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Review

The use of mushrooms as source of protein in market products

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Abstract

To satisfy the needs of a growing population as well as changing dietary habits, the food industry is looking for alternative sources of protein that are sustainable and better for the environment than those of animal origin. Plant protein sources are therefore very promising. However, some of them do not have all the essential amino acids and are therefore considered as low quality protein sources. Nevertheless, mushrooms are plants that generally have a complete profile of essential amino acids. For this reason, edible mushrooms are an interesting protein source for the food industry, which can use them to create high-quality protein-enriched food products. This review will therefore discuss the use of mushrooms as sustainable functional foods and more specifically as an alternative protein source in protein bars.

Keywords mushrooms, protein bars, protein content, vegetables

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Introduction

Today, food products with a specific functional property are more and more in demand. It is the case in particular of the products rich in protein which are strongly present on the food market (MALECKI & al).

Indeed, proteins are basic macronutrients in the human diet necessary for the proper functioning of the body and the maintenance of a good nutritional status. (PAUL & al) They have different functions and properties depending on their structure, such as ensuring growth in children, supporting bone and muscle metabolism, contributing to the maintenance of the nervous system or helping to maintain muscle mass and physical performance in old age. It is therefore necessary to include an adequate amount of protein in the diet every day to maintain good health. Protein is found in meat, fish, eggs, dairy products and vegetables (EWY & al).

Because of this great interest in high-protein products, the food industry is developing protein bars. The objective of these bars is to reinforce the nutrient intake. Its consumption is a simple and effective solution to distribute and complete the daily protein intake. They are strongly consumed by athletes to maintain muscle integrity but can also be recommended to avoid deviations during a vegan or vegetarian diet.

Manufacturers are constantly developing new recipes to meet the consumer demands. Indeed, the eating habits have not stopped changing in recent years. For this reason, alternatives are being thought to replace commonly used protein source ingredients to create innovative products that are in line with current trends in healthy, functional, sustainable, ethical and environmentally friendly nutrition. (SPARKMAN & al)

Nowadays, the protein bars that are mostly found on the market contain animal proteins because of their high nutritional value, desirable sensory properties and excellent functional properties. However, for some time now, alternative protein sources such as vegetable proteins started showing interest. They have been increasingly used as an economical alternative to replace animal sources in the face of a rapidly growing world population, but also as functional ingredients for the formulation of food products. (CONZUELO & al).

More specifically, edible mushrooms are among the promising ingredients as a protein source. Thanks to their nutritional value, which is highly appreciated because they are low in fat, calories and carbohydrates but high in fiber and protein, they are very interesting ingredients for the food industry (ZHANG & al.). Moreover, their proteins have a complete profile in essential amino acids which allows to meet the nutritional needs of the human being and to consider them as a source of high quality protein (GONZALEZ & al). It is also an economical protein source that can be easily produced on a large scale, unlike other plant and animal protein sources. They are now used to develop varieties of food products enriched with high quality protein. (KAUR & al)

The objective of this review paper is therefore to present mushrooms as a new protein source in protein bars.

Materials and Methods

This review paper is based on a literature review that investigated recently published research articles and studies that have been made in the past decade regarding plant protein sources used in order to obtain high protein based vegetables products, such as high protein bars. The research was focused on vegetables like mushrooms, broccoli, asparagus, string beans, eggplants which are a rich protein source for the food industry, which can be used to create high-quality protein-enriched food products.

The content analysis of the reviewed articles was focused on the scope of analysis and classifying them into several directions: proteins as basic macronutrients in the human diet necessary for the proper functioning of the body, health effects of vegetarian diets, current trends in healthy, functional, sustainable, ethical and environmentally friendly nutrition, functional ingredients for the formulation of food products, edible mushrooms as the promising ingredients as a protein source and evaluation of functional and nutritional potential of a protein concentrate from mushrooms.

Web of Science, Wiley, Elsevier and Springer databases were electronically searched for articles and the literature search included document types such as: research articles and reviews.

Discussion

Proteins as basic macronutrients in the human diet necessary for the proper functioning of the body

Vegetable proteins are very interesting from a health and environmental point of view. The health benefits are related to dietary fiber, vitamins, minerals and phytochemicals in plants. However, it is not possible today to distinguish the health benefits of plant proteins in particular from those of plant foods in general (Ahnen et al., 2019).

Proteins are one of the essential macronutrients for human nutrition and health. Depending on their digestibility, amino acid profile, bioavailability or purity, the nutritional quality changes. To meet human needs, ingesting plant proteins can provide enough essential amino acids. In recent years, plant proteins have gained momentum. Due to the in-

creasing needs of the population, proteins of animal origin will no longer be sufficient and it will be necessary to use plant proteins (Kumar et al., 2022).

One of the essential macronutrients of the human body is protein. They are at the origin of many functions and are made up of 20 amino acids, 9 of which cannot be synthesized by the human body. These 9 amino acids must therefore be ingested through food in order to avoid any deficiency (Ewy et al., 2022). They are found in proteins of animal origin but also in small quantities in vegetable proteins. However, more and more people are following diets based on vegetable proteins. Ewy & al. have shown that the consumption of meat in large quantities leads to a high intake of saturated fats and a low intake of fiber.

In Europe, protein-rich foods and food products with specific functional properties are increasingly present on the food market. Malecki & al. show that proteins are increasingly popular ingredients and are attracting more and more attention from the consumer. This is why conventional sources are seeing alternative sources coming in.

Vegan or vegetarian diet

Vegetarian diets are known to improve the health state of the people. Parker & al. qualitatively compared quality of vegetarian and non-vegetarian diets. They showed that vegetarians generally met the nutritional recommendations better than non-vegetarians. Mariotti & al. state that protein-rich foods such as legumes and seeds are sufficient to obtain a complete protein intake in adults following a vegetarian diet. Rogerson 2017 stated that the vegan diet is becoming more and more accepted in the fitness and sports domain.

Health effects of vegetarian diets

Plant-based diets are recommended because of the health benefits, sustainability and ethical aspect. The consumption of meat rich in saturated fats is said to lead to cardiovascular disease and certain types of cancer. However, plant-based diets reduce the risk of chronic diseases, diabetes, obesity. In addition, they have a lesser impact on the environment: reducing land use, greenhouse gas emissions, water consumption. Plant-based diets therefore help reduce chronic diseases while reducing the impact on the environment and cruelty to animals (Lynch et al., 2018; Jenkins et. al., 2019).

Plant-based diets are known to improve health but also to improve the performance of various types of exercises due to the high level of carbohydrates and the high concentration of antioxidants and phytochemicals. However, some plants have anti-nutritional factors. It is therefore necessary to carefully plan plant-based diets to ensure good health (Shaw et al., 2022).

The origin of the dietary proteins we consume determines the degree of insulin sensitivity. In diabetes, it is therefore recommended to reduce meat consumption and increase vegetable proteins to improve insulin sensitivity. Indeed, animal proteins activate the secretion of glucagon and increase insulin resistance while vegetable proteins increase insulin sensitivity (Adeva-Andany et al., 2022). In renal disease, a high-protein diet showed accelerated renal deterioration and a low-protein diet showed protein malnutrition. For this reason, vegetarian diets are a good alternative to stop the progression of kidney disease while maintaining proper nutrition (Bernstein et al., 2007). Bernstein et al., 2007 conducted a review in order to compare the effects of plant and animal proteins on renal function. It has been shown that the effects of animal and vegetable proteins are different but not well understood.

A review made by Herpich et al., 2022 summarizes the results of people following a plant-based diet and presents the limitations of this diet. Studies showed that mortality rates were lower than when the diet was regularly fed with meat. Plant-based diets are shown to be healthier. People show an improved metabolic and inflammatory profile and a lower incidence of cardiovascular disease. However, this type of diet leads to deficiencies and in particular to a lower protein intake. This type of diet is therefore not appropriate for people over 65 years of age who need more protein (Herpich et al., 2022).

Craig & al. state that a properly planned vegetarian diet is nutritionally adequate. In addition, a vegetarian diet can promote health and reduce the risk of chronic disease because of the low intake of saturated fat and cholesterol and the high intake of fiber. Nutrients that are often low in the vegetarian diet are vitamin B, vitamin D, calcium, iron and zinc. Vegetarians often have lower body mass index, total cholesterol and blood pressure than non-vegetarians.

Kahleova et al., 2017 carried out a study regarding cardiovascular diseases, which are a very important health problem today. Vegetarian and vegan diets are an effective prevention strategy to avoid these diseases thanks to the better intake of nutrients. These diets reduce the risk of type 2 diabetes by 50% and coronary heart disease by 40%. Plant-based diets are therefore good for the health of the consumer if they are well planned. They also allow the reversal of atherosclerosis and the reduction of lipids in the blood.

A lot of research on the health effects of plant-based diets is underway. However, it is difficult to assess the effects of nutritional factors on bone. However, plant-based diets can alter the balance of macro- and micro-nutrients as well as alter the inflammatory and immune response. According to various studies, there is no difference in bone health between

vegetarians and non-vegetarians, provided that vitamin D and calcium intake is sufficient. Furthermore, theoretical results have shown that the plant-based diet can reduce the risk of osteoporosis by mechanisms that are not well understood today (Hsu, 2020).

Plant-based diets allow the development of innovative preventive strategies against obesity, eating disorders and associated comorbidities. However, no conclusion can be drawn on the cerebral effects and on cognitive functions because this subject remains unexplored (Medawar et al., 2019).

Current trends in healthy, functional, sustainable, ethical and environmentally friendly nutrition

Vegetables proteins are a possible alternative to animal proteins because they are abundant, inexpensive, sustainable, low in allergens and accepted by consumers. Goldstein & al. showed that the techno-functional properties of legumes varied from one legume to another and could allow to replace animal proteins in food. Today, alternative protein sources are already a global trend in the food industry. Alternative protein sources allow the development of new products in the consumer market (Otero et al., 2022).

Susman et al., 2022 carried out a study in which gluten-free rice cookies from buckwheat and sorghum flour and enriched with pea protein powder were made and their physico-chemical and sensory proprieties were analyzed. The results showed that samples that had 20% and 30% of buckwheat or sorghum flour bad better sensory attributes compared to the control samples. Also, the protein content was higher because of the above mentioned raw materials added.

Functional ingredients for the formulation of food products

Today, legumes are more and more used in the food industry to replace cereals. Thanks to their functional characteristics, legume flours can be used to make healthier snacks by replacing cereal flours. However, it is necessary to find the most appropriate combination of flours so as not to modify the qualitative and sensory properties of the snacks (Tas & Shah, 2021).

Mushrooms have long been used for food purposes. They are sources of compounds that could be used to improve human health. Indeed, mushrooms possess for example ergothioneine. This is an antioxidant molecule that boosts human health and can preserve food. This favors the use of mushrooms as functional foods (Martinez-Medina et al., 2021).

Vadnerker et al., 2022 studied the characterization of an exopolysaccharide (ESP) produced by *Ganoderma* sp for its antioxidant and antitumor activity. The analysis performed in this study were TLC, HPLC, NMR and FTIR and the results showed that the EPS produced by the *Ganoderma* sp positively affects tumor cell growth reduction.

Edible mushrooms as the promising ingredients as a protein source

Mushrooms have always been interesting foods because of their rich composition in nutritive and bioactive compounds. Lopez-Hortas & al. discuss the use of edible mushrooms in food and especially in novel foods.

The recent developments and prospect in the high-valued utilization of edible fungi are discussed and summarized by Zhang, et al. The objective of this review is to improve the understanding of health-promoting properties of edible fungi, and provide reference for the industrial production of edible fungi-based health products. Edible fungi are large fungi with high added value that can be utilized as resources; they are rich in high-quality protein, carbohydrate, various vitamins, mineral elements and other nutrients, and are characterized by high protein, low sugar, low fat and low cholesterol. In addition, edible fungi contain a variety of bioactive substances, such as polysaccharides, dietary fiber, steroids, polyphenols; most of these compounds have antioxidant, anti-tumor and other physiological functions (Zhang et al., 2021).

Trujillo et al., 2021 carried out a study in which they studied the antioxidant capacity and protein content of edible mushrooms on the longevity of the fruit fly (*Anastrepha ludens*).

For thousands of years mushrooms have been used as food or medicine. Mushrooms are good sources of protein. They have no cholesterol and a low fat content. Some mushroom proteins have specific interesting biological activities such as pectins, fungal immunomodulatory proteins, ribosome inactivating proteins or ribonucleases. Some proteins have become natural anti-tumor, anti-viral or anti-microbial agents (Xu et al., 2011).

Spim et al., 2021 carried out a research article which presents the attributes of mushrooms, their relevance for the food industry and the possible development of innovative protein-rich products from mushrooms. In recent years, mushrooms have become interesting functional plants. Thanks to their nutritional composition, notably their low fat content and their high fiber and protein content, as well as their natural source of food molecules such as ergosterol, polyphenols, mannitol, trehalose. These are interesting ingredients for the food industry. They have already been used in formulations of meat and starch based products but not for alternative products without meat for example. Their functional performances must be explored (Spim et al., 2021).

Kaur & al. recognize mushrooms as an excellent source of protein but also of minerals, unsaturated fatty acids, poly-

saccharides and other secondary metabolites. Mushrooms differ from other plant and animal protein sources because their amino acid profile is complete. This complete profile makes mushrooms a good quality protein source that meets the nutritional needs of humans. In addition, mushrooms protect against certain chronic diseases. For example, thanks to their low calorie, carbohydrate, fat and sodium content, they can be used to treat cardiovascular and kidney diseases. In addition, they can be used to treat neurological diseases such as Parkinson's or Alzheimer's or certain cancers. Edible mushrooms are used today in the food industry to improve the functional properties of certain protein-enriched food products.

Evaluation of functional and nutritional potential of a protein concentrate from *Pleurotus ostreatus* mushrooms

Mushrooms have antibacterial activity and are a good immune system enhancer. Also it was discovered that the consumption of mushrooms could lower the cholesterol levels; Additionally, they are important sources of bioactive compounds (Valverde et al., 2015).

Torres-Martínez et, al., 2022 stated in a research article that edible mushrooms as an important source of nutritional and bioactive compounds. The results of research show that *Pleurotus* spp. are an important source of proteins and amino acids, carbohydrates, minerals, and vitamins. In this review, the findings of macronutrients, bioactive compounds, antioxidant activity, and antimicrobials against foodborne pathogens of some *Pleurotus* spp., as well as their potential use as an ingredient in the meat industry are discussed. The presence of some bioactive components, such as polysaccharides (α-glucans, β-glucans, and so on), proteins/enzymes and peptides (eryngin, pleurostrin, and others) phenolic acids (p-coumaric, chlorogenic, cinnamic, ferulic, gallic, protocatechuic, and others) and flavonoids (chrysin, naringenin, myricetin, quercetin, rutin, or the like) has been demonstrated. The use of *Pleurotus* spp. in some meat and meat products (patties, sausages, paste, and suchlike) as a novel ingredient in order to improve their chemical composition and functional health promoting properties, as well as to increase their physicochemical and sensory attributes, was evidenced by several works.

González et al. ., 2021 carried out a study on edible mushrooms. They are an interesting alternative source of protein to conventional sources of protein. The purpose of this article is to characterize and evaluate the nutritional and functional properties of a *Pleurotus* ostreatus mushroom flour protein concentrate. The results showed that the total phenolic content decreased increasing protein digestibility.

To cope with rapid population growth, it is necessary to develop strategies to meet the needs of the population. As far as proteins are concerned, they are mainly provided by proteins of animal origin. For this reason, researchers are interested in alternative, low-cost, under-exploited sources: plant proteins. However, not all plant proteins are considered to be of high quality because they do not contain all the essential amino acids. Mushrooms are one of those foods of vegetable sources that have a complete profile of essential amino acids. They can therefore meet the nutritional needs of humans. Moreover, by processing, it is possible to obtain high quality protein-enriched products from mushrooms. Gonzalez & al. show that mushrooms are sustainable functional foods that can be used in the food industry.

Technology and challenges

The current status of legislation, standard setting, and regulatory acceptance of meat alternatives were reviewed by Zhang et. al., 2022. Considerable progress has been made towards the development and production of meat alternatives, including cultured meat, plant-based meat alternatives, microbial protein, edible fungi, microalgae, and insect protein. Meat production has long suffered from practical problems, such as high resource consumption, pollution, animal antibiotic residues and zoonotic diseases. The meat-based diet has been criticized for a high carbon footprint not only for inefficient production processes, but also for potential nutritional unbalance Considerable progress has been made towards the development and production of meat alternatives, including cultured meat, plant-based meat alternatives, microbial protein, edible fungi, microalgae, and insect protein.

One of the current challenges is the reduction of meat consumption and the development of new sustainable and healthy meat products. Mushrooms are a promising source of bioactive compounds to solve this problem (Gupta et al., 2018). Pérez-Montes & al. show that different species of mushrooms allow the improvement of protein and fiber contents in meat products. Moreover, they show that mushrooms can replace salt, phosphates, proteins and fats in the formulation stage of meat products due to their high fiber content, texture and easily digestible proteins.

Vegetable proteins have major advantages compared to animal proteins: a sustainable origin, an economical low cost, health benefits. Plant proteins have inferior functions that make them less soluble in water, sensitive to environmental stress, pH, temperature and salt. Vegetable proteins are poorly digestible because they are surrounded by hemicellulose or lignin or other polysaccharides. It is possible to modify plant proteins to improve their applications and make them more accessible. It is possible to modify them by

improving their flavor, nutrition or techno-functional attributes to improve their application in plant-based food products (Nasrabadi et al., 2021).

The development of plant-based protein products in place of animal-based proteins must be equally nutritious. However, the quality and protein content in plants varies according to the source. Conzuelo & al. have created a model in order to create food products of plant origin that can be used for long term consumption. Moreover, it is still possible to improve the device in the future. A great potential for expansion is possible for example to model menus or even whole diets.

Known for their low lipid but high protein composition as well as their vitamin and mineral content, *Lentinula edodes* (shiitake mushrooms) are the second most cultivated edible mushrooms in the world. Spim et al., 2021 carried out a research study in which were created and evaluated four food bars (two sweet and two savory) containing this mushroom. To create the bars, the binding ingredients were heated and then the dry ingredients were added. Each bar was developed and then underwent a sensory analysis of texture, aroma, taste, appearance and purchase intention. In addition, an evaluation of the moisture content, lipids, carbohydrates, proteins, chemical elements composition was performed. After analysis of the results, the sweet bars presented very good results. They are an easy alternative to add functional ingredients beneficial to health thanks to their stability, good acceptance and low production cost.

Erjavec & al. consider fungi as a promising source of new bioactive proteins for use in biotechnology, medicine and agriculture. Indeed, mushrooms contain proteins with unique characteristics such as lectins or protease inhibitors. These proteins can be the solution to several current problems: resistance to microbial drugs, low crop yields, and the demand for renewable energy. Mushrooms are therefore a valuable source with great potential.

Xu & al. discuss the potential of these proteins in biomedicine and their future prospects. Many species of mushrooms are excellent sources of protein because of their low fat and cholesterol content. Among these proteins, some are biologically interesting due to their action. We can mention lectins, fungal immunomodulatory proteins, ribosome inactivating proteins, ribonucleases, laccases and other proteins. These proteins have thus become natural antitumor, antiviral, antimicrobial, antioxidant and immunomodulatory agents.

Motta & al. show through experiments the effects of these proteins on human immune cells. The fungal compounds induce biochemical changes in macrophages, dendritic cells, T cells and NK cells. Further studies are needed, but mushroom compounds could be useful for human health.

Conclusions

The use of mushrooms as a protein source in food products is proving to be very interesting. From an environmental, ethical, health and sustainable point of view, proteins from edible mushrooms seem to be a good alternative of sustainable functional ingredient. Indeed, they allow replacement of proteins of animal origin which nowadays do not seem sustainable. Moreover, thanks to their attractive nutritional properties and their specific functional properties, mushrooms are the plants of major interest for their proteins with a complete amino acid profile. They are increasingly used in the food industry to replace mainly proteins of animal origin. They are found in particular in food products enriched with proteins such as protein bars. In a few years, proteins from edible mushrooms will probably be more present on the market. More generally, plant proteins will certainly take over from animal proteins in order to satisfy the overall needs of the growing population and achieve sustainability in terms of protein demand.

Conflict of Interest

The author has no conflict of interest to declare.

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Original paper

Assessing the structure and interaction of sustainable Bio – Composite Films Prepared form deamidated Rice Bran Protein

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Abstract

Rice bran protein (RBP) was utilized to prepare bio-composite films after deamidation modification process with deferent percentages. The physical, mechanical properties, exposure to light properties, FT-IR analysis, surface shape by SEM, thermal stability by DSC and X-ray diffraction were applied to study the potential interaction, structure and stabilizing the thermal property of the prepared films. The deamidation process has proven successfully, depending on the cleared changes of shapes of FT-IR curves resulting from RBP compared with deamidated rice bran protein (DRBP). The deamidation process enhanced tensile strength, elongation at break, haze, transparency, gloss and opacity properties of produced films from DRBP. Additionally, the produced films from DRBP presented smooth and homogenous surface with increasement of thickness, solubility and decrement of water vapor permeability and crystallinity compared with films from RBP.

Keywords

Deamidation, edible films, deamidated rice bran protein, film characteristics

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Introduction

In recent years, the development for newly degradable films from protein isolates has become a must [1]. Therefore, protein films offer the potential for reducing non-renewable resources and may lead to the use of agricultural raw materials for film preparation. Coatings and edible films define as, thin layers of edible substances wrapped on the food products that have an important for distributing, storage and marketing. Some of their functions are to product from mechanical damage, physical, chemical and microbiological activities. The bio-composite films are first molded as solid sheets, which are then applied as a wrapping on the food products [2]. The bio-composite films are analyzed for different applications because of their ability to provide a barrier to mass transfer, carry food ingredients, and improve mechanical integrity of foods. Additionally, it can also enable reduction and simplification of the wrapping material required for a food product. Furthermore, coating material has been utilized in reducing the loss of water, delay of the ripening, reducing the chilling and mechanical damage, reduce decay and added shine, or gloss to the coated commodity [1, 3]. films act films act as a carrier for nutrients and other bioactive compounds that affect the health on a positive way, due to the techniques of microencapsulation or nano-encapsulation. during that, the film substance acts for transporting the bioactive materials to the target sides without losing its activity [4]. The bio-composite films could sustain a modified packaging? passive atmosphere, which encourage some changes in minimally and fresh processed food staff such as : firmness, inhibition of microbial growth, antioxidant properties, color, ethylene production, sensory quality, and volatile compounds as a resulting of anaerobic processes [5].

Polysaccharides, proteins, and lipids are the main polymeric materials to produce edible coatings and films. In many instances two or all of these ingredients could be blended to produce composite coating films [6]. Proteins from plant are more often used than animal proteins for films production because of the availability and lower costs. The by-product from the cereal agro-industrial processing may be a source of protein that can be recovered for formation of protein – based films. Rice bran (RB) is a by-product which derives from the processing of the grain, which proteins were extracted to produce bio – based [7, 8]. The reason why its excellent property as barrier for oxygen, its tightly packed hydrogen bonded structure. Edible coating from protein has good O2 barrier property at low RH. It consists of good organoleptic and physomechanical properties [9]. However, the utilization of rice bran protein (RBP) is still limited because of its low solubility in water. To enhance the food protein solubility various modification approaches, such as physical and chemical modification were made [10, 11]. One method that is commonly used to enhance the functional properties of food proteins is deamidation. Deamidated forms residues of Glutamine and Asparagine to their increase the protein solubility [12]. In this study, RBP was deamidated under various alkaline conditions of temperature, pH, and reaction time. Changes in solubility of protein were examined to determine the degree of deamidation. The objective of the present study was to fabricate new bio-composite films with enhanced manners by utilizing modified RBP

Materials and Methods

Materials

Rice bran (Giza 178) was obtained from Rice Research Center at Sakha, Agricultural Research Center, Egypt. Glycerol, Ascorbic acid, Citric acid, Sodium hydroxide (NaOH), Hydrochloric acid (HCl), N-hexane, Sodium bicarbonate were purchased from El- Gomhoria Co. for Chemicals and Drugs. Teflon plates (20×30) used to cast all the films. All reagents were of analytical grade.

Methods

RBP extraction

RB samples were treated by microwave to inhibit the enzymes action for five minutes. Oil was elicited from RB using n- hexane based on the method outlined by Kahlon [13]. The defatted rice bran was stored at -18oC until used. After that, defatted rice bran was suspended in distilled water (1:10 w/w). slurry's pH was set at 9. 0 using 4 M NaOH solution under continuously stirring for 1 h, then centrifuged for 15 minutes at 12600 g. supernatant protein solution was set to pH 4. 5 using 4 M HCl, stirred for 30 min then left overnight at 4 °C for cold precipitation. The supernatant has been siphoned off then the resulted precipitated protein was washed 3 to 4 times with distilled water. The protein slurry has been set to pH 7 and lyophilized [14].

Production deamidated rice bran protein

Protein deamidation was performed as following; briefly, 0. 25 g of RBP was suspended in 25 mL of 0. 1 M NaHCO3 and pH was set to 8, 10, and 12 with 1 M NaOH or 1 M HCl. The solutions were then heated at 80 or 100°C for 30 or 60 min, or 120°C for 15 or 30 min. Then, samples have been neutralized, dialyzed against water at 4˚C for 48 hrs, and lyophilized.

Determination of protein solubility

Fifty mg of protein sample has been suspended in 5 mL of deionized water, mixed for 30 min at room temperature, and centrifuged at 15000 g for 20 min. The supernatant has

been transported to another tube, after dried at 40 °C, weight of residual protein was determined based on the method outlined by Bradford [15].

Formation of edible film

RBP 1% or DRBP with different concentrations (1, 2 and 3%) were dissolved in distilled water, stirred at 80˚C for 30 min. Glycerol was added and pH was adjusted. Ascorbic and citric acid were added as anti-browning agent and antimicrobial (1. 5% (w/v). Film solution was left in the room temperature for 1h to remove air bubbles and cast into Teflon plates (20×30 cm) then dried at 40˚C in oven. Films have been stored at plastic bags until the measurement were performed.

Mechanical properties

Tensile strength and Elongation

Tensile strength (TS, Mpa) and elongation percentage (%E) at break of film were determined at 22 ± 10C and RH=31% using an Instron Universal Testing Machine (Model 1011, UK), according to Ferreira et al. [16].

Characterization of physical properties.

Film thickness

Thickness of film has been calculated with an accuracy of 0. 01 mm using a digital micrometer (Mitutoyo digimatic indicator corporation, model: pk-1012 E, Tokyo, Japan). The mean thickness have been used to measure the water vapor permeability and tensile strength [17].

Determination of films solubility and swelling power

Solubility and swelling degree of the formed films were measured by using the method of Riaz et al. [18] with a slight modification. The film pieces were cut into 2x 2 cm then dried at 105°C until constant weight to calculate the initial dry mass (M1). Then, those were took place in 100 mL beakers with 50 mL distilled water, covered with plastic wraps and stored at 25°C for 24 h. Later, the films have been dried using filter paper and dried at 105°C until fixed weight to determine final dry mass (M2). The following equation was used to measure film solubility:

$$\text{Film solubility}=((M1-M2)/M1)*100$$

films were placed in beakers (50 mL) that contain distilled water (30 ml) for 24 h at 25°C after weighing (M1). Then, wet film was dried using filter paper then weighed (M2). The swelling degree calculated with this equation:

$$\text{Film swelling degree (\%)}=((M1-M2)/M1)*100$$

Moisture content

Moisture content was determined based on the way of Araujo-Farro et al. [19]. Moisture content was measured by Eqs. 1. film samples have been cut into squares of 2 ×2 and weighted (W1). The samples have been dried at 105°C until constant weighted (W2) obtained. Triplicates of each film samples were tested.

$$\text{Moisture content (\%)}=((W1-W2)/W1)*100$$

Water vapor permeability (WVP)

Modification of the ASTM E96-92 gravimetric method to measure the relative humidity (RH) at the film underside was used for measuring WVP [20]. Distinct glass cups have been used with 4 cm diameter then filled with anhydrous CaCl2 then film samples (5×5 cm) have been sealed above each glass cup. The cups have been placed in a desiccator at 75% RH which was maintained by a saturated sodium chloride solution. The glass cups have been then weighted change against time was determined by linear regression.

The WVP was measured by the following equation

$$WVP = \left(\frac{\text{Slope} \times L}{A \times \Delta P}\right)$$

Where L is the average film thickness (m), A is the transfer area (m2) and ΔP the partial water vapor pressure difference.

Exposure to light properties

Haze and Transparency

Haze and transparency of samples were determined by Hazemeter (BYK- Gardner Gmbh model haze-grad plus, Germany, according to ASTM., (2001).

Film gloss

Gloss is a way to measure of the ability of the film to reflect incident light at angle 450. The sample's gloss has been measured by glossmeter (BYK- Gardner Gmbh model micro- gloss 45, Germany, according to ASTM., (1997). Standard measure for tensile properties of thin plastic sheeting. D882- 97, Annual book of American Standard Testing Methods, ASTM, Philadelphia, PA. Gloss values measurement based on 6 random positions per sample from double faces and three samples per film.

Films opacity

Spectrophotometer (Model PU 8800 UV/VIS, Pye Unicam Ltd., Cambridge, UK) has been used to determine film opacity as described by Sun et al. [21]. Three strips (1 x 4 cm) were cut from the soaked films and placed on the inner side of a transparent plastic cuvette. The adsorption spectrum was determined with a wavelength domain of 400–800 nm, and opacity was taken as the area under the curve, as determined through an integration procedure, and termed as Absorbance Units in nanometers (A nm).

Characterization of RBP films

Analysis of SEM

Scanning electron microscopy (SEM, SU8010, Hitachi, Japan) at 10 K V has been used to study film surface morphology. The films were cut in pieces (10 x 10 mm) then, dried and mounted on aluminum stubs using a double – sided carbon tape and sputter- coated with gold [21].

Analysis of FT- IR

Fourier Transform – Infrared (FT- IR) spectrometry (Nicolet IR200, Thermo, USA) was used to study prepared films structure through KBr module. FT- IR spectra were recorded in the frequency range of 4000 to 400 cm-1.

Differential scanning calorimetry (DSC)

DSC analysis was performed using a Q1000 DSC system (TA instruments, USA) following the way of Akhtar et al. [22]. Film pieces (10 mg) were sealed in a standard aluminum pan and heated at a constant rate of 10 °C / min from 0 to 450 °C at a nitrogen atmosphere.

X-ray diffraction

The crystal structures of films was explored following the way of Ali et al. [17] using x-ray diffractometer (D8 Advance, Bruker. USA) at a voltage of 40kV and 100 Ma. The scattered radiation was detected in the angular range 2ϴ= 10- 40º with scanning speed of 5 º/ min.

Statistical analysis

Data were analyzed statistically by SPSS 20. 0. Analysis of the data (ANOVA) assessed the difference between factors and levels. To identify the significance of differences among mean values. Tukey's multiple range tests were executed. Differences were considered significant when $p< 0.05$.

Results and discussions

Effect of deamidation process on RBP solubility

Different alkaline pH values were utilized with various temperature for different times to modify protein structure by removing amides to characterize RBP solubility. as shown in Table (1).

The results showed that, protein solubility increased from 18 to 93% when deamidation was achieved at pH 12 for 15 min at 120˚C. RBP was deamidated by altering the heating, temperature, pH and period. By raising these parameters, the grade of deamidation has been increased. As expected, solubility with increasing the deamidation degree. That is, Asparagine and Glutamine residues in RBP converted to Aspartate and Glutamate residues upon deamidation, so that the surface polarity of the protein increased to improve its solubility. RBP has high thermo-stability because of its compact structure, with also cause poor solubility. Temperature needed for denaturing of RBP decreased with the deamidation process, which result in unfolding of the proteins. However, deamidation conditions such as high temperature (<120˚C) and high pH are likely to affect the denaturation more strongly. With increasing pH more denaturation and unfolding the protein happened, so deamidation of RBP under alkaline pH improves the solubility of rice bran protein. While, severe alkaline conditions ((pH<12) result in the racemization of amino acids [12].

Table1. The solubility of deamidated RBP under different pH, temperature and time conditions

pH	Temperature (°C)	Time (min)	Solubility (%)
Untreated RBP			18.54
8	80	30	36.53
		60	36.52
	100	30	44.84
		60	41.09
	120	15	54.75
		30	52.10
10	80	30	34.97
		60	30.41
	100	30	39.31
		60	49.83
	120	15	46.09
		30	71.63
12	80	30	51.21
		60	39.52
	100	30	36.71
		60	72.52
	120	15	93.20
		30	92.42

Mechanical properties

Elongation at break (EB) and Tensile strength (TS) are main parameters which indicate film stretchability upon breakage and the strength of the film. Improvement of the mechanical properties of films explains the increase in its endurance versus stress during storage and transportation hence inhibits tearing of composites and perforation [23]. TS and EB values from the films are shown in Figure 1. DRBP with different concentrations films showed significant differences for TS and EB values compared with RBP. The highest TS and EB were found 57. 22 Mpa and 66. 73 %, respectively in DRBP (3%) samples.

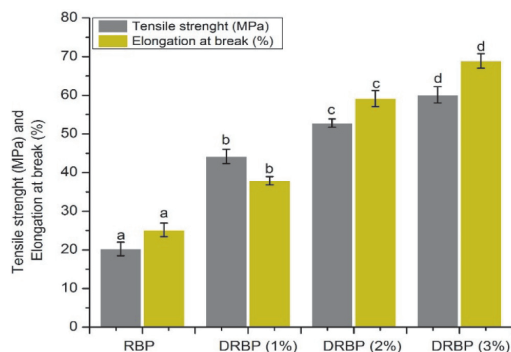

Fig. 1. Mechanical properties of RBP films and films with different concentration from DRBP.

Deamidation process created complex of protein-protein interaction which might make many types of cohesiveness due to changes in electrostatics and hydrophobicity of protein. Additionally, concentration of protein in the film forming solutions affect the self-adhesion and the average of matrix forming on film preparations. On increasing protein concentration, protein – protein interactions increased and resulted high cohesiveness medium [24]. Considering the EB of films depends on the TS [25]., the denaturation case of RBP to produce DRPB in alkaline environment and heating caused ruining of protein structure, breaking existing disulfide intermolecular bonds and exposing sulfhydryl hydrophobic groups, making them available for bonding. The cleavage of di-sulfide bonds results in polypeptide chains with lower molecular weights, destroying elasticity and cohesiveness of RBP. Upon casting and drying, sulfhydryl groups reform disulfide bonds by air oxidation, which leads to elongation of the film structure [26]. On the other hand, RBP was deamidated under various alkaline conditions of temperature, pH and reaction time. These might increase the surface electronic charge and polarity which improve structural flexibility and TS. Deamidation was adopted to modify the structure of RBP with denaturation without causing severe hydrolysis which improve elongation properties [27].

Physical properties

Thickness

The film's thickness significantly increased with increasing concentration of DRBP as show in Table 1 compared with RBP. The deamidation of films by using alkaline medium generated interaction between protein chains and this might lead to denaturation of DRBP which cause tighter binding of protein film [28]. The thicker films are favorable to retard penetrating moisture from surrounded environment to wrapped food.

Moisture content and swelling ratio

Data for moisture content ratio of film samples are shown in Table 2. It provides information about the water affinity, whereas swelling ratio may help to predict quality and stability changes in food product storage [29].

For moisture content and swelling ratio, significant differences were observed in all the films. The results showed that moisture content decreased due to increase protein content, It may be result of hydrophobicity of DRBP [30]. Swelling ratio decreased with increasing DRBP content and this could be because of formation of strong hydrogen bond between the unfolded protein which result from the denaturation process which happen during deamidation. This result improves the swelling property of the film, which need to be resistant to water to make it suitable for use as food packaging material.

Water solubility

Water solubility of the film is an essential property of biodegradable. It showed significant increase for the RBPD films in comparison to control RBP film (Table 2). The higher values of water solubility for RBPD films as for film solubility, it increases due to increase protein concentration from 1 to 3%. Also, alkaline medium led to increase film solubility in water. The higher values of water solubility for DRBP films because of the hydrophilic groups of DRBP that could easily interact with water molecules [31, 32].

Water vapor permeability

The most essential function of the film is to delay the deterioration of food products from the surrounding atmo-

Table 2. Physical properties of RBP and DRBP films

Samples	Thickness	Swelling degree (%)	Water solubility (%)	Moisture Content (%)	Water Vapor permeability (%)
RBP	38.50 ±0.03a	36.56± 0.36d	14.80± 0.45a	17.35±0.44d	5.75±0.77c
DRBP 1%	40.10±0.01b	32.85± 0.25c	19.64± 0.56b	15.90±0.19c	4.40±0.29b
DRBP 2%	42.30 ± 0.15c	28.71± 0.05b	25.82±0.81c	14.30±0.56b	2.80±0.22a
DRBP 3%	44.50± 0.17d	24.95± 0.06a	31.50± 0.51d	12.40±0.26a	2.75±0.08a

Values are mentioned as mean ± standard deviation. Dissimilar letters in the same column show significant differences (p<0. 05)

sphere. The Film is considered better if the moisture transmission between food staff and surrounding environment is as low as possible. Water vapor permeability is one of the main essential characters of bio-composite films for food packaging as it has direct contact with food products hence has high influence on its shelf life. The present results showed that DRBP had significant decreasing effect on WVP of film samples (Table 2). WVP of films decreased from 5. 75 to 2. 75% with increasing concentration of RBPD, because that at higher pH, protein denatures, unfolds and solubilizes, facilitating disulphide bonds by thiol-disulphide interchange and thiol oxidation reactions, resulted in stronger films with less permeability to gases and water [33, 34].

Exposure to light properties

Haze of film is defined as, the percentage (%) of light transferring through the film and refraction by angle more than 2. 5o from incident light. While, known as the percentage of light transfeerring through the film. Appearance of films as (Haze, Transparency and Gloss) are important properties of edible films because it could affect consumer acceptance in potential edible food applications. The consumer preference is greatly influenced by opacity and color of food packaging materials. Data in Table 3 explain result of RBP and rice bran protein deamidated concentration on exposure to light of RBP and DRBP film. Results show that, Haze, transparency and Gloss gradually increase as function of increasing DRPB from 1 to 3% in comparison with the control RBP film, it may be beacuse solubilisation of the protein under the alkaline pH and deamidation of RBP, which yielded in increasing solubility and exposure to light properties [28].

Additionally, Results in Table (3) show that film opacity affected by protein concentration and pH. It could be noted that film opacity increased due to increase protein concentration from 1 to 3 %. It may be because of increasing protein – protein interaction and forming a compact network and increasing insoluble matters [35].

Characterization of fabricated films

SEM

Characterization of fabricated films SEM morphology, homogeneity and Structure of the material have an essential role in the film's permeability [36]. SEM was applied to analyze the micro-structure of the film to resolve and test the film surface voids, smoothness, layer structure and homogeneity.

SEM images of the surface of rice bran protein film and DRBP films are shown in Fig 2. SEM image show that of rice bran protein film has bubbles, crakes and heterogeneous surface. The surface become more smooth, homogenous and has few bubbles with deamidation films. When, DRBP were 2 and 3% the SEM image have best result with smooth, no crake, uniform and homogenous surface. RBP has poor solubility, the solubility of rice bran protein improves with deamidation. Increasing the solubility affect and improve structure, homogeneity and morphology of the matrix, which result in quite smooth, uniform, ordered and homogeneous structure without bubbles or porous with no cracks in contrast to the film with native RBP.

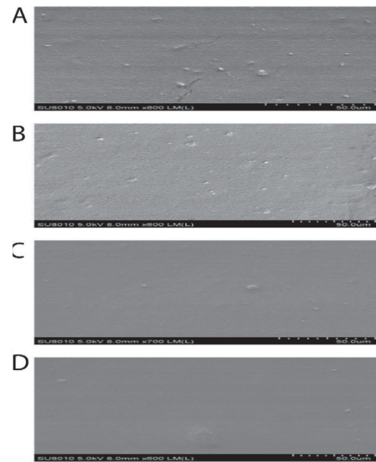

Fig. 2. SEM photographs of (A) RBP films, (B) DRBP 1%, DRBP 2% and DRBP 3% films.

FT-IR spectroscopy

Infrared spectroscopy is a rapid and a nondestructive technique that has been widely used to characterize different biomaterials. Moreover, FT-IR spectroscopy is a powerful

Table 3.Exposure to light properties of RBP and DRBP films

Samples	Haze	Gloss	Transparency	Opacity
RBP	56.07± 0.19a	16.87±0.03a	83.80± 0.25a	0.135 ± 0.01a
DRBP 1%	61.03± 0.23b	19.33± 0.06b	89.90± 0.01b	0.195±0.01b
DRBP 2%	63.46± 0.29c	25.18± 0.02c	91.23± 0.05c	0.325± 0.07c
DRBP 3%	63.46± 0.29c	27.70±0.05d	92.32± 0.15C	0.330± 0.04c

Values are mentioned as mean ± standard deviation. Dissimilar letters in the same column show significant differences ($p<0.05$)

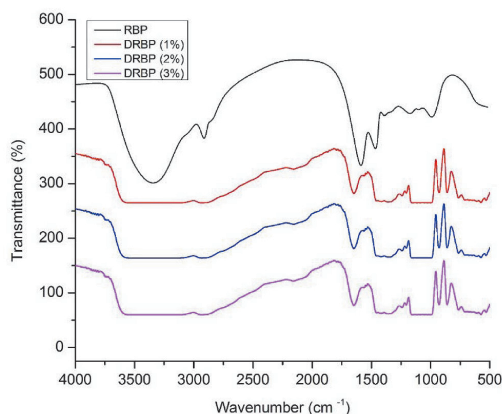

Fig. 3. FT-IR spectra of RBP films and films with different concentration from DRBP.

technique to evaluate polymer blend miscibility. Deamidation result in denaturation and unfolding of protein which facilitating chemical interaction at the molecular level, that increase the hydrogen bonds in protein matrix. These changes can be an indication of good miscibility of matrix. In this work, FT-IR showed that the absorption spectra of films from different formulations had similar absorption regions, differing only in the bands absorption intensity(Fig. 3). The absorption stands on the content of insoluble protein, which decrease with deamidation process [37].

DSC analysis of produced films

In the case of proteins, DSC can be utilized to investigate the thermodynamic stability, folding mechanism, thermal stability and denaturation of protein [38]. DSC thermograms of RBP and DRBP films were observed in Fig4. It was observed that the thermograms changed considerably by deamidation process. One transition with exo-shoulder corresponding to the unfolding of the proteins was observed in all samples. The apparent melting temperatures were 73. 5 °C for RBP films, and 78. 8 °C for DRPP; evidence that the deamidation process increased the thermal stability of proteins and delayed the unfolding process. The thermal property and secondary structure of RBPD determined by DSC and FT-IR was well preserved during the deamidation process [37].

XRD

To identify the crystalline structure of native RBP and study the result of deamidation on RBP. Deamidation process did not change the internal structure of RBP. The X-ray spectrum of the deamidated films showed only a decrease in the intensity of spectrum, and this may be because of decreasing the insoluble matter by deamidation (Fig. 5). The

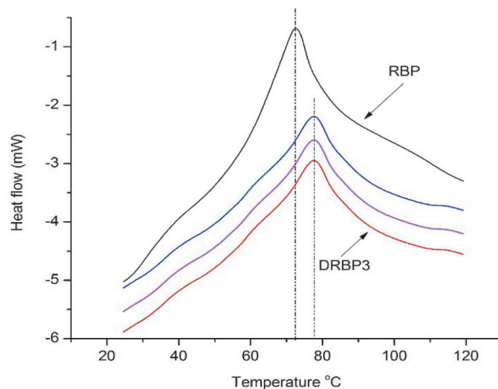

Fig. 4. DSC thermograms of RBP films and films with different concentration from DRBP.

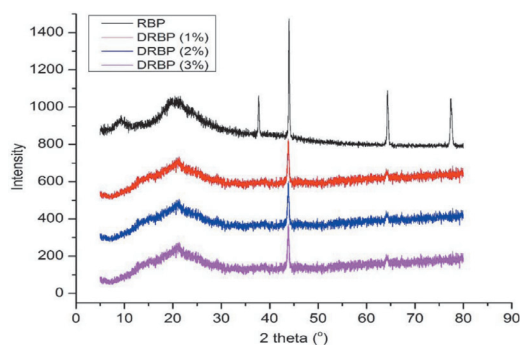

Fig. 5. XRD intensity of RBP films and films with different concentration from DRBP.

interactions that happen during deamidation in the crystalline structure may hinder the intra molecular hydrogen bonds and inter molecular hydrogen bonds in the denaturated unfolded protein, resulting in a low crystallinity. Hence, mechanical properties of the film strongly depend on the crystallites in its structure.

Conclusions

In the present study, solubility of RBP was improved by deamidation process by alkaline conditions. Overall, this study showed that, DRBP has the ability to develop protein films with good physical, mechanical and exposure to light properties. Deamidation process improves protein structure, solubility and increase the strength of hydrogen bonds between protein polymers, hence production of rice bran protein films with suitable physical, mechanical, Exposure to light properties and crystalline structure. That can be widely used as edible film for packaging food products. Hence, deamidation process can be applied on another protein ma-

terial from food and agricultural manufacturing residues to increase their use as distinctive edible coating.

Conflict of Interest

The author has no conflict of interest to declare.

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Original article

Bioactivity of two plant products against *Tribolium castaneum* infesting wheat grains and their impact on some biological parameters in Japanese quail

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Abstract

Several experiments were carried out in the current study to assess the efficacy of Caraway seed (*Carum carvi*) and petitgrain lemon (*Citrus aurantium*) plants against the stored product insect *Tribolium casteneum*. To measure some factors, various plant preparations (powder, oil, extract, and new formulation) with concentrations of 5-10-15- and 20% oils and extracts and 5-10-20- and 40% powder and their formulations were used (biological effect, insect mortality rate). Furthermore, to assess the negative effects of tested materials on certain biochemical parameters of Japanese quail (*Coturnix coturnix japonica*). There have been no significant effects of oral treatment on creatinine level while significant effect were noticed on total protein, total anti-oxidants and Serum glutamic pyruvic transaminase (SGPT) levels in quail. In every treatments for quail, caraway was more effective than petitgrain lemon on some biological parameters. The determination of some biological parameters revealed that treatment after two weeks was affected in comparison to treatment four weeks later. The findings suggest that the tested plant products could be used to protect wheat grains from T. castaneum adults in Egyptian storage facilities.

Keywords

T. castaneum, carawary seed, petitgrain lemon, chemical composition, toxicity, Japanese quail.

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Introduction

Wheat is one of the most important crops in the world because it is the primary source of protein in developing and poor countries (FAO, 2015). *Tribolium castaneum* (Lepidoptera: Tenebrionidae) is a major pest of stored grains, preferring to infect wheat grains in the summer when the temperature and humidity are favourable for infestation. Pest infestation of stored grains results in significant grain weight loss (ABOUELATTA et al, 2016). According to the Food and Agriculture Organization of the United Nations (FAO), agricultural production must increase by 50% by 2050 to meet global food demand (BERES et al, 2020). The quality of the sown seeds is a critical requirement for the successful production of any crop (SABER et al, 2017).

Insect pests reduce the dry weight and nutritional value of stored grains and processed products. More than 600 species of beetle pests attack agricultural stored products around the world. *T. Castaneum* is the most common and prevalent pest species of stored grains, causing quantitative and qualitative losses in warehouses, granaries, and mills. Historically, synthetic insecticides were used to control it. However, the main issues with these insecticides are water and soil contamination, resistance development, and toxicity to non-target species**.** (ADEL et al, 2015).

Many decades ago, insect pest control was entirely based on chemicals, particularly synthetic insecticides such as persistent organochlorine (OC) and organophosphate (OP) insecticides. However, the long-term or heavy use of synthetic pesticides has resulted in serious issues due to factors such as direct toxicity to parasites, predators, pollinators, fish, and humans. (BUCHANAN et al, 2010).

They may continue to poison non target organisms in the environment and increase the risk to humans by disruption in the endocrine, reproductive, and immune systems, cancer, neurobehavioral disorders, infertility and mutagenic effects, although very little is currently known about their chronic effects (BUCHANAN et al, 2010 ; JUREWICZ and HANKE, 2008). Replacement of conventional synthetic insecticides with bio-rational insecticides is a globally acceptable and practical approach. This necessitates ongoing research into the substitution of hazardous synthetic insecticides for the use of less expensive and more environmentally friendly natural plant products with active safe components, such as the use of powdered plant parts, oils, and extracts derived from secondary metabolism in plants. (LALE, 2018). Essential oils are volatile mixtures of hydrocarbons with a variety of functional groups. However, in some cases, these chemicals can work together synergistically, increasing their effectiveness (NERIO et al, 2009) As a result, the researchers focused on finding viable alternatives to antibiotics, such as medicinal plants, which contain effective substances with very few side effects on human health when compared to chemically manufactured medicines (SEGER et al, 2019).

As a result, as part of future alternative feed strategies, new commercial additives derived from plants, such as aromatic plant extracts and their purified constituents, have been investigated. Because they are residue-free, such products have several advantages over commonly used commercial antibiotics. They are also widely accepted as safe and widely used items in the food industry. (ACIMOVIC et al, 2018). To avoid the disadvantages of protective chemicals such as pesticides, the use of natural products, plant extracts and oils has been confirmed as one of the modern trends in controlling stored grain pests (SABER et al, 2021). The purpose of this research is to look into the contact toxicity and biological activity of caraway seeds and petitgrain lemon on *T. castaneum*. Furthermore, the LD50 (Lethal Dose for 50% of the treated pests) value of the tested material on Japanese quail was estimated. Furthermore, the toxic effects of tested oils on some biochemical parameters (liver and kidney enzymes, total protein, and total antioxidants) in Japanese quail blood serum were assessed (*Coturnix coturnix japonica*).

Materials and methods

The tested insects

Tribolium castaneum: The red flour beetles *T. castaneum* used in this study came from a laboratory stock colony at the Plant Protection Research Institute, Sakha Agriculture Research Station, Dokki, Giza, Egypt. In the laboratory of the Stored Grain Pests Department, Plant Protection Research, Sakha Agriculture Research Station, *T. castaneum* was continuously reared free of insecticidal contamination.

Insect breeding conditions

To get a homogenous population, 20 pairs of adult, *T. castaneum* was released in dark glass jars and placed in an incubator at 30±2°C and 60±5% R. H (Relative humidity) (GERKEN and JAMES, 2020). For sexual reproduction and released insects, Whole-meal of wheat and wheat flour were heated or sterilized at 60°C for 6 hours (To get rid of any insect infestation in food(. Wheat flour was provided as a culture medium (ABOUELATTA et al, 2020). Size of glass jars was 14 cm in height & 6 cm in width. Each jar was filled by sterilized wheat flour. Mouth of jars was covered with muslin cloth for good ventilation (OJUMOOLA et al, 2020). Jars were tied with rubber bands to avoid escape of beetles or entry of any other insects from outside (ZIA et al, 2011). Beetles were allowed to lay eggs in the culture medium for three days before being transferred to a new sterilised glass

jar containing food medium using a wire sieve with a diameter of 20 and fine camel hair brushes. After 28-30 days, the population in these jars was considered a homogeneous culture for the experiment. Jars were left in the Pesticides Chemistry and Toxicology Department, Faculty of Agriculture, Kafrelsheikh University's laboratory bench for a month to produce a new generation of adult insects.

Plants

The plants of caraway seeds (*Carum carvi*) and petitgrain lemon (*Citrus aurantium*) were collected from the farm of the Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh Gvrnorate, Egypt.

Oils

The essential oils caraway seeds (*Carum carvi*) and petitgrain lemon (*Citrus aurantium*) used in the study were purchased from Hashem Bothers Company for Essential oils and Aromatic Products (69 Abdel Moneim Riad St. Giza, Egypt).

Preparation of powders

The lemon and caraway seed plants' leaves were collected from the Faculty of Agriculture's farm at Kafrelsheikh University. To remove any dust, the leaves of the tested plants were thoroughly washed with tap water and distilled water. The plants were air dried at room temperature in the absence of sunlight. The samples were dried in an oven at 60°C to confirm drying. The plants were ground several times at room temperature in a stainless steel mill, then samples were sieved through a wire sieve (8-inch diameter) to obtain a very fine powder for packaging the crushed wheat grains. Plant soft powders were stored in a brown jar at -20 m until use. The fine powder processing was done in an ERAP.

Preparation of formulation

Suspensibility test

The test was carried out according to WHO specifications 1973 (EL-HAMADY, 1985), for preparing the wettable powders in the laboratory of Herbicides and Fungicides at Pesticides Chemistry and Toxicology. Five grams from each of two powder samples were weighted and added to beaker 250 ml. Hard water_(hard water contains calcium and magnesium salts principally as bicarbonates, chlorides, and sulfates. Tap water that contains salts when applying it in the field. To aviod diformation) (MCDOUGALL, 2012) was added to at least twice the volume of the additional weight of the sample, and stirred or shaken mechanically for 30 seconds with a glass rod 4. 6 mm diameter including no more than four turns per second

The mixture was quantitatively transferred immediately to a graduated 250 ml cylinder using hardness water under test at 30 ±1 °C until it reached the 250 ml mark, cap closed and stirred. The cylinder was raised and lowered 30 times at a rate of no more than one full revolution every two seconds. The cylinder was placed in a moderate position for 30 min in a water bath at 30 ±1 °C.

After 30 minutes, a glass tube with a pump was inserted into the cylinder and 9/10 of the total volume was withdrawn from the suspension. Then, the remaining suspension solution (1/10) was filtered by weighted filter paper (Wattman No. 10) and dried at 40° C by the furnace. The degree of suspensebility was calculated as follows in the equation:

$$\% \text{ Suspensebility} = (b-a)/b \times 10/9 \times 100 = (b-a)/b \times 111.\ 1$$

Where: a: sample weight in 1/10 suspension solution b: initial weight of sample (5 gm)

Preparation of plant extracts

The fine powdered plants were dissolved in 300 ml of acetone. The tested materials and acetone were macerated for 7 days. Using an electrical shaker, caraway seed and petitgrain lemon powders were shaken for 6 hours per day during the maceration period. Sterilized cotton and filter paper were used to filter the extracts (Wathman No: 101). The extracts were then concentrated and dried with anhydrous sodium sulphate (5gm/100ml) before being evaporated to dryness with a rotary evaporator. To achieve the desired concentration for application, the residue was weighed, dissolved in acetone, and stored in a brown vial at 4°C. **(MURGAN et al, 2007; ISMAIL et al, 2009).**

Contact toxicity

The treatments of wheat grains with the essential oils was performed to determine the contact toxicity against *T. castaneum* adults. Thin film technique was used according to the method of **(METCAL, 1967)** and mixing with diet bioassay (**EL-LAKWAH et al., 1992).** Different concentrations (5, 10, 15 and 20 % w/w) of oils and extracts, while (5, 10, 20 and 40 % w/w) of the formulation and powder were prepared using acetone. Twenty grams of wheat grains were placed in a jar (11. 5 cm x 6 cm, in diameter). One ml of each concentration was dropped in each jar over the surface of grains using micropipette. The jar was shacked to ensure even spread of the materials over the surface of the grains. The treated grains were left for 20 minutes until the solvent evaporation. Each concentration was replicated three times. Grains treated with solvent only served as control. Ten adults of newly emerged of *T. castaneum* were transferred to jar, which covered with muslin cloth and kept under the same laboratory conditions. The control treatment was car-

ried out using water. Morality percentage was recorded after 24h, 72 h and one week after treatment. . The mortality (%) was corrected by **(ABBOTS'S FORMULA, 1925)** equation as follow:

$$\%M = \frac{Mo - Mc}{100 - Mc} \times 100$$

Where, Mo: observed Mortality, Mc: control Mortality.

A series of concentrations in acetone solvent were prepared for all tested materials, and toxicity was drawn Fig 1. It was not acceptable to determine LC_{50} (Lethal Concentration mortality of treatments 50%) LC_{50} values. This step needs concentration more than 20%, so the toxicity was enough, to be appreaed using Fig. 1.

Analysis of essential oils by Gas Chromatography-Mass Spectrometry

The essential oils from each test plant were analysed using a Gas Chromatography coupled with Mass Spectrometry (GC-MS) system manufactured in the United States. The HP5890 column was used in accordance with the specifications (60 metre 0. 25 m film thickness). The MS (Mass Spectrum Selective Detector) was used. Helium was the mobile phase, with a flow rate of 1. 0 mLmin1. The initial temperature was 60 degrees Celsius, and the maximum temperature was 250 degrees Celsius. The temperature of the injector was 240°C. After running the oven temperature programme at 50°C for 2 minutes, the temperatures were raised to 200 °C at a rate of 5 °C min -1. The total area of the peaks is used by the apparatus software to calculate relative percentage amounts. The components of the tested oils were identified by comparing mass spectral data to those stored in a computer library (Wiley275. L). Hashem Brothers' analysis laboratory for Essential Oils and Aromatic Products performed all sample preparation, extraction, drying, concentration, cleaning, and analysis procedures (69 Abdel Moneim Riad St. Giza, Egypt).

Toxicity test

Ethical statement

All the methods, animal care and experimental protocols used in the present study followed relevant guidelines and regulations of Kafrelsheikh University, Egypt

Animals

Japanese quail (3 weeks) birds were obtained from the Faculties of Agriculture and Veterinary Medicine, the poultry Research farm, Department of poultry production, Fac. of Agric., Kafrelsheik University, Sakha, Kafr El-Sheikh, Egypt. Birds were housed in metal cages. Nutritional contents (24% protein, 5. 5% fibre, 3. 5% etherextract, 14% humidity) were fed in the diet (provided by the same source of birds) and water (NARVAEZ et al, 2016). Before participating in any experiments, the birds were acclimatised to the laboratory under natural light-dark cycle conditions for two weeks. All tested groups were subjected to constant temperature, relative humidity, and lighting conditions [255 °C, a relative humidity of 60% 10, 12 h:12 hrs (hours) (light: dark)] (NARVAEZ et al., 2016; RANDALL and BOLLA, 2006, 2008; HAAS et al., 2017). All of the birds were in good health and had never been exposed to any chemicals. Birds were treated with the tested chemicals in the Laboratory of Pesticides Toxicology and Chemistry Fac. of Agric., Kafrelsheikh University, Kafr El-Sheikh, Egypt.

Acute toxicity tests

A serial dilutions of petitgrian lemon and caraway seed oils (10, 50, 100, 200, 400, 800 mg/kg) were dissolved or diluted in corn oil. Birds were oral administrated by the tested materials. Experimental conditions for bird in the test as mention before in animal part. The mortality percentage was recorded after 24 hours of treatment, and LD50 values were calculated according to the method described by (Weill, 1952).

Logm=logDa +d(f+1)

Where:

Log m = log of the LD_{50}

Log Da= log of the lowest dosage.

d= log of the constant factor

f= factor obtained from the tables according to the number of animals dosed For the estimation of confidence limits, the formula used was :

Log m ±2d f

Where: d, f were obtained from the tables.

Animals treatment by sub-lethal dose

Japanese quails were given 1/10 and 1/100 of the tested compounds' LD50 values. Petitgrian lemon and caraway seed oils had LD50 values of 233. 3 and 136 mg/kg, respectively. Sub-lethal The doses were made in corn oil. To begin, exploratory trials were conducted to determine the smallest dose of each plant oil that exhibits toxic effects. The experimental design included two control groups (no tested compounds or no treatment), four groups treated with 1/10 LD50 of the tested compounds, and four other groups treated with 1/100 LD50 of the tested compounds. Blood samples were collected after 14 days of treatment and again after 28 days. As previously stated in the animal section, the environmental conditions of treated animals. Each group consists of five birds. Treatment was performed with 1 ml of treated samples using an injection syringe with a ball in the front to prevent bleeding or injury to the tested animals.

Biochemical assays

Blood samples were centrifuged at 4500 rpm for 20 minutes while being cooled, and serum was collected for enzyme activity determination. Using the kits technique, the colorimetric methods of (GORNAL et al, 1949; SCHIRMESTER et al, 1964; GIARDI et al, 2010) were used to determine the levels of total protein, creatinine, glutamic-pyrovic transaminase (GPT), and total antioxidants, respectively. The enzymes in the serum of rats after UV/VIS treatment were determined using a spectrophotometer (Model: Spectrometer T80+ from PG Instrument Ltd). Quail blood samples were tested at the Alnokhba laboratory in Sadat City, Egypt. .

Statistical analysis

The data were arranged in tabulated form and graph formats. The data were analyzed using a one way (ANOVA) test, using graph pad Prism Version 4 for Windows, Graph Pad Software, San Diego California USA*, (www. graphpad. com).* Results with p<0. 05 were considered statistically significant according **(SPSS, 2012).**

Results and Discussion

The acute toxicity of the tested plants products against *T. castaneum*

Results in Table1 and Fig. 1 showed the toxicity of the tested plants of caraway seeds and petitgrain lemon against *T. castaneum* using the technique of mixing the toxicant with the feeding medium of insects (wheat grains).

The data in Table 1 showed that caraway seeds significantly influenced the number of larvae and adult emergence of *T. castaneum* in oil and extract compared to petitgrain lemon. In the case of powdered and formulated caraway seeds, the performance was significantly inferior to that of petitgarin lemon. After 3 weeks of treatment to wheat grains treated with different concentrations of the tested materials, the reduction percentage (%) (Mean. No. of a larvae and Mean. No. of adults emergence) of *T. castaneum* increased with increasing concentration. Caraway seeds in oil form had a higher reduction percentage (%) in adult emergence than its extract. The same results were evident in the case of petitgrain lemon. While the percentages in both caraway seeds and petitgrain lemon plants were roughly the same in powder form and formulated form. In general, the reduction percentage in adult emergence was clearly greater in powder and formulated forms than in oil and extract forms. When compared to oil and extract, the powder and formulated forms of the petitgrain lemon plant had the highest reduction percentage (%) in adult emergence. The same pattern was observed in the caraway seed plant, but the oil form was more effective in reducing the percentage (%) of adult emergence. . No doubt that the true difference between different forms in the two plants in their effect on reduction percentage (%) in adult emergence in addition to their toxic effect on adult stage of *T. castaneum* are expected to be the real causes of preferable material that must be recommended.

The results showed that the formulated petitgrain lemon extract is preferred for insect control, while the caraway seed extract has the least effect material on *T. castaineum*. The toxicity of the two materials (caraway seeds and petitgrain lemon) against *T. castaneum* when exposed to treated wheat grains containing 10% oils and extract or 20% powder and formulation form is shown in Fig. 1. Lemon appeared to be the most toxic when formulated compared to the other three forms. Caraway seed forms followed the same pattern. Because of the differences in concentrations (10 and 20%) of different forms of lemon and caraway seeds, it is difficult to compare the two materials in general, but the guaranteed result is that lemon is more toxic than caraway seeds in general. This agree with previous studies that suggested by (LEE et al, 2001), that the toxicity of essential oils for stored-product insects was influenced by their chemical composition. The fumigant activity of *A. eryngioides* could be attributed to oil constituents, such monoterpenes, and other components. These constituents had insecticidal activity against several stored-product insect pests.

(YOON et al, 2011) showed that lavender oil had a repellent effect on the spotted lantern fly, Lycorma delicatula (White) (Hemiptera: Fulgoridae) (also known in the literature as “spot clothing wax cicada” or “Chinese blistering cicada”) and that linalool (a monoterpene) was the most effective constituent present in this oil. Similarly, (LUCIC et al, 2015) demonstrated that lavender plant parts and essential oil are toxic to three stored-wheat pests, including *T. castaneum*. Other research has found that plant essential oils from the Linaceae family, such as lavender oil, can inhibit feeding, repel insects, and act as an insecticide to control a variety of insect pests. Moreover, (EL-DIN, 2001) demonstrated that caraway oil was highly toxic to *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) and other pests. (FANG et al, 2013) found that the essential oil of *C. carvi* showed a strong toxicity in controlling *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) and *T. castaneum* with (R)-carvone and D-limonene being the principal toxic constituents for *T. castaneum*. (LEE et al, 2001) stated that the mode of action of bioactive natural monoterpenoids (hydrocarbons, alcohols and ketones) from essential oils (basil, cassia, caraway and spearmint) may be due to inhibition of acetylcholine and reported that 1, 8-Cineole was the most potent inhibitor of Acetylcholinesterase (AChE)

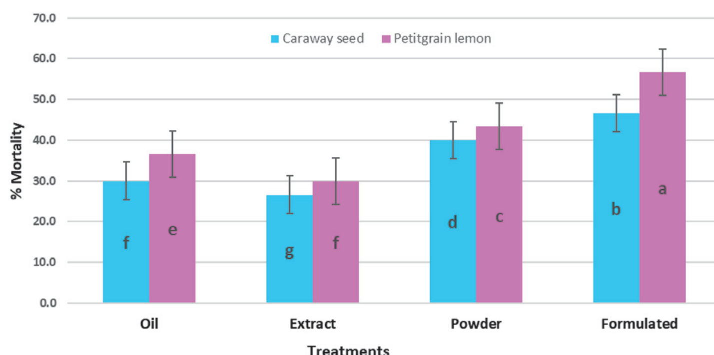

Fig1 The mortality percentage of *T. castaneum* exposed to wheat grains treated previously with 10% oils and extract while 20% of powder and new formulation after three weeks of exposure.

among the monoterpenes tested. This inhibition may be a mode of action for essential oil and monoterpene fumigation toxicity against stored grain insects pests as well. (PARK et al, 2003) reported the insecticidal mode of action of the compounds in spearmint may be largely attributable to fumigant action.

(LAZNIK et al, 2012) also indicated that the essential oil extracted from *Rosmarinus officinalis* has been shown to be highly efficient in fumigation against another pest of the adult *Sitophilus granarius* (grain weevil), and the death rate has reached more than 60% of this stored pest, indicating that the essential oils of *Rosmarinus officinalis* are an effective agent against stored grain pests such as beetles. (PANEZAI et al, 2019) showed that *Rosmarinus officinalis* extract was the most effective against larvae and adult insects of both stored grain pests, as it caused 58. 67% death in *Tribolium castaneum* and 80. 00% in *Trogoderma grananium* in adult stage.

The toxic action may be attributed to penetrating the insect body via the respiratory system. Several researchers

Table (1): Insecticidal activity and biological effects of products with different concentrations from two plants against *T. castaneum* exposed to treated wheat grains.

Tested plant	Con.%	Oils	Extract				
		A	B	C	A	B	C
Petitg rain lemon	5	73c±2.31	63 c±4.04	73.30	50c±3.15	50c±3.15	81.60
	10	70c±2.89	59d±4.04	80.00	43d±2.83	40d±2.25	85.30
	15	56e±2.6	49f±2.02	86.60	29f±1.73	23ef±2.89	91.50
	20	40f±2.02	30g±3.87	91.00	18h±1.73	13h±0.58	95.50
Caraway Seeds	5	83b±1.88	76d±3.75	63.30	90b±2.31	74b±3.46	72.80
	10	63d±2.19	54e±1.62	70.00	52c±1.15	50c±1.15	81.60
	15	40f±2.66	30g±2.66	73.30	37de±1.73	30e±0.58	89.00
	20	30g±2.31	20h±1.15	86.60	26g±1.15	19g±0.29	93.00
Control		281a±2.89	273a±5.2	-	260a±1.73	250a±5.02	-
Petitgrain lemon	5	73c±2.31	63 c±4.04	73.30	50c±3.15	50c±3.15	81.60
	10	70c±2.89	59d±4.04	80.00	43d±2.83	40d±2.25	85.30
	20	56e±2.6	49f±2.02	86.60	29f±1.73	23ef±2.89	91.50
	40	40f±2.02	30g±3.87	91.00	18h±1.73	13h±0.58	95.50
Caraway Seeds	5	83b±1.88	76d±3.75	63.30	90b±2.31	74b±3.46	72.80
	10	63d±2.19	54e±1.62	70.00	52c±1.15	50c±1.15	81.60
	20	40f±2.66	30g±2.66	73.30	37de±1.73	30e±0.58	89.00
	40	30g±2.31	20h±1.15	86.60	26g±1.15	19g±0.29	93.00
Control		281a±2.89	273a±5.2	-	260a±1.73	250a±5.02	-

A= Mean. No. of a larvae after treatment, B= Mean. No. of adult's emergence, C= % Reduction of adult emergence. The numbers followed by the same letter mean that they are not significantly different at the α=0. 05 test level

reported the toxicity and protectant potential of essential oils extracted from different plants against major stored product insects (RAJENDRAN and SRIRANJINI, 2008; USHA et al, 2011).

Essential oil of *Achillea wilhelmsii, Acorus calamus, Allium sativu and Amomum maximum* proved insecticiad activity against *Tribolium castaneum* and *Sitophilus oryzae* (TALUKDER and KHANAM, 2009;YANG et al, 2010; KHANI AND ASGHARI, 2012).

Powder, essential oil and ethanol extract of *Citrus reticulata* proven to be effective against *Tribolium castaneum* (IRAM et al, 2013, LEE et al, 2020).

Essential oil of *Citrus sinensis* showed a high efficacy in controlling of *Tribolium castaneum, Rhizopertha dominica, Sitophilus oryzae* and *Sitophilus zeamais* (CAMPOLO et al, 2014, KIM and LEE 2014 ;ABDELGALEIL et al, 2016;LU (2017). The tested essential oil C. limon were toxic by contact effect against *Tribolium castaneum. C. limon, J. phoenicea, L nobilis, E. tenuifolia ssp. sibthorpiana, O. majorana,* and *O. vulgare ssp*. hirtum were found to be effective against the stored-product insect pests *Tribolium granarium* and *Tribolium castaneum*. *Citrus limon* was found to be effective in controlling *T. castaneum* larvae (PAPANIKOLAOU et al, 2022).

In this investigation, crude leaf extract of *Citrus aurantium* and crude seeds extract of *Carum carvi,* and their formulations showed good potential as repellent agents to larvae and adult stage of *T. castaneum*.

The GC-MS analysis

Tables 2 and 3 show the results of the GC-MS analysis of the two plant products caraway seeds and petitgrain lemon. D-carvone (82. 68%) had the highest concentration in caraway seed oil (as shown in Table2). D-carvone (82. 68%) and Estragole (5. 27%) were the most abundant components in caraway seeds, followed by cyclohexene (6. 38%) and limonene (6. 22%). Furthermore, low concentrations of Cyclohexanone (0. 869%), Beta-selinene (0. 83%), Beta-elemene (0. 445%), Methyl benzoate (0. 36%), and Epi-neointermedol (0. 30%) are present.

These findings are consistent with other studies (ZIA et al, 2011) that found carvone or cis carveol to be the main component of the oil. According to (KASRATI et al, 2015), the insecticidal mode of action of the compounds in spearmint may be due to fumigation action. These compounds may be toxic when they penetrate the insect cuticle via the respiratory system via the stigmata, according to (Park et al, 2003). The main caraway products and their culinary ap-

Table 2.The main constituents of caraway seeds oil.

Main component	Component rate %	Retention time (min)
Estragole	5.27	8.968
D-carvone	82.68	16.922
Cyclohexanone	0.869	14.900
Beta -elemene	0.445	22.770
Beta-selinene	0.83	26.346
Epi-neointermedol	0.30	32.537
Methyl benzoate	0.36	18.112
2-cyclohexene	6.38	17.414
Limonene	6.22	8.379

Table 3.The main constituents of petitgrain lemon oil.

Main component	Component rate %	Retention time (min)
Limonene	34.741	9.269
Beta –ociemene	2.298	9.744
Citronella	2.373	13.964
2, 6 -Octadienal	12.003	18.002
Neryl Acetate	2.159	22.932
2- Citral	15.697	19.420
Caryophyllene	3.489	25.063
Docosane	2.361	55.854
Nerol	6.37	23.851
Beta- Elemene	1.08	23.971
Germacrene	1.35	30.380
1-cycleprop azulen-7-	1.02	33.287

plications are depicted. Several processed products derived from caraway whole seed, including seed powder, essential oil, fatty oil, oleoresins, and caraway carvone, are used in the food and pharmaceutical industries. It can be used as a safe alternative to pesticides in integrated control programmes. (SEGER et al, 2019) mentioned that caraway fruits usually contain around 4% of essential oil, with more than 20 compounds. Among these components, we find the two components, carvone and limonene, constitute in the present study more than 90% of the total oil composition. (MAHBOUBI, 2019) mentioned that carvone and limonene are two major components of oil, which account for 95%.

The data in Table 3 indicate the main components of petitgrain lemon oil. Limonene had the highest content (34. 741%), followed by 2-citral (15. 69%), 2, 6-octadienal (12. 003%), and Nerol (6. 37). Caryophyllene (3. 489%), Citronella (2. 373%), Docosane (2. 361%), Beta-ociemene (2. 298%), Germacrene (1. 35%), Beta- Elemene (1. 08%), and 1-cycleprop azulen-7 (1. 02%) are also present. The use of monoterpenes to protect products with less negative environmental and health impacts than highly effective synthetic pesticides has sparked interest. Essential oils with a high hydrogenated compound content are the most susceptible to oxidation and lose their activity faster than those with a high oxygenated compound content. (REGNAULT-ROGER et al, 2002). Active compounds that sustain essential oils of insecticidal efficiency are monoterpenes (NGAMO et al, 2007). The rhythm of their activity reduction was not the same for all of the tested oils. For compounds such as 1, 8 cineole, the rate of oxidation of hydrogenanted monoterpenes is faster. The oil's insecticidal efficiency is reduced as a result of this oxidation. These findings suggested that the oils' insecticidal mode of action was largely due to fumigant action. Lemonene is an insecticide that is effective against a wide range of insects. Lemonene and other monoterpenes may have been used in the past to treat phytotoxicity in crop plants. (AFLATION, 2008).

Acute toxicity of the tested essential oils on Japanese quail

Data pertaining to the impact of tested materials on Japanese quail are shown in table 4. Perusal of these results clearly indicated the effect of two tested oils on Japanese quail. Bloods samples of control and treated birds (1/10 and 1/100 from LD50) were used to determine the parameters indicated in table 4. The blood samples taken from group (I) were gained 2 weeks after treatment, while blood samples taken from birds in group(II) were gained 4 weeks after treatments. LD_{50} values were evaluated basically to determine the doses introduced to quail birds. Antioxidants are known to help fight free radical substances that inhibit oxidation of some materials, such as vitamin (C) or (E), which remove potentially harmful oxidising agents in living organisms. This may protect cells from the effects of free radicals, which are molecules produced by the body when food is digested or when it is exposed to tobacco smoke and radiation. Free radicals may be the cause of some diseases, such as heart disease and cancer. Antioxidants work by donating electrons to free radicals. The enzyme SGPT (Serum Glutamic Pyruvic Tranceaminase) is found naturally in liver and heart cells. With liver damage, the blood SGPT level rises. Creatinine is a compound excreted in the urine that is produced by creatin metabolism. Creatinine levels in the blood will rise as a result of poor creatinine clearance by the kidneys, eventually leading to kidney failure. After treatment with the two plant oils, the multi antioxidants increased in comparison to the control. The same treatments raised SGPT levels. While the levels of creatinine in all treatments with the two plant oils were not significantly different. In all cases, caraway outperformed petitgrain lemon in increasing levels of the biological parameters measured in this experiment. The data in table 4 pertaining to total protein show that there is a positive correlation between this parameter and SGPT. It is clear that two weeks of treatment (group I) produced more effects than four weeks of treatment (group II). This phenomen could be due to quail recovery (return to normalcy).

The results of the present study were agreed with (BLASZCZYK et al, 2006) recorded high level of cholesterol in the serum quail from the sixth to tenth week of age. that concluded that the addition of *Carum carvi* L. seed powder at a levels of 4, 6 and 8 g/kg to the diet did not affect the growth and development of gonads and the addition led to high lipid profile. (SEGER et al, 2019) mentioned decreased level of fat in the serum maybe due to the low levels of *Carum carvi* L. seed powder used in the study, as the high levels in the study may lead to increased level of fat in the serum. (ACIMOVIC et al, 2018) mentioned plants from *Apiaceae* family have positive effects on many different functions in the poultry organism, but their use is still limited and requires further investigation. (ABOU EL-SOUD et al, 2019) said that the increase in this enzyme after caraway oil treatment could be attributed to the antioxidant effect of caraway oil, which reduces oxygen free radical formation and thus increases kidney enzyme activity. The kidney's susceptibility to oxidative stress during diabetes is an important factor in the development of diabetic nephropathy, where ROS (Reactive Oxygen Species) activates inflammatory pathways that lead to glomerular damage. Thus, increasing antioxidant enzymes (e. g., glutathione peroxidase) and eliminating free

radicals may be involved in the utility of caraway oil to improve the pathology of diabetic nephropathy, though there is no complete reversal of all abnormalities, which may be due to the need for longer treatment duration. (DOSOKY AND SETZER, 2018) mentioned that citrus essential oils are well known for their flavor and fragrance properties, as well as numerous aromatherapeutic and medicinal applications. With the exception of some phototoxicity of expressed oils, they are generally safe to use with negligible toxicity to humans (SAOUDI et al, 2021 ;HASSAN, 2021; HERMAN et al, 2021 ; AZIZA et al, 2019).

Conclusion

The biological activity of the two tested plants, as well as their toxic effects on *T. castaneum* adults, is a common effect that refers to the appropriate material in its formulated form. Biological parameters determined in Japanese quail treated with the two plants revealed that the plant materials are safe, and recovery is expected over a reasonable time period. Plant products (formula) used as insecticides or as an alternative to insecticides are more effective in controlling many insect pests because they are eco-friendly and relatively safe. It is also regarded as one of the most promising compounds for pest control of stored grain and other pests. All of the crude essential oils and components can be used as pesticide substitutes for stored product insects.

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Table (4): Effect of caraway seed and petitgrain lemon oils on some biological parameters in Japanese quail

	Tested plant	Dose mg/kg	Total antioxidant (mmol/ml)	SGPT(u/l)	Creatinine(ml/dl)	Total protein (gm/dl)
Group I	Petitgrain lemon	22.3	0.597 b±0.01	22.11 ab±0.87	0.40a±0.01	3.503 ab±0.23
	Caraway seeds oil	2.23	0.79 ab±0.01	21.53 b±0.87	0.41 a±0.01	4.03 a±0.12
		13.6	0.967 a±0.07	24.67 a±0.58	0.45 a±0.01	4.54a±0.06
		1.36	0.867 a±0.02	24.64 ab±0.58	0.48a±0.01	4.13a±0.08
Control	-	-	0.403 bc±0.18	18.47 c±0.29	0.43 a±0.01	3.99ab±0.29
Group II	Petitgrain lemon	22.3	1.56 ab±0.08	10.225 ab±0.81	0.39a±0.01	3.803a ±0.18
	Caraway seeds oil	2.23	1.44 ab±0.13	8.84 c±0.57	0.405a±0.01	2.8b±0.12
		13.6	1.75 a±0.06	11.415a±0.81	0.465 a±0.01	3.11 ab±0.09
		1.36	1.65 a±0.01	10.415 ab±0.35	0.44a±0.01	3.155 ab ±0.06
Control		-	1.295b±0.06	7.74 cd±0.12	0.38a±0.02	2.97 b±0.06

The numbers followed by the same letter mean that they are not significantly different at the α=0. 05 test level

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Original article

Extending of Shelf Life of Mushroom and Asparagus using Rice bran Protein edible coating

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Abstract

Button mushrooms and asparagus have a very short shelf life after harvesting and are sensitive to mechanical damage, browning, moisture loss and chlorophyll degradation. An edible coating based on rice bran protein (RBP) was tested in order to determine the shelf-life extension period of mushroom and asparagus. Weight loss, color browning, ascorbic acid, total chlorophyll, pH, total titratable acidity, polyphenol oxidase activity and peroxidase activity of mushrooms and asparagus were studied. Also, microbiological analysis of the coated samples was studied. The results revealed that, weight loss and firmness of samples decreased with of using RBP coating compared to the control samples. Also, RBP coating maintain the pH values for samples during storage compared to control. Ascorbic acid decreased during storage for both coated and uncoated while, the uncoated samples decreased more. Also, RBP coating was effective to maintain of the color compared to control. Finally, the study showed also that RBP coating with bacteria, molds and yeasts of mushroom and asparagus samples compared to control.

Keywords

Mushroom; Asparagus; Rice bran protein; Coating; Storage; Quality

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Introduction

Mushrooms are used not only as food but also as functional food and medicines due to high content of proteins and minerals, low cholesterol and starch, and also different bioactive compound (1). Mushrooms have a very short shelf life after harvesting of about 3-5 days at 1- 4 C ºand 70 – 75% relative humidity (2).Thus, it is important to extend the shelf life of fresh mushrooms, while preserving their quality, is desirable to export and import grocery companies (3). Serious problems contribute to the postharvest deterioration of mushroom such as browning, moisture loss, softening, high respiration rate and microbial attack (4). From the point of view of post-harvest physiology, special protection techniques are washing them with citric acid, ethylene diaminetetraacetic acid (EDTA), hydrogen peroxide, and sodium hypochlorite or using ultrasound and high-pressure argon, pulsed light or using coatings required maintaining their quality and freshness (1).

As one of the important fresh vegetables, green asparagus (*Asparagus officinalis L.*) is becoming increasingly popular due to its special flavor, taste, low calories content, high nutritional value and the high economic value in terms of export in recent years**.** Anthocyanins are one of the important groups of phenolic compounds, presenting in fruits and vegetables. They contribute to the characteristic color and have been linked to antihyperglycemic, anticancer, and antimutagenic health benefits. Asparagus has a limited shelf-life of less than 5 days at ambient temperatures, mainly due to its high respiratory rate after harvesting **(5)**. During storage, asparagus undergoes undesirable physiological and compositional changes such as moisture loss, chlorophyll degradation, and lignifications **(5**, 6**)** that lead to a deterioration of the overall quality of the vegetable.

In recent years, the increasing consumption of fresh produce worldwide has led to the necessity for alternative methods with a high efficiency, with a low residue rate, that are non-toxic, environmentally and economically friendly, and which do not threaten human health. Edible coatings applied as a thin layer on the product's surface are biodegradable materials that have no adverse effects on human health and are environmentally and economically friendly. In this respect, edible coating materials are promising treatments for extending the commercial storage life of fresh fruit and vegetables **(7).**

Edible coatings are traditionally used to enhance postharvest food appearance and preservation, as edible coatings provide products with sheen and make them more attractive to consumers **(8)**. Moreover, they maintain the phytochemical (antioxidants, phenolics, and color) and physicochemical (weight loss, respiration rate, and ethylene production) properties for a longer period, and some edible coatings act as a natural antimicrobial and antifungal compound in many vegetables such as mushroom and asparagus **(9).** Edible coatings generally act as a barrier to gas exchange properties and thus prolong the storage life of fruit and vegetables **(10 and 11).** Edible films can be used as semipermeable barriers in food with various purposes, such as: control the respiration rate, retard moisture loss and color variation, improve texture and maintain mechanical integrity, help retain flavor and inhibit growth of micro-organisms (**12**). Thus, the development of edible or biodegradable films arises from the demand for high quality and safe food, as well as from environmental concerns with the disposal of non-renewable materials. It is also an opportunity to create a new market of raw materials for packaging**.** Proteins of plant origin are more often used than animal proteins for films production due to availability and lower cost **(13, 14).** The byproducts from the cereal agro-industrial processing may be a source of protein that can be recovered for the production of protein-based films. An example is rice bran, resulting from the processing of the grain, which proteins were extracted to produce bio-based films (**13**, **15).**

Rice bran Protein has the ability to develop protein films with good physical, mechanical and exposure to light properties. Deamidation process improves protein structure, solubility and increase the strength of hydrogen bonds between protein polymers, hence production of rice bran protein films with suitable physical, mechanical, Exposure to light properties and crystalline structure. That can be widely used as edible film for packaging food products. Hence, deamination process can be applied on another protein material from food and agricultural manufacturing residues to increase their use as distinctive edible coating.

The aim of this study is to evaluate Rice bran protein film to extend shelf life of Mushroom and Asparagus Also, study the changes of some properties during storage.

Materials and Methods

Materials

White mushrooms (*Agricus bisporus*) and green asparagus spears (*Asparagus officinalis L.*) were harvested from a commercial farm in Wadi El-Natrun City, El- Beheira Governorate, Egypt. Samples were transported to the laboratory at 4 Cº. Mushrooms and asparagus with homogeneous color and size and free of injuries were selected. Rice bran protein extracted from rice bran (Giza 178 variety) was obtained from Rice Research Center at Sakha, Agricultural Research Center, Egypt during 2021Season.

All solvents and chemicals such as glycerol, peptone, plate dextrose agar and plate count agar were in analytical grade and purchased from El- Gomhoria Co. for Chemicals and Drugs, Tanta, Egypt.

Methods

Sample preparation

White mushrooms (*Agricus bisporus*) and green asparagus spears (*Asparagus officinalis L.*) of uniform size and free of physical damage, absence of external injuries and fungal infection were chosen and washed with tap water directly before dipping.

Preparation of the edible coating solution

Extracted, freeze-dried rice bran protein at concentration1, 2 and 3% were used. RBP samples were dissolved in distilled water, stirred at 80˚C for 30 min. Glycerol was added at 40% level and pH was adjusted to 8. Citric and ascorbic acids were added as antibrownning and antimicrobial agents at 1% w/v. The solutions were heated under constant agitation until they reached to 80˚C, After that, the coatings were allowed to cool down to room temperature.

Application of coating on samples

Mushroom and asparagus were immersed in a 0.1 % NaClO solution for 1 min for surface-sterilization and air-dried at room temperature for 30 min. Then, the mushrooms and asparagus dipped for 1 min into rice bran protein solutions at levels 1, 2, 3 % (w/v). After coating, the mushrooms and asparagus were left to dry at ambient temperature. All coated samples were placed into macroperforated polypropylene film bag. The treated samples and control were stored in a refrigerator at 5±1˚C and 70 – 75% RH. Samples were stored for 14 and 17 days for mushrooms and asparagus; respectively. Sample withdrawn every 2 days for physical, chemical and microbial properties.

Physical determination

Weight loss

Weight loss was calculated as percentage loss of initial weight, as reported by Han et al., **(16).**

Firmness

The firmness of fresh vegetables was determined by measuring the compression force of the samples using a texture analyzer (QTS-25) fitted with a Kramer shear cell (**17**).

Total soluble solid (TSS)

TSS was measured in the juice pressed from the sample by the refractometeric (RR 12, Nr 05116, 0-35% at 20 ˚C, Made in Poland) method at room temperature according to the methods outlined in the **AOAC** (18 **).**

Chemical properties

Moisture content

Moisture content was determined according to the methods of **(18).**the samples moisture content (approximately 2gm) was determined by measuring the weight loss of samples before and after drying in laboratory oven at 105 Cº until constant weight.

$$\%\text{Moisture content} = W_{O-}W_F/W_O \times 100$$

Where W_O was the sample weight before drying, W_f was its weight after drying.

Measurement of pH

pH meter(Fisher Scientific accument pH meter 25 USA) was used to measure pH the extracted juices as the methods described by **(18).**

Total titratable acidity (TTA)

Total titratable acidity of juices was determined according to the method of **(18).**The (TTA) was expressed as percentage of malic acid in mushroom; while expressed as percentage of galic acid in asparagus.

Ascorbic acid contents

2, 6 dichloro-phenolindophenol titrimetric method was used to determine ascorbic acid in juices as described in **(18).** The results were expressed in milligrams ascorbic acid per 100 ml of vegetables juice.

Chlorophyll content

Chlorophyll content in the asparagus was determined according to the methods described by (**19**) with slight modification. In brief, 2 g asparagus was ground in a mortar and extracted in 10 mL % (v/v) ethanol (95%) and centrifuged at 6000 rpm for 15 min at 4 ºC. The supernatant was used to determine the chlorophyll content. Chlorophyll quantification was performed spectrophotometrically using a spectrophotometer (Varian, Melbourne, VIC, Australia) at 665 and 649 nm, and the chlorophyll content was expressed as chlorophyll mass on a fresh weight (FW) basis (mg/kg FW). The calculation of chlorophyll amount was described by **(20).**

Oxidative Browning

Browning

A colorimeter (CR 300; Minolta, Japan) was used to measure L, a, and b values of the middle part of the A. bisporus cap. The browning index was calculated according to **(21)** the following Equations:

$$\mathbf{X} = a + 1.75L/ (5.645L + a \times 3.012b)$$

$$\text{Browning index} = (100 \times (X-0.31))/0.172$$

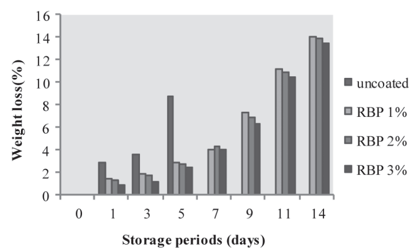

Fig (1): Weight loss (%) of mushroom coated with different levels of RBP coatings, stored at 5±1˚C and 70 – 75% RH for 14 days

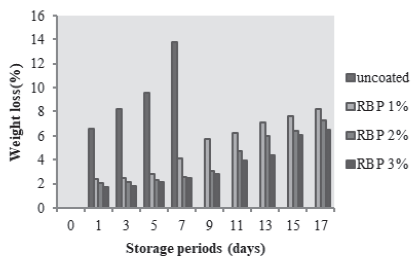

Fig (2): Weight loss (%) of asparagus coated with different levels of RBP coatings, stored at 5±1˚C and 70 – 75% RH for 17 days

Polyphenol Oxidase

Extraction was performed with phosphate buffer at 0.1 M (pH 6.8) using 10 g of each sample; then, 0.5 mL of catechol 0.01M was then added to 5 mL of phosphate buffer solution. The sample was immediately analyzed at an absorbance of 420 nm. Also, absorbance was measured after 2 min. The change in absorbance per minute is 0.01 as a polyphenol oxidase activity unit (U) and the result is expressed in U kg^{-1}1 FW (**22**).

Polyphenol oxidase activity =
= DOD420 × V/ (0.01 × Vs × t × m)

Where:

V = Total volume of enzyme solution (mL).

Vs = volume taken during determination (mL).

T = reaction time (min).

m = fresh weight of sample (kg).

Peroxidase enzyme (POD) activity

POD enzyme activity was determined using the methods reported by **(23).**

Microbiological analysis

Total viable bacteria counts:

Total viable bacteria counts per one of sample were determined using standard techniques on nutrient agar medium. Incubation was carried out for 48 hrs, at 32^{0}C according to **(24).**

Mold and yeast counts

Mold and yeast counts were determined by plating one ml of diluted sample suspension on acidified potato- dextrose agar medium. Triplicate plates were incubated at 25^{0}C for 5 days **(25).**

Results and discussion

Effect of edible coating on physical properties

Weight loss:

The weight loss is indicator for vegetables dehydration process due to transpiration and involves water transfer from the cell to surrounding atmosphere, thus representing a way to evaluate coating efficiency in preservation of quality **(26).**

Data presented in Figures (1 and 2) show the effect of edible coatings prepared using different levels of rice bran protein (1, 2 and 3%) on weight loss of mushroom and asparagus during cold storage for 14 and 17 days, respectively.

The results indicated that weight loss significantly increased during storage of both uncoated and coated samples. All coated samples significantly reduced weight loss during

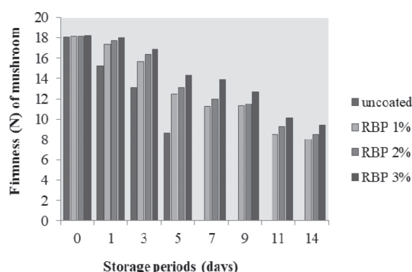

Fig (3): Firmness (N) of mushroom coated with different levels of RBP edible coatings, stored at 5±1˚C and 70 – 75% RH for 14 days

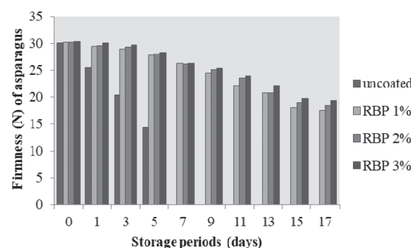

Fig (4): Firmness (N) of asparagus coated with different levels of RBP edible coating of rice bran protein stored at 5±1˚C and 70 – 75% RH for 17 days

cold storage compared to uncoated samples. Coatings are clearly effective in conferring a physical barrier to moisture loss and therefore retarding dehydration and vegetables shriveling **(27).**

The increase in physiological weight loss of fresh mushroom and asparagus during storage period may be due to the loss of moisture content.

It is showed from Figures (1 and 2) that, coated samples with 3% RBP were found to be the best among all of the used coating levels, which reduced the weight loss of mushroom and asparagus to 13.5% and 6.52% after 14 and 17 days; respectively compared to uncoated samples 8.53% for mushroom and 13.80% for asparagus stored for 5 and 7 days; respectively.

Firmness

The data in Figures (3 and 4) revealed that the firmness significantly decreased as a function of storage conditions for both uncoated and coated samples. All coated samples showed significantly beneficial effect on firmness maintenance compared to control.

Firmness measurements of coated and uncoated samples are showed in Figures (3 and 4). Uncoated mushroom lost about 50 % of firmness after 5 days of cold storage; while uncoated asparagus lost about 59% of firmness after 7 days of cold storage, whereas the loss of firmness with other coated samples arranged between 16 to 20 % and 13 to 14 % for mushroom and asparagus after the same period of cold storage. During storage, firmness decreased to 48 % compared to initial of firmness for mushroom and 36% for asparagus; while uncoated samples were removed after 5 and 7 days for mushroom and asparagus; respectively because it were spoiled. The results showed similarity to (29) and (30). According to **(30),** vegetables softening are attributed to the degradation of cell wall components, mainly pectin, due to the action of specific enzymes such as polygalacturonase. The edible coatings showed a good result with respect to the retention of vegetables firmness probably because this coating might be slowed down metabolism and prolonged the storage life, same effect was previously observed by **(31).**

Total soluble solid (TSS)

Data in Figures (5 and 6) shown total soluble solid (TSS) of pressed juices from uncoated and coated mushroom and asparagus during cold storage. The results indicated that TSS which is an indicator of sugar content significantly increased in all samples as a function of storage time. This increase might be due the weight loss during storage especially moisture loss.

TSS of uncoated mushroom and asparagus samples were significantly higher than those of coated samples. It may be explained by decreasing of moisture content of coated samples compared to uncoated samples.

TSS increased during the storage time (Figures 5 and 6) from 9.61 to 12.54% during storage from zero to 5 days for mushroom, while increasing from 13.29 to 34.88% during storage from zero to 7 days; respectively for control asparagus samples (uncoated samples). TSS of samples coated with RBP coating was more than that of control. So, mushroom and asparagus coated with 3% RBP coated can be freshly stored for 14 and 17 days with high juiciness.

TSS increases during storage and ripening of fruits and vegetables. This could be attributed mainly to the breakdown of polysaccharides components into simple sugars during ripening along with a proportional increase in TSS, but further hydrolysis could decrease the TSS during storage and it is caused by a decline in the amount. The results obtained are agreement with investigations by **(32) and (33).** The coated samples showed a lesser increase in TSS than the uncoated samples. This could be due to the reason that coating reduces the production of ethylene and the rate of respiration **(34)** and reduces the rate synthesis and utilization of metabolites, delaying nutrient decomposition and as a result, a lower TSS values in coated samples were there as compared to non-coated samples **(35).** The increment of total soluble solids at the time of storage period is natural as sugar the basic constituent of the TSS is used in respiration process for metabolic activities of the fresh fruits and vegetables **(36).**

Chemical properties

Moisture contents

Figures (7 and 8) show the changes in moisture contents of coated and uncoated mushroom and asparagus during cold storage. Moisture content of mushroom and asparagus significantly decreased as a function of storage time for both uncoated and coated samples.

All coatings provided a beneficial barrier for moisture and prevent weight loss during storage. It may be due to that coatings fill pores and crakes on skin, so prevent moisture loss. The results revealed that uncoated samples can be stored for 5 days and 7 days for mushroom and asparagus; respectively. From the aforementioned results 3% RBP was more effective in reducing moisture loss and low water permeability until day 14th for mushroom and 17th for asparagus followed by 2% and 1%; respectively. The semi permeable barrier provided by edible coatings is aimed to extend shelf life by reducing moisture and solutes migration, gas exchange, respiration and oxidative reaction rates, as well as suppress physiological disorders on fresh vegetables **(37 and 38).**

pH and total titratable acidity (TTA) values

The results are shown in Figures (9 and 11) for mushroom and Figures (10 and 12) for asparagus revealed that pH values of uncoated samples increased as function of storage time. The increase of pH values of coated samples was lower compared to uncoated samples. Increase of pH values (decrease of acidity) demonstrates maturation development **(31).** The coating treatments delayed maturation process. It might be due to the effect of coatings in reducing vegetables respiration rate; therefore they delay the utilization of organic acids as substrates for enzymatic reactions **(39).**

No significant differences in pH values were found between coated samples during storage period, while pH increased in uncoated sample with increasing storage period.

Figure (11) show the total titratable acidity (TTA, expressed as % of malic acid) of both uncoated and coated vegetable. TTA of uncoated sample for mushroom significantly decreased as a function of storage time. No significant

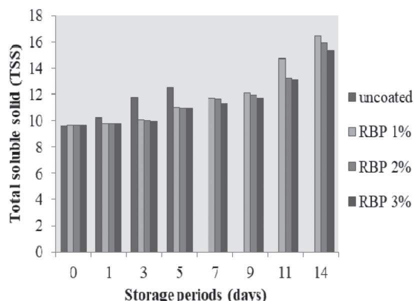

Fig (5): Total soluble solid (TSS) of juices from mushroom coated with RBP edible coatings, stored at 5±1˚C and 70 – 75% RH for 14 days

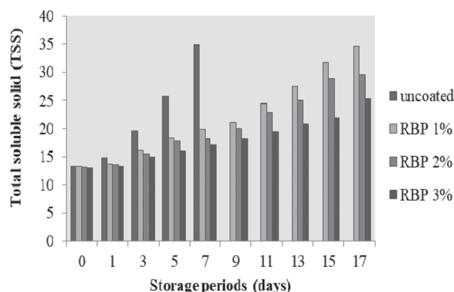

Fig (6): Total soluble solid (TSS) of juices from asparagus coated with RBP edible coatings, stored at 5±1˚C and 70 – 75% RH for 17 days

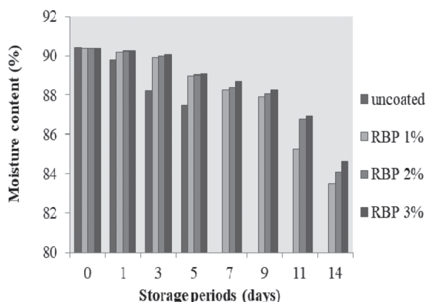

Fig (7): Moisture content of mushroom coated with RBP coating stored at 5±1˚C and 70 – 75% RH for 14 days

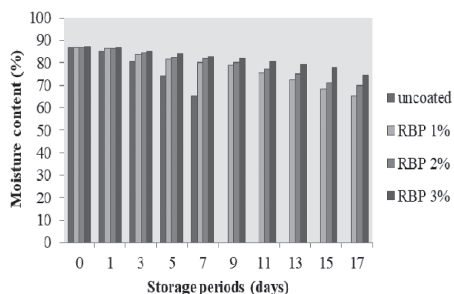

Fig (8): Moisture content of asparagus coated with RBP edible coating stored at 5±1˚C and 70 – 75% RH for 17 days

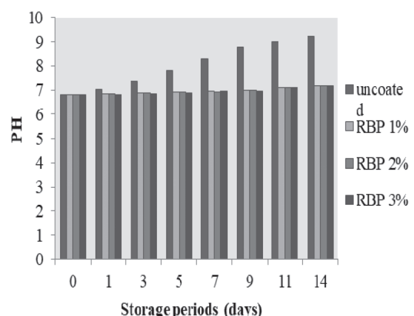

Fig (9): effect of edible coating with different levels of rice bran protein on PH of mushroom at 5±1˚C and 70 – 75% RH for 14 days

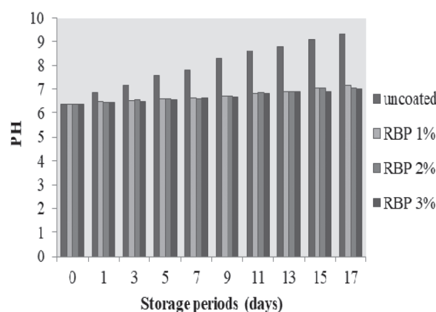

Fig (10): effect of edible coating with different levels of rice bran protein on PH of asparagus at 5±1˚C and 70 – 75% RH for 17 days

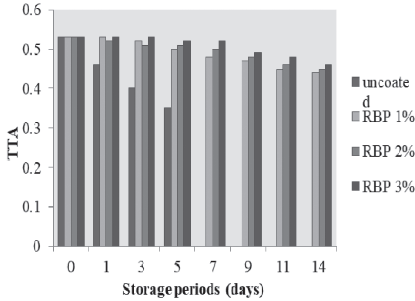

Fig (11): Effect of RBP coating total titratable acidity (TTA) of mushroom stored at 5±1˚C and 70 – 75% RH for 14 days.

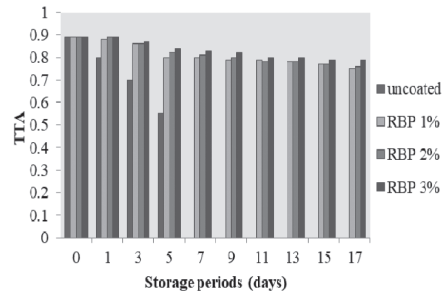

Fig (12): Effect of RBP coating on total titratable acidity (TTA) of asparagus stored at 5±1˚C and 70 – 75% RH for 17 days.

differences were found in pH between all of coated samples and other treated samples during cold storage from zero to 14th.

Results presented in Figures (10 and 12) showed both pH and total titratable acidity values of pressed juices from asparagus samples. No significant differences in pH values were found among coated samples during storage period; while pH increased in uncoated sample with increasing storage period for asparagus samples. Figure (12) show the total titratable acidity of both uncoated and coated samples. TTA of uncoated sample for asparagus significantly decreased as a function of storage time. No significant differences were found in pH among all of the coated samples during storage from zero to 17th.

(40) found that the decrease in titratable acidity and the slight increase in pH of coated samples for mushroom and asparagus might be due to the delay in maturation process and the metabolic activity within the tissue.

Since organic acid are substrates for enzymatic reaction of respiration, a reduction in the acidity and increase in pH values are expected of uncoated samples. Coatings reduce the respiration rate; therefore delay the utilization of organic acids. These results are in agreement of the results of **(41).**

The coating acts as a semi- permeable membrane against respiration, which reduces the rate of respiration and further reduces the consumption of organic acid acids; therefore, the titratable acidity decreased **(42).**

The low change of pH due to the edible coating forming a semipermeable membrane on the surface of fruits and vegetables which modified the internal atmosphere *i.e.,* the endogenous CO_2 and O_2 concentration, thus retarding the ripening process.

(43) reported that the pH seems to be influenced by the concentration of SPI (Soy Protein Isolate) and that of HPMC (Hydroxypropyl methylcellulose). According to this study with the increased edible coating concentration the pH is lowered in pears. **(44)** found that pH of control pears samples significantly increased ($P<0.05$) as compared to coated samples with alginate and carrageenan 0.5% during storage. **(45)** found similar results in chitosan- *Aloe vera* coated cucumbers and cornstarch and carboxy-methylcellulose coated cucumbers, respectively. **(46)** also reported the similar result with guar based edible coating in cucumber decreased the pH as compared to uncoated cucumber. Titratable acidity retention was reported by **(47),** using strawberry fruits coated with gluten film. Slowing down the strawberry respiration rate by means of an edible coating could explain the delay in the use of organic acid in the enzymatic reactions of respiration, **(48).**

Effect on ascorbic acid

It should be noticed from Figures (13 and 14) that ascorbic acid contents in both coated and uncoated mushroom and asparagus significantly decreased during cold storage. Ascorbic acid losses of coated samples were lower than that of uncoated samples during storage. It may be due to adding of ascorbic and citric acids in to coating solutions which plays important role as antioxidants and role of coating in reduction of oxygen permeability. **(49)** mentioned that the decrease of oxygen passes through the edible coating decrease the rate of oxidation of ascorbic acid.

Coating process maintains about 15.36 to 17.74 mg/ 100g of total ascorbic acid after 5 days of cold storage for mushroom, while the remained of ascorbic acid of control after 5 days was about 5.04 mg/ 100g. After 14 days of cold storage, ascorbic acid was 7.16 – 11.50mg / 100 g; while control sample was spoilage after 5 days. As for asparagus, ascorbic acid recorded about 173.95 to 180.97 mg/100g for coated samples; while it recorded 100.77 mg/100g for uncoated samples during storage to 7 days. After 17 days, ascorbic acid reached about 120.66 – 142.12 mg/100g as af-

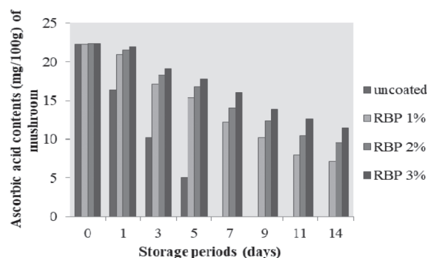

Fig (13): Ascorbic acid contents (mg/100g) of mushroom coated with RBP coating stored at 5±1˚C and 70 – 75% RH for 14 days.

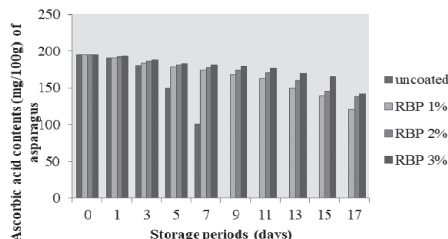

Fig (14): Ascorbic acid contents (mg/100g) of asparagus coated with RBP coating stored at 5±1˚C and 70 – 75% RH for 17 days

fected by coating process, but control sample spoiled after 7 days only of storage.

From the previous results, it found that 3% RBP coated samples were the best in maintenance V.C in mushroom and asparagus. The edible coating acts as a covering layer that prevents the autoxidation process, and maintained of ascorbic acid content **(9).**

Effects on total chlorophyll of asparagus:

In Figure (15) showed that significant difference ($p<0.05$) between coated and uncoated asparagus in total chlorophyll content.

A statistically significant decrease was noticed in total chlorophyll content with time during storage (Figure 15). Data showed that all coated samples had significantly highest chlorophyll content. Coating process maintains about 73.17 to 78.51 mg/ 100g of total chlorophyll after 7 days of cold storage; while total chlorophyll of control after 7 days was about 58.98 mg/ 100g. After 17 days of storage, total chlorophyll was 61.05 to 69.36 mg / 100 g, while control spoiled after 7 days.

From the previous results, with asparagus coated 3% RBP had significantly the highest value of total chlorophyll content among all of others levels during cold storage.

This decrease of chlorophyll content could be attributed to gradual increase of destruction by chlorophyll degrading peroxidase activity and also transformation chloroplasts to chromoplasts by chlorophyllase activity **(50).**

The reduction of chlorophyll loss of asparagus during storage using RBP coating may be attributed to reducing of respiration rate, resulted in lower activity of chlorophylls and consequence reduced color changes **(51).**

Effects on browning in mushroom:

The color of the button mushroom is an important parameter, since color relates directly to the perception of quality and acceptability by the consumer **(52).**

Figure (16) shows of the color parameters (*L*, *a*, *b*, *ΔE* and BI) for coated and control mushrooms.

The *L* values decreases, and *ΔE* and BI increased with storage time for coated and control mushrooms. From the day 3 it is that there was observed differences ($p<0.05$) between two groups, being the coated mushrooms those with higher whiteness, less color difference and browning. Being in agreement with a study realized by **(53),** where the *L* values of mushrooms coated with 1% , 2% and 3% rice bran protein were significantly ($p<0.05$) lower than that uncoated at 5 ºC after 14 days of storage.

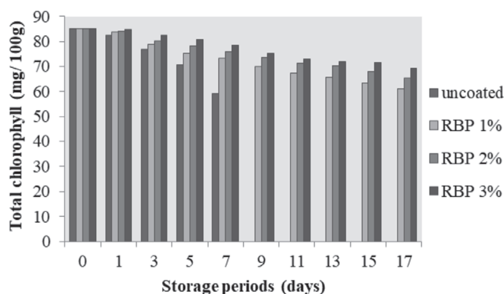

Fig (15): Effect of RBP coating on total chlorophyll of asparagus (mg/ 100g) stored at 5±1˚C and 70 – 75% RH for 17 days.

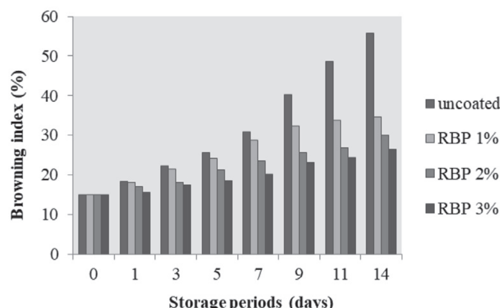

Fig (16): Effect of RBP coating on browning index (%) of mushroom stored at 5±1˚C and 70 – 75% RH for 14 days.

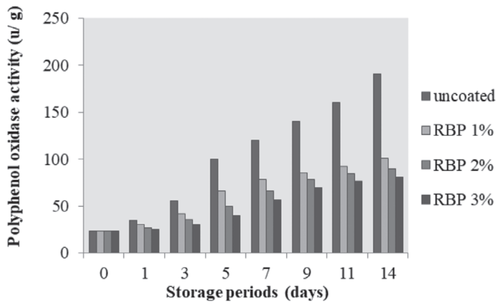

Fig (17): Effect of RBP coating on polyphenol oxidase activity(u/ g) of mushrooms stored s at 5±1˚C and 70 – 75% RH for 14 days

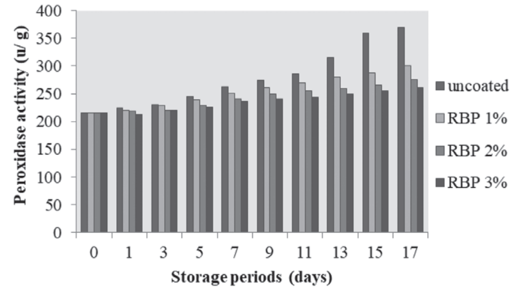

Fig (18): Effect of RBP coating on peroxidase activity (u/ g) of stored at 5±1˚C and 70 – 75% RH for 17 days

During storage, low changes of color were shown of coated mushrooms compared to control. The browning index at the end of storage increased from 15.0 to 55.76, from 15.0 to 34.63,from 15.0 to 30.06 and from 15 to 26.46 for control and coated mushrooms at 1 , 2 and 3% RBP; respectively. Samples coated with 3% RBP was the best reducing browning index for mushroom at the end of storage period, while control spoiled after 5 days of storage.

Protein coatings probably delay browning by preventing the oxidative process. An important factor implied in the inhibition of the oxidative browning is that the coating by protein solution represents an efficient barrier to oxygen.

Effect of RBP coatings on polyphenol oxidase of mushroom

PPO is the major contributor for the browning of fresh white mushrooms, due to their influence in the oxidation of phenolics, resulting in the formation of brown-colored substances. Figure (17) show the PPO activity during storage, which increases for both coated and uncoated samples. Significant difference (p<0.05) were observed between coated and control, being the coated mushrooms is best.

At zero time, no significant differences in PPO activity were found between uncoated and coated samples.

PPO activity increases from 23.67 to 40.15U/g fresh weight for samples coated with 3% RBP, from 23.67 to 49.60U/g fresh weight for coated with 2% and from 23.67 to 66.44 for coated with 1% RBP, while PPO control mushrooms increase from 23.67 to 100.53 after 5 days increased from 23.67 to 190.93 U/g fresh weight at the end of storage. On the other hand PPO activity increased from 23.67 to 80.57, from 23.67 to 89.63 and 23.67 to 100.98 u/g for samples coated with RBP at 1,2 and 3% respectively Generally, 3% RBP coated samples had the best in maintenance PPO activity in mushroom.

It may also be observed that the PPO activity increased more on control than that of coated mushrooms. The rapid increase of PPO activity possibly accelerate the oxidation of polyphenols presents on the mushrooms. However, on coated mushrooms the coating seems inhibit the PPO immediately on the day of coating application (day zero). This difference can be explained by the fact that the coating (that contain an inhibitor of the enzyme tyrosinase, cinnamic acid) inhibited the PPO, reducing drastically its activity.

In addition, and as stated before, the RBP coating forms a protect barrier on the surface of the fresh produce, reducing supply of O2 which can help reducing the PPO activity.

Han (16) found that protein and peptides in rice bran protein can affect the polyphenol oxidase activity by reacting with chelating on copper at active site of PPO, also it has reported that RBP contains anti-oxidative peptides and sulfur amino acids and thus these peptides might be the cause of browning inhibitor.

Effect of RBP coating on peroxidase activity of asparagus:

Figure (18) indicated that peroxidase (POD) activity of asparagus spears increased along storage period; these results are compatible with **(54)** on asparagus. The increasing of POD activity is caused delaying senescence. **(54)** found that POD enzyme cause catalyzes of corruption H2O2, this causes senescence of product. So, POD enzyme destroys pigments of chlorophyll and is considered an indication of senescence and intense stress.

Data reveled that all coating level significantly decreased activity of peroxidase compared with control; however asparagus spears coated with 3% RBP had significantly the lowest e of peroxidase activity, followed by 2% CMC then 1% CMC, while the highest peroxidase activity was obtained from control.

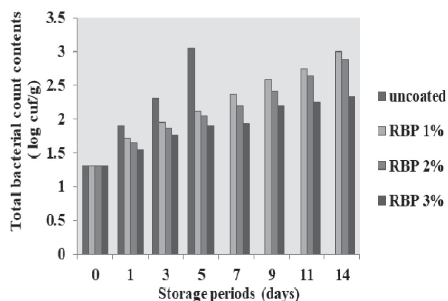

Fig (19): Effect of RBP coating on total bacterial count contents (log cuf/g) of mushroom stored at 5±1˚C and 70 – 75% RH for 14 days.

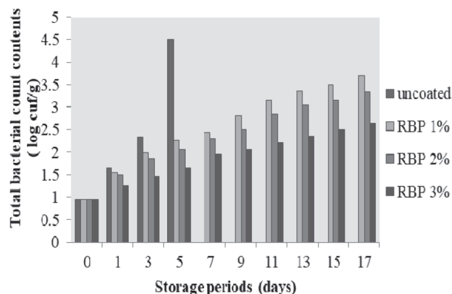

Fig (20): Effect of RBP coating on total bacterial count contents (log cuf/g) of asparagus stored at 5±1˚C and 70 – 75% RH for 17 days.

Effect of coatings on microbial growth

Total bacterial counts

Figures (19 and 20) showed the effects of RBP coating at levels 1, 2 and 3% on total bacterial count of mushroom and asparagus during storage for mushroom and asparagus, at 5± 1ºC and 70 – 75%.

The results pointed to, significant differences were found in total bacterial count between coated and uncoated samples. The same Figure illustrated that, total bacterial count significantly increased gradually with the increasing of storage period in all samples. Where, all coating levels were effective in reducing total bacteria count compared to control. These findings are in agreement with investigations by **(55)**. It should be observed also from Figures that, total bacteria count decreased gradually with the increase of RBP levels comparing to control. However ,samples coating with RBP level 3% was the best among all of the coated samples reducing levels of total bacterial count to 2.33 and 2.65 (Log CFU/g) for mushroom and asparagus; respectively at the end of storage period. These results agreed with those reported by **(56).**

Mold and yeast counts:

Figures (21 and 22) showed the effects of edible coating of RBP at levels 1, 2 and 3% on mold and yeast counts of mushroom and asparagus during storage for mushroom and asparagus, at 5± 1ºC and 70 – 75%. .

The results reflected that, significant differences were found in molds and yeasts count between coated and uncoated samples. The results revealed that molds and yeasts count significantly increased gradually with the increase of storage period in all samples. Where, all coated samples had lower mold and yeast counts than control. These findings are in agreement with investigations by **(56).**

It should be observed also that mold and yeast decreased gradually with increasing of RBP levels comparing to control. Samples coated with 3% RBP was the best coating in reducing levels of mold and yeast to 2.35 (CFU/ g) for mushroom and 2.65 (CFU/ g) for asparagus at the end of storage period; While control sample was spoiled after 5 days for mushroom and 7days for asparagus.

(57) showed higher microbial counts with uncoated sample whereas all coated samples showed low total micro-

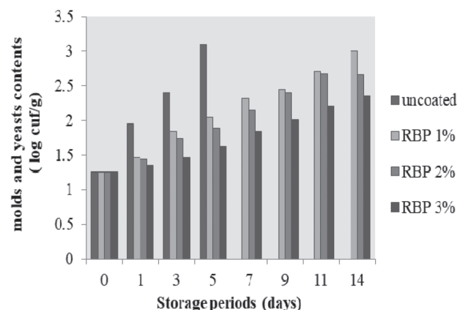

Fig (21): Effect of RBP coating on molds and yeasts contents (log cuf/g) of mushroom stored at 5±1˚C and 70 – 75% RH for 14 days.

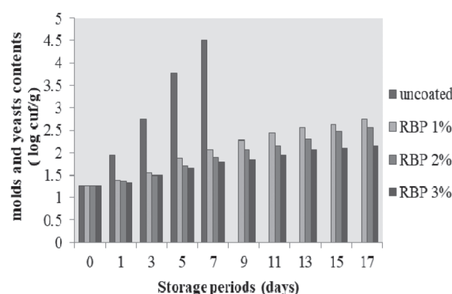

Fig (22): Effect of RBP coating on molds and yeasts contents (log cuf/g) of asparagus stored at 5±1˚C and 70 – 75% RH for 17 days

bial counts. Also, **(45)** showed that during the storage coating decreased aerobic yeast and mold count for cucumber compared with control. Moreover, **(14)** mentioned that edible coating applied on fresh cut apples had marked effect in reducing psychrophilic counts as compared to uncoated apple pieces.

Conclusion

Regarding of this work it can be concluded that the developed coating improved the quality of mushrooms and asparagus. The application of the coating showed to prolong the shelf life of mushrooms and asparagus, decreasing weight loss, reducing changes in color, titratable acidity, pH and TSS during refrigerated storage. The coating was effective as a barrier in the reduction of weight loss during storage and also had beneficial effects in delaying the ripening process and improved the appearance of the coated mushrooms and asparagus when compared with the uncoated.

The color is related to the age of the mushrooms and it has been used as an indicator to quantify the shelf life. The microbial population could affect the color change of fresh mushrooms and asparagus as well as the action of polyphenol oxidase (PPO) in browning for mushroom and peroxidase activity in degradation of chlorophyll for asparagus.

Rice bran protein (RBP) coating at level 3% was more effectively to increase shelf – life of mushroom and asparagus to 14 and 17 days; respectively. Also, protect these samples from microbial contamination compared to control. Also, some physical and chemical properties for coated samples were bitter than that of control. So, 3% RBP coating could be recommendation to maintain of mushroom and asparagus quality during storage for 14 and 17 days at 5 ºC.

These results suggest that the edible coating used in this work may be a promising method of maintaining the quality of the button mushrooms and asparagus that can be used to increase shelf-life during refrigerated storage. The use of this coating may have commercial importance, since it is necessary small amounts of the active compounds to obtain positive results.

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Original paper

Grain Yield Seed Hybrids Maize per Different Way of Detasseling

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Abstract

The experiments were conducted in field conditions, comprising two maize hybrids and two methods of detasseling, in the period 2014-2016 in a randomised block design. The seed yield was 3.64 t/ha, as average for all the hybrids and the methods of detasseling. "Suanito" yielded 2.88 t/ha while "MAS 26K" yielded 4.41 t/ha. The highest yield was obtained in 2015 (3.81 t/ha), then in 2014 (3.47 t/ha), while the lowest yield was obtained in 2016 (3.12 t/ha). Manual detasseling resulted in the seed yield of 3.94 t/ha, whereas a significantly lower yield was achieved with mechanised detasseling (3.34 t/ha). There was a highly significant interaction found between the year and the hybrid, the year and the method of detasseling and the hybrid and the method of detasseling, whereas there was a significant interaction between the year, the hybrid and the method of detasseling.

Keywords

maize, hybrid, grain yield, seed, detasseling

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Introduction

Given that global demands for maize increases each year, the science has to address that challenge. One of the ways to do so is to introduce into production the seeding material that would be more productive, i.e. that would lead to higher and stable yields of satisfactory quality. People are creating hybrids with numerous advantages over cultivars; the first of all is the possibility to obtain higher yields (JOCKOVIĆ & al. [6]).

A shift to hybrid maize breeding and production has enabled us to generate considerable profits and at the same time to achieve astonishing improvement in the field of breeding and seed production, thus making maize seed production become a high-tech industry (DOKIĆ & al. [2], PAVLOV & al. [13], SEČANSKI & al. [15]).

Hybrid maize seed production is also important from the aspect of profits for seed companies, and therefore the bar has been set high in terms of quality, yields and benefits for growing areas so that we could maintain and increase our competitiveness on the world market. The quality and profitability of maize seed production will be ensured by good production management. Seeds must meet high genetic, physical and phyto-sanitary standards (MAC ROBERT & al. [12]).

Detasseling, apart from soil preparations, sowing and removing atypical plants, is one of the key stages in the overall process of seed production, significantly affecting the end result. Unless detasseling is well and timely done, one will not get results they want, which is quality and genetically uniform hybrid maize seeds (KNEŽEVIĆ [8]).

Detasseling has become a common method in production of maize seed. Detasseling either conducted manually or with equipment, always raise a question how much the yields decrease when one or more leaves are removed with the tassel. The aim of the research was to determine which method of detasseling in production of maize hybrid seed ("MAS 26K" and "Suanito") is more effective, manual or with equipment, from the aspect of seed yield per unit area.

Materials and Methods

The field experiment comprised two maize hybrids, Suanito, the product of the German seed company Saaten Union and MAS 26K, the product of the French seed company Maisadour Semences. Both hybrids are early vegetation (Suanito FAO group 230, MAS 26K FAO group 300). The experiment was conducted on the field owned by "AD Poljoprivreda" from Senta, in 2014, 2015 and 2016 in a randomised block design with three repetitions and two variants of detasseling (manual and with equipment). The size of a basic plot was 1,000 m^2 (100 x 10 m). The soil type was chernozem on a loess terrace of a mildly alkaline reaction, with 2.5 % humus in the plough layer, fairly equipped with easily accessible phosphorus and well equipped with potassium. The preceding crop in all three years was barley, and a standard maize cropping practice in the Republic of Serbia was used (MARINKOVIĆ & al. [11], LATKOVIĆ & al. [10]). The both hybrids were sown following the 4:3 seeding scheme. "Mother plants" were sown in four rows, with 70 cm spacing between the rows of and at 55 cm distance from "father plants". "Father" plants were sown in three rows, with 15 cm spacing between the rows (Figure 1). Sowing was done with machinery in the second half of April. The plants were sown in the depth of 5 cm. Detasseling was conducted in the period from late June to early July. The detasseling was done with a high clearance "Frema" machine, type "Aiglon" and manually. After detasseling, three control were done in which the rest of the tassels were manually removed. "Fathers" were removed in early August. The plants were harvested at the moment when the grains contained 35% moisture, at the end of August, after which the seed yield was determined.

The data were statistically analysed by using the analysis of variance, with MSTAT - C software, Michigan State University, Version 1. The year, hybrid and the method of detasseling were taken as factors in the analysis. The results are shown as a three-year average.

Results and Discussions

Based on the trifactorial variance analysis, very significant differences (P≤1%) for the grain yield of seed corn seed were determined, depending on the tested hybrids, the method of detasseling and years. Very significant differences were established for interactions: year x hybrid, year x treatment, hybrid x treatment while significant (P≤5%) differences were established for interaction years x hybrid x treatment (Table 1).

The seed yield per unit area in this three-year research amounted to 3.64 t/ha, regardless of the hybrids and the

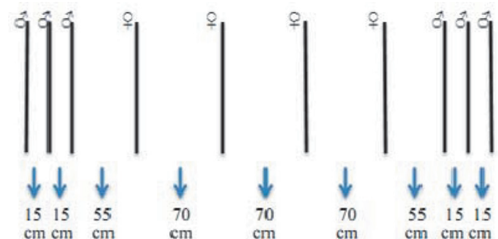


Figure1. Seed production of maize

Table 1. Analysis of variance for grain yield of maize seed

Source	Df	Sum of Squares	Mean Squares	F	
				Value	Significance
Repetition	2	0.002	0.001	0.70	
Year (Y)	2	2.850	1.425	1330.46	**
Hybrid (H)	1	19.010	19.010	17750.06	**
YxH	2	0.134	0.067	62.33	**
Treatment(T)	1	2.970	2.970	2773.10	**
YxT	2	0.022	0.011	10.36	**
HxT	1	4.416	1.416	1322.27	**
YxHxT	2	0.008	0.004	3.94	*
Error	22	0.024	0.001		
Total	35				

method of detasseling. In the three-year average, when observed the hybrids and detasseling, the yield of "Suanito" was 2.88 t/ha and the yield of "MAS 26K" was 4.41 t/ha. This difference is highly significant (Tables 2, 1, Graphs 1 and 2).

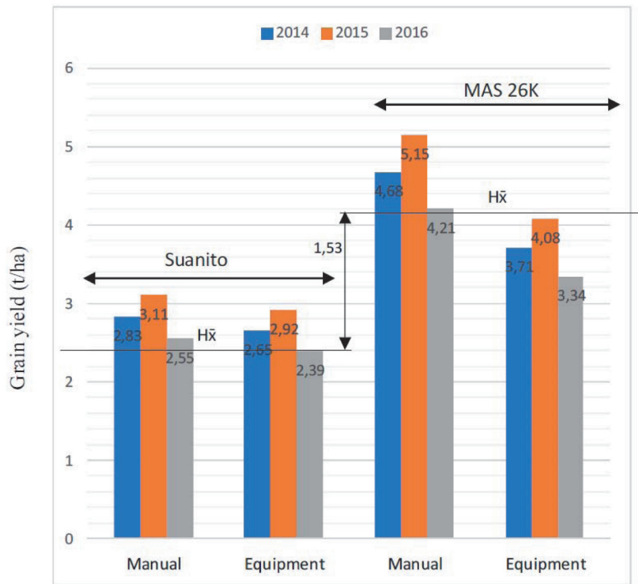
Observed by year, it was shown that the highest seed yield was achieved in 2015 (3.81 t/ha), and then in 2014 (3.47 t/ha), while the lowest yield was achieved in 2016 (3.12 t/ha). The analysis showed a highly significant difference between the years, which often occurs in the agro-ecological conditions of the Republic of Serbia (Tables 1 and 2) (Graphs 1 and 2). The manual detasseling resulted in the seed yield of 3.94 t/ha, while the detasseling with equipment had a significant lower yield of 3.34 t/ha (Tables 1 and 2) (Graphs 1 and 2).

A highly significant interaction was found between the years and the hybrids, between the year and the method of detasseling as well as between the hybrid and the method of detasseling, whereas there was a significant difference between the year, the hybrid and the method of detasselling (Table 1).

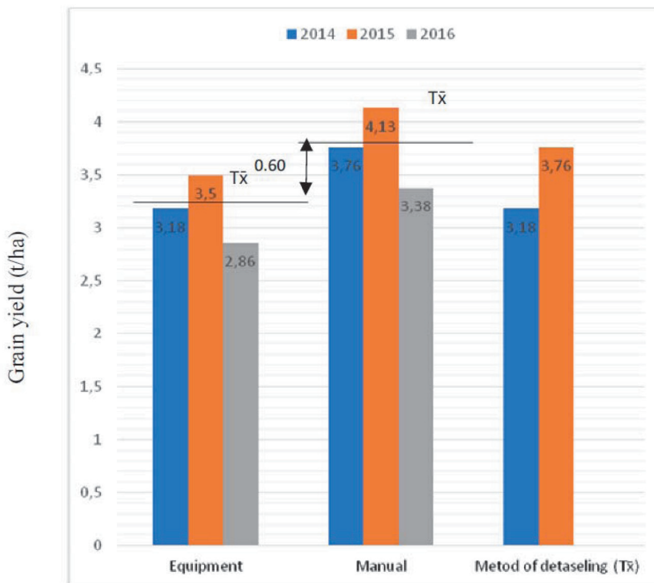
KRIZMANIĆ & al. [9] in their research point out that maize yields significantly vary depending on the method of detasseling but also on hybrids. The highest seed yield was

Table 2. Yield of seeds (t/ha) of different maize hybrids at different detasseling methods (2014-2016)

Years	Method of detasseling	Hybrid (H)					
YTX̄							
YX̄							
(Y)		(T)		Suanito	MAS 26K		
2014	Manual			2.83	4.68	3.76	
	Equipment			2.65	3.71	3.18	3.47
YHX̄				2.74	4.20		
2015	Manual			3.11	5.15	4.13	
	Equipment			2.92	4.08	3.50	3.81
YHX̄				3.01	4.61		
2016	Manual			2.55	4.21	3.38	
	Equipment			2.39	3.34	2.86	3.12
YHX̄				2.47	3.78	T X̄	
	Manual			2.97	4.91	3.94	
TH X̄	Equipment			2.78	3.90	3.34	3.64
HX̄				2.88	4.41		
Level of significance							
	H	Y	T	YH	HT	YT	YHT
LSD 0.05	0.082	0.027	0.040	0.038	0.031	0.038	0.053
0.01	0.095	0.036	0.048	0.051	0.042	0.051	0.073



Graph 1. Grain yield of seed maize by year, hybrid and method of detasseling



Graph 2. Interaction between the years (y) and the methods of detasseling (T)

obtained when detasseling was done manually, and a significantly lower yield when it was done mechanically.

Yield decreasing is directly related to decreasing of the leaf area and it is expressed as a decrease in absolute grain mass and the number of grains per row. When two leaves were removed with the tassel, yield decreased an average of 22–31%, while when plants were cut above the main ear, seed yield decreased an average of 31–66 % (GHEȚE & al. [3]). When it comes to the variants with the second leaf removed and certain maize lines, the yield was not significantly reduced, while in some others the yield was reduced at 5% significance level. By cutting off the leaves at the height of the first tassel the yield will decrease from 11% to 14%. The difference amounts to 7% when the first leaf is removed, 12% when the second leaf is removed, 16% for the third and 21% for the fourth leaf (ĆIROVIĆ & JOCIĆ [1]). Differences in a yield reduction grow with the increase in the number of removed leaves.

Removing the second, third and fourth leaf with the tassel decreases the average yield of seven inbred lines by 11.7, 21.8 and 27.9%, while the average decrease in four individual crossings was 7.1, 8.6 and 19.4% when compared to the decrease without leaf removal (PUCARIĆ & GOTLIN [14]).

A loss of a certain amount of the leaf area was coupled with detasseling, whether manual or mechanised. Studies done by KIESSELBACH [7], HUNTER & al. [4], HUNTER & al. [5], PUCARIĆ & GOTLIN [14], GHEȚE & al. [3] shows that the removal of the tips had different effects, depending on the properties of the studied maize lines. Thus, the number of leaves removed has a varying influence on seed yield. Large differences in yield decrease caused by leaf removal were found among inbred lines and among individual crossbreds. These differences were mostly in the amount of decrease in leaf area per plant.

Conclusions

In three-year research, the maize seed yield of 3.64 t/ha per unit area was recorded, regardless on the tested hybrids and methods of detasseling. The researchers recorded the seed yield of 2.88 t/ha in "Suanito" and 4.41 t/ha in "MAS 26K". The difference is highly significant.

The highest seed yield was recorded in 2015 (3.81 t/ha) and then in 2014 (3.47 t/ha) while the lowest yield was recorded in 2016 (3.12 t/ha). The analysis shows a highly significant difference among the years in which the experiment was conducted.

Manual detasseling resulted in the seed yield of 3.94 t/ha, and mechanised detasseling resulted in a significantly lower yield of 3.34 t/ha.

A highly significant interaction was found between the years and the hybrids, between the year and the method of detasseling as well as between the hybrid and the method of detasseling, whereas there was a significant difference between the year, the hybrid and the method of detasselling.

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Original paper

Research on the action of fenugreek extract on the growth of the pathogen *Monilinia* spp. in vitro

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Abstract

Moniliosis caused by the species of the *Monilinia* genus attacks fruit tree orchards wherever seeds fruits tree and stone fruits tree species are cultivated, producing economically significant losses. *Moniliosis* is also present in Romanian orchards, requiring control of this disease because it can evolve both in the field and in storage conditions, too. The research aimed to test the antifungal in vitro activity of fenugreek extract against the pathogen *Monilinia* spp. *Monilinia* spp was isolated and successively replicated from fresh plant material, represented by apple fruits with specific sporodochia. The fenugreek extract (ska) was tested in concentrations of 3.3% (ska 3,3%) and 10% (ska 10%), comparing the results with the control variant. At the 10% fenugreek extract, the fungus did not grow in the first 3 days, registering a vegetative growth after 6 days of incubation. After 12 days of incubation with the 10% fenugreek extract, the diameter of the mycelium colonies was 6.3 mm compared to the control variant, where the value of the colony diameter was 56.3 mm. The effectiveness of the fenugreek extract at a concentration of 10%, as a percentage of inhibition, was 88.80%.

Keywords

Monilinia spp., antifungal activity, efficacy

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Introduction

Moniliosis is a frequent disease in orchards, being caused by the complex of pathogens belonging to the genus *Monilinia*. The attack of *Monilinia* spp. moulds, responsible for the occurrence of grey mould and fruit rot, represent a serious economic problem, these pathogens determine important losses in fruits vegetation and storage period (OLIVEIRA & al [15]). But the losses caused during the vegetation period can also evolve in storage conditions (LEEUWEN & KESTEREN, [8]). *Monilinia fructigena* is common on apple and quince species, *Monilinia laxa* mainly attacks stone fruits tree species and *Monilinia fructicola* (G. Winter) occur frequently on plum, cherry, apricot and stone cherry (SNYDER & JONES, [5]). The symptoms of the moniliasis attack are visible in the vegetation causing the withering of the shoots and in the flowering stage causing the browning of the flowers. As for the fruits, the attack is different depending on the climatic conditions. In hot and humid weather, the attack appears in the form of brown rot, in wet and cold weather, black rot appears on the fruits and in alternating cold and warm weather, the attack appears in the form of mummified fruits. The identification of the pathogens involved in the occurrence of moniliasis in trees is carried out according to the morphological characteristics of the fungi involved (Gheorghies & Cristea, [2]) and especially through genetic methods (FULTON & BROWN, [3], FULTON & al [4], FORSTER & ADASKAVEG, [9], GELL & al, [9], ZHU & al [21]). The epidemiological studies on the spread of moniliasis have highlighted the importance of biotic and abiotic factors: vectors such as insects and birds, water droplets, fruit lesions, the infectious load on diseased fruit, hail, air currents and man through his activity (LACK, [13]; PAUVERT et al, [17], HELLMAN, [16], BYRDE and WILLETTS [20], BANNON & al [6]). Also, an important role in the transmission of diseases is played by sick fruits and the biological reserve from the orchards, the fallen and mummified fruits from the trees constitute the inoculum for the following year. Field attacks can also evolve under storage conditions (van LEEUWEN [7]). Pathogen survival, tissue colonization, *Monilinia* spore release and infection are related to environmental factors (temperature, relative air humidity, amount of precipitation) (WATSON et al, [19]; BANNON et al, [6]).

Phytosanitary intervention in the control of moniliasis takes into account a lot of prophylactic and therapeutic possibilities, which include crop hygiene, agrophytotechnical measures, genotype resistance, and chemical control (BORVE & STENSVAND, [11]). The administration of fungicides to combat brown rot is the curative measure used in the management of moniliasis, but special care must be given to the phenomenon of resistance, which can occur with the repeated application of products with systemic action. The application of integrated control schemes with the presence of chemical products has shown effectiveness in combating moniliasis in fruit trees (CHITULESCU and CRISTEA, [13]). Research on the biocontrol of *Monilinia* pathogens in the field and laboratory is an alternative to fungicide therapy (GRZEGORCZYK & al, [15]). A researched method is the use of plant extracts with antifungal activity. It was determined under laboratory conditions, the effect of steroidal glycoalkaloids extracted from *Solanum* species, as a component of a bio fungicide, on the growth of *Monilinia* spp., pathogens isolated from plum fruits (CRISTEA & al, [18]).

Materials and Methods

Our research investigated the antifungal action of fenugreek extract on the mycelial growth of the pathogen *Monilinia spp*. It was used the inclusion method in the culture medium, and the PDA (potato-dextrose-agar) culture medium (Carl Roth GmbH +Co) was prepared according to the protocol (autoclaved at 121^{0}C, 1.2 atm, 20 min). The micromycete was isolated directly from the diseased plant material. The affected plant material was passed twice through distilled water. The inoculum was harvested by detaching small portions (2-3mm) from the areas affected with sporodochia and then placing them on the PDA culture medium. Cultures were incubated at 22^{0}C. The isolates were identified based on their morphology and then replanted and kept in pure cultures at a temperature of 22^{0}C. The concentrations used for testing the antimicrobial activity were 3.3% and 10% of fenugreek extract in butylene glycol, these being added to the PDA medium cooled to 45^{0}C. After homogenization, the mixture was distributed in Petri dishes (60 mm diameter) in an amount of approximately 10 ml/dish. After solidification of the medium, the fungal rounds were placed centrally (with a diameter of 7 mm) and executed by an eyelet from the edge of the pure fungal cultures (on the 10th day of growth). The effect of the test concentrations on the mycelial growth of the pathogens was estimated in comparison with a control cultured on a PDA medium. The Petri dishes seeded were incubated at a thermostat at a temperature of 22^{0}C. For all variants, readings were taken 3, 6, 9 and 12 days after the start of the incubation period. The estimation was evaluated by measuring the average diameter of the mycelial growth (the average diameter of the fungal colonies). The effectiveness was calculated, by determining the average diameter (mm) of

Table 1. The influence of test concentrations on the growth of the pathogen *Monilinia* spp.

Pathogen species	Var Ska (%)/ control	The incubation period and diameter were measured in mm			
		3 days - Ø(mm)	**6 days - Ø(mm)**	**9 days - Ø(mm)**	12 days - Ø(mm)
Monilinia spp					
	ska 10%	0.00	0,20	3,30	6,30
	ska 3,3%	11,30	33,80	41,30	53,30
	Control	33,30	49,30	53,30	56,30

Fenugreek extract 10% (ska10%); fenugreek extract 3.3% (ska3.3%)

the vegetative growth around the disc of the pathogen. Efficacy (% inhibition) was calculated according to the formula:

E = [(D var mt- var test)/ D var mt]x100 (%) (Abott formulas).(1)

Results and discussion

The obtained data showed that in the control variant, the fungus grew from the first days, reaching 33.3 mm in diameter after 3 days of observation. After 6 days of incubation, the average diameter of the colonies was 49.3 mm and after 9 days its diameter was 53.3 mm. After 12 days from the start of the experiment, the average diameter of the colonies was 56.3 mm. The fungus registered a rapid growth rate in the first 6 days after incubation, and after 9 days the vegetative growth had a slower growth rate. In the case of the variant containing 3.3% fenugreek extract, the micromycete grew throughout the observation period, registering 11.3 mm after 3 days and 33.8 mm after 6 days. After 12 days of incubation, the colonies reached an average diameter of 53.3 mm.

From the data obtained, the fungus had a faster growth rate, in the monitored interval, with values close to the value of the control variant. Also, in the ska 3.3% variant, a denser growth of colonies was observed than in the control variant, with a slight effect of stimulating vegetative growth, which had a more compact and richer appearance, with growth especially vertically, in culture vessels. The density of the vegetative mass recorded in the case of this extract variant tested (ska 3.3%) led to the conclusion of a possible stimulation of vegetative growth at this concentration. According to the results obtained regarding the variant in which the concentration of 10% fenugreek extract (ska 10%) was tested, the fungus did not develop until after 3 days when slight traces of mycelium were re-

Figure 1. Monitoring the vegetative growth of the micromycete *Monilinia* spp. at 3, 6, 9 and 12 days

Table 2. The effectiveness of the test concentrations on the growth of pathogens

Pathogen species	Var Ska	(%)/ control	3 days / Ø (mm)	Efficacy (%)	6 days/ Ø (mm)	Efficacy (%)	9 days/ Ø (mm)	Efficacy (%)	12 days/ Ø (mm)	Efficacy (%)
Monilinia spp	ska 10%	0	100	0,20	99,59	3,3	93,80	6,3	88,80	
	ska									
3,3%	11,3	66,06	33,8	31,44	41,3	22,51	53,3	5,32		
	Control	33,3	-	49,3	-	53,3	-	56,3	-	

Fenugreek extract 10% (ska10%); fenugreek extract 3.3% (ska3.3%)

corded, so we can say that the micromycete was inhibited in the first 3 days and recorded a significant increase after 6 days of incubation (Table 1, Figure 1). CRISTEA et al., 2017 [18] showed that plant extracts had an inhibitory effect on the mycelial growth of the pathogen *Monilinia* spp. PERISOARA et al [1] demonstrated the antifungal activity of the hydroalcoholic extract obtained from *Tagetes erecta* on the pathogenic species of *Molinilia laxa* and *Fusarium graminearum*, where in the case of the species *F. graminareum*, the highest percentage of inhibition (54.17 ± 5.89%, compared to the solvent, $p < 0.05$) was obtained at the highest tested extract concentration (5%), while in the case of *Monilinia laxa*, the inhibition of the colony diameter was observed (52.29 ± 2.60%, compared with the solvent, $p < 0.05$%) at the lowest tested extract concentration (0.5%).

The efficacity of the tested concentrations on the growth of micromycete colonies (% inhibition) was also calculated and it was found that compared to the control, the efficacy of the ska 10% variant was 88.8 %, which we consider a high percentage of inhibition of the growth of the fungus colonies. For the variant containing ska 3.3%, a very low efficacy was calculated, confirming that at this concentration the fungus was not significantly inhibited, the abundant mycelial growths ensuring a stimulation of the growth of the vegetative mass, the source of subsequent infection (Table 2).

Conclusions

The research on the influence of fenugreek extract on the mycelium growth of *Monilinia spp* showed an inhibition of the vegetative mass of the micromycete at a concentration of 10%. The efficacy of the concentration was 88.80% compared to the control. At the concentration of 3.3%, the percentage of inhibition was insignificant, the vegetative growths of the micromycete being abundant.

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Original article

Study on the opportunity to improve the quality of flour for pastry by adding chemical leavening agents

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Abstract

Food products that do not require practices for processing, and are easy to prepare have attracted the attention of consumers. An example of this type of product is the premixes formulations for cake mix which allows the cake to be made easily and quickly. The consumer of this type of product has the expectation, when following the instructions, to obtain a uniform and quality product. There are many different chemical leavening agents available to the baker. These include baking soda (sodium bicarbonate), ammonium bicarbonate, potassium bicarbonate, baking powder (baking soda, calcium phosphate and sodium aluminum sulfate) and leavening acids. Generating CO2 and neutralizing sodium bicarbonate is the primary role of leavening acids, but, it is important not to forget their secondary role and their effects. Taking into consideration these facts, the objective of experiments was to establish the connection between quantity and quality of leavening agents and the quality of bakery products. As these chemical leavening agents get into flour, it is also important to comprehend how they influence the rheological parameters of flour with direct implication in the quality of the products made. For experiments, backing powder was used, which was added in specific quantities to wheat flour. The flour samples were analyzed both as initial rheological properties and technological behavior. Baking samples were made, and the samples obtained were sensory evaluated in the panel. Correlating Mixolab Profiler indices with the baking samples, and also with the legislation regarding, the best results were obtained for flour with 2% baking powder

Keywords

cake, leavening agents, baking powder, rheological parameters

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Introduction

Being faced with high market demand, segments of the industry should be focused on the production of quality food, which is a determining factor in terms of competitiveness on the international market. Therefore, the quality of raw materials and finished products is essential and it can be influenced by a number of factors. The rheological properties have effects on product quality and nutrition has been thoroughly investigated as they relate to the chemical composition and physical characteristics of foods. [T. M. Souza, 2019] The survival of food industry depends on timing and actions to respond to consumers' desires and needs as well as on monitoring the changes in consumers' behavior. An average modern consumer is interested in the issue of quality, herein several aspects are being considered, such as: ease of preparation, adequate shelf life foods, products containing the least possible amount of additives, food security, products marketed in different servings sizes, and sensory and texture attributes. [VETTER, J. L, 2009] This fact does not apply any longer when a cake is prepared using a homemade recipe, when problems can occur during various stages of its processing. Generally it is about lack of experience and practice of the modern consumer in making cakes, in addition to lack of uniformity of the raw materials used. One of the advantages of using cake mixes is the guarantee of product standardization, since the responsibility for the quality control of the product is being transferred from consumer to industry. During baking, the combined effect of starch swelling and protein denaturation in the presence of other ingredients transforms the liquid batter into solid foam. These events tremendously increase the viscosity of the batter and finally provide structure of the matrix [Hesso et al., 2015]. Cakes are defined as being aerated, chemically leavened bakery products, which are made from many ingredients, such as wheat flour, sugar, egg, fat, leavening agents, salt, nonfat dry milk solids and water. The main parameters of cake quality are the following: volume, firmness, color, and weight loss. Cake quality is influenced by several factors such as quality and level of ingredients [Kahraman et al., 2008] Food quality is defined by a complex set of stimuli beginning with the visual assessment. Once the decision is made to consume food, quality is based not only on the magnitude of flavor, taste and texture, but it also takes into account the temporal coordination of these stimuli. From a historical point of view, food ingredients have been approached from three perspectives regarding their function in foods: formation of structures, stabilization of structures and contribution to sensory quality [Foegeding et al., 2010] Chemical leavens are used to provide cookies, cakes, and other baked goods with characteristic textures. They produce gas resulted from the reaction that takes place when a carbon dioxide source and an acid are mixed together and come into contact with water. The gas forms bubbles that are trapped in the batter or dough and then, it expands during baking to form the holes that are retained in the finished product. The timing of CO_2 release is critical in establishing uniform cell structure. Upon heating, the CO_2 will release and expand, resulting in increased volume and desirable texture characteristic of good tasting, high quality baked goods. [D, Manthey, 2012] Baking soda is the most common carbon dioxide source. It is low in cost, high in purity, easy to handle, and leaves no after taste. Flour and other ingredients are slightly acidic, so baking soda will release some carbon dioxide if added by itself, but more will be produced when more acid is added.[H. I. WIDIASTUTI, 2019]. Baking involves baking soda, one or more leavening acids, and filler. The leavening acids are added under powdered form as salts which do not react until they get dissolved in water. The filler stabilizes the product by keeping the baking soda and leavening acid separate and standardizes it to the desired strength. Leavening acids are selected primarily on the basis of reactivity—how fast they react and at what temperatures. Reactivity depends mostly on solubility, which in turn depends on chemical composition, particle size, and special treatments such as coating. Single-acting baking powders contain a single leavening acid and can be slow acting or fast acting. Slow-acting types are the most common ones and they use a slow-acting acid like SALP (sodium aluminum phosphate) that reacts very little when heated in the oven. Fast-acting types are less common but they use a fast-acting acid such as monopotassium tartrate (cream of tartar) to provide gas production at low temperatures immediately after addition.[J. Brodie, 2006]. Double-acting baking powders contain a mixture of a fast-acting leavening acid like MCP (monocalcium phosphate monohydrate) and a slow-acting leavening acid like SAPP (sodium acid pyrophosphate). They react partially at low temperatures and partially at high temperatures to provide uniform leavening throughout processing. [J. Brodie 2016] In various experimental studies, the influence of different percentages of added leavens on the characteristics of the finished product was highlighted. [G.Pop, 2016]

This study aims to show the influence of the additions of chemical processors on the rheological parameters of flour and the correlation with the quality of the finished products. Taking all into consideration we could say that the key to superior chemical leavening is the selection of the correct type and grade of bicarbonate and acid for the baked goods and the baking process being used.

Materials and Methods

The aim of this experiment is to bring a better understanding of how the addition of baking powder influences the rheological parameters of flour and how the quality of

Table 1. Analytical parameters of Cake control flour

Moisture %	Ash %	Wet gluten %	Protein %	Hydration capacity %
13.9	0.65	27.4	12.2	56.5

the finished products obtained is influenced. It has in view a better understanding of leavening agents' role in batter and cakes' quality and also to apply the mixing techniques-multistage-method.

In order to obtain some available experimental data, witness flour, purchased from the market, from Băneasa - Moara was used. The quality characteristics of this flour are shown in table 1.

The analytical flour quality was determined according to the international standard methods (ash content – ICC104/1, protein content – ICC105/2, wet gluten – ICC106/2, hydration capacity with Farinograph - ICC115/1).

The most popular type of leavening agents based on their properties and level of usage were used: Baking Powder provided by Lallemand, Inc., Montreal, Canada, and based on sodium bicarbonate, MCP and corn starch like filler.

The witness flour was added 1% baking powder for sample P1, 2% baking powder for sample P2 and 3% baking powder for sample P3.

The flour was studied from a rheological point of view based on the Mixolab Chopin protocol.

Baking samples M, P1, P2, P3 were made from these flours following the protocol in Table 2

The sensory analysis was carried out following the work protocol in the panel, following the taste, texture, porosity and color of the peel (those that are also appreciated by potential consumers)

All the experiments are made in the research laboratory of Stefan cel Mare University of Suceava.

Results and discussion

Evaluation of flour samples

In order to be evaluated, the flour samples were marked as follows: M-control flour, P1 –control flour with 1% baking powder, P2 - P1 –control flour with 2% baking powder and P3- control flour with 3% baking powder

The samples were analyzed on Mixolab Chopin, according to the Profile protocol. The curves obtained are shown in figure 1, 2, 3 and 4.

Table2 Formulating sheet

1st STAGE

Ingredients, grams	Control cake	P1 1% baking powder	P2 2% baking powder	P3 3% baking powder
Sugar	250	250	250	250
Eggs	280	280	280	280

Mixing procedures: Start mixing 1minute in 1st speed adding the eggs quickly. Scrape down bowl with a plastic scraper. Continue mixing for 4 minutes in 1st speed

2nd STAGE

Ingredients, grams	Control cake	P1 1% baking powder	P2 1% baking powder	P3 3% baking powder
Milk	150	150	150	150
Oil	100	100	100	100
Salt	10	10	10	10

Mixing procedures: Mix 1 minute in1st speed gradually by adding milk in 3 parts,by avoiding splashing and lumps. Scrape down bowl and continue mixing for approximately 4 minutes in 1st speed, and add oil

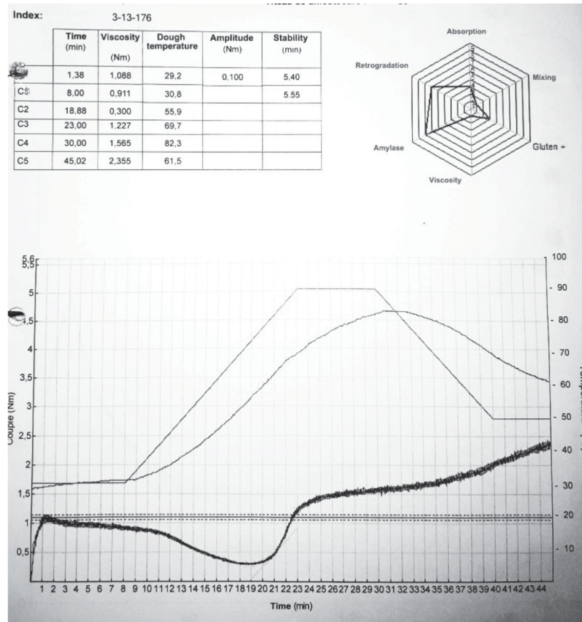
3rd STAGE

Ingredients, grams	Control cake	P1 1% baking powder	P2 1% baking powder	P3 3% baking powder
Baking powder	-	5	10	15
Flour 650	500	500	500	500
Flavor	12,5	12,5	12,5	12,5
Cacao	10	10	10	10

Add the powdered ingredients and mix them for about 5 minutes at 1st spee

The composition was transferred into 2 regular check forms and cocoa can be added to create a marbled look.

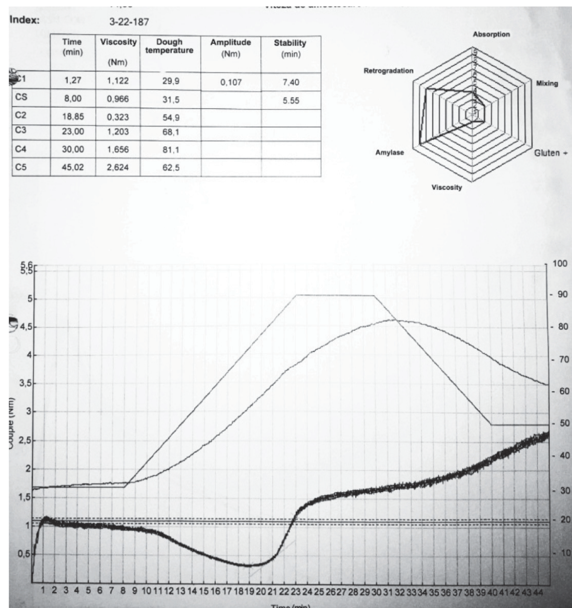
Baking was done at 180 ° C for about 20 minutes, then another 15 minutes at a temperature lower than about 160 ° C. After baking, slice them.



Index: 3-13-176

	Time (min)	Viscosity (Nm)	Dough temperature	Amplitude (Nm)	Stability (min)
[illegible]	1,38	1,088	29,2	0,100	5,40
CS	8,00	0,911	30,8		5.55
C2	18,88	0,300	55,9		
C3	23,00	1,227	69,7		
C4	30,00	1,565	82,3		
C5	45,02	2,355	61,5		

Figure 1 Evaluation curves of control flour M



Index: 3-22-187

	Time (min)	Viscosity (Nm)	Dough temperature	Amplitude (Nm)	Stability (min)
C1	1,27	1,122	29,9	0,107	7,40
CS	8,00	0,966	31,5		5.55
C2	18,85	0,323	54,9		
C3	23,00	1,203	68,1		
C4	30,00	1,656	81,1		
C5	45,02	2,624	62,5		

Figure 2. Evaluation curves of P1 (control flour with 1% baking powder)

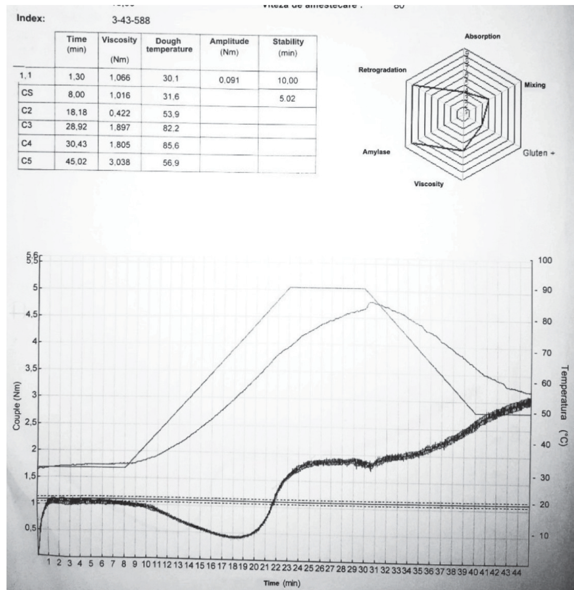


Figure 3 Evaluation curves of P2 (control flour with 2% baking powder)

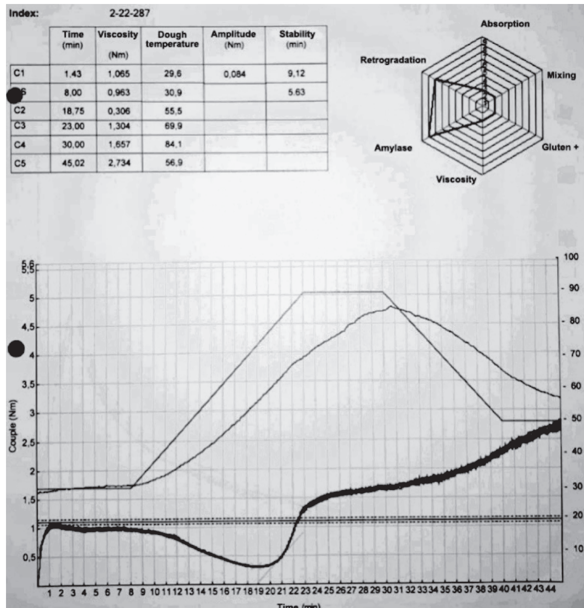


Figure 4 Evaluation curves of P3 (control flour with 3% baking powder)

Based on the results obtained, it is observed that the dough stability increases from 5.4 min to 7.4 (P1) respectively 10.0 (P2) and 9.12 min to P3. The hydration capacity given by the absorption index is constant in all samples, but it decreases from 3 to 2 in P3. Regarding the kneading index, it is observed that it is small regardless of the percentage of baking powder added. A slight increase is observed in P2, which indicates dough stability during kneading. The gluten index is at a minimum in all samples, which indicates that the strength of gluten is low. The addition of baking powder does not improve the strength of gluten. The index expressing the viscosity index is minimal in all the samples 1 and 2, except in sample P2 where it is 5. This means that the enzymatic activity is low and the viscosity increases.

For the other quality indices, the values are close in all the samples and close to the maximum value 8.

Baking test

It was very important to check specific gravity of cake (optimum 0.8-0.85) after 2nd and 3rd stage, to determine the proper mixing time required and record the specific gravity with the final batter temperature and also with viscosity

It could be observed that the viscosities of the samples are almost the same with an exception (sample 4) which is a little grainier. The specific gravity (S.G.) has a direct relationship to volume, grain and texture of cakes and it could be also a measure of incorporated air cells.

In order to observe the influence of different amounts and of leavening agents on batters and cakes quality, 4 samples using the following formulas were made (table 2).

After mixing was completed, 4 cakes pan from each of samples were prepared. Each cake had 370 grams. All the cakes were loaded at one time to better control congestion in the oven. Oven temperature was set at 182 ^{0}C and the time of baking was approximately 24 minutes.

After baking, the cakes were placed on the rack to cool, and after 15-20 minutes they were removed from the cake pans. Also, they were cut for a complete sensory exam.

By visual analysis of the 4 baking samples it is observed that the control has a crumb with uneven pores and it is not well developed. The samples that have added baking powder have a more aerated crumb, and in the sample P2, it is observed that the slice section is more uniform, with no appearance "in the corners". In sample 4 it is observed that although the slice is beautiful in the section, without the appearance of corners, the crumb is colored to reddish, showing an overdose of the tiller. The crust is quite thick and the crumb dense.

As can be seen, in the case of control flour M, the pores are very dense, while at P1 (1% baking powder) and P2 (2% baking powder) the crumb is loose. In the case of P3 (3% baking powder) the pores are large and uneven and the crumb over fermented. A plus to the shape, is at P2 (2% baking powder, where the section is nicely rounded uniform, without corners.

Influence on cakes volume. From the analysis of these four cakes one can observe the fact that each of the four samples with chemical leavening agents had a good final volume. On the other side, the sample without baking powder had a poor volume, almost the same like batter before baking. We can also make the right connection between final cake's volume and specific gravity of this sample (~1) which was more than optimal (0.8).

Influence on cakes texture and mouth feel. Regarding these aspects we can observe (that the sample with high baking powder has got more open porosity and a more grainy aspect.

In the case of sample with 3% baking powder (P3) it is obvious the brown color and the very open porosity. Also this sample had a taste like a soap film on the tongue and as a dry product is unsuitable for chewing. The explanation could be the *p*H.

Taking into consideration these facts, it has become obvious the important role of cake's *p*H on color crust and flavor. And, of course, the level of *p*H is controlled by leavening acids. In these conditions we could say that it is very important to select the proper leavening acid based on type of products and characteristic and when carbon dioxide is aimed at to get released. If too little leavening acid is added, less gas is produced and the residual baking soda raises the

M
Control flour

P1
(1% baking powder)

P2
(2%baking powder)

P3
(3% baking powder)

Photo 1 Cake sections for sensory analysis

pH of the finished product. If too much leavening acid is added, gas production remains the same, but it will leave a bitter aftertaste in the finished product.

Complying with the legislation regarding the maximum percentage of sodium pyrophosphate allowed, the maximum baking powder used can be 2%.

Conclusion

For the analysis of the samples according to the Mixolab Profiler protocol, there was no target profile based on which the flour with the addition of leavening agents to be considered accepted or refused. This is why baking tests have been carried out in parallel, in order to establish an optimal addition of leavening agents and a target profile for the flour that would be added and used in various applications.

The 6 indexes of the Mixolab Profiler for each flour sample were analyzed, in parallel with the baking samples.

By comparing the data, we can analyze:

- regardless of the percentage of added baking powder, the absorption was not significantly inhibited
- Mixing correlated with stability increases when baking powder was used, having a great value when a percentage of 2% baking powder was added.
- Gluten index, the power of gluten worsens when baking powder was added. This translates into the fact that when the baking powder was added, the gluten behavior on heating becomes worse.
- The viscosity index, increases as the percentage of baking powder was increased. This means that the enzymatic activity decreases and the viscosity increases. The highest value is observed at the addition of 2% baking powder, P2.
- in the indices the amylase activity and relegation do not have significant influences.

Correlating Mixolab Profiler indices with the baking samples the best results were obtained with P2, followed by P1.

From the analysis of the results obtained by backing tests on the possible effects of chemical leavening agents on cakes' quality, the following conclusions can be drawn:

- *Taste and texture* are influenced by the choice of leavening acid and ratio of leavening acid to baking soda;
- *Crumb color* is influenced by the pH of the finished product. A low pH in low baking soda levels gives whiter crumb color. A high pH in high baking soda levels gives a darker crumb color that is desirable in chocolate products.

Based on the two types of analysis, practical and laboratory, a target profile for flour with the addition of baking powder was established. The profile can be translated into minimum and maximum values for Mixolab indices: min 322-188 and max 343-588.

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Original article

Microbial Extracellular Enzymatic Activity Unveils the Anthropic Impact on Riparian Ecosystems From Southern Romania

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Abstract

The anthropization processes can cause complex, often irreversible changes in natural ecosystems. Several decades ago, on the upper course of the Argeș River flowing in the Southern Romania, a series of anthropic interventions took place, which resulted in several new accumulation lakes. Above the socioeconomic benefit to the local communities along this watercourse, their construction also influenced the structure and functioning of the existing ecosystems. In the present study, we have analysed the rate of the detrital organic matter decomposition processes achieved by the heterotrophic microbial communities in several ecosystems located on the upper course of the river. The obtained results revealed that the intensity of these processes was influenced both by the type of the anthropic area the sampled ecosystems attain - rural or urban, and by the sampling time.

Keywords extracellular enzymatic activity, detrital organic matter mineralisation, dam reservoirs, Total Dissolved Solids

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Introduction

The riparian ecosystems are often the subject of the anthropic exploitation regarding their wealth in goods and services, perceived as a benefit for the development of human communities – especially the urban ones. Usually, they are characterized by a high degree of biodiversity, specifically harboring numerous endemic species. Their development and spreading are influenced by the presence of watercourses that often isolate them naturally from other types of aquatic ecosystems. Therefore, human interventions on these types of ecosystems can significantly harm the local biodiversity, endangering populations of different plant or animal species. However, the authorities mainly consider riparian ecosystems as important in the socio-economic systems development (based on their exploitation) and rarely impose concrete protection measures, such as the creation of protected areas along the water streams (MAAß et al., [1]; ARIFJANOV [2]; SANTIAGO et al. [3]; DAMO & ICKA [4]). Thus, the assessment of human activities that impact these categories of ecosystems is considered a necessity of high importance in our present context.

Argeș River is a left tributary of the Danube, which flows through the S-SE part of Romania. It has a length of 350 km and the surface of its river basin of 12.550 km^2. The river springs from Făgăraș Mountains, at the confluence of two rivers originating in glacial lakes - Capra and Buda - currently forming the artificial lake Vidraru, from which the river presently flows. It has numerous tributaries with an asymmetrical lateral distribution, the debit of the tributaries on its left side being six times higher in comparison with the right-side ones. It crosses numerous rural and urban localities, the most important in its upper course, being the cities Curtea de Argeș and Pitești. It also serves as an important source of tap water supply for the localities along its course (ION et al. [5]).

Several decades ago, following a national development plan that considered the economic interest of local communities, several dams were built along the Argeș River flow, including: Vidraru, Oești, Cerbureni, Curtea de Argeș, Zigoneni, Vâlcele, Budeasa, Bascov and Golești. These important anthropic habitat alterations, along with the exploitation of the riverbed substrate, poaching, intensive agriculture in the meadows near the stream and different improperly managed household discharges, generated significant changes in the species composition of the previous (unaltered) natural ecosystems. Due to these changes in the structure and functions of aquatic ecosystems, some species of fish disappeared, while other populations developed in the dam areas with the new ”favourable” spawning conditions (TRUŢĂ & DUMITRU [6]; CONETTE [7]; DIACONU & MAILAt [8]; IONESCU [9]).

Monitoring the intensity by which decomposition processes take place can provide important information about the quality and availability of the existing nutritional resources in an ecosystem (FRAINER et al. [10]; HARBOURD et al. [11]) and, indirectly, about its structure.

Microbial extracellular enzymes are involved in the mineralization of detrital organic matter (DOM) and refilling the biogeochemical circuits with chemical elements. Most of the DOM is made up of large polymeric macromolecules, which cannot be introduced directly in the microbial metabolism. As a result, microorganisms synthesize extracellular enzymes that hydrolyze the organic macromolecules into simple molecules that could be then internalized, and thus, the extracellular enzymes play an important role in the transfer of matter and energy through the ecosystem. Taking into account this aspect and the fact that extracellular enzymes are sensitive to anthropogenic changes of the ecosystems (especially pollution), their analysis can provide valuable information about the trophic state of an ecosystem and the impact of the human activity. Nowadays, the activity of the extracellular enzymes is evaluated in most of the studies which assess the ecosystem state (DANG et al. [12]; LEE et al. [13]; KOHLER et al. [14]; STADLER et al. [15]).

Considering the mentioned contextual aspects, we decided to assess the decomposition activity of the aquatic microbial communities along the upper course of the Argeș River, as far as downstream Pitești city. The aim of the present study was to determine whether DOM decomposition processes vary significantly along the reservoirs constructed in the upper course of the Argeș River. Related to the increase of the localities number that the river crosses, they are expected to vary regarding the mineralization of the organic matter. Thus, through this study we want to see if these differences show a gradient pattern from upstream to downstream of the river, or if they have a random variation.

Materials and methods

Sample collection

The area where the study was conducted is geographically located in the central-southern part of Romania, in Argeș County. It is characterized by the presence of a mountainous region in its northern part, that is often populated with mixed and conifer forests. In the southern part of the study region can be found the Subcarpathian hills, mainly dominated by mixed forests.

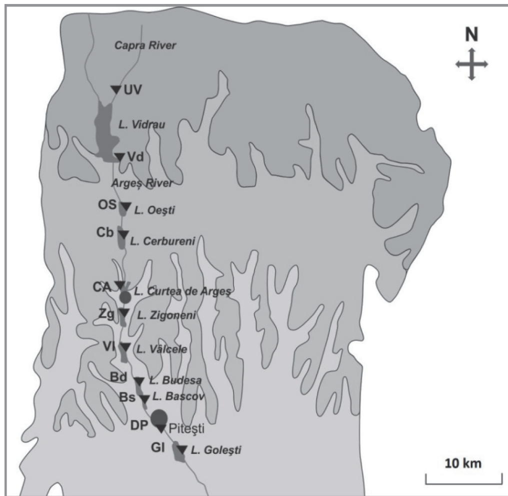

Figure 1. Sampling site locations along the Argeş River (Argeş County, România): UV – upstream Vidraru, Vd – Vidraru Lake, Os – Oesti Lake, Cb – Cerbureni Lake, CA – Curtea de Argeș Lake, Zg – Zigoneni Lake, Vl –Vâlcele Lake, Bd –Budeasa Lake, Bs –Bascov Lake, Gl –Golești Lake, DP – downstream Pitești.

Water samples were taken from 11 stations, the first of them located in a natural ecosystem - the Capra River - upstream the Lake Vidraru (UV). The other ten sampling stations were located along the upper course of the Argeş River - 9 of them corresponding to dam locations: Vidraru Lake L. (Vd), Cerbureni L. (Cb), Oeşti L. (Os), Curtea de Argeş L. (CA), Zigoneni L. (Zg), Vâlcele L. (Vl), Budeasa L. (Bd), Bascov L. (Bs), Goleşti L. (Gl) and one of the river course, downstream Piteşti city (DP) (Fig. 1). Vidraru L. and Capra River sample stations are located in the mountain area, while the sampling points are located in the Subcarpathic Hills area. In order to assess the variation of the enzymatic activity along the areas that were subjected to the anthropic changes, we measured the dynamics of the extracellular enzymatic activity in the accumulation lakes built along the river.

Surface water samples were collected over a 12-month period (September 2017 to September 2018) from all 11 stations, to cover four different seasons. The sampling was done from the upper layer of the water column – in the first 50 cm below the surface, and from the immediate vicinity of the shore. After that, the samples were stored in a refrigerated bag during transportation to the laboratory, where they were subsequently stored at 4°C. Before processing, the samples were filtered through a zooplankton filter of 40 μm, in order to remove the predatory zooplankton organisms.

Measurement of Extracellular Enzymatic Activities

In the present study, we analyzed the activity of several enzymes - α and β glucosidase, alkaline phosphatase and alanine aminopeptidase - involved in the decomposition of some categories of macromolecules present in various types of habitats and involved in biogeochemical circuits of C, P and N, in order to highlight the differences between the selected site regions.

The α glucosidase enzyme (EC 3.2.1.20) catalyzes the hydrolysis of α-1-4-glycosidic bonds in polysaccharides such as starch, a widespread molecule in natural environments (DA COSTA et al. [16]). Instead, β glucosidase catalyzes the hydrolysis of β-1-4-glycosidic molecules, being considered a key enzyme in the mineralization of cellulose, one of the most common molecule on Earth used by many organisms (especially plants and green algae) (KAUSHAL et al. [17]; BRIGHAM [18]). Alkaline phosphatase has a non-specific activity, hydrolyzing a wide range of organic phosphoesters with the releasing of orthophosphate, while alanyl aminopeptidase hydrolyzes the alanyl peptides (PĂCEȘILĂ & RADU [19]; PĂCEŞILĂ et al. [20]).

The activity of four extracellular microbial enzymes involved in the hydrolysis of some organic substrates frequently found in many habitat types was analyzed: α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), alkaline phosphatase (EC 3.1.3.1) and alanine-aminopeptidase (EC 3.4.11.2) (STADLER et al. [15]; PĂCEŞILĂ & RADU [19]; HANC et al. [21]).

The extracellular enzymatic activity was determined colorimetrically, by evaluating the substrate (p - nitrophenyl - α - D – glucopyranoside for α – GLC, p - nitrophenyl - β - D – glucopyranoside for p - nitrophenyl - β - D – glucopyranoside, 4 - nitro phenyl – phosphate for AP and L - alanine - 4 - nitroanilide – hydrochloride for AMP) consumption (PĂCEȘILĂ et al. [22]). Each 0.5 mL of water samples was mixed with 0.5 mL of enzyme substrate solution and incubated for 6 h at 30°C. For each enzyme the substrate solution had a concentration of 1m/mL, dissolved in a 0.14M NaCl solvent solution. After incubation, the enzymatic reactions were stopped by adding 0.5 mL 1M of Na_2CO_3 solution. Prior to spectrophotometer reading, the samples were centrifuged at 2000 rpm for 5 minutes in order to remove any impurities, and the supernatant was used for spectrophotometer reading, using a CECIL CE 1010 spectrophotometer. The absorbance of enzymatic reaction products - p-nitrophenol for the first three enzyme or p-nitroanilline for AMP – was read at 405 nm wavelength, and product concentration was determined by extrapolation on the standard curve. The samples were processed in triplicate.

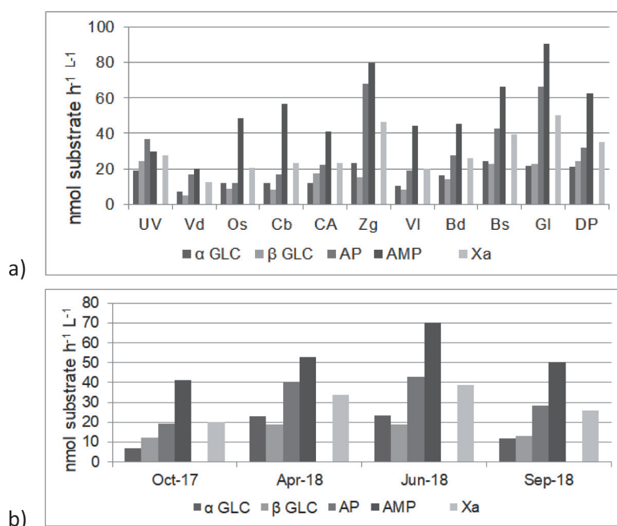

Figure 2. The spatial (a) and seasonal (b) dynamic of the microbial extracellular enzymatic activity in the studied sites (Argeș River, România).

Total Dissolved Solids

Total Dissolved Solids (TDS) was measured directly in the water column using a Sheenzy TDS-3 tester device.

Statistical analysis

The PAST software (Paleontological Statistics) (HAMMER et al. [23]) was used to analyze the logarithm transformed data.

Results

Extracellular Enzyme Activity

The spatial and temporal dynamics of the enzymatic activities intensity along the site locations during the study period is shown in Figure 2.

The α glucosidase activity (α GLC) was not detected in September 2018 in the Cerbureni lake (Cb), but its highest value of intensity was registered in Lake Golești (Gl) in June 2018 (38.45 nmol p-nitrophenol h-1L-1). In the case of β glucosidase activity (β GLC), the minimum value was recorded in Lake Cerbureni (Cb, 1.55 nmol p-nitrophenol h-1L-1) in September 2018, and the maximum - in the station located downstream the Pitesti city (DP, 46.98 nmol p-nitrophenol h-1L-1), in June 2018. The phosphatase activity presented a minimum intensity in Lake Cerbureni (Cb, 5.13 nmol p-nitrophenol h-1L-1), in September 2018, and the highest in Lake Zigoneni (Zg, 105.28 nmol p-nitrophenol h-1L-1), in June 2018. Regarding the aminopeptidase activity, the lowest value was found in the station located upstream of Lake Vidraru (Vd, 10.63 nmol p-nitroanilline h-1L-1), in September 2018, and the highest in June 2018, in Lake Golești (Gl, 163.03 nmol p-nitroanilline h-1L-1) (Fig. 2a).

Also, the enzymatic decomposition processes appeared to be the most intense in June 2018, and the lowest in September 2017 (Fig. 2b). It can be noted as well that α-GLC and β-GLC were also intense upstream the Vidraru L. (UV, on the Capra natural river), which suggests a more abundant presence of polysaccharide molecules in the water column in this area, compared to the following anthropized stations from the upper part of the upper course of the Argeș River (Fig. 2a).

TDS (Total Dissolved Solids) values in the studied ecosystems

In order to enrich the enzymatic activity assessment meaning frame, we also measured the TDS – that include dissolved solids of organic or inorganic nature, present in a molecular, ionized or colloidal state. It is mainly represented by different types of ions (potassium, sodium, carbonates, sulfates, nitrates, etc.) and positively correlates with the water conductivity, influencing as well the pH values (ISLAM et al. [24]).

In the case of our sampled ecosystems, the TDS values varied between 10-89, corresponding to a high quality in terms of drinking water standards (MORAN [25]). The lowest value of this parameter was recorded in Curtea de Argeș L., in April 2017, and the maximum in Golești L., in the same period of time. A weak correlation was found between β GLC values and TDS during the analyzed period (N = 44,

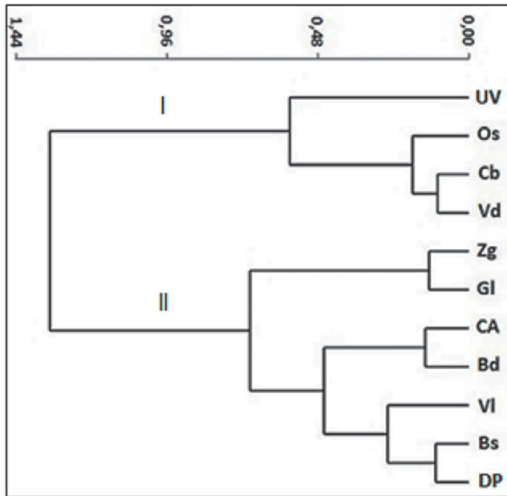

Figure 3. Cluster analysis of the enzymatic activity across the sampling stations (Ward's method, Coph. corr.: 0,7228).

$p < 0.05$), which suggests that the activity of these enzymes was also influenced by the amount of the dissolved particles present in the water column.

Cluster analysis

Using the cluster analysis, we compared the intensity of the hydrolysis processes within the sampling sites (Fig. 3). The obtained clusters (I and II) revealed that the association followed the anthropic area criteria type - the highest similarity being assessed between the urban (CA, BD, BS, Gl, DP) and rural (Os, Cb, Zg, Vl) sampling sites.

The first cluster (I) includes the stations located upstream of the Curtea de Argeș city. It can be observed that they are mainly grouped according to their geographical proximity. Also, the station positioned in the Capra river natural area, upstream the Vidraru dam, belongs to another cluster group. This confirms a different type of ecosystem, a lotic one, and at the same time, the absence of the anthropogenic alterations in the region. For the rest of the stations in this group, the similarity in terms of their bacterial enzymatic activity could be explained by a lower level of the anthropic impact in the territory.

In the second cluster (II), the aggregation based on proximity is less present, except Bs and DP stations. This aspect suggests that each studied lake has a characteristic dynamic of the mineralization processes determined by its unique environmental factors and anthropic influences. It should be noted in this case that Zg and Gl lakes, located downstream of an urban area, had a high degree of similarity in terms of the microbial enzymatic activity.

Discussions

Dams' construction along river courses is often considered a solution to problems related to drinking water supply, irrigation and electricity supply. For a long time, dams were considered an ecological solution, without too many negative effects on the environment. For this reason, their construction has increased in recent decades, including for the large rivers (BINNIE [26]; DOPICO et al. [27]; BIRNIE-GAUVIN et al. [28]).

The most intense enzymatic activity was recorded in Golești Lake which is known for its recreational role. Golești Lake is frequently the subject of eutrophication processes during the summer, with a greatly increased amount of organic matter. This aspect is reflected in the high values of the AP and AMP in this lake (Fig. 2a). In spring and at the beginning of the summer season there are frequent algal blooms occurring here (DUMITRAN & VUTA [29]). The algal blooms increase the amount of DOM, a source of nutrients for the heterotrophic microorganisms (WANG et al. [30]). DOM commonly contains biopolymers, such as polysaccharides and proteins, and humic like substances (YANG et al. [31]). Algal blooms increase the need for phosphorus - an essential element in the algae development, which leads to the synthesis of phosphatases - enzymes that release phosphorus from the organic compounds (ZHANG et al. [32]).

Also, an intense enzymatic activity was shown downstream of Pitesti (Fig. 2a), the most important city located on the upper course of the river, in the hilly region. The sewage water discharge from the settlements frequently determines the nutrient enrichment of water bodies, which leads to the growth of the aquatic organism communities and implicitly to the increase of DOM quantity in these ecosystems. These alterations are frequently associated with intense decomposition processes and the synthesis of extracellular enzymes by the inhabitant aquatic microbial communities (BERRIO-RESTREPO et al. [33]; PEARCE et al. [34]).

Argeș County is characterized by intensive agriculture practices, more than half of its surface being occupied by arrable land, and by horticulture and fruit trees growing. The meadows of the river are traditionally used in agriculture. The practice of intensive agriculture involves the use of artificial and natural fertilizers, but also of other chemical compounds such as pesticides and insecticides. One of the consequences of these treatments is the modification of the soil characteristics, including the quantity and quality of organic matter. Following the elution processes determined by the rain, floods, the organic matter and the chemical compounds existing in these soils reach the river water and influence its quality (MICU et al. [35]; TUDOR et al. [36]). Also, cur-

rently there are not many comprehensive studies about the direct influence of pesticides on the bacterial extracellular enzymatic activity in the aquatic environments. However, most likely, their presence in water does not exert a direct and significant influence on it (STANLEY et al. [37]). Another factor that can influence the extracellular enzymatic activity, and implicitly the rate of the decomposition processes, are represented by the presence of heavy metals (APONTE et al. [38]). Studies carried out along the river on heavy metal pollution have not revealed a very high amount of heavy metals in the water column, not even downstream of Pitești, an important industrial and commercial city (STOICA et al. [39]).

The polysaccharide macromolecules representing an important input of substrate (especially cellulose and starch) are coming most likely of the forest soils located close to the river. The area is largely populated with deciduous trees that sometimes fall in the river, and the organic matter from their litter is eluted by rain in the river water (IFTIMIE [40]; FIORI et al. [41]). It should be noted that in Vidraru Lake (having the largest surface and volume) the enzymatic decomposition processes take place with a lower intensity. In lentic ecosystems the surface water currents transport DOM and nutrients, thus diluting the amount of substrate available for the extracellular enzymes near the shores. In large lakes, the speed of the surface currents is stronger, due to a more powerful wind and to the vertical stratification present in this type of ecosystems (BENNINGTON et al. [42]; HUI et al. [42]).

Zigoneni Lake also recorded high values of the microbial enzymatic activity, compared with Golești Lake. Although no other studies have reported a high concentration of the detrital organic matter in this lake, the dynamics of the mcirobial enzymatic activities determined in the present study suggests that the decomposition processes take place with higher intensity near the lake shores.

Conclusions

The Argeș River is the largest river in the Argeș County that springs from the Vidraru dam reservoir. During the time, important anthropization processes occurred on its upper water course, including the building of several reservoir dams. One of the main occupations of the inhabitants of the county is represented by different forms of intensive agriculture, which are practiced in the meadows of the river (TRUȚĂ & DUMITRU [6]; CONETTE [7]; DIACONU & MAILAt [8]; IONESCU [9]). Despite the important anthropization of the area, the Argeș river water quality preserves high standards regarding its use for the domestic consumption (ION et al. [5]).

The extracellular enzymatic hydrolysis processes taking place at the upper layer of the water column were detected throughout the entire study period in all but one of the sampled stations. They were more intense in the summer, most likely stimulated by the higher temperatures.

The enzymatic activity has shown an increasing gradient from the upstream to the downstream of the river. Its highest values were often observed in the stations from the southern part of the studied area, which correspond to the highest anthropogenic influence exerted by the Pitești city - the most important urban settlement along the Argeș River.

The obtained data reflect the influence of the anthropogenic impact on the decomposition processes performed by the microbial communities present in the river water. This influence becomes more important as much as the river crosses the localities along its watercourse, especially in the urban areas.

The analysis of the mineralization processes carried out by the microbial communities from the Argeș River gives us a better understanding of the way in which the human activity affects the lotic ecosystems. For a better assessment of the anthropogenic impact on the role that heterotrophic microorganism communities play in this ecosystem, additional studies are needed in order to consider other factors, such as the structure of microbial communities and the influence of environmental factors.

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Conflict of Interest

The authors declare to have no conflict of interest.

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Original article

Hairy root induction and plant regeneration of tobacco (*Nicotiana tabacum* L.)

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Abstract

The present study showed the morphogenic potential of an important aromatic and industrial tobacco plants for the development of transgenic plants through *Agrobacterium rhizogenes* (strain 15834) for *rolA, rolB, and rolC* genes. Leaf explants were inoculated with 1/25, 1/30, and 1/50 diluted bacterial culture (OD600= 0.8) for 30 min. containing different acetosyringone doses 250 µM, 200 µM, and 150 µM respectively. The highest number of hairy roots was obtained by 1/25 containing 250 µM acetosyringone was 100%. These hairy roots were then transferred to media containing different combinations of plant growth hormones 0.5, 1.0, and 2.0 mg/L of BAP and NAA. The nutrient medium consisting of MS vitamins supplemented with 2 mg/L BAP and 0 mg/L NAA 91.7 had the highest callus formation. However, the highest callus weight was detected in 0.5 mg/L BAP and 1 mg/L NAA medium, while the highest shoot formation explant rate was 56.7% in 2 mg/L BAP and 1 mg/L NAA medium. Number of shoots per explant was detected in 1 mg/L BAP medium. Putative transgenic plants identified by PCR analyzes. As a result of the study, out of 54 putative transgenic plants 37 samples were confirmed by PCR for primers rolA, rolB, and rolC.

Keywords

A. rhizogenes, hairy roots, *Nicotiana tabacum* L, Ri plasmid, rol genes

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Introduction

A. rhizogenesis belonging to the family *Rhizobiaceae* is a gram-negative bacterium holding a hairy root-inducing plasmid (Ri-plasmid). Hairy roots are capable of exogenous hormone-independent growth, high lateral branching and root development, therefore, called genetically engineered root. In comparison with normal root, hairy roots grow faster than the adventitious roots (K.Y. PAEK & al [1]; K.W. Yu & al [2]) and accumulate higher levels of certain valuable compounds compared with adventitious roots and plant-grown roots (G.P. MIAO & al [3]; X. HAO & al [4]; M. SHI & al [5]). They are usually non-chimeric and similar in phenotype and structure to wild-type root. These kinds of roots can be identified by a reporter gene through PCR analysis (N.I. PARK & al [6]). DNA is transferred and incorporated in the genome (Ri plasmid) of wounded and infected plant explants. The development of hair roots depends on the expression of the *rolA, rolB, rolC,* and *rolD* genes in the T-DNA region leads to the formation of hairy roots emerging at the wounded surface of explants (M.C. CHRISTEY & al [7]). Therefore, *A. rhizogenes*-mediated transformation is used to induce many secondary metabolites and for recombinant protein production, metabolic engineering studies, and the large-scale production of bioactive compounds with medicinal properties (K.D. DEBOER & al [8]; N.N. ONO & al [9]; A. WIŚNIEWSKA & al [10]; C. THILIP & al [11]; R. XUE & al [12]; N. YANG & al [13]). Mechanism of the hairy root production has been carried out a broad spectrum of plant species via *A. rhizogenesis* for many years. Different factors such as genotype, co-cultivation temperature, inoculation time, co-cultivation time, vacuum infiltration, pH, constructs model, age and type of explant and bacterial cell density may affect the transformation frequency of *Agrobacterium* - mediated transformation in plants (S.N. SHEIKHOLESLAM & al [14]; S. URANBEY & al [15]; H.D. JONES & al [16]; B.W. TAGUE & al [17]; M.J. KOETLE & al [18]; E. ANAYOL & al [19]; H.A.A. AHMED & al [20]; H.A.A. AHMED & al [21]; H.A.A. AHMED & al [22]). Transformation frequency is also considerably affected by media composition containing different compounds (S. BARPETE & al [23]). Asetosyringone (AS) was also used as an additive to increase the transformation efficiency of gene transfer by *Agrobacterium* - mediated (T. KUMAR & al [24]; H.A.A. AHMED & al [20]; M. BALASUBRAMANIAN & al [25]; N. ZARE & al [26]). This compound (AS) has a special role as a signal attracting and transforming unique, oncogenic bacteria in the genus *Agrobacterium*. The virA gene on the Ti plasmid of *A. tumefaciens* and the Ri plasmid of *A. rhizogenes* is used by these soil bacteria to infect plants, via its encoding for a receptor for acetosyringone and other phenolic phytochemicals exuded by plant wounds (B. SCHRAMMEIJER & al [27]). AS widely is used on *Agrobacterium* - mediated plant transformation for vir gene induction (K. OZAWA [28]; B. SCHRAMMEIJER & al [27]; M.K. FERIZ & al [29]; T. THOMAS [30]; E.E. Uchendu & al [31]; A. PAUL & al [32]; A.M.P. JONES & al [33]). The efficiency of AS on transfer of T- DNA by inducing Vir genes largely depends on genotype, explant type, culture conditions, and concentrations of AS. Therefore, these types of compounds are important to enhance *Agrobacterium* - mediated genetic transformation efficiency in plants (S.N. SHEIKHOLESLAM & al [14]). The majority common *A. rhizogenes* strains which demonstrated by Ri plasmids are agropine-type: pRi15834, pRi1855, pRiLBA940, pRiHRI and pRiA4, mannopine-type: pRi8196, cucumopine type: pRi2659 and mikimopine-type pRi1724. Even though mikimopine and cucumopine are stereo-isomers, there is no homology between opining biosynthetic genes on the nucleotide equality (A. OUARTSI & al [34]; V. VEENA & al [35]; I.I. OZYIGIT & al [36]). The T-DNA are under the control of the virulence (vir) genes, which function in the process of T-DNA excision. This induction process is driven by the genes virA and virG (S.E. STACHEL & al [37]). VirA gene mainly drives expression of the vir genes encoding a membrane-bound kinase that perceives chemical signals, such as phenolic inducers as acetosyringone (AS), from wounded plant cells (S.E. STACHEL & al [38]; H. CHO & al [39]; J. XI & al [40]). In this study, improvisation of culture media, various combinations of cytokinin/auxin and AS was used for inducing transgenic hairy root on leaf-derived calli of tobacco.

Materials and Methods

Plant material

Turkish local commercial variety of tobacco (*Nicotiana tabacum* L.) cv. Samsun was used for development and optimization of regeneration of *A. rhizogenes*-mediated transformation. The seeds were obtained from Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey. This study was conducted in Department of Field Crops, Faculty of Agriculture, Ankara University, Turkey during 2018 and reconfirm in 2021 for protocol reproducible.

In vitro culture conditions

The Tobacco seeds were kept soaked in 70% ethyl alcohol for 3 min. followed by 1% sodium hypochlorite for 15

min. and then rinsed with double distilled water three times for 5 min. each. Then, the seeds were cultured on MS medium (T. MURASHIGE & al [41]) with 30 g/L sucrose that incubated under 24°C for 16/8 h photoperiod at 16000 lux light intensity Plantlets with a height of 12-14 cm and 6-8 leaves were obtained after four weeks.

A. rhizogenes strain and culture conditions

Wild-type agropine strain of *A.*ATCC 15834 (American Type Cultures Collection, Manassas, USA) were used for plant transformation studies. The bacterial plasmid DNA carries rol genes (*rol ABC*). *A. rhizogenes* strain was inoculated in NA nutrient agar medium (3 g/L yeast extract, 5 g/L peptones, and 1 g/L agar) and grown at 28°C overnight. A single-cell bacterial colony was initiated from the nutrient agar and cultured in 10 ml liquid NB (nutrient broth, Micropoli) medium at 28ºC on a rotary shaker (130 rpm) until OD600 reached around 0.8-1. Thereafter, 10 µl of the fresh culture of *A. rhizogenes* was used for inoculation.

Co-cultivation and genetic transformation

Three different protocols were used for optimization of transformation via *A. rhizogenes*. The leaf discs explants were inoculated with *A. rhizogenes* culture diluted 1:25, 1:30, and 1:50 in liquid MS medium containing 250, 200 and 150 µM acetosyringone respectively for 30 min. After bacterial infection, the explants were transferred to the co-cultivation gelrite solidified MS media for 72 h under dark condition. After co-cultivation, explants were washed with sterile MS medium containing 250 mg/L Cefotaxime to prevent overgrowth of the bacteria. The washed explants were cultured on MS media containing 300 mg/L ticarcillin disodium/clavulanate potassium (Duchefa Biochemie, Hofmanweg, Haarlem, Netherlands). These vessels were incubated at 24±1°C under 16/8 h at 16000 lux light photoperiod condition.

Regeneration of plants from putative transgenic shoots

For callus induction and putative transgenic shoot, hairy roots, were cut into 15-25 mm pieces then cultured on MS media supplemented with different combinations of PGR 0.5, 1.0 and 2.0 mg/L of BAP and NAA, 30 g/L sucrose, 7 g/L agar, and 300 mg/L ticarcillin disodium/clavulanate potassium. All cultures were incubated in growth chamber room at 24ºC under 16/8 h (light/dark) photoperiod cycle.

Rooting and acclimatization of putative transgenic plantlets

Well-developed shoots (5-6 cm length,) grown on selective medium containing ticarcillin disodium/clavulanate potassium were rooted on a selective MS medium containing 0.2 mg/L IBA, 300 mg/L ticarcillin disodium/clavulanate potassium. The rooted plantlets were washed with tap water for 10-15 min. and transferred to pots containing mixture of peat moss, perlite, and soil (1:1:1). The pots covered with polythene bags for 1 week for creating humidity and adaptation purposes then polythene were removed as they adapted to greenhouse conditions.

Molecular screening of rol abc genes by PCR

The presence of *rol* genes in plants was confirmed by PCR analysis *(Phire Plant Direct PCR Kit, Thermo Scientific*) according to manufacturer's instructions. Phire Plant Direct PCR Kit allows use of fresh tissue instead of pure DNA. Approximately 100 mg leaf sample was collected from each putative transgenic plant and grinded with a spatula. A total of 10 µl 2x Phire Plant PCR Buffer (dNTP and $MgCl_2$), 10 pmol Primer mix and 0.4 µl Phire Hot Start DNA II polymerase was added to 0.5 µl leaf extract and final volume of 20 µl done by analyzed grade distilled water. For amplification of the *rolA gene*, the forward 5-GTTAGGCGTGCAAAGGCCAAG-3 and reverse 5-TGCGTATTAATCCCGTAGGTC-3, product length 239-bp, *rolB gene*, the forward 5- AAAGTCTGCTATCATCCTCCTATG-3 and reverse 5-AAAGAAGGTGCAAGCTACCTCTCT-3 product length 348-bp. For amplification of the *rolC gene,* the forward 5-AAATGCGAAGTAGGCGCTCCG-3 and reverse 5-TACGTCGACTGCCCGACGATGATG-3 and the product length 190-bp primers were used as described by S. ZDRAVKOVIC-KORAC & al [42] and A. DI GUARDO & al [43]. Reactions were amplified using the following program: an initial denaturation at 98ºC for 5 min.; 40 cycles at 98ºC for 5 s, 60ºC for 15 s, 72ºC for 30 s; a final extension at 72ºC for 3 min. The amplification products were separated by electrophoresis on 1.5 % agarose gels.

Experimental Design and Statistical Evaluation

The *experimental design used in the study* was completely randomized *with three* replications. The data consisted of the averages of independent measurements. Data given in percentage were subjected to arcsine (√X) transformation (G.W. SNEDECOR & al [44]) before statistical analysis and all data were analyzed with one-way analysis of variance (ANOVA) according to (O. DÜZGÜNEŞ & al [45]) and the differences were compared by Duncan's multiple range test using SPSS programme.

Results and Discussion

Genetic transformation of tobacco leaves

Tobacco (*Nicotiana tabacum* L.) cv. Samsun were used as initial source of leaf explants for gene transformation.

Table 1. Effect *A. rhizogenesis* dilutions and AS on hairy root formation from leaf explant of tobacco

A. rhizogenes dilutions and AS	Root-forming explant rate (%)		Number of hairy roots per explant	
Control	83.3	b	1.2	c
1:25 dilution with 250 µM AS	100.0	a	3.0	a
1:30 dilution with 200 µM AS	56.7	c	1.4	bc
1:50 dilution with 150 µM AS	86.7	b	1.6	b

Values within a column followed by the different letters are significantly different at the 0.05 level of significance using Duncan test

For hairy root formation, explants were inoculated in three different bacterial dilutions of *A. rhizogenes* (1:25, 1:30, and 1:50) supplemented with different levels of acetosyringone concentration (250 µM, 200 µM and 150 µM) for 30 min. to increase gene transformation frequency. Hairy root formation was not seen on non-inoculated control explants (Fig. 1A). The first hairy root structures were observed from the scar tissue of the leaf explants after 6 day of culture inoculation (Fig. 1B). On the other hand, well-developed hairy roots on leaf explants were formed after two weeks of culture inoculation (Fig. 1C and D). The results of the study were statistically significant at the 5% level for *Agrobacterium* and explant type Table 1. The root formation and number of roots per explants ranged from 56.7 to 100% and 1.2-3.0 respectively. *A. rhizogenes* culture diluted to 1:25 containing 250 µM acetosyringone gave the maximum hairy root formation Table 1. The genetic transformation frequency is crucially based on genotype dependent reported by H.A.A. AHMED & al [21] and H.A.A. AHMED & al [22]. Whereas, efficiency of infection was highly influenced by strain of *A. rhizogenes* that plays a significant role in transformation P. TAVASSOLI & al [46]. However, the *A. rhizogenes* host strain ATCC15834 has been commonly used for hairy root culture in Hypericum perforatum, Salvia wagnerian and Lavandula angustifolia M. TSURO & al [47]; B. RUFFONI & al [48]; J. HENZELYOVÁ & al [49].

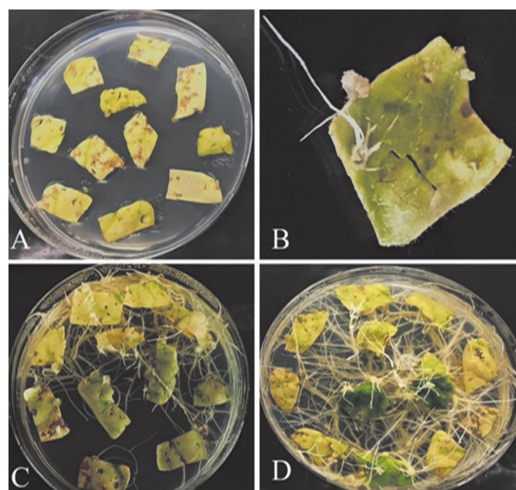

Figure 1. Development of transgenic hairy roots from *N. tabacum* leaf explants by *A. rhizogenes* strain 15834. (A) control (non-inoculated leaf disc), (B) hairy roots formation on leaf explants after 6 day of inoculation, (C & D) hairy roots formed after two weeks and six weeks respectively.

Development of putative transgenic hairy root clones

Putative transgenic hairy roots (by cutting about 20 mm) were cultured on MS medium with different growth regulators combination of BAP (0, 0.5, 1 and 2 mg/L) and NAA (0, 0.5, 1, 2 mg/L) and containing 300 mg/L ticarcillin disodium/clavulanate potassium to eliminate bacteria Table 2 and reliable regeneration. A total number of sixteen growth regulator combinations were used to identify the suitable explant growth. The each treatment was replicated thrice and each Petri dishes contained 20 explants. The culture explants were subcultured to a fresh nutrient medium every two weeks. Non-transgenic control explants was unresponsive for shoot and root regeneration on MS medium containing 300 mg/L ticarcillin disodium/clavulanate potassium after 8–10 weeks (Fig. 2A). Indirect shoot organogenesis was observed on cut surface of explants inoculated with *A. rhizogenes* harboring rol A,B,C genes after four weeks of culture inoculation (Fig. 2B, C, D, E and F). The calli induction ranged from 0.00 to 91.7 % and more than 90% callus induction was observed on MS medium supplemented with 1 mg/L BAP and 2 mg/L BAP devoid with NAA. Table 2. It seems that NAA is inhibiting the callus induction in the culture media. No callus growth was observed on controle medium. The fresh callus weight ranged from 0.00 to 1.264 g, whereas, highest weight was obtained with 2 mg/L BAP and 1 mg/L NAA Table 2. Shoot initiation and formation on putative transgenic hairy roots were developed after 5-6 weeks that ranged from 0.0 to 56.7%. The highest shoot formation was recorded on MS medium containing 2 mg/L BAP and 1 mg/L NAA. (Fig. 2D). In addition, the number of shoot per explant ranged from 0.5 to 2.4 shoot per explant.

Table 2. Influence of different BAP and NAA concentrations on callus and of putative transgenic shoot formation from hairy roots

No	BAP mg/L	NAA mg/L	Regenerative Callus formation explant rate (%)		Callus weight per explant (g)		Shoot induction explant rate (%)		Number of shoot per explant	
1	0.0	0.0	0.0	d	0.000	g	0.0	c	0.0	c
2		0.5	50.0	c	0.418	efg	10.0	bc	0.7	abc
3		1.0	50.0	c	0.419	efg	26.7	abc	1.3	abc
4		2.0	50.0	c	0.236	fg	6.7	bc	0.7	abc
5	0.5	0.0	71.7	b	0.385	efg	55.0	a	2.0	ab
6		0.5	50.0	c	0.995	bc	30.0	abc	2.0	ab
7		1.0	50.0	c	1.658	a	8.3	bc	0.5	bc
8		2.0	50.0	c	1.177	bc	6.7	bc	1.4	abc
9	1.0	0.0	90.0	a	0.232	fg	41.7	ab	2.5	a
10		0.5	50.0	c	0.847	bcde	53.3	ab	1.7	abc
11		1.0	50.0	c	0.946	bcd	30.0	abc	1.3	abc
12		2.0	50.0	c	0.874	bcde	15.0	abc	1.3	abc
13	2.0	0.0	91.7	a	0.486	defg	38.3	ab	2.4	ab
14		0.5	50.0	c	1.221	ab	21.7	abc	1.4	abc
15		1.0	50.0	c	1.264	ab	56.7	a	1.9	abc
16		2.0	50.0	c	0.694	cdef	16.7	abc	1.1	abc

Values within a column followed by the different letters are significantly different at the 0.05 level of significance using Duncan test.

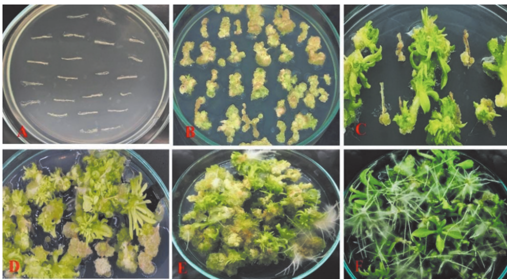

Figure 2. Callus formation and shoot regeneration development in medium control (A), callus indution (B), shoot inition (C), the 5th combination (0.5 mg/L BAP and 0 mg/L NAA) (D), the 9th combination (1 mg/L BAP). and 0 mg/L NAA) (E), The highest callus formation rate in the 13th combination (2 mg/L BAP and 0 mg/L NAA) (F)

Rooting and adaptation of putative transgenic plantlets

The 4-5 cm long shoots developed on hairy roots were cut and rooted on MS medium containing different combination of BAP (0, 0.5, 1, and 2 mg/L), NAA (0, 0.5, 1, 2 mg/L) and 300 mg/L ticarcillin disodium/clavulanate potassium (Fig. 3A and B). The root induction ranged from 0.00 to 58.2% and highest root induction was noted on MS medium containing 1 mg/L NAA Table 3. The well rooted shoots were transferred to pots contained potting mixture (peat and perlite 1:1) for acclimatization in growth chamber (Fig. C and D). The acclimatization rate of putative plants ranged from 0.00 – 65.9% Table 3.

Confirmation of rol gene integrations

A. rhizogenes infection was removed by frequent subcultures with antibiotics in the culture medium. The putative transgenic plants T0 and T1 tobacco plants was confirmed by PCR amplification of the *rol A,B,C* genes. The putative transgenic plants obtained and checked by molecular method whether they carry the role genes. The PCR prim-

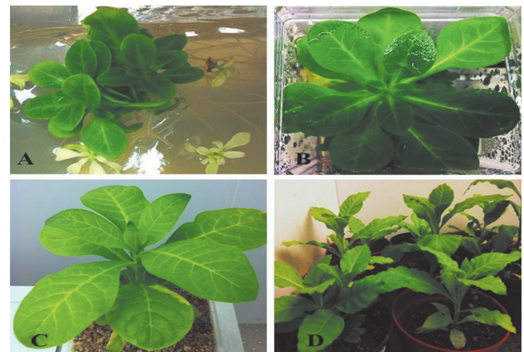

Figure 3. Rooting of transgenic putative shoots grown on (1 mg/L NAA) medium (A,B) adaptation of rooted plants in greenhouses using a mixture of peat moss, perlite, and soil (1:1:1).

Table 3. Influence of BAP and NAA on rooting of putative transgenic shoots and adaptation to the external environment

No	BAP mg/L	NAA mg/L	Number of rooted shoots	Rate of putative transgenic plants acclimatized (%)
1	0.0	0.0	0.0 b	0.0 d
2		0.5	22.2 ab	15.0 bcd
3		1.0	58.2 a	54.8 abc
4		2.0	20.8 ab	16.9 bcd
5	0.5	0.0	37.1 ab	65.9 a
6		0.5	49.7 a	51.7 abc
7		1.0	14.3 ab	18.3 bcd
8		2.0	11.9 ab	13.1 cd
9	1.0	0.0	30.7 ab	58.1 ab
10		0.5	24.9 ab	25.9 abcd
11		1.0	31.9 ab	31.9 abcd
12		2.0	25.4 ab	31.9 abcd
13	2.0	0.0	50.5 a	47.2 abc
14		0.5	28.1 ab	37.4 abcd
15		1.0	41.1 a	46.9 abc
16		2.0	44.6 ab	34.2 abcd

Values within a column followed by the different letters are significantly different at the 0.05 level of significance using Duncan test.

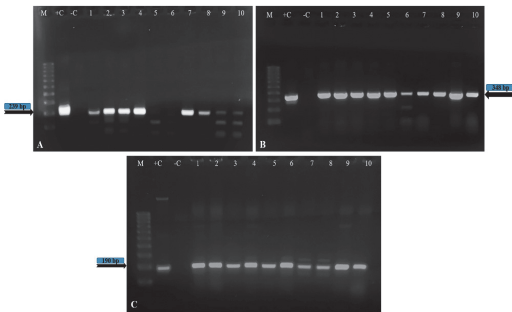

Figure 4. Confirmation of putative transgenic plants by PCR. (A) PCR amplification of the 239 bp rolA gene, (B) 348 bp rolB gene and (C) 190 bp rolC gene in individual transgenic plants developed from hairy root lines transformed by *A. rhizogenes* strain 18455. -C, control (non-transformed roots); +C, positive control (pRi 15834); M, molecular weight 100 bp DNA marker Ladder.

ers previously designed for role genes were used. The Polymerase Chain Reaction (PCR) showed the amplification of band sizes of 239 bp, 348 bp and 190 bp sizes for *rol A, B, C* genes respectively (Fig. 4 A, B and C). These results showed the Ri T-DNA integration into the putative regenerated plant genome. The results obtained specify the previous reports of B. LI & al [50] and Y.A. MOGHADAM & al [51]. Among the genes of rol, rolB plays a critical role in pathogenicity, while rolA and rolC subscribe to the root induction G. SUJATHA & al [52].

Conclusions

Putative transgenic hairy roots were obtained by transferring the rol genes A,B,C to tobacco cv. Samsun via *A. rhizogenes*. The in vitro regenerated explants (leaves) were used as initial explants for culture. The highest hairy root formation (100%) was obtained from 1:25 *A. rhizogenes* dilution containing 250 µM AS. In many plants species, acetosyringone has been reported to increase the rate of transformation with *Agrobacterium* that may be related to the activation of vir genes, which are necessary for the transfer of Ri T-DNA to plant tissue. As a result of the experiment, the highest shoot regeneration achieved in MS media containing 0.5 mg/L BAP, 1 mg/L BAP, and 2 mg/L BAP concentrations 71.7, 90.0, and 91.7 respectively. The putative transgenic plants were confirmed by PCR. As results of PCR, out of 54 putative transgenic plants 37 samples were confirmed by PCR for primers rolA, rolB, and rolC. The *rolA* and *rolC* in bacteria cause stunting and the *rolB* gene causes hairy roots in the plants. *In vitro* regeneration of transgenic hairy roots will result with different physiological and morphological characteristics containing role genes. Moreover, this kind of transgenic plants will play an economically important role in the production of beneficial secondary metabolites through hairy root culture. Due to the role of genes in transgenic plants with Ri lines, it is expected that with the increase of hairy roots, the plant will better adhere to the soil, increase

efficiency in water and mineral substance uptake, and be effective in drought tolerance.

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Review

The members of the RAS–RAF–MEK–ERK signaling pathway and cancer

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Abstract

Cancer is one of the biggest health problems of contemporary humanity, representing the second cause of death after cardiovascular diseases, caused by many contributing factors, such as population growth, ageing, stress, pollution, unhealthy diet, tumor diversity and heterogeneity, difficulties in correct and early diagnosis and inefficiency of current treatments (e.g. cytoreduction, chemotherapy, radiotherapy, etc.). This leads us to look for new ways of dealing with cancer, such as immunotherapy and personalized therapy, which consider the type of tumour, mutations and expression levels of certain genes, making it necessary to detect specific markers. A category of such specific markers is represented by the proteins involved in inter- and intra-cellular signaling which play essential roles in tumor transformation, progression and dissemination. In cancer, some canonical signaling pathways, including RAS–RAF–MEK–ERK, PI3K–PKB/AKT, JAK–STAT, HIF1–VEGF, TGFβ, NOTCH, RAP1, TP53, β-catenin/WNT, HIPPO, KEAP–NRF2, MYC and CDKN2 (cell cycle), may be dysregulated. In this review, we detail the roles of RAS–RAF–MEK–ERK signaling pathway in tumorigenic processes and the types of abnormalities that affect them in cancer.

Keywords

RAS–RAF–MEK–ERK signaling pathway, cancer

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Introduction

The RAS–RAF–MEK–ERK signaling pathway (Figure 1) is a complex intracellular mechanism involved in cell proliferation and differentiation, inflammation, evasion of apoptosis, and support of angiogenesis [1,2]. Transmission of biological signals through the RAS–RAF–MEK–ERK signaling pathway is initiated from outside the cell by binding of numerous cytokines to specific receptors. Such cytokine-receptor pairs involved in the RAS–RAF–MEK–ERK signaling pathway are TGFα and EGF–EGFR/ERBB1/HER1 and ERBB2/HER2, PDGF–PDGFRA and PDGFRB, IGF–IGF1R, FITL–KIT/c-KIT, FLT3L–FLT3, HGF–MET and FGF–FGFR. Receptors activated by ligand binding transmit downstream biological signals through at least four signaling pathways: RAS–RAF–MEK–ERK, PI3K–PKB/AKT, JAK–STAT and PLCγ–PCK. In the RAS–RAF–MEK–ERK signaling pathway, receptors recruit the adaptor protein GRB2 (*Growth factor receptor-bound protein 2*), which plays a pivotal role in signal transduction/cell communication. This protein is well known to bridge the gap between transmembrane receptors and the RAS–RAF–MEK–ERK signaling pathway, its inhibition blocking cell proliferation and transformation and impairing the development of organisms [3,2]. By binding to the SH2- and SH3- domains of the GRB2 adaptor protein, SOS1 *(the human counterpart of Drosophila Son of sevenless 1*), which functions as a RAS-specific guanine nucleotide exchange factor, takes up the signal from it and transmits it directly to members of the *RAS* gene family [4,5,2]. Originally identified in the 1980s as the first isolated human oncogenes, the *RAS* GTP-ase family members *KRAS* (*Kirsten RAS oncogene homolog*) *HRAS* (*Harvey RAS oncogene homolog*) and *NRAS* (*Neuroblastoma RAS oncogene homolog*) constitute the focal point of the RAS–RAF–MEK–ERK signaling pathway, taking up signals from both SOS and PCK, RASGEF and EML4/ALK, and transducing it downstream to RAFs, RALGDS, RASSF1 or PI3K, the latter linking to the PI3K–PKB/AKT signaling pathway [2,6]. In the RAS–RAF–MEK–ERK signaling pathway, members of the RAS family function as mitogen-activated protein kinase kinase kinases (MAPKKKK or MAP4K), transducing the biological signal to RAFs. In humans, the *RAF* (*rapidly accelerated fibrosarcoma*) gene family comprises three members, RAF1/c-RAF, BRAF and ARAF, and acts as Mitogen-activated protein kinase kinase kinase (MAPKKK or MAP3K), the first member of a series of three enzymes (the other two being part of the *MEK–Mitogen-activated protein kinase kinase kinase* and *ERK–Extracellular signal-regulated kinase* gene families; Also known as MAP2K or MAPKK, *MEK* gene family com-

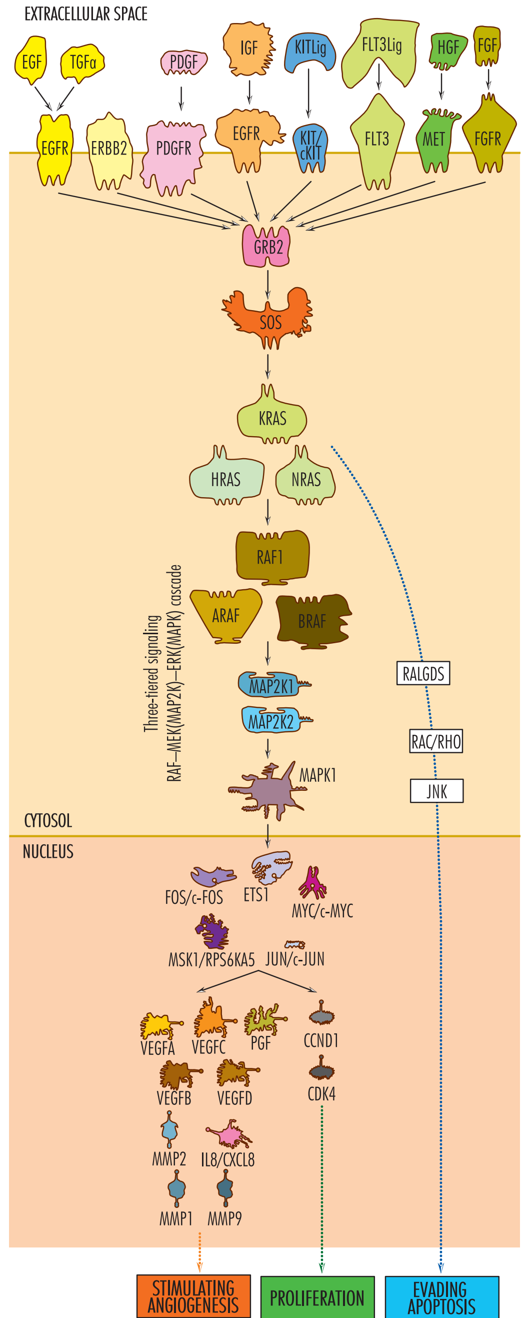

Figure 1. Detail of the signaling pathway RAS–RAF–MEK–ERK in cancer, showing all proteins presented in the text that promote cell proliferation and tumor angiogenesis. On the right, a branch of the RAS–RAF–MEK–ERK signaling pathway, which promotes evasion of apoptosis but whose proteins are not shown in the text, is illustrated.

prises two members, MAP2K1 and MAP2K2, the same as *ERK–Extracellular signal-regulated kinase*, also known as MAPK, with several members, the most important of which are MAPK1 and MAPK3), which constitute the three-tiered RAS-activated RAF–MEK–ERK signaling cascade [7,2]. ERK enzymes act on several nuclear transcription factors, including JUN/c-JUN (*Transcription factor AP-1 subunit Jun*), FOS/c-FOS (*Transcription factor AP-1 subunit Fos*), MYC/c-MYC (*MYC proto-oncogene, BHLH transcription factor*), ETS1 (*ETS proto-oncogene 1, transcription factor*), and MSK1 kinase or RPS6KA5 (*Ribosomal protein S6 kinase A5*). Further, transcription factors stimulate gene expression for the four *vascular endothelial growth factors* (VEGFs), VEGFA, VEGFB, VEGFC and VEGFD, and for PGF (*Placental growth factor*), which stimulates vascular and lymphatic endothelial cell proliferation, and vascular and lymphatic angiogenesis. On the other hand, transcription factors activated by the RAS–RAF–MEK–ERK signaling pathway stimulate the synthesis of members of the *matrix metallopeptidase family* (MMP1, MMP2 and MMP9) and the synthesis of IL8/CXCL8 (*Interleukin 8/C-X-C motif chemokine ligand 8*), promoting cell migration and inflammation, two processes closely associated with tumorigenesis. Moreover, the transcription factors mentioned support *transcription of cyclin D1* (CCND1), overexpressed in cancers, and transcription of *cyclin dependent kinase 4* (CDK4), which, by forming a complex with *cyclin dependent kinase 6* (CDK6), are key players in cell cycle progression, essentially contributing to cell proliferation [2]. Physiologically, the RAS–RAF–MEK–ERK signaling pathway is very active during the embryo-fetal period, when it promotes growth and tissue differentiation of the embryo and fetus, but as these processes slow down, the genes whose products are involved in this signaling pathway become silenced. In the adult stage, their reactivation leads to various diseases, including cancer.

Membrane receptors of the RAS–RAF–MEK–ERK signaling pathway

EGFR/ERBB/HER

Of the four members of the *EGFR/ERBB/HER* gene family, only EGFR/ERBB1/HER1 and ERBB2/HER2 are involved in the RAS–RAF–MEK–ERK signaling pathway, which, after ligand binding (the ligand for ERBB2/HER2 is not known), dimerise, autophosphorylate tyrosine and transmit signals across the transmembrane space to GRB2. The *EGFR/ERBB1* gene is located in the 7p11.2 region and encodes the EGFR/ERBB1 protein of 1210 amino acids and a molecular mass of 134277 Da, which picks up signals from TGFα and EGF ligands and transduces them to the cytosol partner. The *EGFR/ERBB1* gene undergoes abnormalities (e.g. overexpression, activating mutations) in a wide variety of cancers, including oral, esophageal, bladder, cervical, breast, laryngeal, salivary glands, oropharyngeal, pancreatic and appendix cancers, and non-small cell lung carcinoma, choriocarcinoma and glioma [8,9,10,2,11,12,13]. Modern medicine has introduced a number of products directed against EGFR/ERBB1/HER1 into cancer treatment regimens, approved after 2000, including tyrosine kinase inhibitors (Gefitinib, Erlotinib, Afatinib, Dacomitinib, Osimertinib and Vandetanib) and monoclonal antibodies (Cetuximab, Panitumumab, Necitumumab), although for some of these, tumour cells develop resistance. The *HER2/ERBB2* gene has no known ligand, but this does not mean that it cannot exist. It is located in 17q12 and encodes HER2/ERBB2 protein (1255 amino acids; 137910 Da), but by forming a dimer with HER3/ERBB3, it forms a very potent complex [14,15]. Abnormalities in *HER2/ERBB2* gene function occur in a variety of cancers, including gastric, pancreatic, bladder, endometrial, ovarian, cervical, breast, salivary glands, fallopian tubes, pancreatic and appendix cancers, as well as in choriocarcinoma, glioma and cholangiocarcinoma. Treatment regimens for some cancers, which target HER2/ERBB2, include the tyrosine kinase inhibitors Lapatinib, Neratinib and Tucatinib, and monoclonal antibodies or antibody drug conjugates Trastuzumab, Pertuzumab, Trastuzumab emtansine, Trastuzumab deruxtecan, Panitumumab and Margetuximab [16,17;9,18,10,2,20,11,19,13].

PDGFR

The *PDGFR* (platelet derived growth factor receptors) gene family comprises three members, *PDGFRA*, *PDGFRB* and *PDGFRC*, of which only the first two function in the RAS–RAF–MEK–ERK signaling pathway, which, after interaction with PDGF, form a homodimer (by binding PDGFB or PDGFD) or a heterodimer (by binding PDGFA and PDGFB). The *PDGFRA* gene is located in 4q12 and encodes the PDGFRA protein, with chain length of 1089 amino acids and molecular mass of 122670 Da. It undergoes abnormalities (e.g. mutations P130S, W349C, V469A, V536E, F808L, D842V, N870S, G829R, E996K, D1071N, S1049CH) in numerous cancers, including glioma, glioblastoma, lung adenocarcinoma, colon adenocarcinoma, cutaneous melanoma, chronic eosinophilic leukemia, and gastrointestinal stromal tumor [21,22,2,23], and is targeted by Imatinib, Sunitinib, Ripretinib, Avapritinib drugs [24]. The *PDGFRB* gene is located in 5q32 and encodes the PDGFRB protein, with size of 1106 amino acids and molecular mass

of 123968 Da. It is mutated in lung and colon adenocarcinoma, cutaneous melanoma, breast invasive ductal carcinoma, glioma, and melanoma [25,26], being targeted by Sunitinib and Regorafenib drugs [24].

IGF1R

The *IGF1R* gene is located in 15q26.3 and encodes the IGF1R protein, with a size of 1367 amino acids and molecular mass of 154793 Da, which has tyrosine kinase activity and binds insulin-like growth factor with high affinity. Abnormalities of this gene (e.g., missense, nonsense and silent mutations, frameshift, and in-frame deletions) are found in several cancers, including hepatocellular and breast invasive ductal carcinoma, lung, colon, and endometrial endometrioid adenocarcinoma, malignant pleural mesothelioma, synovial sarcoma and cutaneous melanoma [27,2,28]. Among the inhibitors targeting IGF1R in cancer and other conditions are Zykadia, approved in 2014, Tepezza, approved in 2020, and Xentuzumab, Ganitumab, AXL1717, IGF-MTX, W0101 and FPI-1434, which are in clinical trials [29].

KIT/cKIT

KIT/c-KIT gene is located in the 4q12 region and encodes for the tyrosine kinase transmembrane receptor KIT/cKIT (*V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene-Like Protein*), with a size of 976 amino acids and a molecular mass of 109865 Da. KIT/c-KIT is altered in several cancers, including gastrointestinal stromal and appendiceal tumors, lung and colon adenocarcinoma, conventional glioblastoma multiforme, and melanoma [30,18; 2; GeneCards, 2022; 13) and is inhibited by Imatinib, Sunitinib, Regorafenib, Ripretinib, Avapritinib. The V654A mutation confer resistance to Imatinib [24,32].

FLT3

The *FLT3* gene is located in 13q12.2 and encodes the Fms-related tyrosine kinase 3 receptor, FLT3, with a size of 993 amino acids and a molecular mass of 112903 Da, whose ligand binding induces plasma membrane homodimer formation and FLT3 autophosphorylation. FLT3 protein is abnormal in acute myeloid leukemia, colon and lung adenocarcinoma, cutaneous melanoma and invasive ductal carcinoma of the breast [33,18,2]. FLT3 inhibitors are classified as first-generation, type 1 inhibitors (Midostaurin, Lestaurtinib, Sunitinib), first-generation, type 2 inhibitors (Sorafenib, Pexidartinib, Ponatinib), second-generation, type 1 inhibitors (Gilteritinib, Crenolanib, MRX-2843) and second-generation, type 2 inhibitors (Quizartinib) (20,35].

MET

MET – MNNG HOS transforming gene is located in 7q31.2 and encodes MET protein, with size of 1390 amino acids and molecular mass of 155541 Da, a member of the class IV receptor tyrosine kinase family, which, by interacting with hepatocyte growth factor (HGF), dimerizes and becoming active, interacts with the GRB2 carcinoma protein. Somatic MET mutations, such as D1228N, Y1235D, and M1250T and/or amplifications are reported in several cancers, including lung, gastric, esophageal, colorectal, clear cell ovarian and appendix cancers, as well as in gliomas, renal cell carcinomas, hepatocellular carcinomas and head and neck squamous cell carcinoma [36,37,18,2,13]. MET inhibitors in use (Osimertinib, a third-generation drug) or in various phases of clinical testing (multi-target tyrosine kinase inhibitors – Crizotinib, Cabozantinib, Foretinib, Glesatinib and Merestinib, selective MET tyrosine kinase inhibitors – Tepotinib, Savolitinib, Capmatinib, Tivantinib and SAR125844, and anti-MET antibodies – Onartuzumab, Telisotuzumab and JNJ-61186372) are predominantly for non-small cell lung cancer [38,39].

FGFR

The *FGFR* – fibroblast growth factor receptor gene family comprises four members, *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4*, which encode proteins involved in the transduction of the biological signal from FGF in the extracellular space to different molecules in the cytoplasmic space. The *FGFR1* gene is located in 8p11.23 and encodes the FGFR1 protein, with a size of 822 amino acids and a molecular mass of 91868 Da. Mutations of the *FGFR1* gene are activating or can amplify the expression of its product and are identified in a large number of cancers, including breast, ovarian, bladder, prostate and lung cancers, oral squamous cell, esophageal squamous cell carcinomas, colon and appendix adenocarcinomas. The *FGFR2* gene is located in 10q26.13 and encodes FGFR2 protein, with a size of 821 amino acids and a molecular mass of 92025 Da. Abnormalities, such as mutations, amplifications or translocations, activate the *FGFR2* gene and are identified in breast, endometrial and gastric cancers, as well as in cutaneous melanoma, colon adenocarcinoma, appendix and lung adenocarcinoma. The *FGFR3* gene is located in 4p16.3 and encodes the FGFR3 protein, with a size of 806 amino acids and a molecular mass of 87710 Da. Activating abnormalities of the *FGFR3* gene are reported in a wide variety of cancers, including urothelial, bladder, breast, head and neck, lung, brain, gastric, pancreatic, colorectal, kidney, endometrial, ovarian, appendiceal, and cervical cancers. The fourth member of the family is the *FGFR4* gene, located in 5q35.2, which encodes the FGFR3 protein, with a size of 802 amino acids and a molecular mass of 87954 Da. It undergoes missense, nonsense, and silent mutations, and frameshift insertions and

deletions, in lung adenocarcinoma, colon adenocarcinoma, breast invasive ductal carcinoma, cutaneous melanoma, and endometrial endometrioid adenocarcinoma [40–47,18,2]. Among their inhibitors are Pemigatinib, Erdafitinib, Infigratinib, Derazantinib, Futibatinib [48].

Cytoplasmic proteins of the RAS–RAF–MEK–ERK signaling pathway

GRB2

By interacting with the cytoplasmic domains of transmembrane receptors, GRB2 protein is activated, constitutes the first effector of the RAS–RAF–MEK–ERK signaling pathway in the cytoplasm and allows downstream binding of SOS1 protein to its SH2- and SH3- domains, the latter complexing the proline-rich regions of other proteins. The GRB2 protein is 217 amino acids in size and has a molecular mass of 25206 Da and is encoded by the *GRB2* gene in the 17q25.1 region. It appears not to be mutated in cancer, but only to fuse with the RET proto-oncogene in some cases of pheochromocytoma [49,50,2,51].

SOS1

The SOS1 protein, 1333 amino acids in size, with a molecular mass of 152464 Da and encoded by the *SOS1* gene, located in band 2p22.1, binds the SH2- and SH3- domains of its partner in the RAS-RAF-MEK-ERK pathway, GRB2. Further, SOS1 functions as a guanine nucleotide exchange factor for RAS proteins and is regarded as the pacemaker of KRAS. Nucleotide sequence alteration of the SOS1 gene, such as the N233Y point mutation, is very rarely reported in a small number of cases of lung, colon, and endometrial endometrioid adenocarcinoma, cutaneous melanoma, and breast invasive ductal carcinoma [52,53,2]. Having an important function in activating the RAS family member-mediated signaling pathway, SOS1 is targeted by several inhibitors in clinical trials, including Afatinib, Olmutinib, Erlotinib, Refametinib, Trametinib, Nintedanib, Paclitaxel, Abemaciclib, Gemcitabine, BAY293, SAH-SOS1 and BI1701963 [54,55,20].

RAS

The *RAS* gene family comprises three members, *KRAS*, *HRAS* and *NRAS*, which encode central proteins of the RAS–RAF–MEK–ERK signaling pathway. They are activated by SOS1, RASGEF, EML4/ALK, RET/PTK, TRK and PCK proteins and transmit biological signals via four downstream partners: RAF, in the RAS–RAF–MEK–ERK signaling pathway, through which it stimulates cell proliferation and angiogenesis, RASSF1, involved in cell proliferation and apoptosis evasion, PI3K, through which it activates the PI3K–PKB/AKT signaling pathway, involved in angiogenesis and apoptosis evasion, and RALGDS, which takes cells out of the programmed cell death program [2]. Of the three members, *KRAS*, located in the 12p12 region. 1 and encoding the KRAS protein, with a size of 189 amino acids and molecular mass of 21656 Da, undergoes the most activating mutations in numerous cancers [56], especially in gastrointestinal cancers [57], including numerous types of appendiceal cancers [58], pancreatic, colon, colorectal and rectal adenocarcinomas, and lung adenocarcinomas [59]. Other family members are less prone to mutations, probably due to their less important function in cancer signaling pathways. Thus, the *HRAS* gene, located in the 11p15.5 region and encoding the HRAS protein, with a size of 189 amino acids and a molecular mass of 21298 Da, is mutated in several tumor types, such as melanomas, follicular thyroid, bladder, and appendiceal cancers, and oral squamous cell carcinomas [60,13], whereas the *NRAS* gene (1p13. 2), which encodes the NRAS protein, with a size of 189 amino acids and a molecular mass of 21229 Da, is rarely mutated in cancers, these being reported in rectal somatic and follicular thyroid cancers and juvenile myelomonocytic leukemia, but also in a small number of appendiceal cancers [61,13]. Among the many RAS family protein inhibitors are: Tipifarnib, Lonafarnib (farnesyl transferase inhibitors), Sotorasib (AMG-510), MRTX849, MTRX1133, JNJ-74699157, GDC-6036, LY3499446, tergeting KRAS harboring G12C mutation, D-1553, ARS-1620, AMG 404, Trametinib, RMC-4630, Afatinib, Pembro, Panitumumab, Carbo/pem/docetaxel, Everolimus, Palbociclib, Bevacizumab, Adagrasib, TNO155, LY3537982, Abemaciclib, Erlotinib, Sintilimab, Temuterkib, LY3295668, Cetuximab, Atezo, Cetuximab, Bevacizumab, Erlotinib, D-1553, JDQ443, TNO155, Spartalizumab, TNO155, EGF816, RMC-4630, Cobimetinib/osimertinib, BI 1701963, BI 3,011,441, Irinotecan, directed against KRAS and many in various phases of clinical testing [62], Tipifarnib and Salirasib targeting HRAS [63,64], but there are encouraging results on the ability of some MEK (Trametinib) and BRAF (Dabrafenib) inhibitors to inhibit, together or in other combinations, HRAS [65], Trametinib, Binimetinib, Pimasertib, RO4987655 (MEK inhibitor), Alpelisib+binimetinib, GSK2141795 (AKT inhibitor)+Trametinib, Ribociclib+binimetinib, Sorafenib+tivatinib, Axitinib+carboplatin/paclitaxel, and Ulixertinib, used as NRAS inhibitors in various tumor types [66].

RAF

The *RAF* gene family comprises three members, *RAF1*, *ARAF* and *BRAF*, whose products receive signals from two

directions, RAS proteins and PRK proteins, and serve as the first members of the RAF–MEK–ERK three kinase signaling cascade, after which signals are transmitted within the nucleus [2]. The *RAF1* gene is located in chromosomal band 3p25.2, encodes the RAF1 protein, with size of 648 amino acids and molecular mass of 73052 Da, and undergoes fusions, rearrangements, missense mutations, nonsense mutations, and silent mutations in bladder urothelial carcinoma, long adenocarcinoma, endometrial endometrioid adenocarcinoma, colon adenocarcinoma, cutaneous melanoma, and stomach cancers [67–70]. The *ARAF* gene is located in the Xp11 region. 3, encodes the ARAF protein, with size of 606 amino acids and molecular mass of 67585 Da, and is mutated in several cancers, including lung, colon, endometrial endometrioid, and high-grade ovarian serous adenocarcinoma, gallbladder cancers, and breast invasive ductal carcinoma, its mutations being correlated with malignant phenotypes in some cancers, including gallbladder cancer types, or with resistance to some chemotherapeutics [71–73]. The *BRAF* gene is located in the 7q34 region, encodes the serine/threonine kinase BRAF, with a size of 766 amino acids and molecular mass of 84437 Da, and undergoes mutations, most commonly V600E, in several cancers, including non-Hodgkin lymphoma, colorectal and appendiceal cancers, thyroid carcinoma, non-small cell lung carcinoma, hairy cell leukemia and adenocarcinoma of lung [75,76,13]. Several molecules, including LXH254, directed against ARAF [77], Dabrafenib and Vemurafenib, directed against BRAF [65,20], are used in the treatment of cancers which harbor mutations in RAF family genes.

MAP2K/MEK

The *MAP2K* gene family comprises several members, of which, in cancer, only *MAP2K1* and *MAP2K2* are involved in the RAS–RAF–MEK–ERK signaling pathway, as ordinal two protein kinases in the cascade of three such proteins. The *MAP2K1* gene is located in region 15q22.31 and encodes the MAP2K1 protein, with size of 393 amino acids and molecular mass of 43439 Da. Its mutations occur in cutaneous melanoma, lung, and colon adenocarcinoma, colorectal cancers, melanoma, and breast invasive ductal carcinoma [78,79]. In colorectal cancers, mutations in this gene cause poor response to treatment with anti-EGFR compounds [80]. The *MAP2K2* gene is located in band 19p13. 3, encodes the MAP2K2 protein, with size of 400 amino acids and molecular mass of 44424 Da, mutated in several types of carcinomas, including breast invasive ductal carcinoma, cutaneous melanomas and adenocarcinomas, including colon, lung, and high-grade ovarian serous adenocarcinoma [81,82]. Among MEK inhibitors, are used/will be used Alectinib+cobimetinib, Atezolizumab+cobimetinib, AZD8330 (ARRY-424704), Binimetinib, Binimetinib+erlotinib/+encorafenib±ribociclib/+carboplatin or pemetrexed/+Palbociclib/+pembrolizumab, Brigatinib+binimetinib, Carboplatin+pemetrexed+binimetinib, Cisplatin+pemetrexed+binimetinib, Cobimetinib, Cobimetinib+alectinib/+atezolizumab/+vemurafenib, CS-3006, Durvalumab+selumetinib+tremelimumab, E6201, EGF816+trametinib, Encorafenib+binimetinib+docetaxel, FCN-159, GDC-0623, HL-085, HL-085+docetaxel, MEK162, Mirdametinib, PD-0325901, PD-0325901+palbociclib/+dacomitinib, Pimasertib, Refametinib, RO4987655, RO5126766, Selumetinib, Selumetinib+docetaxel/+erlotinib/+vandetanib/+afatinib/+gefitinib/+durvalumab/+paclitaxel/+osimertinib/+durvalumab+tremelimumab/+vandetanib, SHR7390, TAK-733m, TQ-B3234, Trametinib, Trametinib+dabrafenib/+docetaxel/+pemetrexed/+ceritinib/+dabrafenib/+navitoclax/+lapatinib/+pembrolizumab, Trametinib+carboplatin+paclitaxel+radiation therapy, WX-554, some of them still being in various phases of clinical testing, and others are expected to inhibit MEK proteins when there are also mutations in the KRAS, EGFR or BRAF V600E genes [83–85,65,66,86,87].

ERK/MAPK1

The *ERK/MAPK1* gene is located in the 22q11.22 region and encodes the ERK/MAPK1 protein, 360 amino acids in size and molecular mass of 41390 Da, and the third member of the three-protein kinase cascade of the RAS–RAF–MEK–ERK signaling pathway. Activated by MEK/MAP2K proteins, the ERK/MAPK1 protein crosses the nuclear membrane, where it activates several proteins that act as activators of transcription factors [2]. Missense, nonsense, and silent mutations of the *ERK/MAPK1* gene are reported in cervical, and skin cancer, lung, colon, and endometrial endometrioid adenocarcinoma, bladder urothelial, and breast invasive ductal carcinoma [88,89]. ERK/MAPK1 targeting is sometimes seen as the Achilles heel of the RAS–RAF–MEK–ERK signaling pathway, and a number of molecules designed to inhibit ERK/MAPK1 have been clinically tested, including FRI-20, ON-01060, VTX-11e, 25-OH-D3-3--BE, B3CD, Bromoacetoxycalcidiol, FR-180204, AEZ-131, AEZS-131, AEZS-136, SCH-772984, AZ-13767370, BL-EI-001, LY-3214996, LTT-462, KO-947, CC-90003, GDC-0994, RG-7842, MK-8353, SCH900353, BVD-523, and Ulixertinib [90,20).

Nuclear proteins of the RAS–RAF–MEK–ERK signaling pathway

The nuclear proteins of the RAS–RAF–MEK–ERK signaling pathway are grouped in a two-step cascade, where

the first step is activated by the ERK/MAPK1 protein (JUN/c-JUN, FOS/c-FOS, MYC/c-MYC, ETS1 and MSK1/RPS6KA5), and the second, which includes VEGFA, VEGFB, VEGFC, VEGFD, PGF, MMP1, MMP2, MMP9 and IL8/CXCL8 proteins involved in angiogenesis, and CCND1 and CDK4 proteins involved in proliferation, is activated by the first step proteins.

JUN/c-JUN

The *JUN/c-JUN* gene is located in the 1p32 region. 1 and encodes the JUN/c-JUN protein, 331 amino acids in size and with a molecular mass of 35676 Da, a nuclear component of the transcription factor activator protein 1 (AP-1), together with members of the *FOS* gene family (*c-FOS*, *FOSB* and the smaller splice variants FRA1 and FRA2), involved in transcription activation at the TRE/AP-1 element level, and also a key regulator of mitochondrial glutaminase (GLS) levels in cells [91,92,2]. Nucleotide sequence alteration of the JUN/c-JUN gene, through missense, nonsense and silent mutations, as well as frameshift insertions and deletions, is reported in cancers of the gastrointestinal, including colon adenocarcinoma, lung, including lung adenocarcinoma, reproductive, including adenocarcinoma of the prostate and invasive ductal carcinoma of the breast, the latter being associated with cell proliferation and tumour angiogenesis, as well as in skin cancers, including skin cancer, or cancers of fatty tissue, such as dedifferentiated liposarcoma [91,93,94]. Numerous molecules with the property of inhibiting c-JNK (c-Jun N-terminal kinases) are currently being tested, of which JNK-IN-8 shows specificity for JUN/c-JUN [95,96].

FOS/c-FOS

The *FOS/c-FOS* gene is located in the 14q24.3 region and encodes the FOS/c-FOS protein, with a size of 380 amino acids and a molecular mass of 40695 Da. It heterodimerizes with the JUN/c-JUN protein and forms transcription factor activator protein 1 (AP-1), which binds and activates the transcription of TRE/AP-1 elements, involved in the regulation of expression of numerous genes whose transcription products are involved in a wide variety of biological processes, including differentiation, proliferation, and apoptosis [97,2,98]. Activating mutations of the FOS/c-FOS gene are reported in several cancers, including osteosarcomas, endometrial, cervical and thyroid carcinomas, head and neck, and oral squamous cell carcinoma, breast and ovarian cancers, mesotheliomas, lung, colorectal, esophageal and skin cancers, and melanomas [97,99,100]. Of the FOS/c-FOS inhibitors, the best known is T-5224, which is in trials and, at a preclinical level, inhibits some inflammatory diseases, including arthritis [101].

MYC/c-MYC

The *MYC/c-MYC* gene is located in the 8q24.21 region and encodes the MYC/c-MYC protein, 439 amino acids in size and 48804 Da molecular mass, which, through heterodimerization with MAX, folds and becomes transcriptionally active, binding specifically to the consensus DNA sequence CANNTG, which is an Enhancer-box [2,102,103]. The *MYC/c-MYC* gene is aberrantly expressed in approximately 50-70% of human cancers, including invasive ductal breast carcinoma and invasive breast carcinoma, adenocarcinoma of the lung, colon and prostate, as well as endometrial, hematopoietic, lymphoid and stomach cancers [104–107]. Over time, numerous molecules have been developed that target MYC/c-MYC activity, including QN-1, APTO-253, AZD5153, GSK525762 and dBET1, which target *MYC* gene transcription, MLN0128, Silvestrol, eFT226 and BTYNB, which target MYC mRNA translation, [1,2,3] triazolo [4,5-d] pyrimidine derivatives, SZL-P1–41, TD19 and Volasertib, which affect MYC protein stability, MYCMI-6, KI-MS2-008, Omomyc, SaJM589, KJ-Pyr-9 and FPPa-OmoMYC, which target MYC–MAX heterodimer, Sulfopin, ASH2L-derived peptides and C620-0696, which impair accessibility of MYC protein to downstream genes, and B-I09, 8866, Purvalanol A, Berbamine, VX-680 (MK-0457), AZD1152, AZD7648 and Dinaciclib, candidate drugs for alternative selective killing of the dysregulated cells through synthetic lethality conferred by *MYC* overexpression, many of them still being in various phases of preclinical/clinical testing [107–109].

ETS1

The *ETS1* gene is part of a 28-member gene family in humans, located in the 11q24.3 region and encodes the ETS1 protein, 441 amino acids in size and molecular mass of 50408 Da, and, depending on the biological context, acts as an oncogene or a tumour suppressor gene. ETS1 gene aberrations, including fusions, missense, nonsense and silent mutations, as well as frameshift deletions, are associated with endometrial, bowel (colon adenocarcinoma), pleural cancers, but also melanoma, anaplastic oligodendroglioma and basal cell carcinoma [110–112,2,113]. Inhibition of ETS1 activity is pursued through the use of inhibitors of its effectors, in the case of the RAS–RAF–MEK–ERK signaling pathway the use of the MEK inhibitor, Trametinib [114], is proposed, as well as through the use of molecules that target the full range of ETS family members, such as YK-4-279 [115].

MSK1/RPS6KA5

The MSK1/RPS56KA5 gene is located in 14q32.11 and encodes the MSK1/RPS56KA5 protein, with a size of 802 amino acids and molecular mass of 89865 Da, located in the cytoplasm and nucleoplasm and involved in histone-serine

phosphorylation, regulation of histone modification, and regulation of transcription. It is expressed in a wide variety of tissues and, in the RAS–RAF–MEK–ERK signaling pathway, activates the MYC/c-MYC protein [2,116]. MSK1 overexpression is associated with a better survival rate in breast cancer [117] but with increased proliferation and metastasis in uveal melanoma [118].

*VEGF*s

Members of the *VEGF* gene family are directly involved in angiogenesis and lymphangiogenesis by stimulating endothelial cell proliferation, budding and vascular remodeling, processes that lead to the formation of new blood or lymph vessels from pre-existing ones. It is well known that members of this family are some of the most potential angiogenic factors. The *VEGF* gene family comprises four members, *VEGFA*, *VEGFB*, *VEGFC* and *VEGFD/FIGF*, which, in addition to proteins in the RAS–RAF–MEK–ERK signaling pathway, are also activated by HIF1A, as the first molecule synthesized by cells undergoing hypoxia [2]. The *VEGFA* gene is located in 6p21.1 and encodes the VEGFA protein, with size of 232 amino acids, and a molecular mass of 27042 Da. Aberrations of this gene, including missense, nonsense, and silent mutations, and frameshift insertions are present in cancers of the gastrointestinal (esophageal and colon adenocarcinoma), reproductive (endometrial cancer and breast invasive ductal carcinoma), skin cancers, lung adenocarcinomas, and osteosarcomas. The *VEGFB* gene is located in 11q13. 1, encodes VEGFB protein, with size of 207 amino acids, and a molecular mass of 21602 Da, and is rarely mutated, predominantly in adenosquamous lung carcinoma and endometrial endometrioid adenocarcinoma. The *VEGFC* gene is located in 4q34. 3 and encodes the VEGFC protein, with size of 419 amino acids, and a molecular mass of 46883 Da, and is rarely mutated in cancer. The *VEGFD/FIGF* gene is located in Xp22.2 and encodes the VEGFD/FIGF protein, with size of 354 amino acids, and a molecular mass of 40444 Da. Mutations of this gene occur rarely in cancer [119–126].

PGF

The *PGF* gene is located in cytogenetic band 14q24.3 and encodes the PGF protein, with size 221 amino acids and molecular mass of 24789 Da [127]. Abnormalities in PGF gene expression occur in numerous cancers, in renal and liver cancers it is correlated with poor prognosis, and in intrahepatic cholangiocarcinoma, the PGF gene is overexpressed [128]. Promoting angiogenesis, an essential process in tumour development, VEGFs and PGF have long been targeted, against which numerous chemical compounds have been developed, including Apatinib, Bevacizumab, Cabozantinib, Pazopanib, Ramucirumab, Sorafenib, Sunitinib, Vandetanib, Zif-Aflibercept and the mAb 33C3. Although they inhibited the formation of new blood vessels or lymph vessels from pre-existing ones, they had side effects worth considering. Thus, hypertension, artery clots, and slowed or stopped wound healing were common, and less commonly, gastrointestinal perforation and fistula formation. Currently, several therapeutic combinations are being evaluated in solid tumours based on immune-checkpoint inhibitors (ICIs) in combination with anti-angiogenic agents, including ipilimumab+bevacizumab, atezolizumab+bevacizumab, avelumab+axitinib, pembrolizumab+axitinib, cabozantinib+nivolumab+ipilimumab, axitinib+avelumab, atezolizumab+bevacizumab, regorafenib+nivolumab, sintilimab+bevacizumab, atezolizumab+bevacizumab+paclitaxel, nivolumab+axitinib, atezolizumab+RFA+bevacizumab+atezolizumab, and bevacizumab+atezolizumab+paclitaxel [129,130].

*MMP*s

The *MMP* gene family encodes zinc-dependent endopeptidases and the most important proteases involved in extracellular matrix remodeling, with *MMP1*, *MMP2* and *MMP9* involved in the RAS–RAF–MEK–ERK signaling pathway. The *MMP1* gene is located in the 11q22.2 region and encodes the MMP1 protein, 469 amino acids in size and 54007 Da molecular mass, which cleaves type I, II and III collagens. The *MMP2* gene is located in the 16q12 band. 2 and encodes the MMP2 protein, with a size of 660 amino acids and a molecular mass of 73882 Da, which cleaves denatured type IV and V collagen and elastin. The *MMP9* gene is located in the 20q13.12 region and encodes the MMP9 protein, with a size of 707 amino acids and a molecular mass of 78458 Da, which cleaves type IV and V collagens [2,131–133]. Matrix metalloproteinase inhibitors indicated to be used in cancer include Marimastat, Prinomastat, Tanomastat and Neovastat [134], Marimastat being proposed to be used in combination with a cytotoxic agent and delivered via lysolipid-containing thermosensitive liposomes, for the inhibition of tumor metastasis [135].

IL8/CXCL8

The *IL8/CXCL8* gene is located in band 4q13.3 and encodes the CXCL8 protein, with a size of 99 amino acids and molecular mass of 11098 Da, one of the main factors stimulating the inflammatory response, being synthesized by mononuclear macrophages, neutrophils, eosinophils, T lymphocytes, epithelial cells, and fibroblasts. In cancer, IL8/CXCL8 influences the tumor microenvironment, promotes transformed cell survival, stimulates tumor progression, epithelial-to-mesenchymal transition and angiogenesis, and inhibits anti-tumor immune effectors. Aberrations of the *IL8/*

CXCL8 gene, including mutations, amplifications, deletions and copy number amplification of the mRNA are identified in numerous cancers, such as non-small cell lung cancer, colorectal cancer, head and neck cancer, cervical cancer, ovarian cancer, uterine endometrioid carcinoma, breast cancer, pancreatic cancer, lung cancer, endometrial cancer, mature B-cell lymphoma, bladder cancer, esophagogastric cancer, bone cancer, melanomas, hepatobiliary cancer and thyroid cancer [2,136,137].

CCND1

The *CCND1* gene is located in the 11q13.3 region and encodes cyclin D1, with a size of 295 amino acids and molecular mass of 33729 Da. Part of the cyclin family, CCND1 regulates CDK kinase activity. In the RAS–RAF–MEK–ERK signaling pathway, CCND1 and cyclin dependent kinase 4 (CDK4) promote tumor progression by stimulating cell proliferation [2]. Aberrations in *CCD1* gene function, such as mutations, amplification and overexpression, are common in numerous types of human tumors, including endometrial, bowel, stomach non-small-cell lung, endometrial, pancreatic, breast, and colorectal cancers, breast invasive ductal, breast invasive lobular and breast invasive carcinoma, bladder urothelial, and head and neck squamous cell carcinoma, melanoma, and endometrial endometrioid adenocarcinoma [138–140].

CDK4

The *CDK4* gene is located in the 12q14.1 region and encodes cyclin dependent kinase 4, with a size of 303 amino acids and molecular mass of 33730 Da. Together with its partner, CDK6, CDK4 plays a very important role in cell cycle progression from G1 to S phase and in RB1 protein activation, and aberrations in the function of this gene, including missense and silent mutations, are reported in numerous cancers, including long adenocarcinoma, well differentiated/dedifferentiated liposarcomas, conventional glioblastoma multiforme, glioblastoma, endometrial, intestinal, and skin cancers adenocarcinoma [141,142], in which it promotes cellular proliferation and tumor progression (2). Among the second-generation inhibitors tested against CDK4 and other CDKs are: BAY1000394, P1446-05, PD0332991, R547, RGB-286638, ZK304709 [138], and in breast cancer, against CCND1-CDK4-CDK6 complex formation, the combinations Palbociclib+letrozole, Palbociclib+fulvestrant, Ribociclib+letrozole, and Abemaciclib+aromatase inhibitors have been tested, some giving encouraging results [143].

Conclusions

At least 12 canonical signaling pathways are activated in cancer, which are partially or completely inactivated in adults. Among these, a leading place is occupied by the RAS–RAF–MEK–ERK signaling pathway, which uses about 39 proteins to take up biological signals from a plethora of extracellular ligases, transmit them transmembrane through 12 receptors that activate the GRB2 protein, the first cytoplasmic member of the signaling pathway (EGFR/ERBB1/HER1, ERBB2/HER2, PDGFRA, PDGFRB, IGFR, KIT/c-KIT, FLT3, MET, FGFR1, FGFR2, FGFR3 and FGFR4), and eight other receptors, BDKRB1, BDKRB2, EDNRA, EDNRB, NTRK1, TPM3, TPR and TFG, of which the first four activate GNAQ and GNA11 proteins and the other four activate RAS proteins directly. This is followed by 11 cytoplasmic and 16 nuclear proteins, through which the RAS–RAF–MEK–ERK signaling pathway stimulates transcription of factors that promote cell proliferation, evasion of apoptosis and angiogenesis. Apart from GRB2, all other members of the RAS–RAF–MEK–ERK signaling pathway undergo mutations in several cancers that render them inactive and reactivate them, which play important roles in tumour development and their incapacitation is a major challenge for oncologists. To date, products have been developed to block the activity of most members of the RAS–RAF–MEK–ERK signaling pathway, some with encouraging results, by reducing the rate of tumour progression, inhibiting or slowing cell proliferation, halting the angiogenic process or driving transformed cells into the programmed cell death pathway. Some products, including Trametinib, Paclitaxel, Binimetinib, Sorafenib, Sunitinib, Afatinib and Gefitinib, act on multiple protein targets, while others, including JNK-IN-8 and Regorafenib, have target specificity, acting selectively on a single protein. The latter category also includes monoclonal antibody-based products, including Trastuzumab, Pertuzumab, Panitumumab, Margetuximab, Xentuzumab, Ganitumab, Onartuzumab, Telisotuzumab, Sintilimab, Cetuximab, Spartalizumab, which biologically disrupt the signaling pathway of targeted proteins. As some tumour cells have developed alternative mechanisms to circumvent the action of the chemotherapeutic agent, through new mutations or separate pathways to sustain progression, oncology research has sought to introduce innovative 2nd or 3rd generation products into treatment regimens, so that the fight for life for researchers and cancer patients is ongoing and always introducing new challenges.

Conflict of interest

The author has no conflict of interest to declare.

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