2025

FULL TEXT BOOK

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III.International Conference on Agricultural and Veterinary Sciences

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PREFACE

The third International Conference on Agricultural and Veterinary Sciences was organized by Igdir University in collaboration with Rimar Academy. The primary objective of this event was to compile and disseminate valuable scientific knowledge and make a meaningful contribution to the future.

A substantial number of researchers from both local and international backgrounds demonstrated their interest in this conference. The scientific committee meticulously reviewed the submissions and ultimately accepted a select group of applicants—**46** in total—of whom **37** were approved by the scientific committee.

The core of this conference was the presentation of **13** full research papers, while the remaining articles and research findings are set to be featured in forthcoming issues of the MINAR Journal.

I would like to extend my sincere appreciation to all the contributors and scholars who played an essential role in making this conference a resounding success. Your dedication and valuable contributions are deeply respected and acknowledged.

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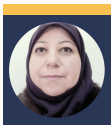

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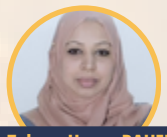

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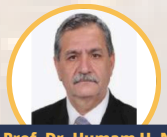

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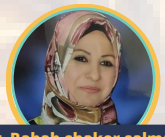

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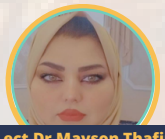

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Study of the possibility of replacing carrot juice waste with wheat flour in the manufacture of gluten-free biscuits and its effect on the sensory and physicochemical properties of the produced biscuits

Ashraq Monir Mahmed [1]

Abdullah Hussein Fathi [2]

Abstract

The aim of this research was to develop gluten-free cookies by substituting wheat flour with carrot waste at varying levels of 0%, 2%, 4%, and 6%, while also examining the chemical composition of carrot waste and identifying its active components. High-performance liquid chromatography (HPLC) was utilized to identify the extracts, and their impacts on the physical, chemical, and sensory attributes of the cookies produced were assessed. Analysis of the chemical composition of carrot waste derived from juices revealed concentrations of protein, fat, ash, and fiber at 2.84%, 3.42%, 4.25%, and 12.7%, respectively, along with a beta-carotene content of 789 ppm. Variations were noted in the chemical composition across the treatments; specifically, as the substitution level rose, the percentages of fat, ash, and protein fiber diminished, while moisture content increased. Sensory evaluations indicated that the biscuit models were comparable, and even though there was a slight edge in controlling the variables, consumers favored the biscuits made with flour alternatives...

Keywords: *Carrot Juice Waste, Biscuit, Protein Concentrate- Gluten-Free.*

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Introduction

Fruits and vegetables account for around 22% of food loss and waste in the supply chain. These goods' byproducts may be converted into flours that are high in minerals, fibres, and bioactive substances, particularly dietary fibre. By adding these components, food items' nutritional value may be raised, leading to goods with a high nutrient concentration that have anti-aging qualities, positive health impacts, and significant nutritional advantages (Santos et al., 2022).

Although they are often used to make salads and soups, carrots are also widely utilised to make nutrient-dense goods including canned carrots, carrot powder, and carrot concentrate (Sharma et al., 2012; Barzi et al., 2019). Up to 50% of the raw material is lost during the juice extraction process, resulting in a usually poor yield of processed carrot juice (Surbhi et al., 2018). Katana et al. (2019) recovered useful components in powdered form from the carrot leftovers produced during the juice making process. The mineral content (K: 668.55-704.73 mg/100 g; Ca: 76.85-86.39 mg/100 g; Mg: 27.86-32.56 mg/100 g; Fe: 2.49-2.97 mg/100 g); ash: 6.84-7.28%; protein: 6.55-6.89%), total carbohydrates: 13.65-16.85%; total fibre: 47.80-51.67%; vitamin C (13.85-15.63 mg/100 g); β-carotene (10.70-13.65 mg/100 g); total polyphenols (265.14-295.85 mg/100 g); and its capacity to prevent the growth of cancer cells (95.54–103.23 mg Trolox equivalent per 100 g) were the components of this powder.

In 2016, Sindhu et al. used blended carrot waste with defatted soy flour to create a variety of cookies. According to their study, adding these useful nutrients significantly raised the final goods' amounts of fibre and β-carotene. The biscuits, which had a great flavour and significant nutritional value, were produced using 17% defatted soy flour and 12% carrot waste powder.

Similarly, Aglawi and Boubaadi (2018) created sweet-fried cookies using defatted soy flour and carrot waste powder in amounts of 5%, 10%, and 15%. As the amount of carrot waste in the cookies grew, so did their protein, ash, and total fibre content. Interestingly, the mixture with 10% carrot juice showed improved flavour characteristics.

Cakes supplemented with carrot waste powder ranged from 4% to 16%, according to Kausar et al. (2018). Ash content increased from 0.42% to 1.16%, fat content increased from 21.43% to 22.64 percent, and crude fibre content increased from 0.2% to 2.35% when carrot waste was added to the soil. The taste and nutritional value of pastries produced with 12% carrot waste were improved.

Materials and Methods

Preparation of Carrot Juice Waste

Carrot juices were wasted in the manner described by Kausar. (2018) with slight alterations. Carrots were scrubbed, juiced, and the waste (pulp) was collected. The trash was heated to 50-60 degrees Celsius for 24 hours and then stored until further utilization..

Measurement of Beta-Carotene of Carrot Juice Waste

The determination of β-carotene concentration in carrot juice waste was conducted using HPLC, following the methodology outlined by Ha et al. (2010). Sample preparation adhered to standard procedures, with the exception of the saponification step. Approximately 2.5 g of the sample was placed into a 50 ml flask, to which 25 ml of anhydrous ethanol and 0.02 g of butylated hydroxytoluene (BHT) were added. For saponification, the flask was heated in a heating block at 100 degrees Celsius for 30 minutes.

A rotary evaporator was used to evaporate petroleum ether at 40 °C, and the resulting concentrate was then adjusted to 200 ml with the mobile phase in a different container. For HPLC analysis, the solution was filtered into a small glass vial using a 0.2 µm filter membrane.

Beta-Carotene Analysis

Using an HPLC system (SYKAM, Germany) equipped with a C18-ODS column (25 cm × 4.6 mm), the concentration of β-carotene was measured. The mobile phase was kept at a flow rate of 0.7 mL/min and included 0.22 mM BHT in addition to ethyl acetate, acetonitrile, and acetic acid at a ratio of 30:68:2 (v/v/v). The detector was configured to operate at 450 nm, an ultraviolet wavelength.

Preparation of Biscuits Fortified with Carrot Juice Waste

The cookies were prepared following the approach outlined by Kausar et al. (2018), with slight modifications. As shown in Table (1), the procedures were divided into four categories. Treatment A acted as the control, whereas treatments B, C, and D substituted wheat flour with spent carrot powder in amounts of 2%, 4%, and 6%, respectively. The samples underwent heating at 200 degrees Celsius for a duration of 7 minutes. The flour utilized in this study was of the deluxe variety.

Table 1. Percentages of biscuit mixture ingredients calculated (1g)

Materials	treatment A	treatment B	treatment C	treatment D
flour	112	109.8	107.5	105.3
sugar	64	64	64	64
Soda	3	3	3	3
Margarine	50	50	50	50
salt	3	3	3	3
water	3 ml	3 ml	3 ml	3 ml

Measurement of chemical properties of carrot and biscuit juice wastes

Moisture for biscuits

Moisture Content Determination

Using the drying procedure outlined by A.O.A.C. (1995), the weight loss before and after drying was used to assess the sample's moisture content. After being precisely weighed (around 3 g), the sample was put in a crucible that had been measured beforehand and dried for 16 hours at 105°C in an electric oven. The following formula was used to calculate the sample's water content:

$$\text{Moisture Content (\%)} = \frac{\text{Weight of sample before drying} - \text{Weight of sample after drying}}{\text{Weight of sample before drying}} \times 100$$

Ash Content Determination

The percentage of ash in the samples was determined by the method of A.O.A.C (1995); where the sample is placed in a previously weighed porcelain crucible and burnt in a muffle furnace at about 525 °C for 16 hours. The percentage of ash value was determined using the following formula:

$$\text{Ash Content (\%)} = \frac{\text{Weight of crucible with ash} - \text{Weight of empty crucible}}{\text{Weight of sample}} \times 100$$

Protein Content Determination

Use Van Dijk et al. (2000)'s Kjeldahl technique to ascertain the samples' protein makeup. A digestion container is filled with the sample, which has a known weight of around 5 g. Add 25 millilitres of concentrated sulphuric acid (H2SO4) and a catalyst combination made of copper sulphate (CuSO4) and potassium sulphate (K2SO4) to this sample.

Digestion Process

TThe sample mixture was processed by heating it until it transformed into a vivid light blue solution..

Distillation Process:

The Kjeldahl apparatus features a distillation device that accepts the mixture in correct proportions. A concentrated sodium hydroxide solution (40% NaOH) is added to the distillation vessel. This setup is linked to a condenser, directing the final output into receiving flasks that contain a known volume of 20% boric acid solution, which also includes a few drops of methyl red and bromocresol blue as color indicators..

Titration:

Approximately 25 ml of the distillate was collected in a receiving vessel and titrated with 0.1 N hydrochloric acid (HCl). A control solution was prepared using the same components, excluding the sample. The percentage of protein was calculated using the formula below:

$$\text{Protein Content (\%)} = \frac{\text{Volume of HCl consumed} \times \text{Normality of HCl} \times 0.014 \times 6.25}{\text{Weight of sample}} \times 100$$

Fat Content Determination

The amount of fat was determined by the AOAC method (1995). A known mass of 10 g of the dry sample was placed in filter paper, which was then wrapped around the sample's sleeve and inserted into the Soxhlet extractor. The receiving container of the device was previously weighed and 250 milliliters of hexane was employed as the solvent..

Extraction Process:

The process of extracting the oil is approximately 5 hours. The solvent is then gathered from the device and the container that contains the fatty debris is removed.

Drying Process:

The flask was positioned in an electric oven set to 80°C for a duration of 30 minutes to guarantee the complete evaporation of all solvents, resulting in only the fat remaining. After this period, the flask was taken out, permitted to cool, and then weighed.

The lipid content was determined with the following formula.:

$$\text{Fat Content (\%)} = \frac{\text{Weight of flask after extraction} - \text{Weight of flask before extraction}}{\text{Weight of sample}} \times 100$$

Determination of Fiber Content

The AOAC (2016) technique was used to determine the fibre content. After weighing and putting around 2 g of the sample powder in a beaker, 30 mL of hexane was added and well stirred to suspend the sample. After that, it was put into a Soxhlet thimble, and for around 90 minutes, petroleum ether was passed through the thimble to remove the fat. After that, the sample-containing thimble was dried for one hour in an oven. After being weighed, the sample and dried thimble were added to a 600 ml Kjeldahl flask along with 200 ml of 1.25% sulphuric acid (H 2 SO4) and 1 g of treated asbestos.

Acid Digestion

The mixture was boiled using a magnetic stirrer under reflux conditions with a drop of methyl alcohol added to prevent foaming. It was boiled for 30 minutes with continuous stirring to prevent solid particles from sticking to the flask walls.

Filtration and Washing

Separate the compost by passing it through a fine mesh liner and cleaning the trash with 50 ml of hot water from a distillery. Repeat the washing process three times with 50 milliliters of hot distilled water to ensure that all of the acidic residues are removed..

Alkaline Digestion

Transfer the waste to another container of size 600 ml and add 200 ml of 1.25% NaOH solution. Reflux the mixture for 30 minutes and filter by using a Gooch crucible containing a filter of the Buchner type.

Neutralization and Final Washing

Wash the waste with 25 mL of 1.25% sulfuric acid, then three 50 mL portions of alcohol. This will serve to neutralize and take away all impurities that might be left..

Drying and Weighing

Position the crucible in an electric oven and dry it for 2 hours at 130° C.±2° C. After drying, cool the crucible in a glass desiccator and weigh it to a constant weight corresponding to the total weight of the fiber and ash (A).

Ashing Process

Place the crucible in an electric oven, heat up to 600 °C and keep it there for 30 minutes. Then take it out, put in a glass desiccator, and cool in a desiccator. After cooling, weigh it again to determine the weight of ash (B).

Estimate the amount of fiber in the following way:

$$\text{Fiber Content (\%)} = \frac{\text{Weight of fiber and ash (A)} - \text{Weight of ash (B)}}{\text{Weight of sample}} \times 100$$

soluble fiber is quantified by utilizing a Buchner funnel to pass the filtrate through and then placing it into a clean, dry beaker of a specific weight. The glass is then placed in a water reservoir until the water is evaporated and the glass is dry. The beaker that contains the sediment is then assessed. The contrast between the two values is the amount of soluble fiber present (A.O.A.C, 2016).

Determination of Carbohydrate Content in Biscuit Sample

Carbohydrate content in the biscuit samples was determined by mathematical subtraction of the AOAC method (2016). Compositions of carbohydrates were calculated using the formula:

$$\text{Carbohydrate Content (\%)} = 100 - (\text{Moisture (\%)} + \text{Ash (\%)} + \text{Protein (\%)} + \text{Fat (\%)} + \text{Fiber (\%)})$$

- **Moisture(%) is** the volume of water in the sample, expressed as a percentage...
- **Ash (%)** is the percentage of inorganic waste remaining after combustion.
- **Protein (%)** is the percentage of protein calculated using the Kjeldahl method.

• **Fat (%)** is the percentage of fats determined by Soxhlet extraction.

• **Fiber (%)** is the percentage of dietary fiber determined using the digestion and ashing process.

The result represents the total carbohydrate content, which includes both digestible carbohydrates (e.g., starches and sugars) and indigestible carbohydrates (e.g., some fiber fractions).

Evaluation of Manufacturing and Physical Properties of Biscuit Samples

The biscuit samples were prepared and their physical properties were recorded as per the procedure described by Omran et al. (2016). These attributes included:

1. **Diameter of the Biscuit:**

Six biscuit pieces were placed side by side, edge to edge, and the diameter was measured. The biscuits were then rotated 90° and the diameter was measured again. The average of these two measurements was calculated (in mm).

2. **Thickness of the Biscuit:**

Stack the cookies on top of each other and measure the thickness of each individual stack. Reorganize the cookies and take a new measurement. Then average the measurements (in mm) listed above.

3. **Expansion Ratio (Spread Factor):**

The expansion ratio (spread factor) was calculated using the following formula:

$$\text{Expansion Ratio (Spread Factor)} = \frac{\text{Diameter}}{\text{Thickness}}$$

This ratio helps assess the spread or expansion of the biscuit dough during baking, which is an important indicator of texture and consistency.

Sensory Evaluation of the Biscuit

The cookies were evaluated sensorially by professors and specialists from the faculty of agricultural engineering sciences at the University of Baghdad. The sensory evaluation attributes were: appearance, surface attributes, texture (crunchiness and hardness), internal color, taste, and aroma. For these characteristics, factors were taken for determining overall acceptance and quality of the cookies.

Results and Discussion

Chemical Properties

Table (2) illustrates the chemical properties of carrot waste. The chart demonstrates discrepancies with the findings of Gopalan et al. (1991) had a protein composition of 0.9%, fat composition of 0.2%, crude fiber composition of 1.2%, and total ash composition of 1.1%. The outcomes of this investigation were more significant than the findings of Gopalan et al. (1991) documented the values. The conclusions are more similar to Katana's. (2019) documented a value for the ash content of 6.84%, a value for the protein content of 6.55-6.89%, a total value for fiber content of 47.80-51.67%, and a value for beta-carotene content of 10.70-13.65 mg/100g (13.66 ppm). However, the beta-carotene content of this research was greater than that documented by Katana et al. (2019) resolved. The researchers demonstrated significant differences in the chemical composition of carrot trash, this is attributed to the different chemical composition of carrots and their various varieties..

Table 2. Chemical properties of carrot juice wastes

β-carotene (ppm)	% protein	Fibers %	fat %	ash %	Moisture %
789	2.84	12.7	3.42	4.25	83.25

Table (3) shows the chemical composition of the biscuits containing carrot juice wastes. The table indicates variations in the nutrient levels, but according to the chemical composition of the biscuit treatments, the addition of carrot juice wastes did not significantly affect the nutrient content, with only slight differences observed

Table 3. Chemical composition of biscuits containing carrot juice wastes

Testes %	A(control)	B (2%)	C (4%)	D (6%)
protein	7.48	7.77	7.90	8.13
fat	19.58	19.77	19.84	19.97
ash	8.15	8.33	8.48	8.60
moisture	7.11	7.08	7.00	6.90
carbohydrates	57.68	57.05	56.78	56.40

Physical Properties

The physical characteristics of the cookies are listed in Table (4). The addition of carrot juiceside did not have a significant effect on the physical properties, except for a minor deviation.

Table 4. Physical properties of biscuits.

Physical properties	A(control)	B (2%)	C (4%)	D (6%)
Height (cm)	0.9	0.8	0.7	1
Diameter (cm)	33.7	35.5	33.1	35.2
Spread Ratio	37.9	44.37	47.28	35.2

Table (5) illustrates the sensory evaluation table for cookies, and the results indicate that the addition of 6% of carrot's juices increased the quality of the cookies compared to the control group. For the treatments (B) and (C), no significant alteration of the cookies' sensory properties was observed..

Table 5. Biscuit Sensory Evaluation Form

Qualitative characteristics	Grade	A(control)	B (2%)	C (4%)	D (6%)
Appearance	20	17	15	16	18
Upper surface qualities	15	13	12	11	15
Textures	15	15	12	13	14
Pulp color	15	13	13	11	12
Taste and flavor	20	17	18	16	17
Total	85	75	70	67	76

appreciation

I yearn for a Ph.D. I wish to take this opportunity to thank and appreciate Ishraq Mounir as well as the faculty.

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Role of Cymbopogon Citratus Leaves Aqueous Extract on Microbial and Sensorial Properties of Fish Ball Under Frozen Storage

Wurood Fawzi Ahmed [1]

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Abstract

Lemongrass (*Cymbopogon citratus*) is a member of the genus *Cymbopogon* of the *Poaceae* family (*Gramineae*). *C. citratus* leaves were subjected to aqueous extraction, in addition to investigating fish balls extended shelf life (for 60 days) after treating it with (5, 10, 15, 20) % of lemongrass aqueous extracts after mixing it with coriander, pepper powder, salt, garlic, cumin, and breadcrumbs at and stored under freezing temperatures and evaluated against control treatment (Lg) (free of aqueous extracts), which were then excluded (after 15 days of frozen storage) due to their contamination with bacteria and due to their poor quality. Fish balls samples (Lg3), which were treated with 15% of aqueous extract, showed high sensorial characteristics (e.g., colour, texture, flavour, taste and general acceptance) Which recorded a rating of 9/9 at the end of the storage period compared to Lg4 treatment (with 20% of aqueous extract), which gained adequate sensorial characteristics after frozen storage.

Keyword: *Cymbopogon Citratus, Frozen Storage, Fish Balls, Shelf Life, Sensorial Properties.*

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Introduction

Lemongrass (*Cymbopogon citratus*) belongs to the genus *Cymbopogon* of the *Gramineae* family, and this genus includes about (50) listed species and various other species under study. Lemongrass is a perennial, herbaceous, aromatic plant (when rubbing its leaves by hand, it emits a scent similar to the scent of lemon), it remains in the ground for up to ten years, inflorescences are small spikelets branched evenly, the fruit is elongated needle resembling rice and barley grains. The plant is tropical, its origin is East Asia, Malaysia or Sri Lanka. Young lemongrass leaves and the basal parts of leafy shoots are used as a seasoning in soups, sauces, and curries. The medicinal part is the shoot of the plant.

One of the main chemicals in the plant is the citral compound (70-80%), in addition to containing myrcene, limonene, geraniol, and traces of hydrocyanic acid, especially in the leaves and roots, in addition to a compound similar to insulin. Some studies mentioned that citral may cause cancer cells to commit suicide without affecting healthy cells (Kholif *et al.*, 2017; Kamona & Alzobaay, , (2021).

Lemongrass oil is used as an antifungal and antibacterial disinfectant, which prevents the transmission of infectious diseases, and is also used in the treatment of intestinal colic and gastric fermentation, and in cases of gastroenteritis and colon, and contains natural antioxidants and is useful in cases of nervous fatigue, exhaustion, nervous spasms, and various skin diseases, and the oil is considered insect repellent (Majewska *et al*, 2019). Lemongrass oil is an amber yellow in colour in which aldehydes, (mostly composed of citral), are the main component of the oil (75-85%) (Hussein *et al*., 2015; Fagbohun *et al*, 2010; Abdulhasan,2021).

Fish meat is one of the sources of meat that can be served as a diet and a primary source of nutritionally integrated proteins compared to red meat, which is easier to digest than other proteins. In addition to being rich in its content of fatty acids and mineral elements, fish meat contains ω-3 and ω-6 and essential and polyunsaturated fatty acids and lower percentages of saturated fatty acids and cholesterol (Gomaa *et al*.,2019; Abed *et al*,2022). Seafood is known to be subjected to rapid spoilage and have a limited shelf life, although stored under refrigerated or frozen storage conditions, as refrigerated or frozen storage is insufficient to prevent the process of fat oxidation or the development of poor and rotten flavours and even to prevent the growth of pathogens (Khidhir *et al*, 2013; Touej,2013).

There is a constant need to develop and improve the quality of seafood and one of these methods is to use plants edible films and coatings suitable for human consumption and add

them to seafood to extend their shelf life (Viji *et al*., 2017). These plant extracts have antioxidant properties in addition to acting as antipathogens as well as acting as a barrier against gases and water (Erkan *et al*., 2015; Yaseen, 2020).

Recently, the trend and reliance on the use of preservatives in the food industry has grown. Preservatives are natural or artificial substances used to maintain food freshness for longer time, as they contain antimicrobials and oxidants, as well as other substances that slow down the natural ripening process. Codex Alimentarius Commission defined food preservatives as "substances that are added to food for maintaining or improving nutritional value" **(**Alimentarius, 2015; Abbas, 2012). The results of a study by (Supardan *et al*., 2016) showed that the coating layer to which lemongrass oil was added had a reducing effect on the bacterial load of meat in addition to prolonging the shelf life of food products compared to products without lemongrass oil. The current study aims to investigates the impact of different concentrations of aqueous extract of *Cymbopogon citratus* leaves on the microbiological and sensory properties of frozen fish balls.

MATERIALS AND METHODS

Extraction of Lemongrass (*C. citratus*) leaves

The method described by Balakrishnan et al. (2014) was modified by the researchers as follows: 100g of C. citratus leaves powder was taken and added to 1000 ml of distilled water, shaken for 8 hours at 40ºC. The mixture was filtered using Whatman No. 1 filter paper. The extract was then collected, poured into Petri dishes, and placed in an electric oven at 40ºC to achieve complete dryness. Afterwards, samples were stored in refrigeration until use.

Fish balls preparation

The method mentioned by Bavitha (2016) and Omar (2003) for the preparation of fish balls was modified by the researchers. Carp meat (Cyprinus carpio) was used after mixing it with coriander, pepper powder, salt, garlic, cumin, and breadcrumbs at concentrations of (100, 0.25, 0.25, 1.5, , 0.4,0.25,10) respectively, adhering to the time concentrations of (5, 10, 15, and 20).% of the aqueous extracts of *C. citratus* was added to all previous samples with continuous manual mixing to ensure the best homogeneity of all mixtures.

The prepared samples and control were stored at a temperature of (-18±2◦C) until the quality was evaluated on the basis of microbial load after sample preparation and after the storage period (1-60) days.

The total number of *Coliform*, *Psychrotrophic*, *Staphylococcus aureus* and *Salmonella* bacteria as well as moulds and yeasts in plates were determined by (APHA, 1978; Speak, 1984; Al-Dulaimi, 1988). On the other hand, the sensory properties of fish balls treated with the aqueous extract of *C. citratus* were evaluated based on 5 scales namely colour, flavour, taste, general acceptability after deep frying with refined sunflower oil and after cooking before serving to the evaluation committee (O'Mahony, 2017).

Results of microbial load of fish balls under frozen storage

The results indicate that Total Plate Count (TPC) for fish balls treated with (5, 10, 15, 20) % of the aqueous extract of *C. citratus* stored for the periods (1, 15, 30, 45 and 60) days are shown in table (1). The results also showed an inverse relationship between the number of microorganisms and the storage period, where it was noted that there was a gradual decrease in the number of microorganisms with increasing storage period, while the number of microorganisms in the control sample (free of aqueous extract of *C. citratus*) reached (78×10^{5} cfu/g) after (30) days of frozen storage. These results exceed the maximum permissible number of microorganisms in fish balls according to (CODEX Alimentarius Commission, 2006). Moreover, in the study by (Salem-Amany *et al*, 2010), results indicated that garlic, thyme and lemongrass oils had a significant effect in reducing the number of microorganisms such as aerobic plate count (APC), *Enterobacteriaceae* count, *Coliform* count and *Staphylococci* count.

The results of the study by (Ibrahim and Salem 2013) on refrigerated chicken patties showed that lemongrass oil has anti microorganism activity due to its content of substances such as citral, which are known to have anti microorganism and antioxidant activity.

Table 1. Total Plate Count (TPC) of fish balls treated with *C. citratus* leaves aqueous extract after freeze storage (-18 ± 2°C)

Storage period (days)	Total Plate Count (cfu/g)				
	Control	Lg_1 (5%)	Lg_2 (10%)	Lg_3 (15%)	Lg_4 (20%)
1	67×10^{3}	32×10^{3}	20×10^{3}	14×10^{3}	12×10^{3}
15	61×10^{4}	0	0	0	0
30	78×10^{5}	0	0	0	0
45	-	0	0	0	0
60	-	0	0	0	0

Table (2) shows the effect of different concentrations of *C. citratus* aqueous extract added to fish ball samples. It is evident from table (2) that number of Psychotropic bacteria was declined after the last day of storage compared to the first day. In addition, control samples showed higher records reaching (12×10^3 cfu/g), which surpasses the maximum limits of microbiological standards of Psychotropic bacteria count in fish balls.

Table 2. Psychrotrophic bacteria count of fish balls treated with C. citratus leaves aqueous extract after freeze storage (-18 ± 2◦C)

Storage period (Days)	Psychrotrophic bacteria count (cfu/g)				
	Control	Lg1 (5%)	Lg2 (10%)	Lg3 (15%)	Lg4 (20%)
1	9.3×10 1	8.7 × 101	7.2 × 101	4.5 × 101	3.9× 101
15	3× 102	0	0	0	0
30	12× 103	0	0	0	0
45	-	0	0	0	0
60	-	0	0	0	0

Table (3) is displaying the decline in *Coliform* bacteria count in fish balls samples treated with (5,10,15, 20) % concentrations of *C. citratus* leaves aqueous extract after freeze storage (-18±2°C) in comparison with control sample (free of the extract). In contrast, *Coliform* bacteria was present in control sample amounting to (2.8×10^2 cfu/g) after 30 days of freeze storage, which surpasses the maximum limits of microbiological standards of *Coliform* bacteria count in fish balls (10^2 cfu/g).

Table 3. Coliform bacteria count in fish balls treated with C. citratus leaves aqueous extract after freeze storage (-18±2◦C)

Storage period (days)	Coliform bacteria count (cfu/g)				
	Control	Lg1 (5%)	Lg2 (10%)	Lg3 (15%)	Lg4 (20%)
1	6× 101	4.5× 101	3.5× 101	3 ×101	2.8 ×101
15	9.7 ×101	0	0	0	0
30	2.8× 102	0	0	0	0
45	-	0	0	0	0
60	-	0	0	0	0

In another study conducted by (Fagbohun *et al*., 2010), results demonstrated that *C. citratus* aqueous extract had an antibacterial characteristic where it exhibited a high inhibitory effect on *Coliform* bacteria. Furthermore, (Salem-Amany *et al*, 2010) study on the effect of lemongrass, thyme and garlic oils (1.5 %), which had a strong adverse effect on the growth of *Enterobacteriaceae* and *Coliform* after refrigerated storage of minced beef. However, (Speak, 1984) reported the presence of Marjoram essential oil effect on chicken drumstick after (12) days under refrigerated storage conditions.

Table (4) exhibits *Staphylococcus aureus* load in all fish balls treatments. The results demonstrate that there is an inverse relationship between the concentration of the added extract and the bacterial load, as with increasing the concentration of the added extract, the bacterial load decreases significantly. Conversely, bacterial load in control sample (free of the extract) reached 4.6×10^3cfu/g after 30 day of freeze storage, which surpasses the maximum limits of microbiological standards of (*Staphylococcus aureus*).

Table 4. Staphylococcus aurous count in fish balls samples treated with C. citratus leaves aqueous extract after freeze storage (-18±2◦C)

Storage period (days)	Staphylococcus aurous count (cfu/g)				
	Control	Lg1 (5%)	Lg2 (10%)	Lg3 (15%)	Lg4 (20%)
1	9.1 ×101	7.8 ×101	4.3×101	3.6 ×101	3.2 ×101
15	8× 102	0	0	0	0
30	4.6 ×103	0	0	0	0
45		0	0	0	0
60		0	0	0	0

Due to biological active compounds in Lemongrass and the use of methanol extracts and essential oil of this plant in antibacterial activity against *Staphylococcus aureus* (ATCC:25923), *Bacillus cereus* (ATCC:1247), *Escherichia coli* (ATCC:25922) *and Pseudomonas aeruginosa* (ATCC: 27853) Jafari *et al*., (2012).

Additionally, table (5) demonstrates moulds and yeasts count in fish balls reaching 44×10^3cfu/g after 30 days of freeze storage in control samples, however, samples treated with lemongrass aqueous extract exhibited a reduction in moulds and yeasts load. Conversely, with

the increase in the concentration of lemongrass essential oil added to yoghurt incubated at 5 or 28 °C for 28 days, a growth repressive influence was observed on all assessed moulds (*Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus ochraceus*). *(*Li *et al*., (2022) .

Table 5. moulds & yeasts count in fish balls samples treated with C. citratus leaves aqueous extract after freeze storage (-18±2◦C)

Storage period (days)	moulds & yeasts count (cfu/g)				
	Control	Lg1 (5%)	Lg2 (10%)	Lg3 (15%)	Lg4 (20%)
1	1.3 × 102	8.4 ×101	7.2 ×101	4.8 ×101	3.3 ×101
15	2× 102	0	0	0	0
30	44× 103	0	0	0	0
45		0	0	0	0
60		0	0	0	0

Salmonella bacteria were not detected in any of the products after the entire period of freeze storage. The products were found to be free from *Salmonella*. Similar results were reported by (Rani *et al*., 2017) after freeze storage. Oregano (*Origanum vulgare* L.) and Lemongrass had an anti- and lethal effect against *Salmonella enterica* in minced beef meat after refrigerated storage at 4±2 °C (De Oliveira *et al*., 2013).

The inhibitory effect of essential oils is not only due to their main components but also because of the existence of a synergistic relationship of their main compounds. In a study by (Falleh *et al*., 2020), it is shown that the efficiency of EOs as anti-microorganisms is essentially attributed to their chemical nature. According to (Kamsu *et al*., 2019), One of the most important components of lemongrass EO is that it contains high levels of linalool acetate and geraniol (41.29 and 32.15) %, respectively.

Moreover, according to (Salih *et al*., 2022), *C. citratus* exhibits a valuable antibacterial, and anti-inflammatory activity, and can promote phagocytic properties against *Salmonella typhi*, although, it is possibly can be used as a replacement therapy for enteric (Typhoid) fever.

Organoleptic properties

Results in table (6) demonstrate elevated sensorial properties in samples treated with *C. citratus* aqueous extract after freeze storage. Lg_3 treatment (15% aqueous extract) showed highest score of colour, texture, flavour, taste and overall acceptability characteristics recording overall score of (9/9) compared to other treatments. Control treatment, (after 30 days) of refrigerated storage, demonstrated low score according to unfamiliar colour, aqueous texture and undesirable flavour and taste.

It is acknowledged that some specifications of a food products are of great importance in the level of consumer acceptance of that product when these standards such as taste, appearance and favour differ from what consumers expect. Among the positive effects of marjoram oil are its contribution to inhibiting colour loss, its effectiveness in fighting lipid oxidation and the formation of unpleasant odour and, most importantly, its antimicrobial activity after freeze storage (Badee *et al*., 2013).

Texture values reduced in treatment Lg_4 (treated with aqueous extract 20%) was reached (4) after 60 days of storage. in Study by (Abd- El Fattah *et al*., 2010), it is pointed out that aldehydes, volatile compounds and high amounts of phenolic compounds available at high levels in lemongrass have an effect on colour fixation, viscous texture and antimicrobial activity.

Conclusion

Due to their content of many biologically active compounds, including flavonoids, alkaloid, tannin and phenolic compounds, terpenoids, in addition to containing monoterpenes hydrocarbons (Citral), which gives it its anti-inflammatory properties, Lemongrass (*Cymbopogon citratus*) has many anti-inflammatory and antioxidant properties, in addition to its antifungal and antibacterial properties. Moreover, dried stems and leaves are mostly used as food flavourings, the pulp of young shoots can be cooked and consumed as vegetables, and the leaves can be used to prepare tea in addition to other uses such as improving sensory characteristics such as (flavour, taste, colour, textures and overall acceptability) after freeze storage.

Table 6. Sensorial characteristics of fish balls treated with C. citratus leaves aqueous extract after freeze storage (-18±2◦C)

Treatment	Storage period	Colour	Textures	Flavour	Test	Overall acceptability
Control	1	6	4	5	5	4
	15	4	3	5	5	5
	30	5	2	3	2	2
Lg1 (5%)	1	7	6	7	8	7
	15	6	5	6	7	6
	30	5	5	6	7	6
	45	5	5	5	6	5
	60	4	4	4	5	4
Lg2 (10%)	1	8	8	7	8	8
	15	7	7	6	7	7
	30	6	7	6	6	6
	45	5	6	5	6	5
	60	5	5	4	5	4
Lg3 (15%)	1	9	9	8	9	9
	15	8	8	8	8	9
	30	8	8	7	9	9
	45	8	9	9	9	9
	60	9	9	9	9	9
Lg4 (20%)	1	7	6	7	4	6
	15	6	6	6	4	5
	30	5	5	5	3	5
	45	4	5	5	4	4
	60	4	4	4	4	4

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Effect of seed stimulation treatments with hydrogen peroxide and sowing dates on some yield traits of Sorghum cultivars

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Abstract

A field experiment during the spring season of the 2023 was Conducted in the agricultural fields of the Agricultural Research Department – Ministry of Agriculture – Abu Ghraib. The aim of this research was to investigate the effect of seed stimulation treatments with hydrogen peroxide and sowing dates on yield traits of Sorghum cultivars . The experiment was Arranged with the split split plots by using a Randomized Complete Block Design (RCBD) with three replicates and three factors. The main plots were allocated for three planting dates (11/3/2023 and 25/3/2023). The sub plots included three varieties of white corn (Al-Khair, Buhooth - 70, and Rabih). The sub sub plots also incorporated a seed stimulation treatment with hydrogen peroxide at a concentration of 9 ml l-1 , additionally, two control treatments were included: seed priming with distilled water only and dry seeds. The results showed that sorghum seeds stimulated with hydrogen peroxide gave highest averages of weight of 500 grain and grain yield . The second planting date 25/3/2023 gave highest averages of number of grains in the head (head - 1) and grain yield . Buhooth-70 cultivar gave highest averages of weight of 500 grain and grain yield. The interaction of seeds stimulated with hydrogen peroxide and planted on first planting 11/3/2023 showed significant superiority in traits weight of 500 grain and grain yield. Buhooth-70 cultivar seeds stimulated with hydrogen peroxide give the highest averages for traits weight of 500 grain and grain yield .

Keywords: *Seed Soaking, Yield Traits , Hydrogen Peroxide, Wide Environmental Rang.*

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Introduction

Seed stimulation has become a common method for reducing the time between seed planting and seedling emergence, as it leads to acceleration and uniformity of germination and field emergence, as well as improving field establishment (obtaining optimal plant density) under a wide range of environmental conditions, and reducing bush competition and the damage caused by other agricultural pests. Early and uniform maturity, which results in reducing losses during harvest and thus increasing yield, as well as improving the vitality of relatively deteriorated seeds. This technique is based on slowly imbibing water, without germination occurring, meaning that cell division and elongation are stopped during the activation process[1,2,3,4,5]

The general purpose of stimulation is to partially moisten the seeds to the point at which metabolic activities begin before germination (without germination occurring, i.e. before radicle emergence) and then re-dry the seeds close to their original weight. This method is effective for improving seed germination and increasing yields for a wide range of crops [6,7], such as wheat, yellow corn, rice, sunflower, and pearl millet, as they were higher in speed and rate of emergence, had higher density, stronger growth, earlier flowering and maturity, and higher yields compared to non-stimulated seeds in India, Nepal, Pakistan, Zimbabwe, and Botswana for field experiments [3,8,9,12,13]

The cultivation and development of sorghum in Iraq faces a number of problems, perhaps the most prominent of which is weak seed germination and heterogeneous growth, which is a major reason for the decline in yield as a result of the plant being exposed to various biotic and abiotic stresses[10,11]. It has become clear that rapid seed germination and a high percentage determine good field establishment in field, which results in an increase in yield. Rapid and homogeneous germination is important in giving a high yield compared to slow and inconsistent seed germination, accompanied by weak emergence of seedlings as a result of weak seed vigor. From this principle, seed stimulation technology has been developed. Seed priming to improve the performance of the seed during the germination and emergence process to stimulate rapid and homogeneous germination and emergence[12,14] .

This technology has given good results in improving the germination and emergence properties of seeds of different types of field crops [14,15], and as a result of the existence of a real problem in the germination and emergence of sorghum seeds represented by the large gap between the rates of laboratory germination and field germination [3,15,16}, and since this problem still exists despite attempts to find solutions to it[17] . this research was carried out

with the aim of improving the germination properties of seeds of different varieties of sorghum and increasing and accelerating their germination rate in the field by treating them with some The aim of this research is to understand the effect of seed stimulation treatments with hydrogen peroxide on the yield traits of sorghum varieties cultivated within different planting periods .

Materials and Methods

A field experiment was conducted during the spring season of the 2023 agricultural season in the agricultural fields affiliated with the Agricultural Research Department – Ministry of Agriculture – Abu Ghraib. The experiment was designed using a Randomized Complete Block Design (RCBD) with three replicates and three factors. The main plots were allocated for three planting dates (11/3, and 25/3). The sub plots included three cultivars of sorghum (Al-Khayr, Research 70, and Rabeh). The sub-sub plots also incorporated a seed stimulation treatment with hydrogen peroxide at a concentration of 9 ml l^{-1}. Additionally, two control treatments were included: seed stimulation with distilled water only and dry seeds. a practical sample was taken from the compound sample of sorghum seeds cultivars , after that it was sterilized with 1% sodium hypochloride solution for three minutes, then washed with distilled water and left on blotting paper to air dry in the laboratory for 6 hours [18,19,20,] , after which the process of seeds priming was carried out by soaking the seeds with the concentrations of the hydrogen peroxide at a concentration of 9 ml l-1 for 24 hours before planting , and after the end of the stimulation periods, the seeds were air-dried in the laboratory for 12 hours, after that the seeds planted in two different planting dates, and measurements were taken for the following yield traits :

1. Weight of 500 grains (gm):

The heads of the five plants of each experimental unit were taken, their inflorescences were studied, and 500 seeds were counted, then they were weighed with a sensitive balance to two decimal places.

2. Number of grains in the head (head - 1):

After weighing the entire grains of the five plants, plus the weight of 500 grains, extract the number of grains for the five plants using ratio and proportion, then extract the average number of grains per plant by dividing the result by five.

3. Grain yield (ton ha-1):

It was calculated from harvesting 6 plants from the middle lines and extracted by multiplying the average plant yield x the actual plant density.

All data were analyzed statistically by analysis of variance (ANOVA) using Genstat software and averages of the main effect and their interactions were compared using the least significant difference test (LSD) at 5 % level of probability [21].

The results and discussion

1. Weight of 500 grain (gm):

Seedling establishment is one of the critical stages in the production of field crops, and it is directly related to the high quality of seeds, which is one of the basic requirements for successful field establishment, as low seed viability and low embryo strength contribute to reducing the growth and productivity of crops, especially crops that face some problems. When planting, which leads to a decrease in productivity, such as the sorghum [22,23,24,25] .

Table (1) indicates significant differences for all study factors and their interactions. The first planting date (D1) showed significant superiority was gave the highest weight of 500 grain average 12.11 gm , while the second date (D2) gave the lowest average 11.38 gm . The superiority of the first planting date may be attributed to the favorable environmental factors, such as temperature and light exposure, that the plants experienced during this date. This positively reflected on improving the synthesis of essential compounds in the chlorophyll biosynthesis pathway. The optimal environmental conditions that the plants experienced in the first date contributed to accelerating the biological synthesis process of chlorophyll by facilitating the respiratory process and electron transport in the respiratory chain. Their role was effective in increasing the rates of photosynthetic synthesis associated with an increase in chlorophyll content in the leaves and maintaining the structural integrity of chloroplasts[26,27].

Table 1. Effect of seed stimulation treatments and sowing dates on weight of 500 grain (gm)

Planting Date (D)	Variety (V)	stimulation treatments (S)			Mean (D x V)
		Dry	Distilled water	hydrogen peroxide	
D1	Al-Khair	9.76	10.93	13.13	11.27
	Rabih	8.63	9.72	11.52	9.96
	Buhooth-70	12.99	15.06	17.28	15.11
D2	Al-Khair	8.61	10.10	11.44	10.05

	Rabih	9.68	11.38	13.32	11.46
	Buhooth-70	10.84	12.84	14.21	12.63
Lsd 0.05		0.54			0.39
D x S					
Planting Date (D)		**Dry**	**Distilled water**	**hydrogen peroxide**	**Mean (D)**
D1		10.46	11.90	13.98	12.11
D2		9.71	11.44	12.99	11.38
Lsd 0.05		0.27			0.27
V x S					
Variety (V)		**Dry**	**Distilled water**	**hydrogen peroxide**	**Mean (V)**
Al-Khair		9.18	10.52	12.29	10.66
Rabih		9.16	10.55	12.42	10.71
Buhooth-70		11.91	13.95	15.74	13.87
Lsd 0.05		0.41			0.32
Mean (S)		10.08	11.67	13.48	
Lsd 0.05		0.21			

Superiority the Buhooth-70 cultivar was statistically significant, giving the highest Weight of 500 grain average 13.87, while the Al-Khair cultivar gave the lowest average 10.66 . The difference in Weight of 500 grain between varieties may primarily be due to genetic variation in this trait, as well as differences in their response to environmental growth factors[32,33].

Regarding seed stimulation treatments, Table (1) shows the superiority of hydrogen peroxide treatment achieving the highest Weight of 500 grain average 13.48 . These results confirm the crucial role of seed stimulation treatments, especially the hydrogen peroxide treatment, and their positive impact on increasing cell division and elongation of cells involved

in the formation of embryonic axes. This led to the development of vigour seedlings that grow and evolve better, positively affecting the vegetative growth, leading to an increase in the products of photosynthesis and the provision of assimilates, thus increasing the total dry matter yield and Weight of 500 grain [8,11,29].

The interaction effect of seeds stimulated with hydrogen peroxide and planted in the first date had a significant impact on the plant height trait, resulting in the highest average 13.98 gm. In contrast, dry seeds planted in the second date gave the lowest average 9.71 gm

The superiority of the dual interaction for Buhooth-70 seeds stimulated with hydrogen peroxide was significant, achieving the highest average 15.74 gm . Meanwhile, dry seeds of the Rabih cultivar gave the lowest average 9.16 gm .

Buhooth-70 cultivar planted in the first date, showed superiority by giving the highest average 15.11 gm. Conversely, Rabih variety, when planted in the first date, gave the lowest average 9.69 gm.

The triple interaction effect for the Buhooth-70 cultivar that planted in the first date and stimulated with hydrogen peroxide, gave superiority by achieving the highest average 17.28 gm . In contrast, the triple interaction for the Rabih that planted in the first date, and with seeds left without stimulation (dry seeds), gave the lowest average 8.63 gm .

2. Number of grains in the head (head - 1):

Table (2) indicates significant differences for all study factors and their interactions. The second planting date (D2) showed significant superiority was gave the highest number of grains in the head average 2501 grian , while the first date (D1) gave the lowest average 2488.2 grian . The reason for the superiority of the second timing in this trait may be attributed to the longer period from planting to maturity, providing an extended opportunity for cell division and elongation planting date and may be attributed to the favorable environmental factors, such as temperature and light exposure, that the plants experienced during this date. This positively reflected on improving the synthesis of essential compounds in the chlorophyll biosynthesis pathway. The optimal environmental conditions that the plants experienced in the second date contributed to accelerating the biological synthesis process of chlorophyll by facilitating the respiratory process and electron transport in the respiratory chain. Their role was effective in increasing the rates of photosynthetic synthesis associated with an increase in chlorophyll content in the leaves and maintaining the structural integrity of chloroplasts and increase number of grains in the head [33,24].

Table 2. Effect of seed stimulation treatments and sowing dates on number of grains in the head (head - 1)

Planting Date (D)	Variety (V)	stimulation treatments (S)			Mean (D x V)
		Dry	Distilled water	hydrogen peroxide	
D1	Al-Khair	2437.3	2455.0	2420.0	2437.4
	Rabih	2621.7	2627.7	2605.0	2618.1
	Buhooth-70	2414.3	2420.7	2391.7	2408.9
D2	Al-Khair	2435.3	2444.3	2420.3	2433.3
	Rabih	2644.3	2655.0	2635.0	2644.8
	Buhooth-70	2432.3	2431.7	2414.0	2426.0
Lsd 0.05		6.1			5.2
D x S					
Planting Date (D)		Dry	Distilled water	hydrogen peroxide	Mean (D)
D1		2491.1	2501.1	2472.2	2488.2
D2		2504.0	2510.3	2489.8	2501.4
Lsd 0.05		3.4			4.4
V x S					
Variety (V)		Dry	Distilled water	hydrogen peroxide	Mean (V)
Al-Khair		2436.3	2449.7	2420.2	2435.4
Rabih		2633.0	2641.3	2620.0	2631.4
Buhooth-70		2423.3	2426.2	2402.8	2417.4
Lsd 0.05		4.7			4.2
Mean (S)		2497.6	2505.7	2481.0	
Lsd 0.05		1.9			

Superiority the Rabih cultivar was statistically significant, giving the highest number of grains in the head 2631.4 grain , while the Buhooth-70 cultivar gave the lowest average 2417.4 grain. The difference in 2417.4 between varieties may primarily be due to genetic variation in this trait, as well as differences in their response to environmental growth factors[1,17,24].

Regarding seed stimulation treatments, Table (2) shows the superiority of distilled water treatment achieving the highest number of grains in the head average 2505.7 gm . This result confirms the significant role of seed stimulation treatments in the cell division and expansion process. They provide the ideal expansion for the cell wall necessary for both growth and division. Additionally, pre-sowing seed stimulation can enhance the capacity to increase DNA and RNA activity. This activity contributes to cell expansion, growth, division, and cellular differentiation. In contrast, hydrogen peroxide gave the lowest average 2481.0 grain[3,10,11,25,29] .

The interaction effect of seeds stimulated with distilled water and planted in the second date had a significant impact on the number of grains in the head resulting in the highest average 2510.3 grain. In contrast, dry seeds planted in the first date gave the lowest average 2472.2 grain .

The superiority of the dual interaction for Rabih stimulated with distilled water was significant, achieving the highest average 2641.3 grain. Meanwhile, hydrogen peroxide of the Buhooth-70 cultivar gave the lowest average 2402.8 grain .

Rabih cultivar planted in the second date, showed superiority by giving the highest average 2644.8 grain . Conversely, Buhooth-70 variety, when planted in the first date, gave the lowest average 2408.9 grain .

The triple interaction effect for the Rabih cultivar that planted in the second date and stimulated with hydrogen peroxide, gave superiority by achieving the highest average 2655.0 grain . In contrast, the triple interaction for the Buhooth-70 that planted in the first date, and seeds stimulated with hydrogen peroxide, gave the lowest average 2391.7 grain .

3. Grain yield (ton ha-1) :

Table (3) indicates significant differences for all study factors and their interactions. The first planting date (D1) showed significant superiority was gave the grain yield average 3.196 ton ha^{-1}, while the second date (D2) gave the lowest average 3.036 ton ha^{-1}. The reason for the superiority of the second timing in this trait may be attributed to the longer period from planting

to maturity, providing an extended opportunity for cell division and elongation, thus contributing to increased grain yield [1,24,30 , 31, 32].

Table 3. Effect of seed stimulation treatments and sowing dates on grain yield (ton ha-1)

Planting Date (D)	Variety (V)	stimulation treatments (S)			Mean (D x V)
		Dry	Distilled water	hydrogen peroxide	
D1	Al-Khair	2.536	2.862	3.389	2.929
	Rabih	2.413	2.724	3.202	2.779
	Buhooth-70	3.345	3.888	4.408	3.880
D2	Al-Khair	2.236	2.633	2.954	2.608
	Rabih	2.731	3.223	3.745	3.233
	Buhooth-70	2.812	3.33	3.658	3.267
Lsd 0.05		0.146			0.107
D x S					
Planting Date (D)		Dry	Distilled water	hydrogen peroxide	Mean (D)
D1		2.765	3.158	3.666	3.196
D2		2.593	3.062	3.453	3.036
Lsd 0.05		N.S			0.075
V x S					
Variety (V)		Dry	Distilled water	hydrogen peroxide	Mean (V)
Al-Khair		2.386	2.748	3.171	2.769
Rabih		2.572	2.973	3.473	3.006
Buhooth-70		3.079	3.609	4.033	3.573
Lsd 0.05		N.S			0.089
Mean (S)		2.679	3.110	3.559	
Lsd 0.05		0.055			

Superiority the Buhooth-70 cultivar was statistically significant, giving the highest grain yield average 3.573 ton ha^{-1}, while the Al-Khair cultivar gave the lowest average 2.769 ton ha^{-1}. The difference in grain yield between varieties may primarily be due to genetic variation in this trait, as well as differences in their response to environmental growth factors [1,17,24].

Regarding seed stimulation treatments, Table (3) shows the superiority of both hydrogen peroxide treatment achieving the highest grain yield average 3.559 ton ha^{-1} . The reason may be that the seed stimulation treatment with hydrogen peroxide achieved the highest average seed weight of 500 seeds (Table 1). In contrast, dry seeds gave the lowest average 2.679 ton ha^{-1} [8,10,35] .

The dual interaction effect of stimulating seeds with distilled water and planting in the first date was statistically significant for the grain yield trait gave the highest average reaching 3.666 ton ha^{-1} , while dry seeds planted in the second date gave the lowest average 2.593 ton ha^{-1}

The dual interaction superiority of Buhooth-70 cultivar seeds stimulated with hydrogen peroxide was statistically significant, achieving the highest average 4.033 ton ha^{-1} , while dry seeds of Al-Khair cultivar gave the lowest average 2.386 ton ha^{-1}

The superiority of Buhooth-70 cultivar planted in the first date was statistically significant, gave the highest chlorophyll content average of 3.880 ton ha^{-1} , while Al-Khair variety planted in the second date gave the lowest average 83.20 ton ha^{-1} .

The triple interaction superiority of the Buhooth-70 planted in the first date, stimulated with hydrogen peroxide, was statistically significant and It achieved the highest average, reaching 4.408 ton ha^{-1} , while the triple interaction for the Al-Khair variety planted in the second date, with seeds left without stimulation (dry seeds), gave the lowest average 2.236 ton ha^{-1}.

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Diagnosis of some fungi in corn seeds and their control using Trichoderma harzianum

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Abstract

Corn is one of the most important agricultural crops globally, which is susceptible to a wide range of fungal species that cause cone rot, often soil and seed-borne pathogens. The studies on the isolation of fungi from corn ears revealed the presence of several fungal genera, including Fusarium spp. (35 %), Penicillium spp. (20%), Aspergillus spp. (33. 9%), Alternaria spp. (8.3%), and Mucor spp. (2.8%). These identifications were based on morphological parameters at the micro and macro level of the fungal colonies. This also evaluated Trichoderma harzianum, a species recognized for its efficiency in controlling the different forms of soil borne diseases . An in vitro analysis of T. harzianum discovered sizable adversarial outcomes towards the pathogens, with boom inhibition charges of fifty eight%, 53mm, 45mm, 33mm and 20mm for Fusarium, Penicillium, Aspergillus, Alternaria, and Mucor, respectively, compared to a manage institution with a 0.00% boom charge for the pathogens. Overall, biological manipulate is important for shielding crops towards diseases. The outcomes of this take a look at suggest for further comprehensive research into the potential programs of this organic control approach in agricultural practices.

Keywords: *Biocontrol, Trichoderma Harzianum, Corn Fungi, Soil-Borne Pathogens, Fungal Inhibition.*

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Introduction

Corn is considered one of the most essential grain crops globally, frequently because of its various packages. It serves as a direct meals source for human beings and is also a essential component in animal feed. In addition to its sizable industrial uses, corn ranks because the third largest crop inside the global, attracting full-size attention from researchers. Its importance is attributed to its excessive production ability, adaptability to distinctive environmental conditions, and the possibility of multiple harvests inside a single (Shevchenko et al. 2021). Corn is defined by its nutritional composition, which consists of 4% oil and 82% carbohydrates, thus making it a staple of sustenance for millions around the world. A study shows that over 80% of the consumed calories worldwide come from crops produced in agriculture, particularly grains, one of which is corn (Yongfeng & Jay-lin 2016). Ingredients used in animal feed, such as cereals, are prone to fungal infections that can result in the production of mycotoxins. Favorable environmental conditions for fungal growth can contaminate cereal crops, food products, and animal feed, leading to huge annual losses and economic problems. In the United States and Canada alone, mycotoxin contamination in agricultural products and food amounts to economic damage in the billions of dollars yearly, whereas the poultry industry worldwide suffers losses in the millions annually from the same kind of contamination problems (Haider & Hussein 2022). A body of research has proven the effectiveness of biological control practices using Trichoderma spp. and other microorganisms, and this has been reaffirmed in more recent scholarship (Elshahawy et al. 2018). A well-studied strategy involves the use of plant growth-promoting rhizobacteria as an organic manipulation mechanism for maize rhizosphere-related Late wilt a disease,(LWD) which may

additionally contribute to advanced plant health, as noted by Ghazy et al. (2020). Another method is the application of species from the Trichoderma genus to address LWD. Members of this genus are able to form mutual endophytic relationships with numerous plant species (Harman et al., 2004). Several species have been developed as biocontrol agents to target fungal phytopathogens (Harman, 2006). in the treatment of fungal plant diseases. Recent research using Elshahawa et al. (2018) revealed that extracts from the microalga Chlorella vulgaris, when mixed with Trichoderma species along with T. Virens and T. Koningii, effectively controlled soil-borne pathogens in both greenhouse and discipline environments.

Materials and Method

Sample Collection

A total of 50 corn ears samples were collected from different agricultural fields in AL-Alam Province, Iraq. The samples were immediately transferred to the laboratory and kept at 4°C for the analysis of fungi.

Isolation and diagnosis of pathogens

In isolating fungal pathogens, a total of 100 corn seeds per sample of collected samples were randomly chosen. Disinfection of these seeds was done with 2% sodium chlorate solution for 2 minutes and followed by two rinses using sterile distilled water to ensure all disinfectant residues are washed off. After drying with sterile filter paper, the seeds were placed using sterile forceps in 9-cm petri dishes on 20 mL of pre-prepared potato dextrose agar (PDA) according to Hoching and Pitt (1991). The isolated fungi were then identified using the taxonomic keys by Seifert.(١٩٩٦)

Morphological identification of fungi

The identification of the isolated fungi was conducted through an examination of colony morphology and microscopic evaluation. To facilitate species identification, fungal colonies were transferred to potato dextrose agar (PDA) slants, as outlined by Leslie and Summerell.(٢٠٠٦)

Results And Disscution

The results shown in Table 1 reveal that researchers identified 100 fungal isolates based on cultural and morphological features obtained from 100 corn samples. The fungal genera obtained are Fusarium spp. (35 %), Penicillium spp. (20 %), Aspergillus spp. (34%), Alternaria spp. (8%) and Mucor spp (3). This is in accordance to the findings of Similar observations were reported by Mohammed et al. (2015); The most common genera isolated were Aspergillus, Penicillium and Fusarium. Among the Aspergillus spp., A. flavus, A. parasiticus, A. niger and A. ochraceus were identified.

Table 1. Most fungal species have been identified in corn samples

Isolates	No.	Percentage %
Fusarium spp.	35	35
Aspergillus spp.	34	34
Penicillium spp.	20	20
Alternaria spp.	8	8
Mucor spp.	3	3

The results in Figure 1 show that T. harzianum fungus inhibited pathogenic fungi strongly compared to the control treatment containing only pathogenic fungi. T. harzianum was especially effective at reducing the growth of the harmful fungi Penicillium spp and Fusarium spp, with inhibition values of 58 mm and 53 mm, respectively. On the other hand, the pathogenic fungus Mucor sp showed the least inhibition of only 20 mm.

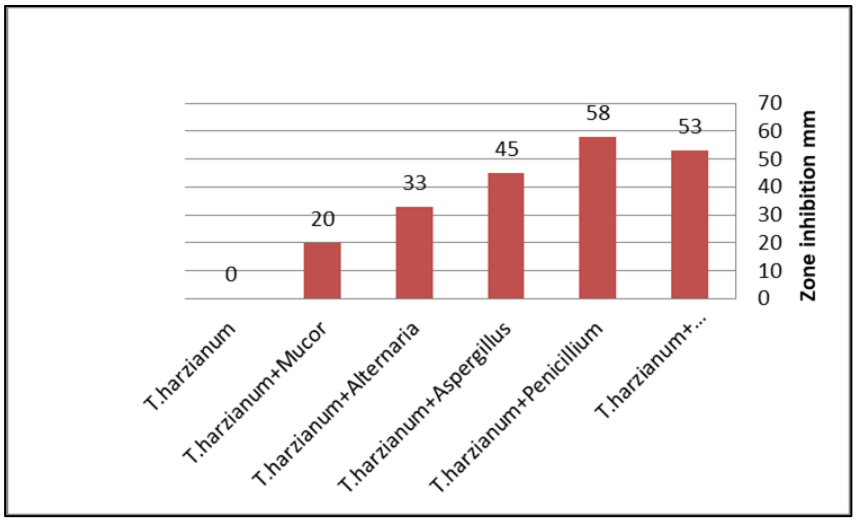

Figure 1. Inhibition of growth mycelial of pathogenic fungi by T. harzianum

The high ability of Trichoderma to adapt to the environment, together with fast growth and reproduction rates, enables it to take hold of nutrients and space in the rhizosphere of plants very effectively. The microorganism consumes oxygen in the air as it suppresses the spread of pathogenic fungi infecting plants (Panchalingam et al., 2022; Xu et al., 2022). Consequently, it causes them to starve by depriving them of nutrients and hampers their growth and reproduction (Guo et al., 2019).

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Isolation and Identification of plant promoting rhizobacteria (PGPR) from Solanum tuberosum in Gypsiferous soil

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Abstract

The potato plant is most famous and widely used agricultural crops in the world. Solanum tuberosum are used in many dishes, whether fried, boiled or mashed, and are a staple food in many countries. They also contain good amounts of carbohydrates, fiber, vitamins such as vitamin C, and minerals such as magnesium.

nine isolated were identified from the rhizosphere of Solanum tuberosum roots by using morphological and biochemical characterization and Polymerase Chain Reaction technology (PCR),

The identified bacterial species were as follows: two isolates (A1, A2) belonged to *Bacillus subtilis*, four isolates (W1, W2, 8, R) belonged to *Pseudomonas fluorescens*, and three isolates (O, G, R1) were identified as *Ochrobactrum sp*.

Several of plant growth promoting properties were measured like indole-3- acetic acid production, Siderophore, nitrogen fixation, phosphate solubility and hydrogen cyanide production.

Six isolates were capable of Siderophores Production, with isolates (W1, W2) showing the highest Production. eight isolates were able to dissolve inorganic phosphorus, with isolate W1 exhibiting the highest dissolution, followed by isolate A1, with dissolution zones around the colonies measuring 9 and 8 mm, respectively.

Six isolates were capable of nitrogen fixation, while isolates (8, R, G) were unable to fix nitrogen.

Six isolates (A1, W1, W2, 8, O, G) produced HCN. The W1 isolate Showed the highest IAA production, hold out 16.28 $\mu g.mL^{-1}$, followed by isolate W2 with a production of 14.88 $\mu g.mL^{-1}$. The lowest IAA production was observed in isolate G, which 2.83 $\mu g.mL^{-1}$.

Therefore, this study indicates that these local species can serve as a basis for effective bio fertilizers that enhance plant growth with increase crop production in gypsiferous soil.

Hence, controlled release of bio fertilizers is an effective and advanced maintain sustainable agriculture yield.

Keyword: *Solanum Turberosum, PGPR, Gypsiferous Soil*.

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Introduction

Solanum tuberosum L. is one of the majority main crops globally. It belongs to the Solanaceae family, the same family that includes tomatoes, eggplants, and peppers. Potato is considered a strategic crop in many agricultural economies (FAO, 2020), contributing to food security and increasing agricultural income. It is a significant food source in many developing countries. In adding to its fresh using up, potatoes are used in many industrial products like chips, and flour. The canned potato industry significantly contributes to the global economy through exports to foreign markets (Monneveux et al., 2013; Nunes-Nesi et al., 2010).

Potatoes are rich in carbohydrates, especially starch, making them a primary energy source. They also contain a group of vitamins and metal, like vitamin C, vitamin B6, and potassium. According to several studies, consuming potatoes is an effective way to improve nutrition and enhance immunity due to their antioxidant content (Zhang et al., 2017). Additionally, potatoes contain dietary fiber, which contributes to improving digestive health.

Plant Growth-Promoting Rhizobacteria (PGPR) is a main research topic in sustainable agriculture, offering an environmentally friendly and sustainable alternative to enhance crop growth **(**Ryan et al.,2008). These bacteria, which naturally exist in the soil, engage in mutual interplay with plants with plants that be able to stimulate their growing and improve their resistance to diseases and harsh environmental conditions (Khan etal., 2020). The role of PGPR extends to a broad range of biological mechanisms, which includes enhancing nutrient absorption, nitrogen fixation, secretion of enzymes and plant hormones, inhibiting the growth of pathogenic fungi, and increasing plants' ability to withstand environmental changes (Glick, 2012). Examples of PGPR include *Azospirillum* (Bashan et al., 2014), *Bacillus*, *Pseudomonas*, and *Rhizobium* (Wang et al., 2021; Lugtenberg and Kamilova, 2009).

With the increasing global population and climate change, potato farmers face significant challenges, including weather fluctuations, water scarcity, and the growing prevalence of diseases and pests. The potato industry needs new technologies to improve productivity while maintaining environmental sustainability. Among these technologies is the use of plant growth-promoting bacteria as a sustainable option chemical fertilizers and pesticides (Waaswa et al., 2022).

Materials and methods

Soil Sample Collection

Sample of soil was collected from the potato plants rhizosphere in the fields of the College of Agriculture, Tikrit University, in Salah-aldin Governorate, in August 2024. The sample was taken from three different sites around the root zone at a depth of 20 cm. The samples were put in plastic cans and transported to the laboratory, where they were stored in a refrigerator at 4°C until the isolation process was conducted.

Isolation of PGPR Bacteria from the Potato Rhizosphere

Bacteria were isolated from the rhizosphere soil of potato plants using the serial dilution method. A 1g sample of soil was append to 9 ml of D.W. Serial dilutions were then performed until a dilution of 10-10 was achieved. A 1 ml aliquot of every one dilution was lay out onto a Petri dish containing N.A medium, and the dishes were incubated at 28°C for 72 hours. After incubation, different colonies were selected and subculture onto slanted Nutrient Agar (NA) medium until pure colonies were obtained. A total of 10 pure isolates were successfully obtained.

Identification of Bacterial Isolates

The chemical and morphological characterization of the isolates was executed through standard tests (Gram staining, Catalase, Oxidase, Motility), according to Bergey's Manual Bacteriology (2005).

Molecular identification of bacteria isolates

DNA extraction

DNA was take out from cultures, young and actively activity growth utilizing Chelex®100 kit. The colony was move into a 1 ml Eppendorf tube and 200 µL of Chelex®100 added. and heated at 95°C for 8 minute. The crude DNA suspension was isolate from cellular fragments by centrifugation at 12,000×g for 5 minute. Then taken away and placed into 0.5 ml tubes.

PCR Amplification

The primer pair 16sRNA F: GTG TAG CGG TGA AAT GCG R: ACG GGC GGT GTG TAC AA using for amplification .PCR amplification was performed in a total volume of 25µl.

mixture consist of 10 µL of master mix, primers 1µL of the forward, and 1µL of the reverse, 3 µL of distilled water and 5 µL of genomic DNA,) denaturation at 95 °C for 3 min followed by 35 cycles of 95 °C for 1min, 58°C for 1 min and 72 °C for 1 min, with final extension at 72 °C for 7 min. PCR results were isolate on 2 % agarose gels hold rad safe under UV light.

Determination of Plant Growth Promotion Properties for Isolated Strains

Estimation of Indole-3-Acetic Acid (IAA) Product

Indole was estimated according to described by Glickmann & Dessaux .,1995. A nutrient broth medium (N.B) was ready and 100 mL of this medium was placed into 250 mL flasks. Then, 0.2% tryptophan was added to each flask, and the medium was sterilized by autoclave at 121°C and 1.5 bar for 15 minutes. After cooling, the flasks were inoculated with bacterial isolates. The flasks were brood on a shaking incubator at 27 ± 2°C / 24 hours. After that, 1.5 mL of the bacterial culture was transferred into test tubes, and centrifugation was performed at 7000 rpm for 7 minutes using a centrifuge. One milliliter of the supernatant was transferred into tubes, and 2 mL of Reagent A (2 mL FeCl3 mixed with 98 mL perchloric acid at 35% concentration) was added. The test tubes were left for 20-25 minutes to allow the reaction and color change to occur. Absorbance was measured by Spectrophotometer on 535nm wavelength. The IAA concentrations were number utilize the curve of indole-3-acetic acid.

The Capacity of isolates to dissolve inorganic phosphorus from the TCP compound

Pikovskaya (1948) method was tested. inoculated the bacterial onto plates containing PVK medium (Glucose 10 g, (NH4)2SO4 0.5g, Yeast extract 0.5g., MgSO4·7H2O 0.1g,, KCl 0.2g, MnSO4·H2O 0.002g., Ca3(PO4)5 5g, FeSO4·7H2O 0.002g, Agar 15g) then incubated at 30°C for 5 day. The ability of microorganisms to dissolve calcium triphosphate was determined based on the forming of a transparent zone on every side the bacterial colony and the extent of dissolution, according to the following equation:

Dissolution diameter = Colony diameter + transparent zone diameter / Colony diameter.

isolates ability to nitrogen-fixing

the nitrogen fixation ability for isolates was determined according to Park et al. (2005) using Petri dishes containing a nitrogen-free solid Burke medium (0.05g Na2SO4, 10g glucose, 0.2g CaCl2, 0.41g KH2PO4, 0.52g K2HPO4., 0.0025g Na2MoO4.2H2O., 0.1g MgSO4.7H2O, 0.005g FeSO4.7H2O, 18g Agar, 1L D.W). The bacteria were cultured then incubated at 30°C

for one day,3 time periods were used to determine nitrogen fixation activity (+++: growth after six h, ++: growth after twelves h, +: growth after one day).

The Capacity of strain to Siderophores Production

According to the Payne (1980); test medium was prepared. prepared One liter of nutrient agar medium and adjusting the pH to 7.2. The medium was then sterilized using an autoclave and let to cool. Next, 2 mg of 2,2-dipyridyl dissolved in 10 milliliters of sterilized distilled water was added. The solution was poured into sterile Petri dishes and allowed to solidify. The isolates was streaked onto the plates and incubated at 30°C for two day. The occurrence of growth on these plates is an indication of ability of the bacteria to output siderophores.

Evaluation of HCN-Producing Isolates

The bacteria were inoculated onto NA medium, and filter paper drenched with picric acid (0.5% picric acid, 2%Sodium Carbonate) were put on the border of Petri dishes. The Petri dishes were close tightly with Parafilm and incubated at 28 ± 2°C / 4 days. after incubation, the yellow filter paper strips turned brown, this considered a positive result. Three-time period were defined for the color change (+++: color change after 6 h, ++: change after 12 h, +: change after 24 h) (Deshwal and Kumar, 2013).

Result and discussion

Diagnoses of bacteria isolates

Nine bacterial isolates were get from samples of soil collected from the rhizosphere of potato plant growth in gypsum soil at the fields of the College of Agriculture, University of Tikrit, Iraq. The isolates were identified using both traditional (Table 1) and molecular (Figure 1) methods. The identified bacterial species were as follows: two isolates (A1, A2) belonged to Bacillus subtilis, four isolates (W1, W2, 8, R) belonged to Pseudomonas fluorescens, and three isolates (O, G, R1) were identified as Ochrobactrum sp.

The results of the vino type and biochemical characteristics tests were showed in Table 1. Among all isolates, two isolates (A1, A2) showed a positive reaction to the Gram stain. Oxidase, catalase, and motility tests were positive for all nine isolates.

Table 1. vino type and biochemical test of bacterial strain in the rhizosphere

Isolates	Gram reaction	Catalase	Oxidase	Motility
B, subtilis	+	+	+	+
B. subtilis	+	+	+	+
P. fluorescens	_	+	+	+
P. fluorescens	_	+	+	+
P. fluorescens	_	+	+	+
P. fluorescens	_	+	+	+
ochrobactrum sp	_	+	+	+
ochrobactrum sp	_	+	+	+
ochrobactrum sp	_	+	+	+

Note: +, positive; -, negative

Molecular identification is an important and sensitive tool that confirms the morphological and biochemical identification of bacteria. It is considered an efficient taxonomic method for determining the origin and phylogeny of bacterial species (Drancourt et al., 2000). Due to the efficiency and sensitivity of molecular diagnostics in detecting bacterial species using specialized primers, it can become a reference for bacterial classification (Clarridge, 2004).

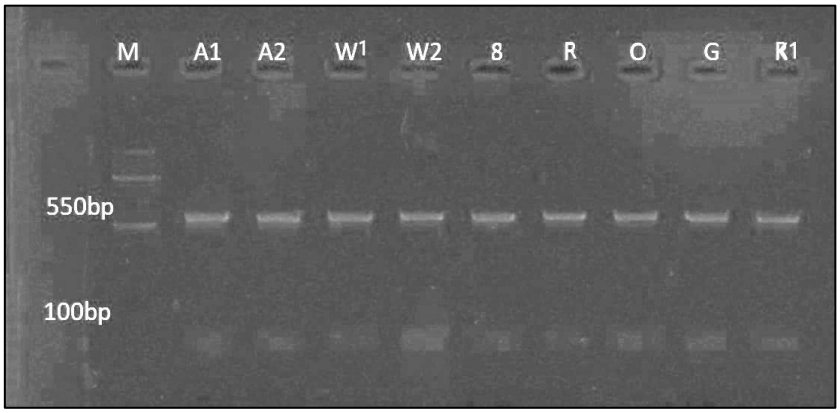

Figure 1. PCR products

M: marker. A1,A2 Bacillus subtilis. W1,W2, 8, R Pseudomonas fluorescens. O,G,R1 ochrobactrum sp.

Plant Growth Promotion Properties for Isolated Strains

plant growth-promoting rhizobacteria (PGPR), benefit plants by producing IAA, siderophores, HCN, dissolving phosphate, and fixing nitrogen. In the pesent study, nine bacterial isolates were tested in the laboratory to determine the characteristics of the isolated strains that promote plant growth. As shown in Table (2), six isolates were capable of Siderophores Production, with isolates (W1, W2) showing the highest Production.

From the same table, the capacity of the bacterial isolates to melt inorganic phosphorus after 7 days of cultivation on the PVK medium is observed. The table shows that eight isolates were able to dissolve inorganic phosphorus, with isolate W1 exhibiting the highest dissolution, followed by isolate A1, with dissolution zones around the colonies measuring 9 and 8 mm, respectively.

Six isolates were capable of nitrogen fixation, while isolates (8, R, G) were unable to fix nitrogen.

Six isolates (A1, W1, W2, 8, O, G) output HCN. The same table that there is variation in IAA production depending on the bacterial isolate. The W1 isolate show the highlevel IAA production, was 16.28 $\mu g.mL^{-1}$, followed by isolate W2 with a production of 14.88 $\mu g.mL^{-1}$. The lowest IAA production was observed in isolate G, which 2.83 $\mu g.mL^{-1}$.

Table 2. divination of plant grown promoting properties of isolate

Isolates	Siderophores Production*	P Solubilization	Biological N Fixation**	HCN Production***	IAA Production
B. subtilis	++	8	+++	+	11.94
B, subtilis	_	6	++	-	7.61
P.fluorescens	+++	9	++	+++	16.28
P. fluorescens	+++	7	+	+	14.88
P.fluorescens	++	6	_	+	8.03
P, fluorescens	+	2	_	_	4.3

ochrobactrum sp.	_	0	+	++	7.02
ochrobactrum sp.	_	2	_	+	2.83
ochrobactrum sp.	++	7	+	_	6.06

***(- No ability , + Low ability , ++ Medium ability, +++ High ability)**

**** (+++: growth after 6 h, ++: growth after 12 h, +: growth after 24 h)**

***** (+++: color exchange later six h, ++: color exchange later twelves h, +: color ex-change later one days)**

the plant rhizosphere is the preferred environment for soil microorganisms due to the availability of nutrients. Plant Growth-Promoting Rhizobacteria (PGPR) settle plant roots and increase plant growth and development through a variety of mechanisms. These mechanisms include the production of plant hormones, biocontrol, phosphate solubilization, siderophore production, and increased absorption of mineral nutrients, all of which contribute to promoting plant growth (Manivannan etal., 2012).

Iron (Fe) cannot be utilized as a nutrient by plants caused it is low solubility in soil. Siderophores are little molecular weight organic complex produced by microorganisms that secrete Fe^{3+} ions. Microorganisms produce iron-binding compounds known as siderophores to survive in the rhizosphere. According to Syed et al. (2023), PGPR strains that produce siderophores stop the grown of plant disease by limit iron absorption, thereby assisting in the biocontrol of plant disease. Several studies have demonstrated the ability of PGPR bacteria to produce siderophores in various plants, including potatoes (Verma and Shahi, 2015), onions (Ahmed et al., 2016), and soybeans (Prasad, 2022) maize (Adjanohoun et al., 2011).

One of the methods to inhibit the development of pathogenic in the rhizosphere is the production of hydrogen cyanide (HCN), which blocks electron transport chains and energy sources in cells, leading to the death of pathogenic organisms (Admassie etal., 2020). In recent years, bacteria present in the rhizosphere utilized as biocontrol factors Because of its ability to produce hydrogen cyanide. (Cucu et al., 2019; Ting et al., 2014).

Indole-3-acetic acid (IAA) has a important role in plants, like cell elongation, division, and differentiation, it is considered the most important natural plant hormone. It is one of the key plant hormones and functions as an essential molecule in regulating plant growth (Yanti et al., 2022).

Phosphorus is an essential element that limits plant productivity after nitrogen, as plants cannot directly utilize phosphorus, which is abundantly available in an insoluble form in the soil. The capacity of bacteria to solubilize phosphate is attributed the organic and inorganic acids they produce. This process is considered one of the most important mechanisms agreed upon by most researchers, as it involves the solubilization of inorganic phosphate compounds. Most soil microorganisms perform this solubilization through the production of inorganic acids (such as sulfuric, and phosphoric) as well organic acids (including fulvic, humic, citric, oxalic, and licinic acids), which grow the dissolution of soil minerals (Silva etal., 2021; Elhosieny etal., 2023). Nitrogen is a necessary nutrient for whole living organisms. Some microorganisms fix atmospheric nitrogen, for the aid of the enzyme nitrogenase, converting it into a form that plants can utilize. Numerous studies have shown that PGPR enhances growth and productivity in various plants. Plants inoculated with PGPR exhibited increases in length of root and shoot, biomass, nitrogen content, with increases of up to 76% in shoot tissues and 32% in roots (Majeed etal. 2015).

Gupta etal (2021) injected onion plants with PGPR strains, which possess growth-promoting properties, and reported that these strains contributed to improvements in both growth and nutritional content.

This study is essential as, it explores the potential of PGPR as inoculants or bio fertilizers. It has been proven that this approach is an effective and environmentally friendly alternative to chemical fertilizers.

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Molecular Differentiation by PCR-RFLP Test of Infectious Laryngotracheitis virus Strains Isolated in Iraq

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Abstract

Background: Avian infectious laryngotracheitis (ILT) is a harsh respiratory sickness in chickens. Polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP) investigation can be effectively used to differentiate field and vaccine strains.

Objective: The aim of this study is to characterize Iraqi isolates by PCR and RFLP to explore the strain's variety.

Methodology: This study of infectious laryngotracheitis (ILT) is based on the molecular characterization of samples collected from 12-layer pullet farms in the suburbs of Baghdad - Iraq. A case history was taken for each flock, including the age of the chickens, clinical signs, mortality, vaccination programs, and any postmortem lesions. A commercial PCR detection kit was used to screen tracheal samples for ILTV.

Results: Four farms (33%) were positive, while four others tested positive in the nested PCR assay for the TK gene. PCR-RFLP for the TK gene and Hae III restriction enzymes produced digestion patterns for field-isolated and reference vaccine strains. Three ILT field isolates showed a digestion pattern of the PCR product to 500 and 400 bp, while the vaccine-controlled strains showed a non-digestive pattern at 1024 bp.

Conclusions: This study is the first trial in Iraq that could differentiate and report the field and vaccine strains.

Keywords: *ILTV, PCR-RFLP, Iraq.*

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Introduction

ILT is an extremely infectious viral disease that affects poultry, particularly chickens, worldwide. The disease, attributable to ILTV, poses important economic challenges to the poultry industry due to decreased production efficiency, increased mortality rates, and costs associated with control measures. Effective management of ILT relies on accurate diagnosis and strain differentiation, allowing for targeted control strategies [1, 2].

The acute form can be characterized by complicated breathing with expectoration of blood and inflamed orbital sinuses. The morbidity rate is high (up to 100%), while the mortality rate is variable, and there was a decrease in the production of eggs. The chronic form of ILT shows the usual respiratory signs [3].

Recent advancements in molecular biology have provided valuable tools for the detection and characterization of ILTV strains [4], understanding of genetic variations, epidemiology, and pathogenicity of ILTV isolates pass locally and contribute to explaining ILTV molecular basis of development of vaccine and pathogenicity. One such technique, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), offers a rapid and reliable method for differentiating ILTV strains based on genetic variations [5]; this approach holds promise for enhancing understanding of ILT epidemiology and facilitating more precise control measures [6].

Recent investigations showed that the virus could also be noticed in organs other than the respiratory tract, such as the spleen, heart, kidney, tongue, liver, thymus, pancreas, proventriculus, small intestine, duodenum, large intestine, cecum, bursa, and brain cecal tonsils [7, 8, 9]. The cause of the disease is the herpes virus (GaHV-1), the subfamily Alpha Herpes virginal of the family Herpes viridae [10]. The genome is linear double-stranded DNA [11].

In Iraq, where the poultry industry plays a crucial role in the economy and food security, ILT poses a significant threat. Understanding the diversity of ILTV strains circulating in the country is essential for implementing effective disease management strategies [12]. A recent study conducted in Iraq aimed to characterize ILTV strains using PCR-RFLP, shedding light on the genetic diversity of the virus and providing valuable insights for local control efforts [13]. meta-analysis of ILT helps in managing the density of breeding, enhancing immunization programs, and constantly observing viruses to avoid the prevalence of ILT [13, 14].

Molecular methods for identifying ILT are more sensitive than virus isolation and can detect acute and dormant infections [14]. The PCR technique combined with restriction

fragment length polymorphism (RFLP) analysis is an effective method for differentiating between field and vaccine strains [15].

The aim of this study is to characterize Iraqi isolates from layer chickens with acute infection from different areas around Baghdad-Iraq by PCR-RFLP to study the variety of the strains.

Materials and methods

Samples collection

Samples were collected from layer flocks showing respiratory clinical signs. The chickens were 29 weeks old, brown Loghman red breed with a mortality rate of 40-50% 35000 birds for batteries not vaccinated with the ILT vaccine, as well as four additional flocks containing 25000 layers, 18000 layers, 18000 layers, and 29000 layers also showed clinical respiratory signs with a mortality rate of 20-30%. The first was vaccinated with the attenuated ILT vaccine while the others were not vaccinated. From each flock, five to ten birds were euthanized, and the tracheas were collected under minimal aseptic conditions and stored at -70°Cuntil virus isolation and molecular characterization were carried out.

Isolation of the virus

Samples (tracheas) from five farms were homogenized and diluted to approximately one in ten in BPS (pH7), which contains penicillin and streptomycin, and then the samples were agitated vigorously. The suspension was centrifuged at 1500 rpm for 50 minutes, then 0.2 ml of the supernatant was inoculated into the chorioallantoic membrane (CAM) of 10-12 day old specific pathogen-free (SPF) embryonated chicken eggs, then incubated at 37°C for 3 to 6 days. The pocks were looked for on the harvested CAM

DNA extraction

Tracheas were homogenized, and viral DNA was extracted and purified using a kit (PROMEGA, USA) as described in the manufacturer's instructions. Briefly, the trachea was homogenized using a pestle and mortar with sand, then the sample was centrifuged, and the supernatant fluid was transferred to the DNAase-free 1.5mL PCR tube. A 600µL lysis buffer was added to the supernatant solution. The solution was incubated for 15 minutes at 60°C, then 3µL RNAase solution was added and incubated for 15 minutes at 37°C. Protein precipitation solution was then added and centrifuged at 14000 g for 10 minutes, then the supernatant was transferred into a new 1.5mL PCR tube, and 600µL of isopropanol solution was added and

centrifuged for 5 minutes at 14000g. After that, the DNA pellet with 70% ethanol was washed and centrifuged in step for 5 minutes at 14000 g. finally, the DNA pellet was re-suspended in DNA rehydration solution (TE buffer) supplied by the kit, and the DNA samples were stored at -70 °C.

Table 1. Special primer for full amplification of TK Gene

PCR assay	Primers	PCR product	Annealingtemp	Reference
TK gene one step of nested PCR TK op	F- CGGATCGATCGTATAGGCCAGCCTT R GCTCTAGACCACGCTCTCTCGAGTAA	1296	59°c	[19]
TK gene two step of nested PCR TK PD2	F- GTTCGAGAACGATGACTCC R GCATTGTAGCGCTCTACTG	1024	59°c	[15]

Polymerase chain reaction

A one-step PCR kit for the detection of infectious laryngotracheitis virus (Intron Biotechnology, Inc. Seoul, Korea) was used to amplify a 477bp, the PCR reaction was incubatedfor43 minutes at 94°C then 40 cyclesat 94°C for 30 seconds (denaturation), at 52°C for 30 sec (annealing) and at 72°C for 40 seconds (extension), with final incubation at 72°C for 5 minutes.The product was analyzed by agarose (1.5 %) gel electrophoresis and a band consistent with the expected size of 477bp.PCR amplification was performed using Promega Go Tag Green Master Mix (PROMEGA, USA), which contains 50 mM KCL, 1.5 mM $MgCl_2$, 0.1wt/vl. Triton X 100, 0.2mg of Bovine serum albumin (fraction IV; Sigma) per ml, and10 mM tris -HCl (pH 8.5), and the procedure carried out as follows:

The total volume of increased PCR was 25µL containing 12.5µL of Gotaq Green Master Mix, 2µL of reverse primers (10 p mol concentration primer(table 1).2µL of $MgCl_2$ .and 4.5µLof nuclease-free water were used. For nested PCR, 2µL of the first round PCR products were used with the same PCR reagent, as mentioned in the first step for the DNA Amplification. Predenaturation was accomplished at 95°C for 3 minutes, followed by 30 cycles of 94°C for 1 minute, 95°C for 1.5 minutes, and 72°C for 1.5 minutes. The cycles were completed by a final extension at 72°C 10 minutes.

Electrophoresis in red save stained 1.5% agarose in TBE buffer, 20 mM Tris-acetate was used to analyze the amplified product, and 1 mM EDTA increase (pH 8.0) was used. The

samples were uploaded on the gel along with a 100 bp DNA molecular weight marker (Intron Biotechnology Inc. Seoul, Korea) positive (CEVAC ITL Hungary Batch no. 0101D4DKA), and negative controls were included. The amplified products were visualized under UV light and then photographed using (alpha Innotech.) image documentation system.

Restriction fragment length polymorphisms (RFLP)

The restriction enzyme Hae III was used to cut the PCR product obtained in the TKPD2 amplification 20 U of Hae III (New England Biolabs. USA) was incubated with the TKPD2 PCR product at 37°C for 2 hours. Digestion products were electrophoresed in 3% agarose gel with red save (Promega) and uploaded on the gel along with 100 bp DNA molecular weight marker (Intron Biotechnology. Inc. Seoul, Korea). Proper positive and negative control was also included, and the result was visualized under UV light and then photographed using the Alpha imager (Alpha Innotech. USA) image documentation system.

Results

The mortality rates were 45%, 35%, 30%, and 25% in farms 1, 2, 3, and 4, respectively. Within 3 days after infection, nasal discharge, watery eyes, and mild severe tracheitis with bloody cough were observed (Figure 1). The farms that tested positive on the VeTek ILTV detection kit are shown (Figure 2), and the number of ILTV-positive stocks with nested PCR is shown (Figure 2). All four positive farms were positive with nested PCR and also tested positive by the ready PCR kit.

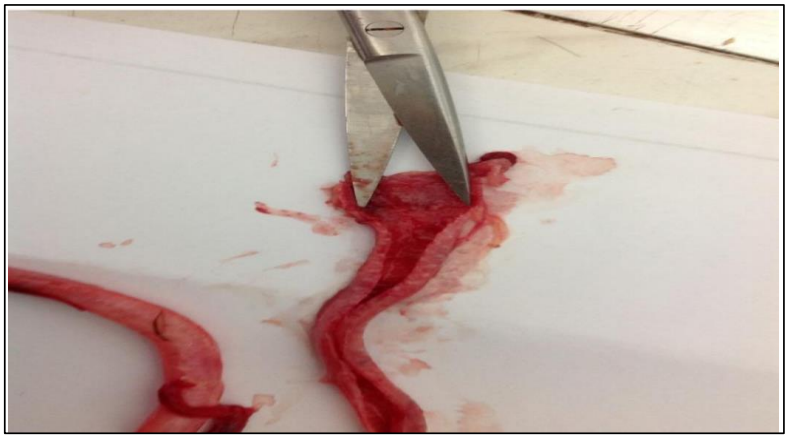

Figure 1. Severe tracheitis with a bloody cough

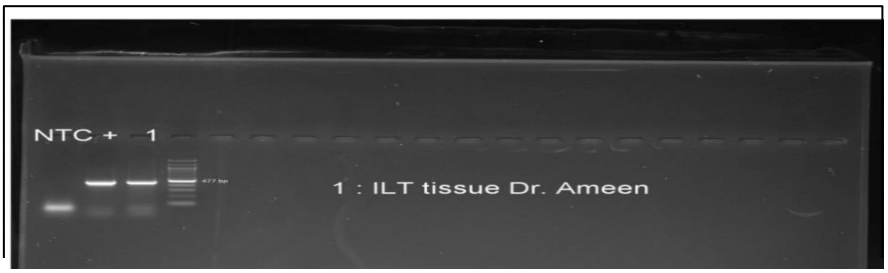

Figure 2. Commercial PCR kit (VeTek) ILTV in lane 1 shows 477bp PCR product fragment of glycoprotein G of ILTV and the positive and negative control

Virus isolation

The isolation of ILTV was carried out from tracheal suspensions obtained from PCR ILTV-positive farms, which were inoculated into 10—to 12-day-old chick embryonated eggs. After incubation, the CAMs showed generalized edema, and the developed lesions varied from scattered pocks to large ones with a diameter range of 4 to 5mm.

Restriction fragment length polymorphism (RFLP)

All positive samples by nested PCR of the TK gene were subjected to HA III digestion ILTV vaccine strain, which resulted in an uncut product, while the virulent results were in 2 bands (400-500bp) as shown in (figure 3).

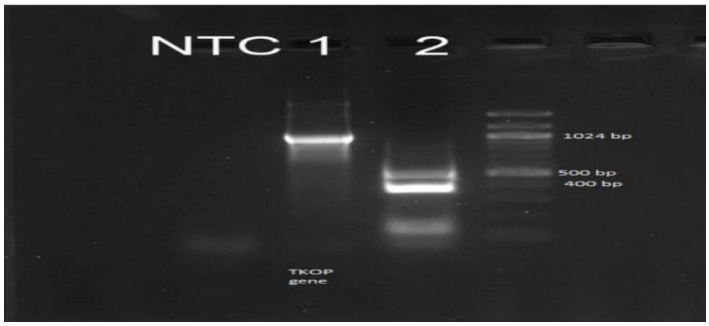

Figure 3. RFLP of the second PCR products, samples lane negative control (NTC) and positive control lane (+), lane 2 (cut pattern) size 500 – 400 bands subjected to Han, M.G. and S.J. Kim, Efficacy of live virus vaccines against infectious laryngotracheitis assessed by polymerase chain reaction-restriction fragment length polymorphism. Avian diseases, 2003. 47(2): p. 261-71. III enzyme of TK gene. Lane 1 shows an uncut 1024 bp band vaccine-like strain

Discussions

This study is the first to distinguish between the ILTV of a vaccination strain and that of an Iraqi virulent field strain.

Tracheal samples were taken from 5-layer flocks in the Baghdad governorate between June and September 2023 to record the isolation, molecular characterization, and differentiation of the field virulent strain of ILTV in layer flocks**.**

Infectious laryngotracheitis is a respiratory disease of chicken, peafowl, and pheasant, usually caused by the jailed herpes virus. This disease results in harsh losses in production due to mortality or decreased egg production. The severe epizootic form of the ILT infection is characterized by mucoid tracheitis, sinusitis, conjunctivitis, general unthriftiness, and low mortality [13, 14]. Clinical signs observed in affected Iraqi farms ranged between the severe epizootic form and the moderate form of the disease.

Over the course of 35 years, 104 field isolates from eight different nations were gathered in Europe. Three genetic groups were identified from the PCR-RFLP analysis of these ILTV isolates focused on the TK gene. The RFLP patterns of (98/104) of these field isolates were identical to those of vaccination strains [19]. A strong genetic connection between field strains and vaccinations derived from chicken embryos was demonstrated by PCR in conjunction with restriction fragment length polymorphism and isolate gene sequencing [27]. Twenty strains' ILTV gG, TK, ICP4, ICP18.5, and ORFB-TK genes were examined in a comprehensive PCR-RFLP investigation conducted in Australia. Based on the restriction patterns for the four genes, these strains could be divided into five genetic groupings [28]. Certain isolates could not be distinguished from vaccination strains, as was the case with isolated ILTV strains [29].

Based on the molecular and phylogenetic analysis of the ICP4 gene, the ILTV closely matched vaccine strains [25]. The initial detection and screening of ILTV in infected chicken in this study used a ready commercial PCR kit obtained from Intron Biotechnology. This kit was able to amplify a 477 bp fragment of glycoprotein G of ILTV, and we were able to identify ILTV-positive samples by using this kit. To confirm this positive result, we used a different PCR assay using specific and published primers for TK genes; the four ILTV-positive samples were also positive in nested TK PCR assays. Nested TKG primers were obtained from [16] with an expected size of 1024bp.

In recent years, the ILTV vaccine has been implicated and caused vaccine-induced ILTV infection [17, 18], and many of the tested layer farms in different studies had been vaccinated with ILTV vaccine and showed clinical signs. The positive result in the TK–PCR test from unvaccinated farms served as a marker to differentiate between the wild-type induced ILTV and the vaccine-type induced ILTV infection from clinical cases [18]. Another crucial point

was to identify the nature of the virus (either field virulent strain or vaccine strain). This was cleared by the digestion of the nested – TK-PCR product with HAI [19, 26].

The ILTV vaccine strain resulted in an uncut product, while the virulent ILTV strain resulted in 2 bands (400 and 500 bp). So, one cut strain, lan1, showed the cut 400 and 500 bp, and the other three strains showed the uncut pattern. The presence of the uncut band in three farms van indicates vaccine-like infection in spite of the unvaccinated farms, which may be due to its breeder farm (which is unvaccinated with ILTV).

Conclusion

- This is the first study to differentiate the ILTV of the Iraqi virulent field and vaccine strains. In this study, isolation, molecular characterization, and differentiation of the field virulent strain of ILTV in layer farms were carried out from 5-layer flocks in areas around Baghdad.

- In conclusion, with the PCR RFLP test, infected farms due to the ILT vaccine could be differentiated from field ILT-infected farms as the sequencing is not enough to differentiate between vaccine-like infection and field strains infection due to the lack of identity between both. This is an original study in Iraq.

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List Of Abbreviations

No.	Abbreviation	Full text
1.	ILT	infectious laryngotracheitis
2.	PCR	Polymerase chain reaction
3.	RFLP	restriction fragment length polymorphism
4.	ILTV	infectious laryngotracheitis virus strains
5.	TBE buffer	Tris-acetate buffer
6.	BPS	Buffer phosphate
7.	CAM	chorioallantoic membrane
8.	GaHV-1	galled herpes virus
9.	SPF	specific pathogen-free

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