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Index Copernicus / IC Value	**:**	**7.61 [2012]**
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Journal Update

Prof. Ghaleb A. Alhadrami

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Dear Colleagues,

2013 was a good year for the Emirates Journal of Food and Agriculture (EJFA) especially in terms of ranking and international audience. The SJR (SciMago Journal Rankings) value for 2012 is 0.153 and SNIP (Source Normalized Impact per Paper) for 2012 is 0.220. The indexing/abstracting for the journal is now 165. There is a drastic increase in the indexing during 2013. The Index Copernicus value (ICV) is increased to 7.61 and NAAS Rating is 4.6 out of 6 for 2011. Google Scholar Metrics also increased in 2013 with h5-index value of 7 and h5-median value of 10. The SCOPUS data indicates that the journal articles are being cited worldwide.

The journal published 12 regular issues. In addition, a Supplementary issue of abstracts was published for the "11th International Ethnobotany Symposio", November 2-5, 2013, Antalya, Turkey. Also, EJFA published two special issues one on Date palm and the other one on Camel Research as promised in the journal update in the January issue of 2013. Similarly the December issue was reserved for papers of a hot current research topic "Modulating Agents of Plant Physiology and Products". As in the previous year, this year also we will consider publishing special issues for international scientific conferences and topics like camel and date palm researches. The editorial board of the journal is revised starting from January issue of 2014.

Sincere thanks to all our reviewers, contributors, and readers who supported the EJFA with important contributions. Special thanks to the editors of the EJFA for their effort during last year in managing the journal.

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NUTRITION AND FOOD SCIENCE

Development and quality assessment of new drinks combining sweet and sour pomegranate juices

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Research Unit on Agrobiodiversity, Higher Agronomic Institute, Chott-Mariem, IRESA-University of Sousse, Tunisia

Abstract

This study was carried out to evaluate the physicochemical properties and the suitability of some parameters for the design of a new beverage combining sour and sweet pomegranate juices as an alternative use for neglected varieties. Sweet pomegranate juices from varieties Zaghouani and Tounsi and sour juices from varieties Mezzi and Garsi were used in this study. Higher titratable acidity (1.6% calculated as citric acid) with a pH of 2.73–2.82 was registered in Garsi and Mezzi juices. The contents of ascorbic acid, anthocyanins and total phenols were in general high, 20-25 mg ascorbic acid, 37.7-56 mg cyanidin and 164.4-181.8 g gallic acid per 100 ml juice, respectively. Zaghouani and Mezzi varieties gave the cleanest juices, while Tounsi gave the less colored juice. The highest antioxidant activity was registered in sour varieties. New polyphenol-rich beverages were prepared using sour and sweet pomegranate juices in different proportions (reaching pH 4.0). The results of sensory tests suggest that mixed beverages are preferred to pure sweet juices. Garsi (18.2%)-Zaghouani (81.8%) mixture provided the most appreciated beverage with good organoleptic properties (color, clarity and flavor) followed by Garsi (16.7%)-Tounsi (83.3%) mixture which was well appreciated for its taste and improved color. Further research is needed to establish new pomegranate juice combinations and to examine the stability of characteristics and bioactive proprieties of mixtures after cold storage in order to develop new healthy beverages.

Key words: Pomegranate, Juice quality, Antioxidant activity, Beverage, Sensory evaluation

Introduction

Over the past few decades, food consumption habits have changed immensely. Consequently, consumers have a broad range of choices for the basic food bundle. The development of healthy food was rated as the most important area of research, followed by developing natural foods (Katz, 2000). The rising interest in maintaining and improving human health and life justify the increasing demand of fruits. One of the most frequently utilized fruit production technologies is juice processing. Consumers demand original juice with minimal processing, a juice with no sugar added, and also a juice which that closely emulates the raw fruit from which it is derived.

Fruits of pomegranate species are not only consumed fresh but also used to produce jam, jelly, syrup and several types of soft drinks (Maestre et al., 2000; Vardin and Fenercioglu, 2003). Recently, there is a huge demand for industrial processing to obtain pomegranate juice because of its benefits toward healthy living. The juice is a rich source of natural components as polyphenols, anthocyanins and mineral nutrients that play an important role in maintaining juice quality and determining nutritive value. Moreover, pomegranate fruit juice has other properties such as antioxidant, antinflammatory, and antiatherosclerotic against some diseases (Malik et al., 2005; Neurath et al., 2005; Sumner et al., 2005). A diet high in pomegranate juice is linked to lower risk for several chronic degenerative diseases, including certain cancers and cardiovascular disease (Lansky and Newman, 2007; Viuda-Martos et al., 2010). In Tunisia, pomegranate has been cultivated traditionally since ancient times and local germplasm is very diverse containing numerous varieties with sweet fruits and others with sour fruits which were more and more neglected. Our research was focused on providing an alternative use of these varieties. The study of organoleptic, chemical and antioxidant properties of some Tunisian varieties and the design

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of new beverages combining sour juice and sweet juice was carried out in this work.

Material and Methods

Plant material

Ripe fresh fruits were collected from four pomegranate varieties growing in the same *ex-situ* collection in Gabès (south Tunisia) (Mars and Marrakchi, 1998). The varieties selected for this study are: Garsi, Mezzi, Tounsi and Zaghouani. Representative fruits samples were collected, at harvesting maturity, for each variety.

Preparation and analysis of raw juice

Fruits were washed in cold tap water and drained. They were manually cut-open and the outer leathery skin was removed. The arils were manually separated from the fruits and juices were obtained by pressing the arils. The following attributes related to juice were evaluated.

Juice color was assessed visually according to grading scale of color intensity (2: light pink: the lightest color) to 18 (reddish purple: the darkest color) according to its intensity. Juice clarity was also evaluated visually through a note scale going from 1 (very hazy) to 5 (limpid).

The pH measurements were performed using a JENWAY model 4320 pH-meter at 20°C. Titratable acidity (TA) was determined according to the AOAC methods (1984) using 0.1N NaOH to the end point of pH 8.1 and expressed as grams of citric acid per liter (Ender et al. 2002). The soluble solids content (TSS) was measured with manual refractometer Atago (Tokyo, Japan) at 20°C and expressed as °Brix. Maturity index was calculated by dividing TSS to TA. Total sugars were determined by the Lane–Eynon (1923) method and expressed as g of sugar per 100 g of juice.

Formol index number (IF), indicator of free amino acids (Fry et al., 1995), was determined using a DL 58 titrator (Mettler-Toledo, Greifensee, Switzerland). The results were expressed in ml 0.25M NaOH 100 ml^{-1} juice. Potassium (K) and sodium (Na) levels were determined using a Jenway flame spectrophotometer after centrifugation in a 201M Sigma centrifuge (AFNOR, 1996). Calcium (Ca) was determined by complexometry using EDTA solution (AFNOR, 1996). All results were expressed in mg 100^{-1} g of juice.

Vitamin C content was determined with iodine and sodium thiosulfate using starch as an indicator according to Arya et al. (2000). Anthocyanin content was determined according to Porter et al. (1989). To 0.5 ml of juice were added 6 ml of n-butanol-HCl (95 : 5, v/v) solution and 0,2 ml of $NH_4Fe(SO_4)_2 12H_2O$-HCl 2M (2%, m/v) solution. The absorbance was determined using an UV/Visible spectrophotometer Philips PU 8800. Results were expressed as mg of cyanidin per 100 ml of juice. Total phenolic content was determined with the Folin–Ciocalteu method (Arnous et al., 2001). Reaction was followed with a spectrophotometer and results were expressed as mg of Gallic acid/100 ml of juice.

Antioxidant activity

The antioxidant activity of pomegranate juices was determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Espín et al., 2000; Llorach et al., 2004). All samples were centrifuged at 10,480 g (model EBA 21; Hettich Zentrifugen) for 5 min at room temperature.

The reaction mixture consisted of 2 µl of the diluted sample and an aqueous methanolic solution (250 µl) containing the free radical (DPPH•). The reduction of the DPPH radical was determined by measuring the absorption at 515 nm after 50 min of reaction, using 96-well microplates (Nunc, Roskilde, Denmark) and Infinite®M200 micro-plate reader (Tecan, Grödig, Austria). The DPPH• radical scavenging activity was expressed as mmol L^{-1} Trolox.

Juice mixture

New beverages were prepared using sour and sweet pomegranate juices in different proportions (reaching pH 4.0) in order to reduce the acidity of sour juices (Carpenter et al., 2000; Mars, 2001).

Juice sensorial analysis

The flavor of a nutriment is a property established using a multitude of sensorial attributes (Martinez et al., 2006). Juice sensorial analysis was conducted by untrained panel of tasters. Fresh pomegranate juices extracted from all the cultivars and the beverages obtained from the mixture were evaluated for their color, taste and the overall appreciation. A hedonic scale going from 1 to 8 was adopted for the taste (1: very acid, 2: acid, 3: acid with a sweet aftertaste, 4: less acid with a sweet aftertaste, 5: less sweet with an acid aftertaste, 6: sweet with an acid aftertaste, 7: sweet, 8: very sweet). Pomegranate juices were served at room temperature (about 20ºC) together with the appropriate questionnaire, one at a time and waiting 5 min between samples. Unsalted crackers and water were provided to consumers for palate cleansing between samples. In each questionnaire, consumers were asked, using 5-point hedonic scales (from 1: dislike, 2: neither like nor dislike, 3: like moderately, 4: like very much and 5: like extremely), about the overall liking of the sample

and their satisfaction degree about pomegranate juice sensory attributes (color and taste).

Statistical analyses

All data were subjected to ANOVA using SPSS 13.0 software. The data shown are mean values and the significance of the differences between juices and mixtures was determined using the Student-Newman and Keuls (SNK) test ($P < 0.05$).

Results and Discussion

Organoleptic characteristics

Color of fruit juice represents key elements of its total appearance. All evaluated pomegranate juices exhibited red color. Mezzi and Garsi juices were rather reddish than Zaghouani while Tounsi gave a red-pink juice. Turbidity is also an important index of clear juice that is a natural juice that is pulpless and do not have cloudy appearance (Sin et al., 2006). Mezzi juice was the clearest one followed by Garsi and Zaghouani, however Tounsi juice is relatively hazy (Table 1).

Regarding TSS content, Garsi yielded the lowest mean of total soluble solids content (14.08 °Brix), and Mezzi had the highest mean (16.28 °Brix). TSS content for all varieties ranged higher than the minimum threshold generally required for commercial use (>12%) and felled into the range of other pomegranate cultivars grown under Spanish (Martinez et al., 2006) and Iranian (Tehranifar et al., 2010) conditions. Total sugars content of studied varieties ranged from 12.56 to 14.23 g/100 g (Table 1). The highest amount was observed for Mezzi and the lowest was in Garsi (Table 1). Similar results were reported by Poyrazoglu et al. (2002), however, Melgarejo et al. (2000) found high amounts over 15 g/100g in some Spanish sweet varieties.

Juice pH variation was high ranging from 2.74 for Garsi to 4.02 for Zaghouani (Table 1). As regards to titratable acidity (expressed as % citric acid), statistically significant differences were noted among varieties. Zaghouani scored the lowest acidity (0.23%) and Garsi the highest one (2.04%). According to Chace et al. (1981), pomegranate was appropriate for fresh market when its acidity content was lower than 1.8%. Hence, Garsi could be considered inappropriate for fresh consumption. Muradoglu et al. (2006) reported that among 53 Turkish cultivars, some of them had an acidity superior to 0.9%.

The maturity index (TSS/TA) is one of the important factors influencing the taste and flavor of pomegranate, which some researcher used to classify the pomegranate cultivars (Kulkani and Aradhya, 2005; Tehranifar et al., 2010). This classification has been optimized for Spanish cultivars: maturity index (MI) = 5-7 for sour, MI = 17-24 for sour-sweet, and MI= 31-98 for sweet cultivars (Martinez et al., 2006). The maturity index values varied from 6.91 to 65.96 (Table 1). According to Martinez et al. (2006), cultivars are classified as: Garsi as sour, Mezzi as sour-sweet and Tounsi and Zaghouani as sweet varieties.

Nutritional properties

There are many mineral elements present in pomegranate juice (Figure 1). Potassium is the most abundant of all the elements investigated in pomegranate juice. The average overall potassium content was 283.88 mg/100g. No cultivar differences were observed for potassium contents. Only juice from Garsi contained markedly higher potassium content. Sodium was also present in pomegranate juices abundantly with amounts ranging from 63 to 72.2 mg/100 g. The highest content was revealed in Tounsi 1 juice while Mezzi 2 has the lower one. As for potassium content, no significant differences were noted between cultivars for their content of calcium. Tounsi1 contained the highest amount (20.52 mg/100g). Thus, pomegranate juice can be a good source of nutrients and variation could originate from the pomegranate cultivar, and agro-climatic conditions (Al-Maiman and Ahmad, 2002). Most of the findings regarding the mineral components of the pomegranate juice are consistent with the results of the other studies (Al-Maiman and Ahmad, 2002; Fischer-Zorn and Ara, 2007).

Table 1. Characteristics of four Tunisian pomegranate varieties.

Variety	JC	TURB	TSS (°Brix)	TS (g/100 g)	PH	TA (%)	MI
Mezzi (Sour-sweet)	14.80±1.79a	4.80±0.45a	16.28±5.40a	14.23±2.23a	2.85±0.12d	1.69±0.59b	9.62b
Garsi (Sour)	13.20±1.79a	4.00±0.0b	14.08±7.56b	12.56±3.99b	2.74±0.03c	2.04±0.14a	6.90c
Tounsi (Sweet)	8.00±0c	3.00±0.0c	14.92±7.95b	13.04±4.64b	3.83±0.10b	0.26±0.12c	56.99a
Zaghouani (Sweet)	10.00±0b	4.00±0.0b	15.00±4.69b	13.07±2.53b	4.02±0.02a	0.23±0.08c	65.96a
Significance level	**	**	**	**	**	**	**

**Means with different letters, in the same column, indicate significant differences at $P< 0.01$.

JC: Juice color, TURB: Juice turbidity, TSS: Total soluble solids content, TS: Total sugars content, PH: juice pH, TA: Titratable acidity, MI: maturity index

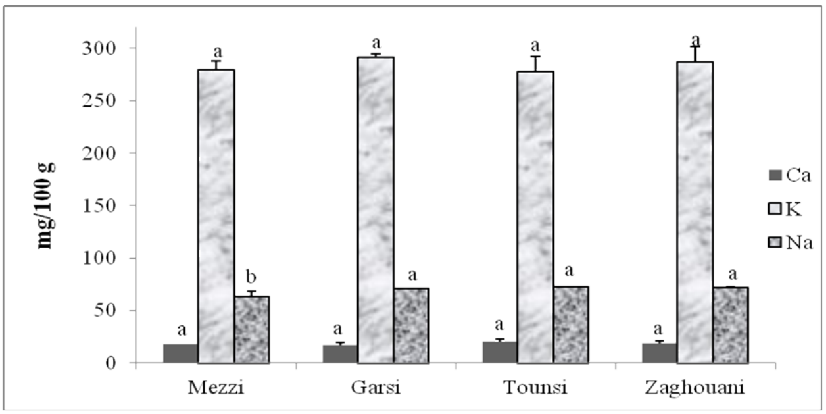

Figure 1. Mineral composition of pomegranate juices prepared from four cultivars.
Means with different letters indicate significant differences at $P \leq 0.01$ according to Duncan range test. Ca: Calcium amount; K: Potassium amount; Na: Sodium amount

Formol index which reflects the amount of free amino acid varied from 2.19 to 3.15 mL 0.1N NaOH/ 100 mL. Our findings related to formal index are lower than those reported by Ekşi and Özhamamci (2009). Ascorbic acid, abundant in pomegranate juice, has biological functions in fruits which include roles in redox control and antioxidant activity that prevent the browning of tissues (Kulkarni and Aradhya, 2005). The concentration of ascorbic acid varied from 20 to 25 mg/100 ml (Table 2). Sour varieties showed higher amounts of ascorbic acid as compared to sweet ones. Our pomegranates exhibited values higher than those reported by Zarei et al. (2010) for Iranian cultivars.

Anthocyanins are water-soluble pigments primarily responsible for the attractive color of many fruits, including pomegranate juices, and they are well known for their antioxidant activity (Noda et al., 2002; Mena et al., 2011). Examination of the total level of anthocyanins in different juices demonstrated that sour varieties exhibited higher content (over than 47 mg/100 ml) than sweet ones (Table 2). This could be explained by dependence of the intensity and stability of the anthocyanin on juice pH (Wilska-Jeszka and Korzuchowska, 1996). The destruction of anthocyanin pigments increases with increase in pH (Laleh et al., 2006). The levels of anthocyanin found with our juices were higher than those reported for Iranian (Alighourchi et al., 2008) and Chilean cultivars (Sepúlveda et al., 2010).

The content of total phenolics is one of the most important parameters for appraising the characterization of pomegranate cultivars, with respect to their nutritional value and potential use for different products. A variation in terms of total phenolic content were observed among the pomegranate varieties (164.47-181.84 mg Gallic acid/100 ml) (Table 2), but no significant differences were revealed between them. Ozgen et al. (2008) reported similar levels of total phenolics for Turkish cultivars (124.5-207.6 mg/100g) whereas, Gil et al. (2000) found higher level exceeding 180 mg/100 ml for Wonderful variety. According to these results, as being a good source of total phenolics, pomegranate can be considered as an important nutrient for human health.

Antioxidant activity

The DPPH method is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. The use of this method DPPH free radical is advantageous because it is more stable than the hydroxyl and superoxide radicals. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. The DPPH scavenging activity of pomegranate juices was significantly different (Table 2). The sour variety Mezzi showed the highest antioxidant activity value (22.50 mmol L^{-1} Trolox) while Tounsi showed the lowest values (16.01 mmol L^{-1} Trolox). These results are closer to those obtained for Iranian (Mousavinejad et al., 2009), Turkish (Ozgen et al., 2008) and Spanish varieties (Mena et al., 2011).

Plant phenolics constitute one of the major groups of antioxidants acting as free radical terminators (Samarth et al., 2008). This result showed a good relationship with the total phenolic content. Many studies reported that high polyphenols content contributes towards high radical scavenging activity (Mena et al., 2011). Nevertheless, antioxidant capacity cannot only be related to the phenolics content but it is the result of multiple factors (Arazo et al., 2011).

Table 2. Formal index, ascorbic acid, total phenolics, total anthocyanins contents and antioxidant capacity in aril juices prepared from 4 Tunisian pomegranate varieties.

Variety	FI	AAC (mg/ 100 ml)	TAC (mg/ 100 ml)	TPC (mg/ 100 ml)	DPPH (mmol L^{-1} Trolox)
Mezzi	3.15±0.11[a]	24.21±0.01[a]	51.01±2.40[a]	181.84±16.71[a]	22.50±3.27[a]
Garsi	3.15±0.14[a]	25.10±0.01[a]	47.66±2.16[b]	174.55±11.30[a]	17.59±2.39[b]
Tounsi	2.64±0.10[b]	21.22±0.01[b]	34.21±1.93[d]	171.21±14.10[a]	16.01±3.76[b]
Zaghouani	2.19±0.04[c]	20.42±0.01[b]	43.11±1.81[c]	164.47±36.19[a]	17.58±3.25[b]
Significance level	**	**	**	NS	**

**Means with different letters, in the same column, indicate significant differences at P< 0.01; NS: Non significant at P>0.05.
FI: Formal index; AAC: Ascorbic acid content; TAC: Total anthocyanins content; TPC: Total phenolics content

Table 3. Volumes of sweet juices added to sour juices.

Sour juice	Tounsi volume added (ml)	Tounsi volume percentage (%)	Zaghouani volume added (ml)	Zaghouani volume percentage (%)
Mezzi (100 ml)	450	81.8	400	80.0
Garsi (100 ml)	500	83.3	450	81.8

Juice mixture

The acidic taste and related flavor are some of the important attributes of pomegranate juice, which contribute to its strong appeal in the food and beverage industry. In this regard, sour varieties represent diversity in local pomegranate genetic resource (Mars, 2001) with very low sugar: acid ratio that is suited for formulation of a wide range of food and beverage products. Providing new beverages mixed with sour pomegranate juices is likely to increase their consumption. Thus, we mixed both sour and sweet juices in different ratios in order to obtain a final pH of 4. With such pH value the juice is considered acceptable (Carpenter et al., 2000). The volumes added were presented in Table 3.

Sensory evaluation

Color

Attractive color is one of the most important sensory characteristics of pomegranate arils and juice products. Significant differences for beverages color acceptance were found ($P < 0.05$). The sensory analysis regarding beverage color recorded mean values between 2.66 (like moderately) and 3.94 (like very much). Zaghouani-Garsi juice mixture was the most preferred according to its color followed by Zaghouani-Mezzi juice mixture and Zaghouani juice (Figure 2).

Flavor

Significant difference was found for the flavor of beverages ($P > 0.05$). Mean values stayed between 1 (very acidic) and 7.66 (very sweet). Mezzi is considered by the panelists as acidic with a sweet aftertaste. The mixture Mezzi-Tounsi was less sweet with an acidic aftertaste while Mezzi-Zaghouani mixture was considered sweet with an acidic aftertaste. Tounsi juice as well as Garsi-Tounsi and Garsi Zaghouani beverages were found sweet. The average scores for flavor acceptance was between 1 (dislike), which can be regarded as unsatisfactory result, considering the bitter flavor of Garsi juice and 5 (like extremely) considering Zaghouani juice as very sweet beverage (Figure 2).

Overall preference

Low overall liking was associated with juices with high intensities of sourness. Mixing the juices elaborated with sour or sour-sweet pomegranates with sweet ones is a solution to simultaneously decrease the sourness of the products and increase its acceptability by consumers. Panelists easily detected significant differences in taste and color. They preferred in general Zaghouani juice beverage as well as beverages made of Garsi juice mixed with Zaghouani which provided good organoleptic properties (color, clarity and flavor) or Garsi mixed with Tounsi which was esteemed for its taste and improved color. The panelists gave such products high ranking (Figure 2).

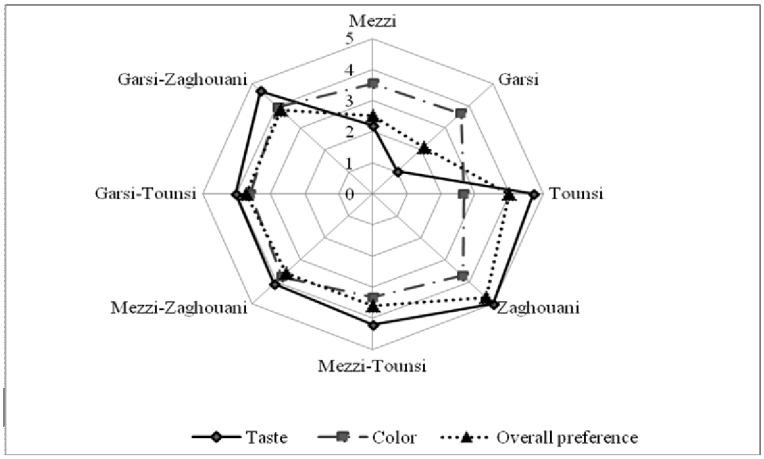

Figure 2. Satisfaction degree scores of consumer panel for main sensory attributes of fresh pomegranate beverages according to taster's hedonic rating.

Conclusion

Though the picture is far to complete, the study has shown that Tunisian pomegranate varieties particularly sour varieties (Mezzi and Garsi) were rich in polyphenols as well as anthocyanins, ascorbic acid and mineral components. This study provides a useful insight into production for new beverages with nutritional intake. Sensory analysis which is often used to determine the acceptability of a newly developed product showed that beverages made by mixing sour and sweet pomegranate juices were well appreciated by the untrained panelists. Further research is needed to establish new pomegranate juice combinations and to examine the stability of characteristics and bioactive proprieties of mixtures after cold storage in order to develop new healthy beverages.

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FOOD SCIENCE AND NUTRITION

Application of colour parameters for assessing the quality of Arabica and Robusta green coffee

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Abstract

This work aims to achieve fast and non-destructive indicators that enable the definition of the quality of green coffee from *Coffea arabica* and *C. canephora*, respectively Arabica and Robusta, in high commercial samples (which displayed, respectively, medium sieve between 17.4 and 19.2, trade homogeneity of 94.3% and 97.0%, mass of 1000 beans with values of 213.16 g and 151.36 g, apparent densities of green coffee not significantly different, showing values of 0.629 and 0.630 g cm-3 and moisture ranging between 9.056 and 9.243%). Being these samples within the established range of the international market, and ensuring good storage conditions, the chromatic parameters L* (lightness), C* (chroma) and Hº (tone or hue angle) of these samples were assessed using illuminants D65 and C. It was concluded that through the application of colour parameters the quality of green coffee can be assessed. Yet, as coordinate a* (contribution of red or green) strongly affects tone of the green coffee, the illuminant type used in the measurement must be defined and/or combined in order to accurately characterize product quality.

Key words: Arabica coffee, Chromatic parameters, Robusta coffee, Technological quality

Introduction

The family *Rubiaceae* includes the *Coffea* genus that has at least103 species, with commercial relevance for *C. arabica* L., *C. canephora* Pierre ex Froehner, *C. liberica* Bull ex Hiern and *C. dewevrei* Wild and Durand cv. Excelsa, that represents, respectively Arabica, Robusta, Liberica and Excelsa types of coffee (Bicho et al., 2011c). *C. arabica* grows in 85% of coffee producing countries, predominantly in the American Continent, accounting for approximately 65-75% of the world coffee production, whereas *C. canephora* is predominantly cultivated in Asia and Africa (this continent with about 80% of total plantation), being responsible for about 25-35% of worldwide coffee yield. The other two species, *C. liberica* Bull ex. Hiern and *C. dewevrei* cv. Excelsa, are cultivated in Africa, accounting for about 1% of world production (Bicho et al., 2011c).

The world green coffee production has been growing since the sixties, with total production varying between *ca*. 4.2 and 7-8 million tonnes, between 1960 and the last decade (ICO, 2009). World consumption of green coffee stabilized at *ca.* 7 million tonnes per year, but only 25% are consumed in producing countries (Bicho, 2005). Also, the global green coffee consumption is not matching the increased production, provoking price depression. This could leads to a quality deterioration of the produced coffee, because the producers tend to limit the financial costs, with less investment in crop and harvest management (Bicho, 2005). Nevertheless, the recognized positive effects on human (Florián et al., 2013) health points to an increase of the trade market in the near future.

Coffee beans quality depends on moisture content, defects, bean size, some chemical compounds and preparation of a sample to perform cup tasting (Bicho et al., 2011b). To perform such physical quality analysis, standard methods are being used to characterize technological quality of green coffee, namely particle size, medium sieve,

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most frequent sieve, share split, cumulative calibration, trade homogeneity, mass of 1000 coffee beans, apparent densities, strange bodies and defects, mass losses on drying and olfactory and visual parameters. However, such physical analysis to characterize technological quality of green coffee should be fast and easy to perform, as well as accurate and with a low cost (Bicho et al., 2011a, 2012). Colour has been used for quality assessment of green coffee beans (Nelson, 2005; Bicho et al., 2012) and there have also been studies using image processing for grain quality inspection (Soedibyo et al., 2010). Also, previous studies of colour parameters underline its importance for roasting coffee degree assessment (Bicho, 2005; Bicho et al., 2012). In this scope, this work aims at to study the use of non-destructive chromatic parameters to help in quality evaluation of green coffee bean that might influence the sensory quality of the drink. For that, using the illuminants D_{65} and C, were analysed two green coffees (Arabica from Brazil and Robusta from India, representative from the two main traded species), having commercial characteristics. The results will be related to grain physical characteristics, in order to determine if a chromatic analysis could constitute a fast, reliable and low cost method for green coffee quality evaluation.

Materials and Methods

Green beans from commercial lots of the two most representative traded species, *Coffea arabica* from Brazil and *Coffea canephora* from India, 4-6 months after yielded, were characterized in terms of its physical quality. The samples were supplied by NovaDelta company (Portugal).

Sampling of *Coffea arabica* and *Coffea canephora* was carried out according to ISO 4072 (1982), as recommended by the ICO for sampling green coffee in bags. The sampling process began with the selection of green coffee bags, at random (a minimum of 10% of the lot). The selected bags were separated from the lot and, with a probe, 30±6 g of coffee were collected in triplicate from three different points in the bag (top, middle and bottom). After collection and standardization of the portions, these were mixed, for an overall take of green coffee, with a minimum mass of 1.5 kg.

The particle size analysis followed ISO 4150 (1991). A sample of each coffee (100.0 ± 0.1 g) was passed through a set of sieves with round holes. After mild shaking for three minutes, the fraction retained on each sieve (mesh 20 through 16) and in the receptacle was weighted (with an accuracy of 100 mg). Data are presented in mass percentage, and corresponds to the average of three trials. The medium sieve, the most frequent sieve, the share split, the cumulative calibration, and the trade homogeneity, were calculated according to Esteves and Oliveira (1970). The share split corresponds to the percentage of the bean mass retained on each sieve. The cumulative calibration expresses the cumulative percentage of the accumulated bean mass retained on each sieve. The medium sieve indicates the weighted average between the share split and the numbers of the sieves used for calibration. The most frequent sieve is the sieve that retained the highest percentage of coffee during the calibration test split. The trade homogeneity considers the maximum percentage of coffee retained in two consecutive sieve numbers, during the calibration test split (Esteves and Oliveira, 1970).

Following Instrução Normativa Nº 8 (2003), the levels of big, medium and small bean were also determined, considering the amounts retained in the sieves 19-17, 16-15 and 14 or smaller, respectively.

The mass of 1000 coffee beans was determined by extrapolation, after weighting 100 beans without defect, following Esteves and Oliveira (1970). Withdrew from the sample, after homogenization, 100 green coffee beans were weighed. Data are the average of five replicates per sample of green coffee.

The determination of apparent density followed NP 2285 (1991), using a container of known volume, with 10 mg. The difference between replicates did not exceed 2% of the average of five replicates.

Foreign bodies and defects were determined according to ISO 4149 (2005). Green coffee beans (300.0 ± 0.1 g) were spread over an orange surface, being observed under diffuse natural light. The defective beans and foreign objects were split up into categories, as proposed by ISO 10470 (2004), being counted and weighed. For each category the number of units and the mass percentage was determined following ISO 10470 (2004). Considering that foreign bodies and defected beans do not have an equal contribution to the sensory profile of a coffee drink, different systems of counting defects, for marketing purposes, have been followed (ISO 10470, 2004; WP Board Nº 1005/06, 2006). As a general pattern, the concept of defect, at a commercial level, was related with the presence of a number of units of each category of foreign or defective beans, allowing the equivalence of defects in accordance with the procedures mentioned above. Following these methods, for each type of defective beans, a score

was assigned, according to tables of defects. The total score pointed the number of defects, for purposes of marketing coffee. This determination was based on the ICC resolution 420 (2004), being considered exportable Arabica or Robusta coffee if there were no more than 86 or 150 defects per 300 g, respectively. In the case of LIFFE classification system (WP Board nº 1005/06, 2006), the defects in the Robusta coffee are analysed in 500 g samples.

The determination of mass losses of green coffee beans by drying, was based on ISO 6673 (2003) and ICC resolution N° 420 (2004). Ten grams of green coffee beans were placed in capsules (previously dried 1 h at 105 ± 1°C, and subsequently allowed to cool down at room temperature in a desiccator) and dried for 16 h ± 30 min at 105 ± 1°C. Data are the average of simultaneous experiments performed in triplicate.

The olfactory and visual assessment followed ISO 4149 (2005). This survey was completed by the analysis of the bean shape, according to Esteves and Oliveira (1970). For the olfactory test, the containers with the samples of green coffee were opened and inhaled, to detect strange or unpleasant smell, being the results recorded as "abnormal" or "normal".

For the visual examination of the beans, samples were spread on a smooth and orange surface, being examined under subdued or artificial light (NP 1795, 1989), in order to determine the: botanical origin of coffee; type of coffee, according to the preparation process, as indicated by NP 1535 (1977); uniformity and dominant colour. The latter was classified, according to NP 1795 (1989), in blue, green, white, yellow and brown. Visual assessment of the beans shape considered the criterion of Esteves and Oliveira (1970).

A Minolta colorimeter CR-300 was used to green coffee colour assessment on beans and grinded coffee, using D_{65} and C illuminants. White tile, used as standard, with the coordinates: Y=93.10, x=0.3161, y=0.3326 or L^*= 97.27, a^*= - 0.01, b^*= 1.98, for the illuminant D_{65}; Y=93.10, x=0.3136, y=0.3197 or L^*= 97.26, a^*= + 0.01, b^*= 1.94, for the illuminant C. The colorimeter was first calibrated to white Yxy coordinates, specific of each. Then, the colour space was chosen to obtain the results expressed in the chromaticity coordinates L^* a^* b^* samples, for the selected illuminant. According to McGuire (1992), the coordinate L^* represents the lightness (contribution of black or white, varying between 0 and 100); a^* indicates the contribution of red or green (when its the value is positive or negative, respectively); b^* the contribution of blue or yellow (when its value is negative or positive, respectively). The coordinate L^* is perpendicular to the plane containing the chromaticity coordinates a^* and b^* (McGuire, 1992). Considering the coordinates $L^*a^*b^*$, the colour is expressed through $L^*C^*H^o$, being: L^* the lightness; C^* the chroma or saturation (Chervin et al., 1996); Hº the tone (or hue angle, which indicates colour variation in the plane formed by the coordinates a^* and b^*) (Chervin et al., 1996). These parameters were determined considering (McGuire, 1992; Chervin et al., 1996): $C^*=(a^2+b^2)^{1/2}$; H^o=(arctg(b/a)/6.2832) x 360 (if $a > 0$ and $b \geq 0$), or H^o=180+(arctg(b/a)/6.2832) x 360 (if $a < 0$ and $b \geq 0$ or $b < 0$), or H^o=360+(arctg(b/a)/6.2832) x 360 (if $a > 0$ and $b < 0$). The overall colour difference, ΔE, was determined using the equation $\Delta E=[(\Delta L)^2+(\Delta a)^2+(\Delta b)^2]^{1/2}$ (Chervin et al., 1996).

Statistical Analysis

Data were statistically analysed using one-way ANOVA ($P \leq 0.05$) applied to the studied parameters. Based on the ANOVA results, a Tukey's test was performed for mean comparison, for a 95% confidence level. Different letters indicate significant differences in a multiple range analysis for 95% confidence level.

Results and Discussion

The particle size of coffee Arabica and Robusta, according to the fractional calibration showed that the most frequent sieve, was number 17 and 19 for Arabica and Robusta coffee seeds, respectively (Figure 1). The result obtained for Arabica green coffee is in agreement with previous works (Bicho, 2005; Bicho et al., 2012). The medium sieve remained between 17.4 and 19.2 for Arabica and Robusta coffees, respectively, thus, according to Instrução Normativa Nº 8 (2003) the samples had big beans but the trade homogeneity was lower in Arabica coffee (94.3%), relatively to Robusta coffee (97.0%).

The cumulative percentage of green Arabica and Robusta coffee accumulated until the 17th sieve was 100% and 99.8%, respectively. Considering that the bean size, coffee samples were found commercially homogeneous, since they offer a level of coffee held in two successive screens larger than 90% (Esteves and Oliveira, 1970). This commercial characteristic resulted of an undergoing calibration stage during the benefit prior to marketing (implicating the separation of

green coffee beans, by the shape and size, using a set of sieves with perforation of variable shape and size), being the homogeneity of the coffee beans, of particular importance for roasting, to avoid the burning of smaller grains (Cortez, 2001; Bicho et al., 2012).

The mass of 1000 beans of green Robusta coffee was significantly higher relatively to coffee Arabica, with values of 213.16 g and 151.36 g, respectively (Table 1). The data was similar to that reported by Coste (1992) for Arabica coffee, but according to Fazuoli (1986) slightly higher for the Robusta coffee.

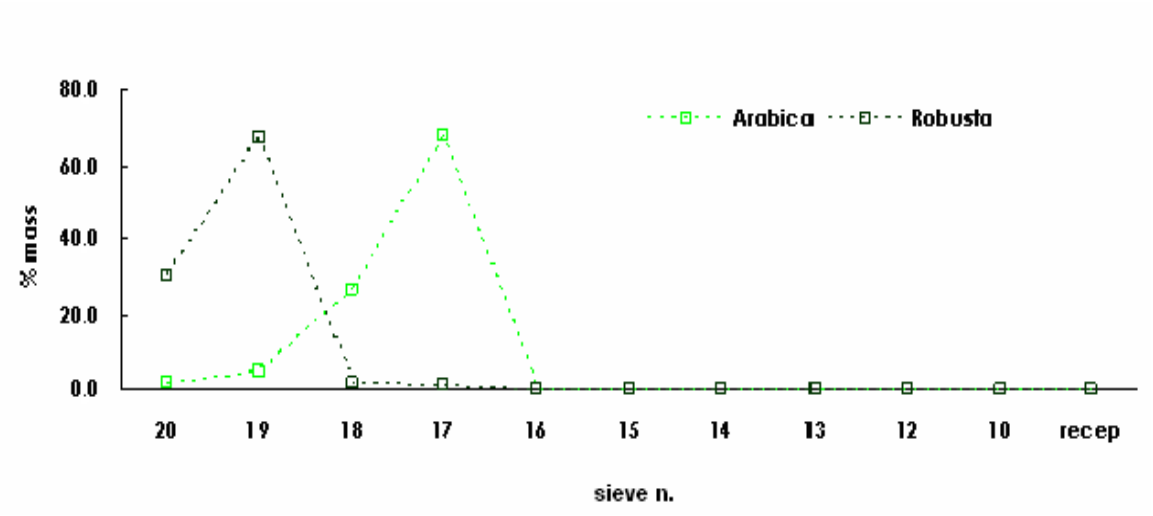

Figure 1. Fractional calibration of Arabica and Robusta green coffee beans.

Table 1. Physical characterization of Arabica and Robusta coffees. For the mass of 1000 beans and apparent densities, each value is the mean ± S.E. Different letters (r, s) indicate significant differences, in a multiple range analysis, for 95% confidence level. NA means "Not Applicable". The analysis of defects considered the systems: Brazil/Nybot (one defect in Arabica coffee equals 3 units of shell bean); Vietnam (one defect of Arabica and Robusta coffee as a coefficient of 0.20); LIFFE (one unit of Robusta coffee equals a defect); ISO-10470 (0 and 0.5 coefficient was applied over the mass percentage of the defect, to determine mass loss and the sensorial impact, respectively).

		Arabica coffee	Robusta coffee
Mass of 1000 coffee beans (g)		$151.36 + 0.47^{r}$	$213.16 + 1.40^{s}$
Apparent density (g cm^{-3})		0.629 ± 0.004^{r}	0.630 ± 0.004^{r}
Defects			
	Name	Shell bean	Shell bean
	Number of units	44	17
	Mass (%)	0.01	0.01
	Brazil/Nybot criterion	14.6	NA
	Vietnam criterion	4.8	3.4
	LIFFE criterion	NA	28.3
	ISO 10470 – Mass losses	0	0
	ISO 10470 - Sensorial	0.01	0.01
Mass loss on drying		9.06 ± 0.03^{r}	9.24 ± 0.03^{r}
Processing type			
Olfactory assessment		Normal	Normal
Visual assessment			
	Colour	yellowish	yellowish
	Uniformity	uniform	uniform

Moreover, the apparent densities of green coffee Arabica and Robusta were not significantly different, showing values of 0.629 and 0.630 g cm^{-3}, respectively (Table 1), but there is a clear difference between the apparent density of Arabica and Robusta coffees, for similar moisture contents. Defects associated with foreign matter to the coffee, or with matters unrelated to the beans from the fruit, were not found in the surveyed coffee, which was a further indication of quality ISO 10470 (2004). Defects associated with irregular beans also could not be detected. Only small quantities of shell beans were found (Table 1). Accordingly, these data further indicated that green Arabica coffee could be exported, with the International Coffee Organization certificate of origin (ICC resolution nº 420, 2004) and additionally could be reported as a coffee "gourmet", according to the classification of ABIC (2007). Considering the defects found, Robusta green coffee could also be exported with the International Coffee Organization certificate of origin, being additionally accepted by LIFFE as green coffee classified with the quality of type I, because had less than 150 defects per 500 g of coffee, thus, presenting a commercial value without financial penalty.

The moisture content of green coffee beans ranged between 9.056 and 9.243%, (Table 1), being therefore within the established range of the international market, and ensuring good storage conditions. Indeed, the moisture content in marketable green coffee bean should not exceed 12.5% according to the technical regulation of identity and quality for classification of the benefited raw green coffee bean (Instrução Normativa Nº 8, 2003). Otherwise the beans will be easily attacked by fungi, allowing the accumulation of ochratoxin A to prohibited levels (in Europe, according to the Regulação (CE) Nº 123, 2005, higher than 5 mg kg^{-1}) and other mycotoxins, undergoing as well a marked colour change (Coste, 1992). Through the prevention of mould development the quality of green coffee is favoured, since its occurrence might produce an undesirable odour and give an unpleasant flavour to the drink, which leads to rejection of these beans for roasting and marketing (Instrução Normativa Nº 8, 2003).

Considering that the absence of any odour in green coffee is considered an additional indicator of quality coffees, during the primary processing, storage or transportation, it is further desirable to minimize the proximity of coffee to be dried or dehydrated, to the peel and pulp or fertilizers, since these materials can develop particularly unpleasant odours (Coste, 1992). Indeed, the odours coming from chemical contamination or other sources persist even after roasting and in the produced drink (Coste, 1992), so they depreciate coffee commercial value (Instrução Normativa Nº 8, 2003). In this context, it was also found that Arabica and Robusta green coffee beans had a normal odour (Table 1), showing no deterioration from chemical or microbiological origin, as well as any contamination with foreign substances or defective beans that could have an impact in the olfactory characteristics. Thus, these results further become favourable indicators of the quality of the analysed samples (Bicho et al., 2011b).

When the colour of the high quality Arabica and Robusta green coffee beans, having optimal characteristics for the international market, was visually analysed, an uniform yellowish was detected (Table 1), suggesting a recent harvest followed by a dry processing. Moreover, one factor that positively contributes to further improve the appearance uniformity and colour of a bean is related to the minimization of the silverskin adherence to the bean by polishing during the benefit. Otherwise silverskin may confer to the seed a strong green tone, when carotenoids and chlorophylls are present in it, or red if only carotenoids prevail (Coste, 1992; Clifford, 1987). In this context, the residual silverskin of the surveyed samples had a reddish-brown colour, being almost absent in the groove of the green coffee beans, which is also an indicator of a dry primary processing. The green coffee samples had still predominantly flaky bean with round shape, according to the classification of Esteves and Oliveira (1970), further supported by Bicho et al. (2011b).

Considering the importance of nanotechnologies (Momin et al., 2013), the determination of the colour of green coffee bean, made with the illuminants D_{65} and C allowed the collection of coordinates systems chromatic CIEL*a*b* and CIEL*C*Hº (Table 2), detecting significant differences between the coffee samples in all parameters except the coordinate L*. The parameters a* and b* (contribution of the green/red, and blue/yellow, respectively) presented positive values, pointing a greater contribution of the red and yellow components. The combined value of these two chromatic parameters justified the yellowish colour visually detected for both coffee

beans. Although the lightness (L*) was not significantly different, the opposite occurred with saturation (C*) and tone (H°). Jointly these three coordinates contribute to the colour observed in each sample. In fact, Arabica green coffee had a greenish yellow, while Robusta green coffee had a brownish yellow colour (with the coordinates a* and b* being significantly higher). Accordingly, the red and yellow colours prevail in this coffee. The values of coordinate b* were similar to those reported by Bicho (2005), for five wet processed coffees, which suggests that a* might be responsible for the colour differences found in the dry or wet processed green coffees. In fact, the wet processed coffee showed negative a* values, pointing to a major green contribution. Therefore, the greenish colour developed in beans by this type of processing, in contrast to the yellowish colour of dry processed green coffee beans (that has a positive a* value). These data show that the coffee industry can use this difference in colour (green or yellow) as a quick way to identify the type of post-harvest processing of green coffee beans. In fact, these data, further supported by previous studies (Bicho, 2005), indicate that the differences in coordinate a* are linked to the green coffee beans processing and variety. Nevertheless, it also must be pointed that the age of the grain can also be affected by the value of a* (Mendes et al., 2001). L*, a* and b* values of arabica sample are similar to those obtained by Nelson (2005) for an aged arabica coffee from Jamaica.

In grinded green coffee, the coordinates CIEL*a*b* and CIEL*C*H°, using illuminant D_{65}, revealed that coordinates a*, b*, C* and Hº did not significantly differ between the two coffee types, unlike the parameter L*, but when we used illuminant C, only b* and C* did not differ significantly (Table 2). These data points to the existence of a significant correlation between the coordinates a* and H° and the coordinates b* and C*. Hº of grinded green coffee would be more influenced by the contribution of a* (that presents a higher variation) than coordinate b*, thus suggesting that a* would determine the colour of green coffee. For C* calculation the b* coordinate assumes a higher importance than a* due to its higher value. Thus the absence of b* variation would justify the absence of significant differences among C* values.

The predominant colour of green coffee depends on factors related with the botanical source and processing type (Coste, 1992). However, bean colour also develops during the green coffee processing, without the contribution of chlorophylls (Clifford, 1987), being mostly associated to fermentation and drying (Coste, 1992). Such colour also depends on the nature of soil and agricultural techniques applied and the duration and conditions of storage (Coste, 1992). The latter two factors do not seem to affect negatively the analysed samples since were freshly collected. After comparing the results of coordinate systems CIEL*a*b* and CIEL*C*H°, for parameters L*, a*, b*, C* and H°, using as variables both illuminants (D_{65} and C), it was found that the samples of green coffee beans (Table 2) did not show clear different values.

Table 2. Chromatic parameters of Arabica and Robusta coffee samples. Each value is the mean of 9 replicates ±S.E. Different letters (r, s) indicate significant differences after a mean comparison by the Tukey's test at a 95% confidence level.

		Chromatic parameters				
		L*	a*	b*	C*	Hº
Entire beans						
		Illuminant D_{65}				
	Arabica	57.54±0.83 ^{r}	2.02±0.17 ^{r}	13.37±0.39 ^{r}	13.53±0.40 ^{r}	81.47±0.63 ^{r}
	Robusta	56.93±0.85 ^{r}	3.50±0.27 ^{s}	16.19±0.36 ^{s}	16.58±0.36 ^{s}	77.79±0.93 ^{s}
		Illuminant C				
	Arabica	57.19±0.70 ^{r}	1.41±0.14 ^{r}	13.50±0.58 ^{r}	13.57±0.58 ^{r}	84.08±0.52 ^{r}
	Robusta	57.27±0.88 ^{r}	3.03±0.25 ^{s}	16.22±0.21 ^{s}	16.52±0.19 ^{s}	79.40±0.92 ^{s}
Grinded beans						
		Illuminant D_{65}				
	Arabica	72.11±0.27 ^{r}	1.06±0.04 ^{r}	15.84±0.12 ^{r}	15.88±0.12 ^{r}	86.19±0.15 ^{r}
	Robusta	75.29±0.16 ^{s}	0.89±0.02 ^{r}	15.83±0.13 ^{r}	15.85±0.13 ^{r}	86.79±0.07 ^{r}
		Illuminant C				
	Arabica	71.69±0.07 ^{r}	0.90±0.01 ^{r}	15.90±0.06 ^{r}	15.93±0.06 ^{r}	86.75±0.04 ^{r}
	Robusta	75.98±0.05 ^{s}	0.66±0.02 ^{s}	15.93±0.09 ^{r}	15.95±0.09 ^{r}	87.62±0.08 ^{s}

However, with the illuminant C, the coordinate a* showed a slightly lower value and the coordinate H^o, was higher. In ground green coffee (Table 2) significant differences were found for parameters a* and H°, whereas the opposite occurred to the other parameters (L*, b* and C*). Also, in this case, using the illuminant C, a similar trend was found to that reported for green coffee beans, occurring a significant lower value for the coordinate a* and a higher value for the coordinate H. Moreover, it was interesting to find that the parameters L* and H^o followed similar patterns using both illuminants, D_{65} and C, to a previous study on roasted coffee grains (Bicho, 2005).

Conclusion

The chromatic parameters, which allow a fast, reliable, low cost and non-destructive analysis that integrates the result of several processes, from plant production until storage, was able to partially differentiate the quality of Arabica and Robusta green coffee samples within the established range of the international market, and having good storage conditions. Yet, as coordinate a* strongly affects tone of the green coffee in the colour analysis, the illuminant type used in the measurement must be defined and/or combined. In fact, our study showed that the variation of spectral composition of incident light leads to a different colour perception.

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PLANT SCIENCE

Effects of fertilization and tillage system on growth and crude protein content of quinoa (*Chenopodium quinoa* Willd.): An alternative forage crop

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Abstract

A field experiment was conducted to determine the effects of fertilization and tillage on growth, yield and quality of quinoa crop (*Chenopodium quinoa* Willd.). The agronomic performance and nutritive value of quinoa was analyzed in order to define alternatives to local forages for dry-season feeding of ruminants in the Mediterranean region. The experiment was laid out in a split-plot design with two replicates, two main plots [conventional tillage (CT) and minimum tillage (MT)] and sub-plots (fertilization treatments: control, cow manure, inorganic fertilization 100 kg ha^{-1} (N1) and inorganic fertilization 200 kg ha^{-1} (N2)). The results indicated that quinoa growth was influenced by both tillage and fertilization. The lowest height and dry weight were found under MT. Moreover, the lowest height and dry weight (8205 kg ha-1 and 8020 kg ha^{-1} for CT and MT, respectively) were found under control treatment (no-fertilization). Concerning the nitrogen content there were no significant differences between CT and MT systems. In addition, the highest quinoa nitrogen content was observed for N2 treatment (200 kg N ha^{-1}). Moreover, the highest nitrogen content was measured at 150 DAS. Concerning the crude protein content, there were no significant differences between CT and MT systems. In addition, there were significant differences in crude protein between fertilization treatments. The greatest value was observed for N2 treatment. Moreover, the highest crude protein yield (2481 kg ha^{-1} and 2356 kg ha^{-1} for CT and MT, respectively) and acid detergent fibre (ADF) were found under N2 treatment. In addition, ash was not influenced by both tillage systems and fertilization treatments. Data indicate that quinoa crop could be used as alternative to legumes for protein production to feed ruminant animals.

Key words: Crude protein, Quinoa, Soil properties, Tillage system, Yield

Introduction

Quinoa (*Chenopodium quinoa* Willd.), is an Amaranthacean, stress-tolerant plant, cultivated along the Andes, for the last 7000 years. Its grains have higher nutritive value than traditional cereals and it is a promising worldwide plant for human consumption and nutrition (Vega-Gálvez et al., 2010). Quinoa is one of the main food crops in the Andean mountains, but during recent times there has been increased interest for the product in the United States, Europe, and Asia (Jacobsen, 2003). Quinoa is a good source of protein and can be used as a nutritional ingredient in food products (Gonzalez et al., 2012). The Organization of the United Nations for Food and Agriculture (FAO) has declared the year 2013 as the year of the quinoa.

Moreover, limited data are available regarding the fodder quality of quinoa crop. Bhargava et al. (2010) reported that the foliage of many species of Chenopodium (*C. album, C. berlandieri, C. bushianum, C. giganteum, C. murale, C. quinoa, and C. ugandae*) is a rich source of minerals like potassium, sodium, calcium and iron. The foliage of quinoa is rich in protein, carotenoid and ascorbic acid (Bhargava et al., 2007). Therefore, members of the genus *Chenopodium* are used as a foliage crop and fodder in many parts of the world (Bhargava et al., 2010).

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In temperate regions, white clover (*Trifolium repens* L.) is the predominant species used for grazing, whereas red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) are cultivated mainly for hay and silage making (Krawutschke et al., 2013). Drought is an adverse factor that forages must cope with in the Mediterranean region. Quinoa is able to tolerate drought (Razzaghi et al., 2011; Razzaghi et al., 2012). Jacobsen (2003) reported that quinoa can grow with only 200 mm of rainfall. Therefore, the aim of this study was to determine the effects of tillage system and organic fertilization on growth and fodder quality of quinoa crop, under Mediterranean semi-arid conditions.

Material and Methods

A quinoa crop (*Chenopodium quinoa* Willd.) was established in the area of Agrinio (western Greece, Lat: 38.35, Long: 21.25) in 2012. The soil was a clay loam (24.9% clay, 61.2% silt, and 13.9% sand) with pH 7.4, organic matter 1.45% and EC 0.63 mS cm^{-1}. Some meteorological data of the experimental sites are presented in Figure 1. The sites were managed according to organic agriculture guidelines (EC 834/2007). The experiments were set up on an area of 650 m^2, according to the split-plot design with two replicates, two main plots (conventional tillage: CT, moldboard plowing at 25 cm, followed by one rotary hoeing at 10-15 cm; minimum tillage: MT, chiseling at 25 cm depth followed by chiseling at 10-15 cm) and four sub-plots (fertilization treatments: control, cow manure (2000 kg ha^{-1}, 1.24% N, inorganic fertilization (fertilizer 26-0-0) with 100 kg N ha^{-1} and 200 kg N ha^{-1}). The main-plot size was 300 m^2. The crop was cultivated before quinoa becomes durum wheat. Quinoa was sown by hand in rows 30 cm apart at a depth of 2-3 cm. Quinoa was sown on 5th of May 2012 at a rate of 10 kg ha^{-1}. Overhead sprinkler system was set up on the field. The total quantity of water was 180 mm.

The organic matter was measured by the Walkey-Black method, for 0-15 cm depth for every plot (Walkey and Black, 1934). Furthermore, the total nitrogen was determined by the Kjeldahl method (Bremner, 1960) using a Buchi 316 device in order to combust and extract the soil samples.

For the computation of dry weight, height and LAI, 10 plants were randomly selected in each plot. The dry weight was determined after drying for 72 h at 70ºC. Leaf area was measured using an automatic leaf area meter (Delta-T Devices Ltd). Root samples were collected 150 DAS (days after sowing) and from the 0–25 cm layer by using a cylindrical auger (25 cm length, 10 cm diameter) at the midpoint between successive plants within a row. For each sample, roots were separated from soil after being in water + $(NaPO_3)_6$ + Na_2CO_3 for 24 h. For the determination of the root density, the root samples were placed on a high-resolution scanner using DT software (Delta-T Scan version 2.04; Delta-T Devices Ltd, Burrwell, Cambridge, UK). The total nitrogen was determined by the Kjeldahl method. Moreover, total protein content was calculated from the nitrogen content using a conversion factor of 6.25 (AOAC, 2009). Acid detergent fibre (ADF) was determined according to the methods of Van Soest et al. (1991).

The data were subjected to statistical analysis according to the split-plot design. The statistical analysis was performed with STATGRAPHICS Plus 5.1 logistic package. Differences between treatment means were compared at P=5% with ANOVA in order to find the statistically significant differences.

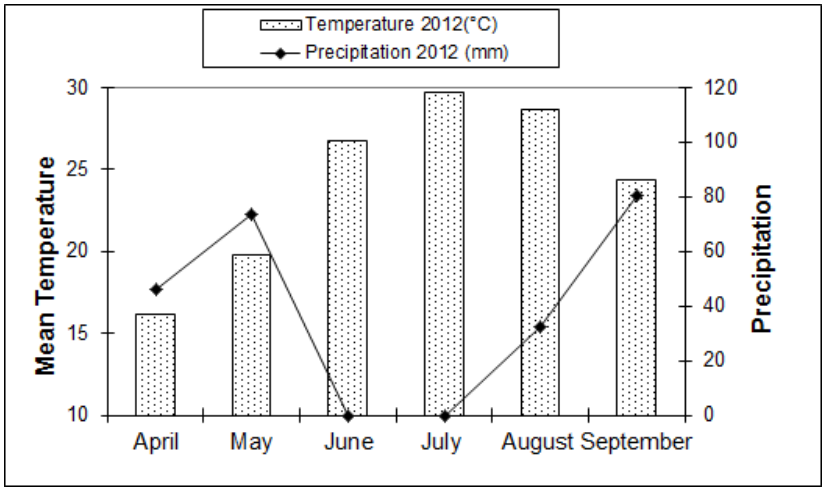


Figure 1. Meteorological data (temperature: °C and precipitation: mm) for the experimental sites during the experimental period (April-September).

Table 1. Effects of tillage system (conventional tillage: CT, minimum tillage: MT) and fertilization (control, cow manure, inorganic fertilization 100 kg ha^{-1} (N1), inorganic fertilization 200 kg ha^{-1} (N2)) on soil porosity (%), soil total nitrogen (%), root density (cm cm^{-3}) and LAI of quinoa crop.

Fertilization	Tillage system			
	CT	MT	CT	MT
Experiment 2011	Organic matter		Soil total N	
Control	1.78	1.62	0.132	0.134
Cow manure	2.69	2.51	0.172	0.195
N1	1.97	1.70	0.144	0.131
N2	1.89	1.62	0.149	0.127
F_{illage}	10.76**(LSD=0.155)		0.13^{ns}	
$F_{fertilization}$	39.45***(LSD=0.219)		16.47***(LSD=0.019)	
Experiment 2012	Root density		LAI	
Control	1.01	0.88	4.30	4.45
Cow manure	1.21	1.11	4.70	4.65
N1	1.15	1.18	4.75	4.35
N2	1.21	1.23	4.25	4.45
$F_{tillage}$	1.20^{ns}		0.03^{ns}	
$F_{fertilization}$	4.67*(LSD=0.219)		1.32^{ns}	

F-test ratios are from ANOVA. Significant at *p=0.05, **p=0.01, ***p=0.001, ns: not significant. The LSD (p=0.05) for tillage systems and organic fertilization are also shown.

Results and Discussion

Tillage and fertilization influences the soil properties. The lowest soil organic matter was found under MT (Table 1). There were statistically significant differences between MT and CT systems. In addition, earlier studies have shown that the adoption of minimum tillage system lead to improvement of soil properties (i.e. organic matter, porosity and total N) (Bilalis et al., 2010, 2012). Furthermore, there were no significant differences in soil total N among tillage systems.

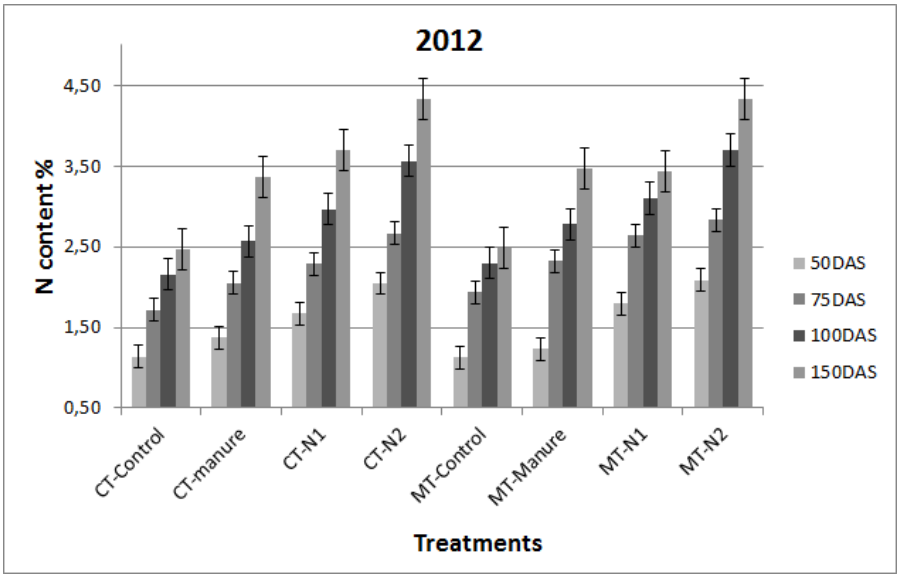
Moreover, there were significant differences between fertilization treatments concerning the soil organic matter and total N. Inorganic N fertilizer had no effect on soil organic matter. Tueche and Hauser (2011) reported that N fertilizer had no effect on soil physical properties. The highest organic matter and total nitrogen content were found under cow manure treatment. Efthimiadou et al. (2010) also observed that organic soil amendments increased the level of soil organic matter and total nitrogen. Furthermore, according to López-Espinosa et al. (2013) the use of organic fertilizers can be considered as an alternative fertilization method for organic crop production. Finally, in soil properties, no tillage x fertilization interaction was found.

Concerning the root density, there were no significant differences between CT and MT systems (Table 1). In a previous study, it was shown that the quinoa root density (1.03-1.21 cm cm^{-3}) was higher in soils subjected to minimum tillage than to conventional tillage (Bilalis et al., 2012). Also, there were significant differences in root growth between fertilization treatments. The lowest root diameter was found under control treatment. Leaf area index (LAI) was not influenced by both tillage systems and fertilization. In addition, Bilalis et al. (2012) observed that the highest LAI (4.47-5.03) and dry weight (8650-9290 kg ha^{-1}) were found in MT. Quinoa responds well to nitrogen fertilization (Berti et al., 2000; Schooten and van Pinxterhuis, 2003; Schulte auf'm Erley et al., 2005). The lowest height (165 cm and 155 cm for CT and MT, respectively) and dry weight (8205 kg ha^{-1} and 8020 kg ha^{-1} for CT and MT, respectively) was found under control treatment (no-fertilization). Dry weight and height had positive and significant correlation with root density ($r=0.741$, $p<0.05$ and $r=0.842$, $p<0.001$, respectively).

Concerning the quinoa nitrogen content there were no significant differences between CT and MT systems (Figure 2). In addition, there were significant differences in quinoa N content between fertilization treatments. All fertilization treatments resulted in values higher than those of the control. The greatest value was observed for N2 treatment (200 kg N ha^{-1}). Schulte auf'm Erley et al. (2005) observed that the N uptake (161 kg ha^{-1}) was nearly doubled by a fertilization of 120 kg N ha^{-1} compared to N 0 (82.6 kg ha^{-1}). The highest nitrogen content was measured at 150 DAS (Figure 1). Quinoa nitrogen content had positive and significant correlation with root density ($r=0.821$,

$p \leq 0.05$). Finally, in quinoa growth traits, no tillage x fertilization interaction was found.

The assessment of quinoa crude protein content is of a great importance for the fodder industry. Forage nutritive value is primarily determined by concentrations of crude nitrogen N protein. There are few data available regarding the fodder quality of quinoa crop. Concerning the crude protein content, there were no significant differences between CT and MT systems (Figure 2). N fertilization consistently had a positive impact on quinoa forage quality. There were significant differences in crude protein between fertilization treatments. The greatest value (27% or 270 g kg^{-1} dry matter) was observed for N2 treatment (200 kg N ha^{-1}). In this context, Kering et al. (2011) reported that application of N increased crude protein of both spring and summer harvested bermuda grass forage. The lowest crude protein yield was found under MT (Table 1). There were no statistically significant differences between MT and CT systems. Crude protein content had positive and significant correlation with root density ($r=0.798$, $p \leq 0.05$).



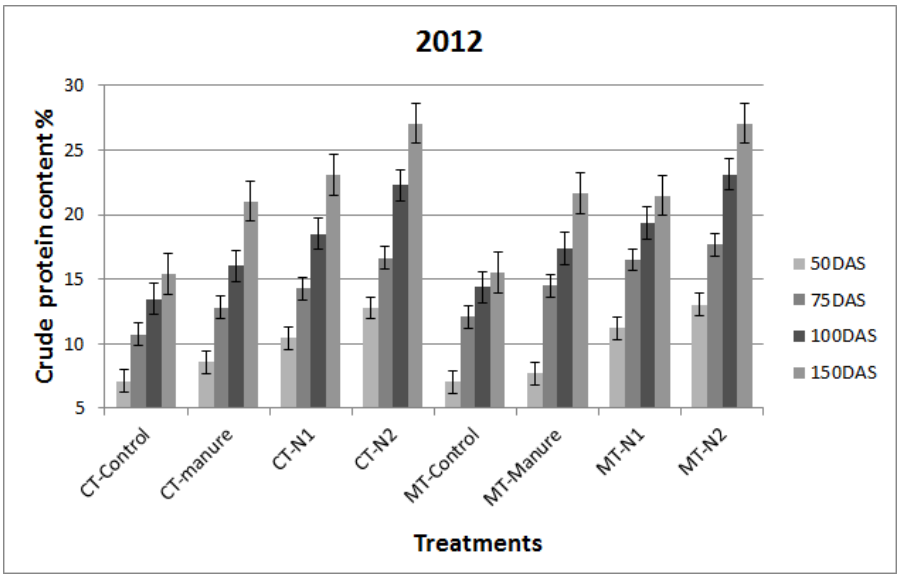


Figure 2. Effects of tillage system (conventional tillage: CT, minimum tillage: MT) and fertilization (control, cow manure, inorganic fertilization 100 kg ha^{-1} (N1), inorganic fertilization 200 kg ha^{-1} (N2)) on nitrogen (%) and crude protein content (%) of quinoa plants (50, 70, 100 and 150 days after sowing (DAS)). Error bars indicate standard error.

There were significant differences between fertilization treatments concerning the crude protein yield. The highest crude protein yield (2481 kg ha^{-1} and 2356 kg ha^{-1} for CT and MT, respectively) was found under N2 treatment. Crude protein yield had positive and significant correlation with root density and soil total N ($r=0.835$, $p\leq0.01$ and $r=0.832$, $p\leq0.05$, respectively).

Forage species differ in their crude protein content. Dugalić et al. (2012) reported that crude protein content in alfalfa plants ranged between 21.7% - 25.9%. The nutritional value of forage crops is also influenced by stage of maturity (Krawutschke et al., 2013). Our results indicated that the highest crude protein content was measured at 150 DAS (Figure 1). In addition, Schooten and van Pinxterhuis (2003) reported that quinoa crude protein content decreased from 190 g crude protein kg^{-1} dry matter at 70 DAS to 155 g at 98 DAS. Additionally, organic matter digestibility increased with growing period, with maximal values of 63.5% for Atlas cultivar and 68.5% g/Kg for Ras1 cultivar at 112 days (Schooten and van Pinxterhuis, 2003). As the plant ages, its morphological and histological development decreases the amount of cell content, which is soluble, and increases the amount of cell walls (Baumont et al., 2000).

Forage intake is affected by crude protein, fibre and ash content (Ibrahim et al., 2012). Acid detergent fibre (ADF) is a major indicator of digestibility, negatively affects feed quality (Han et al., 2003). Therefore, forage with a low ADF content is higher in quality than one with a high ADF content. ADF content in quinoa plants ranged from 24.78% to 39.45% (Table 2). There were significant differences between fertilization treatments concerning the ADF content. The highest ADF content (35.80% and 39.45% for CT and MT, respectively) was found under N2 treatment. In addition, Kering et al. (2011) reported that N fertilization consistently decreased ADF content in berrnuda grass forage. Furthermore, ash was not influenced by both tillage systems and fertilization treatments (Table 2). Finally, in quinoa quality traits, no tillage x fertilization interaction was found.

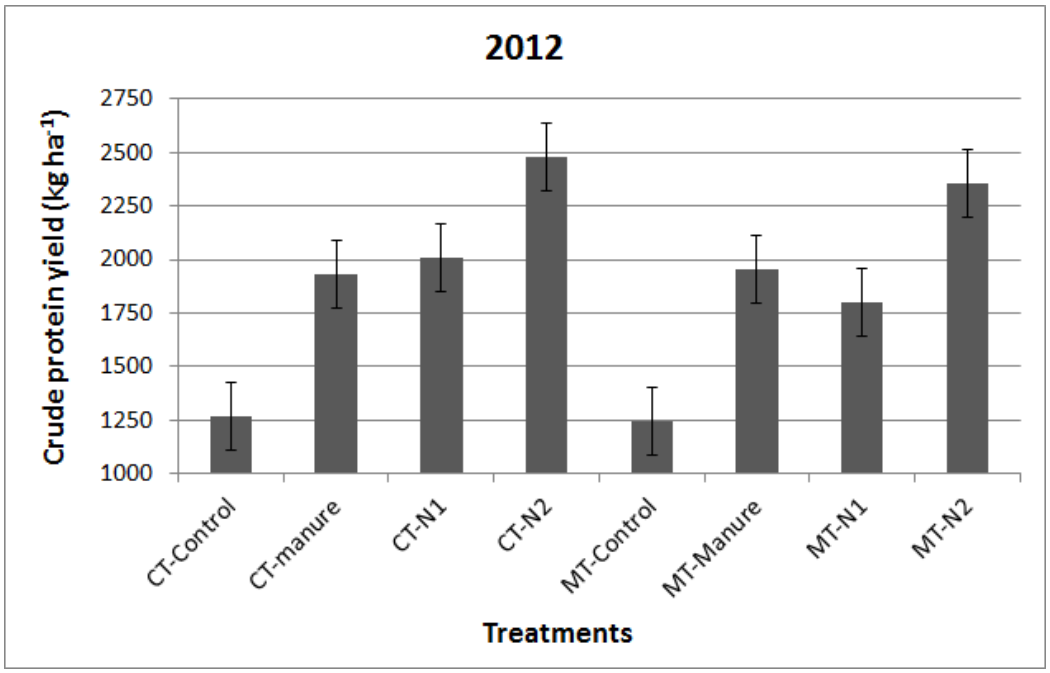


Figure 3. Effects of tillage system (conventional tillage: CT, minimum tillage: MT) and fertilization (control, cow manure, inorganic fertilization 100 kg ha^{-1} (N1), inorganic fertilization 200 kg ha^{-1} (N2)) on crude protein yield (kg ha^{-1}) of quinoa crop. Error bars indicate standard error.

Table 2. Effects of tillage system (conventional tillage: CT, minimum tillage: MT) and fertilization (control, cow manure, inorganic fertilization 100 kg ha^{-1} (N1), inorganic fertilization 200 kg ha^{-1} (N2)) on dry weight (kg ha^{-1}), height (cm), total ash (%) and acid detergent fibre: ADF (%) of quinoa crop.

Fertilization	Tillage system			
	CT	MT	CT	MT
Experiment 2011	Dry weight		Height	
Control	8205	8020	165	155
Cow manure	9170	9010	177	172
N1	8725	8390	171	173
N2	9165	8705	171	168
$F_{tillage}$	5.26*(LSD=143)		10.01** (LSD=5.14)	
$F_{fertilization}$	31.42***(LSD=229)		11.21**(LSD=5.89)	
Experiment 2012	Total ash		ADF	
Control	14.11	14.41	29.72	27.78
Cow manure	13.08	13.86	24.78	27.95
N1	13.88	14.71	30.23	36.80
N2	14.65	14.65	35.80	39.45
$F_{tillage}$	1.23^{ns}		4.96^{ns}	
$F_{fertilization}$	1.34^{ns}		15.25***(LSD=4.19)	

F-test ratios are from ANOVA. Significant at *p=0.05, ***p=0.01, ***p=0.001, ns: not significant. The LSD (p=0.05) for tillage systems and organic fertilization are also shown.

Conclusions

Our results indicate that quinoa growth was influenced by both tillage and fertilization. The lowest height and dry weight were found under MT. The lowest height and dry weight was found under control treatment. N uptake increased with N fertilization. Concerning the crude protein content, there were no significant differences between CT and MT systems. In addition, there were significant differences in crude protein between fertilization treatments.

The highest crude protein yield (2481 kg ha^{-1} and 2356 kg ha^{-1} for CT and MT, respectively) was found under N2 treatment. Our data indicated that quinoa crop could be used as alternative to legumes for dry-season feeding of ruminants in the Mediterranean region. N fertilization consistently had a positive impact on quinoa forage quality.

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PLANT SCIENCE

Drought tolerance, phosphorus efficiency and yield characters of upland rice lines

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Abstract

Objective of this study was to evaluate the characters of drought tolerance, P efficiency and yield of some upland rice lines based on shoot biomass, P accumulation and grain yield. Nine lines of upland rice [viz. aromatic upland rice (Unsoed: G9, G13, G19, G35, G39), upland rice from Rice Research Center Sukamandi (IR-80340-23-B-B-1-B-B, IR 75885-25-1-3-B-5-1-2-B-B, IR 75885-26-2-3-B-18-B-2-1-B), upland rice from University of Mataram (Unram 1E)], four P doses per pot [viz. 0, 0.20, 0.40 and 0.55 kg P2O5] and soil water availability [viz. field capacity (FC) (-10 kPa), 50% FC (-24 kPa), 75% FC (-17 kPa) and 25% FC (-30 kPa) were tested. The study was arranged in randomized complete block design with three replicates. The result showed that upland rice lines of IR 75885-26-2-3-B-18-B-2-1-B, Unsoed G9 and Unsoed G19 resulted higher in grain yield under drought condition than others. Yet, Unram 1E and Unsoed G13 had potency to drought even low in grain yield. Meanwhile, upland rice lines of IR 75885-25-1-3-B-5-1-2-B-B, IR 75885-26-2-3-B-18-B-2-1-B and Unsoed G19 resulted higher in grain yield under low dose of P than others. Unsoed G9 had both characters in efficient and respond to P, and drought tolerance with high in grain yield. Therefore, it needs to evaluate the performance consistency of both characters on those lines through grown under real conditions in the field of rainfed areas.

Key words: Drought tolerance, P efficiency, Upland rice lines, Grain yield

Introduction

Drought is a major limitation to the productivity of agricultural systems and food production worldwide. Among cereal crops that are the major carbohydrate staples for humans, even intermittent water stress at critical stages may result in considerable yield reduction and crop failure. The current environmental problems caused by the sustainability of water consumption have become priority research areas especially in rainfed area where the rainfall is the main source of water or plant growth. The lack of water is also threatening the sustainable production of rice especially in Asia where rice is the most important cereal crop (Bouman and Tuong, 2001). Due to erratic rainfall, drought stress is a serious limiting factor to achieve rice production stability, and a major problem to yield. Drought effect differs according to varieties, growth stage, level and duration to drought stress (Kato, 2004). Drought effect may have different impacts on reducing yield (Forbes and Watson, 1994; Lafitte et al., 2007) and declining rice production generally (Bouman et al., 2005) due to decreasing in growth and photosynthetic rate (Siddique et al., 1999; Zlatev and Lidon, 2012),

Upland rice plays an important role in bringing cleared areas into cultivation by finding characters of tolerance to acid, aluminium-toxic soils (Pinheiro et al., 2006). The problems of acid soils are complex even the major growth-limiting factor for upland rice differs depending on the degree of soil acidity. Phosphorus (P) deficiency is a major abiotic stress that limits rice productivity, particularly under upland conditions in acid soils such as ultisol and alfisol (Kirk et al., 1998). Dobermann et al. (1998) estimated that more than 90% of added fertilizer P may be rapidly transformed to P forms that are not easily available to plants.

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Under conditions of drought due to erratic rainfall and acid soils in which accumulation of toxic element such as Al, Mn and Fe with low of P availability arise and directly impairment the growth of crops so the proper management is a must to improve productivity of upland rice. Erratic rainfall and low soil nutrition of P are specific problems and as the main factor of food production in rainfed dry land area and it has a high risk on production (Fageria and Baligar, 1997; Amberger, 2000).

Rice as a staple food in Indonesia is the first priority to obtain national demand of food and it's mentioned in strategic planning of Indonesian Agricultural Research and Development Agency 2010-2014 due to it has not been met national demand yet. Expansive area of rice production in Indonesia to upland land areas is one alternative to keep and support national food security. Large potency of upland areas could be possible to support upland rice production and enhance contribution to national rice production (Center of Research and Development, 2008).

Selected variety could be possible adaptable grown under specific constraints of limited water and low P availabilities to enhance production. Moreover, those varieties might have the characters of drought tolerance, P efficient and high yield. This is an option and one solution to improve upland rice production to meet national production demand. Several studies were done regarding to P performance in plant (Fageria et al., 1988; Wissuwa, 2003; Akinrinde and Gaizer, 2006; Li et al., 2009), and plant performance under drought condition (Hirayama et al., 2006; Venuprasad et al., 2007; Liu et al., 2007; Lasalita-Zapico et al., 2008). However, study on selection of genotypes with both characters of drought tolerance and P efficient to improve yield has been limited conduct.

Developing rice varieties under acid-soil (low P availability) and drought tolerances (erratic rainfall) would be a way to introduce upland rice in upland cropping systems area as one main objective. The large areas of upland with the some constraints are the potency to develop with emphasis on upland rice production as a main staple food. Therefore, finding out of genotypes of upland rice with the characters of drought tolerance, P efficient and high yield must be done to get the suitable genotypes in specific upland areas.

Materials and Methods

The studies were carried out at Agronomy and Horticulture laboratory and screen house of Faculty of Agriculture Jenderal Soedirman University in July – December 2010. In first study, drought tolerance selection methods were tested by seed germinated level (at laboratory) and seedling level (at screen house), and P efficiency selection was done in screen house on young plant (30 days after sowing) (Ahadiyat et al., 2012). Then, the pot study in screen house was done to evaluate the potency of drought and P efficiency lines of upland rice from previous study.

Upland rice lines of aromatic upland rice (Unsoed: G9, G13, G19, G35, G39), upland rice from Rice Research Center Sukamandi (IR-80340-23-B-B-1-B-B, IR 75885-25-1-3-B-5-1-2-B-B, IR 75885-26-2-3-B-18-B-2-1-B), and upland rice lines from University of Mataram (Unram 1E) in first study were used as materials (Ahadiyat et al., 2012).

This further study was evaluated the potency of drought tolerance and P efficiency by characterization on shoot biomass, P accumulation and grain yield. Three seeds were dibbled in pot (8.5 kg dried soil) and it will be left two plants per pot after 14 days after sowing. The experimental treatments were composed of combinations of nine lines of upland rice [viz. aromatic upland rice (Unsoed: G9, G13, G19, G35, G39), upland rice from Rice Research Center Sukamandi (IR-80340-23-B-B-1-B-B, IR 75885-25-1-3-B-5-1-2-B-B, IR 75885-26-2-3-B-18-B-2-1-B), upland rice lines from University of Mataram (Unram 1E)], four P doses per pot [viz. 0, 0.20, 0.40 and 0.55 g P_2O_5] and soil water availability [viz. field capacity (FC) (-10 kPa), 50% FC (-24 kPa), 75% FC (-17 kPa) and 25% FC (-30 kPa). The study was arranged in randomized complete block design with three replicates.

Each pot received N at dose of 0.40 g and applied at 15 and 30 days after sowing each and K at dose of 0.20 g at 15 days after sowing using urea (46% N) and muriate of potash (50% K_2O), respectively. Super phosphate (18% P_2O_5) was applied following the treatments. The seedlings were thinned out to two plants per hill at 14 days after sowing. There was neither any pest nor diseases also weeds were controlled as need arise.

Dry weight of shoot biomass and grain yield was observed. Grain yield were gathered randomly from two pots as sample from each treatments and replication. Grain per pot was weighed as indicated of yield. Characterization of P efficiencies was calculated in use and absorption as reported by Fageria and Baligar (1997) and Fageria and Santos (2002).

All data were analyzed by using Analysis of Variance procedure (Steel and Torrie 1980) by

using IRRIStat Software (2004). Treatment means were compared using Fisher's Protected LSD method.

Results

Characterization of upland rice lines on drought tolerance

Characteristics of upland rice lines under drought condition resulted in the variation on shoot biomass and grain yield (Table 1). Response of upland rice lines to express adaptability under drought condition could indicate the level of drought tolerance. Drought tolerance is a function of morphological characters i.e. plant biomass and stability of yield. This study showed that shoot biomass declined along with reducing soil water content (Figure 1). Growth of shoot part stunted enough under soil water condition of -30 kPa on upland rice lines of IR-75885-25-1-3-B-5-1-2-B-B, IR-75885-26-2-3-B-18-B-2-1-B, Unsoed G13, G19 and Unram 1E upland rice lines with shoot biomass of less than 20 g.

Grain yield of more than 8 t/ha gained under favorable condition (-10 kPa) on upland rice lines of IR-75885-25-1-3-B-5-1-2-B-B, IR-75885-26-2-3-B-18-B-2-1-B, Unsoed G19 and G9 (Figure 2). However, the grain yield under drought condition (-30 kPa) on almost all upland rice lines gained not more than 4 t/ha. Eventhough, in the same condition some upland rice lines gained grain yield more than 4 t/ha such as IR-75885-26-2-3-B-18-B-2-1-B, Unsoed G19 and G9 (Figure 2). Comparison between stress (-30 kPa) to optimum (-10 kPa) conditions on grain yield showed that some upland rice lines gained more than 75 percent i.e. Unram 1E and Unsoed G13 (Figure 3). This indicated that both upland rice lines still had capacity to produce high grain even under stress condition about 75 percent compared to the yield under optimum condition.

Table 1. Analysis of variance of effect upland rice lines and soil water availability on shoot biomass, P tissue content, P use efficiency (PUE) and grain yield.

Parameter	Upland rice Line (G)	Soil water availability (W)	P dose (P)	G x W	G x P	W x P	G x W x P
Shoot Biomass	**	**	**	*	ns	ns	ns
P tissue content	**	**	**	**	**	**	**
PUE	**	**	**	**	ns	ns	ns
Grain yield	**	**	**	**	ns	ns	ns

ns-non significant, * P<0.05,** P <0.01

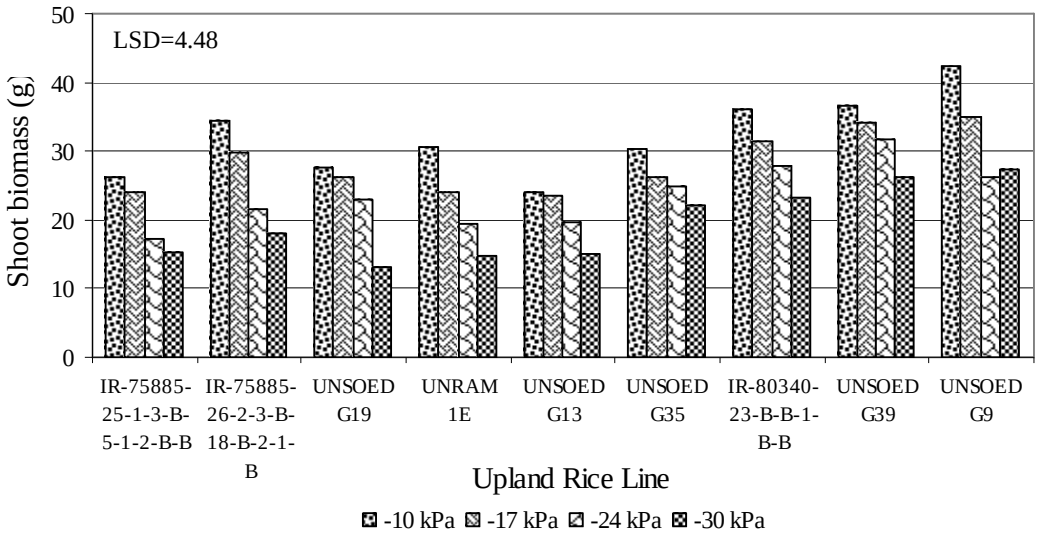

Figure 1. Shoot biomass of upland rice lines at different water availability.

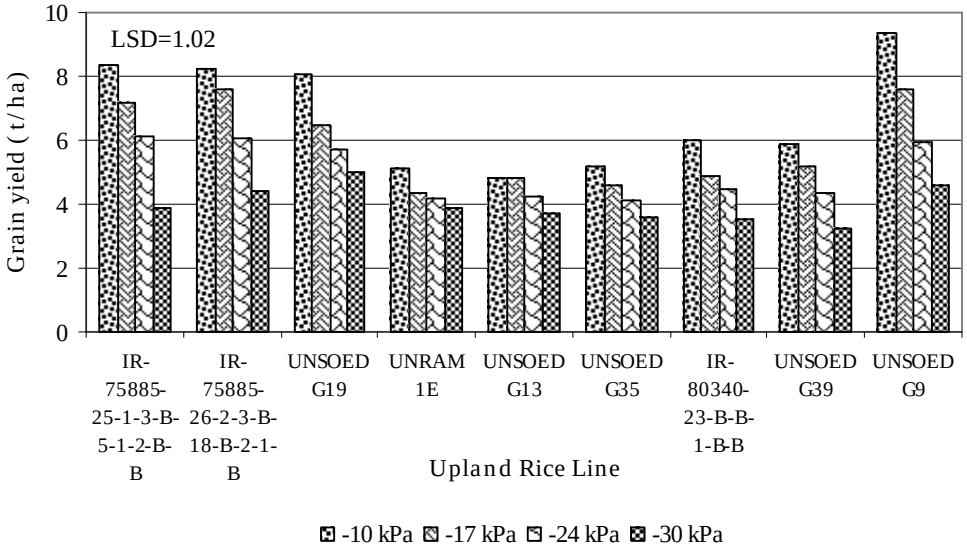

Figure 2. Grain yield of upland rice lines at different water availability.

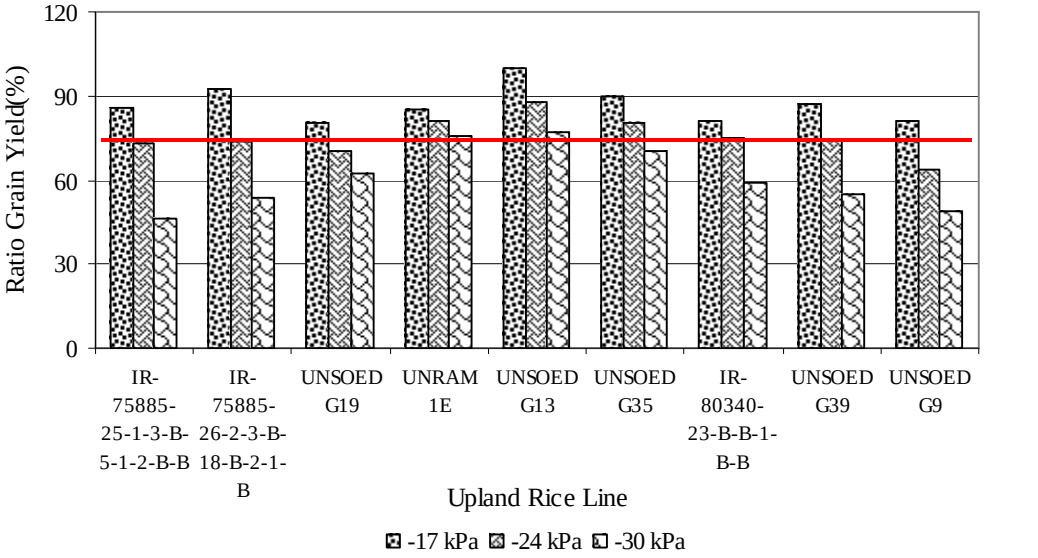

Figure 3. Grain yield ratio of upland rice lines at different water availability.

Characterization of upland rice lines on P efficiency

Characteristics of upland rice lines under different P doses resulted in variation of shoot biomass, P tissue content, P Use Efficiency and grain yield (Table 1). Generally, shoot biomass and P tissue content increased along with increased of P application. Low dose of P (45 kg/ha P_2O_5) obtained more than 25 g of shoot biomass on upland rice lines of IR-75885-26-2-3-B-18-B-2-1-B, IR-80340-23-B-B-1-B-B, Unsoed G9 and Unsoed G39. For high dose of P (90-135 kg/ha P_2O_5) obtained shoot biomass of more than 30 g i.e. upland rice lines of IR-80340-23-B-B-1-B-B, Unsoed G9 and Unsoed G39. Yet, consistency result in application of low to high dose of P (45-135 kg/ha P_2O_5) resulted in high shoot biomass of more than 30 g gained by upland rice lines of Unsoed G9 and Unsoed G39 (Figure 4).

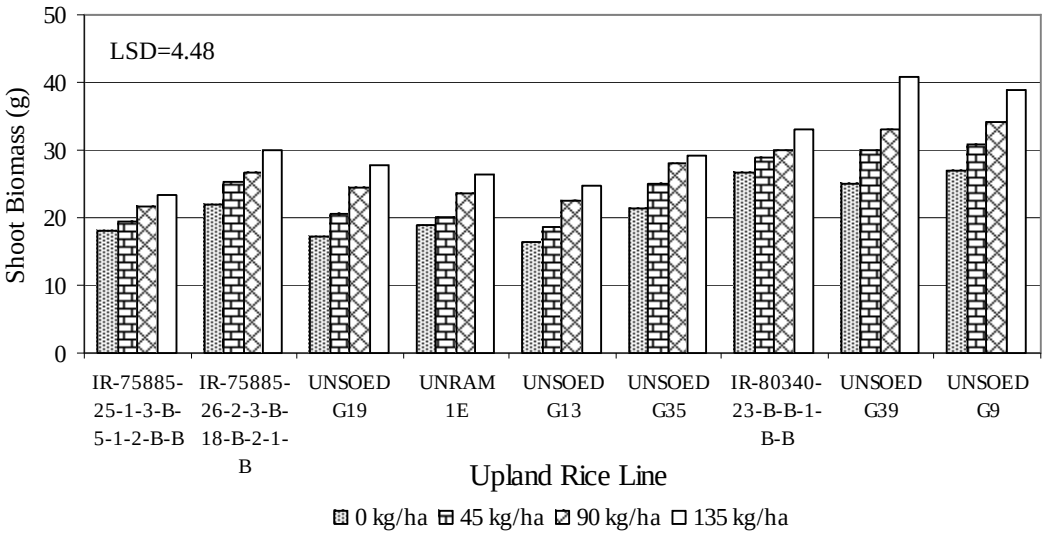

Figure 4. Shoot biomass of upland rice lines at different P doses.

Different upland rice lines had varied responses on P tissue content. Generally, application of P dose of 45-90 kg/ha P_2O_5 accumulated P tissue content between 200 - 300 mg/g. Only upland rice lines of Unram 1E and Unsoed G35 accumulated P tissue content more than 300 mg/g with applied of 135 kg/ha P_2O_5 (Figure 5).

There was contradiction in Absorption Efficiency of P compared to shoot biomass and P tissue content. Increasing dose of P resulted declining of efficiency level. All lines gained level of P efficiency more than 20 mg/mg with dose of P 45 kg P_2O_5/ha. Application dose of P above 45 kg P_2O_5/ha resulted the declining of P efficiency (Fig. 6). Upland rice lines of IR-80340-23-B-B-1-B-B, Unsoed G9 and G39 gained P use efficiency above 120 mg/mg but others gained below 120 mg/mg (Figure 8).

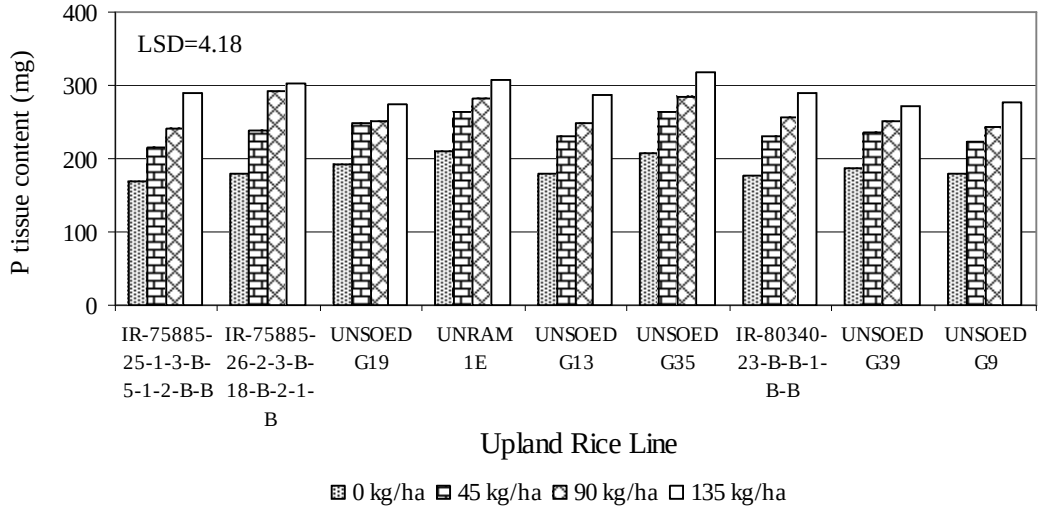

Figure 5. P tissue content of upland rice lines at different P doses.

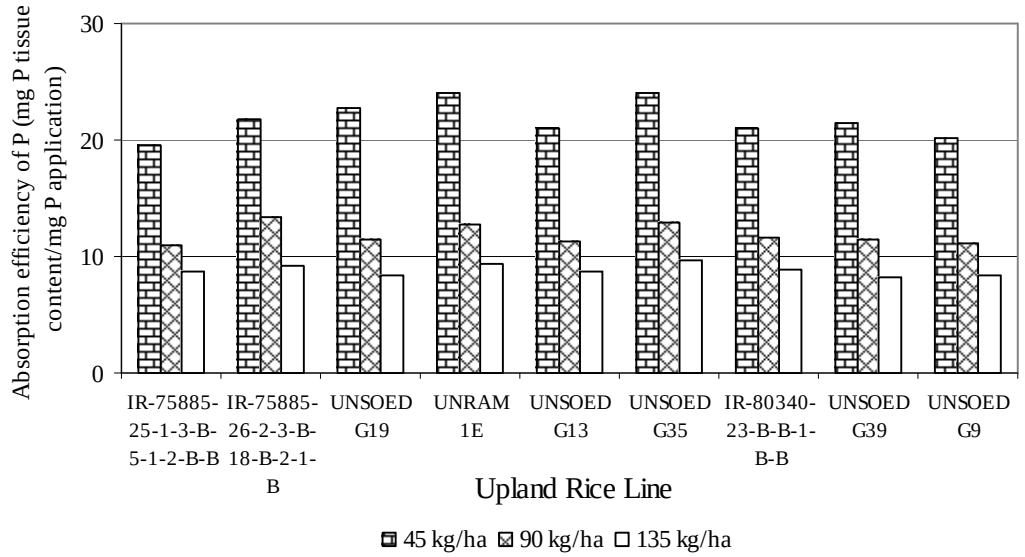

Figure 6. Absorption efficiency of P of upland rice lines.

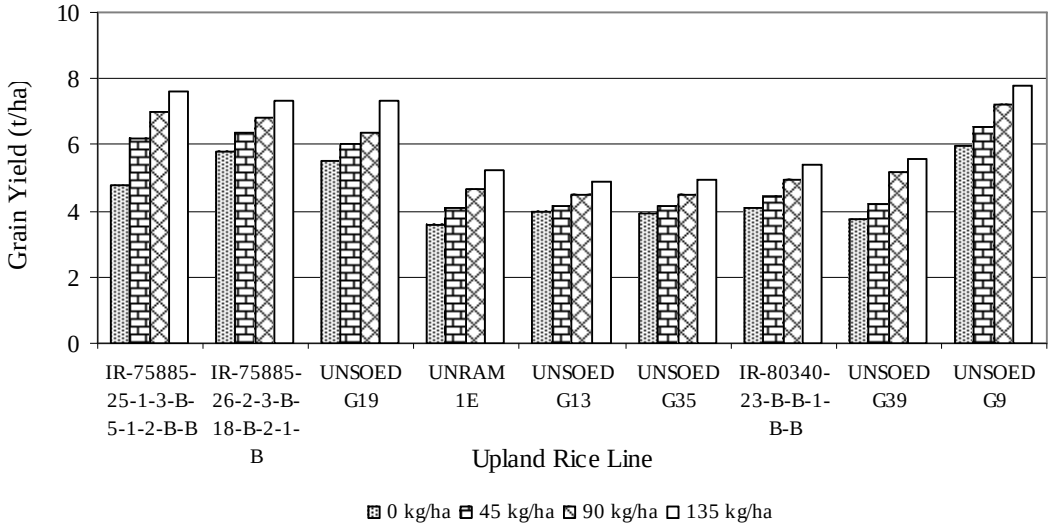

Figure 7. Grain yield of upland rice lines at different P doses.

Grain yield of IR-75885-25-1-3-B-5-1-2-B-B, IR-75885-26-2-3-B-18-B-2-1-B, Unsoed G19, and Unsoed G9 lines had the higher result compared to others under different application of P. In general, all upland rice lines as mentioned above had grain yield potency more than 6 t/ha but others gained less than 4.5 t/ha (Figure 7).

Grouping was arranged to separate different characters of upland rice lines based on P use efficiency and shoot biomass viz. efficient and responsive, efficient but not responsive, not efficient but responsive and either not efficient or responsive (Figure 8). Categorized of upland rice lines as distributed by IR-80340-23-B-B-1-B-B, Unsoed G9 and G39 were efficient and responsive, IR-75885-26-2-3-B-18-B-2-1-B and Unsoed G35 were efficient but not responsive, and others were either not efficient or responsive. There no upland rice lines had character of not efficient but responsive (Figure 8).

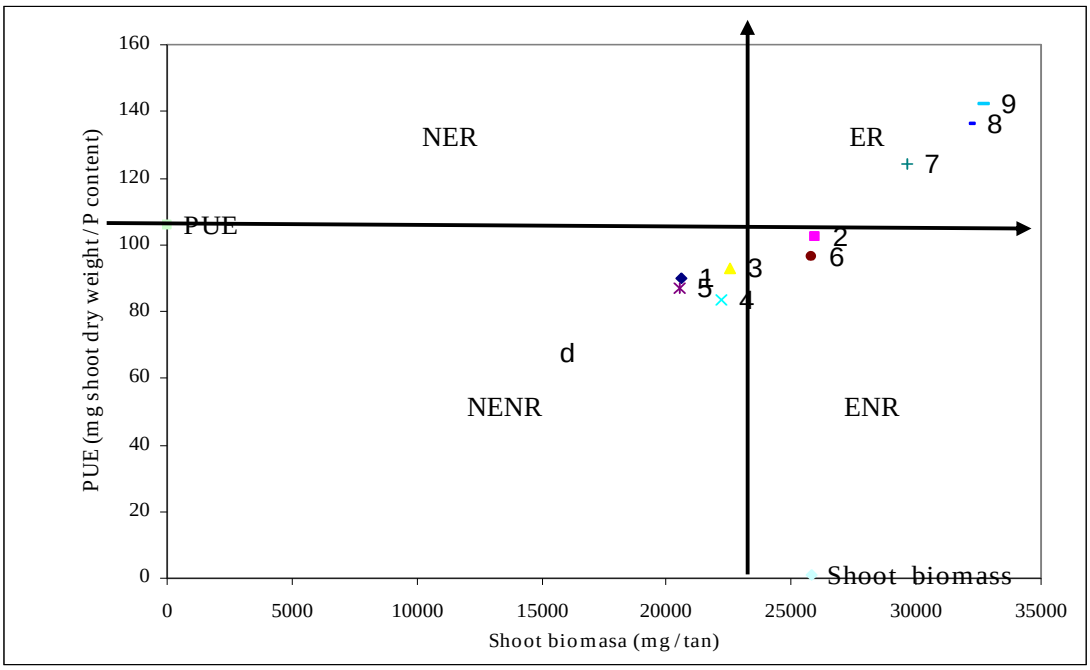

Figure 8. Grouping upland rice lines based on character of efficiency and responses on P use.

PUE = P use efficiency; ER = Efficient and Responsive; NER = Non Efficient and Responsive; ENR = Efficient and Non Responsive; NENR = Non Efficient and Non Responsive.

1. IR-75885-25-1-3-B-5-1-2-B-B
2. IR-75885-26-2-3-B-18-B-2-1-B
3. UNSOED G19
4. UNRAM 1E
5. UNSOED G13
6. UNSOED G35
7. IR-80340-23-B-B-1-B-B
8. UNSOED G39
9. UNSOED G9

Discussion

The variation of results on drought tolerance was evidence that each upland rice lines had different response. Matsuo et al. (2009) reported that morphological characters such as shoot weight tend vary among different conditions. The genotypes may respond different to some factor i.e. age and maturity, biochemical compositions and genetic variability of seeds (Ahmad et al., 2009). The nature of the trade-off between drought tolerance and plant growth rate also constrains the selection for optimal drought-adapted genotypes. The aspects of plant–environment interactions should be considered before establishing selection programs for drought adaptation. Selecting for early maturation also results in selection for drought-stress avoidance (Juliano et al., 2007). Genotypes adapted to particular conditions usually perform poorly when these conditions are absent. Therefore it is expected that the adoption of a single drought-adaptation strategy will not be adequate for all environments (Porporato et al., 2001; Tardieu, 2005; Juliano et al., 2007).

This study was carried out to find out the potential lines with the given typeset and it will be suitable grown under dry land areas especially in rainfed condition. Thus, under rainfed systems, the problems arise from the variability of water regimes, so the soils may drain and re-flood at least once during the season. The resulting water stress is often compounded by nutrient deficiencies especially of P because the soils are often inherently low in P and solubility (Haefele et al., 2006). Identification of rice genotypes in lowland and upland has been done on P efficiency and the differences occur on the ability to capture soil P and utilize it in biomass production as long as the other factors are not limited (Ismail et al., 2007).

Therefore, differences in ability of genotypes of particular species to grow on low P soils can results from quite different phenomena. Some crops grow well on a high or low P soils i.e. potatoes

(Sattelmacher et al., 1991) and *B. oleraceae* (Greenwood et al., 2005). But, commonly dry land areas especially rainfed has a characteristic of nutrient deficiencies, particularly of P (Ozturk et al., 2005) and give adverse effect on rice production (Huguenin-Elie et al., 2009). So, selection of upland rice varieties that grows well on soils with low levels of plant available P is needed to improve productivity.

Selection of P efficiency genotypes refers to Fageria and Baligar (1997) and Fageria and Santor (2002) by which mentioned that biomass weight and P tissue content on shoot part are suitable to use for P efficiency genotypes selection. Other studies of rice were done on P efficient (Ozturk et al., 2005; Gunes et al., 2006) based on ratio of biomass weight between deficient to sufficient conditions. Even though, the results showed the variation under both methods of shoot biomass and P tissue content but some upland rice lines gained the consistent results on shoot biomass and P use efficiency characters such as Unsoed G9 and Unsoed G39. This is the fact that to identify useful traits into broadly adapted genotypes, it is important to understand the mechanisms involved and their genetic basis (Huguenin-Elie et al., 2009) and genotypes develop diverse adaptive responses when they suffer from P deficiency stress (Ozturk et al., 2005).

In general, upland rice lines with the character of tolerance to drought tend low in P efficiency and vise versa. However, Unsoed G9 showed to be superior in drought tolerance and P efficiency. Improving in morphological and physiological characters of plant could enhance level of drought tolerance but not for yield. Previously studies on five upland varieties showed that low in leaf areas and number of panicles gained the low yield (Ahadiyat dan Harjoso, 2010a,b). This is a fact that paddy is sensitive plant to water deficit and would be resulted drought effect and could gain the low in growth and yield.

However, drought character is the important thing for paddy under dry land area especially in rainfed areas with character of low water availability due to low intensity of rainfall during plant growth. Therefore, some genotypes with the character of drought tolerance could be used as important genetic resources for parental and could be possible to breed with genotypes with the character of high yield even low tolerance to drought. Thus, additional character for improving adaptability under acid soils must be found. The characters as mentioned above could be possible to further breed with genotypes characters of P efficient. This effort must be done because of high in P performance of content and efficient resulted low in yield as reported by Ozturk et al. (2005) and Gunes et al. (2006).

Some upland rice lines with the characters of efficient but not respond and not efficient and not respond had high performance in yield potency (>6 t/ha), dry biomass of shoot and root. It could develop by using both characters as genetic resources for adaptable genotypes grown under acid soils (low P availability). As mentioned by Fageria et al. (1988), Fageria and Baligar (1997) and Gunes et al. (2006) that selection of genotypes under low P availability in soils could use dry weight of shoot and root as indicators.

Under control condition in screen house, this study has been found the potential lines with characters of drought tolerance and P use efficiency. Therefore, it needs the further studies to ensure the performance consistency of all upland rice lines through grown in real field conditions of rainfed areas. To achieve the better inform of crop improvement, research must continue and understanding the interactions between plant genotypes and the growing environment under conditions of P deficient (Wissuwa, 2003) and drought (Parry et al., 2005).

Conclusion

Upland rice lines of IR 75885-26-2-3-B-18-B-2-1-B, Unsoed G9 and Unsoed G19 gained higher in grain yield under drought condition than others. Yet, Unram 1E and Unsoed G13 had potency to drought even low in grain yield. For P efficiency characterization, upland rice lines of IR 75885-25-1-3-B-5-1-2-B-B, IR 75885-26-2-3-B-18-B-2-1-B and Unsoed G19 gained in higher grain yield under low dose of P than others along with characters of not responsive and, efficient or not efficient to P. Upland rice line of Unsoed G9 had characters in drought tolerance and P efficiency with high in grain yield. Therefore, for obtaining ideotype variety with the characters of drought tolerance, P efficient and high yield is needed to conduct the further studies and evaluate the performance consistency of upland rice lines through grown under real conditions in the field of rainfed areas for ensure the potency of each lines.

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PLANT SCIENCE

Genetic analysis of three *Amaranth* species using ISSR markers

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Abstract

Amaranthus is one of the underutilized species distributed and utilized worldwide. In the study was used ISSR approach to analyse intra and inter-specific variability of 16 *Amarathus caudatus*, 19 *A. cruentus* and 21 *A. hypochodriacus* accessions. Using of 11 ISSR primers, fragments levels ranged from 15 up to the 24 were obtained. In the dendrogram based on ISSR data set individual species separation is observed except of three accessions. Two *A. caudatus* genotypes originated from India clustered with *A. hypochondriacus* accessions and one *A. hypochondriacus* genotype originated from Nepal clustered with *A. cruentus* accessions. Average similarity index among all 55 analysed genotypes ranged from 0,154 to 1,000 with a mean of 0,522. Highest intaspecific variability is reported for *A. hypochondriacus* accessions when comparing to the *A. caudatus* or *A. cruentus*. This study has demonstared, that a single primer marker system as ISSR is able to generate a sufficient level of informative characters for intra and inter-specific molecular analysis of Amaranthus genus.

Key words: *Amaranthus caudatus, Amaranthus cruentus, Amaranthus hypochondriacus*, ISSR markers

Introduction

The genus *Amaranthus* L. (*Caryophyllales*: *Amaranthaceae*) consists of about 60–70 species. Several of which are cultivated as leafy vegetables or forage, others for grains production and some are planted as ornamental plants. Unfortunately, some are aggressive weeds that affect many agricultural areas of the world. Despite this, cultivated of amaranth plants have a lot of positive. Grain amaranth's balanced amino acid composition is close to the optimum protein reference pattern in the human diet according to FAO/WHO requirements (Mlakar et al., 2010). Presently, amaranth is cultivated in many parts of the world. Mainly, three species of *Amaranthus* L. are commonly cultivated for grain production: *Amaranthus cruentus* L., *Amaranthus caudatus* L. and *Amaranthus hypochondriacus* L. (Williams and Brenner, 1995; Lanoue et al., 1996). Amaranth has been also regarded as relatively drought tolerant, thus, suggesting that reasonable yield can be realized with limited irrigation. So, amaranth (*Amaranthus* spp.) is one of the most important leafy vegetables widely grown in the tropics with a potential to broaden man's food base in Africa (Neluheni et al., 2007). Improvement of amaranth in nowadays breeding programms is realized worldwide (Gajdošová et al., 2008; Hricová et al., 2011) what corresponds to the potential of this underutilized crop. Transgenic manipulation was also suggested as an alternative to irradiation, which can reduce fitness of the males and necessitates removal of females for optimum effect (Heinrich and Scott, 2000; Thomas et al., 2000).

Genus *Amaranthus* consists a large number of species with good facilities so, it has given a signal to increase interest of this plant, one signal to next research of this rediscovered plant. Nowadays, molecular analyses methods provide a base for a wide range of plant biological material assessment. They are involved in different methods from epidemiological changes evaluation (Pochop et al., 2012; Zeleňáková et al., 2012), continuing in authentication of food (Zeleňáková et al., 2008; Židek et al., 2012) up to the analyses of biodiversity of wild and cultivated plants (Gregáňová et al., 2005; Vivodík et al., 2011; Ražná and Žiarovská, 2011).

Nowadays, two basic data sources exist for molecular base of the *Amaranthus* genus. The first genomics - based resources derived specifically

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from *Amaranthus* species are available from BAC library constructed from *A. hypochondriacus* (Maughan et al., 2008). The second *Amaranthus* genomics resource to become available is a set of microsatellite markers (Mallory et al., 2008). Nearly 400 unique microsatellite markers were obtained primarily from microsatellite - enriched libraries but also from BAC - end sequence data. About 180 of these proved to be polymorphic across three grain *Amaranthus* species.

The genus *Amaranthus* L. is known with high inter- and intraspecies variability (Mosyakin and Robertson, 1996; Marhold and Hindák, 1998). Inter alia, molecular tools serve to detect of this inter- and intraspecies variability of organisms, considering they provide valuable data on diversity through their ability to detect variation at the DNA level (Somasundaram and Kalaiselvam, 2011). DNA markers are more suitable for detection of intra- and interspecies variability when compared to the morphological ones, because they are not affected by environment (Gilbert et al., 1999; Duran et al., 2009).

Different techniques are reported as suitable for amaranth germplasm evaluation and screening of genetic diversity using DNA based markers. RAPD (Randomlly Amplified Polymorphic DNA) utilization in studies on genetic diversity in Amaranthus species is reported by Popa et al. (2010) or Tony-Odigie et al. (2012), ITS (Internal Transcribed Spacers), AFLP (Amplified Fragment Length Polymorphism) and double-primer fluorescent ISSR (Inter Single Sequence Repeats Polymorphism) approaches are reported by Xu and Sun (2001) and ISSR markers was also used by Ray and Roy (2007) and Nolah at al. (2010) for different amaranth species molecular studies.

Microsatellites are a common source of polymorphism used for molecular analysis of wide range of plant species. Two basic approached are based on microsattelites - SSR (Gregáňová et al., 2005; Vivodík et al., 2011; Hulín et al., 2012) and ISSR. Conventional ISSR approach is used with oligonucleotides composed of defined short tandem repeat sequences that represented a variety of different microsatellite types. As a marker, in ISSR the oligonucleotide is used in a single primer amplification reaction. Using this approach, 11 of 30 tested ISSR markers were used for estimation of genetic relationships among and within amaranths and chenopods by Ray and Roy (2007). A total of 177 loci were obtained and 42.5% polymorphic bands were reported for leafy amaranth types and 71.6% for grain types.

The objective of presented study was to evaluate and screen genetic diversity of three *amaranthaceae* species - *Amaranthus caudatus* L., *Amaranthus cruentus* L. *and Amaranthus hypochondriacus* L. by ISSR markers. Obtained data were used for dendrogram constructing where the relationships based on the detected length polymorphism are visualized.

Materials and Methods

The seeds of 16 *Amaranthus caudatus* L, 18 *Amaranthus cruentus* L. and 21 *Amaranthus hypochondriacus* L. genotypes (table 1) with different origin were obtained from North Central Regional PI Station (NC 7), Iowa State University, Ames.

The amaranth seedlings were cultivated under *in vitro* conditions on Murashige and Skoog (1962) medium. DNA from fresh young leaves was isolated according to Rogers and Bendich et al. (1994) optimized protocol. Each genotype was represented by ten individuals. The amount of DNA in reaction (10-80 ng in 25 µl total volume), the primer concentration (0.2-1.5 µmol.dm^{-3}), $MgCl_2$ concentration (0.75-1.5 mmol.dm^{-3}), deoxyribonucleotides concentration (0.1-0.2 mmol.dm^{-3}) and the annealing temperature (45-60)°C.

A total of 11 primers were used in analysis (table 2). All reactions were repeated three times. According to the optimization, PCR conditions were assigned 20 mmol.dm-3 Tris-HCl, pH 8,0 (Invitrogen™, Life Technologies), 50 mmol.dm-3 KCl (Invitrogen™, Life Technologies), 1 U Taq polymerase (Invitrogen™, Life Technologies), 3 mmol.dm-3 $MgCl_2$ (Invitrogen™, Life Technologies), 0,1 mmol.dm-3 deoxy ribonucleotides (Promega), 0,2 µmol.dm-3 primer (Invitrogen™, Life Technologies, Table 2), 20 ng DNA in 25 µl total reaction volume.

Table 1. Species and genotypes of amaranth used in the study.

Code	Number	Genotype	Country of origin	Note
Amaranhus caudatus L.	1	Ames 12751 I 667	Nepal	Unknown origin
	2	PI 490440 LSK 19	Peru	Unknown origin
	3	PI 490604 HH 50	Bolivia	Unknown origin
	4	PI 490642 LSK 462	Peru	Unknown origin
	5	PI 480816 IC-38286	India	Unknown origin
	6	PI 480854 IC-38313	India	Unknown origin
	7	PI 511693 Achis	Peru	Cultivated material
	8	PI 511711 HH 77	Ecuador	Cultivated material
	9	PI 568147 coime	Bolivia	Cultivated material
	10	PI 175039 RRC 10	India	Unknown origin
	11	PI 553073 Love-Lies-Bleeding	USA, New Yersey	Cultivar
	12	PI 166045 Chua	India	Unknown origin
	13	PI 632249	USA, Iowa	Unknown origin
	14	Ames 5600	India	Unknown origin
	15	Ames 5685	USA, Pennsyvania	Unknown origin
	16	NSL 109789	Italy	Unknown origin
Amaranthus cruentus L.	17	Ames 1959 RRC 1	Ghana	Cultivar
	18	Ames 5129 RRC 360	Nigeria	Unknown origin
	19	Ames 21948	Papua-Nová Guinea	Unknown origin
	20	PI 566896 Komo	USA, Arizona	Landrace
	21	PI 511719 Niqua, alegria, chang	Guatemala	Cultivated material
	22	PI 511876 Huatle	Mexico	Landrace
	23	PI 527567 IZ 32	Burundi	Landrace
	24	PI 604558 Mapes 821	Mexico	Landrace
	25	PI 612169 Tibet Yellow	China	Cultivated material
	26	Ames 5638 RRC 1139	Mexico, Puebla	Unknown origin
	27	Ames 5648 RRC 1148	Mexico, Sonova	Unknown origin
	28	PI 477913 RRC 1011	Mexico	Cultivar
	29	Ames 5493 RRC 768	Mexico, Morelos	Unknown origin
	30	Ames 5369 RRC 685	Kongo	Cultivar
	31	Ames 5310 RRC 659	Mexico, Sonova	Landrace
	32	Ames 25121 CEN/IB/97/ AMA/003	Nigeria, Oyo	Wild
	33	Ames 2215 RRC 308	Mexico, Sonova	Landrace
	34	PI 566897 Kerala Red	India, Kerala	Cultivated material
Amaranthus hypochondriacus L.	35	Ames 2064 RRC 126	Nepal	Breeding material
	36	Ames 2086 RRC 149	Nepal	Unknown origin
	37	Ames 2061 RRC 124	Nepal	Unknown origin
	38	Ames 21046 Annapurna	India	Cultivar
	39	PI 481464 EC-18626	Nepal	Unknown origin
	40	PI 538794 AJCO74	Russia	Cultivated material
	41	PI 542595	China	Cultivated material
	42	PI 568130 DB 926	USA, Iowa	Breeding material
	43	PI 511731 HH 104	Mexico	Cultivated material
	44	Ames 12744 I 91	Nepal	Unknown origin
	45	Ames 1972 RRC 18 A	Nigeria	Breeding material
	46	PI 274279 RRC 171	India, Himachal Pradesh	Unknown origin
	47	PI 337611 P 373	Uganda	Landrace
	48	PI 477915 RRC 1008	India	Breeding material
	49	PI 477916 RRC 1023	Mexico	Cultivar
	50	PI 477917 RRC 1024	Mexico	Cultivar
	51	Ames 2178 RRC 266	Nepal	Breeding material
	52	Ames 5132 RRC 363	Mexico, Chihuahua	Landrace
	53	Ames 5209 RRC 457	Mexico, Mexico	Landrace
	54	Ames 5321 RRC 539	Mexico, Chihuahua	Landrace
	55	Ames 5467 RRC 720	Mexico, Oaxaca	Unknown origin

Table 2. The list of ISSR primers used in the study.

Primer	Sequence (5′ → 3′)	Primer	Sequence (5′ → 3′)
$(AGC)_4G$	AGC AGC AGC AGC G	ISLA-$(CT)_8TG$	CT CT CT CT CT CT CT CT TG
$(CA)_6AG$	CA CA CA CA CA CA AG	ISLA-$(GA)_6CC$	GA GA GA GA GA GA CC
$(CA)_6GG$	CA CA CA CA CA CA GG	ISLA-$(GAG)_3GC$	GAG GAG GAG GC
$(CA)_6GT$	CA CA CA CA CA CA GT	ISLA-$(GTG)_3GC$	GTG GTG GTG GC
$(CT)_8$	CT CT CT CT CT CT CT CT	ISLA-$(GT)_6CC$	GT GT GT GT GT GT CC
$(CT)_8AC$	CT CT CT CT CT CT CT CT AC		

The PCR cycling conditions were as follows: 94°C for 2 minutes (initial denaturation), then followed by 45 cycles at 94°C for 1 minute (denaturation), 50°C for 1 minute (annealing), 72°C for 2 minutes (polymerisation) with a final 7 min extension at 72°C and then cool down to 4°C. The PCR products were separated by electrophoresis on 2% agarose gel (3: 1, Amresco) containing 0.5 $\mu g.ml^{-1}$ ethidium bromide in a 1 × TBE buffer and then photographed under UV light using KODAK EDAS 290. The ISSR bands were scored using the binary scoring system that recorded the presence and absence of bands as 1 and 0 using KODAK 1 D program. Genetic similarity was calculated on the basis of Nei Li (1979) coefficient. The resulting matrix of genetic similarity was used to construct the dendrogram through UPGMA with statistic program SYNTAX.

Results and Discussion

Many different methods of identification have been used for evaluation of amaranth diversity. RAPD analysis was successful in the investigation of the relationships of four *A. hypochondriacus* varieties (Barba de la Rosa et al., 2009). AFLP markers were successfully used to determine species what demonstrated taxonomic ambiguity at the basic morphologic level (Costea et al., 2006). Other methods such as ITS, ISSR and isozyme profile were used to get exhaustive view of interrelationship and relative closeness among amaranth species (Das, 2011; Xu and Sun, 2001).

As AFLP results in amaranth germplasm analysis are reported as sharing many features in common with the ISSR dentrograms by Xu and Sun (2001), ISSR was chosen for sixteen *A. caudatus* L., eighteen *A. cruentus* L. genotypes and twenty one *A. hypochondriacus* L. genotypes. ISSR markers are more comfortable and lab effective and are able to produce uniquely fingerprints as well as RFLPs, AFLPs, RAPDs and SSRs as reported for *Amaranthus* accessions in Xu and Sun (2001). Not only leafy and grain species of *Amaranthus* are in the center of nowadays molecular markers research. Nolah et al. (2010) reported ISSR as amployed to measure both genetic diversity and the phylogenetic position of *A. pumilus*. Using ISSR markers, genetic variation was detected among and within *A. pumilus* populations, though variability was low. In the study, authors also compared genetic variability of *A. pumilus* to the variation of *A. hypochondriacus* and *A. cruentus*, because of the desirable characteristics of *A. pumilus* in plant breeding trials. *A. pumilus* is reported here as having lower genetic diversity as analysed grain species (Nolah et al., 2010).

Primers used in the study ranged 50–72% GC content. Seven from eleven primers generated consistent profiles. Three of analyzed primers $(CA)_6AG$, $(CT)_8AC$ and $(GA)_6CC$ were used to assess inter and intra-specific diversity. The number of band levels ranged from 15 [primer $(CT)_8AC$] to 24 [primer $(GA)_6CC$]. An average of 6, 11 loci per primer was produced, ranging from a minimum of 4,8 loci using $(CA)_6AG$ to a maximum of 7.9 loci using $(GA)_6CC$.

Cluster analyses performed with the ISSR data matrix generated by all primers could group genotypes at the inter-specific level. *A. caudatus* L. genotypes were clustered with the value of Euclidian Distance Averages of Clusters (EDAC) 0.414; *A. cruentus* L. genotypes clustered at EDAC 0.450 and *A. hypochondriacus* L. at EDAC 0.459. *A. caudatus* L. and *A. cruentus* L. genotypes clustered together at EDAC 0,533 that indicates more genetic relationships at the inter-specific level. *A. hypochondriacus* L. genotypes clustered with genotypes of two grain amaranths at EDAC 0,621 (Figure 1). *A. caudatus* L. PI 480816 IC-38286 and PI 480854 IC-38313 genotypes grouped with *A. hypochondriacus* L. genotypes at EDAC 0.459. Based on ISSR data two of *A. caudatus* L. genotypes originated from India are probably more related to *A. hypochondriacus* L. *A. hypochondriacus* L. Ames 2086 RRC 149 originated from Nepal grouped with *A. cruentus* L. at EDAC 0.471, which is probably more related to *A. cruentus*.

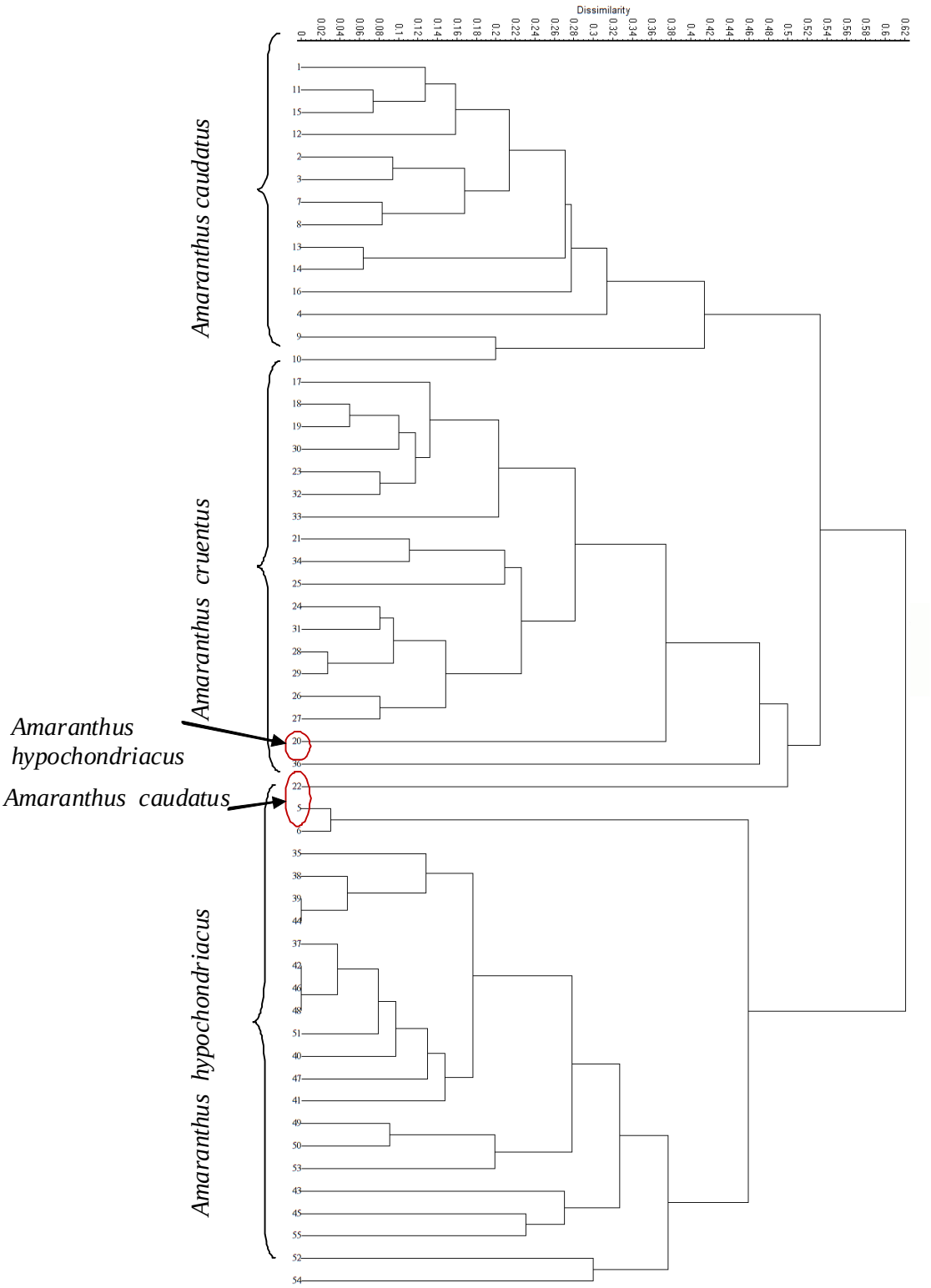

Figure 1. Dendrogram of *Amaranthus* L. genotypes (Table 1) constructed with UPGMA method on the basis of ISSR analysis.

The average similarity index among 55 genotypes ranged from 0.154 to 1.000 with a mean of 0.522 indicating high variation among amaranth genotypes. The cophenetic correlation coefficient was 0.9317. These results are in concordance with finding of Ray and Roy (2007) who reported genetic similarities among *Amaranthaceae* individuals ranged from 0.06 to 0.85. In dendrogram the same authors have reported, *A. caudatus* and *A. cruentus* are grouped into the one cluster at the same level and *A. hypochondriacus* accessions are connected to them at the following closest level. This grouping pattern is repeated fully in the dendrogram obtained in this study (Figure 1).

At the interspecific level, all individual primers with all genotypes used in the study were tested. Genetic diversity of *Amaranthus caudatus* L. was analyzed by eight ISSR primers. The average similarity index was 0.72 and the average resolving power (Rp) value was 6.30. The value of average similarity index in *Amaranthus cruentus* L. was 0.74 and Rp value 5.28. The Rp value in the analysis of *Amaranthus hypochondriacus* L. genotypes was lower than in other amaranth species (4.24) and the average similarity index was 0.73. More than 75% of *Amaranthus caudatus* L. genotypes were differentiated using four ISSR primers $(CA)_6GT$, $(CA)_6AG$, $(CA)_6GG$ and $(GT)_6CC$. Primer $(GT)_6CC$ distinguished 88% genotypes of *Amaranhus caudatus* L. In *Amaranthus cruentus* L., only three primers differentiated more that 75% genotypes and in *Amaranthus hypochondriacus* L. only one primer $(GTG)_3GC$ distinguished 62% genotypes, that indicates less polymorphism at the interspecific level in comparison to *Amaranthus caudatus* L. and *Amaranthus cruentus* L.

Ray and Roy (2007) reported ISSR primers as producing varying numbers of DNA fragments among *amaranthaceae*, depending on their SSR motif. They reported, first - dinucleotide repeats $(CA)_8$ and $(AC)_8$ as having good fingerprint patterns and second – $(CA)_8T$ did not show any amplification by *Amaranthaceae* indicating that CA repeats flanked by either G or A and not by T. Very similar, in this study, primers with the core sequence of CA repeats give the highest polymorphism among all tested species – 88.24% for *A. caudatus* and 100% for *A. cruentus* and *A. hypochondriacus*. Wang et al. (1994) reported that dinucleotide microsatellites are prevalent in plants while mono-, tri- and tetranucleotide repeats are less common. This is confirmed again the results presented here. Dinucleotide cored primers (except of CA repeats) give the polymorphism ranged from 83.33% to 100% while trinucleotite cored primers polymorphism ranged from 61.11% to the 72%.

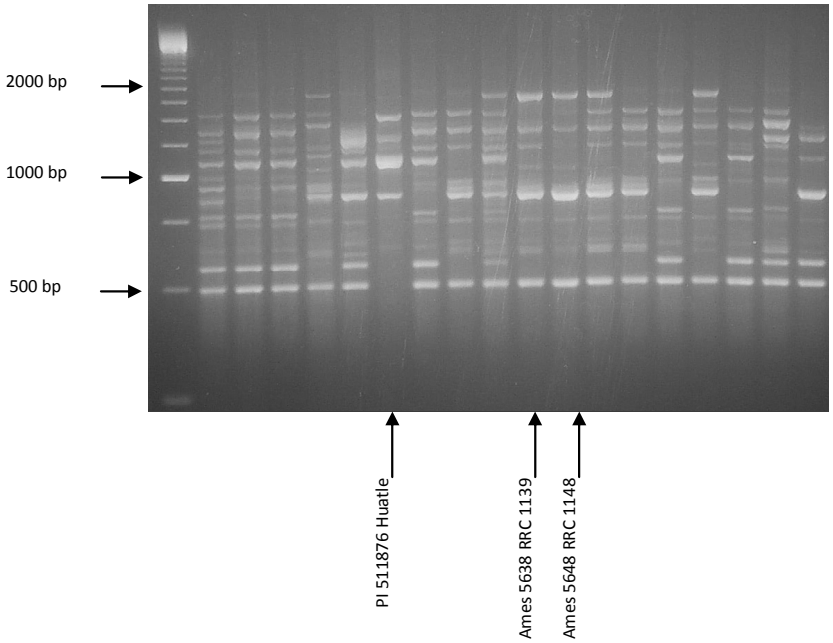

Figure 2. Electrophoreticaly separated DNA fragments of *Amaranthus cruentus* genotypes synthetised with ISLA-(GA)6CC in PCR. Genotypes of *A. cruentus* are from left as in the table1 for this specie.

According to ISSR data, two *Amaranthus caudatus* L. genotypes PI 568147 from Bolivia and PI 175039 RRC from India clustered together using $(GTG)_3GC$, $(GAG)_3GC$, $(GA)_6CC$ and $(CT)_6CC$, indicating higher level of genetic similarity between them. High level of genetic similarity revealed $(CA)_6GG$, $(GTG)_3GC$, $(GAG)_3GC$, $(CT)_8AC$ and $(GA)_6CC$ primers in *Amaranthus caudatus* L. PI 480816 IC-38286 and PI 480854 IC-38313. Two genotypes originated from India clusterd together with 0,000E+00 EDAC value.

On the basis of ISSR data, genotype *Amaranthus cruentus* L. PI 511876 Huatle originated from Mexico clustered as a single cluster using three primers - $(CA)_6GG$, $(CT)_8AC$, $(GA)_6CC$ in PCR, which indicates less genetic relationships between the PI 511876 Huatle genotype and other genotypes. Two primers $(CA)_6AG$ and $(GTG)_3GC$ were able to distinguish four genotypes of *Amaranthus hypochondriacus* L. originated from Mexico from other genotypes used in the study. Genotypes PI 477916 RRC 1023 with Ames 5209 RRC 457 and Ames 5132 RRC 363 with Ames 5321 RRC 539 were clustered with the 0,000E+00 EDAC value using ISLA-$(GA)_6CC$ in PCR (Figure 2). *Amaranthus hypochodriacus* Ames 2086 RRC 149 originated from Nepal clustered as a single cluster with different EDAC values ranging from 0,159E+0,2 to 0,377E+02 with three ISSR primers $(GTG)_3GC$, $(CT)_8AC$, $(GA)_6CC$ indicating less genetic similarity in comparison to other genotypes.

Amaranthus, as well as other crops from central and south America became rediscovered and are studied intensively from different points of view from antioxidant activity through sustainable management up to the molecular based polymorphism (Giuffré et al., 2011; Celis et al., 2011; Milella et al., 2011; Hernández et al., 2012; Martínez at al., 2012). Amaranth, as a promising crop will need a good knowledge about germplasm collections for genetic improvement. However, still only limited information is available on intra- and inter-specific genetic diversity and relationships within *Amaranthus* germplasm collections exist and results of this study report a new insight into it.

Conclusion

ISSR based evaluation of 55 accessions were done. The accessions belong to the three *amaranthaceae* species - *Amaranthus caudatus* L., *Amaranthus cruentus* L. *and Amaranthus hypochondriacus* L. As different origins and breeding stages of the tested species were involved, the ability of inter-species grouping of ISSR markers was proved in the study.

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PLANT SCIENCE

Production and characterization of cellulolytic enzymes by isolated *Klebsiella* sp. PRW-1 using agricultural waste biomass

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Abstract

The efficient cellulolytic microorganism was isolated from soil samples collected from Shivaji University campus. Biochemical test and phylogenetic analysis of isolated culture identified as *Klebsiella* sp. PRW-1. The isolated culture could utilize pure cellulosic substrates (carboxymethylcellulose (CMC) and avicel) and different agricultural wastes like sugarcane bagasse, sugarcane barbojo, sorghum husks, grass powder, corn straw and paddy straw by producing a large amount of endoglucanase, exoglucanase, β-glucosidase, filter paperase (FPU), xylanase and glucoamylase. The reducing sugar production was found higher in the presence of grass powder and sugarcane barbojo. Effects of different physic-chemical parameters to achieve maximum cellulolytic enzymes production were systematically investigated. The effects of supplementation of different metals additives as well as the stability in the presence of higher temperature, pH and commercial detergents, on cellulolytic enzymes were also studied. The foregoing result increases the applicability of the strain for the utilization and bioconversion of lignocellulosic biomass that could be used for bioenergy production.

Key words: Cellulase, Xylanase, FPU, Commercial detergents, Sorghum husks, Sugarcane barbojo

Introduction

The beginning of the industrial revolution and the consequent tremendous usage of fossil fuels showed negative impacts on the environment mostly due to the emission of greenhouse gases (CO_2, CH_4 and CO) resulting in global warming and pollution (Saratale et al., 2008). Nevertheless, large efforts are being conducted worldwide for the development of technologies which could generate clean, sustainable energy sources particularly, lignocellulosic biomass to biofuels, to substitute fossil fuels (Ragauskas et al., 2006; Levin et al., 2006). Biofuels produced from common agricultural wastes biomass, represent CO_2 cycle, ecofriendly, cost competitive with fossil fuels, biodegradable and it also contributes to the sustainability (Saratale and Oh, 2012). The produced biofuels becomes important and promising alternative energy source for fossil fuels to protect the environment and prevents the problem of the pollution (Puppan, 2002). Some recent reports correlated the first-generation biofuels production with food security and market prices of staple food crops such as maize and rice (James et al., 2008; Keeney et al., 2009). Hence it is important to make a fast transition from the first-generation to second-generation biofuels production using the lignocellulosic biomass such as agricultural crops residues (Fischer et al., 2009).

Lignocellulosic biomass is composed of biomolecules such as cellulose (insoluble fibers of β-1, 4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans) and lignin (a complex polyphenolic structure) (Saratale and Oh, 2011). The agricultural wastes or other renewable products are, abundantly available and its production covers more than 180 million tons per year, which can accomplish about two-third of the world's energy requirement (Kim and Yun, 2006). The availability of lignocellulosic biomass is being considered as the largest renewable energy resource all over the world. Thus, it can be considered as the most promising and economically feasible carbohydrate source for

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producing the new generation of biofuels (Kapdan and Kargi, 2006).

The most abundant lignocellulosic agricultural wastes produced every year are corncobs, corn stover, wheat, rice, barley straw, sorghum husks, coconut husks, sugarcane bagasse, switchgrass, pineapple and banana leaves (Demain, 2005). The biodegradation of lignocellulosic materials by microorganisms is affected by some factors, namely porosity of the wastes materials and crystallinity of cellulosic fiber (Zhang et al., 2006). The molecular organization of the plant fiber cell wall, the lignocellulose complex composed of cellulose, hemicellulose, and lignin; among which lignin and hemicellulose limits the biodegradation of this complex (Lo et al., 2008). This problem can be solved by the pretreatment, which reduces crystallinity of cellulose and increases the surface area of the lignocellulosic materials and ultimately improves the biodegradation of cellulose in to fermentable reducing sugars for the production of biofuels (Kumar et al., 2008).

Klebsiella pneumoniae utilises the cellulosic biomass converting it into 2,3-butanediol, thus the conversion of major sugars present in cellulosic biomass (Yu and Saddler, 1982). *K. oxytoca* P2 can utilise the sugars glucose and arabinose to produce ethanol, which are predominant carbohydrates found in sugar beet pulp (Burchhardt and Ingram, 1992). The naturally occurring cellulolytic bacterial strain *K. oxytoca* THLC0409 can degrade micro-sized Napier grass powder producing ethanol by direct conversion (Lin et al., 2010). The genome sequence and analysis of the predicted proteomes of *K. variicola* At-22 codes for enzymes which degrades cellulose polymer is known to be involved in plant polymer degradation, including capacity in the degradation of cellulose (Suen et al., 2010).

In the present study, *Klebsiella* sp. PRW-1 was isolated from soil samples which has the ability to hydrolyse different cellulosic substrates by producing of multiple cellulolytic and hemicellulolytic enzymes under static condition. Various physico-chemical parameters were optimized to achieve maximum enzymes and reducing sugar production. To our knowledge this is the first report on *Klebsiella* sp. for the hydrolysis of different agricultural lignocellulosic substrates by producing multiple cellulolytic enzymes having stability at thermophilic, alkaline conditions and in the presence of detergents which increases the industrial applicability of this strain.

Materials and Methods

Cellulosic substrates

Cellulosic materials (pure and agricultural wastes) such as carboxymethylcellulose (CMC), avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw were chosen as the carbon substrates in this study. The commercial cellulosic materials, such as CMC, birch wood xylan and avicel were obtained from Hi-Media (Mumbai, India). The agricultural wastes materials were air dried, milled and sieved through a 0.2 mm screen before storing at room temperature prior to usage. All other chemicals used were of the highest purity available and of the analytical grade.

Bacteria isolation and morphological tests

To isolate the microorganisms producing cellulases, soil samples were collected from the Shivaji University campus, Kolhapur (16°40'41"N 74°15'19"E / 16.67806°N 74.25528°E) and used as a screening source. The cellulose-hydrolytic bacteria were isolated by using modified Dubos salt medium with CMC as the sole carbon source. The CMC-amended Dubos salt medium consisted of (g l^{-1}): CMC, 10; $NaNO_3$, 0.5; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.001. For isolation, 2 g of different soil samples were transferred to the fresh 400 ml Dubos medium containing CMC as the sole carbon source in 500 ml sealed bottles for incubation at 30ºC for 7 days. After enrichment in CMC-amended medium for more than five times, the inoculum (0.1 ml; serially diluted to 10^{-5} times) was repeatedly streaking on Dubos agar plates containing CMC as a sole carbon source. After certain incubation the plates were stained by Congo red to evaluate the cellulolytic activity of isolated strains (Lo et al., 2009). The cellulase activity of each culture was determined by measuring the zone of clearance on agar plate. The individual colony PRW-1, which showed better growth and higher degradation ability in cellulosic material, was selected and used for the further experiments. The isolated microorganism was further identified on the basis of morphological and biochemical characteristics.

16S rRNA gene sequencing and phylogenetic analysis

The analysis of 16S rRNA gene sequencing and bacterial identification was carried out by Merck Millipore, Bangalore. Genomic DNA was extracted from the isolated cellulolytic bacteria according to the method described by Ausubel et al. (1997). The extracted DNA was then used, as a template, for PCR, to amplify the 16S rRNA gene using PCR

conditions as initial denaturation at 95º C for 5 min (1 cycle); subsequent denaturation at 95º C for 1 min, annealing at 58º C for 30 sec, extension at 72º C for 30 sec (40 cycles); final extension at 72º C for 10 min (1 cycle) and finally hold at 4º C for ∞. The 16S rRNA gene was amplified from the chromosomal DNA, using the universal bacterial primer set 27F (5'-AGAGTTTGATCMTGGCTCAG) and 1525R (5'-AAGGAGGTGWTCCARCC). The 16S rRNA gene sequence obtained after DNA sequencing was blast with 16S rRNA sequences available in the GeneBank. Multiple alignments were performed with the program CLUSTAL X (version 1.83) (Thomson et al., 1997). Evolutionary distances were calculated using the Kimura two-parameter model. Phylogenetic tree was constructed using the neighbour-joining method with the program MEGA 4 (Kumar et al., 2004). Bootstrap values were calculated on the basis of 1000 replications.

Microorganism and culture conditions

The study of the isolated *Klebsiella* sp. PRW-1 growth was carried out using the modified Dubos salt medium containing 1% CMC as carbon source. For the production of cellulolytic enzymes by *Klebsiella* sp. PRW-1, the optimum conditions such as agitation, initial pH of the media, incubation temperature were determined.

Preparation of enzyme source

The isolated *Klebsiella* sp. PRW-1 was grown in the modified Dubos salt medium with 1% CMC, as the sole carbon source, at initial pH, 6.5, 30°C for 3 days, and then a subculture was used once a month and stored at 4°C. *Klebsiella* sp. PRW-1 was grown in Dubos salt medium with carbon sources including carboxymethylcellulose (CMC), avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw (10 g l^{-1}), at 30°C, for 7 days, under static condition, centrifuged at 4000×g for 20 min. The culture supernatant, obtained after centrifugation during the harvesting of cell biomass, was directly used as a source of extracellular enzymes for the determination of the enzyme activities.

Enzyme assay

Endoglucanase activity was determined according to the method described by Saratale et al. (2012), using a reaction mixture containing 1 ml of enzyme solution with 1 ml of 1% carboxymethyl cellulose (CMC) in McIlvaine's buffer (0.1 mol l^{-1} citric acid-0.2 mol l^{-1} phosphate buffer; pH 5) and incubated at 50°C for 30 min. Exoglucanase (avicelase) activity was determined as the reaction mixture containing 1 ml of enzyme solution with 0.5 ml of 1% avicel cellulose in McIlvaine's buffer was incubated, at 50°C, for 2 h, and adding 1 ml of dinitrosalicylic acid reagent (Saratale et al., 2012). β-glucosidase activity was determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-glucoside (PNPG). The enzyme (200 µl) was incubated with 5 mM PNPG in 1 ml of 50 mM citrate buffer, pH 4.5, at 50ºC temperature for 10 min. The enzyme reaction was stopped by addition of 2 ml of 1 M sodium carbonate. One unit of β-glucosidase activity is defined as that amount of enzyme which will hydrolyze 5 mM of PNPG per min (Lymar et al., 1995). Filter paper (FPase) activity was measured according to IUPAC recommendations. FPase activity was determined by measuring the reducing sugars produced from Whatman no. 1 filter paper (50 mg, 1×6 cm). The reaction was carried out in 50 mM citrate buffer at pH 4.5. The reaction mixture was incubated at 50°C for 1 h (Adney and Baker, 2008).

Xylanase activity was determined in a reaction mixture containing 1 ml of enzyme solution diluted in McIlvaine's buffer with 1 ml of aqueous suspension of 1% xylan at 50°C for 10 min (Saratale et al., 2010). Glucoamylase activity was determined in a reaction mixture containing 1 ml of enzyme solution appropriately diluted in McIlvaine's buffer with 1 ml of aqueous suspension of 1% starch at 50°C for 10 min (Anto et al., 2006). In these enzymes test the reaction was terminated by adding 1 ml of dinitrosalicylic acid reagent and heating in boiling water bath for 10 min. One unit of enzyme activity in each case was defined by the amount of enzyme that produces one microgram of reducing sugar from the substrate per minute.

Establishment of optimum operational conditions for cellulolytic enzyme activity

The optimum temperature and pH of the cellulolytic enzymes (endoglucanase, exoglucanase, FPU, β-glucosidase, xylanase and glucoamylase) for *Klebsiella* sp. PRW-1 in the presence of agricultural wastes (10 g l^{-1}) were determined through the incubation of the enzyme and 1% (w/v) substrate at different temperature as 60, 70, 80 and 90°C, keeping constant pH 5.0 for 1 h. The effects of different pH range, from 2 to 10, at 50°C, on the cellulolytic enzymes activities were determined. The hydrolytic efficiency of *Klebsiella* sp. PRW-1 in the presence of different agricultural cellulosic substrates (paddy straw, wheat straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw, at about 10 g l^{-1}) was

determined by measuring the reducing sugar and cellulolytic enzymes production.

Effects of various metal additives on cellulolytic enzyme activity

The effect of various metal ions on the cellulolytic enzyme activity was determined, through the application of 5 mM of $MnCl_2$, $CaCl_2$, $ZnSO_4$, $CoCl_2$, $FeCl_3$ and $HgCl_2$. For all enzyme assays the reaction mixture with 0.5 ml was incubated under optimum temperature (at 30°C and pH of 5.0), being the residual activity of each sample quantified relatively to the control containing no metal ions in the reaction mixture.

Effects of commercial detergents on cellulolytic enzyme activity

The effect of different commercial detergents on the cellulolytic enzyme activity were determined, applying Ariel (Procter & Gamble), Rin (Hindustan, Unilever), Surf excel (Hindustan, Unilever), Tide (Procter & Gamble) and Wheel (Hindustan, Unilever). To simulate washing conditions these detergents were diluted in distilled water up to a final concentration of 7.0 mg mL^{-1} and then boiled for 60 min, for the inactivation of enzymes present in detergent (De Lima et al., 2005). For the determination of the effect of detergent on the cellulolytic enzyme activity, *Klebsiella* sp. PRW-1 supernatant was mixed with the detergents solution (1:1) and incubated under optimum temperature and pH of 5.0 for 1 h. The residual activity of each sample was then quantified with control containing no detergent in the reaction mixture.

FTIR analysis

The FTIR spectra were considered to examine the functional group changes occurred due to the cellulolytic degradation of agricultural wastes material used. FTIR spectra were recorded by the FTIR spectrometer (Perkin Elmer, Spectrum one B; Shelton, WA). The FTIR analysis was performed on the microbial degraded agricultural wastes (after 7 days of incubation), being compared with control agricultural wastes material. The cellulolytic degraded grass powder, sorghum husks and sugarcane barbojo was collected and washed with distilled water for removal of bacterial cells, bound enzymes and reducing sugars. The cellulolytic degraded agricultural wastes were than dried overnight in an oven at 60ºC. The dried samples of cellulosic materials were embedded in KBr pellets with ca.1% of samples in KBr. The background FTIR spectra were obtained using a pure KBr pellet without any agricultural waste material. The FTIR spectra were recorded in the absorption band mode in the range of 4000 – 400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test.

Results and Discussion

Isolation and identification of cellulolytic strain

Colonies of PRW-1 strain on CMC agar plates were white colour, shiny circular colonies grows well within 3 days of incubation. Microscopic examination showed that the isolate was Gram-negative rod and non motile. The nearly full length sequence of 16S rRNA gene for isolate PRW-1, determined and based on the sequence identity of 16S rRNA gene against the Gen Bank database, indicates that the isolate was closely related to the members of the genus *Klebsiella*. The phylogenetic analysis showed about 99.4% similarity between the isolated PRW-1 strain and the *Klebsiella variicola* HUB-IV-005 strain (Figure 1). On the basis of the 16S rRNA sequence, the isolated strain was identified as *Klebsiella* sp. PRW-1. The biochemical characteristics of bacterial isolate is shown in Table 1.

Optimization of growth conditions

The *Klebsiella* sp. PRW-1, incubated in Dubos containing CMC as a sole carbon source, displayed a higher cellular growth, cellulolytic enzymes and reducing sugar production after 6 days of incubation. Better growth of *Klebsiella* sp. PRW-1 was observed under static condition, as compared with under shaking condition (120 rpm) (Figure 2). Thus, for a better cellular growth, the static condition was adopted. This condition was used for the investigation of the cellulolytic enzymes production by *Klebsiella* sp. PRW-1 in the following experiments. The better growth and cellulolytic enzymes production, was observed at 30ºC and initial Dubos medium pH of 6.5 in all cellulosic substrates used in this study.

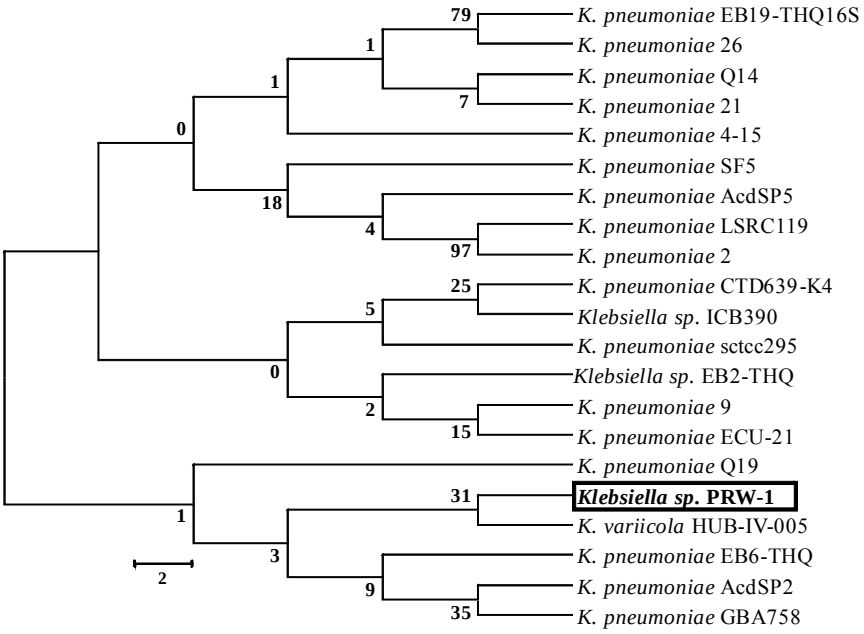

Figure 1. Neighbor-joining showing phylogenetic positions of *Klebsiella* sp. PRW-1 and *Klebsiella* species based on 16S rRNA gene sequence comparisons. *K. pneumoniae* SF5 was used as an out group Bootstrap values are indicated at nodes.

Effects of incubation time on cellulase production

Under the optimum culture conditions, the detailed growth study of *Klebsiella* sp. PRW-1 and the production of cellulolytic and hemicellulolytic enzymes, at different incubation time, showed maximum cellulolytic enzyme activities in Dubos medium containing different cellulosic substrates. The maximum enzyme activities were obtained when it entered in the late logarithmic growth phase (after 6th days of incubation) and continued to secrete enzyme also during stationary phase of growth (Figure 3). After 7 days of incubation *Klebsiella* sp. PRW-1 produced maximum reducing sugar in different cellulosic substrates. These results suggested that *Klebsiella* sp. PRW-1 produced different cellulolytic enzyme activities, which work synergistically on the hydrolysis of cellulosic substrates. The enzyme activities for endoglucanase and glucoamylase were found to be higher in sugarcane barbojo and grass powder at 6th day of incubation (Figure 3 (A) (a) and (A) (c)). The FPU activity remained higher in the grass powder and sugarcane barbojo, at the 6th day, as compare to the pure substrate CMC (Figure 3 (B) (b)).

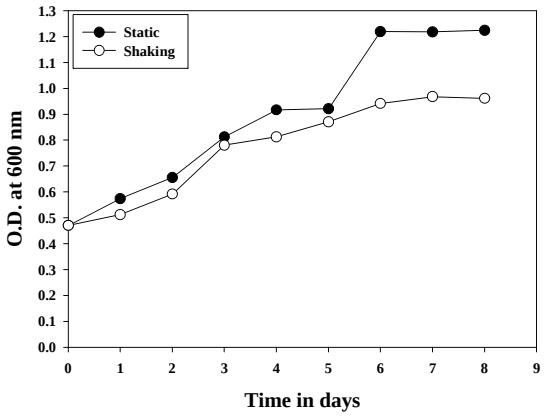

Figure 2. Growth study of *Klebsiella* sp. PRW-1 in Dubos media broth with CMC (10 g l^{-1}).

Table 1. Biochemical characteristics of isolated bacterial strain *Klebsiella* sp. PRW-1.

Biochemical test	Observation
Gram staining	Gram negative
Mobility	Non-motile
Lactose	+ve
Xylose	-ve
Maltose	-ve
Fructose	-ve
Dextrose	-ve
Galactose	-ve
Raffinose	-ve
Trehalose	+ve
Melibiose	-ve
Sucrose	-ve
L-Arabinose	+ve
Mannose	+ve
Inulin	-ve
Sodium gluconate	-ve
Glycerol	+ve
Salicin	+ve
Dulcitol	-ve
Inositol	-ve
Sorbitol	+ve
Mannitol	+ve
Adonitol	+ve
Arabitol	-ve
Erythritol	-ve
α- Methyl-D-glucoside	+ve
Rhamnose	+ve
Cellobiose	+ve
Melezitose	-ve
α- Methyl-D-mannoside	-ve
Xylitol	-ve
ONPG	+ve
Esculin hydrolysis	+ve
D-Arabinose	+ve
Citrate utilization	+ve
Malonate utilization	+ve
Sorbose	+ve

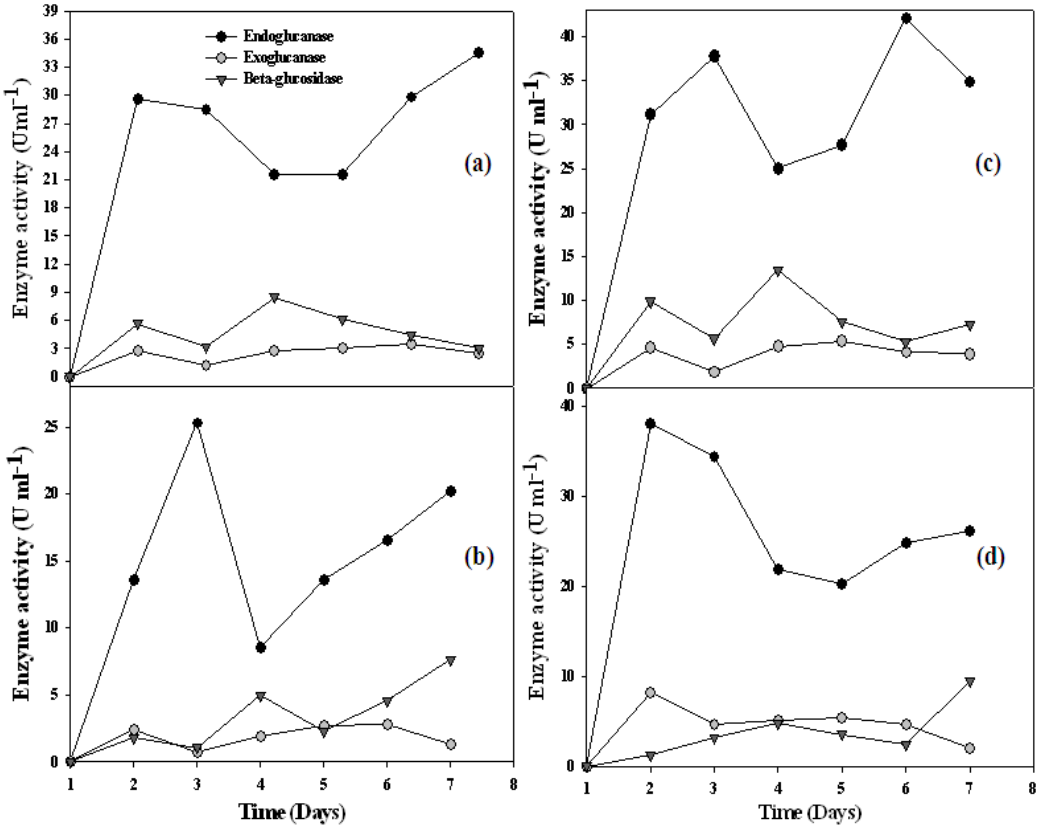

Figure 3 (A). Study of incubation time on endoglucanase, exoglucanase and β-glucosidase production by Klebsiella sp. PRW-1 (a) Sugarcane barbojo; (b) Sorghum husks; (c) Grass powder, (d) CMC.

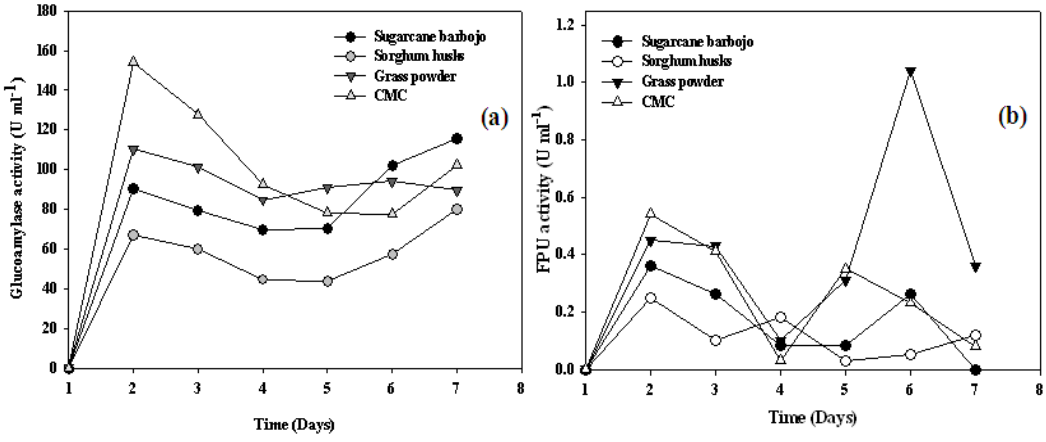

Figure 3 (B). Study of incubation time on cellulase enzyme produced by *Klebsiella* sp. PRW-1 (a) Glucoamylase (b) Filter paperase.

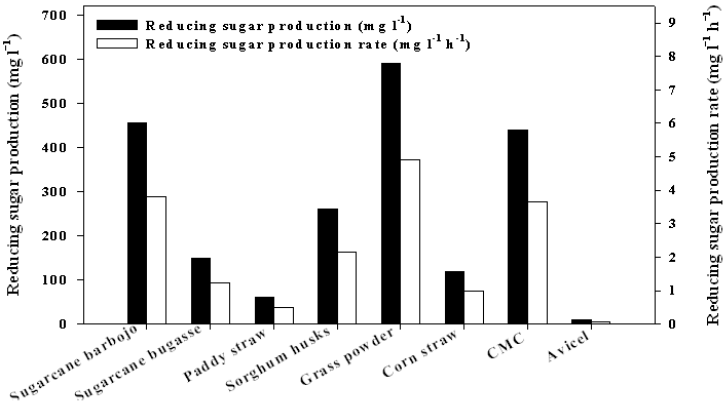

Figure 4. Effect of different cellulosic substrates on cellulose hydrolysis by *Klebsiella* sp. PRW-1 in Dubos media supplemented with 10 g l^{-1} of each cellulosic substrate at 30ºC, with initial pH 6.5 under static condition after 7 days incubation.

Effects of different cellulosic substrates on the production of reducing sugar and cellulolytic enzymes by *Klebsiella* sp. PRW-1

The isolated *Klebsiella* sp. PRW-1 could utilize different cellulosic materials (pure and agricultural wastes including; CMC, avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw). The study by Zhang et al. (2006) showed a positive correlation between the production of cellulase enzyme from cellulolytic microorganisms and different composition of cellulose, hemicelluloses and lignin in different cellulosic materials. In this study we have determined the hydrolytic efficiency of *Klebsiella* sp. PRW-1 grown on the Dubos medium by taking CMC, avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw as the carbon source (all at an initial concentration of 10 g l^{-1}), to explore the effect of carbon sources on cellulose hydrolysis. All cellulosic substrates could be hydrolyzed by *Klebsiella* sp. PRW-1 and produces reducing sugars. Hydrolysis of sugarcane barbojo and grass powder exhibited maximum reducing sugar production rate than that of other cellulosic materials used. Among the different agricultural wastes materials, hydrolysis of grass powder gave the best hydrolysis efficiency with a maximum reducing sugar production (590 mg l^{-1}) and reducing sugar production rate (4.91 mg h^{-1} l^{-1}). After hydrolysis of other cellulosic materials such as sugarcane barbojo, CMC, sorghum husks, sugarcane bagasse, corn straw, paddy straw and avicel exhibited reducing sugar production (455, 440, 260, 150, 120, 60, 10 mg l^{-1}) and reducing sugar production rate (3.79, 3.66, 2.16, 1.25, 1.0, 0.5, 0.083 mg h^{-1} l^{-1}), respectively was observed (Figure 4).

The enzymatic hydrolysis of cellulosic feedstock has several advantages, namely mild experimental conditions, less energy consumption and avoidance of pollution over the chemical processes (Saratale et al., 2008; Zhang et al., 2006). *Klebsiella* sp. PRW-1 has ability to utilise and metabolize different cellulosic substrates for their growth and expresses multiple cellulolytic enzyme activities (e.g., endoglucanase, exoglucanase, β-glucosidase, glucoamylase and xylanase), mainly at extracellular location. In the presence of grass powder *Klebsiella* sp. PRW-1 produces the higher cellulolytic enzyme activities, while other carbon source used substrates showed moderate enzyme activities (Table 2).

Table 2. Cellulolytic and hemicellulolytic enzyme activity of *Klebseilla* Sp. PRW-1 produced extracellularly after 7 days incubation in Dubos media containing different cellulosic substrates.

Cellulosic substrate	Endoglucanase[a]	Exoglucanase[a]	Glucoamylase[a]	Xylanase[a]	FPU[a]	β-glucosidase[a]
Sugarcane barbojo	34.62 ± 0.01	2.43 ± 0.02	115.83 ± 1.18	34.24 ± 0.78	NA	3.00 ± 0.17
Sugarcane bagasse	19.44 ± 0.08	0.23 ± 0.02	62.31 ± 0.63	33.61 ± 0.78	NA	NA
Corn straw	21.04 ± 0.10	0.97 ± 0.02	45.54 ± 0.99	45.65 ± 1.03	NA	NA
Paddy straw	31.42 ± 0.16	0.73 ± 0.02	46.33 ± 0.93	38.04 ± 1.06	NA	NA
Grass powder	34.88 ± 0.04	3.96 ± 0.93	89.47 ± 0.93	51.36 ± 0.07	0.36 ± 0.01	7.24 ± 0.17
Sorghum husks	20.24 ± 0.02	1.33 ± 0.14	79.89 ± 1.04	24.73 ± 0.75	0.12 ± 0.01	7.60 ± 0.17
Carboxymethyl cellulose	26.10 ± 0.08	2.03 ± 0.10	102.25 ± 0.89	53.90 ± 0.78	0.08 ± 0.01	9.54 ± 0.14

Values are mean of three experiments, SEM (±), and by one-way ANOVA with Tukey-Kramer Multiple Comparisons Test; [a]Enzyme activity (U ml^{-1}); NA- No activity.

The maximum the cellulolytic activity of endoglucanase (34.88 U ml^{-1}), exoglucanase (3.96 U ml^{-1}) and FPU (0.36 U ml^{-1}) were observed in the presence of grass powder, as the carbon source in the Dubos media. The hemicellulolytic enzyme activity of glucoamylase (115.83 U ml^{-1}) and xylanase (51.36 U ml^{-1}) was observed when sugarcane barbojo and grass powder was used as carbon source, respectively. Similarly higher exoglucanse, β-glucosidase, xylanase and glucoamylase enzyme activities were detected in the presence of sugarcane barbojo and sorghum husks (Table 2). For the low cost and maximum production of cellulase enzyme different agricultural lignocellulosic wastes were further used as a carbon sources by *Klebsiella* sp. PRW-1. The enzymatic hydrolysis of lignocellulosic biomass produces disaccharides and oligosaccharides, which plays role as strong inducers of cellulases (Kapdan and Kargi, 2006; Saratale et al., 2012). When grass powder was use as a carbon source, the production of endoglucanase (34.88 U ml^{-1}), exoglucanase (3.96 U ml^{-1}), FPU (0.36 U ml^{-1}), β-glucosidase (7.24 U ml^{-1}), xylanase (51.36 U ml^{-1}) and glucoamylase (115.83 U ml^{-1}) was observed, which increases the applicability of this strain (Table 2). In the same contest, production of different lignocellulolytic enzymes from *Lentinus edodes* (Berk.) Sing. has been carried out by Ramkumar et al. (2011). Also Zambare (2011) with the help of response surface methodology, optimized amylase enzyme production from *Bacillus* sp. by using cellulosic material.

Effects of the temperature on the activity and stability of multiple cellulolytic enzymes produced by *Klebsiella* sp. PRW-1

The production of microbial cellulase has long been known to be influence by different factors like the strain type, reaction conditions and substrate types, since the relationship of these factors might affect the production of the cellulase enzymes (Zhang et al., 2006). The cellulases are relatively costly enzymes, and for their commercial use the production cost of cellulase should be significantly reduce for the preparation of cellulosic feedstock. The cellulolytic enzymes (exoglucanase, endoglucanase) and hemicellulolyitc enzymes (glucoamylase and xylanase) by *Klebsiella* sp. PRW-1, in the presence of different pure and agricultural cellulosic substrates, were studied at different temperatures (50–90ºC). These enzyme produced by *Klebsiella* sp. PRW-1 showed the higher performance at 50ºC, by keeping the constant pH 5.0, in the presence of all cellulosic substrates. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Haki and Rakshit, 2003). The thermostability of all enzymes was assessed by incubating the enzyme at different temperatures for 1 h. The endoglucanase and glucoamylase produced in the presence of sugarcane barbojo and sorghum husks are found to be thermostable since it retains 50-60% of its activity. Whereas, in the presence of sorghum husks, enzymes which are produced maintain more than 60% initial activity at higher temperature (80ºC). The glucoamylase and xylanase enzymes produced in grass powder and CMC showed thermo stability up to 80-90% at 70ºC (Figure 5). The thermo stable nature of cellulolytic enzymes added advantages to the lignocelluloses bioconversion processes so that it

remains viable and active at higher temperature. At higher operation temperature of stability of the enzymes significantly influence the bioavailability and solubility of organic compounds. Because of these enzymes can efficiently degrade of cellulosic biomass and increased flexibility with respect to process configuration, and overall improves the economy of the process (Viikari et al., 2007). The thermo stability of this enzyme indicates industrial applicability in the food, sugar, fuel ethanol and agricultural industries where process operation applied with higher temperature (Jang and Chen, 2003).

Effects of pH on the activity and stability of multiple cellulolytic enzymes produced by *Klebsiella sp*. PRW-1

The effect of pH on endoglucanase, exoglucanase, β-glucosidase, glucoamylase and xylanase enzyme activity was studied at different pH (2–10) by maintaining similar standard assay conditions (Figure 6). The optimum pH for these enzymes produced by *Klebsiella* sp. PRW-1 was found to be 5 at temperature 50ºC and in the presence of all cellulosic substrates. The endoglucanse enzyme produced by *Klebsiella* sp. PRW-1 was found to be less stable and residual enzyme activity was only upto 20-30% than its initial activity after incubation at 50ºC for 1 h. The glucoamylase produced using the sugarcane barbojo and sorghum husks remained stable up to 70-80%, at alkaline pH 8. While the xylanase produced in the presence of grass powder showed higher alkalotolerance by retaining more than 70% initial activity of at higher pH 8. The ability of retaining the higher enzyme activity at different pH range is a potentially useful property in the processes employing alkaline delignification.

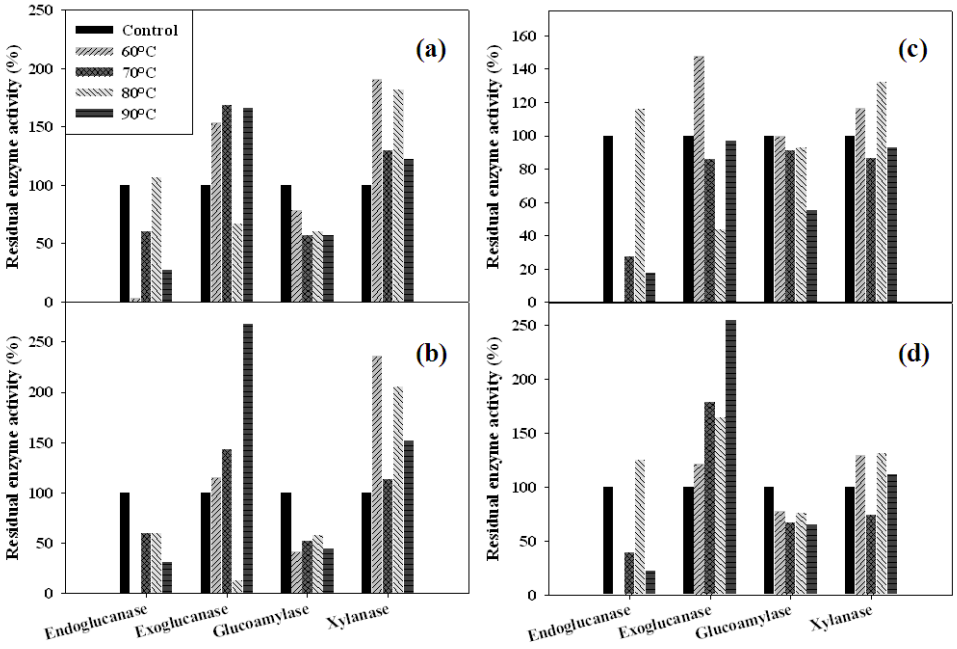

Figure 5. Thermal stability study of endoglucanase, exoglucanase, glucoamylase, xylanase and FPU produced by *Klebsiella* sp. PRW-1 using (a) Sugarcane barbojo, (b) Sorghum husks, (c) Grass powder and (d) CMC.

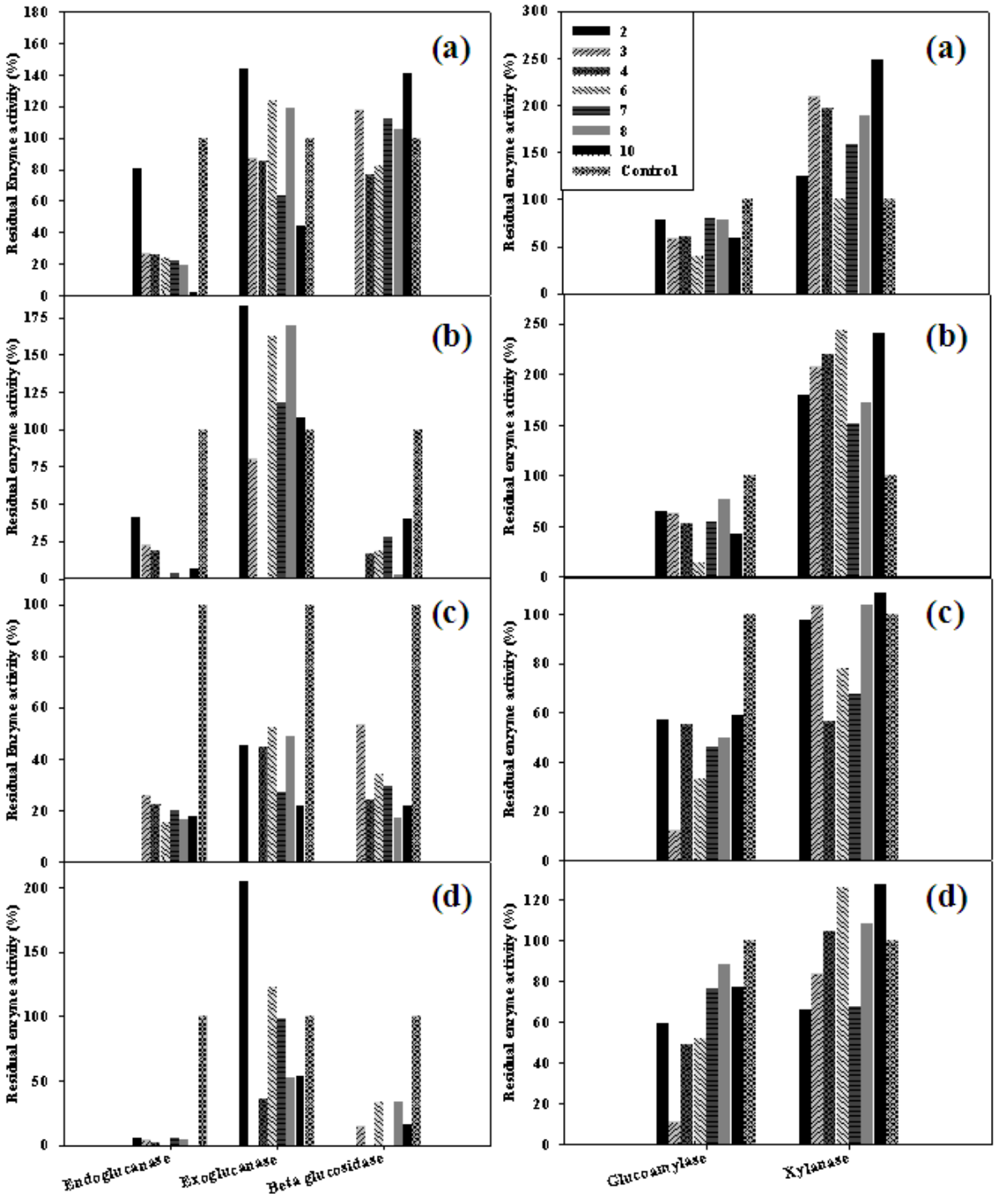

Figure 6. Study of pH stability of cellulase and hemicellulase enzymes produced by *Klebsiella* sp. PRW-1 using (a) Sugarcane barbojo; (b) Sorghum husks; (c) Grass powder and (d) CMC.

Table 3. Effects of different metal additives on endoglucanse, exoglucanse, glucoamylase, xylanase and β-glucosidase enzyme production by *Klebsiella* sp. PRW-1 in the presence of different cellulosic substrates.

Cellulosic substrate	Enzymes	Metal ion concentration (5 mM)						
		Control	$CoCl_2$	$FeCl_3$	$HgCl_2$	$CaCl_2$	$MnCl_2$	$ZnSO_4$
Sugarcane barbojo	Endoglucanase	100	696.30	244.44	NA	NA	774.07	340.74
	Exoglucanase	100	149.57	103.48	74.78	102.61	251.30	106.09
	Glucoamylase	100	277.59	368.97	NA	118.97	370.69	125.86
	Xylanase	100	150.96	75.96	NA	42.31	137.50	72.12
	β-glucosidase	100	110.34	1196.5	100.00	186.21	1041.3	172.41
Grass powder	Endoglucanase	100	204.11	228.08	NA	102.74	288.36	101.37
	Exoglucanase	100	118.32	134.35	61.83	95.42	209.92	118.32
	Glucoamylase	100	140.88	187.59	NA	77.37	229.20	76.64
	Xylanase	100	176.74	113.18	NA	38.76	198.45	113.95
	β-glucosidase	100	185.37	895.12	182.93	178.05	1292.6	214.63
Sorghum husks	Endoglucanase	100	338.24	292.65	360.29	80.88	535.29	76.47
	Exoglucanase	100	377.27	227.27	NA	68.18	854.55	377.27
	Glucoamylase	100	308.22	363.01	NA	165.75	426.03	135.62
	Xylanase	100	153.44	93.13	106.11	89.31	122.90	158.02
	β-glucosidase	100	96.30	1096.3	92.59	92.59	1355.5	166.67
Carboxymethyl cellulose	Endoglucanase	100	461.11	88.89	NA	NA	666.67	266.67
	Exoglucanase	100	238.64	172.73	14.77	159.09	322.73	167.05
	Glucoamylase	100	244.93	227.54	NA	152.17	314.49	111.59
	Xylanase	100	205.62	95.51	NA	NA	211.24	117.98

Effects of different metal additives on cellulolytic enzymes produced by *Klebsiella sp*. PRW-1

Influential studies of metal ions on enzyme are very important because it increases enzymes industrial applications. It has been previously reported that some metals acts as a cofactor of cellulase, inducing or inhibiting the amino acids of the active site of the enzymes (Haki and Rakshit, 2003). The enzyme activities of cellulase produced by *Klebsiella* sp. PRW-1 were assayed under standard optimal conditions in the presence of several metal supplements (at concentration 5 mM each) (Table 3). With the addition of 5 mM $CoCl_2$, $MnCl_2$ and $FeCl_3$ all cellulolytic enzyme activities were sharply induced in the presence of all cellulosic substrates. The enzymes produced by *Cellulomonas biazotea* NCIM-2550, *Streptomyces* sp. MDS showed the induction in $MnCl_2$, $CoCl_2$ and $FeCl_3$, respectively (Saratale et al., 2008; Saratale et al., 2012). The similar results were found to be induced due to the metal additives like $FeCl_3$, $CoCl_2$ and $MnCl_2$ in all enzyme activities in the presence of all cellulosic substrates used in this study. However addition of $HgCl_2$ showed substantial inhibitory effect on all enzyme activities in the presence of all cellulosic substrates (Table 3).

Effects of the commercial detergents on the activity and stability of multiple cellulolytic enzymes produced by *Klebsiella* sp. PRW-1

The study of commercial detergents on the cellulolytic enzyme stability increases its applicability in industry. The enzyme activity of endoglucanase produced by *Klebsiella* sp. PRW-1 using sorghum husks, sugarcane barbojo and grass powder was inhibited in the presence of all the tested detergents. While exoglucanase produced by *Klebsiella* sp. PRW-1 utilising similar substrates retained its activity and stability in the presence of all detergents (Figure 7). The exoglucanase enzyme produced using grass powder retains upto 80% than that of its initial activity. The endoglucanase enzyme found to be totally inhibited in the presence of all commercial detergents used during this study. The xylanase enzyme produced by isolated PRW-1 strain by utilising sorghum husks and CMC shown the inhibition in all detergents. Whereas this enzyme found to be stable in all detergents when enzyme was produced by utilising sugarcane barbojo and grass powder as carbon source. The inhibitory effect of commercial detergent on some enzymes mentioned in above discussion might be because of the active site modifications which alters the substrate specificities (Sukumaran et al., 2005).

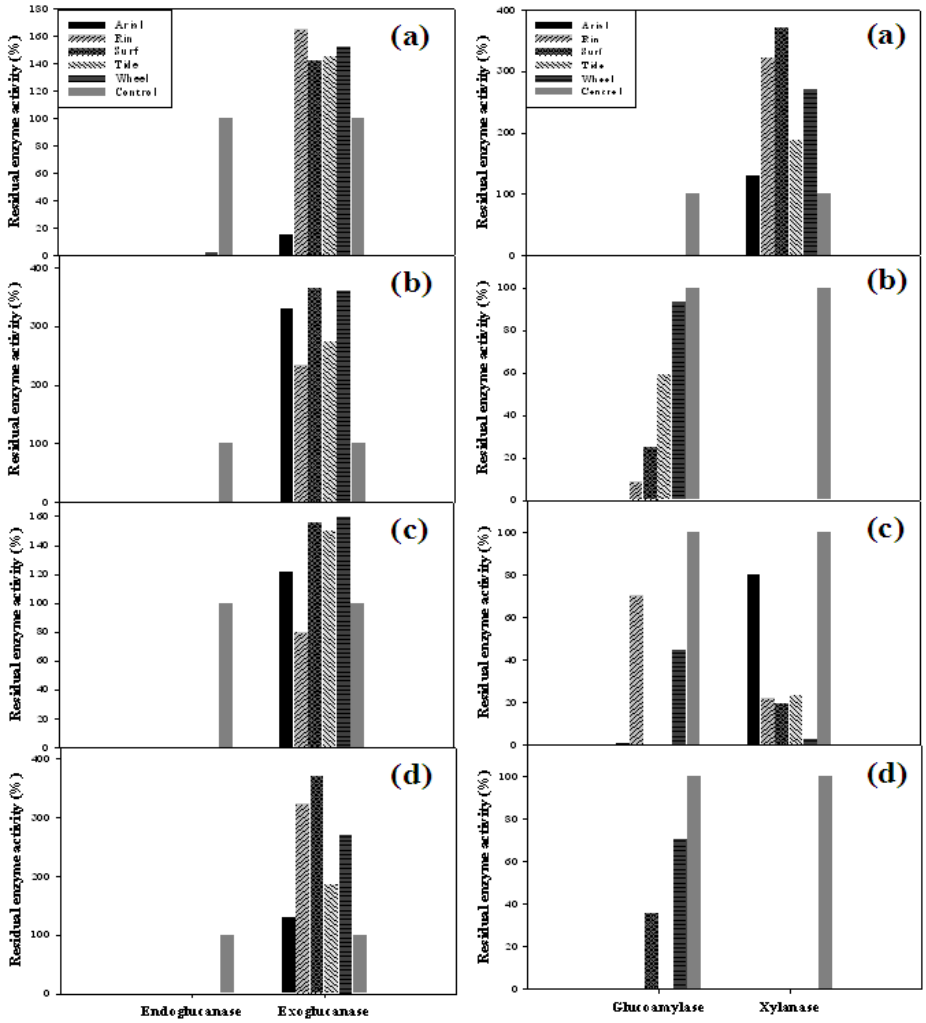

Figure 7. Detergent stability of Cellulase enzyme produced by *Klebsiella* sp. PRW-1 using (a) Sugarcane barbojo; (b) Sorghum husks; (c) Grass powder and (d) CMC.

FTIR analysis

The complex nature of agricultural lignocellulosic waste determines that most of the IR bands were observed in the fingerprint region of FTIR spectra in all substrates. For simplicity the interpretation of IR bands and reflect exact the chemical changes, the four characteristic absorption bands of lignocellulose and two characteristic absorption bands of lignin are used in this study as follows: 1736 cm^{-1} for unconjugated C-O stretch (hemicelluloses), 1372 cm^{-1} for C–H deformation (cellulose and hemicelluloses), 1160 cm^{-1} for C–O–C vibration (cellulose and hemicelluloses), 897 cm^{-1} for C–H deformation (cellulose), 1510 cm^{-1} for aromatic skeletal vibration (lignin) and 1225 cm^{-1} for C–O stretch (lignin) (Pandey and Pitman, 2003).

In the grass powder 1725 cm^{-1} for unconjugated C-O stretch (hemicelluloses), 1508 cm^{-1} for aromatic skeletal vibration (lignin) are removed, 1161 cm^{-1} for C-O-C vibration (cellulose and hemicelluloses) are removed after enzymatic degradation (Figure 8 (a)). While in sorghum husks substrate, the absorbance peak at 1736 cm^{-1} for unconjugated C-O stretch (hemicelluloses) and 1508 cm^{-1} for aromatic skeletal vibration (lignin) are removed after the enzymatic degradation (Figure 8 (b)). In case of sugarcane barbojo 1052 cm^{-1} for C-O stretch in cellulose and hemicellulose was removed while 1107 cm^{-1} for C-OH stretch for secondary alcohol was arised after the enzymatic

degradation (Figure 8 (c)). The removal of most characteristic absorption peaks of cellulose, hemicelluloses and lignin, after the incubation of 7 days showed that the *Klebsiella* sp. PRW-1 has the ability to utilize and metabolise the lignocellulosic biomass as a source of carbon for growth. This showed the potential of *Klebsiella* sp. PRW-1 for cellulolytic degradation of different cellulosic substrates by cellulolytic enzymes produced by it.

Conclusion

Isolated *Klebsiella* sp. PRW-1 strain showed its ability to grow on different cellulosic substrates (pure and agricultural wastes) by producing extracellular cellulolytic and hemicellulolytic enzymes under static submerged condition. Enhancement in the reducing sugar production was observed at the 7th day of incubation using grass powder as substrate. The FTIR analysis confirmes the utilization of cellulosic substrates as a source of carbon. Enzymes produced by *Klebsiella* sp. PRW-1 in presence of different cellulosic substrates showed higher thermal and pH stability which increases the applicability of this strain. The cellulolytic and hemicellulolytic enzymes activities were found to be induced after addition of $MnCl_2$ in the reaction mixture. The findings of this study reveals potential of *Klebsiella* sp. PRW-1 for the preparation of lignocellulosic feedstock will be useful for bioenergy production.

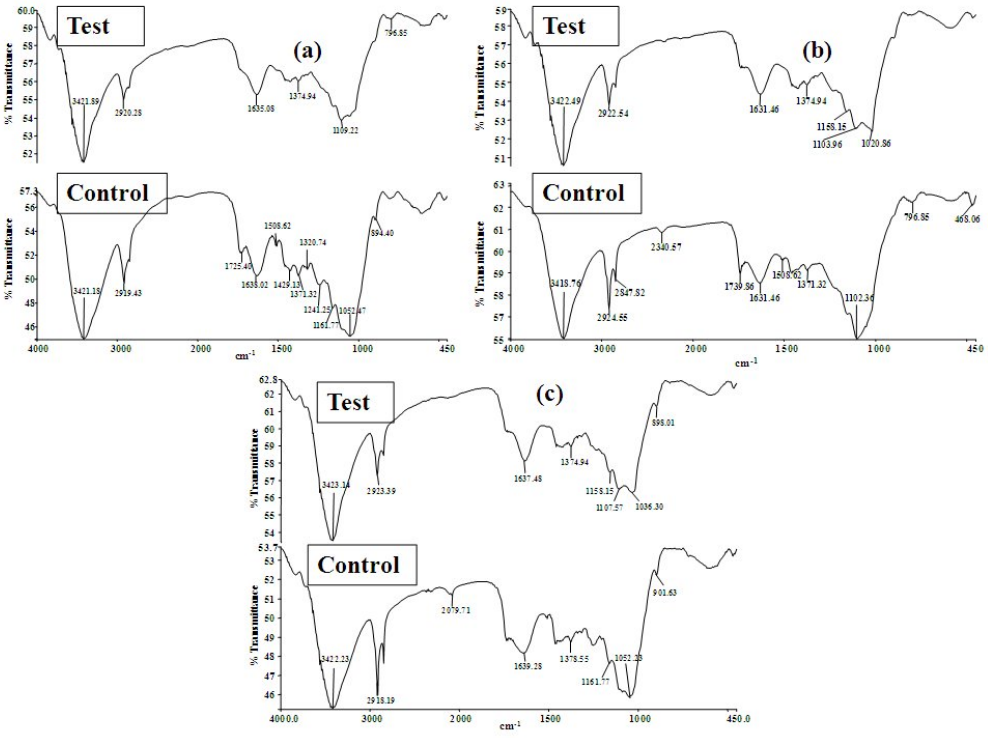

Figure 8. FTIR spectrum of (a) Grass powder; (b) Sorghum husks and (c) Sugarcane barbojo.

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Short Communication

PLANT SCIENCE

Difference on ITS regions among Yacon genotypes and *Smallanthus* spp.

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Abstract

Yacon (*Smallanthus sonchifolius*) is an Andean crop which is very regarded for its medicinal properties. Yacon genetic profiles have been slightly studied using molecular markers. The aim of this study was to prepare the protocol for direct sequencing of the ITS (Internal Transcribed Spacers) regions of yacon DNA genome. The identified ITS regions in three Bolivian yacon's landraces (BOL 20, BOL 22 and BOL 24) were sequenced and then compared with ITS regions found in NCBI database, previously isolated in yacon and in other plants belonging to the genus *Smallanthus*. The analysed ITS regions of studied Bolivian landraces showed differences in the nucleotides 163-164 and 235-236 with the sequence previously isolated in yacon. A dendrogram was constructed by comparing the sequence presented in the manuscript and those in the NCBI database for *Smallanthus*, spp. In the dendrogram, the previously reported ITS sequence of *Smallanthus sonchifolius* was also used. Specific sites of *Smallanthus sonchifolius* ITS region gives a promising base for molecular approach identification of this underutilized crop.

Key words: ITS region, Landraces, Molecular markers, DNA sequence, Yacon

Introduction

Yacon [*Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson - Asteraceae] is a perennial herb, originating in the Andean region. Recently, greater attention has been focused on this plant due to its agronomical, nutritional and pharmacological characteristics (Valentová et al., 2001; Milella et al., 2011). This plant produces tuberous roots rich in inulin. Tubers are consumed as fresh fruit and have a pleasant sweet taste (Manrique et al., 2004) and they are also an excellent natural source of fructo-oligosaccharides (FOS), which can replace sucrose as sweetener for their low caloric value. In different yacon genotypes FOS content can sensibly vary, which offers the possibility of selection and their use for breeding purposes to obtain genotypes with high FOS content (Ishiki et al., 2000). Yacon roots accumulate about 10% of inulin-type FOS, which is characterized as a dietary supplement with positive health effects (Narai-Kanayama et al., 2007). For the assessment of genetic diversity molecular markers have been generally superior to morphological traits, and biochemical markers (Melchinger et al., 1991). Yacon leaf showed also several interesting pharmacological activities. In yacon leaves several compounds as sonchifolin, polymatin A and B, uvedalin, polyphenols and enhydrin were already isolated (Goto et al., 1995; De Pedro et al., 2003). These compounds possess antifungal and anti-inflammatory activities (Valentová et al., 2001; Hong et al., 2008). Yacon tubers also may have potential as a diet food. The human body has no enzyme to hydrolyze inulin, so it passes through the digestive tract unmetabolized, which means that yacon provides few calories. This could be an attractive marketing feature to dieters and diabetics (National Research Council 1989). In fact in South America, Bolivia, Brazil and Argentina, yacon roots and leaves are commonly consumed by people suffering from diabetes or various digestive or renal disorders and this ethno-botanical use was confirmed by recent scientific research (Valentova et al., 2006).

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Yacon was originally cultivated in South America and now has been introduced in New Zealand, Japan, Italy, Germany and the Czech Republic. The productivity and other valuable agronomic traits of yacon strongly suggest that it is a species with a great potential, moreover the cultivation of this plant needs almost no pesticides (Lin et al., 2003).

During yacon evolution, continued vegetative propagation and selection for root yield may have impaired flowering and fruit set. Although yacon is a clonal crop, it shows some morphological and physiological variations. However, this variation may reflect the phenotypic differences expressed due to its growing conditions, rather than genetic variation. On the other hand it is very difficult to differentiate some yacon landraces from a wide geographical range, from Ecuador to Argentina, when they were grown in the same environment (Fernandez et al., 2006; Lebeda et al., 2012).

At the same time molecular markers demonstrated to be useful, in several crops, in order to investigate their genome structure (Milella et al., 2006; Ovesna et al., 2013), germplasm characterization (Garcia-Mas et al., 2000; Žiarovská et al., 2012), or to define crossing combinations and cultivar identification (Karp et al., 1996). Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Karp et al., 1996). Molecular Marker based Genetic Diversity Analysis (MMGDA) also has potential for assessing changes in genetic diversity over time and space. Nowadays, molecular research activities connected to yacon germplasm are focused on the development of different mapping techniques for its genomic characterization. Yacon intraspecific variability was characterized using isozyme polymorphism method and also using some DNA markers. Unlike the morphological and biochemical analysis, molecular characterization of yacon showed to be more efficient (Milella et al., 2011). In fact molecular markers as random amplified polymorphic DNA (RAPD) and amplified fragment length (AFLP) were presented as suitable methods for the analysis of intraspecific genetic variability in *Smallanthus sonchifolius* (Mansilla et al., 2006; Milella et al., 2007). Both methods demonstrated their ability in differentiating among analyzed genotypes and their relationships with yacon phenolic content (Milella et al., 2011). It was demonstrated that phenolic content depends, in this case, not on environmental condition but rather on the genome of a particular landrace (Milella et al., 2011). Svobodová et al. (2011) characterized 14 cultivars native yacon from Peru, where certain polymorphic loci showed a close genetic distance between genotypes. These results demonstrated as molecular markers play an important role in breeding, cultivation and industrial use purposes.

No studies at the moment have investigated Internal Transcribed Spacers (ITS) sequence variation in yacon. The purpose of this study was to develop a protocol for the identification and direct sequencing of ITS regions in yacon genomic DNAs and to compare obtained sequences with those previously reported for *Smallanthus* spp.

Material and Methods

DNA extraction

Three different landraces of yacon [*Smallanthus sonchifolius,* (Poepp. et Endl.) H. Robinson; *Asteraceae*] named BOL 20, BOL 22 and BOL 24, were collected in Bolivia and selected for their different morphological traits. BOL 20 was collected in San Pedro area (2800 m. - Potosí-Bolivia), BOL 22 was collected in Tuquiza (2900 m - Potosí - Bolivia) and BOL 24 was collected in El Locotal (2 500 m- Cochabamba - Bolivia) (Fernández et al., 2005, 2006). The 3 genotypes were then grown under the same environmental conditions in the Czech Republic (Institute of Tropics and Subtropics of the Czech University of Life Sciences in Prague) after vegetative propagation, were their germplasm is deposited.

Genomic DNA was extracted from approximately 500 mg of young leaf tissue. Leaf tissue was ground to a fine powder in liquid nitrogen. Total genomic DNA was extracted using a protocol described by Milella et al. (2011). DNA concentrations were estimated by spectrophotometric assay and then diluted to a final concentration of 25 ng/ul.

PCR reaction and sequencing

Primers were selected for their putative amplification of the DNA sequences that codify for small (18S, **SSU**) and large subunit (28S, **LSU**) ribosomal RNA (rRNA). Primers were selected for their previous use, they are considered as standard primers for the amplification of these regions in various species of fungi and higher plants (White et al. 1990; Gardens and Burns, 1993; Smolik et al. 2011a). Several primer combinations have been selected but only one gave the expected results in the direct sequencing (FWD ITS1 nucleotide sequence 5 '- tccgtaggtgaacctgcgg - 3' REV ITS2 5 '- tcctccgcttattgatatgc - 3'). The polymerase chain reaction (PCR) amplifications of ITS regions were

carried out in an MJ Research PTC-200 thermal cycler under the following conditions: 1X buffer solution containing $MgCl_2$ (2 mM), dNTP (0.2 mM), sense and antisense primer (200 nM) and 1U Taq Green Dream DNA polymerase (Thermo Scientific) in a volume of 15 µl. Following cycling conditions were used: 95°C, 3 min; [95°C, 40 sec; 55°C, 40 sec; 72°C, 40 sec] 33x; 72°C, 7 min. PCR products were run on agarose gel (1.5%), PCR products were purified with Agencourt ® kit Ampure ® XP (Beckman Coulter) according to the producer's instructions. The sequencing reaction took place under the following conditions: 96°C - 20 s, 50°C - 20 s and 60°C 4 min. Free nucleotides purification was performed with Agencourt ® CleanSEQ ® kit (Beckman Coulter) according to the manufacturer instructions. The ITS region sequencing was carried out on the CEQ TM Genetic Analyses System (Beckman Coulter, Inc. Fullerton, California) under standard conditions.

Dendrogram construction

Dendrogram was constructed by hierarchical clustering of selected ITS sequences of *Smallanthus*, spp. and newly sequences one. For the dendrogram construction, Neighbor Joining method was used and the construction was performed fully withing a NCBI software.

Results and Discussion

Currently ITS regions and their sequences have broad application in molecular systematic (molecular phylogenetics) of fungi, to determine relationships among species or within a species, as well as in plants. For ITS region amplification of our DNAs were chosen primer pairs considered as reference and very effective for molecular analysis in previous studies (Alvarez and Wendel, 2003; Feng et al., 2007; Smolik et al., 2011b). After optimization of PCR reaction conditions it was obtained specific monomorphic fragment with an approximate size of 650 bp. The size of the amplified product corresponds to the size of the ITS yacon region previously registered in the NCBI database (AF465902). Amplicons were run on 1.5% agarose gel (Figure 1) then eluted and sequenced as described above.

In total, 3 sequences for each of the accession were obtained in 3 repeated reactions. Any of the sequences didn't show nucleotide difference among them. On the other hand their comparison with *Smallanthus sonchifolius* ITS region previously deposited in the NCBI database (AF465902) showed differences in two nucleotides. More in detail a specific variability in nucleotide 163-164 and 235-236 (Figure 2) was observed. In the data source AF465902 sequence, it is not mentioned the country of origin of sequenced yacon variety but it showed to be different from our germplasm.

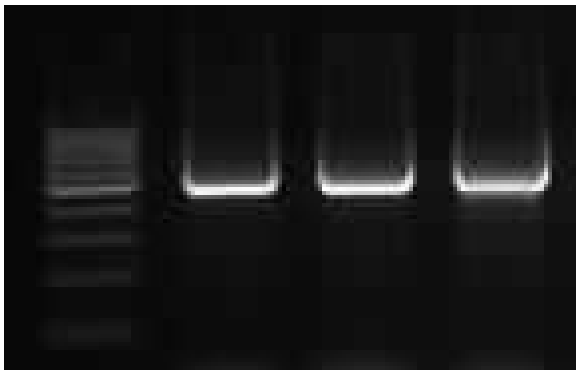

Figure 1. PCR amplification products of ITS regions.

Broadening the effectiveness of ITS regions by PCR is based on multicopy structure. Of equal importance, the ITS regions (ITS1 +5.8 S + ITS2) is the product of an approximate length of 700 bp, which is optimal for specific determination of the chemicals and thermal parameters for PCR reaction.

According to many authors ITS regions, thanks to their variability, gave sufficient molecular information, which can be directly used as identifiers or molecular markers in phylogenetic analyses (Alvarez and Wendel, 2003; Nalini et al., 2007; Smolik, 2011a). In our case the ITS products were not sufficient for differentiation among analyzed genotypes but they resulted as a useful tool for differentiation between bolivian yacon genotypes and the one present in NCBI database. Sequences obtained in our study were also compared with other sequences deposited in NCBI database and obtained from other plant species belonging to the genus *Smallanthus* (Figure 3). For comparison purposes registered NCBI database sequences were used and their sequences were compared with the BLAST algorithm. The dendrogram obtained from BLAST analysis depicting ITS region sequence comparison within the same genus is shown in Figure 3.

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Query  1    TCGAATCCTGCACAGCAGAGCCACTCGTGAACTAGTACTACAAACAGGGCTTAGCGGGGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654  TCGAATCCTGCACAGCAGAGCCACTCGTGAACTAGTACTACAAACAGGGCTTAGCGGGGA  595

Query  61   TCAAGGCTTCTGTTTTGATCCTTGTGAAGCCTCGCTGGCATGCGTTCATGGGCCTCTTTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  TCAAGGCTTCTGTTTTGATCCTTGTGAAGCCTCGCTGGCATGCGTTCATGGGCCTCTTTG  535

Query  121  GGGCGTCATGGACGTAAGTTAGCACAACAACAACCCCCGGCAC-GCATGTGCCAAGGAAA  179
            |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||
Sbjct  534  GGGCGTCATGGACGTAAGTTAGCACAACAACAACCCCCGGCACGGCATGTGCCAAGGAAA  475

Query  180  GCTAAACTTCAAGACCGCCCGTGCCATGTTGCCCCGTTTTTGGTGTGCACATTGTCGGTG  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  474  GCTAAACTTCAAGACCGCCCGTGCCATGTTGCCCCGTTTTTGGTGTGCACATTGTGCGTG  415

Query  240  GCTTCTTTGTAATCTAAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  GCTTCTTTGTAATCTAAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAA  355

Query  300  GAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAATCATCGAGTTTT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAATCATCGAGTTTT  295

Query  360  TGAACGCAAGTTGCGCCCGAAGCCATCCGGTTAAGGGCACGTCTGCCTGGGCGTCACGCA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  TGAACGCAAGTTGCGCCCGAAGCCATCCGGTTAAGGGCACGTCTGCCTGGGCGTCACGCA  235

Query  420  TCACGTCGCCCCCATAAAGTATCTCTTCAAGGGACGCGTTGGGCGGGGCGGAGATTGGTC  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234  TCACGTCGCCCCCATAAAGTATCTCTTCAAGGGACGCGTTGGGCGGGGCGGAGATTGGTC  175

Query  480  TCCCATGCATGTTGCGTGGTTGGCCTAAATAGGAGTCTCCTCAAGAGGGACGTACGACTA  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  TCCCATGCATGTTGCGTGGTTGGCCTAAATAGGAGTCTCCTCAAGAGGGACGTACGACTA  115

Query  540  GTGGTGGTCGAAGTTACTGTCGTCTCGTGTCGTGCGTTTTGATCCTTGAGGAAGAAACTC  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114  GTGGTGGTCGAAGTTACTGTCGTCTCGTGTCGTGCGTTTTGATCCTTGAGGAAGAAACTC  55

Query  600  TTAAGATACCCTGTCGTGTCATCTTCTGATGATGCTTCGATCGC  643
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  54   TTAAGATACCCTGTCGTGTCATCTTCTGATGATGCTTCGATCGC  11
```

Figure 2. ITS sequence variation between ITS sequences of genotypes analyses and NCBI sequence AF465902.

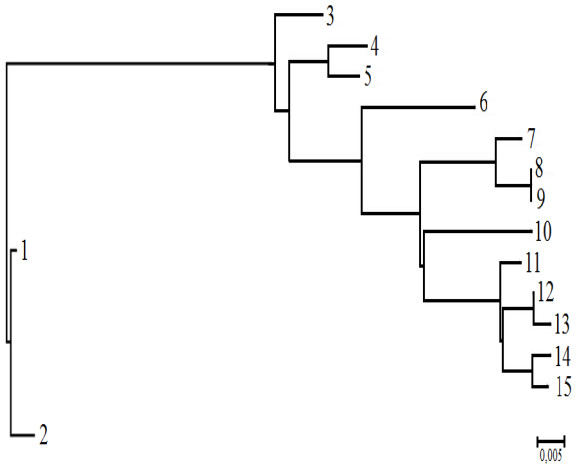

Figure 3. Dendrogram displaying the similarity of ITS sequences obtained from the native cultivars BOL 20 BOL BOL 22 and 24 with gender ITS sequences *Smallanthus*.
Coding ITS region of: 1 *S. sonchifolius* – (sequenced in this study), 2 - *S. sonchifolius* X; 3 - *S. microcephalus*; 4 - *S. jelskii*; 5 - *S. pyramidalis*; 6 - *S. oaxacanus*; 7 - *S. siegesbeckius*; 8 - *S. connatus*; 9 - *S. fruticosus*; 10 - *S. uvedalius*; 11 - *S. maculatus*; 12 - *S. riparius*, 13 - *S. meridensis*; 14 - *S. maculatus*; 15 - *S. quichensis*. Sequences from 2 till 15 were obtained from NCBI database.

The 100% similarity of our ITS sequences (yacon cultivars native from Bolivia) was compared with the existing ITS *Smallanthus sonchifolius*, the homology showed to be 99%. On the other hand the homology with other ITS sequences from 13 species of the genus *Smallanthus* showed to be lowerer, from 92 to 97%. Baldwin et al. (1995) indicate as base substitution ITS regions are a frequent source of polymorphism in a sequence. Results obtained in the yacon ITS region sequencing confirms data previously reported by Baldwin et al. (1995), but for our genotype. On the other hand it is possible to underline as knowledge of nucleotide differences in ITS regions could represent a wider possibility of differentiation and identification of species with a fast and easy PCR screening.

Conclusions

Direct sequencing of PCR products currently represents a useful tool in molecular genetics that present advance in the analysis of anonymous markers. It can result in a high and accurate determination of plant genome characteristics directly at nucleotide sequence level instead their whole genome. ITS regions demonstrated to be able to detect and differentiate among species and also between our germplasm and other germplasm

previously analyzed. The variability in yacon ITS sequences compared with other ITS sequence of plants belonging to the genus *Smallanthus* opens the possibility of a more focused and accurate molecular analysis directed to intraspecific and interspecific *Smallanthus* spp. characterization.

Acknowledgments

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PLANT SCIENCE

Growth suppression of legumes in pyriproxyfen stressed soils: A comparative study

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Abstract

Insecticides are commonly used to combat economically important insect pests in agriculture. These plant-protecting agents severely decline crop productivity by affecting different plant growth parameters. The assessment of phytotoxicity of insecticides are generally, confined to one plant species and broad studies concomitantly evaluating the insecticide effect on more than one crop specifically, the legume are scarce. Hence, this study was designed to assess the effect of technical grade insecticide, pyriproxyfen simultaneously on legumes like chickpea, pea, lentil and greengram. Pyriproxyfen showed the highest toxicity to root and shoot dry biomass, leghaemoglobin, chlorophyll content and seed protein in chickpea, nodule numbers in pea, shoot nitrogen and root phosphorus in greengram, and nodule biomass, root nitrogen, root phosphorus, shoot phosphorus and seed yield in lentil. For instance, pyriproxyfen decreased the number of nodules (percent decline over controls) in each legume in an order: pea (44) > greengram (14) > chickpea (5) = lentil (5). Similarly, pyriproxyfen mediated percent decline in leghaemoglobin occurred in the order like: chickpea (69) > lentil (25) > pea (18) > greengram (12). Generally, pyriproxyfen affected most adversely the growth of both chickpea and lentil. It is concluded that the extent of phyto-toxicity of insecticide and the type of plant organs affected might differ among plant species.

Key words: Insecticide, Pyriproxyfen, Toxicity, Legume, Soil

Introduction

In agricultural fields and farms, considerable amount of pesticides are being used to increase the agricultural production, by controlling insect pests, diseases and weeds as these chemicals act on pests that are detrimental to agricultural output (Ahemad and Khan, 2011a). Pesticides including insecticides accumulated in soils disturb the natural ecological balance by producing toxic effects in recipient environments (Skevas et al., 2012; Pal et al., 2006). The behavior of pesticides in the environment depends on its stability, physico-chemical properties, the nature of the medium into which it is applied, the organisms present, and the prevailing climatic conditions (Ahemad et al., 2009; Ismail et al., 2009; Abou Ayana et al., 2011). Some of the negative effects of pesticides include low crop yield (Fox et al., 2007; Ahemad and Khan, 2012a), destruction of soil micro-fauna and flora (Chowdhury et al., 2008) and their beneficial physiological activities (Madhaiyan et al., 2006; Ahemad and Khan, 2010), undesirable residue accumulation in food crops (Mattina et al., 2000) and decreased soil fertility (Abdalla et al., 2009). Further, they affect the soil microbial communities by adversely decreasing protein synthesis and inhibiting various metabolic enzymes (Boldt and Jacobsen, 1998; Ahemad and Khan, 2012b; Ahemad and Khan, 2012c). Moreover, they also damage the structural proteins by geno-toxicity and by altering the membrane composition (Pham et al., 2004; Kumar et al., 2010).

Among the pesticides used in modern agronomical practices, pyriproxyfen [4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy)propyl ether] (CAS No. 95737-68-1), a broad spectrum insecticide belonging to juvenile hormone mimics is being used in crop production to control many insect pests (e.g., locusts, ticks, whiteflies, houseflies and mosquitoes) both at larval and adult

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stages (Ahemad and Khan, 2011b). Previous studies of assessment of phyto-toxicity of insecticides are generally, restricted to any single crop and comprehensive data evaluating the impact of any specific insecticide on more than one legume in parallel is rare. Experimentation with large number of legume crops with respect to specific insecticide would remove the ambiguity in the reported results consequently would be more informative. Hence, this study aims to evaluate the effect of pyriproxyfen on four commonly grown legumes like, chickpea (*Cicer arietinum* L.), pea (*Pisum sativum*), lentil (*Lens esculentus*) and greengram (*Vigna radiata* L. Wiclzek) simultaneously, to characterize the effects of pyriproxyfen on legumes.

Materials and Methods

Insecticide

The technical grade (a.i. 98%) pyriproxyfen was obtained from Parijat Agrochemicals (New Delhi, India). To prevent the degradation, the stock solution was prepared just prior to each experiment by dissolving insecticide in solvent [dimethyl sulfoxide (DMSO)]. The recommended field dose (1300 µg/ kg soil) of pyriproxyfen was used for the experiments.

Plant growth evaluation

Seeds of the commonly grown legumes like, chickpea var. C235, pea var. arakle, lentil var. K75 and greengram var. K851 were obtained from Indian Agricultural Research Institute (IARI), Pusa, New Delhi, India and surface sterilized with 70% ethanol, 3 min.; 3% sodium hypochlorite, 3 min.; rinsed six times with sterile water and dried. The soil used in pot experiments had the following properties: sandy clay loam, 0.4% organic carbon (C), 0.75 g kg^{-1} Kjeldahl nitrogen (N), 16 mg kg^{-1} Olsen phosphorus (P), 0.44 ml g^{-1} water holding capacity (WHC), 11.7 cmol kg^{-1} cation exchange capacity, 5.1 cmol kg^{-1} anion exchange capacity and pH 7.2. A total of ten seeds of each legume were sown in clay pots (25 cm high, 22 cm internal diameter) using three kg unsterilized soils with a control (without pyriproxyfen) and a treatment with the recommended field rate of pyriproxyfen (in three replicates for each legume). Seeds of chickpea, lentil, greengram and pea were sown in October (2007), November (2007), March (2008) and November (2007), respectively. Plants in each pot were thinned to three plants 10, 10, 7 and 7 days after sowing (DAS) of chickpea, lentil, greengram and pea, respectively. The pots were watered with tap water and were maintained in an open field.

All plants for each treatment were removed at 135 DAS (at harvest stage) of chickpea, 120 DAS (at harvest stage) for both pea and lentil and 80 DAS (at harvest stage) for greengram. The root and shoot of each legume were carefully washed and oven dried at 80°C and weighed. The nodulation in chickpea, pea and lentil was recorded at 90 DAS (pod fill stage) and that of greengram at 50 DAS (pod fill stage). Nodules from the root systems of each legume were separated, counted, oven dried at 80 °C and weighed. The leghaemoglobin (Lb) content in fresh nodules recovered from the root system of each legume crop was quantified at 90 DAS each for chickpea and pea and lentil and 50 DAS for greengram, respectively, by the method of Sadasivam and Manickam (1992).

The total chlorophyll content in fresh foliage of each experimental legume crop was quantified at 90 DAS each for chickpea, pea and lentil and 50 DAS for greengram by the method of Arnon, (1949). The total N and P content in roots and shoots of chickpea (135 DAS), lentil (120 DAS), pea (120 DAS) and greengram (80 DAS) were measured by the micro-Kjeldahl method of Iswaran and Marwah (1980) and the method of Jackson (1967), respectively. Chickpea, pea, lentil and greengram were finally harvested at 135, 120, 120 and 80 DAS, respectively, and seed yield was measured. The protein content in grains of each legume was estimated by the method of Lowrey (1951).

Statistical analysis

The experiments were repeated the next year (2008-2009) with the similar environmental conditions and with the same insecticide treatments to ensure the reproducibility of the results. Since the data of the measured parameters obtained were homogenous, they were pooled together and subjected to analysis of variance (ANOVA). The difference among treatment means was compared by Tukey test (one-way ANOVA) at 5% probability level by statistical software, SPSS 10.

Results and Discussion

The growth parameters of chickpea, pea, greengram and lentil assessed concurrently in the presence of the recommended field rate of pyriproxyfen showed that the root and shoot biomass and the symbiotic attributes (numbers, dry weight and leghaemoglobin content in nodules) varied significantly in pyriproxyfen-amended soils.

In general, pyriproxyfen had a detrimental effect on root and shoot growth. Pyriproxyfen decreased the root biomass of chickpea by highest degree over control. In contrast, the pea plants suffered the least reduction in root biomass

compared to control. Pyriproxyfen declined (percent decline above control) the root biomass of the legumes in the following order: chickpea (46) > greengram (45) > lentil (27) > pea (13). Similarly, the percent reduction in shoot biomass in the presence of pyriproxyfen was observed as: chickpea (46) > greengram (45) > lentil (27) > pea (13).

Nodulation in legumes is an important growth parameter. Therefore, the symbiotic attributes of the tested legumes were also assessed under insecticide-stress. The recommended dose of pyriproxyfen negatively affected on the nodule development for each legume. Pyriproxyfen decreased the number of nodules (percent decline over controls) in each legume in an order: pea (44) > greengram (14) > chickpea (5) = lentil (5). Comparative evaluation of nodule numbers of legume species does not provide an accurate assessment because size of nodules varies from one legume species to another. Both nodule dry biomass and the most importantly their Lb content are the precise parameters to assess the actual impact of any stress factor on nodulation. Therefore, these two symbiotic characteristics for each legume were also determined. The toxic effect of pyriproxyfen on dry biomass (percent decline over controls) was observed in an array as: lentil (33) > chickpea (24) > greengram (19) > pea (17). On the contrary, pyriproxyfen decreased Lb content in nodules of the tested legumes in the following order (percent decline over controls): chickpea (69) > lentil (25) > pea (18) > greengram (12) (Table 1).

The decline in growth of legumes following pyriproxyfen application in our study could be due to the toxic effects of this insecticide on plant organs, especially the function of nodules which consequently disrupts the legume-*Rhizobium* symbiosis and hence, the N_2 fixation and in turn the overall plant growth (Evans et al., 1991). In addition, the inhibitory effect of the insecticide application may possibly be due to (i) the inhibition of enzymes involved in growth metabolisms (Zablotowicz and Reddy, 2004; Ahmad et al., 2003) (ii) disruption of signaling between (legume derived) phytochemicals (luteolin, apigenin) and *Rhizobium* Nod D receptors (Fox et al., 2007). Moreover, plants produced phytoestrogens discourage herbivores and attract insects and also act as recruitment signals for rhizobia. Pesticides have been reported to obstruct the phytoestrogen signaling system that regulates symbiosis between legume plants and rhizobia (Fox et al., 2004). Additionally, pesticides not only inhibit the biochemical signaling between the hosts and the cognate rhizobia but also block the initial attachment of complementary rhizobia to lectins present on root hairs as the recognition sites by protecting them (Musarrat and Haseeb, 2000). As a result, pesticides also adversely affect the legume-*Rhizobium* symbiosis due to competition of pesticides for the rhizobial-binding sites (lectins) on the surface of the legume root hairs.

Table 1. Effect of pyriproxyfen on dry biomass and symbiotic properties of legume crops.

Legumes	Dose rate (µg/ kg soil)	Dry biomass (g/ plant)		Nodulation		
		Root	Shoot	No./ plant	Nodule biomass (mg/ plant)	Leghaemoglobin content [mM (g.f.m.)$^{-1}$]
Chickpea	0 (control)	0.91b	3.80a	21b	180c	0.13c
	1300	0.49e	1.93c	20c	136d	0.04h
Pea	0 (control)	0.92a	2.07b	27a	283a	0.17a
	1300	0.80c	1.84d	15f	236b	0.14b
Greengram	0 (control)	0.47f	2.08b	21b	66e	0.08f
	1300	0.26h	1.33f	18e	54f	0.07g
Lentil	0 (control)	0.55d	1.97c	19d	30g	0.12d
	1300	0.40g	1.66e	18e	20h	0.09e
LSD ($p \leq 0.05$)		0.008	0.05	0.67	5.31	0.003
F value		12.6	106	263.3	489.5	27.9

Values are mean of three replicates where each replicate constituted three plants/ pot. Mean values followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to Tukey test; (g.f.m.)$^{-1}$ = (gram fresh biomass)$^{-1}$

Reports on the effect of insecticides on symbiotic attributes of legumes are, however, contradictory. For instance, Fox et al. (2007) reported a considerable decrease in nodulation, total plant biomass and nitrogenase activity of alfalfa (*Medicago sativa* L.), when grown in soil treated separately with methyl parathion, DDT, bisphenol A and pentachlorophenol. In contrast, monocrotophos, quinolphos and cypermethrin at lower concentration were stimulatory to ammonification process in agricultural soils while toxic at higher concentration (Rangaswamy and Venkateswarlu, 1993). A comparable observation on the effect of insecticides on legumes has been reported. For example, effects of afugan, brominal, gramoxone, selecron and sumi oil on growth and nodulation of soybean (*Glycine max*) were determined by Abd-Alla et al. (2000). Growth of cowpea (*Vigna sinensis* L.), common bean (*Phaseolus vulgaris* L.) and lupin (*Lupinus albus* L.) was inhibited by afugan, brominal, gramoxone and selecron application. However, the effect of insecticides varied with the pesticide applied and plant species. In addition, Aggarwal et al. (1986) evaluated the effect of carbamate on nodulation in *Pisum sativum* and *Vigna sinensis*. They reported that the low concentrations of the insecticides had little effect on nodulation whereas higher concentrations adversely affected it. In a similar study, Alonge (2000) evaluated the phytotoxicity of imazaquin on the growth of soybean plants and found that imazaquin reduced chlorophyll content in leaves, root nodules, shoot growth, whole plant dry weight and grain yield.

In our study, the degree of toxicity of pyriproxyfen to the parameters to each legume however, varied considerably. The variable response of the tested legumes to pyriproxyfen is because the extent of toxicity of any specific insecticide to the plants depends upon the both genetics and physiology of plants that varies from one plant species to another (Ahemad and Khan, 2011c). According to Anderson et al. (2004), insecticides negatively affect the nodulation in legumes by limiting the number of available sites on host plants for the cognate rhizobia by decreasing the carbohydrate supply to existing nodules. Thus, insecticides decrease the rhizobial survival and growth, inactivate the biochemical signaling required to initiate nodule development in plants and inhibit the nodule development by reducing cell division.

Generally, the chlorophyll content in leaves of each legume significantly decreased when grown in pyriproxyfen-amended soils. The most toxic effect of the insecticide was observed on the total chlorophyll of chickpea plants wherein the chlorophyll content decreased by 14% above control. Moreover, pyriproxyfen mediated reduction in the chlorophyll content of lentil was found to be 9% compared to control. Furthermore, marginal decline in the total chlorophyll of pea and greengram was observed (4 and 7% respectively, compared to control) (Table 2).

Table 2. Effect of pyriproxyfen on biological and chemical properties of legume crops.

Legumes	Dose rate (µg/ kg soil)	Chlorophyll content (mg/g)	N content (mg/g)		P content (mg/g)		Seed protein (mg/g)	Seed yield (g/plant)
			Root	Shoot	Root	Shoot		
Chickpea	0 (control)	1.96a	18e	27e	0.17d	0.21f	241c	2.7d
	1300	1.68b	15g	24f	0.17d	0.20g	228e	1.8e
Pea	0 (control)	0.75d	34b	45b	0.21b	0.28c	224f	7.4a
	1300	0.72e	29d	42cd	0.19c	0.25d	223f	7.2a
Greengram	0 (control)	0.82c	36a	50a	0.27a	0.36a	261a	7.4a
	1300	0.76d	31c	43c	0.22b	0.31b	251b	5.5b
Lentil	0 (control)	0.32f	17f	45b	0.21b	0.28c	232d	3.0c
	1300	0.29g	14h	41d	0.17d	0.22e	225f	1. 8e
LSD ($p \leq 0.05$)		0.018	0.72	1.53	0.01	0.005	2.7	0.35
F value		115.4	244	361.8	71.3	117.8	178.5	102.3

Values are mean of three replicates where each replicate constituted three plants/ pot. Mean values followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to Tukey test.

Pyriproxyfen decreased the root N by 17, 15, 14 and 18% in chickpea, pea, greengram and lentil respectively, compared to respective controls. Conversely, the decline in the shoot N of each legume exposed to the insecticide-stress was found to follow the trend as: greengram (14%) > chickpea (11%) > lentil (9%) > pea (7%) (Table 2). In addition, the root P of the tested legumes in response to pyriproxyfen exposure decreased in the following order: greengram (19%) = lentil (19%) > pea (19%) > chickpea (0%) while pyriproxyfen declined shoot P over controls in an array: lentil (21%) > greengram (14%) > pea (11%) > chickpea (5%) (Table 2).

Moreover, pyriproxyfen also significantly decreased the seed protein of chickpea and greengram by 5% and 4%, respectively, compared to control while seed protein in pea and lentil plants was marginally decreased (1% and 3%, respectively over controls). Generally, seed yield of each legume was also adversely affected under pyriproxyfen-stress. The lentil suffered the maximum reduction in seed yield while the least decline in seed yield was observed for pea in the presence of pyriproxyfen. The following decreasing trend (percent decline over controls) was observed: lentil (40) > chickpea (33) > greengram (26) > pea (3).

As reported by Boldt and Jacobsen (1998) that the pesticides adversely affect the metabolic enzymes, therefore, it seems probable that insecticide employed in this study might has inhibited the functioning of the enzymes of photosynthetic carbon reduction (PCR) cycle, such as Rubisco, 3-PGA kinase, NADP, NAD-Glyceraldehyde–3-P-dehydrogenase and aldolase. Nitrogen and P content of the legume plants is one of the most important aspects of legume growth. The nitrogen content in roots and shoots determined at different stages of chickpea, lentil, pea and greengram, differed among treatments. The decrease in N contents of legumes might have been due to the reduction in legume-*Rhizobium* symbiosis, as indicated by a decline in the nodulation in this study. In agreement to this finding, Fox et al. (2007) concluded that agrichemicals including insecticides induce a symbiotic phenotype that inhibits or delays recruitment of rhizobia to host plant roots, fewer root nodules produced, lowers the rate of nitrogenase activity which in turn, reduces N content and decrease the overall plant yields. However, the reduction in P content and seed attributes following insecticide application could probably, be due to inhibition of the enzymes and functional proteins of metabolic pathways involved in protein synthesis and P-uptake (Boldt and Jacobsen, 1998; Nare et al., 2010; Ahemad and Khan, 2011a).

Conclusions

Pyriproxyfen showed varying degree of toxicity to the selected legumes. The highest toxicity of pyriproxyfen was observed on root dry biomass, shoot dry biomass, leghaemoglobin chlorophyll content and seed protein in chickpea; nodule numbers in pea; shoot N and root P in greengram; and nodule biomass, root N, root P, shoot P and seed yield in lentil. In general, the most adverse impact of pyriproxyfen was observed on the growth parameters of chickpea and lentil. These findings demonstrate that an outcome of the effects of a specific insecticide on a specific crop plant species cannot be generalized. The degree of toxicity of any insecticide and the type of plant organs affected may differ from one plant species to another.

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PLANT SCIENCE

Microscopical analysis of *in vitro Mokara* Broga Giant orchid's PLBs

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Abstract

Mokara orchids comes with variety of hybrids that have unique and highly variable types of characteristics such as petal colour, shape, size and other floral characteristics that make it very important economically and aesthetically. This study was conducted to evaluate on its histological and scanning electron microscopy analyses that make its PLBs to be a desirable biotechnological explant in propagation of this orchid as well as its importance in many biotechnological researches. Histological observation in Mokara Broga giant indicated the presence of meristematic tissues that are vital for biotechnological research such as genetic transformation, cryopreservation, micropropagation and others. It also indicated the presence of shoot primordia. Scanning electron microscopy analysis also indicated the developmental characteristics of PLBs in detail. Both analyses showed that the maturation of PLBs and gradual formation of shoot leaf primordial occurs through somatic embryogenesis and PLBs are vital explant in many plant tissue culture researches such as genetic transformation, cryopreservation, micropropagation and others.

Key words: Orchids, PLBs, Histology, Scanning Electron Microscope

Introduction

Orchidaceae, a family with 20,000 to 30,000 species and is the biggest family in the plant kingdom (Godo et al., 2010). Orchids are one of the most attractive clusters of ornamental plants and numerous novel cultivars have been formed by interspecific and intergeneric hybridization in order to produce plants with exotic and elegant flowers. Due environmental disruption, succession of natural habitats and overexploitation of horticultural, many orchid species are threatened today (Godo et al., 2010).

The most widespread use of orchids is as ornamental cut flowers. However, only few orchid plants are attractive enough for direct use as ornamentals. Orchids hybrids are used as ornamental due to they have beautiful foliage, shiny, multicoloured and decorated with prominent veins in elaborate patters. Orchids from several genera such as *Mokara*, *Dendrobium, Anoectochilus*, *Goodyera*, *Ludisia* (Haemaria) and *Macodes* are used widely. Orchids used indoor and outdoor landscapes are often employed to generate a special effect such as exotic surroundings, feeling of luxury and unique environment (Arditti, 1992; Dalayap et al., 2011). Generally, the flowers were made into corsages. In the orchid industry, trade in plants has always been and remains a significant aspect. However, in the past, this trade was limited to species, seedlings and matured plants. The establishment of clonal propagation procedures and hybridization of orchid such as *Mokara* hybrid has improved this market (Arditti, 1992; Dalayap et al., 2011). In return, it had brought a large numbers of outstanding cultivars which are mass-propagated by growers. Orchids are also used as a form of national flower such as *Peristeriaelata* in Panama (Arditti, 1992).

Mokara (*Mokara spp.*), commonly identified as "Smile Orchid" and it is native to Asia, where it has been first discovered and cultivated. It is a *Vandaceous* orchid resulting from a trigeneric hybridization between the *Ascocentrum*, *Vanda* and *Arachnis* orchids. The first *Mokara* hybrid was produced in Singapore in 1969 and was called *Mokara* Wai Liang, named after C.Y. Mok. Since then, several varieties were created where many of the hybrids have exclusive and highly variable star-shaped flowers (Dalayap et al., 2011). This orchid

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has flowers with the largest number of colors compared to other orchids like purple, pink, blue, red, orange, yellow and coral with each and every colour has its own variety. The flower comprises of three sepals, which are usually diverse from the petals in shape but not in colour (Dalayap et al., 2011). There are also three petals but one has been significantly altered into a lip or labellum, which is a very complex structure and quite different from the other two sepals (Soon, 1989; Dalayap et al., 2011). This orchid group is therefore vital economically due to these unique floral characteristic.

In vitro propagation is the utmost popular method used for the multiplication of orchids as this method is found to be more effective than sexual propagation and other vegetative procedures (Talukder et al., 2003). However, tissue culture in orchids via indirect method which produce callus is not favoured due to lower growth rate and regular occurrence of necrosis in culture (Zhao et al., 2008). According to Chugh et al. (2009), the future of orchid propagation is most expected through multiplication of true-to- type plants through *in vitro* techniques. Several methods of propagation have been developed for orchids through *in vitro* culture of various parts including shoot tips, flower stalk nodes, buds, root tips and rhizome segments (Chen et al., 2000; Park et al., 2002b) with varying responses.

The most commonly used micropropagation techniques include direct and indirect shoot organogenesis and somatic embryogenesis. Somatic embryogenesis is more necessary biotechnological technique for large-scale clonal propagation of elite genotypes, development of synthetic seed technology, plant transformation (Bomal and Tremblay, 2000; Bandyopadhyay and Hamill, 2000) and conservation of threatened plant species. The most ideal regeneration systems for genetic transformation are direct and repetitive production of somatic embryos or de novo shoot organogenesis originating from single cells in the epidermis (Rugkhla and Jones, 1998). Whereas, genetic clones created through shoot organogenesis require further manipulation for root initiation, in somatic embryogenesis complete plants with a bipolar axis, vascular system and functional meristems are created in a single step (Bassuner et al., 2007). In somatic embryogenesis, somatic cells develop into plants in similar developmental stages to zygotic embryogenesis (Gomes et al., 2006).

Recently, numerous reports have described shoot organogenesis through somatic embryo-like structures from different explants, mainly leaves and hypocotyls. Histological observations of the embryo-like structures have established the initial globular-shaped morphological features (Haensch, 2004; Tian et al., 2008) during the early developmental stages of their growth, in synchrony with conventional somatic embryogenesis. These embryo-like structures have been mistaken for true somatic embryos (Salaj et al., 2005). Our present study describes the histological approach to determine the anatomical characteristics of somatic cells within the PLBs. To the best of our knowledge this is the first report on the histological analysis in *Mokara* Broga Giant.

Therefore, this study aims to evaluate the importance of the structural conditions by histological analysis and scanning electron microscope analysis of this orchid PLBs that confirms its structure as somatic embryos that makes it vital in the orchid propagation industry.

Materials and Methods

Establishment of *in vitro* culture of PLBs

In vitro culture of protocorm-like bodies (PLBs) of *Mokara* Broga Giant were initiated by aseptically culturing shoot tips of the plant in half strength semi-solid Murashige and Skoog (1962) media supplemented with 1mg/L BAP (BAP; DUCHEFA, Netherlands), 2% (w/v) sucrose and 2.75g/L GelriteTM (DUCHEFA, Netherlands). The pH (CyberScan PC 510 pH/mV/Conductivity/ TDS/°C/°F Bench Meter, Eutech 73 Instruments, Singapore) of all the media in this study was adjusted to 5.8 prior to autoclaving (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan). The resulting PLBs were grown at 25°C under 16 hours photoperiod (Philips TLD, 36 W, 150µmol.m^{-2}.s^{-1}). The PLBs were subsequently subcultured for every 4 weeks in half strength liquid Murashige and Skoog (1962) media supplemented with 1mg/L BAP and 2% (w/v) sucrose. In histological and scanning electron microscope analysis, 4 weeks old *Mokara* Broga Giant PLBs cultures were utilised.

Histological Analysis

Fixation

Mokara Broga Giant PLBs were fixed in FAA (95% ethyl alcohol: glacial acetic acid: formaldehyde: water, 10:1:2:7) for 1 week. Fixation is a process whereby the biological tissues are preserved from decay, stops the biochemical reactions and also increase the mechanical strength or stability of the treated tissues.

Dehydration

The PLBs were rinsed with distilled water a period of 1 hour with every 15 minutes of rinsing. PLBs were then transferred into alcohol tertiary-

butyl alcohol, TBA which was done with varying concentration of (TBA) (50-100%); 50% TBA for 2 hours, 70% TBA for 2 hours, 85% TBA for 2 hours, 98% TBA for 2 hours and 100% TBA for 3 hours. Following that, the PLBs were treated with TBA I and TBA II for 3 hours respectively, and then treated PLBs were left overnight.

Clearing

PLBs were subsequently exposed to xylene for a period of 10 minutes followed by treatment xylene and wax for 30 minutes and subsequently samples were treated with wax I, II, and III (xylene and Shandon Histoplast Pelletised Paraffin Wax, Thermo Scientific) for 1 hour respectively at 60°C in an oven (Memmert, Germany).

Blocking, slicing and staining of samples

PLBs samples were then blocked and sliced using 6 Micron Microtome (Leica RM 2135). Then, the samples were then stained with safranin and fast green. Safranin, a basic dye which stains acidic features of tissue such as nuclei and lignified secondary cell walls that surround mature xylem cells in vascular bundles. Fast Green, an acidic dye that stains the basic components of tissue such as the cytoplasm and cellulosic primary cell walls. Slides were observed by light microscope (Olympus BX41).

Scanning Electron Microscope Analysis

PLBs samples were processed by freeze drying technique and the specimens were observed using Scanning Electron Microscope (Leo Supra 55VP-Ultra High Resolution analytical FESEM).

Results and Discussion

Propagation of orchids by the development of secondary PLBs from protocorms or PLBs has been defined for several orchids including *Phalaenopsis* (Chen and Chang, 2004; Murdad et al., 2006), *Dendrobium* (Saiprasad et al., 2004), *Aerides* (Sheelavanthmath et al., 2005) and *Cymbidium* (Teixeira da Silva and Tanaka, 2006). Numerous comparative analyses of plant responses to various types of plant growth regulators and the addition of organic additives to the culture medium to promote *in vitro* growth and proliferation of orchid is a common practice (Ichihashi and Islam, 1999; Chai et al., 2002; Islam et al., 2003; Rahman et al., 2004; Arditti, 2008, George et al., 2008).

The use of organic additives and PGRs may add toward the development of a simple and economical plant culture (Islam et al., 2003). Numerous comparative analyses of plant responses to diverse types of cytokinin (BA and kinetin) have revealed that the presence of BA in the culture medium produces higher frequencies of induction and proliferation of PLB in some orchids, such as *Dendrobium nobile*, *Dendrobium Densiflorum*, and *Cymbidium aloifolium* (Nayak et al., 2002; Sheelavanthmath et al., 2005; Luo et al., 2008). However, Teixeira da Silva and Tanaka (2006) specified that kinetin was more effective in promoting the formation of PLB in *Cymbidium* hybrids when supplemented together with NAA to the culture medium. In contrast, Chen et al. (2002) described that both BA and kinetin were equally effective for PLB proliferation in *Epidendrumradicans*.

Instead, Chai et al. (2002) described that the addition of a suitable amount of organic additives to the culture media significantly promoted the growth of PLBs in *Phalaenopsis*. This review displayed that varying conditions are desirable for diverse types of orchids to form their optimum PLBs growth. The presence of meristematic cells in PLBs can develop from PLBs wound surface, maturing into shoot primordial and can be converted into plantlets through somatic embryogenesis (Figure 1).

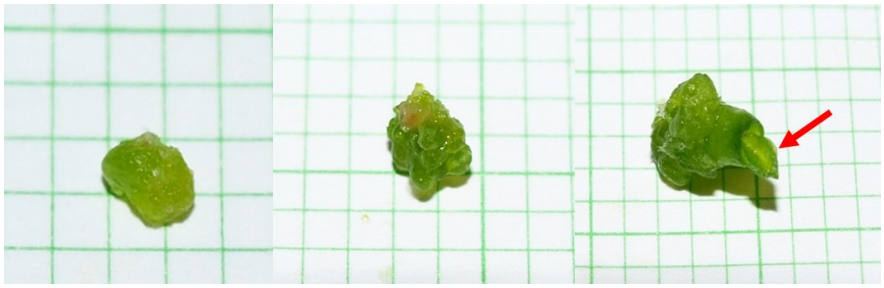

Figure 1. Direct induction and proliferation of protocorm-like bodies (PLBs) of *Mokara* Broga Giant in half strength MS medium (A), Multiplication of PLBs with secondary PLB formation (sPLB) forming cluster of PLBs (B), Proliferating PLBs forming shoots primordial (arrows) from the PLB base (C).

The histological observation showed that the protocorm-like body of *Mokara* Broga Giant orchid comprises of the shoot apical meristem (SAM) and leaf primodia of sheath leaves at the interior region and larger cells act as the storage area at the posterior region (Figure 2). Histological observation presented the presence of an area having dense cytoplasm located on the anterior side of the PLB, showing meristematic cells, embedded below it with parenchymatous tissue with thin-walled cells. This parenchymatic mass of cells protruded from the surface on the upper part of the protocorm and associated with vascular tissue (Figure 3).

Furthermore, dividing cells of the SAM were smaller in size and were more densely organized (Figures 2, 3). Further development of these cells gave SAM a shape of a dome. The shoot primordial (SP) was also noted and is different. They comprised of small cells, undergoing anticlinal and periclinal divisions, still surrounded by large, apparently isodiametric parenchyma cells (Figures 2, 3). SEM study observation using freeze drying method displayed the presence of enlarged globular PLBs shape which consists of epidermal cells with rough surface texture, which gradually tend to become rougher and wrinkled at the further developmental stages (4 weeks old) (Figure 3). The embryo axis also displayed the development of LP after formation of a constriction (C) at the top of the globular head (Figure 4). There are more than one constriction (C) can be detected on a single PLB head (Figure 4). The leaf primordial (LP) was also noted (Figures 4, 5). Even though wide research has been carried out on *in vitro* PLB in cultivated orchids, there are limited complete structural details known on the histological observation of PLBs (Park et al., 2002a).

In the histological analysis, we presented that the presence of parenchyma cells next to the meristematic cells. The parenchyma tissue close to the core played an evident storage function (Sgarbi et al., 2009). The histological and SEM studies carried out in the present work displays that the meristematic core was enveloped by a mass of parenchymatic cells. The existence of meristematic tissue at the leaf apex discloses a characteristic that is inherent to the Orchidaceae family (Churchill et al., 1973). This study offered the morphological development observed with naked eye is substantiated with the details based on histological as well as SEM observations.

Haensch (2004) described that *Pelargonium hortorum* observed that although globular and heart-shaped embryo-like regenerants were formed, histological analysis revealed that they lacked a defined root pole. In contrast to somatic embryogenesis where the globular-stage usually comprises of small cells with dense cytoplasm and large nuclei (Haensch, 2004), globular embryo-like structures had vacuolized parenchymatous cells (Salaj et al., 2005). Similar structures observed in other plant species have also been stated to as meristemoids, promeristems, meristemoid-like precursors (Hicks, 1994), protocorm-like bodies (Young et al., 2000; Tian et al., 2008), nodules (Batista et al., 2000; Xie and Hong, 2001; Ferreira et al., 2009) and nodular meristemoids (McCown et al., 1988). McCown et al. (1988) describe nodules as independent, spherical, dense cell clusters which are cohesively bound together and display consistent internal cell or tissue differentiation and loosely resemble protocorms. In contrast, a meristemoid can be described as a cluster of cells acting together as a meristematic centre (McCown et al., 1988). Hence, a histological approach is vital in distinguishing true somatic embryos and nodular meristemoids. Many published reports on somatic embryogenesis have drawn conclusions on the basis of morphological appearance only and should be reviewed critically (Bassuner et al., 2007). Histological approaches can offer critical information to allow the application of the most suitable *in vitro* plant regeneration methods (Woo and Wetzstein, 2008).

Histological and SEM observations through the presence of meristematic cells clearly presented that PLBs can develop from explants wound surface and can be converted into plantlets through sequential organogenesis or somatic embryogenesis. Since the PLBs comprised of multiple meristematic centres, it can be differentiated into shoot, leaf and then into plantlets gradually. Tian et al. (2008) reported that differentiation of globular cells led to formation of meristematic centers, which developed into PLBs. The SEM observations also definite that PLBs clearly exhibit shoot primordial and a meristem dome. The formation of PLBs is a unique characteristic of Orchidaceae and the term specifies a structure from induction of globular swellings until SP occur, without a root apparatus (Batygina et al., 2003).

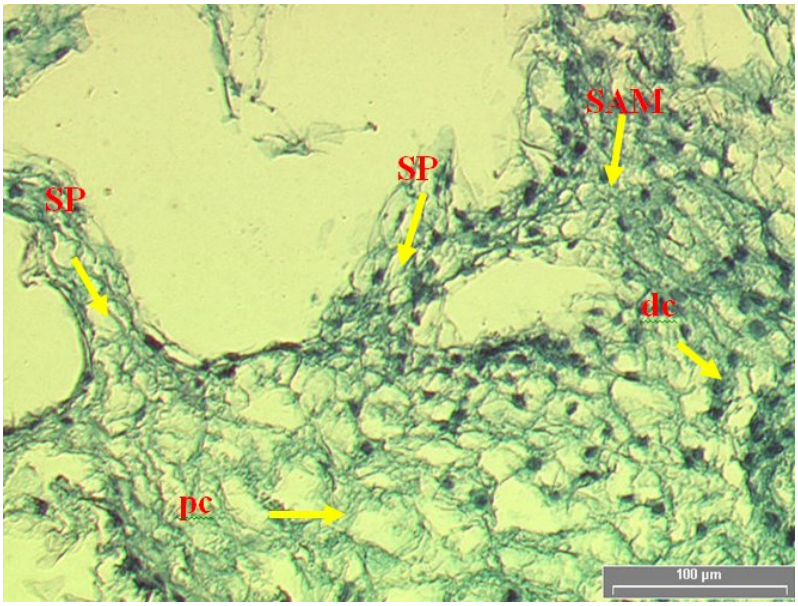

Figure 2. Histological section of *Mokara*Broga Giant's PLBs (100µm).
The cross section of PLBs, n; nucleus, dc; dense cytoplasm, SAM; shoot apical meristem, sp; shoot primordial, pc; parenchyma cell.

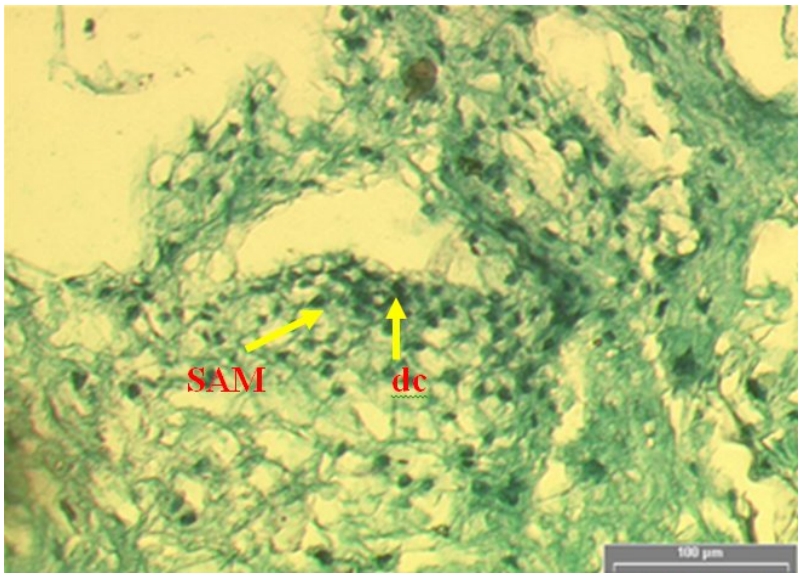

Figure 3. Histological section of *Mokara*Broga Giant's PLBs (100µm).
The cross section of PLBs showingSAM; shoot apical meristem, dc; dense cytoplasm.

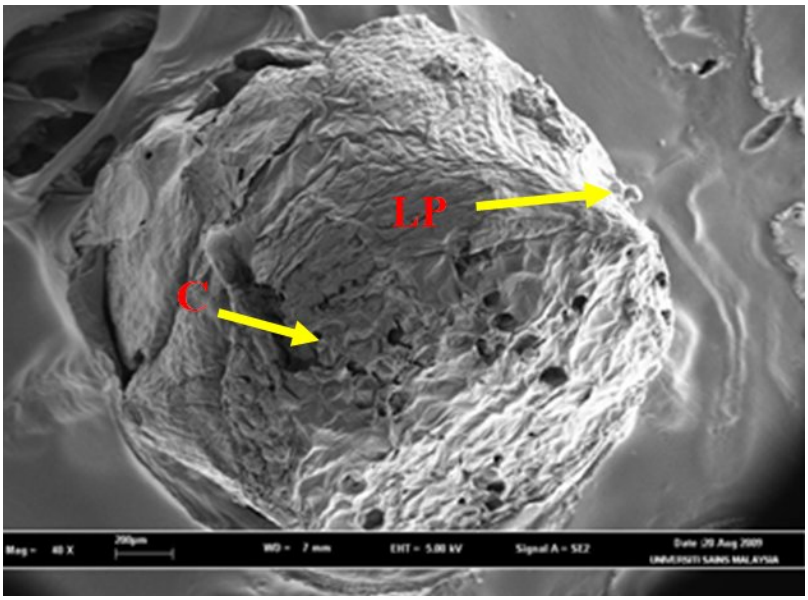

Figure 4. SEM of *Mokara*Broga Giant's PLBs (40X). The top view ofproliferating PLB at 4th week of culture showing constriction (C) and emergence of leaf primordia (LP).

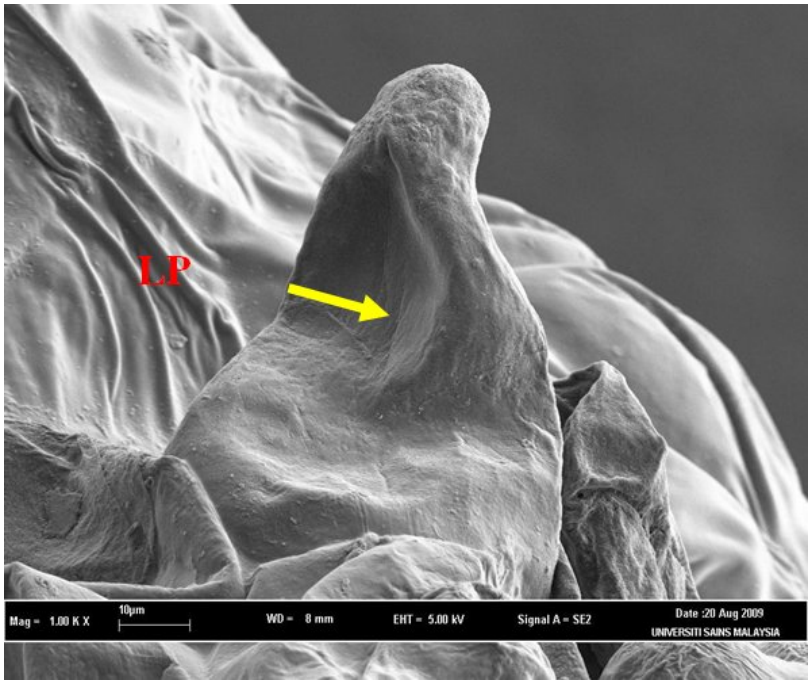

Figure 5. SEM of *Mokara*Broga Giant's PLBs (1.0 KX) showing the leaf primordial (LP).

Therefore, due to the existence of abundant meristematic tissues in PLBs, propagation of orchids using tissue culture techniques has been practiced for more than a century and has resulted in the production of uniform clones in many orchid genera. The formation of protocorms from germinated seed and the successive induction of protocorm-like bodies (PLBs) or callus from the protocorm, stem-node, shoot-tip, leaf, root-tip, or root-tuber explants has become a reliable technique for propagating orchids (Park et al., 2003; Anjum et al., 2006; Hong et al., 2008; Medina et al., 2009). Propagation through PLB formation is preferred by commercial growers of most orchid genera due to

the large number of PLBs that can be obtained within a moderately short period of time. The large-scale propagation of PLBs can also be accomplished using a bioreactor system (Park et al., 2000). PLBs are also the common target tissue for genetic transformation studies in orchids since they can proliferate rapidly and have high abilities to regenerate into complete plantlets (Liau et al., 2003; Sreeramanan et al., 2008). Furthermore, PLBs can also serve as an appropriate plant material for cryopreservation experiment (Yin and Hong, 2009). PLBs are well-differentiated tissues that are sometimes viewed as orchid embryos that develop with two discrete bipolar structures, namely, the shoot and root meristem. Consequently, these structures are capable to convert to plantlets certainly when grown on plant growth regulator (PGR)-free medium. Besides, the PLBs directly formed from meristem tissue will display an advance genetic stability than those produced by callus (Luo et al., 2008; Gantait and Uma Rani, 2012).

Conclusion

Therefore, the presence of abundant meristematic cells within the PLBs displayed importance in orchid propagation through orchid tissue culture which makes it to be a prospective explant for cryopreservation and genetic transformation studies.

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ANIMAL SCIENCE

Physiological effects of endophyte-infected perennial ryegrass straw on female camels in the Middle East

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Abstract

Recently, the United Arab Emirates has increased importation of perennial ryegrass (*Lolium perenne*) straw as part of the grass/hay ration for camels. Unfortunately, perennial ryegrass straw may be infected with the endophyte *Neotyphodium lolii* which produces lolitrem B, a toxic alkaloid responsible for the disease '*ryegrass staggers.*' A range-finding study was conducted using 24 non-pregnant female camels fed four doses (0, 1111, 1478 and 2273 ppb) of lolitrem B-containing perennial ryegrass straw over 56 days to establish a threshold of toxicity in camels so that perennial ryegrass straw can be safely fed as part of their dietary ration. Physiological parameters were evaluated. Neurological deficits were evaluated by videotape and scored according to an established scale for the neurological syndrome ryegrass staggers. Camels in the endophyte-infected groups developed varying degrees of ataxia. Brain edema, degenerative renal and hepatic lesions as well as Purkinje cell vacuolar degeneration were observed in camels receiving the highest dose. To avoid clinical disease in camels, endophyte-infected perennial ryegrass straw should be fed at 500 ppb or lower lolitrem B; straw at 1000 ppb or higher should be diluted to achieve the 500 ppb threshold of toxicity.

Key words: Camel, Lolitrem B, Perennial ryegrass, Ryegrass staggers

Introduction

Camels consume a varied diet, one of the components of which is grass/hay/straw. Recently, the United Arab Emirates has increased the importation of perennial ryegrass (*Lolium perenne*) straw from Italy, Spain, and the United States as part of the grass/hay/straw ration fed to camels in intensive farming system. Unfortunately, perennial ryegrass may be infected with the endophyte *Neotyphodium lolii* which produces lolitrem B, a toxic alkaloid responsible for eliciting the disease known as '*ryegrass staggers*' in domesticated cattle, sheep, horses, alpacas, and deer (Johnstone et al., 2012. Johnston and Mayhew, 2013; Blythe et al., 2007; Gallagher et al., 1982; Tor-Agbidye et al., 2001). 'Ryegrass staggers' is a condition in which animals consuming endophyte-infected perennial ryegrass containing lolitrem B develop stilted gait, ataxia, tremors, and hypersensitivity to external stimuli (Fisher et al., 2004; Gallagher et al., 1981). The molecular site of action of lolitrem B is on large conductance calcium-activated potassium channels (Dalziel et al., 2005), specifically, the ß-4 subunit which is responsible for modulating motor control and is associated with the ataxia observed upon ingestion of this toxin (Imlach et al., 2008). The lolitrems are unique amongst tremorgenic neurotoxins, however, in that they have a long duration of action, yet their neurotoxic effects are completely reversible in most cases (Gallagher et al., 1986; Evans and Gupta, 2012; Fisher et al., 2004; Miyazaki et al., 2007). As such, livestock affected with ryegrass staggers regain normal muscle response within 4-7 days after being removed from infected feed and appear otherwise unaffected. Clinical cases of ryegrass staggers have been observed in camelids such as alpacas; however, it is currently unknown what level of lolitrem B is needed to produce this disease in camels.

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Range-finding experiments examining the threshold of toxicity for lolitrem B in camels are needed so that perennial ryegrass grass/straw can be fed safely as part of the dietary ration to camels. Thus, our objective was to determine the patho-physiological effects of endophyte-infected perennial ryegrass straw on camels containing known amounts of lolitrem B, under the environmental conditions (i.e., high heat) of the Middle East.

Materials and Methods

Animals and diet

This study was approved by the Animal Research Ethics Committee at the United Arab Emirates University, under protocol number A4-13. Twenty-four non-pregnant female camels (*Camelus dromedaries*) of 5-7 years of age were purchased from the Al Ain Livestock Market. Animals were transported to the Faculty of Food and Agriculture Farm, United Arab Emirates University and were given standard camel feed with a salt mineral supplement for 14 days to adapt to the facilities and handling. Animals were then randomly divided into four groups (n=6/group) and fed one of the following diets containing perennial ryegrass straw over 56 days: control group A (0 ppb lolitrem B), group B with 1111 ppb, group C with 1478 ppb and group D with 2273 ppb. Lolitrem B-containing perennial ryegrass (PRG) straw was mixed with protein pelleted supplement to give minimal, balanced nutrition. Forage was provided twice daily (0800 and 1700) at 120% of the average intake for the previous 5 days, with feed refusals from the previous day weighed before feeding fresh forage. A trace mineralized salt mix was available free choice. Animals had continuous access to fresh water and forage throughout the experiment. One camel assigned to the control group A died unexpectedly during the second day of the two week conditioning period. The cause of death determined by necropsy was due to metabolic acidosis with a concurrent impacted bowl. The control group of camels then numbered five.

Nutrient composition of the feed

Dry matter, crude protein, acid detergent fiber (ADF), neutral detergent fiber (NDF) and organic matter were measured at the Animal Nutrition Laboratory of the United Arab Emirates University, according to methods certified by the National Forage Testing Association for the four PRG treatment groups (Table 1). The nutrients levels were consistent with the Arab Centre for the Studies of Arid Zones and Dry Lands requirements for camels.

Analyses of feed for lolitrem B

To establish the lolitrem B concentration for the four treatment groups in this study, plant material was ground in a Cyclotec 1093 sample mill and passed through a 0.5 mm screen (Hovermale and Craig, 2001). Three ml of a 2:1 methanol:chloroform (v/v) mixture was added to 0.2 g of sample, capped and rotated for 18-24 hours in the dark. Samples were then centrifuged at 2,000 x *g* for 10 minutes and 1.6 ml of supernatant was pulled off and dried under nitrogen at ambient temperature. One ml of dichloromethane (DCM) was added to the evaporated supernatant, capped and sonicated for 10 seconds, followed by 10 seconds of mixing; this procedure was repeated with an additional 1 ml of DCM. CUSIL 500 mg/6 ml SPE cartridges (United Chemical Technologies, Bristol PA) were loaded onto a positive pressure manifold and pre-conditioned with 2 ml DCM. The samples were loaded onto the SPE, followed by a 2 ml DCM wash. A 0.5 ml wash of elution solution (4:1 DCM: ACN (v/v)) was added to the cartridges and positive pressure applied. The sample was then eluted with 3.0 ml of elution solution and transferred to amber HPLC vials for analysis by HPLC-fluorescence. The fluorescence detector was set with an excitation wavelength of 268 nm and an emission wavelength of 440 nm. A Zorbax RX-SIL, 5 µ, 4.6 x 250 mm analytical column (Agilent Technologies, Santa Clara CA) was used in conjunction with a hand packed silica guard column, running an isocratic mobile phase (DCM:ACN:H_2O 4:1:0.02 (v/v)) at 0.5 ml/min for 15 minutes (Gallagher et al., 1981; Miyazaki et al., 2004).

Table 1. Nutrient composition of the feed.

Measurement	Diets				
	0 ppb	1111 ppb	1478 ppb	2273 ppb	Concentrate
Dry matter (%)	93.8	93.4	93.7	93.7	94.0
Crude protein (%)	5.0	6.0	6.7	6.6	12.0
ADF (%)	38.1	37.2	37.2	37.1	18.5
NDF (%)	64.2	64.7	65.2	64.3	33.0
Organic matter (%)	88.2	87.9	88.9	88.8	-

Physiological parameters

Each animal was weighed weekly using a livestock scale. Body skin and distal limb temperature were evaluated daily using an infrared thermometer. The feet were examined weekly for any possible lesions. Blood was collected weekly and processed to determine CBC, by the ADFCA Diagnostic Lab in Al Ain, using standardized procedures. The following hematologic parameters were measured: hemocrit (HCT), hemoglobin (HGB), red blood cells (RBCs), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells(WBCs), neutrophils, lympocytes, monocytes, eosinophils and basophils. The following chemistry values were measured: copper (Cu), glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, alkaline phosphatase (ALP), creatine kinase (CK), alanine aminotransferase (ALT), asparate aminotransferase (AST), lactic dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT), calcium, phosphorus, iron, sodium, potassium, chloride, and magnesium.

Monitoring Neurologic Disease

The primary disease condition seen in horses, sheep, cattle, deer, and alpacas being fed endophyte-infected perennial ryegrass is a neurological syndrome called "ryegrass staggers." (Reed et al., 2011; diMenna et al., 2012; Johnstone et al., 2011) The diagnostic criterion for this condition is determined by evaluation of the gait of an animal (Galey et al., 1991). Affected cattle, sheep, horses and alpacas exhibit an onset of muscle tremors, stiffness of gait during walking, base wide stance and gait progressing to clinical signs of ataxia or incoordination. The gait of each of the 24 camels in this study was videotaped prior to the start of the feeding trial and twice a week for the 56 days of the trial period. Subsequently, they were videotaped on 14 days and 41 days after being placed on endophyte-free straw to evaluate degree of recovery in affected animals. Neurological parameters that were evaluated were placement of limbs during stance and walking; presence of muscle tremors in limb, neck and head muscles; presence of hypermetria (exaggerated limb movement); circumduction of a limb on turning; muscle weakness; and ataxia (incoordination) with or without truncal sway. An unexpected finding of "lower lip droop" similar to a lower lip facial paralysis was observed in some camels and noted when present in any camel during its gait evaluation. The scoring of the camels' gait (Table 2) and the aforementioned parameters was done by a specialist in large animal neurology who was blinded to the dose of lolitrem B each animal was receiving. The videotape of each camel had the animal being led by a handler for approximately 20 meters in one direction, turned and then walked back to the starting point. The person doing the videotape then focused in on the major muscle groups of each side of the animal to observe the presence of any muscle tremors post walking. An evaluation of the clinical signs, or lack thereof, was noted for the videotape on each animal independently.

Pathology procedures

The progressive development of the pathological lesions associated with consumption of perennial ryegrass straw was studied by a pathologist in necropsy material from two camels in the high dose group (D) and two camels from the control group (A). Standard necropsy and histopathology procedures were used by the pathologists in the ADFCA Diagnostic Lab in Al Ain.

Table 2. Neurological scoring system used for diagnosis of ryegrass staggers[a].

Score	Clinical signs
0	No clinical signs
1	No resting tremors; low-intensity tremor and/or in coordination with handling; slight stiffness of gait and base wide placement of limbs while walking
2	No resting tremors; moderate-intensity tremors and/or in coordination with handling; marked stiffness of gait and base wide placement of limbs during walking and standing
3	Spontaneous low-intensity tremors at rest; moderate to severe tremors and/or in coordination with handling; marked stiffness of gait; truncal ataxia; difficulty in rising; weakness
4	Pronounced resting tremors and incoordination; convulsive tremors and severe in coordination with handling; extreme spastic gait (hypermetria); truncal ataxia and weakness; falling down into lateral recumbency with difficulty in rising.

[a]Modified from Galey et al. (1991).

Statistics

GraphPad Prism (La Jolla, CA USA) was used for development of graphs and statistical analyses. Physiological parameters and blood values were evaluated by two-way ANOVA, defining $p < 0.05$ as significant.

Results and Discussion

Camels have a rumen-like forestomach and depend, like ruminants, on microbial metabolism of their diet to provide usable products for growth and energy. Observations of abortions, reduced food intake, poor weight gain, lower pregnancy rates, decreased milk production, muscle weakness, tremors, and spasms in camels have become a concern of the camel farmers of the United Arab Emirates (personal observation). The possibility exists that three different fungal diseases of forages may play a role in causing these clinical conditions. These diseases could result from either ingestion of high endophyte infected perennial ryegrass, or high endophyte-infected tall fescue or ergot infected forage. With water becoming a scarce resource in the Middle East, the need for securing high-quality forage is becoming paramount to maintaining optimal herd health and production (Mooney, 2011). Going hand-in-hand with this, however, is the need to ensure that this forage is "safe to feed" throughout the life spectrum of the animals in question. Thus, we focused this initial project on the establishment of threshold of toxicity levels in non-pregnant female camels for lolitrem B.

Physiological parameters

Body weight was adversely affected by consumption of lolitrem B-containing perennial ryegrass straw over 56 days (Figure 1A). Camels fed a diet free of lolitrem B had a larger body weight gain (7.4%) than the other three groups (-2.5%, 0.5% and -8.8% for 1111 ppb, 1478 ppb and 2273 ppb, respectively). Two-way ANOVA was significant for group ($p < 0.001$) but not for date, or the interaction of group x date. However, Figure 1A shows a splitting apart of the four groups as time progresses, with the error bars beginning to become distinct between groups. It is likely that if the experiment were carried out for an additional time period, the weight gain average of the four groups would have continued to drift apart, making date and the group x date interaction significant. This is in contrast to a study on pregnant cows where pre- and post calving body weights were not affected by increasing lolitrem B concentrations (Fisher et al., 2004). The phenomena of decreased weight gain has been seen in numerous other studies of livestock consuming endophyte-infected grasses containing the alkaloid ergovaline, a condition called "summer slump" and is most commonly associated witht ergovaline in tall fescue (*Neotyphodium coenophialum*) (Peters et al., 1992). The perennial ryegrass straw used in this study did contain ergovaline at approximately one-third the level in ppb of lolitrem B.

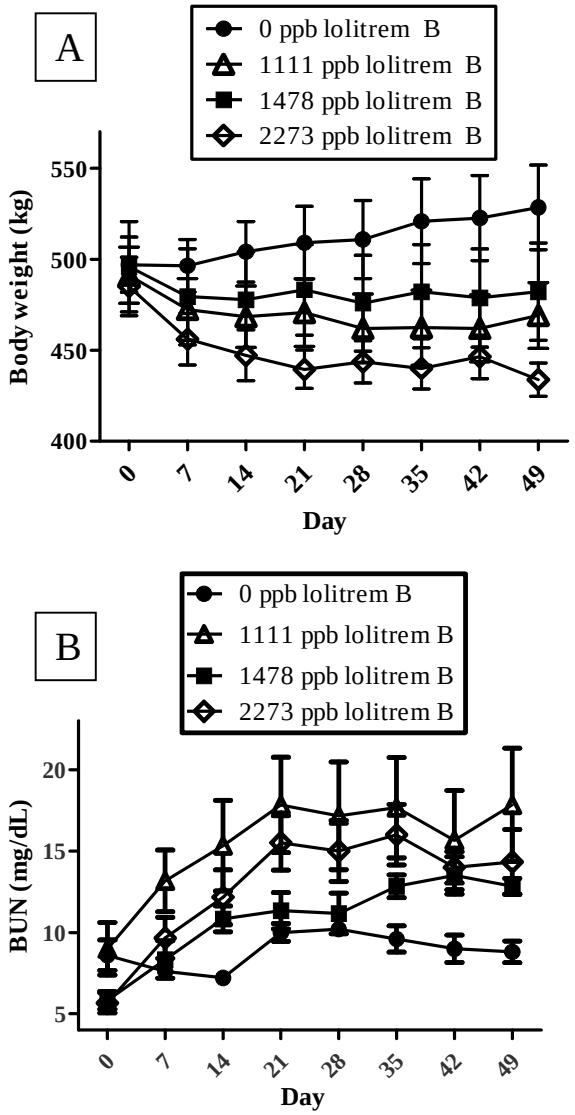

Figure 1. A and B. Body weight in female camels fed varying levels of lolitrem B-containing perennial ryegrass straw over 56 days.

Blood urea nitrogen (BUN) exhibited significant change for group ($p < 0.001$) and date ($p < 0.001$) but not the interaction of group x date by two-way ANOVA. Over the course of the study, the three treatment groups separated out and had higher levels of BUN than the control group

receiving 0 ppb lolitrem B (Figure 1B). Thus, the consumption of lolitrem B appears to increase BUN levels, which is indicative of kidney damage. This was supported by the gross and histopathological findings in the kidneys of both camels in the highest lolitrem B group (Group D) at necropsy.

All other physiological parameters in the blood showed no significant changes, as has been reported by Miyazaki et al. (2007). No dermatologic lesions were noted in any of the camels in this study except D5 and the loss of hair.

Neurological examination results

The mean and range of onset of clinical ataxia in all four groups of camels in this study was as follows: Group B (1111 ppb) had a mean onset of ataxia of 35.8 days +/- 17.0 (SD) (8.7-62.8 = 95% CI); Group C (1478 ppb) had a mean of 43.7 days +/- 11.6 (SD) (31.5-55.9 = 95% CI); and Group D (2273 ppb) had a mean of 19.8 days +/- 8.5 (SD) (10.9-28.8 = 95% CI). The camels in the control group (A) showed no signs of clinical ataxia throughout the study. Table 3 is highlighted with yellow color to illustrate which camels were ataxic in which time period of the study. It also includes details of the clinical evaluations of each camel videotape relative to a base wide stance or base wide limb placement during movement and/ or hypermetria of the rear limbs, weakness, and "lip droop". Weakness of voluntary movement and the presence of a "lip droop" were seen in a number of the camels in each group but rarely in the control camels over the 56 days of the study. Camels that develop the definitive sign of "ryegrass staggers" or clinical ataxia were graded from 1 to 4 and non-affected camels were given a zero. Development of a base wide stance or gait or hypermetria of the rear limbs were labeled Bw. These are seen in red in Table 3. These clinical signs often developed prior to ataxia or were seen intermittent in-between days where there was no clinical signs of ataxia. Each camel video was evaluated independently for that day with the evaluator not being aware of the dose of lolitrem B in the feed or the previous results for each animal. Weakness (w) was evident when the camels had difficulty rising, fell or had "truncal ataxia". The latter describes a sway of the body during forward movement and indicates a more severe ataxia.

At the end of the study, the A group was the control group with an mean of less than 100 ppb (designed as 0 ppb) which is the lower limits of the endophyte laboratory's measurement of lolitrem B; the B group had a mean of 1111 ppb lolitrem B; the C group had a mean of 1478 ppb lolitrem B, and the D group was the highest lolitrem B concentration with an approximate mean of 2273 ppb.

Camel D5 was the first one to start showing signs on day 10 and developed the most serious form of ryegrass staggers than continued until the completion of the study. In the last few weeks, the hair of this animal started falling out, a clinical sign seen only in this camel. While muscle tremors are the earliest clinical sign seen in horses, cattle, and sheep, this was not the case with camels eating moderate to high levels of lolitrem B in endophyte infected ryegrass. Camel D5 developed tremors in the pelvic limbs musculature on day 32 as did camel D15 and D16 on days 32 and 35 respectively. But these were somewhat rare occurrences and occurred late in the disease process rather than early.

By day 35, all six of the camels in the D group had or were showing clinical signs of ryegrass staggers. In contrast, in the C group, the earliest camel to become ataxic was C-3 at 16 days, but by the end of the study, the entire C group of camels had been affected. The B group with the lowest dose of lolitrem B had only 2 camels be affected early, i.e., by days 12 (B1) and 16 (B11) respectively. B1 showed fairly consistent signs of neurological dysfunction with either ataxia or a base wide stance/gait or hypermetria of the rear limbs throughout the study. B11 had onset of signs on day 16 with an intermittent return to normal gait in-between days of ataxia. This may have been related to the amount of feed this camel was eating each day or other unknown adaptive mechanisms. The differences may also be due to the method of evaluation in which each camel's video was viewed without reference to the previous day's results. The evaluator only noted what was evident in the short time of the video tape. By the end of the study, 4 camels in the B group had several days of being ataxic. An interesting note was a "dancing" motion exhibited by camel C17 during rest in pen for a short time during the first few days of the study.

Threshold levels are determined as that amount of toxin that will result in clinical signs. In this 56 day study, 2273 ppb lolitrem B affected all camels, 1478 ppb affected 2 camels between days 16 and 20 with all of the camels affected by day 54 to 56. With the lowest dose of 1111 ppb, 2 camels were particularly susceptible to the toxin at days 12(B1) and 16 (B11). At the end of 56 days, 4 of the 6 camels in the B group were affected. After 56 days, control camels A6 and A7 and two camels from the highest lolitrem B group, D5 and D16 were euthanized and sent to the ADFCA Diagnostic Lab in Al Ain for necropsy.

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Table 3. Summary of clinical signs seen in camels. The ataxia scores are highlighted in yellow and the basewide/hypermetria observations are highlighted in red.

Neurological evaluation
*Ataxia scores = 0-4; Bw = Base wide gait/hypermetria; * Lip droop: w = weak*

	Observation period in days of trial																			*Recovery days*	
	1-9	10	12	14	16	18	20	22	26	28	32	35	40	42	44	48	50	54	56	14	41
Control N=5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	0	0*	1*	1*	1*	1*	1*	1*	Bw*	Bw*	1*	0	Bw	1	Bw*	Bw*	0*	1*	1*	0*	0
B4	0	0	0*	0*	0*	0	0	0*	0*	0*	0*	0*	0*	1	Bw*	1*	0*	0*	0*	0*	0
B11	0	0	0	0	1	1	1	1	Bw	0	0	1	0	0	0	1	1	1w	1	0	0
B18	0	0*	0	0*	Bw	0*	0	0*	0*	0*	0	0*	0*	0*	Bw	1	1	0*	1	0	0
B19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Bw	0	0	0	0
B22	0	Bw	Bw	Bw	Bw	Bw	Bw*	Bw*	0 *	0	Bw*	1	1	1*	1	Bw	1*	1*	Bw*	Bw	Bw
C3	0	0	0	0	1*	1	1	Bw	0	0	1	0	Bw	0	Bw	2	2	2	3	0	0
C9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Bw	1	1	1	Bw	Bw	0
C10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Bw	1	2	1	0
C17	0+	Bw	0	0	0	0	1	1	0	Bw	1	0	2	2	2	2*	1	2w	2	1	Bw
C20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Bw	2	Bw	Bw	1	0
C24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1 *	2	0	0
D5	0	2*	2*	3*	3	3*	1w	3w	3	1*	1*	3w	3w	3*	3w*	4w*	3.5*	3*	3w*	Gone	Gone
D8	0	0	0	0	0	0	1	--	0	0	1	--	1	1	1w*	1w	1w	1	1	0	0
D12	0	Bw	Bw	0	0	0	1	1	1	1	1	1	2w	2w	1	Bw	1	1	1	1	0
D14	0	0	0	0	0	0	1	Bw	1	Bw	0	1	2	2	1	1w*	1	2	2	0	0
D15	0	0	0	0	Bw	0	0	Bw	Bw	Bw	1	2	2	2	1	2w	2w	2w	1	1	Bw
D16	0	0*	Bw*	1*	0	0	1	1*	1*	Bw*	1*	1	2	2*	1w*	2*	1	1*	1	Gone	Gone

+ dances in pen

Videotapes were also made of all the remaining camels during a recovery period on 14 and 41 days after they were placed on control feed with no lolitrem B. On day 14 of recovery, 9 of the remaining camels had normal gait and no sign of neurological deficits. Two camels, B22 and C9 had a base wide placement of limbs during walking and standing, and 5 camels were still grade 1 ataxic. By 41 days, no camels were ataxic and only three, B22, C17 and D15 had a residual base wide stance and gait. This recovery was best seen in the mildly affected camels. All of the camels on control feed (Group A) had no neurological deficits at any time during the study.

The "lip droop" seen most often in affected camels and only rarely in control camels appeared as if the lower lip had lost any tone and fell ninety degrees perpendicular to the lower jaw as if a partial facial nerve paralysis had occurred. This has not been reported in any ryegrass toxicosis study and this is the first report of ryegrass staggers in camels. During the recovery period, only 2 camels, B1 and B4 still had the "lip droop" evident on day 14 of recovery; it had resolved by day 41 of recovery.

Postmortem findings

On necropsy, the two camels from the 2273 ppb lolitrem B group (D5 and D16) showed gross lesions which is in contrast to what is reported in Jubb, Kennedy and Palmer (Maxie, 2007). This included a greenish colored liver compared to control camels (A6 and A7). See Figure 2. The kidneys showed adhesions between the renal cortex and the capsule with an outer layer of detached cortex (Figure 3). The adhesion of the capsule with the renal cortex was severe in one camel (D5) and slight in the other (D 16). The cut surface of the kidney revealed degenerative changes of the cortex and congested renal medullary blood vessels (Figure 2). The brain was edematous and blood vessels were congested (Figure 3). The gastrointestinal tract revealed a slight mucous inflammation. Internal organs of the control group camels were within normal limits. The weights of internal organs of both the control and affected camels, including the brain and divided by the camel weight at time of necropsy are in Table 4. On histopathology, the classic reported lesion of vacuolar degeneration of Purkinje cells in the cerebellum was present (Maxie, 2007). However, in the two affected camels, microscopic lesions were present in the liver and the kidney as well as the cerebellum (Figure 4). This is the first report of kidney dysfunction associated with lolitem B ingestion in camels. In a most recent report (Johnstone and Mayhew, 2013), horses fed 2000ppb lolitrem B in seed and hay developed reduced flow-mediated K+ secretion and had interference with aldosterone production or secretion. These abnormalities in the kidneys' ability to handle electrolytes in horses support this study's findings that lolitrem B at high levels can adversely affect kidney structure and function.

Table 4. The weights of the internal organs of control group camel (A6 and A7) and affected camels (D5 and D16) divided by body weight at the time of necropsy. The weights of the organs for the normal camels were within normal limits while some of the affected camels varied.

Organ / weight	Camel-1 (control)A-6 (gram/bwt kg=ratio)	Camel -2 (control) A-7 (gram/bwt kg = ratio	Camel-3 (treated) D-5 (gram/bwt kg = ratio)	Camel -4 (treated)D-16 (gram/bwt kg = ratio)
Liver	5552.3/438 = 12.7	6967.1/530 = 13.1	4758.1/437 = 10.9	5740.8/437 = 13.1
Left Kidney	547.2/438 = 1.3	678.3/530 = 1.3	712.1/437 = 1.6	577.1/437 = 1.3
Right Kidney	532.3/438 = 1.2	745.3/530 = 1.4	668.5/437 = 1.5	602.7/437 = 1.4
Brain	393.7/438 = 0.9	404.1/530 = 0.8	417.3/437 = 1.0	373.1/437 = 0.9
Age (years)	6-7	6-7	7-8	5-6

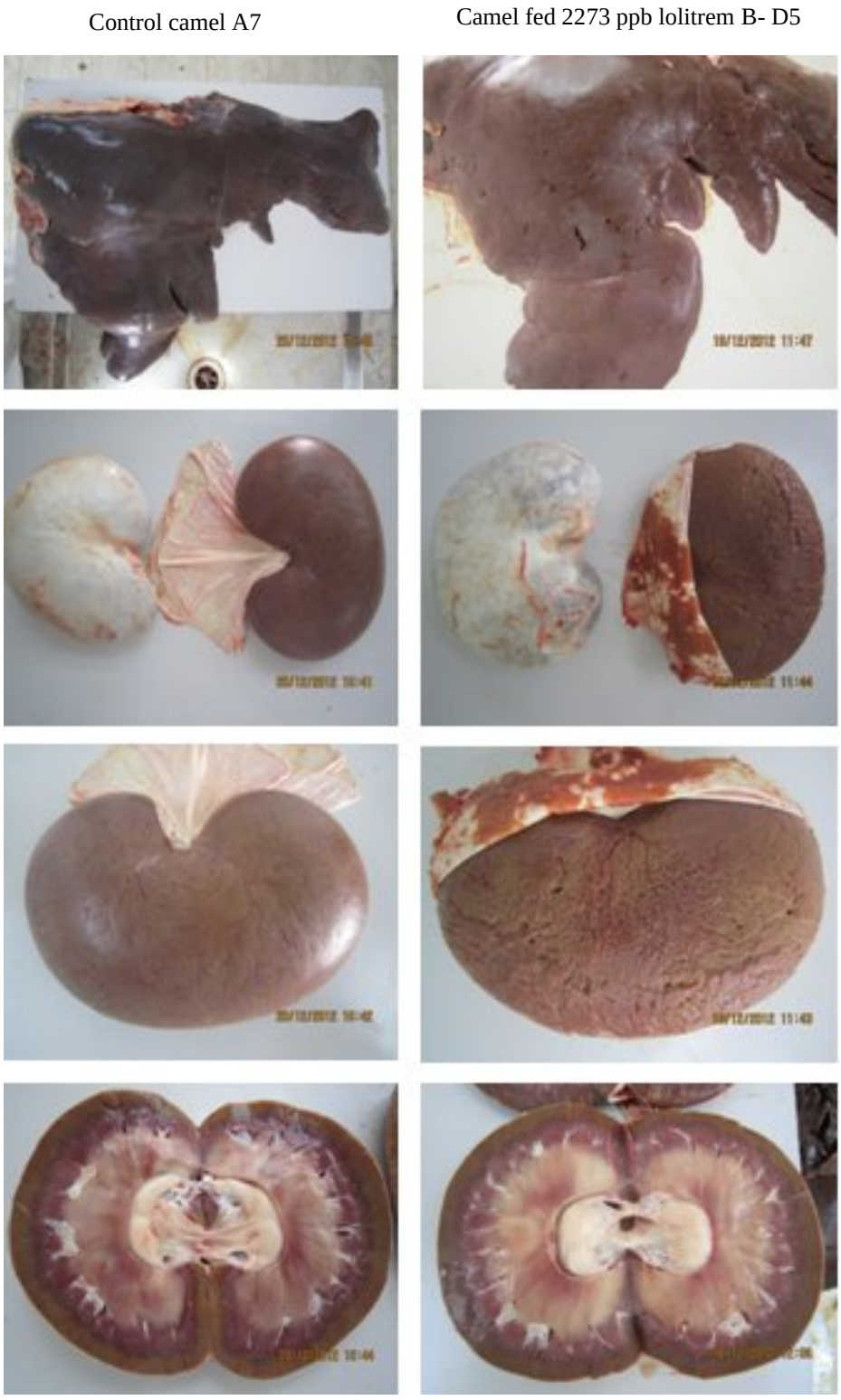

Figure 2. Pathologic examination of internal organs from camels fed perennial ryegrass straw containing 2273 ppb lolitrem B over 56 days.
(Top two photos show hepatic differences; bottom six photos illustrate renal abnormalities between a control animal and one receiving the highest dose of lolitrem B (D5)).

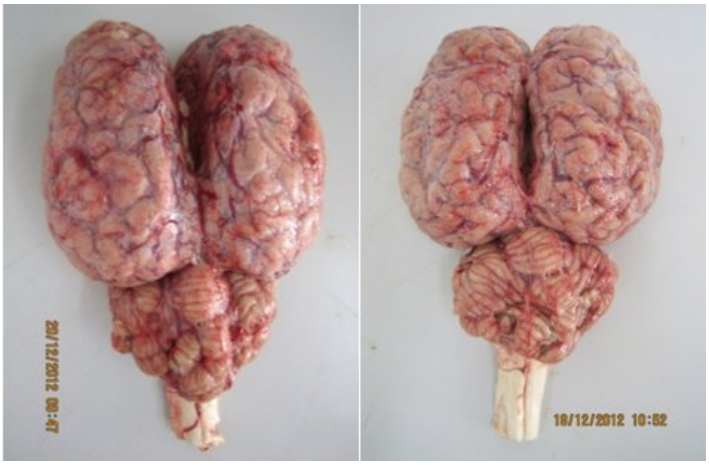

Figure 3. The brain of control camel A7 on the left and affected camel D5 on the right. Note, the edematous appearance of the brain on the right.

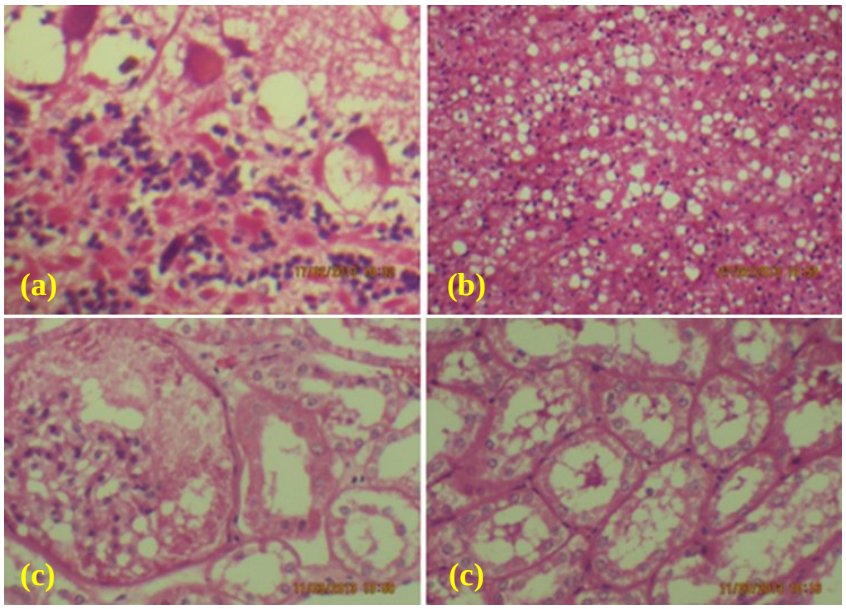

Figure 4 (a). An H & E stain of the gray matter of the cerebellum showing severe vacuolar degeneration of Purkinje cells in camel D5 (fatty necrosis). (b). An H & E stain of the liver of camel D5 showing severe vacuolar degeneration changes. (c). An H & E stain of the kidney of camel D5 showing severe vacuolar degenerative changes in epithelial lining of the convoluted tubules. The glomerular space and tubular lumens were obliterated with casts or proteinase material.

Conclusions

This is the first report on clinical ryegrass staggers in camels. Perennial ryegrass straw with 1000 ppb or greater caused a transient disease condition that resolved with return to "clean" feed. In this study, there was a reduction of clinical signs in 14 days in all camels and after 41 days, no clinical ataxia was evident. With this data over the time period of this study, one would need to say that camels are particularly sensitive to lolitrem B alkaloid. To avoid clinical disease, the authors suggest feeding endophyte infected perennial ryegrass straw at 500 ppb or lower and diluting all straw at 1000 ppb or higher. Imported feed should always be tested for this alkaloid.

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