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QUALITY CHARACTERISTICS OF BISCUIT PREPARED FROM WHEAT AND FLAXSEED FLOUR

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ABSTRACT

Biscuit was produced by substituting 25%, 50% and 75% of wheat flour with full fat (FFF) or defatted flaxseed flour (DFF). Proximate analysis, physical, sensory characteristics and evaluation of acid, peroxide value and antioxidant activity of biscuit produced from FFF and DFF during storage in multilayer flexible packages for 90 days were investigated. The proximate composition of biscuit samples was significantly affected by increasing substitution ratio. There was a significant increase in protein, fat, fiber content and calories of flaxseed biscuit compared with control. Hardness of FFF biscuit decreased with increase level of flaxseed flour and 75% FFF biscuit have the lowest value. Flaxseed biscuit had acceptable sensorial values and DFF at 25% substitution is the most acceptable with reference to all parameters. All flaxseed biscuit exhibited higher antioxidant activity than control. No significant change between biscuits fat with 25 and 50% DFF regarding peroxide and acidity values during storage.

KEYWORDS:
Flaxseed, Biscuit, Chemical analysis, Physical properties, Sensory evaluation, Storage.

INTRODUCTION

The consumer demand has increased for food product quality with taste, safety, convenience and nutrition. Thus, nutrition has emerged as an added dimension in food product chain development [1]. For the reason that competition in the market and increased demand for natural, healthy and functional products, attempts are being made to improve the biscuits nutritive value and functionality by modifying their nutritive composition. Increase biscuit's mineral content and protein for quality and availability often achieved by increasing the ratio of whole grain raw materials other than wheat or different types of dietary fibers [2] or to increase the dietary fiber content and improve prebiotic characteristics [3].

Biscuits are the most consumed bakery products due to its nature, good nutritional quality, affordable cost and availability in different varieties. Different nutritionally rich ingredients can be incorporated in most of bakery products for their diversity. Several health products have now become available [4].

Flaxseed (*Linum usitatissimum L.*) has been used as food and medicines in many countries. It has consumed by humans since the beginnings of the earliest civilizations. It was used for medical purposes, mainly to relieve abdominal pains and as an energy source in ancient Egypt and Greece. Moreover, it has been used in various forms such as seed, flour and oil. Flax seed and flaxseed oil is considered as healthy ingredients due to the presence of various bioactive compounds [5]. Flaxseed is used as a nutritional additive for preparation of baked products, ready to eat cereals and fiber bars which having good health impacts and it has been widely recognized in all parts of the world [6]. Flaxseed is considered as one of the key source of phytochemicals and using of flaxseed in food products will be beneficial to protecting against cancer and lowering cholesterol level, diabetes and heart disease [7].

Flaxseed oilcake, despite its high protein content, has not been used directly in human food. The amino acid profile of flaxseed protein in terms of essential amino acids is similar to that of soybean and is nutritionally more balanced than most other plant proteins [8].

Flaxseed oilcake has also found wide application in feed rations for other livestock. Whole and ground flaxseed has been used extensively in human food products. Furthermore, products include breakfast cereals, pancakes and waffles, as well as in dry mixtures for muffins, powdered drink mixes and other snack foods [8].

Bakery products can be supplemented with whole flaxseed grains to achieve an attractive and appealing form with enhancement in texture of final product. The grinding of flaxseed before its addition to food products can be more useful to obtain the potential health benefits from its active components such as dietary fiber and omega-3 fatty acids. Wheat flour can be substituted with ground flaxseed or whole flaxseed grains for pancake, muffins, bread and cookies production [9,10].

Therefore, the present study had undertaken to produce biscuit supplemented with full fat or

defatted whole meal flaxseed flour as a functional food, and evaluate their chemical composition, physical properties, sensory characteristics as well as effect of different storage periods at room temperature on antioxidant activity, acid and peroxide values of stored biscuit samples.

MATERIALS AND METHODS

Materials. Wheat flour (72% extraction rate), flaxseed and baking ingredients were obtained from local markets in Cairo, Egypt. Chemicals and reagents were analytical grade.

Preparation of flaxseed. The flaxseed was carefully cleaned and freed of extraneous matter. The flaxseed was dried in drying oven at 80±5°C for 3h. Dried flaxseed was milled by using a laboratory mill (IKA-Laboratechnic, Germany) to obtain flour (315 micron). Whole meal flour obtained from raw flaxseed was divided into two batches; first one was left as a full fat flour (FFF), and another one was partially defatted by cold solvent extraction using *n*-hexane. Samples was shacked for 30 min using horizontal shaker and left in dark for 24 h at room temperature. The mixture was filtered and the residue was re-extracted for another two days. The defatted flaxseed flour (DFF) or flaxseed cake were spread on aluminum trays and air dried to expel hexane residual, then full fat and defatted flour were kept at -20°C until further analysis.

Biscuit preparation. Biscuit was prepared according to the method illustrated by Whiteley [11] with minor modification by Omobuwajo [12]. Basic dough formula consisted of 100g wheat flour, full fat or defatted flaxseed flour (flaxseed flour added at level 25, 50 and 75% as partial substitution for wheat flour based on preliminary trials), 30 g fat (milk butter), 32 g sucrose, 2g full fat powdered milk, 0.50 g salt (NaCl), 0.40g sodium bicarbonate, 0.20g vanilla and various proportions of water to make required consistency of dough. The biscuits were baked at 170-180°C for 20 min. The biscuits were allowed to cool, subjected to organoleptical, physical and chemical evaluations, then the high accepted percentage (25% and 50% of DFF or FFF) was packaged in multilayer flexible packages and stored at room temperature for 90 days until further analysis.

Analysis of ingredients and biscuit samples. **Proximate analysis.** Moisture, protein, fat, crude fiber and ash contents of wheat, flaxseed flour and biscuit samples were determined according to the methods of AOAC [13]. Total carbohydrate was calculated by difference. Total calories of samples were calculated according to the formula of James [14] as follows:

Total calories = Fat x 9 + Protein x 4 + Total carbohydrate x 4

Determination of amino acids composition. Amino acids composition of defatted flaxseed flour was determined by using amino acid analyzer (Biochrom 30) according to the method outlined in AOAC [13]. An aliquot sample, were weighed and digested with 25 ml of 6N HCl at 110°C for 24 h. Then HCl was removed by evaporation; the remaining solid fraction was dissolved with 0.2N sodium citrate buffer (pH 2.2). One ml of the solution was filtered through 0.45 µm. The standard amino acids (consist of 17 amino acids) was treated as the same as of the samples. Amino acids were expressed as g/100 g protein on dry weight basis.

Fatty acids determination. Flaxseed oil was extracted by hexane and fatty acids were analyzed by gas chromatography (shimadzu GC-2010, Kyoto, Japan) and reported in relative area percentages. The methyl esters of fatty acids were prepared according to the method of AOAC [13]. The fatty acid methyl esters were identified by comparison their retention times with known fatty acid standard mixture. Peak areas were automatically computed by an integrator. The fatty acid composition was expressed as percentage of total fatty acids.

Physical properties of biscuit . Biscuit samples were evaluated for diameter (mm), thickness (mm) and spread ratio as described by Gaines [15]. Six biscuits edge-to-edge were used for the evaluation and the average was noted. Diameter and thickness were measured using a Vernier Caliper. Spread ratio was calculated by dividing diameter by thickness.

Measurement of biscuit hardness. The maximum force require to break the biscuit was measured as described by Gaines [15]. The hardness of three biscuits was measured by using textural analyzer a three point test on a Universal testing machine (Cometech, B type, Taiwan). The compression strength of biscuit samples was measured at a 20% level of compression with a cross-head speed of 100 mm/min and a flat ended probe (2.50 mm thickness). The hardness was recorded by Newton (N).

Sensory evaluation of biscuit. Sensory analysis of fresh produced biscuit samples was carried out by panelists from Food Technology Research Institute (10 persons; 6 female and 4 male) according to the method of Sudha et al. [16]. Six parameters were examined *i.e.*, appearance, color, odor, texture, taste and overall acceptability.

The panelists were provided with biscuits on a white plate.

Storage experiments. Biscuit samples (25 and 50% flaxseed substitution) were stored for zero, 30, 60 and 90 days at room temperature. Stored biscuits were examined for antioxidant activity, acid and peroxide value.

Antioxidant activity. The antioxidant activity of wheat flour, DFF, FFF and stored biscuit samples was determined based on the radical scavenging ability in reacting with a stable DPPH free radical according to Brand-Williams et al. [17] using methanol extract. Briefly, 2.40mg of DPPH in 100 ml methanol was prepared and 3.90 ml of this solution was added to 0.10 ml of sample extract. The mixture was shaked vigorously using tube shaker for a few second and allowed to stand at room temperature for 30 min in the dark. Then the absorbance was measured at 515nm. The radical scavenging percentage as DPPH was calculated using the following equation:

DPPH radical scavenging (%) = $[(A_0 - A_1/A_0)] \times 100$

A_0= Absorbance of the control reaction (containing all reagents except the test compounds).

A_1= Absorbance in the presence of the tested extracts after 30 min.

Determination of acid and peroxide value. One hundred grams of crushed biscuits were placed in a closed stopper flask, then 300ml of *n*-hexane was added, the flask was shacked for 30 min using horizontal shaker and left for 24 h at room temperature. The homogenized mixture was filtered with suction and the residue was re-extracted twice as mentioned above. The combined filtrates were evaporated under reduced pressure according to AOAC [13]

Acid value. Acid value was determined according to the method outlined in the AOAC [13]. In brief, 2.50g oil was dissolved in 25 ml of petroleum ether: ethanol mixture (1:1 v/v), then heated on a steam bath for two minutes. Two drops of phenol phthalein indicator were used, and then the solution was titrated with potassium hydroxide (0.1N) till pink color appeared.

Acid value (AV) was calculated as (mg KOH/g fat) using the following equation:

$$AV = \frac{V \times N \times 56.1}{\text{Sample weight (g).}}$$

Where: N=Normality of KOH. V=ml. of KOH.

Peroxide value. 5.0g oil was dissolved in 30 ml of glacial acetic acid: chloroform solution (3:2v/v). Then 0.50ml saturated potassium iodide was added with occasional shaking for one min, then 30ml of distilled water was added. The reaction mixture was titrated slowly by using 0.001N sodium thiosulphate ($Na_2S_2O_3$) with vigorous shaking until the yellow color faded. 0.50ml (1%, w/v) of starch indicator was added and the titration was continued until the blue color disappeared [13] and peroxide value (PV) was calculated as meq/kg fat using the following equation:

$$PV = \frac{S \times N \times 1000}{\text{Sample weight (g)}}$$

Where: S= ml of $Na_2S_2O_3$ (blank corrected) N= Normality of $Na_2S_2O_3$

Statistic analysis. For the analytical data, mean values and standard deviation are reported. The data obtained were subjected to one-way analysis of variance (ANOVA) at $P<0.05$.

RESULTS AND DISCUSSION

TABLE 1
Chemical composition of raw materials (on dwt basis)*

Samples	Protein (%)	Crude fiber (%)	Fat (%)	Ash (%)	Total carbohydrate** (%)	Total calorie (kcal/100g)	Antioxidant activity (%)
WF	11.50±0.50^c	0.35±0.02^c	1.09±0.05^c	0.62± 0.02^b	86.79±0.65^a	402.97±1.4^c	3.80±0.05^c
FFF	23.54±0.18^b	5.49±0.29^b	30.28±0.12^b	3.91±0.06^a	42.27±0.29^c	535.76±0.46^a	62.51±0.13^b
DFF	35.41±0.21^a	5.90±0.02^a	6.61±0.40^a	4.02±0.12^a	53.96±0.62^b	416.97±1.93^b	69.29±2.66^a

WF= Wheat flour FFF= Full fat flaxseed flour DFF= Defatted flaxseed flour
*dwt basis= dry weight basis.
**Total carbohydrate was calculated by difference.
Each value is expressed as mean ±standard deviation (n =3), number in the same column followed by the same letter are not significantly different at 0.05 level.

TABLE 2
Amino and fatty acids content of flaxseed

Amino aids	g/100g protein*	Fatty acids	% of total fatty acids
Isoleucine	3.13±0.02	**Palmatic acid**	6.18±0.38
Leucine	5.40±0.03	**Stearic acid**	4.79±0.68
Threonine	3.47±0.02	**Oleic acid**	21.20±2.66
Valine	4.01±0.02	**Linoleic acid**	14.73±1.19
Phenylalanine	4.94±0.03	**Linolenic acid**	51.50±2.92
Lysine	4.00±0.02	**Non identified fatty acids**	1.60±0.12
Methionine	1.52±0.01	**S.F.A****	10.97
Tyrosine	2.85±0.02	**UnSF.A*****	87.43
Total essential amino acids	29.32	**UnS.F.A/S.F.A ratio**	7.97
Alanine	4.21±0.02		
Aspartic	8.99±0.05		
Cysteine	2.20±0.01		
Glutamic acid	18.10±0.11		
Glycine	5.50±0.03		
Arginin	9.00±0.05		
Proline	3.50±0.02		
Serine	4.74±0.03		
Histidine	2.57±0.02		
Total non essential amino acids	58.81		

*Amino acids content in defatted flaxseed flour on dry weight basis.
**S.F.A= Saturated fatty acids.
***UnS.F.A= Unsaturated fatty acids.
Each value is expressed as mean ±standard deviation (n =3).

Proximate composition of full fat (FFF) and defatted flaxseed flour (DFF). Table (1) shows the chemical composition of full fat (FFF) and defatted flaxseed flour (DFF). The results indicated that, DFF had significantly higher content of protein, fiber and total carbohydrate (35.41, 5.90 and 53.96%) than FFF. There are no significant differences between the two types of flaxseed flour in ash content. While fat content and total calorie (30.28% and 535.76 kcal/100g) in FFF was higher than DFF. These results are in agreement with Khorshid et al. [18] who reported that FFF had 23.62% protein, 8.90% crude fiber, 3.61% ash and 36.89% fats. Also, Hassan *et al.* [19] found that FFF contains 23.60% protein, 4.10% ash and 559.29 kcal/100g. Öksüz et al. [20] indicated that flaxseed varieties contain 18.28-25.70% protein and 20.95-35.69% oil.

From results in the same Table, it could be observed that DPPH scavenging activity in DFF and FFF was 69.29 and 62.51%, respectively. Anwar and Przybylski [21] mentioned that the defatted flaxseed extracts showed significant capacity towards scavenging DPPH radicals (42.20-87.50%).

Table 2 shows essential and non essential amino acids content in defatted flaxseed flour (DFF). The data revealed that total essential amino acids in DFF were 29.32g/100g protein and total non essential amino acids in DFF were 58.81g/100g protein, respectively.

The same Table presents percentage of fatty acids of flaxseed oil. The results indicated that flaxseed oil had a high content of unsaturated fatty acids. Furthermore, it could be noticed that the main fatty acids in flaxseed oil were linolenic acid (51.50%), oleic acid (21.20%) and linoleic acid (14.73%), respectively.

The results are in the same trend with Bernacchia et al. [22] who analyzed the amino and fatty acids content in flaxseed.

Chemical composition of biscuit. The chemical composition of different biscuit samples substituted with DFF or FFF at levels 25, 50 and 75% are given in Table 3. It was observed that protein, fiber, fat and ash contents increased by increasing the substitution level of both types of flaxseed flour compared to control sample, this may be attributed to high protein, fiber and fat content in flaxseed. Furthermore, the results indicated that 75% DFF had the highest content of protein, fiber and ash (21.54, 3.85 and 1.82, respectively) relative to other biscuit samples. However, the highest fat content was observed in biscuit substituted with 75% FFF (26.53%).

TABLE 3
Chemical composition of biscuit (on dwt basis)*

Samples	Protein (%)	Crude fiber (%)	Fat (%)	Ash (%)	Total carbohydrate** (%)	Energy (kcal/100g)
Control	9.33±0.21^{g}	0.22±0.00^{g}	14.66±0.40^{e}	0.82±0.03^{g}	75.19 ±0.33^{a}	470.01±0.64^{e}
25% DFF	13.09±0.26^{e}	1.31 ±0.12^{e}	15.28±0.02^{de}	1.32±0.12^{e}	70.31± 0.31^{b}	471.12±0.42^{e}
50% DFF	17.17±0.55^{c}	2.64 ±0.15^{c}	16.22±0.94^{d}	1.53±0.04^{c}	65.08±1.37^{d}	474.98±4.69^{e}
75% DFF	21.54±0.14^{a}	3.85±0.15^{a}	17.72±0.12^{c}	1.82±0.01^{a}	58.92±0.25^{f}	481.33±0.48^{d}
25% FFF	12.1 ±0.08^{f}	0.99±0.01^{f}	18.76±0.38^{c}	1.23±0.05^{f}	67.91±0.45^{c}	488.85±1.79^{c}
50% FFF	15.62±0.1^{d}	2.18±0.1^{d}	22.40±0.10^{b}	1.47±0.04^{d}	60.53±0.16^{e}	506.11±0.42^{b}
75% FFF	18.36±0.29^{b}	3.28±0.07^{b}	26.53±1.57^{a}	1.72±0.02^{b}	53.39±1.81^{g}	525.79±7.67^{a}

Control= 100% Wheat flour FFF= Full fat flaxseed flour DFF= Defatted flaxseed flour
* dwt basis= dry weight basis.
**Total carbohydrate was calculated by difference
Each value is expressed as mean ±standard deviation (n =3), number in the same column followed by the same letter are not significantly different at 0.05 level.

From the results in Table 3, total carbohydrate content was significantly decreased in biscuits substituted with flaxseed compared to control biscuits (100% wheat flour). Moreover, 75% FFF biscuits had the lowest content of total carbohydrate (53.39%) compared with other biscuit samples.

Our work agrees with Khorshid et al. [18] and El-Demery et al. [10] who mentioned that as the level of substitution with flaxseed increased, all compounds increased except total carbohydrate. Ganorkar and Jain [23] revealed that protein, ash and fat content increased in flaxseed flour cookies than control. Hassan et al. [19] revealed that the substitution of wheat flour with 15% flaxseed meals resulted in a considerable improvement in protein, crude fiber and ash of biscuit samples.

Energy significantly increased by addition of flaxseed flour, the increase was higher in case of FFF biscuit which contain the highest fat content.

Physical properties of biscuit. Table (4) shows the physical properties of biscuit samples at different blends of FFF or DFF. The results indicated that diameter and thickness of biscuit (FFF and DFF) were slightly increased with increasing substitution percentage of flaxseed flour compared with control biscuit. 75% FFF revealed the maximum diameter and thickness (47.68 and 7.93mm). The results agree with work done by Hussain *et al.* [24] who found that diameter and thickness of flaxseed cookies showed gradually increase as the level of flaxseed flour substitution.

Moreover, the results of spread ratio of biscuit revealed a reduction in spread ratio from 6.58 to 6.13 mm and from 6.14 to 6.02 mm for DFF and FFF, respectively. It is clear that as the flaxseed level increased, spread ratio for different treated biscuits gradually decreased. These results are on the line with the findings of Ganorkar and Jain [23] who found that the reduction in spread ratio might be due to increase in dietary fiber and protein

TABLE 4
Physical properties of biscuit

Samples	Diameter (mm)	Thickness (mm)	Spread ratio
Control	45.20±0.55^{b}	6.85±0.30^{d}	6.60±0.02^{a}
25% DFF	47.40±0.26^{a}	7.20±0.13^{cd}	6.58±0.09^{a}
50% DFF	47.42±0.20^{a}	7.46±0.26^{bc}	6.36±0.12^{ab}
75% DFF	47.54±0.11^{a}	7.75±0.51^{ab}	6.13±0.41^{bc}
25% FFF	47.48±0.62^{a}	7.73±0.02^{ab}	6.14±0.07^{bc}
50% FFF	47.59±0.20^{a}	7.85±0.12^{ab}	6.06±0.07^{bc}
75% FFF	47.68±0.57^{a}	7.93±0.07^{a}	6.02±0.02^{c}

Control=100% Wheat flour FFF= Full fat flaxseed flour DFF= Defatted flaxseed flour
Each value is expressed as mean ±standard deviation (n =3), number in the same column followed by the same letter are not significantly different at 0.05 level.

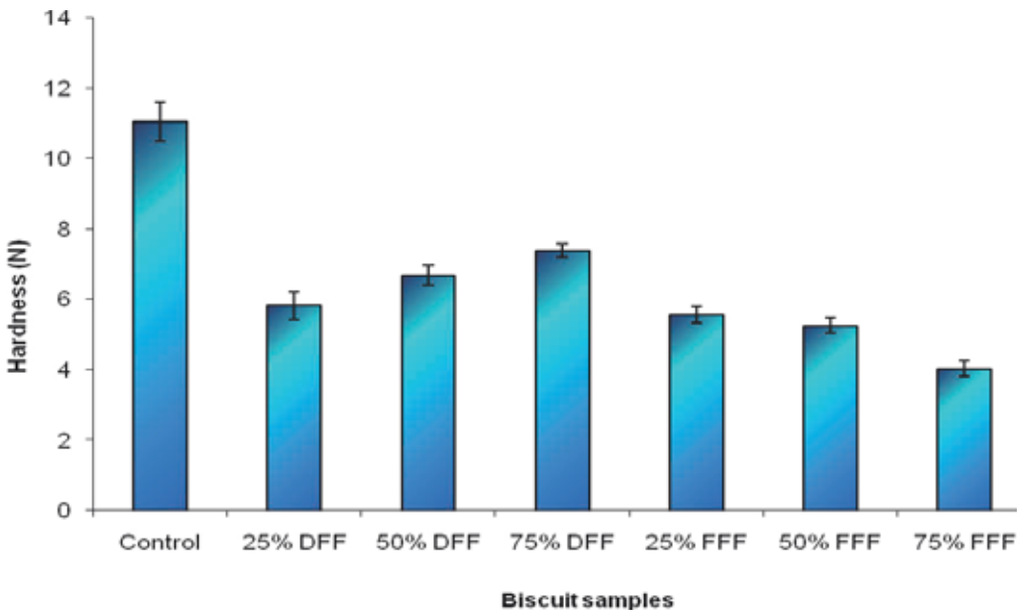

FIGURE 1
Hardness of biscuit

Control= 100% Wheat flour; FFF= Full fat flaxseed flour and DFF= Defatted flaxseed flour, N=Newton
Each value is expressed as mean ±standard deviation (n =3).

percentage with increasing level of flaxseed flour because dietary fiber and protein has more water binding power. Also, when more water is present, more sugar is dissolved during dough mixing [23].

Textural attribute of biscuit. An objective and instrumental method using texture analyzer was used to test the texture of biscuit samples. Hardness results were illustrated in Fig 1. Flaxseed biscuit samples were found to be softer or less hard than that of control biscuit, particularly, in case of whole flax seeds flour substitution. Moreover, hardness of DFF biscuits was increased with increasing flaxseed percentages. In contrast, hardness of FFF biscuits was decreased.

Our results in same line with those obtained by Ganorkar and Jain [23] who reported that the textural parameters were found to decrease with increasing full fat roasted flaxseed flour incorporation. Since flaxseed flour has gum, mucilage (fiber) and protein, high water absorbing capacity components as well as there was a significant level of fat (around 41%) found in flaxseed flour, and both of these factors contributed in sticky dough which reducing dough extensibility. The extensible and cohesive structure is contributed to sugar or water interaction with wheat protein which forming gluten network. With an increase in fat content the flour gets coated and this network gets interrupted thus the properties of cookies are changed and are less hard. At very high fat content the lubricating function is high and has required less water and a soft texture is obtained. Hence the hardness gradually decreased forming softer cookies with an increased level of flaxseed flour [25].

TABLE 5
Sensory evaluation of biscuit

Samples	Appearance (10)	Color (10)	Odor (10)	Texture (10)	Taste (10)	Overall acceptability (50)
Control	9.75±0.42^{a}	9.50±0.55^{a}	9.83±0.41^{a}	9.83±0.40^{a}	9.83±0.40^{a}	48.91±0.66^{a}
25% DFF	9.66±0.52^{a}	9.66±0.82^{a}	9.66±0.52^{a}	9.50±0.83^{a}	9.50±0.54^{ab}	47.83±2.60^{a}
50% DFF	8.66±0.82^{b}	9.00±0.10^{ab}	8.66±0.52^{abc}	8.16±0.98^{b}	8.50±0.54^{bc}	43.00±1.41^{b}
75% DFF	8.33±1.03^{bc}	8.50±0.55^{b}	8.00±1.09^{bc}	8.00±0.80^{b}	6.83±0.40^{d}	39.66±2.25^{cd}
25% FFF	7.66±1.03^{bc}	7.50±1.05^{c}	8.83±0.75^{ab}	8.66±0.51^{b}	9.00±0.6^{ab}	41.66±2.16^{bc}
50% FFF	7.83±0.75^{cd}	7.66±0.82^{c}	8.00±1.89^{bc}	8.16±0.75^{b}	7.66±1.50^{cd}	39.33±3.80^{cd}
75% FFF	6.83±1.33^{d}	7.00±0.89^{c}	7.50±1.87^{c}	8.16±0.75^{b}	7.33±1.96^{d}	37.00±4.19^{d}

Control = 100% Wheat flour FFF= Full fat flaxseed flour DFF= Defatted flaxseed flour
Each value is expressed as mean ±standard deviation (n =10), number in the same column followed by the same letter are not significantly different at 0.05 level.

Organoleptic characteristics. Sensory characteristics of flaxseed substitution at different levels; appearance, color, odor, taste, texture and overall acceptability of produced biscuit was studied (Table 5). The results showed that as the flaxseed ratio increased, all of sensory attributes scores decreased and the color of biscuits become darker compared with control. Furthermore, biscuits containing 25% defatted flaxseed flour showed maximum sensory scores compared to other samples and non-significantly difference with control biscuit. However, above 50% substitution, the product becomes less acceptable to the consumer.

The results were similar with the results obtained by Moraes et al. [26] who found that acceptance of flaxseed, as a dietary ingredient of functional food in cakes, revealed consumer acceptance up to 30% supplementation level.

Non significant difference was observed between different supplementation with flaxseed in textural scores, except for biscuit prepared from 25% DFF. Texture is an important factor of comparing the biscuit as it greatly affects consumer acceptance of the product [27].

Masoodi and Bashir [28] found that the color of the fortified biscuits attained more dark color as the flaxseed supplementation was increased. However, the texture was slightly decreased with supplementation but described no undesirable change.

The overall quality of the biscuits was significantly reduced by addition of flaxseed flour compared with wheat biscuit except for 25% DFF which insignificantly decreased.

The reasoning behind this could be attributed to intensified flaxseed nutty flavor, un-pleasure after taste, dark brownish color, rough surface, less crisp and gritty mouth feel, making them to score low in sensory evaluation [27]. Similarly, organoleptically acceptable cookies can be prepared by supplementing 20% flax in foods as an ingredient [24]. Furthermore, it was observed that biscuits containing 10 and 15% of flaxseed meals recorded higher scores in most sensory characteristics compared to the control sample [29]. Also, flaxseed can be used to improve the nutritive value of bakery products as well as for improving sensory properties [29].

Antioxidant activity of biscuit at different storage periods. The antioxidant activity as DPPH of methanolic extracts of biscuit samples at different storage periods is shown in Table 6. Data indicated that DPPH radical scavenging (%) at zero time in DFF and FFF biscuit samples (25 and 50% substitution) ranged from 15.79 to 23.26% and 11.16 to 19.76%, respectively. Substitution with both types of flaxseeds flour significantly increased their antioxidant activity in comparison with control (4.00%) and antioxidant activity values increased with increasing substitution levels of flaxseed flour. Our results are in the same trend with Masoodi and Bashir [28] who found that antioxidant activity was linearly increased as the flaxseed substitution was increased.

Moreover, antioxidant activity of biscuits after different storage periods has changed. After storage (from 30-90 days), DPPH radical scavenging was significantly decreased from 12.19 to 9.96% and from 18.32 to 12.25% for 25 and 50% substitution of DFF and from 9.24 to 5.14% and from 16.10 to 10.39% for 25 and 50% substitution of FFF in biscuit samples, respectively.

Barthet et al. [30] indicated that the flaxseed antioxidant activities were mainly due probably to more than one group of components of the flax meal and may be involved to provide the seed with its effective and unique antioxidant properties.

Peroxide value (PV) and acid value (AV) of fat extracted from biscuit samples. The changes in fat quality parameters, *i.e.* peroxide value (PV) and acid value (AV) of the flaxseed biscuits were followed throughout the storage period of 3 months at room temperature and the results obtained in Table (7). From Table 6, it could be noticed that PV and AV increased gradually up to the end of the storage time in all samples. PV of lipids extracted from the control sample after baking (at zero time)

TABLE 6
Antioxidant activity of biscuits at different storage periods

Samples	Antioxidant activity (%)			
	Zero time	30 days	60 days	90 days
Control	4.00±0.03^{e}	3.89±0.03^{e}	3.82±0.02^{e}	3.70±0.05^{e}
25% DFF	15.79±0.09^{c}	12.19±0.11^{c}	11.48±0.38^{c}	9.96±0.11^{c}
50% DFF	23.26±2.02^{a}	18.32±0.09^{a}	16.34±0.63^{a}	12.25±0.10^{a}
25% FFF	11.16±0.15^{d}	9.24±0.20^{d}	7.31±0.09^{d}	5.14±0.09^{d}
50% FFF	19.76±0.09^{b}	16.10±0.10^{b}	12.43±0.36^{b}	10.39±0.11^{b}

Control= 100% Wheat flour FFF= Full fat flaxseed flour DFF= Defatted flaxseed flour
Each value is expressed as mean ±standard deviation (n =3), number in the same column followed by the same letter are not significantly different at 0.05 level.

TABLE 7
Peroxide value (PV) and acid value (AV) of biscuits fat at different storage periods

Samples	Storage periods (days)							
	AV (mg KOH/g fat)				PV (meq/kg fat)			
	Zero time	30 days	60 days	90 days	Zero time	30 days	60 days	90 days
Control	0.18±0.01^{a}	0.35±0.01^{bc}	0.53±0.01^{bc}	0.63±0.01^{c}	0.21±0.01^{a}	2.70±0.20^{b}	4.50±0.08^{c}	5.62±0.18^{c}
25% DFF	0.18±0.01^{a}	0.33±0.01^{bc}	0.48±0.08^{bc}	0.62±0.01^{c}	0.20±0.03^{a}	2.62±0.14^{b}	4.38±0.09^{c}	5.55±0.16^{c}
50% DFF	0.17±0.01^{a}	0.30±0.01^{c}	0.46±0.01^{c}	0.60±0.03^{c}	0.20±0.01^{a}	2.58±0.17^{b}	4.22±0.10^{c}	5.40±0.11^{c}
25% FFF	0.22±0.02^{a}	0.42±0.01^{b}	0.62±0.08^{b}	0.77±0.01^{b}	0.26±0.04^{a}	3.00±0.16^{b}	5.60±0.02^{b}	6.18±0.08^{b}
50% FFF	0.24±0.05^{a}	0.70±0.08^{a}	0.94±0.02^{a}	1.30±0.04^{a}	0.27±0.02^{a}	3.94±0.20^{a}	7.20±0.12^{a}	10.50±0.03^{a}

Control= 100% Wheat flour FFF= Full fat flaxseed flour DFF= Defatted flaxseed flour
Each value is expressed as mean ±standard deviation (n =3), number in the same column followed by the same letter are not significantly different at 0.05 level.

was 0.21 meq/kg fat which did not differ considerably from those of other biscuit samples, which ranged between 0.20 and 0.27 meq/kg fat. The increase in PV was observed in all the biscuit samples; however biscuits produced by 50% whole flaxseed which had the highest value (10.50 meq/kg fat) after 90 days, and the PV of the other biscuit samples ranged from 5.40 to 6.18 meq/kg fat. The least stable macro components in food are the lipids and biscuits become unacceptable and are rejected by the panelists as a result of rancidity development after storage [31]. Jeyasanta et al. [32] reported that peroxide values in biscuit fat were within the acceptable limit of 10-20 meq/kg of fat throughout the storage period.

At the same time, an increase in AV value was observed in all biscuit samples after storage. The increase was considerably higher in biscuits prepared with 50% whole flaxseed. AV of lipids extracted from the control biscuit (at zero time) was 0.18 mg KOH/g fat which did not differ considerably from those of other variations, which ranged between 0.18 and 0.24 mg KOH/g fat. Also, control and biscuit samples with 25 and 50% DFF showed no significant change in peroxide and acidity values of extracted fat after storage of biscuits samples up to 90 days and this may be because of the low fat content in these samples and high antioxidant activity compared to other samples. Rajiv et al. [25] found a marginal decrease in PV and acidity of cookies with 15% roasted and ground flaxseed was observed when stored up to 90 days. Moreover, Vadukapuram et al. [33] mentioned that peroxide values of extruded bean snack increased with increased flaxseed levels and over a storage period.

CONCLUSION

Based on our results, it could be concluded that flaxseed flour can be incorporated in biscuit as a partial substitution up to 50 % of wheat flour without negatively affecting physical and sensory qualities. Substitution with DFF at 25% recorded the highest score in all sensory attributes relative to other flaxseed biscuit samples. Biscuit made from 25 and 50% DFF can be stored up to 90 days without any significant change with respect to acidity and peroxide value of extracted fat compared to control. Flaxseed appears to have a potential role as an extender in bakery products as well as a functional food.

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PHYTOHORMONES INFLUENCES THE MICROPROPAGATION OF *STEVIA REBAUDIANA* BERTONI THROUGH CALLOGENESIS

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ABSTRACT

In this study the effect of different Plant Growth Regulators (PGRs) on efficient regeneration of *Stevia (S.) rebaudiana* (Bert.) from the leaf and nodal explants has been investigated. Suitable leaf and nodal sections were inoculated onto Murashige and Skoog (MS) medium for callus induction. Maximum callus induction (80%) was observed when leaf portions were treated with 2.5 mg L-1 of 2, 4-dichlorophenoxyacetic acid (2, 4-D). However, nodal explants showed lower callogenic potential (46.6%) when exposed to 1.5 mg L-1 of 2, 4-D. Viable callus was sub-cultured into fresh medium for shoot organogenesis. Maximum shooting response (45%) with 6.76 shoot buds and 3.34 cm mean shoot length was observed on MS-medium supplemented with 2.0 mg L-1 of 6-Benzyl amino-purine (BAP). Maximum number of shoots per explant (8.60) and mean shoot length (4.45 cm) was recorded on an MS-medium augmented with a combination of BAP (1.5 mg L^{-1}) and Kinetin (Kn; 0.25 mg L^{-1}). Suitable micro-shoots were excised from shoot regeneration medium and transferred to a new medium for root induction. Higher root induction response (>98%) with 30.47 number of roots and 2.66 cm mean root length was observed when the MS-medium was augmented with 2.0 mg L-1 of Indole butyric acid (IBA). In this study efficient regeneration system was optimized for healthy biomass production of *Stevia rebaudiana.* Such *in vitro* systems should be exploited for consistent plantlets production for commercial applications.

KEYWORDS:
Stevia rebaudiana Bert. PGRs; 2, 4-D; callus; BAP; shoots; IBA

INTRODUCTION

S. rebaudiana (Bert.) is a medicinal and more recently an economical important plant in family *Asteraceae [1].* The medicinal and economical importance of this specie is due to the presence of sweet major Steviol Glycosides in the leaves that is 300 times sweeter than commercially available sugar [2]. Major developing countries approved steviosides extracts of this species as additive for different food-stuffs. Steviosides play an important role in controlling obesity and diabetes. Commercial sugar obtained from sugar cane and sugar beets enter into the blood stream due to the presence of receptors in the small intestine. But the receptors for absorption of Steviosides are missing and hence cannot enter into the blood stream which helps in controlling obesity and diabetes [3]. Stevia extracts have no reported side effects and can be used as an alternative to sugar and other synthetic sweeteners [4]. This species is indigenous to Brazil and Paraguay and recently replaced cane and beet sugar in major developing countries due medicinal efficacy [5]. A recent studies showed that the extract from *in vitro* and *in vivo* grown tissues and organs of *S. rebaudiana* have the ability to detoxify free radicals, inhibit the growth of toxic metabolites producing strains and containing larvicidal activities against *Anopheles stepensi [6-10].* Stevia has gain attention as an alternative for low-carbohydrate, low-sugar food with the rise in demand for it and is very attractive as a nature controlled diets [11]. In 1899, The Swiss botanist Moises Santiago Bertoni first described the plant and the sweet taste in detail. But only limited research was conducted on the topic, until 1931, when two French chemists isolated the glycosides that give stevia, its sweet taste. These compounds were named stevioside and rebaudioside, which were 300 times sweeter than sucrose, heat stable, pH stable, and non-fermentable [12]. Stevia can be used as medicinal tea for treating heartburn and other ailments [13]. More recent medical research has shown promise in treating high blood pressure, and hypertension [14]. Stevia has a negligible effect on blood glucose, even enhancing glucose tolerance. Therefore, it is attractive as a natural sweetener on carbohydrate-controlled diets [15]. Stevia improves insulin sensitivity in rats [16] and may even promote additional insulin production [17] helping to reverse diabetes and metabolic

syndrome. Millions of Japanese have been using Stevia for over thirty years with no harmful effects reported. Similarly, Stevia leaves have been used for centuries in South America as an ethno medical tradition treatment of type II diabetes [18]. *Stevia rebaudiana Bertoni* that conventionally propagated by seeds, cuttings or clump division which has a limitation of quality and quantity seed material. Seed germination of Stevia is often poor [19]. Therefore; there are basically two options for multiplication, the tissue culture and stem cutting. But in Pakistan lack of mass availability for propagation through cutting is prime limitation. So keeping in view the importance of Stevia plant and the limitation of propagating through seed and cutting the current research studies are design to propagate Stevia plant through callus culture technique.

MATERIALS AND METHODS

The research work was conducted at Pakistan Council of Scientific and Industrial Research (PCSIR) laboratories Peshawar.

Treatments for micropropagation. The following set of treatments was applied for micropropagation of *S. rebaudiana.* Callus induction (table-1), shoots induction from callus cultures (table-2), shoots elongation and

TABLE 1
Treatments used for callus induction from leaf and nodal explants

Callus induction		
Treatments	**MS + PGRs**	**Explants**
T1	MS0	Leaf+ nodal
T2	2, 4-D (0.5 mgL^{-1})	Leaf+ nodal
T3	2, 4-D (1.0 mgL^{-1})	Leaf+ nodal
T4	2, 4-D (1.5 mgL^{-1})	Leaf+ nodal
T5	2, 4-D (2.0 mgL^{-1})	Leaf+ nodal
T6	2, 4-D (2.5 mgL^{-1})	Leaf+ nodal

TABLE 2
Treatments used for shoots regeneration from callus cultures

Shoots induction		
Treatments	**MS + PGRs**	**Callus**
T1	MS0	Leaf+ nodal
T2	BAP (0.5 mg L^{-1})	Leaf+ nodal
T3	BAP (1.0 mg L^{-1})	Leaf+ nodal
T4	BAP (1.5 mg L^{-1})	Leaf+ nodal
T5	BAP (2.0 mg L^{-1})	Leaf+ nodal
T6	BAP (2.5 mg L^{-1})	Leaf+ nodal

TABLE 3
Treatments used for shoots multiplication and elongation

Shoots multiplication and elongation		
Treatments	**MS + PGRs**	**shoots**
T1	MS0	Leaf+ nodal
T2	BAP (0.5 mg L^{-1}) + Kn(0.25 mg L^{-1})	Leaf+ nodal
T3	BAP (1.0 mg L^{-1}) + Kn(0.25 mg L^{-1})	Leaf+ nodal
T4	BAP (1.5 mg L^{-1}) + Kn(0.25 mg L^{-1})	Leaf+ nodal
T5	BAP (2.0 mg L^{-1}) + Kn(0.25 mg L^{-1})	Leaf+ nodal
T6	BAP (2.5 mg L^{-1}) + Kn(0.25 mg L^{-1})	Leaf+ nodal

TABLE 4
Treatments used for roots induction from excised in vitro shoots

	roots induction	
Treatments	**MS + PGRs**	***In vitro* shoots**
T1	MS0	Leaf+ nodal
T2	IBA (0.5 mg L^{-1})	Leaf+ nodal
T3	IBA (1.0 mg L^{-1})	Leaf+ nodal
T4	IBA (1.5 mg L^{-1})	Leaf+ nodal
T5	IBA (2.0 mg L^{-1})	Leaf+ nodal
T6	IBA (2.5 mg L^{-1})	Leaf+ nodal

multiplication (table-3) and roots induction (table-4).

Micropropagation. Callus induction response was studied at different concentration of 2, 4 - D . The data was recorded aft er 4 to 5 weeks of explants culture (leaf; 0.7 cm; nodal; 1.0 c m) . Shoot induction response was studied at different concentration of BAP when added to MS medium. The data was noted at 6 weeks after inoculation of callus cultures. Shoot proliferation response was observed while using various concentration of BAP in combination with Kn. The data was collected after 4 weeks of inoculation. Rooting response was studied in MS medium containing different concentration of IBA, and data was recorded after 5 weeks interval.

Culture Medium. Media used in plant tissue culture contained nutritional components which are essential for the growth and development of the cultured tissues. The most frequently used medium for callus and cell culture, plant regeneration and micro-propagation contained basic salts of Murashige and Skoog [22] media was used. For the preparation of one liter MS medium the following volumes of stock solution were used: 100 ml of macronutrients, 10 ml of micronutrients, 10 ml of vitamins, 10 ml of Iron EDTA, Growth regulators were added according to the requirement, 30 g of sucrose was dissolved in distilled water. Final volume was made up-to one liter by adding distilled water after mixing well all the ingredients of the medium, pH was adjusted to 5.8 using 1N NaOH and 1N HCl solutions. Agar (0.8%) was added to the medium for solidification. The mixing of agar in the medium was done by warming and continuous stirring. Then 10 ml per test tube and 50 m1 per flask were poured. The mouths of glassware were closed with cotton plugs and covered with tin foils and autoclaved at 12 1°C and 15 psi pressure for 15 minutes. After autoclaving, culture glassware were cooled under room temperature and stored at 25°C till use.

Explants sterilization and inoculation. Leaves of about 0.5-0.7cm and un-sprouted node of about 0.5 to 1cm were collected from two to three years old well growing open field plants, of PCSIR nursery. Unwanted portion was removed from the explants with the help of sterile blades. Explants were exposed to different sterilization reagents for surface sterilization by using the methods of Ahmad et al. [20-2 1]. For inoculation hands were washed with soap and then ethanol was scrubbed on hands and finally dried inside laminar air flow cabinet. Inoculation was done in sterile environment inside laminar air flow cabinet. The mouths of flasks were uncovered over the flame and then heated by holding in angled position. One explant was inoculated alone in the culture flask with sterile forceps. Care was taken to avoid forceps touching with MS medium and also not to dip the whole explant in the medium. Then the flask mouth was covered with cotton plugs and tin foil. The same procedure was repeated for all the flasks. Forceps and scalpels were dipped in ethanol and then flamed and cooled and then used. After inoculation all the cultured flasks were transferred to growth chamber.

Growth conditions. The cultures inoculated with explant were grown under carefully regulated temperature and light conditions. All the culture were grown under eight hours dark and sixteen hours photoperiod in incubator. The light intensity was regulated at 2000 Lux. The incubator temperature was adjusted at 25± 2 0C.

Statistical analysis. The experiment was designed on Completely Randomized Design (CRD). For callus induction six treatments was used and two explants was cultured in culture flasks. For shoot induction and multiplication 12 treatments were applied and each flask containing single callus or shoot for regeneration. Six treatments were used for root induction. Each treatment had 3 replications with 5 units per treatment. There were total 450 experimental units or test tubes used during the course of the whole study.

RESULTS AND DISCUSSION

Effect of 2, 4-D on callus induction from leaf

TABLE 5
Effect of 2, 4-D at different concentration on callus induction from leafand nodal explants of Stevia rebaudiana Bertoni.

Treatment (m g L^{-1})	Callus induction from Leaf explants		Callus induction from nodal explants	
2, 4-D	Callus initiation	Percent response	Callus initiation	Percent response
Basal (MS)	0.00 d	0.00	0.00 d	00.00
0.5	1.67c	33.33	0.67 cd	13.33
1.0	2.33 bc	46.67	1.33 bc	26.67
1.5	3.00 ab	60.00	2.33 a	46.60
2.0	3.33 ab	66.67	2.00 ab	40.00
2.5	4.00 a	80.00	0.67 cd	13.13
LSD	**1.257**		**0.834**	

Means followed by different letter are significantly different at 5% level of significance

explants. A medium without PGRs (MS0) was not found inductive for callus formation. Callus induction from leaf explant was varied significantly with addition of 2, 4-D in basal MS media. It was found that MS + 2.5 mg L-1 2, 4-D resulted in maximum callus from leaf explants (80%) and the poorest response (33.33%) was recorded at MS + 0.5 mg L^{-1}. The percent response of callus induction from leaf explant of *Stevia rebaudiana* Bertoni also indicated explant compatibility with 2, 4-D concentration (Table 5). Initially at low concentrations less callus formation was observed, but as 2, 4-D concentration was increased the callus induction response also increased. When 2.5 mg L-1 of 2, 4-D was added to the basal MS medium, the maximum callus response (80%) was recorded. The results are in line with the findings of Uddin et al. [23] who investigated the effect of different concentrations of 2, 4-D on callus induction from leaf explants and recorded the maximum amount of callus on MS medium containing 3.0 mg L-1 of 2, 4-D. Thomas and Maseena [24] also investigated the effect of different concentrations of 2, 4-D and reported that 2, 4-D as an effective auxins for callus induction from leaf and nodal segments of *Cardiospermum halicacabum* L.

Effect of 2, 4-D on callus induction from nodal explants. No response was observed in case of both leaf as well as nodal explants on MS0. Basal (MS) media did not initiate callus from nodal explants however with addition of 2, 4-D various responses were observed at various concentrations. The highest callus response (46.60%) from nodal explant was observed at MS + 1.5 mg L-1 concentration of 2,4-D significantly followed by callus induction (40%) at MS + 2.0 mg L-1 2, 4-D, while the poorest response (13.33%) was recorded at MS + 2.5 mg L-1 2, 4-D. Here also compatibility was observed with 2, 4-D concentration and callus induction from nodal explant of *Stevia rebaudiana* Bertoni in percent response (Table 5). Increase in concentration of 2,4-D, callus induction was increased first up to MS + 1.5 mg L-1 at which maximum callus induction was recorded and then with further increase in its concentration callus induction response declined which indicated that auxin (2,4-D) became toxic to the tissues of nodal explant. The results were also supported by the findings of Gopi and Vatsala [25] who noted that 2, 4-D was an effective for callus induction in *Gymnema sylvestre* and also with those of Uddin et al. [23] who reported that 2, 4-D as an efficient auxins for callogenesis from nodal segments in *Stevia rebaudiana* Bertoni. Thomas and Maseena [24] also investigated the effect of different concentrations of 2, 4-D and reported addition of 2, 4-D as an effective auxins for callus induction from leaf and nodal segments of *Cardiospermum halicacabum* L.

Effect of BAP on shoots regeneration from callus cultures. MS0 media did not induce shoot initiation but the explants remained green for few weeks. By contrast supplementation of BAP to basal (MS) medium initiated shoot induction and at various concentration various responses were observed. It was observed that MS + 2.0 mg L-1 BAP gave the maximum shoot regeneration response (45%) from callus cultures and the poorest response (21.20%) was recorded at MS + 0.5 mg L-1 BAP. The percent shoot induction also indicated explant compatibility to cytokinin concentration (Table 6). Linear increase in shoot induction was observed with the increase in concentration of BAP. Shoot induction was increased first up to MS + 2.0 mg L-1 BAP at which maximum shoots induction was observed and with further increase decline in shoot induction was observed. The difference in shoot induction might be due to endogenous cytokinin in the explants and culture conditions, as cytokinin promotes chloroplast development, chloroplast synthesis, increases cell expansion in leaves and in dicots, increase nutrient sink activity, promotes cell

TABLE 6
Effect of BAP at different concentrations on shoots induction and average shoot length from callus explants

Treatment (mg L^{-1})	Shoots induction from callus		Average shoots length from	
BAP	Shoot buds per explant	Percent shoot response	Average shoot length	Percent response
Basal MS	0.00 f	00.00	00.00 e	00.00
0.5	3.18 e	21.20	2. 10 cd	14.02
1.0	4.34 d	28.90	2.30 bc	15.31
1.5	5.51 b	36.70	2.41 b	16.04
2.0	6.76 a	45.00	3.34 a	22.24
2.5	5.13 c	34.20	2.00 d	13.33
LSD	**0.177**		**0.253**	

Means followed by different letter are significantly different at 5% level of significance

TABLE 7
Effect of BAP + Kn in combination at different concentrations on shoots induction and shoot length from shoot tip explants.

Treatment(mg L^{-1}) BAP + Kn	shoots induction from shoot tip explants		shoots length from shoot tip explants	
	Shoot buds per explant	Percent shoot induction	Average shoot length	percent response
Basal (MS)	0.00 d	00.00	0.00 e	00.00
0.5+0.25	3.07 c	20.44	2.29 d	15.29
1.0+0.25	5.13 b	34.22	3.37 b	22.28
1.5+0.25	8.60 a	57.33	4.45 a	29.64
2. 0+0 .25	3.27 c	21.78	3.33 b	22.18
2.5+0.25	3.13 c	20.89	3.14 c	20.93
LSD	**0.550**		**0.099**	

Means followed by different letter are significantly different at 5% level of significance

division and organ development [26]. The results are in line with the findings of Rajore and Batra [27] who investigated the effect of different concentrations of BAP alone and in combination with other phytoharmones for efficient regeneration of *Jatropha curcas* and recorded the maximum shoot buds in alone BAP at 2 mg L^{-1}.

Effect of BAP on shoot elongation in shoot induced from callus cultures. Alone addition of BAP was found the source of variation in average length of shoot induced from callus because no response was observed from MS0 media. The data testify that at MS + 2.0 mg L-1 BAP the maximum shoot length (3.34 cm) from callus explant was observed however with further increase the poorest response (2.00 cm) was noticed at MS + 2.5 mg L-1 BAP. The percent response for average shoot length also indicated explant compatibility to cytokinin concentration (Table 6). As the concentration of BAP was increased up to MS + 2.0 mg L-1 BAP shoot length was also increased but with further increase in its concentration shoot length response was decreased which indicated that cytokinin (BAP) became toxic to the tissues of explant which resulted in less response in term of both shoot induction as well as average shoot length. These results are supported by Aamir et al. [28] who investigated the effect of BAP on shoot regeneration and average shoot length. They observed the maximum number of shoots i.e., 8.6 shoots per culture with average shoot length of 6.0 cm after 20 days of inoculation, on MS basal medium having 1.0 mg L-1 of BAP.

Effect of BAP + Kn on shoot prolife ration. Combination of both BAP + Kn in basal (MS) media showed significant effects on shoot proliferation. The maximum numbers of shoots (8.60) from micro shoot were produced when MS medium was supplemented with 1.5 mg L-1 BAP + 0.25 mg L-1 Kn, followed by (5.13), when MS medium was enriched with 1.0 mgL^{-1} BAP + 0.25 mg L-1 Kn. The poorest multiplication response (3.00 shoots per culture) was recorded for 0.5 mgL^{-1} BAP + 0.25 mg L-1 of Kn application. No multiplication was observed at control treatment (Table 7). The shoots showed stunted growth as well as browning of leaf tissues, which might be due to the PGRs toxicity towards the multiplying tissues. The large numbers of adventitious shoot produced in the presence of Kn is the evident of the fact that Kn triumphs over apical dominance, release lateral buds from dormancy and upholds formation of shoots. The results were also supported by the findings of Smitha et al. [29] who

TABLE 8
Effect of IBA at different concentrations on root induction and average root length from shoot explants.

Treatment (mg L^{-1}) IBA	Root induction from shoot explants		Average root length from shoot explants	
	Root induction $explants^{-1}$	Percent root induction	Average Root length	Percent response
Basal (MS)	0.00 f	00.00	0.00 f	00.00
0.1	10.27 e	68.44	3.00 c	20.00
0.5	20.53 d	36.89	3.18 b	21.33
1.0	27.37 c	81.56	3.80 a	25.33
1.5	29.33 b	95.56	2.77 d	18.44
2.0	30.47 a	98.11	2.66 e	17.73
LSD	**0.777**		**0.063**	

Means followed by different letter are significantly different at 5 % level of significance

reported that when 0.05 mg L-1 kinetin was used in combination with 1.0 mg L-1 of BAP on MS medium shoot multiplication was enhanced.

Effect of BAP + Kn on average shoot length during multiplication. The maximum shoot length (4.45 cm) was obtained by applying MS + 1.5 mg L-1 BAP + 0.25 mg L-1 Kn. The poorest response was observed at 0.5 mg L-1 BAP + 0.25 mg L-1 Kn treatment which showed an average (2.29 cm) shoots length. In the control treatment, neither multiplication response nor any effect in length was observed (Table 7).

STAGE IV: Effect of IBA on root organogenesis. The in-vitro grown shoots obtained in stage-II were transferred to MS media supplemented with different concentrations of auxins (IBA) for rooting. In this stage plantlet require either no cytokinin or very small amount for normal development of plantlets. No root induction was observed in basal (MS0) media but addition of IBA cased root induction and that was varied at various IBA concentrations. The maximum number of roots (30.47) produced shoots-1 of *Stevia rebaudiana* Bertoni at 2.0 mg L-1 IBA in MS medium. The minimum numbers of roots (10.27) were observed at MS + 0.1 mg L-1 IBA (Table 8). Auxin is a rooting hormone and the addition of IBA promotes adventitious root development on stems [26]. The findings are also supported by the investigation of Tadhani et al. [30] who observed the maximum number of roots on medium supplemented with 1.0 mg L-1 of IBA. The results were also in agreement with Ferreira and Handro, [31] who reported that addition of auxin to the rooting medium (especially 0.1 mg L-1 of IBA) favored root formation in *Stevia rebaudiana*. It was observed that the average root length was significantly varied with supplementation of IBA to the basal (MS) medium. However without IBA to the basal medium no root induction was observed. In response to addition of IBA, the maximum root length (3.80 cm) was recorded at 1.0 mg L-1 IBA in MS medium. The minimum roots length (2.6 cm) was observed at MS + 2.0 mg L-1 IBA (Table 8). The addition of IBA enhanced the average root length because auxin promotes adventitious root development on stems [26]. The results are also in agreement with Debnath [32], who investigated the effect of IBA on root elongation. He reported 2.0 mg L-1 IBA concentration is best for root elongation.

CONCLUSIONS

On the basis of findings achieved in this research study, it is concluded that Leaf explant give maximum callus induction response at 2.5 mgL-1 of 2, 4-D in Ms-Medium. Results also revealed that Maximum shoots induction and shoots elongation was observed when 2.0 mgL-1 of BAP added to MS medium. Combination of 1.5 mgL-1 BAP + 0.25 mgL-1 Kn in MS medium induced highest shoot proliferation response. IBA concentration 2.0 mgL-1 MS medium resulted in maximum roots per in-vitro grown shoot.

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EVALUATING OF FARMYARD MANURE APPLICATIONS ON GERMINATION, SEEDLING GROWTH AND CERTAIN HORMONE CONTENTS OF SILAGE MAIZE (*ZEA MAYS L.*) UNDER SALT (NACL) STRESS

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ABSTRACT

Different farmyard manure (FM) application (0-20-40 t ha^{-1}) mixed with different salinity (0-50-100 mmolNaCl L^{-1}) levels (S) had an effect on germination, seedling growth and certain hormone concentrations including GA_3, IAA and Zeatin in silage maize (*Zea mays* L.). As seedling growth; germination rate (%), stem and root length (cm), stem and root dry weight (g) were recorded. This is to be expected that increased S levels affected the measured parameter; in contrast, the more FM applied the less hazardous affects of S are measured. Sharp decrease in the stem length, root length, stem and in root dry weight by the higher doses of NaCl was recorded, but the benefits of FM especially on the 100 mmolNaCl was recorded. Regarding to hormone, GA3, IAA and Zeatin concentrations tended to increase with the elevated S, but to get lower by the elevated FM doses. Finally, higher doses of FM tended to mitigate not only for the germination and plant growth as well as the GA3, IAA and Zeatin concentrations which is a proof of the positive effects of FM on the hazard affects of S has been found.

KEYWORDS:
Salinity, Farmyard Manure, Maize, Germination, Seedling Growth, Hormones.

INTRODUCTION

Salinity is well known and the most common problems in arid and semi-arid areas. According to the FAO (2015), over 6% of the world's land is affected by either salinity or sodicity.Salf effected soils cover nearly 955 million ha and they generally originate in arid and semi-arid regions [1]. This is equal to 10% of the agricultural area in the world [2] and nearly 20 million ha area are left due to the salinity problem [3].

Any plants are sensitive to salinity especially at the beginning of the growth, germination. Thus; seed resistance to increased salinity during germination is essential for plant survival in the field, consequently for its further development and high yield performance [4]. Furthermore, salt stress adversely affects plants at all stages of their life cycle, but many plants are most sensitive to salt during seed germination. As stated by Greenway and Munns (1980) [5] high concentrations of soluble salts in soil cause reduction in the germination percentage and delay germination of seeds of many plant species. Therefore, eliminating the hazardous affects of salinity is of importance not only for growing the plants effectively but also ecologically.

Maize (*Zea mays* L.), being of the most important cereal crops growing in the world, is commonly used as food and corn oil for human consumption, and secondly is a silage material used for livestock and poultry and raw material for agro-based industries [6]. Moreover, maize ranks third in term of the growing area of cereals that are grown a wide range of environmental conditions [7]. Rahman et al. (2000) [8] reported maize to be more tolerant to salt stress at germination than at later stages of growth. In addition, maize is classified as moderately salt sensitive crops [9].

Some research reports indicated that indole acetic acid (IAA) responded to salinity in crop plants [10]. IAA also responds to salinity in crop plants. However, little information seems to be available on the relationship between salinity stress and auxin levels in plants and the role of auxin in alleviating salt stress. Gibberellic acids (also called Gibberellin A3, GA, and GA3) are generally involved in growth and development; they control seed germination, leaf expansion, stem elongation and flowering [11].

Organic amendments are known to be beneficial for amelioration the soil conditions. Therefore this work focuses on the evaluation of beneficial effects of organic manure on germination, early seedling growth and certain hormone concentrations of silage maize in the seedling stages.

MATERIALS AND METHODS

The experiment was laid out at the open field conditions under the shade at Akdeniz University, Faculty of Agriculture, Field Crops Research field in

TABLE 1
Chemical and some physical parameters of the FM and soil

Farmyard Manure		Soil	
N,%	1.24	N,%	0.08
P,%	0.40	P,ppm	15.90
K,%	0.72	K,ppm	100.22
Ca,%	2.17	Ca,ppm	1450.00
pH	9.10	pH	7.92
EC, dS m^{-1}	6.16	EC, dS -1	0.22
Org. Matter,%	50.00	Org. Matter, %	1.10
		Texture	Sandy-Clay-Loam

2015. Silage maize cultivar EGE F_1 by which was bred by BATEM was used as test plant. FM was taken from the husbandry facilities located in the Akdeniz University. FM was well fermented before the use. The seeds were subjected to 0 (control-pure water)-50-100 mmolNaCl salinity levels with different FM containing soils, 0 (control-no application)-20-40 ton ha^{-1}. The soils were taken from the open field, as it is and were filled to the pots. Table 1 indicates the chemical analysis results of that soil and FM.

The soils well mixed with the FM to get desired doses and than were transferred to the pots having 1,2 kg of soils, and were kept 15 days for incubation of the fermented FM. After that, all pots were watered when reaching to its field capacity (FC). The water status of the soils was kept to 75% of its FC during the experiment and irrigation was started when the soils reached to 50% of its FC. For salinity, NaCl were used at different concentrations and all solutions preparations and also the irrigation were conducted with pure water

Experimental. The experiment begun on June 15, 2015 and maize seeds were directly sown to the pots in which were adjusted to have 10 seeds per each. In the study, 3 salinity levels, 3 FM doses were tested with 4 replications, so 36 pots were used. All Pots were transferred under the shade on the field. After that, the water was applied to reach the 75% of its FC as mentioned above. Germination was observed every day and was noted. Having reached the seedling stages the study was terminated at the end of the 11th day.

Measured parameters. The following parameters were recorded during/at the end of the study as described below.

Germination Percentage

The emergence of plumule was taken daily during the 11 days and was converted to the percentage via the formula given below.

Germination percentage (%) = Number of germinated seeds per given day *100
Number of total seeds

Growth Parameters

At the end of the 11th day of the experiment, 3 plants were selected randomly and root and stem length of them were measured (cm). Moreover, dry weights of the stems and roots were also measured after having dried at 70°C for 24 h.

Hormone analysis. Analyses of plant hormones were determined according to Atmaca (2015). One-gram samples of maize seedling (whole of the plant aerial part without root) were homogenized in cold methanol: chloroform (14:6 v/v) mixture at room temperature, and stored at 20 C for 1 week. The extracts were filtered through Whatmann No. 5 filter paper and the residue re-homogenized with the same solution mixture, and the extracts were combined. The aqueous residue was adjusted to pH 8.5 with 1 N NaOH, and transferred to a separating funnel to separate chloroform from the methanol. The chloroform phase was discarded. The methanol phase was reduced at 40 C to an aqueous phase under reduced pressure on a rotary evaporator. It was then adjusted to pH 2.5 with 1 N HCl and extracted with ethyl acetate (3 volumes). The aqueous phase was adjusted to pH 7 with 1 N NaOH and extracted with ethyl acetate (3 volumes), and then the acidic and neutral ethyl acetate phases including free hormones were combined. In order to release conjugated hormones, the aqueous phase was adjusted to pH 11 with 1 N NaOH and incubated in a water bath at 70 C for 1 h. The hydrolysate was adjusted to pH 7 with 1 N HCl and extracted with ethyl acetate (3 volumes). The aqueous phase was then adjusted to pH 2.5 with 1 N HCl and extracted with ethyl acetate (3 volumes). Acidic and neutral ethyl acetate phases were combined, and then this combined hormone conjugated extract was combined with the previous free hormone extract and reduced to dryness in vacuum at 40 C.

TABLE 2
Variance analysis results on the seedling growth parameters

Sources	Stem Length (cm)	Root Length (cm)	Stem Dry Weight (g)	Root Dry Weight (g)	GA_3 (ppm)	IAA (ppm)	Zeatin (ppm)
Farmyard Manure (FM)	N.S	N.S.	N.S.	N.S.	*	**	NS
Salinity (S)	***	***	***	N.S.	***	***	NS
FMXS	N.S	N.S.	N.S	N.S.	*	*	NS

Significance: * 0.05, ** 0.01, *** 0.001, N.S. Not Significant

The residue was dissolved in 1 mL methanol, and transferred to a microcentrifuge tube. The methanol was reduced to 100 lL under vacuum, and then line-loaded onto a 20 · 20 cm, 0.25 mm thick silica gel 60 F_{254} TLC plate (Merck Plc, Darmstadt, Germany). Standard IAA, GA_3 and zeatin were also spot-loaded in scored strips at both edges of the plates. The plate was allowed to develop for 15 cm in the vertical direction using methanol: ammonia:water (84:8:8 v/v) as the solvent system. After development, the position of IAA, GA_3 and zeatin were detected under UV light (254 nm wave-length) and marked. A band of silica corresponding to the R_f values of standards was scraped off, dissolved in 0.5 ml methanol in a microcentrifgue tube, and then dried under vacuum. The purified samples were methylated with diazomethane [12] and dissolved in ethyl ether and methanol (9:1 v/v). The derivatives were dried under vacuum and re-dissolved in 100 ll ethyl acetate for GC analysis. Levels of the IAA, GA_3 and zeatin were determined with a Fisons 8560 HRGC Mega 2 series equipped with FID, and using a SPB-l (30 m · 0.32 mm I.D.) capillary column. Injection and detector temperatures were 200 and 300 C, respectively. Samples (1 lL) were injected into the column at 80 C. The temperature was programmed 5 C min^1 until the column was at 280 C. Helium flow rate was 1 mL: min^1 and inlet pressure was 22 psi. The amounts of IAA, GA_3 and Z levels were determined using peak areas. The ratio of response of detector to putative IAA, GA_3 and Z peaks in the plant samples were compared to the response ratio of the detector for authentic IAA, GA_3 and Z standards (SIGMA).

Statistical. The experiment was conducted by using 2 factorial (salinity and farmyard manure) randomized parcel design with 4 replications. The data of germination percentage was given as % by days. Results were analyzed using the SAS software package to statically significant difference among treatments. Mean values were compared by Duncan's multiple range tests in SAS statistical package program.

RESULTS

Table 2summarize variance analysis results of the experiment, indicating the statistical relations among the treatments. Results differ from each of the measured parameters. S had profound effects nearly all the parameters, except for root dry weight and zeatin.

The inhibition of the germination by the increased salinity levels was observed clearly for all pots. However, the mitigation of the hazardous affects of the NaCl on the germination was briefly observed in the presence of FM (Figure 1). After 7-9^{th} days, nearly the all seeds germinated depending on the NaCl and also FM applications. The more the salinity increase, the lower seed germinate is monitored.

The 20 ton ha^{-1} application was superior on the 50 mmolNaCl not on the 100 mmol; however, 40 ton ha^{-1} application gave the better results in the 100 mmol application (Figure 1).Naturally, 0 mmolNaCl (NaCl-control) gave the highest levels of GR in all treatments, but the response of supplemental FM applications differs among the NaCl doses. In addition, elevated NaCl doses inhibited the GR clearly, but not for all. Nonetheless, 40 ton ha^{-1} FM application could mitigate the hazardous effects of NaCl especially in 100 mmolNaCl L^{-1} as mentioned above.

Different NaCl and FM applications affected the stem length distinctly. As seen Table 3, stem length decreased significantly by the NaCl, but this trend was inhibited by the FM.

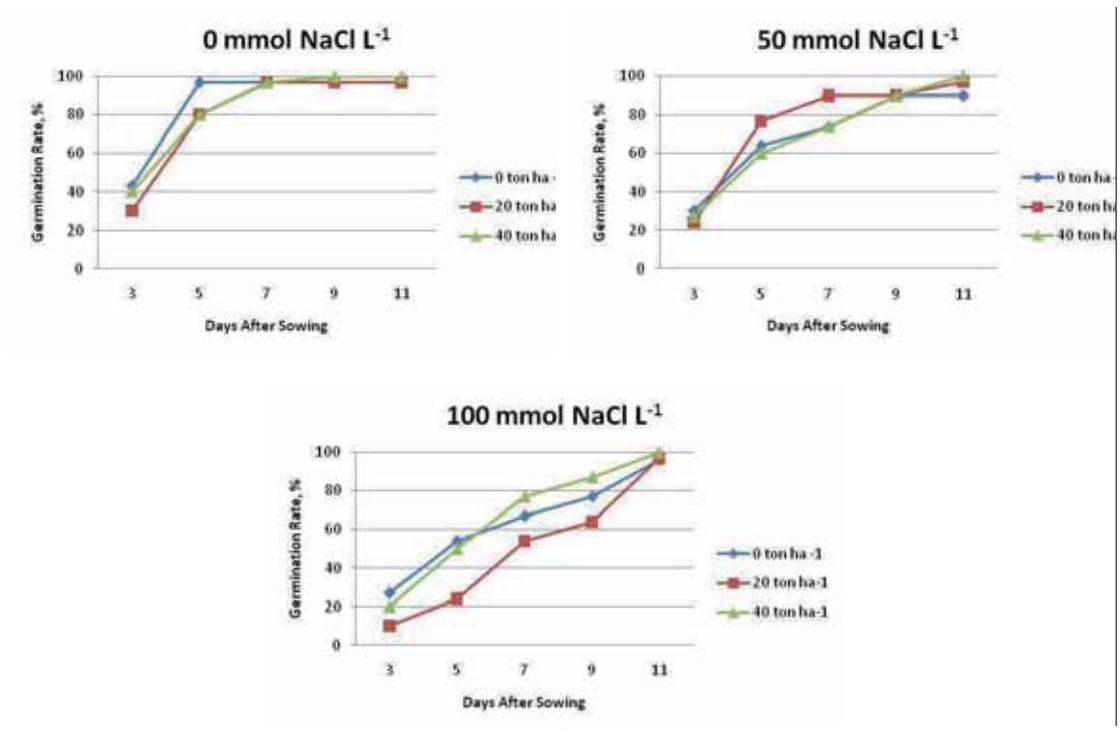

FIGURE 1
Germination rate of maize plants exposed to different salinity and FM applications.

TABLE 3
Effects of different FM and NaCl treatments on stem length (cm)

	NaCl (mmol L^{-1})^Z			FM
FM (ton ha^{-1})	**0**	**50**	**100**	**mean**
0	28.89 a	18.55 b	10.89 c	19.44
20	31.11 a	18.66 b	8.11 c	19.29
40	29.89 a	19.11 b	12.33 c	20.44
NaCl mean	29.69 A^Y	18.77 B	10.44 C	

Significance: Farmyard Manure(FM) : N.S.: Salinity (S) : ***: FMxS : N.S.
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

TABLE 4
Effects of different FM and NaCl treatments on root length (cm)

	NaCl (mmol L^{-1})^Z			FM
FM (ton ha^{-1})	**0**	**50**	**100**	**mean**
0	32.67 ab	26.88 bc	18.88 c	30.26
20	37.11 a	24.77 bc	19.55 c	27.14
40	36.33 a	29.00 ab	25.55 bc	26.14
NaCl mean	35.37 A^Y	26.88 B	21.33 C	

Significance: Farmyard Manure(FM) : N.S. Salinity (S) : *** FMxS : N.S
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

The root length (Table 4) also responded as similar as the stem length in which the obvious decrease was realized by the elevated NaCl doses. Similar to the stem length, FM and FMXS interaction was not statistically significant; however, S gave the statistically significant results among the applications.

Table 5 gives the dry weight of the stem, which represents the statistically significant differences among the NaCl doses. The decreasing the stem dry weight in relation to NaCl doses, and the beneficial effects of FM could be fixed; however, there was no significance relation. This event indicates the supplemental FM addition could be useful, which could be clearly seen in the combination of 100 mmolNaCl and 40 ton FM ha^{-1}.

TABLE 5
Effects of different FM and NaCl treatments on stem dry weight (g)

FM (ton ha^{-1})	NaCl (mmol L^{-1})^{Z}			FM mean
	0	75	150	
0	0.18 a	0.12 bc	0.08 cd	0.13
20	0.19 a	0.12 bc	0.05 d	0.12
40	0.20 a	0.13 b	0.08 cd	0.13
NaCl mean	0.19 A^{Y}	0.12 B	0.07 C	

Significance: Farmyard Manure(FM) : N.S. Salinity (S) : *** FMxS : N.S
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

TABLE 6
Effects of different FM and NaCl treatments on root dry weight (g)

FM (ton ha^{-1})	NaCl (mmol L^{-1})^{Z}			FM mean
	0	75	150	
0	0.18	0.21	0.18	0.19
20	0.19	0.20	0.15	0.18
40	0.24	0.22	0.21	0.22
NaCl mean	0.20 ^{Y}	0.21	0.18	

Significance: Farmyard Manure(FM) : N.S. Salinity (S) : N.S. FMxS : N.S.
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

TABLE 7
Effects of different FM and NaCl treatments on GA3 (ppm)

FM (ton ha^{-1})	NaCl (mmol L^{-1})^{Z}			FM mean
	0	50	100	
0	0,436 b	1.066 b	2.060 a	1.368 A
20	0.913 b	0.563 b	2.966 a	1.481 A
40	0.206 b	0.810 b	1.060 b	0.692 B
NaCl mean	0.518 B^{Y}	0.813 B	2.21 A	

Significance: Farmyard Manure(FM) : N.S. Salinity (S) : N.S. FMxS : N.S.
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

The similar response of the maize to the elevated NaCl doses was taken for root dry weight as shown in Table 6. However, higher FM application had a beneficial effect on root dry weight, as given the other parameters, but no significant relation was recorded.

GA_3 concentrations varied among the applications, however; GA3 increased with the elevated S levels; however, in contrast to S, an increased FM dose lowers the GA_3 clearly (Table 7).

Regarding to IAA (Table 8), in similar to GA3, IAA tended to increase by elevated S levels, but the more FM applied, the higher concentration of IAA were recorded.

Zeatin concentrations showed insignificant responses to S and FM applications (Table 8). However, inhibition of Zeatin concentrations by the increased FM doses could be viewed clearly, especially in 100 mmolNaCl. In addition to this, elevated FM doses also rise the Zeatin concentrations in control (0 mmolNaCl) application.

TABLE 8
Effects of different FM and NaCl treatments on IAA (ppm)

FM (ton ha^{-1})	NaCl (mmol L^{-1})^{Z}			FM mean
	0	50	100	
0	52.05 f	138.01 cde	173.27 bc	121.11 B
20	94.10 ef	175.74 bc	199.82 b	156.55 AB
40	100.99 def	152.71 bcd	283.21 a	178.97 A
NaCl mean	82.38 C	155.48 B	218.77 A	

Significance: Farmyard Manure(FM) : N.S. Salinity (S) : N.S. FMxS : N.S.
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

TABLE 8
Effects of different FM and NaCl treatments on Zeatin (ppm)

FM (ton ha^{-1})	NaCl (mmol L^{-1})^{Z} 0	50	100	FM mean
0	0.503	0.863	1.476	0.947
20	0.696	1.083	1.086	0.955
40	1.796	0.660	0.823	1.093
NaCl mean	0.998	0.868	1.128	

Significance: Farmyard Manure(FM) : N.S. Salinity (S) : N.S. FMxS : N.S.
Z: Different letters in the same column represents a difference at 0.05 according to Duncan's multiple range test
Y: Different letters in the same row represents a difference at 0.05 according to Duncan's multiple range test

DISCUSSION

The main reason why the seed germination is inhibited under the high saline conditions is mainly related to the repression of water uptake [13]. Radic et al., (2007) [4] studied maize and found that the increased concentration of NaCl negatively affected the germination and development of maize seedlings.

Combination of organic amendments and soil washing reduced salt effects but did not insure satisfactory plant growth conditions. In addition, Under saline media, [14] Masciandaro et al. (2002) found that the addition of humic substances (HS) to the solution of NaCl (NaCl+HS) at high electrical conductivity (4.0 mS cm^{-1}) increased the germination index (GI) from 68.5 to 118.0%, suggesting that HS could reduce the inhibitor effects induced by salinity on *Lepidiumsativum* and *Zea mays*. This result could be a proof of our findings about the beneficial effects of FM on germination.

On checking the former studies, similar results were reported by Ashraf and O'leary (1997) [15]. Regardless of the germination rate, FM application could not respond well in term of seedling growth statistically. This situation was addressed by Kaddah and Gowail (1964) [16] found that salt had a higher impact on seedling growth than on field germination capacity.

Regarding to seedling growth in the early stages, though increased NaCl doses gave rise to a decrease on root length; higher application of FM caused stem length to increase obviously, especially in 100 mmolNaCl with 40 ton ha FM application, in contrast to 20 ton ha^{-1} (Table 3). The sharp decrease on stem length in dependence with elevated NaCl doses is clear and is in agreement with former studies.. Banaras et al. (2002) [17] pointed out that FM addition was useful for saline soils.

Stem dry weight showed statistically significant responses to the applications; however, root dry weight not. Consequently, on the one hand higher S caused a tendency to decrease the dry weight of the stem and root; FM addition had a beneficial effect for the adverse outcome of the S on the other. As stated by Giaveno et al. (2007) [18] reported that salt treatments affected root and shoot fresh weight.

Rohanipoor et al. (2013)[19] studied Maize (Zea mays) under the salt stress and reported salinity to have a negative affect on root and stem dry weight. Hoque et al. (2015) [20] studied early seedling stages of maize under the salt stress and found similar results, reporting the adverse affects of salinity on the stem and dry weight as well as root and stem length.

Regarding to GA3, IAA and Zeatin concentrations, all of them increased with S stress and tended to get lower by the FM addition clearly. This to be expected that FM addition could lower the stress conditions which in turn resulted in the decreasing concentrations of GA3, IAA and Zeatin.

Akhiyarova et al. (2005) [21] reported that an increase in IAA contents promotes the formation of an attraction signal in the leaf growth zone in response to salt stress. Therefore, an increase in IAA concentrations is an outcome of salt stress; thus, a decrease of this addresses the benefits of FM clearly.

CONCLUSION

That the elevated dose of NaCl inhibits the germination as well as the seedling growth is obvious; however, supplemental FM could partly mitigate the negative effect of NaCl, especially in the germination stages. The other growing parameters such as plant length and root length etc., were negatively influenced by the NaCl. Regarding the beneficial effect of FM application, 40 ton ha-1 rather than 20 ton ha-1 application appeared to be better, even though there were no statistical significance on seedling growth parameters, regardless of the interaction.

As stated the former studies, the hazardous affects of S is not only related to physical growth parameter, but also related to endogenous hormone such as GA3, IAA and Zeatin. FM clearly inhibits all the hormones and this could be attributed to FM applications. These hormones are produced under the stress conditions; thus, any treatments that lowers the concentrations of them might regarded as anti-

stress materials. As noted, the higher doses of FM could give possibly better results in mitigating the salinity hazards and further studies should be carried out in order to understand well the relationships between FM and NaCl. Usage of FM is also ecologically friendly way to overcome this problem in which have also many benefits to the soil for example increased soil organic matter and water holding capacity those of which have also positive impact on detrimental affect of soil salinity.

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SOME KINETIC AND INHIBITION PROPERTIES OF DEEPWATER PINK SHRIMP FROM AEGEAN SEA: pH, TEMPERATURE, KINETIC AND INHIBITION

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ABSTRACT

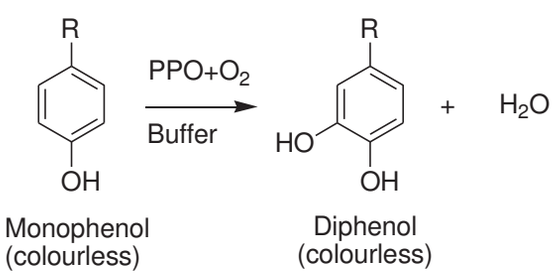
The aim of this study was to identify the tyrosinase activity in the extract of deepwater pink shrimp (*Parapenaeus longirostris*) using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate. Therefore, some kinetic properties of polyphenol oxidase (PPO) from deepwater pink shrimp and the inhibitory effect of gallic acid and L-cysteine on its PPO activity were investigated. The enzyme showed maximal activity at pH 4.0 and 35 ^{0}C. It was stable in a wide pH range of 3.0-6.5 but unstable at a temperature greater than 55 ^{0}C. Kinetic constants were K_M = 10 mM and V_{max} = 10.000 EU/mL min. Gallic acid showed the mixed type reversible inhibition with a K_I value of 0.96 mM, whereas L-cysteine exhibited the uncompetitive reversible inhibition with a K_I value of 6.70 mM. Thus, gallic acid and L-cysteine could inhibit melanosis in deepwater pink shrimp with different modes of inhibition towards PPO.

KEYWORDS:
Deepwater pink shrimp, melanosis, polyphenol oxidase, kinetics, inhibition.

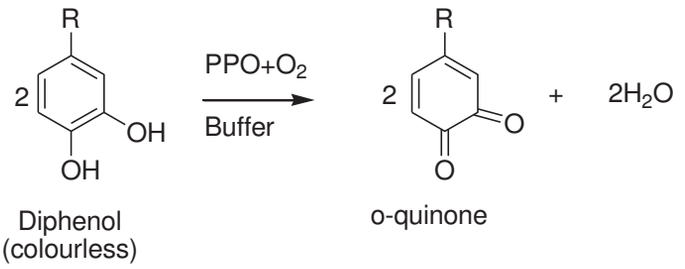
INTRODUCTION

Deepwater pink shrimps (*Parapenaeus longirostris*) show a wide geographic distribution around the world. In 2003, annual landings of pink shrimp in the Sea of Marmara in Turkey were 4059 tonnes, which constitutes approximately 72% of the total shrimp landings in Turkish waters. It is also known that this species has a high market value in France, Spain, Italy, Algeria, Tunisia and Greece [1,2]. Black spot formation occurring as they are postmortem handled and stored is very perishable and vulnerable for this high value shrimp [3]. Because of the long storage periods some problems related with the quality of the products (the shrimps) occur [2]. Microbial contamination, chemical changes and melanosis are the reasons of the quality loss of this product [4].

During postmortem handling and storage of crustaceans, one of the most important troubles is melanosis. Melanosis in shrimp causes a decreasing in consumer acceptability and product's market value, resulting a great deal of financial loss [5]. Polyphenol oxidase (PPO), also known as phenoloxidase, tyrosinase and phenolase, induces the melanosis in crustacean by catalyzing the biochemical mechanism [6,7]. PPO is a copper-containing metalloenzyme and in the presence of molecular oxygen, catalyzes the o-hydroxylation of monophenols to o-diphenols [equation 1] and the oxidation of o-diphenols to o-quinones [equation 2], which rapidly polymerize to produce black, brown or red pigments (polyphenols). These reactions have been given as following [8]:



[1]



Diphenol (colourless) o-quinone [2]

The standard ways to decrease quality deterioration in seafood due to the activity of PPO enzyme are i. freezing, ii. heating, iii. ultrafiltration, iv. high pressure treatment, v. ultrasonication, vi. treatment with supercritical carbon dioxide, vii. microwave heating, viii. gamma irradiation and ix.browning inhibitors [8,9]. Although a heating process can inactivate the enzyme, it renders a product that is no longer fresh, but processed. Cooling has also been used to significantly reduce PPO activity. However, if the temperature is around 0°C, accelerated endogenous enzyme activity occurs and the rate of decomposition increases. To inhibit the activity of PPO, freezing process at -18°C or lower is needed. On the other hand, freezing may be the reason of changes in quality of the products and can result in reduced values. High hydrostatic pressure application, in the range of 900 MPa at 45°C, is another method to inactivate PPO enzymes but it can only be achieved in very small devices and it is not useful for commercial purpose. In addition, high pressures make the products cooked because every 100 MPa pressure causes a 3°C rise in temperature. Low dose gamma irradiation is known to increase PPO activity in shrimp, while medium doses are known to inhibit the activity. Due to its undesirable side reactions, irradiated foods are not preferred by many consumers [9].

PPO activity or ezymatic browning may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. Complete elimination of oxygen is difficult because oxygen is ubiquitous. Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Browning inhibitors have been classified in accordance with their primary mode of action. These are i. reducing agents such as sulphiting agents, ascorbic acid and analogs, glutathione; ii. acidulants such as citric acid, phosphoric acid; iii. chelating agents such as phosphates, EDTA, organic acids; iv. complexing agents such as cyclodextrins; v. enzyme inhibitors such as aromatic carboxylic acids, aliphatic alcohol, anions, peptides, substituted resorcinols; and vi. enzyme treatments such as oxygenases, *o*-methyl transferase, proteases [8].

Little is known the role of PPO in the action of melanosis and the inhibiton mode of deepwater pink shrimp PPO in Turkey. Thus, the aims of this investigation were to study some properties such as optimum pH, temperature and kinetic parameters of deepwater pink shrimp PPO using L-DOPA as a substrate and to elucidate the effect of some chemicals such as gallic acid and L-cysteine on PPO activity. Using sulphites in any of their forms (sulphur dioxide, sodium or potassium metabisulphite and sodium or potassium bisulphite) is a method used more often for controlling browning [10,11]. Sulphites are not only economical and functional for the control of enzymatic/nonenzymatic browning but also they can function as antimicrobials, bleaching agents, reducing agents, and antioxidants. Therefore, they are unique and multifaceted compounds [11,12]. However, the Food and Drug Administration (FDA) has prohibited their use in raw fruits and vegetables because of the adverse effects on consumers' health [11,13]. Therefore, gallic acid and L-cysteine have been used as inhibitor in this study.

MATERIALS AND METHODS

Chemicals. L-3,4-dihydroxyphenylalanine (L-DOPA), Brij-35, disodiumhydrogenphosphate ammonium sulfate, L-cysteine, gallic acid, NaCl, glycine, HCl, and other chemicals were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich. All above mentioned chemicals were of analytical grade.

Materials. Deepwater pink shrimps (*Parapenaeus longirostris*) were caught off the Aegean coast of Turkey (Altınoluk) by trawl in September 2014 and placed in polystyrene boxes, dipped in liquid nitrogen, covered with ice and transported to Balikesir University Molecular Biology and Genetics Laboratory within 2 h. Non sulphited shrimps were frozen in liquid nitrogen and ground into a fine powder using a Waring Blender at high speed for 2 min. The homogeneous powder was stored at -80 ^{0}C until further analysis.

Methods. Extraction of PPO. The powder obtained was used for extraction of PPO. Shrimp PPO was extracted according to the procedure of Simpson et al. (1988) [14] with slight modification. Shrimp powder (10g) was added to 0.1 M sodium phosphate buffer (30 mL) (pH 7.2) containing 1 M NaCl (extraction buffer) and 0.2% Brij 35. The

extract was stirred at 4^0C for 3 h. The extract was filtered and the suspension was centrifuged at 3.000 × g at 4^0C for 5 min. The supernatant was brought to 40-70%$(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitated tyrosinase was separated by centrifugation at 23.500 × g at 4^0C for 30 min. The precipitate was dissolved in a small amount of 0.05 M phosphate buffer (pH 7.2) and dialysed at 4^0C in the same buffer for 24 h with three changes of buffer during dialysis. The dialysed sample was used as the PPO enzyme source in the following experiments.

Measurement of PPO Activity. PPO activity was assayed by measuring the rate of increase in absorbance at 420 nm wavelength for L-DOPA using a double beam model of PerkinElmer Lambda 35 UV-Visible Spectrophotometer, as described previously [15]. The desired temperatures were provided by using a Tempette Junior TE-85 temperature controller attached to the cell holder of the spectrophotometer. Total reaction volume was always maintained at 3.0 mL. The sample cuvette contained 0.1 mL of the enzyme, 2.3 mL of 0.1 M buffer solution and 0.6 mL of 0.1 M substrate solution. The blank sample contained only 0.6 mL of 0.1 M substrate and 2.4 mL of 0.1 M buffer solution. The 0.1 M concentration was chosen to avoid the influence of enzymatic extract ionic strength on PPO activity, described by Angleton and Flurkey (1984) [16]. The reaction was carried out in a 1 cm light path quartz cuvette. The reaction was initiated by adding aliquots of enzymatic extract in the assay medium. Activity was determined by measuring the maximal slope from the linear part of the curve. Unless otherwise indicated, PPO was added last. Enzyme activity data are averages of three measurements. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per minute for 1 mL of enzyme at 35 ^{0}C. Unless otherwise specified, experiments were conducted at three times [17]. Error bars belonging to the experimental data have been given on the curves.

Effects of pH and Temperature on PPO Activity. The pH and temperature optima of deepwater pink shrimp PPO were determined using L-DOPA as a substrate. All buffer concentrations were 0.1 M. The buffers used were 0.1 M glycine-HCl (3.0-3.5), 0.1 M acetate (pH 4.0-6.0) and 0.1 M phosphate (pH 6.0-9.0) adjusted with 0.1 M NaOH and HNO_3 [17]. For determining the optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the ranges of 25-75^{0}C. The effect of temperature on the activity of PPO was tested by heating the standard reaction solutions (buffer and substrate) to the appropriate temperatures before introduction of the enzyme. The desired temperatures were provided by using a Tempette Junior TE-85 temperature controller attached to the cell holder of the spectrophotometer. Once temperature equilibrium was reached, enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals. The reaction mixture contained 0.6 mL of substrate, 2.3 mL of 0.1 M buffer solution, and 0.1 mL of enzyme solution. All assays were performed at three times [15].

Determination of Kinetic Parameters. The kinetic constants (K_M and V_{max}) were estimated in the partly purified PPO extract from deepwater pink shrimp under optimum assay conditions. L-DOPA with seven different concentrations (0.33-2.33 mM) was used as the substrate. The kinetic parameters were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph.

The Effect of L-cysteine and Gallic Acid on PPO Activity. Gallic acid and L-cysteine inhibitors for inhibiting of PPO activity were tested using L-DOPA as a substrate. The inhibitory effects of gallic acid and L-cysteine on PPO activity were determined at different concentrations. Three millilitres of the reaction mixture contained the substrate solutions at various concentrations in 0.1 M phosphate buffer (pH 6.5), 0.1 mL enzyme solution and the inhibitor solution at fixed concentrations. Inhibition constants (K_I and K_I') were deduced from the Lineweaver–Burk plots for each inhibitor [18].

RESULTS AND DISCUSSION

It is well known that uncontrolled oxidative reactions mediated by PPO are responsible for quality deteriorationin several food products derived from plant and shrimp sources [19]. The major biocatalytic properties, which influence the activity of PPOs were pH, temperature, substrate and inhibitors. Firstly, in this study, the effect of pH is discussed, followed by temperature, substrate and inhibitors on PPO activity.

Optimum pH. Because pH affects the ionization of amino acid chains or substrate, it is a determining factor in the expression of enzymatic activity [20]. The enzyme activity with increasing pH values reaches a maximum value and then drops to zero in the alkaline region. Optimum pH values for deepwater pink shrimp PPO were determined in pH ranges of 3.0–9.0. Deepwater pink shrimp PPO

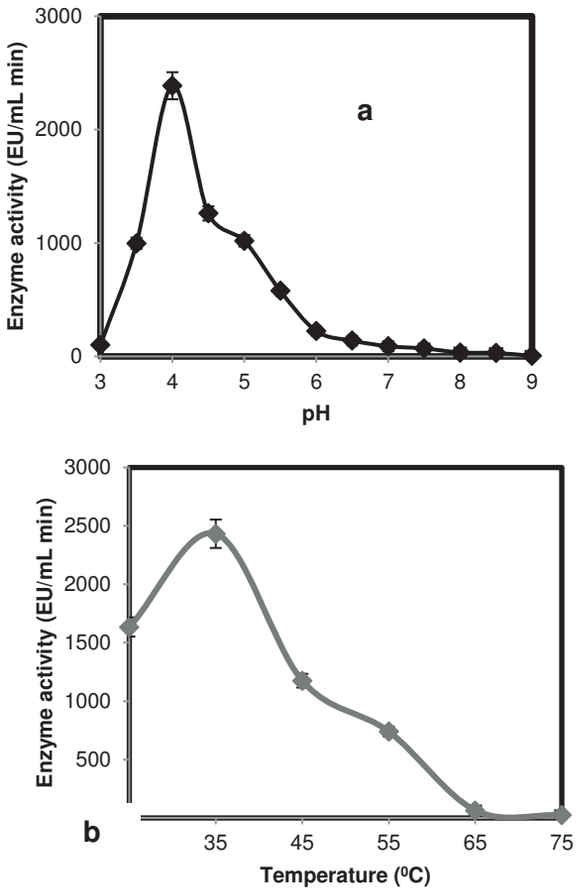


FIGURE 1
The change of deepwater pink shrimp PPO activity with a. pH and b. temperature

showed a clear maxima pH around 4.0 with L-DOPA as substrate (Figure 1a). This result was similar to that of Adachi et al. (2001) [21] who reported that maximal activity of PPO from tiger shrimp hemocytes was found at pH 4.9; Montero et al. (2001) [22] who reported that maximal activity of PPO from carapace was found at 5.0; and Zamorano et al. (2009) [23] who reported that maximal activity of PPO from Atlantic coast deepwater pink shrimp was found at pH 4.5. In this study, deepwater pink shrimp PPO was stable in the pH range of 3 to 6.5 but unstable at a pH above 6.5 and below 3. As seen from Table 1, different optimum pHs for PPOs obtained from various sources are reported in the literature. The optimal pH of PPO were found different among the crustacean species and their anatomical locations [6]. PPO activity was markedly decreased in either acidic or alkaline pH range. At extreme acidic or alkaline pH conditions, unfolding of enzymes might occur due to disruption of electrostatic bonds stabilising enzyme molecules, thereby making PPO inactive.

Optimum Temperature. Enzyme activity is generally measured as the amount of some specific substrates converted per unit time. This activity, as observed by activity measurements, is a combination of a true concentration of the enzyme, multiplied by its specific reaction rate constant. According to Arrhenius law, the specific rate constant increases if the temperature is increased. The enzyme starts to denature at higher temperatures, so the amount or concentration of the active enzyme configuration decreases effectively. The entire activity of the enzyme is ultimately lost [24]. Figure 1b showed that the activity of the PPO gradually increased from 25 °C to 35 °C and decreased from 35 °C to 75 °C, thus the optimum temperature of PPO from deepwater pink shimp was about 35 °C. After optimum temperature, PPO activity showed very little activity at

TABLE 1
Kinetic and inhibition properties of some PPO sources

PPO sources	Substrates	Optimum pH	Optimum temperature (^{0}C)	Kinetic parameter K_M (mM)	Inhibitors	Inhibition types	Inhibition consatant (mM)	References
Deepwater pink shrimp	L-DOPA	4.5	30-35	1.85	-	-	-	**[23]**
Pacific white shrimp	L-DOPA	6	55	2.43	-	-	-	**[3]**
Tokyo kuruma prawn	L-DOPA	6.5	35	-	-	-	-	**[6]**
Pacific white shrimp	L-DOPA	-	-	-	Mimosine	Mixed	3.7	**[5]**
Pacific white shrimp	L-DOPA	-	-	-	Catechin	Mixed	1.4	**[7]**
Pacific white shrimp	L-DOPA	-	-	-	Ferulic acid	Non-competitive	37	**[7]**
Tokyo kuruma prawn	L-DOPA	-	-	-	Cysteine	Competitive	0.45	**[6]**
Tokyo kuruma prawn	L-DOPA	-	-	-	Glutathione	Competitive	0.46	**[6]**
White shrimp	L-DOPA	-	-	3.48	Kojic acid	Mixed	0.15	**[34]**
Spiny lobster	L-DOPA	-	-	3.27	Kojic acid	Mixed	0.07	**[34]**
Grass prawn	L-DOPA	-	-	3.64	Kojic acid	Mixed	0.05	**[34]**
White shrimp	Catechol	-	-	4.27	Kojic acid	Mixed	0.18	**[34]**
Spiny lobster	Catechol	-	-	4.98	Kojic acid	Mixed	0.10	**[34]**
Grass prawn	Catechol	-	-	5.29	Kojic acid	Mixed	0.07	**[34]**
Ocimum basilicum L.	4-methylcatechol	-	-	-	Gallic acid	Uncompetitive	0.10	**[35]**
Deepwater pink shrimp	L-DOPA	4	35	10	Gallic acid	Mixed	0.96	In this study
Deepwater pink shrimp	L-DOPA	4	35	10	L-cysteine	Uncompetitive	6.70	In this study

higher temperatures. As seen from Table 1, the variation in optimal temperature of different shrimp PPOs was most probably related to their habitat temperature. Further increase in temperature above 35°C resulted in the decrease in PPO activity, more likely due to the thermal denaturation of PPO.

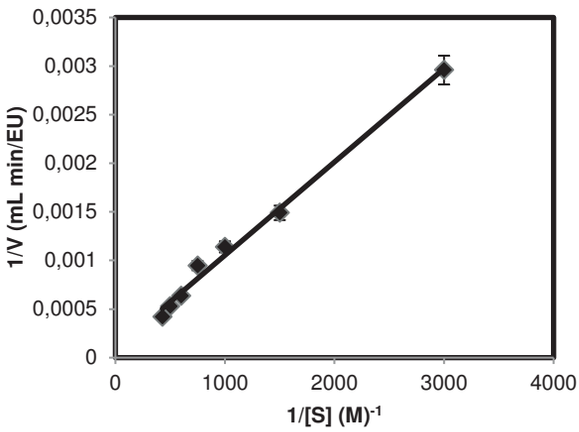


FIGURE 2
The Lineweaver-Burk plot for deepwater pink shrimp PPO

Enzyme Kinetics. The kinetic characterization of PPO from deepwater pink shrimp using L-DOPA as a substrate led to the determination of V_{max} and K_M values. In order to investigate of enzyme kinetics, Lineweaver–Burk graphs are formed to calculate the K_M and V_{max} values. The effect of substrate concentration on the rate of PPO-catalyzed reaction was tested. Increasing the concentration led to a greater enzyme activity. The enzyme kinetics was measured for a period of 3 min. The slope of the straight line was used to compute the enzyme reaction rate throughout the experiment. The Lineweaver–Burk plots, from which the kinetic parameters were derived, are shown in Figure 2. K_M and V_{max} values of PPO were calculated from a plot of 1/V versus 1/[*S*]. The Lineweaver-Burk plot showed a correlation coefficient of $R^2 = 0.9918$. The K_M value of deepwater pink shrimp PPO for L-DOPA was calculated as 10 mM. K_M values reflect the affinity of enzymes to their substrates. A lower K_M value indicates a higher catalytic efficiency of the enzyme towards its substrate. PPO from deepwater pink shrimp showed a higher catalytic affinity than PPO from the carapace with K_M value 19.40 mM [25]. Besides a relatively high affinity towards its substrate as compared to other crustaceans PPO, the PPO characterised in this study showed an V_{max}= 10.000 EU/mL min. The relatively high affinity of PPO from deepwater pink shrimp towards its substrate is indicated by the low K_M value, as well as a high reaction rate once the substrate is bound by the enzyme (as indicated by the V_{max}). The differences in K_M and V_{max} of PPO from different species were plausibly owing to the differences in molting stage, method of capture, handling and storage conditions [26]. As previously mentioned, in the presence of molecular oxygen, PPO catalyses the hydroxylation of monophenols to diphenols, then the oxidation of diphenols to quinones, and finally this colorless quinone subsequently undergoes polymerization, giving rise to black high molecular weight pigments or melanins. This case has clearly been shown in Figure 3. It can be said that the reactions in Figure 3 occures at pH 4 and 35 ^{0}C in which PPO has the highest activity.

Inhibition Kinetics of PPO. There are pH, heat, light, some physical factors, enzyme concentration, substrate concentration, time, reaction products, various ions, hormones, some biochemical factors, inhibitors

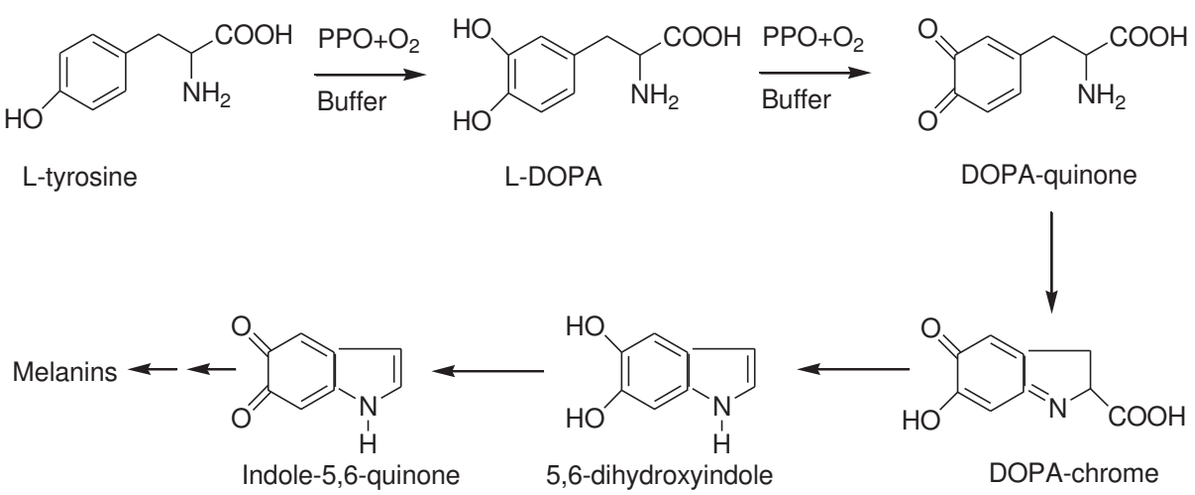


FIGURE 3
Melanin biosynthesis in the presence of PPO from L-tyrosine [8].

among the factors affecting the rates of the reactions catalysed by enzymes [27]. A lot of compounds change the enzyme activity affecting the binding of substrate to the enzyme and/or the turnover number of the enzyme. Substances that reduce an enzyme's activity in this way are known as inhibitors [28]. The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or be the result of an interplay of two or more mechanisms of inhibitor action.

The effect of gallic acid and L-cysteine on PPO activity is shown in Figure4a and b. In the absence of inhibitor, as seen from Figure 3, PPO is the enzyme, which uses molecular oxygen to catalyze the hydroxylation of monophenols to o-diphenol and their further oxidation to coloured and highly reactive o-quinones [29]. Phenolic compounds inhibit PPO activity by interacting with the active site of the enzyme. Also, phenolic derivatives act as chelating agents of Cu^{2+}, a metal necessary for the activity of PPO, either by bonding the hydroxyl group to the active center of enzyme, or by forming a Schiff base trough its aldehyde group, although the earlier mechanism is predominant [30]. Inhibition kinetics of gallic acid and L-cysteine toward deepwater pink shrimp PPO using L-DOPA as a substrate was studied from Lineweaver -Burk plot. In inhibition of mixed type, presumably a mixed inhibitor binds to enzyme sites that participate in both substrate binding and catalysis. The reaction schema for mixed-type inhibition has been given in Figure 5a.

The Lineweaver-Burk equation for mixed-type inhibition from Figure 5a is

$$\frac{1}{V} = \left(\frac{\alpha K_M}{V_{max}}\right) \cdot \frac{1}{[S]} + \frac{\alpha'}{V_{max}} \qquad [3]$$

where

$$\alpha = 1 + \frac{[I]}{K_I} \qquad \text{and } \alpha' = 1 + \frac{[I]}{K_I'} \qquad [4]$$

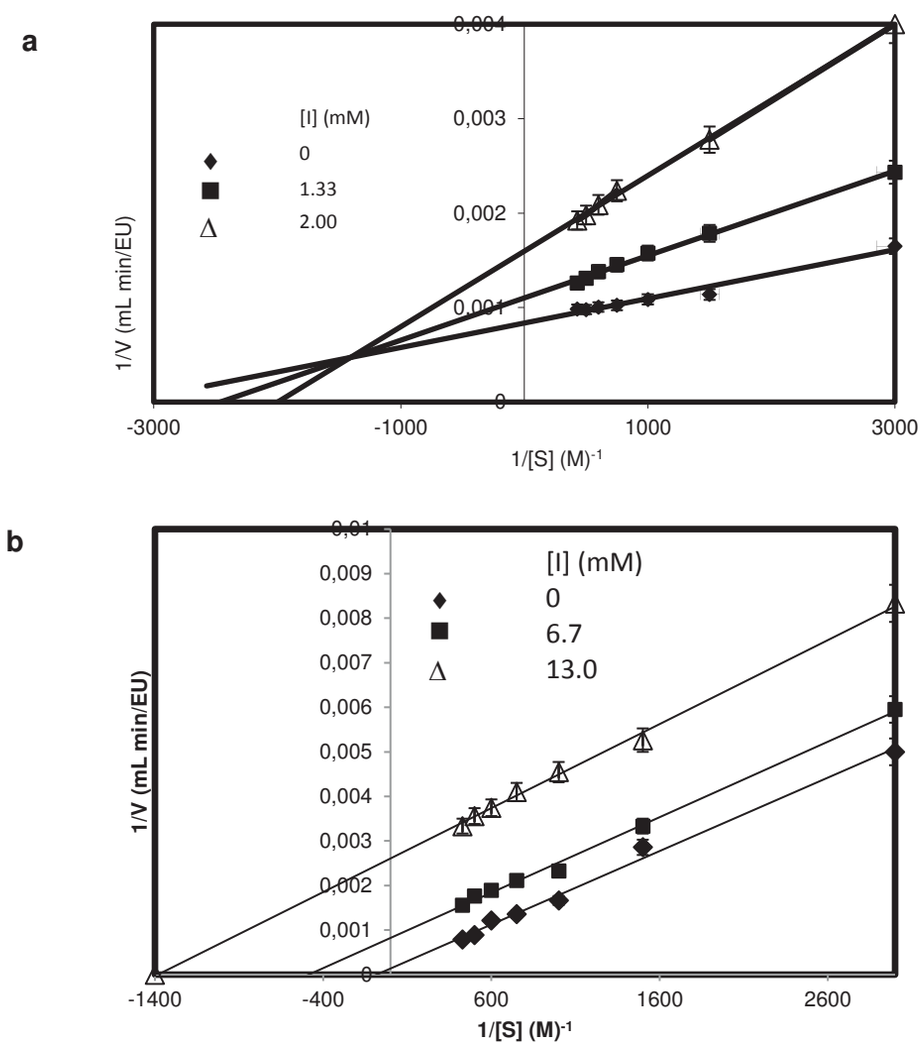


FIGURE 4

Effect of a. Gallic acid and b. L-cysteine inhibitors on deepwater pink shrimp PPO using L-DOPA as a substrate

The plot of this equation consists of lines that have slope $\alpha K_M/V_{max}$ with 1/V intercept of α'/V_{max} and a 1/[S] intercept of $-\alpha'/\alpha K_M$. Algebraic manipulation of this equation for different values of [I] reveals that this equation describes a family of lines that intersects to the left of the 1/V axis [31]. A typical example of mixed-type inhibition is shown in Figure 4a for gallic acid inhibitor using L-DOPA as a substrate. The plots of the enzyme activity vs the concentrations of substrate in the presence of different concentrations of gallic acid showed a series of lines, which intersect to the left of the vertical axis and above the horizontal axis, with a decrease in V_{max} and, conversely, an increase in K_M. Inhibitory activity of gallic acid toward PPO was in a dose dependent manner. Gallic acid at different concentrations affected both the K_M and V_{max} values of PPO. Since the K_M value increased and the V_{max} value decreased with increasing gallic acid concentrations, the inhibitory mode of gallic acid was found to be mixed type. Results indicated that gallic acid could bind with both the enzyme and enzyme-substrate complex, but with different affinities. The effect of various inhibitors at different concentrations on different shrimp PPOs is shown in Table 1. Similar result was obtained for *C. scolymus* L. PPO [15].

In uncompetitive inhibition, inhibitors bind to the ES complex, forming an inactive ESI complex. The binding of the uncompetitive inhibitor distorts the active site of the enzyme rendering it catalytically inactive without affecting substrate affinity [28]. The reaction schema for uncompetitive inhibition has been given in Figure 5b.

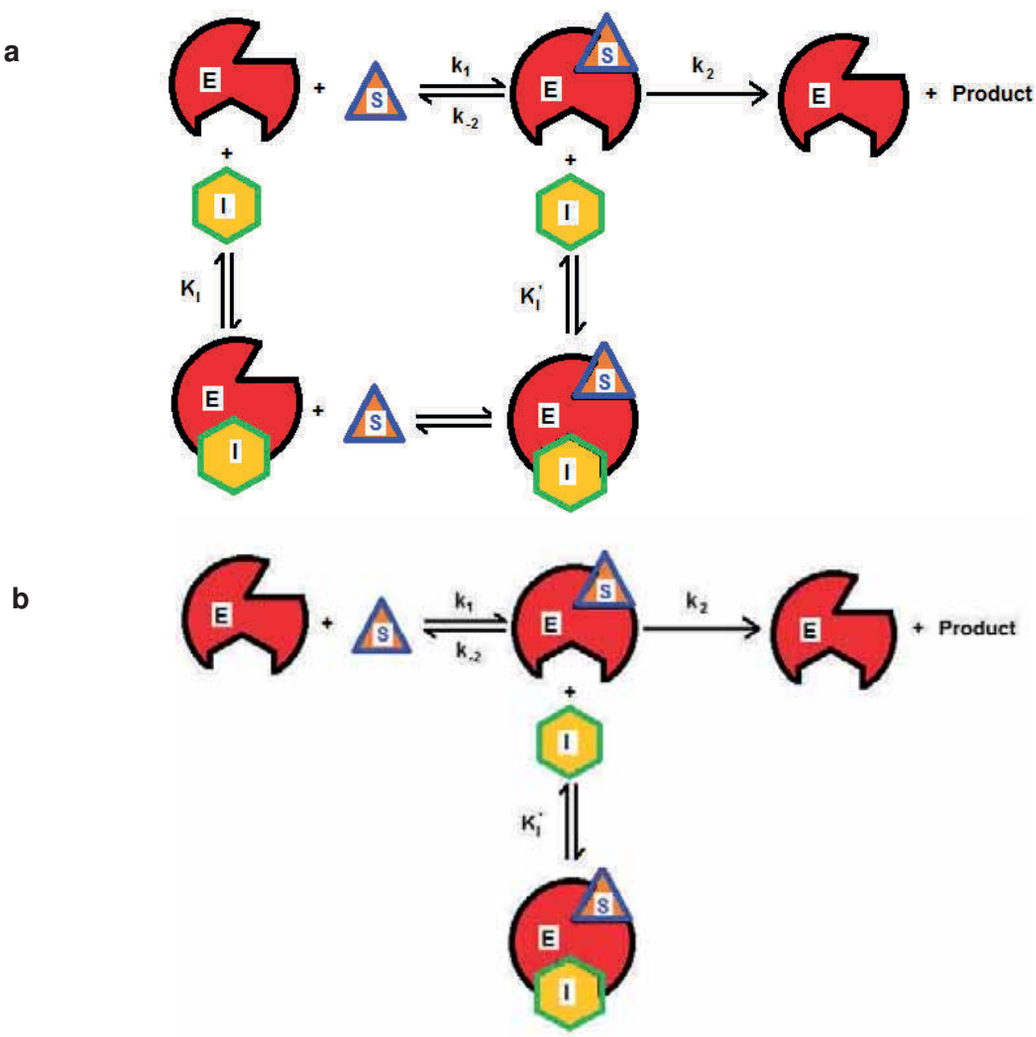


FIGURE 5
Reaction schema for a. mixed-type and b. uncompetitive inhibitions

Lineweaver-Burk equation for uncompetitive inhibition from Figure 5b is given by the following equation:

$$\frac{1}{V} = \left(\frac{K_M}{V_{max}}\right) \frac{1}{[S]} + \frac{\alpha'}{V_{max}} \quad [5]$$

where

$$\alpha' = 1 + \frac{[I]}{K_I'} \quad [6]$$

The increased substrate concentrations do not reverse the effect of uncompetitive inhibition. Lineweaver-Burk plots for different concentrations of an uncompetitive inhibitor yield a family of parallel lines which are diagnostic for uncompetitive inhibition. A plot of 1/V versus 1/[S] for uncompetitive inhibition of deepwater pink shrimp PPO with L-cysteine inhibitor using L-DOPA as a substrate is shown on Figure 4b. The binding of the uncompetitive inhibitor, which needs to resemble the substrate, is envisaged to cause structural distortion of the active site, thereby rendering the enzyme catalytically inactive. Uncompetitive inhibition requires that the inhibitiors affect the catalytic function of the enzyme but not its substrate binding [28]. L-cysteine containing sulphur group might be involved in the PPO inhibition. Thiol reagents might interact with copper at the active site of PPO, leading to the loss of the activity [32]. L-cysteine can react with o-quinones by forming cysteinyl adduct, a colourless compound [33]. Therefore, oxidation of L-DOPA by deepwater pink shrimp PPO was inhibited by L-cysteine due to the various inhibitory mechanisms of L-cysteine towards PPO.

As a result, from K_I values in Table 1, it can be said that gallic acid is a more effective inhibitor than L-cysteine due to lower K_I values. From the result, PPO from deepwater pink shrimp might be inhibited by both substances, gallic acid and L-cysteine, with multiple mechanisms. A lowered absorbance in the sample treated with gallic acid and L-cysteine indicated their inhibitory activity against deepwater pink shrimp PPO (Figure 4a and b). Gallic acid exhibited a slightly greater inhibition, toward deepwater pink shrimp PPO, than did L-cysteine

CONCLUSIONS

The some kinetic and inhibition properties of deepwater pink shrimp PPO in Aegean coast of Turkey has been reported for the first time. Deepwater pink shrimp PPO showed a clear pH optimum and temperature for L-DOPA substrats. The Michaelis-Menten constants, K_M and V_{max}, were 10 mM and 10.000 EU/mL min. The results of this study showed that partially purified PPO obtained from enzymic extract of deepwater pink shrimp was effectively inhibited with gallic acid and L-cysteine using L-DOPA as a substrate. Gallic acid and L-cysteine showed dose dependent inhibitory activity toward PPO. Gallic acid and L-cysteine also affected the intermediate browning product by reduction of quinone to diphenol or by formation of yellow colour complex. Gallic acid exhibited higher inhibitory activity towards PPO, compared with L-cysteine. Gallic acid exhibited mixed type reversible inhibition on PPO when L-cysteine exhibited uncompetitive reversible inhibition. The increase in the inhibitory activity of gallic acid and L-cysteine toward PPO was coincidental with the increase in the reducing power, intermediate formation as well as browning development. Thus, gallic acid and L-cysteine could be used as a potential inhibitor source to inhibit PPO and subsequently prevent melanosis formation in deepwater pink shrimp.

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