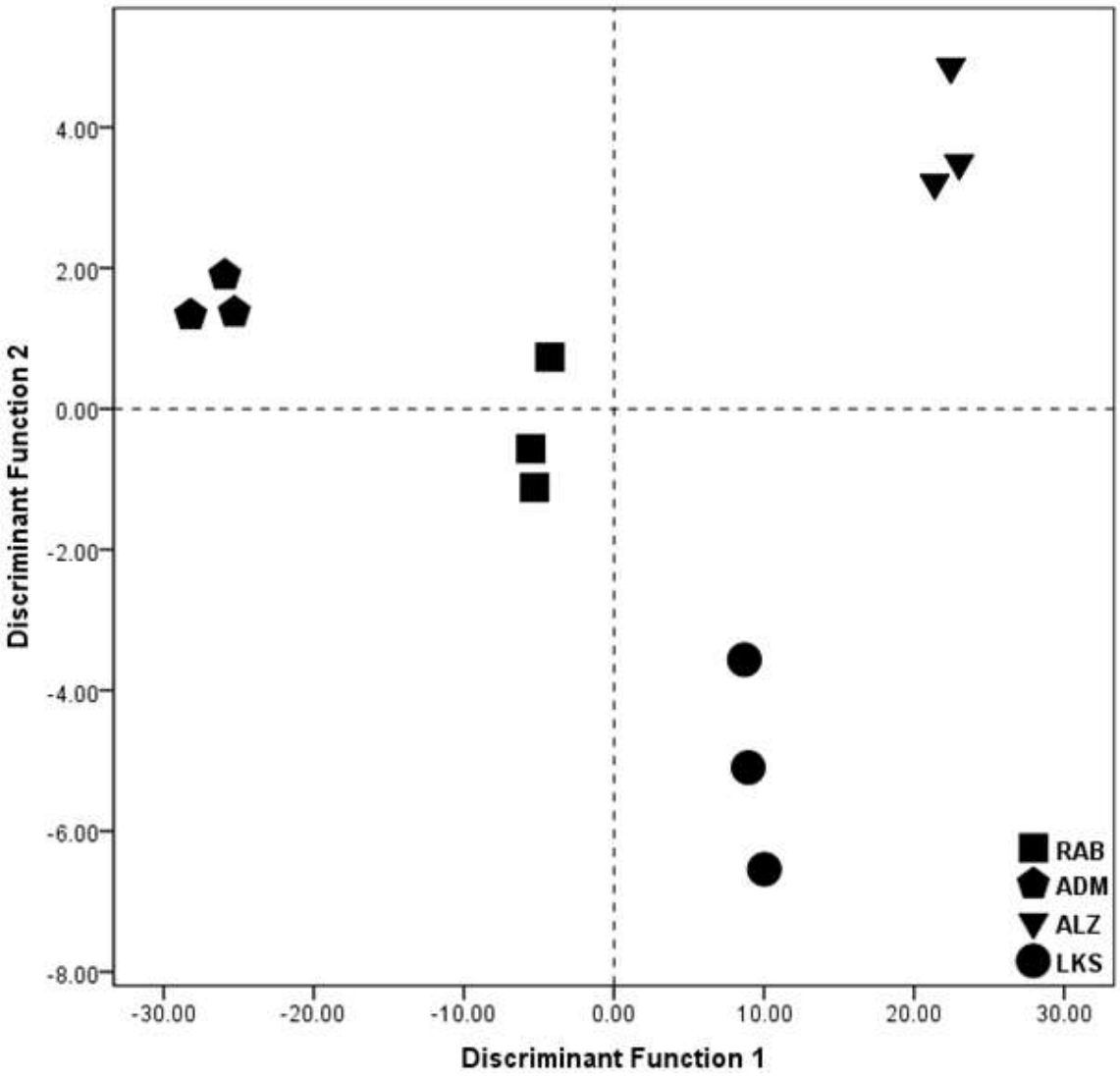

Figure 6. 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits under rehydration conditions.

plants affect the soil moisture by forming a biological pathway for the water transport from the soil to the atmosphere by their root system (Wang et al., 2010). When the seasonal or interannual drought occurs, the photosynthesis and transpiration responses in the ecosystem strongly depends on the total amount of water stored in the soil, such that the plants have the ability to extract water and push it towards the aerial parts, hence the ability to withstand stress-induced by depletion of moisture and on the decrease of soil water potential (Jipp et al., 1998).

The accumulation of various ions during drought stress period is of great interest. The *A. spinosa* plants showed significant accumulation in leaf concentrations of inorganic ions K^+, Ca^{2+} and Mg^{2+} after withholding watering. As a necessary element for the physiological and molecular processes, Ca^{2+} plays an important role in the regulation of plant metabolism, growth and development. Yuan-Yuan et al. (2009) reported that Ca^{2+} can improve the hydrophobicity of the cell membrane, while reducing its permeability by direct effects as a structural basis to ensure a plant resistance to drought. K^+, the famous osmoticum, was strongly accumulated in stressed *A. spinosa* plants. Patakas et al. (2002) also reported a significant accumulation of K^+ in vine plants subjected to drought conditions and showed the contribution of this ion in the adjustment of osmotic potential. In addition to its role as an osmotic substance in maintaining osmotic balance and osmoregulatory strategy of plants, the increase in K^+ concentration can also lead to increased stomatal conductance since it plays an important role in the regulation of stomatal oscillations (Mahajan and

Tuteja, 2005; Nasri et al., 2008). A significant accumulation of the foliar concentration of Mg^{2+} in stressed plants during drought stress period was also recorded. Mg^{2+} is an essential macronutrient for plant growth, because it allows the activation of over 300 enzymes (as cofactor) and the synthesis of organic molecules necessary for the growth of plants (Wilkinson et al., 1990).

In fact, the high foliar concentrations of K^+, Ca^{2+} and Mg^{2+} recorded in stressed argan plants can be suggested as a physiological mechanism of tolerance that their role is to mitigate the negative effects of drought. In terms of comparison, both inland ecotypes seem be more tolerant considering their constitutive high concentrations of these ions. After rehydration, leaf concentrations of K^+, Ca^{2+} and Mg^{2+} were significantly reduced, by referring to the concentrations noted in drought stress period, before reaching levels converging to those noted in the control plants. The argan plants showed an alternative and quicker strategy to promote drought tolerance by accumulating sufficiently high levels of inorganic ions. Indeed, the energy cost of the osmotic adjustment using inorganic ions is much lower than that of the use of organic molecules synthesized in cells (Hu and Schmidhalter, 1998; Patakas et al., 2002). High concentrations of these elements in the leaves can help *A. spinosa* plants to overcome the negative effects of drought.

The glutathione-related enzymes GP, GR and GST showed a significant increase in their activity in *A. spinosa* plants subjected to drought stress. GR is a key enzyme in the ascorbate-glutathione cycle that protects cells against oxidative damage by maintaining a high ratio of GSH/GSSG. This enzyme has been shown to be activated in response to abiotic stress (Anderson and Davis, 2004). In our study, drought stress induced a significant increase in GR activity reflecting its important role in H_2O_2 scavenging according to Asada-Halliwell pathway in plant cells (Noctor et al., 2002). This response is intended to maintain a high ratio of $NADP^+$/NADPH, thus ensuring $NADP^+$ availability to accept electrons of photosynthetic electron transport chain and facilitate the regeneration of ascorbate oxidized (Noctor et al., 2002; Yang et al, 2008). Sofo et al. (2005) reported a significant increase in GR activity proportional to the severity of drought stress in plant leaves of four interspecific hybrids of *Prunus*.

GST isoenzymes in plants are known to function in the detoxification of herbicides, hormonal homeostasis, phytohormones transport, vacuolar sequestration of anthocyanins, tyrosine metabolism, detoxification of hydroperoxides, the regulation of apoptosis as well as in plant responses to biotic and abiotic stress (Dixon et al., 2002, 2010; Gill and Tuteja, 2010). Some GST isoforms exhibit activity of glutathione peroxidase, which suggests that their primary function may be to reduce toxic lipid peroxidation products and the maintenance of the membrane integrity under stress conditions, for example: osmotic stress (Dixon et al., 2003). In this study, a significant increase was detected in GST in plants of four contrasting ecotypes during drought stress confirming the protective role of GST in the argan tree.

Furthermore, the glutathione peroxidase activity increased significantly under drought stress conditions in plant leaves of all ecotypes. As GR and GST, the GP is a large family of various isoenzymes that use glutathione to reduce H_2O_2 and organic and lipid hydroperoxides, and therefore, protect plant cells against oxidative stress (Noctor et al., 2002). The overexpression of GP was found to improve abiotic stress tolerance in transgenic plants (Gill and Tuteja, 2010). The activity levels of GR, GST and GP appear to link directly to the degree of stress. Their activities increased in parallel with the increase of drought intensity and subsequently they reduced during rehydration. This response could limit cellular damage caused by ROS during drought stress period. Down regulation of these enzymes observed in rehydrated plants is probably due to a reduced need for the elimination of ROS.

During drought stress period, the content of total thiols in the leaves of *A. spinosa* plants increased significantly in the four ecotypes. This increase is mainly due to the significant increase in thiol protein content. The rate of protein thiols is a relevant marker of oxidative stress. However, the non-protein thiol content decreased significantly under drought stress conditions. The thiols are antioxidants that act through various mechanisms (Deneke, 2000). High levels of glutathione and other thiols have been associated with an increased tolerance to oxidative stress under abiotic stress conditions (Szalai et al., 2009; Nazar al., 2011). According to our results, the increase of the total thiols content could have signed the occurrence of oxidative stress and reflected the antioxidant capacity of thiols in the argan tree. These thiols seem to efficiently allocate antioxidant defense system in the argan tree under drought stress conditions. After rehydration, the decrease of the protein thiols can be explained by the fact that the thiol groups are oxidized by reducing free radicals and these denatured proteins are removed.

A good separation between the four contrasting ecotypes of *A. spinosa* was obtained taking into account the canonical plots (Figures 5 and 6). The vertical separation in the four analyses was established by the first DF which it quantifies the greatest degree to which all ecotypes differ in their physiological and biochemical traits. This has allowed us to make a connection between the studied traits and the registered differences among ecotypes in their tolerance to drought stress. According to the canonical plot, the first discriminant function has separated the inland ecotypes from the coastal ecotypes, while the second discriminant function has been attributed to distinguish between both coastal ecotypes on one hand and another hand between both the inland

ecotypes under drought and rehydration conditions, respectively. Both inland ecotypes (Lks and Alz) were clearly separate from both coastal ecotypes (Adm and Rab), under drought conditions, especially by high concentration of non-proteins thiol and Ca^{2+} and high GST activity. These traits reflected a significant antioxidant capacity and good membrane stability, justified by the high Ca^{2+} content, in both inland ecotypes. This permits us to suggest that the inland ecotypes studied are more tolerant to drought stress than coastal ecotypes. During rehydration conditions, both inland ecotypes have shown good recovery, compared to other ecotypes, but in different way. In fact, these both ecotypes have reacted differentially under rehydration condition. Taking into account the second discriminant function and the traits having the most discriminating power: GR and GST; Lks ecotype seems to possess a very effective antioxidant defense system ensuring a good and quick recovery.

Conclusion

The results obtained in this study in pots showed significant differences in physiological and biochemical responses of the four contrasting ecotypes of *A. spinosa*. These ecotypes experimentally exposed to edaphic drought induced by cessation of irrigation followed by rehydration in order to evaluate the potentialities and the flexibility of tolerance in the argan tree. Intra-specific differences were observed in the traits referring to the ionic state, antioxidant system and oxidative damage. Indeed, the drought stress induced significant changes in the studied traits. However, the kinetics of recovery in *A. spinosa* plants after rehydration was rapid; it was manifested by significant levels close to those recorded in the control plants only after four days. According to the canonical discriminant analysis, the inland ecotypes, especially Lks, were clearly distinguished from others ecotypes by high antioxidant capacity switch connected with the high activity of glutathione system enzymes. In terms of degree of drought tolerance, Lks ecotype showed a better upregulation of its protective mechanisms compared to other ecotypes. The research results are constructive for contribution to select tolerant ecotypes in order to develop the Argan sector.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular detection of disease resistance genes to powdery mildew (*Blumeria graminis* f. sp. *tritici)* in wheat (*Triticum aestivum*) cultivars

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A study was conducted to detect the presence of disease resistance genes to infection of wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) in selected wheat cultivars from China using molecular markers. Genomic DNA of sixty cultivars was extracted and tested for the presence of selected prominent resistance genes to the fungal disease using molecular markers linked to *Pm* genes. Results showed that 17 cultivars were detected with *Pm2* gene, 24 cultivars were detected with *Pm4b* gene, two cultivars were detected with *Pm6* gene while 24 wheat cultivars were detected with *Pm8* gene. Multiple genes were also detected in the study. Cultivars Xinxuan2039, Lankao008 and Zhengmai366 were detected with possible multiple *Pm2+Pm4b+Pm8* genes while Yumai368 was detected with possible multiple genes *Pm2+Pm4b+Pm6*. The results of this study provide a significant contribution to breeding for resistance to wheat powdery mildew disease since the identified cultivars detected with *Pm* genes will contribute to further studies on improving wheat resistance to the disease. Also, the continued resistance of cultivars with designated resistance genes demonstrates that the responsible *Pm* genes are still effective in overcoming powdery mildew infections.

Key words: *Blumeria graminis* f. sp. *tritici*, wheat powdery mildew, disease resistance, *Pm* genes, *Triticum aestivum*, molecular marker.

INTRODUCTION

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici (Bgt)*, is one of the most damaging foliar diseases of wheat in most parts of the world, especially in cool and humid areas (Cao et al., 2013; Li et al., 2013; Peng et al., 2014; Mandal et al., 2015; Wang et al., 2015). Countries such as China, United Kingdom, Germany, Japan, Russia, South and West Asia, North and East Africa and the Southeastern United States are

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highly affected by the damage caused by the disease (Bennet, 1984; Alam et al., 2011). The disease causes devastating effects both in grain yield and quality (Everts et al., 2001; Conner and Kuzyk, 2003; Asad et al., 2014). The affected area for powdery mildew from 2001 to 2006 in China was 5.9 to 9.4 million hectares resulting in 0.26 to 0.32 billion kilograms reduction in grain yield (Xue et al., 2009).

Currently, the yield losses range from 13 to 34% under high infestation in the field but during epidemic seasons, the disease can cause up to 50% yield loss (Yao et al., 2007; Zhang et al., 2008; Li et al., 2011; Alam et al., 2013; Quijano et al., 2015; Yu et al., 2015). The shift in pathogenic virulence structures range from the southwest and the southeast coastal regions of China to almost all the wheat growing areas throughout the country and the damage exceeds any other known wheat disease (Liu and Shao, 1994; Luo et al., 2009; Cao et al., 2013; Zhao et al., 2013; Shen et al., 2015).

In an attempt to control the diseases, use of resistant or tolerant cultivars has been one of the effective methods widely used by scientists as it is economical as well as environmentally friendly (Song et al., 2009; Ben-David et al., 2010). Several wheat cultivars are being developed and tested for resistance to wheat powdery mildew but their resistance is easily broken down due to continuous development of new and more virulent strains through genetic recombination (Piarulli et al., 2012; Hurni et al., 2014). Some of the developed cultivars carrying resistance genes to wheat powdery mildew lose their resistance even before they are made available for commercial production (Hao et al., 2015; Ma et al., 2015). It is, therefore, important that the development of resistant cultivars should target multiple resistances to disease isolates so that effective and lasting control can be attained. Assessment of disease resistance on new wheat cultivars can be effective when the cultivars are subjected to existing as well as new strains of powdery mildew isolates over a considerable period of time (Li et al., 2012).

In developing resistant cultivars, several powdery mildew resistance genes have been identified and mapped in wheat. The mapping has helped in locating and subsequent introgression of the genes into susceptible cultivars carrying other desirable traits (Huang and Roder, 2004; Xiao et al., 2013; Li et al., 2014). To date, about 78 formally designated *Pm* genes (*Pm1–Pm54, Pm1c = Pm18*, *Pm1e = Pm22*, *Pm4c = Pm23*, *Pm21 = Pm31*) have been catalogued at 50 loci (Hao et al., 2015; Petersen et al., 2015). Among them, loci *Pm1*, *Pm3*, *Pm4*, *Pm5* and *Pm24* have 5, 17, 4, 5 and 2 alleles, respectively (Xie et al., 2012; Mohler et al., 2013; McIntosh et al., 2013, 2014).

Sources of these genes have been the cultivated or wild relatives of *Triticum* species and then they got transferred to common wheat. For example, resistance gene *Pm2* originated from the wild species *Ae. tauschii* that got introgressed into common wheat *T. aestivum* (Lutz et al., 1995). Powdery mildew resistance gene *Pm4a* originated from *T. diccocum* while *Pm4b* originated from *T. carthlicum* (Briggle, 1969; Law and Wolfe, 1966). Resistance gene *Pm6* was transferred from *T. timopheevii* (Jorgensen and Jensen, 1972). Another distant wild cultivar, *S. cereale*, is where resistance gene *Pm8* came from (Hsam and Zeller, 1997) while gene *Pm21* and *Pm30* came from wild relatives *H. villosa* and *T. dicoccoides*, respectively (Chen et al., 1995; Liu et al., 2002). These genes are common in cultivars grown in Asian region and gene *Pm21* has been very effective against a broad-spectrum of wheat powdery mildew isolates found in China (Song et al., 2009). Currently, over 56 cultivars being grown in China possess *Pm4b* and *Pm8* genes (Wang et al., 2005; Zeng et al., 2014), while most red soft winter cultivars carrying gene *Pm8* are widely grown in southeastern USA (Cowger et al., 2009; Hao et al., 2012).

This study, therefore, was aimed at detecting the presence of some prominent wheat powdery mildew resistance genes in selected wheat cultivars using molecular markers linked to the genes.

MATERIALS AND METHODS

Sixty wheat cultivars from different parts of China were identified for the study (Table 1). Ten seeds of each cultivar were sown on trays in greenhouse located at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. Around 15 days after sowing, when three to four leaves had been developed, seedling leaves were detached and their DNA was extracted following cetyltrimethylammonium bromide (CTAB) extraction method (Zheng, 2010). Chancellor, a susceptible cultivar with no known *Bgt* resistance genes, was used as negative control.

Seven additional cultivars with known wheat powdery mildew resistant genes were selected and used as positive controls (Table 2). Hence, a total of 67 cultivars were selected for the molecular study using various molecular markers linked to wheat powdery mildew resistance genes. To test their purity, the extracted DNA was run on 2% Agarose gel of 1% TAE (Tris-Acetate-EDTA) buffer solution and the image was captured using Gel Documentation and Image Analysis System after staining in ethidium bromide solution for 10 min.

Following extraction and dilution to a concentration ranging between 50 and 80 ng/µl, the genomic DNA samples were amplified in a polymerase chain reaction (PCR) machine using the following mixture: 5 µl containing PCR Master mix, 2 µl of double distilled water, 1 µl reverse primer and 1 µl of forward primer for SSR markers (or as described in Table 3 for non-SSR markers). Genomic DNA amounting to 1 µl was added, making up a total of 10 µl of the PCR reaction mixture. A drop of paraffin oil was, thereafter, added to prevent the reaction mixture from evaporation during the amplification.

PCR protocol was done using Bio-Gener Technology, Gene explorer PCR machine as follows: 94°C for 3 min, 35 cycles of 94°C for 40 s, a range of 55 to 61°C depending on primer annealing temperature (Table 3) for 30 s, 72°C for 40 s and a final extension of 72°C for 10 min before soaking at 4°C. The PCR products were, thereafter, run on 2% Agarose gel immersed in 1% TAE (Tris-Acetate-EDTA) buffer solution and the image was captured using Gel Documentation and Image Analysis System after staining in

Table 1. Names of wheat cultivars, their pedigree information and origin.

Cultivar designation	Name of cultivar	Pedigree information*	Origin
S	Chancellor	Carina/Mediterranean//Dietz/ Carina/3/ P-1068/3×Purplestraw	-
1	Tian0015	-	Gansu
2	Longjian101	8487/85-173-12-2	Gansu
3	Tian96-86	863-13/8560-2-2-1	Gansu
4	Tian03-160	0037-1-2 / 9938-2-2-1	Gansu
5	Yujiao0338	-	Henan
6	Tian00296	9362-13-3-4/8748-0-2-1	Gansu
7	Zhengnong01059	-	Henan
8	Xinyumai836	-	Henan
9	Tian03-142	9589-8-1-2-1/Qing 95-111	Gansu
10	Tian00127	(Baidatou/C184-3-4-1)F2//85-173-4	Gansu
11	Tian9681	863-13/87148-1-1-2-2-2	Gansu
12	Lantian093	Lantian23/Zhou92031	Gansu
13	AvocetYrA	Avocet	USA
14	Pu02056	Zhoumai16/ Yumai24	Henan
15	Tian01-104	93R177 / 912-2-1-2	Gansu
16	Tian02-195	Wenmai8/Tian96-1c1	Gansu
17	05bao1-1	Zhongliang22+ gDNA of oil sunflower	Gansu
18	Tian02-204-1	Wenmai8/ 9157-3-2-2-1	Gansu
19	Lantian095	-	Gansu
20	Longjian127	7402/Lv419//7415	Gansu
21	Tian989	9362-13-4-4/lantian1	Gansu
22	Punong1	-	Henan
23	Longjian102	Lin87-4535/81168-4-3//Longyuan932	Gansu
24	Tian98101	9362-13-4-4/Tian94-3	Gansu
25	N. Strampelli	LIBERO//S.Pastou/C.Jrometh.lig	Italy
26	Zhongliang 27	90293///Zhongliang12/Zhongsi// Bulgaria10/Xiannong4	Gansu
27	03bao1-1	Lantian10+ DNA of oil sunflower	Gansu
28	Zhongzhi4	Mianyou2/Zhongzhi1	Beijing
29	Lantian097	92R137/87-121 //Shan167	Gansu
30	Zhongzhi1	Shan167/C591	Beijing
31	Zhoumai19	Neixiang185 / Zhoumai9	Henan
32	Kenya Kongoni	C18154/2×Fr/2/Romm/3/WIS.245-II-50-7/C8154/2/2×Fr	USA
33	Keyuan5	-	Henan
34	Xinmai19	(C5/xinxiang3577) F3d1s/Xinmai9	Henan
35	Lantian20	CappelleDesprez/Lantian10	Gansu
36	Yumai368	-	Henan
37	Taikong06	Space-flight mutation from Yumai49	Henan
38	Guoan368	-	Henan
39	Guomai301	G883/ Pumai9	Henan
40	Yangao03710	-	Henan
41	Zhou99233	-	Henan
42	Zhongzhi2	Shan167/ Guinong22 / *T. Spelta* album	Beijing
43	Longchun26	Yong3263/Gaoyuan448	Gansu
44	Xinxuan2039	-	Henan
45	Lantian23	SXAF4-7/87-121	Gansu
46	Zhoumai32	Zhoumai12/ Wenmai6 // Zhoumai13	Henan
47	Zhongxin01	-	Henan
48	Zhongyu885	-	Henan
49	04zhong70	-	Henan
50	Zheng366	-	Henan

Table 1. Contd.

51	Lankao008	-	Henan
52	Tianmin198	R81/Bainong64//Yanzhan4110	Henan
53	Zhengyumai9989	Benyumai21/Yumai2//Yumai57	Henan
54	Zhengmai9023	[Xiaoyan6/Xinong65//83(2)33/84(14)43] F3/3Shan213	Henan
55	Zhengmai366	Yumai47/PH82-2-2	Henan
56	Zhoumai16	Zhoumai9/Zhou8425B	Henan
57	Yanzhan4110	[(C39/Xibei78(6)9-2) /(FR81-3/ Aizao781-4)] /Aizao781-4	Henan
58	Bainong160	Duokang893/Wenmai6//Bainong64/Wenmai6	Henan
59	Lantian15	Lantian10 /Ibis	Gansu

*Cultivars with a dash (-) indicate that their information could not be traced.

Table 2. Wheat cultivars used as positive control and their known powdery mildew resistance genes.

Cultivar designation	Wheat cultivar	Known resistance gene present
1	Ulka/8Cc	*Pm2*
2	Khapli/8Cc	*Pm4a*
3	Armada	*Pm4b*
4	Coker 747	*Pm6*
5	Kavkaz	*Pm8*
6	Yangmai 5/Sub.6V	*Pm21*
7	5P27	*Pm30*

Table 3. Description of molecular markers used for *Bgt* resistance gene identification.

Marker	Type of marker	Gene	Marker Sequence	Chromosomal location	Annealing Temp. (°C)	Reference
Whs350-1 Whs350-Re	STS	*Pm2*	AGCTGTTTGGGTACAAGGTG GCCATCGTTTTCTACTAG	5D	58	Mohler and Jahoor 1996
Xgwm356 F Xgwm356 R	SSR	*Pm4a*	AGCGTTCTTGGGAATTAGAGA CCAATCAGCCTGCAACAAC	2A,6A,7A	55	Luo et al., 2005
P1 P2	STS	*Pm4b*	ACGAGTGATGCTCCAGGATATGG GATCCACCTTTTCCTTGACAAGC	2A	61	Luo et al., 2005
Pm6 L Pm6 R	STS	*Pm6*	GCTCCGAAGCAAGAGAAGAA TCTGCTGGTCCTCTGATGTG	2B	58	Ji et al., 2008
Pm8 L Pm8 R	SCAR	*Pm8*	GGAGACATCATGAAACATTTG CTGTTGTTGGGCAGAAAG	1B	55	Mohler et al., 2001
Pm21 C Pm21 D	SCAR	*Pm21*	CACTCTCCTCAAACCTTGCAAG CACTCTCCTCCACTAACAGAGG	6A	61	Luo et al., 2005
Xgwm159 F Xgwm159 R	SSR	*Pm30*	GGGCCAACACTGGAACAC GCAGAAGCTTGTTGGTAGGC	5B, 5D	60	Liu et al., 2002

ethidium bromide solution for 15 min.

Seven molecular markers of the following types: Three Sequence Tagged Sites (STS) markers, two Simple Sequence Repeats (SSR) markers and two Sequenced Characterized Amplified Region (SCAR) Markers were used for the study (Table 3).

RESULTS

Among the sixty wheat cultivars, the results showed that 17 cultivars contained possible *Pm2* gene (Figure 1). The cultivars included Tian0015, Yujiao0338, Tian00127, Zhengnong01059, Xinyumai836, Pu02056, Tian01-104, Lantian095, Punong1, Zhoumai19, Yumai368, Guomai301, Xinxuan2039, Zhongyu885, Lankao008, Zhengyumai9989 and Zhengmai366. Positive control wheat cultivar, Ulka/8Cc was used to determine the size of the gene and it showed that the resistance *Pm2* gene was detected with a molecular weight of 480 bp (Figure

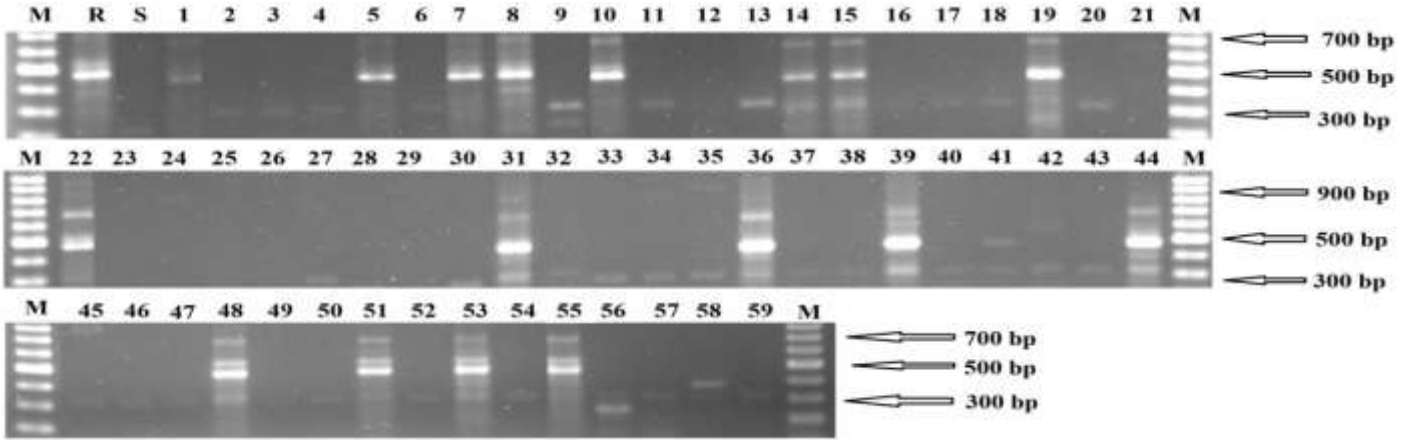

Figure 1. Wheat powdery mildew gene *Pm2* as identified in the wheat cultivars at 480 bp. Lanes 1-59 represent cultivars designation. R is resistant cultivar Ulka/8Cc, S is susceptible cultivar Chancellor. M is a 100 bp DNA ladder.

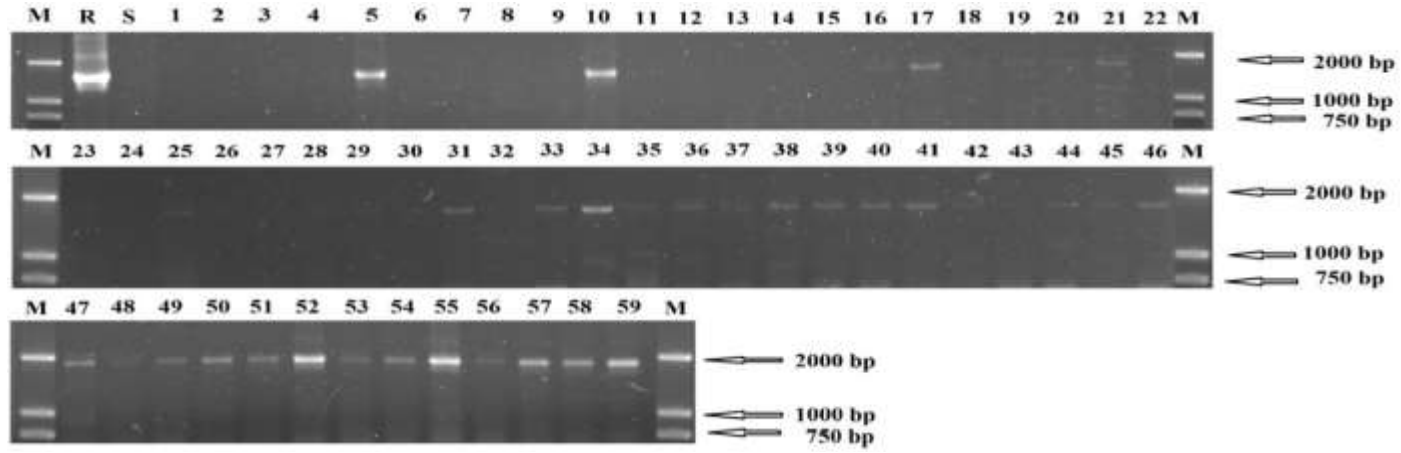

Figure 2. Wheat powdery mildew gene *Pm4b* as identified in the wheat cultivars at 1800 bp. Lanes 1-59 represent cultivars designation. R is resistant cultivar Armada, S is susceptible cultivar Chancellor. M is a D2000 DNA ladder.

1). This was confirmed due to the absence of the alleles in the susceptible cultivar Chancellor.

A total of 24 wheat cultivars were detected with possible wheat powdery mildew *Pm4b* gene. The cultivars were; Yujiao0338, Tian00127, 05bao1-1, Zhoumai19, Keyuan5, Xinmai19, Yumai368, Guoan368, Guomai301, Yangao03710, Zhou99233, Xinxuan2039, Zhongxin01, Zhoumai32, 04zhong70, Zheng366, Lankao008, Tianmin198, Zhengyumai9989, Zhengmai9023, Zhengmai366, Yanzhan4110, Bainong160 and Lantian15 (Figure 2). Positive control cultivar, Armada detected the gene as having a molecular size of 1800 bp when a D2000 DNA ladder was used (Figure 2).

Only two wheat cultivars; Yumai368 and Guomai301 were detected with possible *Pm6* gene. The gene had a molecular size of 140 bp detected in cultivar Coker747, which was used as positive control (Figure 3).

A total of 24 wheat cultivars were detected with possible *Pm8* gene. Among them were cultivars Tian0015 and Tian00127, which both had faint but positive alleles for *Pm8* gene. Other wheat cultivars were 05bao1-1, Tian01-104, Lantian095, Tian989, Punong1, Xinmai19, Lantian20, Taikong06, Guoan368, Guomai301, Yangao03710, Zhou99233, Longchun26, Xinxuan2039, Zhongyu885, 04zhong70, Lankao008, Zhengmai9023, Zhengmai366, Zhoumai16, Yanzhan4110 and Lantian15 (Figure 4). The gene was detected using a positive control cultivar Kavkaz and it had a molecular size of 1300 bp.

Multiple genes were also observed in some wheat cultivars during the study. For example, four cultivars; Tian00127, Xinxuan2039, Lankao008 and Zhengmai366 were detected with three possible multiples genes for *Pm2*, *Pm4b* and *Pm8* while one cultivar, Yumai368 had a possible combination of three multiple genes for *Pm2*,

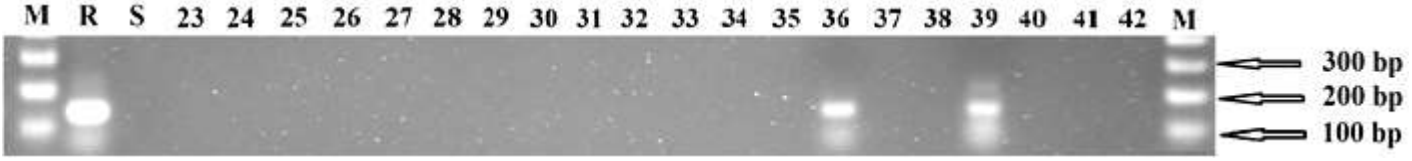

Figure 3. Powdery mildew gene *Pm6* as identified in the tested cultivars at 140 bp. Lanes 23-42 represent cultivars designation. R is resistant cultivar Coker 747, S is susceptible cultivar Chancellor. M is a 100 bp DNA ladder.

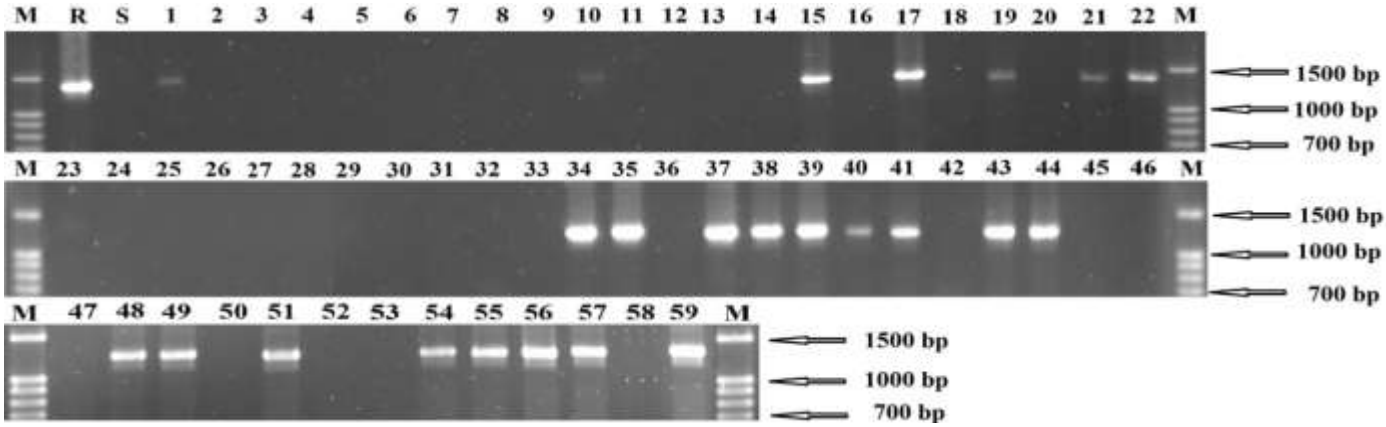

Figure 4. Powdery mildew gene *Pm8* as identified in the tested cultivars at 1300 bp. Lanes 1-59 represent cultivars designation, R is resistant cultivar Kavkaz, S is susceptible cultivar Chancellor, M is a 100 bp DNA ladder.

Pm4b and *Pm6*. Sixteen cultivars were detected with two possible multiple wheat powdery mildew resistance genes (Table 4).

Overall, 22 wheat cultivars did not show presence of any of the seven powdery mildew genes. Among them were; Longjian101, Tian96-86, Tian03-160, Tian00296, Tian03-142, Tian9681, Lantian093, AvocetYrA, Tian02-195, Longjian127, Tian02-204-1, Longjian102, Tian98101, Strampelli, Zhongliang27, 03bao1-1, Zhongzhi4, Lantian097, Zhongzhi1, Kenya kongoni, Zhongzhi2 and Lantian23 (Table 4).

The molecular study showed that *Pm4b* and *Pm8* were the powdery mildew genes present in the highest number of wheat cultivars. A total of 24 wheat cultivars carried these genes. Molecular marker detected possible *Pm2* in 17 cultivars while *Pm6* was the lowest detected in two cultivars. Three powdery mildew resistance genes *Pm4a*, *Pm21* and *Pm30* were not detected in any of the tested cultivars using the molecular markers (Figure 5).

DISCUSSION

In this study, 17 cultivars were detected with possible *Pm2* gene. This resistance gene had been widely used and had been highly effective in different parts of Europe and China years ago (Liu et al., 2000). Though the growing number of virulent strains has been reported in some parts of China, the gene occurs in high frequency in so many commercial cultivars as it is easily transferred from resistant to susceptible cultivars (Parks et al., 2008; Gao et al., 2012). Recently, Ma et al. (2015) reported the presence of a new gene *Pm2b* in the cultivar KM2939, a Chinese breeding line, which exhibits high resistance to powdery mildew at both the seedling and adult stages. This gene was reported to carry a single dominant powdery mildew resistance allele of *Pm2*, designated as *Pm2b* and was mapped on chromosome 5DS, rendering the previous allelic designation *Pm2* to be re-designated as *Pm2a*.

Resistance genes *Pm4b* and *Pm8* were genes detected in highest frequency among the cultivars used in the present molecular study. This is consistent with the finding reported by Li et al. (2012) that these genes are the most widely distributed among wheat cultivars in China. *Pm8* cultivars have been grown worldwide since the 1980s and due to wide use, their effectiveness has since declined (Parks et al., 2008; Tang et al., 2014; Zeng et al., 2014). Several virulent strains of powdery mildew have emerged rendering the gene ineffective. In fact, it is one of the major reasons that an epidemic boom of wheat powdery mildew was recorded in China in the early 1990s (Graybosch, 2001; Ryabchenko et al., 2003).

Among the 59 cultivars, only two cultivars Guomai301 and Yumai368 were detected with possible *Pm6* gene apart from other multiple genes also suspected to be present in the cultivars. *Pm6* has been widely and successfully used in breeding for wheat powdery mildew

Table 4. *Bgt* resistance genes detected using molecular markers.

Cultivar designation	Name of cultivar	Detected *Bgt* resistance genes using molecular markers
S	Chancellor	Susceptible (no gene)
1	Tian0015	*Pm2+Pm8*
2	Longjian101	-
3	TianTian96-86	-
4	Tian03-160	-
5	Yujiao0338	*Pm2+Pm4b*
6	Tian00296	-
7	Zhengnong01059	*Pm2*
8	Xinyumai836	*Pm2*
9	Tian03-142	-
10	Tian00127	*Pm2+Pm4b+Pm8*
11	TianTian9681	-
12	Lantian093	-
13	AvocetYrA	-
14	Pu02056	*Pm2*
15	Tian01-104	*Pm2+Pm8*
16	Tian02-195	-
17	05bao1-1	*Pm4b+Pm8*
18	Tian02-204-1	-
19	Lantian095	*Pm2+Pm8*
20	Longjian127	-
21	Tian989	*Pm8*
22	Punong1	*Pm2+Pm8*
23	Longjian102	-
24	Tian98101	-
25	Strampelli	-
26	Zhongliang27	-
27	03bao1-1	-
28	Zhongzhi4	-
29	Lantian097	-
30	Zhongzhi1	-
31	Zhoumai19	*Pm2+Pm4b*
32	Kenya Kongoni	-
33	Keyuan5	*Pm4b*
34	Xinmai19	*Pm4b+Pm8*
35	Lantian20	*Pm8*
36	Yumai368	*Pm2+Pm4b+Pm6*
37	Taikong06	*Pm8*
38	Guoan368	*Pm4b+Pm8*
39	Guomai301	*Pm2+Pm4b+Pm6+Pm8*
40	Yangao03710	*Pm4b+Pm8*
41	Zhou99233	*Pm4b+Pm8*
42	Zhongzhi2	-
43	Longchun26	*Pm8*
44	Xinxuan2039	*Pm2+Pm4b+Pm8*
45	Lantian23	-
46	Zhoumai32	*Pm4b*
47	Zhongxin01	*Pm4b*
48	Zhongyu885	*Pm2+Pm8*
49	04zhong70	*Pm4b+Pm8*
50	Zheng366	*Pm4b*

Table 4. Contd.

51	Lankao008	*Pm2+Pm4b+Pm8*
52	Tianmin198	*Pm4b*
53	Zhengyumai9989	*Pm2+Pm4b*
54	Zhengmai9023	*Pm4b+Pm8*
55	Zhengmai366	*Pm2+Pm4b+Pm8*
56	Zhoumai16	*Pm8*
57	Yanzhan4110	*Pm4b+Pm8*
58	Bainong160	*Pm4b*
59	Lantian15	*Pm4b+Pm8*

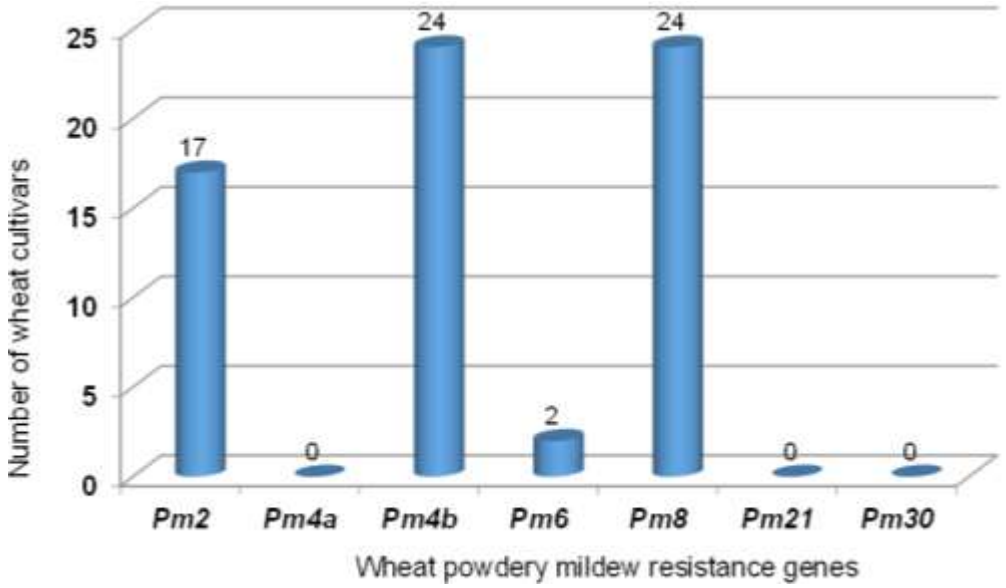

Figure 5. Total number of wheat cultivars detected with wheat powdery mildew resistance genes.

resistance for long time. The gene usually exhibits its best expression from the third leaf stage of wheat growth and thereafter, it is moderately effective, but recognizable at the seedling stage (Bennett, 1984; Qin et al., 2011; Li et al., 2014). Virulence matching the *Pm6* gene has occurred in many regions but still, the gene remains effective in some areas, especially when other genes such as *Pm2* and *Pm4b* are combined during their use (Cai et al., 2005; Costamilan, 2005; Shi et al., 2009; Purnhauser et al., 2011). This could be one of the possible reasons why the two cultivars were resistant during the study (data not presented) as the genes such as *Pm2*, *Pm4b* and *Pm8* were also possibly present in these cultivars.

Conclusion

This study detected 24 cultivars likely carrying *Pm4b* and *Pm8* while two cultivars carried likely *Pm6* gene. Despite the recent findings on these genes indicating that they are being overcome by emerging virulent isolates, their abundant availability in the currently produced wheat cultivars signifies their continued contribution to wheat production in China. The cultivars detected with these genes can still be useful in various breeding programs for disease resistance. The detection of likely multiple resistance genes in some wheat cultivars in this study showed that where multiple genes were involved in conferring resistance to powdery mildew disease, the cultivars could effectively resist infection from isolates that would have otherwise caused virulence if a single gene was involved.

Conflict of interest

The authors have not declared any conflicts of interest.

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Full Length Research Paper

Isolation and characterization of heavy metal tolerant bacteria from Panteka stream, Kaduna, Nigeria and their potential for bioremediation

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Panteka stream is a flowing stream polluted with wastes from the activities of mechanics. Water samples collected at different points of the stream were analysed in order to determine the level of heavy metal contamination and bacteria diversity with the view to elucidating the bioremediating potentials of the bacteria isolates. Four bacteria, tolerant to heavy metals, were isolated from Panteka stream. These were identified by morphological and biochemical techniques as *Staphylococcus epidermidis, Serratia marcescens, Proteus mirabilis* and *Escherichia coli.* The 16S rRNA gene sequencing and Basic Local Alignment search tool (BLAST) result confirmed *E. coli* and *Staphylococcus* spp. as heavy metal tolerant bacteria. Heavy metal tolerance analysis of the isolates exposed to nickel, zinc, lead, cadmium and iron showed that the isolates had maximum tolerance to the four heavy metal. Studies on bioremediation potential of the isolates to heavy metals in the stream revealed that mixed bacteria culture completely removed lead, nickel, zinc and cadmium. Analysis of pure isolates revealed *S. epidermidis* to be the most effective in removing lead (100%), nickel (100%), cadmium (90.29%), zinc (84.95%) and iron (54.82%). The results obtained from this study show that all four bacteria species isolated from Panteka stream have potential for bioremediation of heavy metals in contaminated water.

Key words: Panteka stream, mechanic workshop, heavy metals, bacteria isolates, bioremediation.

INTRODUCTION

The recent expansion of human industrial activity, including mining, smelting, and synthetic compound creation, has led to an exponential increase in the amounts of heavy metals released into the atmosphere, water, and soil (McConnell and Edwards, 2008). Many countries have regulatory guidelines for heavy-metal presence and exposure as well as remediation and treatment options. Screening of soil and water sources is conducted frequently to prevent overconsumption, but many of these programs and technologies are not readily available in developing nations (Li et al., 2006).

The term toxic heavy metal have particular application to cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As), all of which appear in the World Health

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Organisation's list of 10 chemicals of major public concern. Other examples include manganese, chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se), silver (Ag), antimony (Sb) and thallium (Ti). These heavy metals are non-degradable and must be reduced to acceptable limits before discharging into environment to avoid threats to living organisms (Alam et al., 2012).

The challenge of ensuring usable water in sufficient quantities to meet the needs of human and ecosystems emerged as one of the primary issues of the 21st century (Lawford et al., 2003). For example, inadequate water supply and poor water quality give rise to health and other societal issues, limit agricultural productivity and economic prosperity, and pose national security risk in some countries (Nwidu et al., 2008). The effect is damaging not only to individual species and populations but also to the natural biological communities and it accounts for the deaths of more than 14,000 people daily (WHO, 2007).

The interaction of bacterial species with metals and their use to remove metals from contaminated sites represent a unique process. Heavy metals are natural elements and in the most basic level are just atoms; degradation and metabolism are not possible. Instead, microorganisms have evolved coping strategies to either transform the element to a less-harmful form or bind the metal intra- or extracellular, thereby preventing any harmful interactions in the host cell. Plus, they are able to actively transport the metal out of the cell cytosol (Hamlett et al., 1992; White and Gadd, 1998).

Microbes deal with poisonous chemicals by applying enzymes to convert one chemical into another form and taking energy or utilizable matter from this process. The chemical transformations generally involve breaking of large molecules into several small molecules in simpler form. Microbial activity plays a key role in detoxification of metals in water. In view of the interest in water and wastewater treatment, the response of microorganisms towards toxic heavy metals is of importance. In some cases the by-products of microbial remediation are not only harmless but may prove useful (Gupta et al., 2003). There is therefore, need to search for such metal tolerant, metal absorbent as well as moderate thermophilic acidophilic organisms for bioremediation applications (Martin-Gonzalez et al., 2006; Umrania, 2006).

Metal toxicity results from alterations in the conformational structure of nucleic acids, proteins or by interference with oxidative phosphorylation and osmotic balance (Yaoa et al., 2008). Use of bio-adsorbents such as bacteria, fungi, algae and some agricultural wastes that emerged as an eco-friendly, effective and low cost material option could offer potential inexpensive alternatives to the conventional adsorbents (Valls and Lorenzo, 2002).

No previous work on bioremediation has been done on Panteka stream. The contaminated stream may find its way into domestic sources of water located around the communities which can be hazardous to human health, hence the need to carry out research work for the presence of bacteria species with the potential for removal of heavy metals and providing a template for the decontamination of Panteka stream as well as other contaminated streams.

MATERIALS AND METHODS

Sample collection

Panteka is a place in Kaduna where all kinds of cars and motorcycle spare parts are sold, and their maintenance are carried out. The stream in Panteka is located at the Northern part of Kaduna, Nigeria. There were four sampling points in the course of the study. Point A, the entry point of Panteka stream which is at Rafin Guza (upstream), Point B, C and D are the part of the stream that flows from Panteka through National Eye centre (Downstream). Samples were collected with caution into sterile bottles. The lid of the bottle was removed, held by its base and was completely submerged below the surface of the water. The bottle was filled by holding it upstream in the flowing water and in sweeping motion, to prevent any water which has come in contact with the hand from entering the bottle. Samples were taken to NDA (Nigeria Defence Academy) Medical Centre Laboratory for culture and analysis.

Isolation of heavy metal tolerant bacteria

Using the pour plate method, water samples were inoculated into nutrient agar plates containing different concentrations (0.5 and 3 mg ml^{-1}) of the heavy metals in their salt form such as cadmium nitrate (Cd NO_3), nickel chloride ($NiCl_2$) zinc sulphate ($ZnSO_4$), ferric chloride ($FeCl_3$), and lead sulphate ($PbSO_4$). The plates were incubated at room temperature for 3 days. The bacteria were isolated and sub cultured to obtain their pure cultures.

Identification of isolated bacteria

Pure cultures of the isolates were identified based on colony characteristics like shape, colour, texture, form, elevation, gram staining and biochemical tests. Molecular methods were used to confirm the identities of the isolates.

Isolation of genomic DNA

200 µl of the bacteria cells was added in a 1.5 ml Eppendorf tube. 400 µl of lysis buffer and 25 µl proteinase k was added to the sample in 1.5 ml Eppendorf tube. The tube was placed on heat block at 60°C for minimum of 1 h. 400 µl of phenol chloroform (1:1) was added to the lysate and vortexed briefly. This was spun at 10000 rpm for 10 min to separate the phases. The upper layer was carefully removed with a micropipette into a new 1.5 ml Eppendorf tube.

Equal volumes of 100% ethanol was added with 20 µl of 3 M sodium acetate and mixed by inverting the tube several times which was incubated at -20°C overnight. The tube was spun at a maximum speed for 30 min. 70% ethanol was added and spun at a maximum speed for 5 min at 4°C. All traces of ethanol were removed by spinning for another 30 s. The DNA was dried by leaving the tube open for 10 min. The pellet was resuspended in 20 to 50 µl sterile water and the DNA quality was determined using a

Table 1. Morphological characterization of the heavy metal tolerant bacteria from Panteka stream.

Parameters	P3m1	P3m2	P2m3	P2m4
Form	Grape	Circular	Swarming	Circular
Colour	Cream	Cream with red pigment	Cream	White
Elevation	Raised	Umbonate	Raised	Convex
Shape	Cocci	Rod	Rod	Rod
Gram staining	+	-	-	-
Likely bacteria	*Staphylococcus epidermidis*	*Serratia marcescens*	*Proteus mirabilis*	*Escherichia Coli*

spectrophotometer (Jyothi et al., 2012)

Amplification of 16S rDNA genes by polymerase chain reaction (PCR)

The isolated DNA was amplified using 16S rDNA universal primers, (Forward, GGACTACAGGGTATCTAAT) and (Reverse, AGAGTTTGATCCTGG) (Kenneth et al., 1990). The PCR conditions were Pre- Denaturation: 5min at 94°C, Denaturation: 1min at 94°C, Annealing: 1 min at 52°C, Extension: 1 min at 72°C, Final extension: 5 min at 72°C for 35 cycles. The PCR products were first analysed by electrophoresis in 1.5% agarose gel which was stained with ethidium bromide and visualized under short wavelength UV light.

Nucleotide sequencing and alignment

16S rDNA sequencing of the isolated strain was carried out by Dye terminator cycle sequencing (Quick start kit). The gene sequences of each isolate obtained in this study was compared with known nucleotide database at the National Center for Biotechnology Information (NCBI) by using their world wide website, and the BLAST (Basic Local Alignment Search Tool) algorithm (Jyothi et al., 2012).

Maximum tolerance concentration (MTC)

The four bacteria isolates each were inoculated into a nutrient broth containing different concentrations of heavy metals. The concentrations of heavy metals used were determined based on the maximum amount each bacteria isolate could tolerate. The concentrations for nickel, cadmium, zinc and lead ranged from 0.5 to 4 mg $ml^{-1.}$ The concentrations for Iron were 0.5, 0.7, 0.8, 0.9 and 1 mg ml^{-1}. The growth rate was monitored with a spectrophotometer at an absorbance of 600 nm against a nutrient broth (blank) containing the same amount of heavy metals (Pandit et al., 2013).

Bioremediation potentials of the heavy metals

100 ml of the water sample was poured into six 250 ml conical flasks containing 100 ml of nutrient broth each. The Stream water and nutrient broth was sterilized in an autoclave at 120°C for 15 min. Flask A was not inoculated with bacterium (Control), flask B, C, D and E were each inoculated with, *Staphylococcus epidermidis, Serratia marcescens, Proteus mirabilis* and *Escherichia coli,* respectively. Flask F was inoculated with a mixed Bacterial culture (MBC) of the four organisms. The samples were kept at room temperature. The concentration of cadmium, iron, lead, nickel and zinc was measured after 14 days of treatment with the isolates using atomic absorption spectrophotometer (AAS). Comparison was done on the values obtained before (control) and after treatment.

Statistical analysis

Data obtained for mixed bacteria culture and the most efficient pure isolate (*S. epidermidis*) in the removal of heavy metals from Panteka stream was compared using student t-test.

RESULTS

Identification of Bacteria by gram staining, morphological and biochemical characterization of Isolates P3M1, P3M2, P2M3, and P2M4 (Tables 1 and 2) identified *S. epidermidis, S. marcescens, P. mirabillis* and *E. coli* as heavy metal tolerant bacteria.

PCR amplification of 16S rRNA gene produced fragments of approximately 800 base pairs in size for the four bacteria isolates (Plate 1). Full length sequence of 16S rDNA gene was deduced for two isolates showing tolerance to high concentrations of the metals. Sequences were aligned and the closest match was detected using BLAST. Identification of strains by biochemical tests and 16S rRNA gene sequence analysis revealed that strain P2M4 showed 99% similarity to *E. coli* and strain P3M1 showed 100% similarity to *Staphylococcus* species.

Heavy metal tolerance of isolated bacteria

S. epidermidis and *E. coli* had MTC of 1.5 mg ml^{-1} for nickel, 3 mg ml^{-1} for zinc, 3 mg ml^{-1} for lead, 1.5 mg ml^{-1} for cadmium and 0.9 mg ml^{-1} for iron (Figure 1 to 5). *S. marcescens* and *P. mirabilis* had MTC of 1.5 mg ml^{-1} for nickel, 3 mg ml^{-1} for zinc, 4 mg/ml for lead, 1.5 mg ml^{-1} for cadmium, and 0.9 mg ml^{-1} for iron (Figure 1 to 5). The bacteria isolates showed similar pattern of heavy metal tolerance and this could be attributed to the fact that isolates are closely related genetically as the gram staining results showed that, most of the isolates were gram negative and belonged to the enterobacteriaceae family.

Table 2. Biochemical Characterization of heavy metal tolerant bacteria from Panteka stream.

Biochemical test	P3m1	P3m2	P2m3	P2m4
Mannitol	-	+	+	+
Orthinine decarboxylate	-	+	+	-
Nitrate reduction	-	+	+	+
Urease	+	-	+	-
Citrate	-	+	+	-
Methyl red	-	-	+	+
Catalase	+	+	+	+
Coagulase	-	-		-
Indole	-	-	-	+
Lactose	+	-	-	+
Hydrogen sulphide	+	-	+	-
Oxidase	-	-	-	-
Motility	-	+	+	+
Likely bacteria	*Staphylococcus epidermidis*	*Serratia marcescens*	*Proteus mirabilis*	*Escherichia coli*

+, Positive; -, negative.

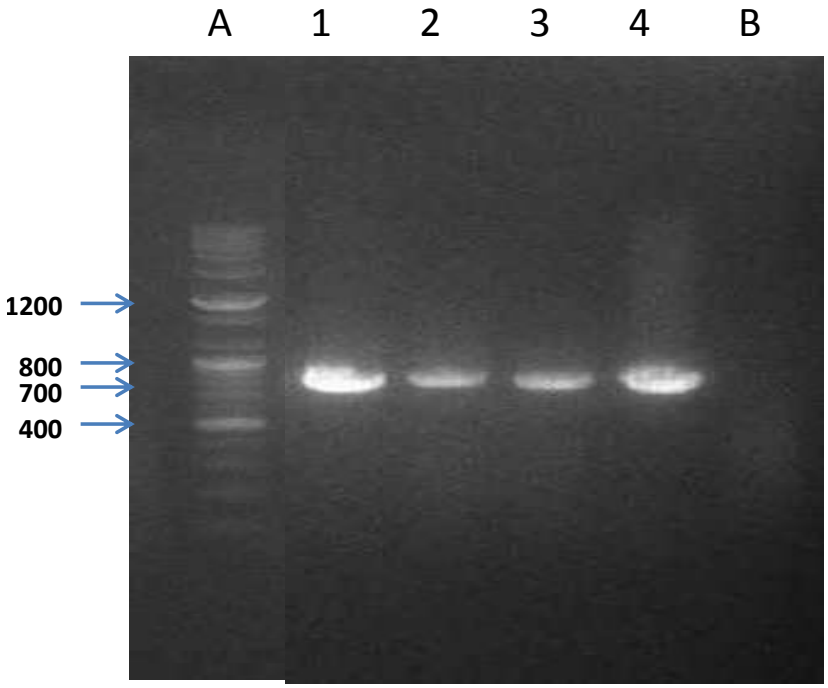

Plate 1. PCR amplicons of rRNA genes in four bacteria isolates from Panteka stream. Lane A, Ladder; Lane 1, P3M1; lane 2, P3M2; lane 3, P2M3; lane 4, P2M4; lane B, negative control.

Bioremediation potentials of the isolates to nickel, lead, zinc, cadmium and iron

Nickel and lead concentration was completely removed (100% reduction) by four bacteria isolates and mixed bacteria culture (MBC). Zinc concentration was reduced by *S. epidermidis* (84.95%), *S. marcescens* (58.73%), *P. mirabillis* (34.0%), *E. coli* (52.23%), and mixed bacteria culture (100%) (Figure 7). Cadmium concentration was reduced by *S. epidermidis* (90.29%), *S. marcescens* (66.82%), *P. mirabillis* (25.40%), *E. coli* (74.91%), and mixed bacteria culture (100%) (Figure 8). Iron concentration was reduced by *S. epidermidis* (54.82%), *S. marcescens* (52.01%), *P. mirabillis* (53.51%), *E. coli*

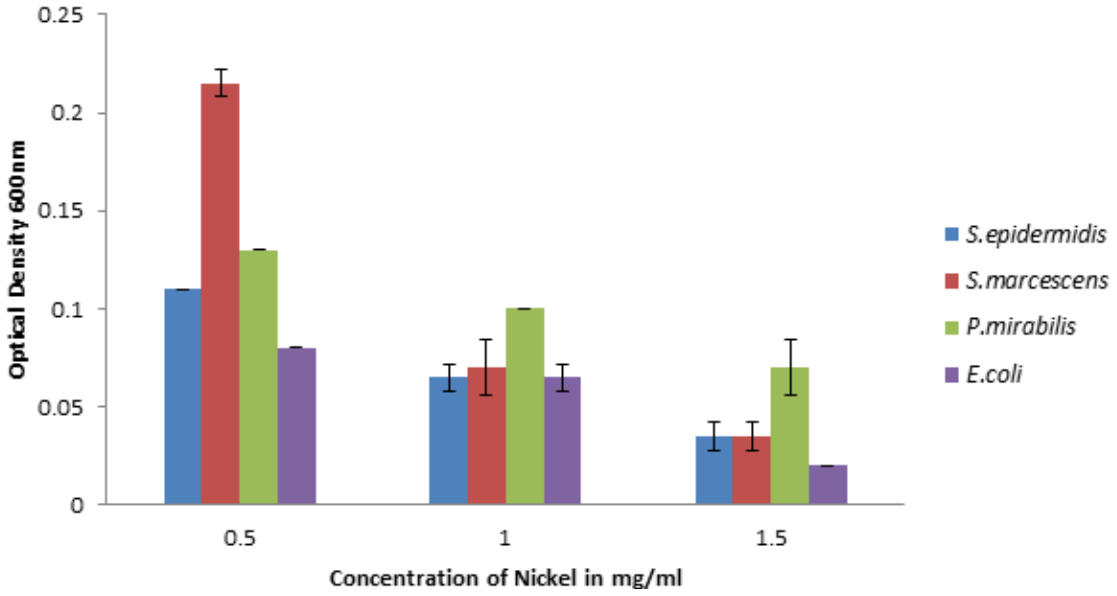

Figure 1. Effect of different nickel concentrations on the growth rate of bacteria isolates from Panteka stream. Data used are from two biological repeats. Error bars indicate ±SEM.

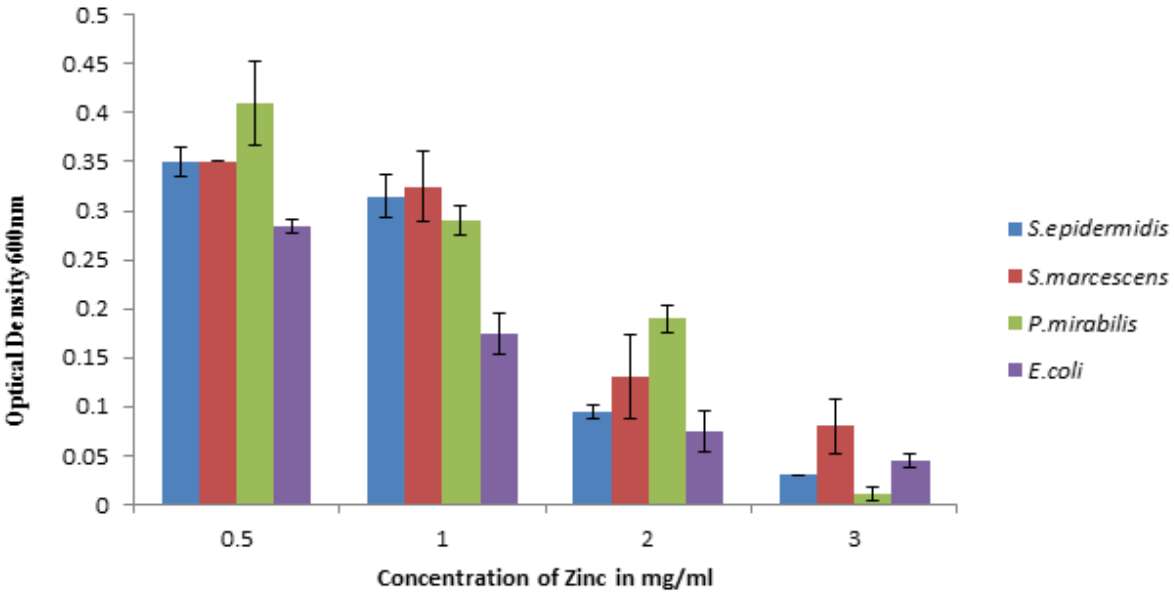

Figure 2. Effect of different zinc concentrations on the growth rate of bacteria isolates from Panteka stream. Data used are from two biological repeats Error bars indicate ±SEM.

(54.10%), and mixed bacteria culture (73.88%) (Figure 6). The best isolates for heavy metal reduction in Panteka stream in increasing order were *P. mirabilis, E. coli, S. marcescens,* and *S. epidermidis*. Mixed bacteria cultures had a highest percentages reduction of each heavy metal analysed in Panteka stream.

T-test data showed a significant difference between MBC and *S. epidermidis* in the removal of heavy metal; lead, nickel, cadmium, iron and zinc ($P < 0.05$).

DISCUSSION

The four heavy metal tolerant bacteria isolated were *S.*

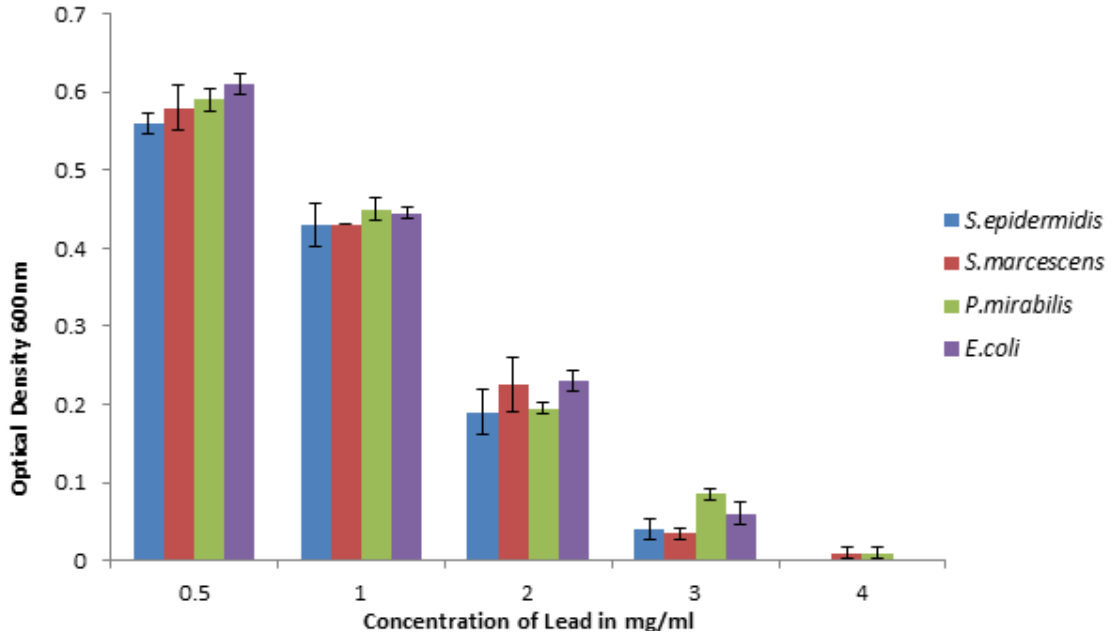

Figure 3. Effect of different lead concentrations on the growth rate of bacteria isolates from Panteka stream. Data from two biological repeats. Error bars indicate ±SEM.

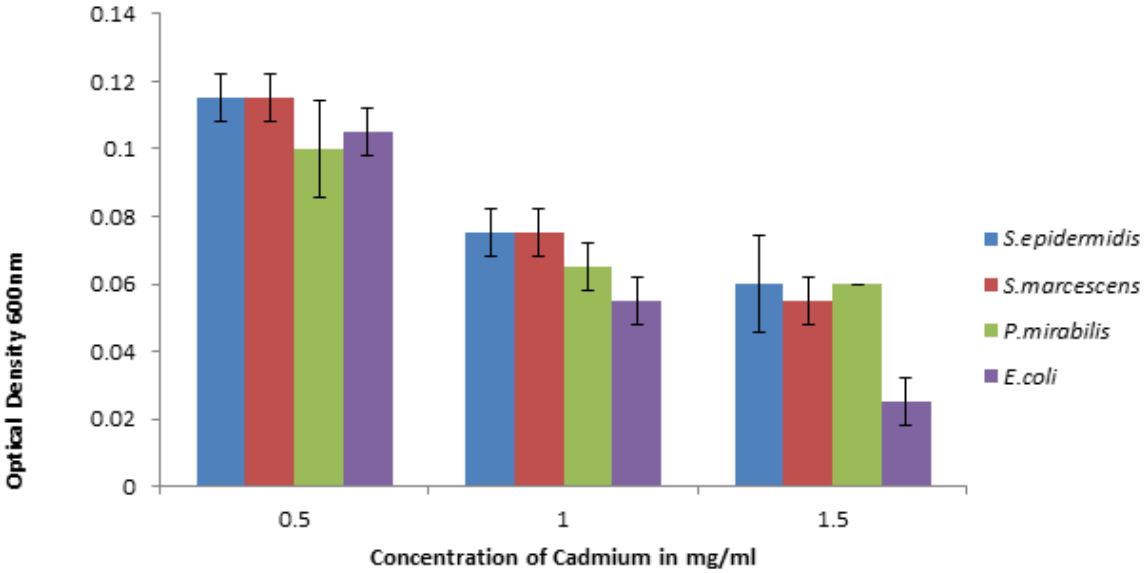

Figure 4. Effect of different cadmium concentrations on the growth rate of bacteria isolates from Panteka stream. Data used are from two biological repeats. Error bars indicate ±SEM.

epidermidis, S. marcescens, P. mirabilis, and *E. coli.* This result is similar to the report of Kolawole and Obueh (2015) that isolated *Staphylococcus spp., E. coli, Klebsiella, Salmonella* and *Pseudomonas* spp. as heavy metals tolerant bacteria. *Serratia marcescens* was reported to have been isolated as heavy metal tolerant bacteria (Nageswaran et al., 2012)

S. marcescens and *P. mirabilis* were the only isolates that showed maximum tolerance to lead at 4 mg/l. This compared favourably to the report of Owolabi and Hekeu (2015) who indicated in their works the high tolerance ability of *S. marcescens* and *Proteus* spp. to lead in a bioremediation study. The growth of the four heavy metals tolerant bacteria as reflected in their $O.D_{600}$ readings in increasing order was iron, cadmium, nickel, zinc, lead. Figure 3 shows that heavy metal tolerant

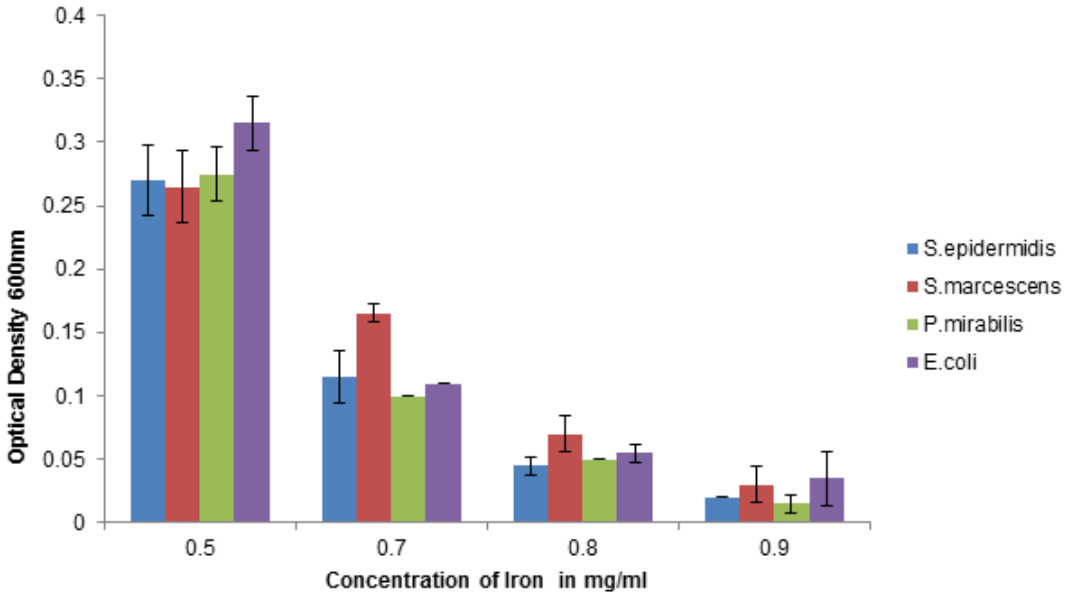

Figure 5. Effect of different iron concentrations on the growth rate of bacteria isolates from Panteka stream. Data used are from two biological repeats. Error bars indicate ±SEM.

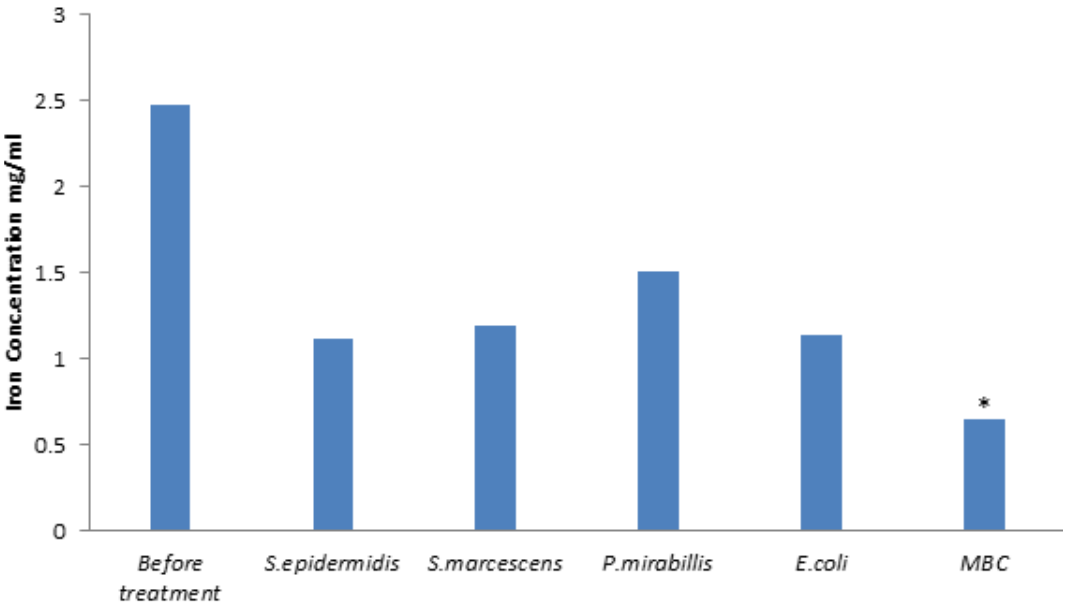

Figure 6. Iron reduction by bacteria isolates in Panteka Stream (* denotes significance difference from the most efficient single isolate, *S. epidermidis,* $p<0.05$).

isolates had high $O.D_{600}$ in the presence of lead as compared to other metals. The high tolerance to zinc could be attributed to being one of the essential trace metal ions for living organisms. Grass et al. (2001) stated that *E. coli* contains at least four zinc transport pumps, two zinc transporters (importers), ZnuABC and ZupT, and two zinc exporters, ZntA and ZitB. Fosmire (1990) however, reported that excess zinc can be harmful. Excessive absorption of zinc suppresses copper and iron absorption. Iron was poorly tolerated by the isolates. Simon (1998) stated that, iron is an essential nutrient for living agents due to its noticeable activity in electron transport reactions in biological systems, but its insolubility and reactivity lead to problems of poor availability and toxicity, respectively.

The bioremediation potential of the four heavy metal

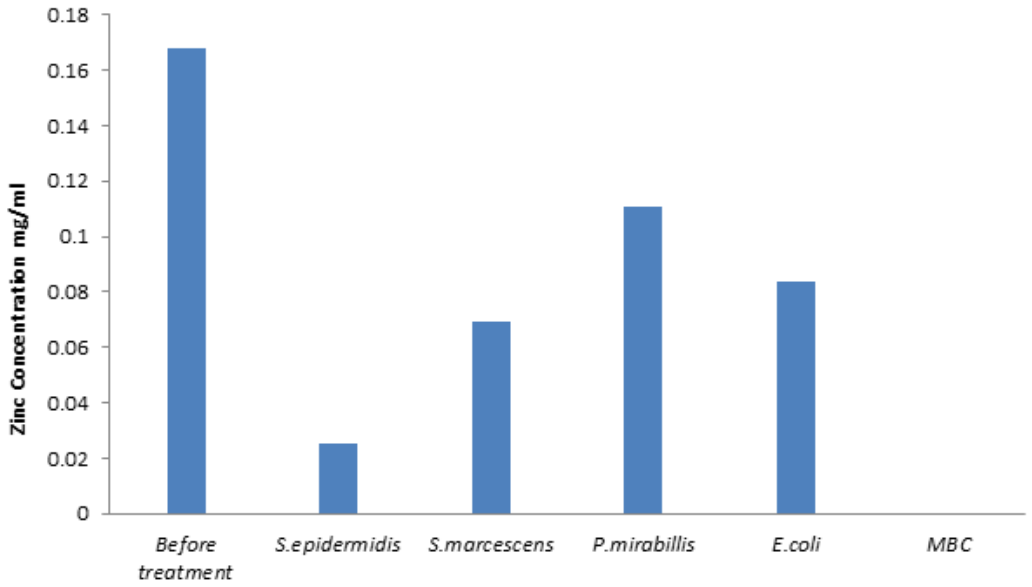

Figure 7. Zinc reduction by bacteria isolates in Panteka Stream.

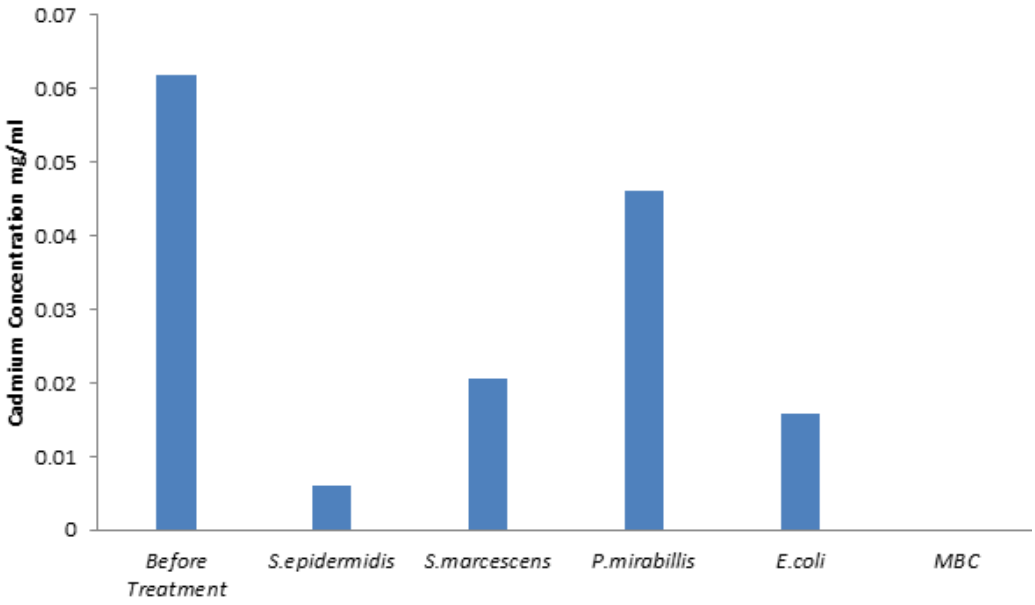

Figure 8. Cadmium reduction by bacteria isolates in Panteka Stream.

tolerant bacteria revealed that, *S. epidermidis* and *S. marcescens* show high potential for the removal of heavy metals in Panteka stream for pure isolates but mixed bacteria culture as being more effective than single cultures. The mixed bacteria culture completely removed lead, nickel, cadmium and zinc. This result compares favourably with the report of Oaikhena et al. (2016), who indicated in a bioremediation study of a petroleum refinery effluent that, mixed culture consortium was more efficient in the removal of heavy metals than pure isolates. Although lead is known for its toxicity as reported by Manton et al. (2000), it was observed that, the four heavy metal tolerant bacteria isolated from Panteka stream thrived well in the presence of lead.

Conclusion

The presence of bacteria capable of tolerating heavy metals from contaminated Panteka stream was

investigated. Bacteria that are capable of tolerating heavy metals were isolated in pure cultures, where three isolates were identified to belong to the enterobacteriaceae.

In summary, the results presented in this work revealed that the four isolates, characterized with remarkable tolerance to heavy metals could be potential agents for the development of inoculants applicable in bio augmentation of heavy metals polluted industrial sites. The genetic capacity of the isolates can also be exploited for the remediation of heavy metal polluted sites. The result obtained from the research proved that, mixed bacteria culture was more effective for bioremediation than pure culture bacteria isolates.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Generation of bioethanol from common date by-products, "Teggaza and Lebghel" in Southern Algeria

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Date by-products constitute the principal food for the oasis populations in Middle East and North Africa. Dates contents consist of 70 to 80% of reducing sugars, and do not require an intensive energy and labour for thermophysical pre-treatment. They can serve as a good feedstock for bioethanol generation through fermentation and distillation. Algeria is among the top sixth producers of dates in the world with more than 250,000 tons/year; from these, more than 30% can be lost for different reasons and may be of low quality. In the laboratory, after an alcoholic fermentation of the substrate of the date varieties, *Teggaza* and *Lebghel* (T & L) using bakery yeast at 30°C for 72 h, the distilled and rectified date juice generated the highest ethanol (88° and 90°) with acceptable productions of 2.5 and 2.78 mL/kg/h, and assessed scale efficiencies of 23.57 and 26.2%. This is unlike the one (ethanol; 50%) directly generated by chemical reaction using the same quantity of sugar. The efficiencies that were obtained seem satisfactory and encourage the great scaling development of bioethanol generation using date waste biomass abundant in Algerian Sahara.

Key words: Algerian Sahara, alcoholic fermentation, bioethanol, bakery yeast, dates by-product, distillation.

INTRODUCTION

Due to the variability in oil-market and increase in air pollution, there is the need to discover novel alternatives for renewable and sustainable energy sources to ensure energy security in the future. Bioethanol produced from bioenergy feedstock is one of the sustainable, economic and ecologic solutions to these issues. It can be produced from reduced sugars and stiff biomass or from Lignocellulosic biomass (Sims et al., 2008). Typical alcohol applications include chemicals, food and fuel products, fungicides, laboratory reagents, plastics, antiseptic, preserving solutions, refrigeration, solvents and others (Simo et al., 2009). Bioethanol was suggested as biofuel substitution; it is economical and ecofriendly and can be produced from many biomass sources including wood ships, corn husks, dates and other

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agricultural by-products. Using bioethanol instead of gasoline leads to the reduction of carbon emission by 80% and overall gasoline consumption by more than 30% (Elsanhoty et al., 2012). The production of bioethanol from lignocellulose raw materials requires generally the incorporation of an efficient pretreatment, followed by a scarification of the carbohydrates to obtain satisfactory effectiveness (Acourene and Ammouche, 2012). Compared to the use of gasoline, bioethanol helps to reduce CO_2 emission to about 80% (Li et al., 2008).

"D*actylifera L*." constitutes the central harvest and adapted sapling in arid and semi-arid areas of the globe. It constitutes the principal food of their inhabitants and animals since one kilogram of dates offers about 3,000 calories, income and economy sources for Saharan people in middle-East and North Africa (Boufis et al., 2014). The economy of the these regions is based principally on date palm cultivation and the use of its fruit by-products to prepare pasta, flours, syrups, vinegars, alcohols, yeasts, and confectioneries. It provides major sources of revenue for these oasis populations. The date palm is completely used, including its trunks, leaves for basketry and house structure. The date fruit is used in fresh and dry forms, and transformed into syrup (rub of tamr), (Amani et al, 2013), or fermented to produce metabolite (wine and vinegar); its leaflets and seeds are used in animal feed (Abbès et al., 2011). The world's potential production of dates is increasing in some countries like Egypt (17.2%), Saudi Arabia (13.7%), Iran (13%), United Arab Emirates (9.8%), Pakistan (9.6%), Algeria (9%), Iraq (7.2%), Sudan (5.4%), Oman (3.5%) and Libya (2%) (Chandrasekaran and Bahkali, 2013).

Algeria "*Phoenicia*" had an important progress in date-palms cultivars: it got 18,000,000 palms, covering more than 350,000 ha, where 11,000,000 trees are productive (FAO Statistic, 2015). The Algerian harvest attained 500,000 tons. The leftover dates that constitute the common dates reach 250,000 tons, in which 30% are of low quality dates. Only Adrar Province produced 86,500 tons of dates in 2012, coming from 2,000,000 of date-palms. This important production is commercialized in large quantities to foreigners in border countries while a few quantities are locally consumed.

Dates are rich in biodegradable sugars of about 73 to 83% on dry mass basis in two inverted forms, glucose and fructose (FAO statistics, 2015). They have long conservation and constitute a basis for various products, sugar-liquid, juice, alcohol, vinegar etc. Various products can be derived from dates feedstock like bio-polymers (Louhichi et al., 2013), organic and amino-acids (Radwan et al., 2010), bakery-yeast (Qureshi et al., 2012), probiotics and antibiotics (Abd-Alla and El-Enany, 2012), enzymes (Mussatto et al., 2010) and biofuels (hydrogen, butanol) (Nigam and Singh, 2011).

Algerian economy is based principally on fossil fuel. Algeria imports about 30,000 to 50,000 hl of ethylic alcohol per year for its proper uses. To reduce the dependency on fossil fuels and importations of chemical products, the Algerian Government has developed a national program from 2011 to 2030 to promote concrete-actions in the fields of energy efficiency and renewable energy (MEM, 2011; Stambouli et al., 2012).

The objective of the present work is to study the feasibility and productivity of generating bioethanol of highest quality in laboratory from the transformation of two common varieties of date by-products, Teggaza and Lebghel (T & L) of low quality in Adrar Province using anaerobic fermentation and distillation processes.

MATERIALS AND METHODS

Raw material and microorganisms

The date by-product (Figure 1) used in the present study to generate bioethanol is composed essentially of (T & L) varieties of dates originated from Algerian Sahara. It was obtained from Adrar conditioning unit of dry dates feedstock at the Agronomy Research National Institute of Algeria (INRAA, elmouchir.caci.dz). The dates were dried, kept in bags and stored at room temperature. They were sold in few quantities at local markets or served as feed for animals. The microorganism *S. cerevisiae* used in the fermentation process of date juice was provided by the industrial plant of bakery yeast production, from Oued-Semar in Algeria.

Bioethanol generation medium

Dates were washed, plunged in water, rubbed, and rinsed to eliminate sand, pebbles, insects and leftover plants. Then the seeds were separated from the coats and then petted (Figure 2a). Dates substrate was imbibed in hot water at 90 to 95°C to facilitate sugars extraction. Then 250 g of dates was diluted into 1 L of tap water, and simultaneously sulfuric acid was added and adjusted to obtain pH between 4.3 and 4.7, to inhibit bacteria and favor overgrowth of yeast (Wei-Hao et al., 2016). The anaerobic medium was inoculated with 1 g/L by *S. cerevisiae* model SII esaffre 59703 which is available in local markets; it was reactivated during 60 to 90 min under 30°C into an aqueous solution in glucose with 12% V/V.

Two bioreactors were prepared for each date variety studied. The first bioreactor is a glass bottle of 3 L capacity and used to follow the fermentation process while the second is a plastic jug with a great capacity of 30 L used to assess the system scaling efficiency. For both bioreactors, the inoculum size is 3% from the active dry yeast. The inoculum was prepared in a 3 L bioreactor containing 2 L of date substrate. It was incubated at 30°C, 10 rpm and air flow rate of 1 vvm for 30 to 60 min and then stored at 4°C.

During the fermentation process, the bioreactor was equipped with a manual agitator shaft (Figure 2b). The main objective of aeration is to provide microorganism growth in submerged cultures with appropriate oxygen for their metabolic needs. Agitation guarantees a homogeneous distribution of microorganisms and nutrients in the broth.

The density of sugars consumed, pH and the alcohol concentration of the date juice are controlled using Dubois method given in DuBois et al. (1956). The pH was measured by a digital pH-meter model Mettler Toledo methods (ISO11289, 1993; AOAC, 98, 1.12) and the date juice temperature during the alcoholic fermentation was recorded using thermocouples K type connected to the data logger model Fluke 2635A. Reducing sugars RS and total sugars TS are assessed by titration using a spectrometer UV (Siddiq et al., 2013). Saccharose content was estimated with the following formula (Reynes et al., 1994).

Figure 1. Date's variety by-product used in bioethanol generation.

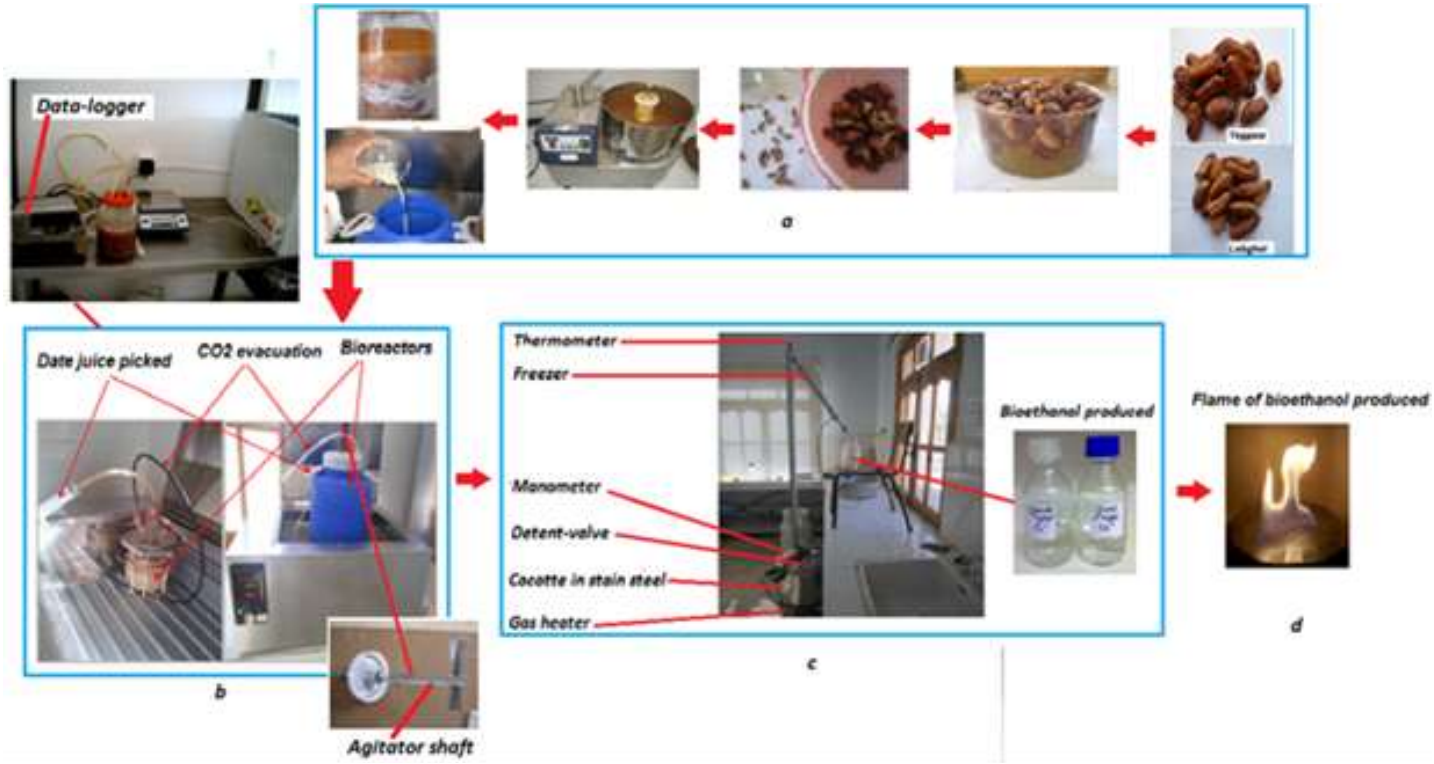

Figure 2. Bioethanol generation process in laboratory.

$$Saccharose\ (\%) = (TS - 0.95RS)\%. \quad (1)$$

Different analyses were performed on dates including water content, sugars content, pH, consistency, protein rate, cinder rate and the concentration of ethanol. The physicochemical proprieties of the date by-product (T & L) used to produce the bioethanol at URERMS laboratory are summarized in Table 1. The moisture containing MC% in the fresh date was determined as the difference between the fresh mass FM and the dry mass of date DM at 105°C, until a constant mass was attained. This was done with a digital balance model SKU: US- TRADER-PRO UPC: 878285001193 and the following formula (Siddiq et al., 2013):

$$MC(\%) = \frac{FM - DM}{FM} 100 \quad (2)$$

Based on the water content, dates are classified as soft if MC>30%, dry if MC<10 and semisoft or semidry if 10%<MC<30%. The consistency of dates was determined as the ratio of total sugar/water content (Reynes et al., 1994). Dates with consistency up to 3.5 are classified as dry, those between 2 and 3.5 are considered as semisoft or semidry, and those with ratio less than 2 are soft dates

Table 1. Physicochemical characteristics of dates studied.

Characteristics	Teggaza	Lebghel
Color	Brown Reddish	Yalow
Total date mass (g)	6.05	6.2
Seed mass/pulpy mass (%)	15.9	19.46
Date large/date length (%)	55.93	45.65
Pulpy mass/date mass (%)	86.2	83.71
Seed mass /date mass (%)	13.79	16.29
pH	5.44	5.7
Water content (%)	7.46	9.4
Dried substance (%)	92.54	89.6
Cinder rate (%)	3.29	4.38
Organic substance (%)	96.71	95.65
Reduced sugars rate (%)	41.23	41.52
Protein rate (%)	2.19	1.75
Azote rate (%)	0.35	0.28
Consistency	2.3	1.1
Refractive index	1.3409	1.34

(Lakkana et al., 2009).

After 72 h of alcoholic fermentation (Figure 2b), the substrate juice was used to filter the bioethanol (Figure 2c). At the beginning of the distillation process, the degree of alcohol is measured every 30 min, and once the process is slowed, the alcohol is recorded every one hour. The distillation process is stopped when the concentration of the alcohol became very feeble. The distillation temperature was kept at 78°C.

The deposit after distillation (Figure 2c) is composed of a cocotte of 30 L capacity, built in stain steel; its cover is made of a manometer, detent-valve, and a vertical colon tube of 3.5 cm diameter and 1.5 m height built in cooper. The cocotte is rumpled at 75% of its capacity with date substrate juice. The liquid mixture is evaporated at 78°C by heating the cocotte at the bottom and the vapor crosses the distillation colon by density gradient. The ethanol vapor is the condensed one traversing the sloped tube retriggered to accelerate the ethanol condensation process. The distillate produced was recuperated in a bottle at the end of the cooling system and rectified in order to increase the alcoholic degree.

RESULTS AND DISCUSSION

S. cerevisiae yeast has an optional anaerobic respiration in the fermentation process. In anaerobic phase, glucose was converted to ethanol by fermentation effect. Firstly, the process is active particularly between 24 and 55 h, where the yeast population reaches 37,719 and 35,088 cells/µL. The ethanol produced increased during the last 40 h of the process and an important degradation of the sugar is observed after 72 h (Figure 3). The density of the date juice (Figure 4) decreased considerably during the fermentation process from 1.07 to 0.99 g/cm^3, due to the conversion of sugar into alcohol and the loss of mass under CO_2 form. Also, the diminution of the protein rate in the date juice (Figure 5) represents an additional azotic source for the yeast to grow. In addition, azote has an important role in alcohol transformation; it ensures the transport of sugars to the external of the biologic cells to generate the fermentation process. The typical period of the fermentation process of the date juice varied between 48 and 72 h under similar conditions. The glucose is not consumed entirely due to the cessation of yeast growth caused by accumulation of toxic substances into the date juice, particularly the octane and decane in the date juice (Benziouches, 2011).

The total solvable solids in the date juice (Figure 6**)** were measured by a handheld refractometer for viniculture, using refractometer, model Atago NAR-3T (°Bx). This corresponds approximately to the total sugar concentration in (g/L) (DuBois et al., 1956). The continuous diminution of the refractive index (Figure 7) indicated the augmentation of the light speed through the date caused by the reduction of the date density. The concentration of the ethanol in the alcohol produced (Figure 8) is performed using liquid chromatography (Benziouches, 2011). After distillation of the date juice, a significant specific production of the bioethanol reached 180, and 200.5 mL/kg of dates at 90°C was obtained, representing bioconversion efficiencies of 23.57 and 26.2% for both variety of dates (T & L) respectively (Ahmed et al., 2016). Finally, the vibration sign of the biofuel produced (Figure 9) is identified using Infra-Red Spectra. It showed different vibrations bands characterized by the following wave numbers:

3339.08, 2974.29, 2888.12 and 1380.91/1380.5, 1087.06/1087.18, 1044.53/1044.66 cm^{-1}, corresponding

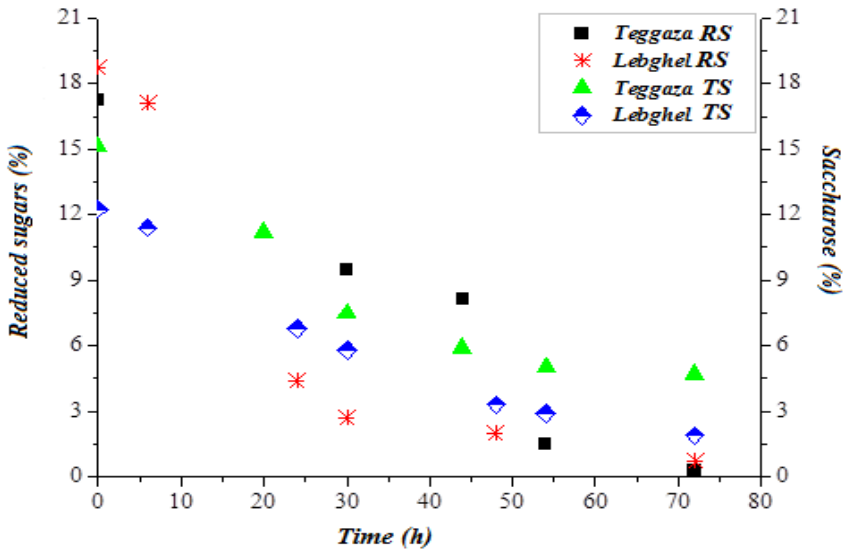

Figure 3. Sugars consumed during the fermentation process.

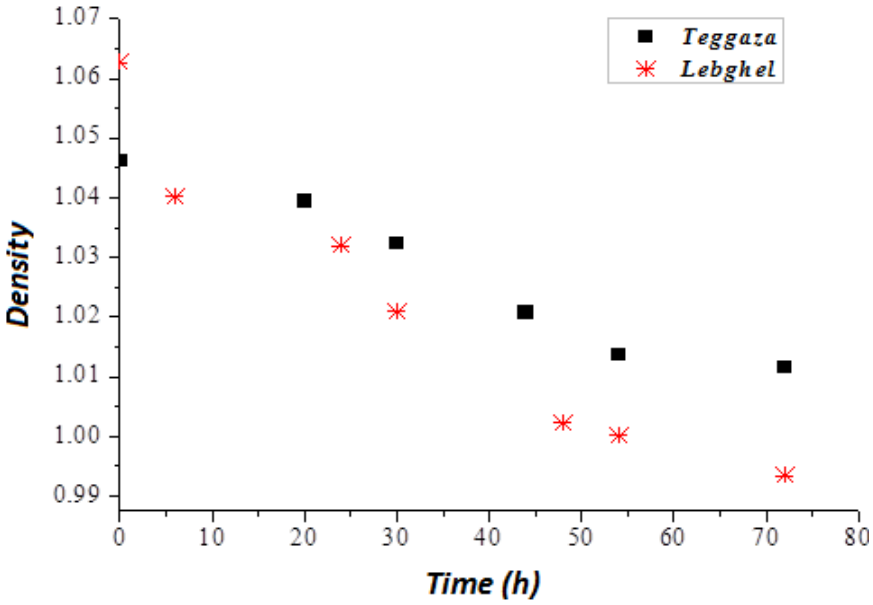

Figure 4. Density of date's juice during the fermentation.

to the molecule groups C-H, O-H and C-O for the two varieties (T & L) respectively. This is compared to the results reported by Khaled and Segni (2014), Ghanim (2013) and Chniti et al., 2014), where 1 kg of date produces 300 to 350 mL of ethanol at 95°. The production obtained in the present study appeared acceptable since the price of 1 L of ethanol at 90° in the worldwide market is about 10€ on web site (http://www.servilab.fr/, 2015). The price of 1 kg of date crude is about 0.25€ and when it is transformed into bioethanol its cost is 1€. This corresponds to an income of about 6€/1L of ethanol produced by fermentation process at 90°, which it is equivalent to a benefit of 60%.

Conclusion

The current study in laboratory shows that the date by-product of the varieties, T & L, abundant in Algerian Sahara, constitutes an important biomass and a favorable

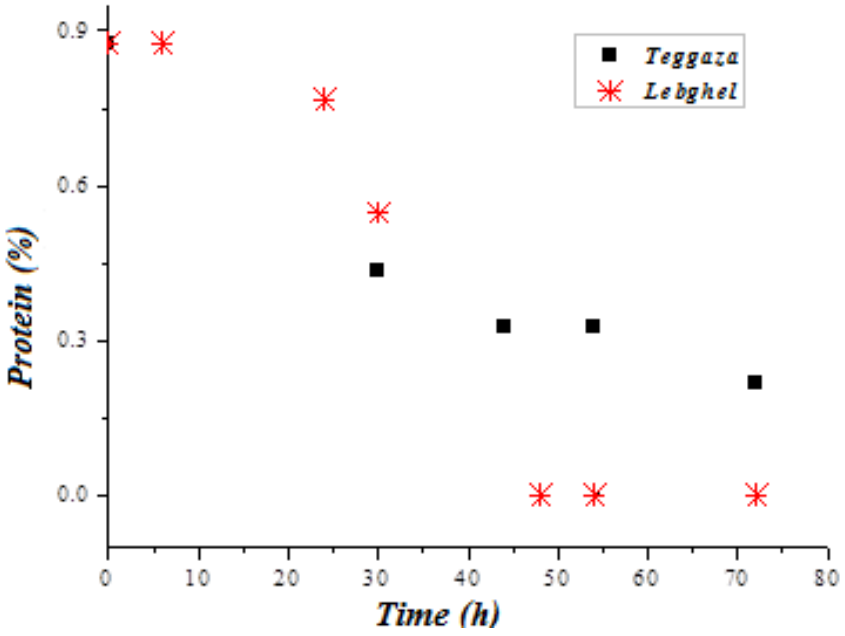

Figure 5. Protein rate during the fermentation process.

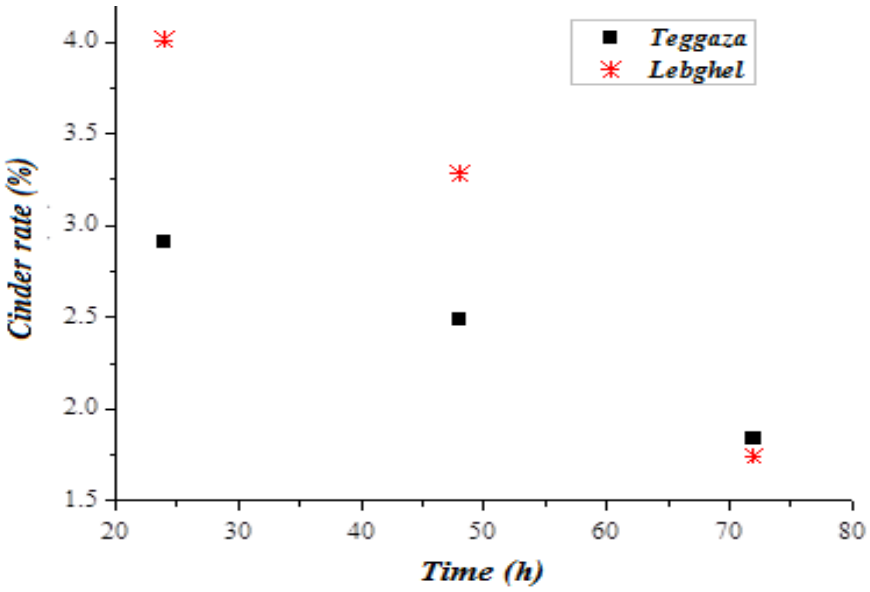

Figure 6. Cinder of dates during the fermentation process.

medium for bakery yeast growth for alcoholic fermentation due to its sugar content, cheap pretreatment and it is an attractive biomass. Dates are used to generate bioethanol at relatively moderate cost, without negative effects on air and water resources. After an alcoholic fermentation, the distilled and rectified date juice generated the highest ethanol concentration of about 88 and 90°; they had an acceptable production of 180 and 200.5 mL or 2.5 and 2.78 mL/kg/h, and assessing scale efficiencies of 23.57 and 26.2% for both varieties studied (T & L) respectively. This is compared to the theoretical ethanol efficiency obtained from a chemical reaction using the same sugar quantity, which is 50%. The present results obtained encourage the continuation of research and development in this clean and sustainable energy field and prospect novel bio-resources, bio-technologies and microorganisms that are more economical and efficient. They are useful because they enhance ethanol productivity, shorten

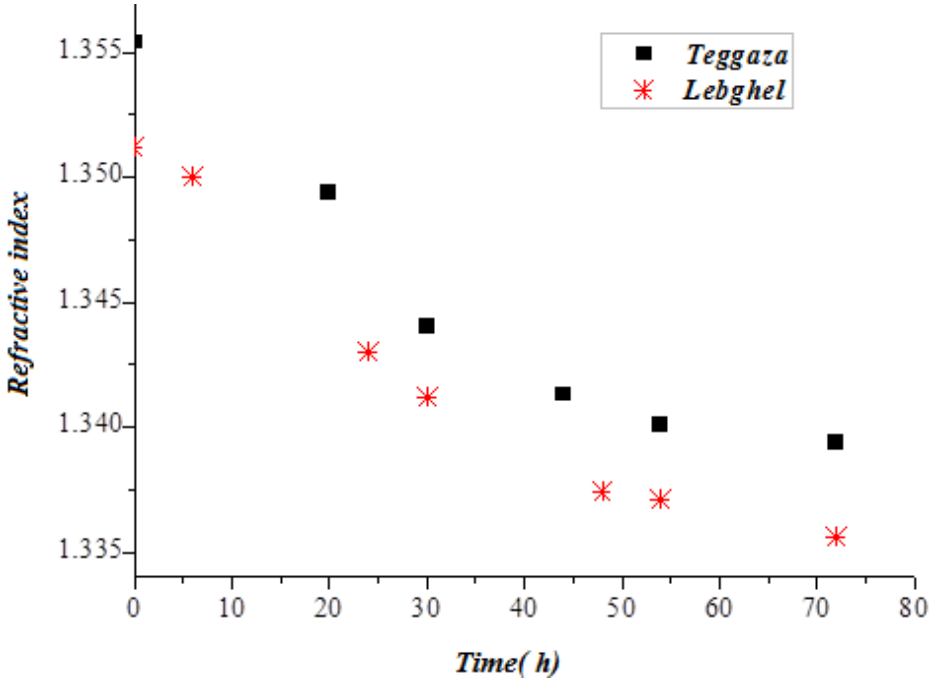

Figure 7. Refractive index during the fermentation process.

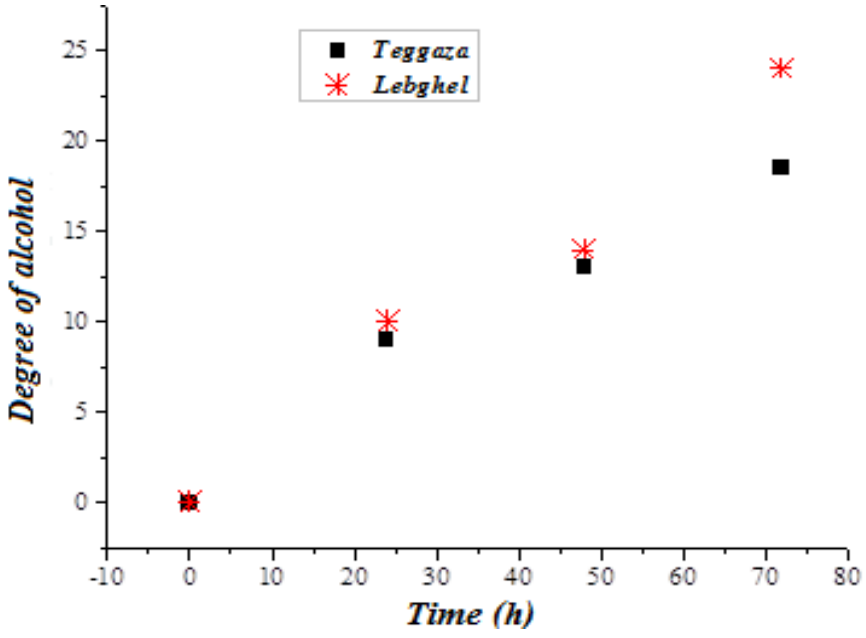

Figure 8. Alcohol concentration during the fermentation process.

ethanol development process, and reduce the energy consumed to lower the final cost of the product. The amount of 250,000 tons/year of date by-product seems favorable for developing a biofuel industry in South Algeria. However, firstly it is necessary to build bioethanol pilot installations to confirm the results obtained in laboratory before transposing the experience into industrial scales.

Conflict of Interests

The authors have not declared any conflict of interests.

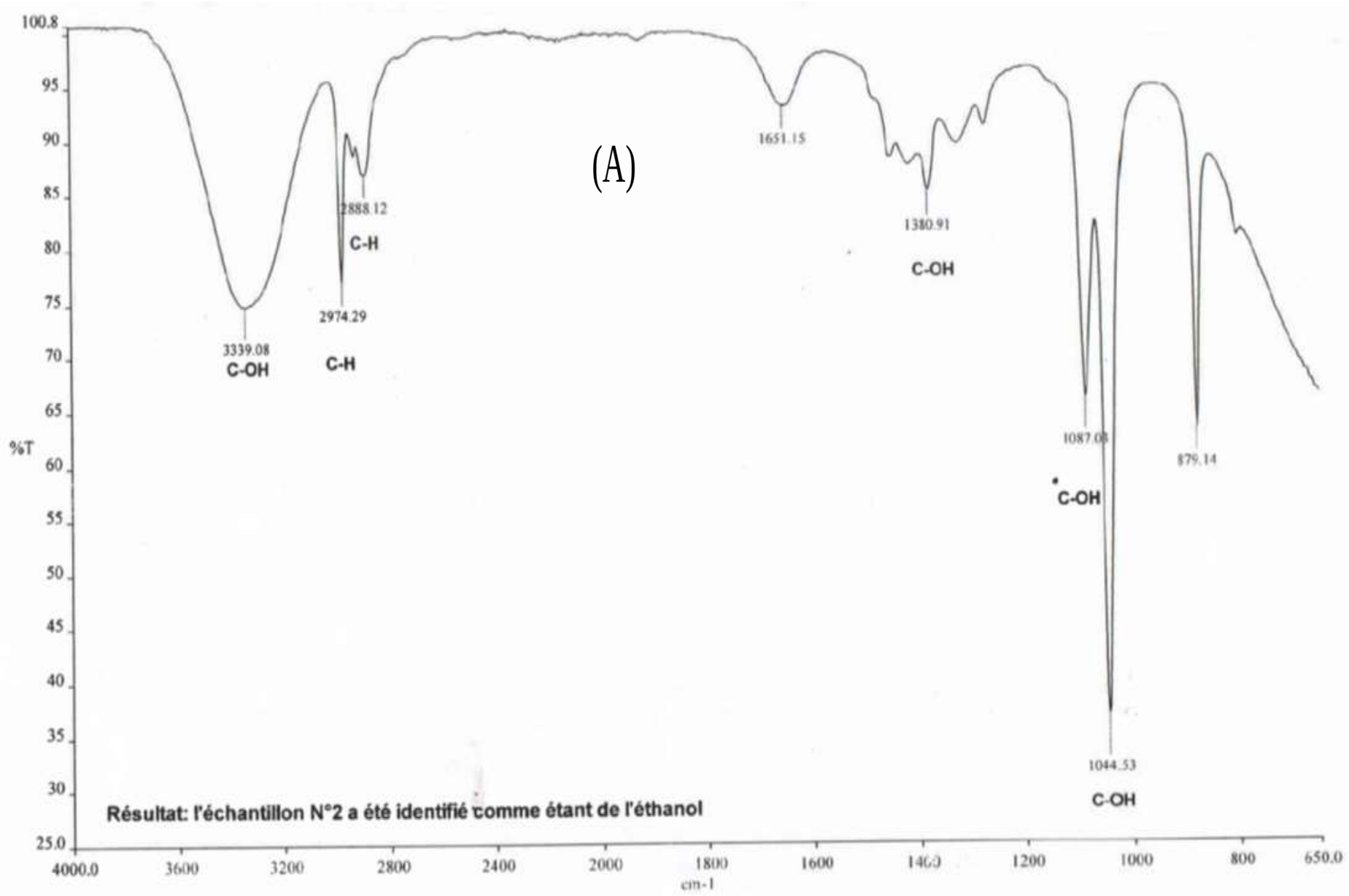

Figure 9. Infra-Red Spectra of the bioethanol produced. (A) Teggaza; (B) Lebghel.

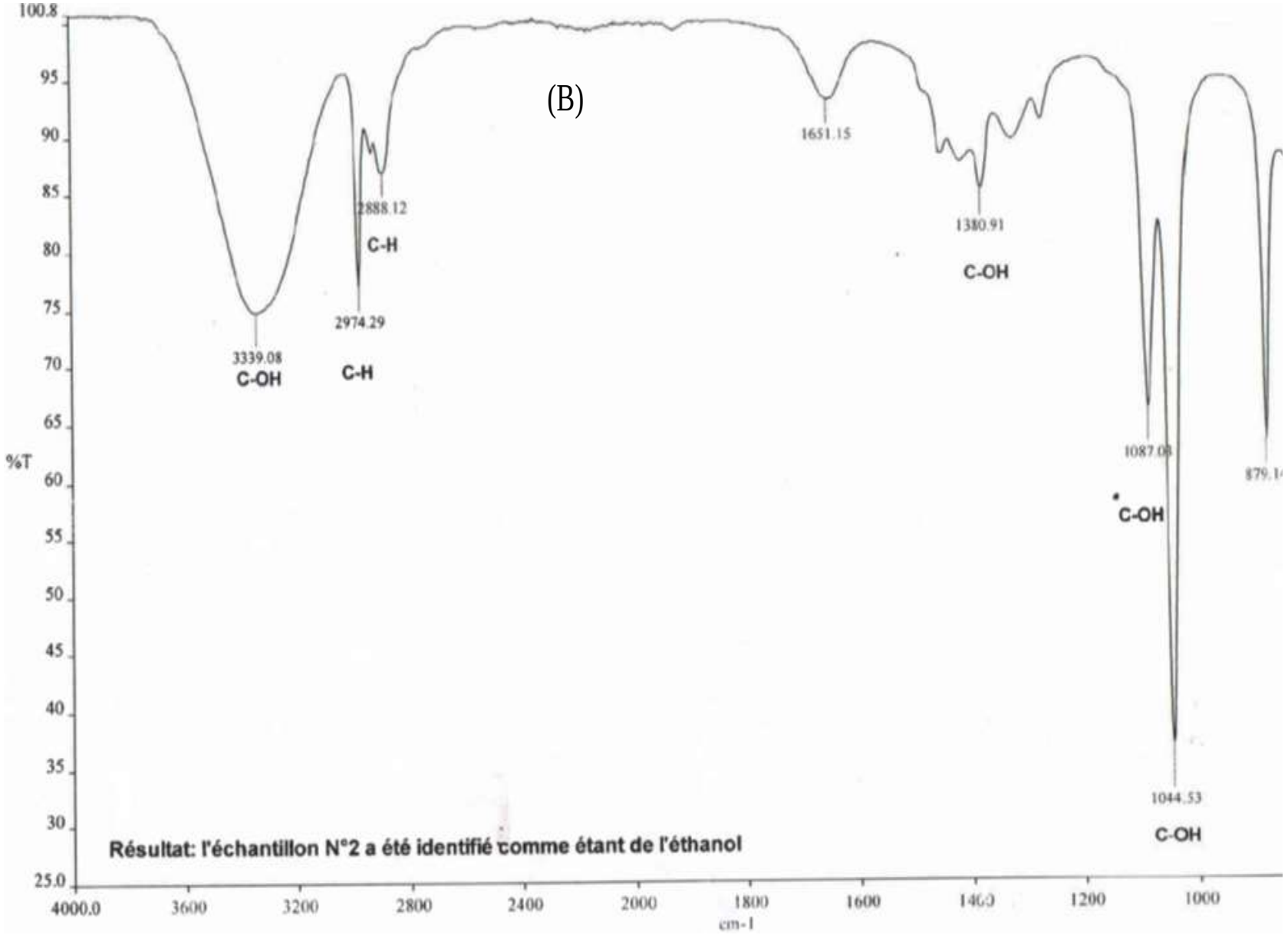

Figure 9. Contd.

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Full Length Research Paper

Comparison of growth and yield adaptability indicators of two maize (*Zea mays* L.) cultivars under planting basin technique in Zimbabwe

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In eastern semi-arid Zimbabwe, planting basins are mostly used to boost maize yields, but still low yields are often obtained due to poor choices of varieties to grow. A comparative study of growth and yield of the only two locally and commonly grown Pioneer cultivars (P2859W and PHB3253) under planting basin technique (PBT) was carried out to determine a more adaptable cultivar in Guhune, eastern Zimbabwe (NR IV). The study was done in 2012/2013 and 2013/2014 growing seasons. An experiment was laid out in a Randomized Complete Block Design (RCBD), with the two cultivars, each replicated four times. Yield, leaf length and plant height were measured and averaged. Results showed that PHB3253 had growth of 75.2 cm after measuring at 2 weeks interval for 10 weeks. Its growth was significantly ($p<0.05$) higher in terms of plant height than P2859W. Leaf length of P2859W was not significantly ($p>0.05$) greater than PHB3253. Grain yield of PHB3253 (35.1 kg) was significantly ($p<0.05$) greater than that of P2859W (26.6 kg). PHB 3253 is therefore more adaptable to semi-arid conditions under basin technique as signified by its higher growth and yield than P2859W. It is therefore recommended that farmers who use planting basins in semi-arid areas for growing short season maize cultivars (P2859W and PHB3253) should opt for PHB3253 for better productivity.

Key words: Early maturing cultivars, PHB3253 and P2859W performance, adaptation.

INTRODUCTION

Maize (*Zea mays* L.) is the most important grain crop in Southern Africa. It is the staple food of 12.5 million inhabitants of Zimbabwe, which is part of Southern Africa. The crop is grown in all natural regions (NR) of Zimbabwe, including dry marginal areas of NRs IV and V that receive between 450 to 650 mm rainfalls annually (Chitagu et al., 2014). These regions, is dominated by the highest proportion of the smallholder population (Chimhowu et al., 2009; Mehretu and Mutambirwa, 2006), which have the least agricultural potential (Vincent

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Table 1. NR differences according to rainfall and length of growing season in Zimbabwe.

Parameter	Influence of rainfall (mm) per annum on LGP				
	Natural region				
	I	II	III	IV	V
Rainfall	1000	1000-700	700-550	600- 450	500
LGP (days)	165	150-165	135-150	105-135	105

Adapted from Mugandani et al. (2012). LGP, length of growing period.

and Thomas, 1960).

Zimbabwe has five (I to V) major natural regions. The quality of the land resource declines from NR I through NR V (Moyo, 2000; Vincent and Thomas, 1961). Rainfall, temperature and soil type, among other factors, influence the agricultural potentials within the zones (Vincent and Thomas, 1962). For example, in NR IV the soils are poor, temperatures are usually high in summer, and rainfall is low and erratic. Moisture availability (rainfall) and temperature also influence the length of growing period (LGP) (Table 1) (Mugandani et al., 2012).

They, thus affect crop growth and yield. Against this background, manipulation of the biophysical environment influences adaptability of a cultivar. Farming communities in NR IV manipulate the environment by practicing various conservation techniques which include planting basin technique (PBT) to enhance maize growth and yield.

Southern Africa indicates a decline in mean annual rainfall of 40%; the region is also expected to experience a 3.7°C temperature increase in summer and a 4°C increase in winter (Intergovernmental Panel on Climate Change-IPCC, 2007). Zimbabwe is already in serious stress of such semi-arid conditions (Nyabako and Manzungu, 2012). Smallholder farmers, who form the bulk of the farming population in southern Africa, will be worst affected because of a lack of capacity to use the right technologies which are adaptable to such conditions (Nyabako and Manzungu, 2012).

Mean maize yield levels have been reported in declining over the last 20 years under conventional farming where whole fields were ploughed. Mean maize grain yields fluctuate around 1 tha^{-1} in high potential areas such as in natural region II and III, while in NR V, the yields are mostly below 500 $kgha^{-1}$ (Food and Agricultural Organization/World Food Program (FAO/WFP) 2010; Musiyiwa et al., 2014). In a yield comparative study of maize yields in Zimbabwe between conventional tillage (1 t was produced on 5 ha and PBT (1 ton 1.2 ha). PBT produced higher yields per unit area than conventional tillage (Jerie and Mugiya, 2010). Low yields obtained in Southern Africa from conventional tillage are largely due to low soil fertility and moisture content, which have inadequate inputs and increasingly unreliable rainfall patterns [International Maize and Wheat Improvement Center, commonly called by its Spanish acronym CIMMYT for *Centro Internacional de Mejoramiento de Maíz y Trigo*- (CIMMTY, 1992)].

Coping and adaptation options for smallholder farmers in low rainfall areas include crop choices (Kurukulasuriya and Mendelsohn, 2008) and cultivar choice. However, farmers in NR IV usually try to expand cropping areas use to compensate for low yields. Unknowingly, these farmers end up growing crop cultivars that are even not adaptable to their specific farming areas. It is recommended that on selecting cultivars, one has to consider the agro-ecological region and growing season length (Agronomy Research Institute - ARI, 2002).

The aforementioned environmental problems compelled the government, Non-Governmental Organizations, farmers and all other related stakeholders to search for alternative production methods that are based on sound programs for conservation and management of natural resources to boost rainfed maize yields in the rural sector. Reports in literature indicate that in Zimbabwe, farmers in NR IV obtained maize yields of 3 tha^{-1} under tied-ridges as compared to the average of about 1.5 tha^{-1} under conventional tillage treatments (Motsi et al., 2004). Work done by Nyamangara and Matizha (2010) showed that PBT was one of the best alternatives which were found to utilize land and other related resources in dry areas in the most sustainable manner. and Giller (2012) found that PBT is highly needed in southern Africa because it enhances water harvesting and targeted application of fertilizers. PBT in some parts of southern Africa has been adopted by a large number of farmers (Mazvimavi and Twomlow, 2009).

PBT falls under specific components of conservation farming (CF) (Protracted Relief Programme 2005; Mazvimavi and Twomlow, 2009). A pit, which is dug and formed into a basin-like structure, is the planting station where seed is sown. The rest of the other space is left untilled. This pit went through several stages before being refined to a plant basin. The concept was first coined by Oldreive in Zimbabwe (1993). It filtered into Zimbabwe as a modification of the traditional pit system which was started as a variation of the Zai Pit system from West Africa (Twomlow et al., 2008). It was subsequently further modified and promoted in Zambia by the Zambian Farmers Union Conservation Farming Unit (Haggblade and Tembo, 2003). The technology was then received in Zimbabwe after undergoing these stages

Table 2. Classification of maize varieties according to days to maturity.

Variety	Minimum days	Maximum days
Late	145	160
Medium	135	145
Early	115	135

Source: Nyabako and Manzungu (2012).

of modifications.

One of the known advantages of PBT under CF is moisture conservation (Zimbabwe Conservation Task Force-ZCATF, 2009). PBT also enables precision application of both organic and inorganic fertilizers as they are applied directly into the pit and not broadcasted (ZCATF, 2009). In marginal areas, the technique serves as the best solution to low yields of cultivars which could be well adapted in specific locations of semi-arid regions. Zimbabwe has the three main seed houses namely: Seed-co, Pannar and Pioneer. Generally, they supply late, medium and short season varieties which are classified according to days of maturity (Table 2).

Among them, Pioneer has become the commonest supplier of early maturing varieties to communal farmers in dry parts of the country. Early maturing varieties which are mostly short season varieties (SSVs) have improved characteristics which include drought tolerance and heat resilience. Pioneer company gives benefits like seed and fertilizer free to farmers in Chimanimani who grow its cultivars and offers competitions, where winners are given machinery and large amounts of the company's short season varieties. After realizing this, communal farmers in semi-arid parts of Chimanimani, Zimbabwe have resorted to grow, mainly and solely, the Pioneer short season maize cultivars (P2859W and PHB3253). It takes 115 to 135 days respectively to mature (Table 2). Farmers complement these characteristics with planting basins practice so as to, hopefully, obtain sustainable yields.

Planting basins have been reported in literature to increase maize yields in semi-arid regions when manure and fertilizers are added (Mashingaidze et al., 2012; Nyamangara et al., 2014). The two technologies in planting basins and fertilizer application in the basins can, increase yields if the correct choice of well adapted cultivar to a particular environment is done. Most communities do not use planting basins because of several reasons which include; some farmers not being aware of the advantages of planting basins and the labour involved in preparing the basins. Mrs Mboto and some farmers in NR IV and V had not been using planting basins to grow their maize. The current research used growth and grain yield as adaptability indicators of P2859W and PHB3253 cultivars in Guhune, a core semi-arid part of Chimanimani District, Zimbabwe. The cultivars were evaluated under PBT. The study, herewith, determined a more adaptable cultivar between the only two cultivars when grown.

MATERIALS AND METHODS

Study area; NR 1V

Location

The study was carried out in Chimanimani District, in eastern part of Zimbabwe. The country is located between 15°37' S to 22°24' S and from longitudes 25°14' E to 33°04' E; and covers an area of 390 580 km^2 (Mugandani et al., 2012). The coordinates of the town of Chimanimani are: 19°48' 0.00"S, 32°51' 36.00"E (Latitude: 19.8000; Longitude: 32.8600). The research was done in Guhune communal rainfed area, ward 4, at Mrs Jane Mboto`s plot. The plot site is 65 km from Chimanimani.

Rainfall and temperature

The research area, which is in NR IV, is characterized by low range of annual rainfall 450 to 550 mm with less than 14 rain pentads each season, and experiences dry spell during summer. In this NR, rainfall intensity, reliability and distribution are significantly not conducive for maize production (Igbekele, 1975). Temperature ranges from 32 to 40°C in summer while winter temperatures range from 15 to 30°C.

Soil type and conditions

The pH of the soil is 6.0. The soils are sandy soils derived from granite rock and contain 75% sand and less than 20% clay (Nyamapfene, 1991). The study area has reddish coloured soils which fall in Fersiallitic group, under the family 5G which is dominated by coarse grained sandy soils (Thompson and Purves, 1978). The soils are degraded, and hence, have low nutrient status and water holding capacity. They develop low organic and base status and are prone to erosion. The site has a south facing with a slope of ±5% which further increases run-off and soil drainage.

Treatments and experimental design

Performance comparison was carried out between two cultivars (P2859W and PHB3253). The two cultivars were the only commonly grown in early maturing cultivars, advocated by Pioneer in Guhune, under PBT (Agriculture Technical and Extension Services-AGRITEX, 2012). What the community critically needed was a more adaptable cultivar, in their area specially earmark for planting basins. The area experiences low rainfall, and has poor soils. This area was earmarked for maize cropping under planting basins. In such conditions, farming community in Guhune was in dilemma as to which is to be chosen among the two to be grown, after considering the better one in terms of growth rate and yield performance. Therefore, P2859W and PHB3253 were used as treatments in this study (Treatment 1 and Treatment 2 respectively); both were white, early maturing hybrids. Each treatment was replicated 4 times. The 4 plots of each cultivar measured 10 m length x 5 m width. The design was Randomized Complete Block Design (RCBD).

Planting basins and planting procedure

Basins were 15 cm long x 15 cm wide and 15 cm deep, spaced at

Table 3. Growth (plant height) of P2859W and PHB3253 in Guhune dryland in 2012/13 and 2013/ 14 growing seasons.

Treatment	Mean growth (cm) of maize in all plots after emergence					Mean of means for the 10 weeks
	Week 2	Week 4	Week 6	Week 8	Week 10	
P259W	13	30	52	120	150	73
PHB3253	13.5	30.5	55	122	155	75.2

Table 4. Growth (mean leaf length) of P2859W and PHB3253 in 2012/2013 and 2013/2014 growing seasons.

Treatment	Mean leaf length (cm) of maize in all plots					Mean of means for the 10 weeks
	Week 2	Week 4	Week 6	Week 8	Week 10	
P2859W	16	54	67	80	90	61.4
PHB3253	16.5	54.5	67.5	84	95	63.5

90 cm × 60 cm (Protracted Relief Programme, 2005; Twomolw et al., 2008).

In NR 1V, growing season in rainfed conditions starts usually in November and ends in March. Planting starts in the first effective rains. The maize for the current experiment was grown under rainfed conditions. An amount of 5 g compound D, using a cup size of 5, was applied per each planting basin and mixed with 500 g of local cattle manure, which was well decomposed. Planting was done in planting basins on 16 November 2012 to 2013 growing season, on plot area of 200 m^2. Each cultivar was planted on an area of 100 m^2. Planting depth was 5 cm. Three seeds were planted per station (basin) and then thinned out for 7 days post emergency to remain with one healthy plant per basin. The procedure was repeated in 2013 to 2014 growing season, where planting was done on 25 November on the same planting stations or basins.

Main agronomic practices done

Weeding

Weeding was done in all plots on the same day, whenever weeds appeared inside the basins. Within the field, areas without basins were also weeded, but less frequently in basins.

Top dressing

Split application of ammonium nitrate (34.5%N) fertilizer was done at 4 and 9 weeks post emergence at 4.5 g per basin. 2.5 g were applied at 4 weeks post emergence and the remaining 2 g were applied at 9 weeks post emergence, instead of 8 weeks. This was due to lack of moisture at that stage. Ammonium nitrate application rate was 90 $kgha^{-1}$

Assessing growth rate and yield adaptability factors

Measurements of growth parameters

Leaf length and plant height for each cultivar were taken from four replicates/plots for each treatment at every two week interval (from 07/12/2013 and 07/12/2014/2015), from 2 weeks post emergence up to 10 weeks post emergence. Four plants from the center row of each plot were measured and the data were averaged. Leaf number 5 was measured for each plot. This measurement determines growth.

Harvesting and measurement of grain yield

Performance evaluation was done over 18 weeks from day of planting of each cultivar. Grain weights of the two maize cultivars P2859W and PHB3253 were recorded in tha^{-1}on a digital scale after harvesting at 14% moisture content. A grain moisture tester meter was used after drying and shelling. All plants from each plot were measured and the data was recorded and averaged.

Data analysis

Data was analyzed by comparing means of growth rate as well as grain yields of the two cultivars using “t”-test. Least significance difference (LSD) was used to separate differences between treatments. Analysis was done at 5% significance level.

RESULTS

Growth rates of PHB3253 and P2859W

P2859W had a plant height of 73 cm while PHB3253 had 75.2 cm when measured at 2 to 10 weeks (Table 3). The percentage (%) difference was 3. The results for leaf length showed that P2859W had growth of 61.4 cm and PHB3253 had a growth of 63.5 cm for 10 weeks (Table 4). The percentage (%) difference was 3.3. PHB3253 had slightly greater leaf length than that of P2859W and also had significantly ($p<0.05$) greater growth than P2859W.

Grain yields of P2859W and PHB3253

The harvest was done on 20/03/2015. Table 5 shows the yield for each of the two cultivars. The total of P2859W was 2.66 tha^{-1} and PHB3253 was 3.51 tha^{-1}. There was a

Table 5. Total grain yields of P2859W (treatment 1) and PHB3253 (treatment 2) obtained at Mrs Mboto's plot.

Treatment	Replication	Grain yield (kg 100 m^{2-1})
1	1	6.6
1	2	6.9
1	3	8
1	4	5.1
Total		26.66
2	1	9.1
2	2	10
2	3	7.8
2	4	8.2
Total		35.18
Expected yield		37.8
Difference of the two treatments totals		8.5

significant difference ($p<0.05$) on grain yield. Results showed that PHB3253 had higher yield than P2859W (Table 5). The percentage difference was 24.2 %. Each yield level was obtained from an area of 200 m^2.

DISCUSSION

Growth rate of P2859W and PHB3253 (plant height)

The study shows that PHB3253 had a mean plant height of 75.2 cm while P2859W had 73 cm (Table 3). This is in agreement with the study of Rockstrom et al. (2009) who reported that PHB3253 is more adaptable; is more droughts tolerant; uses nutrients more efficiently; grows taller than most short season cultivars in dry areas. Growth for the two cultivars was almost the same for the first two weeks. As from week 6 to week 8, PH3253 had faster growth than P2859W. This could have been due to the advantage of the better height which could enhance efficient trap and use sunlight for effective growth. Basins had soil amendments which include ammonium nitrate. According to the literature, ammonium nitrate increases the growth of a more adaptable cultivar than a non-adaptable cultivar in a particular area (Sawi, 1993).

Comparing growth of P2859W and PHB3252 (leaf length)

Leaf length for both treatments was almost the same for the first 6 weeks. There was a slight difference as from week 8 to 10, although statistically insignificant ($p>0.05$) to the parameter was measured. If PH3253 had better growth than P2859W then, it will have greater leaf length as well as height and leaf which have proportional growth (Vanlalhluna and Sahoo, 2011). However, in the current study, PH3253 plant height did not really influence leaf length as much as P2859W. This could have been influenced by the genotype of the cultivar. This is in harmony with the work of Muchow and Sinclar (1994) which revealed that, there is a genetically determined upper limit to leaf growth when inorganic fertilizers and manure have been applied.

Determining grain yield of P2859W and PHB3253

PHB3253 produced greater grain yield (3.51 tha^{-1}) than P2859W (2.66 tha^{-1}) (Table 5). Statistical results also showed that PHB3253 had significantly higher yields ($p<0.05$). One of the reasons which contributed to better yields was of better height which had better potential of capturing more sunlight for photosynthesis than the shorter cultivar P2859W. The total biomass built from the superior height help in reducing runoff, evapo-transpiration, (Jerie and Mugiya, 2010), and also helps generate more shade which conserves moisture. High runoff levels, high drainage, and high evapo-transpiration rates contribute about 60 to 70 % loss of water from a rainfall event, (Jerie and Mugiya, 2010), hence highly recommended techniques combined with conservative crop growth characteristics are needed to wisely utilize the scarce soil, water and nutrient resources available in semi-arid regions. PHB3253 has been reported in literature to be a high yielding, resource intensive hybrid maize cultivar which needs more water and more fertilizer (Mourice et al., 2014) with a high yielding potential of 5 to 8 tha^{-1}.

Results indicate that there is a positive relationship between maize grain yield and its components. This is in agreement with the study of Cross (1991) whose report clarified that high maize grain yield is attributed to longer and thicker cobs with many kernel rows. This researcher

also agrees with the study of Heisey and Edmeades (1999), which suggest that, yields are higher in CA both in the year of drought and of good rains. This evidence was shown when PHB3253 yielded 5.2 tha^{-1}. In an experiment done, Kirway et al. (2000) found out that the potential yield of PHB3253 was 8 tha^{-1}. P2859W can yield 3.9 tha^{-1} in a drought year. Like PHB3253 (Mourice et al., 2014), P2859W is also known for its good yielding characteristic which is 5 to 8 $tha^{-1.}$ The cultivar is known for its average cob sizes with big kennels. The yields in various literatures can be in disagreement with the yields obtained in the current study because of the variations in the biophysical factors impacting on the type of cultivar(s) grown. Effect of basin tillage practice on yield can also be affected by soil type, amount of rainfall, management practices (which include inorganic fertilizers and organic type and rates), mulching and prevailing temperature (Nyamangara et al., 2013).

The fertilizer rates of 100 $kgha^{-1}$ compounds D and 90 $kgha^{-1}$ ammonium nitrate contributed much on the yield. The results of this research also agrees with those of Lowe (2011) who indicated that, CA reduces inputs costs, while yields are increased. In Guhune, mulch and manure were locally available. Finally, higher yields obtained under CA practices through good choice of a variety can improve the household food security of Guhune dryland.

Conclusion

The study compared growth and yield of two commonly and solely grown Pioneer short season cultivars in Guhune, to determine a more productive cultivar in the area. Growth and yield of PHB3253 were found greater than those of P2859W under basin technique. However, there was no significant difference between the leaf length of PHB3253 and that of P2859W. The study has shown that PHB3253 performs well under planting basin technique, implying that PHB3253 is more adapted to semi-arid conditions of Southern Africa as compared to P2859W.

Conflict of interests

The authors did not declare any conflict of interest.

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