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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Biotechnology

Full Length Research Paper

Controlling water deficit by osmolytes and enzymes: Enhancement of carbohydrate mobilization to overcome osmotic stress in wheat subjected to water deficit conditions

Asthir, B.*, Gulati, A. and Bains, N. S.

Department of Biochemistry and Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana-141004, Punjab, India.

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The present study demonstrates the effect of polyethylene glycol-6000 (PEG) induced water-deficit stress on growth, physiological and biochemical responses in six wheat (*Triticum aestivum* L.) cultivars viz. C 306, C 273, PBW 175 (drought tolerant), PBW 534, PBW 343 and PBW 550 (drought susceptibile) at seedling stage for six days. Stress caused short stature in shoot and elongated roots in all cultivars. Significant reduction in chlorophyll content and increase in membrane injury index and lipid peroxide content were observed in all the cultivars. Water stress accentuated sucrose synthase/phosphate synthase activities while sucrolytic enzymes namely; acid, neutral and sucrose synthase (cleavage) got repressed. Higher build up of sucrose and lower activity of amylase in root and shoot further decreased reducing sugar (glucose, fructose) content in stressed seedlings. Though the sugar supply (sucrose) under stress was not a limiting factor for germination but the poorer 'metabolic conversion efficiency' via sucrolytic enzymes in root and shoot resulted in their favoured utilization in amino acid, proline and glycine betaine. It may therefore be concluded that certain traits namely: membrane stability parameters, sugars, enzymes and osmolytes might help the cultivars to tolerate water deficit conditions.

Key words: Carbohydrates, chlorophyll, sucrose metabolism, membrane stability parameters, proline, protein, starch, *Triticum aestivum,* water-deficit.

INTRODUCTION

Water deficit is one of the most common environmental limitations affecting growth and productivity of plants. Exposure of plants to water deficit conditions induces several physiological and biochemical changes which enable plants to adapt to limited environmental conditions (Basu et al., 2010). Reduction of photosynthetic activity, changes in carbohydrate metabolism, accumulation of organic acids and osmolytes are typical responses

*Corresponding author. E-mail: b.asthir@rediffmail.com. Tel/Fax: 00 91 161 2400955.

affected by stress (Valliyodan and Nguyen, 2006; Saeedipour and Moradi, 2011). Genotypic variation exists for water deficit tolerance and identification of key metabolic steps will enhance our understanding and fill our knowledge gaps.

One of the earliest responses against water stress is stomatal closure that limits CO_2 diffusion towards chloroplast, which in turn alters carbohydrate metabolism. Another group of compounds which may be affected are amino acids and proline that are often increased in water stressed-leaves (Pinheiro et al., 2004).

Proline, an osmoprotectants, plays an important role in the protection of subcellular structures and scavenging of free radicals. Whereas, glycine betaine is effective in the protection of membranes and the quaternary structures of complex proteins and enzymes. Accumulation of protecttive solutes like soluble sugars acts as osmoprotectant during stress.

Biochemical conversion of starch to sucrose in the endosperm during germination and seedling growth is a fast process and the sucrose formed is transported to roots and shoots, where it is utilized by invertase and sucrose synthase to provide hexoses for growth and sugar nucleotides for cell wall synthesis (Rosa et al., 2009). Sucrose cleavage catalysed by sucrose synthase is an easily reversible reaction. Apparently, hydrolysis of sucrose by invertase is an irreversible reaction, producing twice as many hexoses. A key function of invertase is a regulation of turgor and the control of sugar balance (Bogdan and Zagdanska, 2009).

The functional significance of sucrose synthase is related to the carbon direction toward both biosynthesis of polysaccharide and respiration (Weschke et al., 2003). Under water deficit, the conversion of starch to sucrose is inhibited and sucrose transport to growing tissues is also decreased, leading to increase in sucrose content in residual endosperm (Regier et al., 2009). In addition, water stress inhibits photosynthesis causing changes in chlorophyll content and thereby damage to photosynthetic apparatus (Nikolaeva et al., 2010). Measurement of such processes in response to drought stress may provide valuable information on the various strategies of the plant that intends to remove or to reduce the harmful effects of water-deficit in soil or plant tissues.

The prerequisite for a successful breeding program for drought tolerance is the availability of tolerant cultivars (Kosturkova et al., 2008). Therefore, the present investigation was aimed to characterize the tall traditional cultivars C 306 and C 273 suitable for rainfed agriculture and is known for drought tolerance but is low yielding cultivars (Gupta et al., 2010). Other cultivated varieties PBW 343, PBW 534 and PBW 550 are widely grown with high yield but not water stress tolerant.

The obtained results would complement the ongoing efforts at our institute for creating drought tolerant varieties derived from crosses of tall traditional varieties with modern semi-dwarf wheat (C 306/PBW 534//PBW 534 and C 273/ PBW 343// PBW 343). This will pave the way for tagging of drought resistance genes.

The present study was examined for differential response to water deficit in six wheat cultivars C 306, C 273, PBW 175 (drought tolerant), PBW 534, PBW 343 and PBW 550 (drought susceptible) based on growth, physiological and biochemical parameters.

MATERIALS AND METHODS

Plant materials and treatment

Wheat (*Triticum aestivum* L.) cultivars, namely: C 306, C 273 (pre dwarfing era varieties and suitable for rainfed agriculture), PBW 343 (widely grown wheat variety for irrigated conditions), PBW 534 (an advanced breeding line for irrigated conditions), PBW 550 (recently released variety for irrigated conditions), PBW 175 (presently cultivated under rainfed conditions) were evaluated for differential sensitivity to water stress during germination. The seeds of above genotypes were collected from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The seeds were sown in pots filled with field soil under two sets, that is, control and drought set, for characterization of various drought tolerance related biochemical indices. Artificial drought conditions were generated in pots using 6, 8, 10, 15% polyethylene glycol (PEG 6000) solution. The root and shoot length (cm) of seedlings under control and drought set were measured at 10th day post germination (DPG) whereas other parameters were studied at 6th day stage of germination. The plants were watered twice a week with PEG solution in drought set and with distilled water in control set.

Relative water content

The observations on relative water content (RWC) of leaf were taken. RWC of the leaf was determined by using the formula:

Relative water content = (Fresh weight – dry weight)/ (Turgid weight –dry weight)

Where, turgid weight = weight after the leaf was kept immersed in distilled water for 16 h.

Estimation of membrane injury, lipid peroxide and chlorophyll content

Membrane Injury and lipid peroxide content were estimated as described by Valentovic et al. (2006). The concentration of lipid peroxide products were determined from the thiobarbituric acid reactive substance (TBARs) contents resulting from the thiobarbituric acid reaction.

Chlorophyll content was estimated according to the method of Hiscox and Israelstam (1979).

Estimation of soluble sugars and starch

Total soluble sugars and starch were extracted and estimated colorimetrically using the reaction with phenol as described by Rosa et al. (2009), sucrose by the method of Roe (1934) and fructose by Williard and Slattery (1945).

Extraction and assay of enzymes

From freshly collected samples, soluble acid invertase (EC 3.2.1.26;

pH 4.8), soluble neutral invertase (EC 3.2.1.27; pH 7.5), sucrose synthase (synthesis, EC 2.4.1.13) and sucrose phosphate synthase (EC 2.4.1.14) were extracted and estimated by the procedure employed by Rosa et al. (2009). Total amylase was extracted with 50 mM sodium acetate buffer (pH 5.0) containing 1 mM $CaCl_2$ and contents centrifuged at 10 000 *g* for 10 min. Amylase activity was determined by estimating reducing sugars as described by Rosa et al. (2009). In all enzyme assays, the condition for linear rates with respect to substrate concentration, time, optimum temperature and pH were determined in preliminary assays.

Estimation of amino acid protein, proline and glycinebetaine

Total free amino acids and soluble proteins were extracted and determined as described by Good and Zaplachinski (1994) and proline contents by Valentovic et al. (2006). Soluble proteins were extracted in 0.1 M NaOH and precipitated with trichloroacetic acid (TCA) and estimated by the standard procedure. For estimating proline content, fresh tissue (0.5 g) was extracted in 3% sulphosalicylic acid and the homogenates were centrifuged at 10 000 *g* for 10 min. Two ml of the supernatant was made to react with 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C and the reaction terminated in an ice bath. To this 4 ml of toluene was added and mixed vigorously. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance measured at 520 nm using toluene as blank. The glycinebetaine content was determined using the method of Grieve and Grattan (1983)

Statistical analysis

Data was subjected to two-way analysis of variance (ANOVA) to compare genotypic variations and environmental conditions and the LSD at $p < 0.01$. Standard errors were computed from the values of two independent experiments with three replicates. The data was analyzed statistically according to Duncan's multiple range tests.

RESULTS

Effect of water stress on growth parameters

PEG did not affect the germination percentage, but the rate of germination was markedly improved with 8% PEG than with 10 and 15% PEG in seedlings. An increase in root length and decrease in shoot length was recorded in all the cultivars under stress conditions compared to control (Figure 1A). It was observed that C 306, C 273 and PBW 175 varieties had longer root system compared to PBW 534, PBW 343 and PBW 550 ones reflecting their inherent capability to drought tolerance. Relative water content was found to decrease under water deficit conditions in all the studied cultivars.

Effect of water stress on membrane injury, lipid peroxide and chlorophyll content

Membrane injury index and lipid peroxide content measured in form of thiobarbituric acid reactive substances (TBARS) content increased both in root and shoot (Figure 2A and B) in the stressed seedlings, but the overall increase was more in root as compared to shoot. Roots of C 306 and C 273 had lower membrane injury and lipid peroxide content over PBW varieties both under control and stressed conditions. PBW 175 showed highest membrane injury compared to PBW 550. Total chlorophyll content decreased in shoot of stressed plants in all the cultivars (Figure 2C). Comparatively, chlorophyll a content was significantly high than chlorophyll b although both had reduced proportionally under stress. Cultivar PBW 534 had maximum chlorophyll a content (297.11 $\mu g\ g^{-1}$ FW) while minimum content was observed in PBW 175 (213.65 $\mu g\ g^{-1}$ FW) under stress. C 306 and C 273 cvs had lesser amount of total chlorophyll content over others both under control and stress conditions.

Effect of water stress on sucrose metabolizing enzymes

Acid invertase activity predominated over sucrose synthase (cleavage) in root and shoot (Figure 3). Water deficit caused a marked decrease in all sucrolytic activities in both root and shoot while activities of sucrose synthase and sucrose phosphate synthase were increased (Figure 3). PBW cultivars had higher invertase activities in root while sucrose synthase and sucrose phosphate synthase activities predominated in C cvs in both roots and shoot. Neutral invertase was too low in comparison with acid invertase and its interaction between cultivars and environment was statistically non significant showing little contribution towards sucrose hydrolysis.

Effect of water stress on starch content and amylase activity

Starch content was found to increase under stress conditions in endosperm as compared to control, in correspondence with a decrease in amylase activity (Figure 4). However, converse was found to be true in root and shoot as the content of starch decreased in root and shoot while amylase activity increased (Figure 4). Total amylase activity was significantly high in residual endosperm and was negligible in root and shoot (Figure 4) and it decreased with stress in endosperm. Maximum decrease of amylase activity was recorded in endosperm of C cultivars. Shoot and root of C cultivars maintained higher amylase activity over PBW cultivars under stress conditions showing that the hydrolysis of starch in these tissues generated higher assimilates in form of glucose as carbon source for its subsequent growth.

Effect of water stress on proline, glycine betaine, protein, amino acids and sugars

Enhanced accumulation of cytosolic proline, amino acid

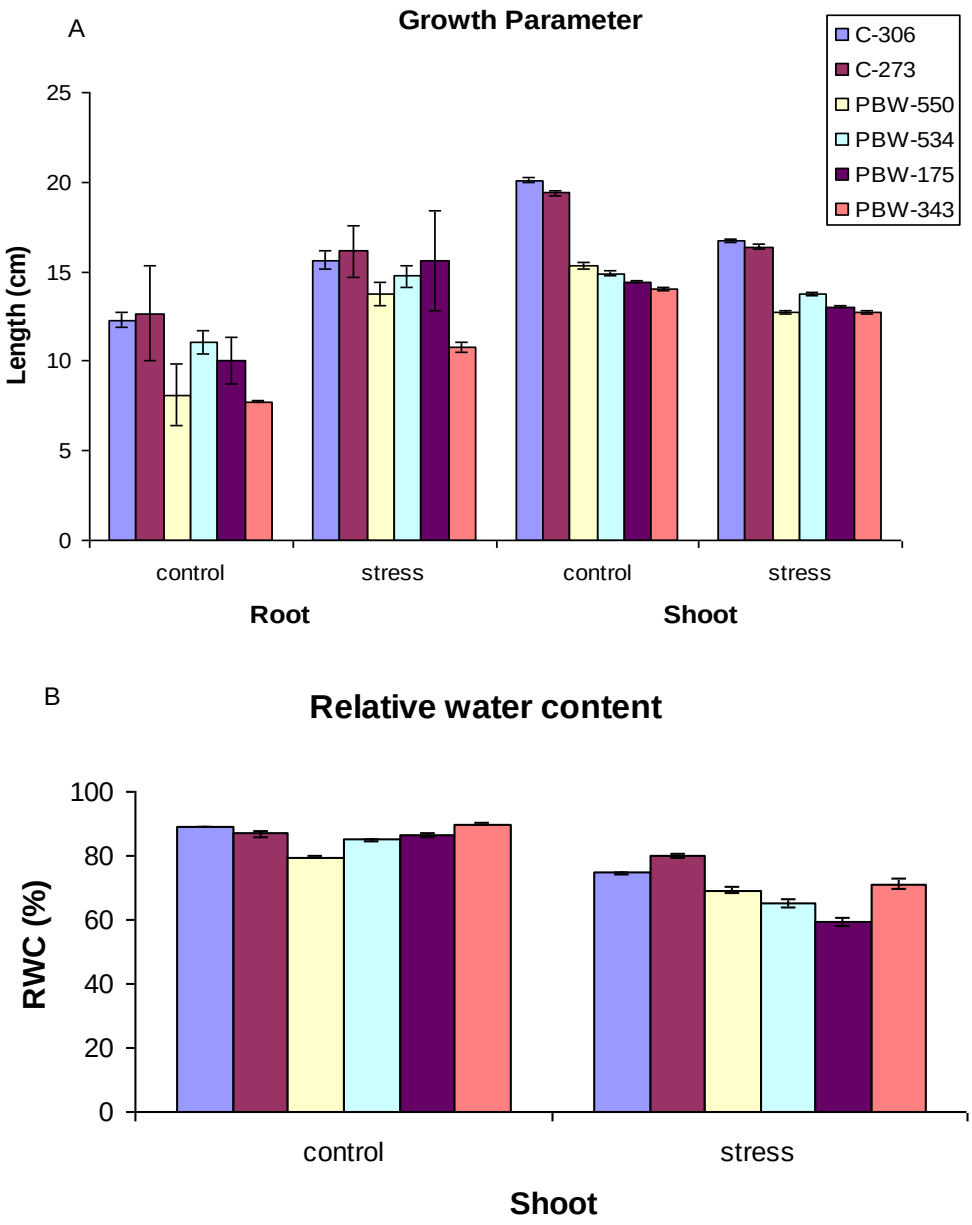

Figure 1. Effect of water deficit on growth parameters (A), relative water content (B) in root and shoot of germinating seedlings of six wheat genotypes. Vertical bars represents ± SD based on three independent determinations.

and glycine betaine was observed in response to water stress in both root and shoot (Figure 5). On the other hand, protein content was decreased under water deficit conditions. Shoot maintained higher protein content over root under both control and stress conditions (Figure 5). In root, the highest increase in amino acid content was found in C 273 (5.13 mg g^{-1} FW) followed by C 306 and amongst PBW cvs, it was maximum in PBW 343 (3.17 mg g^{-1} FW) followed by PBW 175. Whereas in shoot; it was highest in PBW 534 followed by PBW 550. C cvs possessed lower level of amino acid than PBW ones both under control and stressed seedlings (Figure 5). Free sugar content was higher in shoot as compared to root under control and stressed conditions (Figure 6). C cultivars maintained higher sugar content especially in roots over shoot. Sucrose was the predominant sugar present followed by glucose and fructose and its content increased both in root and shoot under stress (Figure 6).

DISCUSSION

A drought/water deficit condition is the main constraint for crop production in the world and variations among plants

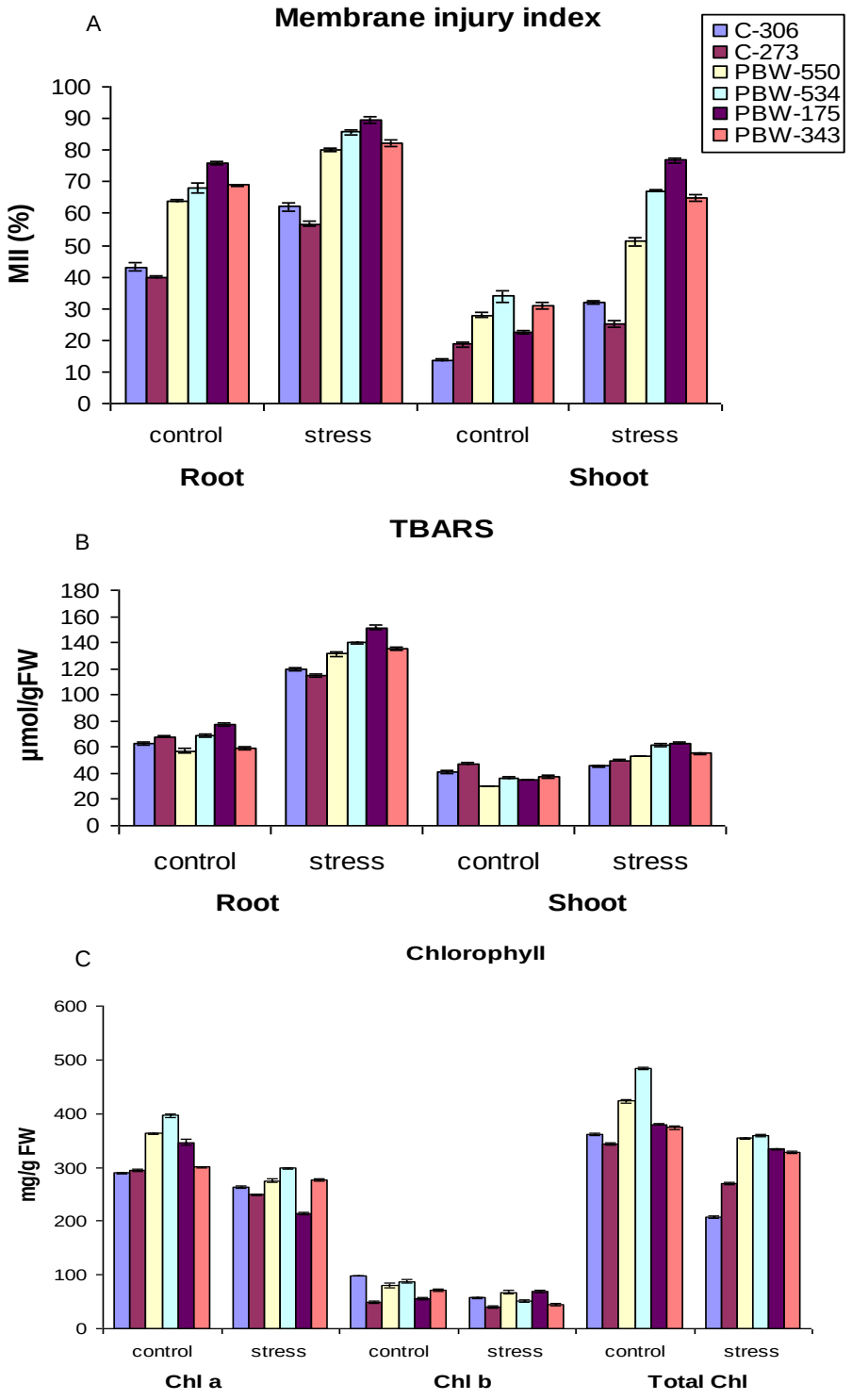

Figure 2. Effect of water deficit on membrane injury index (A), thiobarbituric acid reactive substances (TBARs, B) and chlorophyll content (C) in root and shoot of germinating seedlings of six wheat genotypes. Vertical bars represents ± SD based on three independent determinations.

provide a valuable tool in the selection of cultivars with desirable traits. Measurement of physiological and bio-chemical responses induced by the application of PEG-6000 under laboratory condition is considered as an

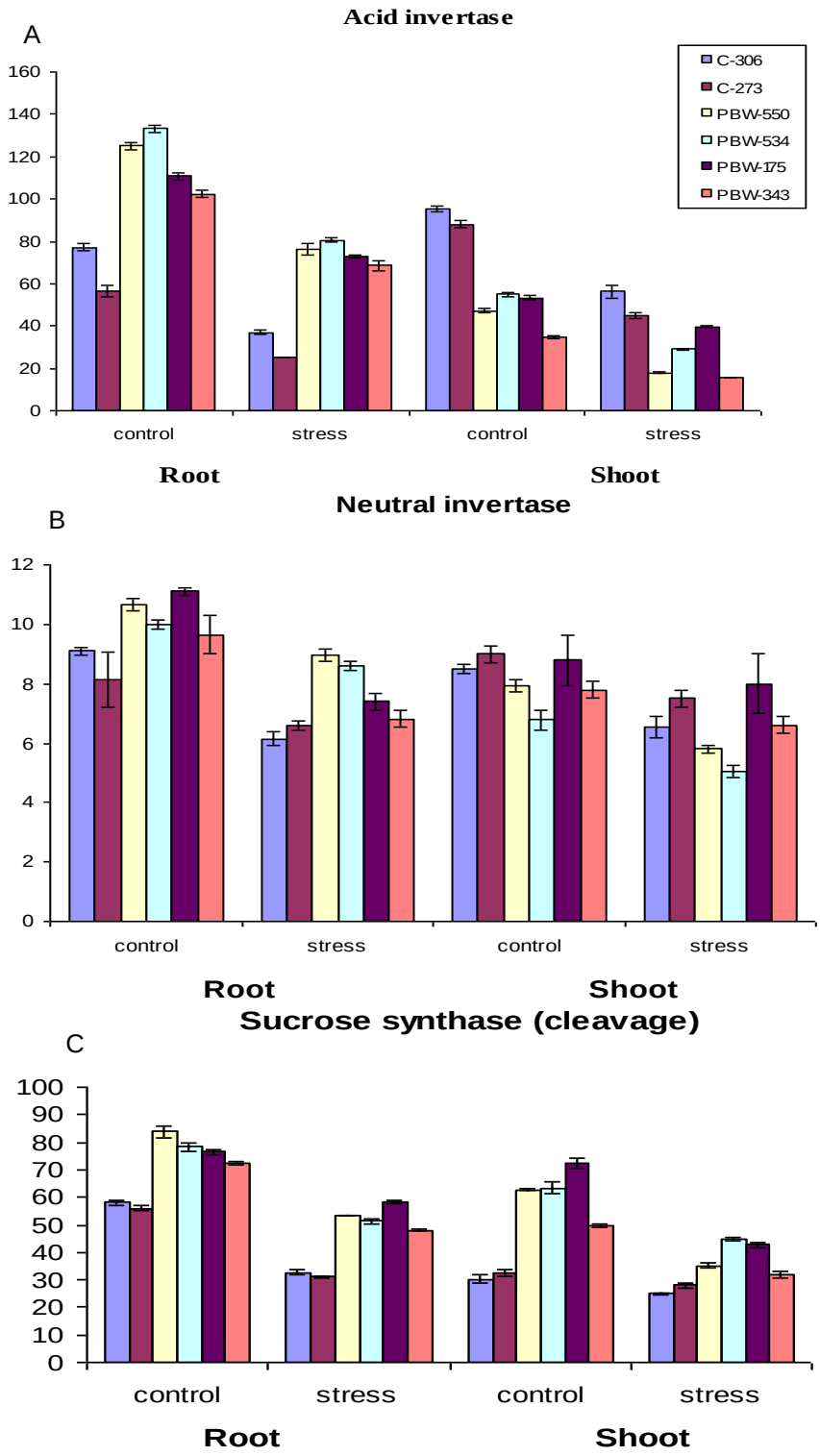

Figure 3a-c. Effect of water deficit on sucrose metabolizing enzymes viz. acid invertase (A), neutral invertase (B), sucrose synthase (cleavage, C), sucrose phosphate synthase. Vertical bars represents ± SD based on three independent determinations.

alternative of judging the performance of plants under field conditions (Basu et al., 2010). Although water stress induced by PEG develops faster creating an osmotic shock, metabolic responses to such osmotic treatment by

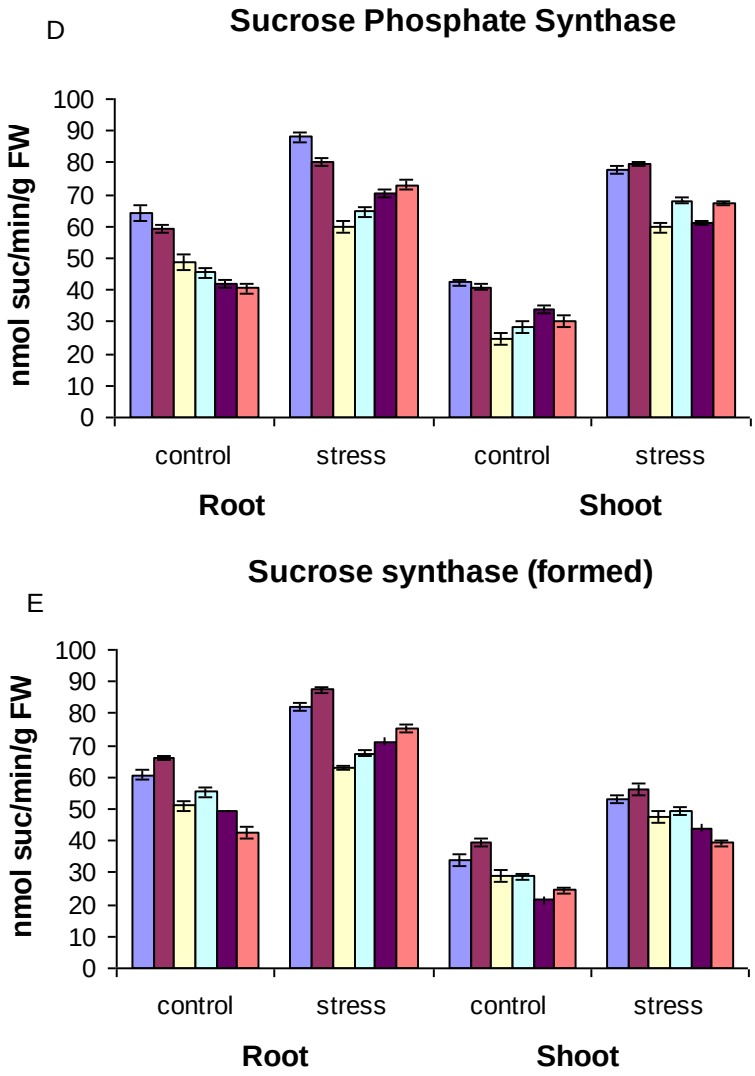

Figure 3d-e. Effect of water deficit on sucrose metabolizing enzymes viz. sucrose phosphate synthase (D) and sucrose synthase (synthesis, E) in root and shoot of germinating seedlings of six wheat genotypes. Vertical bars represents ± SD based on three independent determinations.

exposing the roots at least is indicative of the relative potential of the different wheat cultivars to tolerate this stress at physiological and biochemical level.

Under water deficit conditions, the extent of increase or decrease in the rate of germination varied in a genotype-specific manner. Germination percentage was decreased under water deficit conditions but the rate of germination in form of root lengths was markedly improved especially in C 306 and C 273 cvs. C cvs had longer roots which emerged as a primary sensor of water stress by absorbing water from deeper soil layers. Cellular water status measured in form of relative water content (RWC) was used to quantify the extent of dehydration which decreased under stress conditions. Many researchers have identified the genotypes of crop species as sensitive and tolerant based on RWC (Boughalleb and Hajlaoui, 2011).

Water deficit crops experiences higher membrane disruption as evident from the increased electrolyte leakage and lipid peroxide content in PBW cvs. C 306, C 273 cultivars had lower contents of lipid peroxide and membrane injury index (MII) over PBW ones suggesting a correlation between these two parameters. Chlorophyll content also decreased under stress however, less decrease was observed in PBW cultivars. Our results suggests that C 306 and C 273 cvs which are stress tolerant due to their inherent inbuilt genetic ability had more stable membranes than PBW cvs which otherwise had higher chlorophyll content. Decreased level of chlorophyll under stress might be due to peroxidative enzyme activity which

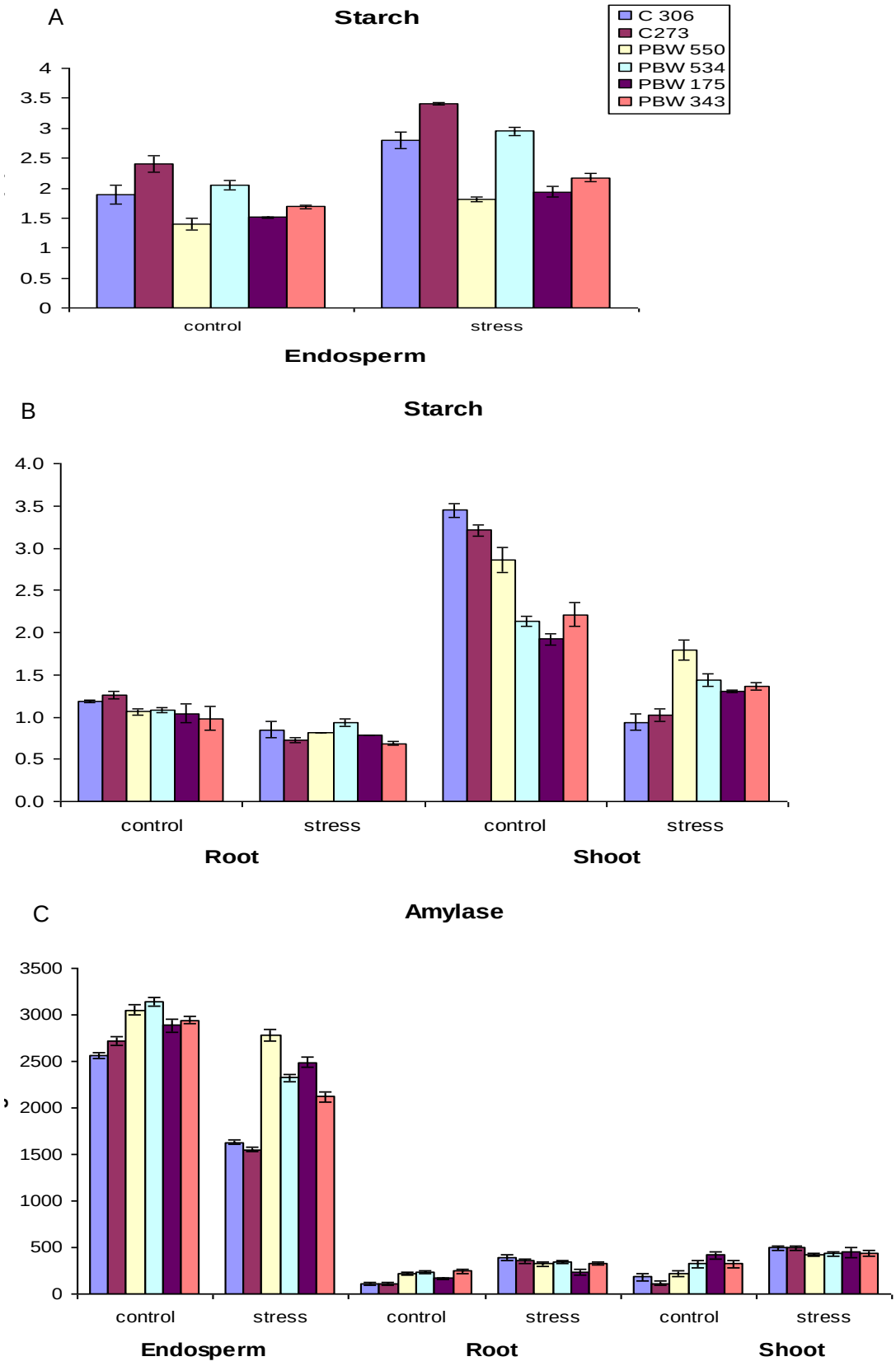

Figure 4. Effect of water deficit on starch content and amylase activity in endosperm, root and shoot of germinating seedlings of six wheat genotypes. Vertical bars represents ± SD based on three independent determinations.

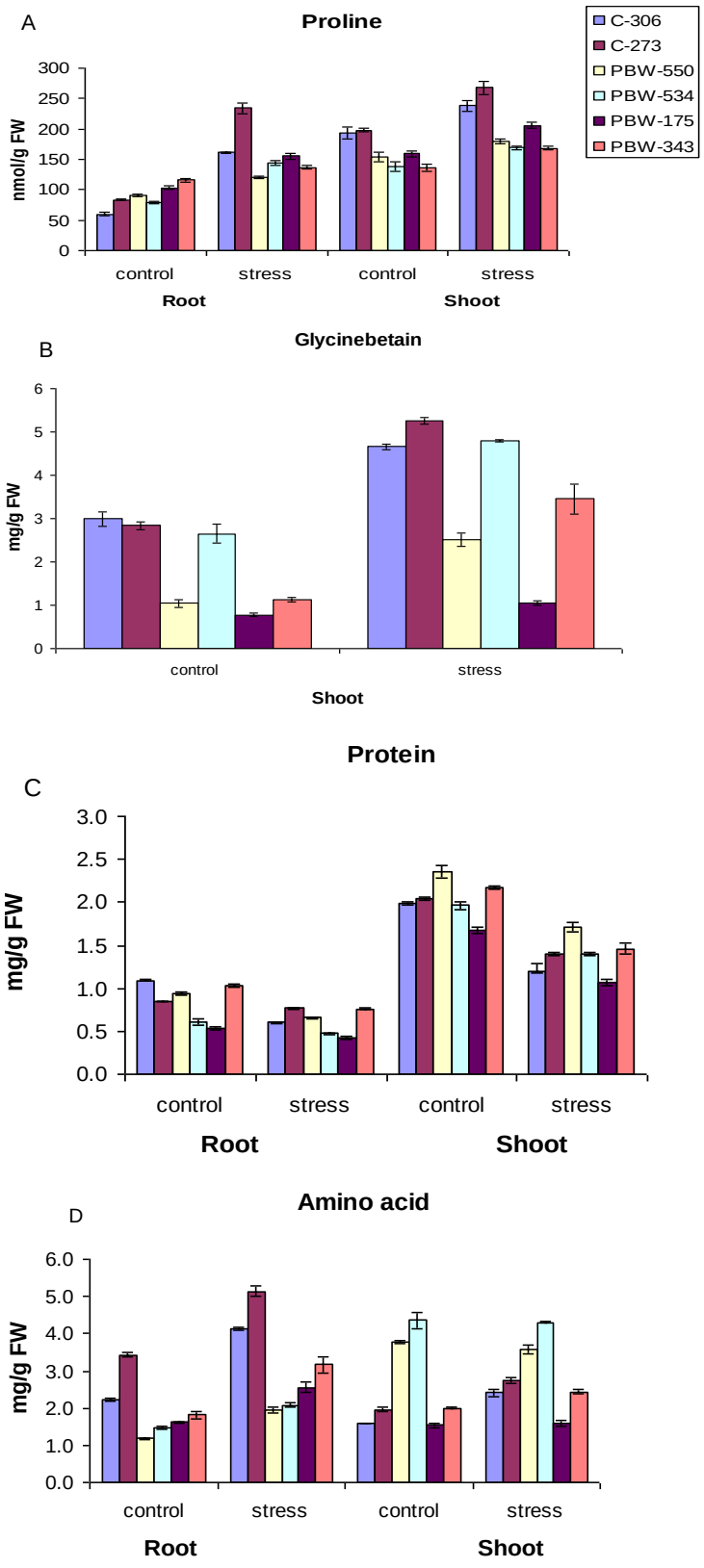

Figure 5. Effect of water deficit on proline (A), glycine betaine (B), protein (C) and amino acid (D) contents in root and shoot of germinating seedlings of six wheat genotypes. Vertical bars represent ± SD based on three independent determinations.

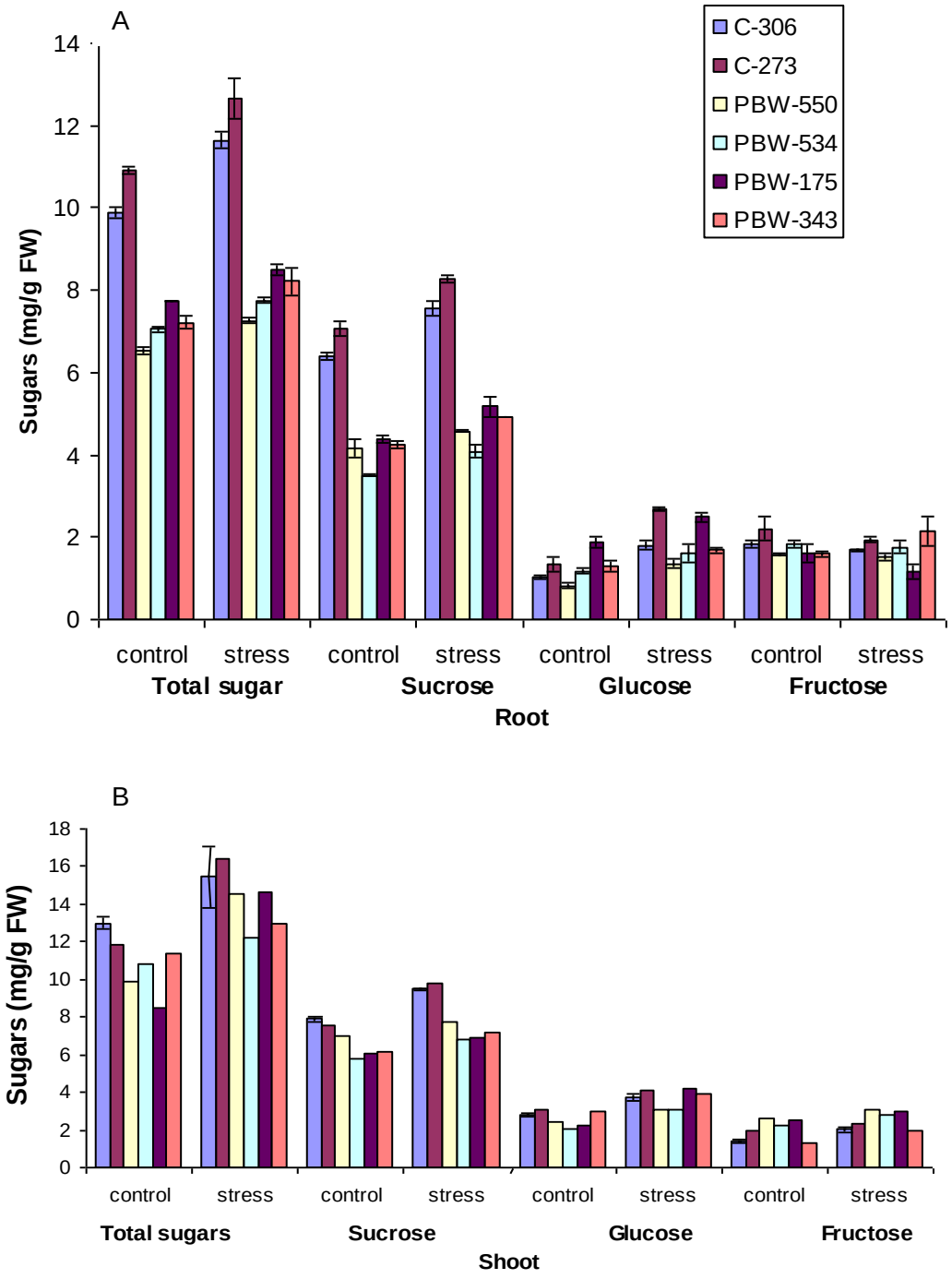

Figure 6. Effect of water deficit on total sugars, sucrose, glucose and fructose contents in root (A) and shoot (B) of germinating seedlings of six wheat genotypes. Vertical bars represents ± SD based on three independent determinations.

promotes thylakoid membrane damage (Rojas et al., 2004).

Water soluble sugars and starch contents also revealed differential response in cvs under water deficit conditions. For instance, total soluble sugars (sucrose, glucose and fructose) were increased in correspondence with a decrease in starch content in root and shoot under stress conditions thus, implying a transformation of starch to sugars. However, in residual endosperm, the levels of starch increased under stress conditions. Our data showed the highest induction of sucrose level in C cultivars over PBW ones suggesting that the induced sugar

production upon stress in the tolerant varieties helped the cvs in effective osmoregulation, which appeared to be central to the development of desiccation tolerance. According to Chaves and Oliveira (2004), accumulation of sucrose promotes osmotic adjustments that act as osmolyte under stress by increasing the gradient for water flux into the cell for maintaining turgor (Kerpesi and Galiba, 2000). Consequently, water stress tolerance was enhanced by faster hydrolysis of starch in shoot and root due to stimulation of amylase activity, so as to maintain the concentration of low molecular weight carbohydrates, which helped plants to retain turgidity and protect protoplasmic constituents. A drought-induced decrease in starch contents was correlated with inhibition of starch synthase activity (Geigenberger et al., 1997). It has been suggested that under water stress, the products from starch hydrolysis could be used as substrate for sucrose biosynthesis (Lee et al., 2008).

Sucrolytic activity catalyzed by invertase and/or sucrose synthase enzyme is the first step in carbon utilization by the majority of tissues in plants, and either enzyme can predominate depending upon the tissue or developmental stage involved. Comparatively, soluble acid invertase activity was higher over other sucrolytic enzymes indicating its predominant role in sucrose catalysis. Higher activities of sucrose synthesizing (SPS) enzymes and low activity of invertases under water stress conditions corresponds to higher build up of sucrose in C varieties. Excess sucrose formed might also be utilized for the growth of seedlings of C cultivars, basically a tall variety. The sugars are taken up into the scutellum, converted into sucrose and then loaded into the phloem for transport to root and shoot. Significance of SPS in drought stress tolerance has earlier been reported by Leprince et al. (2004). Sugars that accumulate in response to stress can function as osmolyte to maintain cell turgor and have the ability to protect membranes and protein from stress damage. It is believed that the continual cycling of its degradation and synthesis is one of the common features of sucrose metabolism in many plant systems (Hill and Rees, 1995).

Proline is a low molecular weight osmoprotectant that helps to preserve structural integrity and cellular osmotic potential within different compartments of cell (Mohammadkhani and Heidari, 2008). Water stress mediated higher accumulation of proline in shoot enables it to maintain osmotic balance than root where the level decreased compared to non stress conditions. The proline level was reached, the highest level in C cvs over PBW ones. The decreased level of proline in roots of stressed wheat seedlings may be due to increased rate of proline utilization over synthesis (Marjorie and Nicholas, 2002). Total amino acid pool was increased by water deficit in all the genotypes and marginal change in protein contents reflects the mode of adjustment to water stress in wheat seedlings. Alternatively, it suggests that pathways of carbon (starch) and nitrogen (protein) biosynthesis were inversely correlated under water stress. Good and Zaplachinski (1994) reported that free amino acid increased under stress which in turn stimulate protein biosynthesis for osmotic adjustment. Increase in soluble protein, proline, glycine betaine might be correlated to synthesis of osmotin-like protein or structural protein which are involved in modification of cell wall (Chaitanya et al., 2010; Zhang et al., 2011).

In summary, our results indicate that sensitivity to water stress is associated with reduced utilization of sucrose and sugars in seedling tissues and considerable differences that exist in sucrose metabolizing enzymes, amylase activity, starch, sugars, proline that could be associated with differential tolerance of cvs to water stress in pre dwarfing era and cultivated varieties. C 306 and C 273 seem to be relatively more tolerant to water deficit than cultivated ones at least partly due to its higher expression levels of sucrose metabolism and osmolytes and lower levels of TBARS content. Results from this study thus provided baseline information and a system necessary to conduct further studies related to the physiological bases of PEG-induced dehydration tolerance.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Application of bioinformatics to optimization of serum proteome in oral leukoplakia and oral squamous cell carcinoma

Hong He[1], Gang Sun[1], Feiyun Ping[2*], Zhijian Xie[1], Huiming Wang[1*], Yining Li[1] and Kai Zhang[1]

[1]Stomatology Hospital, School of Medicine, Zhejiang University. 395 Yan an Rd, Hangzhou, 310006, China.
[2]Second Hospital Affiliated to Medicine College of Zhejiang University 88 Jie fang Rd, Hangzhou 310009, China.

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Intervention to the evolution and metastasis of oral squamous cell carcinoma (OSCC) from oral leukoplakia (OLK) out of normal mucosa is currently far from ultimate. New technologies and insights are reckoned on for etiology and clinical protocol of OSCC and mucosa lesions. SELDI-TOF-MS technology, Support vector machine, Discriminate Analysis, and CM10 protein chip were applied to study the sera proteomes of 32 healthy volunteers, 6 patients with oral mucosa leukoplakia, 28 OSCC patients, and 8 patients with metastatic OSCC. Ratios of protein mass to its charge (m/z) showed in valve peak value were delivered and discriminated out of the huge amount of protein data as group markers for identifications. Protein peak values 4181 and 4651 were high in volunteers serum while low in patients with OLK, the sensitivity of which was 100.00% (32/32), specificity was 83.33%(5/6), accuracy was 97.37%(37/38). And m/z 4162, 6886 of 87.82, 92.86 and 66.67%; 4289, 5661, 6195, 4352, 5072 of 97.22, 100.00 and 87.5% were discriminates between OLK and local OSCC, between local OSCC and regionally metastatic OSCC, respectively. Conclusively, researches are encouraged to launch a proteomics assistance and guidance in modern molecular diagnostic approaches for understanding and controlling the mucosa lesions especially in conquering the malignant progress.

Key words: Oral squamous cell carcinoma (OSCC); oral leukoplakia (OLK), ZUCI-PDAS (Zhejiang University Cancer Institute ProteinChip Data Analysis System), bioinformatics technology, discriminate analysis, proteomes of optimization.

INTRODUCTION

Mucosa lesions have never ceased threatening the human's health even with the development of modern science and technology. Oral leukoplakia (OLK) is the first one among the precancerous oral mucosal lesions, with a canceration rate of 7 to 15%, and 14 to 50% in non-homogeneous leukoplakia. Although, studies have

*Corresponding authors. E-mail: honghehh@zju.edu.cn. Tel: +86-571-87217431. Fax: +86-571-87217423.

Abbreviations: Oral squamous cell carcinoma (**OSCC**), oral leukoplakia (**OLK**)

illuminated that there exist in biomarkers as cytokines, genes, and antigens in the precancerous lesions to escape the failure in intervening the marching of malignance and metastasis of tumor. The reality is that 80% of oral malignant tumors, which rank the sixth in the queue of human malignant tumors, are the oral squamous cell carcinoma (OSCC), whilst the deformation and recurrence rate after surgery treatment are high and the prognosis is out of anticipation (Chen et al., 2004; Ferlito et al., 2002). To search for any meritorious biomarkers to trace the lesions evolution, scientists found that in the mass spectrometer, samples could be respired by laser irradiation and dissociated into gasified ions, in the uniform electric field conditions these ions perform an accelerative out fly, fly through a vacuum tube with no electric field, and eventually captured by the ion detecting receiver. The square of the ion flight time is in inverse proportion to the ratio of molecule size and molecular charge, when the numbers of charge (ions evoked by Surface Enhanced Laser Desorption/Ionization SELDI (He et al., 2009; He et al., 2011; Hu et al., 2005) are usually a single charge are equal, the time of flight associates with the molecule size, the smaller the molecule is, the shorter the flight time would be, hence small molecules were first to reach the receiver. If reflected in a mass spectrum diagram, the peak position corresponds to molecule weight of the relevant protein compositions; and the spectral peak height corresponds to the number of the relevant protein compositions. Thereby proteins could be identified with the peak position and height. This is what the protein fingerprint is. This research is aimed to obtain a sequence of fingerprints of serum protein between OLK and OSCC.

MATERIALS AND METHODS

Serum sample collection and processing

Under informed consent, sera samples of patients and healthy volunteers from the Affiliated Stomatology Hospital and 2nd Hospital of Zhejiang University during Feb 2010 to March 2014 were obtained before any treatment was implemented, and collected in the early morning before breakfast, then immediately separated and stored at -80°C until use, with diagnoses confirmed by post surgical pathology. There were 19 males and 9 females in the 28 cases of local OSCC (L. OSCC) group with a median age as 58.6 years (35 to 88 years range). Four males and 2 females with a median age as 53 years (44 to 59 years range) constituted the OLK group. Seven males and 1 female with a median age as 56.4 years (37 to 71 years range) constituted the regional metastasis group (R. OSCC). 22 males and 10 female with a median age as 50.9 years (34 to 71 years range) constituted the volunteers group (N.).

Proteins bind chip and spectrometer detection

After thawing and 2 min of centrifugation (10,000 r/min), 5 µl serum sample was added into 10 µl 0.5% U9 (9 mol/L urea, 0.2% CHAPS (3[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate), 0.1% DTT (DL-dithiothreitol) in a 96-well plate and incubated for 30 min at 4°C with 600 r/min vigorous shaking. The ProteinChip array cassette was put into a 96-well bioprocessor and 200 µl NaAc (50 mmol/L, pH 4.0) was put into each well, also incubated for 5 min at 4°C with 600 r/min vigorous shaking. Supernatant was collected and the procedure is repeated once. Then, 185 µl NaAc was added into each well in the 96-well plate (600 r/min, 2 min) and 100 µl samples of different patients disposed as above were separately added into different well of the ProteinChip array cassette (600 r/min, 1 h). After the content from each well was removed, each well was washed with 200 µl NaAc (pH = 4.0, 600 r/min, 5 min). This procedure was repeated two more times. Each spot was washed with 200 µl HPLC water, which was removed immediately. This was repeated once. After natural air drying, 1 µl SPA (sinapic acid) was applied to each spot. After natural drying for another 5 min, another 1 µl SPA was applied. Naturally dried again.

American Ciphergen SELDI Protein Biology System II plus (PBS II plus) and ProteinChip Software (Version 3.0, Ciphergen Biosystems) were used to read the chips and analyze the data. Setting parameters: laser intensity 165, 65 laser shots per sample, detector sensitivity 7, automatically detected peaks from 2,000 to 30,000 *m*/*z*. Mass accuracy was calibrated to less than 0.1% using the All-in-1 peptide molecular mass standard chip (Ciphergen Biosystems). The peaks were normalized and noises were filtrated (first Signal to Noise ratio >2.5). Peak clusters were completed using second-pass peak selection (Signal to Noise ratio>4, within 0.3% mass window) and estimated peaks were added. Discrepant mass peaks of different groups were identified by support vector machines (SVM) applying radially based kernel function with Gamma value as 0.6, Penalty score function C as 19, each m/z peak was proved with Wilconxon rank test ($p < 0.05$).

Database analysis and statistical validation

Bioinformatics studies were integrated in the ZUCI-PDAS (Zhejiang University Cancer Institute ProteinChip Data Analysis System) available at www.zlzx.net. Samples of group models from different stage were developed and validated by SVM, discriminate analysis and time-sequence analysis. These statistical analysis tools were implemented by Matlab-nn Tools software. Training was conducted to converge on the training data and to minimize the biases. Discriminate analysis and SVM models introduced random perturbations in multiple runs to test the consistency of the top 10 ranked peaks, measured by the *P* value of *m*/*z* peaks of computed ranks from multiple runs. Stage models were built using the selected peaks. Moreover, leave-one-out cross-validation approach was applied to estimate the accuracy of the classifier to determine the misclassification rate. For each step of the cross-validation, one sample was left out. The possibility of obtaining a small cross-validated misclassification rate by chance was obtained by repeating the entire cross-validation procedure using *n* random permutations of the class labels for the clinical criteria being evaluated. The ultimate candidate biomarkers of the highest Youden's index are selected out during group validation. The models established are based on these selected biomarkers should be further validated independently. In such studies, validation datasets should be preferably derived from sources different from that of the original training dataset. This is one way to ensure that the performance of the selected biomarkers is not influenced by systematic biases between different groups. Time-sequence analysis was used to distinguish different stage groups.

RESULTS

After the tremendous comparison data from the mass spectrums, 112, 108, 120 discrepant protein mass peaks between sera of 32 healthy volunteers and 6 OLK patients,

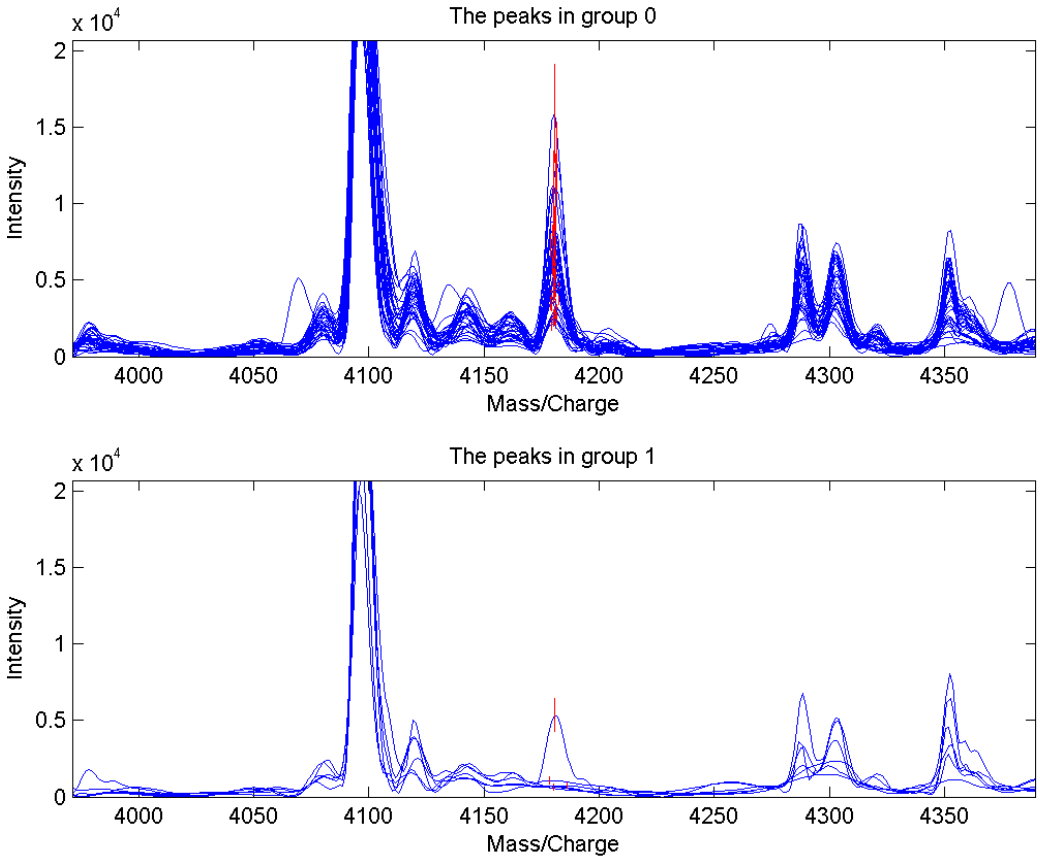

Figure 1a. Serum protein mass/charge wave peak *4181* expressed significantly high in the healthy volunteers group (group 0), while remarkably low in the group of patients with OLK (group 1), *P*=0.0001307483.

28 OSCC patients, 28 locally sited OSCC patients and 8 regionally metastatic OSCC were respectively exhibited as significant difference, among which ten of the lowest *p* value of the integrated proteomes constituted respectively of 2, 2, 5 protein peaks (Figure 1a to c, Tables 1 to 4) were eventually selected out as the highest Youden's index via SVM Discriminate Analysis and as common key sense upon corresponding and simultaneous changes. They were 4181 mutually together with 4651; 4162 together with 6886; 6195, 5661, 4289~~ and 4352 together with 5072. These ultimately obtained individual protein peaks and integrated discrepant serum proteomic selections with sensitivity, specificity, and accuracy and with some of the peak figures were presented as shown in Figures 1a to c.

DISCUSSION

The expression profiling of serum proteins from human with OLK or OSCC and healthy volunteers was investigated by a combination of proteomic techniques in this study. Different to Panicker's research (Kordy et al., 2012), which is currently lack of diseases links such as to carcinoma with metastasis or not, and in some cases to pre-invasive disease, and although our study is temporally lack of protein identification, our present research can be used to further some understanding of the serum proteomic behaving from individual to universal of oral mucosal lesion for tumorgenesis etiology as well as tumor evolution, and provide a snapshot of the proteome for diagnosis, prognosis and prophylaxis of oral mucosal disease. Validated protein biomarkers could be useful in early detection of disease and any possibility to be getting malignant or metastatic, monitoring disease progression or monitoring response to treatment. Upon neoplasia arises, it is likely to study proteins produced by the local lesion as well as by the host reaction in serum with response to the lesion. Advances in proteomic technologies have greatly accelerated the field of protein biomarker discovery (Nemet et al., 2005; Panicker et al., 2009; 2010). High-throughput technology Surface Enhanced Laser Desorption and Ionization Time of Flight Mass Spectrometry (SELDI-TOF-MS) has achieved tremendous harvests from different cancerous field to infectious diseases or drugs for researches (Sun et al.,

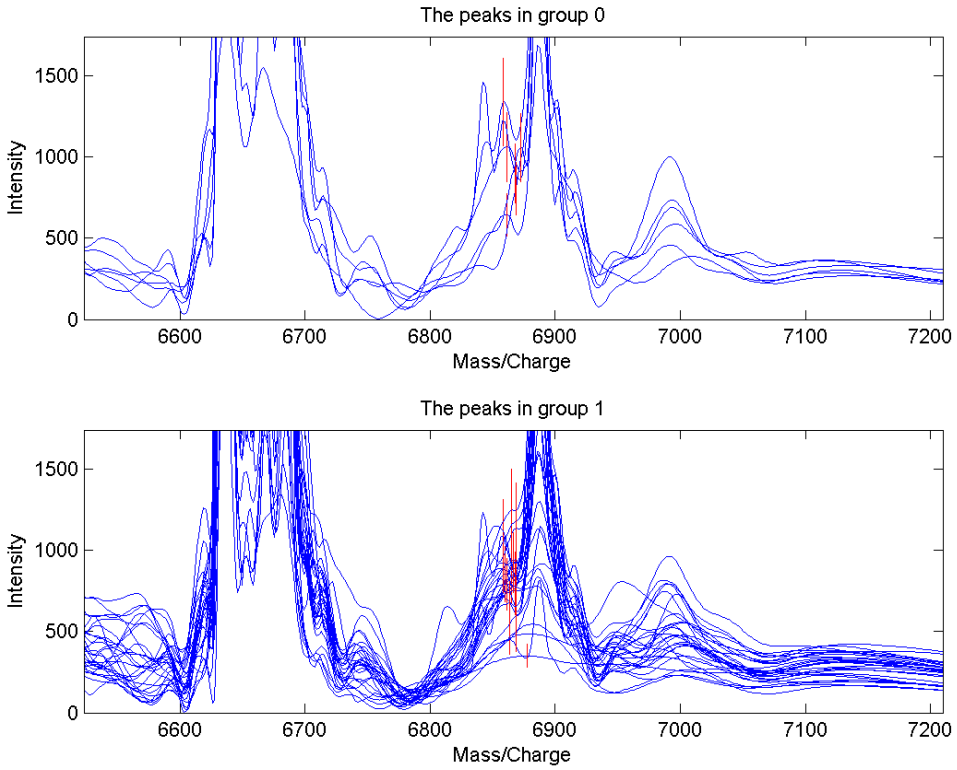

Figure 1b. Serum protein mass/charge wave peak 6886 expressed statistically different between the group of patients with OLK (group 0) and the group of patients with OSCC (group 1), *P*=0.0015641816, together with m/z 4162 protein, established the distinguished proteome with a highest Youden's index.

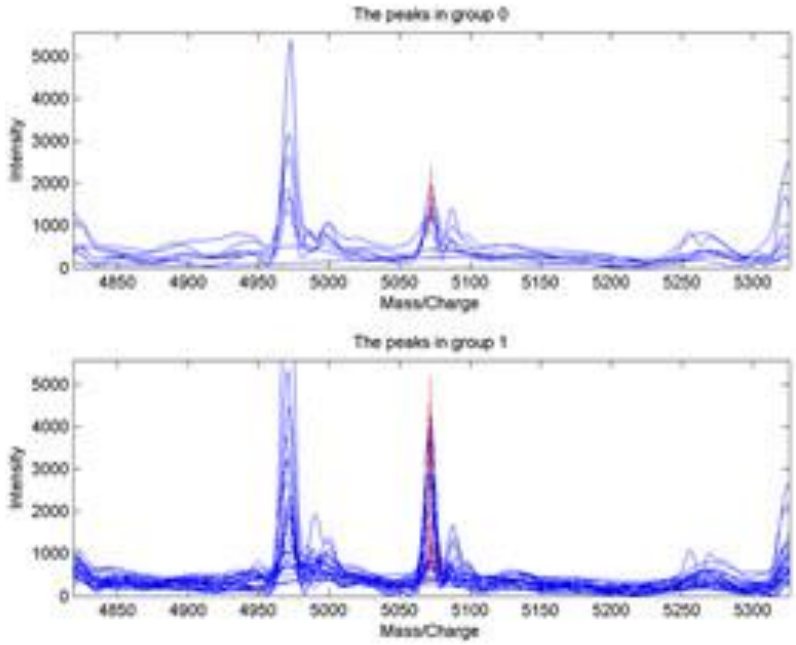

Figure 1c. Serum protein mass/charge wave peak 5072 expressed significantly low in the group of patients with metastasis OSCC (group 0), while remarkably high in group of patients with local OSCC tumor (group 1), although *P*=0.1529314178, it was one of the distinguished proteome with a highest Youden's index between the two compared groups.

Table 1. Discrepant protein peaks between OLK patients and healthy volunteers (x±SD).

m/z Peak	*p* value	Healthy volunteer (group 0)	OLK patient (group 1)
4651	0.0000086626	1905.30±548.37	686.69±386.12
4181	0.0001307483	5901.24±2548.82	1333.55±1042.86

Table 2. Discrepant protein peaks between OLK patients and local OSCC patients (x±SD).

m/z Peak	*p* value	OLK patient (0)	Local OSCC patient (1)
6886	0.0015641816	3551.45±1833.51	1818.32±921.62
4162	0.0365123759	1101.78±373.05	1644.09±579.45

Table 3. Discrepant protein peaks between local OSCC patients and regional metastatic OSCC patients (x±SD).

m/z Peak	*p* value	R. metastatic OSCC (1)	Local OSCC patient (0)
6195	0.0015387984	893.32±382.21	578.99±165.48
5661	0.0061131375	771.95±255.40	1167.44±355.59
4288	0.0289773483	1865.87±772.37	2891.56±1196.07
4352	0.0540207442	3663.07±946.03	2708.56±1249.09
5072	0.1529314178	1010.51±384.44	1518.43±952.59

Table 4. The sensitivities, specificities and accuracies of integrated discrepant serum proteomes in OLK, local (L) and regional (R) metastatic OSCC patients, and healthy volunteers.

Sample (0 vs 1)	m/z Peaks proteome	Sensitivity (%)	Specificity (%)	Accuracy (%)
N. vs OLK	*4181*，*4651*	97.37	83.33	100.00
OLK vs L. OSCC	4162, 6886	87.82	92.86	66.67
L. vs R. OSCC	4289, 5661, *6195*, *4352*, 5072	97.22	100.00	87.5

Note: Italic values of protein peak represent high expressions in group 0; regular scripts represent high expression in group 1.

2009; Weinberger et al., 2000; Xu et al., 2006; Yu et al., 2004; Yu et al., 2005; Zhou et al., 2013). Of the current proteomic tools, no single method can resolve an entire proteome. Combination of several methods like Zhejiang University Cancer Institute ProteinChip Data Analysis System (ZUCI-PDAS) with SELDI-TOF-MS (He et al., 2009; 2011; Hu et al., 2005) and SVM established the Bioinformatics. One of the merits of this study protocol is the Discriminate Analysis derived from ZUCI-PDAS and SVM has launched the other merit of it to link to the development of diseases for the biological meaning of the different preferential expressions (He et al., 2009; 2011; Hu et al., 2005).

On the one hand, SVM technique is proper to solve the limitation of samples, and on the other hand, our future work still would take into consideration to enlarge the samples as well as about the necessity to identify the proteins in the optimized group peaks. Current study indicates that progressive study of such prognostic biomarkers which were based on tumor phenotype and biologic behavior, no matter proteomes of protein mass peaks or characterization of individual protein, would allow clinicians not only to diagnose a disease involving OSCC as well as precancerous lesion like OLK, but also to select the most efficient treatment modalities other than absolutely radical surgery.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Geochemical aspects of *Meretrix casta* (bivalve) shells of Vellar estuary, southeast coast of India

Shoba, K. and Senthilkumar, G. R*

Department of Earth Sciences, Annamalai University, Annamalainagar, 608 002, India.

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The bivalve mollusk, *Meretrix casta* shells are abundant in the Vellar estuary along the East coast of India, they are economically important and used as chief raw material for many lime based industries. Their shells are harvested in large quantities for meat and lime production. The present study focused on understanding the chemical characteristics of *M. casta* shells, collected at eight different locations in the marine zone of Vellar estuary. Slica, alumina, iron, calcium and magnesium were determined by wet analysis method. The concentrations of Cu, Fe, Zn, Cd, Hg and Mg were analysed by inductive coupled plasma - optical emission spectroscopy (ICP-OES). The *M. casta* shells consists of calcium which is up to 54%, silica, aluminum, iron and magnesium constituents are very small. Metal concentrations in the shells were in the following order: Fe>Mg>Zn>Cu. The results of heavy metal concentrations was Fe (1.822 mg/l), Mg (0.420 mg/l), Zn (0.026mg/l) and Cu (0.017 mg/l), the elements Cd and Hg were below detection limit in all the eight locations. The result evidenced that the shells were suitable for industrial applications especially for lime based industries.

Key words: Raw materials, bivalve, *Meretrix casta*, ICP-OES, heavy metals, Vellar estuary.

INTRODUCTION

Meretrix casta (Chemnitz) (Bivalvia: Family Veneridae) occurs in extensive and dense beds all over the East coast of India (Abraham, 1953). It is fairly abundant in Pulicat Lake, Kovalam backwaters, Muthupet swamps, Vellar estuary and Vaigai estuary (Nayar and Mahadevan, 1980). Clam production in Vellar estuary is about 730 t/year (Silas et al., 1982). In Vellar estuary, among the bivalves, *M. casta* and *Tellina* sp. were commonly encountered; the former accounting for 80 to 90% of the total benthic population (Chandran, 1987). *M. casta* is harvested in large quantities from Vellar estuary region for meat and provides a source for proteins, lipids and minerals. Sugesh and Mayavu (2013) studied that *M. casta* are rich in protein and antimicrobial compounds. *M. casta* shells (without animal) are used as a raw material for pulp making, bleaching, pharmaceuticals, leather tanning, shell grit, lime and cement manufacturing industries (Rao, 1969; Alagarswami and Narasimham, 1973; Rasalam

*Corresponding author. E-mail: gr_senthilkumar@yahoo.com.

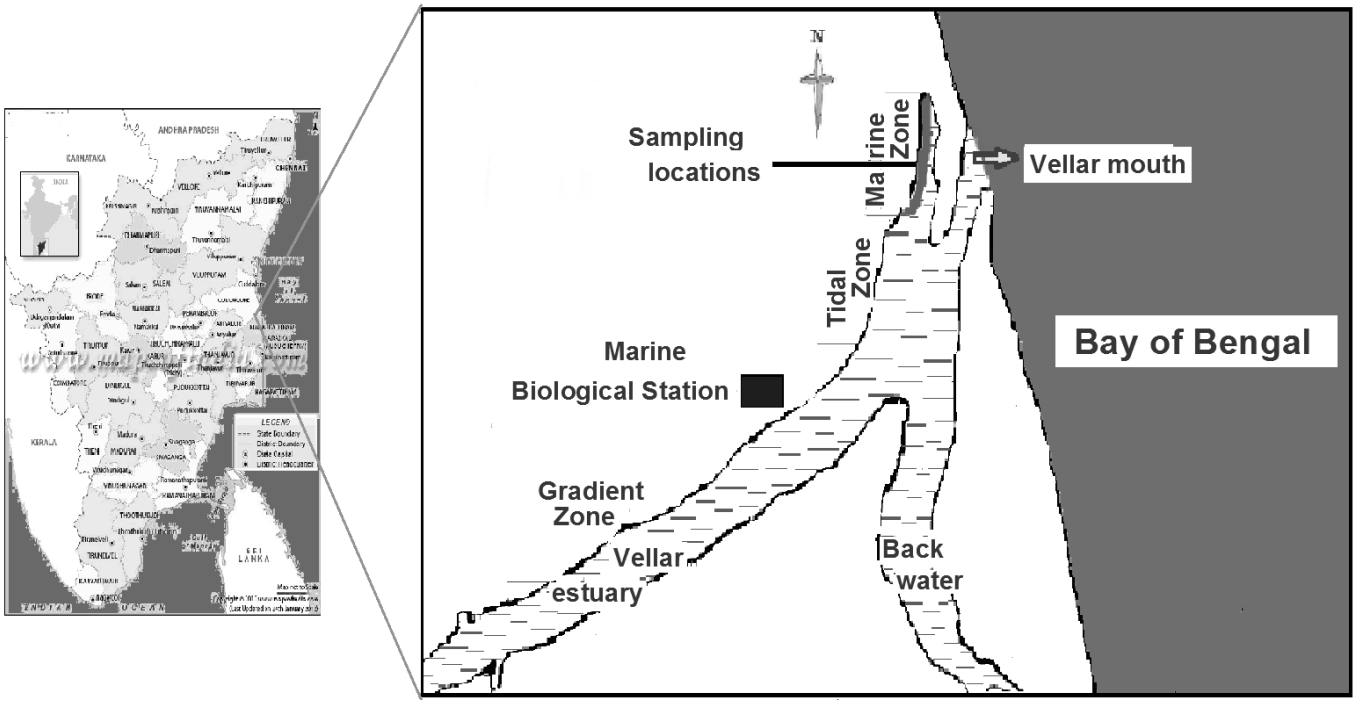

Figure 1. Map showing the study area and sample locality.

and Sebastan, 1976). In agriculture, it is used as pesticide, manure in plantation and poultry-feed. Until recently, shells were burnt merely for mortar and plaster in building work. But, now it is vastly used in paper mills, rayon fibre, calcium carbide, white cement manufacturing and biomaterials for orthopaedic applications (Awang-Hazmi et al., 2007).

In fact, mineral resources are finite and non-renewable resources. Many important minerals are depleting very drastically by mining activities including limestone resource, which is the chief raw material for many lime based industries. In the future, *M. casta* shells will play significant role in sustainable industrial development as well as in countries economic growth, because these are replenishing resources. Fewer studies have been done on economic valuation of bivalves (Boominathan et al., 2008) and the use of *M. casta* shells of east coast of India for preparation of lime (Panda and Misra, 2007). M/S. Travancore Cements Ltd. (TCL), Kerala, India is reportedly the only cement manufacturing industry in the country using lime shell as raw material and mining from Vembanadu backwaters. The white cement made from lime shell is considered to be highly durable and superior in quality, due to absence of magnesium oxide. This product enjoys good reputation in the country and the yearly requirement of lime shell for white cement production by TCL is about 40,000 tonnes (Ravindran et al., 2006). Consequently, the requirement of raw material for lime based industries depends on the lime shell resources in the backwaters/estuaries, river mouths and lagoons along the coastal tract. Geochemical aspects of *M. casta* shells found in the study area in literature are inadequate, particularly with reference to major and trace elemental variations. Comprehensive analysis has shown the chemical composition of shells of this species. To the best to our knowledge, this kind of study may be the first in the study area. Hence, the present study was done to assess the geochemical aspects of the *M. casta* shells from Vellar estuary along the southeast coast of India.

MATERIALS AND METHODS

Study area

The Vellar estuary lies between (latitude 11°17’23” N and longitude 78° 50’24” E), the southeast coast of India, which originates from the Shervarayan hills of Salem District in Tamil Nadu, India. After traversing a distance of about 480 km, it forms an extensive estuarine system at Parangipettai, before it joins with the Bay of Bengal. The Vellar estuary is always open with the Bay of Bengal and it is said to be a “true estuary” as there is no complete closure of the mouth. In terms of salinity characteristics, the estuary is demarcated and divided into four zones based on Rochford (1951) classification of estuarine environment, viz. marine zone, gradient zone, tidal zone and freshwater zone (Ramamoorthi, 1954; Antony Fernando et al., 1983). Geologically, Vellar estuary belongs to quarternary period. The estuarine complex is bestowed with a variety of biotopes such as mangroves and backwaters (Kesavan et al., 2010). The hard clam has a thick, moderately large shell with a brown horny periostracum. A dark greyish band is present at the posterior margin of the shell. The clam reaches a height of 48.7 mm and a width of 38.7 mm in about 18 months. A map of the study area is shown in Figure 1.

Table 1. Microwave parameters for *M. casta* shells digestion.

Maximum power	Power used	Ramp time	Temperature	Holding time
1600 W	100%	15:00 min	200°C	15:00 min

Table 2. Elements symbol detection Wavelength (nm) by ICP-OES.

Cu	Fe	Zn	Cd	Hg	Mg
327.393	238.204	206.200	228.802	253.652	285.213

Table 3. Chemical composition of *M. casta* shells of Vellar estuary.

Location number	SiO_2 (%)	CaO (%)	MgO (%)	Fe_2O_3 (%)	Al_2O_3 (%)	LOI
1	0.10	54.03	1.59	0.08	0.04	44.15
2	0.10	54.04	1.51	0.08	0.04	44.10
3	0.11	54.07	1.53	0.09	0.04	44.14
4	0.10	54.05	1.57	0.08	0.03	44.16
5	0.11	54.06	1.55	0.08	0.04	44.15
6	0.12	54.09	1.52	0.09	0.03	44.10
7	0.11	54.08	1.54	0.09	0.04	44.12
8	0.10	54.20	1.55	0.09	0.03	44.15
Total	0.85	432.68	12.36	0.68	0.29	353.13
Mean	0.11	54.09	1.55	0.09	0.04	44.14

Sample collection

M. casta shells were collected along the marine shoreline (this zone extends up to 0.8 km upstream from the mouth of the river) of Vellar estuary during the month of May, 2012. The shells were collected within eight 1 x 1 m squares in the entire study area. Distance between sample spots was 100 m. In the demarcated area, available *M. casta* shells were collected by hand digging and picking irrespective of size. The shells were then washed with seawater at the point of collection and placed into clean plastic bags and packed separately with sample numbers.

Sample preparation

In the laboratory, the samples were washed thoroughly with clean water and dried. Then, the shells were crushed and pulverized into fine powder with an iron-mortar, agate-mortar and pestle. The well mixed powder samples were tagged separately according to the location numbers for major and trace elemental analysis.

Chemical analysis

Major elemental analysis of *M. casta* was done by wet analysis method and results are shown in compound percentage. The loss on ignition (LOI), silica (SiO_2), iron (Fe_2O_3), alumina (Al_2O_3), calcium (CaO), and magnesium (MgO), were determined. Loss on ignition is about 44.15% which is common in organic shells.

Trace metal concentrations were measured by inductive coupled plasma - optical emission spectroscopy (ICP-OES) using a Perkin Elmer, Optima 5300; 0.5 g of shell powder was taken for analysis and one time aliquots were measured. A total of eight samples were taken for trace metal concentration studies. The samples were digested with 5 ml of concentrated HCl; after digestion, insoluble remains were not found. After the digestion, the solution was transferred and filled into 50 ml with de-ionized water, the clear solution was analyzed. No double correction, bulk analysis was made and organic matrix in the shell was not determined.

The concentrations of Cu, Fe, Zn, Cd, Hg and Mg in the shells of *M. casta* were determined. To analyse trace metal concentration in bivalve shells, the widely used method was used (Lazareth et al., 2003; Gillikin et al., 2006; Uysal et al., 2008; Ravera et al., 2009: Schöne et al., 2010; Voslooa et al., 2012; Yesudhason et al., 2013). The samples were digested with 5 ml of aquaregia in CEM microwave digester using MARSX Press (self-regulating microwave vessel) microwave digester under the following conditions (Table 1), the wavelengths used for determination of elements is shown in Table 2. The values are expressed as mg/l dry sample.

RESULTS

Major elements

The *M. casta* shells are one of the best sources of high grade lime. They are also easy for calcinations. Major analytical results of *M. casta* shells collected in marine zone of Vellar estuary is shown in Table 3.

The average CaO is 54.09% of the total mineral consti-

Table 4. Heavy metal concentration of *M. casta* shells of Vellar estuary (concentrations in mg/l).

Location	Cu	Fe	Zn	Cd	Hg	Mg
1	0.018	1.56	0.03	BDL	BDL	0.369
2	0.012	2.041	0.022	BDL	BDL	0.274
3	BDL	0.947	BDL	BDL	BDL	0.24
4	BDL	1.249	BDL	BDL	BDL	0.268
5	BDL	1.173	BDL	BDL	BDL	0.241
6	BDL	1.308	BDL	BDL	BDL	0.248
7	0.014	3.477	BDL	BDL	BDL	0.911
8	0.023	2.823	BDL	BDL	BDL	0.808
Total	0.067	14.578	0.052	-	-	3.359
Mean	0.017	1.822	0.026	-	-	0.420

*BDL, Below detectable limit.

tution. The average silica content in the shells is about 0.11%. The total iron has been estimated in the form of Fe_2O_3. The Fe_2O_3 content is very small (0.08-0.09%) and the Al_2O_3 compound percentage range between 0.03-0.04% and average is 0.04%.

Heavy metals

The mean heavy metals concentration of *M. casta* shells of Vellar estuary is shown in Table 4.

DISCUSSION

Iron (Fe), Mg, Zn and Cu were the main abundant heavy metals; Cd and Hg were below detectable level (BDL). Fe concentrations (1.822 mg/l) were significantly higher in *M. casta* shells of Vellar estuary as compared to Mg (0.420 mg/l), Zn (0.026 mg/l) and Cu (0.017 mg/l), concentrations of Cd and Hg in the shells of *M. casta* showed below detection limit in all eight locations, indicating that these metals are imprecise in the shells.

Maximum of the Fe concentration is 3.477 mg/l, which is highest concentration in the shells when compared with other heavy metal concentrations. Ramanathan et al. (1999) observed that higher concentration of Fe in the mangrove sediments might be a result of the textural and mineralogical characteristics of the mangrove sediments. In this context, it is important to note that clays and feldspar were the dominant mineral species present in the sand and silt size populations of the sediments. Kesavan et al. (2010) identified the source of Mg concentration in tissue and shells of Vellar estuary. The authors stated that agricultural activities and the release of fresh water from reservoirs which contain high heavy metal concentrations and drains into estuary and mix up with the seawater and also hundreds of boats are available in the study area, which is also an additional source of Mg. Srilatha et al. (2013) found that the Cu concentration in *M. casta* tissue of Vellar estuary were 2.33 mg/l, whereas in the present study, Cu level in shells was 0.017 mg/l, which indicates that Cu concentration is not distributed to shells significantly. Among the eight locations, Zn was reported in only three locations with an average concentration of 0.026 mg/l, Cd and Hg were not reported in all the eight locations. The studied shells heavy metal concentrations are low and they are only in reporting level.

Conclusion

The result revealed that the shells are potential source of lime with low amount of silica, alumina, iron and magnesium. Based on ICP-OES analysis, the concentration of Fe, Mg, Zn and Cu were in least amount and they are only in noticeable levels. The Cd and Hg were below detectable limits. The present study has shown that enough scope for the development of lime based industries in the study area, owing to the availability and quality of *M. casta* shells which are rich in calcium content and poor in Mg content is most suitable for lime making.

Conflict of Interests

The author(s) have not declared any conflict of interests

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Protamine 3 expressions in crossbred bull spermatozoa may not be a prognostic marker for differentiating good and poor quality semen

Sushil Kumar, Umesh Singh, Indrajit Ganguly#, Rajib Deb*, Rani Singh, Sandeep Mann, G. Sengar, D. K. Mandal, Mahesh Kumar and Arjava Sharma

Molecular Genetics Laboratory, Project Directorate on Cattle (ICAR), Meerut, Uttar Pradesh-250001, India.

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Protamines are short and highly basic sperm-specific nuclear proteins. Though the expression profile of protamines 1 and 2 is well known, but there is little knowledge of protamine 3 expression in bovine semen. In this study, Frieswal (HF x Sahiwal) crossbred bulls were categorized into two groups (good and poor quality) based on the initial progressive motility of semen and other seminal parameters. The mRNA expression of PRM3 gene among two groups was evaluated by real time quantitative polymerase chain reaction (PCR) using TaqMan chemistry, where peptidylprolyl isomerase A (PPIA) was used as an internal control. Our finding revealed that expression of PRM3 was down regulated in poor quality semen producers as compared to good quality semen producing group, but unfortunately no significant difference of transcript abundance was observed between the groups. To shed light on present findings, it can thus assume that PRM3 may not be a prognostic marker to differentiate good and poor quality bull semen in Frieswal cattle.

Key words: Protamine 3, Frieswal, semen, expression.

INTRODUCTION

To differentiate finally into spermatozoa, haploid spermatids undergo complex morphological and physiological changes during spermatogenesis. These changes include chromatin remodeling and condensation mediated through the replacement of somatic histones by transition proteins and protamines (Wykes and Krawetz, 2003). The first step in this process occurs in haploid round spermatids and involves replacement of somatic histones with the transition proteins (TNP1 and TNP2). Subsequently, in elongating spermatids, the protamines (PRM1 and PRM2) replace TNP1 and TNP2. The resulting chromatin is highly condensed and transcriptionally silent. In bovine, PRM1, PRM2, and TNP2 genes encode basic chromosomal proteins and are located in a compact gene cluster as observed in mouse, rat and human (Balhorn et al., 2000; Engel et al., 1992; Ferraz et al., 2010; Schluter et al., 1992; Singh and Rao, 1988; Le Lannic et al., 1993). The protamine gene cluster contains a fourth gene, designed protamine 3 (PRM3), located between the PRM2 and TNP2 genes in rat, human and mouse

*Corresponding author. E-mail: drrajibdeb@gmail.com. Tel: +911212645598. Fax: +911212656021.

Table 1. Seminal qualities (least square mean values) of good vs poor quality semen producing Frieswal bulls for the present study.

Particular	Good	Poor
Volume (ml)	$4.39 \pm 0.27_a$	$3.11 \pm 0.26_b$
Concentration (millions/ml)	$923.433 \pm 106.21_a$	$692.23 \pm 99.65_b$
Number of sperm/ejaculate	$3905.66 \pm 94.62_a$	$2039.24 \pm 198.01_b$
Motility (%)	$57.61 \pm 1.41_a$	$18.45 \pm 1.61_b$
PTM (%)	$40.24 \pm 6.24_a$	$26.88 \pm 5.92_b$

a,b Row wise means with different subscripts differ significantly at $p < 0.05$. PTM: Post thaw motility.

(Singh and Rao, 1988; Schluter and Engel, 1995; Kramer and Krawetz, 1998; Cho et al., 2001; Schluter et al., 1996). The timing of PRM3 mRNA translation is developmentally regulated in spermiogenesis and it codes for a small intronless protein localized in the cytoplasm of elongated spermatids (Kleene, 1989; Schluter et al., 1996). It has also been established that the PRM3 gene is conserved in diverse mammalian species including bovine and PRM3 –/– knockout experiment showed a reductions in sperm motility of male mice, lacking PRM3 protein, without compromising their fertility (Kleene, 1989). To date, the PRM3 gene has not been studied adequately and there is a paucity of published literature describing its function, expression profile, regulation pattern and association with sperm motility and fertility in bull. Recently, we have observed higher PRM1 transcripts in normal crossbred Frieswal bulls compared to motility impaired group ($p<0.05$) as well as a non-significant difference of PRM2 transcripts between the groups (Grzmil et al., 2008). The present research was undertaken to elucidate the PRM3 transcript abundance in ejaculated spermatozoa of normal and impaired crossbred Frieswal (HF X Sahiwal) bull semen using Taqman-real time PCR based quantification.

MATERIALS AND METHODS

Fresh semen samples were collected from categorized crossbred Frieswal bulls into normal (good) and impaired groups (poor) according to their seminal quality parameters viz. volume, concentration, number of sperm/ejaculates, progressive motility (>40% considered as good) and post thaw motility (PTM). The concentration of sperm was estimated by using a photometer (Accucell, IMV- France). The testis tissues of Buffalo (*Bubalus bubalis)* were collected in RNA later and stored at -80°C before use. Assessment of membrane integrity (HOST test) of spermatozoa was performed as per procedure described elsewhere (Grzmil et al., 2008). To rule out the possibility of spermatozoa and contaminating somatic cells, the semen samples were purified through a discontinuous Percoll (Sigma-Aldrich) gradient (40:80) centrifugation (20 min at 300 *g*, 25°C) as described earlier (Grzmil et al., 2008). The motile spermatozoa were kept at -80°C in RNA later (Ambion, Austin, TX, USA) until RNA extraction. The quantity and purity of total RNA isolated from purified spermatozoa and buffalo testis were measured by using ND-1000 spectrophotometer (Nano-Drop, Fisher Thermo, and Wilmington, DE, USA).

Total RNA isolated from crossbred bull spermatozoa and buffalo (*B. bubalis)* testicular tissues were reverse transcribed to complementary DNA using Random primers and M-MuLV reverse transcriptase (Protoscript, NEB) according to the manufacturer's instructions. The cDNA product was stored at -20°C. Genomic DNA contamination was checked by PCR, using intron spanning primer specific to bovine PRM1 (Forward 5'AGATACCGATGCTGCCTCAC3', Reverse 5'GTGGCATGTTCAAGATGTGG3) gene. Bovine genomic DNA isolated from blood by GenElute™ Blood Genomic DNA Kit (Sigma–Aldrich) was used as a positive control. A diluted 1:10 solution of the cDNA was used to elucidate the differential expression of PRM3 in mature spermatozoa of good and poor quality crossbred Frieswal (HF X Sahiwal) bull. The expression of PRM3 mRNA was quantified by real-time PCR (Step One, Applied Biosystems, Foster City, CA, USA) using TaqMan probe chemistry. The PRM3 (FAM, PN4351372) and PPIA (VIC, PN4448489) gene probes were obtained from Applied Biosystems (Foster City, CA, USA). PPIA gene was used as an endogenous control. All the PCR reactions were performed in optical 48-well reaction plates in triplicate. The amplification was carried out in 10 µl volume containing 5 µl TaqMan 2X Universal PCR Master mix, 0.5 µl Probe (20X), 2 µl of cDNA template and 2 µl DNase/RNase free sterile water. PCR cycling conditions were: initial denaturation of 95°C for 10 min followed by 40 cycles of denaturation 95°C for 30 s; annealing for 60°C for 60 s and extension 60°C; 55 s. For gene of interest negative and positive controls were included. Samples were quantified by the ΔΔCt method (Martin-Coello et al., 2011).

Data are presented as mean ± SEM and analyzed by using SPSS statistical program (SPSS 10.0 for Windows; SPSS, Inc., Chicago, IL, USA). The experiment was reproduced thrice. Significant differences were determined by one-way ANOVA using the SPSS program. Gene expression pattern of the PRM3 gene between good and poor quality semen producers were compared using Student *t*-test.

RESULTS AND DISCUSSION

Bulls were categorized into normal (good) and impaired (poor) groups based on basic semen parameters like volume, sperm concentration, number of sperm/ejaculates, initial progressive motility and post thaw motility (PTM). The overall seminal attributes of bull spermatozoa categorized as good and poor are shown in Table 1. Presence of genomic DNA, the intron-spanning primers of PRM1 gene produced an amplicon of 334 bp, whereas a single band corresponding to the size of 234 bp observed in case of pure cDNA without genomic DNA contamination (Figure 1). Sperm cDNA samples, devoid

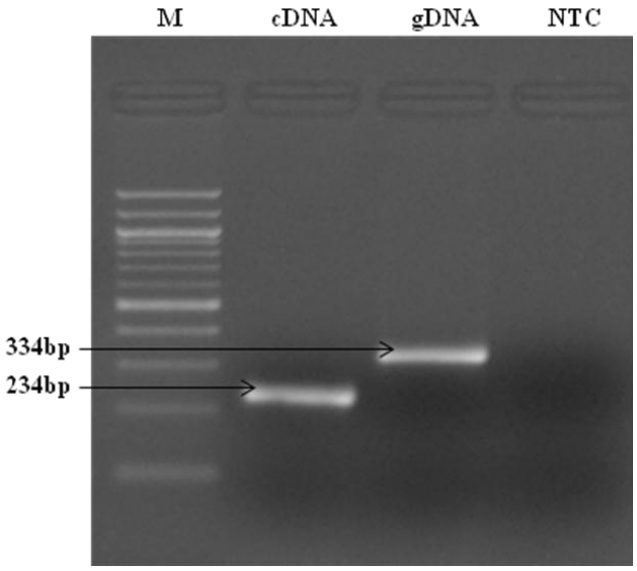

Figure 1. The intron-spannig primer of PRM1 was used to detect the genomic DNA contamination. Lane 1 = M, 100 bp DNA ladder, Lane 2 = PRM1 produced 234 bp amplicon size with cDNA sample, Lane 3 = PRM1 produced 334 bp amplicon size with gDNA sample and Lane 4 = NTC, No template control.

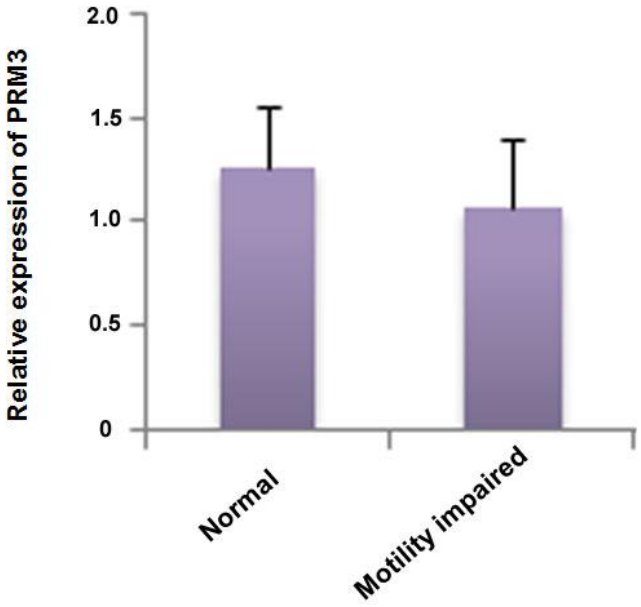

Figure 2. PRM3 mRNA expression in normal (good) (n=10) and motility impaired (poor quality) (n= 9) semen producing Frieswal bulls. Expression of mRNA was normalized using PPIA (Peptidyl Prolylisomerise A) as housekeeping gene. Data of two independent experiments indicating mean mRNA expression ± standard error for triplicates.

of genomic DNA contamination, were used for down-stream qPCR. The mRNA expression of PRM3 was monitored in good (normal) and poor (motility impaired) quality semen producing crossbred Frieswal bulls. In the present investigation, we observed the relative transcript abundance of PRM3 was down regulated in poor quality crossbred Frieswal bulls, however, the difference was not significant ($p>0.05$) between the groups (Figure 2). Similarly, we also analyzed the different relative mRNA expression profile of PRM3 in Frieswal bull semen categorized according to semen volume, concentration, number of sperm/ejaculates and post thaw motility (PTM). The results are shown in Table 2.

The sperm cell is a highly differentiated cell type that results from a specialized genetic and morphological process termed spermatogenesis. During spermatogenesis, various modifications occur which is associated with the DNA condensation of the chromatin. Concomitant with these visible changes in chromatin organization, the histone and non-histone proteins are removed from the DNA and replaced for a period of time by several transition proteins. These proteins are subsequently replaced by protamines during the final stages of spermatid maturation, chromatin reorganization and condensation. In spermatozoa formation, the histone-to-protamine transition plays an important role. It is well known that the protamines are the most abundant basic sperm-specific proteins present in sperm nuclei. During spermatogenesis, protamines are replaced by somatic histones that are essential for sperm formation and function (Domenjoud et al., 1990). The PRM1 and PRM2 genes contain a single intron whereas; PRM3 is an intron-less gene that is conserved in mammals. The expression of the PRM3 gene sequence was first reported in rat testis (Singh and Rao, 1988), located in the cytoplasm instead of the nucleus (Kleene, 1989). The genomic sequences of the PRM1 and PRM2 genes are organized in the form of a loop domain together with the transition protein 2 gene (TNP2) and a sequence called PRM3 (Choudhary et al., 1995; Schluter et al., 1996; Singh and Rao, 1988; Krawetz and Dixon, 1988; Wykes and Krawetz, 2003; Nelson and Krawetz, 1993). Due to their spatial organization, they may allow a coordinated expression of these genes during spermiogenesis. In humans, while the protamine (PRM1 and PRM2) and transition protein (TNP2) genes are expressed at high levels, the potential role of PRM3 is expressed at very low levels (Schluter et al., 1996; Singh and Rao, 1988; Krawetz and Dixon, 1988). The predicted amino-acid sequence of PRM3 is not at all related to other protamines, as it lacks arginine clusters and, instead, is rich in glutamic acid. Therefore, PRM3 is not likely to bind DNA. In mouse, PRM3 exhibits severe clear difference from the other protamines, that is, PRM1 and PRM2 (Martins et al., 2004). PRM3 –/– knock-out male mice, lacking PRM3 protein, showed a reduction in sperm motility without compromising their fertility (Kleene, 1989). The role of PRM3 related to bovine fertility and sperm motility is not yet clear and attempted till today. In the present investigation, we could observe

Table 2. Least square mean values for mRNA relative expression of PRM3 gene in Frieswal bulls categorized according to semen volume, concentration, number of sperm/ejaculate and post thaw motility percentage.

Parameter	Good	Poor	p Value
Volume (ml)	1.34±0.48 (n=8)	1.16±0.45 (n=9)	ns
Concentration (millions/ml)	1.54±0.39 (n=8)	1.34±0.36 (n=9)	ns
Number of sperm/ejaculate	1.50±0.36 (n=7)	1.43±0.41(n=6)	ns
PTM (%)	1.29±0.47 (n=9)	1.13±0.44 (n=9)	ns

ns: Non-significant; PTM: post thaw motility.

non-significant difference of PRM3 transcript abundance between normal and motility impaired groups. Our previous study showed a significantly decreased level of PRM1 mRNAs in spermatozoa of motility impaired group compared to normal crossbred Frieswal bulls. On the other hand, the expression of PRM 2 transcript level was not significantly different between the groups (Grzmil et al., 2008). The lowest mRNA expression of PRM3 was found in bovine testis as compared to other protamines (Ganguly et al., 2013). Together with these findings, our results indicate that protamine 3 expression does not vary significantly among good and poor quality matured bull spermatozoa of Frieswal crossbred cattle. The present preliminary claim must be verified by further experiments to find out the exact role of Protamine 3 to differentiate good and impaired spermatozoa among bulls of different cattle breeds need to be imitated.

Conclusions

The present study although revealed a down regulation of PRM3 transcripts in impaired semen producers as compared to normal crossbred bulls, however, significant difference of transcripts abundance between good and poor quality semen producers could not be observed. The present results thus suggest very little probable role of PRM3 gene in sperm motility as well as other seminal parameters among Frieswal crossbred bulls. However, further studies to understand detailed molecular function, expression profile, regulation pattern of PRM3 gene and its association with sperm motility and fertility in bulls need to be undertaken.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Genetic variability assessment of accessions of *Corchorus olitorius* L. using sodium dodecyl sulphate polyacrylamide gel electrophoresis

Chinyere Chioma Isuosuo and Florence Ifeoma Akaneme*

Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Nigeria.

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Corchorus olitorius **is one of the neglected indigenous leafy vegetables in Nigeria. Majority of the accessions of this species stored at the National Centre for Genetic Resources and Biotechnology (NACGRAB) Moor Plantation, Ibadan, Nigeria, have only been characterized morphologically. To provide further information on the extent of genetic diversity, this study was initiated to assess the genetic variabilities among 14 accessions of the species using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Seven polypeptide bands were obtained ranging from 9.73 to 88.79 KDa thus depicting variations in the banding patterns of the accessions. The unweighted pair group method with arithmetic mean algorithm (UPGMA) dendrogram grouped the 14 accessions into two clusters and five groups with 50% of the accessions in one group. Accession 3 which was obtained from Lagos state was observed to be a very distant relative of the other accessions and so could be combined in a breeding programme with any of the others.**

Key words: Genetic, variability, *Corchorus olitorius,* accessions.

INTRODUCTION

The genus *Corchorus* has undergone many taxonomic revisions. It was originally placed in the family Tiliaceae, subsequently in the family Malvaceae but it has now been placed in the family Sparrmaniaceae (Heywood et al., 2007). *Corchorus olitorius* otherwise known as Bush Okro or Jew's mallow has been found to be very useful in the following industries among others: interior decoration, accessories sector of apparel and textile industry, footwear industry, pulp and paper industry as a source of non-wood fibrous material (Khan, 2008). The leaves are generally rich in protein, β-carotene, iron, calcium, vitamin B, vitamin C and folic acid (Sinha et al., 2011; Mavengahama et al., 2013) and they form part of the meals of people of Asia, Middle East and parts of Africa (Fondio and Grubben, 2011; Sinha et al., 2011). Adebooye et al. (2003) listed the species as one of the seven highly valued indigenous leafy vegetables (ILV) in Nigeria. Certain problems, however, limit its production

*Corresponding author. E-mail: ifeoma.akaneme@gmail.com. Tel: (234) 803 6698 201.

Abbreviations: SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; **UPGMA,** unweighted pair group method with arithmetic mean algorithm.

and improvement (Fondio and Grubben, 2011). These include absence of improved seeds, impermeability of seed coat and flower drop after emasculation.

The authors called for the generation of improved cultivars and improved seeds. Any crop improvement programme, however, can only succeed on the strength of the genetic diversity available to breeders (Keatinge et al., 2008). Estimation of genetic diversity involves germplasm collection, characterization and evaluation. Some of the local landraces of *C. olitorius* which are maintained at National Institute for Horticultural Research (NIHORT), Ibadan Nigeria have been characterized morphologically, based on variations in leaf shapes. Opabode and Adebayo (2005), however, are of the opinion that the genetic improvement of ILV's including *C. olitorius* urgently requires the application of biotechnological techniques, such as molecular breeding.

Due to the cost of using molecular markers for germplasm characterization, many authors have advocated for the use of biochemical markers such as (protein or isozyme) particularly Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE technology) as a reliable tool for economic characterization of germplasm (Iqbal et al., 2005; Stoyanova and Bolla, 2010; Kakaei and Kahrizi, 2011). SDS-PAGE, a technique for analyzing protein contents, provides information on the level of relatedness of accessions of a species on the basis of similarity or dissimilarity of protein bands of the different accessions (Ejele and Osuagwu, 2003). Singh et al. (2004) also endorsed the use of protein markers for germplasm characterizations and pointed out that since storage proteins are the third- hand copy of DNA, they reflect the genetic make-up of the plant and as such could be used to distinguish genetically different varieties or accessions of a species.

Little or no molecular studies have been performed to estimate the diversity of *C. olitorius* in Nigeria (Ogunkanmi et al., 2010). Most genetic diversity studies had been focused on vegetative and physiological characteristics. In order to fully achieve the objectives of Raw Materials Research and Development Council (RMRDC, 2007) of Nigeria, which include the boosting of the production of jute fiber for textile and wearing apparel, there is need to characterize further the various accessions of *C. olitorius* being kept in the seed banks of the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria and National Institute for Horticultural Research in Nigeria (NIHORT).

This study was therefore, undertaken to evaluate the genetic variability among fourteen accessions of *C. olitorius* obtained from NACGRAB with the aid of SDS-PAGE.

MATERIALS AND METHODS

The *C. olitorius* accessions which were characterized in this study are shown Table 1. The method of Laemmli (1970) was used in carrying out the SDS-PAGE. During the process of protein extraction, 20 seeds of the respective accessions were macerated with mortar and pestle. The resulting powder (0.2 g) were homogenized thoroughly using a vortex with an extraction buffer containing 0.5 M Tris-HCl (pH 6.8), 2.5% sodium dodecyl sulphate (SDS), 10% glycerol and 5% 2-mercaptoethanol. The respective samples were centrifuged at 10,000 rpm for 5 min. The supernatants (500 µl) were collected in separate vials. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out using 4% stacking gel and 12% resolving or separating gel. The runs were performed on a mini gel apparatus in Tris-glycine (pH 8.3) buffer. Bromophenol blue (BPB) was added to the sample buffer as tracking dye to monitor the movement of protein molecules in the gel. The gels were run at 90 V for 2 h in an Ominipac mini- vertical gel apparatus using promega protein as a standard marker. They were gently removed and washed with 500 ml of the gel fixing solution and subsequently covered with 400 mls of Coomassie blue stain at room temperature for 3 to 4 h and were gently agitated. The coomassie stains were removed after staining by covering gels with 250 mls of the destaining solution. The destaining solution was changed severally until the protein bands were seen clearly without background staining of the gel.

Table 1. *C. olitorius* accessions and their states of origin.

S/N	Accession number	State of Origin
A_1	NG/AA/SEP/09/173	Osun
A_2	NG/TO/AUG/09/008	Ogun
A_3	NG/AO/AUG/09/003	Lagos
A_4	NG/OE/10/002	-
A_5	NG/MR/MAY/09/004	Ogun
A_6	NG/SA/DEC/07/0403	Niger
A_7	NG/SA/DEC/07/0402	Niger
A_8	NG/AO/MAY/09/018	Osun
A_9	NG/SA/07/189	Osun
A_{10}	NG/OA/JUN/09/001	Oyo
A_{11}	NG/SA/JAN/09/142	Niger
A_{12}	NG/OCT/09/001	-
A_{13}	NG/AO/MAY/09/013	-
A_{14}	NG/SA/07/203	Ondo

Banding patterns of the 14 accessions were examined and photographed. Each band was considered as a character and presence or absence was coded for analysis. In order to estimate genetic diversity, a dendrogram (a tree - like diagram that shows the degree of relatedness among organisms) was constructed using numerical taxonomic and multivariate analysis system software (NTSYS-pc) version 2.2.

RESULTS AND DISCUSSION

The electrophorogram of the protein bands are shown in Figures 1 and 2. Seven polypeptide bands ranging from 9.73 to 88.79 KDa were recognized among the 14 accessions screened in this study (Figures 1 and 2, Table 2). The bands showed variability on the basis of intensity and presence/absence of any of them among the accessions. There were no bands 2, 4 and 6 in accessions 1 to 7 and no bands 1, 3 and 7 in accessions 8 to 14 (Table 2). The protein band 3 was present in accessions 3 and 5

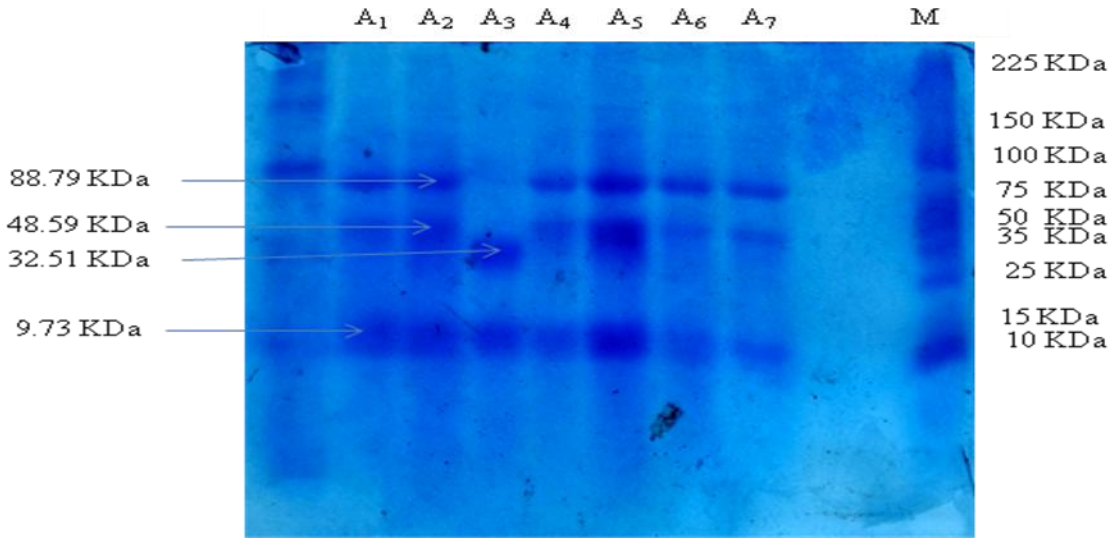

Figure 1. SDS - PAGE electrophorogram of the seed protein for accessions 1 to 7 of *Corchorus olitorius.* M is the Promega standard molecular protein marker.

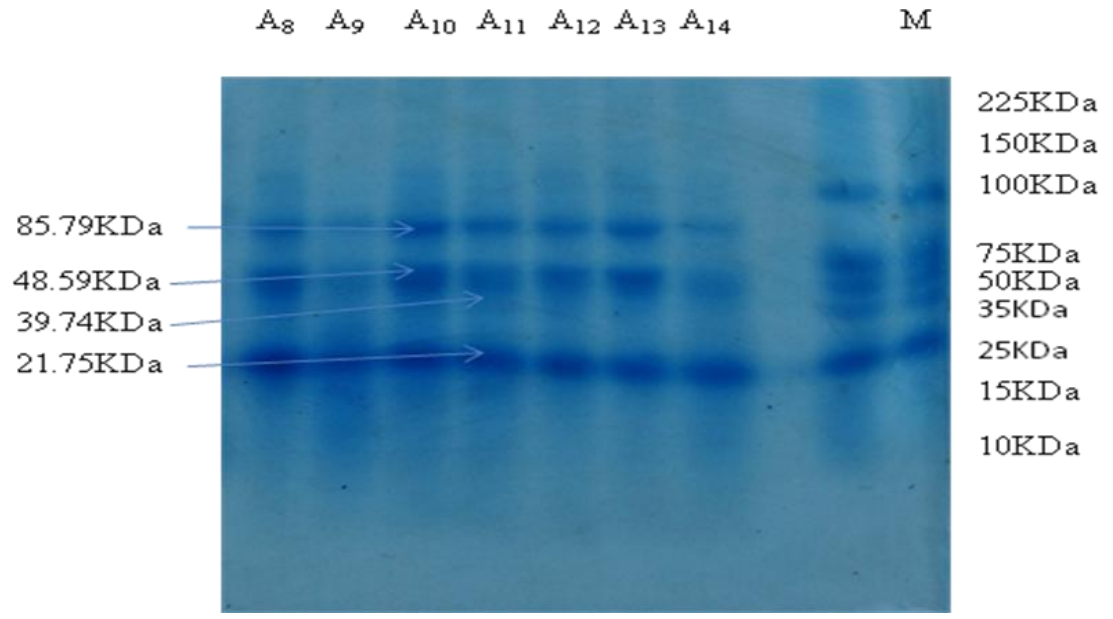

Figure 2. SDS - PAGE electrophorogram of the seed protein for accessions 8 to 14 of *Corchorus olitorius.* M is the Promega standard molecular protein marker.

only. Band 5 (48.59 KDa) was present in all accessions except accession 3 and the degrees of intensities in the 13 accessions differed (Table 2).

The banding patterns across the accessions were moderately dissimilar indicating moderate heterogeneity of the seed storage proteins. This corroborates the report of Hossain et al. (2002) that genetic variability in *C. olitorius* is limited due to self-pollination. Self pollination generally generates offsprings that closely resemble the parents genetically. Some investigators (Soetan and Fafunsho, 2009; Vishwanth et al., 2011) noted that differences in bands could be used as basis for identification and to check for variation among accessions since the bands observed show the distinc-tiveness of the plants. Mehlhorn (2008) observed that protein migration differences correspond to amino acid composition differences which in turn correspond to the differences in gene sequences although according to him, silent point mutations cannot be detected. Inspite of this, he concluded that protein electrophoresis gives the first indication of the existence of genetically different populations or species.

Table 2. Intensities of bands present in each *Corchorus olitorius* accession.

Band number	Molecular weight	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{12}	A_{13}	A_{14}
1	9.73	++++	++++	+++	+++	++++	+++	++	0	0	0	0	0	0	0
2	21.75	0	0	0	0	0	0	0	+++	++	++	++	+++	+++	++++
3	32..51	0	0	+++	0	++	0	0	0	0	0	0	0	0	0
4	39.74	0	0	0	0	0	0	0	0	0	+	+	0	+	0
5	48.59	+++	+++	0	++	++++	+	+	+++	+++	++++	+++	+++	+++	++++
6	85.79	0	0	0	0	0	0	0	++	+	+++	++	++	++	+
7	88.79	++	++	0	++	+++	++	++	0	0	0	0	0	0	0

+, Band present; 0, band absent.

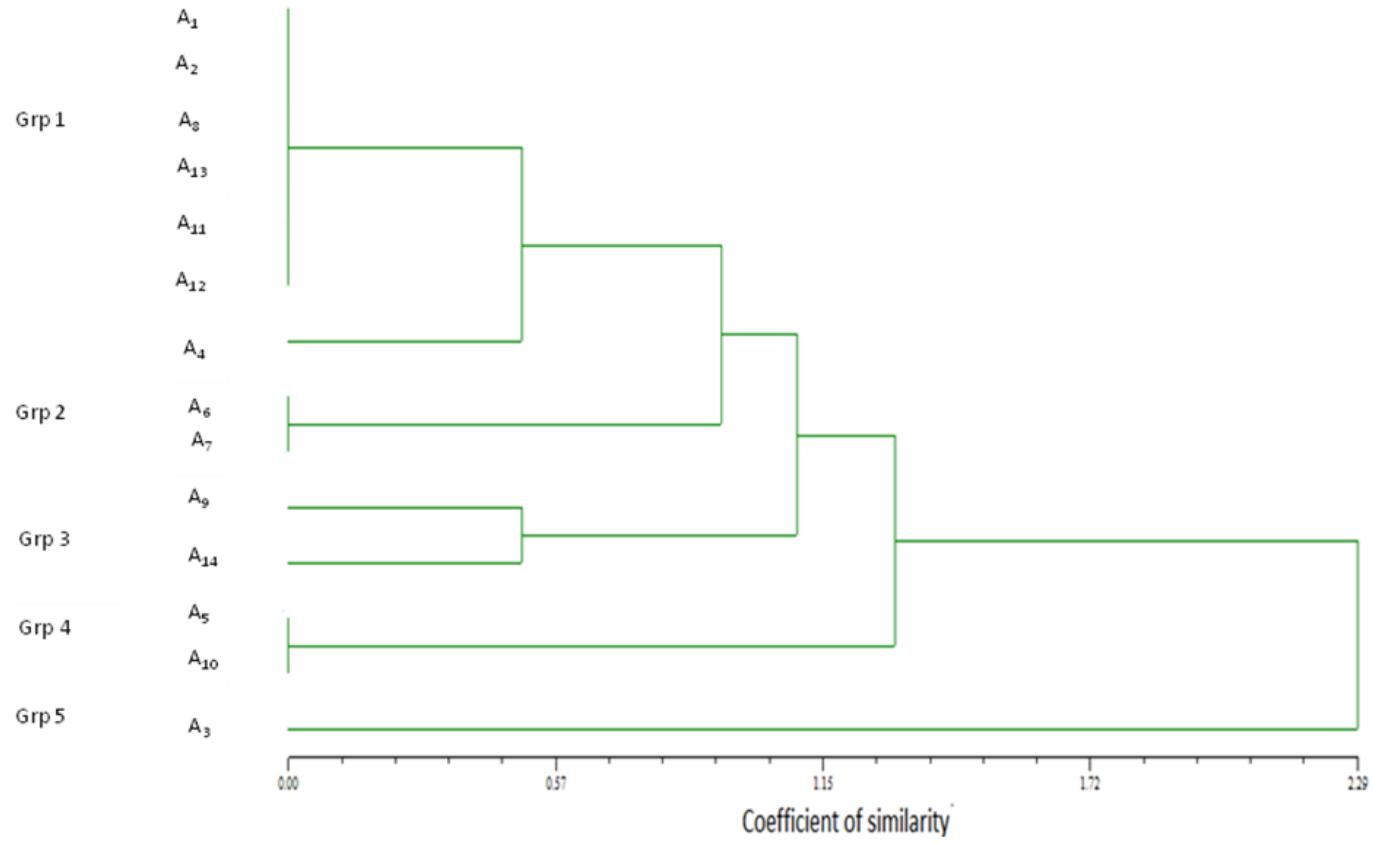

Figure 3. Dendrogram showing the relationship among the 14 accessions of *C. olitorius*.

Amersham BioSciences (1999) opined that the position a protein occupies along the separation lane gives a good approximation of its size and after staining, the band intensity is a rough indicator of the amount present in the sample. Uniform or similar banding patterns observed in some accessions could be due to these proteins being conserved (Ullah et al., 2009). Torkpo et al. (2006) noted that differences in bands which could possibly be associated with economically important traits such as diseases resistance, nematode, parasitic weeds and drought resistance should be a viable research option.

The unweighted pair group method with arithmetic mean algorithm (UPGMA) clustering method based on Jaccard's similarity coefficient separated the 14 accessions into two clusters and five groups at an UPGMA distance coefficient range of 0.48 to 2.29 (Figure 3). Groups 1 to 4 formed the 1st cluster at similarity coefficient of 1.32 while group 5 formed the second cluster at a coefficient of 2.29. Clustering observed in the dendrogram could indicate close genetic proximity or relatedness. Accessions 6, 7 and 11 originated from Niger state but while accessions 6 and 7 were found in group 2, accession 11 was found in group 1. The accessions from

Osun State (A_1&A_8; A_9) were also found in two different groups: group 1 (A_1 & A_8) and Group 3 (A_9) - that were distant from each other. Ogun state was the home of accessions A_2 and A_5 which were part of groups 1 and 4, respectively. The groups (1 and 4) were really distant from each other. It can be deduced from Figure 3 that all the accessions in groups 1 and 5 had the least similarity and maximum distance. Accessions 9 and 14 may be closely related even though they were collected from Osun and Ondo states, respectively. Relative closeness (Yi et al., 2008) could be due to the fact that there is no cross boundary check among divisions or states and seed exchange between farmers may disseminate plants from one region to the other. This could be an indication that these accessions may have been moved from one state to the other, therefore, implying that the seeds may be the same genetically. Accession 3 originated from Lagos State and it was observed to be an independent group and not related closely to other accessions. Accessions on different groups could create wider variation when crossed because they are dissimilar genetically. Maity et al. (2009) had earlier noted that based on distance between accessions of different clusters, contrasting parents may be identified and used in the crossing programme for generating wider variability for selection and crop improvement.

Conclusion

In conclusion, seed protein electrophoresis was able to show the differences and relationship among the 14 accessions of *C. olitorius* evaluated in this study. This result agrees with the report of Patra and Chawla (2010) that electrophoretic analysis of total soluble proteins is widely recognized as a technique for cultivar identification. Thus, it can be deduced from the present study that accession 3 (A_3) can be combined in a breeding programme with the other accessions because it is a very distant relative, while accessions A_1, A_2, A_8, A_{13}, A_{11} and A_{12} may not be used together in a breeding progamme since they appear to be duplications of the same material, just as A_6 and A_7; A_5 and A_{10} appear to be duplications of each other, respectively.

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Conflict of Interests

The authors wish to declare that there are no conflicts of interest with respect to this article.

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Full Length Research Paper

Analysis association of milk fat and protein percent in quantitative trait locus (QTLs) on chromosomes 1, 6, 7 and 20 in Iranian Holstein cattle using ten micro satellite markers

A. Attar[1]*, G. Rahimi[1], M. R. Nassiri[2] and B. Sadeghi[2]

[1]Department of Animal Genetics and Breeding of Sari University, Sari, Iran.
[2]Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.

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Protein and fat percent as content of milk are high-priority criteria for financial aims and selection of programs in dairy cattle. In order to incorporate information on marker- quantitative trait locus (QTL) associations, QTL detection and parameter estimation should also be performed. Microsatellite markers are most valuable markers in association studies between different loci with the production traits. With the aim of analysis markers previously identified by other researchers in QTLs of milk fat and protein percent traits in Iranian Holstein dairy cattle sample population, we use ten microsatellite markers on chromosomes 1, 6, 7 and 20. All microsatellite markers were genotype in 156 dairy cattle which have at least one year data of milk fat and protein record using 6 to 8% polyacrylamide gel electrophoreses determined with silver nitrate staining. We find 7, 5 and 8 out of 10 markers showing significant effect on milk fat percent in 1 to 120, 120 to 240 and 240 to 360 milking days, respectively. Our results show 6, 8 and 9 out of 10 markers have significant effect on protein percent in 1 to 120, 120 to 240 and 240 to 360 milking days respectively.

Key words: Fat percent, Iranian Holstein cattle, microsatellites, milking days, protein percent, quantitative trait locus (QTL).

INTRODUCTION

Traits like milk fat and protein percent usually have a complex determinism. They are affected by an unknown number of genes and environmental factors. By the way, these traits are economically interesting. However, advances in molecular genetics in the last decade have made it possible to find out genetic variability of complex traits into quantitative trait loci. Moreover, even if the genes involved are still unknown, individual quantitative

*Corresponding author. E-mail: aminattar60@gmail.com. Tel: +989158094726. Fax: +985118672279.

Abbrviations: QTL, Quantitative trait locus; **PCR,** polymerase chain reaction.

Table 1. Primer sequence microsatellite markers.

Chr. Number	Microsatellite marker name	Annealing temperature (C°)	Primer sequence
1	BM1824	57	ARKMKR00006708 [1]
	BM4307	57	ARKMKR00006776
6	FBN13*	56	Forward ACTTTCATTAGATTGCTGCAAATAG
			Reverse AAATATGGAAACGACCTGTGG
	ILSTS97*	56	Forward AAGAATTCCCGCTCAAGAGC
			Reverse GTCATTTCACCTCTACCTGG
	BM143	57	ARKMKR00006693
7	BMS1979	58	ARKMKR00007053
	ILSTS006	60	ARKMKR00007780
20	AGAL29*	57	Forward AGGAAGCCGAGTGAGATATGTAAGC
			Reverse TTACAGCCTGTGTGAATGTCCTCTA
	ILSTS72*	52	Forward ATGAATGTGAAAGCCAAGGG
			Reverse CTTCCGTAAATAATTGTGGG
	BM5004	57	ARKMKR00006793

[1] Marker ID in ArkDB. http://www.thearkdb.org, *Reinecke et al. (2005).

trait locus (QTL) information could enhance selection efficiency and is known to be particularly beneficial when the trait is difficult or expensive to measure, when each individual performance brings a few information or when the polygenic approach has a limited efficiency or a high cost.

Numerous studies on QTL mapping of milk production traits of dairy cattle have been reported. Zhang et al. (1998), Georges et al. (1995), Vittalla et al. (2003), Huglund et al. (2009) and Arranz et al. (1998) reported significant QTL for milk fat and protein on chromosome 20. Heyen et al. (1999), Boichard et al. (2003) and Mosig et al. (2001), find out QTL on chromosome 7 affected milk fat. Freyer et al. (2003), Szyda and Komisarek (2007), Chen et al. (2006), Zhang et al. (1998), Viitalla et al. (2003), Olsen et al. (2004), Nadesalingam et al. (2001), Ashwell et al. (2004), Mei et al. (2009), Ron et al. (2002) and many other researchers have shown most significant QTL on chromosome 6 for milk fat and protein. Nadesalingam et al. (2001), Bagnato et al. (2008) and Zhang et al. (1998) said some QTL on chromosome 1 affected milk fat and protein.

This research was carried out to check out if significant QTLs markers found by other researchers have the same results in Iranian Holstein cattle sample population which is not done yet.

MATERIALS AND METHODS

Animals and data

Blood samples were randomly collected from tail vein of 156 dairy cattle which have at least one year milking record in herd H125 of Astan-e Qods Razavi by salting out method. Four year data of milk fat and protein percent were collected daily, automatically for statistical analysis. Based on the positional information derived from the previously mentioned QTL regions, we select 10 microsatellite markers in 4 chromosomes of cattle with primer sequence mentioned in Table 1.

Genotyping

Simple polymerase chain reaction (PCR) has been done for each primer at suitable situation mentioned in Table 1. All microsatellite markers were genotyped and PCR products were run on 6 to 8% polyacryamide gel electrophoreses to determine genotype of each sample using silver nitrate staining. The frequency of allele and genotype for each marker was determined by direct counting of alleles.

Statistical design

Effect of each marker on milk fat and protein percent in different sections was calculated using a General Linear Model (GLM) program of SAS 9.1 software (SAS 9.1 software, SAS Institute Inc., Cary, NC). We only use cattle's data which have 1 to 6 lactation periods. Any lactation period which has more than 360 days of milking was omitted. We have classified the lactation period (360 days) to three sections: Section one (1 to 120 days), two (120 to 240 days) and three (240 to 360 days); and these sections were used for statistical analysis, separately.

To investigate the association of candidate microsatellite markers polymorphisms with percent of milk fat and protein, the following model was applied in the Statistical Analysis System (SAS):

$$Y_{ij} = \mu + G_i + L_i + S_i + (GL)_i + (GS)_i + e_{ij}$$

Where, Y_{ij} is a vector of phenotypic observations (DYD) for cattle; μ is the overall mean; G_i is the fixed effect of the marker genotype of

Table 2. Population analysis of genetic parameters for markers.

Shannon index	Effective allele	Null allele Frequency	PIC	Expected He	Expected Ho	He	Ho	Obs. Allele	Total	Loci
1.2897	3.3915	-0.1854	0.651	0.7074	0.2926	1	0	4	312	AGLA29
0.9588	2.3309	-0.2918	0.479	0.5728	0.4272	1	0	4	312	ILSTS72
1.5443	3.9623	-0.1519	0.710	0.7501	0.2499	0.9808	0.0192	6	312	BM143
1.4215	3.4865	-0.1828	0.665	0.7155	0.2845	1	0	6	312	FBN13
1.2581	3.1935	-0.2039	0.635	0.6891	0.3109	1	0	4	312	ILSTS97
1.2020	2.7722	-0.2434	0.572	0.6413	0.3578	1	0	5	312	BM4307
1.0731	2.5825	-0.2422	0.535	0.6147	0.3853	0.9679	0.0321	4	312	BMS1979
1.3269	3.2344	-0.1995	0.636	0.6930	0.3070	1	0	5	312	BM1824
1.2487	2.8927	-0.2482	0.597	0.6564	0.3436	1	0	6	312	BM5004
1.2691	3.2653	-0.1935	0.637	0.6960	0.3040	1	0	4	312	ILSTS006
1.2611	3.1141	--	0.612	0.6739	0.3261	0.9949	0.0051	4.9	312	Mean
0.1639	0.4770	--	--	0.0526	0.0526	0.0112	0.0112	0.8756	--	SD

Table 3. P-Values of loci and fixed effects on milk fat percent and their Interactions.

Loci fixed effect	Fat (%)			Interaction between fat and lactation period (%)			Interaction between fat and season of giving Birth (%)		
	1 -120	120 -240	240-360	1 - 120	120-240	240-360	1 - 120	120-240	240-360
AGLA29	0.01	ns	0.0001	0.0001	0.0002	0.0001	0.0001	0.0002	0.0001
ILSTS72	ns	0.008	0.0001	ns	ns	0.0001	0.01	0.04	0.0001
BM5004	0.001	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
BMS1979	ns	ns	0.0001	0.003	0.0001	0.0001	0.0001	0.0001	0.0001
ILSTS006	0.05	ns	0.003	ns	0.001	0.0001	ns	0.0003	0.0001
FBN13	ns	ns	ns	0.05	0.0001	0.0001	0.001	0.0001	0.0001
ILSTS97	0.02	0.0001	ns	0.0001	0.0001	0.0001	0.01	0.0001	0.0001
BM143	0.002	0.0001	0.0001	0.0001	0.0001	0.0001	0.001	0.0001	0.0001
BM1824	0.004	0.0001	0.0001	0.001	0.0001	0.0001	0.0001	0.004	0.0001
BM4307	ns	0.002	0.0001	0.0003	0.0001	0.0001	ns	0.0001	0.0001
Season of giving birth	0.0001	0.002	0.0001				--		
Lactation period	ns	0.0003	0.0001				--		

animal i; L_i is the random effect of lactation period of animal i; S_i is the random effect of calving season of animal i; $(GL)_i$ is interaction effect between genotype and lactation period of animal i; $(GS)_i$ is interaction effect between genotype and calving season of animal i; e_{ij} is the random residual.

Analysis of population parameters was done by POPGENE 1.32 (Yeh et al., 1999) and CERVUS 2.0 (Marshall et al., 1998).

RESULTS AND DISCUSSION

Analysis of association of candidate microsatellite markers polymorphisms with fat percent is shown in Table 3 and results for protein percent are shown in Table 4. Population analysis of genetic parameters for each microsatellite marker shows variety in our population (Table 2). Velmala et al. (1999) reported the effects of BM143 on protein and fat percent of milk. Ron et al. (2002) find significant effect of BM143 on milking traits except milk fat yield. Freyer et al. (2003) show the effects of FBN13 on protein and fat yield. Reinecke et al. (2005), Ron et al. (2001), Zhang et al. (1998) and Georges et al. (1995) confirmed that, chromosome 6 and 20 have more QTL loci affecting milking traits. Szyda and Komisarek (2007) reported significant QTL loci on chromosome 6 affecting milking traits such as protein and fat yield. Ashwell et al. (2004) stated that some QTL loci affected protein and fat yield on chromosomes 6, 7, 11, 14 and 17. Olsen et al. (2004) and Nadesalingam et al. (2001) confirmed that QTL loci affecting protein percent and fat yield is near BM143. They show significant QTL affecting fat yield and fat percent on chromosome 1. Olsen et al. (2004) find out significant effect of FBN13 and BM143 on protein and fat percent. Zhang et al. (1998) reported significant QTL loci on chromosome 3, 6, 20 and 28 affecting milk protein and on chromosome 6, 20, 26 and 28 affecting milk fat, respectively.

Table 4. P-Values of Loci and Fixed Effects on Milk Protein Percent and their Interactions.

Loci fixed effect	Protein (%)			Interaction between protein and lactation period (%)			Interaction between protein and season of giving birth (%)		
	1 - 120	120 - 240	240 - 360	1 - 120	120 - 240	240 - 360	1 - 120	120 - 240	240 - 360
AGLA29	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.001	0.0001	0.0001
ILSTS72	ns	0.0008	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
BM5004	0.0002	0.01	0.0001	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001
BMS1979	0.0005	0.005	0.0001	0.0001	0.0001	0.0001	0.02	0.0001	0.0001
ILSTS006	ns	ns	0.0001	ns	0.0001	0.0001	ns	0.0005	0.0001
FBN13	ns	0.01	0.0001	0.0009	0.0001	0.0001	0.0001	0.0001	0.0001
ILSTS97	0.0001	ns	0.0001	0.0005	0.0001	0.0001	0.0001	0.0001	0.0001
BM143	0.0001	0.0002	0.0001	0.004	0.0001	0.0001	0.001	0.0001	0.0001
BM1824	0.009	0.0002	0.0001	0.004	0.0001	0.0001	0.0001	0.0001	0.0001
BM4307	0.05	0.0005	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Season of giving birth	ns	0.0001	0.0001				--		
Lactation period	0.001	0.0001	0.0001				--		

We find out significant effects of all markers on fat and protein percent on chromosome 20. Arranz et al. (1998), Viitalla et al. (2003) and Georges et al. (1995) confirm that, AGLA29 has significant effect on protein percent. Olsen et al. (2004) and Hugland et al. (2009) show significant effect of AGLA29 and BM5004 on protein yield. Ashwell et al. (2004) and Viitalla et al. (2003) show significant effect of ILSTS97 and BM5004 on protein percent.

We find out significant effect of BMS1979 on protein yield on chromosome 7, the same as Heyen et al. (1999). Ehud et al. (1998) show effect of ILSTS006 on milking traits that is not the same with our result. Heyen et al. (1999) confirmed the effect of ILSTS006 on protein yield that is not found in our study. But Mosig et al. (2001) reported significant effect of ILSTS006 on protein percent that was reviewed in this research.

Significant effect of FBN13 on protein yield was found out by Freyer et al. (2003), Szyda and Komisarek (2007) and Olsen et al. (2004). Although, Chen et al. (2006), Szyda & Komisarek (2007), Reinecke et al. (2005), Velmala et al. (1999), Zhang et al. (1998) and Ehud et al. (1998) reported significant effect of ILSTS97 on milking traits, our study does not confirm it. Most studies such as Liu et al. (2004) show significant effect of BM143 on milking traits such as protein and fat percent as seen in this study.

Significant effects of milk protein and fat on chromosome 1 have been shown by Nadesalingam et al. (2001), Viitalla et al. (2003), Bagnato et al. (2008) and Zhang et al. (1998) researches.

Comparison of our results with other reports shows that most of the markers used in this study have significant effect on fat and protein percent. Because our data are separated in different lactation periods and seasons of giving birth to calves in each cattle, we need to find out the effect of these two parameters and in this way, data show significant effect of lactation period and season of giving birth to calves as seen in Tables 3 and 4. Interaction effects between lactation period and season of giving birth with each marker are also shown in Tables 3 and 4.

It seems that FBN13 has no significant effect on fat percent in any lactation days, as it has no effect on total kilograms of fat in our last investigation. Therefore, significant effects of interaction between FBN13 and lactation periods and season of giving birth must not be described by the effect of FBN13. Our results show that most of these markers have more significant effects on fat and protein percent on 240-360 days of lactation. Also it seems most of the markers used by this research have more significant effect on protein percent. According to our results, we can say that AGLA29, ILSTS006 and BMS1979 might have more significant effect on protein and fat percent in 240-360 lactation days. ILSTS72 has significant effect on fat and protein percent in 120-240 days of lactation. BM5004 must have effectsand BMS1979 might have more significant effect on protein and fat percent in 240-360 lactation days.

ILSTS72 has significant effect on fat and protein percent in 120-240 days of lactation. BM5004 must have effects on fat and protein percent traits in 1-120 and 240-360 days of lactation. ILSTS97 might have significant effect on both fat and protein percent on 0-120 days of lactation. Two markers BM143 and BM1824 might have significant effect on both fat and protein percent on all days of lactation. And at last BM4307 might have effects on fat and protein percent in 120-360 days of lactation.

Conclusion

Based on this study, it was reviewed that markers associated with QTL of milk fat and protein have significant effects on the Iranian Holstein cattle population. Although, Iranian crossbreed Holstein cattle are unknown to the scientific society, markers with peak of QTL effects on milk fat and protein found out by other researchers have shown the same result in our sample population. We need more exploration in our future researches based on QTL analysis to check out if QTLs are significant. Anyway, due to Iran's major policies for dairy production which is quantity and milk fat, we can use these markers to identify potential individuals for breeding.

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Full Length Research Paper

Effect of growth regulators and explant types on callus induction in *Telfairia occidentalis* Hook F

Sakpere, A.M. A.[1]*, Ajayi, S. A[2] and Adelusi, A.A.[3]

[1,3]Department of Botany, Obafemi Awolowo University, Ile – Ife, Nigeria.
[2]Department of Crop Production and Protection, Obafemi Awolowo University, Ile – Ife, Nigeria.

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Different concentrations of growth regulators and three types of explants were investigated for their efficiency on callus induction in *Telfairia occidentalis* with a view of providing baseline information for the development of a callus initiation protocol. Three concentrations of kinetin (KN) (0.1, 3.3 and 5.0 mg/L) in combination with two concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) (2.0 and 5.0 mg/L) and two concentrations of naphthalene acetic acid (NAA) (0.25 and 0.5 mg/L) in combination with 0.25 mg/L benzyl adenine (BA) were tested for their effect on callus induction from stem, leaf and nodal explants collected from field-grown *Telfairia* plants. Media supplemented with 2,4-D in combination with kinetin gave the highest cumulative percent callus induction. With regards to cumulative percentage callus induction and total callus produced, media supplemented with BA alone was better than media supplemented with kinetin alone. Irrespective of the growth regulator type, percent callus induction was not significantly different among explant types. The study concluded that, 2,4-D is a better auxin for high callus induction in *T. occidentalis* explants as compared to NAA. However, there is still a need to test the effect of 2,4-D in combination with BA on callus induction.

Key words: *Telfairia occidentalis,* callus initiation, 2,4-D, BA, kinetin.

INTRODUCTION

Telfairia occidentalis Hook F. commonly called fluted pumpkin is one of the native vegetables of Nigeria and found in the moist coastal areas of West Africa (Ajayi et al., 2007; Odiaka et al., 2008). It belongs to the family Cucurbitaceae. The edible leaves and young shoots have a high nutritional and medicinal value. The leaves are rich in protein (29%), fat (18%) as well as minerals and vitamins (20%) (Akanbi et al., 2007). The leaves are also rich in iron as a result of which fresh leaf concoction is used as a health tonic for the treatment of anaemia (Akoroda, 1990; Schippers, 2000). Other parts of the plant have several important uses in the production of marmalade,

*Corresponding author. E-mail: aasakpere@oauife.edu.ng. Tel: +234 803 403 3610.

Abbreviations: 2,4-D, 2, 4-Dichlorophenoxy acetic acid; **BA,** benzyl adenine; **NAA,** naphthalene acetic acid; **KN,** kinetin.

fodder (Egbekun et al., 1998; Odiaka and Schippers, 2004); food, fats and oil for soap making, cooking, margarine production, and also in production of drying oil for paints and varnishes (Badifu and Ogunsua, 1991; Giami and Isichei, 1999; Akwaowo et al., 2000; Fashina et al., 2002; Steinkraus, 2002; Giami et al., 2003; Agatemor, 2006; Akanbi et al., 2007) among other uses. Many biological constraints have become potent threats to the existence of *T. occidentalis* necessitating an urgent need to collect and conserve the existing narrow genetic diversity (Ajayi et al., 2006b). However, conservation by seed storage in spite of itsrelatively low cost is impossible because the seed is recalcitrant (seeds lose viability due to desiccation when stored for a considerable length of time) (Odiaka and Schippers, 2004; Ajayi et al., 2006b). The availability of seeds for planting is also limited by its recalcitrance and the fact that seeds are utilized for a variety of purposes.

Micropropagation, the propagation of plants *in vitro*, is an alternative means of vegetative propagation. According to Debeaujon and Branchard (1993), strategies based on the application of biotechnologies to crop improvement programs generally require regeneration of whole plants from cells or tissues cultivated *in vitro*. In order to maximize the potential benefits of micropropagation, protocols have to be developed for different plants and often times explants from different parts of the same plant to suit their peculiar needs. According to Pierik (1999), there are great differences in cell division and regenerative capacity between plants even within a single species.

Earlier attempts on tissue culture and micropropagation of *T. occidentalis* were carried out using shoot tips, nodal cuttings and roots (Balogun et al., 2002; Ajayi et al., 2006a; Balogun et al., 2007; Sanusi et al., 2008) but the possibility of somatic embryogenesis has not been explored. As a first step towards this, callus initiation protocol has to be in place for the supply of callus for induction of somatic embryos. The present study was initiated to investigate the influence of different concentrations of auxins and cytokinins on callus induction from different explants.

MATERIALS AND METHODS

Fruits of *T. occidentalis* landraces were collected from the area around Ile-Ife, Osun state, Nigeria (Latitude: 7° 467' 0 N, Longitude: 4° 567 ' 0 E) and plants were raised in nursery. Leaf, stem and nodal explants were obtained from nursery grown *Telfairia* plants. They were washed under running tap water to remove dirt and reduce microbial load. The explants were then surface sterilized for 10 min with 10% (v/v) sodium hypochlorite solution to which two drops of Tween 20 was added as a surfactant. The explants were then rinsed three times in sterile distilled water. The leaves were thereafter trimmed into pieces of about 2 cm^2 and the stems were cut into about 2 cm long pieces before inoculation. Five replicates of leaf, nodal and stem explants were used per treatment. All protocols were carried out under the laminar airflow chamber.

Murashige and Skoog's (1962) (MS) medium supplemented with 3% (w/v) sucrose was used as the basal medium in all the experiments. The media was solidified with 0.8% (w/v) agar and the pH adjusted to 5.7 ± 0.1. The media was dispensed into McCartney bottles which were sealed with cotton wool and thereafter wrapped with aluminum foil before autoclaving at 121°C and 15 lb/in^2 for 15 min. Cultures were maintained at 25°C ± 2°C in the dark. The following concentrations of growth regulators were tested for their effect on callus initiation and embryogenic callus induction from stem, leaf and nodal explants: 5 mg/L 2,4-D + 3.3 mg/L kinetin; 2 mg/L 2,4-D + 5 mg/L kinetin; 5 mg/L 2,4-D + 0.1 mg/L kinetin; 0.25 mg/L BA + 0.25 mg/L NAA; 0.25 mg/L BA + 0.5 mg/L NAA; 0.5 mg/L BA; 0.5 mg/L kinetin.

The cultures were monitored weekly for the effect of each treatment on the induction of callus or organogenesis. The explants were visually observed for the presence and type of callus (Remotti and Loffler, 1995; Mencuccini and Rugini, 1993). Responses of explants to callus induction were expressed as percentages (%) of induced explants. Where applicable data collected were subjected to analysis of variance and means were separated with Duncan's multiple range test (DMRT), using system analysis software (SAS) version 9.2.

RESULTS

Callus was induced by all the growth regulator treatments. However, not all explants produced callus on the different growth regulator treatments. For example, callus was not induced on leaf explants inoculated on media supplemented with 5.0 mg/L 2,4-D and 3.3 mg/L kinetin but, there was 60% callus induction on leaf explants inoculated on media supplemented with 2.0 mg/L 2,4-D in combination with 5.0 mg/L kinetin (Figure 1A). Percentage callus induction of stem explants was highest on media supplemented with 0.25 mg/L BA and 0.25 mg/L NAA.

For nodal explants, however, the highest percentage callus induction occurred on media supplemented with 5.0 mg/L 2,4-D and 3.3 mg/L kinetin. Leaf explants produced highest percentage callus induction on media supplemented with 2.0 mg/L 2,4-D in combination with 5.0 mg/L kinetin and on media supplemented with 5.0 mg/L 2,4-D in combination with 0.1 mg/L kinetin (Figure 1A).

Stem and leaf explants generated biggest callus size on media supplemented with 5.0 mg/L 2,4-D and 0.1 mg/L kinetin. Nodal explants on the other hand, generated the largest callus on media supplemented with 2.0 mg/L 2,4-D and 5.0 mg/L kinetin (Figure 1B). Averaged over all treatments, media supplemented with 5.0 mg/L 2,4-D in combination with 0.1 mg/L kinetin gave the best results for callus induction and size of callus (Figure 2A and B).

Stem and leaf explants generated more callus than nodal explants (Figure 3). With regards to growth regulator type, 2,4-D in combination with kinetin was more effective than BA in combination with NAA for callus induction and callogenesis (Figure 4A and B). Effect of different growth regulators and explants on morphology

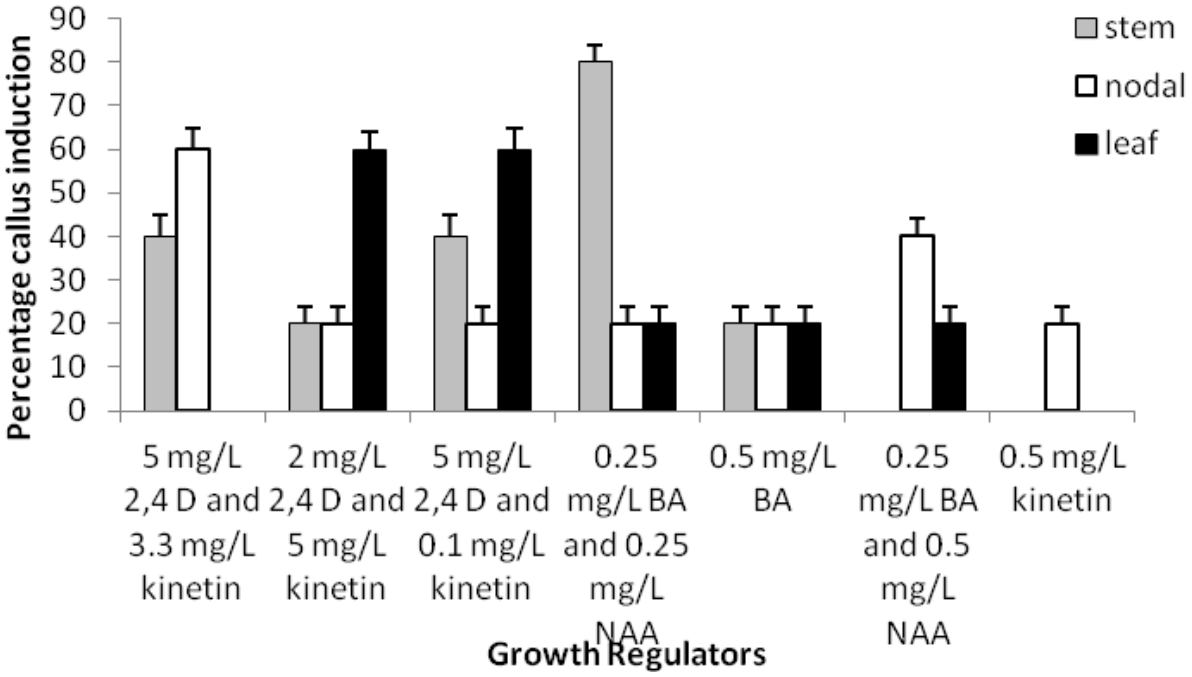

Figure 1A. Effect of different concentrations of growth regulators on percentage callus induction from different explants of *T. occidentalis*.

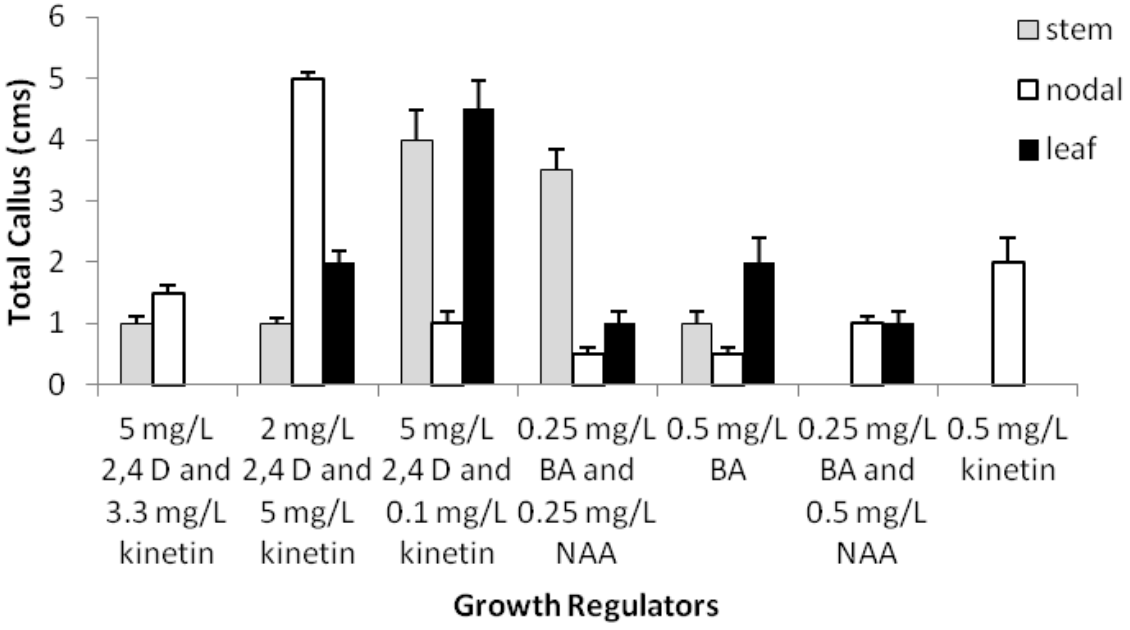

Figure 1B. Effect of different concentrations of growth regulators on total callus produced on different explants of *T. occidentalis*. **Note:** Vertical bars represent mean ± SE.

of callus is presented in Plate 1. The calli induced from leaf were soft, creamy, and nodular both on medium supplemented with 2,4-D + kinetin and BA + NAA; stem calli were cream and loose on medium supplemented with BA + NAA and greenish on 2,4-D + kinetin while calli from nodal explants were white and fuzzy.

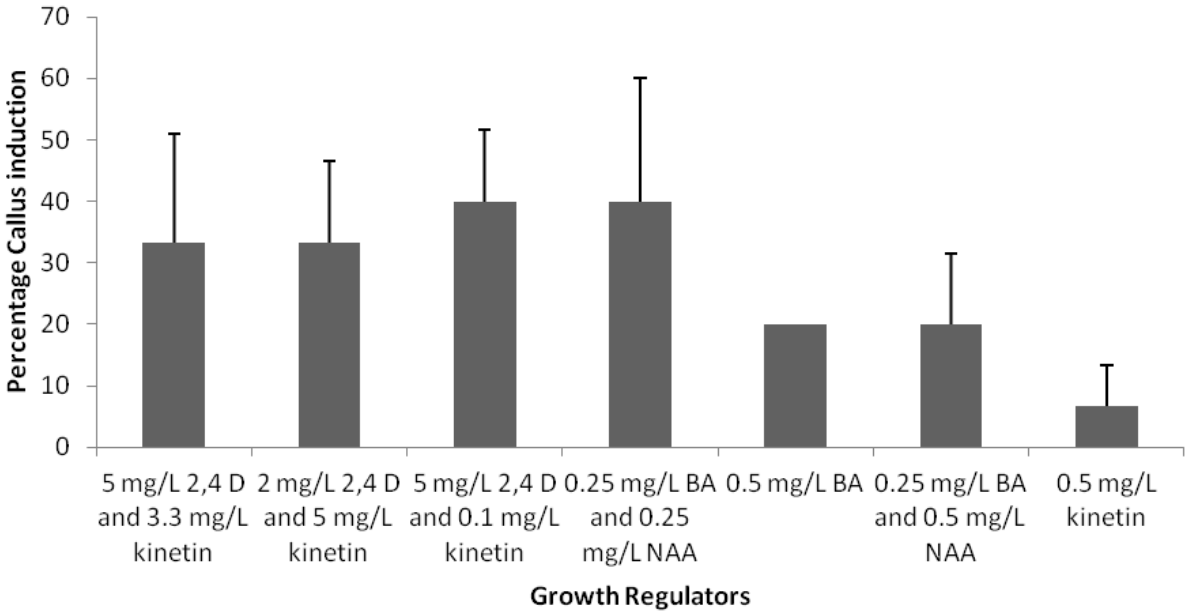

Figure 2A. Effect of different concentrations of growth regulators on percentage callus induction from a combination of all explant types.

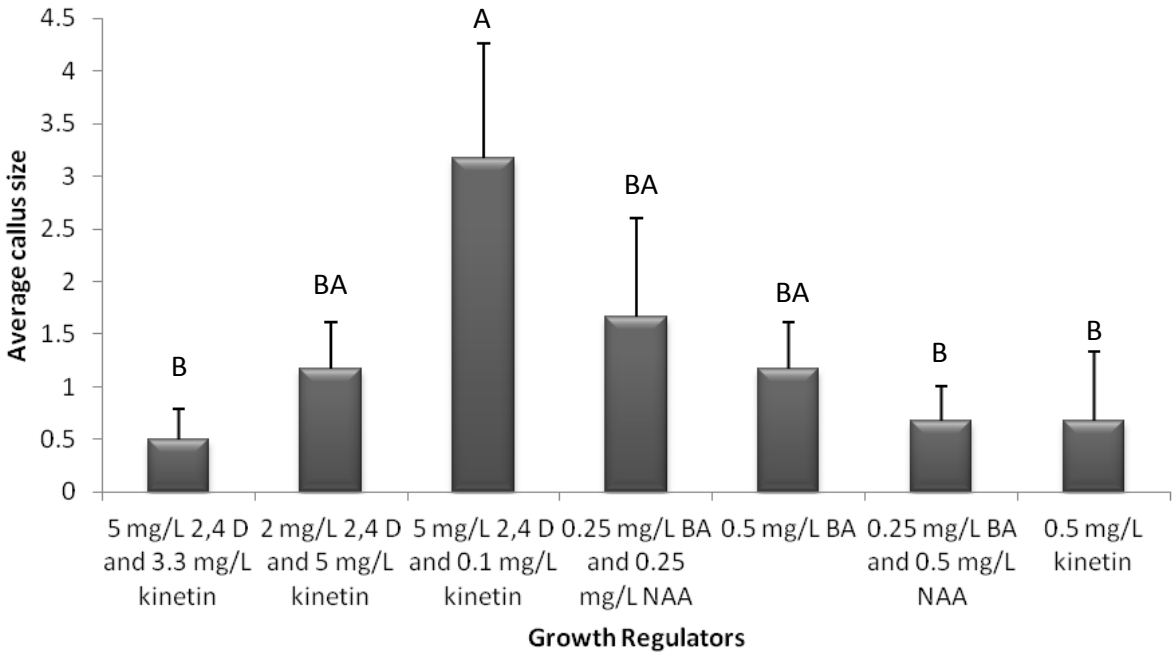

Figure 2B. Effect of different concentrations of growth regulators on callus size from a combination of all explants types. **Note:** Vertical bars represent mean ± SE, and values with the same superscript letters are not significantly different at P< 0.05.

DISCUSSION

For a given growth regulator to be useful, it must not only induce callus but, the degree of callusing must also be appreciable. In this study, highest callus induction and degree of callusing occurred on medium supplemented with 5 mg/L 2,4-D and 0.1 mg/L kinetin.

Liu et al. (2006) reported that auxin 2,4-D by itself or in combination with cytokinins has been widely used to enhance callus induction and maintenance.

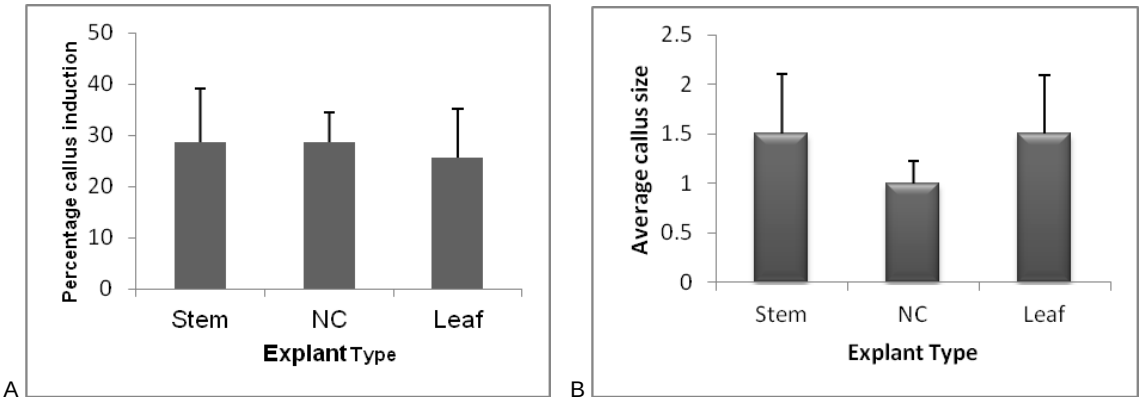

Figure 3. Effect of different explants types on (A) percentage callus induction and (B) callus size. **Note:** Vertical bars represent mean± SE, and the unit of measurement was cm.

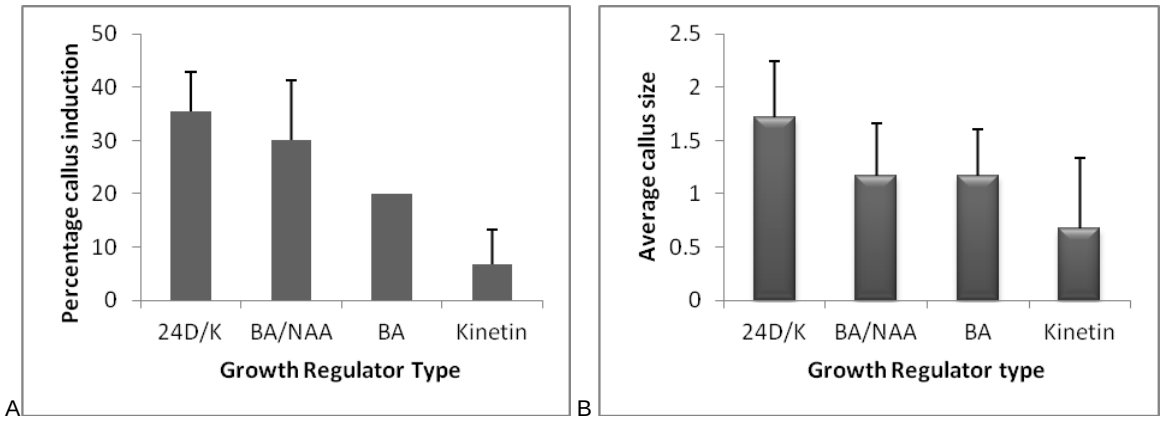

Figure 4. Effect of different growth regulator types on (A) percentage callus induction and (B) callus size. **Note:** Vertical bars represent mean± SE, and the unit of growth regulators was mg/L.

Moreover, many researchers have reported the use of 2,4-D for callus induction in other Cucurbits (Selvaraj et al., 2006; Usman et al., 2011; Thiruvengadam et al., 2012). Overall, among different explants, the highest callus induction was observed on MS medium containing 2,4-D for different explants from cucumber cultivars explored for callus induction in response to different media (Usman et al., 2011). Pal et al. (2007) also reported that for *Cucurbita pepo*, callus was induced on medium containing 2,4-D from both explants utilized, but not on 2,4-D-free medium. Optimum level of callus from *Trichosanthes dioica* was likewise found on medium supplemented with 1.0 mg/l of 2, 4-D (Malek et al., 2010).

Initiation of callus using cytokinin alone has not been promising in this study. Abd Elaleem et al., (2009) working on *Solanum tuberosum* reported that BA alone was not efficient for callus initiation except in combination with 2,4-D. Arivalagan et al. (2012) also reported that when kinetin alone was used as media supplement, callus was not induced.

Cytokinins, such as BA and kinetin, at low concentrations, in combination with auxins have been reported (Chai and Mariam, 1998; Arivalagan et al., 2012) to be frequently used in plant species to promote callus initiation. Efficient callus was induced from leaf and stem explants of *Citrullus colocynthis* on MS medium containing 1.5 mg/l 2 4-D + 1.0 mg/l BAP and 2.0 mg/l 2,4-D + 1.0 mg/l BAP (Savitha et al., 2010). According to Fellner and Lebeda (1998), a combination of growth regulators such as 2,4-D, BAP or Kinetin seems to be necessary for the formation and differentiation of calli from *Cucumis sativus* and *Cucumis melo* explants. Zouzou et al. (2008) also reported that auxin and cytokinin combination is suitable to obtain more vigorous and friable callus and that 0.1 mg/l 2,4-D and 0.5 mg/l kinetin was the proper combination of hormones to induce callus in cotton (*Gossypium hirsutum* L.). Media supplemented with BA alone significantly induced more callus on *T.*

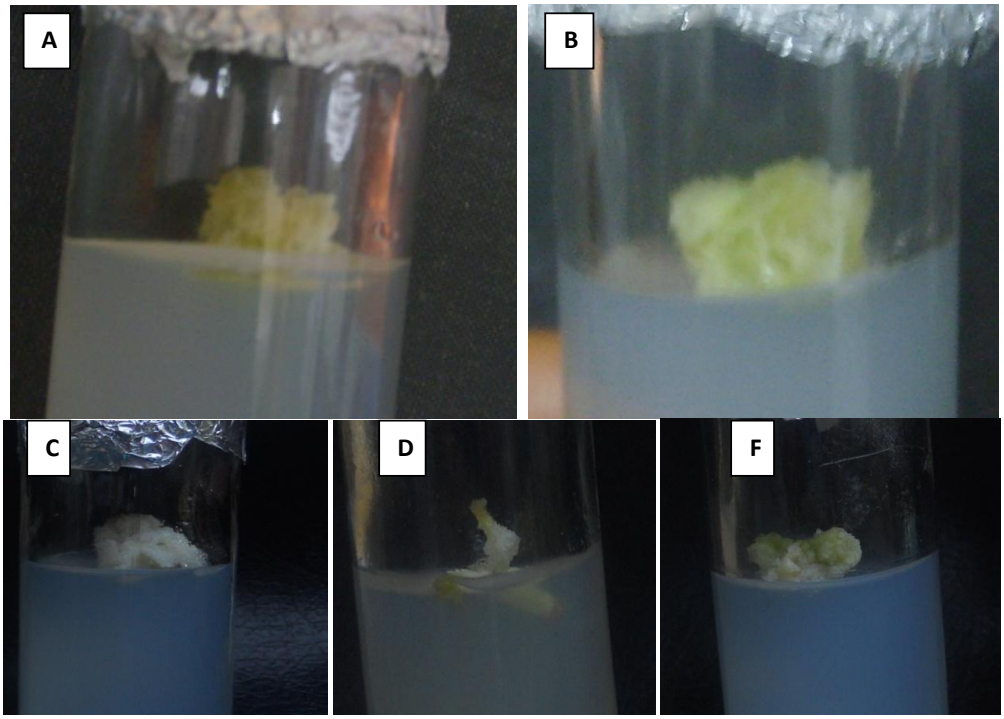

Plate 1. Effect of different concentrations of 2,4-D, Kinetin, NAA and BAP on callus morphology in *T. occidentalis*.
Note: (A) Yellowish callus on stem explant of *T. occidentalis* inoculated on 5 mg/L 2,4-D + 0.1 mg/L kinetin; (B) Yellowish green callus on stem explant of *T. occidentalis* inoculated on 5 mg/L 2,4-D + 0.1 mg/L kinetin; (C) Cream callus on stem explant of *T. occidentalis* inoculated on 0.25 mg/L BA +0.25 mg/L NAA; (D) White callus on nodal explant of *T. occidentalis* inoculated on 0.25 mg/L BA + 0.25 mg/L NAA; (E) Nodular callus on leaf explant of *T. occidentalis* inoculated on 0.5 mg/L BA

occidentalis explants than media supplemented with kinetin alone. However, there was more significant increase in callus induction and proliferation with the addition of kinetin to 2,4-D than with the addition of BA to NAA. This could be due to the resulting ratios of auxin to cytokinins in each situation; the endogenous hormones are present within the explants and the sensitivity of the explants to the hormones. Arivalagan et al. (2012) in their study of the effect of growth hormones on callus induction of *Sauropus androgynous* observed that when auxins alone were used as media supplements, callus was not induced. Introduction of kinetin along with any of the auxins used however, promoted induction of callus.

Equal concentrations of BA and NAA elicited more callus formation than BA in combination with higher concentration of NAA. This finding is supported by the observations of Malek et al. (2010) where he reported that the highest amount of callus from *Trichosanthes dioica* was observed in combination of 0.5 mg/l BAP + 0.5 mg/l NAA when leaf or inter-node explants was cultured in the medium. In this study, low concentration of Kinetin in the medium, in combination with 2,4-D, stimulated the induction and proliferation of callus in *T. occidentalis*. For cotton, low concentration of 2,4-D and high concentration of Kinetin stimulated the proliferation of callus (Zouzou et al., 2008).

The callus formed in *T. occidentalis* was different amongst the explants, with stem and leaf explants generating more callus than nodal explants. These results are in agreement with other results published by several authors who showed that stem (internode) explants are more callogenic (Lou and Kako, 1994; Savitha et al., 2010; Malek et al., 2010). Leaf and internode explants have also been used to induce callus in other cucurbits (Savitha et al., 2010; Malek et al., 2010; Lou and Kako, 1994). According to Zouzou et al. (2008), variation in callus forming ability of different explants types has been reported in many other plants and callogenesis specificity of explants type could be explained by their differential reactivity to media components. The calli color observed in this study were similar to those obtained by Lou and Kako (1994) where they reported that the first leaf- and cotyledon-derived calli of *C. sativus* were yellowish and whitish and the internode-derived callus was larger and greenish.

The results reported in this research work are expected to contribute to the scientific baseline data necessary for the callus initiation of *T. occidentalis*; a *sine qua non* for

its somatic embryogenesis and conservation. Pumpkin cells *(C. pepo* L.) synthesize a tetracylic terpenoid – *cucurbitacin* (Lavie and Glotter, 1971) that could be of interest to the pharmaceutical industry (Katavic and Jelaska, 1991). This terpenoid or its equivalent may be present in *T. occidentalis* and this study provides preliminary information on optimization of media content and hormonal concentration that may provide desired source of pharmacologically active plant constituents through callus culture.

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Morpho-physiological characterization of Indian wheat genotypes and their evaluation under drought condition

Naresh Pratap Singh*, Pramod Kumar Pal and Sandeep Kumar Vaishali

Department of Biotechnology, SVP University of Agriculture and Technology, Meerut, 250110, India.

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To study the morpho-physiological and genetic diversity analysis of Indian wheat cultivars a total of 10 genotypes were collected namely: HD-2133, HUW-825, R-54, K-9533, V-110, V-70, HUW-213, V-23, VWTH-08-07 and HUW-37 to evaluate the genotypes under drought stress the seeds of 10 varieties were treated with polyethylene glycol (PEG) for inducing stress condition. Among the morphological characteristics of wheat genotypes under controlled and treatment condition the characters as; leaf area, numbers of productive tillers/plants, days of maturity were recorded at pre harvest stage and seeds per spike, length of spike and grain yield per plant were recorded at post harvest stage. In the present study, significant reduction in yield components like seeds per spike, number of filled and unfilled seeds per spike and final grain yield was observed in all the test genotypes when drought was imposed at seed stage by treating with PEG. After PEG treatment, the wheat variety V-110, performed better under artificially imposed drought condition and can be considered as drought tolerant variety.

Key words: Wheat, morphological parameters, physiological growth attributes, proline.

INTRODUCTION

Wheat (*Triticum aestivum)* belonging to family *Poaceae* (*Gramineae*) is the major cereal crop of the world. In last few years, climatic conditions have been drastically changed and most part of the world is under low water availability especially in South Asia and Africa. Drought imposes one of the commonest and most significant constraints to agricultural production, seriously affecting crop growth, gene expression, distribution, yield and quality (Zhu, 2002; Zheng et al., 2010; Almeselmani et al., 2011). Drought affects wheat productivity in dry and semi arid areas, and reduces plant yield more than any other environmental stress (Ali et al., 2013). The extent of modification depends upon the cultivar, growth stage, duration and intensity of stress (Araus et al., 2002). Hence, even at the same level of moisture stress condition, different genotypes show different responses as per their genetic potential for adaptation. Drought stress induces a range of physiological and biochemical responses in plants so that plants are able to develop tolerance mechanisms towards environmental stress (Shinozaki and Shinozaki, 2007*;* Gholamin et al., 2010). These responses include stomata closure, repression of cell growth and photosynthesis and activation of respiration, decreesed in the water relation, nutrient uptake and grain yield of the wheat cultivars (Fahimnavaz et al., 2012).

Morphological and agronomic traits of wheat have a

*Corresponding author. E-mail: naresh.singh55@yahoo.com. Tel: +9808388674.

special role in determining the importance of each trait in increasing yield; therefore, these traits were used in breeding programs which at least led to improving yield and introducing commercial varieties that can withstand seasonal drought stress condition (Ahmadizadeh et al., 2011). The biological yield, spike length, number of grains per spike and harvest index showed more direct positive effects on yield (Ahmadizadeh et al., 2011).

Improving drought tolerance and productivity is one of the most difficult tasks for researchers because under drought prone conditions, plant themselves adopts diverse strategies to combat drought stress depending on the timing, severity and stage of crop growth. In addition to this, the meager availability of drought resistant cultivars further adds to the problem. It is therefore, necessary to appraise and screen wheat genetic resources to identify the sources of the resistance for breeders to construct new wheat varieties. In this view, the major wheat varieties grown in Western Uttar Pradesh were evaluated for their performance in response to drought with a special focus given to morphological, physiological and biochemical parameters.

MATERIALS AND METHODS

Ten (10) major grown varieties of wheat (*T. aestivum*) in the area of Western Uttar Pradesh were selected for the present study. For this, seeds of all varieties viz. HD-2133, HUW-825, R-54, K-9533, V-110, V-70, HUW-213, V-23, VWTH-08-07 and HUW-37 were collected and treated with polyethylene glycol (PEG) for imposing drought condition (Figure 1a). Thirty (30) seeds of each variety were placed on the moist Whatman germination papers in Petri dishes and 5 ml of 15% PEG solution was applied on each day up to seven days along with control. After seven days, the germinated seeds were transferred into field for overall comparative study of morpho-physiology of wheat. The crop was maintained in the field using conventional agronomic practices to keep the crop in good condition (Figure 1b) and data was recorded timely.

Morphological evaluation of wheat genotypes

Observations for morphological parameters were recorded from randomly selected five plants from each introgression lines in each replication at maturity. The data was recorded for pre-harvest characters like number of productive tillers (at 30 to 45 days after sowing depending upon the growing condition), leaf area (length and width of flag leaf), and days to maturity (number of days taken from sowing to the browning of ears). For post harvest characters, the data was recorded for length of spike (ear length is measured in centimeter from tips of apical spikelet (excluding awns) to the bases or collar of ear), number of grains per spike (mean number of seeds counted from 10 randomly sampled spikes at maturity), and grain yield per plant (weight of seed per plant expressed in grams).

Physiological evaluation of wheat genotypes

To study the physiological changes after imposing the drought condition, the data for related water content and chlorophyll content was recorded. The relative water content (RWC) of flag leaf was measured following the method of Turner (1981). Fresh weight (FW) of flag leaf was determined immediately after harvest, and then allowed to float in distilled water until fully rehydrated and weighed for turgid weight (TW). The turgid leaf was dried in a hot oven at 80°C to constant weight, and dry weight (DW) was recorded. The RWC of the first leaves, coleoptiles and roots was calculated as: RWC (%) = (FW - DW) / (TW - DW) * 100.

Chlorophyll meter (SPAD 520) was used to measures the relative chlorophyll content of the leaves. Five readings were taken from single plant leaves and their average was considered for determination of chlorophyll content. The chlorophyll content was recorded in terms of percentage.

Biochemical evaluation of wheat genotypes

Prolone is a major biochemical signal of drought tolerance. For estimation of Proline content, 100 mg of fresh leaf tissue was taken from normal plants and treated plants. Grind the leaf tissue in aqueous sulphosalisylic acid (3%) and centrifuged at 7000 rpm for 5 min. The supernatant was mixed with equal volume of Glacial acetic acid and 0.5 ml of ninhydrin was added. The tubes were incubated for 30 min in boiling water bath and placed for 5 min in ice bath for cooling. To stop the reaction, 2 ml of toluene was added in each tube. The reaction mixture was mixed properly and aqueous phase was transferred in new tube. The reaction mixture was warm at 25°C and chromophore was measured at 520 nm.

Statistical analysis

The experimental data were compiled by taking mean values over randomly selected plant from both replications and subjected to the statistical analysis. The analysis of variance for the design of the experiment was carried out according to the procedure outlined by Panse and Sukhatme (1978).

RESULTS AND DISCUSSION

Morphological characteristics of wheat genotypes under controlled and treatment condition

PEG is an osmotic agent, which plays an important role in the regulation of mineral elements harmones, protein metabolism and effects on signal transduction. The main function of PEG is to slow down the moisture rate of impact and export seeds, which benefit to reduce membrane system injury in process of seed imbibitions and repair impaired membrane system. Therefore in the present study, the PEG has been used in seed priming and simulated water stress to study the effect of drought on various aspects like photosynthesis (Guo et al., 2004).

Pre harvest characteristics

Leaf area of flag leaf is directly related to higher photosynthesis and high chlorophyll content. For measuring the leaf area of flag leaf, five plants of each variety was taken and their mean is presented in Table 1. The leaf area was varied from 30.88 to 62.71cm^2 of genotype R-54 and V-23, respectively. Total leaf area of flag leaf of wheat genotypes was decreased significantly after PEG

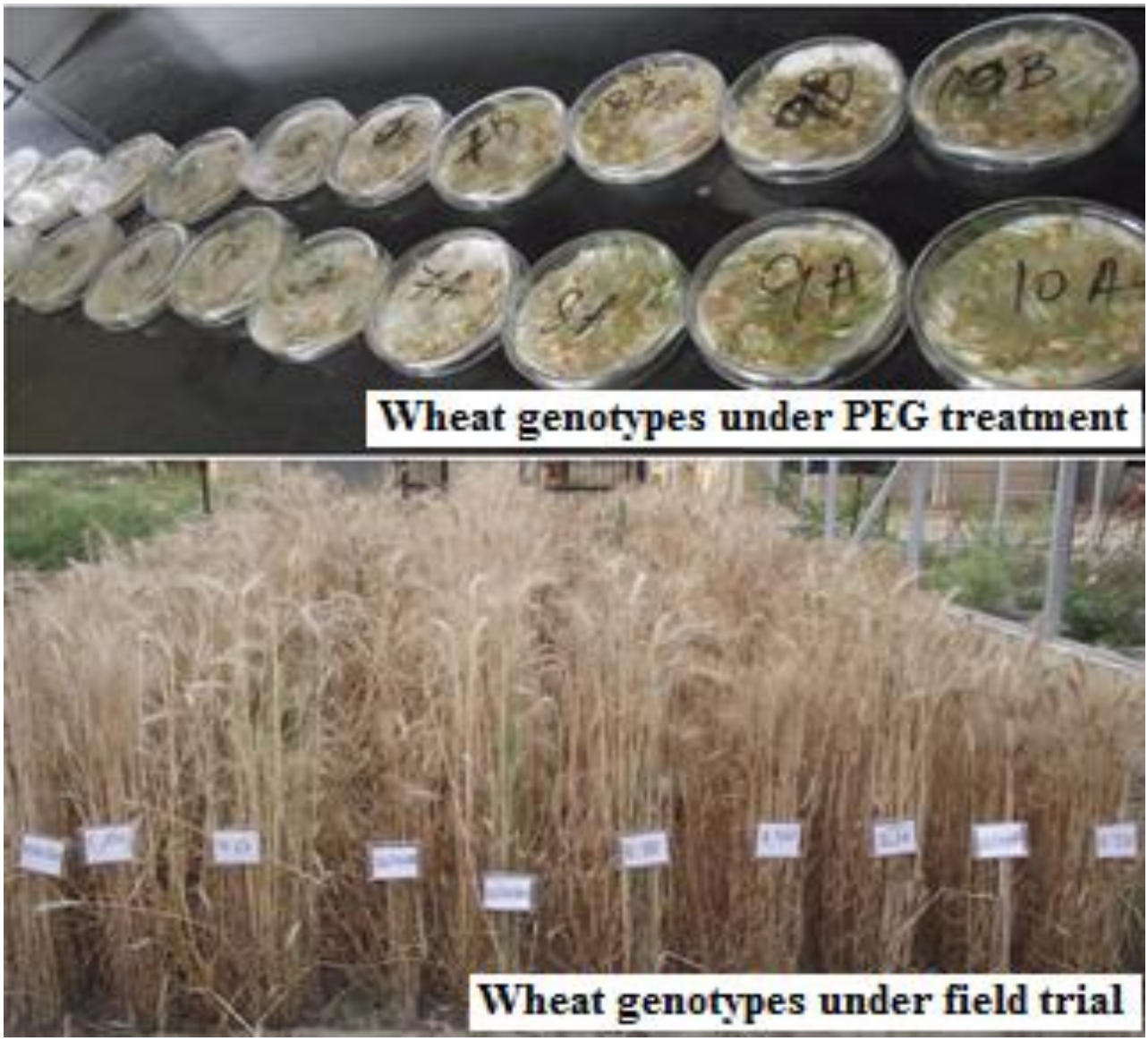

Figure 1. Wheat genotypes under PEG treatment and mature crop under field trial.

treatment. However, genotype V-110 and V-70 showed a least decline in leaf area and considered to be of better performance under drought condition (Figure 2).

The numbers of productive tillers per plants were noted at the time of maturity (Table 1). The number of productive tillers per plant was varied from 5 in HD2133 to 8.8 in V110 in controlled plants. The treatments of PEG, reduces the number of productive tillers per plant. After the treatment, the number of productive tillers reduced and varied from 3.6 to 8.5. The genotype V-110 and V-70 showed lesser effect of PEG treatment and performed better under artificially induced drought conditions; whereas the genotype HD-2133 showed a significant effect of PEG treatment.

All the wheat genotypes were maintained in good condition using recommended agronomic practices; although, all the varieties took different time to reach maturity. The days of maturity of each variety were noted down at the time of 50% grain maturity. Overall, the genotypes took 105 to 130 days to reach maturity in field (Table 1). Under artificially imposed drought condition by treating them with PEG, the crop took longer time in field to reach maturity as compared to controlled plants. How-ever the genotype V-110 shows the less effect of PEG treatment and matures along with the controlled plants.

Post harvest characteristics

In the present study, the significant differences were reported in total yield per plant and numbers of grains per spike amongst different varieties under simulated drought

Table 1. Morphological characteristics of wheat genotypes under normal and treatment of PEG

Genotype	Leaf area (cm)^2		No. of tiller		Days To maturity		Length of spike		N. of grain/ spike		Grain yield/ Plant (gm)	
	C	T	C	T	C	T	C	T	C	T	C	T
HD-2133	55.77	54.90	5.00	3.60	130	134	7.10	6.50	45.00	42.00	11.32	9.18
HUW-825	35.81	28.24	6.80	6.10	115	119	9.50	9.60	51.00	50.40	13.36	12.62
R-54	30.88	29.59	6.60	6.80	118	122	9.20	8.94	52.80	48.60	13.89	12.98
K-9533	51.95	39.61	6.40	6.60	116	117	8.60	8.74	49.80	45.30	11.42	10.82
V-110	58.23	58.29	8.80	8.50	105	104	10.50	10.00	61.40	63.00	16.37	16.32
V-70	53.16	52.81	8.10	8.00	108	108	9.04	9.04	54.00	53.10	15.23	15.02
HUW-213	43.04	38.01	6.80	5.90	115	113	9.04	8.97	51.00	48.00	13.36	13.01
V-23	62.71	58.65	7.80	7.00	110	115	8.93	8.52	60.00	58.80	15.71	14.62
VWTH-08-7	47.66	42.61	6.40	5.20	120	119	7.04	8.20	52.20	51.30	14.52	13.95
HUW-37	40.54	35.78	7.20	7.10	108	112	8.70	8.70	53.20	51.00	15.26	15.13

C, Control; T, treatment.

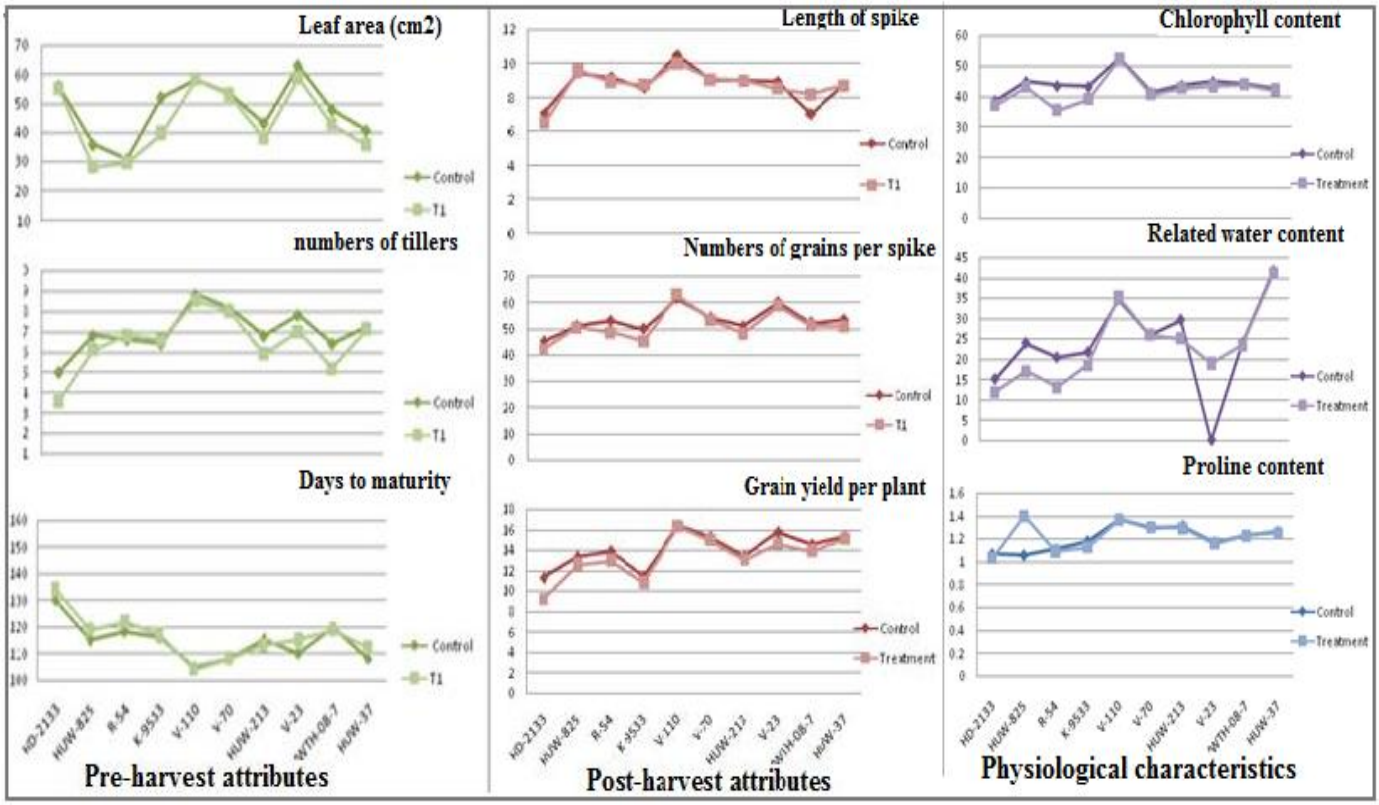

Figure 2. Pre-post harvest and Post-harvest growth attributes of wheat genotypes.

stress condition. Drought stress negatively affect length of spikelet yield per plant and number of grains per spike. The length of spikelets is directly contributed to yield component. The result shows that the length of spike varied from 7.04 cm in VWTH-08-7 to 10.5 cm in V-110 genotype (Table 1). The treatment of PEG affects the length of spike a little in all the genotypes except genotype V-110 (Figure 2).

Seeds per spike are a direct measure of yield/plant, hence it is an economically important post harvest characteristic. Number of seeds/spike varied from a lower value of 45 in HD-2133 to a higher value of 61.4 in V-110

Table 2. Physiological performance of wheat genotypes under normal and treatment of PEG.

Genotype	Chlorophyll		RWC		Proline	
	Control	Treatment	Control	Treatment	Control	Treatment
HD-2133	38.484	36.82	15.02	12.00	1.07	1.03
HUW-825	44.776	42.98	23.90	17.05	1.06	1.40
R-54	43.464	35.40	20.33	13.10	1.11	1.09
K-9533	43.152	39.000	21.66	18.61	1.18	1.13
V-110	52.420	52.31	34.66	35.21	1.37	1.37
V-70	41.228	40.54	25.87	26.00	1.30	1.30
HUW-213	43.584	42.53	29.62	25.09	1.31	1.29
V-23	44.776	43.28	20..49	19.01	1.17	1.16
VWTH-08-7	44.200	43.83	23.86	23.37	1.23	1.23
HUW-37	42.558	41.98	41.56	40.98	1.26	1.25

(Table 1). The number of seeds per spike was decreases after PEG treatment in almost all the genotypes except genotype V-110 where it increases from 61.4 to 63 after the treatments of PEG (Figure 2).

The grain yield per plant of each genotype were recorded and found to be varied from 11.32 in HD-2133 to 16.37 in V-110 in controlled plants (Table 1). After the treatment of PEG, the HD-2133 genotype showed a remarkable decrease in grain yield per plant (Figure 2). On the other hand, the grain yield of rest of the genotypes showed a little effect of PEG treatment.

This is also supported by Chander and Singh (2008) and Ali et al. (2013) that numbers of grains per spike were decreased under drought stress. Water stress has been reported to affect all the yield components, mainly the number of grains per spike and the number of pikes per plant (Giuanta et al., 1993; Simane et al., 1993). It has been recognized that decrease in yield and yield component under drought stress is a key concern in developing countries of the world (Guo et al., 2004).

Physiological characteristics of wheat genotypes under controlled and treatment condition

The chlorophyll content showed variation in control and in both treatments of PEG. Among all the genotype, V-110 shows highest chlorophyll content; that is, 52.42 µg and lowest in HD-2133; that is, 38.48 µg (Table 2). The treatment of PEG reduces the total amount of chlorophyll as compared to control and varied from 35.40 µg in R-54 to 52.31 µg in V-110 (Figure 2).

The plant free proline massive accumulated when the plants was subject to drought stress, the reason is, proline dehydrogenase activity is decreased and made the proline oxidation weakened; drought suppressed the protein synthesis, proline utilization is decreased and to increased accumulation in plants (Zhan et al., 2011). Therefore, the proline content is positively correlated with drought resistance in wheat seedling; hence the accumulation of proline contents could be use as physiological indicators of stress resistance (Gabor et al., 2004). In this view the total proline content was estimated among the studied genotypes. The proline content is present in significant amount in the leaf of wheat genotypes and varied from 1.06 µg/gfw in HUW-825 to 1.37 µg/gfw in genotype V-110 (Table 2, Figure 2). Proline content increased under treatment of PEG; this is quite understandable as proline is known to be produced in higher amount under stress condition as it helps in resisting plants against stress condition.

The RWC of the leaves indicate the water condition of the cells and have important correlation with biotic and abiotic stress tolerance (Almeselmani et al., 2011). It has been reported that, RWC of the leaves has strong association with drought tolerance (Kaur et al., 2011) and it is a good indicator of drought stress than other physiological and biochemical characteristics of the crop plants (Colom and Vazzana 2003). Our results reveal significant differences in RWC among varieties at three different stages and showed that, retention ability of the plant was significantly different at different growth stages. The relative water content (RWC) was estimated for the present 10 wheat genotypes under controlled condition and after the treatment of PEG (Table2). The RWC in controlled plants varied from 15.02% in HD-2133 to 41.56% in HUW-37. The higher RWC was estimated after PEG treatment in cultivar HUW-37(41.56%) and V-110 (35.21%) and thus can be considered as drought tolerant. The lowest RWC estimated after PEG treatment were obtained in K-9533 (12.00%) and can be considered as drought sensitive (Figure 2).

This variation in RWC of leaf may be due to the ability of the tested wheat genotypes to absorb more water from soil and also to control water loss through the stomata (Sinclair and Ludlow1985). It may also be due to the variation in the ability of wheat genotypes to avoid stress by maintaining tissue turgor osmotically. These results were supported by Schonfeld et al., (1988) and Ali et al., (2013) that RWC may be used as a selection criterion in

breeding for improved drought resistance in wheat genotypes (Schonfeld et al., 1988).

Results showed significant variation among the genotypes, traits and their interactions. Artificial induction of drought by treatment of PEG caused a substantial reduction in growth related attributes in most of the wheat genotypes except in V-110 genotype. In the present study, significant reduction in pre-harvest and post-harvest characteristics which are directly related to yield like leaf area, number of productive tillers, days to maturity, length of spike, number of filled and unfilled seeds per spike and final grain yield per plant was observed in all the test genotypes when drought was imposed at seed stage by treating with PEG. Overall the genotype V-110sows least effect of PEG treatment in term of leaf area, number of tillers and maturity time, therefore can be considered as drought tolerant genotype. On the other hand the variety HD-2133 shows lesser leaf area, less no of productive tillers and stays a long in field to get mature after the PEG treatment and therefore said to be sensitive against drought condition. Bayoumi et al. (2008) observed that water stress caused 43% reduction in grain yield of wheat varieties and subjecting the seeds of wheat varieties to artificial osmotic stress condition in the laboratory (treating with PEG solution) is an adequate tool for the presumption of their thriving against water stress field condition. The parameters related to plant growth envisaged as prominent characteristics for drought resistant screening process of wheat varieties (Foito et al., 2009).

This study allows us to recognize those physiological characteristics that are associated with drought stress, and screen out appropriate wheat genotypes, which can be introduced in arid area to produce high yield in drought conditions and can be further used in breeding programs to produce a stress tolerant genotype.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Cloning and tissue expression of cytochrome P450 *1B1* and *1C1* genes from Javanese Medaka, *Oryzias Javanicus*, under environmental stress conditions

Trieu Tuan[1]*, Yoshino Kaminishi[1], Aki Funahashi[1], Abeer A. I. Hassanin[2] and Takao Itakura[1]

[1]Laboratory of Marine Biotechnology, Faculty of Fisheries, Kagoshima University, 4-5-20 Shimoarata, Kagoshima 890-0056, Japan.
[2]Department of Animal Wealth Development, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

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Cytochrome P450 1 (CYP1) is widely used as an indicator of exposure to environmental contaminants. In the study, two full-length complementary DNAs encode for CYP1B1 and CYP1C1 were cloned from medaka liver exposed to 500 ppb β-naphthoflavone for 24 h. *CYP1B1*, having 1984 bp, contains an open reading frame of 1551 bp encoding a protein of 517 amino acids; while, *CYP1C1* having 2601 bp consists of an open reading frame of 1578 bp encoded for 525 amino acid residues. The highest levels of these CYP1 genes transcript were observed in intestine and the lowest in liver from the fish fed on fuel oil-contaminated feed. Javanese medaka CYP1B1 and CYP1C1 transcripts were detected in the gill, muscle and liver when transferred from seawater to freshwater with the highest level of expression in gill and muscle. Starvations for one week tended to down- regulate the Javanese medaka CYP1 expression.

Key words: Cytochrome P450, Javanese medaka, salinity, starvation, heavy fuel oil, cloning, expression.

INTRODUCTION

Cytochrome P450 (CYP) super family can be found in nearly all organisms, including animals, plants, fungi, lower eukaryotes and bacteria(Bernhardt, 2006; Nebert and Dalton, 2006; Nelson, 2010). Cytochrome P450 is widely used as an indicator of exposure to environmental contaminants (Hahn et al., 1998). CYP proteins play a critical role in the oxidative metabolism of endogenous compounds, and xenobiotic (exogenous) compounds, in-cluding pharmaceuticals and environmental toxins (Diotel et al., 2010). The cytochrome CYP1 family consists of four known subfamilies of vertebrate species (CYP1A, CYP1B, CYP1C and CYP1D) but only CYP1A and CYP1B enzymes appear to be present in all vertebrates and have been investigated the most. Mammals have twoCYP1A genes (*CYP1A1* and *CYP1A2*) while most fish have a single CYP1A gene (Goldstone et al., 2007). Almost all vertebrates have a single CYP1B gene, the *CYP1B1*. There are two CYP1C genes found in fishes

*Corresponding author. E-mail: tttuanbl@gmail.com. Tel/Fax: +81-99-286-4220.

(*CYP1C1* and *CYP1C2*), while all non-mammalian vertebrates have only a single CYP1C1 (Goldstone et al., 2007). Given their various degree of responsiveness to pollutants, expression patterns of the *CYP1A*, *CYP1B*, and *CYP1C* genes in fish could become useful biomarkers in environmental monitoring (Jonsson et al., 2010).

Javanese medaka (*Oryzias javanicus*) is used extensively as experimental test fish because it has several advantages; it has a unique osmotic adaptation mechanisms, and a wide range of salinity tolerance (Inoue and Takei, 2002). Another advantage is that medaka can naturally tolerate to low temperature, it can survive at 40°C in summer and 4°C in winter without any thermostatic regulator (Kinoshita et al., 2009). Therefore, the fish has been widely used in assessing environmental risk, toxicity test for new pollutants in various environments, biological response testing and sensitive molecular biomarkers testing (Koyama et al. 2007; Woo et al. 2009).

Salinity changes are considered as aquacultural and environmental problem worldwide. Euryhaline fish, such as the Javanese medaka, can live in both fresh and seawater. In freshwater, these fish are hyperosmotic relative to their external medium. Many changes in gene expression and protein activity observed at least 12 to 18 h after exposure to a hyperosmotic environment, suggesting that these effects may be mediated by immediate early gene transcriptional factors (Fiol and Kültz, 2005). Indeed, the involvement of such transcriptional factors displaying rapid changes to gene expression after hyperosomotic stress has recently been described in tilapia (*Oreochromis mossambicus*) transferred from freshwater to seawater(Fiol and Kültz, 2005). Functional genomic studies of fish stress responses, particularly the identification of a core set of stress-related transcripts of CYP1 genes, are crucial for better understanding in their physiological and toxicological functions.

Heavy fuel oil products have been reported to contain several toxic organic and inorganic components such as polycyclic aromatic hydrocarbons, atoms of sulfur, nitrogen, oxygen as well as metals such as; iron, vanadium, sodium, nickel, chromium and other metals present in small amounts (Baker, 1970; Williams et al., 1994; Irwin et al., 1998) which constitutes a significant health risk to both people and other organisms. The ingestion of petroleum hydrocarbon has been reported to induce oxidative stress (Val and Almeida-Val, 1999) generating free radicals, (Achuba and Osakwe, 2003) which leads to lipid peroxidation (Halliwell, 1994) that damages critical cellular macromolecules like DNA, lipids and proteins (Breimer, 1990; Romero et al., 1998; Souza et al., 1999). Heavy fuel oil also causes an increased prevalence of morphological abnormalities, and reductions in growth and recruitment causing Blue Sac Disease (BSD) in fish, with symptoms of edema, hemorrhaging, deformities and induced CYP1A enzyme(Marty et al., 1997). Fish affected in this way were unable to feed and eventually starved or were consumed by predators.

All animals face the possibility of limitations in food resources that could ultimately lead to starvation-induced mortality. Starvation is a situation undergone and tolerated by many species of fish in their natural environments in response to several factors. To survive these periods of unfavorable feeding conditions, fish reduce their energy expenditures, which in a high percentage are derived from protein synthesis, and mobilize their endogenous reserves to obtain the energy required to maintain the vital processes (Miriam and Ana, 2011). Starvation has been reported to have pro-oxidant effects, and both the inadequate neutralization of the reactive oxygen species (ROS) generated by oxidative metabolism and the reduced level of antioxidant defenses, both enzymatic and non-enzymatic, may be responsible for some of the detrimental effects of starvation. In addition, starvation may influence 7-ethoxyresorufin-O-deethylase (EROD) activities in fish (Andersson et al., 1985; Jorgensen et al., 1999). Starvation promotes the mobilization of lipids from adipose tissue and/or liver lipid droplets, and, thus the mobilization of the lipophilic toxicants is stored in these tissues (Sancho et al., 1998). The effects of starvation on EROD activities are contradictory; in rainbow trout starved for 6 or 12 weeks, a single intraperitoneal injection of benzo[a]pyrene decreased renal EROD activities after six weeks and hepatic EROD activities after 12 weeks of starvation (Andersson et al., 1985). However, in a study by Vigano et al. (1993), three weeks of starvation had no influence on hepatic EROD activities, (Vigano et al., 1993) whereas increased liver EROD activities were observed in starved Arctic charr (*Salvelinus alpinus*) previously exposed to the PCB mixture Aroclor 1260 (Jorgensen et al., 1999).

Real-time PCR (qRT-PCR) is a sensitive, reproducible and high-throughput method that can show subtle changes in relative quantities of a large number of genes, consuming small sample amounts (Bustin, 2002; Bustin and Nolan, 2004). Expression patterns of CYP1 genes in fish could become useful biomarkers in environmental monitoring (Jonsson et al., 2010). In the study, we report on cDNA cloning and sequence analysis of the dominant isoforms of cytochrome P450 genes, CYP1B1 and CYP1C1 from Javanese medaka exposed to 500 ppb BNF over a period of 24 h, and examined their level of inducibility by environmental stress factors in various fish organs using real-time PCR.

MATERIALS AND METHODS

Treatment of fish

Javanese medaka was held in the aquarium facility at the Marine Biotechnology Laboratory, Faculty of Fisheries, in Kagoshima University. The fish were cultured in laboratory conditions for one week before the experiment, during which they fed twice daily on a fine fish commercial diet. After the acclimatization period, eight

Table 1. Oligonucleotide primers used for CYP1B1, 1C1 cloning and real time PCR.

Primer name	Description	Location	Primer sequence (5'-3')
Dgp_CYP1B 1F	Degenerate PCR	697-715	GTGGAYGTGATGCCYTGGC
Dgp_CYP1B 2R	Degenerate PCR	1230-1250	TGRTTSAHRGACCACTGGTTG
Dgp_CYP1B 3F	Degenerate PCR	646-665	SAGGTGGTGGGYAGRAAYGA
Dgp_CYP1B 4R	Degenerate PCR	1107-1127	ACYTCRTARAYGAARGCCATG
Dgp_CYP1C 1F	Degenerate PCR	697-715	GTGGAYGTGATGCCYTGGC
Dgp_CYP1C 2R	Degenerate PCR	1230-1250	TGRTTSAHRGACCACTGGTTG
Java_CYP1B_GSP 1F	RACE PCR	3-28	GGATGTGATGCCTTGGCTCCAGTATT
Java_CYP1B_GSP 2R	RACE PCR	335-359	CCCACCACTCTGTCCACCTCCTTCT
Java_CYP1B_GSP 3F	RACE PCR	21-46	CCAGTATTTCCCCAACCCCATCAAGA
Java_CYP1B_GSP 6R	RACE PCR	309-333	CAGACGCCGCTGCATCTCAGGATAC
Java_CYP1C_GSP 1F	RACE PCR	131-155	ACCCCGAGGTGACCCGAGACATAAG
Java_CYP1C_GSP 2R	RACE PCR	458-483	AATGGTGACGTCAGAGGTGGTGGAGT
Java_CYP1C_GSP 3F	RACE PCR	172-196	GTGATTGAGCACGGAGAGGACAGCA
Java_CYP1C_GSP 4R	RACE PCR	404-429	GAAGCGCATGGTCTCGTAGATGAAGG
Java_CYP1B 1F	Real-time PCR	1188-1207	GAGCTACACCATCCCCAAGA
Java_CYP1B 2R	Real-time PCR	1301-1320	CTTGTTCAGCTTCCCCTCTG
Java_CYP1C 1F	Real-time PCR	1244-1263	ACCAGTTCTCCGTCAACCAC
Java_CYP1C 2R	Real-time PCR	1362-1381	GCCGTTTACCTGTGGAGAAA
Java_actin 1F	β-actin RT PCR	346-365	AGGGAGAAGATGACCCAGAT
Java_actin 2R	β-actin RT PCR	447-466	CAGAGTCCATGACGATACCA

adult fish were exposed to 500 ppb β-naphthoflavone for 24 h. Liver was dissected, immediately frozen in liquid nitrogen, and stored at -80°C until further use.

RNA preparation and cDNA synthesis

Total RNA was extracted from liver using ISOGEN reagent (Nippon Gene, Japan) according to the manufacturer's protocol. The concentration of RNA was determined by a spectrophotometer (Gene Spec V, Hitachi, Japan). Total RNA concentration and purity were determined spectrophotometrically as described by (Sambrook and Russell, 2001), and the A260/A280 ratio was between 1.7 and 1.9. Poly $(A)^+$ RNA was purified using an Oligotex™ -dT30 <Super> mRNA Purification Kit (Takara Bio, Japan). First strand cDNA was synthesized by the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Japan).

cDNA cloning and sequencing of CYP1 family cDNAs

Degenerate inosine-containing primers were designed from highly conserved regions, based on the alignment of CYP1B and CYP1C sequences from other fish species. All the primers are shown in Table 1. PCR was performed using an Astec PC320 PCR system (Astec Bio, Japan) and TaKaRa Ex Taq polymerase (Takara Bio, Japan) using the following PCR program: initial denaturation step at 94°C for 2 min 30 s and subsequent 35 cycles of amplification (94°C, 30 s; 50°C, 30 s; 72°C, 30 s) and a final extension step for 2 min at 72°C. The 5' and 3' ends of the CYP1 family cDNA were obtained by rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA Amplification Kit (Clontech Takara Bio, Japan) following the supplier's protocol. Gene-specific primers (GSP) were designed based on the sequence obtained from PCR with degenerate primers (Table 1). For cloning, DNA bands of the expected size were excised from the gel, purified using the illustra GFX DNA and Gel Band Kit (GE Health Care, UK), and sub-cloned using pT7 Blue T vector (Novagen, USA) with the Ligation-Convenience Kit (Nippon Gene, Japan). Ligated DNA was transformed into JM109 *Escherichia coli* cells. Purified plasmids were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA).

DNA sequencing data was retrieved and edited by Lasergene sequence analysis software (DNAStar ver5.2). Sequence homology searches were carried out using the Basic Local Alignment Search Tool (BLAST) program at http://www.ncbi.nlm.nih.gov/BLAST/, whereas sequence alignment was performed using the ClustalW program with MegAlign in DNAStar. DNA sequences with their GeneBank accession numbers that were retrieved from the database and used in the analysis are shown in Table 2. Phylogenetic and molecular evolutionary analyses were constructed by web-based software using the neighbor-joining method (Dereeper et al., 2008; Dereeper et al., 2010). MatGAT (James et al., 2003) was used to calculate similarity and identity of deduced amino acid sequence of Javanese medaka CYP1s with other CYP1 family members.

Expression of CYP1B1 and CYP1C1 in various organs of Javanese medaka

Animal and experimental design

Javanese medaka fish were acclimatized to laboratory conditions for one week before the experiment during which they were fed

Table 2. Gene Bank accession numbers of the CYP1 cDNAs used.

Species	cDNA	Accession number
Tilapia (*Oreochromis niloticus*)	CYP1B1	HQ829968
	CYP1C1	HQ829969
Scup (*Stenotomus chrysops*)	CYP1C1	AF131885
Plaice (*Pleuronectes platessa*)	CYP1B1	AJ249074
Rat (*Rattus norvegicus*)	CYP1B1	U09540
Human (*Homo sapiens*)	CYP1B1	U03688
Rainbow trout (*Oncorhynchus mykiss*)	CYP1C1	NM_001185031
Zebrafish (*Danio rerio*)	CYP1C1	NM_001020610
Chicken (*Gallus gallus*)	CYP1C1	JN656933
	CYP1B1	XM_001233594
Japanese eel (*Anguila japonica*)	CYP1B1	AY518340
	CYP1C1	AY444748
Carp (*Cyprinus carpio*)	CYP1B1	AB048942
	CYP1C1	AY437776
Killifish (*Fundulus heteroclitus*)	CYP1B1	FJ786959
	CYP1C1	DQ133570

twice daily on the medaka commercial diet (Kyorin, Japan); all fish were feeding well at the initiation of the treatments. Natural seawater (33 to 34 ppt) was used for all the experiments. The water temperature was kept at 23±0.5°C. Water pH value ranged from 7.4 to 7.6. The experimental systems were continuously aerated to ensure that the dissolved oxygen levels were adequately maintained.

Oil-contaminated feed experiment: Heavy fuel oil (bunker C) was used in the experiment. The oil contents of carbon and sulfur residues were <4 and <2%, respectively (Koyama and Kakuno, 2004). Javanese medaka was fed with an oil-contaminated feed at levels of 0% (control) and 1%. Thirty medaka fish were divided into two groups and kept in a 12 L tank. Feed was given *ad libitum* during the experiment cycle. Tissue samples were collected after 24 h.

Salinity shock experiment: Adult Javanese medaka cultured in seawater were starved for 2 days prior to freshwater transfer, and the change in salinity was ensured by direct transfer from seawater to either freshwater or seawater (control group). Tap water was dechlorinated, and aerated several days prior to its use in the salinity shock experiment. Fifteen medaka fish per group (control and treated) were kept in a 12 L tank. During the freshwater transfer experiments, fish either kept in seawater or transferred into freshwater were sampled after 24 h.

Starvation experiment: The fish were acclimated to laboratory conditions for one week prior to the study and all fish were feeding well at the initiation of the treatments. Two groups (15 medaka fish per group) of adult Javanese medaka were either starved or fed (control group) for 1 week. Fish were kept in a 12 L tank. Fish in the control group were fed twice daily with medaka commercial pellets (Kyorin, Japan).

Reverse transcription, primer design and real-time PCR

Total RNA was isolated from liver, gill, muscle, and intestine using QuickGene RNA Tissue Kit S II (RT-2) (Fujifilm, Japan), according to the manufacturer's instruction. Reverse transcription of mRNA was performed with PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, Japan), following the supplier's protocol. Gene-specific primers for CYP1s and β-actin, as an internal control gene (accession no. JQ905607), were designed by the web-based software Primer3Plus (Andreas et al., 2007) with a product size between 50 to 150 bp, T_m ranging from 57 to 63°C and all the default parameters (Table 1). Real-time PCR was performed using FastStart Essential DNA Green Master Kit and a LightCycler® Nano system (Roche Applied Science). For each sample, gene expression was analyzed in triplicate with the following protocol: initial holding at 95°C for 10 min, 3-step amplification in 45 cycles (95°C for 10 s, 60°C for 10 s and 72°C for 15 s), holding stage at 95°C for 30 s and melting at 60°C for 20 s and 95°C for 20 s. Melt curve analysis was performed at the end of each PCR run to assure that the single product was amplified. The $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) was used to compare the expression levels of the different CYP1s within a given organ, and to calculate changes in fold-induction in response to treatments, oil-contaminated feed, salinity and starvation, using β-actin as a reference gene.

RESULTS AND DISCUSSION

Cloning of CYP1B1 and CYP1C1 from Javanese medaka

Two full-length CYP1 cDNAs were cloned from Javanese medaka liver exposed to 500 ppb β-naphthoflavone for 24 h. CYP1B1 from Javanese medaka contains 1984 bp, a 5' noncoding region of 106 bp, an open reading frame of 1551 bp, and a 3' noncoding region of 300 bp including the polyA tail. The deduced protein sequence has 517 amino acid residues with an estimated molecular weight of 58.64 kDa. Three putative polyadenylated signal sites (AATAAA) can be found in the long 3' untranslated region

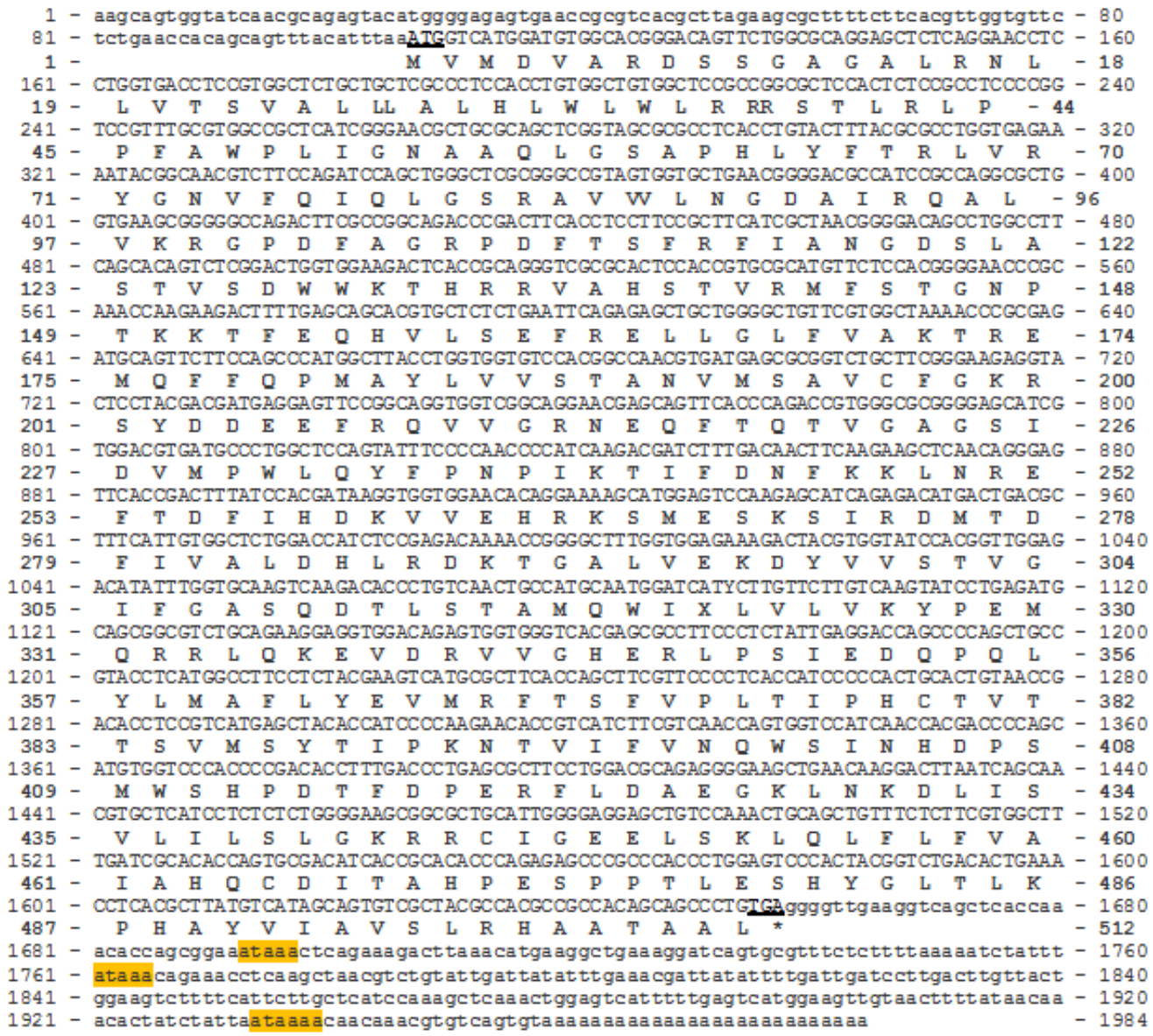

Figure 1A. Full-length cDNA of CYP1B1 and its deduced amino acid residues. The coding sequences are shown in uppercase letter whereas lowercase for untranslated regions. The predicted amino acid sequences are in bold letters. The translation start codon and termination codon are underlined and the putative polyadenylation signal (aataaa) is colored in orange.

(Figure 1A). The full-length cDNA of CYP1C1 from Java medaka contains 2601 bp with an open reading frame of 1578 bp. The deduced protein sequence has 525 amino acid residues with an estimated molecular weight of 59.21 kDa. A 216 bp 5' untranslated region precedes the start codon, and 807 bp 3' untranslated region follows the stop codon. Three putative polyadenylated signal sites (AATAAA) were found in the long 3' untranslated region (Figure 1B). Table 3 shows the percentage of similarity and identity of deduced amino acid sequences of Javanese medaka CYP1B1 and CYP1C1 with other published cytochrome P450 sequences. Results indicates that Javanese medaka CYP1B1 showed highest similarity and identity to CYP1B1 from tilapia (*Oreochromis niloticus*) 69.3 and 84.5%, respectively. The Javanese medaka CYP1C1 deduced amino acid sequence shows highest similarity and identity to the tilapia CYP1C1 82.3 and 95.4%, respectively. The phylogenetic tree, based on the amino acid sequences was used to assess the relationship of CYP1 of Javanese medaka with those in other fish species (Figure 2). The phylogenetic tree, with representatives of full-length CYP1 protein sequences, indicates

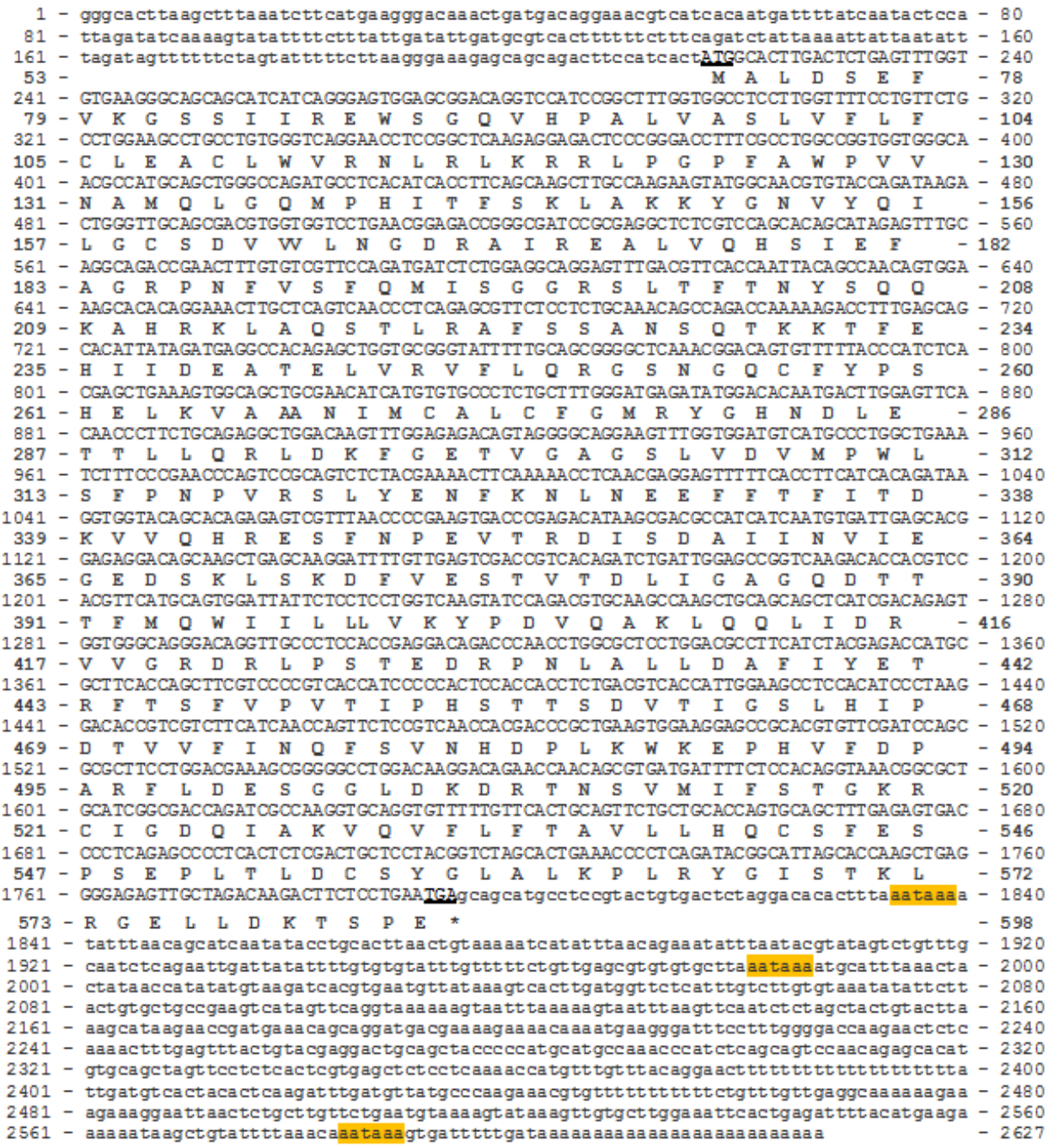

Figure 1B. Full-length cDNA of CYP1C1 and the deduced amino acid residues. The coding sequences are shown in uppercase letter whereas lowercase for untranslated regions. The predicted amino acid sequences are in bold letters. The translation start codon and termination codon are underlined and the putative polyadenylation signal (aataaa) is colored in orange.

that Javanese medaka CYP1B1 and tilapia (*O. niloticus)* CYP1B1 formed a cluster in the phylogenetic tree, indicating the evolutionary relatedness. The phylogenetic tree of Javanese medaka CYP1C1 and other CYP1 families and subfamilies revealed that CYP1C1 belonged to a cluster with CYP1C1 from scup (*Stenotomus chrysops*) and tilapia.

The deduced amino acid sequences of CYP1B1 and

Table 3. Percent similarity and identity (upper triangle) of deduced amino acid sequence of Javanese medaka CYP1s with other CYP1family members.

	1	2	3	4	5	6	7	8	9	10	11	12
Java_CYP1B1		61.9	63.3	69.3	68.3	69.1	51.5	49.7	49.1	50	48.6	48.7
Carp_CYP1B1	78.1		66.1	63.2	60.9	60.6	51.5	48.4	49.5	49.6	48.9	49.4
Jeel_CYP1B1	78.7	80.9		65.9	65	64.4	50.2	48.4	49.5	48.7	49	47.8
Tila_CYP1B1	84.5	80.2	79		72.2	68.2	48.8	48	49	48.7	48.6	46
Plaice_CYP1B1	79.3	77.1	80.8	83.3		68.9	47.8	47.3	48.2	47.1	47	45.6
Killi_CYP1B1	81.2	77.3	79.6	81.4	83.2		49.5	48.7	49.4	48.5	48.4	46.6
Java_CYP1C1	70.1	71.7	70.1	70.3	69.2	69.8		74.9	78.7	82.1	82.3	69.4
Carp_CYP1C1	71.4	71.1	70.7	71	69.2	71.3	89.5		82.5	80.6	78.2	67.9
Jeel_CYP1C1	70.3	71.1	70.7	71.9	69.8	71.1	90.7	92.8		82.5	80.1	70.2
Scup_CYP1C1	71.4	71.1	69.9	71.2	69.8	72.1	93	92.4	94.7		85.9	72.3
Tila_CYP1C1	70.3	71.7	70.9	70.3	68.9	70.2	93.2	91.3	92.6	95.4		68.6
Killi_CYP1C1	69.8	70.4	69	68.7	67.8	69.8	85.7	84.7	84.6	86.3	85	

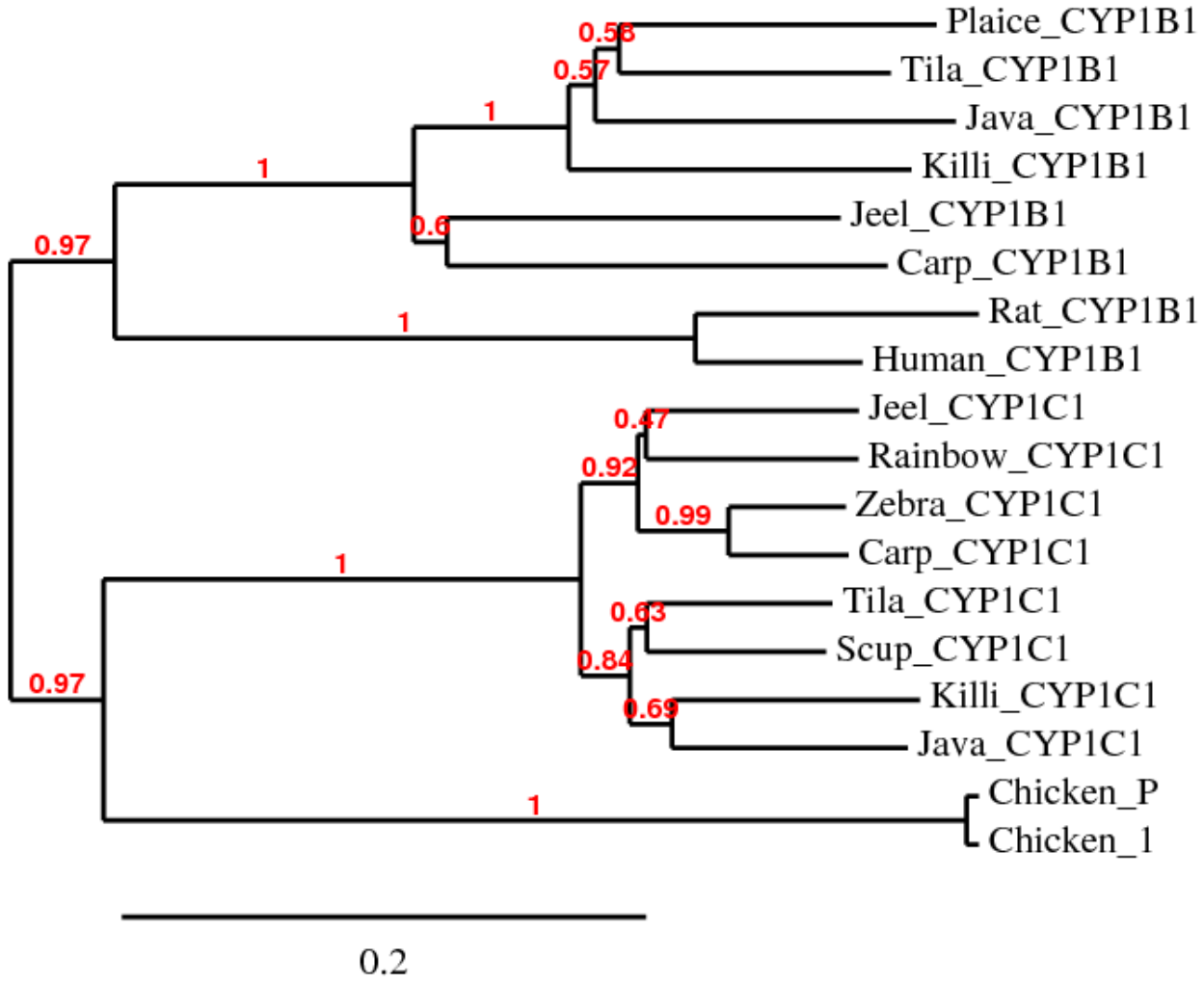

Figure 2. Phylogenetic tree of Javanese medaka CYP1s family genes constructed by the neighbor-joining using percent identity of deduced amino acid sequences of other species with accession numers. The building of the tree also involves a bootstrapping process repeated 100 times to generate a majority consensus tree.

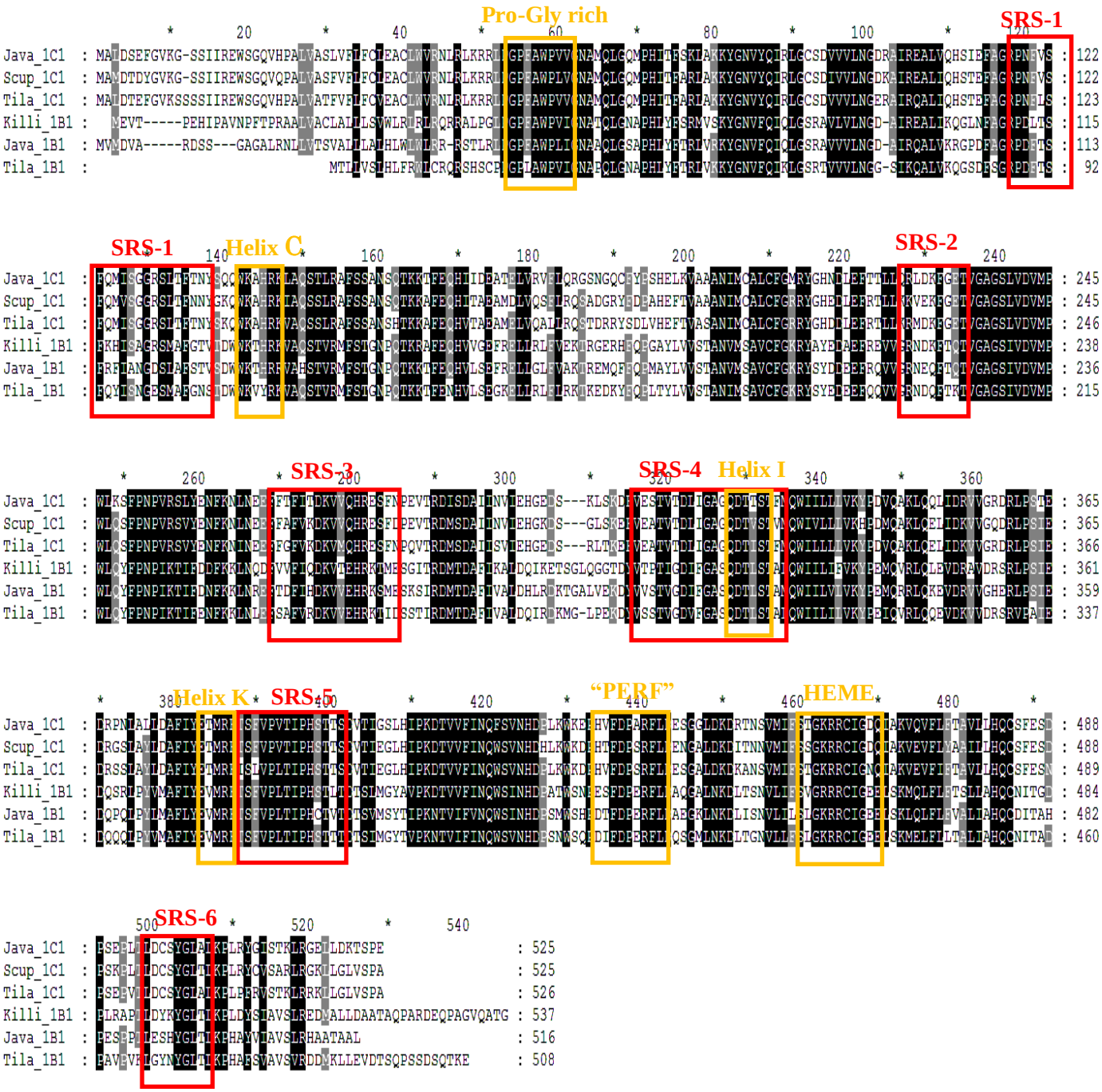

Figure 3. Amino acid sequence alignments of Javanese medaka CYP1s with orthologues. The alignment was constructed using the CYP1s amino acid sequences of tilapia (*Oreochromis niloticus*) and Japanese eel (*Anguilla japonica*) by ClustalW program. Orange boxes indicate the conserved motif region and red boxes indicates substrate recognition sites (SRS).

CYP1C1 shares a number of characteristic domains with other cytochrome P450s. The N-terminal of the Javanese medaka CYP1s consists of a proline-glycine rich region as PGPFAWPL in CYP1B1 and PGPFAWFV in CYP1C1. In addition, sequence alignment of Javanese medaka CYP1 enzymes with those of CYP1 family indicated that Javanese medaka CYP1B1 and 1C1 contain the five structural motifs around heme-binding core for all cytochrome P450 and six separate substrate recognition sites (SRSs) (Figure 3). The signature motif (FxxGxRxCxG) of the heme-binding core appeared as LSLGKRRCIR in CYP1B1 and FSTGKRRCIR in CYP1C1. The heme-inte-

Table 4. CYP1B1 mRNA expressions in Javanese medaka by environmental conditions.

Sample	Fold induction		
	Oil-contaminated feed	Salinity shock	Starvation
Liver	0.28 ± 0.04	1.44 ± 0.06	0.00 ± 0.00
Gill	7.24 ± 1.66	13.42 ± 0.70	1.00 ± 0.23
Muscle	2.84 ± 0.15	27.82 ± 1.51	0.15 ± 0.01
Intestine	11104,45 ± 76.67	0.60 ± 0.02	2.11 ± 0.33

Amount of CYP1B1 mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3).

racting region of Helix C (WxxxR) was presented as WKTHR in CYP1B1 and WKAHR in CYP1C1. The highly conserved residues in Helix I ((A/G)GxxT) showed as SQDTL in CYP1B1 and GQDTT in CYP1C1. The structural motif played a role in the stabilization of the core structure of cytochrome P450s by hydrogen bond, Helix K (ExxR), was found in all of the Javanese medaka P450 proteins: EVMR in CYP1B1 and ETMR in CYP1C1. The invariant sequence (PxxFxPE/DRF) proximal to the heme-binding motif is demonstrated as PDTFDPERF in CYP1B1 and PHVFDPARF in CYP1C1. The characteristics of the N-terminal region of medaka CYP1s proteins exactly match that of the microsomal P450 proteins. At the N-terminal region of medaka CYP1s, a proline-glycine rich region was found, which allows CYPs to function at the ER-membrane. For efficient folding and proper assembly of the P450 proteins, the subsequent proline-glycine rich region acts as a rigid hinge for connecting the membrane anchor and the large catalytic domain, and hence designates the orientation of the catalytic domain in the cytoplasmic side of the ER membrane (Kusano et al., 2001; Kemper, 2004). Moreover, several conserved structural elements can be identified from the deduced protein sequences of the Javanese medaka CYP1 members. The structural conservation around the heme-binding core includes: the heme-binding motif (FxxGxRxCxG), helix C (WxxxR), helix I ((A/G)GxxT), helix K (ExxR), and the invariant sequence prior to heme-binding motif (PxxFxPE/DRF) (Feyereisen, 2005). CYP protein have a strongly conserved region surrounding the heme core structure and possesses poorly conserved N- and C-termini region (Hasemann et al., 1995). The more conserved regions include helices D, E, K, L, J and C-termini of helix I (Hasemann et al., 1995; Graham and Peterson, 1999; Rewitz et al., 2006d).

Rewitz et al. (2006d) stated that these characteristic signatures sequence are conserved for all P450s due to functional significance (Rewitz et al., 2006d). Therefore, these structural consensus sequences have been used as a guideline for cytochrome P450 identification. Previously, Gotoh et al. (1992) identified six regions on CYP2 that are putative substrate recognition site (SRS) for many further studies involving the determination of SRS regions (Godard et al., 2005; Wang et al., 2006). The flexible substrate recognition sites are located near the substrate access channel and catalytic site (Wade et al., 2004). Conformational changes of these regions indicated different kind of substrates in their catalytic centers and facilitate the enzymatic reaction (Gotoh, 1992; Johnson, 2003; Pylypenko and Schlichting, 2004), this feature explains the ability of an individual cytochrome P450s to catalyze a broad spectrum of substrate. In this study, Figure 3 shows the location of the

SRS regions within the Javanese medaka CYP1B1, and CYP1C1 proteins; results shows that SRS1, SRS4, SRS5, SRS6 were highly similar between these CYP1 genes. These SRSs have showed to be conserved among CYP1 orthologous. In contrast, SRS1, SRS2 and SRS3 exhibit low sequence similarity. These results are similar to the variation in sequence similarities observed between zebrafish CYP1A and CYP1D1 (Goldstone et al., 2009) and in CYP1 genes in killifish (Zanette et al., 2009). The six SRSs may differ in their relative importance among CYPs, but are likely to correspond to regions containing substrate-contacting residues in most CYP genes. These SRS regions have been proposed as crucial for defining substrate specificity for individual CYP isoforms (Gotoh, 1992).

CYP1 mRNAs expression in Javanese medaka

Effect of oil-contaminated feed on CYP1s expression

Real-time PCR results showed that the highest expression rates of the CYP1s gene from the Javanese medaka transcript in response to 1% heavy fuel oil-contaminated feed were observed in intestine, and the lowest in liver (intestine>gill>muscle>liver for CYP1B1; intestine>muscle>gill>liver for CYP1C1). Although the liver is a major site of expression, not all genes were predominantly expressed in this tissue (Tables 4 and 5). The induction of the CYP1s in intestine may reflect functions associated with the role of the organ in nutrient uptake and processing of body waste products, that is, detoxification of endogenous metabolites and providing a

Table 5. CYP1C1 mRNA expressions in Javanese medaka by environmental conditions.

Sample	Fold induction		
	Oil-contaminated feed	Salinity shock	Starvation
Liver	0.44 ± 0.37	1.08 ± 0.33	0.00 ± 0.00
Gill	1.51 ± 0.35	7.32 ± 1.07	0.49 ± 0.13
Muscle	1.60 ± 0.29	2.94 ± 0.50	0.02 ± 0.01
Intestine	7490,14 ± 131.01	0.88 ± 0.04	0.41 ± 0.09

Amount of CYP1C1 mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3).

defensive mechanism against the pollutants entering from the external environment (Nebert et al., 2000; Hassanin et al., 2009). Abeer et al. (2012) determined the expression patterns of CYP1C1 gene in liver, intestine, and muscle of tilapia, and found that there was a large increase in CYP1C1 mRNA in liver, intestine and muscle of tilapia injected benzo[a]pyrene (100 mg/kg body weight) (Abeer et al., 2012). The induction of CYP enzymes in fish liver was first seen as an indicator of aquatic contamination in the 1970s (Payne, 1976; Zanette et al., 2009). Since then, many studies have shown that CYP genes in vertebrate liver are strongly induced by certain organic contaminants that represent a risk to humans and wildlife (Bucheli and Fent, 1995; Zanette et al., 2009). Although the liver is a major site of expression, not all genes were predominantly expressed in this tissue. In our studies, the CYP1s constitutive expression was down-regulated in liver. In carp, CYP1C1 has been reported by Northern blot in gills but not in kidney, liver, or intestine (Itakura et al., 2005). In the present study, we found that CYP1s mRNA was significantly induced by 1% heavy oil-contaminated feed in all tissue selected except liver. While, the lack of induction in the liver was unexpected, it may be because the fish were fed with contaminated food in a short time and thus rapid biotransformation of heavy oil in the liver lowered the concentrations in this tissue. Similar to the present study, previous studies have shown that the *Fundulus heteroclitus* exposed to BaP caused the lowest induction of CYP1C1 in female and male liver (Wang et al., 2006). Jonsson et al. (2007) found that CYP1B1, and CYP1C1 transcripts were induced in gills but not in liver of zebrafish exposed to β-naphthoflavone in the water, while similar exposure to PCB126 induced these genes in both organs (Jönsson et al., 2007). Comparing the levels of expression, each CYP1 in different organs of zebrafish, killifish, and mouse showed similar patterns for the Javanese medaka CYP1B1, with lower level in the liver and gill (Choudhary et al., 2005; Jönsson et al., 2007; Zanette et al., 2009). The relative levels of expression of CYP1B1 in brain, gill, kidney and liver observed by Gao et al. (2011) in three-spined stickleback were explored to aryl hydrocarbon receptor (AHR) which is similar to the CYP1B1 results presented here (Gao et al., 2011). The relatively low inducibility in liver tissue was supported by Hoffmann and Oris (2006) demonstrating that zebrafish exposed to 1.5 and 3.1 g/L waterborne BaP for 56 days, there was a dose-related increase in CYP1B mRNA expression in heads but not in liver (Hoffmann and Oris, 2006).

Effect of salinity shock on CYP1s expression

Javanese medaka CYP1B1 and CYP1C1 transcripts were detected in most of tissues examined including gill, muscle and liver (Tables 4 and 5). For CYP1B the highest and lowest levels of expression were found in muscle and liver, respectively (muscle>gill>liver>intestine). The highest level of CYP1C1 transcript expression was found in gill, while the lowest was found in the intestine (gill>muscle>liver>intestine). Many changes to gene expression and protein activity are observed at least 12 to 18 h after exposure to a hyperosmotic environment, suggesting that these effects may be mediated by immediate early gene transcriptional factors (Fiol and Kültz, 2005). In this study, we directly transferred the fish from seawater to freshwater and kept them in the stressful condition for 24 h. This result is also consistent with several previous studies. The involvement of such transcriptional factors displaying rapid changes in gene expression after hyperosomotic stress has been described in the gills of tilapia (*O. mossambicus*) acclimated to seawater from freshwater (Fiol and Kültz, 2005). The findings of the present study clearly show that CYP1 genes expression is up-regulated after freshwater transfer, suggesting that this biotransformation enzyme (Stegeman and Hahn, 1993; Buhler and Wang-Buhler, 1998; Rifkind, 2006) may be involved in the acclimation of Javanese medaka to freshwater. Fiol and Kültz (2007) indicated that euryhaline fish can sense and quantify changes in external salinity and activates appropriate compensatory responses (Fiol and Kültz, 2007). After seawater transfer, some parameters are expressed diffe-

rently with salinity, such as transcription factors, and blood parameters, the levels of which increased when salinity increased (Fiol and Kültz, 2005; McGuire et al., 2010). The outcome of the study presented that the expression levels of the biomarker family genes also increased when salinity decreased. Following transfer to freshwater, CYP1 mRNA levels rose, suggesting that this enzyme may play an important role in the salinity stress response developed at the level of the gill and muscle. The present study clearly suggests CYP1 genes involvement in another unexpected physiological function of the Javanese medaka, that is, acclimation to changes in salinity.

Effect of starvation on CYP1s expression

Real-time PCR results showed that starvation of the Javanese medaka for one week tended to be down-regulated in CYP1s expression (Tables 4 and 5). CYP1B1 expressed in intestine and gill, but not in muscle and liver. CYP1C1 gene expressed in all the tissues analyzed was down- regulated in Javanese medaka starved for one week. There are many reports on the effects of starvation on the content of nutritional components and on energy production pathways in fish (Shul'man, 1974; Love, 1980). Vertebrates differ in their ability to tolerate starvation. Some small birds and mammals may only tolerate one day of starvation (Baggott, 1975; Mosin, 1984; Blem, 1990), whereas some snakes and frogs are reported to survive nearly two years of starvation (Grably and Peiery, 1981; de Vosjoli et al., 1995). The mechanism by which starvation exerts its effects on these parameters is often discussed in terms of metabolic reorganization in response to changes in nutritional state. It is well- known that a substantial part of the dietary requirement of fish is derived from protein sources, whereas a larger proportion of carbohydrates and fat are necessary in homeothermic animals (Cowey and Sargent, 1972). Furthermore, several continuous days of starvation in mammals is a physiological abnormality, whereas fish are generally adapted for extensive periods of starvation. Therefore, the mechanism by which starvation exerts its effects on mammals is probably quite different from that of fish (Andersson et al., 1985; Quabius et al., 2002). The results of the present study shows that Javanese medaka starved for one week tends to down- regulate on CYP1 genes expression. Experimental induction of cytochrome P450 in fish by several xenobiotics is well- studied. Several researches have demonstrated that the induced activity may vary with sex, stage of sexual maturity, food availability and ambient temperature (Hansson et al., 1980; Forlin et al., 1984; Forlin and Haun, 1990; Quabius et al., 2002). However, the present study indicates that the level of induction of the CYP1 genes in Javanese medaka was down-regulated by a one-week period of starvation. Nutritional status (that is, long-term food deprivation) influenced both tissues concentrations and biomarker responses. Food deprivation did not appear to influence hepatic EROD activities or CYP1 gene content in *S. alpinus* held for 141 days either under a restricted feeding regime or without food (Jorgensen et al., 1999). Cytochrome P450-dependent activities towards selected substrates were decreased to varying extents, whereas the liver cytochrome P450 content was not affected by starvation (Andersson et al., 1985). The lower levels of CYP1 induction observed in this study may have results from a non-specific loss of microsomal proteins due to an increased demand for proteins needed for energy production (Quabius et al., 2002).

According to Barclay et al. (1983); Dall and Smith (1986), muscle protein is the main protein reserve during starvation periods (Barclay et al., 1983; Dall and Smith 1986). Most studies on nitrogen metabolism during starvation of decapods indicate that total protein values generally show a significant reduction, confirming that shrimp, as other crustaceans, are well adapted to use protein as a source of energy (Claybrook, 1983). The transcription rate of housekeeping genes or those expressed in a constitutive manner, such as h-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytochrome c oxidase (COX), is constant by food deprivation (Yamada et al., 1997). In this study, the transcription rate of the CYP1 genes was down- regulated by starvation for a 1-week period. This might be a response to the acclimatization strategy in which organisms were fed at certain times each day, so that they prepared themselves for food digestion (Sánchez-Paz et al., 2003).

Conclusion

In summary, we cloned two CYP1 family genes, *CYP1B1* and *CYP1C1* in Javanese medaka, an important model fish widely used in environmental toxicology studies. Javanese medaka CYP1B1 and CYP1C1 transcripts were detected in all the gill, muscle and liver with the highest transcript levels found in gill and muscle, when the fish transferred from seawater to freshwater. The highest transcript levels were found in intestine and the lowest in liver in response to 1% heavy fuel oil-contaminated feed. Starvation of Javanese medaka for one week was down- regulated on CYP1 genes induction in most of tissue analyzed. Cloning of the CYP1 genes and given their various degrees of responsiveness to pollutants, expression patterns in fish could become useful biomarkers in environmental monitoring.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Effect of extracts of *Trichilia silvatica* C. DC., on development and reproduction parameters of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

Alessandra F. Freitas[1]*, Anelise S. N. Formagio[2], Fabricio F. Pereira[1], Jéssica T. Lucchetta[1] and Maria Do Carmo Vieira[2]

[1]Universidade Federal da Grande Dourados (UFGD), Faculdade de Ciências Biológicas e Ambientais (FCBA), Dourados-MS, Brazil.
[2]Universidade Federal da Grande Dourados (UFGD Faculdade de Ciências Agrárias (FCA), Dourados-MS, Brazil.

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The objective of this study was to evaluate the effects of the methanolic extracts from the leaves, bark and flowers of *Trichilia silvatica* on *Spodoptera frugiperda*. Also, it was use in evaluating the total phenolic and flavonoid content of methanolic extracts. We also reported chemical study on the most active extract. Corn leaves were immersed in a 1% methanolic extract solution and fed to second instars of *S. frugiperda*. The extract of the *T. silvatica* (LTS) leaves decreased the viability of the larva, prolonged larval duration, affected the pupal biomass, decreased the period of oviposition and the number of eggs as well as affected the egg viability. The methanolic extract of the *T. silvatica* (BTS) bark decreased the larval viability, oviposition period, number of eggs and egg viability. The flower extract of *T. silvatica* (FTS) decreased the larval viability and period of oviposition. In relation to the constituent contents, the methanolic extract of the leaves showed highest total phenol (233.37 mg gallic acid/g of extract) and flavonoid (53.17 mg quercetin/g of extract) content. The chemical study of the FTS resulted in α-tocopherol, sitosterol 3-O-glucopyranoside, mustakone and N-metilproline. Our results indicate that the extracts affected the biology of *S. frugiperda*, with LTS being the most promising.

Key words: Meliaceae, methanolic extracts, insecticides, plant-derived compounds.

INTRODUCTION

The *Trichilia* genus consists of about 70 species, mainly distributed in tropical America and Africa, of which 43 species occur in Brazil. Chemical investigations have revealed the presence of limonoids as the main bioactive

*Corresponding author. E-mail: a_ffreitas@yahoo.com.br. Tel: (67)3410-2198.

Abbreviations: LTS, Leaves of *Trichilia silvatica*; **BTS,** bark of *Trichilia silvatica*; **FTS,** flower of *Trichilia silvatica*.

agents (Champagne et al., 1992). The limonoids of Meliaceae are very complex with a high degree of oxidetion and rearrangement exhibited in the parent limonoid structure. Compounds belonging to this group express a wide range of biological activities like insecticidal deterrence, insect antifeedant and growth regulating capacity on insects (Champagne et al., 1989; Kubo and Klocke, 1982; Mikolajczak and Reed, 1987; Mikolajczak et al., 1989; Nakatani et al., 1981; Simmonds et al., 2001; Xie et al., 1994) as well as antiviral and analgesic, and several other pharmacological effects on humans (Romin et al., 1992; Vaz et al., 1997).

Biological studies conducted on plant extracts of *Trichilia connaroides*, *Trichilia prieureana*, *Trichilia roka*, *Trichilia triphyllaria*, *Trichilia casaretti, Trichilia catigua*, *Trichilia claussenii*, *Trichilia elegans and Trichilia pallid* demonstrated insecticidal activity on *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) (Bogorni and Vendramim, 2003; 2005; Mikolajczak and Reed, 1987; Roel and Vendramim, 1999; Roel et al., 2000a; 2000b). Some previous studies have focused on nortriterpenoids (limonoids) from the family Meliaceae because of their potent effects on insect pests linked to low toxicity (Carpinella et al., 2002; 2003; Champagne et al., 1989; Klocke et al., 1989; Kubo and Klocke, 1982).

Trichilia silvatica C. DC., is a small evergreen tree occurring in Brazil, commonly known as "*cachuá*", "*cachuá-blank*" in Santa catarina and "*rosa-blank*" in Bahia, Brazil. Chemical studies performed on the extracts of the *T. silvatica* leaves described the isolation of the sesquiterpenes spathulenol, veridiflorol, humulene oxide, (2*S*,3*S*,6*R*,7*R*)-humulene-2,3;6,7-diepoxide, (2*R*,3*R*,6*R*,7*R*)-humulene 2,3;6,7-diepoxide, mustakone, the steroid, and the triterpenes*α*-amyrin, *β*-amyrin, pseudotaraxasterol and lupeol (Soares et al., 2013). The extract *n*-butanol of leaves demonstrated growth inhibition of *Streptococus salivarius* and *Streptococus mutans* (Figueiredo, 2010). Recently, we reported the chemical composition, relating to the anti-edematogenic and antioxidant activity of the essential oil from the *T. silvatica* leaves in particular, being characterized by the GC-MS high number of sesquiterpenes, which exhibited antioxidant and *in vivo* anti-inflammatory effects (Formagio et al., 2012).

Although the literatures show several studies about the limonoids isolation and insecticidal effect of different species of *Trichilia* species, there is no investigation of the effect of *T. silvatica* on *S. frugiperda*. Thus, a search for new plant-derived extracts to develop alternatives to conventional insecticides and reduced negative impacts on health and the environment, we continue our investigation of this plant.

The main goal of the present study was to evaluate the total phenolic and flavonoid content and the effect of the methanolic extract of the leaves, bark, and flowers of *T. silvatica* on the development and survival of *S. frugiperda.* The active extract was also investigated and the chemical composition was reported with the use of chromatographic methods. The compounds thus isolated were characterized by NMR spectral data and compared with those reported in the literature.

MATERIALS AND METHODS

Plant materials

The leaves, bark and flowers of *T. silvatica* were collected in May 2012 from Dourados (22°14′16″S E 54°48′02″W, average elevation of 452 m), in the southern part of Mato Grosso do Sul State, Brazil. The species were identified by Dra. ZefaValdevina Pereira, and the voucher of *T. silvatica* (DDMS 4662) was deposited at the Herbarium of the Federal University of Grande Dourados, Dourados, MS.

Preparation of crude extracts, fraction and isolation of the constituents

The dried plant materials (leaves, bark and flowers) were successively extracted via maceration with methanol. The extract was then filtered, concentrated under pressure in a rotoevaporatorat and lyophilised to obtain methanol crude dry extract from the leaves (LTS), bark (BTS) and flowers (FTS). The most active extract in the development and survival of *S. frugiperda*, was dissolved in MeOH-H_2O 1:1 and separated with *n*-hexane, chloroform and ethyl acetate, which was then tested for insecticidal activity. The solvent was evaporated to give the hexane (5.5 g), chloroform (5.4 g), ethyl acetate (6.2 g) and hydromethanol (7.5 g) fractions. The hexane fraction (2.58 g) was purified on chromatographic column of silica gel, eluting with a mixture of *n*-hexane: ethyl acetate in increasing polarity, to afforded α-tocopherol (6.2 mg). The chloroform fraction (3.8 g) was placed in a chromatographic column on silica gel and eluted with a mixture of *n*-hexane, ethyl acetate and methanol in increasing polarity, to give sitosterol 3-*O*-glucopyranoside (145 mg) and mustakone (14.5 mg). The hydromethanol fraction (3.4 g) was purified on Sephadex LH-20 (25 g) using H_2O, H_2O-MeOH (1:3, 1:1) and MeOH, affording N-prolinebetaine (17 mg).

Determination of total phenol content

The total phenolic content in the samples, methanol extract leaves crude (LTS), bark (BTS) and flowers (FTS), was determined using the Folin-Ciocalteu method (Meda et al., 2005). Specifically, 100 µL of samples in methanol (1 g/L) was mixed with 1.0 mL of distilled water and 0.5 mL of Folin-Ciocaleu's (1:10 v/v) reagent. After 3 min, 1.5 mL of a saturated solution of Na_2CO_3 (2%) was added. After 30 min, the absorbance was measured at 765 nm using a spectrophotometer. Quantification was carried out using a standard curve of gallic acid prepared in 80% methanol, and the results were expressed in milligrams of gallic acid equivalent per gram of extract. The equation for the gallic acid curve was y= 0.1073x + 6.2733 with a correlation coefficient of R =0.9912. The methanol solution was used as a blank. All of the assays were carried out in triplicate.

Determination of total flavonoids

To determine the level of flavonoids, 500 µL of samples (methanol extract leaves crude (LTS), bark (BTS) and flowers (FTS) was mixed with 1.50 mL of 95% ethanol, 0.10 mL of 10% aluminium chloride ($AlCl_3.6H_2O$), 0.10 mL of acetate sodium ($NaC_2H_3O_2.3H_2O$)

(1 M) and 2.80 mL of distilled water. The tubes were maintained at room temperature for 40 min. The optical density was measured at 415 nm using a spectrophotometer. The same procedure was used for the analysis of the blank (Lin and Tang, 2007). To calculate the concentration of flavonoids, we prepared a calibration curve (2.5, 5.0, 10.0, 20.0, 25.0, 50.0, 100.0 and 125.0 µg) using quercetin as the standard. We used these data to generate a linear regression model, and an equation for the line was obtained and used for the calculation of the experimental samples. The results are expressed in milligrams of quercetin equivalents per gram of extract. The equation of the quercetin curve was y= 0.04372x + 11.8202 with a correlation coefficient of R = 0.9989. All of the assays were carried out in triplicate.

Insects

Larvae of *S. frugiperda* were obtained by rearing on an artificial diet in the Laboratory of Entomology of Faculdade de Ciências Biológicas e Ambientais (FCBA), according to the methods described by Parra (2001).

Effect of extracts on the development of *S. frugiperda*

Aqueous solutions (1%) were prepared and used in assays according to the method of Roel and Vendramim (1999). For better solubilization, 9.4 µl of Tween 80, per 100 ml of distilled water, was added to the solution. Hybrid maize leaves XB 6012 (about 28 cm^2) aged 50 to 60 days were immersed in the prepared solution for approximately 2 s and then maintained under ambient conditions for the evaporation of excess fluid, per the method used by Bogorni and Vendramim (2003). Corn leaves treated with extracts and distilled water were placed in Petri dishes (90 × 15 mm) containing a second instar larvae of *S. frugiperda* per plate. We opted for the second instar because the first instar was more sensitive to a variety of factors that caused mortality. The leaves were replaced daily with new leaves treated with 1% aqueous solution per the instructions of Roel and Vendramim (1999). The larval viability (percentage of larvae that reached the pupal stage), larval duration (duration of the larval stage in days), pupal viability (percentage of pupae that reached adulthood), pupal duration (duration of the pupal phase in days), and pupal biomass (weight in milligrams) were evaluated in the bioassays. The experimental design was completely randomized with four treatments (three plant species and the control) and five replications, each consisting of 10 larvae, totaling 50 larvae per treatment (Figure 3).

Effect on the reproductive stage

Adults from larvae previously treated during the larval stage were used to assess the following parameters: pre-oviposition period (duration in days from adult emergence of the female until the first day of oviposition), oviposition period (duration in days from the first day until the last day of oviposition), post-oviposition period (duration in days of the last day of oviposition until mortality of the female), total number of eggs per female (number of eggs laid during the oviposition period of a female), egg viability (percentage of hatched larvae), incubation period (number of days from egg laying to hatching of eggs), and adult longevity (duration in days from emergence to death for males and females). The experimental design was completely randomized with four treatments (three plant species and the control) and five replications per treatment, each mating pair of *S. frugiperda* was considered as a replication.

Data analyses

The data were expressed as means ± standard error of mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by the *Tukey's* test. The differences were considered statistically significant when $P< 0.05$.

RESULTS

Crude extracts, total phenolic and flavonoids content

By maceration, the leaves, bark and flowers of *T. silvatica* produced the highest yield with 27.60, 44.34, and 15.33% of efficiency, respectively. Figure 1 shows the total phenolic and flavonoid methanolic extracts of *T. silvatica*. The extracts of the leaves and bark, on analysis, showed the highest total phenolic content with values of 233.37 and 177.62 mg gallic acid/ g of extract, respectively (Figure 1). By comparison, the extracts also presented a higher content of flavonoids (leaves, 53.17 mg quercetin/ g from the extract; bark, quercetin/41.13 mg/ g from the extract).

Isolation of the constituents of active extract

The chemical study of the hexane, chloroform and hydro-methanol fraction, resulting to liquid-liquid partitioning of methanolic extract of leaves at the time it resulted in α-tocopherol, sitosterol 3-*O*-glucopyranoside, mustakone and N- methylproline. The compounds were identified by comparison of their NMR data with those reported in the literature (Alam et al., 1996; Ayres et al., 2009; Daniewski et al., 1996; Nyasse et al., 1988). α-Tocopherol: ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.62 (t, J=6.7 Hz, 2H), 2.20 (s, 3H), 2.15 (s, 6H); ^{13}C NMR (75.5 MHz): δ146.5, 143.5, 122.9, 121.6, 118.4, 117.4, 74.8, 39.8, 39.4, 37.5, 32.8, 31.5, 28.0, 24.8, 24.4, 23.9, 22.7, 21.5, 20.6, 19.7, 12.5, 11.8, 11.3. sitosterol 3-*O*-glucopyranoside: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.37 (d, J=5.1Hz, 1H); 4.41 (d, J=7,5Hz, 1H), 2.41 (dd, J=12.3 e 4.1Hz), 1.02 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (75.5 MHz): δ 140.1, 122.0, 100.9, 78.9, 77.9, 77.1, 75.6, 69.8, 61.5, 56.5, 55.8, 50.0, 45.6, 42.1, 39.5, 38.4, 37.0, 36.5, 35.9, 33.7, 31.6, 31.4, 29.3, 28.9, 28.0, 25.8, 24.0, 22.9, 20.9, 19.5, 19.0, 18.7, 18.5, 11.6, 11.4. mustakone: ^{1}H NMR (300 MHz, $CDCl_3$): δ2.63 (dd, J=6.5 and 1.5, 1H), 5.76 (m, J=1.5, 1H), 1.94 (d, J=6,5 e 1.5, 1H), 2.60 (s, 1H), 1.64 (m, 1H), 1.50 (m, 1H), 1.73 (m, 2H), 1.85 (m, 1H), 1.73 (m, 1H), 1.54 (dd, J=6.5 and 6.5, 1H), 0.86 (d, J=6.5, 3H), 0.85 (d, J=6.5, 3H), 2.00 (d, J=1.5, 3H), 0.97 (s, 3H). ^{13}C NMR (75.5 MHz): δ56.9, 203.4, 120.8, 171.4, 55.8, 54.7, 45.6, 22.8, 35.9, 57.8, 32.4, 19.6, 20.6, 20.5, 24.3. N-methylproline: ^{1}H NMR (300 MHz, D_2O): δ 2.93, s, CH_3N+; 3.91 (dd, J= 11:5 and 7.5 Hz, 1H), 2.55 (m, 1H), 1.94-2.24 (m, 3H), 3.74 (m, 1H), 3.16 (m, 1H). ^{3}C NMR (75.5 MHz): δ 43.9, CH_3N+; 73.6, 25.9, 31.8, 58.8,

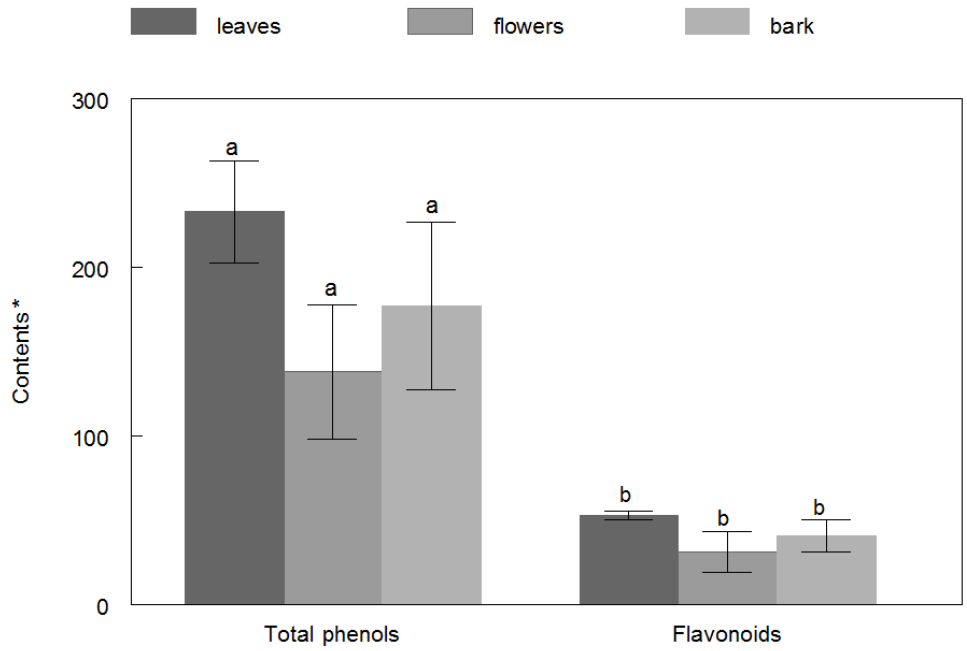

Figure 1. Total phenoic and flavonoid contents of different *T. silvatica* parts. *Total phenolic were expressed by mg GAE/g of extract and flavonoid contents were expressed by mg QE/g of extract. Data are given as mean ± SD (n=3). The data marked with the different lower case letters in the histograms of each category share significant differences at $p < 0.05$ (Tukey test).

Table 1. Mean (+SE) biomass of pupae, duration and viability of the larval and pupal stages of *S. frugiperda* (J.E Smith) (Lepidoptera: Noctuidae) fed on corn leaves treated with methanol extracts of medicinal plants of the family meliaceae. temp.: 25 ± 1°C, RH: 70 ± 10% and photoperiod: 14 h.

Parameter	Pupal biomass (mg)	Larval duration (day)	Larval viability (%)	Pupalduration (day)	Pupalviability (%)
Control	198.80±2.67^{a} *n*=47	16.57±0.13^{b} *n*=50	94.50±0.93^{a} *n*=50	10.11±0.11^{ab} *n*=47	93.77±2.46^{a} *n*=47
LTS	104.00±6.95^{b} *n*=12	20.34±0.49^{a} *n*=50	24.00±6.78^{c} *n*=50	10.90±0.61^{ab} *n*=12	60.00±18.70^{a} *n*=12
BTS	183.13±11.90^{a} *n*=16	17.04±0.72^{b} *n*=50	32.00±4.89^{bc} *n*=50	9.30±0.91^{b} *n*=16	61.99±3.26^{a} *n*=16
FTS	201.78±7.31^{a} *n*=27	16.74±1.07^{b} *n*=50	54.00±10.77^{b} *n*=50	11.90±0.63^{a} *n*=27	86.06±11.08^{a} *n*=27

Different letters in the same column differ at 5% probability by *Tukey* test. SE = standard error. *N* = number of insects.

177.5. However, the isolation of N-metilproline is reported for the first time in *T. silvatica*.

Development and reproductive stage of the *S. frugiperda*

Larval viability was affected in all the treatments (LTS 24.00±6.78, BTS 32.00±4.89 and FTS 54.00±10.77%) when compared with the control (94.50±0.93%). The LTS also prolonged the duration of the larval stage (Table 1). The duration of the pupal phase and viability revealed no significant difference between the treatments and the control (Table 1). The *S. frugiperda* larvae fed on the corn leaves dipped in the LTS solution showed a lower average pupal biomass (104.00±6.95 mg) when compared with the control (198.80±2.67 mg). The other treatments

Table 2. Mean (+ SE) of pre-oviposition, oviposition and post-oviposition, number of eggs, period of incubation and egg viability and longevity of females and males of adult *S. frugiperda* (J.E Smith) (Lepidoptera: Noctuidae). temp.: 25 ± 1°C, RH: 70 ± 10% and photoperiod: 14 h.

	Pre-oviposition (day)	Oviposition (day)	Post-oviposition (day)	Number of eggs (unit)	Incubation period (day)	Eggviability (%)	Longevity of females (day)	Longevityof male (days)
Control (*n*=5)	4.00±0.54^{a}	6.80±0.40^{a}	2.10±0.71^{a}	939.99±214.75^{a}	3.24±0.18^{a}	87.79±2.57^{a}	10.99±0.59^{a}	9.33±0.69^{a}
LTS (*n*=4)	4.00±1.00^{a}	3.50±1.50^{b}	1.50±0.50^{a}	87.50±50.51^{b}	3.30±0.30^{a}	6.25±6.25^{b}	7.25±1.03^{a}	6.00±0.81^{a}
BTS (*n*=3)	6.00±0.00^{a}	1.50±0.76^{b}	1.33±0.33^{a}	124.66±32.63^{b}	3.75±0.38^{a}	-	8.33±0.88^{a}	8.00±1.52^{a}
FTS (*n*=5)	7.00±1.00^{a}	1.37±0.55^{b}	2.00±0.00^{a}	358.80±110.44^{ab}	4.33±0.46^{a}	46.59±22.09^{ab}	9.20±1.35^{a}	7.00±0.94^{a}

Different letters in the same column differ at 5% probability by *Tukey* test. SE = standard error. *n*= number of couples of *S. frugiperda*. -, Data were not sufficient to conduct the analysis due to insect mortality.

Figure 2. Effect of extract of *T. silvatica* on adults of *S. frugiperda.*

showed no statistical difference with respect to the biomass of the pupae compared with the control (Table 1). The leaf, bark and flower extracts of *T. silvatica* also adversely affected the biological characteristics of the *S. frugiperda* adults. The oviposition period was reduced in length (LTS 3.50±1.50; BTS 1.50±0.76; FTS 1.37±0.55 days), when compared with the control (6.80±0.40 days). The periods of pre-oviposition and post-oviposition, however, were not affected by the extracts (Table 2). The *S. frugiperda* females oviposited less numbers of eggs in the BTS and LTS treatments (Table 2). These eggs revealed reduced viability when compared with the control (Table 2). Female and male longevity as well as the incubition period of the eggs was statistically similar in all the treatments compared with the control (Table 2). In adulthood, deformation was also observed in moths, especially in their wings (Figure 2).

DISCUSSION

Effect of the *T. silvatica* extracts on development and reproductive stage of the *S. frugiperda*

The extracts of the different plant parts of *T. silvatica* reduced the larval viability of *S. frugiperda*. Such results have to confirm the insecticidal activity of this genus ever reported for *S. frugiperda* and other insects (Bogorni and Vendramim, 2003; Bogorni and Vendramim, 2005; Conceschi et al., 2011; Cunha et al., 2006; 2008; Mikolajczak and Reed, 1987; Nebo et al., 2010; Roel et al., 2000b; Thomazini et al., 2000; Wheeler and Isman, 2000). Reports regarding the biological activities of the substances and / or extracts from plants of this genus, such as "antifeedant" and insecticidal properties, comparable to azadirachtin are available (Bogorni and Vendramim, 2005; Cunha et al., 2006; Mikolajczak and Reed, 1987).

The LTS was found to affect not only the survival of *S. frugiperda*, but also other biological characteristics, such as the prolonged duration of the larval phase (20.34±0.49 days) when compared with the control (16.57±0.13 days). This could be related to the presence of the compounds that interfered in metamorphosis, not only causing mortality but also hindering the development of the surviving larvae (Bogorni and Vendramim, 2005). The *Trichilia T. pallens*, *T. casaretti* and *Trichilia pallida* leaves and *T. pallid* branches also induced significant lengthening of the larval stage of *S. frugiperda* (Bogorni and Vendramim, 2005). The prolonged stage of the larval may be associated with the effect antifeedant reported in the

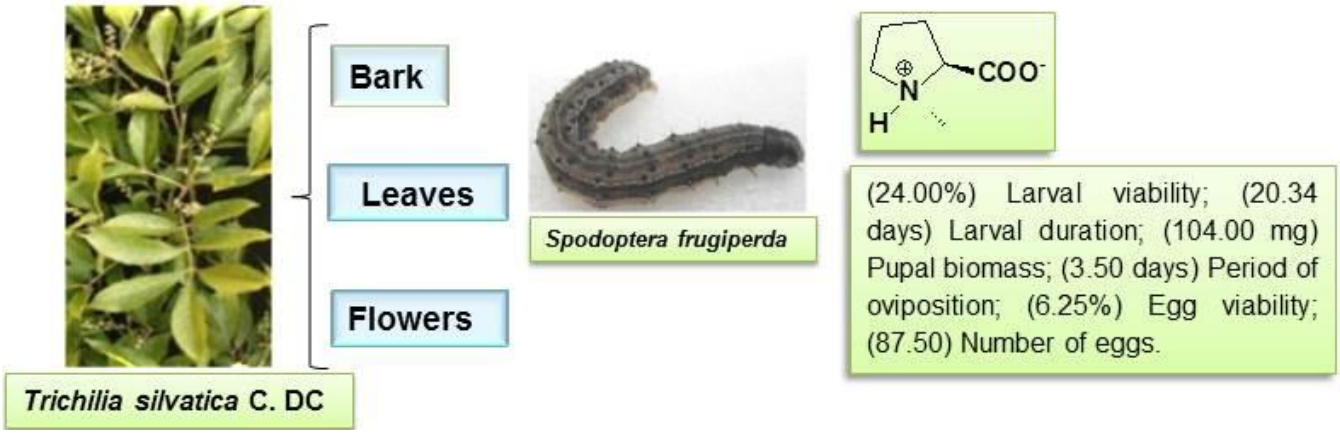

Figure 3. The larval viability (percentage of larvae that reached the pupal stage), larval duration (duration of the larval stage in days), pupal viability (percentage of pupae that reached adulthood), pupal duration (duration of the pupal phase in days), and pupal biomass (weight in milligrams) of *Spodoptera frugiperda.*

Trichilia genus (Mikolajczak and Reed, 1987), and in compounds family Meliaceae (Mossini and Kemmelmeier, 2005). Antifeedants can be described as allomone substances which inhibit feeding and do not kill the insect pests directly, but rather limit its developmental potential considerably (Gokulakrishnan et al., 2012).

Torrecillas and Vendramim (2001) also reported that the branches of *T. pallida* 0.1% affected not only the survival but also the duration of the larval stage of *S. frugiperda.* This fact is attributed to the presence of the growth inhibitors or toxic substances in the extract and emphasize that theelongation of the larval stage is important in the field, as it can increase the exposure time of the insect to natural mortality factors (Torres et al., 2001; Alves et al., 2012). McMillian et al. (1969) observed growth inhibition in *S. frugiperda* by the use of plant extracts as well as reported the prolongation of the larval stage on an artificial diet containing achloroform extract from the *Melia azedarach* leaves. The pupal viability and duration of the stage were not affected by the extracts (LTS, BTS and FTS). Other authors attribute the lower toxicity of chemical agents to the pupa stage to the lower activity of the insect in such stage (Jbilou et al., 2006). Probably, the effect of the plant extracts is felt more intensely in the larval stage because it is at this stage that the insect ingests the chemicals present in the food (Rodríguez and Vendramim, 1996). Maroneze and Gallegos (2009) also found no negative effects of *M. azedarach* on the duration and viability of the pupal stage of *S. frugiperda*. The results concur well with those obtained by Bogorni and Vendramim (2005); Rodríguez and Vendramim (1996, 1997), working with the *Trichilia* species, also found no negative effect of the extracts on the pupal stage of *S. frugiperda*. The biomass of the pupae was reduced (104.00±6.95 mg) in the LTS treatment when compared with the control (198.80±2.67 mg). The biomass of the pupae is directly related to the performance of the insect in the larval stage, increasing the leaf consumption by the larvae, the pupae also increases biomass (Lima et al., 2006); however, in some extracts, the feed inhibitor compounds are present which limit the food intake during the larval stage and thus reduce the pupal biomass (Carpinella et al., 2002; Huang et al., 1996).

The *T. silvatica* extracts also interfere with the biological characteristics of the *S. frugiperda* female adults. The oviposition period was reduced in all treatments (with LTS, BTS and FTS). Probably, the plant species possess substances capable of interfering in the reproduction of insects. A similar result was described for the extract of *M. azedarach*, which reduced the oviposition period and the fertility of *S. littoralis* females (Schmidt et al., 1997). *Azadirachta indica*, considered the most promising plant species in pest control, has substances capable of interfering with the reproduction of insects, either by reduction of the fecundity or complete sterilization (Schmutterer, 1990).

The number of eggs per female was also reduced by treatment with LTS and BTS. According to Costa et al. (2004), the quantity and quality of the nutrients obtained during larval feeding can influence the number of ovarioles per ovary and, by extension, reduce the potential for egg production, that is, larvae which consume protein-rich diets produce heavier pupae and adults, which in turn produce more eggs than those insects which fed on poor diets. The results of the LTS concur with this fact, the larvae fed on treated corn leaves, if fed less than the control, would yield smaller pupae and adults which produce fewer eggs. Reducing the number of eggs and inhibiting oviposition are important effects that plant extracts exert on insect reproduction (Costa et al., 2004). The reduction in the egg viability induced by LTS and BTS demonstrates that the extracts may have a transovarial action (Pratissoli et al., 2004). In the field, reduced egg

viability results in a significant reduction in the damage to the maize crop (Maroneze and Gallegos, 2009).

The malformation in adults was also observed with others species of the *Trichilia*. Bogorni and Vendramim (2005) found that when the aqueous extracts of the *Trichilia* branches and leaves were applied to the corn leaves fed to the larvae, they affected not only the larval and pupal stages, but also the emergence of adults. The main defect observed by them in the *S. frugiperda* adults was the malformation of the wings and antennae. Considering the parameters evaluated during the various developmental stages of the pest (Tables 1 and 2), all the extracts (LTS, BTS and FTS) were seen to affect the parameters of the juvenile and adult stages of *S. frugiperda*. However, based on the results obtained, it was concluded that the methanolic extract from the *T. silvatica* leaves is the most promising for use in the control of *S. frugiperda*. The effect of the extracts varies according to the plant part used in their preparation (Alves et al., 2012). This result is in accordance with the described metabolic variation between plant parts (François et al., 2009). Gobbo-neto and Lopes (2007) reported that plant parts can not only influence the total quantity of the metabolites produced, but also the relative proportions of the components of the mixture.

Constituents of the *T. silvatica*

The chemical composition of the most active extract of *S. frugiperda* was exploited in order to elucidate the main secondary metabolites present in the extract of *T. silvatica* collected in Dourados-MS and correlate with the effect observed from *T. silvatica*. Future studies to report the effect of sitosterol 3-O-glucopyranoside, tocopherol, mustakone and N-metilproline isolated compounds in development and reproductive stage of the *S. frugiperda* is in process. In the literature, the sitosterol 3-*O*-glucopyranoside, tocopherol, mustakone and N-metilproline isolated compounds were not evaluated with insecticidal activity. Sterols and triterpenes have an important biological function as key compounds in the acquirement of cholesterol by insects. Mammals obtain cholesterol either by dietary absorption or by biosynthesis from mevalonate. Based on the fact that insects have no capacity for de novo sterol synthesis, they rely exclusively on exogenous sources for their normal growth, development and reproduction (Ikekawa et al., 1993).

Flavonoids and phenolic compounds in plants exert protection against ultraviolet rays, and protection against insects, viruses and bacteria, while they attract the pollinators, reveal antioxidant activity, are allelopathic agents and express enzyme inhibition (Zuanazzi and Montanha, 2004). In insects, the flavonoids interfere in reproduction; feeding and behavior stimulate oviposition or act as feeding deterrents (Bernays et al., 1991; Harborne and Grayer, 1994; Matsuda, 1978; Morimoto et al., 2000; Musabyimana et al., 2001; Reyes-Chilpaet al., 1995; Simmonds, 2001). The contents of flavonoids and phenolic compounds reported in the leaves from *T. silvatica* may have promoted the insect growth inhibition. Several studies have highlighted the dual nature of specific flavonoids as both pest-feeding deterrents and stimulants.

Our study demonstrates the effect of the *T. silvatica* on growth inhibition of *S. frugiperda* and the presence of the metabolites in leaves extract, for the moment demonstrate the possible metabolites responsible for activity. Based on the results, we suggest that insect growth inhibition caused of leaves extract could be attributed mainly to flavonoids and phenolic compounds. However, studies of the isolated compounds and of polar fractions (ethyl acetate and hydromethanolic) are in progress to verify development of the *S. frugiperda*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Physicochemical properties of lignocellulosic biofibres from South Eastern Nigeria: Their suitability for biocomposite technology

Agu, Chidozie Victor[1, 2, 3]*, Njoku, O. U.[1], Chilaka, F. C.[1], Agbiogwu, D[2], Iloabuchi, K. V.[1] and Ukazu, B.[1]

[1]Department of Biochemistry, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Centre for Composite Research and Development, JuNeng Nigeria Limited, Nigeria.
[3]Biotechnology and Fermentation Group, Department of Animal Sciences, The Ohio State University and Ohio Agricultural Development Center, Wooster OH, 44691, United States.

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Five plant raw materials collected from South Eastern part of Nigeria were used for biofibre extraction and analysis to assess their suitability for biocomposite production. Lignocellulosic biofibres were extracted from young stems of *Adenia lobata, Ampelocissus leonensis*, *Cissus palmatifida, Morinda morindoides* and *Urena lobata* through natural water retting process for a period of 14 - 16 days and the resulting fibres were uniform with almost flat or circular cross sections. Phytochemical contents and extractives were determined on the untreated and treated fibres respectively. The %w/w cellulose contents of the pretreated biofibres were found to be 48.97± 1.33% for *A. leonensis* and 43.22±0.95% for *A. lobata*. The cellulose content of *M. morindoides* and *C. palmitifida* were found to be 55.76±1.40% and 55.20±1.59%, respectively. In all the plants studied, *U. lobata* had the greatest %w/w cellulose content of 58.94±1.05% while *A. lobata* had the least cellulose content of 43.22±0.95%. Estimation of %w/w hemicellulose contents showed *A. leonensis* to be 21.22±0.89% whilst the hemicelluloses content in *A. lobata* and *U. lobata* were observed to be 18.22±2.18% and 12.38±0.33% in that order. Lower hemicelluloses contents were obtained in *C. palmitifida* and *M. morindoides* as 10.32±1.27, 9.32±0.58 and 8.62±1.67%, respectively. The klason lignin contents were found to be 31.33±1.05% for *C. palmitifida*, 31.22±0.97% for *M. morindoides*, 28.22 ± 1.96% for *A. lobata*, and 24.91±0.61% for *A. leonensis*. The lignin content of *U. lobata* was found to be the least at 22.26±0.55%. Acid soluble lignin (ASL) content was greater in *A. lobata* (2.17±0.08%) while *A. leonensis* had the least value of 1.74±0.34%. ASL-derived products (vanillin, p-coumaric acid and ferulic acid) ranged between 0.50±0.12% and 1.41±0.02% for vanillin; 0.03± 0.02% and 0.65±0.14% for p-coumaric acid; and ferulic acid was only detected in *A. leonensis* as 0.41±0.11%. The mechanical properties of most fibres used in this study are comparable to those of other biofibres already used in manufacturing and can even match those of some synthetic fibres. Results obtained revealed that fibres used in this study had comparable properties with those already established for manufacturing in biofibre industries.

Key words: Biofibre, biocomposite, cellulose, lignin, hemicelluloses.

INTRODUCTION

In 1989, the German DLR Institute of Structural Mechanics developed an innovative idea. By embedding natural reinforcing fibres for example flax, hemp, and ramie into biopolymeric matrix made of derivatives from cellulose,

starch, lactic acid; new fibre reinforced materials called biocomposites were created and are still being developed (Mohanty et al., 2000). Biocomposites consist of biodegradable polymer as matrix materials and usually biofibres as reinforcing element. Biofibres (natural polymers) are generally biodegradable but they do not possess the necessary thermal and mechanical properties desirable for engineering plastics (Mohanty et al., 2000). A lot of research and development efforts had been carried out on biofibre reinforced synthetic polymers. The composites of biodegradable natural fibres and non-biodegradable synthetic polymers may offer a new class of materials which however are not completely biodegradable (Mohanty et al., 2000).

Biocomposites are finding applications in many fields ranging from the construction to automotive industries. The use of plant fibres in composites had increased due to their relative low cost, recyclability and the fact that they compete well in terms of strength per weight of material (Maya and Sabu, 2008). Natural fibres are considered as composites consisting mainly of cellulose fibrils embedded in lignin matrix. The cellulose fibrils are aligned along the length of the fibre which renders maximum tensile and flexural strengths thereby providing rigidity to the fibre. Thus, the reinforcing efficiency of natural fibre is related to the nature of cellulose and its crystallinity (Maya and Sabu, 2008). Most plant fibres, except for cotton, are composed of cellulose, hemicellulose, lignin, waxes, and some water-soluble compounds, with cellulose, hemicelluloses, and lignin as the major constituents (Taj et al., 2007). Depleting natural resources, regulations on using synthetic materials, growing environmental awareness and economic considerations are the major driving forces to the utilization of annually renewable resources such as biomass for various Industrial applications (van Wyk, 2001). Approximately 2×10^{11} tons of lignocellulosics are produced every year, compared with 1.5×10^{8} tons of synthetic polymers (Mohanty et al., 2000). These lignocellulosic agricultural byproducts could be principal sources of fibres, chemicals and other industrial products.

The various applications of lignocellulosic materials depend on their chemical composition and physical properties. Wheat, rice straw and corn stalks to a limited extent, have traditionally been used for pulp and paper making while coconut fibre (coir), pineapple and banana leaves have been used as natural cellulose fibre source for making textiles, composites and paper (Majumdar and Chanda, 2001). Recently, natural cellulose fibres suitable for textile and other industrial applications have been produced from corn husks and corn stalks (Reddy and Yang, 2004). Rice and wheat straw have also been used to produce regenerated cellulose fibres as an alternative to wood for cellulose-based materials (Lim et al., 2001). Increase in fuel costs and scarcity of petroleum sources led to the use of lignocellulosics to produce ethanol and other sugars by fermentation; biomasses can also be converted into carbon, hydrogen and oxygen to produce various chemicals, enzymes and proteins (Reddy and Yang, 2005).

In our former study, the biocomposite potentials of *Ampelocissus cavicaulis* a highly fibrous plant domestically used as twine was highlighted (Agu et al., 2012). In the present study, novel plant fibres locally used for various applications were sourced, identified, characterrized and their potentials for use in biofibre technology were investigated. Fibre bundles from the stems of these plants are traditionally used in making sponge, mat and twine; thereby emphasizing their immense potential as industrial raw materials.

MATERIALS AND METHODS

Raw materials extraction

Five species of woody plants were selected for the study: *Adenia lobata, Ampelocissus leonensis*, *Cissus palmatifida, Morinda morindoides* and *Urena lobata*. The stems of the above species are locally made into twine in south eastern Nigeria. The parent plants were identified and the vouchers were also deposited at the Bioresources Development and Conservation Programme (BDCP) Research Centre, Enugu, Nigeria. Stems freshly cut from young plants (< one year old) were allowed to ret in a flowing stream (natural water retting) for a period of 14-16 days. During the retting process, the tissue that interconnects the single fibres that is the middle lamella are degraded by microbial activity to yield strands of natural plant fibre after which the materials were macerated to remove the remaining stem bark and other foreign materials. Extracted fibres were sun dried after which they were milled into powder using Safar miller SNE-200 machine.

Pretreatment of natural fibres

Pretreatment of the natural fibres was done using the Soxhlet technique. This procedure was carried out in order to remove lipophilics (gums and waxes) and residual phytochemicals remaining after the retting process (especially tannins) that could interfere with the determination of the structural components of the biofibres. This method also allows for the determination of extractives (lipophilic and alcohol) at the same time. It is based on the initial preatment of the samples with n-hexane to remove the lipohilics and then methanol to remove the polar substances. Milled biofibres (100 g) were properly packed into the thimble of the soxhlet extractor, and n-hexane (300 ml) was poured into the round bottomed flask of the soxhlet extractor. The complete soxhlet extractor (ithat with its condenser) was then mounted on a heating mantle which had its temperature guage set at 70°C. The above experiment was repeated using methanol (300 ml; 40 - 60°C) as the extracting solvent. The percentage yields of the lipophilic and methanol extractives

*Corresponding author. E-mail: agu.2@osu.edu.

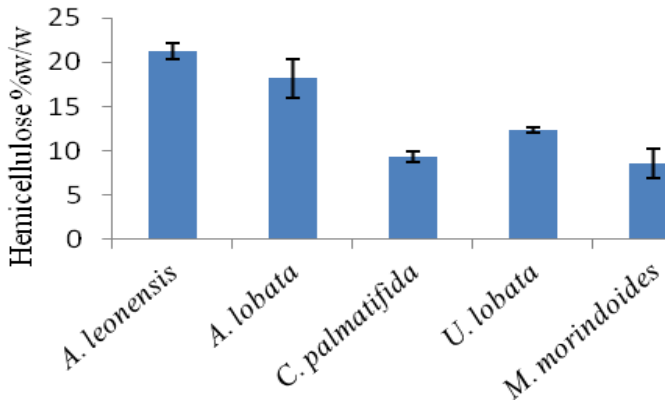

Figure 1. Hemicellulose content of fibres in %w/w basis (Error bars refer to 95% JSCI).

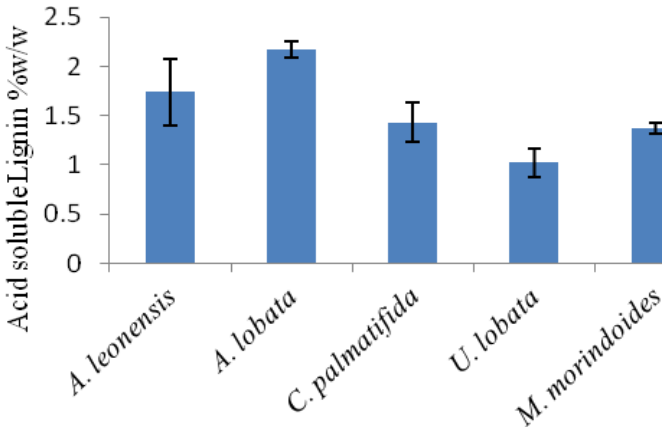

Figure 2. Acid soluble lignin contents of fibres in %w/w basis (Error bars refer to 95% JSCI).

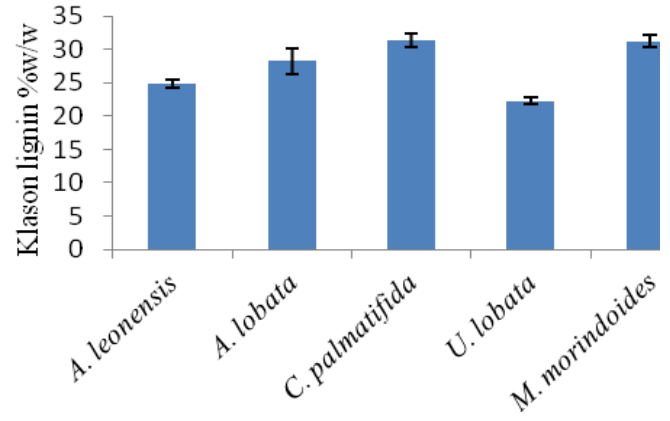

Figure 3 Acid insoluble (Klason) Lignin contents of fibres (Error bars refer to 95% JSCI).

were determined after triplicate run and the mean values reported.

The pretreated samples were removed from the thimble, oven dried at 90°C for 3 h to remove solvents and subsequently characterized.

Chemical analysis

Phytochemical analysis

The untreated fibres were analysed for the presence of phytochemicals including tannins, alkaloids, flavonoids, saponins, glycosides, proteins, reducing sugars terpenoids and steroids. This was done using the methods developed by Harborne (1998).

Analysis of chemical (structural) compositions

Lignin, ash, cellulose, hemicellulose contents were determined as follows; Kurshner and Hoffer cellulose was determined using the method described by Kurschner and Hoffer (1933) as adopted by Beakou et al. (2008). A quantity (0.7 g) of the pretreated sample was added with a 95% solution of nitric acid and ethanol. The mixture was filtered and the residue washed first with hot water then with absolute ethanol to completely remove the acid. The cellulose corresponds to the insoluble fraction of the mixture. The residue was oven dried at 100°C to a constant weight. The test was run in triplicates and the mean value reported.

The neutral detergent fibre method of Goering and van Soest (1975) was adopted for determining the hemicellulose content of the samples. Neutral detergent fibre was prepared by refluxing for one hour a quantity, 0.7 g of each fibre sample with 10ml of cold neutral detergent solution and 0.5 g of sodium sulfite (Figure 1). The mixture was subsequently filtered through sintered glass crucible (G-2) after which the residue was washed with hot distilled water and ethanol. The residue was subsequently oven dried to constant weight at 100°C for 8 h. The weight obtained is the neutral detergent fibre weight. The test was run in triplicates and the mean value taken. Hemicellulose content was calculated as the difference in weight of neutral detergent fibre and the acid detergent fibre prepared from acid hydrolysis of the same mass of sample. The hemicellulose was determined in triplicate run and the mean value was reported.

The standard method described by the Technical Association of Pulp and Paper (1998) was adopted for the estimation of acid soluble (or Klason) lignin and ash contents (Figure 2). A quantity, (0.7 g) of each pretreated fibre sample was boiled with 5 ml of 72% w/w H_2SO_4 solution for 4.5 h in order to hydrolyse the cellulose and hemicellulose. The suspension remaining after the above treatment was filtered through a crucible and thoroughly washed with hot distilled water and absolute ethanol to completely remove the acid present. The solid residue was dried at 105°C for 24 h and weighed (W1). This residue is known as the acid detergent fibre. The residue was then transferred to a pre-weighed dry porcelain crucible and heated at 600°C for 5 h. After cooling, it was weighed (W2) and ash content (%) was determined. Acid insoluble lignin was then calculated by the difference (W1 - W2). The test was run in triplicates and the mean value reported (Figure 3).

The method described by Hyman et al. (2008) was adopted for determination of acid insoluble lignin and its derived products. The method is based on boiling a quantity; 0.7 g of each pretreated fibre sample with 5 ml of 72% w/w H_2SO_4 solution for 4.5 h in order to hydrolyse the cellulose and hemicellulose. The suspension remainning after the above treatment is filtered through a crucible. The filtrate from acid hydrolysis above will be diluted with distilled water and the dilution factor was noted. Using UV-Visible Spectrophotometer, the absorbance at a wavelength of 205 nm was taken and the ASL calculated using the formula below. ASL-derived products-Vanillin, p-coumaric acid and ferulic acid were determined at different wavelengths of 230, 308 and 322 nm corresponding to

a: *A. leonensis* b: *A. lobata* c: *C. palmitifida* d: *M. morindoides*

Figure 4. Retted bast fibre bundles.

their wavelengths of maximum absorption respectively. Each parameter was run in triplicate and the mean value was recorded. The acid soluble lignin was calculated on extractives free basis using the formular;

$$\%\,ASL = \frac{UVabs \;x\; Volume_{filtrate} x\; Dilution}{\varepsilon\, x\; ODW_{sample}}\; x\; 100$$

Where, UVabs = average, UV – V is absorbance for the sample at specified wavelength, $Volume_{hydrolysis\ liquor}$ = volume of filtrate, 87 ml, ε = absorptivity constant of biomas at specific wavelength in L/g-cm.

The absorptivity constant ε was obtained using the Beer's law; $A_\lambda = \varepsilon bc$; A_λ = average UV-Vis absorbance at a specific wavelength; ε = absorptivity constant at a specific wavelength in L/g-cm; b is the path length through the sample in cm; c is the concentration of a single analyte in mg/ml.

Analysis of mechanical properties of the fibres

Three mechanical properties (tensile strength, Young's modulus and elongation at break) were selected for the study. Hounsfield Tensometer testing machine (model 5566) was used to determine the tensile strength, Young's modulus and elongation at break of the specimens. After finding the average diameter (D) of the fibres, the cross sectional area of each individual fibre can be determined using the formular;

Cross sectional area, $A = \pi r^2$, Where A = cross sectional area; r =D/2; D = diameter; π = 3.142

Chemical modification of fibre surface hydroxyl group

Alkali treatment is a chemical method, which can change the constituents of fibres. The procedure described by Bledzki and Gassan (1999) and Cao et al. (2007) was used. Fibres were soaked in 15 wt% NaOH solutions at room temperature for 2 h, maintaining a liquor ratio of 20:1. The fibres were washed several times with water to remove any alkali solution sticking to the fibres surface, neutrallized with dilute acetic acid and then washed again with water. Finally, the resulting fibres were dried at 70°C for 72 h.

Design and production of composites

The method of Yuhazri et al. (2010) was adopted. Productions of the composite samples were prepared using facilities at the Center for Composite Research and Development, JuNeng Nigeria Limited, Nsukka. Randomly oriented fibre reinforced bio-composites were prepared by taking different dimensions and percentages of the untreated, silane treated and alkaline treated fibres. Fibre/resin matrix composite laminates were prepared using a combination of hand lay-up and compression moulding method. The surfaces of moulds were first coated on the inside with universal mould release wax to avoid adhesion of the mixture and to allow easy removal of the composites. After thorough mixing of the resin with 0.4 wt-% methyl ethyl ketone peroxide (MEKP) solution with dimethylpthalate as catalyst and 0.3 wt% of cobalt derivative as accelerator, the mixture was poured into the moulds and the fibres added. The moulds were then closed and kept under pressure with a load of about 50 kg for 24 h. Subsequently, this cast is post cured in the air for another 24 h after removing the mould. Specimens of suitable dimension are cut using a diamond cutter for mechanical testing. Neat resin composites were also made as a control sample.

RESULTS AND DISCUSSION

Extracted fibre bundles

The following results were obtained after extraction of fibre bundles using natural water retting technique. The fibre bundles were uniform with almost flat and circular cross sections (Figure 4a, b, c and d).

Phytochemical analysis of untreated fibre bundles

The results obtained from the phytochemical analysis of the untreated plant fibres presented in Tables 1 and 2 shows moderate presence of steroids, tannins, proteins and alkaloids, and total absence of flavonoids and reducing sugar. Further analysis showed that *C. palmitifida* had the highest alkaloids concentrations of 7.09±0.04 mg/g. Moderate amount of residual tannins and steroids were obtained, with the values ranging between 0.01 - 0.03 mg/g in all the fibres analysed. Determination of phytochemical content has economic value during bio-fibre process. It gives an idea of how much pretreatment will be required. The absence of reducing sugars in this study suggests that the retted fibre were well protected from hydrolytic activity of the ambient environment and may explain the non-easily hydrolysable fibre materials

Table 1. Qualitative phytochemical analysis of the plant fibres.

Sample	Alkaloid	Flavonoid	Tannin	Steroid	Reducing Sugar	Protein
Ampelocissus leonensis	+	-	++	+	-	+
Adenia lobata	+	-	+	+	-	-
Cissus palmitifida	++	-	+	+	-	-
Urena lobata	+	-	++	-	-	-
Morinda morindoides	+	-	+	+	-	-

Table 2. Quantitative phytochemical analysis of the plant fibres.

Sample	Common name	Alkaloid (mg/g)	Tannin (mg/g)	Steroid (mg/g)
Ampelocissus leonensis	Okpaowoko	5.61 ± 0.071	0.02 ± 0.001	0.01 ± 0.001
Adenia lobata	Usoro	4.60 ± 0.028	0.01 ± 0.002	0.02 ± 0.001
Cissus palmitifida	Okpote	7.09 ± 0.04	0.01 ± 0.007	0.01 ± 0.002
Urena lobata	Abari/ udo	n.d	0.03 ± 0.001	n.d
Morinda morindoides	Ogbuebo	4.10 ± 0.11	0.01 ± 0.002	0.02 ± 0.002

n.d = not determined.

Table 3. %w/w Extractives, ash and moisture contents of retted fibre bundles.

Sample	Common name	Lipophilic extractives (%w/w)	Alcohol extractives (%w/w)	Ash (%w/w)	Moisture content (%)
Ampelocissus leonensis	Okpaowoko	0.27 ± 0.001	0.98 ± 0.002	0.57 ± 0.10	3.21 ± 0.05
Adenia lobata	Usoro	1.86 ± 0.61	5.64 ± 0.32	2.57 ± 0.08	5.50 ± 0.01
Cissus palmitifida	Okpote	0.12 ± 0.01	0.26 ± 0.001	2.14 ± 0.14	0.51 ± 0.18
Urena lobata	Abari/Udo	4.07 ± 0.02	10.22 ± 0.31	0.42 ± 0.06	2.61 ± 0.02
Morinda morindoides	Ogbuebo	0.21 ± 0.01	2.12 ± 0.11	2.46 ± 0.14	0.43 ± 0.21

Results expressed as mean ± S.D; n = 3.

obtained in this study.

Results for chemical analysis of the extracted fibres

Extractives, moisture and ash contents

From the results in Table 3, the highest amount of lipophilic extractives and alcohol extractives were found in *U. lobata* (4.07 ± 0.02 and 10.22 ± 0.31%) respectively whilst the least values were found in *C. palmatifida* (0.12 ± 0.01 and 0.26 ± 0.001%) and *A. leonensis* (0.27 ± 0.001 and 0.98±0.002%) respectively. *A. lobata* and *A. leonensis* had the greatest moisture contents at 5.60±0.071, 5.50±0.01, and 3.21±0.05%, respectively. Lower moisture contents were recorded for *M. morindoides* (0.43 ± 0.21%), and *C. palmitifida* (0.51 ± 0.18%). The ash content of *C. palmitifida* (2.14 ± 0.14%) was found to be greater than *A. leonensis* (0.57 ± 0.10%) and *U. lobata* (0.42 ± 0.06%) but lower than the values obtained for *A. lobata* (2.57 ± 0.08%) and *M. morindoides* (2.46 ± 0.14%). The determination of the extractive contents was necessary because surface waxes and encrusting substances make fibre surfaces smooth and interfere with adhesion of the fibres to polymer matrices when used as reinforcement materials (Eichhorn et al., 2001; Saha et al., 1990). The extractive content of *A. lobata* is comparable to an earlier study on curaua (5.3%) using acetone as the extracting solvent (Marques et al., 2010). *Ampelocissus leonensis, C. palmitifida, and M. morindoides* had lower total extractives contents when compared with fibres from barley straw (5%) and corn stover (10%). *U. lobata* had higher total extractives content when compared with fibres from barley straw (5%), corn stover (10%). Thus, pretreatment is required before composite reinforcement with this fibre.

The determination of the moisture content of fibres is very important because fibre dimensions and properties vary with the moisture content (Mohanty et al., 2000). Such properties affected by the moisture content include

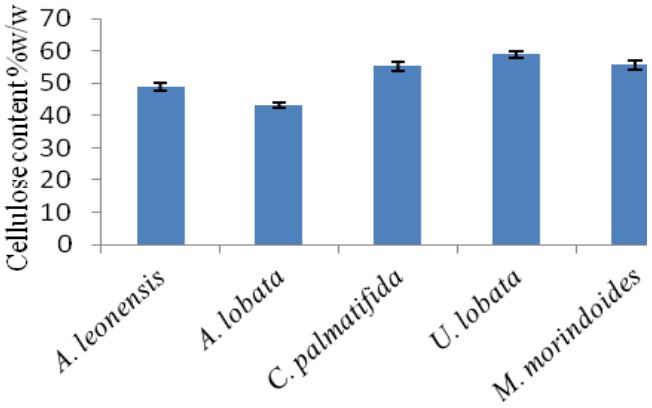

Figure 5. Cellulose contents of fibres in %w/w basis (Error bars refer to 95% JSCI).

the degree of crystallinity, crystallite orientation, tensile strength, swelling behavior and porosity (Sukumaran et al., 2001). Also, increased moisture content decreases electrical resistance and this affects the dimensional stability (Mohanty et al., 2000; Sukumaran et al., 2001). The strong hydrophilic nature of plant fibres means that precautions must be taken to improve the water-related dimensional stability of the fibres, and to enhance the low compatibility between the fibres and the hydrophobic polymeric matrix (Madsen, 2004). The moisture content of *A. lobata* in this study is comparable to that of rice straw (6.5%), abaca (5 - 10%) and hemp (6.2 - 12%) but are lower than the moisture contents of flax (8 - 12%), jute (12.5 - 13.7%) and sisal (10 - 12%), an indication that the fibre materials may not readily absorb water (Taj et al., 2007). The moisture contents of the other samples are even lower.

Ash present in lignocellulosics contains silica that has many undesirable effects (Reddy and Yang, 2005). Silica blunts cutting machinery, reduces the digestibility of straw, interferes with the pulping process by forming scales on the surface of the reactors and makes combustion more difficult (Reddy and Yang, 2005). The ash contents (%w/w) of the fibres used in this study were comparable to the bast fibres of flax and hemp (1 - 2%) but lower than jute (8%), ramie (5%) and cotton (0%) (Averous and Digabel, 2006; Mwaikambo, 2006).

Kurshner-Hoffer cellulose

The results of Kurshner - Hoffer cellulose in % w/w basis are presented in Figure 5. The cellulose contents of the woody fibres *C. palmifitida* (55.20±1.59%), *U. lobata* (58.94±1.05%) and *M. morindoides* (55.76±1.40%) are comparable to those of kenaf (45 - 57%) and abaca (56 - 63%) (Taj et al., 2007). Also, the cellulose contents of *A. leonensis* (48.97±1.33%) and *A. lobata* (43.22±0.95%) are comparable to those of coir (36 - 43%w/w), Norway spruce (49%), barley straw (43%w/w) and corn stover (33%w/w) (Majumdar and Chanda, 2001; Rowell and Han, 2000). However, the type of cellulose (amorphous or crystalline) influences the properties and applications of the fibre such that fibres with higher crystalline cellulose would be suitable for composite reinforcement while those with higher amount of the easily hydrolysable amorphous cellulose would be suitable for pulp/paper making and bioethanol production (Madsen, 2004). Woody fibres contain a higher proportion of crystalline cellulose (60 - 70%) when compared with non-woody fibres (40 - 45%) (Madsen, 2004). Thus, lower cellulose content in woody fibres when compared to non woody ones such as cereal straws used as raw materials for bioethanol production, does not mean that such fibres will not support load bearing materials. The cellulose content is critical in order to dictate the specific use of a certain fiber (Shimizu, 2001). This also is influenced by the lignin content. For instance, fibres with high cellulose and lignin content may not be suitable for pulp/paper making or in textile industries since they will require delignification and more severe pulping conditions (Omotoso and Ogunsile, 2009). However, they may be suitable for composite production (Maya and Sabu, 2008).

Hemicellulose content

Hemicellulose contents of the fibres range from 8.62±1.67% for *M. morindoides* to 21.22±0.89% for *A. leonensis* as shown in Figure 3. These values are lower when compared with biofuel producing-lignocellulosic agro wastes such as straws of barley (27 - 38%), rice (23 - 28%), and wheat (26 - 32%) (Han, 1998; Gressel and Zilberstein, 2003; Reddy and Yang, 2005). The low hemicellulose content of the plant fibres used in this study implies that their water absorbing capacity will be low since hemicelluloses is the cell wall polymer with the highest water sorption capacity (Madsen, 2004). This is responsible for the high moisture absorption of natural fibre leading to swelling and presence of voids, which results in poor mechanical properties and reduces dimensional stability of composites (Maya and Sabu, 2008). This particular property reaffirms the potentials of the plant fibres used in this study for biocomposite technology. The low water retention capacity of the fibres decreases the activities of micro organisms when used in composite reinforcement (Maya and Sabu, 2008).

Lignin content

The lignin content of the plant fibres selected for this study (estimated as Klason lignin) ranges from 22.26±0.55% for *Urena lobata* bast fibre to 33.21±2.76% for *Ampelocissus cavicaulis* (Figure 5). These values are higher when compared with non woody bast fibres of flax (2.2%), hemp (3.7 - 5.7%) and kenaf (8 - 13%) (Taj et al.,

Table 4. Result of acid soluble lignin and its derived products.

Sample	ASL (%w/w)	% w/w ASL-derived products		
		Vanillin acid λ_{230}	p-Coumaric acid λ_{308}	Ferulic acid λ_{322}
A. leonensis	1.74 ± 0.34	0.50 ± 0.12	0.65 ± 0.14	0.41 ± 0.11
A. lobata	2.17 ± 0.08	0.57 ± 0.02	0.96 ± 0.002	0.59 ± 0.08
C. palmifitida	1.43 ± 0.20	1.41 ± 0.02	0.04 ± 0.01	N.D
U. lobata	1.02 ± 0.14	0.20 ± 0.001	N.D	N.D
M. morindoides	1.37 ± 0.05	0.91 ± 0.28	N.D	N.D

Table 5. Mechanical properties of the fibres.

Sample	Diameter (mm)	Tensile strength (MPa)	Young's modulus (GPa)	Elongation @break (%)
A. leonensis	0.23 ± 0.02	67.65 ± 0.73	0.42 ± 0.33	8.0 ± 0.01
A. lobata	0.40 ± 0.10	588.94 ± 2.00	2.03 ± 2.20	6.75 ± 0.01
C. palmifitida	0.14 ± 0.03	1022.73 ± 2.30	20.05 ± 8.24	2.55 ± 0.23
U. lobata	0.19 ± 0.02	2587.5 ± 4.70	51.75 ± 4.90	2.5 ± 0.50
M. morindoides	0.28 ± 0.10	1201.79 ± 2.04	11.95 ± 2.78	5.03 ± 0.11

Results expressed as mean ± S.D; n = 3.

2007); but lower when compared with the woody fruit fibres of coconut (41 - 45%) (Reddy and Yang, 2005). The high lignin contents of most of the fibres seem to be disadvantageous for their use in paper, pulp and bioethanol manufacturing, as they would require higher amount of chemicals and more drastic conditions during pulping and bleaching (Marques et al., 2010). Since lignin provides fibres with compressive strength, stiffens the fibre and protects the cellulose and hemicellulose from chemical and physical damage (Saheb and Jog, 1999), the biofibres used in this study will be suitable for composite reinforcement. Acid soluble lignin (ASL) content (Table 4) was greater in *A. lobata* (2.17 ± 0.08%) but *A. leonensis*, *C. palmitifida*, and *M. morindoides* were found to be 1.74 ± 0.34, 1.43 ± 0.02, and 1.37±0.05%, respectively. ASL-derived products (vanillin, p-coumaric acid and ferulic acid) ranged between 0.50 ± 0.12% and 1.41 ± 0.02% for vanillin; 0.04 ± 0.01% and 0.96 ± 0.002% for p-coumaric acid; and ferulic acid was detected in *A.leonensis* (0.41±0.11%) and A. lobata (0.59±0.08%).

Result of mechanical properties

Tensile strength is a measure of a material's resistance to being pulled apart. Young's modulus is a measure of the material's stiffness while elongation at break is a measure of its ability to extend linearly without breaking when subjected to pulling forces. The percentage elongation obtained for *A. leonensis* and *A. lobata* are comparable to that of cotton (7.0 - 8.0%) but lower than that of coconut fruit fibre (17 - 47%) (Beakou et al., 2008). Also, the values obtained for *C. palmitifida* (2.55 ± 0.23%) and *U. lobata* (2.5±0.50%) are comparable to those of Jute (1.5 - 1.8%), flax (2.7 - 3.2%), sisal (2.0 - 2.5%) and the synthetic fibres carbon (1.4 - 1.8%) and Glass-E (2.5%) (Taj et al., 2007; Beakou et al., 2008). The tensile strength of *U. lobata* (2587.5 ± 4.70 MPa), *C. palmifitida* (1022.73±2.30 MPa), and *M. morindoides* (1201.79±2.04 MPa) are comparable to that of flax (345 - 1035 MPa) and the synthetic glass-E fibre (2000-3500 MPa) (Bledzki and Gassan, 1999). The Young's modulus of *U. lobata* (51.75±4.90 GPa) and *C. palmifitida* (20.05±8.24 GPa) are comparable to that of jute (26.5 GPa) and flax (27.6 GPa) but higher than that of cotton (5.5 - 12.6 GPa) and coir (4 - 6 GPa). In brief, the mechanical properties of most fibres used in this study are comparable to those of other biofibres already used in manufacturing and can even match those of some synthetic fibres (Table 5).

Conclusion

The results show that plant fibres used in the present study have properties that are comparable with those of common biofibres such as kenaf, hemp and flax; and even synthetic fibres. Plant fibres are renewable resources with production requiring little energy and are biodegradable. This particular property of plant fibres is attributed to the presence of their biocomponents which include cellulose, hemicellulose and lignin that possess the necessary functional groups which can enable microorganisms to degrade them with ease. When used in the production of reinforced materials such as floor tiles, gas

cylinder, hot water tank, inner panel of cars, or when delignified for use in pulp making, these fibres may not result in severe environmental consequences. This is unlike non-biodegradable synthetic fibres which usually cause skin irritation during composite manufacturing or environmental hazard when any of its products is disposed. Plant fibres have low density, so when used in the construction of car parts such as the door panels and roof may reduce fuel consumption since it would require less energy to propel a lighter object than a heavier one.

Finally, to fully exploit the potentials that composites offer, education at Universities and other Technology Institutes is required. Sufficient knowledge of materials and manufacturing processes is required. Concerning materials, one requires the knowledge to quantify properties and to use these properties in the best way. The research and development circle for composite technology shown below requires the collaborative input of Universities, local fibre industries and enterprises to develop concepts, materials and processes in the product aspect. The natural fibre composites offer benefits to the society in different aspects including economy, ecology and technology transfer.

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Changes in protein solubility, fermentative capacity, viscoelasticity and breadmaking of frozen dough

Elisa Magaña-Barajas[1], Benjamín Ramírez-Wong[2]*, Patricia Isabel Torres-Chavez[2], Dalia Isabel Sánchez-Machado[3] and Jaime López-Cervantes[3]

[1]Universidad Estatal de Sonora. Ley Federal del Trabajo Final. Col. Apolo. Hermosillo, Sonora, México.
[2]Departamento de Investigación y Posgrado en Alimentos. Ave. Rosales y Blvd. Luis Encinas s/n o al Apartado postal 1658, C.P. 83000, Hermosillo, Sonora México.
[3]Centro de Investigación e Innovación en Biotecnología Agropecuaria. Instituto Tecnológico de Sonora. Calle Antonio Caso s/n, Col. Villa ITSON, C.P. 85130. Obregón, Sonora, México.

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The use of frozen dough remedied availability of fresh bread. However, bread elaborated from frozen dough has less volume and texture is firmer. This study evaluates how storage affects the protein solubility, fermentative capacity and viscoelasticity of frozen dough. In addition to examining the effects of storage on the quality of the final baked bread. Dough was frozen at a rate of -0.146°C/min and stored at -18°C for 42 days. Protein solubility was measured using the SE-HPLC method. A dynamic measurement method was used to determine the viscoelastic parameters of dough: storage and loss modulus (G´ and G´´), and phase angle (δ). The most drastic changes in the frozen dough occurred during the first seven days of storage. The weakening of frozen dough correlated with the hydrolysis of insoluble polymeric proteins, which is associated with the increase in the concentration of the protein soluble polymer. The viscous (δ) of the frozen dough increased to 25.88% after 28 days of storage, and the soluble polymeric protein concentration increased by 10.12% in this period. Frozen dough should be stored for fewer than 21 days; time in which the loaf volume of bread made from frozen dough was approximately 40.84% smaller than that of fresh bread dough formulation.

Key words: French type bread, frozen dough, protein solubility, baking quality, viscoelasticity.

INTRODUCTION

Frozen bread dough was developed with the goal of obtaining products that are similar to "fresh" bread made according to a traditional recipe. However, developing an adequate freezing step in the continuous process of bread-making, still presents a number of challenges. The diminished loaf volume of bread produced from frozen dough in comparison to bread made from fresh dough remains a challenge for the bread-making industry. The reduction in the volume of bread made from frozen dough can be attributed to decrease in yeast viability and

*Corresponding author. E-mail: bramirez@guaymas.uson.mx. Tel: (62): 59-22-07 and 59-22-09.

changes in the structure of the dough (Gant et al., 1990; Esselink et al., 2003; Cauvain and Young, 2008).

Wheat-based bread dough is a viscoelastic material that exhibits both viscous and elastic behavior. Wheat is the only cereal grain that has the ability to retain gas and that can be converted into a spongy product called bread. The gluten network that facilitates this ability forms as a result of the increased hydrophobic interactions, and disulfide bonds between the protein polymers found within the flour (gliadins, GLI and glutenins, GS) as well as noncovalent disulfide bonds (Godón and Herard, 1984; Shewry et al., 1995; Weiser, 2007; Kontogiorgos, 2011).

Proteins can be isolated on the basis of their solubility in alcohol-water solutions. The GLI fractioninclude monomers linked by noncovalent disulfide bonds. GS fraction comprise a heterogeneous group of high-molecular-weight polypeptides linked by disulfide bonds (Shewry et al., 1995; Weiser, 2007; Kontogiorgos, 2011). Pro-teins in the GLI fraction give the dough extensibility and viscous, whereas GS mainly influence functional properties of the dough such as strength and elasticity (Lu and Grant, 1999). The GS fraction is composed of a mix of high- and low-molecular weight (HMW and LMW, respectively) GS (Weiser, 2007; Kontogiorgos, 2011). The HMW-GS in wheat flour contribute greatly to the elastic behavior of wheat dough (Shewry et al., 1995; Lefebvre and Mahmoudi, 2007). Lu and Grant (1999) found that the GS fraction has a substantial effect on the baking characteristics of dough that has been frozen; the GLI and starch fractions affect the baking characteristics of the dough to a lesser extent. These authors suggest using strong flours (those with high levels of GS) to produce frozen dough. Borneo and Khan (1999) found a direct and inverse relationship between soluble polymeric protein fractions and albumin+globulin (A+G) with loaf volume.

Each protein fraction has a different degree of solubility, and the solubility of each fraction varies depending on the stage of the bread-making process. In general, the solubility of various proteins decreases during mixing and fermentation. During mixing, the polymers bind covalently to water and subsequently produce a continuous macromolecular viscoelastic material. During fermentation, an oxidative process that involves the crosslinking of these polymers occurs (Hoseney et al., 1979; Borneo and Khan 1999; Cuq et al., 2003). Dough freezing results in further changes to the gluten-based polymeric structures coupled with changes in protein solubility that occurs during bread preparation. Lei et al. (2012) used size chromatography (SEC) to determine the degree of depolymerization of the frozen gluten proteins stored at -18°C. They found a 40.83% decrease of the GS-HMW fraction in the gluten stored for 120 days. These authors discuss that there is a greater degree of depolymerization in the gluten protein fractions of higher molecular weight and are increasing during frozen storage. These changes on gluten contribute to viscoelastic changes in the dough that disrupt its ability to be baked. The impacts of these changes can increase with storage time, and they often result in the comparative weakening of frozen dough evidenced by the results of viscoelastic tests, diminished fermentative capacity, and decreased loaf volume associated with the use of frozen dough.

The freezing process results in mechanical damage to the gluten network that result from the formation of ice crystals. Ice crystal formation provokes ruptures in the gas cell membrane (Gant et al., 1990), and the increase in crystal size within the gas cells results in water redistribution that ultimately causes dehydration of the dough (Esselink et al., 2003) and in protein that changes the original structure of the dough. During storage, changes in the number, sizes and shapes of the ice crystals occur; this phenomenon is called of "re-crystallization" (Baier-Schenk et al., 2005a). Kontogiorgos et al. (2008) agreed that both the formation of ice crystals and the re-crystallization phenomenon cause disruptions in the structure of the gluten network and change its morphology. During thawing (rehydration), the process of water transfer occurs in reverse, and the water molecules bind to different sites on the dough proteins than those they initially occupied, thereby changing the conformation of the dough.

The structural changes in the dough that occur during freezing and storage modify the viscoelastic properties of the dough and thereby alter its behavior during baking. During storage, both the elastic and viscous (G' and G") moduli of frozen dough decay over time, and the viscous behavior prevails in comparison to that of fresh dough (Ribotta et al., 2004), which reduces the degree to which frozen dough is able to retain gas (Selomulyo and Zhou, 2007). The loss of elastic behavior in frozen dough is attributed to the fragmentation of the polymeric protein chains.

SDS gel electrophoresis has provided evidence of increased protein solubility in frozen dough that was thought to result from glutenin degradation (Kennedy, 2000; Ribotta et al., 2001). Leray et al. (2010) concluded that, during storage, changes in the viscoelasticity of the dough occur and are related to the observed reduction in the volume of the final baked bread (Ribotta et al., 2001).

In relation to the frozen dough, changes that occur in wheat dough during freezing and storage have been evaluated using empirical and fundamental rheological methods (Bhattacharya et al., 2003; Giannou and Tzia, 2007; Angiolini et al., 2008; Leray et al., 2010). It has also been shown that the freezing conditions (Aibara et al., 2005) may alter the structure of frozen dough, and the effect of ingredients and additives (Sharadanant and Khan, 2006; Selomulyo and Zhou, 2007) have also been studied. The freezing-induced weakening of the dough can be attributed to damage to the gluten network that result from the formation (Shelton and Freeman, 1991) and growth (Ribotta et al., 2004; Selomulyo and Zhou,

2007) of ice crystals, but there is still a need for information about the interrelationship between the changes in the protein solubility and viscoelasticity that occur in frozen dough and the quality of the bread made from it (Kennedy, 2000; Ribotta et al., 2001; Sharadanant and Khan, 2006). To date, there is no clear understanding of the ways in which the effects of both freezing process and storage time on the protein solubility and viscoelastic properties of frozen dough affect the quality of the baked product.

Currently, no research has been done employing to identify changes in proteins during frozen storage of dough. In addition, there is no evidence of the degree of hydrolysis of proteins detected by the technique of SE-HPLC and how it affects changes in the viscoelasticity of frozen dough, which are reflected in poor baking quality.

The aim of the present study was to evaluate the way in which storage time affects the protein solubility and viscoelasticity of frozen bread dough in addition to the quality of the baked product.

MATERIALS AND METHODS

Raw materials

We used a formulation for French type bread to prepare the frozen dough. Commercial high-protein flour (13.64%, dry basis) was supplied by Molino la Fama S.A. de C.V. The remaining ingredients were: salt (Sea of Cortez, Sales del Valle SA de CV), shortening (Inca, Food Capullo, S. de R. L de CV), instant yeast (Nevada, SAFMEX SA de CV/FERMEX SA de CV) and white bread improver (Magimix 40, SAFMEX SA de CV / FERMEX SA de CV). The yeast used remained under freezing conditions in order to increase its cryotolerance and preserve their fermentative power (Wolt and D'Appolonia, 1984; Ribotta et al., 2003).

Flour quality evaluation

Proximate composition of the flour was determined by the methodology of A.A.C.C. (2000): protein content (method 46-13), ash content (method 08-03), and moisture content (method 44-40). The water absorption, stability and the development time of the dough were evaluated using the farinographic method (54-21) proposed by the A.A.C.C. (2000). The extensibility and deformation energy of the dough were tested using the alveographic method (53-30) established by the A.A.C.C. (2000).

Processes for freezing and thawing the dough

Formulation

Preliminary, we tested three formulations of ingredients for frozen dough in which was varied only the yeast content (2, 3 and 5%, dry basis), and the rest ingredients remaining constant. The frozen dough formulation that resulted in baked bread with similar in quality to fresh bread included the following ingredients: high-protein flour (13.54%) (100%, weight basis flour), salt (1.5%), lyophilized yeast (3%), shortening (5%), white bread improver (2%) and 200 ml of water (the appropriate water volume calculated using a farinograph).

Dough preparation

The dough was prepared according to the method described by Magaña-Barajas et al. (2011). Briefly: the dry ingredients were mixed in a blender (MFG Lincoln, NE, USA) for 1 min, after which the dough was mixed for 3 min upon incorporating appropriate volume of water obtained via the farinograph.

Molding

Fifty-gram dough samples were rounded and set aside for 5 min, after which they were manually molded into bread loaves. In addition, larger dough samples (315 g) were used to evaluate the fermentative capacity of the dough and were rounded and molded for bread-making according to the same procedure.

Preproofing

The dough samples were preproofed for 10 min in a controlled environment (30°C, 85% relative humidity) using a proofing cabinet (MFG National brand, Lincoln, NE, USA).

Freezing and storage

Preproofed dough samples were frozen using a slow-rate freezing method that appears to minimize damage to the gluten structure and yeast viability of the dough (El-Hady et al., 1996; Codón et al., 2003). The loaves were frozen in a freezer at a temperature of -18°C (Frigidaire brand, model GLFC1526FW, Mississauga, Ont., Canada). The total freezing time was 5 h and 44 min; dough was frozen at a rate of -0.146°C/min. Samples were stored at -18°C for up to 42 days. The freezer was calibrated during 24 h by monitoring the temperature using a thermocouple (Digi Sens). Every 7 days, frozen dough samples were removed from the freezer and subjected to a series of evaluations.

Thawing

The dough samples were thawed under refrigeration conditions (4°C) (Ribotta et al., 2001, 2003; Karaoğlu et al., 2008). The thawing time and rate were determined by measuring temporal changes in the temperature of the dough using a thermocouple (Digi Sens). Thawing occurred at a rate of 0.062°C/min, and the samples reached the equilibrium temperature (4°C) after thawing for 4 h and 15 min.

Proofing

The thawed dough was fermented for 50 min in a proofing cabinet. The temperature in the proofing cabinet was 30°C and the relative humidity was 85% (MFG National brand, Lincoln, NE, USA).

Frozen dough evaluations

Assessments of the protein solubility, fermentative capacity, viscoelasticity and baking quality of frozen, thawed and fermented dough samples were carried out in triplicate. Samples of the frozen dough were assessed after each storage period (0, 7, 14, 21, 28, 35 or 42 days).

Protein solubility

For each storage time, 300 mg samples of dough were used to eva-

luate the changes in protein solubility. Protein solubilities were determined via molecular exclusion liquid chromatography (SE-HPLC). Soluble proteins were extracted using a 50% propanol solution. An SE-HPLC system (Varian ProStar equipment, Model 410, Palo Alto CA) with a diode array detector (Varian, Palo Alto CA) and an autosampler (Varian, Palo Alto CA) was used for all of the analyses. Detections using a chromatography column were performed at a wavelength of 210 nm (Biosep-SEC-S-S4000, Phenomenex, Torrence, CA). The mobile phase was an acetonitrile/water (50:50) mixture with 0.1% TFA (Lookhart et al., 2003). The flow rate was 0.5 ml/min, the temperature of the column was 40°C and, the conditions remained isocratic. The chromatograms were evaluated, and each peak represented one of the protein fractions: the soluble polymeric protein (SPP) fraction, the gliadin (GLI) fraction, and the albumin and globulin (A+G) fraction. The aforementioned proteins are listed by the order in which they were excluded.

Fermentative capacity

The fermentative capacities of the dough were determined by placing 315 g samples into a rheofermentometer (Chopin, type Rheo F3), and the protocol provided in the equipment manual was followed. The results were read after 3 h of fermentation at a constant temperature (28.5°C). Values for the volume of the total gas production (CO_2T, ml) and the volume of retained gas (CO_2R, ml) were obtained.

Viscoelasticity

Dough viscoelasticity was evaluated using a 2.6 g sample of thawed proofed dough. A controlled deformation rheometer (Rheometrics Scientific brand, model, RSF III, Piscataway, NJ, USA) equipped with parallel plates that were 25 mm in diameter was used for this purpose, and a Peltier system was used to maintain a sample temperature of 25°C. The dough was maintained on the appropriate plate with a 2 mm separation between the plates. Any leftover dough was removed from the apparatus, and the part of the sample that was exposed to the environment was covered with petroleum jelly to prevent dehydration. The sample was allowed to stand for 15 min in order to set. Oscillatory tests in a frequency sweep were measured at 0.1% strain in a linear regime; the frequency range was 0.1 to 100 rad/s (Magaña-Barajas et al., 2011). The storage modulus (G ', Pa), loss modulus (G'', Pa) and phase angle (δ, °) parameters were calculated using an appropriate software program (RSI Orchestrator, Rheometrics Scientific).

Bread quality of frozen dough

The molded thawed dough was fermented in a proofing cabinet with a fermentation temperature of 30°C and a relative humidity of 85% (MFG National brand, Lincoln, NE, USA). After fermentation, bread samples were obtained by baking the dough for 12 min at 250°C in a Partlow oven (National brand, MFG, Lincoln, NE, USA). Fully baked loaves were cooled for two hours at a temperature of 25°C (Magaña-Barajas et al., 2011) after the specific volume and crumb firmness of the bread was measured.

Specific volume

The displacement principle was used to determine the loaf volume of the bread; volume measurements were made using rapeseed and a volume meter (National brand MFG Company, PUP) that had been calibrated to a volume of 400 cm^3. Each loaf was weighed using a balance (OHAUS, 2610 g capacity), and the specific volume of the bread was obtained using its volume/weight ratio.

Firmness

The maximum firmness of the bread crumbs were evaluated using a universal testing machine (Instron Corp, model 4465, USA), and a modified version of A.A.C.C. method 74-09 (A.A.C.C., 2000); the modifications have been described by Magaña-Barajas et al. (2011). The modification of the method consisted of using geometry referenced to a 30 mm diameter. The sample bread crumbs were obtained by slicing the bread into pieces that were 25 mm thick. Square samples with side lengths of 30 mm were extracted from the center of the crumb and were used in the subsequent firmness evaluation. The measured parameter was the maximum force (kg-f) of the bread after two hours of storage.

Experiment design and statistical analysis

A randomized experimental design was performed in which the independent variable factor was the time over which the frozen dough was stored, and the levels of the variable were: 0, 7, 14, 21, 28, 35 or 42 days. To determine the effects of storage time on the various measured parameters, analyses of variance (ANOVA) were conducted. A 95% significance level was chosen to indicate significant differences. Tukey tests with the same statistical significance level were conducted to identify differences between specific experimental manipulations. In addition, simple correlations (r) among the various evaluations were made. The ANOVA was conducted using the Statistical Analysis Software System (SAS Institute, Inc. Cary, NC, 2002).

RESULTS

Flour quality evaluation

Flour with high protein content (13 to 15%) is recommended for production of frozen dough (Mesas and Alegre, 2002). Table 1 shows the results of the physicochemical and rheology of the flour quality. The flour showed high protein content, farinogram water absorption and alveogram extensibility (P/L) values 13.64%, 63.84% and 1.9. These parameter values are consistent for a high-quality bread flour (Mesas and Alegre, 2002), that is suitable for the production of frozen dough to make French type bread.

Frozen dough evaluation

Protein solubility

Several researchers associate frozen dough deterioration with the degradation of protein fractions (Kennedy, 2000; Ribotta, et al., 2001; Li et al., 2012). Figure 1 shows chromatograms of bread dough after 0 and 42 days of storage at -18°C. The chromatograms were obtained using an SE- HPLC technique and outline three soluble protein fractions. Peak I corresponds to the soluble polymeric protein (SPP) fraction; peak II corresponds to the gliadin (GLI) fraction; and peak III corresponds to a fraction

Table 1. Physicochemical and rheological characteristics of the flour used to make french type bread.

Determination	Value
Proximal	
Moisture[1] (%)	11.11
Protein[1] (%)	13.64
Ash[1] (%)	0.94
Farinograph	
Water absorption (%)	63.84
Stability (min)	7.50
Development time (min)	7.17
Alveograph	
Extensibility, P/L	1.91
General strength, W (10^{-4}J)	248.50

1, Dry basis; P, Maximum height of the curve or stretch resistance; L Length of the curve or dough extensibility; W, Strain energy.

containing two proteins that are not related to gluten, albumin and globulin (A+G) (Borneo and Khan, 1999).

Soluble protein

Figure 2 shows changes in solubilities of the soluble protein fractions (calculated as the areas under the curve) that occurred during the storage of frozen dough. Figure 2 shows that the average of soluble polymeric protein (SPP) solubility in frozen dough increased by approximately 8.09% during the first 14 days of storage. In general, after this time period (21 to 42 days) the average of SPP content of the frozen dough increase more slow. This reveals the degradation of high molecular weight glutenin results in gluten weakening.

The increase in degradation of SPP suggests that there may be structural breakdowns in protein polymers that promote the formation of new smaller and/or more soluble polymers or the reassociation of more soluble polymers to a new polymer with similar molecular weight at SPP. This coincided with the negative correlation found between SPP and GLI (r=-0.96) content, which indicates that higher levels of SPP hydrolysis were associated with increases in the amount of GLI. These results are in agreement with Lei et al. (2012) who observed a decrease of higher molecular weight proteins during storage of the frozen gluten. It could indicate that there is degradation in gluten of frozen dough. The general increase in SPP degradation explains the observed changes in the elastic behavior of the dough and in the quantity of retained gas.

Dough elasticity has been attributed to the SPP fraction, and the type(s) of SPP subunits in the dough determine its functionality (Field et al., 1983 in Tatham et al. 1995). Cornec et al. (1993) used SE-HPLC to characterize sub-fractions of fresh gluten by evaluating their individual rheology and relationships to the viscoelasticity of the dough. Gluten subunits can be classified into three groups; the HMW-GS group contains the gluten subunits that are responsible for dough elasticity. The elasticity conferred by the HMW-GS group appears to result from three aspects of these protein subunits. The first relates to their potential to form cysteine residue cross-links. The second is their spiral structure, and the third aspect is their high capacity to form intra- or intermolecular hydrogen bonds due to high levels of residual glutamine (Field et al., 1983 in Shewry et al., 1995). Belton (1999) describes a new model for the elasticity of the HMW-GS, indicating that viscosity is due to the high density of attached groups by hydrogen bonds provided by the long chain polymer itself. At the end, there are cysteine residues. The chains are joined together in the absence of water.

When hydrated promoted protein interactions by hydrogen bonds, without promoting the breakdown in existing hydrogen bonds. There will be a balance between interchain bonds and bonds with water. This promotes the formation of the region of loops and train region. This region is likely related to the β sheet formation. With increasing hydration of the region, the loop increases thereby decreasing the train region. The structure can be deformed first by the loops then by the train region. When this occurs the entropy of the loops is lost due to the formation of inter-chain hydrogen bonds, and is partially substituted by increasing the entropy of the hydrogen bonds of water released**.** Because HMW-GS subunits appear to determine the elasticity and baking quality of the dough, maintaining a certain quantity of these subunits in frozen dough designed for French type bread would be ideal (Shewry et al., 1995. Baier-Schenk et al. (2005b) used laser scanning microscopy to observe changes that occurred in gluten that had been isolated during freeze-thaw cycles. This group observed changes in the gluten fibrils that resulted from the water fusion-mediated cryo-concentration of protein polymers. Although the observed changes appeared to be reversible during thawing, the distribution of water in the resulting dough had changed by the end of the freeze-thaw cycle. Some authors explain that the degree to which the structural rearrangement of gluten is reversible depends on the origination of the structure and the types of links between the polymers that are present in it (Evans et al., 1996; Goff et al., 1999; Lozinsky et al., 2000 in Baier-Shenck et al*.,* 2005b).

Figure 2 also shows changes in the solubility of the Gliadin (GLI) fraction of frozen dough during storage. In general, during the first 21 days was observed a decrease on GLI fraction. This can be explained as function of a possible association between this polymer and other one to form a new polymer with similar molecular weight to the SPP. After this period, this fraction was reduced by an average of 10.55%, indicating the degradation of these polymers. Borneo and Khan (1999) evaluated changes in protein solubility in fresh dough during the baking process,

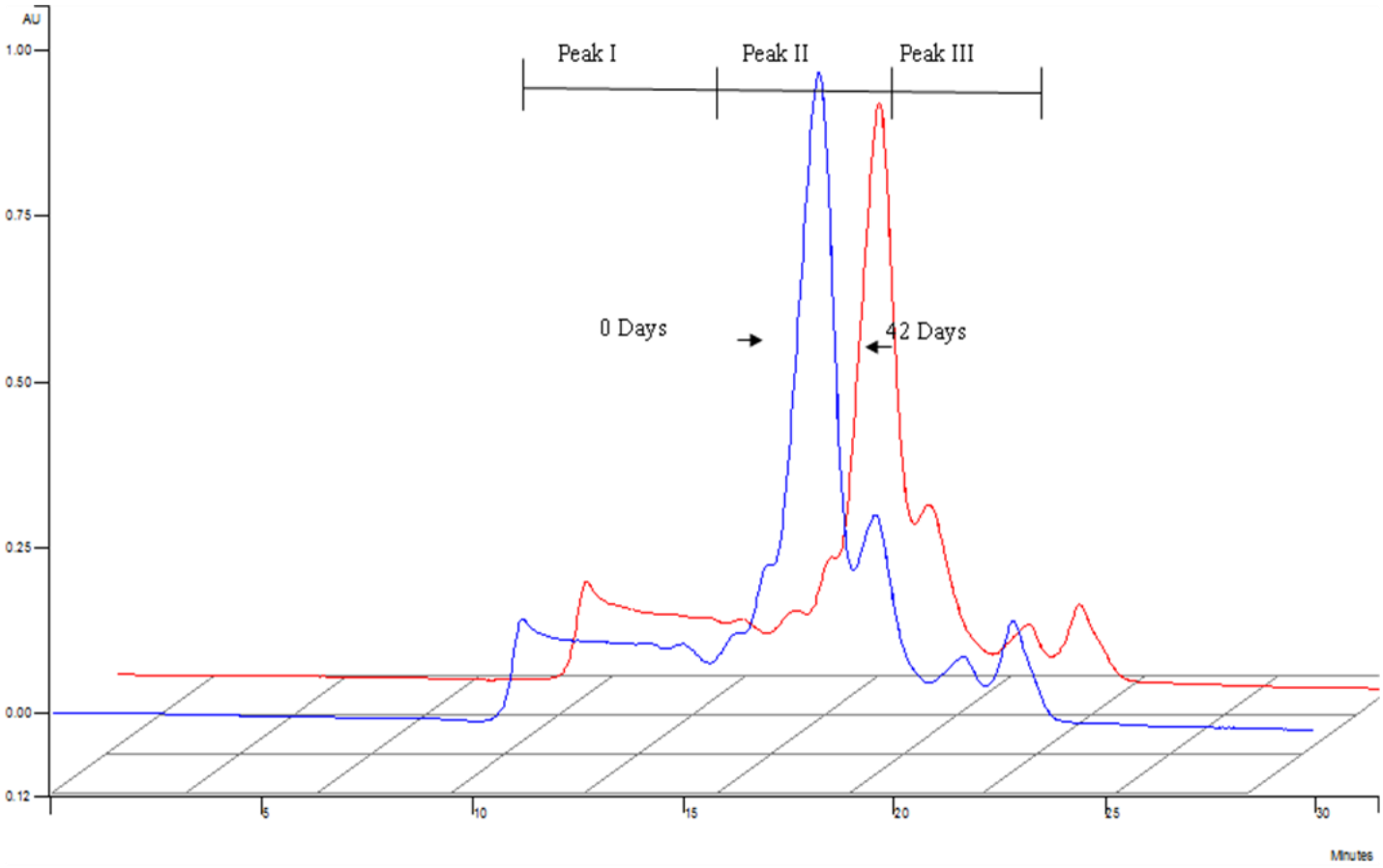

Figure 1. Chromatograms of french type bread dough at 0 and 42 days of storage at -18ºC.

and only they found evidence of changes in the SPP and A+G fractions. They interpret their findings as suggesting that large branching polymers composed of higher molecular weight subunits are more susceptible to changes during the baking process than smaller polymers, and the aforementioned changes are reflected in protein solubility; these changes appear to be similar to the changes that occur during the process of freezing and thawing dough.

Figure 2 shows also the changes in solubility of the albumin and globulin (A+G). In general, this coincides with changes in GLI fraction solubility. This reveals the degradation of gluten polymers associated with weakening of dough, and the poor breadmaking quality of frozen dough. Fluctuations in SPP, GLI and A+G contents of frozen dough evaluated by SE-HPLC after various period of frozen storage demonstrate the occurrence of the dissociation and/or reassociation of protein polymers during freezing and storage steps associated with increase of viscoelastic behavior of frozen dough. In general, the solubilities from all of the soluble protein fractions changed after 21 days of storage, which shows that a restructuring of protein polymers occurs in frozen bread dough. More specifically, a shift in the direction of the mass balance occurred that was oriented toward soluble protein degradation, and the size of this shift increased over time. Ribotta et al. (2001) observed a similar trend when using electrophoresis to evaluate changes in the concentration of various proteins in frozen dough protein that had been stored for as long as 7 days.

Clearly, the observed increase in the presence of non-gluten polymers in the frozen dough that occurred during storage corresponds to the weakening of the frozen dough, and this increase is likely also related to the loss of bread quality. In general, the SPP fraction (0.73%) was more affected by the freezing and storage of the dough than the other fractions was (0.63% GLI, ≈ A+G). Sharadanant and Khan (2003) used an SDS (sodium dodecyl sulfate) technique to study protein concentrations, and they also observed a direct relationship between soluble protein concentrations and storage. Shewry et al. (1995) mentioned a loss of dough elasticity that appears to occur when the SPP fraction dissociates into monomers because of the activity of reducing agents such as b-mercaptoethanol and dithiothreitol.

The frozen dough have intensified their use in recent decades, however, the low quality of their products has been associated with several factors. One of them is

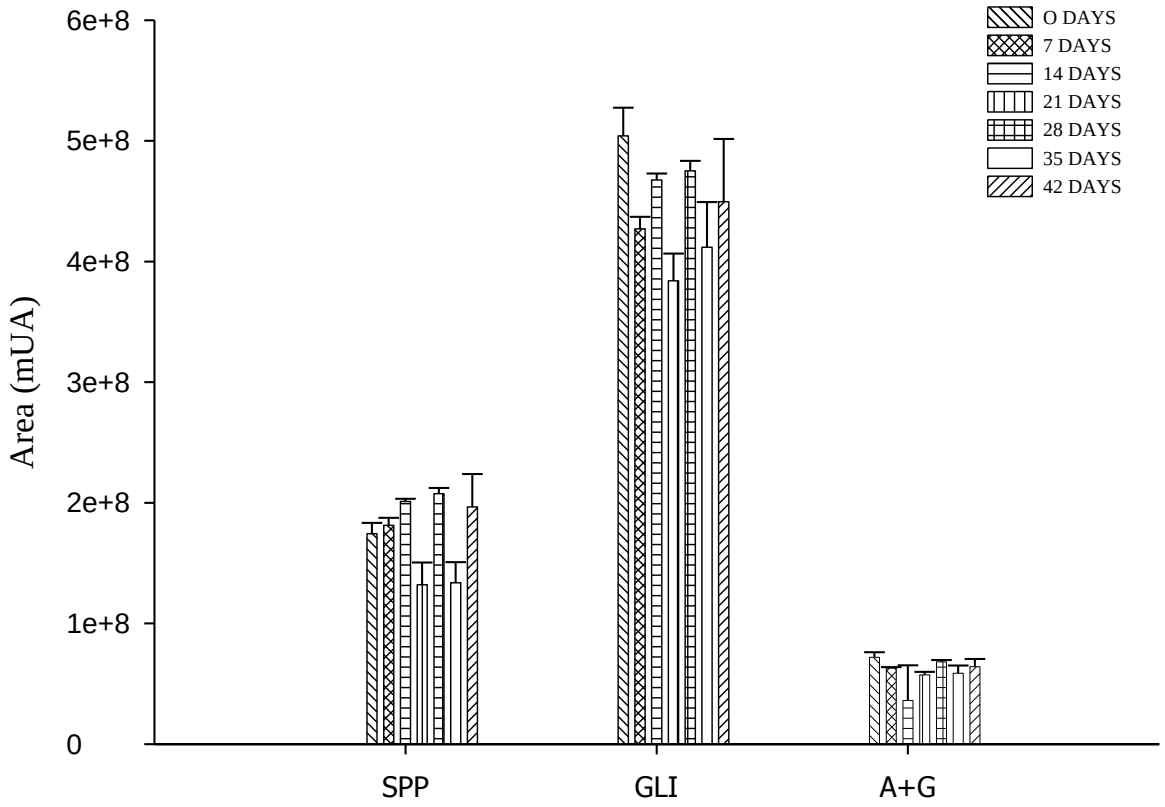

Figure 2. Changes in the solubilities of the soluble protein fractions in frozen french type bread dough during storage. SPP, soluble polymeric protein (peak I); GLI, gliadin (peak II); A+G, albumin + globulin (peak III). Bars indicate standard deviations.

attributed to possible breakings in the gluten protein polymers. However, there is little evidence of this. With our study, it was established that the SE-HPLC technology is suitable for detecting changes in frozen dough protein during storage. The main protein fraction affected was SPP, which is expected to be associated with the possible loss of the elastic behavior of dough and the low baking quality.

Fermentative capacity

The low amount of CO_2 produced and retained in frozen dough is attributed to the weakening of the dough relevant mainly to the gluten network, and a deficient activity of the yeast. Changes in the fermentative capacity of frozen dough with respect to the storage time are shown in Figure 3. The variability of both the total gas production and the gas retention (TCO_2 and RCO_2, respectively) of the frozen dough during storage were evaluated using a rheofermentometer. Storage time had a significant effect ($p < 0.01$) on both parameters.

During the first 7 days of storage (day 0 to day 7), the fermentative capacity values of the frozen dough were reduced by 20% (for TCO_2) and 15% (for RCO_2). Between 7 and 42 days of storage, there were slight decreases in both parameters and the fermentative capacity of the frozen dough was still reduced compared to 0 days. During mixing, gas cells form in the dough, and the resulting increase in gas production results in a spongy product called bread. The freezing process apparently modifies the structure of the bread dough, thereby alters the fermentative capacity of it. The gas pressure exerted on the damaged dough exceeds its capacity to support gas cell formation, resulting in diminished bread volume. The observed decrease in the fermentative capacity of the dough used in the present study, particularly in terms of the TCO_2 (14.43%) value, was less substantial than that reported by El-Hady et al., (1996) (54% after 28 days of storage). This difference is most likely due to differences in the specific formulation and type of flour used in each study.

Some authors (Kontogiorgos, et al., 2008; Phimolsiripol et al., 2008) have attributed the observed reductions in gas retention to damage on the gluten network, damage caused by recrystallization. After damage to the gluten network occurs, the diffusion of both nutrients and the byproducts of yeast metabolism is limited (Yi and Kerr, 2009). This results in an increase in the crumb firmness of the fully baked bread and a decrease in the volume of it (Aibara et al., 2001). The observed reduction in RCO_2 also coincided with the measured protein degradation,

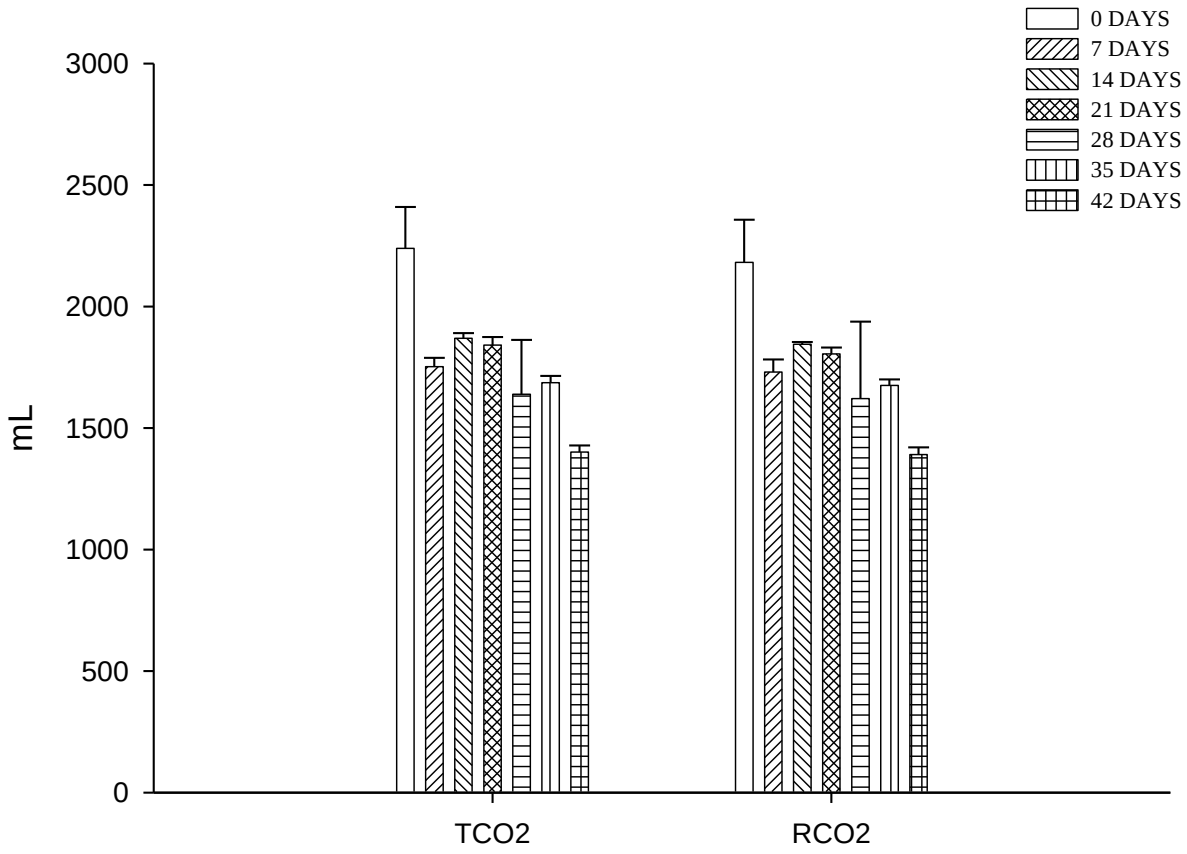

Figure 3. Changes in the fermentative capacity of frozen french type bread dough during storage. TCO2, total gas production; RCO2, retained gas. Bars indicate standard deviations.

particularly with the degradation of SPP, so a lower bread volume was expected. A conclusion is that the relationship between low levels of RCO_2 and decreased TCO_2 affect the specific volume of bread baked from frozen dough more than they affect its maximum crumb firmness.

The most dramatic changes in the fermentation capacity of the frozen dough were observed during the first 28 days of storage and appeared to coincide with the breaking and regrouping of protein chains. Both gas production and retention were adversely affected by increases in storage time, which further demonstrates the loss of the fermentative capacity associated with the use of frozen dough. These results agree with the findings of Aibara et al. (2001), who observed reduced CO_2 production by frozen yeast that had been stored at sub-zero temperatures that were reflected in the volume and firmness of the finished bread. An attempts to preserve and extend the freshness of French type bread dough by storing it at sub-zero temperatures affects both the viability of yeast and the viscoelastic structure characteristic of the gluten network. The temperature decrease creates pressure gradients in the dough that put osmotic pressure on the yeast and thereby compromises their ability to function normally. Protein hydrolysis that occurs in the frozen dough also coincides with its poor fermentative capacity. During freezing and storage, the polymer chains in the dough break down, which is demonstrated by the observed increase in the degradation of soluble protein. This weakening of the dough reduces its gas retention capacity.

Viscoelasticity

Loss of the integrity of frozen dough has been reported as a general viscous behavior increase during storage (Ribotta et al., 2004). All of the viscoelastic parameters of the frozen dough were significantly affected ($p < 0.01$) by storage time. The values of the viscoelastic parameters used to evaluate the effect of storage time on the viscoelasticity of the frozen dough were recorded at a frequency 5 rad/s; the viscoelastic properties behave linearly at this frequency. Figures 4a, b, and c show the values of the storage modulus (G´), loss modulus (G´´) and phase angle (δ), respectively, obtained from the frozen dough after various periods of storage.

Figures 4a and 4b show that all of the storage durations had similar effects on the viscous (G´´) and elastic (G´) moduli of the frozen dough. Increases in the test

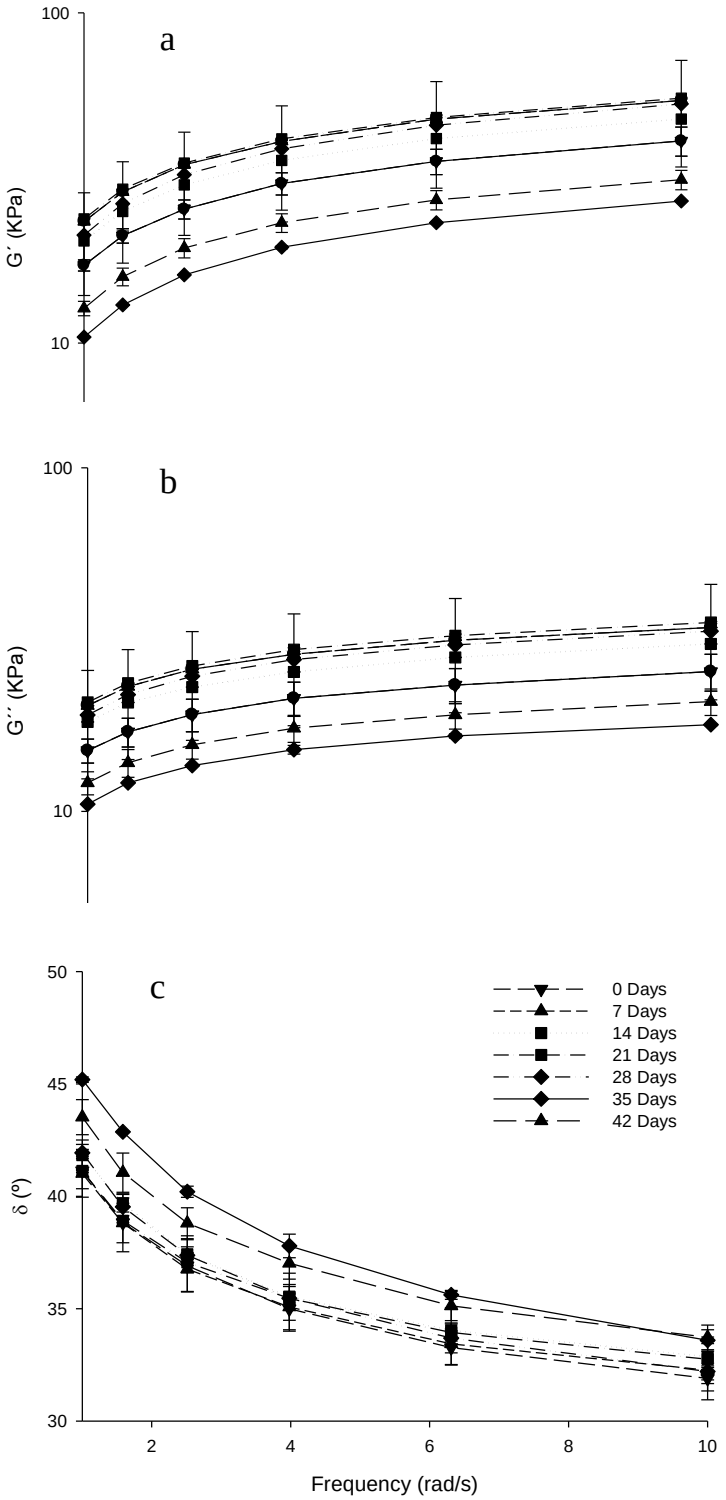

Figure 4. Behavior of the: a) storage modulus (G´), b) loss modulus (G´´), and c) phase angle (δ) of frozen french type bread dough during storage. Bars indicate standard deviations.

frequency were accompanied by increases the values of storage (G´) and loss (G´´) moduli. The G´ values were greater than the G´´ values, which agree with the findings of other authors (Ribotta et al., 2004; Angiolini et al., 2008; Leray et al., 2010). Moreover, both Figures (4a and 4b) show that for any frequency, the intermediate values

of both moduli were observed after 0 days of storage (G´=30.41 KPa and G´´=21.32 KPa, at a frequency of 5 rad/s). Furthermore, the largest increases in the G´ and G´´ values were observed between 0 and 7 days of storage (34.13 and 34.38% increases for G´ and G´´, respectively), coinciding with changes on SPP protein solubility.

From day 7 to 14, both the G´ and G´´ moduli of the frozen dough decreased to half of the respective values that had been obtained from frozen dough that had only been stored for seven days. However, the values were still greater than the values observed in dough that had been stored for 0 days (Figures 4a and 4b). From day 14 to day 21, the stored frozen dough had G´ values that were similar to the G´ values that had been obtained from frozen dough that had been stored for 7 days; the same trend was observed in the relevant values of G´´ (Figures 4a and 4b). This observation is consistent with both the increase in protein degradation and the reduction in gas retention capability that have been discussed in the preceding sections. Additionally, this observation shows that the reorganization of protein polymers that occurs during freezing affected the viscoelasticity of the dough.

Frozen dough that had been stored from 35 to 42 days showed lower values in two of the measured viscoelastic parameters (G´ and G´´). For this storage duration, the observed results of the viscoelastic evaluation of the dough were in agreement with those of other researchers who observed decreases in both G´ and G´´ during storage (Kenny et al., 1999; Leray et al., 2010). Between 28 and 42 days of storage, the average decreases in these parameters were 57.22 and 42.97% for the G´ and G´´ moduli, respectively. Progressive decreases in the values of G´ and G´´ during storage were expected to occur after day 35 (Ribotta et al., 2004; Angiolini et al., 2008; Leray et al., 2010), but the expected decreases occurred after 28 days of storage in this case.

The *Phase angle (δ)* value was used as a measure of the viscous behaviour of the frozen bread dough (Figure 4c). The structure of the frozen dough deteriorates during storage, and a clear increase in the value of δ with storage time can be observed. This increase in the δ value is consistent with the hydrolysis of the glutenin polymer and the low fermentative capacity of frozen dough. Frozen dough had the most elastic behavior at 0 days of storage (δ=35°), and this value was higher than that of dough made with fresh dough formulation (2% yeast and 2% shortening, data not show) (Magaña-Barajas et al., 2011), which concurs with the findings of other authors (Ribotta et al., 2004; Leray et al., 2010).

The decrease in the elastic behavior of frozen dough associated with structural changes in its gluten network may be caused by mechanical damage that occurs during the formation and growth of ice crystals. This damage results in smaller loaves. Phimolsiripol et al. (2008) further suggested that the damage to the gluten network was associated with decreased rates of gas retention.

Using transmission electron microscopy, Jiang et al. (2008) observed that the changes in the morphology of the gluten structure that occurred during freezing were caused by the formation of ice crystals and the re-crystallization phenomenon. This observation supported the notion of a direct relationship between structural changes in protein polymers that occur during freezing and the relatively poor quality of bread baked from frozen dough (Ribotta et al., 2001; 2004).

The viscous character of frozen dough increased between 0 and 42 days of storage, which is also in agreement with several researchers (Autio and Sinda, 1992; Ribotta et al., 2004, Angiolini et al., 2008). The transition of water from liquid to a glassy state and an increase in crystal size occurs during storage breakdown of protein polymers. This weakens the structure of the frozen dough and thereby increases the viscous behavior of it.

Thus, the weakening of the dough is associated with both an increase in SPP degradation and a loss of gas retention capacity. The most substantial loss of elastic behavior (25.88%) occurred after 28 days of storage, and most likely resulted in the most profound changes in the baking characteristics of the dough. Moreover, this temporal effect coincided with the observed loss of fermentation capacity, which is consistent with the findings of Leray et al. (2010).

The effect that freezing process has on bread dough is largely reflected in both the observed decreases in the G´ and G´´ moduli and the increase in the δ of frozen dough. Taken together, these effects indicate that the dough has been damaged, and they are consistent with Leray et al. (2010).

The weakening of the frozen dough during storage was expected and was reflected in the increase in the viscous behavior of the dough at the expense of the associated decrease in elastic behavior. In general, ice crystals tend to breakdown of various protein structures that alter the viscoelastic properties of the dough may be related to the observed loss of baking quality. The observed increase in the δ of the frozen dough (Autio and Sinda, 1992; Angiolini et al., 2008) and the loss in the extensibility of it (Yi and Kerr, 2009) that occur during storage associated with the development a gluten network containing more ruptures and fewer continuous, disintegrated remains of starch granules.

This result in the weakening of the dough was shown in the present study.

Bread quality of frozen dough

The poor quality of bread obtained from frozen dough bread is associated with the loss of dough structure, increasing its effect during extended storage time. Figures 5 and 6 show the average values of the specific volume (SV) and the maximum crumb firmness (MF) of bread baked from frozen dough. Structural changes in

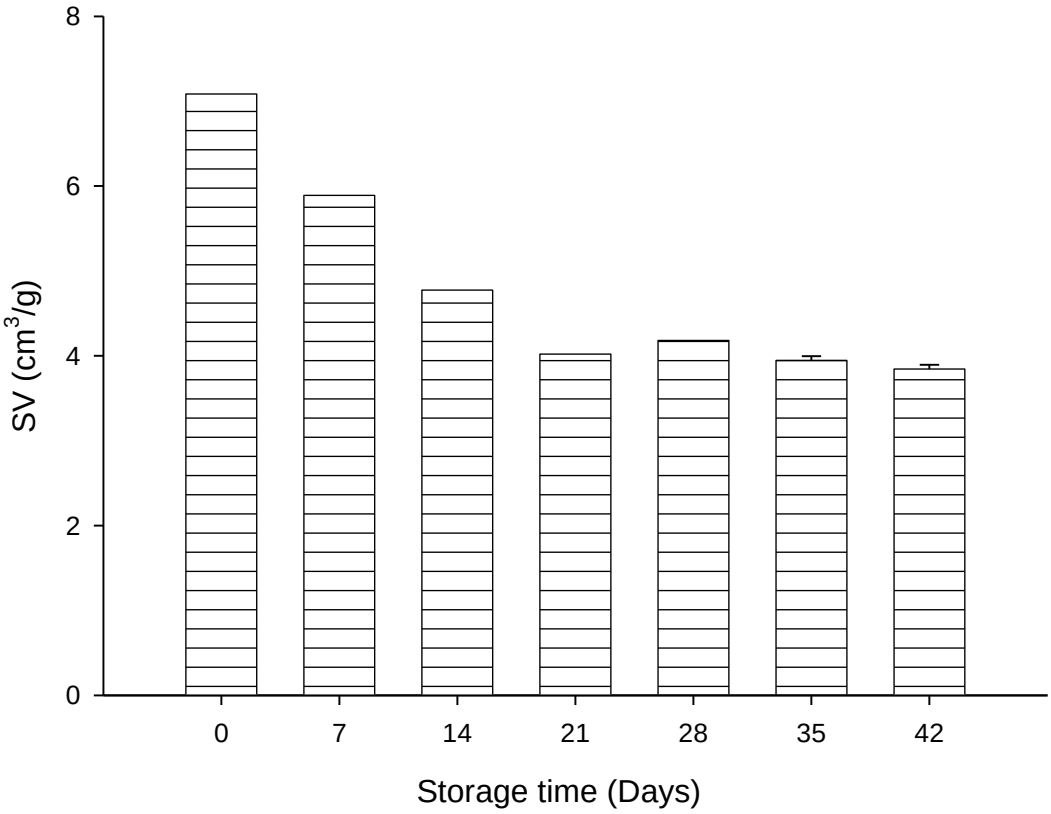

Figure 5. Effect of storage duration on the loaf volume of bread made from frozen french type bread dough. SV, specific volume. Bars indicate standard deviations.

the protein polymers and variations in the viscoelasticity of the frozen dough that occur during storage can result in variations in bread quality. Two research groups (El-Hady et al., 1996; Gianno and Tzia, 2007) have shown that the loaf volume is the main quality of bread affected by the liquid-glassy transition of water that occurs during the freezing process. The ANOVA showed that storage time had a significant ($p < 0.01$) effect on both of the bread quality parameters evaluated.

Specific volume (SV)

Bread volume made of frozen dough, is considered the main quality parameter that is affected. Figure 5 presents the effect of storage time on SV. Bread made with dough with 0 days of storage had the highest SV (7.10 cm^3/g) of the breads made from dough that had been stored for any of the durations we tested. The largest decrease in the SV (10.47%) occurred between 0 and 7 days of storage. The isolation of proteins led to an increase in the viscous behavior of frozen dough that had been stored for 7 days (0.28%, $\delta = 35.10°$). Because the gas pressure exceeded the rate of gas cell rupture in the weakened frozen dough, some of the gas produced during baking was released, thereby reducing the SV of the bread. This observation is consistent with Kenny et al. (1999) who suggested that the poor gas retention capacity of the frozen dough was associated with damage to the gluten network. The results are also consistent with Gabric et al. (2011). They observed an inverse relationship between the SV of bread made from frozen dough and the duration of dough storage, and they attributed the loss of SV to the phenomena of the coalescence and disproportionation (Kokelaar and Prins, 1995) of the gas cells in the dough. Those authors related the observed reduction in the SV of bread made from frozen dough with breaks in the structure of the gluten network and CO_2 diffusion within the frozen dough. Borneo and Khan (1999) identified relationship between the bread volume and the SPP and GLI protein fractions ($r=0.73$ and $r=-0.64$, respectively) found in fresh dough. Lu and Grant (1999) increased the content of the GS fraction in frozen dough and found that doing so had a positive effect on the bread volume.

The SV of the bread made from frozen dough in the present study decreased by 46.05% between 0 and 21 days of storage. During days 21 to 28 and 28 to 42 of storage, bread made from frozen dough had a SV decrease of 1.5%. Kenny et al. (1999) reported that rolls that were smaller in volume than the frozen dough itself exhibited more viscous behavior. This progressive loss in

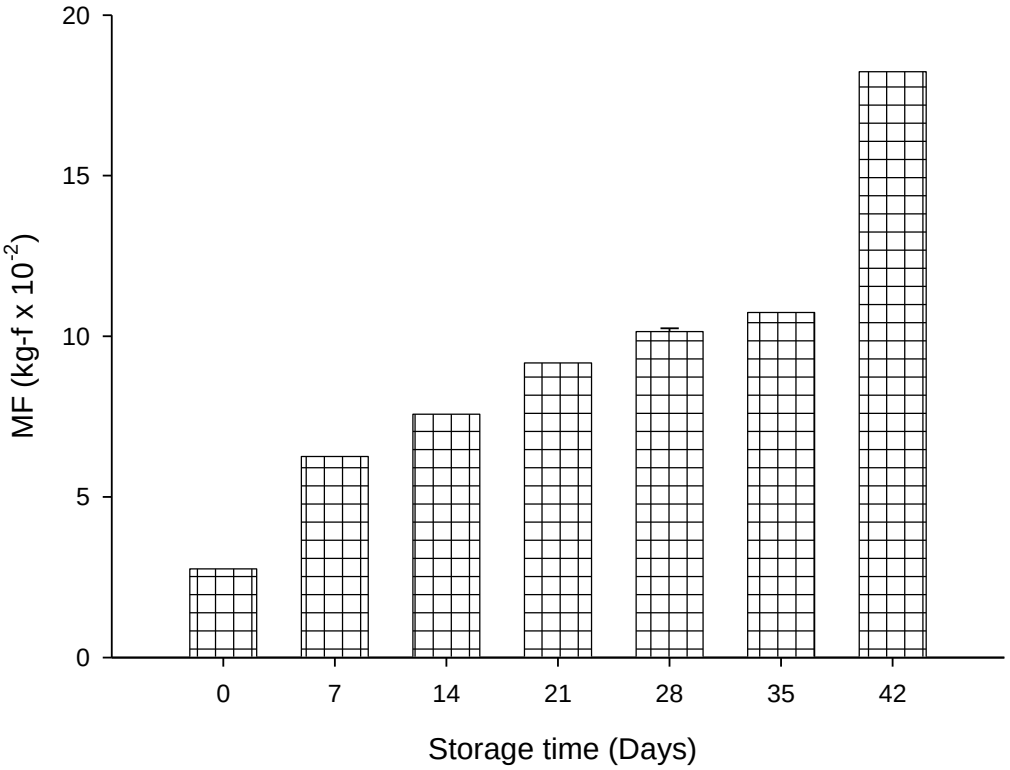

Figure 6. Effect of storage duration on the crumb firmness of bread made from frozen french type bread dough. MF, maximum firmness. Bars indicate standard deviations.

the SV of bread made from frozen dough is consistent with the observed protein degradation and weakening of the dough during storage. It also concurs with the observations of Matuda et al. (2008), who concluded that the compressive stress caused by the ice-water transition caused a reduction in both the number and size of the gas cells. Further, Ribotta et al. (2001) suggested that a reduction in gas retention efficiency in frozen bread dough was associated with the depolymerization of the gluten proteins. In the present study, SV losses associated with the storage of frozen dough appear to result from the increased viscous behavior of the dough caused by the degradation of proteins within it and the poor gas retention capacity of it.

Mezaize et al. (2010) observed a 24% reduction in the SV of bread made from frozen dough in comparison to bread made from fresh dough. In the present investigation, a lower average difference of 10.47% was observed and can be attributed to the formulation and flour that were used. We identified a significant correlation between the SV and RCO_2 ($r = 0.79$, $p < 0.05$). Reductions in the concentrations of certain protein polymers that occur during both the freezing and storage of frozen dough cause distinct structural reorganization of the native protein polymers. This reorganization contributes to the formation of less elastic gas cells, so the resulting bread is less likely to harbor large amounts of gas and therefore has a lower SV. These results are also consistent with Yi and Kerr (2009), who observed that the SV (specific volume) of bread tends to diminish with the duration of dough storage. However, in one study, a loaf in which the SV increased after storage at -18°C was observed, and the increase was thought to be associated with the decreased extensibility of the dough (which is thought to determine the ability of the dough to retain gas during fermentation (Sharadanant and Khan, 2003).

Firmness

The decrease in cell size of gas and its heterogeneous distribution in the crumb are parameters related to damage occurring during freezing of the dough, which is reflected in an increase in bread firmness. Figure 6 shows the effect of storage on the maximum crumb firmness (MF) of bread made from frozen dough. Bread made from dough with 0 days of storage had both the softest MF (2.68×10^{-2} kg-f) and the highest SV.

Moreover, the greatest increase in MF occur between 0 and 7 days of storage about a factor of two occurred, but this value is similar at MF of bread made with the formulation of fresh bread (2% yeast and 2% shortening, data not show) (Magaña-Barajas et al., 2011). The MF is inversely related to the SV. During the first 7 days of sto-

rage, structural changes occur in the gluten network of frozen dough and result in three-dimensional changes in it. The prevailing view is that these changes likely results in a reduction in the formation of gas cells that contributes to the observed increase in MF. Between 7 and 14 days of storage, there was an increase of 20% of the MF, the same occur during 14 and 21 days of storage. During 21 and 28 days of storage, there was an average MF increase of 10%. The next seven days the MF there were not a significative change. At 42 days of storage there was an average increase in MF about a factor of seven.

The MF was the baking quality parameter that was most affected by the storage of the frozen dough, and this observation is in accordance with the negative correlation between RCO_2 and MF ($r = -0.90$, $p < 0.01$). This correlation results from the low gas retention capacity of the frozen dough, which is also associated with the structural rearrangement of the proteins that contributed to the observed MF increases. In bread made from frozen dough, both the capacity to retain gas and the amount of retained gas decrease, thereby reducing the interstitial spaces within the matrix formed by gluten and other ingredients and ultimately resulting in a bread material that is denser than fresh bread. Therefore, compressing bread made from frozen dough requires more force than compressing fresh bread, and the measured MF increases. Some authors have also observed an increase in the MF that results from the storage of frozen bread dough (Ribotta et al., 2004; Phimolsiripol et al., 2008; Yi and Kerr, 2009). One group (Ribotta et al., 2004) suggested that the observed increase in bread firmness that was associated with storage resulted from a high degree of glutenin depolymerization that was also linked to the retrogradation of starch present in frozen dough.

The bread made from frozen dough that had been stored for more to 7 days had a lower SV and higher MF than bread made from fresh dough. These changes were associated with protein degradation, a decline in the fermentative capacity of the dough and increased viscous behavior during storage at -18°C, and the greatest differences among the dough were observed for these three measures.

Conclusions

The technique of SE-HPLC found that freezing and storing bread dough causes protein polymer restructuring which affects fermenting capacity (gas retention), viscoelasticity and consequently the baking quality of the bread. Stress caused by freezing the dough increased both the size and number of water crystals that formed during storage, which then weakened the network of gluten. The observed decay in the elastic behavior of the frozen dough is related to the breakdown of gluten polymers; this decay is indicated by the increase in protein solubility, particularly that of the soluble polymeric protein fraction. Damage to the structure of the gluten network in the frozen dough also lowered the capacity of the dough to retain gas produced during fermentation. Thus, freezing French type bread dough results in a lower loaf volume and increased crumb firmness.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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