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RESEARCH PAPER

Isolation, identification and characterization of three new strains of *Bacillus sphaericus* as mosquito pathogen

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Abstract

In the present study, three entomopathogenic strains of *Bacillus sphaericus* (MBI5, MBI6, and MBI7) were isolated from greenhouse pest and larval habitat of Istanbul, Turkey. All of the MBI groups tested in laboratory bioassays were able to kill larvae, but not pupae and adults of *Culex* spp. in water. The most efficient larvicidal activity of the strains was found in the presence of two toxin genes, *binA* and *binB* for MBI5, MBI6, and MBI7 strains. Each *B. sphaericus* strains identified in this study were characterized as unique and novel in terms of fatty acid methyl ester (FAME) profiles and 16S rRNA sequencing data. The results of this study suggest that the three strains of *B. sphaericus* may be new sources of potential biocontrol agent of mosquito.

Introduction

Mosquitoes are vectors of many diseases such as mosquito-borne arboviruses, malaria, filariasis and Japan encephalitis. Generally, the control of mosquitoes has been carried out more by chemical pesticides than biopesticides in the world. These chemical pesticides are known as dichlorodiphenyltrichloro ethane (DDT), gammaxene, malathion, chlordane, and organophosphates (van den Berg, 2009). All of them have high toxic range for both human health and environment (Casida & Quistad, 1998; Costa, 2006; Didia et al., 1975; Hurlbert et al., 1972). Compared to chemical pesticide, microbial insecticides are often species specific and do not contaminate environment, therefore, safe to non-target organisms in the nature. Among various microbial pesticides, *Bacillus thrungiensis* and *B. sphaericus* are being widely used.

Mosquitocidal bacteria are environmentally friendly alternatives to chemical pesticides for controlling water mosquitoes (Federici et al., 2006). *B. thrungiensis* subs. *israilensis* (*Bti*) is the most extensively used mosquito larvicidal bacteria in the world. *Bti* produces different type of insecticidal proteins during its sporulation, which are known as Cry or Cyt toxins (Schnepf et al., 1998; Soberón et al., 2007). Cry toxins have been widely used in the control of broad range of mosquito and blackfly species as well as nematodes, mite, and protozoa (Marroquin et al., 2000; Pinto et al., 2003; Schnepf et al., 1998). Another potential microbial insecticide, *B. sphaericus*, is known to be effective against *Culex* spp. and *Anopheles* spp. species and has better residual activity in polluted waters by production of binary toxin (Bin) and mosquitocidal toxins (Mtx). Mosquito resistance to some of *B. sphaericus* strains have been reported in many countries (Berry, 2012; Broadwell & Baumann, 1986; Park et al., 2010; Rao et

al., 1995; Silva-Filha et al., 1995; Wirth et al., 2007; Yuan et al., 2000; Lacey et al., 2015). Therefore, there is always great need and force for the scientists to search for new natural mosquitocidal bacterial strains, which can be used for development of new strains for development of new commercial microbial insecticide.

In the present study, three new strains of *B. sphaericus* (MBI5, MBI6, MBI7) isolated from larval habitat and some greenhouse pests have been identified and characterized in terms of phenotypic features as well as larvicidal activity in laboratory conditions.

Materials and Methods

All the *B. sphaericus* strains were isolated (as described below) from unhealthy mosquito larvae and aphid samples, which were collected from the district of Istanbul, Turkey. *Bti* ATCC 35646, *Bti* 4Q4, and *B. sphaericus* serotype H were obtained from the culture collection unit in Department of Genetics and Bioengineering at Yeditepe University, Istanbul, Turkey.

Isolations of *B. sphaericus* strains from mosquito larvae and aphid samples

Fifty-three *Culex* spp. larvae samples were collected from natural habitat in Istanbul, Turkey. In addition, 18 samples of unhealthy aphids were taken from tomato plants in grown greenhouse. Each of the unhealthy larvae and aphid samples were crushed in sterile water and then plated on nutrient agar (NA) media for isolation of the microorganisms. All inoculated plates were incubated at 27°C for 4 days, and then individual bacterial colonies grown on the plates were selected and purified on NA. Totally, 157 and 22 bacterial strains were isolated from mosquito unhealthy larvae and aphid samples, respectively. *B. thrungiensis* ATCC 35646, *B. thrungiensis* 4Q4 and *B. sphaericus* serotype H were also used as reference strains in this study. All bacterial strains were stored in 15% glycerol at -80°C for further studies.

Bioassays

Single colonies of newly isolated bacterial strains and reference strains (*Bti* 4Q4, *Bti* ATCC 35646, *B. sphaericus* serotype H) were cultivated on nutrient yeast salt mineral agar (NYSM) containing 5 g glucose (bacteriological), 5 g peptone, 5 g NaCl, 3 g beef extract, 5 g yeast extract, 203 mg $MgCl_2$, 10 mg $MnCl_2$ and 103 mg CaCl and incubated for 48 h at 30°C. Each strain was harvested and re-suspended in 10 mL of distilled water. Absorbance was adjusted to 0.2 with distilled water and then 0.5 mL of suspension was added to 500 mL of fresh water/polluted water in 1000 mL flasks containing 100 larvae (at the stage of 3rd instar) of *Culex* spp. The larvae were taken with polluted water, which has rich organic matter, from natural habitat in Istanbul, Turkey. The inoculated flasks were maintained on laboratory bench and observed for 48 h at room temperature. The positive and negative control flasks (treated with reference strains and sterile water, respectively) were kept at the same conditions to determine larvicidal bacterial strains capable of killing 90% of larvae. The numbers of alive larvae were counted, and the percentage viability was reported from the average of four replicates.

Morphological, physiological and biochemical characterizations of isolates

Bacterial strains selected as larvicidal in bioassay test were identified based on morphological biochemical, physiological features according to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986). Phenotypic characteristics of the strains include cell and colony shape on NA, while spore formation on NYSM media. Biochemical tests are composed of gram reaction, oxidase, catalase, capsule staining. Physiological characteristics of natural strains were determined in terms of growth ability at different conditions. To do the hemolysis test, *Bti* ATCC 35646, *Bti* 4q4, *B. sphaericus* serotype H and MBI group were cultivated onto Sheep Blood Agar. They were incubated for 24 h in CO_2 incubator. Moreover, all bacteria were grown on 20 mL nutrient agar at 25°C for 48 h for anaerobic test. On the other hand, MBI group and *B. sphaericus* serotype H were grown aerobically on 20 mL nutrient agar with penicillin disk at 25°C during 48 h for penicillin susceptibility test. To perform temperature test, high (50°C) and low (4°C) temperature were chosen to incubate bacteria in order to understand growth range.

Extraction and analysis of fatty acid methyl ester (FAME) profiles

Extraction and analysis of FAME from whole cell fatty acids of bacterial strains were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA) (Miller & Berger, 1985). FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m×0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of the strains were identified by the commercial TSBA6 database with Microbial Identification System (MIDI, Inc., Newark, Delaware, USA) software (MIS version no: 6:0). The cellular concentrations of the fatty acids for each strain were determined and strains were identified at species level.

DNA extraction from bacterial strains

Total genomic DNA from bacterial strains was extracted according to methodology described by Jimenez et al. (2000) with some modifications.

PCR amplification and purification of 16S rRNA

16S rRNA genes of the bacterial DNA isolates (TABLMBI5, MBI6, MBI7 and *B. sphaericus* serotype H

for control) amplified by the PCR (Bio-Rad, Italy) using purified DNA and primers 27f and 1492r (Weisburg et al., 1991). PCR amplifications were carried out in total volume of 50 µL reaction mixture containing 0.2 mM of 27f and 1492r primers for total 16S, 1 U of *pfu* DNA polymerase (Fermentas, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 mM $MgSO_4$, 10 mM Tris and 50 ng template DNA. PCR conditions were as follows: pre-amplification 94°C for 5 min; denaturation at 94°C for 30 s; annealing at 55°C for 40 s; elongation at 72°C for 2 min repeated 34 cycles and then post amplification for final extension 10 min at 72°C.

We designed specific two new primers for *B. sphaericus* like members of Bacillaceae family. We amplified 550 bp of 16S rRNA gene fragments of the DNA from bacterial isolates (MBI5, MBI6, MBI7, and *B. sphaericus* serotype H for control) by the PCR (Bio-Rad, Italy) using purified DNA and primers FAM1 and FAM2. PCR amplifications were carried out in total volume of 50 µL reaction mixture containing 0.2 mM of FAM1 and FAM2 primers for 550 bp of 16S, 1 U of *pfu* DNA polymerase (Fermentas, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 mM $MgSO_4$, 10 mM Tris and 50 ng template DNA. PCR conditions were as follows: pre-amplification 94°C for 5 min; denaturation at 94°C for 30 s; annealing at 51°C for 40 s; elongation at 72°C for 45 s repeated 34 cycles and then post amplification for final extension 10 min at 72°C. All the primer sequences are given in Table 1.

The amplified DNA products were detected by using Bio-Rad image analyzing system (Bio-Rad, Italy) after electrophoresis of PCR amplicons in a 1% agarose gel stained with ethidium bromide.

Table 1. Primers used for PCR amplification of 16S rRNA of MBI group and toxin gene sequencing

16S universal primers (1500 bp) 16S (550 bp)	27F	AGAGTTTGATCCTGGCTCAG
	1492R	CGGCTACCTTGTTACGAC
	FAM1	CTCTGTTGTAAGGGAAGAAC
	FAM2	CCATGCACCACCTGTCACCG
Toxin Gene Primers	51 kDa F	CGCTAAATACTACTCCTACAAGCC
	51 kDa R	GGATACGATTGTATACCTGCC
	42 kDa F	CCCACAGAAGGAAAGTACATTCGC
	42 kDa R	CCTAGTAAAGGTTCACTTGC
	Mtx1 F	CAAGCTGCTTCACTTACATG
	Mtx1 R	GTCCAGTTACATCTTGAGCC
	Mtx2 F	GGAGACTAATTGAATTTTCGGTTTCC
	Mtx2 R	GCGATGCTGGGCTATGTTCGTTGTTA

16S rRNA gene sequencing and phylogenetic analysis

Pure amplification products were sequenced with Prism ABI 3100 Genetic Analyzer 16 capillary, dideoxy terminator cycle sequencing kit (Applied Biosystems). The manufacturers' recommendations were followed for sequencing. Sequences were determined with an automated DNA sequencer (model: Prism ABI 3100; Applied Biosystems). Both strands were sequenced using the primers 27f, 1492r, FAM1 and FAM2 (Roberts et al., 1996). The Clustal Omega program (Higgins et al., 1992) was used to align the 16S DNA sequences generated with sequences of *B. sphaericus* like members from GenBank NCBI (Larsen et al., 1993). Genetic distance was computed by using Kimura's two-parameter model (Maruyama & Kimura, 1980) and used for neighbor-joining analysis. Phylogenetic trees were constructed using neighbor-joining and maximum-parsimony methods provided by CLC Genomics Workbench_2_1_1 both methods produced trees with similar topologies.

Determination of toxin genes

Toxin genes investigated according to methodology described by Nishiwaki et al. (Nishiwaki et al., 2007). PCR amplification was performed of the toxin genes using MBI5, MBI6, MBI7, and *B. sphaericus* serotype H for control as template to find the mosquitocidal binary toxin genes, *mtx1*, and *mtx2.* Toxin gene primers are given in Table 1. PCR was constructed according to the following conditions: pre-amplification 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min 30 s. The master mix consisted of 1 U of TSG polymerase (Biobasic, Canada), 1 mM $MgSO_4$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 20 ng of template DNA, and 5 pmol of each primer in total volume of 50 µL reaction mixture. The amplified DNA products was detected by using Bio-Rad image analysing system (Bio-Rad, Italy) after electrophoresis of PCR amplicons in a 1% agarose gel stained with ethidium bromide.

Scanning Electron Microscope

The bacterial samples (MBI5, MBI6, MBI7, and *B. sphaericus* serotype H) were grown axenically and aerobically for 24 h at 27°C on 20 mL nutrient agar. The bacteria were collected with sterile plastic inoculating loops solid culture plate. The collected samples were added into 1 mL deionized water, vortexed, and centrifuged for 5 min at 7500 rpm. The supernatant was discarded. This procedure was repeated three times. Finally, the washed bacteria were diluted 20 times with sterile water. Then, 5 µL of the bacteria solution was spotted and dried on a scanning electron microscope (SEM) specimen stub (Carl Zeiss, Germany). The accelerating voltage was in the range of 5 to 10 kV.

Results and Discussion

Bioassay results

Investigation of the larvicidal features of three bacteria were carried out in fresh and polluted water that contained 100 larvae (Table 2). *Bti* ATCC 35646, *Bti* 4Q4 and *B. sphaericus* serotype H were used as positive control. The *Bti* were less effective on 3rd instar larvae with respect of strains of *B. sphaericus*. The MBI group were re-isolated from dead mosquito larvae and re-identified after the bioassay treatments (Figure 1). Based on the larvicidal activity of three strains (MBI5,

MBI6, MBI7) were selected and characterized. Bioassay test results clearly show that MBI5, MBI6, and MBI7 have a potential to be toxic for larvae of *Culex* spp.

Table 2. Bioassay results of MBI5, MBI6, MBI7, *B. sphaericus* serotype H, *Bti* ATCC 35646 and *Bti* 4Q4 on of *Culex* spp. larvae in polluted/fresh water

Bacteria Name	The number of alive larvae of *Culex* spp. (Total number : 100 in 500 mL)		
	Polluted water		Fresh water
	24h	48h	24h
B. sphaericus serotype H (0.5 mL)	10	4	0
MBI5 (0.5 mL)	6	4	0
MBI6 (0.5 mL)	7	3	0
MBI7 (0.5 mL)	9	2	0
*Bti*ATCC35646 (0.5 mL)	20	20	16
Bti 4Q4 (0.5 mL)	34	32	24

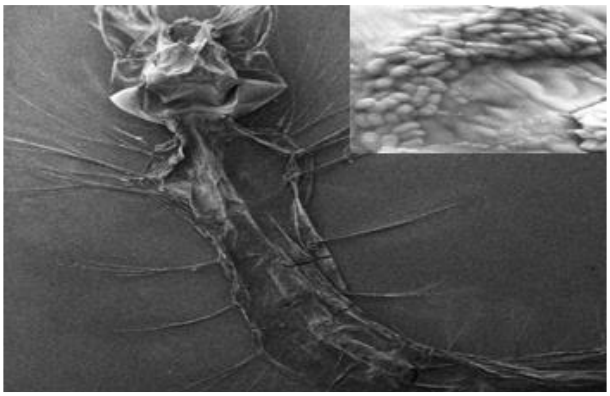

Figure 1. Scanning electron microscope image of dead mosquito larvae.

Morphological, physiological, and biochemical characterizations of newly isolated strains

The physiological characteristics of MBI5, MBI6, and MBI7 were summarized and selective characteristics with related model as *B. sphaericus* were compared in Table 3. According to the results; the new strains of *Bacillus* spp. MBI5, MBI6, and MBI7 were aerobic, gram-positive, spore-forming, capsulated and rod-shaped bacteria (Figure 2). Also, they have penicillin susceptibility. The optimum growth temperatures were 27-30°C. There is no growth at 4°C and 50°C on NA. All of the tested strains were oxidase and catalase negative. However, they were hemolysis positive.

Table 3. Phenotypic characteristics of strains MBI5, MBI6, MBI7 compared with *B. sphaericus* serotype H

Characteristics	MBI5	MBI6	MBI7	*B. sphaericus* serotype *H*
Gram reaction	+	+	+	+
Oxidase	-	-	-	-
Catalase	-	-	-	-
Capsule staining	+	+	+	+
Endospore staining	+	+	+	+
Hemolysis	+	+	+	+
Anaerobic test	-	-	-	-
Penicillin	+	+	+	+

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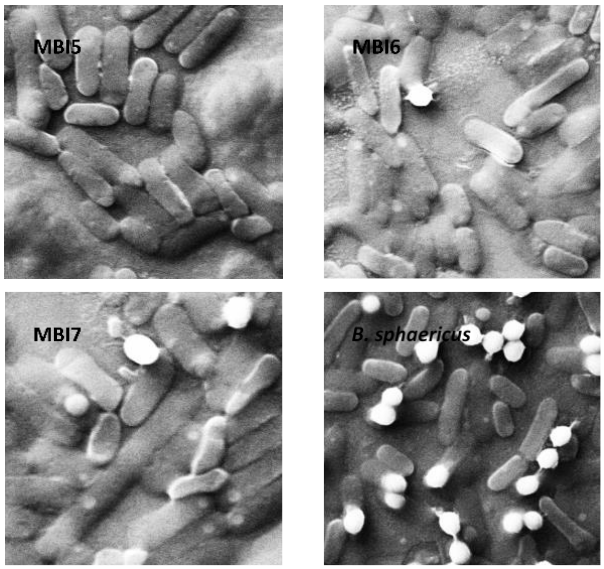

Figure 2. Scanning electron microscope images of MBI5, MBI6, MBI7 and *B. sphaericus* serotype H.

FAMEs analysis

The cellular fatty acid profiles of MBI group (MBI5, MBI6, and MBI7) and *B. sphaericus* serotype H were listed in Table 4. The major cellular fatty acids in MBI5 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45%) and $C_{16:0}$ iso, 12.65%, minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.60%), $C_{16:0}$ (1.72%), $C_{17:1}$ iso ω10c (1.43%). The major cellular fatty acids in MBI6 included iso-pentadecanoic acid ($C_{15:0}$ iso, 44.99%), and $C_{16:0}$ iso, 15.24%. Minor amounts of the fatty acids $C_{16:0}$ (0.78%), $C_{17:1}$ iso ω10c (1.40%). The major cellular fatty acids in MBI7 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45.84%), and $C_{15:0}$ anteiso, 13.13%. Minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.68%), $C_{18:1}$ iso ω9c (1.03%). The peak of 14:0 iso 3-$OH^{(-)}$ was found only *B. sphaericus* serotype H. The bacteria of MBI group do not exist this peak. Consequently, significant similarities in fatty acids profiles were found between *B. sphaericus* serotype H and MBI group. All of the groups MBI and *B. sphaericus* serotype H were identified with MIDI as *B. sphaericus* GC subgroup E.

Table 4. Cellular fatty acid composition of MBI5, MBI6, MBI7, and *B. sphaericus* serotype H

Fatty acids	Fatty acid concentration (%)			
	MBI5	MBI6	MBI7	*B. sphaericus* serotype H
14:0 iso	2.02	4.38	1.51	1.26
14:0	0.60	-	0.68	0.85
15:0 iso	45.00	44.99	45.84	46.61
15:0 anteiso	10.87	9.22	13.13	7.89
14:0 iso 3OH	-	-	-	1.05
16:1 ω7c alcohol	9.93	12.38	9.55	6.80
16:iso	12.65	15.24	8.14	5.48
16:1 ω11c	3.31	2.04	3.31	5.62
16:0	1.72	0.78	1.78	1.64
17:1 iso ω10c	1.43	1.40	2.35	4.92
Sum In Feature 4	1.65	1.72	2.32	2.58
17:0 iso	6.11	4.67	5.69	10.86

PCR of 16S rRNA amplification

We carried out PCR amplification of *bin* and *mtx* toxin genes of MBI group and *B. sphaericus* serotype H. Our analyses revealed that *B. sphaericus* serotype H, MBI5, MBI6, and MBI7 all had the *binB* gene (Figure 3). Besides, *binA* toxin gene is present in *B. sphaericus* serotype H, MBI5, and MBI6, except MBI7. At the same time, MBI5, MBI6, and MBI7 did not have *mtx1* and *mtx2* toxins. In addition**,** commercial *B. sphaericus* serotype H has both *bin* and *mtx* toxin genes (Figure 4).

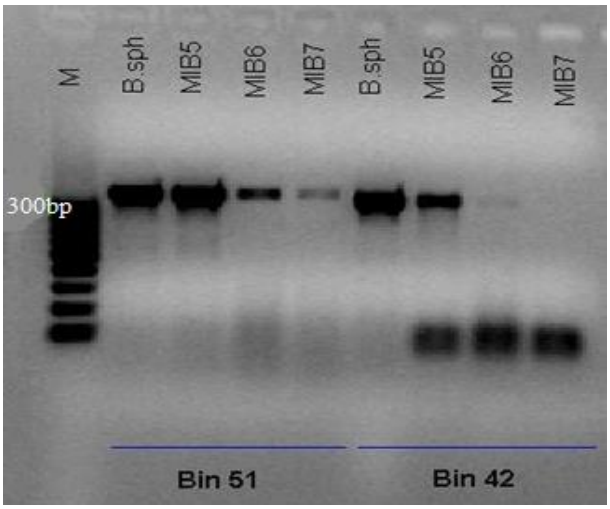

Figure 3. PCR amplicon of *bin 51* and *bin 42* toxin genes of B.sph: *B. sphaericus* serotype H, MBI5, MBI6, and MBI7.

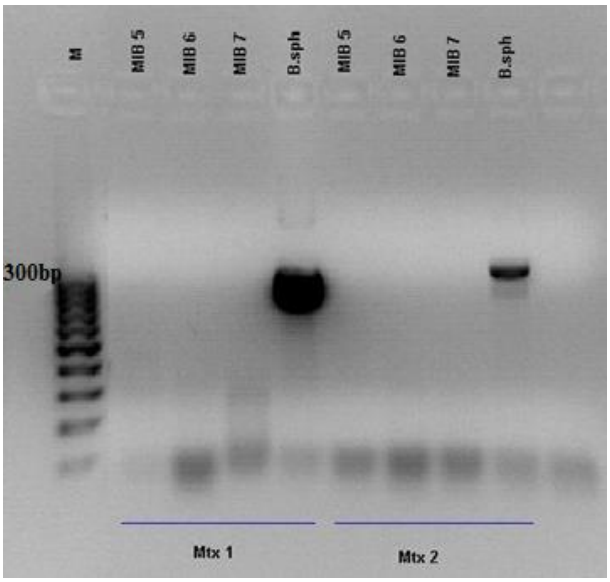

Figure 4. PCR amplicon of *mtx 1* and *mtx 2* toxin genes of B.sph: *B. sphaericus* serotype H, MBI5, MBI6, and MBI7.

Another study was neighbour-joining tree analysis that is based on nucleotide sequences of MBI group from 16S rRNA gene sequencing data (Figure S1). Confidence limits estimated from bootstrap analyses (100 replications) appear at the nodes. A maximum-parsimony tree generated from the sequence data exhibited similar topology to this tree. In the phylogenetic tree; MBI5, MBI6, and MBI7 clearly belonged to the strains of *B. sphaericus*, as shown by the high bootstrap value (Figure 5). Moreover, the MBI group was located at different nodes within the *B. sphaericus*-like strains in the phylogenetic tree (Figure 5).

Generally, bacteria that are used for biological control of mosquitoes have effects on varied mosquitoes. This variety derives from specificity of bacteria toxins and their host range. *B. sphaericus* is more active against *Anopheles* and *Culex* spp. and less active against *Aedes* spp**.,** on the contrary *B. thrungiensis* subsp*. israilensis* is more active against *Aedes* and *Culex* spp., whileless active against *Anopheles* spp. In addition, *B. sphaericus* has ability of larvacidein polluted aquatic environments. But *B. thrungiensis* subsp*. israilensis* has lost its activity due to organic components of polluted aquatic environments (Suryadi et al., 2016; Wirth et al., 2010).

These bioassays compromise the determination of the lifecycle of the *Culex* spp. larvae after the bioinsecticide bacteria were inoculated in their natural habitats. Here, we have observed their life cycle at the laboratory condition, which is explained at the experimental section. MBI5, MBI6, MBI7, *B. sphaericus* serotype H, *Bti* ATCC 35646, and *Bti* 4Q4 were examined on larvae of *Culex* spp.. Each bacterium was inoculated in 0.5 mL using flasks. In fresh/polluted water, experiment results showed that MBI5, MBI6, MBI7, and *B. sphaericus* were effective as a biological control agent of larvae of *Culex* spp. Due to loss of activities of *B. thrungiensis* subsp. *israilensis* ATCC 35646 and 4Q4, the observed number of died larvae were decreased as compared to *B. sphaericus* (Table 2). Eventually MBI5, MBI6, MBI7, and *B. sphaericus* serotype H had mosquito pathogen ability in both environments (Table 2). In addition, MBI groups were more effective in fresh water for 24 hours. These results demonstrated that MBI5, MBI6, and MBI7 have potential to be used as bacterial insecticides because of their high toxicity on larvae. After this study, we identified mosquito pathogen bacteria through widely used techniques that are based on morphology, FAME, and PCR.

According to FAME analysis of MBI5, MBI6, MBI7, and *B. sphaericus,* they have similar results due to their fatty acid compositions**.** Due to the fact that, the MIDI library is not sensitive between sub strains of bacteria. According to our findings, MBI5, MBI6, MBI7, and *B. sphaericus* serotype H are the same bacteria, which are named as *B. sphaericus* GC subgroup E. Our analyses revealed the fatty acid structure differences between MBI5, MBI6, MBI7, and *B. sphaericus* serotype H (Table 4). However, the cell and colony morphology, and the physiological properties of MBI5, MBI6, and MBI7 were similar to *B. sphaericus* serotype H. Therefore, we performed 16S rRNA gene sequencing of MBI5, 6 and 7 for obtaining detailed information about their exact classification under the genus *Bacillus*. The 16S rRNA gene sequencing of MBI5, MBI6, and MBI7 showed that they are new members of *Bacillus* genus.

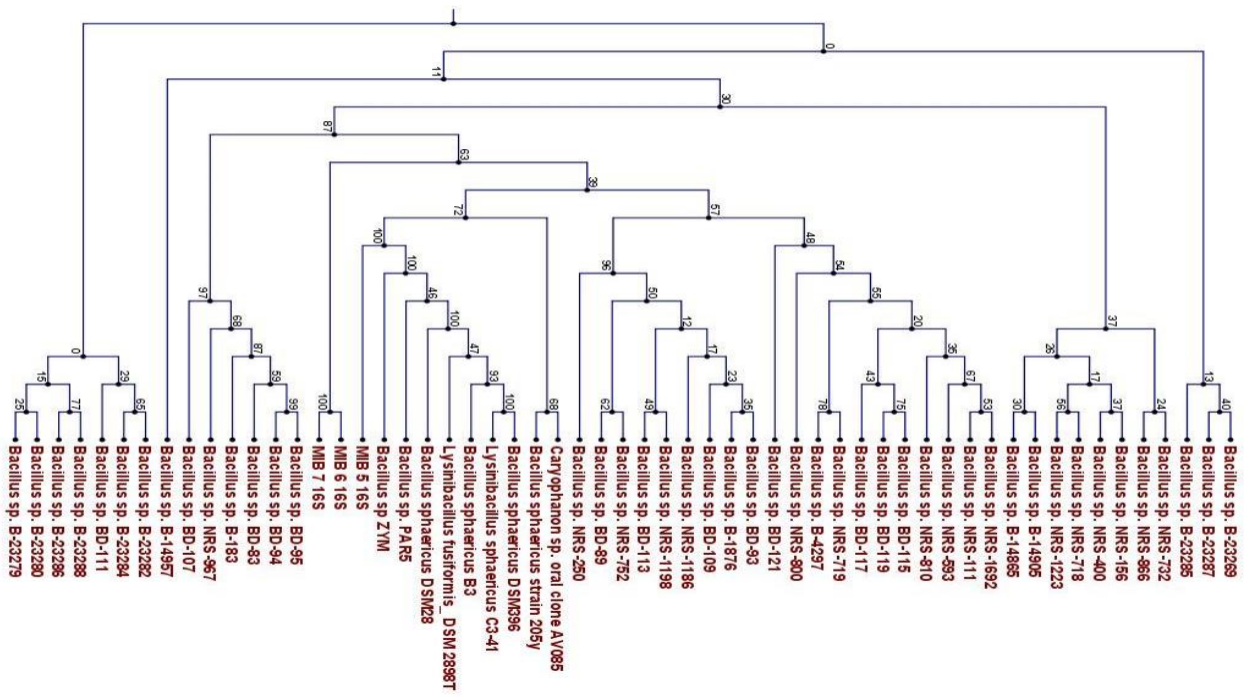

Figure 5. Neighbour-joining tree: the phylogenetic relationships among the *B. sphaericus*-like strains.

The *B. sphaericus* produces proteins that are toxic to mosquito larvae and the mosquito pathogen strains are divided into two groups depending on their toxicity. Highly active strains of *B. sphaericus* produce the crystalline binary toxin Bin during sporulation which is extremely toxic to *Culex* species. Bin is composed of BinA (41.9 kDa) and BinB (51.4 kDa). BinB binds to a specific receptor in *Culex pipiens* on the mosquito larvae midgut. Whereas BinA binds to a receptor only present on BinB. Both BinA and BinB are responsible for insecticidal activity (Park et al., 2010).

On the contrary, the low-activity-showing strains of *B. sphaericus* lacked Bin. Insecticidal activity of these strains is provided by proteins known as Mtx toxins (mosquitocidal toxins such as Mtx1 and Mtx2). Mtx1 and Mtx2 increase the toxicity of low activity strains of *B. sphaericus*. Several Mtx toxins exist in *B. sphaericus* strains which have both high and low insecticidal activities (Wirth et al., 2007). The three new isolated bacteria have bio insecticidal activity on *Culex* spp. larvae.

We have done PCR amplification of *bin* and *mtx* toxin genes of MBI5, MBI6, MBI7, and commercial *B. sphaericus* serotype H has both of *Bin* and *Mtx* toxin genes (Figure 3 and Figure 4). However, MBI5 and MBI6 have *BinA* and *BinB* toxin genes but not *Mtx*. Furthermore, MBI7 has only *BinB* toxin gene. The data of the amplicon of toxin genes has demonstrated that the bacteria of the MBI group are different from the point of toxin genes in comparison with *B. sphaericus* serotype H.

Conclusion

According to FAME, phenotypic characteristics and PCR-based 16S rRNA gene sequencing results, MBI5, MBI6, and MBI7 have been identified as new *B. sphaericus* like strains. Therefore, MBI5, MBI6, and MBI7 were deposited into the Agricultural Research Service (ARS) Patent Culture Collection (USDA) as NRRL B-50199, NRRL B-50200, NRRL B-50201, respectively.

Bioassay applications have proved that the three newly isolated bacteria are more effective in a short time than commercial *B. sphaericus* on the larvae of *Culex* spp. This study demonstrates a new isolated *B. sphaericus* like strains can be used as a mosquito pathogen against to *Culex* spp..

Acknowledgments

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Additional Information

Supplementary data accompanies this paper at http://biotechstudies.org/uploads/BIO-84_Supp1.pdf.

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BIOTECH STUDIES

RESEARCH PAPER

In vitro androgenesis in pepper and the affecting factors on success: I. Carbon source and concentrations

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Maltose
Sucrose

Abstract

In vitro androgenesis methods are significant in rapid production of completely homozygous lines. Factors such as nutrient medium composition, types and concentrations of carbohydrate sources are effective on androgenetic success. Different sugar types were tested at different doses in anther culture of pepper to elucidate their influence on the yield of androgenesis. In this study, maltose was used as a source of sugar at 1.5, 3 and 6% doses. 3% sucrose was used as the control treatment. Murashige & Skoog (1962-MS) medium and 0.25% activated charcoal, 15 mg L^{-1} silver nitrate ($AgNO_3$), 4 mg L^{-1} naphthaleneacetic acid (NAA), and 2 different amounts of 6-benzylaminopurine (0.5, 1 mg L^{-1} BAP) with three breeding lines of pepper were used to know the genotypic effects on haploid embryo formation. When comparing 1.5, 3 and 6% maltose and 3% sucrose with each other in the medium, embryo response increased with maltose concentration. Maltose, which was generally compare to sucrose as carbohydrate source for anther culture, the embryo response had significantly affected at maltose concentrations. The combination with the best effect among the trial parameters was 30 g L^{-1} maltose and 0.5 mg L^{-1} BAP for obtaining embryos from anther culture. Also, it was found that genotype is one of the main factors affecting success in pepper anther and microspore culture.

Introduction

Pepper which belongs to the *Capsicum* genus and Solanaceae family is a domesticated species originated from the tropical and sub-tropical central regions of South America known as Bolivia (Olmstead et al., 2008). All pepper populations are diploid and have the same chromosome number (*2n=2x= 24*). *Capsicum annuum* L. is widely grown and produced all over the world. It was domesticated from wild bird pepper or 'Chiltepin' in Mexico (Perry et al., 2007; Yildiz et al., 2020). However, it is the most broadly distributed and economically crop of the world. Red pepper cv. Capia (*C. annuum*) type is an essential and widely used among other vegetables all over the world. Turkey, due to its geographical location and soil fertility has played an important role in the distribution of different plant species. It is one of the most important country having great cultivation of peppers. Annual production and harvested area of dry peppers and green peppers in Turkey were reported to be 16,355 tones and 6,749 ha and 2,608,172 tones and 94,444 ha, respectively (FAOSTAT, 2017).

One of the most important and original aims of the breeding programs in capia type pepper is development of new marketable cultivars. The most commonly used method by pepper breeders is pure line and pedigree selection. Biotechnological methods such as doubled haploid (DH) plant production, are intended for reducing the breeding process of these new homogenous and totally pure varieties (Lantos et al., 2012; Thomas et al., 2003). Obtaining high quality homozygous pure lines by classical methods is very time consuming (nearly 6-7

years) and labor intensive. Time can be shortened up to 1 year using androgenesis to obtain haploid plants via tissue culture. Microspore embryogenesis is an important and useful culture technique to obtain full homozygous lines from only the male gametes in pepper breeding (Comlekcioglu & Ellialtioglu, 2018). The first successful haploid embryo development from *C. annuum* anthers was happened by Wang et al. (1973) in China, George, and Narayanaswamy (1973) in India. In Turkey, the first *in vitro* androgenesis studies on domestic pepper genotypes were started by Abak (1983). Afterwards, many scientists in different parts of the world have investigated the factors affecting anther culture and published their valuable experiences. Some of the essential factors such as growing condition of donor plants, collection of donor buds in the optimal stage (Ari et al., 2016 a,b; Kim et al., 2004; Supena et al., 2006), pre-treatments of buds or anthers (Dumas de Vaulx et al., 1981), nutrient media (Comlekcioglu et. al., 2001; Dumas de Vaulx et al., 1981; Dumas de Vaulx, 1990; Irikova et al., 2011; Ozkum-Ciner & Tipirdamaz, 2002; Qin & Rotino, 1993; Supena et al., 2006), temperature treatment (Dolcet-Sanjuan et al., 1997; Koleva-Gudeva et al., 2009), culture season (Buyukalaca et al., 2004; Ercan et al., 2006; Gonzalez-Garcia, 2002; Rodeva & Cholakov, 2006; Taskin et al., 2011), incubation conditions (Ellialtioglu et al., 2001), plant growth regulators and genotype of the donor plant (Ari et al., 2016a,b; Buyukalaca et al., 2004; Comlekcioglu et. al., 2001; Wang & Zhang, 2001) which significantly influence the usefulness of haploidy could affect androgenetic responses.

Among the various factors used to increase the rate of haploid plants and cross-species hybrids of *C. annuum*, carbohydrate sources as nutrient components play a very important role. Many researchers have reached substantial results by changing source and concentration of carbohydrates (sucrose, maltose, and glucose) in the nutrient medium, in order to increase the effectiveness of microspore embryogenesis. Sucrose at different concentrations is commonly used as a carbohydrate source for *in vitro* cultivation of pepper anthers by Binzel et al. (1996), Morrison et al. (1986), and Supena et al. (2006). Maltose also used by Dolcet-Sanjuan et al. (1997), Gémesné et al. (2000), and Supena et al. (2006). There are very few research about using honey as a carbohydrate source instead of sucrose. Gebologlu et al. (2017) stated that the use of honey contributes positively to embryo formation, but this effect varies depending on genotypes. Consequently, different experiments are carrying out to create new protocols for effective embryo production in pepper.

In order to be used as parents in F1 hybrid variety breeding, 3 genotypes of sweet capia type pepper were studied to developed pure lines. The main propose of this work was to investigate the effect of different concentrations of maltose and sucrose as a carbohydrate sources in anther culture.

Materials and Methods

This research was carried out in the Tissue culture Laboratory owned by United Genetics Seed. A.Ş Company in Bursa, Turkey.

Plant material

In the current study, three different breeding lines (G-23, G-24, and G-26) of capia type peppers were used as a plant material. Pepper seeds were planted in styrofoam containing peat: perlite with the ratio of 2:1 in the greenhouse conditions, of the United Genetics Turkey Vegetable Seeds Company at Mustafakemalpaşa/Bursa location. Seedlings (42 day-old) were transferred into a controlled greenhouse to be grown as donor plants (Figure 1a). Due to the importance of healthy growth and development conditions of the donor plant, they were subjected to growth free from any stress like insects, weeds, diseases, water and nutrient deficiencies (Figure 1b).

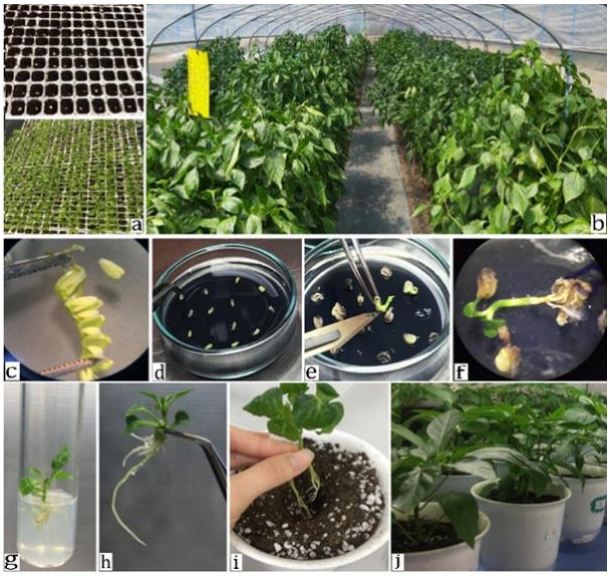

Figure 1. (a) Planting donor plants seeds and growing the seedlings, **(b)** donor plants in the controlled greenhouse, **(c)** isolation of anthers from buds under aseptic conditions, **(d)** putting the anthers into the petri dishes, **(e)** transferring the embryos to hormone-free MS medium, **(f)** haploid plantlet under stereomicroscope, **(g)** growing of embryos *in vitro*, **(h)** removing advanced embryos from the test-tube, **(i)** acclimatization of pepper plantlets, **(j)** healthy androgenic pepper plants.

Anther culture studies

Flower buds were collected in the morning. In the phase when the corolla and the calix are at the same height or when the the height of corolla is a little above the height of the calix and also when there is an anthocyanin production, mostly late uninucleate and mid uninucleate microspore stage were found alongside anthers that contain pollen in the young binucleate stage (Comlekcioglu & Ellialtioglu, 2018).

Surface sterilization and anther extraction is also critical in anther culture. In the laboratory this method

started with washing flower buds with sterile distilled water very quickly. Then 70% ethanol sprayed on buds and kept in 2.5% sodium hypochlorite and a drop of Tween-20 for 10 minutes and washed in sterile distilled water 3 times or more to remove the traces of sodium hypochlorite. And later all buds are carefully cut (Figure 1c) and examined with using a microscope inside laminar flow cabinet (anther should not be damaged during cutting) on a sterile paper and cultured horizontally on the pepper agar medium (Figure 1d).

Treatments and observations

Anthers of three capia (G-23, G-24, and G-26) pepper genotypes (breeding lines) were cultured on MS (Murashige & Skoog, 1962) media including vitamins and plant growth regulators. It was previously reported (Buyukalaca et al., 2004; Keles et al., 2015 and Taskin et al., 2011) that MS nutrient media supplemented with NAA, BAP, activated charcoal, sucrose and $AgNO_3$ were successful in the androgenesis of pepper. Actually, 8 different types of culture media were prepared supplemented with 4 mg L^{-1} NAA, 15 mg L^{-1} $AgNO_3$, 0.25% activated charcoal, and 8 g L^{-1} agar for all under the same condition. Variable factors were BAP with (0.5, 1 mg L^{-1}) doses and carbon source and concentrations as maltose with 15, 30 and 60 g L^{-1} and sucrose 30 g L^{-1} as a control treatment.

The cultures were incubated in the dark conditions for 2 days at 35°C, later at 25°C for 4 to 6 weeks to induce direct embryogenesis continuously. Embryo formation (Figure 1e and 1f), development, germination and transformation into a complete plantlet were regularly observed. After growing embryos were transferred to MS medium without any plant growth regulator (Figure 1g) and were kept in light with 16 hours photoperiod at 25°C. After few days the embryos were transferred into sterile coco peat pots (Figure 1h and 1i) for shoot and root elongation (Figure 1j).

Experiment design and statistical analyses

The experiment was carried out in a "completely randomized design" with four replications and 5 petri dishes per repetition. Five anthers obtained from a flower bud were planted in each petri dishes (20 Petri dishes and 100 anthers were used in each medium). Data were subjected to analysis of variance (ANOVA, Tarist Statistical Software) and means were separated by LSD test (A probability level $P < 0.01$ was used to test significance of differences between means).

Results and Discussion

In this study, for evaluating of embryo induction of anthers of three Capia pepper genotypes (G-23, G-24, and G-26) were cultured on MS medium including (0.5 and 1.0 mg L^{-1}) BAP with different concentrations of maltose (15, 30 and 60 g L^{-1}) and sucrose (30 g L^{-1}) as a carbon source.

The data presented in Table 1 show the effect of different carbon sources and BAP doses on number of embryos per 100 anther obtained and developed embryos obtained on pepper anther culture. According to data, G-26 genotype produced significantly higher embryos at each carbon and BAP level than the other two genotypes. This technology consists of stimulation and germination of haploid plants via anther or

Table 1. Number of embryos from different carbon sources, doses and two different doses of BAP in the culture medium

Carbon Source	BAP content (mg L^{-1})	Donor genotype	Number of embryos obtained	Total obtained embryos		Number of developed plantlets	Total developed plantlets	
Sucrose 30 g L^{-1}	0.5	G-23	4	18	55	2	5	16
		G-24	0			-		
		G-26	14			3		
	1.0	G-23	3	37		1	11	
		G-24	4			-		
		G-26	30			10		
Maltose 15 g L^{-1}	0.5	G-23	2	43	69	-	8	12
		G-24	0			-		
		G-26	41			8		
	1.0	G-23	2	26		-	4	
		G-24	0			-		
		G-26	24			4		
Maltose 30 g L^{-1}	0.5	G-23	6	44	84	-	13	24
		G-24	3			-		
		G-26	35			13		
	1.0	G-23	0	40		-	11	
		G-24	19			6		
		G-26	21			5		
Maltose 60 g L^{-1}	0.5	G-23	1	36	46	-	11	17
		G-24	2			-		
		G-26	33			11		
	1.0	G-23	3	10		1	6	
		G-24	1			1		
		G-26	6			4		

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microspores culture. Anther culture is one of the most interested method because of its high yield performance and number of male gametes existing in each bud. The genotype has the main role and often restricting aspect in the pepper androgenesis (Buyukalaca et al., 2004; Koleva-Gudeva et al., 2007; Rodeva et al., 2004). According to Morrison et al. (1986), the donor's plant genotype is the most important factor influencing the embryo production from anther culture of pepper. In our experiment, G-26 was the most responsive genotype for anther culture.

As a result of variance analysis, it was determined that two-way and three-way interaction among all studied factors (carbon source, BAP dose, and genotypes; CS×BAP×G) statistically were significant. This significance was realized for both the number of embryos obtained and the number of embryos developed. ($P \leq 0.01$) (Table 2, Table 3).

The highest average percentage of embryo induction rate in MS medium was obtained from G-26 with 10.25% including 15 g L^{-1} Maltose + 0.5 mg L^{-1} BAP per anther and this was followed by the same genotype created higher embryos than other genotypes with 8.75% embryos in 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP, and 8.25% embryos in 60 g L^{-1} Maltose + 0.5 mg L^{-1} BAP (Table 2).

Table 2. Average of obtained embryos according to different carbon sources, BAP doses and genotypes

Carbon source	BAP (mg L^{-1})	Genotype			Carbon×BAP Mean	Carbon Mean
		G-23	G-24	G-26		
Sucrose	0.5	1.00	0.00	3.50	1.50	**2.29^{c}**
30 g L^{-1}	1.0	0.75	1.00	7.50	3.08	
Mean		**0.88^{a}**	**0.50^{b}**	**5.50^{c}**		
Maltose	0.5	0.50	0.00	10.25	3.58	**2.88^{b}**
15 g L^{-1}	1.0	0.50	0.00	6.00	2.17	
Mean		**0.50^{a}**	**0.00^{c}**	**8.13^{a}**		
Maltose	0.5	1.50	0.75	8.75	3.67	**3.50^{a}**
30 g L^{-1}	1.0	0.00	4.75	5.25	3.33	
Mean		**0.75^{a}**	**2.75^{a}**	**7.00^{b}**		
Maltose	0.5	0.25	0.50	8.25	3.00	**2.04^{d}**
60 g L^{-1}	1.0	0.75	0.25	2,25	0.86	
Mean		**0.50^{a}**	**0.38^{bc}**	**5.25^{c}**		
BAP	0.5	**0.81^{a}**	**0.31^{b}**	**7.69^{a}**		
Mean	1.0	**0.50^{a}**	**1.50^{a}**	**5.25^{b}**		
Genotype Mean		**0.66^{c}**	**0.91^{b}**	**6.47^{a}**		

Means were separated by LSD test at $P \leq 0.01$. Column having different letter(s) are statistically significant.

Factor A: Carbon source; Factor B: BAP dose; Factor C: Genotype.
LSD (%)
Factor A: 0.240, Factor B: 0.170. A×B: 0.339,
Factor C: 0.208,
A×C: 0.415, B×C: 0.294 , A×B×C: 0.587

It was followed by G-24 including 30 g L^{-1} Maltose + 1.0 mg L^{-1} BAP with the highest average percentage of 4.75 embryos per anther. The lowest average of 1.5 embryos per anther was observed in G-23 genotype including 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP (Table 2).

Considering to the carbon source showed that the highest amount of developed embryo number occurred in 30 g L^{-1} Maltose medium. Although, there are significant differences among BAP doses according to genotypes. The obtained average of 2.94 and 2.42 embryos were determined for 0.5 and 1.0 mg L^{-1} BAP in turn. Genotype averages were 0.66, 0.91, and 6.47 for G-23, G-24, and G-26, respectively (Table 2).

These results show that the genotype is the main limiting factor in androgenesis and that the success of androgenesis for the same genotype is also affected by growth regulators. Therefore, experimental determination of optimum culture conditions for each genotype is important.

In anther culture, the number of embryos obtained from different genotypes and their conversion rates to plant differs. There are problems in the development and transformation of some of the embryos formed into plants, or abnormal plant formation can be observed.

The highest rate of developed into plantlets according to the obtained embryos was determined from the G-26 genotype with 37.1%, in 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP contained medium. The highest total number of developed plantlets in all MS medium including maltose (15, 30, and 60 g L^{-1}) and sucrose (30 g L^{-1}) was obtained from G-26 (Table 1). The ratio of the total number of developed embryos into the plantlets obtained is proportional calculated 37.1%, 30.0%, and 33.3%, respectively.

In terms of the average percentage number of embryos per anther showed significant difference between genotypes, G-26, G- 24, and G-23 in 0.5 mg L^{-1} BAP medium respectively were determined 7.69, 0.31 and 0.81 embryos, and in 1 mg L^{-1} BAP medium it was 5.25, 1.50, and 0.50 embryos, respectively (Table 2).

According to carbon sources, the highest average of embryo at the genotypes is in the G- 26 genotype with 8.13, 7.00, and 5.50 embryos, including 15 g L^{-1} Maltose, 30 g L^{-1} Maltose and 30 g L^{-1} Sucrose medium and the lowest average of embryo with 5.25 related to 60 g L^{-1} Maltose media (Table 2).

The highest number of the developed plantlets at G-26 genotype in three-way interaction was determined from medium including 0.5 mg L^{-1} BAP and 15 g L^{-1} Maltose (10.25 embryo). Other combinations followed this medium respectively (30 g L^{-1} Maltose: 8.75 embryo, 60 g L^{-1} Maltose: 8.25 embryo, and 30 g L^{-1} Sucrose: 7.50 embryo) (Table 2).

Genotypes have shown different performance in terms of transformation of regenerated embryos from different MS media and BAP doses into complete plants. In G-26 genotype, 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP medium showed the highest number of plantlets with an average of 3.25 per anther. The highest number of average plantlets per anther was 0.93 for G-23 in 30 g L^{-1} Sucrose + 1.0 mg L^{-1} BAP contained medium and 1.5 for G-24 in 30 g L^{-1} Maltose + 1.0 mg L^{-1} BAP included medium (Table 3).

As an important result of this research, 30 g L^{-1} of maltose with both 0.5 and 1.0 mg L^{-1} BAP doses showed significantly higher "number of embryos obtained" and

"developed embryo into the plantlets" compared to other culture media and control. This was followed by maltose 15 g L^{-1}, sucrose 30 g L^{-1} and maltose 60 g L^{-1}. Thereby, the performance of genotypes differed significantly from each other, and also it varied according to different media culture. G-26 has been identified as the most successful genotype and the lowest performance occurred in G-23 genotype.

Table 3. Average of developed androgenetic plantlets number according to different carbon sources, BAP doses, and genotypes

Carbon source	BAP (mg L^{-1})	Genotype			Carbon×BAP Mean	Carbon Source (Mean)
		G-23	G-24	G-26		
Sucrose	0.5	0.50	0.00	0.75	0.42^{c}	**0.78^{ab}**
30 g L^{-1}	1.0	0.93	0.00	2.50	1.14^{a}	
Mean		**0.71^{a}**	**0.00^{b}**	**1.63^{b}**		
Maltose	0.5	0.00	0.00	2.00	0.67^{bc}	**0.50^{c}**
15 g L^{-1}	1.0	0.00	0.00	1.00	0.33^{b}	
Mean		**0.00^{b}**	**0.00^{b}**	**1.50^{b}**		
Maltose	0.5	0.00	0.00	3.25	1.08^{a}	**1.00^{a}**
30 g L^{-1}	1.0	0.00	1.50	1.25	0.92^{a}	
Mean		**0.00^{b}**	**0.75^{a}**	**2.25^{a}**		
Maltose	0.5	0.00	0.00	2.75	0.92^{ab}	**0.71^{bc}**
60 g L^{-1}	1.0	0.25	0.25	1.00	0.50^{b}	
Mean		**0.13^{b}**	**0.13^{b}**	**1.88^{ab}**		
BAP	0.5	**0.13^{a}**	**0.00^{b}**	**2.19^{a}**		
Mean	1.0	**0.29^{a}**	**0.44^{a}**	**1.44^{b}**		
Genotype Mean		**0.21^{b}**	**0.22^{b}**	**1.81^{a}**		

Means were separated by LSD test at $P \leq 0.01$. Column having different letter(s) are statistically significant.

Factor A: Carbon source; Factor B: BAP dose; Factor C: Genotype.
LSD (%)
Factor A: 0.259, Factor B: 0.183, A×B: 0.366
Factor C: 0.224,
A×C: 0.449, B×C: 0.317, A×B×C: 0.634

The addition of auxins, cytokines, or their combinations is very important for the production of microspore derived haploid embryos, especially in stubborn plant species (Germenà, 2011).

Some researchers reported successful results in combination with 4 mg NAA and 0.1 mg BAP (Alremi et al., 2014; Buyukalaca et al., 2004; Comlekcioglu et al., 2001). Some researchers reported that higher haploid embryos were obtained with the combination of 4 mg NAA and 1 mg BAP (Ozkum-Ciner & Tipirdamaz, 2011; Taskin et al., 2011). On the other hand, Keles et al. (2015) found that the combination of 4 mg NAA and 0.5 mg BAP successful.

The effects of many critical factors in anther culture studies have been determined to affect successful embryo formation. In this research, the genotype of the donor plants, the sugar composition of the nutrient medium, growth regulators microspore development stage, physiological condition and growing conditions of the donor plant, preliminary applications to the anthers and the incubation conditions of the cultures greatly affect the response to androgenesis. Dolcet-Sanjuan et al. (1997) used maltose, malt extract and sucrose in different concentrations in anther of pepper anther culture. They reported that sugar type and concentration had significant effects on the total number of embryos obtained and the best result was obtained from 40 g L^{-1} Maltose. These results are compatible with the literature information (Comlekcioglu & Ellialtioglu, 2018; Irikova et al., 2011; Koleva-Gudeva et al., 2007; Segui-Simarro et al., 2011).

Conclusion

The perfect potential of haploidy and doubled haploidy in breeding programs for the development of new standard and hybrid varieties with unique characteristics of homozygous genotypes is clearly evident. Plant regeneration from microspore derived embryos is one of the most critical steps in pepper microspore culture. Among the many factors affecting the success of haploid androgenesis, such as genotype of donor plants, carbon source and concentrations, growth regulator combinations are also important factors in anther culture. Comparing the effects of genotype and the content of nutrient medium on the direct embryogenesis, a higher influence of interaction was observed between two factors.

After the evaluation of our results, we have also found that the factors such as genotype and carbon source play an important role in direct embryogenesis in capia pepper.

Embryogenesis responses in pepper was found to be genotype-dependent. So, the different responses of genotypes to the applied nutrient media will enable the selection of the proper medium for each genotype.

In conclusion of this research, we showed that genotype is one of the main factors affecting success in pepper anther and microspore culture. Also, it was found that the use of maltose as a carbon source had significant effect on embryo formation frequency in pepper anther culture. Future studies on different concentrations and doses of carbon source in combination with BAP might shed more light on improving the androgenesis in peppers.

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BIOTECH STUDIES

RESEARCH PAPER

Impact of titanium dioxide nanoparticles (TiO_2-NPs) on growth and mineral nutrient uptake of wheat (*Triticum vulgare* L.)

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Abstract

Titanium dioxide nanoparticles (TiO_2-NPs) among metallic NPs are one of the most produced and consumed NPs in the world. Thus, TiO_2-NPs release into the environment is inevitable. Their impact on food source cereals is still unclear. The purpose of this study was to assess the phytotoxic impacts of TiO_2-NPs on growth and some mineral nutrient (zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), nitrogen (N), phosphorous (P), potassium (K)) uptake of the wheat (*Triticum vulgare* L.) plant. The TiO_2-NP suspensions at increasing concentrations (0, 5, 10, 20 and 40 mg L^{-1}) were applied to wheat plant Yunus cultivar grown in hydroponic culture under controlled conditions for 21 days. The results indicate that the TiO_2-NPs have not statistically significant effects on plant growth. The chlorophyll content was decreased with increasing TiO_2-NP treatment, except for 5 mg L^{-1} TiO_2-NPs treatment. The higher N (4.58%), P (0.78%), Zn (87.50 mg kg^{-1}), and Cu (12.90 mg kg^{-1}) concentration were recorded at 40 mg L^{-1} TiO_2-NPs treatment. On the other hand, K concentration was decreased at 20 and 40 mg L^{-1} TiO_2-NPs treatments. This study has shown that exposure to TiO_2-NPs causes no significant phytotoxic effect on wheat.

Introduction

In the last decade, the fast development in nanotechnology and its impacts have raised concerns about the application of nanoparticles in agriculture and environment (Rafique et al., 2014). The most commonly used metallic NPs in industries are TiO_2, ZnO, CuO, Au, Fe_3O_4 and CeO_2. Among these NPs, TiO_2-NPs are one of the most produced and consumed materials in the world. Up to 2 million tons per year of TiO_2-NPs are produced in the worldwide (Larue et al., 2011). According to the group of Swiss Federal Laboratories for Materials Testing and Research (Empa)-ETH and the University of Zurich, the estimated annual production of TiO_2-NPs in Europe is 39.000 metric tons (SNSF, 2016). Today, TiO_2-NPs are widely used as the pigment in paint, paper, ink and plastics industries (Larue et al., 2011), and also in sunscreen due to their UV protective properties in cosmetic products (Mahmoodzadeh et al., 2013, Fan et al., 2016). This extensive production is postulated to result in the release into the environment with subsequent contamination of water, air, soils and plants (Aliabadi et al., 2016; Gottschalk et al., 2009; Shafea et al., 2017). It has been expected that these amounts will increase every year and they will accumulate in the ecosystems.

Plants may provide a potential pathway for NPs to enter the food chain. The accumulation of NPs in plants can cause a potential risk for human health and ecosystems due to their gross contamination into plant-based materials (Larue et al., 2018).

The metallic NPs play an essential role in plant growth and development at very low concentrations within the tolerance limits of plants. The response of plants to metal NPs varies according to the characteristics of NPs (size, shape, concentrations, exposure time and so on), the plant species and the growth stage of plants (Aliabadi et al., 2016; Aslani et al.,

2014; Hasanpour et al., 2015). Some researchers reported that the excessive of TiO_2-NPs in the long term caused a phytotoxic effect on seed germination, chlorophyll content, photosynthesis activity, plant growth and so on (Dogaroglu & Koleli, 2017a; Samadi et al., 2014; Yaqoob et al., 2018). In literature, there are contradictory reports about the potential toxicity of TiO_2-NPs in plants. Some reports proved that it has positive effect (Feizi et al., 2013; Landa et al., 2012; Servin et al., 2013; Singh et al., 2012; Song et al., 2013), some of them mentioned its adverse effects (Asli & Neumann, 2009; Cai et al., 2017; Castiglione et al., 2011; Du et al., 2011) and the others reported even no effect (García et al., 2011; Jacob et al., 2013; Landa et al., 2012; Larue et al., 2011, 2012; Seeger et al., 2009; Song et al., 2013; Zheng et al., 2005). Most studies on metallic NPs effects have been done in petri dishes through one week (Dogaroglu & Koleli, 2017a, 2017b). On the other hand, studies related to TiO_2-NPs phytotoxicity and accumulation in crop plants in hydroponic culture are still lacking (Lin & Xing, 2008; Song et al., 2013; Yang et al., 2006; Zhu et al., 2008). However, to simulate results closer to actual field conditions, the hydroponic or pot experiments should be verified in the controlled conditions. Hydroponic culture makes it possible to study with more plants in a short time, even if not as much as toxicity test in the petri dishes.

Wheat among cereals is the most essential food source for more than half of the world's population. Despite the fact being an important food source for human consumption all over the world, the impact of TiO_2-NPs on growth and some mineral nutrient uptake of cereals, including wheat is still unclear. Therefore, there is a need for a better understanding of the current knowledge about the effects of TiO_2-NPs on not only growth but also some mineral nutrient uptake in wheat under hydroponic conditions. In the present study, we examined the effects of TiO_2-NPs on growth and some mineral nutrient uptake in wheat (*Triticum vulgare* L.) in hydroponic culture.

Materials and Methods

Nanomaterial

The TiO_2-NPs (~30-50 nm) were prepared with the aqueous sol-gel method (Gokhale et al., 2009), which was modified by Dr. Karakaya. The identification of TiO_2-NPs as one of the primary nanomaterials by the Organization for Economic Cooperation and Development (OECD) is the most essential criterion in selecting the NPs to be used in this study (OECD, 2013).

The size, morphology and elemental analysis of TiO_2-NPs were determined by using Scanning electron microscopy (SEM) and energy - dispersive X-ray spectroscopy (EDX) (Carl Zeiss, Supra 55). The particle size and size distribution of the TiO_2-NPs in the solution was analyzed by using Zetasizer (Nano ZS, Malvern Instruments, UK).

Plant material

The seeds of Yunus cultivar (bread wheat) were used as plant material in this study. The seeds were purchased from the Transitional Zone Agricultural Research Institute, Eskisehir Province, Turkey.

Hydroponic experiment

The wheat seeds were germinated in a mixture of peat and perlite (1:1 w/w) until the seedlings were getting hairy rooted and 2-3 leaves. Afterwards, twenty-five of the wheat seedlings (5 plants each branch × 5 branches, each pot) were transferred into the 4.5 L polyethylene pots supplied with the Hoagland nutrient solution medium. The nutrient solution was contained as essential macro (1 mM KH_2PO_4, 3 mM KNO_3, 0.25 mM $MgSO_4 \cdot 7H_2O$, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$ and $2.5x10^{-2}$ mM KCl) and micronutrients (1 µM $MnSO_4 \cdot H_2O$, 1 µM $ZnSO_4 \cdot H_2O$, 0.25 µM $CuSO_4 \cdot H_2O$, 0.25 µM $(NH_4)_6Mo_7O_{24}$, and 0.125 µM H_3BO_3). The pH of the nutrient solution was adjusted to 5.2. Increasing doses (0, 5, 10, 20 and 40 mg L^{-1}) of TiO_2-NPs were added to the nutrient solution 5 days after transferring of seedlings into the pots. The suspensions of each pot were changed every 2-3 days. The experiment was designed as a randomized block method with three replications. The hydroponic experiment was conducted under controlled environmental conditions (16/8h light/dark period, 25/20°C, 60% humidity and 10 Klux light intensity) for 21 days.

Morphological observations and chlorophyll contents

The morphological changes of wheat plants under TiO_2-NPs exposure were observed throughout the experiment. Additionally, the effects of TiO_2-NP treatment on the leaf chlorophyll contents were measured by the Konica Minolta SPAD-502 chlorophyll meter as soil plant analysis development value (SPAD value) (Dağhan, 2018).

Elemental analysis in plants

The plants were harvested as shoots and roots separately 21 days after planting. Then, all plant samples were washed with distilled water and dried at 65 °C in an oven. When the samples reached a constant weight, their dry weights (DW) were taken and then, they were ground in the agate mill (Retsch MM301 Mixer Mill, Retsch, Nordrhein-Westfalen, Germany). Grounded plant samples were digested using a wet digestion method with HNO_3 and H_2O_2 in the microwave oven (MarsXpres CEM, Matthews, USA) for elemental analysis. Total essential element (potassium (K), phosphorus (P), zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn)), and Ti concentrations of the extracted samples were determined using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7500ce, Agilent Technologies, Santa Clara, USA).

The Ti contents of roots were calculated by multiplying root dry weights and Ti concentrations as below;

Ti content (µg root^{-1}) = Ti concentration in the root (mg kg^{-1} DW) × root DW (g)

The nitrogen (N) concentration of the shoots was determined, according to Nelson & Sommers (1980). Certified reference materials (SRM 1573A, SRM 1547) were analyzed to check the accuracy of the extraction technique.

Statistical analysis

The variance analysis of the data was statistically analyzed by using the SPSS-20 statistical analysis package program to determine the significance of levels and grouped by Duncan test at the 0.05 probability level.

Results and Discussion

Characterization of TiO_2-NP

The SEM image of the synthesized TiO_2-NPs is shown in Figure 1. The average size of TiO_2-NPs was measured at about 30-50 nm. Besides titanium (Ti), carbon (C) and oxygen (O) elements were detected in the EDX spectrum (Figure 1). The SEM images spectrums showed that the TiO_2-NPs were round in shape and tend to agglomerate; this tendency might be based on its hydrophobic surface properties (Dogaroglu & Koleli, 2017a; Nia et al. 2015).

The particle size and size distribution of TiO_2-NPs were measured by using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The Zetasizer results showed that the mean particle size of TiO2-NPs was around 50nm (Figure 2).

Hydroponic experiment

Among the plants, wheat, lettuce, cucumber, red clover and radish are the most recommended plants to determine the effects of NPs toxicity, by the US Environmental Protection Agency (US EPA), the US Food and Drug Administration (US FDA), and OECD (Faraji & Sepehri, 2018). Therefore, the wheat plant (*Triticum vulgare* L. var. Yunus) was chosen as a model and widely used to evaluate the effect of the TiO_2-NPs.

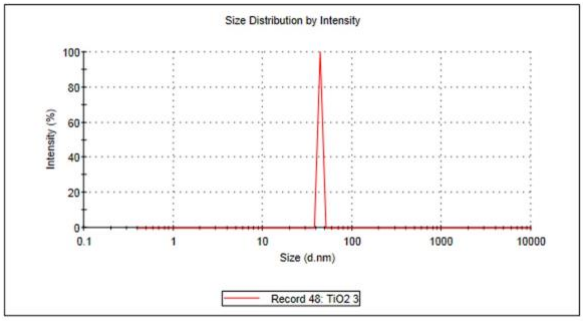


Figure 2. Particle size distribution by intensity of TiO_2-NPs.

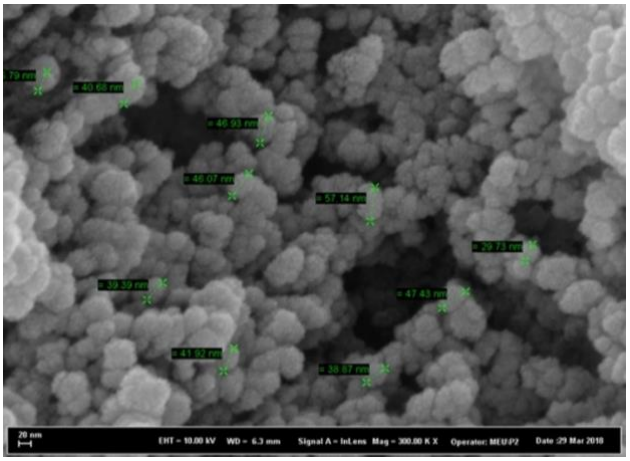
Morphological observations and chlorophyll contents

The effects TiO_2-NPs on morphological changes of wheat plants were observed throughout the experiment. The wheat plants did not show any visual symptoms such as chlorosis or necrosis under the increasing doses of TiO_2-NPs treatments (Figure 3).

Chlorophyll, which gives green color to plants, is a vital and the most abundant pigment for plants. The primary function of chlorophyll is absorbing light to provide energy for photosynthesis (Tan et al., 2018). Determination of chlorophyll content, which is a vital pigment molecule for photosynthesis, is an important indicator for observation of plant growth, especially under stress conditions. In this study, the chlorophyll content of leaf was measured for determination of the effect of TiO_2-NPs treatment on plant growth.

The 5 mg L^{-1} TiO_2-NP treatment increased chlorophyll content compared to control. On the other hand, the chlorophyll contents of plant decreased post 5 mg L^{-1} TiO_2-NP treatments ($p<0.01$). The highest chlorophyll content (43.1 SPAD Unit) was obtained at 5 mg L^{-1} TiO_2-NP treatment and the lowest chlorophyll content (40.9 SPAD Unit) was obtained at 40 mg L^{-1} TiO_2-NP treatment (Figure 4).

a



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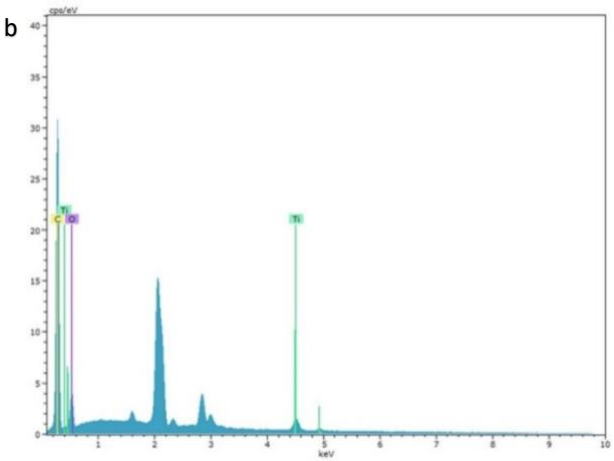

Figure 1. SEM image and EDX spectrum of synthesized the TiO_2-NPs. Scanning Electron Microscopic image of TiO2-NPs **(a)** and elemental analysis of the TiO2-NPs **(b)**.

Many conflicting results in the literature reported decreasing or increasing chlorophyll content in wheat under TiO_2-NPs treatments. Dogaroglu & Koleli (2017a) notified that the chlorophyll contents of barley decreased at 20 mg kg^{-1} TiO_2-NPs treatments. They also found that the chlorophyll content of wheat was negatively affected by increasing doses of TiO_2-NPs treatments (Dogaroglu & Koleli, 2017b). We had a similar result with Dogaroglu & Koleli (2017a, b). Aliabadi et al. (2016) investigated the effects of nano TiO_2 (0, 100, 1000, 2000 mg L^{-1}) and nano Al_2O_3 (0, 100, 500, 1000 mg L^{-1}) on plant growth as a spray to the wheat leaves two times in a week. Compared to the control treatment, nano TiO_2 and Al_2O_3 treatments significantly reduced chlorophyll a and b at high concentrations. The chlorophyll content (chlorophyll a and b) of wheat increased only at 100 mg L^{-1} nano TiO_2 treatment. The photosynthetic abilities may be reduced decrease because of increased reactive oxygen species (ROS) and lipid peroxidation, thus the chlorophyll content decreases (Hou et al., 2018).

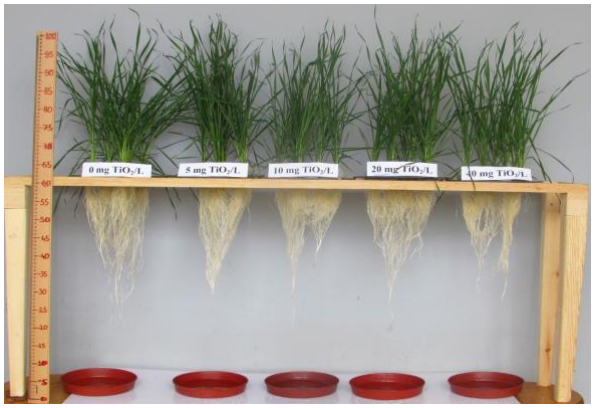

Figure 3. The effects of increased TiO_2-NP suspensions on the shoot and root biomass of wheat plant grown in hydroponic culture after 21 days.

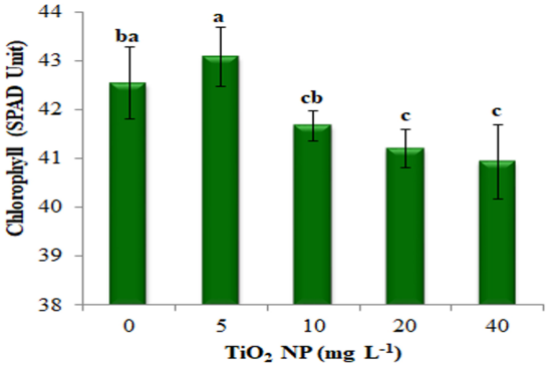

Figure 4. The effects of increased TiO_2-NP suspensions on the chlorophyll content of wheat plant leaves (n=3). Letter(s) on each bar show significant level ($p<0.01$).

On the contrary to our result, many studies reported that TiO_2-NP treatments increased photosynthetic activity and chlorophyll content of plants such as spinach (Hong et al., 2005; Yang et al., 2006), cucumber (Servin et al., 2012, 2013), Arabidopsis (Lenaghan et al., 2013), rapeseed (Li et al., 2015). Yang et al. (2006) and Siddiqui et al. (2015) reported the TiO_2-NPs enhanced the chlorophyll formation and light absorption, so the photosynthetic time of chloroplast and plant growth/development increased. Besides that, Hong et al. (2005) and Jaberzadeh et al. (2013) reported that TiO_2-NPs positively affected photosynthetic activity and so the chlorophyll content of spinach and corn, respectively.

Plant dry weight (DW)

TiO_2-NP treatments did not significantly affect shoots and roots dry weights (DW) of wheat plants (Figure 5). The recorded maximum and minimum shoot dry weights were obtained as 0.157 g at the control group and 0.137 g at 10 mg L^{-1} TiO_2-NPs treatment respectively. The effects of increased dose of TiO_2-NPs on dry weights of shoots were not statistically significant. However, the shoot produced higher biomass than the roots (Figure 4). The lowest root dry weight (0.0383 g) was obtained in control and the highest root dry weight (0.0417 g) was recorded at 40 mg L^{-1} TiO_2-NPs treatment.

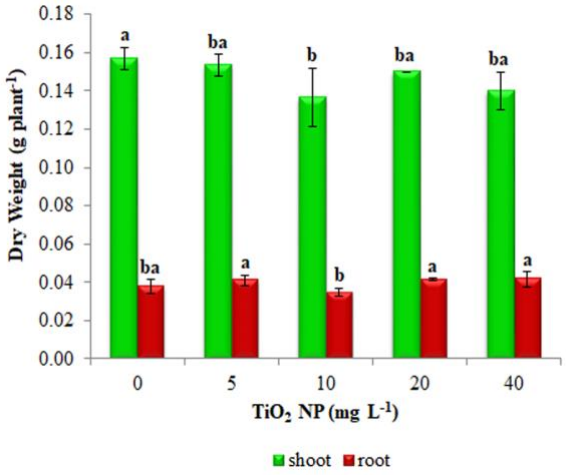

Figure 5. The TiO_2-NP treatments effects on shoot and root DWs of the wheat plant. Letter(s) on each bar show significant level (n=3).

Studies in the literature showed that TiO_2-NPs had positive effects sufficient or low concentrations and had adverse effects in high concentrations (Mahmoodzadeh et al., 2013; Rafique et al., 2014). Du et al. (2011) reported that the application of 10 mg kg^{-1} TiO_2-NPs into the soil reduced wheat growth and soil enzyme activities under field conditions. Feizi et al. (2012) showed a promoting effect of low concentrations of TiO_2-NPs for seed germination of wheat. Jacob et al. (2013) did not observe differences in the wheat dry matter upon exposure to TiO_2-NPs in a hydroponic system.

On the other hand, Du et al. (2011) have reported reduced wheat dry matters at a concentration of approximately 90 mg kg^{-1} TiO_2-NPs.

Ti concentration

There is limited information about uptake, transport, accumulation and tolerable level of Ti and TiO_2NPs by plants in literature. Lyu et al. (2017) reported that the concentration of Ti in above-ground parts of some plant species, which are grown in soils can vary between 1 to 578 mg kg^{-1}. Kelemen et al. (1993) were found that Ti accumulates in roots and only a small amount of it transported to the shoots.

The Ti concentrations were not detected in the parts of the shoot in all treatments because it was under the detection limits (data not shown). However, the Ti concentration of roots was increased with increasing concentration of TiO_2-NPs (Table 1).

Table 1. The TiO_2-NP treatment's effects on Ti concentration (mg kg^{-1} DW) and content (µg $plant^{-1}$) of wheat roots (n=3)

Plant	TiO_2 NP (mg L^{-1})	Dry matter (g $plant^{-1}$)	Ti concentration (mg kg^{-1} DW)	Ti content (µg $plant^{-1}$)
	0	0.0383^{ba}	n.d.	n.d.
	5	0.0409^{a}	1980^{d}	80.98^{b}
Wheat	10	0.0347^{b}	2285^{c}	$79,29^{b}$
	20	0.0414^{a}	2431^{b}	100.64^{a}
	40	0.0417^{a}	2603^{a}	$108,55^{a}$
Dose	F	$3.272^{n.s.}$	578**	285**

n.d.: not determined, n.s.: not significant, **: p≤0.01

The highest Ti concentration (2603 mg kg^{-1}) was obtained in 40 mg L^{-1} TiO_2-NP treatment, whereas the lowest (1980 mg kg^{-1} DM) was obtained in 5 mg L^{-1} TiO_2-NP treatment (Table 1). This result may be indicated that TiO_2-NPs could not be transferred from roots to the shoots of wheat due to its size or the lower exposure concentration relative to other studies (Larue et al., 2012). Larue et al. (2012) indicated that the upper threshold diameter of TiO_2-NPs for root uptake was 140 nm and translocation in the shoot of wheat was 36 nm. Tan et al. (2017) reported that the TiO_2-NPs, which have smaller particle sizes than 36 nm, could translocate from root to shoots.

Ti content

The Ti content refers to total Ti concentration of root or shoot and it is directly related to the total dry mass of plant parts and Ti concentration.

The root Ti content was increased with the increasing TiO_2-NP treatments (p≤0.01). The highest Ti content (108.55 µg $plant^{-1}$) was shown in 40 mg L^{-1} TiO_2-NP treatment, whereas the lowest (79.29 µg $plant^{-1}$) was shown in 5 mg L^{-1} TiO_2-NP treatment (Table 1). The results were the same with results reported by Feizi et al. (2012). Consequently, due to TiO_2-NPs accumulation in roots and less transfer to the shoots of wheat may not have caused any toxic effects on plants. Cox et al. (2017) reported similar results. Plants shown response to hydroponics exposure to NPs may differ from response to NP-contaminated soil exposure (Larue et al., 2012).

Mineral nutrition uptake

In the literature, the metallic nanoparticle effects on plant mineral nutrition uptake were studied very few (Larue et al., 2018). However, any phytotoxicity may affect the plant mineral nutrient uptake as a synergistic, antagonistic or neutral. For this reason, the effects of increasing TiO_2-NPs doses on macro (N, P, and K) and micro (Cu, Fe, Mn, and Zn) nutrient element concentrations of the shoot (Table 2) and root (Table 3) were investigated. This study ensured our understanding of the effects of the TiO_2-NPs on some essential mineral nutrition uptake, such as N, P, K, Fe, Zn, Cu, and Mn of wheat.

The highest N (4.58%), P (0.78%), Zn (87.5 mg kg^{-1}) and Cu (12.90 mg kg^{-1}) concentrations were recorded at 40 mg L^{-1} TiO_2-NP treatment in shoots of wheat. However, K concentration was decreased at 20 and 40 mg L^{-1} TiO_2-NPs treatments and Mn concentration was not affected by TiO_2-NP treatments. Except Mn concentration of shoot, mineral nutrient concentrations of shoot results were statistically important (*p≤0.01*). According to Jones et al. (1991), the shoots of wheat plant nutrients concentrations were determined to be sufficient Zn (21-70 mg kg^{-1}) except for 40 mg of TiO_2 L^{-1}, Fe (10-300 mg kg^{-1}), Mn (16-200 mg kg^{-1}), and Cu (5-50 mg kg^{-1}). On the other hand, macronutrients (N (>3%), P (0.5-0.8%) and K (>5%)) concentrations were determined higher than the critical concentration of these nutrients in wheat. These results indicated that TiO_2-NPs application was promoted N, P and K uptake of the plant shoots. Hong et al. (2005) and Yang et al. (2006) reported similar positive effects. Potassium, Zn, Mn, and Fe concentration of the roots were significantly decreased with TiO_2-NP treatments compared to the control plant (*p≤0.01*). The higher K (4.94%), Zn (195 mg kg^{-1}), Fe (3491 mg kg^{-1}), and Mn (134.4 mg kg^{-1}) were obtained in control treatments (Table 3). The highest P (2.21%) and Cu (32.1 mg kg^{-1}) concentrations were obtained at 40 mg L^{-1} TiO_2-NP treatment. Other researchers reported

Table 2. The effects of increased TiO_2-NP suspensions on some mineral nutrient concentration of wheat shoot (n=3)

Plant	TiO_2 NP	N	P	K	Zn	Fe	Mn	Cu
	(mg L^{-1})	(%)			(mg kg^{-1})			
	0	4.31^{b}	0.65^{d}	6.03^{a}	46.4^{b}	165^{c}	147.3^{a}	9.70^{c}
	5	4.53^{a}	0.70^{b}	6.01^{a}	41.2^{c}	204^{a}	140.3^{b}	10.57^{b}
Wheat	10	4.35^{b}	0.69^{cb}	6.05^{a}	35.9^{d}	190^{b}	141.3^{ba}	10.00^{c}
	20	4.27^{b}	0.67^{dc}	5.82^{b}	41.6^{cb}	170^{c}	139.7^{b}	10.60^{b}
	40	4.58^{a}	0.78^{a}	5.72^{b}	87.5^{a}	158^{c}	141.3^{ba}	12.90^{a}
Dose	F	20.2**	39.5**	17.1**	222**	27.9**	$2.67^{n.s.}$	67.3**

**: p≤0.01, n.s.: not significant

Table 3. The effects of increased TiO_2-NP suspensions on some mineral nutrient concentration of root (n=3)

Plant	TiO_2 NP (mg kg^{-1})	P	K	Zn	Fe	Mn	Cu
		(%)		(mg kg^{-1})			
Wheat	0	1.87^{d}	4.94^{a}	195^{a}	3491^{a}	134.4^{a}	28.3^{cb}
	5	2.05^{c}	4.72^{cb}	105^{b}	1855^{b}	129.7^{b}	26.6^{c}
	10	1.82^{e}	4.80^{b}	94.0^{c}	1808^{b}	129.4^{b}	28.6^{cb}
	20	2.11^{b}	4.79^{b}	100^{cb}	710^{c}	129.3^{b}	29.3^{b}
	40	2.21^{a}	4.66^{c}	96.1^{c}	355^{d}	130.4^{b}	32.1^{a}
Dose	F	231**	10.5**	365**	7251**	7.12**	11.0**

n.s.: not significant **: p≤0.01

similar results. The application of increasing doses of TiO_2-NP (250, 500, and 750 mg kg^{-1}) to the soil was rising the accumulation of K and P concentration in cucumber fruit (Servin et al., 2013).

Mattiello & Marchiol (2017) reported that the TiO_2-NP treatments (0, 500, 1000, and 2000 mg kg^{-1}) to the soil affect mineral nutrition uptake of the barley (*Hordeum vulgare* L. var. Tunika) plant compared to the control treatment. The N, S, Ca, Fe, Mn, and Zn concentrations of barley seeds increased compared to the control treatment while K concentration decreased with TiO_2-NP treatments. On the other hand, P, and Mg, B, and Cu concentrations did not affect the incensement of TiO_2-NP treatments (Mattiello & Marchiol, 2017). Tan et al. (2017) reported that the unmodified TiO_2-NPs enhanced Cu (104%) and Fe (90%); hydrophilic TiO_2-NPs enhanced Fe (90%). However, Mn (339%) concentration raised while, Ca (71%), Cu (58%), and P (40%) concentration of basil plants decreased with 500 mg kg^{-1} hydrophobic TiO_2 particles treatment to the soil. Pošćić et al. (2016), investigated that the application of increasing doses (0, 500, and 1000 mg kg-^{1}) of CeO_2 and TiO_2-NPs effects on the nutrient concentration of barley (*Hordeum vulgare* L). They significantly were obtained with increased Ca and significantly decreased sulfur (S) in both TiO_2-NP treatments. Potassium concentration was decreased at 1000 mg kg^{-1} TiO_2-NP. Mn and Zn concentrations were increased at 500 mg kg^{-1} TiO_2-NPs treatment. All these results showed that increasing dose TiO_2-NPs application positively affected nutrient uptake of wheat plant.

Conclusion

Among metallic NPs, TiO_2-NPs is the most produced and consumed materials by far. This extensive production and consumption are postulated to result in the release into the environment with subsequent contamination of soils and plants (agroecosystems). Thus, plants may be a potential entry point for TiO_2-NPs in the food chain. Today TiO_2-NPs contamination and accumulation in plants may be a severe problem around the world in the future due to the potential threat to food safety and its detrimental effects on the agroecosystems and human health.

This study provides new information about the effects of TiO_2-NPs on growth and mineral nutrient uptake of shoots and roots of wheat (*Triticum vulgare* L.) in a hydroponic culture trial. The possible phytotoxic effect of TiO_2-NPs on chlorophyll content, plant growth and mineral nutrient uptake of the wheat plant was investigated in this study. The TiO_2-NPs reduced chlorophyll content over 5 mg L^{-1} TiO_2-NP treatment. Plant growth was not statistically affected by increasing doses of TiO_2-NPs treatments. The dose of increased TiO_2-NPs resulted in increased N, P, Zn, and Cu concentrations. Nevertheless, it caused K and Mn concentrations of the plant to reduce. Further study of the effects of lower and higher dosage TiO_2-NPs on cereals and food crops should be needed to be undertaken.

Furthermore, for clarification of possible phytotoxic effects of TiO_2-NPs on the wheat plant, more studies need to investigate genotypic differences of wheat plants. Besides these, the mobility, transportation, accumulation and risk assessments of NPs on the environment and living organisms should be urgently studied both in the field and in hydroponic culture experiments.

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BIOTECH STUDIES

RESEARCH PAPER

Can bioactive peptides of *Lagocephalus sceleratus* be evaluated in the functional food industry?

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Abstract

Observations of Lessepsian migrant *Lagocephalus sceleratus* has been increasing along the Turkish coastline. Because of its toxins, it is known as a poisonous fish and not recommended to consume. To overcome this problem, an *in silico*-based biotechnological approach is proposed to evaluate the bioactive peptides from this species. The bioactive peptide contents of cytochrome oxidase subunit 1 in *L. sceleratus* with BIOPEP parameters were investigated in this study. The results show that there are many bioactive peptides such as the peptides with DPP-IV, ACE, alpha-glucosidase inhibition activities, antioxidant and antiamnestic and their levels are comparable in well-consumed species such as *Gallus gallus domesticus* and *Bos taurus*. In conclusion, after removal of the toxin, the biomass of the *L. sceleratus* can be used to produce bioactive peptides for the production of functional foods which will be very important for food industry to provide multi-functional properties to foods. The paper can be used as a model methodology to exploit new bioactive peptides when new proteins are explored from *L. sceleratus*.

Introduction

Introduction of invasive species into the new ecosystem creates important problems on the indigenous species. The Mediterranean Sea is under threat of introduction of invasive species due to the Suez Channel and also heavy maritime traffic (Galil, 2009). Although *Lagocephalus sceleratus* (Gmelin, 1789) is a very common species in the tropical waters such as Indian and Pacific Ocean, it is being one of the well invaded species in the Mediterranean Sea via Suez Channel (Leonardo et al, 2019). This alien fish has been commonly observed in almost all the Mediterranean Sea region (Akyol et al., 2005; Kalogirou, 2013; Kasapidis et al., 2007). Since this species has poisonous compounds in its tissues, it is not under stress of human consumption and fisheries (Leonardo et al, 2019). Therefore, this species has increased its habitat along the Mediterranean Sea. The scientific reports and also observations by fishermen revealed that this species consumes the eggs of local fish species and destroys the fisheries in the Mediterranean Sea (Kalogirou, 2013; Yaglioglu et al., 2011). According to Ulman et al. (2015), while 5% of fish caught in 2003 were *L. sceleratus*, this increased up to 50% after 5 years. *L. sceleratus* is a successful invasive species as it has a high reproduction rate, can hunt natural predators, benefit from food resources and adapt well to environmental conditions (Yaglioglu et al., 2011). They can reach 15-60 cm in length and mostly live at depths of 18-100 m (Yaglıoglu et al., 2011). *L. sceleratus* is very difficult to be hunted by its predators since it can inflate itself with the water and air in the environments and is highly poisonous (Golani et al., 2006). *L. sceleratus* includes tetrodotoxin (TTX) and this toxin is considered as one of the most effective chemicals among the marine toxins. Since no developed antidote or antitoxin related to TTX is existed, it is very dangerous to touch or consume *L. sceleretus*.

According to scientific publications, *L. sceleratus* in the Mediterranean Sea also includes the toxin at lethal doses (Bentur et al., 2008). As the fish grows in size, its toxicity increases (Katikou et al., 2009). Katikou et al. (2009) reported that while liver, gastrointestinal system and gonads had the highest level of toxicity in all of their samples, the toxicity in muscles and tissues was lower. The toxicity distribution is species-specific, but within the same species, local, seasonal and individual variations and also variations in toxin composition are observed (Katikou et al., 2009; Noguch & Arakawa, 2008; Yu & Yu; 2002). Kosker et al. (2019) investigated TTX levels in different *L. sceleratus* and found the TTX level in tissues in the range of 0.69-35.6 µg/g and most in gonad and liver tissues.

TTX is used as a channel blocker in physiological and neurological studies in many laboratories (Narahashi, 2001; Saoudi et al., 2010). TTX has an analgesic effect in advanced patients, shows an antitumor effect and is used for the treatment of drug addiction (Bragadeeswaran et al., 2010; Haque et al., 2008; Saoudi et al., 2010; Schwartz et al., 1998; Yu, 2008). Also, TTX is used for rheumatism treatments in Japan (Noguchi & Arakawa, 2008). Due to the possible inhibitory effect of TTX on neural conduction, it is examined for developing anesthetic drugs (Schwartz et al., 1998).

Food derived bioactive peptides are the hot research topics in functional food industry. Bioactive peptides are important sources for essential amino acids, and they have biological activities such as antioxidant, antimicrobial, antihypertensive, anticancer and antihyperglycemic (Karami et al., 2019; Tonolo et al., 2020; Valencia-Mejía et al., 2019; Zhou et al., 2020). Bioactive peptides can be obtained through the enzymatic hydrolysis, fermentation or food processing (Liu et al., 2020). Most of the bioactive peptides consist of 2-20 amino acids and they display different activities based on their amino acid compositions and secondary structures (Bechaux et al., 2019; Ryan et al., 2011). Bioactive peptides have been classified via *in silico* or *in vitro* studies from dairy products, mushroom, fish, meat, and seaweed etc. (Barati et al, 2020; Cipolari et al., 2020; Lafarga et al., 2020; Sayd et al., 2018; Zhou et al., 2020). The number of bioactive peptides according to BIOPEP-UWM is 4056 and this number is increasing with the new studies (Minkiewicz et al., 2019). Since they are easily absorbed in the intestine and can go into the circulatory system, they can exhibit physiological effects (Martínez-Sánchez et al., 2020). Moreover, bioactive peptides have been the subject of research in the development of functional foods and medicines (Jauset & Beaulieu, 2019; Tadesse & Emire, 2020). Fish based bioactive peptides are reviewed by Cipolari et al. (2020). They underlined that fish venoms and poisons are still undiscovered resources for bioactive peptides.

In this study, we aimed to investigate bioactive content of *L. sceleratus* by selecting a model protein (cytochrome c oxidase subunit 1) and then the results of *L. sceleratus* were compared with the traditional protein sources. This model biotechnological method proposes a novel *in silico* approach to evaluate the biomass of *L. sceleratus.*

Methods

The proteins were searched by using the keyword "*Lagocephalus sceleratus*" in Uniprot.org (The UniProt Consortium, 2019). 45 entries were found in uniprot.org related to *L. sceleratus*. From the entries, it is understood that they are unreviewed that means "*records that await full manual annotation*" according to Uniprot.org. The min and max length of amino acids in the entries were found in major capsid protein and NADH-ubiquinone oxidoreductase chain 5 (EC 7.1.1.2) to be 51 and 612, respectively. Cytochrome c oxidase subunit 1 was selected as a model protein for our research due to *i)* it contains relatively high number of amino acids, *ii)* it is also found in well-consumed animal sources such as bovine (*Bos taurus*) and chicken (*Gallus gallus domesticus*), iii) it is an important protein in electron transport chain. Bovine and chicken sequences were selected for comparison purposes. Moreover, this protein is one of the vital components of the mitochondria. The catalytic activity is based on the electron transfer and it catalyzes the reduction of oxygen to water via reduced cytochrome c. The aim of this report is to exhibit a model study for *L. sceleratus*. When new proteins from this fish species are discovered, bioactive peptide contents can be identified by using this methodology. The flowchart of the study was given in Figure 1.

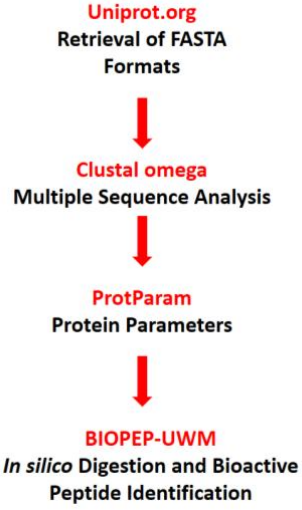

Figure 1. Flowchart of the methodology.

Amino acid sequence in FASTA format of cytochrome c oxidase subunit 1 protein found in *Lagocephalus sceleratus* (F2EN11)*, Bos taurus* (P00396) *and Gallus gallus* (P18943) were retrieved from Uniprot.org (The UniProt Consortium, 2019; Morgat et al., 2019).

Multiple sequence analysis of cytochrome c oxidase proteins was performed using Clustal omega

(Sievers et al., 2011). The similarity between sequences and preserved regions were shown by using the outputs of Clustal omega.

ProtParam tool was used for defining the physical and chemical characteristics of studied proteins (Gasteiger et al., 2005). Molecular weight, percentage and number of amino acids, instability index, net charge and theoretical pI value were determined via this tool.

Bioactive peptides found in *L. sceleratus* were investigated using BIOPEP-UWM database (Minkiewicz et al., 2019). Chymotrypsin, trypsin and pepsin (pH=1.3) enzymes were used for *in silico* digestion of the proteins studied in the report. These enzymes were chosen in the study because they are involved in the gastrointestinal track of humans. Cytochrome c oxidase proteins of *B. taurus* and *G. gallus domesticus* were also *in silico* digested by using the same methodology in the present study.

Results

Clustal omega database was used for determining the similarity between *Lagocephalus sceleratus, Bos taurus* and *Gallus gallus domesticus*. The asterisk symbol (*) shows conserved residues while colons (:) indicates groups with similar characteristics and period (.) shows groups with little similar characteristics. The comparisons between cytochrome c oxidase subunit 1 proteins of *L. sceleratus, B. taurus* and *G. gallus domesticus* can be seen in Figure 2a-b. The multiple sequence analysis results of the cytochrome c oxidase subunit 1 proteins found in *L. sceleratus, B. taurus* and *G. gallus domesticus* were shown in Figure 2a-b. From Figure 2a and b, it could be said that there is very high similarity among these species.

Physical and chemical characteristics of cytochrome c oxidase subunit 1 proteins found in *L. sceleratus, B. taurus* and *G. gallus domesticus* were determined by using ProtParam tool (Gasteiger et al., 2005). According to Table 1, most abundant amino acids found in cytochrome c oxidase subunit 1 protein of *L. sceleratus* are leucine, alanine and glycine to be 12.10, 9.20, and 9.10%, respectively. There is a similar order for the number of amino acids found in cytochrome c oxidase subunit 1 proteins of *B. taurus* and *G. gallus domesticus*. The theoretical pI values of cytochrome c oxidase proteins found in *L. sceleratus* and *G. gallus domesticus* were found nearly the same as 6.21 and 6.23 (Table 2).

Table 1. Number and percentage of amino acids found in cytochrome c oxidase subunit 1 proteins of *Lagocephalus sceleratus*, *Bos taurus* and *Gallus gallus domesticus*

	Lagocephalus sceleratus		***Gallus gallus domesticus***		***Bos taurus***	
	#	**%**	**#**	**%**	**#**	**%**
Ala (A)	48	9.20	46	8.90	40	7.80
Arg (R)	9	1.70	9	1.70	8	1.60
Asn (N)	14	2.70	15	2.90	19	3.70
Asp (D)	14	2.70	15	2.90	16	3.10
Cys (C)	1	0.20	1	0.20	1	0.20
Gln (Q)	7	1.30	9	1.70	6	1.20
Glu (E)	12	2.30	10	1.90	9	1.80
Gly (G)	47	9.10	46	8.90	47	9.10
His (H)	21	4.00	19	3.70	17	3.30
Ile (I)	37	7.10	41	8.00	37	7.20
Leu (L)	63	12.10	62	12.00	59	11.50
Lys (K)	9	1.70	9	1.70	9	1.80
Met (M)	27	5.20	26	5.00	34	6.60
Phe (F)	42	8.10	43	8.30	42	8.20
Pro (P)	28	5.40	31	6.00	28	5.40
Ser (S)	28	5.40	26	5.00	30	5.80
Thr (T)	37	7.10	40	7.80	38	7.40
Trp (W)	17	3.30	17	3.30	17	3.30
Tyr (Y)	18	3.50	18	3.50	19	3.70
Val (V)	40	7.70	32	6.20	38	7.40

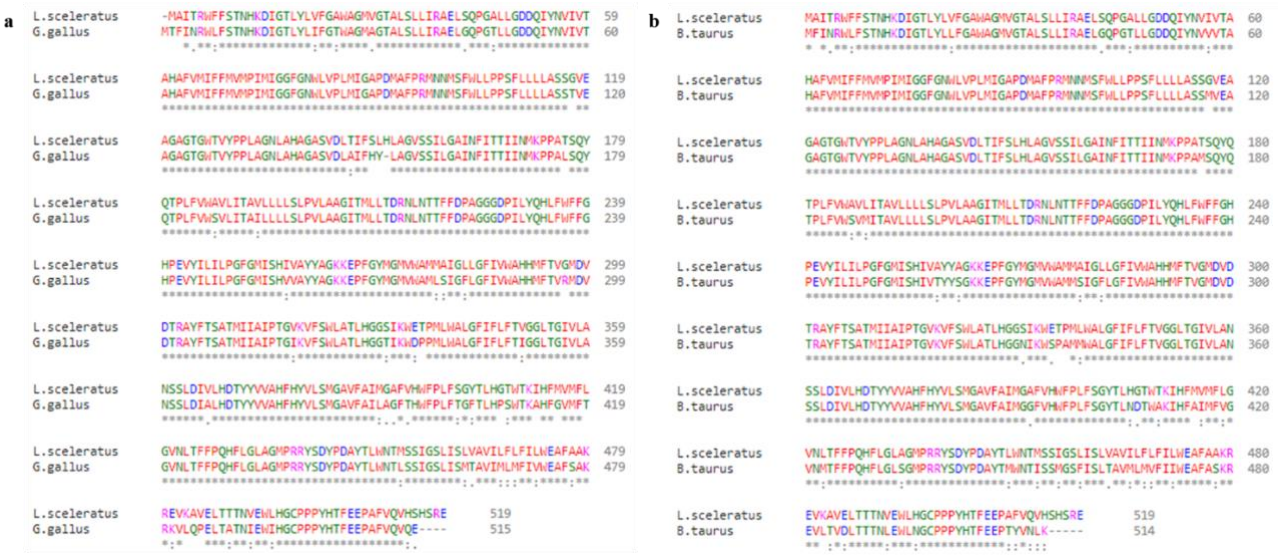

Figure 2. Multiple sequence analysis of **(a)** *Lagocephalus sceleratus* and *Gallus gallus domesticus* **(b)** *Lagocephalus sceleratus* and *Bos taurus.*

Table 2. Protein parameters of cytochrome c oxidase subunit 1 proteins found in *Lagocephalus sceleratus*, *Bos taurus* and *Gallus gallus domesticus* (aa: amino acids)

Species	# aa	Mw (Kda)	Theoretical pI	Negatively Charged Residues (Asp+Glu)	Positively Charged Residues (Arg+Lys)	Net Charge	Instability Index
Lagocephalus sceleratus	519	57314.59	6.21	26	18	-8	26.54
Gallus gallus domesticus	515	57051.23	6.23	25	18	-7	29.60
Bos taurus	514	57032.31	6.06	25	17	-8	25.81

The net charge of cytochrome c oxidase subunit 1 protein of *L. sceleratus* was found to be -8 while cytochrome c oxidase proteins of *B. taurus* and *G. gallus domesticus* were determined as -7 and -8, respectively. Instability index is a value that corresponds to the stability of the protein found in the test tube. Protein is stable when the stability index is below 40 (Guruprasad et al., 1990). According to Table 2, the instability index of cytochrome c oxidase proteins found in *L. sceleratus* is 26.54 and other studied proteins have similar instability index values.

Table 3. *In silico* hydrolysis of cytochrome c oxidase subunit 1 protein of *Lagocephalus sceleratus* using BIOPEP-UWM database (CaMPDE: Calmodulin-dependent phosphodiesterase 1; ACE: Angiotensin Converting Enzyme)

DH_t [%] 35.1393					
No	**Activity**	**A_E**	**W**	**B_E**	**V**
1	Antiamnestic	0.0015	0.3261	3.28850E-5	1
2	Inhibitor	0.0015	1.0000	0	
3	ACE inhibitor	0.0495	0.1266	0.00404	0.14986
4	Stimulating	0.0077	0.1158	0	
5	Antioxidative	0.0108	0.1247	0	0
6	Renin inhibitor	0.0062	0.2672	0.00017	0.98743
7	CaMPDE inhibitor	0.0015	1.0000	0	
8	Dipeptidyl peptidase IV inhibitor	0.0634	0.1100	0.00013	0.21510
9	Alpha-glucosidase inhibitor	0.0031	0.1179	0.00014	0.65774
10	Dipeptidyl peptidase III inhibitor	0.0124	0.1542	0	

In silico hydrolysis of studied proteins were performed using BIOPEP-UWM tool and chymotrypsin, trypsin and pepsin (pH=1.3) enzymes were used. DH_t value is a theoretical degree of hydrolysis and in this study DH_t value of cytochrome c oxidase subunit 1 proteins found in *L. sceleratus* is 35.1393 (Table 3). A_E value corresponds to the frequency of release of fragments by selected enzymes (Minkiewicz et al., 2019). According to Table 3, A_E value was found for dipeptidyl peptidase IV inhibitor as 0.0634, ACE (Angiotensin-converting enzyme) inhibitor as 0.0495 and dipeptidyl peptidase III inhibitor as 0.0124. Similar activities and A_E values were obtained with the hydrolysis of cytochrome c oxidase proteins of *B. taurus* and *G. gallus domesticus* (Table 4-5).

Table 4. *In silico* hydrolysis of cytochrome c oxidase protein of *Gallus gallus domesticus* using BIOPEP-UWM database (CaMPDE: Calmodulin-dependent phosphodiesterase 1; ACE: Angiotensin Converting Enzyme)

DH_t [%] 35.1823					
No	**Activity**	**A_E**	**W**	**B_E**	**V**
1	Immunostimulating	0.0016	0.5000	0	
2	Antiamnestic	0.0016	0.3404	3.36655E-5	1
3	ACE inhibitor	0.0522	0.1320	0.00382	0.13687
4	Stimulating	0.0079	0.1280	0	
5	Antioxidative	0.0127	0.1639	0	
6	renin inhibitor	0.0079	0.2500	0.00017	0.98746
7	CaMPDE inhibitor	0.0016	1.0000	0	
8	Dipeptidyl peptidase IV inhibitor	0.0633	0.1102	0.00018	0.29338
9	Alpha-glucosidase inhibitor	0.0032	0.1190	0.00014	0.49511
10	Dipeptidyl peptidase III inhibitor	0.0142	0.1547	0	

Table 5. *In silico* hydrolysis of cytochrome c oxidase protein of *Bos taurus* using BIOPEP-UWM database (CaMPDE: Calmodulin-dependent phosphodiesterase 1; ACE: Angiotensin Converting Enzyme)

DH_t [%] 36.2041					
No	**Activity**	**A_E**	**W**	**B_E**	**V**
1	Antiamnestic	0.0016	0.3333	3.38799E-5	1
2	Inhibitor	0.0016	1.0000	0	
3	ACE inhibitor	0.0478	0.1261	0.00391	0.14212
4	Stimulating	0.0064	0.1087	0	
5	Antioxidative	0.0096	0.1257	0	0
6	Renin inhibitor	0.0064	0.3092	0.00017	0.98743
7	CaMPDE inhibitor	0.0016	1.0000	0	
8	Dipeptidyl peptidase IV inhibitor	0.0621	0.1068	0.00013	0.20641
9	Alpha-glucosidase inhibitor	0.0032	0.1339	0.00014	0.65819
10	Dipeptidyl peptidase III inhibitor	0.0127	0.1564	0	

Discussion

The importance of bioactive peptides is being increased since the several health effects of bioactive peptides are shown in numerous papers. Bioactive peptides occur via enzymatic catalysis, digestion in

gastrointestinal track or fermentation via several microorganisms (Chalamaiah et al, 2018; Gorguç et al., 2020). Bioactive peptides are generally characterized via wet lab studies. On the other hand, *in silico* tools developed under the umbrella of bioinformatics have been providing significant contributions to wet-lab studies and also, they have been accelerating the discoveries of novel bioactive peptides. One of the most important and well known in silico tools is found in BIOPEP-UWM Database. (Minkiewich et al., 2019). By using BIOPEP-UWM Database, several bioactive peptides such as ACE inhibitor, activating ubiquitin-mediated proteolysis, alpha-glucosidase inhibitor, antiviral, dipeptidyl peptidase IV inhibitor, heparin binding, HMG-CoA reductase inhibitor, Protein Kinase C inhibitor and vasoconstrictor can be obtained under *in silico* conditions. More activities are listed by Minkiewich et al. (2019). The tool provides scientists to compare the protein sources efficiently before wet-lab conditions. From this perspective, the bioactive peptide contents of cytochrome c oxidase subunit 1 from invasive *L. sceleratus* are compared with cytochrome c oxidase subunit 1 from *Bos taurus* and *Gallus gallus domesticus*.

Red meat consumption is becoming very problematic due to many factors such as the contribution to increased emission of greenhouse gases, environmental pollution problems and also health problems caused by processed meat products (Gonzales et al., 2020). Instead of increasing red meat consumption from terrestrial livestocks, the use of *Lagocephalus sceleratus* may provide an alternative source after removal of its toxin.

In this study, protein parameters of cytochrome c oxidase subunit 1 protein from *L. sceleratus* were compared with *G. gallus domesticus* and *B. taurus*. From the results, it could be said that essential amino acids are almost same levels in these species. Therefore, *L. sceleratus* could be used to supply essential amino acid resources for functional food industry. However, it is important to note that the toxin should be removed before use of the bioactive peptides or the essential amino acids from *L. sceleratus*. The concentration of TTX varies based on many different factors such as season, length and also organs (Kosker et al., 2016; Kosker et al., 2019). Therefore, optimized conditions should be developed before utilization of the bioactive peptides from this alien species in the Mediterranean Sea.

The model protein, cytochrome oxidase subunit 1, from *L. sceleratus* was hydrolyzed in the study by using digestion enzymes such as chymotrypsin, trypsin and pepsin. Many bioactive peptides such as antiamnestic, inhibitor, ACE inhibitor, stimulating, antioxidative, renin inhibitor, Calmodulin-dependent phosphodiesterase 1 (CaMPDE) inhibitor, dipeptidyl peptidase IV inhibitor, alpha-glucosidase inhibitor, dipeptidyl peptidase III inhibitor were obtained after *in silico* hydrolysis. The results were also compared with *B. taurus* and also *G. gallus domesticus* (Table 3-5).

Although we observe slightly increased DHt (%) in *B. taurus*, the values of *L. sceleratus* and *G. gallus domesticus* were very close. It is very interesting to note that A_E value related to ACE inhibition in *G. gallus domesticus* was significantly higher than those of other studied animals in the study. Stimulating bioactive peptides are defined as the peptides that stimulate various biological processes. Since the values of *G. gallus domesticus* and *L. sceleratus* were very close, *L. sceleratus* can be used as an alternative source for *G. gallus domesticus*. Antioxidant property of *L. sceleratus* showed similar trend, AE value was very close to *G. gallus domesticus*, on the other hand, it was higher than that of *B. taurus*. Since there are many well defined antioxidant molecules in scientific literature, antioxidant property of an industrial food component can be supplied from different plant based sources. Renin-angiotensin system (RAS) has important physiological role for blood pressure and related homeostasis (Crowley et al., 2012). Increased activity of renin is associated with elevated blood pressure (Fu et al., 2017). Renin inhibitor property of *L. sceleratus* was found to be close to *B. taurus,* on the other hand, it was lower than that of *G. gallus domesticus*.

The bioactive peptides with CaMPDE inhibitor property may be of importance in the diseases associated with excessive inflammatory signaling since CaMPDE plays critical roles in cyclic nucleotide metabolism (O'Brien et al., 2020). Obtaining similar values compared to *G. gallus* and *B. taurus* might show the importance of *L. sceleratus*.

For example, DPP-IV is one of the target enzymes in the therapy of Type II Diabetes. Its inhibition increases the insulin stimulation (Craddy et al., 2014). Similar inhibition parameters were found within degraded protein of *L. sceleratus* confirms that the isolated bioactive peptides (TTX-free) from this alien can be proposed for consumption in diabetes therapy. Another medicinally important enzyme is ACE. This enzyme is associated with hypertension and its inhibition may provide a contribution to the therapy of hypertension. We also observed that ACE inhibitor property is also existed in the results (Table 3).

Alpha-glucosidase inhibition is an important topic in therapeutic approaches for diabetes mellitus due to involvement of alpha-glucosidase in the digestion of dietary starch into glucose (Papoutsis et al., 2020). The A*E* values related to alpha-glucosidase inhibition were found quite similar. Therefore, it could be said that there is no difference among the species studied in this study for alpha-glucosidase inhibition.

At first glance, it could be thought that use of this alien species for the Mediterranean Sea could be dangerous due to TTX. On the other hand, several studies reveal that the tissues of the fish even with TTX may be used for the treatment of various diseases.

In an interesting study carried by Hong et al (2018), it is shown that oral TTX pellets inhibited resiniferatoxin-induced postherpetic neuralgia in a rat model. TTX was

also reported as an analgesic for various pains and also cancer (Nieto et al., 2012). TTX combined with lidocaine is also proposed for severe arrhythmias by Hong et al. (2019). From these publications, it could be inferred that bioactive pellets including bioactive peptides mentioned in this study can be exploited in these diseases.

As it is mentioned in the materials section, limited numbers of protein sequences exist in the protein databases related to *L. sceleratus*. Liver and gonads of this species may include novel proteins and also novel bioactive peptides. After wet lab-based techniques such as 2D-electrophoresis, the novel proteins in this fish can be explored. After sequence analysis, enzymatically digested proteins can be studied *in silico* and *in vitro* to understand their functional properties. However, it must be noted that TTX should be removed from *L. sceleratus* based products for possible human consumption because of its toxic effects on human metabolism. Alternatively, TTX containing formulas like in Hong et al. 2018 and Hong et al. 2019 may be prepared to evaluate the biomass of the species (for therapeutic purposes).

Conclusion

Observation of *L. sceleratus* in the Mediterranean coastline of Turkey has been in increasing trend. Since no human consumption is proposed due to its highly effective toxin, an alternative utilization method should be proposed to create a stress on the *L. sceleratus* population. The present paper reveals that there are many bioactive peptides and their BIOPEP parameters are in comparable levels with well-consumed *B. taurus* and *G. gallus domesticus*. Moreover, TTX have been recently associated with promising therapeutic effects (Hong et al., 2018; Hong et al., 2019; Nieto et al., 2012). Therefore, more wet lab-based studies are strongly recommended to exploit the idea on the utilization of widely distributed *L. sceleratus* in the Mediterranean Sea.

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BIOTECH STUDIES

REVIEW

Bigger, stronger, better: Fish transgenesis applications and methods

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Abstract

Transgenesis has been applied to several species to benefit from them in different fields. As natural fish stocks decline and the world population increase, the application of transgenesis on commercial fish species takes more attention to reduce the limitations of aquaculture and meet the increasing food demand. Transgenesis has been applied to obtain a stable transgenic line with improved traits to date. In aquaculture, growth rate, cold resistance, and disease resistance of commercial fish species were enhanced by transgenesis and even one of them, AquAdvantage Salmon, took place markets in the North America. Also, transgenic fish were developed to evaluate the health impacts of chemicals in ecotoxicology and provide more options with new color variants in ornamental fisheries. Different approaches for generating transgenic fish have been performed successfully, but they still require some developments. More transgenic fish could take place in the market by developing more efficient techniques and informing consumers about these techniques to reduce their concerns. This review discusses the application fields of transgenic fish with examples and provides an overview of gene delivery techniques and transgenesis methods.

Introduction

Transgenic animals are genetically modified organisms (GMOs) with heritable changes to the genome by integration of exogenous DNA (transgene) into the host genomic DNA. Since the human growth hormone gene has been successfully transferred to goldfish (*Carassius auratus*) genome to produce the first transgenic fish, more than 35 species had been genetically modified in research laboratories worldwide (Zbikowska, 2003; Zhu et al., 1985). Most transgenesis studies were conducted on fish traits for sustainable aquaculture by various gene delivery and transgenesis techniques (Durham et al., 1987; Ju et al., 2003; Sarmasik et al., 2002; Shears, 1991). Transgenic fish with enhanced traits could provide a great benefit as food for the growing world population while it could be a dangerous product of biotechnology upon escaping accidentally to the environment. However, aquaculture could more contribute to the food demand of the increasing world population by advanced biotechnology. There are many factors related to the environment (water supply and quality, environmental impact, climate change, farming technologies) and also with the cultured organism itself (feed supply, diseases) that can affect the development of aquaculture (Gómez, 2018).

Certain characteristics of the fish could be improved through transgenesis for more sustainable and profitable aquaculture. It is possible to save enormous money by increasing the growth rate of the fish as well as cold and disease tolerance. In addition to aquaculture, transgenic fish could be developed as experimental models for biomedical research (Goldman

et al., 2001; Ward & Lieschke, 2002). The use of transgenic fish in research laboratories as an alternative to rodents could considerably reduce the exploitation of mammals, decrease costs, and accelerate the research process (Zbikowska, 2003). Furthermore, transgenic fish could be used in environmental monitoring, and ornamental fisheries (Amanuma et al., 2000; Ju et al., 2003). In this review, we described application areas of transgenic fish in aquaculture and mentioned some gene delivery techniques. Furthermore, we attempt to provide an overview of both traditional and modern transgenesis methods.

Applications of Transgenic Fish

Transgenic fish in aquaculture

Growth enhancement

Insertion of genes for growth enhancement has been applied to many fish species using different DNA constructs relating to the origin of the growth hormone gene and the promoter. During initial experiments conducted on fish in the 1980s, DNA constructs comprised of mammalian or viral promoters and mammalian growth hormone genes, but then the genes and promoters of piscine origin were used (Durham et al., 1987; Nam et al., 2001; Rahman et al., 1998; Zhu et al., 1985). Although transgenes derived from very distantly related species had no or only modest effects on growth in transgenic fish, transgenes from piscine species were shown to be more efficient (Betancourt et al., 1993; Penman et al., 1991). Significant growth enhancement by genetically modifying fish with an exogenous growth hormone gene is achieved for a few species including nile tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), mud loach (*Misgurnus mizolepis*), coho salmon (*Oncorhynchus kisutch*), and rohu carp (*Labeo rohita*) (Barman et al., 2015; Devlin et al., 1994; Nam et al., 2001; Rahman et al., 1998; Zhang et al., 1990). Effect of transgene on growth rate could varied from 35-fold increase in size compared with the non-transgenic counterparts, to almost no difference depending on the species and DNA constructs (Nam et al., 2001; Pitkänen et al., 1999a).

Considerably high growth rate effects (typically 6- to 14-fold compared to controls) were observed for some salmonid species comprising Atlantic salmon (*Salmo salar*), Coho salmon, and Arctic charr (*Salvelinus alpinus*), but levels of growth enhancement were variable among transgenic salmonid lines (Devlin et al., 1994; Du et al., 1992; Pitkänen et al., 1999a). Extreme growth in salmonids carrying a transgene could be because of experiencing important seasonal reductions in growth while warm-water fish species naturally grow at near maximal rates during the year (Mori & Devlin, 1999).

Although constructs containing permissive viral or piscine constitutive promoters usually increase growth rate, some constructs could not affect growth performance. For example, a construct consisting of the growth hormone 2 gene from Atlantic salmon and the homologous promoter (SsGH2) did not affect the growth rate of rainbow trout (*Oncorhynchus mykiss*) expressing growth hormone only in the pituitary, the normal site of growth hormone expression (Pitkänen et al., 1999a). In a study, it was showed that growth enhancement could depend on the intrinsic growth rate and genetic background of the host strain (Devlin et al., 2001). Wild strains of rainbow trout naturally grow slowly while the growth rate is higher in selectively bred domesticated strains. Growth hormone construct was introduced into a wild and domesticated strain of rainbow trout, yet the introduction of the growth hormone construct into the domesticated strain did not cause further growth enhancement because this strain reaches maximal rate with the different genetic background (Devlin et al., 2001).

Upon DNA constructs were applied to fish, besides growth enhancement, other phenotypic changes in fish morphology, and some physiological abnormalities were observed in some species (Devlin, 1997). These could be an alteration of skin color, modifications of skull shape, acceleration of smoltification in salmonids, precocious sexual maturation, decreased fertility or even sterility, and reduced viability.

Several studies suggested that transgenic fish display considerable metabolic differences compared to non-transgenic siblings and their metabolism is more efficient (Krasnov et al., 1999; Martinez et al., 2000). For example, the juveniles of transgenic tilapia demonstrate increased protein synthesis and growth rate concomitant with enhanced glycolysis and oxidation of amino acids (Martinez et al., 2000). Also, transgenic charr showed enhanced metabolic activity and utilization of dietary lipids (Krasnov et al., 1999).

The story of transgenic Atlantic salmon:

The most well-known transgenic fish with an increased growth rate is the AquAdvantage Salmon developed by the group of Fletcher at Memorial University of Newfoundland (Gómez, 2018). This transgenic Atlantic salmon was created via microinjection of a DNA construct containing an antifreeze protein gene promoter from ocean pout (*Macrozoarces americanus*) and a Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone cDNA (opAFP-GHc2) into fertilized eggs of wild Atlantic salmon (Du et al., 1992). Therefore, the line of transgenic Atlantic Salmon has a copy of transgene expressing continuously along with its salmon growth hormone gene expressing seasonally. Transgenic Atlantic salmon reach market size (4-5kg) from eyed-egg stage in nearly 18 months compared to 36 months for conventionally farmed Atlantic salmon and also consume 25% less feed than conventionally farmed Atlantic salmon during the growth period (Gómez, 2018).

After a long journey through the US regulatory system, AquAdvantage Salmon is now produced by the company AquaBounty Technologies (Massachusetts,

USA). The journey started in 1993 when AquaBounty first approached the U.S. Food and Drug Administration (FDA) to find out the requirement for approval of AquAdvantage Salmon as food, but a regulatory pathway did not exist for genetically engineered (GE) animals (Van Eenennaam & Muir, 2011). The company appealed for regulation under FDA since they thought the difficult pathway for approval would contribute to addressing public concerns about food from GE animals. AquaBounty established an Investigational New Animal Drug (INAD) file with the Center for Veterinary Medicine (CVM) of the U.S. FDA to pursue the development of AquAdvantage Salmon in 1995. During the assessment period, AquaBounty Technologies constructed a land-based aquaculture facility (AquaBounty Panama) in the highlands of Panama to conduct trials of the Company's AquAdvantage Salmon (Aquabounty, 2020).

FDA released Guidance 187 for the regulation of GE animals in 2009 (Van Eenennaam & Muir, 2011). Based on this guidance, the FDA applies a hierarchical risk-based approach to evaluate GE animals and their edible products in seven-step by investigating the safety of the recombinant DNA construct for the animal, the safety of food from the animal, and any environmental impacts and efficiency claimed for the animal in the seven-step (Sanderson and Humphries, 2015). Molecular characterization of the rDNA construct should be conducted to detect whether it includes DNA sequences from viruses or other organisms that could cause health risks to the GE animal or those consuming the animal (Van Eenennaam & Muir, 2011). Also, molecular characterization of the GE animal lineage should be conducted to control the inherited stability of the rDNA construct in the next generations. Furthermore, the health state and development process of GE animals should be evaluated by phenotypic characterization comparing with non-GE animals (Sanderson & Humphries, 2015). If the GE animal is proposed as a source of food as with the AquAdvantage salmon, FDA evaluates the composition of edible tissues and risk of allergenicity compared to their non-GE counterparts (Van Eenennaam & Muir, 2011). FDA finally requires the preparation of an environmental assessment of the animal and of conditions suggested for raising the GE animal as stated in the product definition and the sponsor data supporting the claimed efficiency of GE animal (Van Eenennaam & Muir, 2011).

Following the release of the Guidance, AquaBounty Technologies submitted its final regulatory study to the FDA completing all tests for evaluation (Aquabounty, 2020). After that, AquaBounty's Panama site was investigated and approved by the FDA for the production of AquAdvantage Salmon. FDA concluded that AquAdvantage Salmon is safe to eat; and poses no threat to the environment under its raising conditions and then FDA convened its Veterinary Medicine Advisory Committee (VMAC) in a public meeting to review its findings (Van Eenennaam & Muir, 2011). The VMAC agreed with the FDA; AquAdvantage Salmon is safe to consume, and safe for the environment, therefore FDA completed a food safety assessment in 2010. The FDA consults with the National Marine Fisheries Service of NOAA and the U.S. Fish and Wildlife Service for more investigation on environmental impacts of AquAdvantage salmon in 2011(Aquabounty, 2020). These organizations agreed with the findings of the FDA that the AquAdvantage Salmon do not pose a threat to the environment. The FDA completed the environmental assessment and published in the Federal Register a preliminary Finding of No Significant Impact (FONSI) for AquAdvantage Salmon in 2012 (Gómez, 2018).

In 2011, AquaBounty Technologies also completed a New Substance Notification (Organisms) for AquAdvantage Salmon and applied it to Environment Canada. AquaBounty Technologies had continued its story in Canada by applying to the Canadian Food Inspection Agency in 2012 for registration of AquAdvantage Salmon as a Novel Feed and to Health Canada for a Novel Foods Pre-Market Submission (Aquabounty, 2020).

Environment Canada published a Significant New Activity Notice indicating AquAdvantage Salmon is not considered to be a risk to the environment in 2013 (Aquabounty, 2020). Therefore, AquaBounty Technologies receives authorization for the production of eggs at AquaBounty Canada's hatchery for commercial sale. However, Ecojustice on behalf of Ecology Action Centre and Living Oceans Society files lawsuit against the Canadian federal government (Health Canada, Environment Canada) and AquaBounty for permission to produce genetically modified salmon in Canada. Similar actions by non-governmental organizations also occurred when the FDA evaluating the environmental impacts of AquAdvantage salmon (Van Eenennaam & Muir, 2011).

The main environmental concern about AquAdvantage fish is the possibility of escape and cause the collapse of wild salmon populations. Environmental concerns could be mitigated by land-based production with physical confinement barriers and also biological measures resulting in 99% sterility and 100% female production stocks (Van Eenennaam & Muir, 2011). In principle, there is no difference between potential environmental risks related to the escape of transgenic fish and those related to the annual escape of a lot of farmed selectively bred fish. Matings between escaped farmed salmon and wild native fish could cause a substantial risk of extinction for natural populations, but the comparative risk of sterile transgenic AquAdvantage salmon is probably to be less than that of fertile, selectively bred, Atlantic salmon (Van Eenennaam & Muir, 2011).

AquAdvantage Salmon was approved by the FDA for consumption in the USA in 2015 (Gómez, 2018). After a while, the import of AquAdvantage Salmon into the USA was prohibited until labeling requirements were announced by FDA (Van Eenennaam, 2017). The

U.S. Congress passed a GMO food labeling bill and give 2 years to USDA to establish a labeling language and guidelines. Meanwhile, Health Canada approved the production, sale, and human consumption of AquAdvantage Salmon in Canada. Following those approvals, AquaBounty Technologies purchased certain assets of the Bell Fish Company LLC farm site in Albany, Indiana to establish the first commercial-scale production facility in the United States in 2017 (Figure 1) (Aquabounty, 2020).

The FDA approved to raise AquAdvantage Salmon at the Company's Indiana farm in 2018 (Aquabounty, 2020). After establishing of labeling language and guidelines for GMO food, the FDA allowed the Company to start farming AquAdvantage Salmon in Indiana in 2019. Also, the company gained permission from Environment and Climate Change Canada (ECCC) for the commercial production and grow-out of AquAdvantage Salmon in Rollo Bay facility. At the end of the tortuous journey, AquAdvantage Salmon reached to market in the USA as well as in Canada. The market of AquAdvantage Salmon would probably extend through projects conducted by AquaBounty Technologies in Brazil, Argentina, Israel, and China starting new journeys (The Fish Site, 2020).

AquAdvantage Salmon could also continue its journey in Europe. First, it should take proper scientific opinion from the European Food Safety Authority (EFSA) agency which provides independent scientific advice on current and emerging risks to food safety in the European Union (EU) (Slyck, 2017). A guidance on the environmental risk assessment of GE animals were published by EFSA to present information for applicants and risk assessors on placing GE animals on the EU market. EU regulatory system adopts precautionary principle for releasing of GE animals in constrast to FDA adopting prevention principle. Precautionary principle aims a high standard of environmental protection via preventative decision-making if risks are reasonably suspected. Based on this principal difference, AquAdvantage salmon is ban now in the EU, but this situation could change with the positive results of long-term effects of GE animals on enviroment and food safety, and also trade agreements (Debode et al., 2018).

Cold resistance

Many species of polar and northern fish inhabiting frigid water such as winter flounder (*Pleuronectes americanus*) and ocean pout produce antifreeze proteins to protect them from freezing (Lee et al., 2013). Based on their structural features, four types of antifreeze proteins (AFPs; type I, II, III, and IV) were characterized from teleosts as well as antifreeze glycoproteins (AFGPs) (Lee et al., 2013). These proteins bind to the ice surface to prevent the growth of ice crystals by decreasing the freezing temperature. Generation of freeze-tolerant transgenic salmon or other species via the introduction of an AFP gene could greatly improve fish farming in northern latitudes.

The idea of producing transgenic fish with cold tolerance was actually motivated by the possibility of culturing Atlantic salmon along the East coast of Canada (Gómez, 2018). The Atlantic salmon is incapable to survive in sub-zero seawater temperature due to the lack of any of these AFGPs or AFPs gene(s) (Hew et al., 1995). This inability cause one of the major problems of sea cage farming in the northern Atlantic coast severely limiting the selection of suitable sites for operation. To solve this problem, winter flounder type I AFPs that have two isoforms, liver-type, and skin-type, could be good candidates for gene transfer. The former type mainly is produced in the liver as precursor proteins (preproAFPs) that need to be further processed while the latter type is produced in peripheral tissues as intracellular, mature AFPs (Hew et al., 1999). The AFP gene for type I AFP from winter flounder were inserted into Atlantic salmon genome under its promoter and expressed at a level of 0.1–50 µg/ml (Shears, 1991). Another study generated a stable transgenic line of Atlantic salmon by incorporating one copy of the winter flounder liver-type AFP gene into the genome of Atlantic salmon and the expression of the transgene was stable (approximately 250 µg/ml) up to the F3 generation (Hew et al., 1999). ProAFP was expressed only in the liver and display seasonal variations similar to those in winter flounder.

Figure 1. AquaBounty Technologies farm in Albany, Indiana established as first commercial-scale production facility of AquAdavantage Salmon.

The antifreeze activity was found in the sera of F3 offspring despite the lack of necessary processing enzymes to process proAFP precursor into a mature protein, but low-level production of AFP remained a problem (Hew et al., 1999).

Similar approaches were also conducted on goldfish by integrating the type III AFP gene from ocean pout encoding a mature protein into its genome (Wang et al., 1995). In this model, a mature AFP was produced in F1 and F2 offspring, and the transgenic goldfish showed better tolerance to cold water compared with the control group. Likewise, nile tilapia become resistant to temperatures as low as 13°C by injection or oral administration of AFP to juveniles or adults (Wu et al., 1998). This suggests that transgenic tropic species could be farmed in cold areas by the integration of AFP into their genome from other species, but low-level production of AFP decreases the efficiency of this approach. Whilst the expression level of AFP in winter flounder is generally approximately 10–20 mg/ml, all transgenic fish for AFP only express in the µg/ml range (Zbikowska, 2003). An increase in the copy number of the transgene or the use of constructs with other AFPs could increase expression and help to enhance freeze-resistance in farm fish.

Disease resistance

One of the promising application areas of gene transfer in aquaculture is the development of disease resistance. In aquaculture, diseases are an important problem disrupting animal welfare and leading to great economic losses. Transgenic fish with increased disease resistance could improve the profitability, production, efficiency, and welfare of the cultured fish (Dunham, 2009).

One approach to increase resistance against bacterial pathogens is to transfer antibacterial peptide genes. This approach was applied to Channel catfish (*Ictalarus punctatus*) by the transfer of a DNA construct containing a lytic peptide, cecropin B, driven by a cytomegalovirus (CMV) promoter and transgenic fishes showed two and four-fold increases in resistance against *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively (Dunham et al., 2002). Also, no difference in growth rate was observed between the transgenic and non-transgenic siblings. Another example of increasing bacterial resistance through transgenesis is the transfer of cecropin genes to medaka (*Oryzias latipes*) using various DNA constructs (Sarmasik et al., 2002). At the F2 generation, transgenic medaka from different families were challenged with *Psuedomonas fluorescens* and *Vibrio anguillarum,* and then transgenic lines showed 0–10% and 10–30% cumulative mortality, respectively. The enhanced disease resistance showed a difference between transgenic families, which means family variation could be important due to differences in the genetic background (Dunham, 2009). This emphasizes combining gene transfer with selection to provide maximum genetic gain from the gene transfer. Besides antibacterial peptides, other proteins with antimicrobial properties could be used to enhance disease resistance. B actin-human lactoferrin gene was transferred to grass carp (*Ctenopharyngodon idellus*) and then P1 individuals were more resistant to *Aeromonas* with increased phagocytic activity (Mao et al., 2004).

Another approach is to enhance the expression of a piscine lysozyme as an antibacterial agent against some fish pathogens. Yawaza et al. (2006) produced F2 transgenic zebrafish (*Danio rerio*) using a DNA construct including hen egg white (HEW) lysozyme gene and green fluorescence protein (GFP) gene driven by Japanese flounder (*Paralichthys olivaceus*) keratin promoter. Expression of both HEW lysozyme gene and GFP gene was detected in the liver and protein extracts from the liver of F2 transgenic fish showed 1.75 times higher lytic activity than in the controls. In a challenge experiment with *Flavobacterium columnare*, 65% of the F2 transgenic fish survived while 100% of the control fish were killed (Yawaza et al., 2006). Similarly, 60% of the F2 transgenic fish survived during a challenge test with *Edwardsiella tarda*, while 100% of the control fish died.

To prevent viral diseases in aquaculture, the common gene transfer approach is to use viral antisense RNAs or DNA-vaccines. However, the application of these techniques does not create exactly transgenic fish because these DNA/RNA constructs could not be passed to the next generation, instead, these techniques play a role in the gene expression of fish. Antisense RNA approach was applied to prevent infectious pancreatic necrosis virus pathogenicity by hammerhead ribozyme cleavage in vitro (Chen et al., 2000). The first DNA vaccine was produced against infectious hematopoietic necrosis using its the glycoprotein gene and tested on rainbow trout (Anderson et al., 1996). Since then, mono and multivalent DNA vaccines were applied to many fish species to protect against viral and bacterial pathogens (Kumar et al., 2008; Pereiro et al., 2012; Sun et al., 2012).

Transgenic fish in ecotoxicology

Transgenic fish, particularly transgenic zebrafish, have significant potential use in aquatic ecotoxicology as biosensors and models providing information on health effects of chemical exposure, but the use of transgenic fish in environmental toxicology is not common (Lee et al., 2014). Biosensor fish work in the principle of stimulation of specific genes, often enzymes or receptors, by certain chemicals/pollutants (Zbikowska, 2003). Transgenic fish could detect environmental pollutants in water and then induce a reporter gene driven by an element activated by low levels of pollutants. Transgenic fish lines developed to research contaminants and other environmental stressors include cadmium and copper toxicity by induction of heat-shock protein gene, oxidative stress via the induction of an electrophile-responsive element (EpRE), various organic chemicals interacting with the aryl hydrocarbon receptor-mediated toxicity, and estrogenicity (vitellogenin, choriogenins, estrogen

receptor-responsive elements) generally using either luciferase or GFP as reporter genes (Blechinger et al., 2002; Kusik et al., 2008; Lee et al, 2014; Mattingly et al., 2001; Petersen et al., 2013; Zeng et al., 2005). Transgenic biosensor fish were created by using the heat-shock protein (*hsp*) promoters, promotors of *hsp70* and *hsp27,* stimulated by various environmental stressors such as temperature and heavy metals (Lee et al., 2014). Transgenic zebrafish using *hsp 70* gene promoter to control eGFP (enhanced green fluorescent protein) as the reporter gene was exposed to cadmium and was sensitive at concentrations as low as 0.2 µM (22.5 µg/L) (Blechinger et al. 2002).

The promoter of the *cyp1a1* gene was used to control a GFP reporter gene for detection of exposure to organic chemicals in transgenic zebrafish and medaka (Hung et al. 2012; Kim et al. 2013; Lee et al, 2014; Ng & Gong 2013). *Cyp1a1* is a member of the cytochrome P450 superfamily and has a role in the oxidative metabolism of diverse organic substances including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Ma & Lu, 2007). Transgenic *Cyp1a - GFP* medaka embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*–dioxin (TCDD) for 24 hours and then GFP expression was detected in kidney, liver, and gut at an exposure concentration of only 0.005 nM (1.6 ng/L) (Ng & Gong, 2013). Also, this transgenic medaka expressed the GFP reporter gene in the liver and kidney when exposure to other PAHs including 3-methylcholanthrene (3-MC) and benzo[a] pyrene (BaP).

Furthermore, transgenic fish could be used for mutation assays to evaluate potential DNA damage after exposure to chemicals in aquatic environments. Transgenic medaka developed by Winn et al. (2000) harbors bacteriophage λ LIZ vector containing the *lacI* or *cII* bacterial gene as mutational targets. Also, mutation assays based on plasmid vectors were designed such as zebrafish carrying the pML4 plasmid vector fused to *rspL* gene (Amanuma et al., 2000). Thanks to the valuable features of fish as test organisms, transgenic fish could make important contributions to ecotoxicology studies.

Transgenic fish in Ornamental Fisheries

Ornamental aquaculture is a growing commercial sector with more than 4500 freshwater species and 1450 marine species traded worldwide (Stevens et al., 2017). Ornamental fish trade is a significant source of income for a lot of countries including Singapore, Malaysia, Thailand, and Indonesia while USA is the largest importer of Ornamental fish (Satam et al., 2018). Although accurate information about total value of the sector is lack, estimated total value of the sector varies between U.S. $800 million and $30 billion annually and the amount of traded fish is considered to be between 350 million and 1.5 billion live fishes (Stevens et al., 2017). Development of species with new features by transgenesis could contribute to the growth of this sector presenting customers with new options.

Transgenesis is applied on ornamental fish species to produce transgenic fish with different color forms. Color genes showed stable expression in zebrafish embryos injected with GFP expression constructs under the control of the zebrafish muscle-specific promoter of the *myosin light polypeptide 2* (*mylz2*) gene (Ju et al., 2003). Then fluorescent white skirt tetra (*Gymnocorymbus ternetzi*), medaka, and farmed rohu carp were successfully produced using the zebrafish *mylz2* promoter (Mohanta et al., 2014; Pan et al., 2008; Zeng et al., 2005). 'GloFish' with six attractive fluorescent color combinations, including Starfire red, cosmic blue, electric green, galactic purple, sunburst orange and moonrise pink have been already presented to the market as commercial value-added aquarium fishes by transgenesis (Figure 2) (Vick et al. 2012).

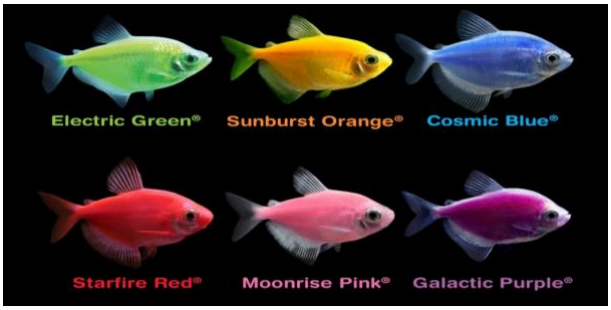

Figure 2. 'GloFish' with six attractive fluorescent color combinations, including Starfire red, cosmic blue, electric green, galactic purple, sunburst orange and moonrise pink as ornamental transgenic fish (Spectrum Brand Pet, 2020).

Gene Delivery Techniques in Fish

Microinjection

Microinjection of DNA into eggs or embryos at the one-cell stage is the most commonly applied gene transfer method in aquaculture (Pitkänen et al., 1999b; Cheers and Ettensohn, 2004). This technique was first used on goldfish for injection of foreign DNA into embryos and then applied on a variety of fish species such as common carp, atlantic salmon, medaka, tilapia, rainbow trout, and zebrafish to improve traits by gene transfer (Dunham et al., 1987; Hew et al., 1992; Ozato et al., 1986; Penman et al., 1991; Xu et al., 2008; Rahman et al., 1998; Rasal et al., 2016; Zhu et al., 1985).

Characteristics of eggs affect the application of microinjection and gene transfer efficiency. Fish eggs could have tough and opaque chorion which prevents insertion of glass micropipettes leading to low gene transfer efficiency rate (Dunham et al., 1987; Sin et al., 1997). Also, transgene could be injected into the cytoplasm of the egg because the nuclei of eggs could be small and hard to visualize. Limitations related to tough and opaque chorion dealed with injection into oocyte nuclei after making a hole in salmonids called two-step injection (Rasal et al., 2016). Eggs could be dechorionated manually or using trypsin or pronase to ease the insertion of pippets. Injection pipettes are chosen depending on the egg size of species to prevent

mortality because of mechanical damage on fertilized eggs (Tonelli et al., 2017a). In order to enhance the efficiency of the selection of transgenics, genetic markers could be co-injected with the transgene to monitor zygotes as using of GFP as a marker in zebrafish (Peters et al., 1995).

Beyond problems related to characteristics of eggs, the microinjection method is time-consuming since microinjection of a transgene is operated on only one embryo after another (Singh et al., 2019). It even requires a great deal of technical skill and comparatively expensive types of equipment including micromanipulators and microinjectors. Despite these mentioned limitations, microinjection is a favored technique for gene transfer with a success rate ranging from 10% - 70% in fishes (Powers et al., 1991).

Electroporation

Electroporation, the use of high-voltage electric shocks to introduce DNA into cells, is a procedure that is popular for introducing foreign genetic material into the cells of many different organisms (Potter and Heller, 2018). The standard protocol for all organisms involves cells being suspended in an appropriate, conducting buffer with the foreign gene being transfected, and then high-voltage electric shocks are used to make the cells more porous, allowing the introduction of the foreign gene into the cell. Transfected cells are then diluted and initially cultured in a non-selective medium. Afterward, appropriate selection is added, and cells are then separated and assessed for the introduction of the transgene (Potter and Heller, 2018).

Electroporation can be used to introduce transgenes in aquaculture. Either by inserting the transgene into spermatozoa (Celebi et al., 2003) or oocytes (Grabarek et al., 2002) before fertilization or by direct insertion into fertilized embryos (Kera, Agerwala and Horne, 2010). These have been applied with success *in vivo* results in salmon with observed mosaicism of the fish (Sin et al., 2000), shrimp (Arenal et al., 2000), nile tilapia (Lin, Chang and Chen, 2016), and medaka with changes being transferred through the germline (Hostetler, Peck and Muir, 2003).

The success of electroporation has shown to be extremely influential for zebrafish developmental biology studies. One example involves introducing a CRISPR/Cas9 plasmid to knockdown the gene *Mctp2p* to characterize its effect on neuronal and muscular development in zebrafish embryos (Espino-Saldaña et al., 2020). Electroporation is a diverse method with many potential applications for the development of transgenic fish.

Transgenesis Tools

Transposon Vectors

Transposable elements are a valuable tool to integrate genes into chromosomes to provide new traits. A transposon system usually contains a transgene sequence flanked by transposon-inverted repeats and the transposase-coding sequence (Tonelli et al., 2017a). *Sleeping Beauty*, *Tol2*, and *piggyBac* are commonly applied transposons for fish.

Transposon systems first were used on fish when a *Caenorhabditis elegans* transposon (*Tc3* element from the Tc1/mariner family) system was injected into one-cell-stage eggs to integrate GFP into the zebrafish genome (Raz et al., 1997; Tonelli et al., 2017a). *Tol2* element, derived from the medaka genome, was also used on zebrafish to deliver various genes (CFP/YFP/RFP or Gal4 cassettes) and this study provided new protocols to rapidly apply *Tol2* mediated zebrafish transgenesis (Bussmann & Schulte-Merker, 2011). Application of *Sleeping Beauty* transposon on zebrafish to transfer a blue-shifted GFP variant and a red fluorescent gene in a tissue-specific manner enhanced the transgenesis and expression rate sixfold (from 5 to 31%) compared to standard, plasmid injection-based transgenesis methods (Davidson et al., 2003). Applicability of the transposon *piggyBac* for transgenesis was showed on goldfish and loach (Hu et al., 2012).

Transposon vectors have a few advantages for transgenesis in fish compared to plasmid and viral vectors. Transposon vectors provide the insertion of a single, defined DNA sequence into the genome without absolute size restrictions instead of a multi-copy of sequence observed use of plasmid vectors (Hackett et al., 2004). However, possible active copies of the corresponding transposases might prevent insertion and enhanced stability of transgenes in fish such as salmonids (Tafalla et al., 2006).

Viral Vectors

Infection by viruses is a well-established gene delivery tool still used for transgenesis due to efficient integration and usually single-copy insertion of genetic material into the host genome. When retroviruses and lentiviruses are internalized into the target cell, their genetic material (RNA) is transformed to DNA, thereby their genetic information is inserted into the host genome (Tonelli et al., 2017a). After transduction efficiency of pseudotyped viruses was displayed on zebrafish in 1994, this method was applied on various fish species including nile tilapia and live-bearing fish (*Poeciliposis lucida*) to integrate transgene into the fish genome (Lin et al., 1994; Sarmasik et al., 2001; Tonelli et al., 2017b).

In order to improve this method for transgenesis in aquaculture, a number of viral gene delivery studies were conducted on zebrafish and medaka due to their transparent embryos (Tonelli et al., 2017a). Zebrafish cells were infected by vesicular stomatitis virus (VSV)-glycoprotein envelope including a genome obtained from the Moloney murine leukemia virus (MLV) and studies showed the degree of transduction efficiency expanded as the titer elevated and transmittable insertions could be enhanced in zebrafish by selecting virus-producer cell lines (Chen et al., 2002; Gaiano et al.,

1996; Lin et al., 1994). Also, transgenic zebrafish could be produced by applying viral gene delivery into the sperm culture and then performing in vitro fertilization (Kurita et al., 2004). Baculovirus system as that used on zebrafish could be used as an alternative to retrovirus and lentivirus because they provide increased control of the transduced region and exact determination of gene expression time using various promoters on DNA constructs (Wagle & Jesuthasan, 2003).

Despite the higher transduction efficiency of gene delivery by viral vectors, this method has some important disadvantages. Transgenes transferred by a viral vector could show unstable expression or even complete silencing of the transgene (Rasal et al., 2016). The reason for silencing of the transgene is that activation of gene repression machinery in the host cells through the promoter and enhancer sequences of the retroviral long-terminal-repeats (LTRs), followed by hypermethylation of the viral promoter sequences by *de novo* DNA methylation (Jahner and Jaenisch, 1985). Owing to the small size of viral vectors, generally, 10 kb transgene could be package into viral vectors, which restrict the transfer of larger genes by this method (Robl et al. 2007). Also, infection of early embryos usually shows a delay in transgene integration, thus producing mosaic animals that is because the breakdown of the nuclear membrane during mitosis is necessary for infection by retroviruses (Robl et al. 2007). Even though limitations of this method could be solved, the public may not accept transgenic fish as food produced by an integration of viral sequences because of biosafety and ethical issues (Rasal et al., 2016).

Tools for site-specific integration

In contrast to transgenesis, which involves the transfer of a gene from one organism to another, genome editing allows specific, targeted, and often minor changes to the genome of the species of interest. Three methods have been predominantly utilized to conduct genome editing. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR).

The earliest developed programable method of gene editing involves utilising a group of nucleases called zinc-finger nucleases (ZFNs). ZFNs are hybrids between a nonspecific DNA-cleavage domain and a DNA-binding domain composed of Cys_2His_2 zinc fingers. These nucleases have been used to stimulate homologous recombination of DNA, allowing the introduction of mutations (Bibikova et al., 2001). ZFNs have increased the efficiency of introducing foreign genetic material to the DNA of higher eukaryotes from approximately 1 for each 10^6 cells treated to nearly 100% of all cells treated. Gene editing is conducted by co-injecting to nuclease and DNA into the cells. ZFNs then cut specific restriction sites within the cell DNA and the introduced DNA is introduced via homologous overhangs, allow specific, site-directed mutagenesis (Bibikova et al., 2001). This technique has been used to introduce targeted mutations *in vivo* in many species including *Drosophila* (Bibikova et al., 2002) and zebrafish with mutations being carried through the germline for zebrafish (Foley et al., 2010). More recently, ZFNs have been applied *in vivo* to species of interest to aquaculture such as editing the luteinising hormone in channel catfish to create sterile fry (Qin et al., 2016) Notable limitations of gene editing through use of ZFNs include the need for specific restriction sites to be present within the gene of interest as well as two restrictions sites being located within 6 to 18 bp of each other (Bibikova et al., 2001). This restricts the capacity for developing point mutations and small edits within the genome using this technique and necessitates the application of other techniques to achieve these changes.

Transcription activator-like effector nuclease (TALENs) was later developed after the discovery of the transcription activator-like (TAL) family of proteins in the plant pathogenic bacteria *Xanthamonas (Boch and Bonas, 2010).* TALENs could be used for genome editing by inducing double-strand breaks (DSB), which activate repair mechanisms of cells (Joung & Sander, 2012). Non-homologous end joining (NHEJ) ligates DNA from either side of a double-strand break where there is little, or no sequence overlap for annealing. This induces errors in the genome via indels (insertion or deletion), or chromosomal rearrangement, resulting in a non-functional gene (Miller et al., 2011). TALENs has successfully been applied *in vivo* to edit the genome of aquaculture species including editing oestrogen production in nile tilapia (Li et al., 2013). A major limitation to the application of TALENs in aquaculture is that the mechanism of NHEJ only ablates genes and cannot create point mutations (Miller et al., 2011). This restricts gene editing to those genes where loss of function is beneficial while other methods of gene editing will be required where change or increase in function is required.

The most novel method of gene editing methods is CRISPR which uses the Cas family of proteins to introduce breakage to the DNA of the target organism allowing the introduction of genetic edits (Jinek et al., 2012). CRISPR and accompanying Cas proteins constitute an adaptive antiviral immune system in bacteria and archaea (Barrangou, 2015). The CRISPR defense system allows bacteria and archaea to recognize specific sequences and degrade them to prevent viral infection. CRISPR-Cas immune responses proceed in three stages: adaptation, where pieces of DNA are sampled from the invasive genetic material and are acquired into CRISPR loci for the purposes of immunity and immune memory, expression through transcription and processing of interfering CRISPR RNAs (crRNA), and interference through Cas directed cleavage of the invasive genetic material.

A subset of the CRISPR immune system response which utilizes mature crRNA base-paired to trans-

activating tracrRNA to form an RNA structure that directs CRISPR-associated protein Cas9 has been exploited for the purposes of gene editing (Jinek et al., 2012). The CRISPR/Cas9 gene-editing system creates site-specific double-stranded breaks in target DNA. Genes are edited by incorporating a target sequence with a neighbouring protospacer adjacent motif (PAM) specific to Cas9 into the guide RNA (gRNA) of the Cas9 vector. The Cas9 vector is then inserted into the target cells where it encodes for the gRNA and the Cas9 protein. The gRNA directs the Cas9 to the target DNA where the Cas9 creates a double-stranded break at the PAM site and the cells naturally repair the DNA using NHEJ. This mechanism is often inaccurate, leading to random insertions and deletions, and causing frameshifts in the target gene, which can knock out the entire gene. More targeted mutagenesis can be achieved by utilizing homology-directed repair (HDR). This process requires a homology-containing donor DNA sequence to be coinserted with the Cas9 vector to facilitate repair (Zhang et al., 2014). This sequence can contain specific mutations which will then be incorporated into the modified DNA as it repairs, creating potentially "error-free" mutagenesis.

Multiple gRNAs can also be incorporated into a single CRISPR/Cas9 vector, inducing multiple mutations within a genome or a single gene (Sakuma et al., 2014). This multiplexing system has been demonstrated in mammalian genomes as well as those of zebrafish, drosophila, *Caenorhabditis elegans* and bacteria (Zhang et al., 2014). However, CRISPR/Cas9 can also risk cleavage of highly homologous sites other than those intended, creating off-target mutations, and is entirely dependent on the location of a PAM site. This makes the selection of the gRNA sequence extremely important to minimize these risks.

CRISPR editing has successfully been applied *in vivo* to many species including those relevant to aquaculture research (Table 1). These include modification of immune function in rohu carp (Chakrapani et al., 2016) and growth in channel catfish (Khalil et al., 2017) and rainbow trout (Cleveland et al., 2018) and the pacific oyster (*Crassostrea gigas*) (Yu et al., 2019) with germline transmission of these gene edits being observed in channel catfish (Khalil et al., 2017).

Conclusion

Classical genetic studies were used to improve fish stocks providing valuable information about genetic traits. Nevertheless, scientists started to engineer a particular genetic trait in a directed way with the discovery of recombinant DNA technology and the development of gene transfer techniques. Therefore, transgenesis for enhancement of traits in a directed fashion started a new era in aquaculture. Transgenic fish have many significant biotechnological applications in several fields including aquaculture. Transgenic fish have been generated with enhanced growth rate, cold tolerance, and disease resistance for aquaculture. Also, transgenic fish have been developed for environmental monitoring and ornamental fisheries. Microinjection was used commonly as a gene delivery method despite its some disadvantages. Viral vectors or transposons are efficient transgenesis techniques, but DNA sequence randomly integrates into the genome that might disrupt a functional gene in the host using these techniques. This problem could be solved by using tools such as ZFNs, TALENs, CRISPR/Cas to achieve site-specific integration. These tools have successfully been applied to improve traits of fish stocks, but they have important disadvantages like off-target effect. Following biotechnological advances, new techniques with the increased efficiency and effectiveness will be proposed to generate transgenic fish solving problems related to current methods.

Besides technical problems, environmental risk and human safety aspects of transgenic fish should be considered. Environmental risk could be mitigated using inland farms and sterile animals as AquAdvantage salmon. Food-safety issues are dealing with regulatory agencies, but the fate of transgenic fish as food will be determined by consumers in the long term. While research on the generation of transgenic fish continues, consumers could be informed by education and campaign on technology for transgenic fish production to reduce their concerns.

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Table 1. *In vivo* CRISPR editing of organisms relevant to aquaculture studies

Species	Trait of interest	Method of introduction	NHEJ/HDR	Germline transmission	References
Labeo rohita	Immune fuction	Microinjection	HDR	No	(Chakrapani et al., 2016)
Ictalurus punctatus	Growth	Microinjection	NHEJ	Yes	(Khalil et al., 2017)
Crassostrea gigas	Growth	Microinjection	NHEJ	No	(Yu et al., 2019)
Pagrus major	Growth	Microinjection	NHEJ	No	(Kishimoto et al., 2018)
Salmo salar	Sterility	Microinjection	NHEJ	No	(Wargelius et al., 2016)
Oncorhynchus mykiss	Growth	Microinjection	NHEJ	No	(Cleveland et al., 2018)
Exopalaemon carinicauda	Molting	Microinjection	HDR	Yes	(Gui et al., 2016)
Oreochromis niloticus	Reproduction	Microinjection	NHEJ	Yes	(Li et al., 2014)

(*Litopenaeus schmitti*) by electroporation of single-cell embryos and injection of naked DNA into adult muscle. *Biotecnologia Aplicada*, *17*(4), 247–250.

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