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Research Article

UPREGULATION OF NEUROGLOBIN PROMOTES TM3 LEYDIG CELL VIABILITY

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Abstract: Neuroglobin (NGB) is a globin family member protein expressed in the nerve system and prevents oxidative stress and apoptosis induced death in neurons. The anti-oxidant role of NGB against oxidative stress and reactive oxygen species (ROS) brings up its promising protective role in other tissues. The overexpression of NGB in testosterone producing Leydig cells might be a solution to hypoxia related male infertility. In the current study, NGB was overexpressed in Leydig cells by using viral transduction methods and the cell proliferation, gene expression and anti-oxidant enzyme levels were analyzed. Upregulation of NGB increased anti-apoptotic Bcl-2, cell proliferation and anti-oxidant enzyme levels and decreased the expression of apoptotic genes BAX, p53 and caspase 3. In addition, NGB transduced cells proliferated and expressed less apoptotic genes after H_2O_2 exposure. In conclusion, NGB might be a target for androgen deficiency related male infertility and could be used in clinics in the future.

Key words: Neuroglobin, Leydig cell, oxidative stress, anti-oxidant enzymes, male fertility.

Özet: Globin ailesi proteinlerinden bir tanesi olan Neuroglobin (NGB) sinir sisteminde ifade edilmektedir ve oksidatif stres ve apoptoz kaynaklı nöron ölümünü engellemektedir. Oksidatif stres ve reaktif oksijen türlerine karşı gözlemlenen anti-oksidan aktivite, NGB'nin diğer dokulardaki potansiyel koruyucu rolünü gündeme getirmektedir. Testosteron üreten Leydig hücrelerinde NGB'nin fazla ifade edilmesi hipoksi ile ilişkili erkek infertilitesi için bir çözüm olabilecektir. Bu çalışmada, NGB proteinin viral aktarım metodu ile Leydig hücrelerinde aşırı ifade ettirilmiş ve hücre çoğalması, gen anlatımı ve anti-oksidan enzim seviyeleri analiz edilmiştir. Artan NGB, anti-apoptotik Bcl-2, hücre çoğalması ve anti-oksidan enzim seviyelerini arttırmış, BAX, p53 ve kaspaz 3 gibi apoptoz genlerinin ifadesini azaltmıştır. Ek olarak, H_2O_2 uygulaması sonrasında NGB aktarılan hücreler çoğalmış ve apoptotik genlerin ifadesi azalmıştır. Sonuç olarak, NGB androjen eksikliğine bağlı erkek infertilitesi için hedef olabilir ve gelecekte klinikte kullanılabilir.

Introduction

Neuroglobin (NGB) was identified as a vertebrate nerve globin in neural tissues by Burmester *et al.* (2000). The NGB protein consists of 150 amino acids and is similar to myoglobin structurally (Burmester *et al.* 2000, Dewilde *et al.* 2001). Expression of NGB is observed in central and peripheral nervous system and some endocrine tissues (Reuss *et al.* 2002, Burmester *et al.* 2000).

The neuroprotective activity of NGB through prevention of apoptosis and oxidative stress is well documented (Amri *et al.* 2017). NGB is able bind to O_2, CO or NO and prevents oxidative stress (Dewilde *et al.* 2001). The potential pathways and regulatory roles of NGB are providing O_2 supply, preventing reactive oxygen species (ROS) damage and hypoxia (Burmester & Hankeln 2009). NGB acts as a respiratory protein with an O_2 binding affinity which resembles to myoglobin oxygen-binding capacity (Hundahl *et al.* 2006). Localization of NGB in specific tissues and cellular regions that are metabolically active supports its O_2 binding ability (Schmidt *et al.* 2003). Interaction of NGB with O_2 enables the regulation of NGB expression in hypoxic conditions (Schmidt-Kastner *et al.* 2006). NGB acts as NO-dioxygenase when O_2 levels are low and react with NO_2 to form NO (Petersen *et al.* 2008). NGB expression in human cell cultures is not only triggered by hypoxia (Haines *et al.* 2012) but also induced by H_2O_2 (De Marinis *et al.* 2013) indicating the regulatory role in O_2 metabolism. 17β-estradiol (E2) induced NGB upregulation in neurons has been shown to be neuroprotective (De Marinis *et al.* 2013). Moreover, NGB was shown to translocate into mitochondria upon hormone and H_2O_2 stimulation and prevent cytochrome C release to help overcoming stress induced programmed cell death (De Marinis *et al.* 2013, Brittain *et al.* 2010). NGB overexpression in transgenic animals prevented heart and brain injury after ischemia or stroke indicating the tissue protective role of NGB after oxidative stress (Sun *et al.* 2003, Khan *et al.* 2006, Jin *et al.* 2010). Endogenous NGB in neural tissues exerted

neuroprotective roles against oxidative stress indicating the possible protective role of NGB in neurodegenerative disorders (Ye *et al.* 2009, Fiocchetti *et al.* 2013).

The harmful effects of oxidative stress are not only observed in neural tissues but also disrupt functions in many tissues of the systems of the body one of which is the reproductive system. Oxidative stress induced DNA damage in sperm function is one of the most important reasons for male fertility (Bisht *et al.* 2017). In addition to spermatogenesis, Leydig cell steroidogenesis is also sensitive to oxidative stress. A number of internal and external factors, e.g. age, infection, diabetes, temperature, testis diseases, toxin exposure and hormonal changes cause ROS production in the testes leading to male fertility problems (Asadi *et al.* 2017). The antioxidant defense system in Leydig cells protects testicular tissue and provides appropriate testosterone production and sperm generation (Aitken & Roman 2008). Leydig cells are the primary sources for testosterone production which is required for male reproduction. Therefore, identification of new protective mechanisms that control oxidative stress mechanisms in Leydig cells might be valuable for future therapeutic applications. Although large amounts of NGB were detected in the nervous system and brain, endocrine organs including testis and pituitary and adrenal glands also express NGB (Burmester *et al.* 2000, Reuss *et al.* 2002, Zhang *et al.* 2002). Because NGB is expressed in testis tissue, we hypothesized that overexpression of NGB might protect testis cells against stress. Protective activity of NGB against oxidative stress in tissues such as that of the nervous system where high amounts of localization are observed gave rise to the idea of potential protective effect of NGB in other tissues. In the current study, the protective role of NGB in TM3 mouse Leydig cells was evaluated by using a genetic manipulation approach.

Materials and Methods

Cell line

TM3-Leydig cells #CRL-1714 were purchased from ATCC (Rockville, MD). Cells were incubated in a humidified chamber at 37 °C and 5% CO_2 in 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's Medium (Invitrogen, Gibco, UK) supplemented with 5% horse serum, 2.5% fetal bovine serum (FBS, Invitrogen, Gibco, UK) and 1% Penicillin/Streptomycin/ Amphotericin (PSA, Invitrogen, Gibco, UK).

Viral production

The coding sequence of mouse NGB was ligated into pLenti-III-2A-GFP (Abm, Richmond, CA, USA, Fig. 1A). pLenti-III-2A-GFP was used as the control vector. Lenti viral vector stocks for pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP expressing vectors were produced by calcium phosphate transfection of 293T cells. pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP were transfected into 293T cells together with pCMVDR8.2DVPR (Addgene, Cambridge, MA, USA) and pMD2.G (VSVG, Addgene, Cambridge, MA, USA) for packaging and viral supernatants were collected at 24, 48 and 72 h post-transfection (Fig. 1B). Lenti viral supernatants were filtered and concentrated by ultracentrifugation. HeLa cells were transfected for viral titer calculation and GFP positive cells were analyzed by flow cytometry (FACS Aria, Becton Dickinson, San Jose, CA). $4x10^4$ transducing units/mL was determined as titer of pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors.

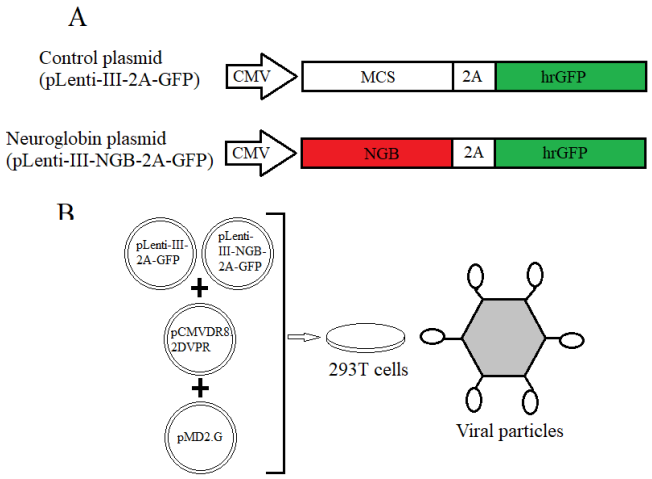

Fig. 1. Plasmid constructs and viral production method. (A) Structure of pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors. (B) Packaging process of pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors in 293T cells.

Cell transduction

TM3 cells were transduced with the viral supernatants of pLenti-III-2A-GFP and pLenti-III-NGBB-2A-GFP in the presence of 8µg/mL of polybrene for 24h. GFP positive cells were sorted by flow cytometry and NGB overexpression was confirmed by qPCR analysis. Cell populations were referred as TM3-NGB and TM3-GFP for further experiments.

Cell viability (MTS) analysis

Cell viabilities of TM3-NGB and TM3-GFP cells and H_2O_2 applied TM3-NGB and TM3-GFP cells were measured by the 3-(4,5-di-methyl-thiazol- 2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)- 2H-tetrazolium (MTS)-assay (CellTiter96 Aqueous One Solution; Promega, Southampton, UK) as described previously (Doğan *et al.* 2014). Briefly, $5x10^3$ TM3-NGB and TM3-GFP cells were seeded onto 96-well plates and cell viability was measured by MTS assay for 24, 48, and 72 hours. Absorbances were measured at 490 nm by using an ELISA plate reader (Biotek, Winooski, VT).

Determination of H_2O_2 toxic dose

TM3 Leydig cells were seeded onto 96-well plates at a cell density of 5000 cells/well. One day later, cells were treated with various concentrations (100µM, 200µM, 300µM, 400µM) of H_2O_2 to determine the toxic dose for further experiments. Cell viability was measured by MTS assay and absorbance was measured at 490nm using an ELISA plate reader.

TUNEL assay

TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed in TM3-NGB and TM3-GFP cells by using a TUNEL Assay kit (Roche Molecular Biochemicals, Indianapolis, IN) to detect the apoptotic cell number. Briefly, transduced cells were collected and suspended in 50µl of TUNEL reaction mixture (Labeling solution + Enzyme solution, supplied with the kit), incubated at 37°C and suspended in PBS for flow cytometry analysis using Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometry system.

Gene expression analyses

Quantitative RT-PCR experiments were conducted according to the previously described protocol (Apdik *et al.* 2015). Primer sequences for NGB, p53, caspase3, Akt, BAX, Bcl-2 and β-actin were designed by IDT primer Quest software. β-Actin was used as housekeeping gene for normalization of the data. Total RNA was isolated form transduced cells and H_2O_2 administered cells by using a RNA-easy plus mini kit (Qiagen, Hilden, Germany) and cDNA was synthesized using High Fidelity cDNA synthesis kit (Roche, USA). qPCR experiments were conducted by SYBR Green using the CFX96 RT-PCR system (Bio-Rad, Hercules, CA).

SOD and GPx activity measurements

SOD (19160, Sigma, MO, USA) and GPx (CGP1, Sigma, MO, USA) enzyme activities were determined in TM3-NGB and TM3-GFP cells according to the manufacturer's instructions. Protein samples were isolated from TM3-NGB and TM3-GFP cells by RIPA buffer and used for enzyme activity analysis. Absorbances of SOD and GPx assays were measured at 450nm and 340nm, respectively by using an ELISA plate reader.

Statistical analysis

Results are expressed as mean ± standard deviation. Standard errors and t-test values were calculated using the GraphPad Prism 5 (GraphPad, La Jolla, CA) software. Differences were considered to be statistically significant at P values of less than 0.05 ($P < 0.05$).

Results

Cell transduction and proliferation analysis

TM3 cells were successfully transduced by pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP lentiviral vectors. pLenti-III-2A-GFP transduction was conducted as control of viral transduction. GFP expressing cells were visualized by a fluorescence microscope (Fig. 2A). NGB expression was approximately 10 fold higher in TM3-NGB-GFP cells (10±0.0197) compared to TM3-GFP (1±0.0056) cells demonstrating the successful overexpression by lentiviral vectors (Fig. 2B). In order to determine the effect of NGB upregulation on cell proliferation, MTS analyses were performed. Cell proliferation of TM3-NGB-GFP cells was significantly higher for 24, 48 and 72 h compared to TM3-GFP cells (Fig. 2C). Although absorbance values for TM3-GFP at 490 nm were 0.290±0.0051, 0.350±0.0167 and 0.410±0.0197; absorbances of TM3-NGB-GFP were 0.367±0.0201, 0.520±0.0198 and 0.630±0.0265 for day 1, day 2 and day 3 respectively.

TUNEL assay

The apoptotic status of NGB overexpressing cells were detected by TUNEL assay. Although 27% of the TM3-GFP cells were apoptotic after transduction, only 12% of the TM3-NGB-GFP cells were positively stained (Fig. 2D).

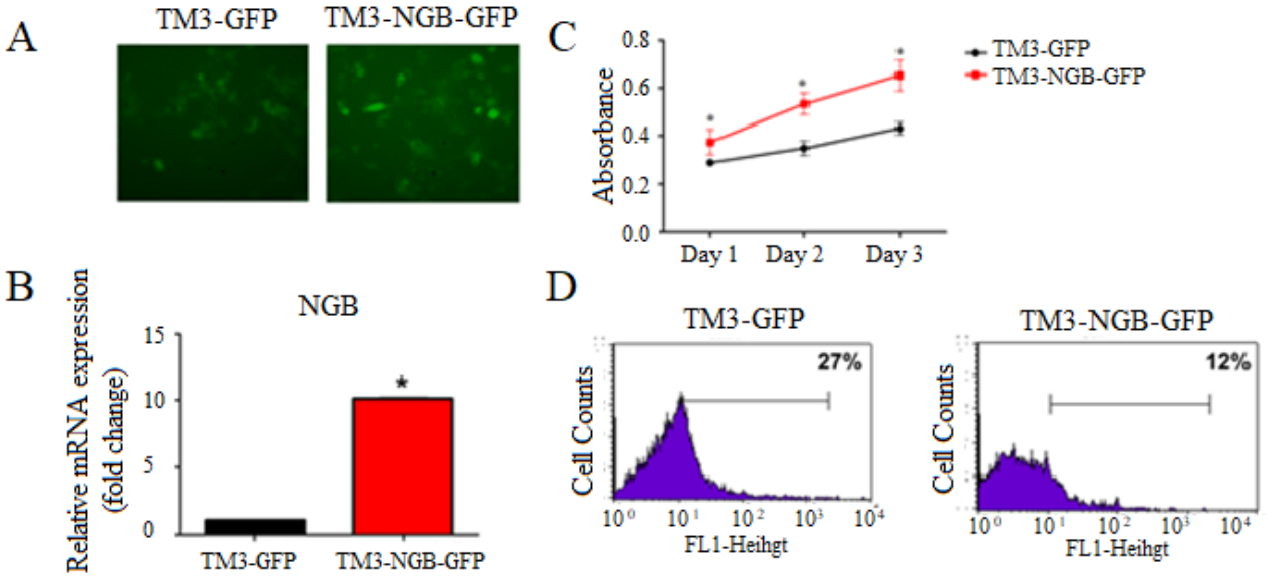

Fig. 2. Transduction of TM3 cells with pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors and viability analysis. (A) Successful transduction of TM3 cells. Cells were GFP positive under fluorescence microscope indicating the viral transduction. (B) Confirmation of overexpression of NGB by qPCR analysis. (C) Cell proliferation of TM3-NGB-GFP and TM3-GFP cells. (D) TUNEL staining of TM3-NGB-GFP and TM3-GFP cells. *P < 0.05.

Gene expression analyses

Pro-apoptotic and anti-apoptotic gene expression levels were detected in NGB overexpressing TM3 cells. The apoptotic genes BAX, Caspase-3 and p53 were downregulated in TM3-NGB-GFP indicating the protective role of NGB in TM3 cells. Bcl-2 as an anti-apoptotic gene was upregulated in NGB overexpressing TM3 cells (Fig. 3). The expression in TM3-NGB-GFP expressed almost 2-fold higher Bcl-2 (1.964±0.0298) compared to TM3-GFP (1±0.1390). BAX expression was not significantly different in TM3-NGB-GFP (0.764±0.0299) and TM3-GFP (1±0.1470) cells. Caspase-3 and p53 were downregulated in TM3-NGB-GFP (0.56±0.0210 and 0.58±0.0231) compared to GFP expressing TM3 cell line.

Protective role of NGB against H_2O_2 toxicity

Four different doses (100 µM, 200 µM, 300 µM, 400 µM) of H_2O_2 were applied to determine the toxic concentration for cell viability and qPCR analysis. Significant toxicity was observed starting from 200µM H_2O_2 (0.164±0.0080) compared to the control group (0.669±0.0205). 200 µM H_2O_2 significantly reduced cell viability and was selected for further analysis (Fig. 4A). TM3-NGB-GFP cell viability was higher compared to TM3-GFP cells for 24, 48 and 72h. The cell viability showed a time dependent increase in TM3-NGB-GFP cells for 3 days and was 2 fold higher compared to TM3-GFP by day 3 (TM3-NGB-GFP: 0.205±0.0148, TM3-GFP: 0.093± 0.0265) (Fig. 4B). BAX, Caspase-3 and p53 genes were downregulated in TM3-NGB-GFP indicating the protective role of NGB in of TM3-NGB-GFP for p53, BAX, Bcl-2 and Caspase-3 were detected as 0.864±0.0299, 0.800±0.0100, 1.100±0.0300 and 0.812±0.0400, respectively and no statistically significant difference was measured for these values (Fig. 4C).

SOD and GPx enzyme activity

Potential role of NGB overexpression on antioxidant enzyme activities was detected by SOD and GPx activity measurements. NGB overexpression increased the antioxidant enzyme activities significantly compared to TM3-GFP cells. TM3-NGB-GFP cells exerted approximately 2 fold higher antioxidant enzyme activity. SOD and GPx enzyme activities of TM3-GFP cells were determined as 60%±3.1 and 57%±3.2, respectively while activities of both were 100% in TM3-NGB-GFP cells (Fig. 5).

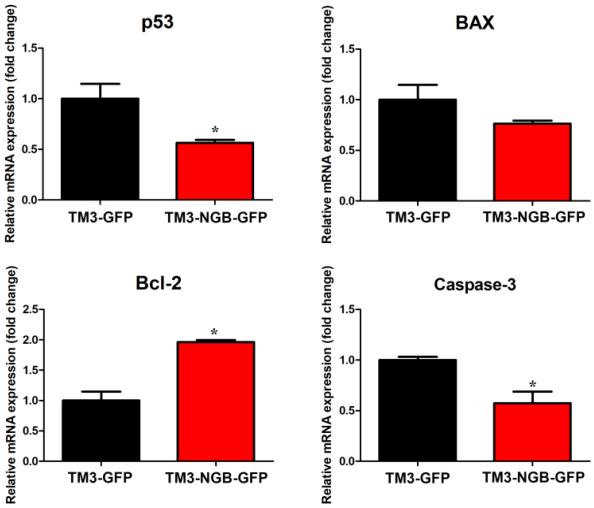

Fig. 3. Gene expression analysis of TM3-NGB-GFP and TM3-GFP cells for pro- and anti-apoptotic genes. *P < 0.05.

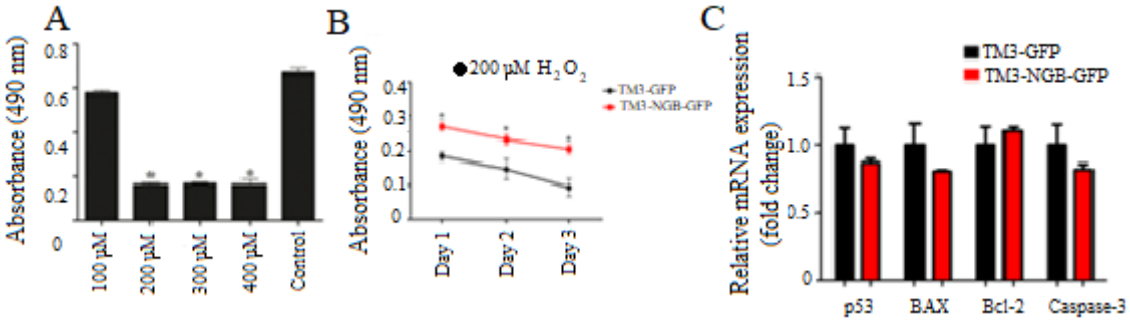

Fig. 4. Protective role of NGB against H_2O_2 stress. (A) Determination of H_2O_2 toxic dose based on cell viability. (B) Cell proliferation of TM3-NGB-GFP and TM3-GFP cells after 200µM H_2O_2 exposure. (C) Expression levels of pro- and anti-apoptotic genes in TM3-NGB-GFP and TM3-GFP cells after 200 µM H_2O_2 exposure. *P < 0.05.

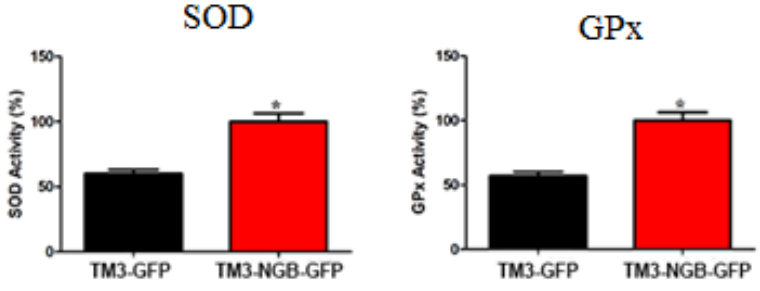

Fig. 5. SOD and GPx enzyme levels in TM3-NGB-GFP and TM3-GFP cells. *P < 0.05.

Discussion

Free radicals generated through cellular metabolism cause oxidative stress in tissues such as testis and induce cell death. Oxidative stress induced ROS disrupts reproductive system, prevents androgen (testosterone) secretion and spermatogenesis (Aitken *et al.* 2008). Leydig cell dysfunction due to membrane lipid peroxidation upon Lipopolysaccharide (LPS) application has been observed previously (Husain & Somani 1998). As Leydig cells produce androgens such as testosterone which binds to the androgen receptor and activates sperm generation (Dohle *et al.* 2003), they are potential targets for oxidative stress related male fertility treatments. Understanding the protective mechanisms against oxidative stress and identifications of new potential pathways are among the recent aims of studies addressing development of new therapeutic targets.

In the present study, we evaluated the role of NGB in Leydig cells by using an overexpressing gene editing approach. Because NGB protects many tissues against oxidative stress and are expressed in testis tissue, overexpression of NGB in Leydig cells could be promising to prevent harmful effects of oxidative stress. Proliferation of NGB overexpressing mouse TM3 Leydig cells were higher compared to control cells indicating the potential protective activity. The activity of NGB on cell proliferation and protection has previously been shown in neurons and cancer cells (Fiocchetti *et al.* 2017, Greenberg *et al.* 2008, Zhang *et al.* 2018) which supports our results. NBG overexpressing transgenic mice can rescue under hypoxic stress and NGB induces neural protection in vitro against hypoxic injury (Greenberg *et al.* 2008). Overexpression of NGB increased cell viability in MCF-7 breast cancer cells against nutrient deprivation through an anti-apoptotic Bcl-2 expression (Fiocchetti *et al.* 2017). Similarly, both overexpression and knockdown of NGB in glioblastoma cells regulated the cell proliferation by activating the PI3K/AKT pathway (Zhang *et al.* 2018). Promoting activity of NGB in cell proliferation and viability might be useful to increase Leydig cell number and to treat oxidative stress related male fertility in the future clinical applications. Additionally, low levels of TUNEL positive cells in NGB transduced cells demonstrated the anti-apoptotic role of NGB in Leydig cells. Knockdown of NGB in neurons increased TUNEL positive cells in arsenite induce toxicity indicating the protective role of NGB against apoptosis (Liu *et al.* 2015). Anti-apoptotic activity was confirmed by gene expression analysis of pro- and anti-apoptotic genes including BAX, p53, caspase-3 and Bcl-2. Enhanced Bcl-2 and low levels of BAX and caspase-3 have been reported after spinal cord injury in NGB overexpressing animals (Lan *et al.* 2014). Because Leydig cells are responsible for testosterone production and highly crucial for spermatogenesis, anti-apoptotic function of NGB to block apoptosis is highly important for potential therapeutic options. Same observations for cell proliferation and gene expression were reported after H_2O_2 exposure indicating the protective effect against ROS and oxidative stress. Induction of SOD and GPx in testis tissue (Kaur *et al.* 2006) and Leydig cells (Baek *et al.* 2007) is a defense mechanism after oxidative stress. Therefore, we evaluated the effect of NGB overexpression on anti-oxidant enzyme activities. NGB overexpression upregulated the SOD and GPx levels as reported in the literature. Although there is not an evidence in the literature for NGB overexpression in Leydig cells, transgenic mice overexpressing NGB showed high SOD and GPx levels in hippocampal tissues (Li *et al.* 2010).

In conclusion, we demonstrated, for the first time, the promising protective role of NGB in Leydig cells against oxidative stress. NGB gene could be a potential target for oxidative stress induced male infertility and might be used for therapy in future. Further experiments explaining the molecular mechanism of NGB in Leydig cells should be conducted both *in vitro* and *in vivo*.

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ResearchArticle

OPTIMIZATION OF ULTRASONIC-ASSISTED EXTRACTION PARAMETERS FOR ANTIOXIDANTS FROM *Curcuma longa* L.

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Abstract: In this study, ultrasonication was used to extract antioxidant compounds such as polyphenols, flavonoids, and curcuminoids from turmeric (*Curcuma longa* L.) The influences of time, ethanol concentration and temperature as three independent factors on the extraction of the total phenolic content were evaluated by the Folin-Ciocalteu method and the antioxidant capacities by the ABTS and chromium reducing antioxidant capacity (CHROMAC) methods. The central composite design (CCD) with a multi-response surface methodology (MRSM) was used for the statistical modeling of the response data followed by the regression and analysis of variance (ANOVA) to determine the significance of the model and the factors. The response predictions obtained at optimum extraction conditions of an extraction time of 64min, an ethanol concentration of 82% (v/v) and an extraction temperature of 32°C were 47.32mg GAE/g (for Folin-Ciocalteu), 29.15 (for ABTS) and 5.17mg TE/g (for CHROMAC). The predicted values obtained from the multi-response surface methodology agreed with the experimental values data 95% confidence level. These data indicate that the multi-response surface methodology is applicable for optimizing the ultrasonic-assisted extraction of antioxidant compounds from *C. longa*.

Keywords: *Curcuma longa* L., ultrasonic-assisted extraction, antioxidant capacity, phenolic compounds, multi-response surface methodology.

Özet: Bu çalışmada ultrasonikasyon, *Curcuma longa* L.'da bulunan polifenol, flavonoid ve kurkuminoid gibi antioksidan bileşiklerin ekstraksiyonu için kullanılmıştır. Zaman, etanol derişimi ve sıcaklık gibi 3 bağımsız değişkenin toplam fenolik madde (Folin yöntemi) ve antioksidan kapasite (ABTS ve CHROMAC yöntemi) üzerine etkisi değerlendirilmiştir. Merkezi kompozit dizayn ile çok yanıtlı yüzey analiz yöntemi, sonuçların istatistiksel modellenmesi, model ve faktörler arası etkileşimi belirlemek için regresyon ve ANOVA analizinde kullanılmıştır. Belirlenen optimum koşullarda (64dk ekstraksiyon zamanı, %82 (v/v) etanol derişimi ve 32˚C ekstraksiyon sıcaklığı) toplam fenolik madde miktarı 47,32mg GAE/g, antioksidan kapasite 29,15 (ABTS) ve 5,17 (for CHROMAC) mg TE/g bulunmuştur. Bu deneysel değerler ile tahmini değerler %95 güven aralığında birbiri ile uyumludur. Buna göre çok yanıtlı yüzey analiz tekniği (MRSM), *C. longa*'dan antioksidan bileşiklerin ultrasonik-destekli ekstraksiyon optimizasyonu için güvenle kullanılabilir.

Introduction

Curcuma longa L. (turmeric) distributed in East and South-East Asia (Xu *et al.* 2017)is generally used as a functional food and an herbal medicine (Péret-Almeida *et al.* 2005). The curcuminoids (curcumin-Cur, demethoxycurcumin-DMC, bisdemethoxycurcumin-BDMC) are natural and active phytochemicals in turmeric (Xu *et al.* 2017). A large number of studies reported that curcuminoids possess strong antioxidant (Martínez-Morúa *et al.* 2013), anti-inflammatory (Kant *et al.* 2014), antimicrobial (Kiamahalleh *et al.* 2016), and anticarcinogenic (Riela *et al.* 2014) properties and some other pharmaceutical activities (Lima *et al.* 2011, Mourtas *et al.* 2014).

Curcuminoids can be extracted from plants using a variety of methods. Non-conventional extraction techniques include ultrasound-assisted (Mandal *et al.* 2009), supercritical fluid, pressurized liquid and microwave extractions (Mandal *et al.* 2008). Among these techniques, ultrasound-assisted extraction (UAE) is widely used for the extraction of phytochemicals and is a simple, inexpensive, energy-saving, and efficient method when compared with other extraction techniques (Nasır *et al.* 2017). UAE was used for the extraction of antioxidant compounds from plants with higher yields (Şahin *et al.* 2013).

Different methods have been suggested for the extraction of phytochemicals from plants and the optimization of the extraction conditions due to the differences in the chemical and physical properties, concentrations, and matrix complexity related to the extractable phytochemical. The extraction solvent, pH, temperature, time, solid/liquid ratio, pressure, and particle

size are typical factors that contribute to the yield of extraction. Optimizing one factor at a time has been a generally used approach (Lai *et al*. 2014), but it is time-consuming and expensive and does not allow the analysis of possible interactions among the extraction variables (Matshediso *et al*. 2015). Therefore, a multi-response surface methodology (MRSM) is considered an efficient method for evaluating multiple individual parameters and their interactions (Baş & Boyacı 2007). The extraction of antioxidant compounds has been recently studied using different solvents such as dichloromethane, hexane, methanol, ethanol, acetone, dimethyl ether, diethyl ether, dimethyl sulfoxide, toluene, 2-propanol, and n-butanol. Due to the high solvent costs, the toxicity resulting from the solubility of the bioactive compounds in the solvent and low extraction efficiency, these solvents have been used regularly for the extraction of antioxidant compounds. Therefore, ethanol is one of the appropriate extraction solvents for antioxidant compounds due to its low cost, "green" characteristics, ease of access and safety for direct use in foods and pharmaceuticals (Xu & Bao 2014, Dailey & Vuong 2015).

The present study is focused on the application of UAE using a multi-response surface methodology to optimize the antioxidant extraction parameters for *C. longa*. Three extraction factors -the ethanol concentration, extraction temperature, and extraction time- were optimized by the MRSM. A three-variable and five-level, central composite design was used for simultaneously maximizing the antioxidant capacity and the total phenolic content.

Materials and Methods

Chemicals and reagents

Trolox, Folin–Ciocalteu reagent and ABTS were purchased from Sigma. Ethanol and methanol (HPLC grade), formic acid, phosphoric acid, sodium dihydrogen phosphate and HCl were purchased from Merck. Potassium dichromate, 1,5-diphenylcarbazide, sodium carbonate, potassium sodium tartrate, sodium hydroxide, copper(II) sulfate pentahydrate, and gallic acid in HPLC grade were obtained from Sigma.

Plant material

Dried *Curcuma longa* L. was purchased from a medicinal market in Bursa-Turkey and was stored at 4°C until extraction.

Ultrasound-Assisted Extraction (UAE) method

An ultrasonic cleaner (United model, Bursa, Turkey) was used for the extraction. The temperature was controlled using a resistance thermometer. *Curcuma longa* (0.5g) was placed in a vial, ethanol (30mL) was added, and the solution was placed in an ultrasonic cleaning bath. The operation was performed at 40 Hz ultrasonic wave frequency. The extraction parameters are given in Table 1.

Table 1. Range of coded and actual values for central composite design.

Factor	Level				
	-2	**-1**	**0**	**1**	**2**
Extraction time (min)	19	30	45	60	71
Ethanol concentration (%, v/v)	23	30	40	50	57
Extraction temperature (°C)	15	30	50	70	85

Antioxidant capacity

The antioxidant capacities of the extracts were determined with the chromium reducing antioxidant capacity (CHROMAC) (Işık *et al.* 2013) and ABTS (Re *et al.* 1999) methods with slight modifications (Şahin *et al.* 2013, Nasır *et al.* 2017). In the ABTS method, 3.8ml of ethanol was added to the *C. longa* sample (0.2ml). Then, 1ml of the ABTS solution (diluted with ethanol at a ratio of 1:10) was added. The absorbance of the sample was measured at 734nm against a blank sample after 6min by Varian Cary-50 UV/VIS spectrophotometer (Melbourne, Australia). The resulting antioxidant capacity was expressed as mg of Trolox equivalents (TE) per gram of sample. In the CHROMAC method, approximately 0.2ml *C. longa* sample, 0.3ml distilled water, 3.5ml phosphate buffer solution (pH 2.8) and 0.5ml $K_2Cr_2O_7$ (50mg l^{-1}) were added into a test tube. The $K_2Cr_2O_7$ solution was allowed to react with the sample. After incubation for 1min, approximately 0.5ml of 1,5-diphenylcarbazide (3.4×10^{-4}mol L^{-1}) was added and mixed thoroughly. The absorbance of the solution was measured at 540nm against a reagent blank after 50 min. The reagent blank at pH 1.2 was prepared with 0.1M citric acid and 6M HCl. A standard calibration curve was prepared using various concentrations of Trolox. The results were expressed as mg TE per gram of sample.

Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method (Singleton *et al.* 1999) with slight modifications (Şahin *et al.* 2013). 50mL of Lowry A solution (2% aqueous Na_2CO_3 in 0.1M NaOH) was mixed with 1mL of Lowry B solution (0.5% $CuSO_4$ solution in 1% $NaKC_4H_4O_6$ aqueous solution) to produce Lowry C solution. Briefly, 1.8ml of distilled water and 2.5ml of Lowry C solution were added to *C. longa* sample (0.2ml), and the mixture was left for 10min. After 10min, Folin reagent (0.25ml) was added and the blue color of the mixture was allowed to stabilize under darkness for approximately 30min. The absorbance of the sample was measured at 750nm in triplicate. The total phenolic contents of *C. longa* extracts were expressed as mg gallic acid equivalent (GAE) per g of *C. longa*.

Response surface methodology

A central composite design (CCD) was selected for the optimal extraction conditions for the most successful RSM design (Aybastıer & Demir 2010). A three-factor, four-level central composite design experimental design matrix with factors such as the extraction time (min), ethanol concentration (%, v/v) and extraction temperature (°C) (Table 1) was formed, and the responses were

selected as the maximum antioxidant capacity and the total phenolic content. Twenty experiments were performed to optimize the parameters (Aybastıer & Demir 2010). Design Expert 7.0.0 software (Stat-Ease Inc., USA) was used for the statistical analysis.

Results

MRSM model

The MRSM was used to determine the extraction conditions that give the best results in terms of the antioxidant capacity and total phenolic content. The experimental and predicted total phenolic content and antioxidant capacity values are shown in Table 2. The extraction time, ethanol concentration and extraction temperature were studied with respect to the UAE of *C. longa*. The results indicate good correlations between the parameters because of a satisfactory *R*-squared value (R^2 =0.902) (Table 3). The *F*-value and *p*-values are indicated by larger corresponding coefficients. The x_1 (extraction time), x_2 (ethanol volume), x_3 (extraction temperature), x_1x_3, x_2x_3 and x_2^2 variables were the most significant factors ($p \leq 0.05$) (Table 4). However, x_1x_2, x_1^2 and x_2^2 had less effect (where $p > 0.05$) on the UAE in terms of the total phenolic content. The effects of the extraction factors on the total phenolic content were studied using response surface plots (Fig. 1). The MRSM of the data in Table 3 also showed good correlation (R^2 = 0.895 for ABTS and 0.826 for CHROMAC). The x_1, x_2, x_3, x_1x_3, x_2x_3, x_1^2, x_2^2 and x_3^2variables for ABTS and the x_1, x_2, x_3, x_2x_3, x_1^2 and x_3^2variables for CHROMAC were the most significant factors ($p \leq 0.05$) (Table 4) for the UAE of antioxidant compounds from *C. longa*. The $x_1x_2$variable for ABTS and x_1x_2, x_1x_3, x_2^2 for CHROMAC had minor effects on the antioxidant capacity derived from the UAE. The effects of the factors and antioxidant capacity values determined with the ABTS and CHROMAC methods were analyzed using response surface plots from the MRSM in Fig. 2.

Optimization of the extraction parameters

Validation tests were used to verify the reliability of the model by comparing the experimental and the predicted values for the MRSM. The optimum UAE conditions were presented in Table 5. An extraction time of 64min, an ethanol concentration of 82% (v/v), and an extraction temperature of 32ºC produced the maximum antioxidant capacities (29.15mg TE/g for ABTS and 5.17mg TE/g for CHROMAC) and the total phenolic content (47.32mg GAE/g) from *C. longa*. After comparing the predicted and experimental results, the RSM was more stable with good correlation (R^2>0.95) for *C. longa*.

Table 2. Central composite design of factors with experimental and predicted values.

Total phenolic content (mg GAE/g dried plant)			Antioxidant capacity (mg TE/g dried plant)			
			ABTS		CHROMAC	
Treatment	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	42.32	43.68	7.63	5.33	2.16	1.33
2	18.34	17.95	5.56	5.21	0.16	0.17
3	44.21	47.70	19.92	22.36	3.51	4.36
4	42.93	42.36	11.05	10.81	2.55	2.47
5	46.55	48.12	9.49	9.84	3.62	3.74
6	42.74	42.79	5.43	7.49	2.62	3.26
7	41.49	43.68	4.81	5.33	1.09	1.33
8	38.54	43.68	6.33	5.33	0.67	1.33
9	46.35	43.68	2.86	5.33	1.51	1.33
10	46.77	46.01	12.91	10.58	4.09	3.34
11	32.46	32.23	14.57	15.04	2.93	2.90
12	47.37	43.68	6.63	5.33	1.25	1.33
13	36.57	38.62	5.46	8.58	0.94	1.78
14	46.45	41.98	13.67	10.64	3.97	3.06
15	46.00	43.68	3.70	5.33	1.31	1.33
16	45.34	46.86	13.05	13.19	1.62	1.72
17	20.78	24.47	6.65	7.53	0.18	0.39
18	45.80	43.37	20.76	20.66	2.10	1.96
19	33.46	30.98	10.64	9.91	1.38	1.22
20	41.00	39.98	19.56	16.90	3.45	2.76

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters.

Source	Folin (R^2 = 0.902)					ABTS (R^2 = 0.895)					CHROMAC (R^2 = 0.826)				
	DF	SS	MS	*F* value	*p* value	DF	SS	MS	*F* value	*p* value	DF	SS	MS	*F* value	*p* value
Model	9	1188.05	132.01	10.30	0.0006	9	519.61	57.73	9.55	0.0008	9	23.99	2.67	5.28	0.0079
Lack of fit	5	68.98	13.80	1.17	0.4354	5	43.45	8.69	2.55	0.1633	5	3.82	0.76	3.13	0.1178
Pure error	5	59.19	11.84			5	17.01	3.40			5	1.22	0.24		

Table 4. Second order polynomial equations and regression coefficients of the response variables (the extraction time; x_1; the ethanol concentration; x_2, the extraction temperature; x_3).

Responses	Second order polynomial equations
Total phenol content (mg GAE/g dried plant)	$y=43,68+2,13x_1+5,40x_2+4,67x_3-3,05x_1x_3-4,29x_2x_3-3,19x_2^2$
ABTS (mg TE/g dried plant)	$y=5,33+0,58x_1+3,75x_2-1,53x_3-1,96x_1x_3-2,29x_2x_3+1,42x_1^2+2,86x_2^2+2,46x_3^2$
CHROMAC (mg TE/g dried plant)	$y=1,33+0,45x_1+0,45x_2+0,25x_3-0,98x_2x_3+0,41x_1^2+0,69x_3^2$

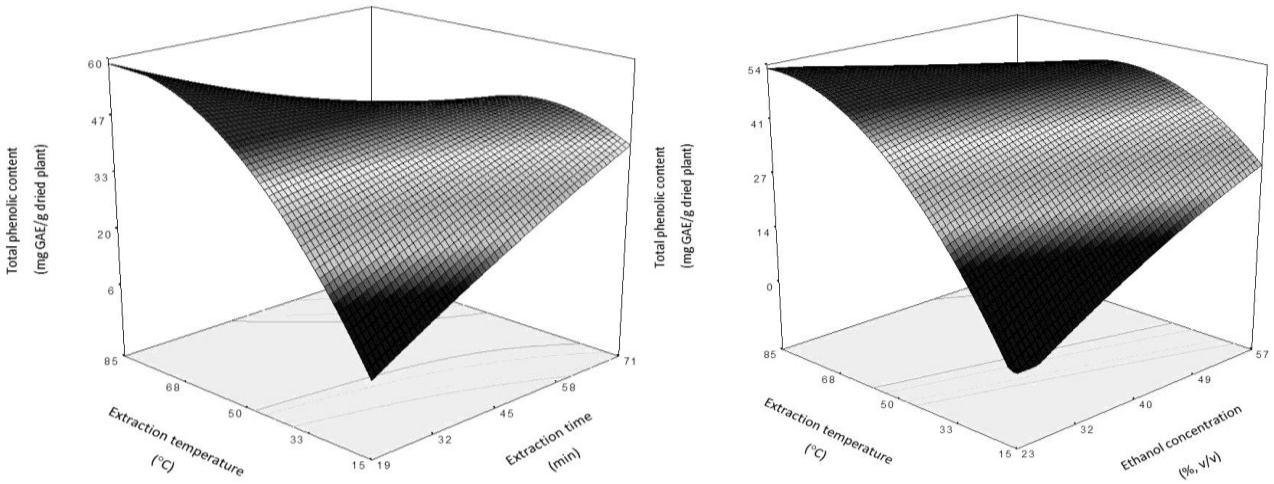

Fig. 1. Response surface plots of *Curcuma longa* showing the effects of (A) Extraction time and extraction temperature, (B) Ethanol concentration and extraction temperature on total phenolic content.

Discussion

Among the 15 runs (Table 3), experiment 12 (45min, 23% (v/v) of ethanol, 50ºC) produced the highest total phenolic content (47.37mg GAE/g), and experiment 10 (19min, 40% (v/v) of ethanol, 50ºC) produced the highest antioxidant capacity (4.09mg TE/g) for the CHROMAC method. For both the Folin-Ciocalteu and CHROMAC methods, experiment 2 produced the lowest values (18.34mg GAE/g and 0.16mg TE/g). For the ABTS method, experiment 18 produced the highest antioxidant capacity (20.76mg TE/g), but experiment 9 produced the lowest antioxidant capacity (2.86mg TE/g).

ANOVA revealed that the total phenolic content and antioxidant capacity values were described by quadratic polynomial models. The analysis showed that the large model *F*-values (10.30, 9.55 and 5.23 for Folin-Ciocalteu, ABTS and CHROMAC methods, respectively) and the lack of fit *p*-values (0.4354, 0.1633 and 0.1178 for Folin-Ciocalteu, ABTS and CHROMAC methods, respectively) were statistically significant at a 95% confidence level (Table 4). The larger *p*-values were statistically non-significant relative to the pure errors. The R^2 values were 0.902, 0.895 and 0.826 for the Folin-Ciocalteu, ABTS and CHROMAC methods, respectively. It was shown that the linear relationship between the extraction parameters and the responses were significant and the model was appropriate for optimization.

The three-dimensional images of the response surface methodology showed the interactions between the extraction parameters and the total phenolic content (Fig. 1). At higher extraction temperatures and time, the

highest total phenolic content was observed (Fig. 1a). The diffusion and solubility rates of the antioxidant compounds increased with a higher extraction temperature. When experiments 13 and 17 were compared (Table 3), the total phenolic content increased from 20.78 to 36.57mg GAE/g dried plant at a higher extraction temperature. Consequently, the effect of increasing the extraction temperature was significant for extracting antioxidant compounds in *C. longa.* Unfortunately, the extraction temperature was an important factor affecting the activity of the extracts due to both the degradation or loss of the antioxidant compounds (Yap *et al*. 2009, Dorta *et al*. 2012) and reactions with other components. However, the highest total phenolic content and antioxidant capacity values were obtained at 32°C by using the MRSM.

Ethanol was selected as the solvent for the UAE of antioxidant compounds from *C. longa.* A higher total phenolic content was observed at higher ethanol concentrations (Fig. 1b.). When experiments 6 and 8 are compared (Table 3), it appeared that the total phenolic content increased from 38.54 to 42.93mg GAE/g dried plant with increasing ethanol concentrations. It was reported that the higher ethanol concentration gave the highest extraction yield of the antioxidant compounds (Kwang *et al*. 2010). Therefore, the extraction yield of the antioxidant compounds was higher when the total phenolic content and antioxidant capacity of the extract was higher, as shown in our results.

It was clear that when the temperature was increased with a shorter extraction time, the antioxidant capacity was also increased for the ABTS method. The highest antioxidant capacity was observed at the shortest extraction time (19min) and the highest temperature (85°C). Fig. 2a-b shows the interactions between the ethanol concentration and extraction temperature on the antioxidant capacity for the ABTS and CHROMAC methods. The antioxidant capacity increased with increasing temperature at a low ethanol concentration. At lower ethanol concentrations and higher temperatures, the highest antioxidant capacity was observed. It was reported that the extraction yield of antioxidant compounds increased with increasing extraction time (Xu *et al.* 2015). Thus, an extraction time ranging from 19 to 71min was used in the present study, and the extraction yield of antioxidant compounds was affected by the extraction temperature. A high temperature may improve the solubility of antioxidant compounds (Liang *et al.* 2017), but there is an important risk of thermal degradation of antioxidant compounds. The solvent concentration is an important factor influencing extraction yield of antioxidant compounds and extraction yield of antioxidant compounds increased with increasing solvent concentration (volume) (Xu *et al.* 2015).With the antioxidant capacity as the reference value from Fig. 2, the optimum conditions obtained by the MRSM analysis were an extraction temperature of 85°C, an extraction time of 19min and an ethanol concentration of 23% (v/v). However, with the antioxidant capacity and the total phenolic content values as the reference value, the optimum conditions obtained by the MRSM analysis were an extraction temperature of 32°C, an extraction time of 64min and an ethanol concentration of 82% (v/v). The optimum conditions were obtained from the MRSM analysis to predict the maximum values for the antioxidant capacities and the total phenolic content. Multi-response surface methodology is the most suitable method for optimizing the ultrasonic-assisted extraction of antioxidant compounds from *C. longa* when compared a single response surface methodology. Because, multi-response surface methodology ensured the optimum extraction parameters with limit of time and solvent concentration and without no thermal degradation. Furthermore, the antioxidant capacity is dependent on the synergistic effect of the extracted antioxidant compounds. However, the synergistic effect will decrease when the total phenolic content increase (Thoo *et al.* 2010). Additionally, when compared with the available published data, similar results were found showing that the antioxidant capacity increased with increasing extraction time (Xu *et al.* 2015).

UAE was also used successfully for the extraction of antioxidant compounds from *C. longa* with yields higher than previously reported (Xu *et al.* 2015, 2017). The multi-response surface methodology was successful in optimizing the antioxidant compound content using UAE from *C. longa.*

Table 5. Optimum conditions, predicted and experimental values of responses.

Responses	Optimum Ultrasonic-assisted extraction conditions			Maximum values	
	Extraction time (min)	Ethanol concentration (%, v/v)	Extraction temperature (°C)	Predicted	Experimental
Total phenolic content (mg GAE/g dried plant)	64	82	32	47.63	47.32±0.02
ABTS (mg TE/g dried plant)				29.50	29.15±1.05
CHROMAC (mg TE/g dried plant)				5.13	5.17±0.08

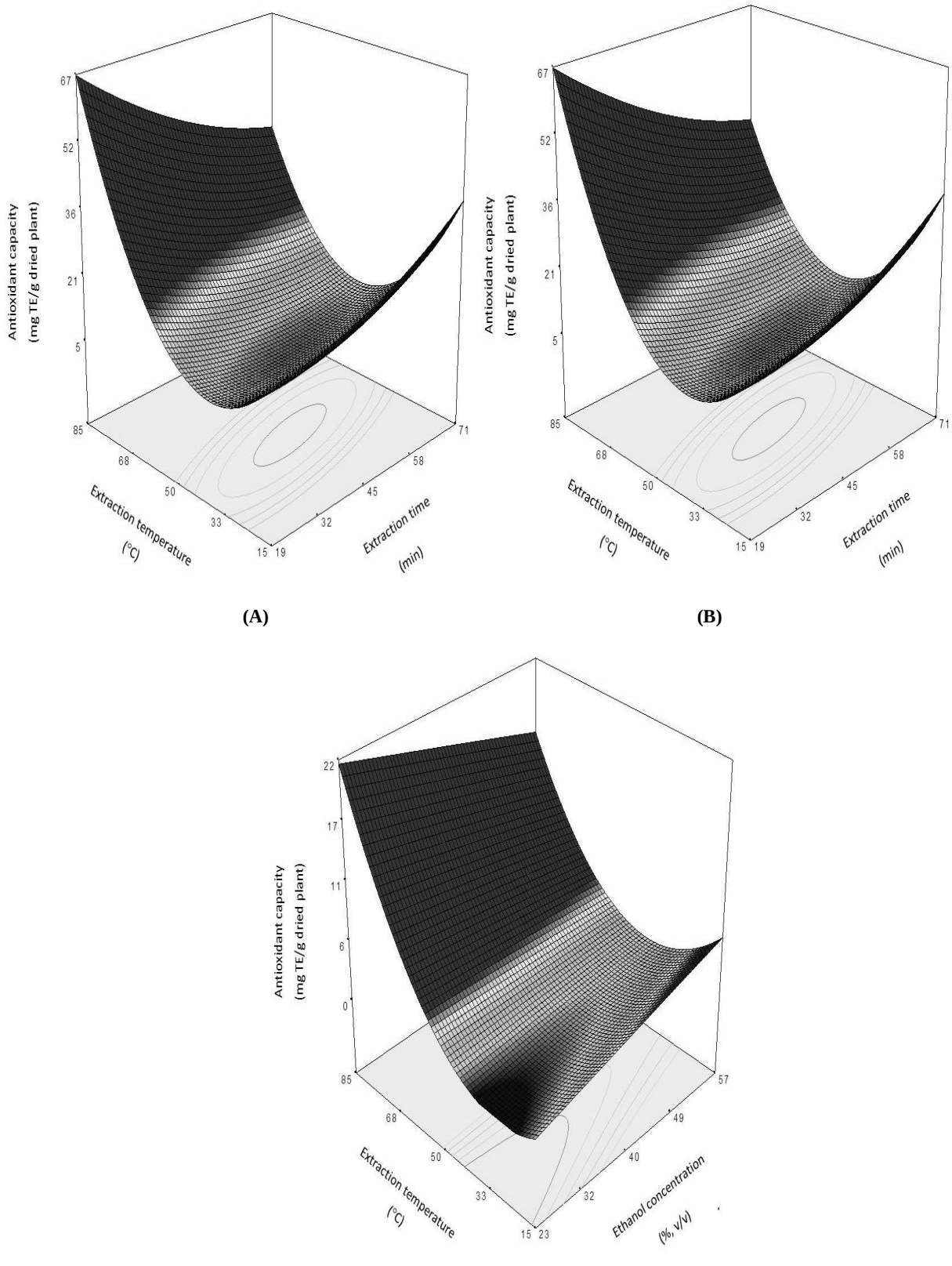

Fig. 2. Response surface plots of *Curcuma longa* showing the effects of (A) Extraction time and extraction temperature, (B) Ethanol concentration and extraction temperature on ABTS values, and (C) Ethanol concentration and extraction temperature on CHROMAC value.

The optimum conditions using UAE of antioxidants from *C. longa* are shown in Table 5.The study of Xu *et al.* (2017) with *C. longa* have demonstrated that phenolics such as curcuminoids are major contributors to antioxidant properties. Therefore, the total phenolic content and antioxidant capacity have been selected as the response of the MRSM model. All responses from each extraction factor were combined into a single set of optimum conditions. When the multi-response surface methodology was used, the total phenolic content of the extract increased with antioxidant capacity (Nasır *et al.* 2017). The present study is the first study about determination of the antioxidant capacity of *C. longa* by the CHROMAC method. It can be concluded that the MRSM is accurate and reliable within a 95% confidence interval to predict the results obtained with *C. longa*.

Conclusions

The multi-response surface methodology was used successfully for the optimization conditions of UAE of antioxidant compounds from *C. longa*. The CCD provided a powerful design for the optimization conditions using UAE. The extraction factors strongly influenced the extraction of the antioxidant compounds from *C. longa.* We conclude that *C. longa* is a good and reliable source of antioxidant compounds.

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Research Article

GENETIC DIVERSITY IN SODIUM AZIDE INDUCED WHEAT MUTANTS STUDIED BY SSR MARKERS

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Abstract: Mutations induced artificially way are one of the tools used to increase genetic variation in populations where genetic variation has been shrinking especially due to various reasons one of which is domestication. In this study, Simple Sequence Repeats (SSRs) markers were used to screen genetic diversity in sodium azide (NaN_3) induced fourteen fourth-generation advanced wheat mutant lines. The mean values of polymorphism rate (29.44%), polymorphic information content (PIC; 0.82), marker index (MI; 1.95) and resolving power (Rp; 1.31) were calculated according to SSR marker profiles. Two SSRs, Xwmc170 and Xcfd6, were detected as the most polymorphic markers, Xgwm626 proved the highest PIC and MI values, and Xcfd6 gave the highest Rp value. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram classified 15 plants into four groups. The Principle Component Analysis (PCA) showed 88.9% of the total genetic variation. The results obtained in the present study might be useful for determining the efficiency of NaN_3 for creating mutant wheat lines with enough genetic variability to implement wheat-breeding programs as germplasm resources.

Key words: Bread wheat, sodium azide mutagenesis, somatic embryogenesis, SSR markers, PCA, UPGMA.

Özet: Yapay yolla indüklenen mutasyonlar, genetik varyasyonun özellikle ıslah gibi çeşitli nedenlerden dolayı küçüldüğü popülasyonlarda, çeşitliliği arttıran araçlardan biridir. Bu çalışmada, sodyum azid (NaN_3) kullanılarak indüklenen on dört dördüncü jenerasyon ileri mutant buğday hatlarında, genetik çeşitliliği taramak için Basit Dizi Tekrarları (SSR) belirteçleri kullanıldı. SSR belirteç profillerine göre ortalama polimorfizm oranı (% 29,44), polimorfik bilgi içeriği (PIC; 0,82), belirteç indeksi (Mİ; 1,95) ve belirteç çözünürlük gücü (Rp; 1,31) hesaplandı. İki SSR belirteci, Xwmc170 ve Xcfd6, en yüksek polimorfizm oranına sahip belirteçler olarak tespit edildi. Xgwm626 en yüksek PIC ve Mİ değerlerini, Xcfd6 de en yüksek Rp değerini verdi. Ağırlıksız Çift-Grup Yöntemi ile Aritmetik Ortalama (UPGMA) dendrogramı 15 bitkiyi dört gruba ayırdı. Temel Bileşenler Analizi (PCA) toplam genetik varyasyonun % 88,9'unu gösterdi. Bu çalışma, buğday ıslah programlarında genetik kaynak olarak kullanılmak üzere yeterli genetik çeşitliliğe sahip mutant buğday hatlarını oluşturmak için sodyum azitin etkinliğinin gösterilmesi hususunda yararlı olabilir.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crops contributing substantially to food and nutritional security. Production and productivity of wheat are affected by several abiotic and biotic stress factors (Shewry 2009). Although some measures are taken to protect plant production and productivity against these stress factors, the ultimate solution is to develop novel lines, which are tolerant/resistant to these factors. In addition to the abiotic and biotic stress factors, loss of genetic diversity duo to monoculture farming or domestication is one of the distinct disadvantages, which put long-term yield production potential at risk. Grain yield is an important trait as it is considered as a measure of economic productivity in wheat. However, yield and its component traits are polygenic traits with low inheritance (Abdipour *et al*. 2016). On the other hand, induced genetic variability with mutation techniques brings about heritable changes in plants and offer new genetic varieties resistant to effects of climate change and tolerant to environmental stresses (www.iaea.org/topics/plant-breeding) or germplasm recourses to implement breeding programs. Chemical mutagens, like sodium azide (NaN_3) are frequently used in studies based on induced mutations (Al-Qurainy & Khan 2009). Sodium-azide generally induces mutation bias on AT→GC base pair substitutions resulting in amino acid changes which in turn change the function of proteins and alter phenotypes (Olsen *et al*. 1993). Wannajindaporn *et al*. (2014) reported that 28 Dendrobium mutants were generated after exposure to NaN_3 under *in vitro* conditions.

DNA based molecular markers can be used to investigate the effects of mutagens. Among the molecular markers, microsatellites or SSRs are multiallelic, chromosome-specific, evenly distributed along chromosomes, and have been developed and widely used for studies of wheat genetic diversity (Mason 2015) and genetic mapping, such as association to salt tolerance in *Triticum* species (Genc *et al*. 2010, Xu *et al*. 2013, Sardouie-Nasab *et al*. 2013, Ghaedrahmati *et al*. 2014, Turki *et al*. 2015).

The aim of the present study was to identify genetic diversity in fourteen-fourth generation candidate wheat mutants induced with NaN_3 application with SSR markers. Polymorphism rate (%), polymorphic information content (PIC), marker index (MI), and resolving power (Rp) were used to calculate according to SSR markers profile and the genetic distance among the candidate mutants was assessed by Principle Component Analysis (PCA) plots and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms.

Materials and Methods

Materials

Fourteen fourth generations advanced wheat mutant lines were used to screen genetic diversity via SSR markers. The cultivar used was the bread wheat cultivar Adana 99 (*Triticum aestivum* L. cv.) and was obtained from the Eastern Mediterranean Agricultural Institute in Adana, Turkey. To obtain the mutant lines, two different concentrations (3 and 4 mM) of NaN_3 (pH 3.5) were applied under *in vitro* conditions to embryonic calli for 30 minutes *in vitro* conditions to induce genetic variations on the genome. Treated and non-treated embryonic calli were screened for salinity tolerance by putting 125 mM NaCl in indirect regeneration media. Then, the mutant lines were segregated up to fourth generation by combining *in vitro* and *in vivo* NaCl screening techniques. The treatment concentrations of NaN_3 and NaCl were determined according to the results of preliminary experiments (Sen and Sarsu unpublished results).

Genetic variability analysis

DNA isolation

Frozen leaf bulks mixed from five plants from each mutant line (500 mg) were ground in a mortar and pestle with liquid nitrogen. The isolation of the genomic DNA was performed with the CTAB method (Doyle & Doyle 1990). The quality and quantity of the purified genomic DNA were estimated with spectrophotometry and gel electrophoresis. Each sample was diluted to 25 ngμL^{-1} in TE buffer and stored at 4°C.

Table 1. SSR primers, localized chromosome, total number of alleles, polymorphic alleles, polymorphism ratios, PIC, MI and Rp.

No	Primer Name	Localized Chromosomes	Total Number of alleles	Polymorphic alleles	Polymorphism rate (%)	PIC	MI	Rp
1	Xubc853b[a]	1B	3	1	33.33	0.50	0.50	0.93
2	Xgwm10[b]	2A	9	3	33.33	1.17	3.51	1.60
3	Xwmc261[b]	2A	6	3	50.00	1.03	3.09	1.47
4	Xgwm294b[b]	2A	8	2	25.00	0.94	1.88	1.33
5	Xfca2121b[b]	2A	8	2	25.00	0.94	1.88	1.33
6	Xwmc296[b]	2A	5	2	40.00	0.55	1.10	0.67
7	Xgwm515[b]	2A	9	3	33.33	0.83	2.49	1.33
8	Xgwm95[b]	2A	8	2	25.00	0.94	1.88	1.33
9	Xwmc170[be]	2A	5	3	60.00	1.26	3.78	2.04
10	Xwmc445d[a]	2B	4	2	50.00	0.80	1.60	1.20
11	Xwmc11[b]	3A	5	1	20.00	0.50	0.50	0.93
12	Xgwm403[c]	3A	8	0	0.00	0.00	0.00	0.00
13	Xgwm6[a]	3D	7	3	42.86	1.26	3.78	1.73
14	Xgwm538[a]	4B	9	2	22.22	0.82	1.64	1.33
15	Xwmc413[a]	4B	7	2	28.57	0.55	1.10	0.67
16	Xgwm194[b]	4B	6	2	33.33	0.69	1.38	0.80
17	Xgwm624[b]	4D	8	0	0.00	0.00	0.00	0.00
18	Xgwm540[d]	4D	9	3	33.33	0.76	2.28	1.07
19	Xcfd080[e]	5B	8	2	25.00	0.96	1.92	1.60
20	Xgwm626[d]	5D	6	3	50.00	1.40	4.20	1.86
21	Xgwm88[d]	5D	7	2	28.57	0.96	1.92	1.60
22	Xwmc416[b]	6A	4	1	25.00	0.50	0.50	1.07
23	Xwmc83[a]	6B	10	2	20.00	0.98	1.96	1.73
24	Xgwm4[c]	6B	8	3	37.50	0.92	2.76	1.73
25	Xcfd6[c]	6D	5	3	60.00	1.32	3.96	3.86
26	Xubc853b[a]	7A	9	2	22.22	0.80	1.60	1.20
27	Xgwm10[b]	7A	12	3	25.00	0.94	2.82	1.33
28	Xwmc261[b]	7A	4	1	25.00	0.50	0.50	1.07
	Total		**197**	**58**	**-**	**-**	**-**	**-**
	Mean		**7.04**	**2.07**	**29.44**	**0.82**	**1.95**	**1.31**

[a] (Xu *et al*. 2013), [b](Sardouie-Nasab *et al*. 2013), [c](Turki *et al*. 2015), [d](Ghaedrahmati *et al*. 2014), [e](Genc *et al*. 2010).

PCR Amplification

Genomic diversity was analyzed through 28 SSR markers previously determined to be related with salt tolerance (Table 1). In SSR analysis, the reaction mixture, 25 µL in total, consisted of 400nmol of forward and reverse SSR primers, 0.2mM of each nucleotide, 2.5 mM $MgCl_2$, 10x Polymerase Chain Reaction (PCR) buffer, 1 unit of *Taq*-DNA polymerase, and 75 ng of genomic DNA. The PCR conditions, separation of the amplified fragments on 2.5% agarose gel electrophoresis and gel staining were carried out following Shahzad *et al*. (2012).

Data analyses

Comparison of mutants was carried out based on the presence or absence of fragments produced by SSR primer amplifications. The Multivariate Statistical Package v3.1 (Kovach 1999) was used to estimate the genetic diversity with Nei and Li's similarity coefficients to construct a UPGMA dendrogram with a neighbor-joining algorithm (Nei & Li 1979) and control and mutant scores were plotted with a PCA from a matrix according to Nei and Li's (1979) genetic similarity. Dissimilarity (*D*) matrices were based on the equation $D=1-S$, where *S* is genetic similarity. PIC, MI, and Rp were calculated according to Anderson *et al*. (1993), Powell *et al*. (1996), and Prevost & Wilkinson (1999), respectively.

Results

SSR based diversity in wheat mutants

The PCR analysis showed that a total of 197 alleles were amplified and 58 of these were polymorphic (29.44%). The number of alleles per locus ranged from 3 (Xubc853b) to 12 (Xgwm10) with an average of 7.04 alleles per locus (Table 1).

The mean values of PIC, MI, and Rp were detected as a 0.82, 1.95, and 1.31, respectively. The highest PIC and MI values were obtained with the primer Xgwm626 and the highest Rp value was obtained with the primer Xcfd6. Most of the PIC values were recorded above 0.5, indicating that they were informative and useful (Table 1).

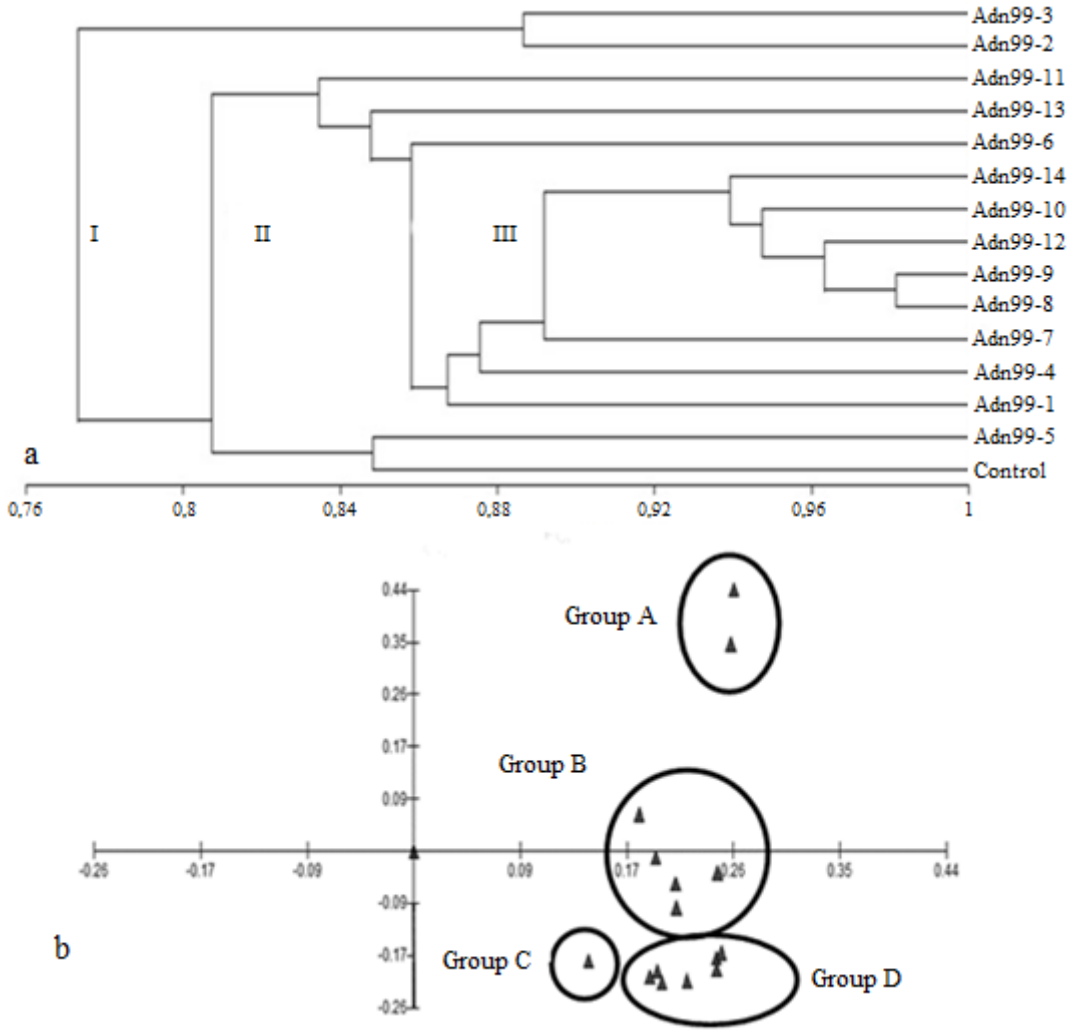

Fig. 1. UPGMA dendrogram of SSR data using Nei and Li's coefficient (a) and PCA plot of SSR data (b) showing relationships between the control and the mutant lines.

Table 2. The genetic distances between the control and the mutant lines based on Nei and Li's coefficient using banding patterns of SSR primers.

Experimental Groups	Control	Adn99-1	Adn99-2	Adn99-3	Adn99-4	Adn99-5	Adn99-6	Adn99-7	Adn99-8	Adn99-9	Adn99-10	Adn99-11	Adn99-12	Adn99-13	Adn99-14
Control	0.000														
Adn99-1	0.174	0.000													
Adn99-2	0.255	0.236	0.000												
Adn99-3	0.223	0.189	0.113	0.000											
Adn99-4	0.149	0.179	0.274	0.228	0.000										
Adn99-5	0.152	0.183	0.264	0.200	0.142	0.000									
Adn99-6	0.168	0.150	0.248	0.216	0.142	0.161	0.000								
Adn99-7	0.197	0.148	0.274	0.243	0.139	0.174	0.125	0.000							
Adn99-8	0.198	0.116	0.213	0.182	0.124	0.175	0.143	0.107	0.000						
Adn99-9	0.181	0.098	0.227	0.164	0.106	0.158	0.125	0.089	0.019	0.000					
Adn99-10	0.216	0.117	0.231	0.167	0.142	0.193	0.161	0.125	0.056	0.037	0.000				
Adn99-11	0.284	0.189	0.223	0.173	0.212	0.263	0.232	0.196	0.131	0.112	0.096	0.000			
Adn99-12	0.203	0.123	0.250	0.187	0.114	0.149	0.133	0.097	0.046	0.028	0.064	0.138	0.000		
Adn99-13	0.231	0.169	0.308	0.248	0.176	0.240	0.210	0.192	0.130	0.113	0.132	0.202	0.137	0.000	
Adn99-14	0.210	0.147	0.257	0.211	0.122	0.188	0.157	0.122	0.055	0.054	0.073	0.145	0.062	0.112	0.000

Table 3. Eigenvectors, eigenvalues, and percentage of variance for six principal components based on SSR data from the control and the mutant lines.

	Eigenvectors					
Genotype	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**	**PC 6**
Control	0.185	0.062	-0.498	-0.127	-0.313	0.133
Adn99-1	0.215	-0.055	0.013	-0.563	0.059	-0.065
Adn99-2	0.263	0.437	0.074	0.092	0.123	0.377
Adn99-3	0.261	0.346	0.100	0.071	-0.085	-0.394
Adn99-4	0.200	-0.201	-0.229	0.366	-0.025	-0.005
Adn99-5	0.198	-0.011	-0.295	0.093	0.243	-0.513
Adn99-6	0.216	-0.094	-0.176	-0.052	0.420	0.106
Adn99-7	0.194	-0.211	-0.025	-0.016	0.435	0.174
Adn99-8	0.248	-0.178	0.143	0.019	0.007	0.139
Adn99-9	0.249	-0.199	0.121	-0.005	-0.024	-0.011
Adn99-10	0253	-0.170	0.201	-0.019	-0.114	-0.041
Adn99-11	0.249	-0.037	0.323	0.085	-0.170	-0.033
Adn99-12	0.225	-0.216	0.119	0.060	0.111	-0.091
Adn99-13	0.143	-0.184	0.174	-0.033	-0.512	0.106
Adn99-14	0.204	-0.220	0.157	0.120	-0.091	0.124
Eigen-values	1.883	0.349	0.286	0.157	0.109	0.096
Percentage	58.137	10.765	8.882	4.851	3.386	2.956
Cumulative %	58.137	68.902	77.721	82.573	85.940	88.896

Phylogenetic relationship as revealed by SSR markers

The data from all 15 plants and 197 alleles produced by SSR primers were compiled and analyzed using Nei and Li's coefficient. The analysis showed that genetic differences between pairs of mutants ranged from 0.019 (Adn99-8 and Adn99-59) to 0.308 (Adn99-13 and Adn99-2) with an average difference value of 0.203 between control and mutant lines (Table 2).

The results obtained with the cluster analysis are given in Fig. 1a. The UPGMA dendrogram classified the mutants into four major clusters, the first with two mutant lines (Adn99-2 and Adn99-3), the second with three mutant lines (Adn99-11, Adn99-13 and Adn99-5) and the control, the third with three mutant lines (Adn99-6, Adn99-1, and Adn99-4) and the fourth with six mutant lines (Adn99-7, Adn99-8, Adn99-9, Adn99-10, Adn99-12 and Adn99-14).

In order to complement the cluster analysis, a PCA plot was constructed with SSR data. Eigenvectors, eigenvalues, and proportion of accounted variance for each variable are shown in Table 3. The Principle Component (PC) 1 had an eigenvalue of 1.883, the PCs 2, 3, 4, 5, and 6 had eigenvalues of 0.349, 0.286, 0.157, 0.109, and 0.096, respectively. The first six variables accounted for 88.896% of the total variance (Table 3). The control and the mutant lines were grouped into four groups (termed as group A-D) according to the six principal components (Fig. 1b). Group A had two mutant lines (Adn99-2 and Adn99-3), group B had five mutant lines (Control, Adn99-1, Adn99-5, Adn99-6, and Adn99-11), Group C had one mutant line (Adn99-13), and Group D had seven mutant lines (Adn99-4, Adn99-7, Adn99-8, Adn99-9, Adn99-10, Adn99-12 and Adn99-14). Both the cluster analysis and the principle component analysis classified the control and the mutant lines in four clusters/groups but the groupings were not identical.

Discussion

In this study, fourteen fourth-generation advanced mutant lines were used to screen genetic diversity induced by NaN_3 application via SSR markers. Chemical mutagens tend to cause random point mutations (transitions and transversions) and small insertions/deletions (InDels) in whole target genome. As a result, loss- or gain-of-function mutations can occur leading to formation of different phenotypes. A number of techniques have been developed to detect natural or mutagen induced Single Nucleotid Polymorphisms (SNPs) and InDels in whole genome. Among these techniques, PCR based molecular marker techniques have been widely used in genotyping in mutants. For instance, Wannajindaporn *et al*. (2014) reported a 22.5% polymorphism ratio in NaN_3 induced *Dendrobium* "Earsakum" mutants using inter simple sequence repeats (ISSR) markers. Polymorphism ratio in our present study was measured as 29.44%.

Marker attributes such as PIC, MI, and Rp indicate the informativeness of primer combinations and are widely used in genetic diversity studies in natural populations

instead of mutant lines. The average PIC and MI values for Turkish wheat varieties, as revealed by SSR markers, were detected as 0.52 and 1.84, respectively (Akfirat & Uncuoglu 2013). These average values are lower than values determined in our study. Turki *et al*. (2015) reported that wmc633 detected the highest number of alleles and PIC value among 119 durum wheat varieties. The average of PIC, MI and Rp values for Iranian wheat cultivars and breeding lines were calculated as 0.22, 1.34 and 12, respectively using 10 ISSR markers (Najaphy *et al.* 2011).

Wu *et al. et al*. (2011) reported in their study based on ISSR molecular maker technique that NaN_3 applied *Chlorophytum* showed genetic differences ranging from 0.141 to 0.402 with an average of 0.2715. In the Turkish wheat population, the rate of genetic differences was reported between 0.367 and 0.521 (Akfirat & Uncuoglu 2013) and these values are higher genetic difference values determined in the present study.

PCA is a multivariate analysis method that allows the simultaneous evaluation of numerous variables, reduces variations to a manageable level, and visualizes the results in 2- or 3-dimensional plots for interpretation and analysis. In other words, PCA makes it possible to extract rich information from genetic data, assign individuals to the groups, make visual assessments of between-population differences, and assess the contribution of individual alleles to population structuring (Lasalita-Zapico & Aguilar 2014). In our study, the results of both UPGMA and PCA classified the control and mutant lines in four major groups but the groupings were not identical. Akfirat & Uncuoglu (2013) reported that UPGMA based on genetic distance estimates produced two groupings according to SSR data results.

In conclusion, overall results demonstrated that chemical mutagenesis has an effect on generating mutant lines for implementing genetic resource in wheat breeding programs.

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Research Article

ANALYSIS OF ATMOSPHERIC POLLEN GRAINS IN DURSUNBEY (BALIKESİR), TURKEY

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Abstract: In this study, airborne pollen grains in the atmosphere of Dursunbey (Balıkesir, Turkey) were collected using a gravimetric method. The pollen grains were investigated by light microscopy and a total of 6265 pollen grains per cm^2 were counted. 42 different pollen types were identified of which 24 belonged to the arboreal plants (86.17% of the annual pollen index) and 18 to non-arboreal plants (13.16% of the annual pollen index). A small portion of the pollens (42 grains, 0.67%) were not identified. The most frequent pollen types, which constituted more than 1% of annual pollen count were regarded as the predominating pollen types for the region. The predominating group was determined to be consisted of pollens of *Pinus* L. (48.23%), Cupressaceae Rich. ex Bartl./Taxaceae Gray (16.74%), Poaceae Barnhart (8.32%), *Quercus* L.(5.31%), *Acer* L. (4.07%), *Platanus* L. (3.10%), *Juglans* L. (2.26%), *Abies* Mill. (1.75%), *Plantago* L. (1.25%), Amaranthaceae Juss. (1.22%) and *Olea europaea* L. (1.16%). The highest pollen count was determined in May and it is striking that most of the determined predominant pollen types have previously been reported as main causes of pollinosis.

Key words: Pollen fall, atmospheric monitoring, gravimetric method, pollen calendar, allergy.

Özet: Bu çalışmada, Dursunbey (Balıkesir, Türkiye) atmosferindeki polenler gravimetrik yöntem kullanılarak toplanmıştır. Toplanan polenler ışık mikroskobu ile incelenmiş ve cm^2 başına toplam 6265 polen sayılmıştır. 24 tanesi odunsu bitkilere (yıllık polen indeksinin % 86,17'si), 18 tanesi ise otsu bitkilere (yıllık polen indeksinin %13,16'sı) ait olan toplam 42 farklı polen tipi tanımlanmıştır. Polenlerin az bir kısmı ise (42 polen, %0.67) tanımlanmamıştır. Sıklıkla rastlanılan ve yıllık toplam polen sayısının %1'inden fazlasını oluşturan polen tipleri bölge için dominant polen tipleri olarak kabul edilmiş olup *Pinus* L. (%48,23), Cupressaceae Rich. ex Bartl./Taxaceae Gray (%16,74), Poaceae Barnhart (%8,32), *Quercus* L. (%5,31), *Acer* L. (%4,07), *Platanus* L. (%3,10), *Juglans* L. (%2,26), *Abies* Mill. (%1,75), *Plantago* L. (%1,25), Amaranthaceae Juss. (% 1,22) ve *Olea europaea* L. (%1,16) polenleri atmosferik polen spektrumunun ana bileşenleri olarak kaydedilmiştir. Atmosferdeki en yüksek polen miktarı Mayıs ayında tespit edilmiş olup, belirlenen dominant polen tiplerinin çoğunun daha önce polinosisin ana nedenleri olarak rapor edilmeleri dikkat çekicidir.

Introduction

Pollen is the male gametophyte that plays an important role in pollination of flowering plants. The movements of pollens from one flower to the others can take place through different ways but wind and insects are the two vectors with the greatest share in pollination. Atmospheric occurences of pollens of wind pollinated plants are considered to be very important in terms of human health. Pollens found in the atmosphere may cause allergic symptoms in susceptible individuals like mucous membrane irritation, chronic bronchitis, allergic rhinitis and asthma, extrinsic allergic alveolitis (hypersensitivity pneumonitis), inhalation fever, humidifier fever or organic dust toxic syndrome, and immunological response impairment (Lacey & Dutkiewicz 1994). It has been reported that the ratio of individuals complaining from pollinosis in Europe reached up to 40% (D'Amato *et al.* 2007). The changing meteorological conditions and climate along with the increasing air pollution in urbanized areas increase the allergenicity of pollen grains in the atmosphere. For this reason, pollen calendars have been prepared in many countries (Giner *et al.* 2002, Peternel *et al.* 2003, Ianovici *et al.* 2013, Ribeiro & Abreu 2014, Puljak *et al.* 2016) and in Turkey (Bicakci *et al.* 2002, Guvensen & Ozturk 2003, Altunoglu *et al.* 2008, Tosunoglu *et al.* 2009, Çeter *et al.* 2011, Tosunoglu & Bicakci 2015, Uguz *et al.* 2018).

This study was performed *i*) to determine airborne pollen types and their densities in the atmosphere of

Dursunbey in Balıkesir province in Turkey, *ii*) to show seasonal variations of pollen types and *iii*) to prepare a pollen calendar for the sampling area.

Materials and Methods

The study area

Dursunbey is located at 39° 34.8' N, 28° 37.8' E in northwest of Turkey at an altitude of 639 m above sea level. It covers an area in the eastern part of Balıkesir and geographically occupy a place in Marmara region of the country. The hilly characteristic of the study area is notable and most parts of the area is covered with *Pinus nigra* Arn. forests, making Dursunbey a famous region with its timber and a well-known exporter of it. The region has a Mediterranean climate type and the floristic structure shows transitional features between Euro-Siberian and Mediterranean phytogeographic regions. The major vegetation in the study area and its surroundings consists of *Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe, *Abies nordmanniana* (Stev.) subsp. *bornmuelleriana* (Mattf.) Coode & Cullen, *Cupressus sempervirens* L., *Juniperus communis* L. subsp. *saxatilis* Pall., *Juniperus oxycedrus* L. subsp. *oxycedrus*, *Alnus glutinosa* (L.) Gaertner subsp. *antitaurica* Yalt., *Carpinus betulus* L., *Carpinus orientalis* Miller, *Fagus orientalis* Lipsky., *Fagus sylvatica* L., *Quercus pubescens* Willd., *Quercus cerris* L. var. *cerris* L., *Corylus avellana* L., *Populus tremula* L., *Fraxinus excelsior* L., *Fraxinus ornus* L., *Acer campestre* L., *Ulmus glabra* (Hudson), *Cistus laurifolius* L., *Arbutus unedo* L., and *Robinia pseudoacacia* L. (Dirmenci 2006, Açar & Satıl 2014). Afforestation areas of *Cedrus libani* A. Rich. can also be seen. The southern parts are are dominated by Mediterranean maquis elements in addition to the natural vegetation of *Acer* sp., *Betula* sp., *Cupressus arizonica* Green, *Elaeagnus angustifolia* L., *Juglans regia* L., *Malus domestica* Borkh., *Morus* sp., *Olea europaea* L., *Platanus orientalis* L., *Populus* sp., and *Prunus* species as members of parks, gardens, and streets.

Palynological study

A Durham sampler was used as the gravimetric sampler which was operated from January to December 2012. The sampler was placed on the roof of a building at a height of 9 m above ground level. The slides of the sampler were covered with glycerine jelly mixed with basic fuchsin (Charpin & Surinyach 1974) before exposure and were changed weekly with new ones. Weekly slides were examined by light microscopy (Olympus BX51 trinocular light microscope) and the raw data was converted to pollen number in cm^2. For pollen assignments, Uludağ University Palynology Laboratory reference collection was used.

Results

A total of 6265 pollen grains from 42 taxa were recorded in the atmosphere of Dursunbey annually. Most of the pollen grains (5399 grains of 24 taxa, 86.17%) were found to be arboreal, while 824 pollen grains of 18 taxa (13.16%) were non-arboreal. A small portion of the pollens (42 grains, 0.67%) were unidentified (Table 1).

Pollen grains were recorded every month except December (Fig. 1, Table 2). During the first six months of the sampling, arboreal pollen grains dominated the atmosphere, and in July, August, September and October non-arboreal grains were dominant. Arboreal grains were not sampled in November and non-arboreal grains were not sampled during the first three months (Fig. 1, Table 2). The most common arboreal pollen producers were found as *Pinus* (48.23%), Cupressaceae/Taxaceae (16.74%), *Quercus* (5.31%), *Acer* (4.07%), *Platanus* (3.10%), *Juglans* (2.26%), *Abies* (1.75%), *Olea* (1.16%) constituting 82.62% of the total pollen number (Fig. 3, Table 1). The most frequently recorded non-arboreal pollen grains belonged to Poaceae (8.32%), *Plantago* (1.25%) and Amaranthaceae (1.22%), which constituted 10.73% of the total pollen number (Fig. 2, Table 1).

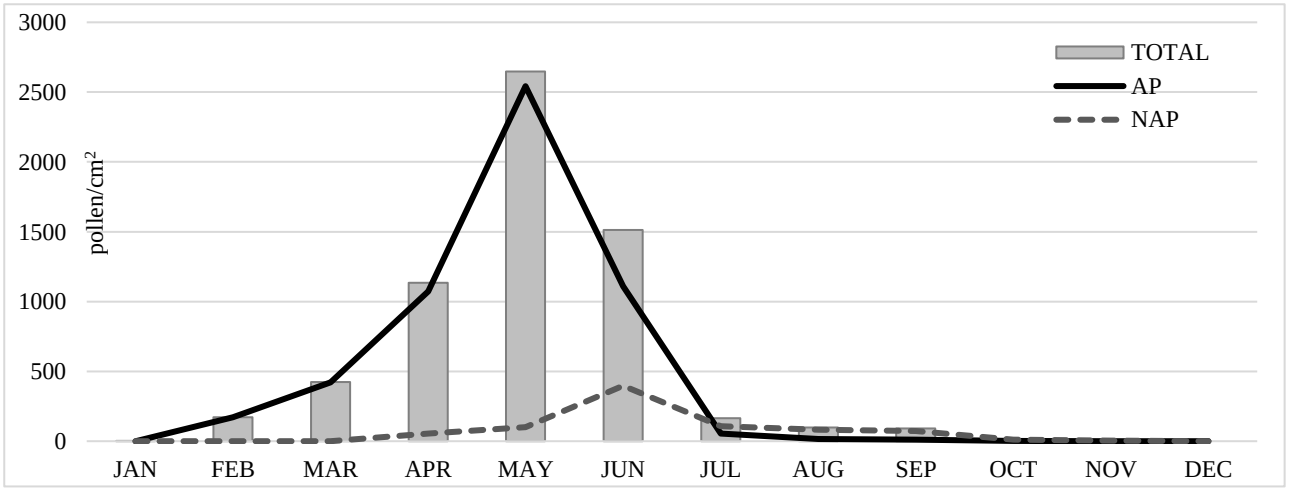

Fig. 1. Monthly distribution of the pollen grains sampled based on their numbers per cm^2. AP; arboreal pollen grains, NAP; non-arboreal pollen grains.

Table 1. The annual pollen numbers and percentages of arboreal plants (AP) and non-arboreal plants (NAP) sampled in the atmosphere of Dursunbey.

ARBOREAL PLANTS	Annual number	%
Pinus	3022	48.23
Cupressaceae/Taxaceae	1049	16.74
Quercus	332	5.31
Acer	255	4.07
Platanus	194	3.10
Juglans	142	2.26
Abies	110	1.75
Olea europaea	73	1.16
Fagus	42	0.67
Fraxinus	37	0.58
Alnus	31	0.49
Populus	31	0.49
Betula	20	0.31
Ericaceae	14	0.22
Pistacia	14	0.22
Carpinus	6	0.10
Rosaceae	5	0.09
Tilia	5	0.08
Ulmus	5	0.07
Morus	4	0.06
Salix	4	0.06
Cedrus	3	0.05
Corylus	2	0.03
Acacia	1	0.02
Total (AP)	**5399**	**86.17**
NON-ARBOREAL PLANTS	**Annual number**	**%**
Poaceae	521	8.32
Plantago	78	1.25
Amaranthaceae	76	1.22
Xanthium	34	0.54
Boraginaceae	19	0.30
Apiaceae	17	0.28
Asteraceae	13	0.21
Lamiaceae	11	0.17
Fabaceae	10	0.16
Urticaceae	10	0.15
Taraxacum	8	0.13
Brassicaceae	7	0.10
Artemisia	6	0.09
Campanulaceae	5	0.08
Juncaceae	4	0.07
Cyperaceae	3	0.04
Papaveraceae	2	0.03
Caryophyllaceae	1	0.02
Total (NAP)	**824**	**13.16**
Unidentified	42	0.67
TOTAL	**6265**	**100.00**

The most common arboreal pollen producers were found as *Pinus* (48.23%), Cupressaceae/Taxaceae (16.74%), *Quercus* (5.31%), *Acer* (4.07%), *Platanus* (3.10%), *Juglans* (2.26%), *Abies* (1.75%), *Olea* (1.16%) constituting 82.62% of the total pollen number (Fig. 3, Table 1). The most frequently recorded non-arboreal pollen grains belonged to Poaceae (8.32%), *Plantago* (1.25%) and Amaranthaceae (1.22%), which constituted 10.73% of the total pollen number (Fig. 2, Table 1).

The earliest airborne pollen grain recorded with 1 pollen (0.01% of the total pollen grains) was in January and belonged to *Alnus* (Table 2). In February, 172 pollen grains, constituting 2.75% of the annual count, were recorded. The dominating pollen grains (1.88%) in February belonged to Cupressaceae/Taxaceae (Table 2). The number of airborne pollen grains and the number of pollen types these grains belonged to increased in March and April with 424 (6.77%) and 1134 pollens (18.09%), respectively. Dominating taxa were Cupressaceae/Taxaceae (5.44%) in March and *Pinus* (6.55%), Cupressaceae/Taxaceae (6.47), *Platanus* (1.61%), and *Quercus* (1.22%) in April (Table 2).

The highest pollen level during the sampling period was recorded in May with 2648 pollen grains (42.26%) originating from *Pinus* (26.02%), *Quercus* (3.64%), *Acer* (3.61%), Cupressaceae/Taxaceae (2.35%), *Juglans* (1.93%), *Platanus* (1.49%), and Poaceae (1.34%) (Fig. 1, Table 2). The highest pollen load of the air was observed in the 3rd week of May (20th week) with 894 pollen grains. In June, during which 1514 pollen grains were recorded, the dominating pollen grains belonged to *Pinus* (14.54%), Poaceae (4.70%), and *Olea* (1.02%) making 24.16% of the total pollen load. The numbers of pollen grains started to decrease by July and the decrease continued in August and September. In July, 165 pollen grains were recorded (2.63%) followed by 99 pollen grains (1.58%) in August and 90 pollen grains in September (Table 2). The pollen load was very low in October (0.23%) and November (0.07%) and no pollen grains were recorded in December (Fig. 1, Table 2).

Pollen types that comprised more than 1% of the annual total pollen number were considered to be dominant. Weekly variations of these dominated taxa are shown in Fig. 3 and pollen calendar that shows pollination seasons of all identified taxa are shown in Fig. 4.

The pollen season of *Pinus* started by the beginning of March (9th week) and lasted in the 4th week of September (38th week). The highest level of *Pinus* pollens was recorded in the 4th week of April (17th week) with 558 pollen grains (Figs. 3-4). Cupressaceae/Taxaceae pollens started to appear in the 1st week of February (5th week), the peak value was recorded in the 4th week of April with 267 pollen grains and disappearance took place in the 3rd week of October (42nd week) (Figs. 3-4). The relatively short pollen season of *Quercus* started in the 1st week of April (14th week) and lasted in the 3rd week of June. The highest levels of oak pollen grains were recorded in the 2nd week of May (19th week) with 112 grains (Figs. 3-4). Pollen grains of *Acer* were found as a predominating pollen type with an annual percentage of 4.07 (Table 1).

Table 2. Monthly percentages of airborne pollen grains of arboreal plants (AP) and non-arboreal plants (NAP)in the atmosphere of Dursunbey.

		JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
ARBOREAL PLANTS	*Abies*	-	-	-	0.07	0.58	0.97	0.10	0.04	-	-	-	-
	Acacia	-	-	-	0.02	-	-	-	-	-	-	-	-
	Acer	-	0.04	0.13	0.26	3.61	0.03	-	-	-	-	-	-
	Alnus	0.01	0.45	0.04	-	-	-	-	-	-	-	-	-
	Betula	-	0.22	0.09	-	-	-	-	-	-	-	-	-
	Carpinus	-	-	0.06	0.03	-	-	-	-	-	-	-	-
	Cedrus	-	-	-	-	-	-	-	-	0.05	-	-	-
	Corylus	-	0.01	0.02	-	-	-	-	-	-	-	-	-
	Cup./Taxaceae	-	1.88	5.44	6.47	2.35	0.46	0.07	0.02	0.03	0.02	-	-
	Ericaceae	-	-	-	0.05	0.10	0.05	-	-	0.02	-	-	-
	Fagus	-	-	-	0.43	0.24	-	-	-	-	-	-	-
	Fraxinus	-	0.04	0.21	0.02	0.31	-	-	-	-	-	-	-
	Juglans	-	-	-	0.27	1.93	0.05	-	-	-	-	-	-
	Morus	-	-	-	0.05	0.02	-	-	-	-	-	-	-
	Olea europaea	-	-	-	-	0.14	1.02	-	-	-	-	-	-
	Pinus	-	-	0.20	6.55	26.02	14.54	0.67	0.18	0.08	-	-	-
	Pistacia	-	-	0.02	0.06	0.10	0.04	-	-	-	-	-	-
	Platanus	-	-	-	1.61	1.49	-	-	-	-	-	-	-
	Populus	-	0.08	0.41	-	-	-	-	-	-	-	-	-
	Quercus	-	-	-	1.22	3.64	0.44	-	-	-	-	-	-
	Rosaceae	-	-	-	-	-	0.07	0.02	-	-	-	-	-
	Salix	-	-	0.05	0.01	-	-	-	-	-	-	-	-
	Tilia	-	-	-	-	0.05	0.03	-	-	-	-	-	-
	Ulmus	-	0.02	0.06	-	-	-	-	-	-	-	-	-
	Total (AP)	**0.01**	**2.73**	**6.72**	**17.13**	**40.58**	**17.71**	**0.86**	**0.24**	**0.17**	**0.02**	**-**	**-**
NON-ARBOREAL PLANTS	*Artemisia*	-	-	-	-	-	-	-	0.05	0.04	-	-	-
	Boraginaceae	-	-	-	-	-	0.23	0.05	0.02	-	-	-	-
	Campanulaceae	-	-	-	0.05	0.02	0.01	-	-	-	-	-	-
	Caryophyllaceae	-	-	-	0.02	-	-	-	-	-	-	-	-
	Amaranthaceae	-	-	-	-	-	0.13	0.23	0.33	0.42	0.07	0.03	-
	Asteraceae	-	-	-	-	0.04	0.06	0.04	0.03	0.03	0.02		
	Berassicaceae	-	-	-	0.02	0.02	0.03	0.02	0.02	-	-	-	-
	Cyperaceae	-	-	-	-	-	0.02	0.02	-	-	-	-	-
	Poaceae	-	-	-	0.64	1.34	4.70	0.77	0.39	0.36	0.08	0.04	-
	Juncaceae	-	-	-	0.02	0.03	0.02	-	-	-	-	-	-
	Lamiaceae	-	-	-	0.02	0.01	0.02	0.10	0.02	-	-	-	-
	Fabaceae	-	-	-	0.03	0.02	-	0.05	0.03	0.03	-	-	-
	Papaveraceae	-	-	-	-	-	0.03	-	-	-	-	-	-
	Plantago	-	-	-	0.06	0.08	0.79	0.24	0.08	-	-	-	-
	Taraxacum	-	-	-	0.02	-	0.02	0.04	0.02	0.05	-	-	-
	Apiaceae	-	-	-	0.02	-	0.14	0.10	0.02	-	-	-	-
	Urticaceae	-	-	-	-	-	0.10	0.05	-	-	-	-	-
	Xanthium	-	-	-	-	-	-	-	0.31	0.23	-	-	-
	Total (NAP)	**-**	**-**	**-**	**0.87**	**1.56**	**6.31**	**1.71**	**1.31**	**1.16**	**0.17**	**0.07**	**-**
	Unidentified	-	0.02	0.05	0.10	0.13	0.14	0.06	0.03	0.10	0.05	-	-
	TOTAL	**0.01**	**2.75**	**6.77**	**18.09**	**42.26**	**24.16**	**2.63**	**1.58**	**1.44**	**0.23**	**0.07**	**-**

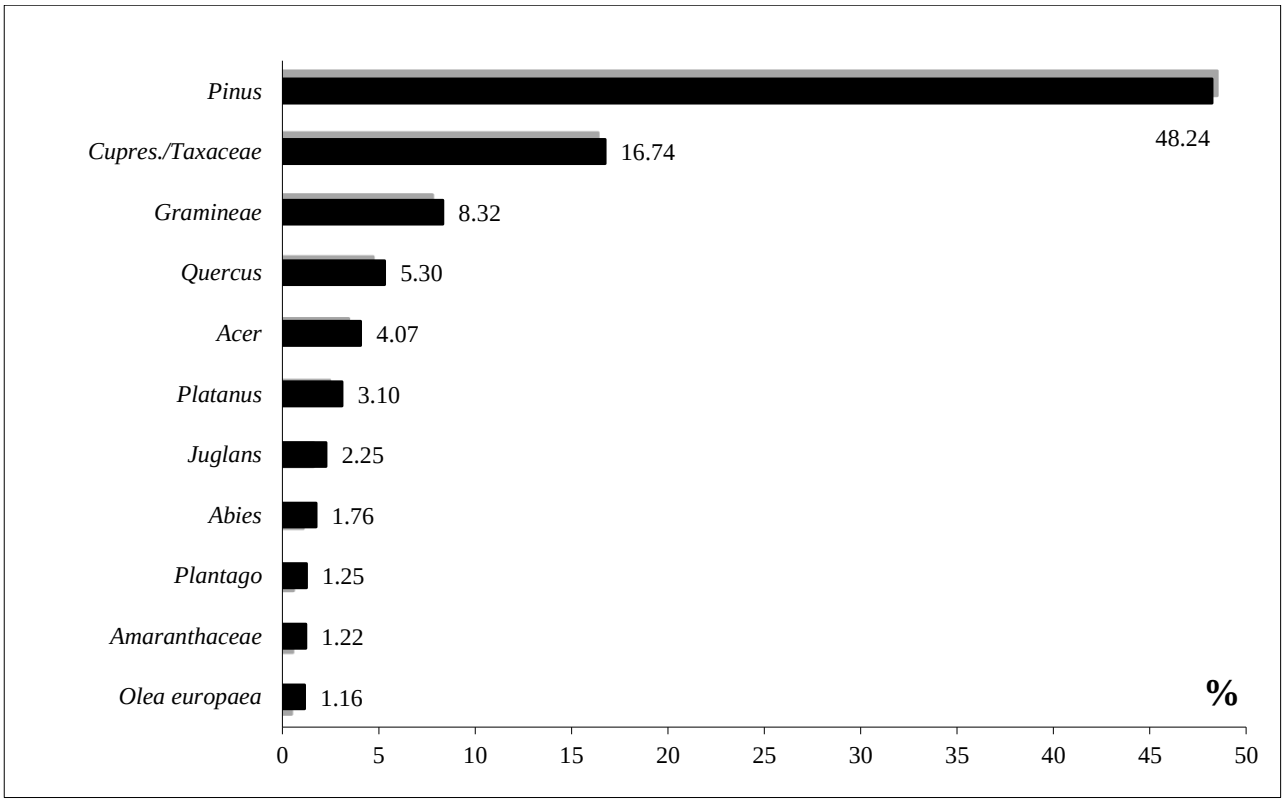

Fig. 2. The annual percentages of main pollen types determined in the atmosphere of Dursunbey.

The pollen season of *Acer* started in the 2nd week of February (6th week) and lasted in the 1st week of June (22nd week). The highest number of *Acer* pollen grains (117 pollen grains) were recorded in the 3rd week of May (20th week) (Figs. 3-4). The pollen season of *Platanus* was very short and started in the 2nd week of April and lasted in the 4th week of May (21st week). The highest pollen number of this genus (73 pollen grains) was recorded in the 4th week of April (Figs. 43-4). The pollen season of *Juglans* began in the 3rd week of April (16th week) and lasted by the beginning of June. The highest levels of *Juglans* pollens were observed in the 2nd week of May with 66 pollen grains (Figs. 3-4).

Juglans appeared to be one of the main pollen producers with an annual value of 2.26%. The pollen grains of *Abies* were sampled from the 3rd week of April until the 3rd week of August (33rd week). Maximum pollen load of *Abies* was recorded in the last week of June (26th week) with 32 pollen grains (Figs. 3-4). The last dominating pollen type was of *Olea europaea* and showed a very short pollen season, which started in the 4th week of May (2st week) and lasted in the last week of June. The highest level of *Olea europaea* pollen grains (39 grains) was recorded in the 1st week of June (Figs. 3-4).

Poaceae was the most common herbaceous plant whose pollen grains started to be seen from the 2nd week of April (15th week) to the end of November (47th week) with its highest value in the 3rd week of June (24th week) with 92 pollen grains (Figs. 3-4). The pollen season of *Plantago* started in the 2nd week of April and lasted at the end of August. The highest number of *Plantago* pollen grains was recorded in the 3rd week of June with 16 pollen grains (Figs. 3-4). The pollen season of the family Amaranthaceae was long and started by the beginning of June and lasted in the 1st week of November (44th week). The highest number of pollen grains (10 grains) was recorded in the 1st week of September (35th week) (Figs. 3-4).

Discussion

Airborne pollen grains of arboreal plants were found to be predominant in the atmosphere of Dursunbey with a ratio of 86.17% most probably as a result of the geographical location, climate and vegetation of the study area. The same dominancy for arboreal pollen types have previously been reported in Balıkesir (70.92%) (Bicakci and Akyalcin 2000), Gemlik (82.39%) (Saatcioglu *et al.* 2011), Konya (61.29%) (Kizilpinar *et al.* 2012) and Büyükorhan (87.46%) (Tosunoglu *et al.* 2015b) in Turkey and in Zagreb (69.14%) (Peternel *et al.* 2003) in Crotia.

The pollen grains of pine was the most frequent pollen type during the investigation period due to widespread pine forests present at higher altitudes of the study area and its surroundings. Pine pollen has previously been recorded as a predominated pollen type with high percentages also in İzmir (57.30%) (Guvensen *et al.* 2003), Sivrihisar (48.13%) (Potoglu Erkara 2008), Köyceğiz (48.01%) (Tosunoglu *et al.* 2009) and Sakarya (14.10%) (Bicakci 2006) in Turkey. Pine pollens were commonly identified at family level in many

aerobiological studies and were thought as a group of vesiculate type pollen grains because of their similar and low allergenic potential. On the contrary, there are different hypotheses about the remarkable allergenicity in high levels of Pinaceae pollen in the air (Harris & German 1995, Marcos *et al.* 2001). Pollen grains of *Abies*, another conifer genus, were also found to dominate the total pollen load of atmosphere of our study area. Similarly, *Abies* pollen grains have previously been to predominate in Savaştepe (Bilisik *et al.* 2008b), Bilecik (Türe & Böcük 2009) and Büyükorhan (Tosunoglu *et al.* 2015b) in Turkey.

Cupressaceae/Taxaceae type pollens were shown to be an important aeroallergen and a major cause of winter and early spring pollinosis around the Mediterranean basin (Charpin 2005, D'Amato *et al.* 2007). Cupressaceae/Taxaceae pollen was the second dominating pollen type in our study and previously was reported as the main pollen type in Balıkesir (15.73%) (Bicakci & Akyalcin 2000), Kuşadası (30.04%) (Tosunoğlu *et al.* 2013), Büyükorhan (20.69%) (Tosunoglu *et al.* 2015b) and Antalya (38.33%) (Tosunoglu *et al.* 2015a) in Turkey and in Cax do Sul (7.7%) (Vergamini *et al.* 2006) in Brazil.

Quercus, one of the main allergenic pollen types (Spieksma 1990, D'Amato *et al.* 1991) was found as a predominated pollen type with the annual percentage of 5.31% in the atmosphere of our study area (Table 1). *Quercus* has previously been reported as the main pollen type from many regions in Turkey including Çanakkale (9.28%) (Guvensen *et al.* 2005), Karabük (5.89%) (Kaplan and Özdoğan 2015), Fethiye (2.34%) (Bilisik *et al.* 2008a), Antalya (4.58%) (Tosunoglu *et al.* 2015a) and Bodrum (15.95%) (Tosunoglu & Bicakci 2015). Pollen grains of *Platanus* are another important allergen (Subiza *et al.* 1994, Varela *et al.* 1997) and were also reported as a predominant pollen type from Kuşadası (Tosunoglu *et al.* 2013), Denizli (Guvensen *et al.* 2013) and Antalya in Turkey (Tosunoglu *et al.* 2015a). . *Olea europaea* has been reported as a predominated pollen type and the main cause of pollinosis around the Mediterranean basin (D'Amato & Liccardi 1994, Liccardi *et al.* 1996, Diaz de la Guardia *et al.* 2003, Gioulekas *et al.* 2004). On the other hand, *Acer* and *Juglans* pollen were not reported as highly allergenic pollen types in previous studies (Esch *et al.* 2001, D'Amato *et al.* 2007).

Poaceae, Amaranthaceae and *Plantago* pollens were the most frequent herbaceous pollen types in Dursunbey atmosphere. Poaceae pollens have previously been reported as an important aeroallergen (Bousquet *et al.* 1984, D'Amato & Spieksma 1992, D'Amato *et al.* 2007, Mandal *et al.* 2008). Pollens of members of this family are frequently seen in high levels during summer periods according to the variable vegetation period in many studies and were reported as a predominant pollen type in Kayseri(20.44%) (Ince *et al.* 2004), Yalova (10.01%) (Altunoglu *et al.* 2008), Gemlik (10.67%) (Saatcioglu *et al.* 2011) and Van (20.94%) (Bicakci *et al.* 2017) in Turkey and in Calcutta (12.98%) (Mandal *et al.* 2008) in India. *Plantago* pollens were mostly reported as an important cause of pollinosis (Bicakci *et al.* 2011) and were found to predominate in the atmosphere of our study region, as in the case reported in previous studies (Guvensen *et al.* 2005, Altunoglu *et al.* 2008, Tosunoglu & Bicakci 2015). Another important aeroallergen is Amaranthaceae pollens (Fang *et al.* 2001) which were found to dominate in the atmosphere of our study area with a percentage of 1.22% (Table 1).

The results of former studies in various countries showed that *Platanus*, Poaceae, *Acer*, *Cupressus*, Chenopodiaceae, Urticaceae, *Morus*, *Plantago* and Oleaceae pollens were the dominant pollen types in Santiago, Chile (Villegas & Nolla 2001), *Betula*, *Corylus*, *Ambrosia*, Urticaceae pollens in Zagreb, Croatia (Peternel *et al.* 2003), Cupressaceae, Pinaceae, Urticaceae, Anacardiaceae, Oleaceae and Polygonaceae pollens in Cagliari, Italy (Ballero & Maxia 2003), Taxus/Cupressaceae, *Quercus*, Poaceae, *Pinus*, *Betula*, Urticaceae and *Fraxinus* pollens in Neuchâtel, Switzerland (Clot 2003), Cupressaceae, Poaceae, Hamamelidaceae, Pinaceae, Urticaceae, *Quercus*, *Acer*, Myrtaceae, Caryophyllaceae, Oleaceae, Betulaceae and *Plantago* pollens in Porto region, Portugal (Abreu *et al* 2003), *Betula*, Pinaceae, *Alnus*, Poaceae and *Urtica* pollens in Lublin, Poland (Weryszko-Chmielewska and Piotrowska, 2004), Cupressaceae, *Quercus*, Urticaceae, Oleaceae, Pinaceae, Poaceae, Platanaceae, *Corylus*, Chenopodiaceae and *Populus* pollens in Thessaloniki, Greece (Gioulekas *et al.* 2004), *Pinus*, Cupressaceae, Poaceae, *Platanus*, *Quercus*, *Artemisia*, Amaranthaceae and Urticaceae pollens in Isparta, Turkey (Bicakci *et al.* 2000), *Pinus*, Cupressaceae/Taxaceae, Gramineae, *Platanus*, *Quercus*, *Olea*, *Salix*, Urticaceae, Moraceae, *Plantago*, Chenopodiaceae/Amaranthaceae, *Ailanthus*, *Juglans*, *Carpinus* and Rosaceae pollens in Balıkesir (Bicakci & Akyalcin 2000); *Pinus*, *Quercus*, Cupressaceae/Taxaceae, *Salix*, *Platanus*, *Populus*, *Carpinus*, *Fagus*, Moraceae, *Corylus*, *Fraxinus*, Gramineae, Chenopodiaceae/ Amaranthaceae, *Xanthium* and Urticaceae pollens in Sakarya (Bicakci 2006) and *Olea europaea*, Cupressaceae/ Taxaceae, *Pinus*, *Platanus*, Poaceae, and *Morus* pollens in Kuşadası, Turkey (Tosunoğlu *et al.* 2013).

In conclusion, the annual sampling of the airborne pollen grains in Dursunbey atmosphere showed presence pollens of 24 arboreal and 18 non-arboreal plants. A total number of 6265 pollen grains per cm^2 were counted during the sampling period and the main pollen producers were recorded as *Pinus*, Cupressaceae/Taxaceae, Poaceae, *Quercus*, *Acer*, *Platanus*, *Juglans*, *Abies*, *Plantago*, Amaranthaceae, and *Olea* in Dursunbey atmosphere. Most of the predominated pollen types have previously been reported as important allergenic pollen types. We hope the calendar designed by us will be helpful for medical treatment of patients complaining from pollen allergy in Dursunbey and its surroundings.

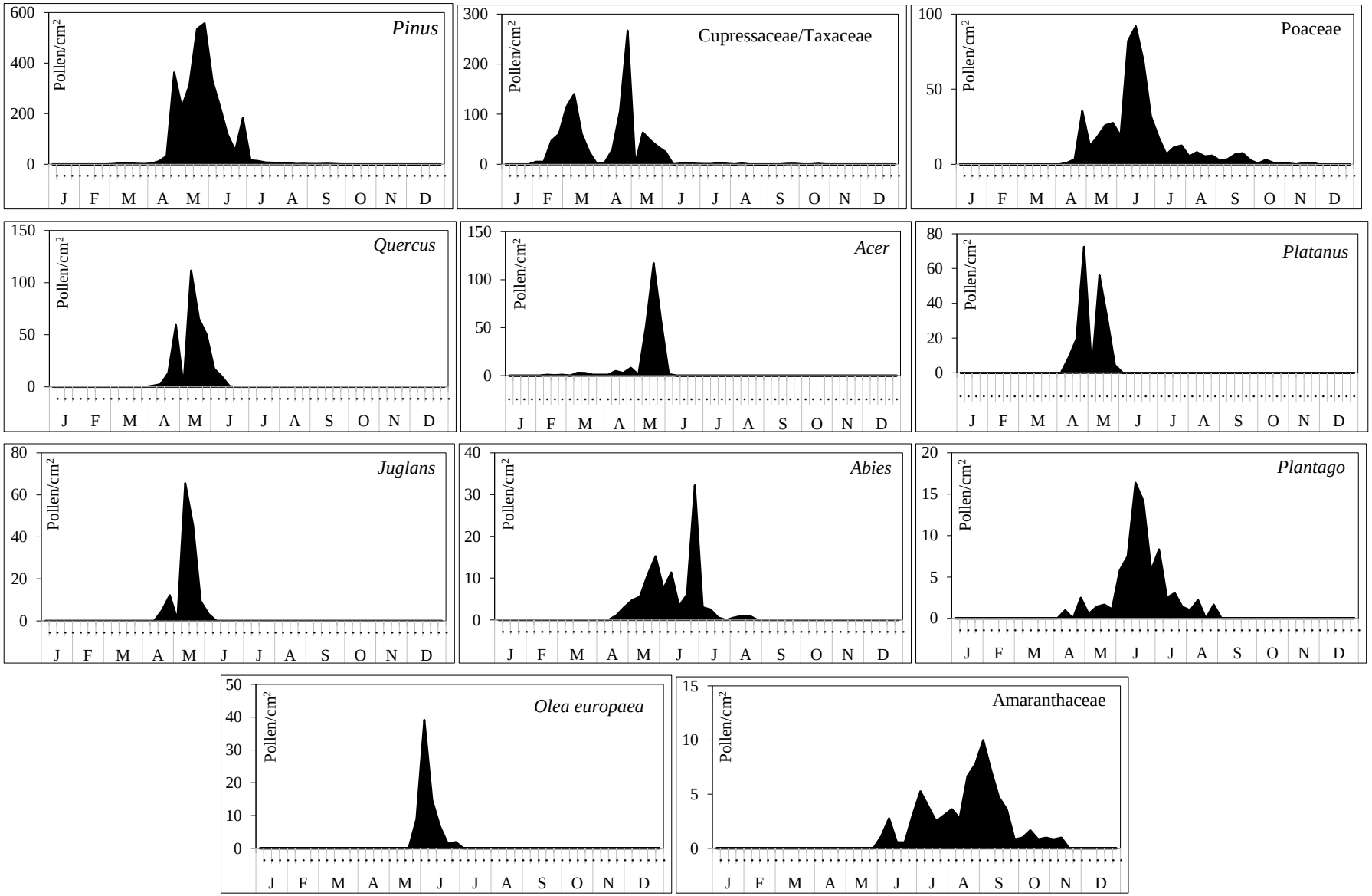

Fig. 3. Main pollen producers and their weekly variations in the atmosphere of Dursunbey. The letters in the *X* axis correspond to the sampling months.

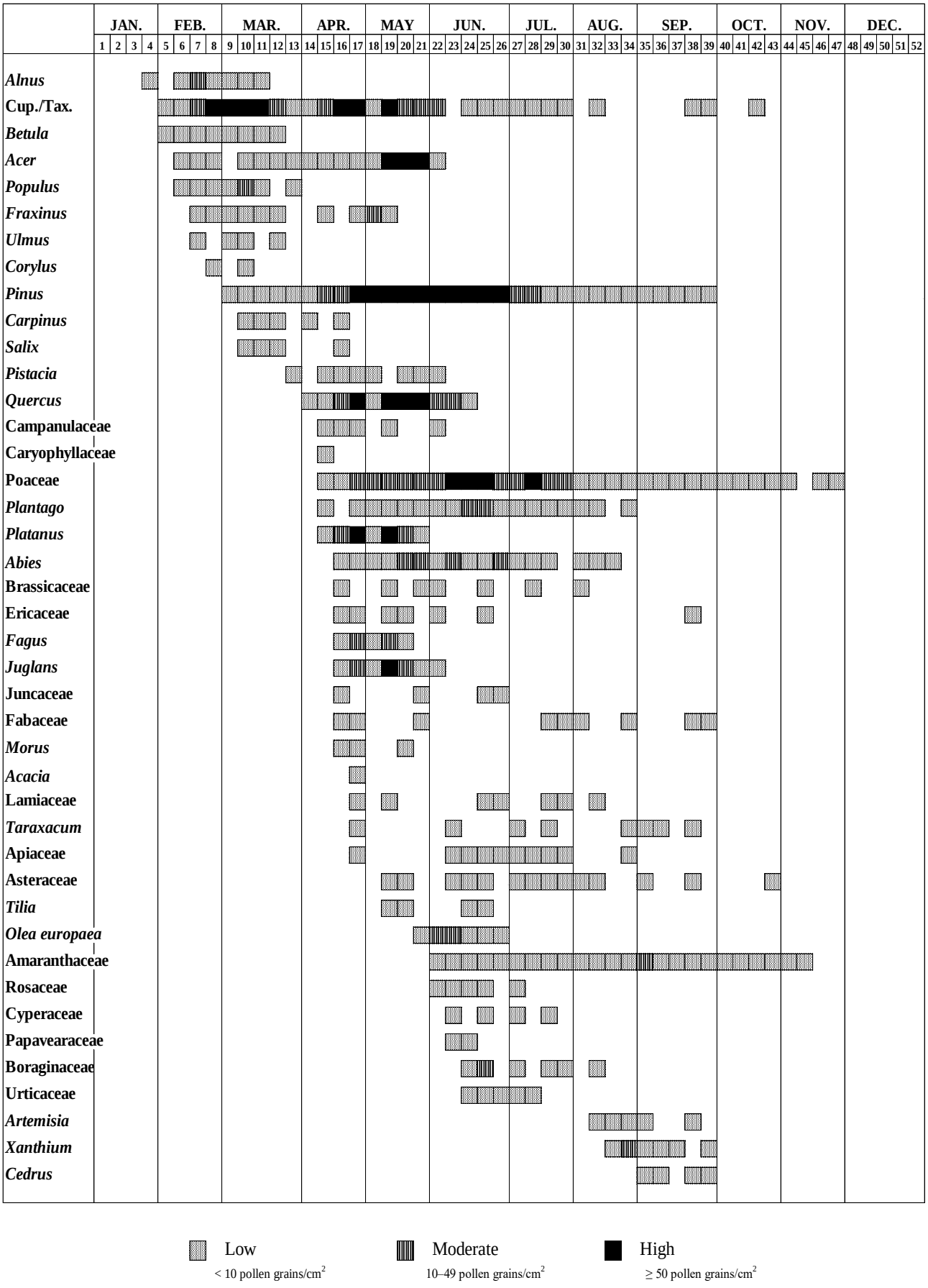

Fig. 4. Annual airborne pollen calendar of Dursunbey.

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Research Article

INTRASPECIFIC VARIATIONS STUDIED BY ISSR AND IRAP MARKERS IN MASTIC TREE (*Pistacia lentiscus* L.) FROM TURKEY

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Abstract: In this study, intra-specific variations in naturally growing and cultivated mastic tree (*Pistacia lentiscus* L.) samples obtained from western parts of Turkey were examined using ISSR and IRAP marker techniques. Samples from Crete and Chios were also included in the study. Morphological measurements of some leaf characteristics of the samples were performed and the measured data was evaluated statistically with a Pearson Correlation analysis to reveal the correlations between character pairs. ISSR primers produced 81 bands between 161-1884bp with 96.3% polymorphism and IRAP primers produced 72 bands between 124-2027bp with 91.67% polymorphism. Polymorphism information content (PIC) values were 0.458 and 0.418 for ISSR and IRAP, respectively. Genetic similarity matrix was examined with Jaccard's coefficient. Maximum similarity was found between the Cretan samples (LG2 and LG3) with the ISSR analysis (0.933) and between L25A (C1, Bodrum) and L29A (C1, Milas) with the IRAP analysis (0.593). Unweighted pair group method with arithmetic mean (UPGMA) dendrogram was divided into 12 and 4 groups by ISSR and IRAP methods, respectively. Specimens were segregated on 3 main different clusters by the Principal Component Analysis (PCA) based on the combined marker systems. The results showed that *P. lentiscus* has very high ratios of intraspecific variation. The present work is an original study in terms of large sampling including wild genotypes, cultivated specimen, Chios and Cretan varieties, use of ISSR and IRAP combination, determination of relations between culture and wild genotypes and the use of *Bagy-1* retrotransposons in intraspecific polymorphism. This study may be considered as a reference study for studies on gene pools of *P. lentiscus* and phylogenetic relationships within the species and may contribute to species concept and agricultural breeding programs.

Key words: IRAP, ISSR, Phylogenetics analysis.

Özet: Bu çalışmada, ISSR ve IRAP markör teknikleri kullanılarak Türkiye'nin batı kesiminde doğal olarak yetişen ve kültürü yapılan sakız ağacı (*Pistacia lentiscus* L.) örneklerindeki tür içi varyasyon analizi yapılmıştır. Girit ve Sakız Adası örnekleri de çalışmaya dahil edilmiştir. Bazı yaprak özelliklerinin morfolojik ölçümleri gerçekleştirilmiş ve aralarındaki korelasyon Pearson Korelasyon analizi ile belirlenmiştir. ISSR primerleri 161-1884bç arasında %96,3 polimorfizm ile 81 bant üretmiştir. IRAP primerleri ise 124-2027bç arasında %91,67 polimorfizm ile 72 bant üretmiştir. Polimorfizm bilgi içeriği (PIC) değerleri 0,458 (ISSR) ve 0,418 (IRAP) arasında bulunmuştur. Genetik benzerlik matriksleri Jaccard katsayısıyla oluşturulmuştur. ISSR sonuçlarında en yüksek benzerlik Girit örnekleri (LG2 ve LG3) arasında (0,933) ve IRAP sonuçlarında ise L25A (C1, Bodrum) ve L29A (C1, Milas) arasında bulunmuştur. UPGMA yöntemiyle kurulan dendrogramlarda sırasıyla ISSR için 12 grup ve IRAP için 4 grup ayrılmıştır. Her iki markör sistemi için ortak kurulan temel bileşen analizi (PCA) grafiğinde 3 farklı küme oluşmuştur. Sonuçlar göstermiştir ki, *P. lentiscus* yüksek oranlarda tür içi varyasyona sahiptir. Bu çalışma, ISSR ve IRAP markörlerinin yabani genotipler, kültür türleri, Sakız Adası ve Girit çeşitlerinin analizinde kullanımı, kültür varyeteleri ve yabani genotipler arasındaki ilişkilerin belirlenmesi ve tür içi polimorfizmde *Bagy-1* retrotranspozonlarının kullanımı açısından özgün bir çalışmadır. Söz konusu çalışma, *P. lentiscus* gen havuzunun ve filogenetik ilişkilerinin araştırılması, tür sınırlarını saptamaya yönelik yapılacak tarımsal ıslah çalışmaları için referans niteliğindedir.

Introduction

The genus *Pistacia* L. within the family Anacardiaceae is represented with 11 species worldwide and with six naturally growing species in Turkey (Stevens 2008, Kafkas & Perl-Treves 2001, Kokwaro & Gillet 1980, Whitehouse 1957, Yaltırık 1967, Zohary 1952). Members of the genus grow naturally in various areas of the northern hemisphere including the Middle East, Canary Islands and the Mediterranean region (Ak & Parlakçı 2009). *Pistacia* genus is believed to have originated in Central Asia 80 million years ago (AL-Saghir 2010, Parfitt & Badenes 1997). Mastic tree (lentisk) (*Pistacia lentiscus* L.) is an economically

important species of the genus in terms of mastic resin which has a wide spectrum of biomedical usage and cultivated for its aromatic resin. As an evergreen dioecious maquis element with a height ranging from 1 to 5m and a strong smell of resin, *P. lentiscus* bears fleshy drupe with an initial red colour which becomes black after ripening. The leaves are compound paripinnate, alternate, and leathery. The high ecological tolerance of the species allows it to resists heavy frosts and drought (Correia & Catarino 1994) and to grow well in all types of soils ranging from limestone areas to saline environments around sea (Zohary 1952). Its resin, known as mastic, is harvested as a spice from cultivated mastic trees and used in food industry, cosmetics and medicine with therapeutic effects such as gastrointestinal ailments, and antibacterial and antifungal properties. Mastic resin is reported to be effective in inhibition of cell lines of some cancer types including colon (Balan *et al*. 2007), prostate (He *et al*. 2007), and erytrolosemi (Loutrari *et al*. 2006). Analysing gene pools and conservation of genetic resources of *P. lentiscus* are important strategies for selection of high yield genotypes adapted to various environmental conditions and for increasing product potential in breeding programmes. *Pistacia* has formerly been divided into four groups as *Lenticella, Eu-Lentiscus, Butmela* and *Eu-terebinthus* including 11 species in total based on some morphological observations of Zohary (1952) and Whitehouse (1957). In the first molecular study based on chloroplast DNA, two groups, *Terebinthus* and *Lentiscus*, represented by deciduous and evergreen plants were suggested to be placed in *Pistacia* (Parfitt & Badenes 1997). The group *Terebinthus* was also supported by some molecular studies carried on Mediterranean *Pistacia* species (Golan-Goldrish *et al*. 2004, Kafkas and Perl-Treves 2001, Kafkas & Perl-Treves 2002, Kafkas 2006). Some morphological characters such as features of rachis and compound leaves, shape, colors and venation of the leaflets, shape of fruits are used in taxonomical delimitations of *Pistacia* species in Turkey (Yaltırık 1967). Some studies were performed on Turkish *Pistacia* species based on random amplified polymorphic DNA (RAPD) (Kafkas & Perl-Treves 2002) and amplified fragment length polymorphism (AFLP) markers (Kafkas 2006) explaining some taxonomical relations of the species and suggesting some nomenclatural combinations. In a recent work carried on *P. Lenticus* in Turkey, Inter Simple Sequence Repeat (ISSR), Simple Sequence Repeat (SSR), RAPD and Internal Transcribed Spacer (ITS) markers were used to evaluate patterns of genetic variation and phylogenetic relationships in 24 wild-type mastic trees (Abuduli *et al*. 2016). The ISSR results indicated that male and female genotypes were distinctly separated from each other and that ISSR markers were useful for analysis of intraspecific variations in mastic trees.

ISSRs are defined as inter-gene regions of microsatellites and ISSR markers are used as molecular markers in polymorphism studies. Microsatellites occuring at thousands of locations within a genome are widely used for DNA profiling, genetic diversity, genetic linkage analysis and marker assisted selection (MAS) to locate a gene or a mutation responsible for a given trait. They are also used, owing to their high polymorphism rates, in phylogeographic studies (Nagy *et al*. 2003) and to measure the levels of relatedness between infraspecific taxa, groups and individuals (Fritz *et al*. 2005). As an alternative valuable retrotransposon-based marker, inter-retrotransposon amplified polymorphism (IRAP) markers are also used to determine genotypes, measure diversity, establish taxonomical relations or reconstruct phylogeny (Kalendar *et al*. 1999). Retrotransposons which are mobile genetic elements can induce mutations by inserting near or within genes. The copy number of retrotransposons exhibit diagnostic patterns among closely related plant taxa (Tenaillon *et al*. 2011) and are useful for determining genetic diversity within a species (Kolano *et al*. 2013) in addition to genomic changes associated with retrotransposon activity in abiotic stress conditions (Fan *et al*. 2014). Retrotransposons are divided into two groups according to the presence or absence of long terminal repeats (LTR). The LTR retrotransposons were well analyzed at different taxonomical categories of plants (Park *et al*. 2007, Ma *et al*. 2008).

In this study, intra-specific variations in 35 samples of naturally growing and culture varieties of *P. lentiscus* obtained from various localities in Turkey, Chios and Crete were examined using ISSR and IRAP marker techniques in order to provide data for elucidating the gene pool of the species in the sampling region, contribute its revision, taxonomical delimitation and its phylogeographical relations. For phylogenetic analysis, dendrograms were generated based on ISSR and IRAP data and the marker results were evaluated by PCA analysis. Morphological measurements of the collected specimens were also carried out to determine similarity coefficients of phenotypic characters at population level.

Materials and Methods

Plant material

Pistacia lentiscus specimens were collected from 31 native populations distributed in İstanbul (A2(E)), İzmir (B1), Muğla and Aydın (C1 and C2) according to the grid system of Turkey. Four specimens collected from Chios (1) and Cretan (3) were also included in the study (Table 1). The collected specimens were prepared as herbarium materials and are kept in ISTF (Herbarium of İstanbul University Faculty of Sciences). Leaf samples of the collected specimens were transported to the laboratory in polypropylene bags and kept at -80ºC conditions until the analysis.

Morphological analysis

The lengths of leaflet tips, widths and lengths of rachis, leaf and leaflets were measured and leaflet numbers and the shapes of leaflets were determined using binocular stereomicroscope. Each measurement was performed as five replicates for each character. A variance

analysis (ANOVA) was performed with the obtained data to determine phenotypically differences. The Pearson correlation test was performed to determine the relationships between measured characters.

DNA isolations

Genomic DNA isolations of *P. lentiscus* specimens were performed by the CTAB method of Lodhi *et al.* (1994) with minor modifications. The concentration and purity of the genomic DNA samples were evaluated in the Nanodrop 2000c instrument. Qualities of the genomic DNAs were analyzed by running on 0.8% agarose gel using 1xTBE buffer and ethidium bromide (EtBr). Scanning of the gels was carried out under UV (GELIANCE 200 Imaging System with GeneSnap software).

PCR Amplification of ISSR and IRAP

Polymerase chain reaction (PCR) amplifications of the DNA samples were carried out in Thermo Scientific Arktik Thermal Cycler using 12 ISSR and 14 IRAP primers (Table 2). The primers showing clear bands on gel images were selected. The ISSR amplifications were analyzed in volumes of 20µl including 1xPCR buffer, 2.5mM $MgCl_2$, 0.4mM dNTP mix (dATTP, dGTP, dCTP and dTTP), 1U Taq DNA polymerase, 0.4mM for each ISSR primer, 50ng DNA sample and DNase/RNase free water. The PCR reactions for ISSR markers were programmed with the following conditions: after initial denaturation of DNA at 95°C for 5min, 35 cycles of denaturation at 95°C for 30sec, annealing (T_a°C is different for each primer) (Table 2) for 1min, 72°C for 1min and final extension at 72°C for 5min. The IRAP

Table 1. The details of the localities of *Pistacia lentiscus* L. specimens included in the study.

No	Accessions	Grid no	Altitude	Coordinates	No	Accessions	Grid no	Altitude	Coordinates
1	L2A	C1	19m	37º41'46.33"N 27º41'44.62"E	**19**	L23A	C1	16m	37º01'45.05"N 27º25'02.05"E
2	L2B	C1	19m	37º41'46.33"N 27º41'44.62"E	**20**	L24A	C1	3m	37º08'11.80"N 27º34'51.94"E
3	L11A	C1	118m	37º27'39.68"N 27º22'38.99"E	**21**	L25A	C1	3m	37º08'11.80"N 27º34'51.94"E
4	L11B	C1	118m	37º27'39.68"N 27º22'38.99"E	**22**	L26A	C1	3m	37º08'11.80"N 27º34'51.94"E
5	L16A	C1	172m	37º19'44.74"N 27º39'07.03"E	**23**	L30A	C1	141m	37º43'51.71"N 27º23'18.03"E
6	L16B	C1	172m	37º19'44.74"N 27º39'07.03"E	**24**	L33A	C2	62m	36º45'39.44"N 28º56'04.64"E
7	L16C	C1	172m	37º19'44.74"N 27º39'07.03"E	**25**	L33B	C2	62m	36º45'39.44"N 28º56'04.64"E
8	L17A	C1	0m	37º16'55.88"N 27º35'21.58"E	**26**	L34A	C2	62m	36º45'39.44"N 28º56'04.64"E
9	L17B	C1	0m	37º16'55.88"N 27º35'21.58"E	**27**	LEU	B1	50m	38º45'58.63"N 27º22'89.37"E
10	L17C	C1	0m	37º16'55.88"N 27º35'21.58"E	**28**	LCHIA	-	-	38º37'24.71"N 25º92'42.12"E
11	L18A	C1	0m	37º16'55.88"N 27º35'21.58"E	**29**	LCES1	B1	10m	38º32'35.56"N 26º28'31.48"E
12	L19A	C1	43m	37º17'01.10"N 27º34'32.55"E	**30**	LCES2	B1	10m	38º32'35.56"N 26º28'31.48"E
13	L28A	C1	8m	37º15'32.72"N 27º31'28.48"E	**31**	LG1	-	200m	35º10'11.0"N 24º58'38.8"E
14	L28B	C1	8m	37º15'32.72"N 27º31'28.48"E	**32**	LG2	-	200m	35º10'11.0"N 24º58'38.8"E
15	L29A	C1	8m	37º15'32.72"N 27º31'28.48"E	**33**	LG3	-	200m	35º10'11.0"N 24º58'38.8"E
16	L21B	C1	16m	37º01'45.05"N 27º25'02.05"E	**34**	LZB	A2(E)	10m	40º58'00.59"N 28º53'00.59"E
17	L22A	C1	16m	37º01'45.05"N 27º25'02.05"E	**35**	LBB	A2(E)	69m	41°0'43.56"N 8°57'50.21"E
18	L22B	C1	16m	37º01'45.05"N 27º25'02.05"E					

Table 2. The list of the ISSR and IRAP primers with their corresponding Ta°C values and 5'-3' sequences.

No	Primer code	References	Sequences (5'-3')	Annealing temperature (Ta°C)
1	ISSR1	UBC-864	ATG ATG ATG ATG ATG ATG	49.8
2	ISSR2	UBC-828	TGT GTG TGT GTG TGT GA	45.7
3	ISSR3	UBC-815	CTC TCT CTC TCT CTC TG	56.2
4	ISSR4	UBC-827	ACA CAC ACA CAC ACA CG	56.2
5	ISSR5	UBC-823	TCT CTC TCT CTC TCT CC	51.0
6	ISSR6	RAMP-TAG	TAG AGA GAG AGA GAG AGA G	59.0
7	ISSR7	UBC-807	AGA GAG AGA GAG AGA GT	52.2
8	ISSR8	UBC-813	CTC TCT CTC TCT CTC TT	50.4
9	ISSR9	UBC-861	ACC ACC ACC ACC ACC ACC	57.6
10	ISSR10	UBC-862	AGC AGC AGC AGC AGC AGC	64.7
11	ISSR11	UBC-844A	CTC TCT CTC TCT CTC TAC	53.7
12	ISSR12	17899A	CAC ACA CAC ACA CAC AG	43.4
13	IRAP1	560LTR, *Wis2*	TTGCCTCTAGGGCATATTTCCAACA	60.0
14	IRAP2	2107LTR, *Wilma*	AGCATGATGCAAAATGGACGTATCA	60.0
15	IRAP3	2109LTR, *Daniela*	TAC CCC TAC TTT AGT ACA CCG ACA	60.0
16	IRAP4	2114LTR, *Fatima*	GGACACCCCCTAATCCAGGACTCC	60.0
17	IRAP5	728LTR, *Sabrina*	TGTCACGTCCAAGATGCGACTCTATC	60.0
18	IRAP6	432LTR, *Sukkula*	GATAGGGTCGCATCTTGGGCGTGAC	60.0
19	IRAP7	LTR *Sukkula*LARD	TAGGGTCGCATCTTGGGCGTGACA	60.0
20	IRAP8	2123LTR, *Wham*	GGAAAAGTAGATACGACGGAGACGT	60.0
21	IRAP9	552LTR, *Bagy1*	CGATGTGTTACAGGCTGGATTCC	60.0
22	IRAP10	1369LTR, *BARE1*	TGCCTCTAGGGCATATTTCCAACAC	60.0
23	IRAP11	LTR6149, *BARE-1*	CTCGCTCGCCCACTACATCAACCGCGTTTATT	60.0
24	IRAP12	LTR4, 1111←1133	AGCCTGAAAGTGTTGGGTTGTCG	59.0
25	IRAP13	LTR7, 460 → 486	CACTTCAAATTTTGGCAGCAGCGGATC	60.0
26	IRAP14	LTR2, 8 ← 30	CTTGCTGGAAAGTGTGTGAGAGG	55.0

reactions were performed in 25µl volumes containing 1xPCR tampon, 2.5mM $MgCl_2$, 0.4mM dNTP mix, 2U Taq DNA polymerase, 1µM primer, 50ng DNA sample and water using the protocol of Kalendar *et al.* (2011) with minor modifications: initial denaturation at 95°C for 3min, 35 cycles of denaturation at 95°C for 1min, annealing (T_a°C is different for each primers) (Table 2) for 45sec, 68°C for 3min and final extension at 72°C for 5min. The PCR products stained with the loading dye were analyzed by running on a 1.5% agarose gel with using 1xTBE and EtBr. Visualisation of the gels with fragmented DNA was carried out under UV (GELIANCE 200 Imaging System with GeneSnap software).

Evaluation of the datasets

The "Thermo Scientific myImage Analysis v2.0" software was used to determine the band molecular sizes using the gel images. The band fragments were visually scored for presence (1) or absence (0) on their gel patterns (additional data are given at the journal's web page as Supplemantary Material Table 6). The polymorphism information content (PIC) value was calculated according to the formula;

$$\mathrm{PIC} = 2\mathrm{P}i\,(1 - \mathrm{P}i)$$

where Pi represents the frequency of polymorphic bands present for a primer (Bhat 2002). The similarity coefficient matrix of the analyses was made based on the "Jaccard" similarity formula (Jaccard 1908) and was generated with the PASW18 software. The results were evaluated by constructing dendrograms for ISSR and IRAP according to the Jaccard's similarity coefficients. In order to determine phylogenetic relationships, unweighted pair group method with arithmetic mean (UPGMA) algorithm was produced using the XLSTAT package program. The distribution of the genotypes in the *P. lentiscus* gene pool was assessed by the principle component analysis (PCA) based on ISSR and IRAP data. The PCA analyses were also performed using the XLSTAT package program.

Results

Morphological analysis

The results of the morphological measurements (see Table 3) showed that the minimum and maximum values were 0.31mm and 1.42mm for the tip length of leaflets. The minimum rachis length was measured as 1.93cm for L28A and the maximum as 5.71cm for LZB and the rachis width ranged from 1.10mm (L16B) to 2.69mm (L11A). The lowest leaf size was measured as 3.66cm for L28A and the highest value as 7.68 cm for LZB. The average value of the leaflet lenght in the population was calculated as 2.73cm (Table 3). The results of the Pearson correlation analysis were given in Table 4. A high correlation level ($p<0.01$) was determined between the pairs of the morphological characters, and the highest correlation ($r=0.901$) was measured between leaf width and leaflet size.

ISSR and IRAP polymorphism analysis

Although a total of 12 different primers were used for ISSR analysis, 9 of them were included in the band analysis. A total of 81 bands and 1444 amplicons were

obtained between 161 bp and 1884 bp per ISSR primer and 3 of them were monomorphic band (Fig. 1). The lowest and the highest PIC values were 0.368 and 0.495, respectively. The mean PIC value was 0.458 and the polymorphism rate was found to be 96.3% (Table 5).

14 different primers were tested in the IRAP-PCR. *Bagy*-1, *BARE*-1 and *Sukkula* LTR-retrotransposon primers were determined after the PCRs. *Bagy*-1 retrotransposon primer (IRAP9), LTR4 primer (IRAP12) and LTR2 primer (IRAP14) were selected based on the clearly produced band profiles in agarose gel electrophoresis, and the other primers were not included in the assays since they were determined to be monomorphic.

Table 3. The average values of each morphological measurement and the leaflet shape details of the specimens. a; oblanceolate, b; oblong-obovate, c; oblanceolate-obovate, d; ovate-oblanceolate, e; oblong-oblanceolate, f; orbicular-oblanceolate, g; ovaet, h; oval-obovate.

		Tip	Rachis		Leaf		Leaflet			
No	**Samples**	**Length (mm)**	**Length (cm)**	**Width (mm)**	**Length (cm)**	**Width (cm)**	**Length (cm)**	**Width (cm)**	**Number**	**Shape**
1	L2A	0.62±0.21	2.23±0.55	1.40(0.36)	3.8 ±1.15	4.14±0.45	2.53±0.19	1.02±0.24	3.6	d
2	L2B	0.37±0.09	3.16±0.59	1.65±0.33	4.44±0.65	5.30±0.84	3.19±0.57	1.31±0.12	5.6	a
3	L11A	0.48±0.05	2.62±0.45	2.69±0.24	4.82±0.88	5.52±0.53	3.10±0.34	1.40±0.59	3.2	a
4	L11B	0.44±0.06	3.82±0.43	1.92±0.37	6.18±0.81	5.10±1.08	3.24±0.49	1.35±0.29	6.0	b
5	L16A	0.91±0.23	3.20±1.02	2.04±0.09	5.00±0.52	3.94±0.44	2.32±0.28	1.30±0.14	6.6	f
6	L16B	1.42±0.24	2.90±0.24	1.10±0.14	4.88±0.63	4.10±0.14	2.52±0.22	0.98±0.15	6.0	c
7	L16C	0.75±0.22	3.43±0.68	1.64±0.30	4.78±0.63	3.92±0.29	2.08±0.49	0.87±0.29	6.6	b
8	L17A	0.43±0.06	2.57±0.53	1.45±0.17	4.40±0.61	4.03±0.67	2.43±0.30	1.18±0.33	5.6	a
9	L17B	0.53±0.10	2.85±0.80	1.41±0.23	4.86±0.77	3.77±0.25	2.43±0.57	0.78±0.07	5.6	e
10	L17C	0.43±0.07	2.56±0.53	1.80±0.27	4.54±0.72	4.28±0.72	2.57±0.13	1.34±0.20	5.0	h
11	L18	0.47±0.13	2.18±0.50	1.62±0.21	4.40±0.90	4.56±0.56	2.82±0.27	1.12±0.19	4.4	e
12	L19A	0.46±0.03	2.89±0.41	1.88±0.21	4.42±0.46	4.77±0.44	2.44±0.42	0.96±0.04	4.4	a
13	L21B	0.46±0.05	2.35±0.21	2.04±0.26	3.90±0.26	4.62±0.69	2.72±0.25	1.33±0.10	4.0	a
14	L22A	0.68±0.24	2.23±0.44	2.27±0.24	4.47±0.36	5.27±0.52	2.87±0.49	1.64±0.19	3.8	d
15	L22B	0.43±0.10	2.56±0.33	1.59±0.38	4.48±0.76	3.54±0.45	2.28±0.23	0.84±0.20	5.2	b
16	L23A	0.43±0.13	3.40±0.80	1.96±0.34	5.18±1.00	4.87±0.76	2.80±0.36	1.22±0.31	6.2	d
17	L24A	0.46±0.18	2.29±0.98	2.12±0.37	4.86±1.45	4.31±0.77	2.35±0.61	1.25±0.41	6.4	a
18	L25A	0.52±0.03	4.17±0.29	1.76±0.25	6.21±0.77	4.98±1.05	2.94±0.50	1.27±0.38	6.4	c
19	L26A	0.48±0.09	2.78±0.63	2.26±0.56	4.45±0.9	5.04±0.36	2.75±0.38	0.98±0.13	5.1	a
20	L28A	0.51±0.07	1.93±0.33	1.77±0.23	3.66±0.22	3.40±0.59	2.08±0.13	0.94±0.09	5.6	a
21	L28B	0.51±0.01	3.13±0.62	1.78±0.22	4.77±0.64	3.83±0.62	2.36±0.09	1.05±0.16	5.2	a
22	L29A	0.55±0.14	3.34±0.73	2.02±0.21	5.26±1.41	5.48±0.67	3.31±0.12	0.96±0.11	5.2	b
23	L30A	0.31±0.17	3.59±0.55	1.85±0.22	4.90±0.82	4.72±1.08	2.64±0.68	1.14±0.27	5.6	c
24	L33A	0.53±0.14	5.55±2.80	2.02±0.31	7.55±2.80	7.78±0.81	3.62±0.83	1.17±0.29	4.4	e
25	L33B	1.06±0.09	2.7±0.64	1.89±0.22	4.74±0.72	5.56±0.41	3.11±0.32	1.27±0.23	3.8	b
26	L34A	0.67±0.17	3.48±0.95	2.30±0.27	5.19±1.22	6.14±1.03	3.46±0.58	1.22±0.25	7.2	e
27	LEU	0.44±0.09	3.15±0.40	1.72±0.33	4.65±1.08	4.12±0.21	2.33±0.19	1.23±0.48	5.4	a
28	LCHIA	0.66±0.18	3.35±0.96	2.23±0.57	6.50±0.79	5.56±1.64	3.10±1.03	1.93±0.35	5.6	f
29	LCES	0.54±0.15	3.70±1.29	1.94±0.26	6.06±1.82	4.84±0.50	3.05±0.67	1.60±0.42	6.4	a
30	LG	0.70±0.14	4.84±0.91	1.90±0.51	6.30±0.72	4.18±0.58	2.28±0.34	0.85±0.18	8.8	e
31	LZB	0.74±0.08	5.71±0.69	2.60±0.65	7.68±0.78	5.40±0.23	2.72±0.31	1.56±0.23	6.6	a
32	LBB	-	3.16±0.77	2.1±0.22	6.64±1.08	5.84±0.42	3.20±0.39	1.71±0.21	6.0	g
	Arithmetic mean	0.58±0.22	3.18±0.89	1.90±0.34	5.13±1.00	4.78±0.89	2.73±0.41	1.21±0.27	5.49±1.17	
	Standard deviation of the population (σ)	0.21	0.88	0.33	0.94	0.88	0.40	0.27	1.15	
	Standard deviation variance of the population (σ2)	0.04	0.77	0.11	0.97	0.78	0.16	0.07	1.32	

Table 4. The correlation values between the character pairs.

		Tip	Rachis		Leaf		Leaflet		
		L	L	W	L	W	L	W	N
Tip	**L**	-	0.082	-0.158	0.126	-0.007	-0.046	-0.003	0.141
Rachis	**L**	0.082	-	0.286	0.877**	0.497**	0.331	0.130	0.493**
	W	-0.158	0.286	-	0.397*	0.552**	0.426*	0.554**	-0.033
Leaf	**L**	0.126	0.877**	0.397*	-	0.600**	0.484**	0.431*	0.423*
	W	-0.007	0.497**	0.552**	0.600**	-	0.901**	0.471**	-0.173
Leaflet	**L**	-0.046	0.331	0.426*	0.484**	0.901**	-	0.500**	-0.190
	W	-0.003	0.130	0.554**	0.431*	0.471**	0.500**	-	-0.083
	N	0.141	0.493**	-0.033	0.423*	-0.173	-0.190	-0.083	-

L (length), W (width), N (number). *Level of significance of correlation, 0,05. **Level of significance of correlation, 0,01.

Table 5. ISSR and IRAP analysis reaults for each primer.

Primer	Range size (bp)	Total no of amplicons	Total no of bands	% of polymorphism	PIC
1. ISSR1	446-1071	215	10	90.00	0.474
2. ISSR2	470-1197	137	11	72.73	0.458
3. ISSR3	1008-1676	35	4	50.00	0.375
4. ISSR4	564-1884	120	9	66.67	0.470
5. ISSR5	369-1880	187	9	66.67	0.486
6. ISSR6	271-1333	228	13	92.31	0.499
7. ISSR7	282-1689	201	10	70.00	0.494
8. ISSR9	287-533	111	7	71.43	0.495
9. ISSR10	161-593	212	8	87.50	0.368
TOTAL	-	1444	81	-	-
MEAN	-	-	-	96.30	0.458
10. IRAP9	164-1426	192	20	85.00	0.398
11. IRAP12	124-2027	319	35	94.29	0.384
12. IRAP14	217-1844	226	17	94.12	0.471
TOTAL	-	737	72	-	-
MEAN	-	-	-	91.67	0.418

The band results of the IRAP-PCR revealed a total of 737 amplicons from 72 band fragments between 124 bp and 2027 bp (Fig. 2). The mean PIC value for IRAP marker assay was found to be 0.418 (Table 5).

Genetic similarity analysis

The percentages of similarity obtained as a result of whole ISSR and IRAP analyses were calculated with Jaccard's coefficient. According to ISSR datasets (additional data are given at the journal's web page as Supplemantary Material Table 7), similarity ratios varied between 0.080 and 0.933. The lowest similarity was found to be 8% between LBB and L33A, and the highest (93%) was between LG2 and LG3. The lowest polymorphism was found in LBB genotype with an average value of 0.127 and the highest polymorphism was in L28B genotype with an average value of 0.628. The average values of the highest and lowest polymorphism values were calculated as 0.712 and 0.125, respectively. According to IRAP datasets (additional data are given at the journal's web page as Supplemantary Material Table 8), similarity ratios were found to between 0.087 and 0.593. The lowest (0.087) and highest (0.593) similarities were found between L11A and L33A, and between L25A and L29A, respectively. The minumum polymorphism was determined in L33A genotype with an average value of 0.2222 and the maximum polymorphism in L25A genotype with an average value of 0.404. The average value of genetic similarity according to IRAP markers in all specimens was calculated as 0.323.

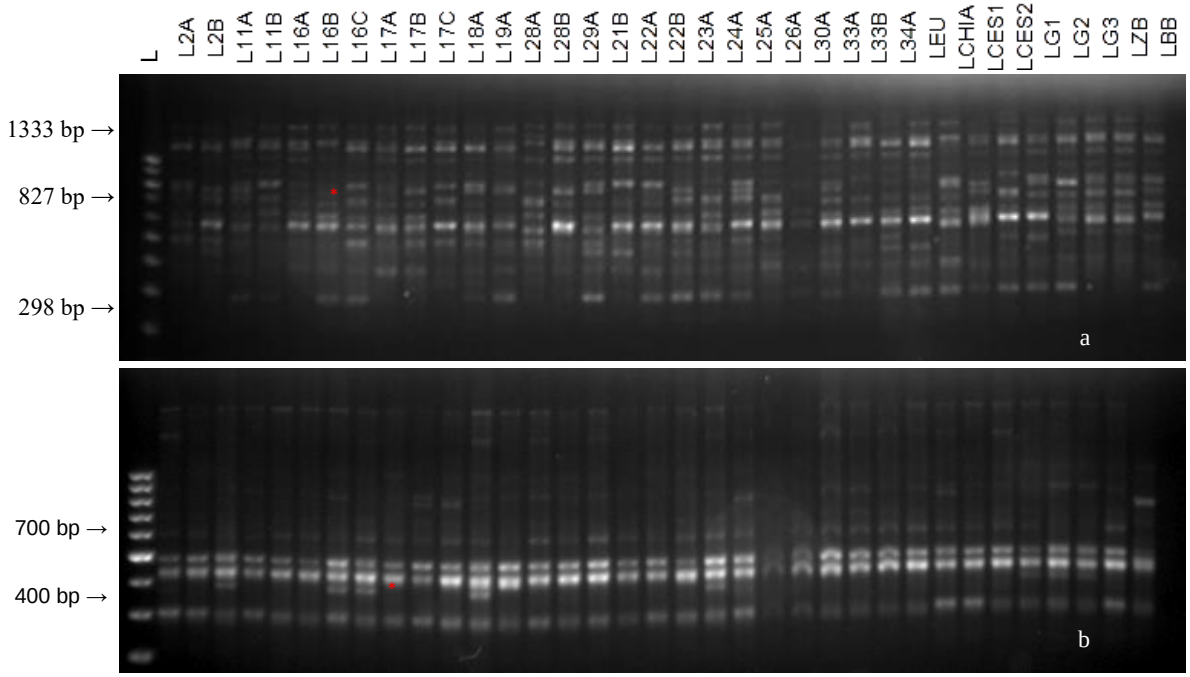

Fig. 1. Agarose gel electrophoresis of ISSR6 (a) and ISSR7 (b) L; 100bp DNA Ladder, red solid circle; polymorphic bands.

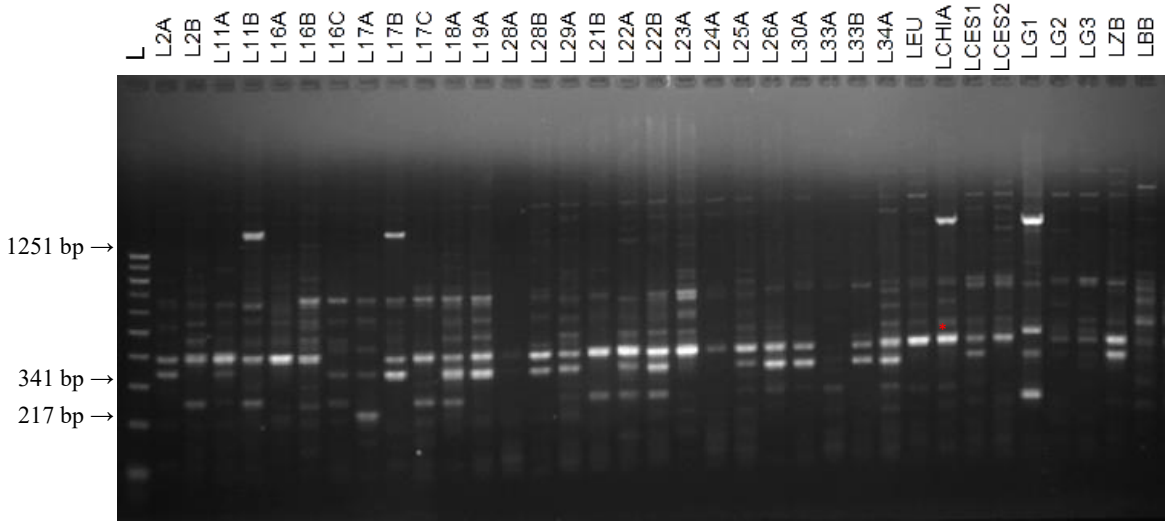

Fig. 2. Agarose gel electrophoresis of IRAP14. Red solid circle; polymorphic band.

Phylogenetic analysis

For phylogenetic analysis of the 35 *P. lentiscus* samples, phylogenetic trees were constructed by UPGMA method according to the data obtained from ISSR and IRAP. Dendrograms were constructed based on the Jaccard's similarity coefficient. The ISSR dendrogram was characterized with 12 different groups (Fig. 3). The first group (G1) consisted of L2A and L2B genotypes and the intra-group variance value was found to be 5.000. The second group (G2) included L11A, L16A, L17A, L17B, L17C and L33B genotypes and the group variance value was detected to be 8.867. The third group (G3) was only represented by the L11B genotype. The fourth group contained G4 (L16B), G6 (L28A), G7 (L23A), G8 (L26A), G9 (L33A), G10 (LG1), G11 (LZB) and G12 (LBB). G5 was divided into 16 branches containing L16C, L18A, L19A, L28B, L29A, L21B, L22A, L22B, L24A, L25A, L30A, L34A, LEU, LCHIA, LCES1, LCES2, LG2 and LG3. The in-class variance value of G5 was calculated as 9.556. Some genotypes were separated according to their location records. In the analysis of variance for the best grouping, the values were calculated as 9.208 (78.42%) within the group and 2.533 (21.58%) among the groups. The total value was found to be 11.741.

The IRAP dendrogram revealed presence of 4 groups (Fig. 4). The first group (G1) contained 26 genotypes (L2A, L11B, L17B, L17C, L18A, L19A, L28B, L29A, L21B, L22A, L22B, L23A, L24A, L25A, L26A, L30A, L33B, L34A, LEU, LCHIA, LCES1, LCES2, LG1, LG2, LG3, LZB), the second group (G2) contained six genotypes (L2B, L11A, L16A, L16B,

L16C, L17A), the third group (G3) contained L28A and L33A genotypes, the fourth group (G4) contained only the LBB genotype. The variance values were calculated as 10.385 (G1), 8.767 (G2) and 4.000 (G3). The variance values calculated for the best classification of the genotypes were found to be 9.918 (92.19%) intra-group and 0.840 (7.81%) among the groups, the total value being 10.758.

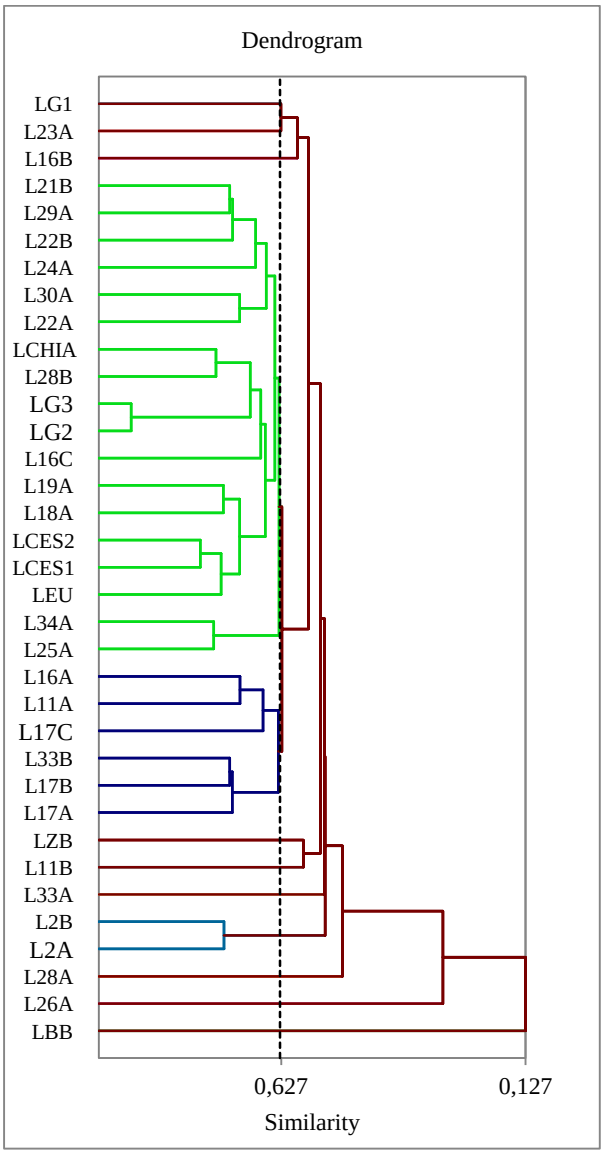

Fig. 3. UPGMA Dendrogram of 35 P. lentiscus specimens based on ISSR data.

Principle component analysis (PCA) was performed to assess the distribution patterns of the populations based on ISSR and IRAP marker systems (Fig. 5). The PCA results showed that the two axes (F1, 43.62% and F2, 5.09%) as the main component represented a total of 48.71% of the variation in the population. It appeared that there were 3 distinct groups. Two of the groups were the clusters of independent genotypes of LBB and L24A and one group was the sum of the other specimens. This last group, represented by a large number of genotypes, was divided into 4 subgroups among themselves.

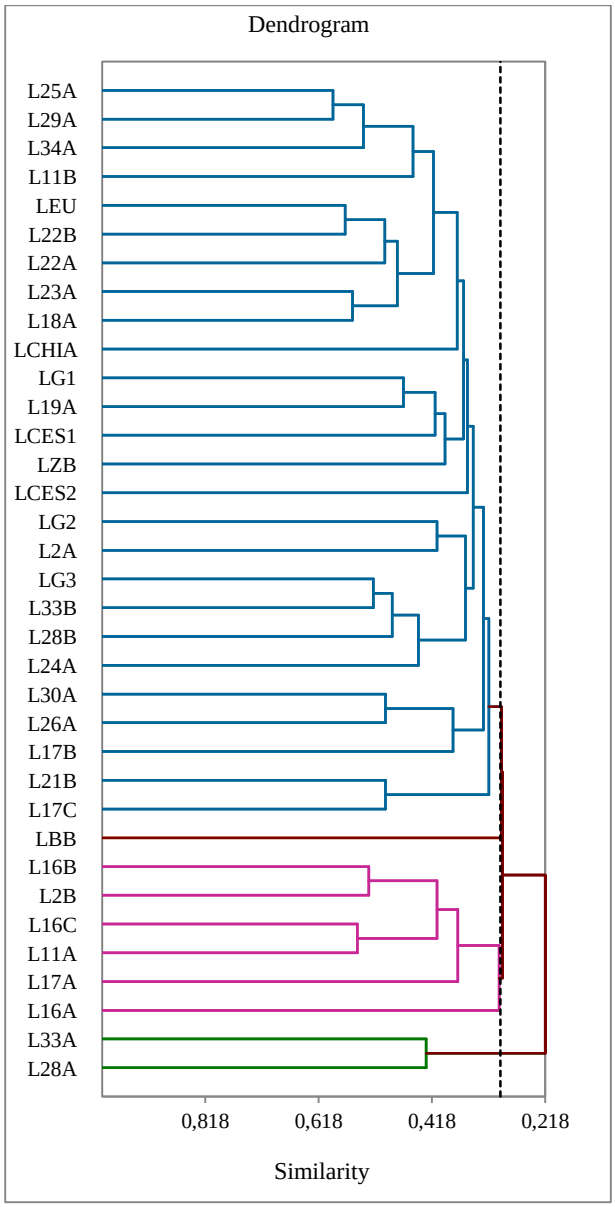

Fig. 4. UPGMA Dendrogram of 35 *P. lentiscus* speciemens based on IRAP data.

Discussion

The average PIC value was calculated as 0.458 according to the ISSR assay. If a PIC value is >0.5, then polymorphism is at a high level, while if it is between 0.25 and 0.5, the polymorphism is normal. However, when the PIC value is 0.25, it is necessary to mention the weakness of the polymorphism (Botstein *et al.* 1980). The highest genetic similarity was found between the Cretan genotypes (LG2 and LG3), indicating that the biogeographic distribution is the factor for microsatellite polymorphism. It was remarkable that the similarity between Çeşme culture varieties (LCES1-2) and Cretan samples (LG1-2-3) was 0.629, indicating the closeness of the phylogenetic and genetic relations. The similarity value was pointed to the biogeographic source of the material used in culture studies. LCHIA (Chios variety) also showed the highest similarity (0.760) to L28B (C1,

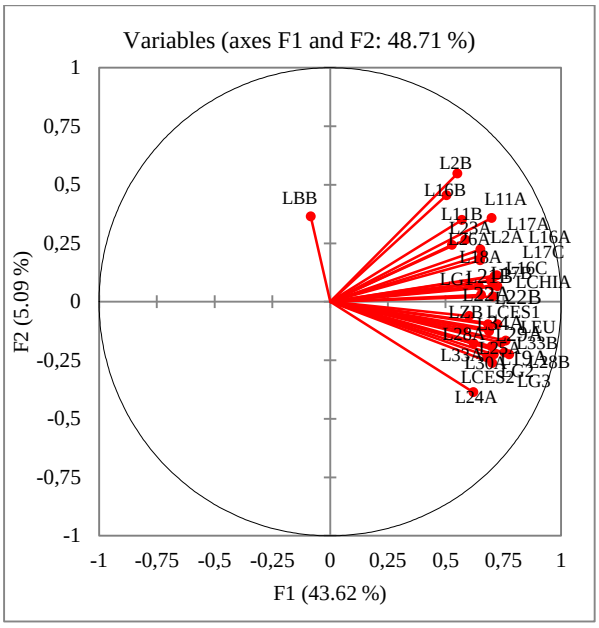

Fig. 5. PCA output of the populations based on ISSR and IRAP data.

Kıyıkışlacık village / Milas). The highest ISSR polymorphism of the L28B (wild genotype) may be due to the change in primer binding sites, the independent alleles to occur and mutations in the microsatellite gene (Freudenreich *et al*. 1997). The phylogenetic tree constructed with the ISSR data shows that the specimens are separated according to their biogeographic positions. From the phylogenetical data, it was observed that the genotypes were divided into 12 groups and 9 of them were independent branches in the dendrogram. It can be explained by the emergence of isolated and differentiated genotypes that grow in various habitat conditions as adapted ecotypes. In the study of Zografou *et al*. (2010), UBC842, UBC850 and UBC856 primers were used, and the similarity ratios ranged from 68% to 12.5%. However, the similarity coefficient between the Chios varieties and the other specimens in our study was calculated between 0.760 and 0.093. This result indicates a wider distribution range compared to the results of Zografou *et al*. (2010). In another study with *P. lentiscus* (Abuduli *et al.* 2016), gene pool was investigated using SSR, RAPD and ISSR markers, and ITS regions in 24 different *P. lentiscus* wild genotypes, and the highest polymorphism was obtained by ISSR compared to other molecular markers (SSR, RAPD and ITS region) and the PIC value was calculated as 0.887. In the study using ISSR, the genotypes examined were divided into 5 subpopulations. As a result, the use of the ISSR markers was demonstrated to be a useful tool for genetic diversity analysis in wild genotypes of *P. lentiscus* and in future breeding studies (Abuduli *et al*. 2016). Considering the genetic similarity rates of the intraspecific variation obtained by the IRAP method, it was determined that LG1 (Cretan) was the closest specimen to the LCHIA (Chios variety. In terms of genetic similarity, the nearest sample to LCHIA genotype was L26A (C1, Güvercinlik village / Bodrum, 3m) and the furthest sample was L33A (C2, sea edge, Göcek / Fethiye, 62m), showing that species locations along seaside or in the lower parts of the mountains were not effective for genetic similarity. The dendrogram of IRAP data was examined in order to evaluate phylogenetic relations and it was shown that 3 main groups and 1 genotype (LBB) were separated, and retrotransposon mobilities resulting in high polymorphism were affected by the geographical locations of the plant samples. The results of Kılınç *et al*. (2014) and Koç *et al*. (2014) on *P. lentiscus* based on IRAP and AFLP techniques were supported with the findings of amplification data obtained in our present study in relation to the LTR2 region. Kırdök & Çiftçi (2016) used iPBS, REMAP and IRAP techniques of retrotransposon markers for genetic diversity analysis of *Pistacia* genus. They found high genetic similarity between *P. vera* - *P. khinjuk*, *P. atlantica* - *P. mutica* and *P. terebinthus* - *P. palaestina* and it was reported that the IRAP technique of retrotransposon-based DNA markers for 7 Mediterranean *Pistacia* species (total 35 samples) could be used in molecular characterization and molecular breeding trials (Kırdök & Çiftçi 2016). In addition, retrotransposon marker systems have also been used in taxonomic studies of the Persian species of the genus *Pistacia* (*P. vera*, *P. khinjuk*, *P. atlantica*) (Ghaemmaghami *et al*. 2013). In another study, IRAP analysis resulting in high polymorphism was performed on *P. vera*, *P. khinjuk*, *P. eurycarpa* and *P. atlantica* (Amirbakhtiar & Sorkheh 2015). Genetic diversity analysis using SCoT and IRAP markers in wild *Pistacia* species found an average PIC value of 0.32 for IRAP (Sorkheh *et al.* 2016). In conclusion, the applications of molecular markers are useful not only in the intraspecific variations, but also in interspecific analysis of *Pistacia* species. In a study using RAPD and AFLP markers, *Pistacia* was divided into two groups in a dendrogram segregated as evergreen *P. lentiscus* and the other group as deciduous (Golan-Goldhirsh *et al*. 2004). UPGMA analysis using AFLP markers showed clustering of *P. terebinthus* with *P. mexicana* and *P. lentiscus* implying their phylogenetic relations (Kafkas 2006). Golan-Goldhirsh *et al*. (2004) also reported that close genetic relationships in *Pistacia* species gave more reliable results in sex taxonomy compared to morphological classification. In the present study, the PCA analysis identifed different groups of 35 accessions in close proximity to each other. The results from PCA analysis based on ISSR and IRAP data showed that the variation in the *P. lentiscus* gene pool in Anatolia was high, genotypes were separated as groups, geographically isolated accessions were clearly separated from the others, and geographically related specimens were grouped together. On the other hand, significant differences were calculated among the accessions based on morphological parameters. Supportingly, phytochemical and morphological characters of *P. lentiscus* genotypes collected from

different locations (Spain, Tunisia, Cyprus and Israel) showed a tendency of high phenotypic variation (Barazani *et al*. 2003). The results obtained with the combination of the data produced by both marker systems revealed that the genotypes distributed in the Aegean region constitute separate groups.

Conclusion

Retrotransposon-based molecular markers produce high polymorphism and high solubility in identifications of plant genetic resources, determining the relationships between various plant groups and plant taxa of agricultural potential and the evolutionary history of plant genome (Gribbon *et al*. 1999). ISSR has been used successfully to analyze the level of genetic diversity within and between species in wild accessions (Reddy *et al*. 2002). In the present study, the high polymorphism in western Turkey genotypes of *P. lentiscus* determined at both morphological and molecular levels indicates that this region is one of the important origin centers of *P. lentiscus*. In conclusion, the results of our study showed that *P. lentiscus* has high intraspecific variations for the wild genotypes in Anatolia and combined marker systems reveal high polymorphism and detailed phylogenetic relations in this species. More detailed investigations on molecular variation in the gene pool of this species are needed in a wider range of samples using various marker systems to provide molecular databases useful in agricultural rehabilitation programs and molecular taxonomic studies to identify infraspecific boundaries.

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Research Article

OPTIMISATION OF *Bacillus amyloliquefaciens* FE-K1 EXTRACELLULAR PEPTIDASE PRODUCTION BY RESPONSE SURFACE METHODOLOGY

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Abstract: In this study, it was aimed to optimise the extracellular peptidase production of *Bacillus amyloliquefaciens* FE-K1, previously isolated from ropy wholemeal bread, by using response surface methodology (RSM) based on central composite design (CCD). The temperature (20-45°C), initial pH of the enzyme production medium (pH 5-9) and inoculation level (1-5%, v/v) were used as the factors for RSM, and the fermentation time was determined for each trial separately. Results showed that the optimum peptidase production occurred at 33.4°C, pH 6.62 and 2.3% inoculation. It was determined that the fermentation time was only 7h, the crude enzyme had a peptidase activity of 49.17U/mL and a specific activity of 504.77U/mg under the optimised conditions.

Key words: *Bacillus*, central composite design, peptidase, response surface methodology.

Özet: Bu çalışmada, daha önce sünmüş ekmekten izole edilmiş olan *Bacillus amyloliquefaciens* FE-K1'in ekstraselüler peptidaz üretiminin, merkezi kompozit tasarıma (MKT) dayalı yanıt yüzey yöntemi (YYY) ile optimize edilmesi amaçlanmıştır. Yanıt yüzey yönteminde sıcaklık (20-45°C), enzim üretim ortamının başlangıç pH değeri (pH 5-9) ve inokülasyon seviyesi (%1-5, v/v) faktör olarak kullanılmış, fermentasyon süresi her deneme için ayrı ayrı belirlenmiştir. Sonuçlar optimum peptidaz üretiminin 33,4°C, pH 6,62 ve %2,3 inokülasyon seviyesinde elde edildiğini göstermiştir. Optimum koşullar altında fermentasyon süresinin sadece 7 saat, ham enzimin aktivitesinin 49,17U/mL, spesifik aktivitesinin ise 504,77U/mg olduğu tespit edilmiştir.

Introduction

The enzyme group that converts proteins or large polypeptides into smaller peptides or free amino acids by hydrolysing peptide bonds are peptidases. Although typically known as proteases, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends using the terms 'peptidase' or 'peptide hydrolase' for any enzyme that hydrolyses peptide bonds (Rawlings *et al.* 2007). Therefore, the term 'peptidase' has been used throughout this paper.

Bacillus (Cohn) species have been the most commonly used microorganisms in microbial fermentation applications. The ability to produce and secrete large quantities (20–25g/L) of extracellular enzymes into the culture medium, a high adaptability to environmental changes, high growth rates that lead to short fermentation cycles, GRAS (generally recognised as safe) status, and the vast amount of information about these species have placed them among the most important enzyme producers (Schallmey *et al.* 2004).

Bacillus species are widely used for peptidase production and most of the commercially used neutral and alkaline peptidases are produced by these species (Outtrup *et al.* 1990, Vetter *et al.* 1995, Sandhya *et al.* 2005, Leong 2006, Kolkman *et al.* 2016). They secrete extracellular peptidases principally at the end of the exponential growth phase (Simonen & Palva 1993), in the early stationary phase or during the stationary growth phase (Mabrouk *et al.* 1999), but peptidase production at the beginning of the exponential phase and death phase has also been reported (Beheshti Maal *et al.* 2011). Peptidase secretion by *Bacillus* species may be associated with nutrient depletion (particularly, nitrogen and carbon sources) during the stationary phase, as well as to the sporulation (Hanlon & Hodges 1981, O'hara & Hageman 1990). Thus, strategies of prolonging the stationary phase can be useful to increase the peptidase activity (Gupta *et*

al. 2002). According to Hamoen *et al*. (2003) if the nutrients are limited in the stationary growth phase, *Bacillus subtilis* (Ehrenberg) Cohn secrete degradative enzymes, such as peptidases, to liberate nutrients from alternative sources that are usually difficult to access. Prolonged nutritional stress results in the development of competence and, ultimately, in sporulation of the bacterial population.

Members of the genus *Bacillus* secrete the proteins across their single membrane system directly into the culture medium at high concentrations. *B. subtilis* produce two groups of extracellular peptidases, known as "quality control" and "feeding" peptidases. While quality control peptidases have major roles in the removal of potentially detrimental molecules, such as cleaved signal peptides, incompletely synthesised proteins and misfolded proteins, feeding peptidases provide nutrients to the cells by degrading the proteins or peptides in the environment (Harwood & Cranenburgh 2008, van Dijl & Hecker 2013).

In some of the studies related to production of *Bacillus* peptidases, strains have been isolated, particularly, from soil (Gupta *et al*. 1999, Kumar *et al*. 1999, Singh *et al*. 2001, Beg & Gupta 2003, Shafee *et al*. 2005, Chu 2007, Nadeem *et al*. 2008, Reddy *et al*. 2008, Kim *et al*. 2016, Hussain *et al*. 2017), soil mixed seawater (Patel *et al*. 2006), wastewater near a milk processing plant (Chu 2007), slaughterhouse waste water (Hammami *et al*. 2018), tannery waste (Anandharaj *et al*. 2016), marine sediment (Uttatree & Charoenpanich 2016, Uttatree *et al*. 2017), raw milk (Matta & Punj 1998), sugarcane molasses (Johnvesly & Naik 2001), leather by-products (Genckal & Tari 2006, Tari *et al*. 2006), compost (Denizci *et al*. 2004), industrial waste discharge (Gerze *et al*. 2005, Orhan *et al*. 2005), leather, soil and horse faeces (Akbalik *et al*. 2004), cheese (Molva *et al*. 2009) or obtained directly from culture collections (Yang *et al*. 2000, Beg & Gupta 2003).

Response surface methodology (RSM) is an experimental strategy applied to evaluate the interactions among different parameters and to find the best combination parameters for multivariable systems. RSM is widely used for the optimisation of microbial fermentation parameters such as medium composition, pH, temperature, inoculation level, agitation rate etc. The major advantage of RSM is to minimize the number of experiments and time (Lakshmi & Hemalatha 2016, Ahsan *et al*. 2017).

The *Bacillus* strain used for peptidase production in this study (*Bacillus amyloliquefaciens* FE-K1) has previously been isolated from ropy wholemeal bread (Erem *et al*. 2009). Rope is a type of bread disease seen in regions where the climate is warm and moist. The causative agent is commonly *B. subtilis*, and some other *Bacillus* species, such as *B. licheniformis* (Weigmann), *B. megaterium* (Bary), *B. pumilus* (Meyer & Gottheil) and *B. cereus* (Frankland & Frankland), which may contaminate bread through the raw materials and bakery equipment used (Kirschner & von Holy 1989, Collins *et al*. 1991, Bailey & von Holy 1993). Although most of the vegetative forms of these bacteria are killed during the baking process, the spores can survive the baking process and germinate during storage of bread, causing the disease (Volavsek *et al*. 1992, Thompson *et al*. 1993). The liquefaction that occurs in the centre of the bread results from the degradation of starch and protein molecules by the amylases and peptidases secreted from the germinated spores (Kirschner & von Holy 1989, Voysey 1989, Rosenkvist & Hansen 1995, Ellis *et al*. 1997, Thompson *et al*. 1998, Pepe *et al*. 2003, Erem *et al*. 2009). Hence, this study was performed to optimise the extracellular peptidase production of *B. amyloliquefaciens* FE-K1, considering the probability that *Bacillus* species isolated from ropy bread can be a good source for peptidase production.

Materials and Methods

Materials

Bacillus amyloliquefaciens FE-K1 has previously been isolated from ropy wholemeal bread (Erem *et al*. 2009), and its 16S rRNA gene sequence was determined by Gene Research and Biotechnology, Ankara, Turkey (REFGEN). The strain was also investigated for haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) production and verified as an HBL- and NHE-negative strain (unpublished data). Nucleotide sequence data for *B. amyloliquefaciens* FE-K1 are available in the GenBank databases under the accession number MH045777.

Preparation of stock culture and pre-culture

A single colony of *B. amyloliquefaciens* FE-K1, grown overnight on nutrient agar, was inoculated into 50mL of nutrient broth (in 250-mL baffled flask) and incubated at 37°C and 250rpm for 18h. Then, the culture was mixed with 50% glycerol (1:1, v/v) and 1-mL aliquots were distributed into 2-mL sterile cryo-tubes and maintained at -80°C.

For pre-culture preparation, the whole contents of one tube stock of culture were inoculated into a 250-mL baffled flask, containing 50mL of growth media and incubated at 37°C, with shaking (250rpm), until reaching the mid-log phase (7.5h). The growth medium contained 2.0g/L glucose, 10.0g/L yeast extract, 1.0g/L KH_2PO_4, 3.0g/L $K_2HPO_4.3H_2O$, 2.0g/L Na_2SO_4 and 0.1g/L $MgSO_4.7H_2O$.

Some of the parameters used in this study for the production of *B. amyloliquefaciens* FE-K1 peptidase have previously been determined with one factor at a time approach (Erem & Certel 2018). Carbon (glucose) and nitrogen (yeast extract) sources, which provide the maximum peptidase activity, were selected among different sources and they used in the growth medium. Furthermore, a carbon/nitrogen ratio of 1:5 and agitation rate of 250rpm were determined as the best values in terms of obtaining higher peptidase activity.

Measurement of optical density (OD)

Samples taken from the fermentation medium were diluted with sterile distilled water, and the absorbance was measured at 600nm by using the sterile fermentation medium as the blank.

Determination of the growth curve

In order to determine the growth curve of *B. amyloliquefaciens* FE-K1, the pre-culture was inoculated into 50mL of growth media by setting the initial OD value at 0.1 and the OD was measured at 1-h intervals during 24h of fermentation. The growth curve was obtained by plotting the OD values versus fermentation time.

Experimental design

Temperature (20-45°C), initial pH of the growth medium (pH 5–9) and inoculation level (1-5%, v/v) were chosen as factors for RSM. A central composite design (CCD), which was generated with Minitab statistical software (version 17, Minitab Inc. USA), was implemented to identify the optimum values of these factors. The minimum and maximum values for the factor levels, which represent the axial points in the CCD, were determined according to preliminary experiments. Central Composite Inscribed (CCI) design which is one of the three types of CCD was used to limit the factor levels within the values given above in the text. A total of 40 fermentation trials were conducted by using duplicate samples and triplicate analyses. Duplicate samples were evaluated as blocks in the CCD, and six centre points were used per block. Table 1 shows the experimental design.

Peptidase activity values obtained were evaluated with the analysis of variance (ANOVA), and the regression coefficients (R^2) and equations were determined. Factors with a p value of $p<0.05$ were evaluated as statistically significant. The polynomial regression equation (Eq. 1) depending on the factor levels was then fitted to the data by the software:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad \text{(Eq. 1)}$$

where Y, predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, squared coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients; A, B, C, factors.

Response surface graphs were then generated by the Minitab statistical software using this model.

Peptidase production

The pH of the growth medium (50mL) was adjusted with 6N HCl and 6N NaOH, according to the pH values in Table 1, before sterilisation. The sterilised medium was inoculated with the determined level of pre-culture, as seen in Table 1, for each trial. Fermentation experiments were conducted in 250-mL baffled glass flasks, at 250rpm, using a shaking incubator (Sartorius Certomat IS, Germany) until the OD of the supernatant reached its maximum value. Then, the culture broth was centrifuged at 20,000*g* and 4°C for 15min (Biochrom Libra, Cambridge, England) to obtain the cell-free crude enzyme solution. Peptidase activity, protein content and glucose determination were assayed in the cell-free supernatants obtained at 1-h intervals during the fermentation.

Peptidase assay

Peptidase assay was performed as described by Cupp-Enyard (2008), with a slight modification, in that the transparent solutions were obtained by centrifugation (10000*g* and 4°C for 10min) instead of using syringe filters and the volume of samples and all solutions used in the analysis were decreased with a ratio of 5:1. The peptidase activity of the cell-free clear supernatant was assayed at 37°C in potassium phosphate buffer (50mM, pH 7.5) by using 0.65% (w/v) casein as the substrate. One unit of peptidase was equivalent to the amount of enzyme required to release 1mg of tyrosine/mL/min under standard assay conditions.

Protein content

The total protein content of the enzyme solution was determined by the Bradford method (Bradford 1976), using a Coomassie Plus (Bradford) assay kit (Thermo Scientific, Pierce Biotechnology, USA) with bovine serum albumin as the reference standard. Accordingly, 100µL of crude enzyme solution was mixed with 3mL of Bradford reagent. The mixture was incubated at 25°C for 10min, and the absorbance was measured at 595nm. Protein analysis was done immediately after obtaining the enzyme solution.

Glucose assay

Glucose was determined by modifying the method of Miller (1959). Briefly, 600µL of cell-free supernatant was mixed with 600µL of 3,5-dinitrosalicylic acid reagent. The mixture was kept at 90°C for 15min and, then, 200µL of 40% potassium sodium tartrate was added. Next, the mixture was cooled in an ice bath for 5min, and the absorbance was measured at 575nm. A glucose calibration curve was established.

Table 1. Central composite design used for the optimisation of *Bacillus amyloliquefaciens* FE-K1 peptidase.

	Experimental values				
Variables	-α (-1.682)	-1	0	+1	+α (1.682)
Temperature (°C)	20.00	25.07	32.50	39.93	45.00
pH	5.00	5.81	7.00	8.19	9.00
Inoculation level (%)	1.00	1.81	3.00	4.19	5.00

Results and Discussion

Growth curve of B. amyloliquefaciens FE-K1 and variation in its peptidase production

The growth curve and peptidase production of *B. amyloliquefaciens* FE-K1 (Fig. 1) revealed that the strain grew rapidly after the lag phase and reached a high OD level of 8.0. Then, a prompt decrease in the OD value occurred, and there was no observable stationary phase. It is known that metabolites produced towards the end of the log phase could prevent the growth of the bacteria (Tunail 2009). Therefore, the pH of the fermentation medium was measured to check whether the strain produced acid that could hinder its proliferation. However, the measured pH of the sterile medium increased from pH 6.86 to neutral levels during the growth of *B. amyloliquefaciens* FE-K1. It was also thought that due to the rapid growth, the sugar could be insufficient to sustain the cells. However, although the glucose level decreased, there was still some sugar present that the cells could utilise. Therefore, it was decided that either the bacteria needed a specific nutrient or due to the high OD level there was a competition for the nutrients between the living cells. Consequently, the cells which could not access the nutrients were lysed, and the OD quickly decreased. In order to verify cell lysis, gram staining was employed (not shown). The results showed that after the highest OD level, cells began to seem pink which is the result of cell wall degradation. This was probably due to the intracellular peptidoglycan hyrolases released from the lysed cells. Nevertheless, with the intention to obtain a satisfactory peptidase activity over a short fermentation time, the study was continued, and the reason for the drop in the OD level was considered to be the subject of another study.

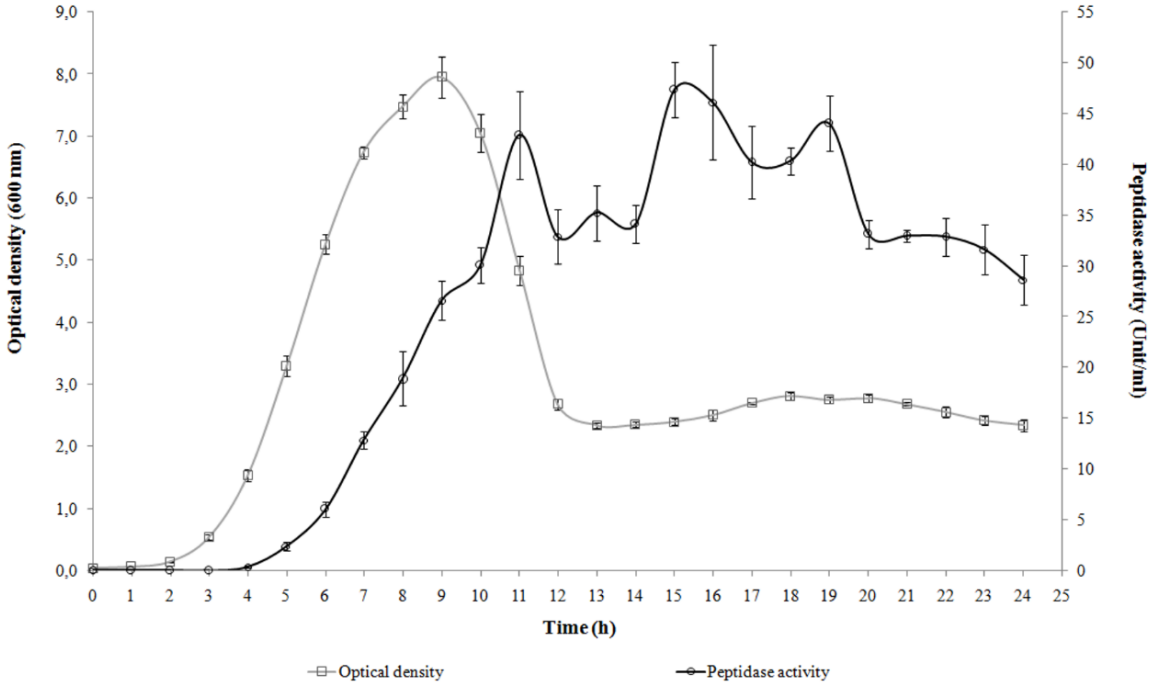

Fig. 1. Growth curve and peptidase production of *Bacillus amyloliquefaciens* FE-K1 at 30°C.

Optimisation of peptidase production by RSM

As seen in Fig. 1, even though the OD decreased, the peptidase activity kept increasing. This behaviour was most probably because of the intracellular peptidases secreted during cell lysis. However, although the actual reason for the cell lysis was not known, the main interest of this study was to optimise the extracellular peptidases of *B. amyloliquefaciens* FE-K1. Therefore, in RSM, the maximum peptidase activity reached before cell lysis, that is, the peptidase activity obtained at maximum OD level was used as the response, to exclude intracellular peptidases. Nonetheless, the time necessary to reach the maximum OD level was variable, depending on the factor levels. For accurate determination of the incubation time of each fermentation experiment, it was necessary to measure the OD and peptidase activity at short intervals. Thus, after inoculating the fermentation media with pre-culture, samples were withdrawn from the fermentation medium at 1-h intervals to perform the glucose and protein content analysis in addition to the OD and peptidase activity measurements. The glucose and protein contents were determined to obtain information about the nutrient content during fermentation, particularly to observe the nutrient content change at the time the OD began to decrease.

In contrast to the results of Gençkal & Tari (2006) and Razak *et al.* (1997), the OD and peptidase activity values obtained under various conditions (Fig. 2) showed that peptidase production is closely linked to the growth of *B. amyloliquefaciens* FE-K1. Even at different temperatures, pH values and inoculation levels, the strain started to produce peptidase consistently shortly after the beginning

of the log phase, and the peptidase activity increased with the growth of the strain. However, due to the sudden cell death observed after reaching a high cell density, the association between the growth of the strain and its extracellular peptidase production could not be determined after the log phase.

The glucose contents measured under various fermentation conditions demonstrated that glucose was not entirely consumed at the point where the fermentation was terminated. Sugar consumption by the bacteria was relatively low, particularly at lower temperatures, such as 20.00 and 25.07°C. Sugar consumption differed when the fermentation was performed at the same temperature but with different pH values. For instance, at 25.07°C, the bacteria consumed more glucose when the initial pH value was higher. When the fermentation media had an initial pH of 5.81, the glucose consumption rate at maximum OD level was 50 and 47% for the inoculation levels of 1.81 and 4.19%, respectively. However, the glucose consumption rate was 84 and 77% under the same conditions, when the fermentation started at pH 8.19. It can be understood from the results that at a given pH, the inoculation level had low influence on glucose consumption. For fermentation at 32.5°C and 3% inoculum, the glucose consumption rates were 56, 76 and 89%, at pH 5, 7 and 9, respectively. Pantamas *et al*. (2003) also found that *B. licheniformis* and *B. coagulans* (Hammer) consumed more glucose at higher alkali conditions.

For all fermentation trials, protein content and its rate of increase were quite low, until the OD reached its maximum value. It is considered that at this stage, the protein content was based on the peptidases secreted by the bacteria into the fermentation medium because no protein was detected in the sterile enzyme production medium (it contains low molecular weight of peptides and amino acids) and the protein content could be determined just after the peptidase activity analysis started to give positive results. Typically, a sudden increase was observed in the protein content under all fermentation conditions after the maximum OD level had been reached. This tendency was also probably due to the intracellular proteins secreted by the bacteria depending on the cell lysis. While living cells kept secreting extracellular proteins, principally peptidases, intracellular proteins were also secreted into the medium because of the lysed cells, increasing the protein content.

The specific activity values at maximum OD and 1h after maximum OD levels were reached (Table 2) indicated that the specific activity of the peptidase started to decrease, generally more than 50%, only at 1h after the maximum OD level, most probably due to secretion of the intracellular proteins during cell lysis. Peptidase activity also increased after cell lysis but the decrease in the specific activity suggested that proteins other than the peptidases were responsible. Therefore, peptidases which have a relatively high specific activity will be obtained by using the peptidase activity level corresponding to the maximum OD level as the response in RSM.

Table 2. Specific activity of peptidase at different fermentation conditions.

Factor levels	Specific activity (U/mg)[a]	Specific activity (U/mg)[b]
25.07°C, pH 5.81, 1.81%	1056.57	474.15
39.93°C, pH 5.81, 1.81%	1318.22	550.62
25.07°C, pH 8.19, 1.81%	760.96	392.21
39.93°C, pH 8.19, 1.81%	663.40	701.63
25.07°C, pH 5.81, 4.19%	795.53	823.70
39.93°C, pH 5.81, 4.19%	1466.99	644.38
25.07°C, pH 8.19, 4.19%	477.69	266.48
39.93°C, pH 8.19, 4.19%	1423.25	579.85
20.00°C, pH 7.00, 3.00%	1508.35	761.18
45.00°C, pH 7.00, 3.00%	1447.31	320.27
32.50°C, pH 5.00, 3.00%	754.18	489.61
32.50°C, pH 9.00, 3.00%	782.15	148.63
32.50°C, pH 7.00, 1.00%	563.02	268.09
32.50°C, pH 7.00, 5.00%	859.87	440.24
32.50°C, pH 7.00, 3.00%	921.70	419.36

[a] Spesific activity at maximum OD level; [b] Spesific activity at 1 hour after the maximum OD level.

Peptidase activity values obtained at maximum OD level were evaluated with analysis of variance (ANOVA), by using Minitab statistical software (version 17, Minitab Inc. USA), and the regression coefficients (R^2) and equations were determined. The polynomial regression equation (Eq 2), depending on the factor levels of temperature (t), initial pH of fermentation medium (p) and inoculation level (i) was as follows:

$$\text{Peptidase activity} = -283.5 + 6.79t + 63p + 1.12i - 0.0853t\text{^}2 - 4.219p\text{^}2 - 1.366i\text{^}2 - 0.214\, t \times p + 0.148\, t \times i + 0.007p \times \text{i} \quad \text{(Eq 2)}$$

The results of the CCD experiments, for the effects of temperature, initial pH of fermentation medium and inoculation level on peptidase production, are presented in Table 3. The predicted values were determined by the statistical software program according to the regression equation.

The ANOVA results in Table 4 verify that according to the p-value, the effect of "block" on peptidase activity is not statistically significant. In this study, the "blocks" represent the duplicate samples (fermentations carried out under the same conditions but at different times). These fermentations were performed to determine the effects of the possible differences that may arise during the preparation of the fermentation medium at various times, although, the same boxes of the enzyme production medium components were used throughout the study. Based on the ANOVA results, however, there were no differences in peptidase activity between the fermentations done under the same conditions but at different times.

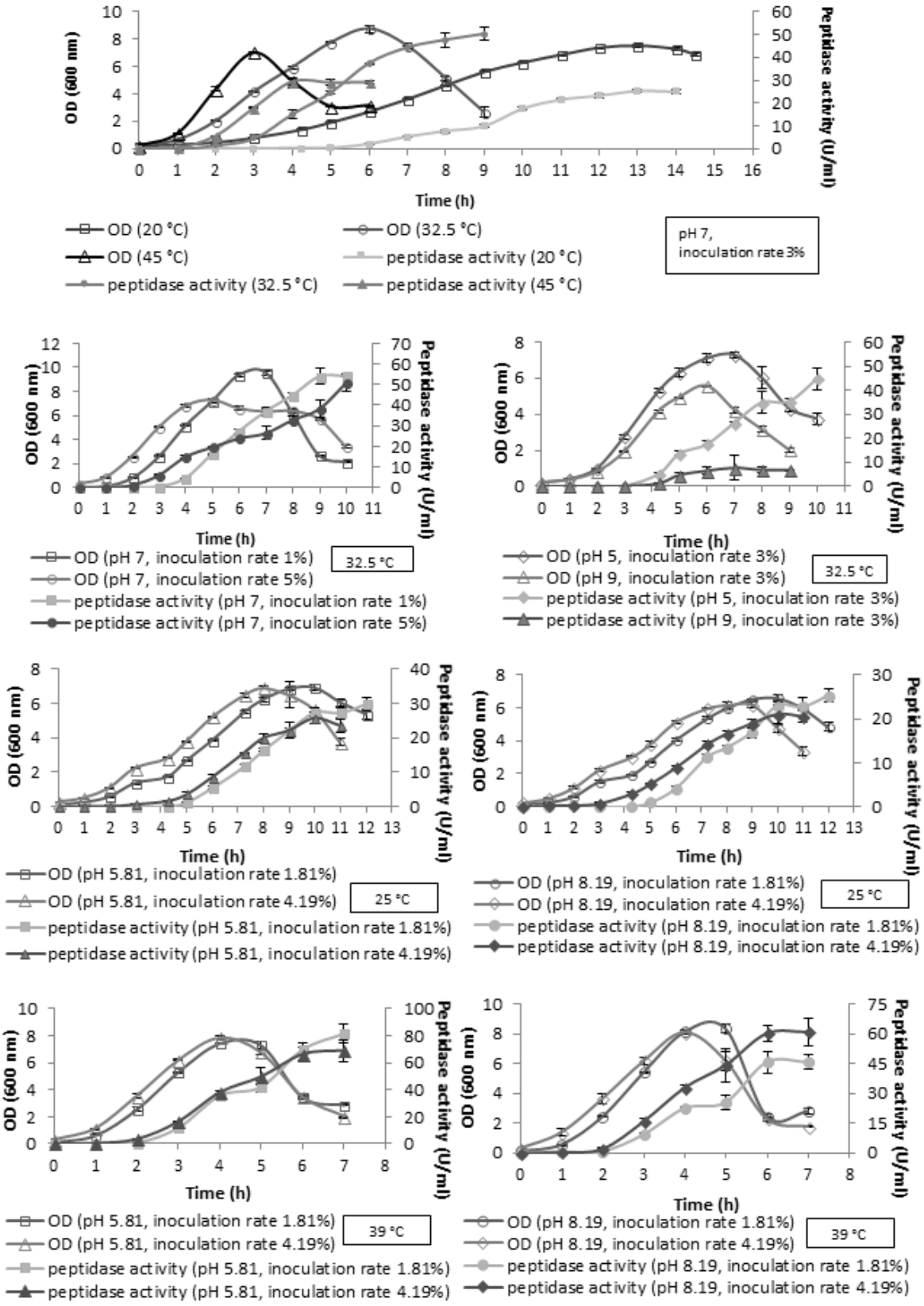

Fig. 2. Changes in optical density (OD) and peptidase activity values in different fermentation trials.

Table 3. Observed and predicted peptidase activities of *B. amyloliquefaciens* FE-K1 depending on the factor levels obtained with CCD.

Run order	Block	Factors (actual levels)			Time (h)[a]	Peptidase activity (U/mL)	
		Temperature (°C)	pH	Inoculation level (%)		Predicted response	Observed response
1	1	25.07	5.81	1.81	10	29.64	27.61 ± 1.25
2	1	39.93	5.81	1.81	4	33.66	35.84 ± 1.65
3	1	25.07	8.19	1.81	10	26.17	23.00 ± 0.87
4	1	39.93	8.19	1.81	5	22.62	25.29 ± 3.61
5	1	25.07	5.81	4.19	8	21.74	19.97 ± 1.26
6	1	39.93	5.81	4.19	4	31.00	38.05 ± 1.13
7	1	25.07	8.19	4.19	9	18.32	18.97 ± 0.94
8	1	39.93	8.19	4.19	4	20.01	32.91 ± 0.94
9	1	20.00	7.00	3.00	13	22.28	25.30 ± 0.38
10	1	45.00	7.00	3.00	3	27.08	17.79 ± 0.25
11	1	32.50	5.00	3.00	7	27.21	26.40 ± 0.66
12	1	32.50	9.00	3.00	6	15.05	6.36 ± 1.46
13	1	32.50	7.00	1.00	7	36.96	36.95 ± 1.96
14	1	32.50	7.00	5.00	5	28.12	19.62 ± 0.61
15	1	32.50	7.00	3.00	6	38.01	37.49 ± 0.08
16	1	32.50	7.00	3.00	6	38.01	37.40 ± 4.85
17	1	32.50	7.00	3.00	6	38.01	44.43 ± 4.92
18	1	32.50	7.00	3.00	6	38.01	43.53 ± 4.71
19	1	32.50	7.00	3.00	6	38.01	39.86 ± 0.11
20	1	32.50	7.00	3.00	6	38.01	38.12 ± 2.90
21	2	25.07	5.81	1.81	8	29.64	26.48 ± 0.72
22	2	39.93	5.81	1.81	4	33.66	35.44 ± 0.52
23	2	25.07	8.19	1.81	9	26.17	27.05 ± 1.05
24	2	39.93	8.19	1.81	5	22.62	30.37 ± 1.77
25	2	25.07	5.81	4.19	7	21.74	22.23 ± 0.24
26	2	39.93	5.81	4.19	4	31.00	35.38 ± 2.44
27	2	25.07	8.19	4.19	8	18.32	22.84 ± 0.33
28	2	39.93	8.19	4.19	4	20.01	21.43 ± 1.51
29	2	20.00	7.00	3.00	12	22.28	25.80 ± 0.26
30	2	45.00	7.00	3.00	3	27.08	16.91 ± 1.45
31	2	32.50	5.00	3.00	6	27.21	27.14 ± 0.65
32	2	32.50	9.00	3.00	7	15.05	11.71 ± 0.18
33	2	32.50	7.00	1.00	7	36.96	37.27 ± 4.03
34	2	32.50	7.00	5.00	6	28.12	23.41 ± 2.84
35	2	32.50	7.00	3.00	7	38.01	35.51 ± 0.37
36	2	32.50	7.00	3.00	7	38.01	35.83 ± 0.34
37	2	32.50	7.00	3.00	7	38.01	38.67 ± 0.99
38	2	32.50	7.00	3.00	7	38.01	34.35 ± 1.42
39	2	32.50	7.00	3.00	7	38.01	35.81 ± 1.87
40	2	32.50	7.00	3.00	7	38.01	37.29 ± 2.36

[a] Fermentation time= time necessary to reach maximum OD level.

Table 4. The results of ANOVA analysis of peptidase activity.

Source	Total Degrees of Freedom	Adjusted Sums of Squares	Adjusted Mean Squares	F-value	P-value
Model	10	2238.35	223.84	7.58	<0.001
Block	1	4.87	4.87	0.16	0.688
Linear	3	601.43	200.48	6.79	0.001
Temperature (*t*)	1	55.62	55.62	1.88	0.180
pH (*p*)	1	357.16	357.16	12.10	0.002
Inoculation level (*i*)	1	188.65	188.65	6.39	0.017
Square	3	1547.28	515.76	17.48	<0.001
*t***t*	1	639.86	639.86	21,68	<0.001
*p***p*	1	1025.85	1025.85	34.76	<0.001
*i***i*	1	107.58	107.58	3.65	0.066
2-way interaction	3	84.78	28.26	0.96	0.426
*t***p*	1	57.31	57.31	1.94	0.174
*t***i*	1	27.47	27.47	0.93	0.343
*p***i*	1	0.00	0.00	0.00	0.994
Error	29	855.85	29.51		
Lack-of-fit	19	795.88	41.89	6.99	0.002
Pure error	10	59.96	6.00		
Total	39	3094.20			
Model Summary	**Standard deviation**	**R^2**	**R^2 (adjusted)**		**R^2 (predicted)**
	5.43249	**72.34%**	**62.80%**		**38.85%**

While the effects of initial pH and inoculation level on peptidase activity were significant ($p<0.05$), the temperature had no effect on peptidase activity (Table 4). Furthermore, *t***t* and *p***p* interactions were also significant ($p<0.05$), suggesting that there is a quadratic relationship between the peptidase activity and these factors.

The ANOVA results indicated a relatively low satisfactory adjustment of the model to the experimental data, due to the R^2 value of 72.34%. The generated model could only explain 72.34% of the variability in the peptidase activity. For a good adjustment of the model, the R^2 value should be higher than 75% (Puri *et al*. 2002). Moreover, the lack-of-fit value was significant ($p<0.05$) which was also an indicator of the inadequacy of the model. Determining the temperature as statistically insignificant factor was probably the main reason of obtaining a relatively incompatible model. Several studies have reported that fermentation temperature is a major factor regarding peptidase activity (Puri *et al*. 2002, Chauhan & Gupta 2004, Gorlach-Lira *et al*. 2010). Finding temperature as an insignificant factor in the present study was probably because of using the peptidase activity values at maximum OD level as the response in RSM, which caused different fermentation times under different fermentation conditions. Fig. 2 illustrates how the temperature had a greater effect on the fermentation time than the pH and inoculation level. For example, the time necessary to reach maximum OD at 32.5°C and pH 7 was 7 and 5h for inoculation levels of 1 and 5%, respectively. In other words, when the other factors were constant, a 5-fold increase in the inoculation level resulted in a 2h decrease in the fermentation time. Conversely, at a fixed pH 7 and 3% inoculation level, the OD reached its maximum value after 13 and 3h at 20 and 45°C, respectively. Therefore, the temperature was an important factor regarding the growth of the bacteria which was also closely associated with its peptidase production. As a result of selecting the peptidase activity corresponding to maximum OD level as the response, the peptidase activities obtained at 20 and 45°C were quite similar to each other despite the different fermentation times. Thus, the temperature was determined as an insignificant factor in the model. A constant fermentation time for all fermentation conditions would solve the problem. However, it would be impossible to determine the extracellular peptidase activity of *B. amyloliquefaciens* FE-K1 in such conditions due to the reasons explained

above. Another possible reason for the model incompatibility is considered to be the composite structure of the crude enzyme solution. It may contain several different enzymes and also various types of peptidases. A peptidase that is active under one fermentation condition may lose or decrease its activity under another condition. If this is the case, it is probable to obtain a variation in the measured response values (peptidase activity), compared to the predicted values.

The contour and surface plots of peptidase production are presented in Figs. 3 and 4, respectively. Given the plots can show the interaction of two factors, the third factor was fixed at the centre points. The circular and elliptical shapes of the contour plots indicated that peptidase activity had reached its maximum level. The same result could be seen in the surface plots (Fig. 4). When the temperature was close to the centre point (approximately 30°C), maximum peptidase activity could have been obtained and a decrease was noted in the activity at lower and higher temperatures. Gençkal and Tari (2006) determined that 30 or 37°C were the optimum temperatures both for the growth of bacteria and enzyme production, and the decrease in enzyme activity, particularly at 45°C or higher, was associated to the protein denaturation and degradation caused by the proteolytic activity of the peptidase produced. Previous studies have documented the optimum temperature for peptidase production as 25°C (Jaswal *et al*. 2008), 34.58°C (Rao *et al*. 2007), 37°C (Chauhan & Gupta 2004, Ali *et al*. 2016) and 40°C (Anadharaj *et al*. 2016).

It is well-known that the culture pH strongly affects many enzymatic processes (Bhunia *et al*. 2012). The surface plots (Fig. 4) confirmed that maximum peptidase activity occurred when the initial pH was around 6.5-7.0, and a substantial decrease in the activity appeared at higher initial pH values.

Bacillus amyloliquefaciens FE-K1 is a bacterial strain isolated from bread and, therefore, it was not expected to grow at high pH values. However, considering that the

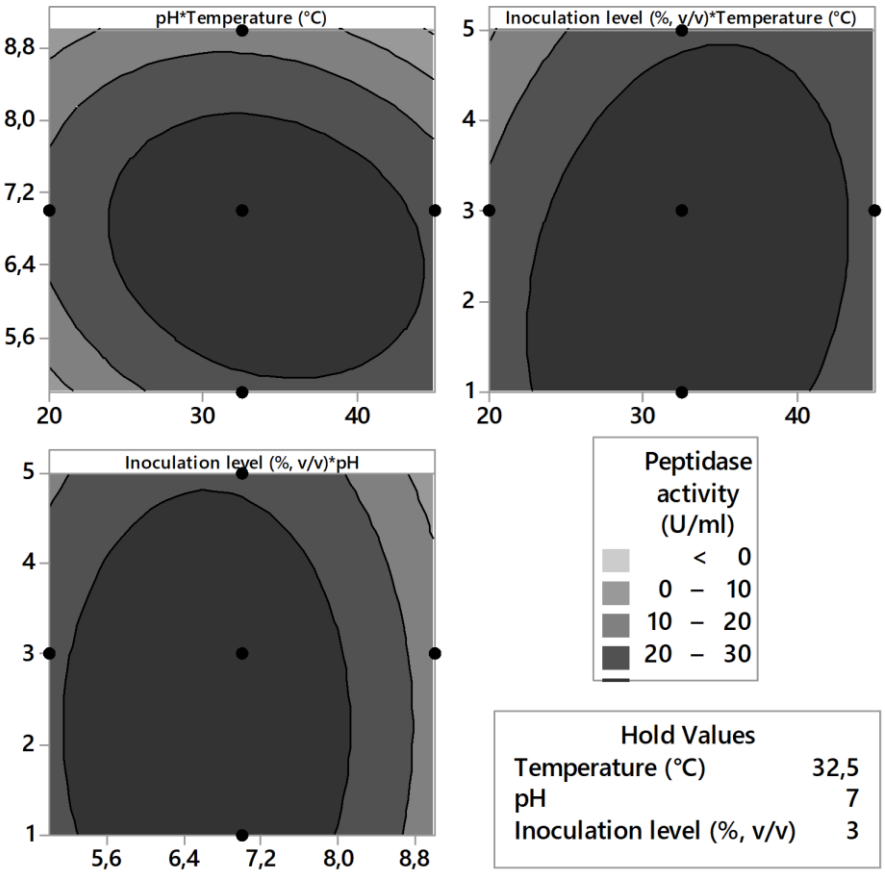

Fig. 3. Contour plots of peptidase activity (U/mL).

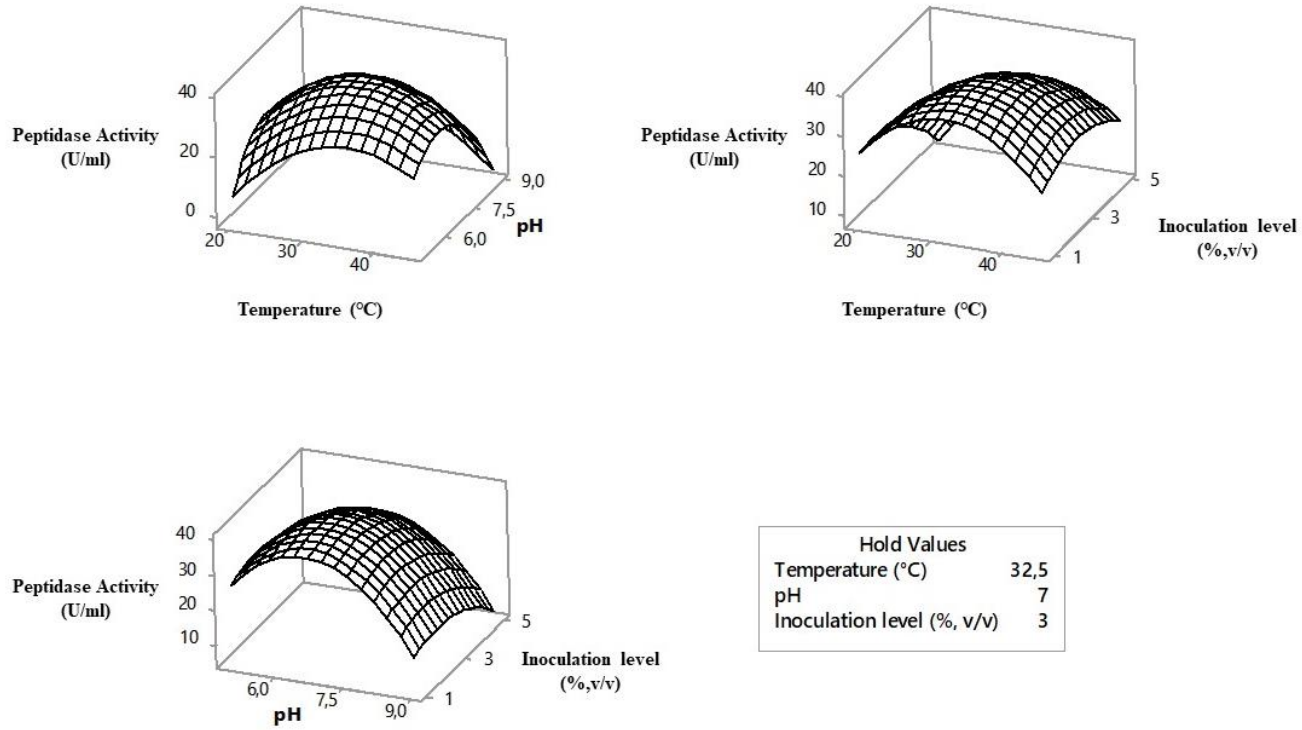

Fig. 4. Surface plots of peptidase activity (U/mL).

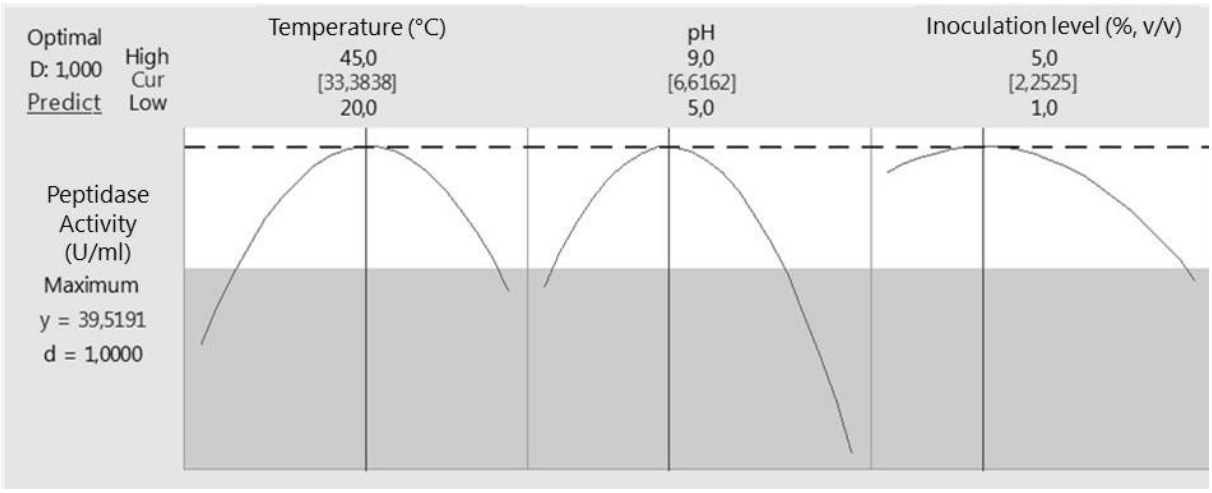

Fig. 5. Optimisation plot for peptidase activity of *Bacillus amyloliquefaciens* FE-K1.

correlation between growth and peptidase production of this strain was not known at the beginning of the study, high pH values (i.e., pH >7) were also added to the CCD. The pH was measured at the end of each fermentation. Irrespective of whether higher or lower initial pH values were established, the pH of the medium was always close to neutral levels at the end of all fermentations. The final pH was between 6.7 and 8.0, depending on the initial pH. This finding indicates that the bacteria have created neutral conditions by producing acidic or alkali metabolites to sustain its growth. Qadar *et al*. (2009) noticed an increase in the pH value of the fermentation medium and proposed that the increase was due to metabolite accumulation. Several studies determined the optimum pH of the culture as 10.5 (Jaswal *et al*. 2008), 10.0 (Ali *et al*. 2016), 7.0 (Qadar *et al*. 2009) and 4.8 (Gorlach-Lira *et al*. 2010).

The optimum inoculation level was approximately 2.5% (Figure 3 and 4). Prakasham *et al*. (2006) applied an inoculum value between 1–5% and found that the optimum level for peptidase production was 3%. Puri *et al*. (2002) selected 1–3% inoculum for *Bacillus* sp. and noted that the inoculation level did not affect the peptidase production. Suganthi *et al*. (2013) documented an optimum inoculation of 1%, for *B. licheniformis* peptidase. The authors reported that lower inoculum doses (<1%) caused a reduction in enzyme production due to the insufficient number of bacteria while higher inoculum levels (>1%) led to the same reduction effect because of

reduced dissolved oxygen and increased competition towards nutrients.

The dashed line in the optimisation plot, generated by the Minitab 17 software (Fig. 5), represents the maximum peptidase activity and the vertical lines show the necessary factor levels to reach this maximum value. This plot indicates that temperature, pH and inoculation level should be 33.4°C, 6.62 and 2.3%, respectively, to provide the maximum peptidase activity of 39.519U/mL. Furthermore, the *d*-value of 1.0 shows that the optimum factor levels have been reached.

Validation of optimisation

In order to validate the optimisation results, triplicate fermentations trials were undertaken at 33.4°C and pH 6.62 with 2.3% inoculum. The change in OD, peptidase activity, and glucose and total protein contents during the fermentations (Fig. 6) demonstrated that the maximum OD was reached at 7h and corresponded to 49.172±1.014U/mL peptidase activity. There were almost 10U difference between the measured and predicted (39.519U/mL) optimum peptidase activity values. The most important reason for this difference is the inadequacy of the model. However, it is possible to evaluate the results from another perspective. Given the fermentation time is not a factor in the CCD or the time is not constant for all the fermentations, it is not possible for the software to determine the fermentation time, namely, the time necessary to reach maximum OD for the optimised conditions. Therefore, the conditions in CCD, which were very close to the optimum factor levels (33.4°C, pH 6.62, 2.3% inoculum level), should be considered. In any case, the software determines the optimum activity level according to the values in the CCD. Fermentation conditions closest to the factor levels that gave the optimum peptidase activity were located at the centre point (32.5°C, pH 7, 3% inoculum level) in the CCD (Table 3). When the fermentations were done at the centre points, the fermentation time was determined as 6 and 7h, respectively, in the different blocks. Therefore, it is possible to accept a 6 or 7h fermentation time for the optimum factor levels. If the time is selected as 6h, it can be seen from Fig. 6 that the corresponding peptidase activity is 40.566U/mL, and this is within proximity to the predicted optimum value (39.519U/mL).

When the optimum peptidase activity of 49.172U/mL was compared to the optimum activity values of similar studies (Table 5), it was determined that a relatively high value was obtained even though the fermentation time was short. In this study, one unit of peptidase was equivalent to the amount of enzyme required to release 1mg of tyrosine/mL/min under standard assay conditions. On the other hand, in most of the other studies related to peptidase production of *Bacillus* species, one unit of peptidase was defined as the amount of enzyme required to release 1µg of tyrosine/mL/min under standard assay conditions. One of the highest activity values encountered for crude enzyme solution was 8320U/mL for *B. circulans* (Jordan) peptidase (Rao *et al*. 2007). When one unit of peptidase activity was evaluated in terms of 1µg tyrosine/mL/min released, the activity value for *B. amyloliquefaciens* FE-K1 peptidase was calculated as 45607U/mL which is almost 5.5-fold higher than the activity value of *B. circulans*. Furthermore, Rao *et al*. (2007) reached the mentioned value after 24 hour fermentation which is almost 4 times longer than the fermentation time of *B. amyloliquefaciens* FE-K1. Peptidase activity value of *B. amyloliquefaciens* FE-K1 in terms of 1µmol tyrosine/mL/min released is equal to 0.272U/mL which is lower than the activity value of 808.68U/mL determined by Jaswal *et al*. (2008) who used relatively longer fermentation time.

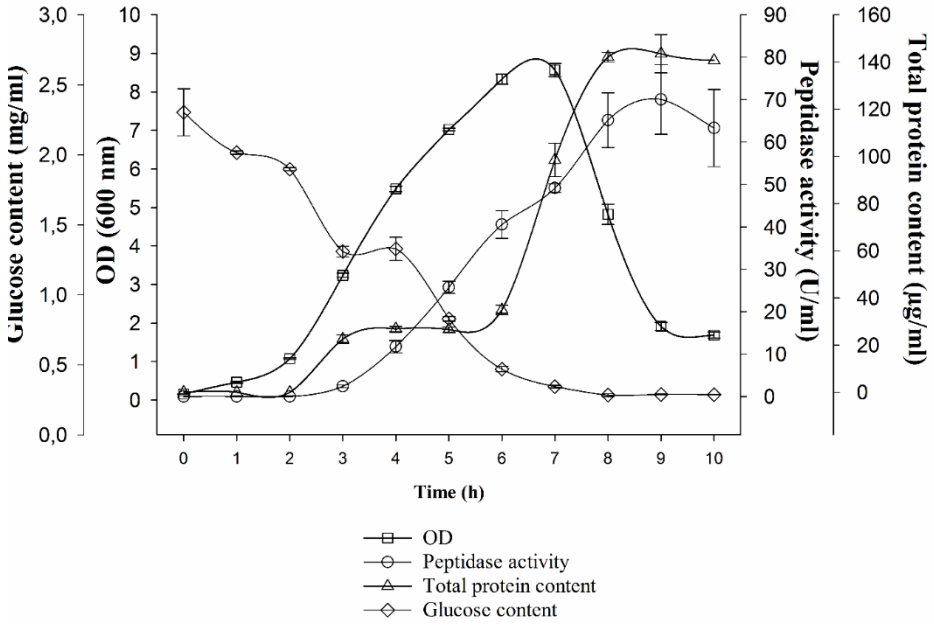

Fig. 6. OD, peptidase activity, total protein content and total glucose content variation with respect to the optimised factor levels.

Table 5. A comparison of results of some studies related to peptidase production by *Bacillus* species

Bacillus strain	Type of peptidase	Enzyme activity*	Fermentation time (h)	References**
Bacillus sp.	Alkaline	1939U/mL[a]	96	Puri *et al*. (2002)
Bacillus sp. RGR-14	Alkaline	3996U/mL[b]	96	Chauhan & Gupta (2004)
Bacillus sp.	Alkaline	222U/mL[a]	96	Gençkal & Tari (2006)
Bacillus sp.	Alkaline	2560U/mL[a]	48	Chu (2007)
Bacillus sp. HS08	Neutral	6804U/mL[a]	18	Guangrong *et al*. (2008)
Bacillus sp. RKY3	Alkaline	939U/mL[a]	24	Reddy *et al*. (2008)
Bacillus sp. PCSIR EA-3	Neutral	~0.7 U/mL[c]	48	Qadar *et al*. (2009)
Bacillus cereus SV1	Metallo-peptidase	5700U/mL[a]	48	Manni *et al*. (2008)
Bacillus circulans	Alkaline	8320U/mL[a]	24	Rao *et al*. (2007)
Bacillus circulans	Alkaline	808.68U/mL[c]	101	Jaswal *et al*. (2008)
Bacillus clausii GMBAE 42	Alkaline	45U/mL[a]	65	Denizci *et al*. (2004)
Bacillus licheniformis N-2	Alkaline	991U/mL[a]	24	Nadeem *et al*. (2008)
Bacillus licheniformis	Alkaline	185.40U/mL[a]	72	Lakshmi & Hemalatha (2016)
Bacillus megaterium	Alkaline	6.57U/mL[a]	15	Uttatree *et al*. (2017)
Bacillus mojavensis	Alkaline	2389U/mL[a]	12	Beg *et al*. 2003
Bacillus mojavensis SA	Alkaline	1467.27U/mL[a]	24	Hammami *et al*. (2018)
Bacillus subtilis FBL-1	Neutral	578.55U/mL[a]	36	Kim *et al*. (2016)

One unit of peptidase was defined as the amount of enzyme required to release a: 1µg of tyrosine/mL/min, b: 1mg of tyrosine/mL/min, c: 1 µmol of tyrosine/mL/min under standard assay conditions

* The given activity values are the maximum peptidase activity reached in crude enzyme solution.

**The studies were selected irrespective of whether they were carried out by using shaking incubator or bioreactor.

Conclusion

This study showed that *B. amyloliquefaciens* FE-K1 grew rapidly to a high OD level and then underwent cell lysis, most probably due to nutrient competition. Therefore, maximum peptidase activity before cell lysis was selected as the response for RSM, to eliminate intracellular peptidases. It was concluded that owing to the different and relatively short fermentation times (3-13h) encountered in this study, depending on the fermentation trials in the CCD, the present experimental design (selecting the peptidase activity of maximum OD level as response) was not an effective method to optimise the extracellular peptidase production of *B. amyloliquefaciens* FE-K1. Particularly, although a significant effect of temperature was evident from the raw data, determining the temperature as an insignificant factor for the peptidase production in the model, due to the different fermentation times, resulted in an incompatible model (R^2 = 72.34%) with a significant lack-of-fit value. However, despite the incompatible model, maximum peptidase activity (49.172U/mL) was obtained compared to the factor levels used in the CCD by applying the optimised factor levels determined by Minitab software according to RSM. This activity was relatively high compared to the literature data, even though the fermentation time was brief. If the cell lysis can be prevented, for instance, by conducting the fermentation in a fermenter, it is possible to improve the extracellular peptidase activity by increasing the fermentation time.

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Research Article

SCREENING FOR ANTI-QUORUM SENSING AND ANTI-BIOFILM ACTIVITY IN *Viscum album* L. EXTRACTS AND ITS BIOCHEMICAL COMPOSITION

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Abstract: Many opportunistic pathogenic bacteria use the Quorum Sensing (QS) system to coordinate their virulence expressions. Thus, QS can likely be a new approach to control bacterial infections. The aim of this study was to evaluate the antimicrobial, anti-biofilm, and anti-quorum sensing activities of ethanol, chloroform, and dichloromethane: methanol extracts of leaf, stem, and fruits of the European mistletoe *Viscum album* L. on 2 Gram-positive and 7 Gram-negative pathogenic bacteria. The extracts at concentrations ranging from 50 to 250mg/ml were tested depending on the extracts of the plant parts and the test bacteria. The extract with 50mg/ml concentration, in which no antimicrobial activity was observed, was used for anti-quorum sensing and antibiofilm studies. The dichloromethane: methanol extracts were found to show the highest biological activities. QS activities of the plant extracts were also determined using the recently established *Chromobacterium violaceum* CV026 reporter strain and the signaling molecule *N*-(ß-ketocaproyl)-L-homoserine lactone (3-oxo-C6-HSL) agar well diffusion assay. Biofilm was quantified using the microtiter plate test and the crystal violet assay. Anti-microbial, anti-biofilm, and anti-quorum sensing activity of leaf and stem extracts showed higher efficiency than fruit extracts. It was concluded that the extracts of *V. album* had the potential to treat microbial infections by biofilm inhibition or inhibition of QS.

Key words: Anti-quorum sensing, Anti-biofilm activity, Antibacterial activity, *Viscum album* L.

Özet: Fırsatçı patojenik bakteriler, virülans ifadelerini koordine etmek için Quorum Sensing (QS) sistemini kullanır. Dolayısıyla QS sistemi, bakteriyel enfeksiyonların kontrolü için yeni bir yaklaşım olarak tercih edilebilir. Bu çalışmanın amacı, 2 Gram-pozitif, 7 Gram-negatif patojenik bakteri üzerinde analiz edilen *Viscum album* L. bitkisinin gövde, yaprak ve meyve gibi bölümlerine ait etanol, kloroform ve diklorometan:metanol ekstraktlarının antimikrobiyal, anti-biyofilm ve anti-quorum sensing aktivitelerinin değerlendirilmesidir. Kullanılan bitki parçası ekstraktına ve test mikroorganizmasına bağlı olarak, 50-250mg/ml arasında değişen konsantrasyonlarda ekstraklar test edildi. 50mg/ml'lik konsantrasyonda antimikrobiyal aktivite görülmediği için anti-quorum sensing ve antibiyofilm çalışmalarında bu konsantrasyon kullanılmıştır. En iyi biyolojik aktivitenin görüldüğü çözücünün ise diklorometan:metanol olduğu saptandı. Ekstraktların anti-quorum sensing aktiviteleri, *Chromobacterium violaceum* CV026 biyosensör suşu ve sinyal molekülü N- (β-ketokaproil) -L-homoserin lakton (3-okso-C6-HSL)'nün bulunduğu besiyerinde agar difüzyon deneyi kullanılarak da tespit edilmiştir. Biyofilm, mikrotiter plaka testi ve kristal viyole kullanılarak ölçülmüştür. Yaprak ve gövde kısımlarının antimikrobiyal, anti-biyofilm ve anti-quorum sensing aktivitesi, meyve ekstraktına göre daha yüksek verimlilik göstermiştir. *V. album* özütlerinin, biyofilm ya da QS inhibisyonu yoluyla mikrobiyal enfeksiyonları tedavi etme potansiyeline sahip olduğu kanısına varılmıştır.

Introduction

Viscum album L. subsp. *album* (mistletoe), belonging to the family Santalaceae, is an evergreen plant growing semi-parasitically on its host. It is a Eurasian and North African species and its distribution in Turkey covers mainly the north, west, and south-west of Anatolia. Its leaves are opposite and parallel-veined, and its fruits are viscid berries. The epithet name of *Viscum album* in Latin was assigned to the plant considering the white color of the fruits (Ergun & Deliorman 1995). Seed distribution of *V. album* is mediated by birds who achieve this task by eating the fruits and leaving their stools on trees. The seeds germinating on the host trees send out their rootlet to penetrate into the bark and absorb nutrients and water from the trees which are used by the growing plant. The

high amount of loss in volume and diameter of the host trunk occurs due to this parasitic feature of *V. album* (Eroğlu & Usta 1993).

Viscum album has long been known as one of the most magical, mysterious, and sacred plants in nature. Therefore, the use of *V. album* as a herbal medicine probably dates back to prehistoric times. The Druids of Britain used to harvest *V. album* from their sacred oaks to use them in rituals and medicine. Dioscorides (15–85 AC) and Hippocrates (460–377 BC) used it to treat diseases of the spleen and complaints of menstruation. Plinius (23–79 AC) was also reported to cure epilepsy, infertility, and ulcers using this plant. Celcus the Platonist remarked that mistletoe was used in the treatment of swellings or tumors (around 150 AC). Tabernaemontaunus stated that its leaves healed hepatitis, leprosy, and mumps. At the end of the 19th century, mistletoe-containing ointment was reported to be effective in the treatment of eczema, burn diseases, and some wounds (Bussing 2003). *V. album* has also drawn attention as a possible anti-cancer agent since the 1920s (Habeck 2003). Although it is a parasitic plant, its medical importance has been gradually increasing due to the diverse phytochemicals that the plants contain such as lectins, viscotoxins, alcholoids, amines, amino acids, flavonoids, glycosides, lignans, carbocyclic acids, phenylpropanes, polypeptides, polysaccharides, sugar alcohols, polyphenolic, and terpenoid compounds all which are known to have rich biological activities (Deliorman *et al*. 2001, Arda *et al*. 2003, Sengul et al. 2009, Nazaruk & Orlikowski 2016). However, the use of the European mistletoe *V. album* is common in medicine since the American mistletoe *Phoradendron flavescens* (Pursh Nutt.) is toxic (Ogunmefun *et al*. 2013). The compounds obtained from *V. album* are prevalently used for gastro-intestinal, diabetes, blood pressure problems, treatment of cancer, the hepatitis C virus (HCV), HIV, and human parainfluenza virus type 2 (HPIV-2) (Stoss & Gorter 1998, Tusenius *et al*. 2001, Karagöz *et al*. 2003), but the field of the use of the plant may vary based on the chemical properties of host tree which in turn affect compounds present in *V. album*.

Viscum album has been successfully used for the treatment of infection diseases in consequence of the effects of plant on microorganisms (Hussain *et al*. 2011). A bacterial infection begins with the organization of bacteria acting in unison (Rumbaugh *et al*. 2009, Antunes *et al*. 2010). This system is known as "Quorum-sensing" (QS) system (Waters & Bassler 2005). Microorganisms cannot activate their resistance mechanisms if this step is blocked in the treatment of infection (Adonizio *et al*. 2006, Musthafa *et al*. 2010). Biofilms are thought to be associated with microbial infection and their formations are considered to be regulated by QS (Brackman & Coenye 2015). Therefore, maturation and eradication of biofilms have great importance in fighting infection (Chung & Toh 2014).

In the present study, we evaluated the anti-biofilm activities of ethanol, chloroform, and dichloromethane: methanol extracts of different parts of *Viscum album* against *Listeria monocytogenes* ATCC 7644, *Staphylococcus epidermitis* wt, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Proteus vulgaris* Hauser, *Pseudomonas aeruginosa* PA14, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157: H7, and *Bacillus cereus* RSKK 863 and the interaction of these plant extracts with bacterial QS. *Viscum album* was selected as the study material because of the wide use of natural and easily available herbal drugs obtained from it.

Materials and Methods

Collection of V. album samples and extract preparation

Viscum album samples collected at an altitude of 1475m around Ihlara-Kulaköy in Aksaray province in October and November 2015 were used. The Flora of Turkey and The East Aegean Islands (Davis 1965) and The Checklist of the Flora of Turkey - Vascular Plants (Güner 2012) were used for identification of the plant specimens. Fresh plant samples were separated as leaves, stems, and fruits in sterile conditions in the laboratory and were left to dry at room temperature. Dried plant parts were grinded by a pulverizer, and 100gr/500ml of each dry plant part was extracted with ethanol, chloroform, and dichloromethane:methanol (w/v) (at 60°C for 6h) in soxhlet apparatus. The extracts were then concentrated in a rotary evaporator after the residues had been centrifuged (at 3.000g) and washed with physiological saline solution for 5 minutes. The supernatants of the extracts were kept at 4°C and used for future investigations. The residues were used for anti-quorum sensing and anti-biofilm experiments.

Bacterial Strains and Culture Conditions

The microorganisms used in the study were produced from the microbial culture collection kept in microbiology laboratory of Scientific and Technological Research Center of Aksaray University. *Chromobacterium violaceum* ATCC 12472 and *C. violaceum* CV026 strains used for anti-quorum sensing were kindly provided by Prof. Dr. Robert Mclean in Department of Biology, Texas State University-San Marcos, USA. *Listeria monocytogenes* ATCC 7644, *Staphylococcus epidermitis* wt, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Proteus vulgaris*, *Pseudomonas aeruginosa* PA14, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157: H7, and *Bacillus cereus* RSKK 863 were grown using the Brain Heart Infusion Broth (Merck) medium. Dichloromethane: methanol extracts were tested to determine if they had an effect on anti-quorum sensing and anti-biofilm activity of the strains.

Phytochemical Analysis with GC-MS

GC-MS analysis of *V. album* extracts (100mg/ml) was carried out by 5975 Mass Selective Detector with Agilent 6890 GC, using HP-5 MS capillary column (30m x 250µm x 0.25µm) at a flow rate of 3mL per minute, split mode injection (1:20), GC/MS interface at 280°C

temperature, column temperature program as 50°C (2 min) -300°C to 5°C min^{-1} (16 min). 1.0µL of the diluted sample was manually injected in splitless mode. Identification of the compounds was made by NIST 14 and Wiley Libraries within the mass spectral databases of the device at ASÜBTAM (Aksaray University Scientific and Technological Application and Research Center).

Antibacterial Activity Assays

The antibacterial influence of *Viscum album* leaf, stem, and fruit extracts on the nine pathogenic bacteria were investigated by the agar well diffusion method. Each purified extract was dissolved in dimethyl sulfoxide and stored at 4°C. The antibacterial activity of the substances was shown by a clear zone of inhibition around the application point. All bacterial strains were grown in Brain Heart Infusion Broth (Merck) for 24h at 37°C. The concentration of bacterial suspensions was adjusted to 10^8cells/ml in Brain Heart Infusion Broth and 100µl of each culture of bacteria was spread on agar plate surfaces. Wells 5mm in diameter were opened on the agar with a cork borer to load 20µl of each sample incubated at 25°C for 3h. Gentamycin (10UI) was used as positive control, while Dimethyl sulfoxide (DMSO) was used as the negative control. The plates were incubated at 37°C for 24h before they were examined for inhibition zones of growth. All tests were performed in triplicate.

Antiquorum Sensing Activity Assay

Plant extracts at a concentration of 50mg/ml were used depending on the results of the antibacterial effects. The bacterial culture of *C. violaceum* CV026 biosensor strain, which was grown at 30°C for 15 hours, was adjusted to a McFarland standard 0.5 (10^6CFU/ml). The ideal wavelength of the absorbance was established, the absorbance of the standards was measured, and the cell counting in UV-Vis spectrophotometrically was performed. *Chromobacterium violaceum* CV026 and the extract of 50µl *N*-(ß-ketocaproyl)-L-homoserine lactone (3-oxo-C6-HSL) on 10ml soft Luria Bertani (LB) agar medium was added for agar diffusion test. LB agar medium was prepared with 0.9% agar, 100µl *C. violaceum* CV026, and 50µl *N*-(ß-ketocaproyl)-L-homoserine lactone (3-oxo-C6-HSL) extract and after solidification, 4mm diameter wells were scooped out from the LB agar medium. The wells were filled with the plant extracts (50mg/ml) in different solvents. The agar plates were incubated at 30°C for 48 hours. Agar diffusion tests were also conducted in triplicate (Adonizio *et al*. 2006, Bezek *et al*. 2016, Oliveira *et al*. 2016).

Violacein Pigment Isolation

100µl of 20ml of fresh *C. violaceum* CV026 strain grown LB medium in 15 hours was inoculated according to the procedure given in Table 1 for the extraction of violacein pigment from the liquid culture. The bacterial culture was incubated at 30°C for 24 hours and then vortexed. 200µl of the culture from the tube was transferred to a 1.5ml Eppendorf microcentrifuge tube. 200µl of 10% sodium dodecyl sulfate (SDS) solution was added in the culture for cell fractionation, and the culture was kept at room temperature for 5 minutes after vortexing for 5 seconds. 900µl of water-saturated butanol (50ml n-butanol + 10ml distilled water) was added into the tube for violacein pigment isolation, and the tube was vortexed for 5 seconds. It was then centrifuged at 10,000rpm for 5 minutes. The upper phase was transferred to a new tube. It was read spectrophotometrically at 595nm, and the amount of viola was determined (Khan 2009).

Table 1. *Viscum album* extract application procedure.

	Group A	Group B	Group C	Final concentration
Luria Bertani Broth	895µl	890µl	890µl	
***C. violaceum* CV026**	100µl	100µl	100µl	1x10^8cfu/ml
3-oxo-C6-HSL	50µl	50µl	50µl	150µmol/ml
***Viscum* extracts**	-	-	5µl	50mg/ml
DMSO or Ethanol	-	5µl	-	
Total volume	1045µl	1045µl	1045µl	

*Group A and B are the control groups. Group C is the experimental group.

Anti-biofilm Activity Assays

Biofilm formation was confirmed with the Crystal Violet Method. The test bacteria which were incubated in Tryptic Soy Broth at 37°C for 24 hours were diluted according to Mc Farland 0.5. 100µl of the bacterial cultures was inoculated in 5ml Tryptic Soy Broth (TSB) in a shaking incubator at 120rpm for 24 hours. Following the incubation, polystyrene microplates with 24 wells were filled with 900µl TSB medium + 50µl plant extract (50mg/ml) + 50 µl test bacteria, and left at 37°C for 48 hours. The mediums were removed from microplates, and washed by 1xPBS buffer three times. The microplates were dried at 65°C in an incubator, and then were left to be dyed with 1% Crystal Violet for 2 minutes. The microplates were washed three times with distilled water at the end of the staining process and dried at room temperature. The Crystal Violet solutions in microplates solved by 30% acetic acid solution were read in the spectrophotometer at 595 nm against the control group. Thus, the ability of biofilm formation of the test bacteria was determined (Stepanović *et al*. 2000, Hoffman *et al*. 2005, O'Toole 2011, Kaya *et al*. 2016).

Statistical analyses

Arithmetic means and standard deviations of the obtained data were calculated in Microsoft Excel 2016.

Results

In recent years, despite the development of technology and medicine, the fight against the infection factors remains inadequate. This leads the scientists to the use of natural resources. The use of plant extracts in healthcare industry has recently gained popularity, and it is a prevalent application in not only in Turkey but also in

many developing countries worldwide. Scientists have been interested in the availability of new compounds in plants which have not yet been discovered for the inhibition of anti-quorum sensing recently. The effects of compounds in plant extracts on microorganisms have been reported to vary according to the plant species, amount of plant parts in the extract, and their maturation.

The utilization of *V. album* in traditional medicine implies that it can also be used in modern medicine, especially in cancer and cardiovascular diseases (Gray & Flatt 1999). According to epidemiological studies, essential oil and phenolic compounds have inhibitive effects against the progress of bacterial infections (Ghuman *et al*. 2016, Martinelli *et al*. 2017). GC-MS chromatogram analysis of leaf extracts of *V. album* showed different peaks, which indicates the presence of 8 main phytochemical constituents (Fig. 1), whilst the stem of *V. album* was found to include 10 main components (Fig. 2). These constituents were characterized and identified by comparing their mass spectra with the NIST library (Tables 2,3).

The GC-MS chromatogram analysis of fruit extracts showed that the content of fruit extracts appeared to be rich in some compounds such as 5-hidroximetilfurfural, glucose, Inositol, Palmitic acid, Stearic acid, p-coumaric acid, and phenolic components (Fig. 3). The identification and quantification of phenolic compounds in fruits were analyzed by the GC-MS analysis (Table 4). The overall results of the GC-MS analysis showed that both stem and leaf extracts contained polyphenolic compounds that are biologically more important compared to compounds in fruits. Although the fruit extracts of *V. album* appeared to be rich in antioxidant content, they should be used with caution because they have cytotoxic activity. The pharmacologically active chemical content of the *V. album* extracts varies depending on the host plant and the harvesting time (Büssing & Schietzel 1999). In conclusion, this study revealed the anti-quorum sensing, antibiofilm, and antimicrobial activities of the main chemical components of the *V. album.*

Viscum album is known to possess healing properties and was successfully used in treatment applications of various infectious diseases in humans. The antibacterial activity of *V. album* stem and leaf extracts in our study was determined on different pathogenic bacteria. The results of the antimicrobial screening of fruit, stem and leaf extracts are shown in Tables 5 and 6. The extracts prepared at concentrations of 150mg/ml, 200mg/ml, and 250mg/ml in methanol: dichloromethane, ethanol, and chloroform were shown to stop the bacterial growth.

The dichloromethane: methanol extract prepared at a concentration of 50mg/ml inhibited only on *Proteus vulgaris*. In general, it was found that the effectiveness of chloroform and ethanol extracts prepared at 150mg/ml concentration on bacteria was lower, but the efficiency of the same concentration of dichloromethane: methanol extract on bacterial growth was higher.

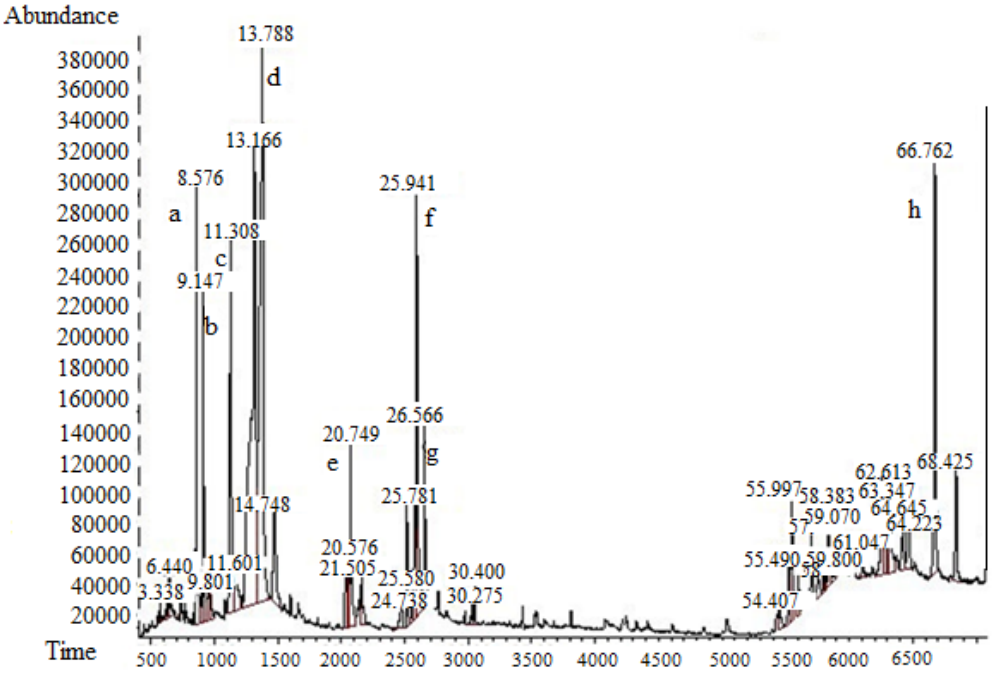

Fig. 1. GC-MS analysis of *V. album* leaf extracts. Main components: (a) Trans-cinnamic acid, (b) 3,5-Dimethoxyphenol, (c) Thiophane, propyl-, (d) Ethoxycitronellal, (e) Palmitic acid, (f) Alpha-linolenic acid, (g) Stearic acid, (h) β-Amyrin.

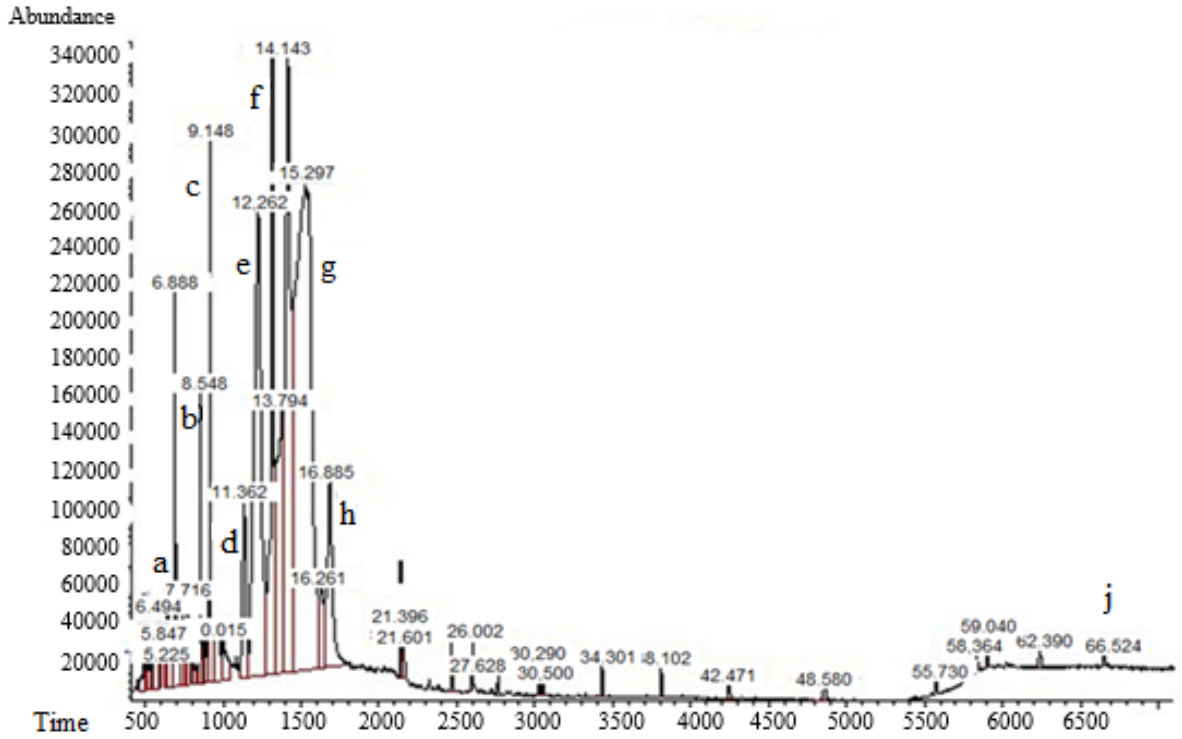

Fig. 2. GC-MS analysis of *V. album* stem extracts. Main components: (a) Phenol, 2,6-dimethoxy, (b) trans-cinnamic acid, (c) Phenol, 3,5-dimethoxy-, (d) Spiro[1,3-dioxolane-2,2'-[7]oxabicyclo[2.2.1]hept[5]ene], (±)-, (e) 1,3,4,5-tetrahydroxy-cyclohexane carboxylic acid, (f) 2-ethyl-1-thia-cyclopentane, (g) Octadecamethylcyclononasiloxane, (h) Mome Inositol, (i) Eicosane, (j) Gibberellic acid.

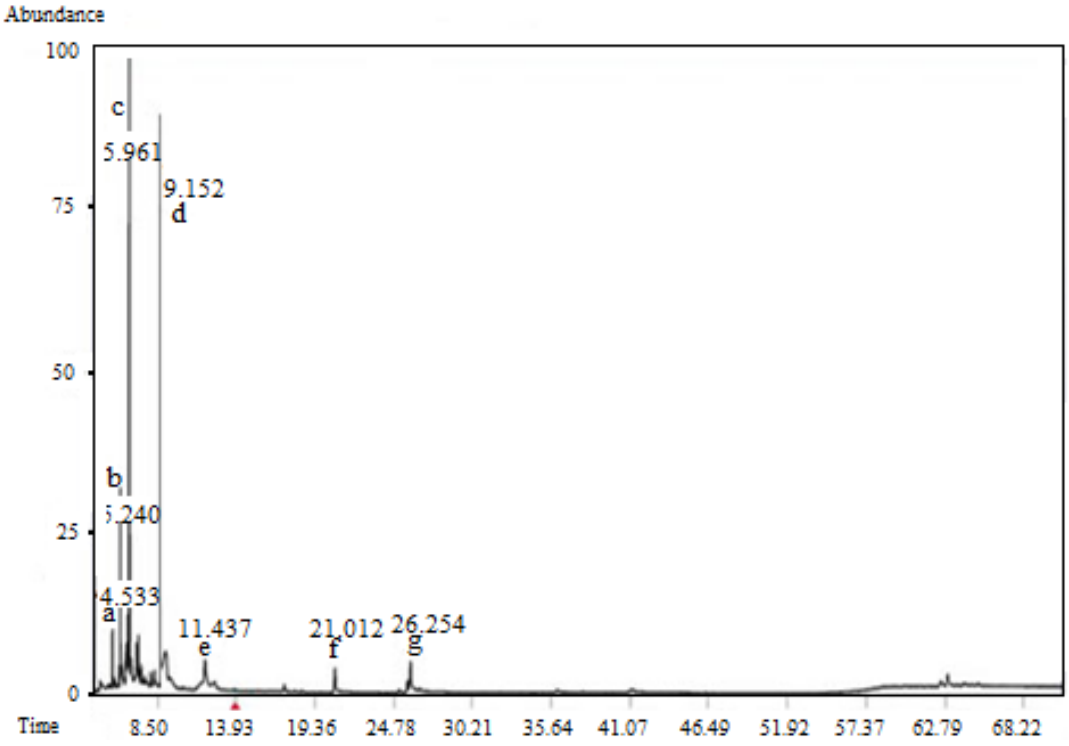

Fig. 3. GC-MS analysis of *V. album fruit* extracts. Main components: (a) 5-Hidroximetilfurfural, (b) Glucose, (c) Inositol, (d) Palmitic acid, (e) Stearic acid, (f) p-Coumaric acid, (g) Phenols.

Table 2. GC-MS Analysis of *V. album* leaf extracts.

Peak	RT	Area %	Library ID	Molecular formula
1	8.577	4.08	Trans-cinnamic acid	$C_9H_8O_2$
2	9.146	2.63	3,5-Dimethoxyphenol	$C_8H_{10}O_3$
3	11.298	8.49	Thiophane, propyl-	$C_7H_{14}S$
4	12.897	10.59	Thiophene D3	C_4H_4S
5	13.159	4.58	Xanthoxylin	$C_{10}H_{12}O_4$
6	13.789	25.23	Ethoxycitronellal	$C_{12}H_{24}O_2$
7	14.727	3.23	methyl β-D-mannoside	$C_7H_{14}O$
8	20.355	1.47	Palmitic acid	$C_{16}H_{32}$
9	20.739	2.43	Palmitic acid	$C_{16}H_{32}$
10	25.168	1.75	Neophytadiene	$C_{20}H_{38}$
11	25.767	1.90	cis-Linoleic acid	$C_{18}H_{32}$
12	25.937	8.26	Alpha-linolenic acid	$C_{18}H_{30}O_2$
13	26.567	2.82	Stearic acid	$C_{18}H_{36}$
14	55.305	1.46	4-Cyclohexene-1,2-dicarboximide, N-butyl-	$C_{12}H_{17}NO_2$
15	55.490	1.75	5-Nitro-2-benzofurancarboxylic acid	$C_9H_5NO_5$
16	55.997	0.87	Zierone	$C_{15}H_{22}O$
17	59.057	1.76	Propiophenone, 2'-(trimethylsiloxy)-	$C_{12}H_{18}O_2Si$
18	62.594	2.19	Gibberellic acid	$C_{19}H_{22}O_6$
19	63.240	1.23	5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)-	$C_{15}H_{22}O$
20	66.761	7.81	β-Amyrin	$C_{30}H_{50}O$
21	68.421	2.37	Hexamethylcyclotrisiloxane	$C_6H_{18}O_3Si_3$

Table 3. GC-MS Analysis of *V. album* stem extracts.

Peak	RT	Area %	Library ID	Molecular formula
1	4.902	0.29	Phenol	C_6H_6O
2	5.056	0.32	3-Phenoxypropionic acid	$C_9H_{10}O_3$
3	5.225	0.19	Ethoxybenzene	$C_8H_{10}O$
4	5.701	0.64	Phenol, 2-metoxy-	$C_7H_8O_2$
5	5.917	0.21	Thiophene, tetrahydro-3-methyl-2-propyl-, cis-	$C_8H_{16}S$
6	6.347	0.44	Pyrocatechol	$C_6H_6O_2$
7	6.501	0.89	(Ethenyloxy)-benzene0	C8H8O
8	6.885	2.74	Benzohydroquinone	$C_6H_6O_2$
9	7.362	0.60	2-methoxy-4-vinyl phenol	$C_9H_{10}O_2$
10	7.716	0.55	Phenol, 2,6-dimethoxy	$C_8H_{10}O_3$
11	8.085	0.25	3-Phenyl-2-Propenoic Acid Methyl	$C_{10}H_{10}O_2$
12	8.546	1.13	Trans-cinnamic acid	$C_9H_8O_2$
13	8.731	0.33	2-Propenoic acid,3-phenyl-	$C_9H_8O_2$
14	8.977	0.47	Thiacyclopentadeca-3,13-diyne	$C_{14}H_{20}S$
15	9.146	2.21	Phenol, 3,5-dimethoxy-	$C_8H_{10}O_3$
16	11.360	2.54	Spiro[1,3-dioxolane-2,2'-[7]oxabicyclo[2.2.1]hept[5]ene], (±)-	$C_8H_{10}O_3$
17	12.267	14.39	1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{12}O_6$
18	13.174	5.96	2-Hydroxyl-4,6-dimethoxy-acetophenone	$C_{10}H_{12}O_4$
19	13.789	6.67	3,4-Di-O-methyl-L-arabinopyranose	$C_7H_{14}O_5$
20	14.143	14.23	2-Ethyl-1-thia-cyclopentane	C_9H_{18}
21	15.296	33.80	Octadecamethylcyclononasiloxane	$C_{18}H_{54}O_9Si_9$
22	16.265	1.47	Alpha-d-mannofuranoside,methyl	$C_7H_{14}O_6$
23	16.865	4.34	Mome Inositol	C7H14O6
24	21.401	0.50	Eicosamethylcyclodecasiloxane	$C_{20}H_{60}O_{10}Si_{10}$
25	21.601	0.28	Eicosane	$C_{20}H_{42}$
26	24.660	0.26	Heneicosane	$C_{21}H_{44}$
27	26.014	0.32	Hexadecamethylcyclooctasiloxane	$C_{16}H_{64}O_8Si_8$
28	27.628	0.10	Nonadecane	$C_{19}H_{40}$
29	30.288	0.18	Hexadecamethylheptasiloxane	$C_{16}H_{48}O_6Si_7$
30	30.504	0.12	Hexatriacontane	$C_{36}H_{74}$
31	34.301	0.17	Silikonfett SE30(Grevels)	-
33	42.466	0.17	Silicone grease,siliconfett	-
34	48.586	0.18	Tetracosamethylcyclododecasiloxane	$C_{24}H_{96}O_{12}Si_{12}$
35	55.736	0.16	4H-Dibenz[de,g]isoquinoline,5,6,6a,7-tetrahydro-1,2,9,10-tetramethoxy-5-methyl-	$C_{21}H_{25}NO_4$
36	58.365	0.26	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$
37	62.394	0.17	1,2-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$
38	66.515	0.16	Gibberellic acid	$C_{19}H_{22}O_6$

Table 4. GC-MS *Analysis* of *V. album* fruits extracts**.**

Peak	RT	Area %	Library ID	Molecular formula
1	4.533	15.16	5-Hidroximetilfurfural	$C_6H_6O_3$
2	5.240	10.05	Glucose	$C_6H_{12}O_6$
3	5.961	15.70	Inositol	$C_7H_{14}O_6$
4	9.152	1.53	Palmitic acid	$C_{16}H_{32}$
5	11.437	1.80	Stearic acid	$C_{18}H_{36}$
6	21.012	1.72	p-Coumaric acid	$C_9H_8O_3$
7	26.254	1.78	Phenol	C_6H_6O
8	62.765	0.30	Gibberellic acid	$C_{19}H_{22}O_6$

The dichloromethane: methanol extract at concentrations of 150, 200, and 250mg/ml showed the highest inhibition on *E. faecalis* ATCC 29212. All extracts prepared at concentrations 150, 200, and 250 mg/ml were found to be effective on *P. aeruginosa* PA14. We found that the dichloromethane: methanol extract at concentrations from 100 to 250mg/ml prevented spread of *Klebsiella pneumoniae* ATCC 700603 with the zones ranging from 11-15mm. The efficacy of dichloromethane:methanol extracts at concentrations of 100, 150, 200 and 250mg/ml was higher on *Klebsiella pneumoniae* ATCC 700603 strain than *E. coli* O157: H7. Different concentrations of *V. album* extracts were also found to stop the growth of *L. monocytogenes* ATCC 7644 (8-13mm), *B. cereus* RSKK 863 (7-13mm), *S. epidermitis* wt (9-13mm), and *P. aeruginosa* ATCC 27853 (10-13mm) (Table 6).

According to the antimicrobial activity analysis results, the highest antimicrobial effect was observed in dichloromethane: methanol extracts for all plant parts tested. Therefore, anti-biofilm and anti-quorum sensing assays were performed using dichloromethane: methanol extracts. The lowest concentration of fruit extract for biological activity was determined as 150 mg/ml. Therefore, anti-quorum sensing and anti-biofilm tests were performed at a concentration of 100mg/ml in accordance with the concentration of leaf and stem extracts. When we analyzed the antimicrobial activity of dichloromethane: methanol extracts of fruit, we found that the maximum effect was on *E. faecalis* ATCC 29212 with a zone diameter of 15 mm. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* O157: H7 are among the most inhibited species by the fruit extracts.

The fruit extract (150mg/ml) was found to be less effective against *P. aeruginosa* PA14 (10 mm inhibition zone). The fruit extracts of prepared with ethanol and chloroform resulted in an inhibition of 5-9mm. It was also proved that DMSO solution selected as the control had no effect on any of the strains. When our results are evaluated in general, it is clear that the extracts whose antibacterial activities were tested have inhibitory effects on the bacterial strains included in the study. The antimicrobial activities of different concentrations of N-hexane extracts of *V. album* L. subsp. *abietis* (Wiesb.) was investigated on *Candida albicans* (Robin) Berkhout, *Bacillus subtilis* (Ehrenberg) Chon, *Staphylococcus aureus* Rosenbach, *Escherichia coli* Escherich, *Pseudomonas aeruginosa* (Schröter) Mihgula, *Enterobacter cloacae* (Jordan) Hormaeche & Edwards, and *Proteus vulgaris* and the 6th and 7th hexane fractions were found to be effective (Ertürk *et al*. 2004).

The antimicrobial activities of dichloromethane, chloroform, and water extracts of *Viscum capense* L. were tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* (Amabeoku *et al*. 1998). The results showed that the chloroform extract inhibited the growth of *S. aureus*, whereas it was not effective on other microorganisms.

As a result of the present study, *V. album* extracts were found to be effective on 3-oxo-C6-HSL which is a bacterial communication molecule used by many Gram-negative pathogenic bacteria. *C. violaceum* CV026 is a mini Tn-5 mutant of *C. violaceum* ATCC 31532 (Mc Clean *et al.* 1997). The mutant produces violacein pigment when 3-oxo-C6-HSL is in the medium. It was seen that violacein pigment production was reduced using both agar diffusion test (zone formation) and the extraction of violacein pigment from broth medium when the *Viscum* extract was released into the medium. We came to the conclusion that this molecule was affected as a result of these tests. The dichloromethane: methanol extracts of stem, leaf and fruit of *V. album* showed promising anti-quorum sensing activity, and a clear white opaque zone of inhibition was observed in the biosensor plate containing the reference strain *C. violaceum* CV026 (Fig. 4).

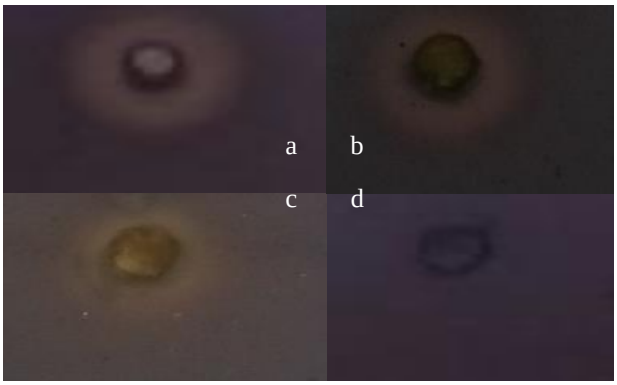

Fig. 4. Inhibition of violacein production by (a) fruit, (b) leaf, and (c) stem extracts in dichloromethane: methanol using *Chromobacterium violaceum* CV026 biomonitor strain and the agar well diffusion method. The inhibition was detected by a colorless, opaque halo around the discs. (d) DMSO was used as control. In accordance with the data acquired from well diffusion studies, the pigment isolation of violacein is carried out to understand the inhibition of the signal molecule (Fig. 5).

Therefore, it is possible to suggest that the interception of bacterial communication by *V. album* extracts can bring a new perspective to antimicrobial studies.

The production of violacein by *C. violaceum* CV026 was inhibited by dichloromethane: methanol extracts of leaf, stem, and fruits (Fig. 5). The presence of plant extracts had no influence on the growth of *C. violaceum* CV026. The violacein production and cell counts were similar in the control group with solvents (Group B) and the group without solvent (Group A).

Viscum album was reported to be highly effective on both bacteria and fungi (Dulger & Gonuz 2004). The components of *V. album* were proved to be effective as anti-diabetic and anti-hyperlipidemic in diabetic rats, which supports the use in traditional medicine (Adaramoye *et al*. 2012). The positive effect on lipid profile in diabetic rats eliminates secondary complications of diabetes. Önay-Uçar *et al*. (2012) stated that chloroform extracts of *V. album* can inhibit oxidative DNA damage, and that biological activity of *V. album* depends on its host tree.

The biological activity (anti-tumor and anti-bacterial) of the European mistletoe *V. album* collected from 13 different host trees was evaluated by (Turker *et al.* 2012). The water extract of the plants collected from *Prunus divaricata* Ledeb. showed the best antitumor activity (87.3% inhibition). The anti-quorum sensing activity of *E. angustifolia* extracts were determined in another study (Erdonmez *et al*. 2016). In our study, *V. album* was also collected from *Elaeagnus angustifolia* L. as the host plant. When the results of effects of *V. album* extracts are compared with the effects of *E. angustifolia* extracts, it is seen that the extracts of *V. album* are more efficient than the extracts of *E. angustifolia* on QS process. Even so, *V. album* may contain the chemical components of its host and these components may increase the biological activity of *V. album*.

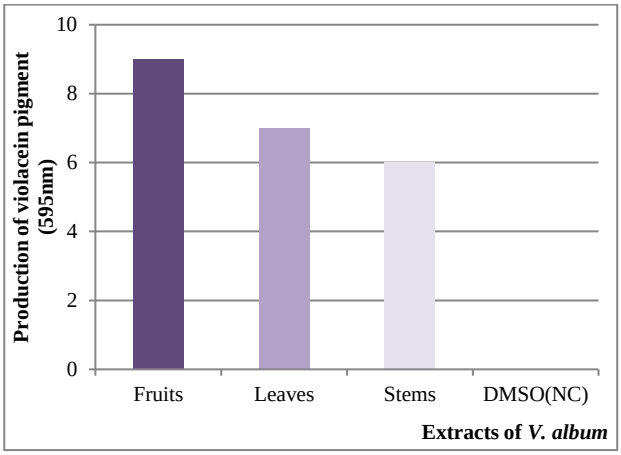

Fig. 5. Inhibition of violacein production by *Chromobacterium violaceum* CV026.

In future, the prevention of bacterial communication by the help of plant extracts instead of anti-microbial activity will increase the chance of success in infectious diseases. Anti-quorum sensing activity of *V. album* may play an important role in antibacterial activity, and therefore, it ensures an extra strategy in the struggle against bacterial infections. However, molecular researches are needed to explain the mechanism of antibacterial activity completely.

Many studies showed that the biofilm formation by pathogens leads to an increase in their virulence (Vuong *et al.* 2004, Antunes *et al.* 2010). Therefore, if the biofilm formation is inhibited, the bacterial infection can be prevented.

Table 5. Growth inhibition activity of *V. album* fruit exracts against the pathogenic bacteria tested.

Exracts (150mg/ml)	Zone of inhibition (mm)								
	Listeria monocytogenes ATCC 7644	*Staphylococcus epidermitis* wt	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumoniae* ATCC 700603	*Proteus vulgaris*	*Pseudomonas aeruginosa* PA14	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* O157: H7	*Bacillus cereus* RSKK 863
Ethanol	7±0.40	5±0.50	7±0.04	6±0.24	8±0.27	6±0.24	6±0.33	8±0.20	5±0.08
Chloroform	8±0.24	7±0.33	7±0.04	8±0.40	9±0.20	7±0.040	8±0.27	6±0.24	7±0.04
Dichloromethane: methanol	10±0.51	12±0.50	11±0.13	14±0.90	13±0.33	10±0.55	15±0.40	14±0.50	12±0.48
DMSO	0	0	0	0	0	0	0	0	0

0, no inhibition, DMSO, dimethylsulfoxide, ± Standard deviation

Table 6. Growth inhibition activity of *V. album* stem and leaf extracts against the pathogenic bacteria tested.

Microorganisms			*Bacillus cereus* ATCC 10876	*E. coli* O157 :H7	*Enterococcus faecalis* ATCC 29212	*Staphylococcus epidermitis* wt	*Pseudomonas aeruginosa* ATCC 7853	*Proteus vulgaris*	*Listeria monocytogenes*	*Klebsiella pneumoniae* ATCC 700603	*P. aeruginosa* PA14	*DMSO*
Zone of inhibition (mm)	Chloroform extracts	50mg/ml	-	-	-	-	-	-	-	-	-	0
		100mg/ml	-	-	-	-	-	-	-	-	-	0
		150mg/ml	7 ±0.51	-	8 ±0.40	9 ±1.00	10 ±1.00	12 ±0.13	8 ±0.47	-	14 ±0.20	0
		200mg/ml	8 ±0.44	-	8 ±0.52	10 ±0.27	12 ±0.03	12 ±0.40	9 ±0.85	-	14 ±0.62	0
		250mg/ml	8 ±0.33	-	9 ±0.40	10 ±0.13	12 ±0.38	12 ±0.03	9 ±0.08	-	15 ±0.04	0
	Ethanol extracts	50mg/ml	-	-	-	-	-	-	-	-	-	0
		100mg/ml	-	-	-	-	-	-	-	-	-	0
		150mg/ml	7 ±0.04	-	8 ±0.24	9 ±0.10	10 ±0.13	12 ±0.16	8 ±0.27	-	14 ±0.33	0
		200mg/ml	8 ±0.50	-	8 ±0.26	10 ±0.65	12 ±0.27	12 ±0.12	9 ±0.40	-	14 ±0.66	0
		250mg/ml	8 ±0.03	-	9 ±0.01	10 ±1.00	12 ±0.90	12 ±0.50	9 ±0.33	-	15 ±0.10	0
	Methanol: dichloromethane extracts	50mg/ml	-	-	-	-	-	12 ±0.07	-	-	-	0
		100mg/ml	10 ±0.27	6 ±0.44	21 ±0.28	11 ±0.55	13 ±0.24	16 ±0.25	8 ±0.33	11 ±0.27	18 ±0.34	0
		150mg/ml	12 ±0.04	8 ±0.33	25 ±0.28	13 ±0.70	13 ±0.56	17 ±0.8	10 ±0.66	13 ±0.27	19 ±0.48	0
		200mg/ml	13 ±0.80	8 ±0.66	26 ±0.56	13 ±0.27	13 ±0.48	17 ±0.33	10±0.27	13±0.13	19±0.40	0
		250mg/ml	13±0.40	8±0.13	26±0.33	13±0.62	13±0.04	17±1.00	13±0.40	15±0.33	20±0.27	0

0, no inhibition, DMSO, dimethylsulfoxide, ± Standard deviation

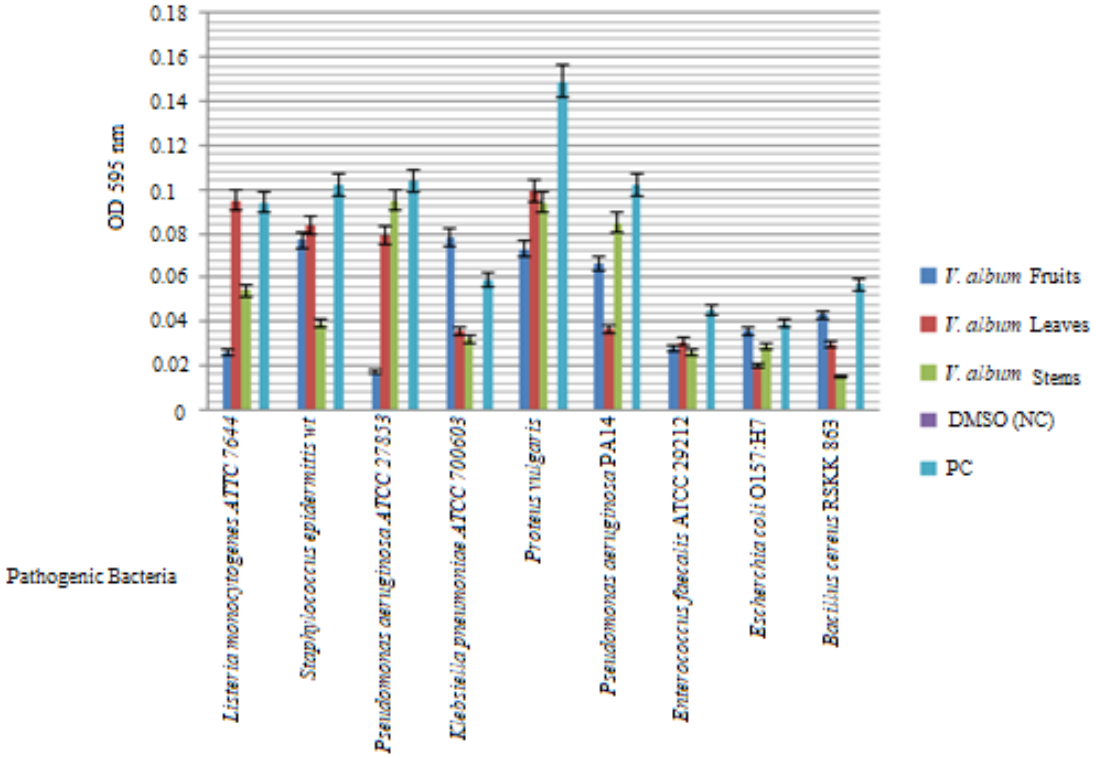

Fig. 6. The effect of *Viscum album* extracts in reducing biofilm formation in *Listeria monocytogenes* ATCC 7644, *Staphylococcus epidermitis* wt, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Proteus vulgaris*, *Pseudomonas aeruginosa* PA14, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157: H7, *Bacillus cereus* RSKK 863.

Nowadays, biological materials are mostly preferred for use in the inhibition methods of biofilm formation and also in protection against bacterial infections (Rutherford & Bassler 2012). Although many antimicrobial studies of *V. album* are available in literature, no studies related to anti-biofilm and anti-quorum sensing activity of this species has been carried out (Chandrashekhara *et al*. 2010, Hussain *et al*. 2011, Kotan *et al*. 2013, Nawrot *et al*. 2014, Sadananda *et al*. 2014).

Some plant extracts were reported to show anti-biofilm activity by inhibiting the initial phase of biofilm formation and growth (Sandasi *et al*. 2008). Our results showed that *V. album* extracts were highly effective in degrading the biofilm activity of *Listeria monocytogenes* ATCC 7644, *Staphylococcus epidermitis* wt, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Proteus vulgaris*, *Pseudomonas aeruginosa* PA14, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157: H7, and *Bacillus cereus* RSKK 863 (Fig. 6). The formation of bacterial biofilm decreased after the addition of plant extracts. A decrease ranging from 20% to 80% depending on the extract used was recorded in the spectrophotometric measurements. The effects of biofilm inhibition of leaf and stem extracts were higher than effects of fruit extracts.

Bazargani & Rohloff (2016) investigated the vitro anti-biofilm activities of essential oils and plant extracts of *Coriandrum sativum* L. through Crystal Violet (CV) and XTT tests and found that the plant extracts and their essential oils could reduce the bacterial biofilm at a rate of 50% and that the activity of essential oils of coriander was particularly high. The ethanol extract of stem bark of *Terminalia fagifolia* Mart. showed a distinct biofilm inhibition, and it could block biofilm formation of some strains above 80% (de Araujo *et al*. 2015). Stem, leaf and fruits of *V. album* contain very large amounts of phytochemicals such as flavonoids, alkaloids, steroids, glycosides, and phenols showing that *V. album* is suitable for medical use.

In conclusion, this study demonstrated that *V. album* extracts are remarkable anti-biofilm agents against biofilm formed by pathogens and inhibit signal molecules, which form the basis of bacterial communication mechanism.

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Research Article

CORRELATION PROFILES OF THE ACCUMULATED METALS IN SEAWATER, SEDIMENT AND *Pachygrapsus marmoratus* (Fabricius) TISSUES IN BLACK SEA (ORDU, TURKEY)

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Abstract: This study was performed in order to investigate the interactions of accumulation patterns of some metals (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) in exoskeleton, gill, hepatopancreas and muscle tissues of the marbled crab *Pachygrapsus marmoratus* (Fabricius) sampled from near streams and domestic discharge points along the coastal region of Black Sea along Ordu in Turkey as well as patterns in sediment and seawater samples of the crab sampling sites. The study area covering a coastal stretch of 28 km along Black Sea lies between the latitudes 41°03'46.42"-41°07'42.35"N and longitudes 37°28'45.63"-37°41'15.29"E. The metal contents of the samples were analysed by the inductively coupled plasma - optical emission spectrometry (ICP-OES) technique. The results showed that the accumulation orders of the metals, in a descending order, were Fe>Al>Mn>Zn>Cr>Pb>Cu>As>Ni>Cd in the sediment, Fe>Mn>Cr>As>Cd in the water, and Al>Fe>Cu>Mn>Zn in crab tissues when evaluated together**.** In the crab samples, the amount of the accumulation of all metals was ordered as gills>exoskeleton>hepatopancreas>muscle. Al and Fe were the predominant metals in the sediment and crab tissues but Al was not detected in the seawater samples. A correlation test was performed to reveal the interaction of accumulation in the sediment, water and crab tissues. Metal-metal interactions and their co-accumulation was detected by correlation test. These interactions which exist in the crab tissues but not in the seawater and sediment were the main point of this study. Gills and exoskeleton displayed the greatest number of significant correlations between metal–metal interactions. Also, metal concentrations were found to be higher in the gills and exoskeleton. Strong correlations between Mn-Al (r=0.954, correlation p<0.001), in the exoskeleton, Al-Fe (r=0.849, correlation p<0.001), Mn-Zn (r=0.854, correlation p<0.001) in the gills, Al-Zn (r=0.882 correlation p<0.001) in the hepatopancreas were determined. Moderate correlations between Zn-Cu were found in the hepatopancreas. These metal-metal interactions may have been a result of metallothionein activity. No significant relations were found between metal levels in sediment samples and crap tissues (p>0.05). The results also showed that metals present in the seawater and sediment did not directly transform to tissue accumulation. This result showed that metal amounts in the tissues of the *P. marmoratus* did not reflect environmental contaminations and that sediment accumulated higher amounts of metals than seawater and tissues.

Key words: Correlation, metabolic pathways, transferrin, metallothionein.

Özet: Bu çalışma, bazı metallerin (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb ve Zn) Karadeniz (Ordu, Türkiye) kıyısı boyunca dere ve evsel atık bölgelerine yakın noktalardan örnekleme yapılmış mermer yengeci *Pachygrapsus marmoratus* (Fabricius)'un dış iskelet, solungaç, hepatopankreas ve kas dokularındaki birikim ilişkilerinini etkileşimlerini araştırmak amacıyla yapılmıştır. Karadeniz boyunca 28 km'lik bir kıyı şeridini kapsayan çalışma alanı 41°03'46.42"-41°07'42.35"N enlemleri ve 37°28'45.63"-37°41'15.29"E boylamları arasındadır. Numunelerin metal içerikleri indüktif eşleşmiş plazma - optik emisyon spektrometrisi (ICP-OES) tekniği ile analiz edilmiştir. Elde edilen sonuçlar birlikte değerlendirildiğinde, sedimentte Fe>Al>Mn>Zn>Cr>Pb>Cu>As>Ni>Cd, suda Fe>Mn>Cr>As>Cd ve yengeç dokularında Al>Fe>Cu>Mn>Zn şeklinde gözlenmiştir. Yengeç örneklerinde, tüm metallerin dokularda birikim miktarının sırası solungaç>dış iskelet>hepatopankreas>kas şeklindedir. Al ve Fe, sediment ve yengeç dokularında baskın metaller iken, deniz suyu örneklerinde Al tespit edilmemiştir. Sediment, su ve yengeç dokularında birikimin etkileşimini ortaya koymak için bir korelasyon testi yapılmıştır. Metal-metal etkileşimleri ve bunların birlikte birikimi korelasyon testi ile tespit edilmiştir. Yengeç dokularında gözlenen, ancak deniz suyu ve sedimentte bulunmayan bu etkileşimler çalışmanın temel noktasını oluşturmuştur. Metal-metal etkileşimleri arasında en fazla anlamlı korelasyon solungaç ve dış iskelette gözlenmiştir. Ayrıca, solungaçlarda ve dış iskeletlerde metal konsantrasyonları daha yüksek bulunmuştur. Dış iskelette Mn-Al (r=0,954, korelasyon p<0,001), solungaçta Al-Fe (r=0,849, korelasyon p<0,001), Mn-Zn (r=0,854, korelasyon p<0,001), hepatopankreasta Al-Zn (r=0,882 korelasyon p<0,001) arasında kuvvetli korelasyonlar tespit edilmiştir. Hepatopankreasta Zn-Cu arasında ise orta dereceli bir korelasyon bulunmuştur. Bu metal-metal etkileşimleri metallothionein aktivitesinin bir sonucu olabilir. Sediment örnekleri ve yengeç dokuları arasında anlamlı ilişki bulunamamıştır (korelasyon p>0,05). Hatta sonuçlar göstermiştir ki deniz suyu ve sedimentindeki metaller, dokudaki birikime direkt dönüşmemiştir. Bu sonuç *P. marmoratus*'un dokularındaki metal miktarının çevresel kontaminasyonu yansıtmadığını göstermektedir ve aynı zamanda sediment sonuçlarının deniz suyu ve yengeç dokularından daha fazla metal birikimi miktarını gösterdiği tespit edilmiştir.

Introduction

The coastal areas and estuaries which connect land and sea, provide spawning, nesting and feeding area, and transport nutrient and organic materials for crabs are critically important transition zones (CTZs) (Kasmin 2010). The CTZs fulfil fundamental ecological functions such as decomposition, nutrient cycle and nutrient production. Crabs living in such areas and the sediment present here are important components of these fundamental functions. The most critical function of crabs is achieved by enriching the sediment by processing organic material. Considering the fact that sea pollution is mostly caused by industrial, domestic and agricultural wastes, the major threats to biodiversity in the CTZs appear to be due to anthropogenic effects such as non-traditional pollutants (heavy metals, pesticides, new generation contaminants, drugs, nanoparticles, etc.), species invasions, overfishing, and climate change under the influence of industry, urbanisation and agricultural areas (Kasmin 2010).

CTZs and crabs in marine ecosystems can potentially be exposed to sub-lethal concentrations of a wide range of chemicals originating from anthropogenic sources. Considering the environmental conditions, the most dangerous environmental pollutants are heavy metals. It's a matter of vital importance to understand the existence, accumulation and distribution of heavy metals in marine ecosystems for which heavy metal pollution is a danger. Heavy metal accumulation in crab tissues can be higher than accumulation levels in seawater and sediment (Rainbow 2007), which may imply that there is an acceleration of environmental toxicity. This, in turn, raises the need of certain sensitive monitoring methods to be used to prevent, or least minimize, destructive effects of metal pollutions on marine species (Bresler *et al.* 2003).

There exist some monitoring systems which include chemical analysis of abiotic factors such as seawater and sediment. Although these monitoring systems produce accurate results, it is impossible to provide sufficient data on ecological situation when the number and range of contaminants exceed the ability of the chemical tests applied or when there are possible synergistic effects of contaminants on each other (Phillips & Rainbow 1994).

Metals and metalloids are important environmental pollutants and may show severe acute or chronic effects on living organisms (Ullah *et al.* 2015). Species dispersed in various habitats can play an important role for ecosystems. For instance, any variable that affects crabs can have a major impact on the habitat and the ecosystem (Siddon & Witman 2004, Pandya & Vachharajani 2011, Trivedi *et al.* 2012, Arya *et al.* 2014). Crabs are a vital component of food chain in marine ecosystems, and control ecological function by feeding on both detritus and organic matter (Parsa *et al.* 2014). *Pachygrapsus marmoratus* (Fabricius) is the most common rocky shore inhabitant of the Mediterranean, Black Sea and East Atlantic coasts, and is active both under and above seawater for long periods of time (Cannicci *et al.* 1999). Organisms need metals in varying quantities. A high concentration of any metal is toxic to organisms. Metal transporters, i.e. transferrin, natural resistance-associated macrophage protein (NRAMP) family, ZIP (ZRT and IRT-like protein) family, etc. are involved in the procurement of metals as in the case of transportation, storage and remobilization of metals to proteins that need metals in their structures (Kramer *et al.* 2007). Metals can accumulate in organisms through different metabolic pathways. These metabolic pathways are not selective about the metal since they can carry more than one metal (Menon *et al.* 2016). The lack of selectivity of the metabolic pathways leads to a competition between the metals and forms the basis of metal accumulation in organisms.

The studies performed with the aim of determination of metal levels in Black Sea region mostly used various fish species as an indication of accumulation of metal pollution in living organisms. In one of these study, Filazi *et al.* (2003) evaluated levels of Cu Pb, Cd, Cr and Ni in liver and muscle tissues of *Mugil auratus* Risso (syn. *Chelon auratus* (Risso)) collected from Sinop-Icliman, Black Sea, Turkey and found that the highest level of accumulation was of Pb. Tüzen (2003) determined concentrations of Pb, Cd, Fe, Cu, Mn and Zn in the fish species *Alosa caspia* (Eichwald), *Engraulis encrasicholus* (L.), *Trachurus trachurus* (L.), *Sarda sarda* (Bloch), and *Clupea sprattus* (L.) sampled from the middle Black Sea Region and found that levels of the essential metals in the samples were higher than those of the non-essential metals. Altas & Büyükgüngör (2007) investigated the heavy metals levels in water samples of the Middle Black Sea region of Turkey from May 2000 to October 2001 and determined that Cd and Cu levels generally and Pb and Zn levels sometimes exceeded the levels reported in the Marine General Quality Criteria, while Ni concentrations were at desired levels. Makedonski *et. al.* (2017) were investigated Cd, As, Hg, Pb, Zn and Cu levels in edible parts and gills of seven Bulgarian fish species ((*Sprattus sprattus* (L.), *Trachurus mediterraneus ponticus* Aleev (syn. *Trachurus mediterraneus* (Steindachner)) *Neogobius melanostomus* (Pallas), *Alosa pontica* (Eichwald) (syn. *Alosa immaculata* (Bennett)), *Sarda sarda*, *Pomatomus saltatrix* (L.), *Mugil cephalus* L. collected from north-east coast of Black Sea and found that the metal concentrations were highest in the gills for all fish species, showing that metal accumulation varied between fish species, tissues and the metals analysed.

In the present study, the marbled crab *Pachygrapsus marmoratus* (Fabricius) sampled from different streams and domestic discharge points along the coastal region of Black Sea in Ordu, Turkey was investigated for accumulation levels of Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn and in exoskeleton, gill, hepatopancreas and muscle tissues. The sediment and seawater samples from the crab sampling sites were also analysed for the same purpose. *Pachygrapsus marmoratus* was chosen as the

study material because it has previously been reported as a bioindicator for metal accumulation (Álvaroa *et al.* 2016). The sampling localities were selected from the coastal region of Ordu where no former study has been performed addressing metal accumulation levels. The results were analysed based on metal–metal interactions and co-accumulations and the obtained data was discussed with regard to the effects of metabolic pathways in co-accumulation.

Materials and Methods

Sample collection

The samples included in the study were obtained in February 2015 from 8 different stations along the coastal region of Black Sea in Ordu in Black Sea Region of Turkey. Stations were selected near from streams (Bolaman, Elekçi, Ilıca and Çalış) and domestic discharge points near human settlements (Fig. 1).

Table 1. Latitude and longitude of the sampling stations.

Station	Latitude	Longitude
S1	41°03'46.42"N	37°28'45.63"E
S2	41°02'18.59"N	37°29'33.06"E
S3	41°01'17.57"N	37°32'13.97"E
S4	41°02'01.92"N	37°35'02.09"E
S5	41°02'57.34"N	37°36'35.45"E
S6	41°04'51.87"N	37°37'44.11"E
S7	41°06'30.37"N	37°39'06.73"E
S8	41°07'42.35"N	37°41'15.29"E

The study area covered a coastal stretch of 28 km along Black Sea (Figure 1, Table 1). The 10 male crab samples from each location (N=80) were randomly collected directly by hand picking. Only male individuals were studied to prevent the results will not affect from gender. 2 surface seawater and 2 surface sediment samples from each station (N=16) were also sampled from the crab sampling sites.

Metal Analysis

Male crabs were immediately transported live to Toxicology Laboratory of Ankara University in frosted plastic storage boxes and stored at -20°C until the analyses performed. All samples were dissected immediately to obtain the tissues for metal analysis. Approximately 2g of tissue samples were taken from hepatopancreas, muscles, exoskeleton and gills through dissection and all were digested for the inductively coupled plasma/optical emission spectroscopy (ICP-OES) (Agilent, 5110) analysis. Tissue samples were placed in porcelain crucibles, dried at 50-60°C for 24h and then weighed to determine their net dry weights. Approximately 1g of each tissue sample was used for the measurements. 3mL of nitric acid (HNO_3) (Merck) were added to the dried tissue samples. The tissues were kept at 100°C temperature for 20min on a heating table to achieve a thorough digestion (Núñez-Nogueira *et al.* 2013). After the samples were cooled, 5mL of distilled water (pure water) was added on each. The samples were then filtered with a 0.45µm pore size glass microfiber Whatman filter paper with the help of a syringe.

Sediment samples were taken superficially from the same coordinates with a Bridge-Ekman grab sampler from sampling stations and samples were put in plastic containers. Both seawater and sediment samples were kept at 4°C prior to analysis. The water samples were acidified with HNO_3 at pH 2 and filtered before analysis. The samples were read in triplicate. Prior to analysis, the sediment samples were combined in a FRITSCH tungsten carbide mortar, mixed with the binder material (Wachs) at ICP-OES measurements. The sediment and seawater samples were analyzed by ICP-OES. It was service procurement from Cinar Engineering Consulting Co. for the reading of the samples. Reference materials (CRM-Mess 4 for sediment and LUTS-1 for tissues) were also analysed to avoid any error with samples. The reference material values and control samples results displayed good harmony between each other.

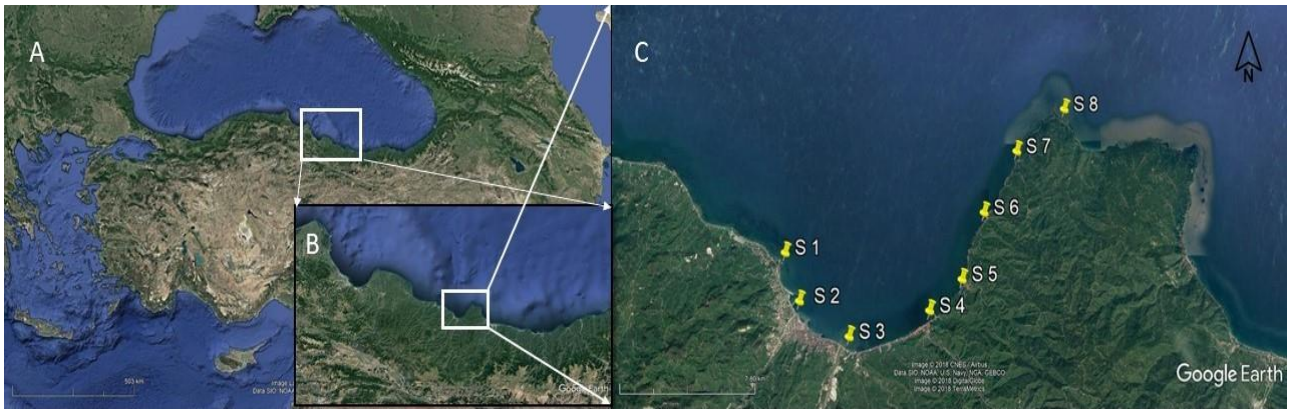

Fig. 1. The map showing the sampling locations. (A) Turkey, (B) Ordu province (C) Sampling Stations

Statistical Analyses

All statistical analyses were performed by the SPSS 21.0v. software. Prior to analysis, all results were subjected to the Kolmogorov–Smirnov test to observe the normality of data distribution. Data sets that fit a parametric distribution were analysed by the Pearson's correlation test, whereas those which did not fit a parametric distribution were analysed by the Spearman's correlation rank test. Mann-Whitney U-test test was used to investigate the accumulation differences in non-parametric data.

Results

The results showed that among the tested metals, only Al, Cu, Fe, Mn and Zn were detected in the crab tissues. When the accumulation levels of these metals were evaluated in all the tissues as a whole, the highest accumulation value was determined for Al followed by Fe, Cu, Mn and Zn (Table 2**).** The correlation analysis results revealed different correlation values for metal pairs for each tissue (Table 3). The correlations were strong and very strong for most of the pairs. The tissues were examined to investigate the metal co-accumulation tendency (Table 3). Gills and exoskeleton were found to show the greatest number of significant correlations of metal-metal interactions (Table 3). Strong correlations between Mn-Al ($r=0.954$, $p<0.001$), in the exoskeleton, Al-Fe ($r=0.849$, $p<0.001$) and Mn-Zn ($r=0.854$, $p<0.001$) in the gills and Al-Zn ($r=0.882$, $p<0.001$) in the hepatopancreas were noted.

The results showed that metal concentrations were found to be very lower in seawater samples than sediment samples (Table 4). The predominant metals in the sediment were Al and Fe.

Table 2. Mean values of metal concentrations in crab tissues (μg/g dry weight).

Tissues	Al	Cu	Fe	Mn	Zn
Hepatopancreas	123.4±12.9 (25.5-325.9)	12.1±3.2 (1.7-35.6)	9.1±1.4 (1.3-13.8)	2.2±1.3 (1.1-4.1)	4.2±2.1 (1.4-7.9)
Exoskeleton	711.6±28.4 (74.3-1658)	14.3±5.2 (4.7-42.2)	79.2±11.7 (10.2-161.8)	6.4±2.3 (1.8-10.7)	6.5±2.3 (4.2-8.8)
Muscle	20.9±12.7 (6.0-35.7)	7.4±1.7 (1.04-26.02)	4.0±1.1 (0.6-8.2)	1.7±0.5 (0.5-,3.6)	2.0±0.7 (0.6-3.04)
Gills	806.0±100.6 (97.4-1674.8)	17.8±8.6 (10.8-23.6)	120.0±12.5 (9.8-241.7)	11.5±3.1 (3.9-20.9)	10.9±4.9 (8.4-15.3)

Table 3. Correlations of accumulation levels of metal pairs in crab tissues.

All tissues	Fe	Cu	Zn	Al
Cu	.604**			
p	.000			
Zn	.576**	.760**		
p	.000	.000		
Al	.840**	.560**	.582**	
p	.000	.000	.000	
Mn	.431**	.483**	.435**	.487**
p	.001	.000	.001	.000

Exoskeleton	Fe	Cu	Zn	Al
Cu	.628*			
p	0.007			
Zn	.654**	.700**		
p	.008	.004		
Al	.664**	0.496	.714**	
p	.007	.060	.003	
Mn	.752**	.588*	.758**	.954**
p	.001	.021	.001	.000

Hepatopancreas	Fe	Cu	Zn	Al
Cu	.782**			
p	.001			
Zn	.618*	.782**		
p	.014	.001		
Al	.632*	.679**	.882**	
p	.011	.005	.000	
Mn	-0.504	-0.175	-0.375	-0.439
p	.056	.533	.168	.101

Gills	Fe	Cu	Zn	Al
Cu	.571*			
p	.026			
Zn	.618*	.764**		
p	.014	.001		
Al	.849**	0.481	.549*	
p	.000	.070	.034	
Mn	.679**	.725**	.854**	.690**
p	.005	.002	.000	.004

Muscle	Fe	Cu	Zn	Al
Cu	-.150			
p	.593			
Zn	-0.271	.729**		
p	.328	.002		
Al	.575*	.048	-.100	
p	.025	.864	.723	
Mn	-.150	.445	.421	.343
p	.594	.096	.118	.211

*The mean difference is significant at the 0.05 level ($p < 0.05$).
**The mean difference is significant at the 0.01 level ($p < 0.01$).

Table 4. Metal concentrations in sediment (µg/g) and seawater (µg/L) samples.

Sediment	Al	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
S1	12560.2	12	0.08	18.9	25.8	15432	316.2	12.8	20.8	30.9
	14499.4	8	0.12	19.1	20	12774.2	400.6	14	17.6	81.3
S2	13002.2	23.7	0.09	38.9	28.3	10937.8	432.2	17.3	24.7	79.8
	9050.4	12.3	0.11	41.1	10.1	31156.8	554	12.1	20.9	59.6
S3	8931.3	22.8	0.18	33	16.1	21537.6	602.3	16.1	27.3	81.2
	7415.9	14.8	0.22	22.8	15.1	12830.8	552.1	14.1	23.5	57.8
S4	14992.9	14	0.05	17.5	10.7	21823.9	389	14.7	26.3	76.5
	12767.9	7	0.15	7.5	14.3	10190.7	507.2	16.1	12.1	106.5
S5	14322.8	12.8	0.1	14.2	12.3	18348	332.5	20	20.6	27.4
	14295.6	6	0.1	12.2	15.1	10456	381.9	15.2	17.6	77
S6	11657.8	21.3	0.19	21.3	18.9	20962.6	496.9	14	25.2	37.9
	12268	14.7	0.21	30.1	12.1	20136.2	476.5	12	14.4	67.3
S7	17891.7	12.1	0.08	32.4	22.1	23656.9	666.8	13.3	27.3	101.7
	6850.7	26.3	0.12	34	16.3	22247.7	484.4	13.1	18.5	89.7
S8	8449.5	17.2	0.31	17.1	25.5	18932.8	382.3	17.2	18.4	62.3
	14578.5	22	0.09	13.1	22.9	22766.2	498.7	18	31	57.3
Mean±SD	12095.9 ± 3035.5	15.4±6.01	0.14±0.07	23.33±9.9	17.9±5.5	18386.9 ± 5664.2	467.1 ± 94.8	15±2.2	21.6±4.99	68.4±22.3
Seawater	**Al**	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Pb**	**Zn**
S1	N.D.	1.2	1.3	0.7	N.D.	658.7	145.8	N.D.	N.D.	N.D.
	N.D.	0.6	1.1	0.5	N.D.	564.9	211.8	N.D.	N.D.	N.D.
S2	N.D.	1.3	0.3	1.7	N.D.	892.6	159.3	N.D.	N.D.	N.D.
	N.D.	0.5	0.7	1.1	N.D.	816.8	91.7	N.D.	N.D.	N.D.
S3	N.D.	0.8	1.1	0.8	N.D.	554.2	102.9	N.D.	N.D.	N.D.
	N.D.	0.6	0.7	1	N.D.	359.3	90.9	N.D.	N.D.	N.D.
S4	N.D.	0.9	0.3	0.3	N.D.	743.6	103	N.D.	N.D.	N.D.
	N.D.	1.7	0.7	0.5	N.D.	721.4	117.6	N.D.	N.D.	N.D.
S5	N.D.	0.8	0.9	0.9	N.D.	1021.6	129.5	N.D.	N.D.	N.D.
	N.D.	0.6	0.7	0.7	N.D.	518.2	108.7	N.D.	N.D.	N.D.
S6	N.D.	1.1	0.3	0.2	N.D.	903.2	192.3	N.D.	N.D.	N.D.
	N.D.	0.5	0.5	0.6	N.D.	579.8	78.7	N.D.	N.D.	N.D.
S7	N.D.	0.8	0.6	0.8	N.D.	432.2	129	N.D.	N.D.	N.D.
	N.D.	0.8	0.6	0.4	N.D.	442	126	N.D.	N.D.	N.D.
S8	N.D.	0.9	0.3	0.3	N.D.	506.3	120.2	N.D.	N.D.	N.D.
	N.D.	1.1	0.5	0.5	N.D.	602.1	100	N.D.	N.D.	N.D.
Mean±SD	N.D.	0.9±0.3	0.7±0.3	0.7±0.4	N.D.	644.8 ± 183.6	125.5 ± 35.4	N.D.	N.D.	N.D.

N.D.: Not Detected.

Correlation analyses were also performed to reveal metal-metal accumulation interactions, if any, in seawater and sediment samples (Table 5). Some correlations determined in the crab tissues but not in seawater and sediment samples were considered as indicators of metal–metal interactions and co-accumulation. Significant correlations in the sediment samples were among Mn-Pb (r=0.743, p=0.035), As-Pb (r=0.916, p=0.01), As-Fe (r=0.790, p=0.02), Cr-Mn (r=0.738, p=0.37), Al-Pb (r=-0.874, p=0.005) and Al-Mn (r=-0.714, p=0.047). The level of correlation between metal concentrations in crab tissues and the sediment samples were also tested but no significant correlation was determined whereas significant correlations were observed between concentration values in crab tissues (Table 6). The results showed that metals present in the seawater and sediment did not directly transformed to accumulations in crab tissues.

Discussion

The accumulation orders of metals were determined as Al>Fe>Cu>Mn>Zn in crab tissues, Fe>Mn>Cr>As>Cd in the seawater and Fe>Al>Mn>Zn>Cr>Pb>Cu>As>Ni>Cd in the sediment. Al and Fe were found to be predominant metals in the sediment but Al was not detected in the water samples. This may be related to the fact that sediment particles tend to absorb metals in the water, and this metal with high molecular weight tend to precipitate at the bottom (Table 2, 4).

Metal concentrations in the sediment and seawater samples were measured to determine whether accumulation levels in crab tissues signify environmental contamination. Compared to the crab tissues and

seawater, there were higher levels of metal concentrations in the sediment. This can be attributed to the fact that the sediment in wetlands assumes a storage role in terms of heavy metal accumulation. It has been reported that sediment accumulates metals at higher amounts compared to water and tissues (Mendil & Uluözlü 2007, Yildiz & Yener 2010, Oner & Celik 2011, Kır & Tumantozlu 2012, Fikirdeşici Ergen *et al.* 2015).

Although a positive strong correlation between As-Pb (r=0.916, p=0.001) and a negative strong correlation between Al-Pb (r=0.874, p=0.005) were determined in sediment samples, there was no correlation among the metals in seawater samples. This means that the metal concentrations in the sediment were not affected by the changes in the seawater samples. This can also be explained by the fact that metals are under the influence of processes such as absorption and desorption (Hung *et al.* 2001).

Among the investigated metals, only Fe, Cu, Zn, Al and Mn were detected in crab tissues. The amount of accumulation of these metals in crab tissues revealed an order as gills>exoskeleton>hepatopancreas>muscle (Table 2) most probably because gills and exoskeleton tissues are in contact with the external environment (Tunca *et al.* 2013a). The accumulation level of metals in the muscle tissue is less than the other tissues. This can be explained by the limited occurrence of metal binding proteins in muscle tissue (Guner 2007). Al was determined at high levels in all tissues. Gills in particular accumulated A1 at higher levels compared to other tissues. The levels of Al accumulation in hepatopancreas and muscle were lower than in the gills and exoskeleton as reported in previous studies (Alexopoulos *et al.* 2003, Kurun *et al.*2010, Tunca *et al.* 2013b). The gills are the primary sites for the uptake of soluble metals from the aquatic environment (Walton *et al.* 2010). Gills were found to be the prime site of Al accumulation in this work. There was higher accumulation level of Al in the tissues ($p<0.05$) since it is as an essential element (Woodburn *et al.* 2011) and also essential metals were reported to be transported to other organs by the gills (Nott 1991, Guner 2007). The results of these studies also showed that gills play an important role in Al intake and this intake cause excessive mucus secretion.

Metal uptake and accumulation can be facilitated by respiration and digestion in aquatic animals. Metals have a point of entry from the environment to the animal's body. Gills and exoskeleton are the primary point of entry and they revealed the greatest number of significant correlations of metal–metal interactions in the present study (Table 3).

Table 5. Correlations between metal pairs in seawater and sediment samples.

Sediment	**Cu**	**Pb**	**Zn**	**Ni**	**Mn**	**Fe**	**As**	**Cd**	**Cr**
Pb	0.494								
p	.213								
Zn	0.072	0.431							
p	.866	.286							
Ni	-0.096	-0.084	-0.156						
p	.820	.843	.713						
Mn	0.012	.743*	0.643	-0.455					
p	.978	.035	.086	.257					
Fe	0.323	0.647	0.571	-0.287	0.643				
p	.435	.083	.139	.490	.086				
As	0.524	.916**	0.479	-0.096	0.611	.790*			
p	.182	.001	.230	.820	.108	.020			
Cd	0.17	0.624	-0.282	0	0.282	0.169	0.567		
p	.687	.099	.499	1.000	.499	.689	.143		
Cr	0.395	0.563	0.357	-0.611	.738*	0.667	0.443	0.056	
p	.333	.146	.385	.108	.037	.071	.272	.895	
Al	-0.443	-.874**	-0.238	0.18	-.714*	-0.548	-0.695	-0.62	-0.69
p	.272	.005	.570	.670	.047	.160	.056	.101	.058

Seawater	**Mn**	**Fe**	**As**	**Cd**
Fe	0.214			
p	.610			
As	0.012	0.073		
p	.977	.864		
Cd	0.108	-0.241	-0.442	
p	.798	.565	.273	
Cr	0.22	0.39	-0.522	-0.173
p	.601	.339	.185	.682

*The mean difference is significant at the 0.05 level ($p < 0.05$).
**The mean difference is significant at the 0.01 level ($p < 0.01$).

Table 6. Correlations between metal levels in crab tissues and the sediment samples.

	Hepatopancreas	Exosceleton	Muscle	Gills
Exosceleton	.818**			
p	.004			
Muscle	.842**	.927**		
p	.002	.000		
Gills	.903**	.794**	.782**	
p	.000	.006	.008	
Sediment	.073	-.220	-.134	.152
p	.841	.542	.712	.674

Exposure time and metal concentrations are some of the important reasons for bioaccumulation (Anderson *et al.* 1997). Therefore, different tissues in an organism or the same tissues in different individuals of the same organism can accumulate high concentrations of the same metal. So, it is possible to find examples for that different tissues accumulate same metals. For instance, Chagas *et al.* (2009) found that hepatopancreas was the main tissue to accumulate Mn, whereas in the present study, the results indicated gills as the principal site of accumulation for Mn. The strongest correlation was found between Mn-Al ($r=0.954$, $p<0.001$) in the exoskeleton. Moderate correlation was determined between Mn-Al ($r=0.690$, $p=0.004$) in the gills but not in hepatopancreas and muscle (Table 3). This suggests that Mn and Al can have the same natural origin and there are similarities between the adsorption chemistries of these metals. In addition, the correlation between Mn-Al might be due to the transportation of these metals by transferrin protein because common use of metabolic pathways can lead to strong correlations in tissues (Cohen *et al.* 1998). However, negative moderate correlation between Mn-Al ($r=-0.714$, $p=0.047$) was determined in sediment samples which points out that the sighted effect is not a reflection of metal background in the environment (Table 5). This might be related with the fact that there is an antagonism between these metals during absorption and it may be related with the competitive effects over transferrin binding. Transferrin has an important role especially in the transport of Fe (Chua *et al.* 2007) but it also plays a role in transports of Al^{3+}, Cr^{3+}, Cu^{2+}, Ga^{3+}, Ni^{2+}, Ti^{4+} and Zn^{2+} (Quarles *et al.* 2011). A positive strong correlation between Mn-Zn ($r=0.854$, $p<0.001$) in the gills and a moderate correlation ($r=0.758$, $p=0.001$) in the exoskeleton were found while there were no correlations between these metals in the hepatopancreas and muscle. Bervoets *et al.* (2001) found the same strong correlation between Mn-Zn in the gills of the *Gastrosteus aculeatus* L.

A strong correlation was determined between Al-Fe in the gills ($r=0.849$, $p<0.001$) whereas a moderate correlation was determined between Al-Fe in other tissues. All correlations between Al and Fe were found to be positive. Kurun *et al.* (2010) reported similar correlations between Al and Fe accumulations in the crayfish *Astacus leptodactylus* (Eschscholtz). This result may be due to one or more of the following items: (1) similarities of Fe and Al in metal adsorption kinetics, (2) similar chemical properties of Fe and Al, (3) the major protein, transferrin which plays a major role in the Fe transport and Al absorption (Moshtaghie & Taher 1993). The correlation results of Al accumulation with Cu and Zn are remarkable in hepatopancreas since hepatopancreas is the central place for accumulation of various metals and detoxification in decapod crustaceans (Tunca *et al.* 2013a).

Moderate correlation between Zn and Fe accumulations in hepatopancreas ($r=0.618$, $p=0.014$), exoskeleton ($r=0.654$, $p=0.008$), and gills ($r=0.618$, $p=0.014$) were determined whereas there was no correlation between Zn and Fe accumulations in muscle. Zn is an important essential metal for decapods and serves as a cofactor for the enzymatic systems. Additionally, metalloenzymes use Zn as the active core (Alcorlo *et al.* 2006). A moderate correlation was determined between Cu and Fe accumulations in hepatopancreas ($r=0.782$, $p=0.001$), exoskeleton ($r=0.628$, $p=0.007$) and gills ($r=0.571$, $p=0.026$) whereas there was no correlation present between Cu and Fe accumulations in muscle. The exoskeleton serves as a structural support and has biomineral composite, so it tends to accumulate copper from the water environment (Soedarini *et al.* 2012). Gills, the respiratory organs of crabs, were indicated to be sensitive to changes in copper concentrations in seawater. Copper in the water binds to hemocyanin by respiration and then circulated to all organs of the crustacean.

Some of the metal–metal correlations found in gills, exoskeleton and hepatopancreas were not present in the muscle tissues. A metal should gain an entry point from the gills and the exoskeleton to be transported to another tissue. For this reason, high correlations in these tissues are expected. Furthermore, especially gills are primary site of metal entry because they are rich in blood vessels. Gills are under effects of a blood-borne transfer protein such as transferrin so they may be related to metal-metal interactions more than other tissues. Another reason may be that the binding proteins are more prominent in the gills (Guner 2007). The lack of correlations in the muscle tissue can be explained by the lack of these proteins in the muscle tissue.

A moderate correlation between Zn-Cu was found in all tissues but the correlation in hepatopancreas was slightly higher ($r=0.782$, $p=0.001$). Metallothioneins are one of the most important metal sequestrating protein families and hepatopancreas is a main site of metal sequestration in crustaceans. Metallothioneins plays an important role in preventing undesirable reactions in the transfer of IB and IIB metals such as Zn, Cu and Hg (Naji *et al.* 2014). For this reason, they are important proteins in preventing metal toxicity. If they are found in hepatopancreas at high concentrations, they help detoxify dangerous metals (Pourang *et al.* 2005). The production of the metallothionein in hepatopancreas is correlated with Zn and Cu accumulation in this tissue. Bochenek *et al.* (2008) found relationships between tissues (kidney, liver, gill and muscle) concentrations of Zn and Cu of the

Rutilus rutilus (L.) and the sediment. In the present study, no correlation between Zn-Cu was determined in sediment samples.

When the correlations among tissues were examined, it was determined that the highest correlation was between muscle-exoskeleton ($r=0.927$, $p<0.001$) and gills-hepatopancreas ($r=0.903$, $p<0.001$). These results were similar to study of the Fikirdesici Ergen *et al.* (2015). There was also a high correlation between hepatopancreas-muscle and gills, and moderate correlations between gills-muscle and hepatopancreas (Table 6).

Conclusion

In conclusion, as a result of examining the metal accumulation profiles of crab tissues, it can be concluded that metal-metal interactions and metabolic pathways play important roles in accumulation of specific metals. Metal concentrations in the sediment and seawater samples showed that the metal amounts and correlations in the crab tissues did not reflect any environmental contamination. Very low metal concentrations were found in seawater samples. No correlation was found between metal concentrations in crab tissues and the sediment samples whereas significant correlations were determined among the crab tissues, meaning that metals present in the seawater and the sediment did not directly transform into accumulation in crab tissues. In order to examine the existing metal deposits in an aquatic environment and to have an understanding of the metal pollution, it is absolutely necessary to conduct analysis on the sediment. It was concluded that the sediment sample results reflected reality more than those of the seawater and the tissues analysed in this study.

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Review

CURRENT CANCER STEM CELL BIOMARKERS IN TONGUE SQUAMOUS CELL CARCINOMA

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Abstract: Tongue squamous cell carcinoma (TSCC) is known to be the most malignant cancer type amongst other oral cancers with increasing incidence and mortality rates in the past five years. Since the life expectancy for TSCC patients is limited and the current chemo-radiotherapy treatments are not curative, novel biomarkers are urgently needed. As many other solid tumors, TSCC has a heterogeneous cancer cell population, which includes a small subpopulation identified as cancer stem cells (CSCs) that are considered as the driving force for tumor initiation, development, spread, recurrence, and resistance to chemo-radiotherapy. Although the underlying molecular mechanisms of how CSCs are involved in the carcinogenesis are not completely understood, scientists and clinicians aim to utilize those cells as therapeutic tools in fight against different cancer types including TSCC. Here, we reviewed and summarized important findings and the most current literature to shed light on the potential of cancer stem cells markers in TSCC. Possible functions of CSCs biomarkers in TSCC pathogenesis during cancer initiation, progression, invasion or metastasis are also summarized.

Key words: Tongue squamous cell carcinoma, cancer stem cell, biomarker.

Özet: Dil skuamöz hücreli karsinomu (DSHK) son 5 yılda artan insidans ve mortalite oranları ile birlikte diğer ağız içi kanserleri arasında en kötü huylu kanser tipi olarak bilinmektedir. DSHK hastalarının yaşam süreleri sınırlı olduğundan ve mevcut kemo-radyoterapi yaklaşımlarının başarılı olmamasından dolayı acilen yeni biyolojik belirteçlere ihtiyaç duyulmaktadır. Diğer solid (katı) tümörlerde olduğu gibi, DSHK'da da, tümörün başlaması, gelişmesi, yayılması, nüksetmesi ve kemo-radyoterapiye direnç göstermesi noktasında itici bir güç olarak kabul edilen "kanser kök hücreleri" (KKH) olarak tanımlanan küçük bir alt popülasyonu da kapsayan heterojen bir hücre popülasyonuna sahiptir. KKH'nin kanser oluşum sürecinde nasıl bir katkı yaptıklarının altında yatan moleküler mekanizmalar tamamen anlaşılamamış olmasına rağmen, bilim insanları ve klinik tedavi uzmanları DSHK da dâhil olmak üzere kansere karşı mücadelede bu hücreleri tedavi edici araçlar olarak kullanmayı amaçlamaktadır. Burada, DSHK'daki potansiyel kanser kök hücrelerinin belirteçlerine ışık tutmak için önemli bulguları ve en güncel literatürü gözden geçirdik ve derledik. Ayrıca, DSHK kanser başlangıcı, ilerlemesi, invazyonu ya da metastazı sırasında KKH biyo-belirteçlerinin olası fonksiyonları özetlenmiştir.

Introduction

Oral cancer is the sixth most frequent cancer type worldwide with an annual global incidence of 300.000 cases and 130.000 deaths (Brockton *et al.* 2017, T.W. Chen *et al.* 2017 Daigo *et al.* 2018, Iqbal *et al.* 2017, Yu & Li 2016). Tongue squamous cell carcinoma (TSCC) is a very aggressive disease and is the most common cancer type of the oral cavity making up almost 40% of all oral carcinomas (Cui *et al.* 2017). TSCC is also known to be the most malignant cancer type amongst other oral squamous cell carcinomas and causes speech, mastication and deglutition problems (J. Chen & Li 2017, Zhang *et al.* 2017). Although overall cancer incidence and mortality rates reduced in the past five years, incidence and mortality rates of TSCC increased significantly within the same period (Siegel *et al.* 2016). In addition, contrary to improvements in multimodal diagnosis and treatment techniques in the past few decades, the 5-year survival rate for TSCC stayed almost unchanged, which makes it one of the most lethal cancer types seen in head and neck region (Xing *et al.* 2013). Therefore, novel early diagnostic and prognostic markers are urgently needed for TSCC since the life expectancy for patients is very limited and the current chemo-radiotherapy treatments mostly fail to give positive clinical outcomes (Alam *et al.* 2017 Boldrup *et al.* 2015).

As in many other solid tumors, TSCC has a heterogeneous cancer cell population (Weng *et al.* 2017) which includes a small subpopulation identified as cancer stem cells (CSCs). These stem cells are thought to be responsible for tumor initiation, development, spread,

recurrence, and resistance to chemo-radiotherapy. Therefore, their identification, isolation, and characterization are of paramount importance to enhance the success of treatment of TSCC through development of CSCs targeted therapy techniques.

Here, we reviewed and summarized important findings and the most current literature to shed light on the potential CSCs markers in TSCC. Possible functions of these CSCs biomarkers in TSCC pathogenesis during cancer initiation, progression, invasion or metastasis were summarized.

Presence of CSCs in TSCC

CSCs were initially identified in leukemia through isolation of a subpopulation with tumor initiating potential, which suggested that tumors are comprised of hierarchically organized heterogeneous cell populations (Vlashi & Pajonk 2015). Further studies conducted with pediatric brain tumors demonstrated the presence of CSCs for the first time in solid tumors (Hemmati *et al.* 2003).

CSCs were shown to have essentially similar properties as normal stem cells in terms of their self-renewal, unlimited proliferation, and differentiation potentials. CSCs and normal stem cells utilize similar signaling pathways (Dawood *et al.* 2014) and both have the ability to activate anti-apoptotic signals and have increased telomerase activity (D'Arcangelo *et al.* 2015).

CSCs are often the main source of genetic abnormalities for malignant transformation (Li *et al.* 2015) and unlike normal stem cells, they initiate and propagate tumor when they are transplanted into immunodeficient model organisms and take an active role in metastasis, recurrence, and resistance of tumor to chemo-radiotherapy. As a result of these features, CSCs are referred as "tumor-initiating cells" or "tumorigenic cells". Surface markers such as CD133, CD24 and CD44 are typically used for isolation and identification of CSCs (Dawood *et al.* 2014).

In the last decade, researchers demonstrated the presence of CSCs in TSCC through isolation and characterization of specific cell populations expressing certain surface markers such as CD133 and CD44 (Sun *et al.* 2012). CD133 and CD44 expressing cells were demonstrated to have increased tumorigenic potential both *in vitro* and *in vivo* (Saleem *et al.* 2014, Wang *et al.* 2016). Another recent study identified two different cancer stem cell subpopulations, p63+/NANOG+/SOX2+/SALL4+/pSTAT3+/OCT4- and p63-/NANOG-/SOX2-/SALL4-/pSTAT3-/OCT4+ in moderately differentiated TSCC (Baillie *et al.* 2016). Besides, ALDH1 positive cells exhibited increased proliferation and differentiation capacity compared to ALDH1 negative cells in Tca8113 cell line, which confirmed the presence of cell clusters showing CSCs properties in human TSCC (Zou *et al.* 2012).

However, further efforts are needed for elucidation of genetic and epigenetic circuits modulating the stem cell features of CSCs to help understanding the molecular basis of carcinogenesis.

CSCs Surface Markers

CD44

CD44 and its variants are among the well-described and studied biomarkers in TSCC (Kunishi *et al.* 1997, Sato *et al.* 2000). CD44, located on chromosome 11, encodes for a conserved transmembrane glycoprotein which is necessary for the maintenance of tissue integrity. It has been shown to be deregulated in several cancers including TSCC (Lindquist *et al.* 2012, Yanamoto *et al.* 2014). CD44 has important roles during carcinogenesis and metastasis in TSCC and is involved in several carcinogenic processes like proliferation, migration, cell-cell interactions, and apoptosis in TSCC cells (Saleem *et al.* 2014, Wu *et al.* 2017). Its expression shows significant correlation with several other critical biomarkers such as CD24 (Baillie *et al.* 2017), E-cadherin (Bánkfalvi *et al.* 2002), MMP-9 (Kosunen *et al.* 2007), ABCG2 (Patel *et al.* 2014), CD117 and CD133 (Mărgăritescu *et al.* 2011) during initiation of tumor formation and induction of metastasis. CD44 was also proposed as a stem cell marker for various types of cancer such as breast cancer and head and neck squamous carcinomas (Bourguignon *et al.* 2017, Cruz Paula & Lopes 2017). Recent studies showed that CD44 expression is increased in tumor cells (Lindquist *et al.* 2012, Yanamoto *et al.* 2014) and some other studies demonstrated that CD44 expression has a negative correlation with tumorigenic potential of cancer cells (González-Moles *et al.* 2004, Sato *et al.* 2000).

The expression of CD44 in TSCC samples was initially investigated in 1996, where 83 head and neck squamous cell carcinoma (including 12 TSCC specimens) samples were used for immunohistochemical analysis of CD44 variants (v5, v6, v7, v7-v8, and v10) in normal, dysplastic squamous epithelia, primary and metastatic squamous cell carcinoma samples. CD44 v7, v8, and v10 were reported to be significantly downregulated in primary tumor tissues and were not detectable in most of the metastasis-derived specimens. On the other hand, v5 and v6 of CD44 displayed no significant alteration between normal and tumor samples (Herold-Mende *et al.* 1996). However, in the study of Kunishi (1997), expression level of CD44v6 was found to be significantly reduced in tumor samples when compared to normal healthy mucosa. Decreased expression of CD44v6 was also associated with regional lymph node metastasis. Interestingly, although no significant correlation was found between the expression of CD44v6 and level of differentiation, poorly differentiated carcinoma samples tended to express reduced level of CD44v6 (Kunishi *et al.* 1997). In the following years, reduced expression of different CD44 variants was associated with different clinical parameters such as CD44H with late nodal metastases following interstitial brachytherapy (Masuda *et al.* 2000), CD44v9 with lymph node metastasis and poor survival of patients (Sato *et al.* 2000), CD44 v3, v4-5, and v6 with cell differentiation, tumor grade, and the pattern of neoplastic invasion (Fonseca *et al.* 2001), CD44v6 with high histological

grade (Cruz *et al.* 2009) and CD44 with cervical lymph node metastasis (Mostaan *et al.* 2011). CD44 expression was also demonstrated to be profoundly reduced in TSCC compared to other tumors within the oral cavity (Krump & Ehrmann 2013). Furthermore, loss of CD44 expression in normal epithelia of tongue was suggested as an early biomarker for tongue carcinogenesis (González-Moles *et al.* 2004) and its expression was demonstrated to be strongly reduced in tongue tumors in comparison to other sub-sites within the oral cavity (Krump & Ehrmann 2013).

The first findings about the elevated expression of CD44 in TSCC were obtained in the study of Järvinen *et al.* (2008) where the authors characterized genome-wide copy number and gene expression alterations on microarrays for 18 TSCC cell lines. They identified several high-level amplifications including 11p12-p13 region where CD44 gene is localized. In addition, high intensity CD44 staining was demonstrated as a strong indicator of poor prognosis (Lindquist *et al.* 2012) and was significantly associated with regional lymph node metastasis, pattern of invasion, depth of invasion, perineural invasion and local recurrence (Yanamoto *et al.* 2014).

CSCs were reported to be enriched in the highly invasive UM1 cell line which is strongly positive for CD44 expression but were not present in the less invasive UM2 cell line (Misuno *et al.* 2013). In a recent study, where 4-Nitroquinoline 1-oxide was used to induce tongue cancer in mice, CD44 expression along with CD133 was found to be slightly overexpressed in dysplasia group compared to normal rats (Lim *et al.* 2014). Furthermore, CD44 and CD133 expressions were found to be strongly overexpressed in 4-Nitroquinoline 1-oxide induced TSCC in addition to several other cancer stem cell markers including ALDH1, Nanog and OCT-4. These findings point out the importance of CD44 as well as other cancer stem cell markers in the multistep carcinogenesis process of TSCC (Lim *et al.* 2014).

In another recent study, a cell line called UM-SCC-103 was developed by isolating highly aggressive tumor cells from a pregnant woman diagnosed with TSCC. Sorted CD44+ cells from UM-SCC-103 were able to induce carcinogenesis, whereas, CD44- cells failed to produce the overall heterogeneity of the primary tumor. This study suggested that CD44 is an important putative marker for tongue CSCs (Owen *et al.* 2014).

Saleem *et al.* (2014) used biopsy specimens from TSCC and its neck nodules for development of primary cell cultures and examined the growth and sphere formation capacity of those cells. They found the lowest CD44 expression in hyperplastic tongue tissue and the highest expression in neck node positive TSCC specimens. Cells with increased CD44 expression showed much higher sphere-forming capabilities compared to the corresponding CD44- cells. Interestingly, CD44+ cells from hyperplastic or non-cancer tongue tissue did not produce spheres. Besides, primary cultures from metastatic TSCC specimens showed stronger CD44/CD24 expression and produced much more spheres in significantly shorter duration compared to those of node negative TSCC and normal tongue specimens (Saleem *et al.* 2014).

Besides, injection of CD44+ sorted and labeled TSCC CSCs into the tongue of nude mice resulted in formation of highly metastatic tumors which were larger in size compared to CD44- and unsorted SCC-9 cells. Tumor formation efficiency was 100% in CD44+ group, but CD44- group showed slightly reduced tumor formation efficiency. Wu *et al.* (2017) also demonstrated that CD44 expression in human clinical samples was significantly higher in metastatic tumors than in primary tumor samples.

The evidence presented here demonstrates that CD44 expression has the potential to suppress and induce cancer progression. Discrepancies reported in the literature might stem partially from differences in the cell lines used, antibody variability, culture conditions, and other experimental variability. However, the conflicting data about the potential of CD44 as a stem cell marker needs further studies and clarification for its successful utilization in therapeutic approaches against TSCC.

CD133

Cluster of differentiation (CD) 133 (also known as AC133; human homologue of mouse Prominin-1) is a highly conserved pentaspan transmembrane glycoprotein encoded by *PROM1* on chromosome 4 (4p15.32) (Yanagisawa *et al.* 2005). It consists of 865 amino acids and has a total weight of 120 kDa. Its expression has been associated especially with progenitor/stem cells and differentiation (Li 2013). Subsequently, CD133 has been identified as a promising CSCs surface marker in several cancer types including larynx, lung, brain, skin, colon carcinoma and TSCC (Li 2013, Major *et al.* 2013, Suer *et al.* 2014, Wang *et al.* 2016, X. Yu & Li 2016) and it is currently considered as a universal marker of tumor-initiating cells (Li *et al.* 2014, Shrivastava *et al.* 2015). CD133 positive (CD133+) cells promote tumorigenesis, invasion, metastasis, and they are significantly related with drug resistance and disease relapse (Major *et al.* 2013, Wang *et al.* 2016).

CD133+ cells were initially identified in TSCC Tca8113 cell line by Kang *et al.* who showed that a small portion of Tca8113 cell line were positive for CD133, and these CD133+ cells represented high proliferation capacity compared to corresponding CD133 negative (CD133-) control Tca8113 cells (Kang *et al.* 2010). This study pointed the importance of CD133 as a putative CSCs surface marker for tumor-initiating cells of human TSCC (Kang *et al.* 2010). In another study carried out with Tca-8113 cell line, it has been shown that proliferative capacity and differentiation potential of CD133+ cells were profoundly higher than those of CD133- cells *in vitro* (Wang *et al.* 2016).

CD133+ cells were also characterized with their relatively strong tumorigenic potential *in vivo* (Wang *et al.* 2016). In a 4-Nitroquinoline 1-oxide-induced rat tongue carcinogenesis model, CD133 expression during the progress of multistep carcinogenesis was slightly increased in the dysplasia group compared with normal rats, but the expression level was significantly increased in rats with TSCC along with other important CSCs markers. This study proposed CD133 as a crucial CSCs surface marker for identification of CSCs with a high potential of oral carcinogenesis (Lim *et al.* 2014).

In a recent clinical study conducted by Mascolo *et al.* (2012) the expression level of CD133 in the oral squamous cell carcinoma samples mostly comprised of TSCC was significantly higher compared to normal oral mucosa samples.

Taking all these findings into account, CD133 has been suggested to serve as an important CSCs surface marker for isolation, identification and characterization of CSCs in TSCC patients, which might help development of novel targeted therapeutic approaches to enhance the quality of life and survival of patients.

Stemness Related Markers in TSCC

Several transcription factors including the reprogramming so-called 'the Yamanaka Factors' have been shown to play critical roles in acquisition and maintenance of self-renewal and pluripotency characteristics in embryonic stem cells via their interaction with other transcription factors as well as important signaling molecules (Boyer *et al.* 2005, Loh *et al.* 2006, Patel *et al.* 2014). Interestingly, the gene expression profiling of CSCs and the rest of the tumor burden pointed the differential expression of those stemness-associated genes in CSCs (Chen 2009, Zhang *et al.* 2012).

SOX2 and OCT4

Qiao *et al.* (2014) investigated the expression status of stemness markers SOX2 and OCT4 in the tumorigenesis process of oral mucosa by immunohistochemistry using both rat and human samples. They demonstrated that individual expression and co-expressions of SOX2 and OCT4 were detected in normal oral mucosa, premalignant diseases, primary and metastatic sites of oral squamous cell carcinoma. However, co-expression of SOX2 and OCT4 in transforming oral mucosa cells were suggested to contribute to the malignant transformation of oral mucosa (Qiao *et al.* 2014). In another study performed in 2016, lentiviral transduction of Sox2 and Oct4 together into immortalized oral epithelial cells triggered formation of neoplasms in immunodeficient mice, but their individual introduction into immortalized oral epithelial cells did not result in tumor formation. Furthermore, injection of Cal27 cells, with simultaneous knockdown of Sox2 and Oct4, into immunodeficient mice caused reduced tumor size. Besides, positive expressions of OCT4 and SOX2 were preferentially located in the nucleus of tumor cells obtained from 51 TSCC patients (Jiang *et al.* 2017). These findings strongly indicate that both SOX2 and OCT4 might be required for reprogramming of cancer stem cells for induction of oral carcinogenesis (Cai *et al.* 2016).

Quantitative proteomic analysis of sphere-forming CSCs of highly invasive UM1 and low invasive UM2 cells both obtained from the same tongue squamous cell carcinoma patient demonstrated that UM2 cells did not possess sphere-forming CSCs. On the other hand, sphere-forming CSCs of UM1 cells were strongly positive for several stem cell factors including SOX2 and OCT4. Along with those markers, many proteins in cell cycle, metabolism, G protein signal transduction, translational elongation, development, and RNA splicing pathways were differentially expressed among the two cell phenotypes, which might be associated with elevated levels of stem cell factors like SOX2 and OCT4 (Misuno *et al.* 2013).

KLF4

Interestingly, another reprogramming factor, KLF4, has been found to play Janus-faced roles in oral cancer carcinogenesis, acting both as a tumor suppressor and as an oncogene. The expression of KLF4 was found to be lower in the poor-differentiated oral cancers compared to the well-differentiated cancers. KLF4 functions as a tumor suppressor *in vitro* and/or *in vivo* through suppressing cell proliferation, cell cycle progression, cell colony formation and by inducing apoptosis (W. Li *et al.* 2015). It was also shown to inhibit cellular proliferation and induce differentiation to help maintenance of epithelial homeostasis. In a recent study in which an inducible oral-specific mice model was utilized to knockout Klf4 specifically in the oral cavity, dysplastic lesions with increased cell proliferation and abnormal differentiation were reported in the tongue cells four months after induction (Abrigo *et al.* 2014). Utilization of 4-nitroquinoline 1-oxide along with Klf4 knockout in the oral cavity resulted in development of more severe dysplastic lesions in the oral cavity and a tendency for increased incidence of oral squamous cell carcinoma (Paparella *et al.* 2015). These findings support the potential of KLF4 as a tumor suppressor for TSCC, however, upregulation of KLF4 induced TSCC cell migration and invasion *in vitro*. In addition, although knockdown of KLF4 in TSCC cells increased cellular proliferation and colony formation, it significantly resulted in inhibition of cell migration and invasion. Contradictory to its tumor suppressor function, these recent findings assign an oncogenic role for KLF4 in TSCC. Therefore, further studies are necessary to clarify the roles of KLF4 as a CSCs marker during tongue carcinogenesis process.

NANOG

Overexpression of Nanog has been previously reported in several types of tumors including oral squamous cell carcinoma (Bourguignon *et al.* 2012, Ezeh *et al.* 2005, Meng *et al.* 2010, Tsai *et al.* 2011) and it has

been suggested to play critical roles during carcinogenesis, tumor-progression, and metastasis (Ratajczak *et al.* 2007, Trosko 2006). Nanog expression was found to be significantly higher in tongue tumor tissues than in corresponding normal oral mucosa tissues (Fu *et al.* 2016). Nanog expression together with ALDH1 and OCT4, which are other CSCs markers, were reported to significantly increase during multistep carcinogenesis in a 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis model (Lim *et al.* 2014). Besides, long-term exposure of the carcinogenic 4-methylnitrosamino-1-3-pyridyl-1-butanone resulted in promoted migration and invasion in a dose-dependent manner induced overexpression of several CSCs markers including Nanog (Nieh *et al.* 2015). Nanog expression together with Oct3/4 were also reported to be significantly upregulated in side population compared to remaining cell population and their expressions were found to be strongly correlated with development of delayed neck metastasis (Habu *et al.* 2015). Taking into account these findings, Nanog might be considered amongst important CSCs biomarkers for TSCC to be utilized for identification and characterization of CSCs present in TSCC tissues.

BMI1

Bmi1, which plays critical roles in the functioning and maintenance of both endogenous stem cells and CSCs (Tanaka *et al.* 2013), was demonstrated to be significantly overexpressed during tongue carcinogenesis starting from very early stages (He *et al.* 2015). A recent study reported that knockdown of Bmi1 in TSCC cell lines stimulated cell apoptosis and senescence along with suppression of cell proliferation and migration. Interestingly, its inhibition also reduced the colony formation efficiency and the proportion of CD44+/CD133+ sub-population with CSCs features (Li *et al.* 2014). Besides, Bmi1 positive (Bmi1+) lingual epithelial stem cells were reported as the origin for tongue cancer in 4-nitroquinoline-1-oxide induced tongue cancer models since certain Bmi1+ cells were found to proliferate constantly, which eventually resulted in the formation of tumors derived from single Bmi1+ cells. This *in vivo* model provided invaluable data about the presence of CSCs within TSCC specimens and pointed out the importance of Bmi1 as a critical CSCs marker (Tanaka *et al.* 2016).

NOTCH

A recent meta-analysis study explored the role of Notch signaling pathway in human oral squamous cell carcinoma to understand the role of Notch signaling in during oral carcinogenesis (Osathanon *et al.* 2016). The researchers utilized 13 Gene Expression Omnibus datasets including those carried out with TSCC samples and found that oral squamous cell carcinoma specimens showed significant overexpression of several genes in Notch signaling pathway including *JAG1*, *JAG2*, *ADAM17*, *NCSTN*, *PSEN1*, *NCOR2*, N*UMB*, *DVL3*, *HDAC1*, and *HDAC2*, pointing out the importance of Notch and its effectors for TSCC carcinogenesis (Osathanon *et al.* 2016). Notch is reported to be amplified and overexpressed in significant proportion of early stage TSCC tissue samples. Overexpression of Notch, which is characterized as inducer of stem cell maintenance in potential cancer-initiating cells, in TSCC cell lines caused enrichment of stem cell markers and formation of spheroids with CSCs features. Conversely, its inhibition suppressed spheroid forming capacity, transformation, survival and migration of TSCC cells, indicating an oncogenic role for Notch in TSCC through regulating stem cell characteristics (Upadhyay *et al.* 2016).

EMT Markers

Accumulating evidence shows that epithelial mesenchymal transition (EMT), which is a significant physiological process where epithelial features are lost and mesenchymal properties are acquired (Iwatsuki *et al.* 2010, Thiery *et al.* 2009), is a crucial process playing critical roles during embryogenesis and carcinogenesis (Vered *et al.* 2010). It has been recently proposed that CSCs undergo an EMT for acquisition of migratory and invasive phenotype, which is necessary for progression of tumor (Pan *et al.* 2016).

In a recent study, treatment of Tca-8113 cells with Bone morphogenic protein 4 (BMP-4) was reported to promote EMT through acquisition of stem cell associated phenotypes (Qiao *et al.* 2011). BMP-4 significantly upregulated mesenchymal markers like Snail, Slug and Vimentin, and downregulated the epithelial marker E-cadherin along with overexpression of stemness gene which was not synchronous with the expression of EMT markers (Qiao *et al.* 2011). Another study investigating the expression profile of Bmi1, which is involved in self-renewal of stem cells, and that of ZEB1, a transcription factor that is associated with EMT, in TSCC cells and tongue specimens revealed that Bmi1 and ZEB1, both in mRNA and protein levels, were observed at the invasive front of TSCC cells, which were accompanied by the downregulation of epithelial markers and overexpression of mesenchymal markers both *in vitro* and *in vivo* (Kurihara *et al.* 2015). In addition, induced expression of Snail and Slug, two important EMT markers, resulted in a mesenchymal phenotype and morphology and induced cell invasion and stem cell related features in TSCC cells (Zheng *et al.* 2015).

Biomarkers in TSCC Prognosis

In addition to their tumor initiating and promoting properties, CSCs are significantly associated with cancer progression and clinical outcome and they are thought to be responsible for tumor development, metastasis, and recurrence as well as tumor initiation (Guzel *et al.* 2014). In addition, since CSCs and normal tissue stem cells share many similarities and CSCs are significantly involved in the malignant progress of tumors (Table 1), they are considered among the critical targets for future therapies.

Table 1. TSCC CSCs biomarkers associated with prognosis of disease.

CSCs Biomarker	Expression Level	Association	Reference
CD44	Low	Regional late nodal metastases	(Kunishi *et al.* 1997, Masuda *et al.* 2000)
		Poor survival of patients	(Sato *et al.* 2000)
		High histological grade	(Cruz *et al.* 2009)
		Cervical lymph node metastasis	(Mostaan *et al.* 2011)
		Lymph node metastasis	(Kunishi *et al.* 1997)
		5-year disease-free survival	(Dunkel *et al.* 2013)
	High	Poor prognosis	(Lindquist *et al.* 2012)
		Tumor local recurrence	(Yanamoto *et al.* 2014)
SOX2	High	Large tumor size, poorer overall, cancer-specific and disease-free survival of patients	(Du *et al.* 2011)
		Histological grade	(Jiang *et al.* 2017)
OCT4	High	Histological grade, lymph node metastasis	(Jiang *et al.* 2017)
		Vascular invasion, delayed neck metastasis	(Habu *et al.* 2015).
BMI1	High	Poor survival A positive node metastasis	(He *et al.* 2015)
		Cervical node metastasis and reduced overall survival	(Li *et al.* 2014)
	Low	Risk for recurrence	(Häyry *et al.* 2010)
CD147	High	Tumor diameter, clinical stage, poor overall survival	(Huang *et al.* 2009)
		Poor prognosis, a higher T stage	(Yu *et al.* 2015)
		Recurrence, node metastasis	(Huang *et al.* 2012)

The expressions of ALDH1, CD44, OCT4 and SOX2, as important cancer stem cell markers, were investigated in a recent clinical study by immunohistochemistry utilizing 66 TSCC tissue specimens (Huang *et al.* 2014). This study demonstrated that all of these CSCs markers were significantly overexpressed in TSCC samples, however, only the expression of SOX2 along with recurrence and distant metastasis were found as individual independent prognostic factors of overall survival in TSCC patients (Huang *et al.* 2014). Moreover, in another study, where tumor tissue samples from 82 histologically node-negative TSCC patients were evaluated with immunohistochemistry, SOX2 expression was significantly associated with large tumor size, and its increased expression was related with poorer overall, cancer-specific and disease-free survival of patients (Du *et al.* 2011). In a different study, paraffin embedded tissue specimens of 51 patients with TSCC were examined immunohistochemically, and OCT4 and SOX2 expressions were found to be significantly associated with histological grade of TSCC and poor overall survival of patients (Jiang *et al.* 2017). Moreover, OCT4 expression, but not SOX2, was also significantly correlated with lymph node metastasis. In the meantime, SOX2 was suggested as an independent prognostic factor for overall survival (Jiang *et al.* 2017). In addition, along with vascular invasion, expression of OCT4 was reported to be a potential predictor for identification of patients with high risk of delayed neck metastasis (Habu *et al.* 2015). Taking these findings into account, certain CSCs markers including SOX2 and OCT4 might be proposed as independent prognostic factors for patient survival in TSCC.

Apart from SOX2 and OCT4, deregulation of another important stem cell marker, Bmi1, was demonstrated to be a common event during TSCC progression. Its overexpression was significantly associated with poor

survival of TSCC patients. Late stage patients and patients with a positive node metastasis tended to have significantly higher Bmi1 expression compared to early stage patients and patients with a negative node metastasis, respectively (He *et al.* 2015). Its overexpression was also reported in a major fraction of tumor samples obtained from 52 patients with no prior history of chemotherapy or radiotherapy, and the overexpression was significantly associated with cervical node metastasis and reduced overall survival, revealing Bmi1 as an independent prognostic marker for TSCC patients (Li *et al.* 2014). On the other hand, an earlier study showed that negative or reduced Bmi1 expression was related to recurrence of patients and suggested negative Bmi1 expression as a biomarker for poor prognosis in TSCC patients (Häyry *et al.* 2010). The contradictory findings of the present studies question the power of Bmi1 as a prognostic marker and require further investigations to enlighten its roles in the progression of TSCC.

In several studies, reduced expression of different isoforms of CD44, as a widely investigated CSCs biomarkers in TSCC, was reported to be correlated with different clinical parameters including regional lymph node metastasis (Kunishi *et al.* 1997), late nodal metastases (Masuda *et al.* 2000), cervical lymph node metastasis (Mostaan *et al.* 2011), poor survival of patients (Sato *et al.* 2000), 5-year disease-free survival (Dunkel *et al.* 2013), and high histological grade (Cruz *et al.* 2009). However, increased expression of CD44 in TSCC samples, in accordance with its potential for contribution to CSCs features, was also associated with poor prognosis (Lindquist *et al.* 2012) and tumor local recurrence (Yanamoto *et al.* 2014). In the meanwhile, there are reports showing no statistically significant association between the expression of CD44 and different clinical or histological parameters (Krump & Ehrmann 2013, Yu *et al.* 2015). Thus, the exact role of CD44 for prediction of TSCC progression is not clear yet and further efforts are needed for clarification of its power for estimation of patients' prognosis in TSCC.

Expression of CD147, a potential oncogenic CSCs marker in TSCC, was significantly correlated with tumor diameter, clinical stages of the TSCC tumor samples and poor overall patient survival (Huang *et al.* 2009). CD147 expression level was also demonstrated to have significant association with poor prognosis, a higher T stage (Yu *et al.* 2015), recurrence, and node metastasis (Huang *et al.* 2012).

Future Potential

Experiencing a recurrence after a successful therapy is quite frequent in various cancer types including TSCC. Besides, current conventional treatment options mostly fail to result in a positive clinical outcome in advanced cases, which ultimately results in cancer related death due to disease progression and related organ failure. Recent studies indicated that CSCs are critical contributors of tumor initiation, progression, metastasis, recurrence, and chemo-radioresistance. Although underlying molecular mechanisms of how CSCs contribute to cancer pathogenesis is not completely clarified and understood, scientists and clinicians aim to use those cells as therapeutic tools to fight against cancer. Unraveling both genetic and epigenetic machineries of CSCs is necessary to develop effective and successful therapeutic options.

One of the major challenges of CSCs research is the accurate identification and characterization of CSCs, which is needed for their specific targeting. Therefore, to develop a therapeutic approach aiming at preferentially killing CSCs, the initial goal needs to be the identification of true surface marker(s) and stemness genes in CSCs. To do so, further development of anti-CSCs agents will help overcoming the tumor chemoresistance or radioresistance.

Furthermore, early detection and accurate diagnosis are especially crucial for determination and application of effective cancer therapy options in the clinical decision making process. Detailed characterization of circulating tumor cells, especially CSCs, will give opportunity to predict the prognosis of the cancer and decide the personalized therapy strategies for each patient.

In conclusion, the area of CSCs research is in its infancy and current understanding of the CSCs biomarkers in tongue pathogenesis is quite limited. Therefore, further detailed investigations are essential for better understanding of the roles and functions of CSCs biomarkers in TSCC biology.

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(Trakya Univ J Nat Sci)

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Yazı aşağıdaki sıraya göre düzenlenmelidir:

Yazarlar: Yazının ilk sayfasında sadece yazar isimleri ve adresleri bulunmalıdır. Adlar kısaltmasız, soyadlar büyük harfle ve ortalanarak yazılmalıdır. Adres(ler) tam yazılmalı, kısaltma kullanılmamalıdır. Birden fazla yazarlı çalışmalarda, yazışmaların hangi yazarla yapılacağı yazar ismi altı çizilerek belirtilmeli (sorumlu yazar) ve **yazışma yapılacak yazarın adres ve e-posta adresi yazar isimlerinin hemen altına yazılmalıdır. Bu sayfaya yazı ile ilgili başka bir bilgi yazılmamalıdır. Yazı, takip eden sayfada bulunmalı ve yazar-iletişim bilgisi içermemelidir.**

Başlık: İngilizce olarak Kısa ve açıklayıcı olmalı, büyük harfle ve ortalanarak yazılmalıdır.

Özet ve Anahtar kelimeler: Türkçe ve İngilizce özet 250 kelimeyi geçmemelidir. Özetin altına küçük harflerle anahtar kelimeler ibaresi yazılmalı ve yanına anahtar kelimeler virgül konularak sıralanmalıdır. Anahtar kelimeler, zorunlu olmadıkça başlıktakilerin tekrarı olmamalıdır. İngilizce özet koyu harflerle "Abstract" sözcüğü ile başlamalı ve başlık, İngilizce özetin üstüne büyük harflerle ortalanarak yazılmalıdır. Yazıdaki ana başlıklar ve varsa alt başlıklara **numara verilmemelidir.**

Giriş: Çalışmanın amacı ve geçmişte yapılan çalışmalar bu kısımda belirtilmelidir. Yazıda SI (Systeme International) birimleri ve kısaltmaları kullanılmalıdır. Diğer kısaltmalar kullanıldığında, metinde ilk geçtiği yerde 1 kez açıklanmalıdır. Kısaltma yapılmış birimlerin sonuna nokta konmamalıdır (45 m mesafe tespit edilmiştir). Kısaltma cümle sonunda ise nokta konmalıdır (… tespit edilen mesafe 45 m. Dolayısıyla…).

Materyal ve Metod: Eğer çalışma deneysel ise kullanılan deneysel yöntemler detaylı ve açıklayıcı bir biçimde verilmelidir. Yazıda kullanılan metod/metodlar, başkaları tarafından tekrarlanabilecek şekilde açıklayıcı olmalıdır. Fakat kullanılan deneysel yöntem herkes tarafından bilinen bir yöntem ise ayrıntılı açıklamaya gerek olmayıp sadece yöntemin adı verilmeli veya yöntemin ilk kullanıldığı çalışmaya atıf yapılmalıdır.

Sonuçlar: Bu bölümde elde edilen sonuçlar verilmeli, yorum yapılmamalıdır**.** Sonuçlar gerekirse tablo, şekil ve grafiklerle de desteklenerek açıklanabilir.

Tartışma: Sonuçlar mutlaka tartışılmalı fakat gereksiz tekrarlardan kaçınılmalıdır. Bu kısımda, literatür bilgileri vermekten çok, çalışmanın sonuçlarına yoğunlaşmalı, sonuçların daha önce yapılmış araştırmalarla benzerlik ve farklılıkları verilmeli, bunların muhtemel nedenleri tartışılmalıdır. Bu bölümde, elde edilen sonuçların bilime katkısı ve önemine de mümkün olduğu kadar yer verilmelidir.

Teşekkür: Mümkün olduğunca kısa olmalıdır. Teşekkür, genellikle çalışmaya maddi destek sağlayan kurumlara, kişilere veya yazı yayına gönderilmeden önce inceleyip önerilerde bulunan uzmanlara yapılır. Teşekkür bölümü kaynaklardan önce ve ayrı bir başlık altında yapılır.

Kaynaklar: Yayınlanmamış bilgiler kaynak olarak verilmemelidir (*Yayınlanmamış kaynaklara örnekler: Hazırlanmakta olan veya yayına gönderilen yazılar, yayınlanmamış bilgiler veya gözlemler, kişilerle görüşülerek elde edilen bilgiler, raporlar, ders notları, seminerler gibi*). Ancak, tamamlanmış ve jüriden geçmiş tezler ve DOI numarası olan yazılar kaynak olarak verilebilir. Kaynaklar, yazı sonunda alfabetik sırada (yazarların soyadlarına göre) sıra numarası ile belirtilerek verilmelidir.

Yazıların ve kitapların referans olarak veriliş şekilleri aşağıdaki gibidir:

Makale: Yazarın soyadı, adının baş harfi, basıldığı yıl. Makalenin başlığı, *derginin adı*, cilt numarası, sayı, sayfa numarası. Dergi adı italik yazılır.

Örnek:

Tek yazarlı Makale için

Soyadı, A. Yıl. Makalenin adı. (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin açık ve tam adı*, Cilt(Sayı)**:** Sayfa aralığı.

Kıvan, M. 1998. *Eurygaster integriceps* Put. (Heteroptera: Scuteleridae)'nin yumurta parazitoiti *Trissolcus semistriatus* Nees (Hymenoptera: Scelionidae)'un biyolojisi üzerinde araştırmalar*. Türkiye Entomoloji Dergisi*, 22(4): 243-257.

İki ya da daha çok yazarlı makale için

Soyadı1, A1. & Soyadı2**,** A2. Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt(Sayı): Sayfa aralığı.

Lodos, N. & Önder, F. 1979. Controbution to the study on the Turkish Pentatomoidea (Heteroptera) IV. Family: Acanthasomatidae Stal 1864. *Türkiye Bitki Koruma Dergisi*, 3(3): 139-160.

Soyadı1, A1., Soyadı2, A2. & Soyadı3, A3**.** Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt (Sayı): Sayfa aralığı.

Önder, F., Ünal, A. & Ünal, E. 1981. Heteroptera fauna collected by light traps in some districts of Northwestern part of Anatolia. *Türkiye Bitki Koruma Dergisi***,** 5(3): 151-169.

Kitap: Yazarın soyadı, adının baş harfi, basıldığı yıl. Kitabın adı (varsa derleyen veya çeviren ya da editör), cilt numarası, baskı numarası, basımevi, basıldığı şehir,toplam sayfa sayısı.

Örnek:

Soyadı, A., Yıl. *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Önder F., Karsavuran, Y., Tezcan, S. & Fent, M. 2006. *Türkiye Heteroptera (Insecta) Kataloğu*. Meta Basım Matbaacılık, İzmir, 164 s.

Lodos, N., Önder, F., Pehlivan, E., Atalay, R., Erkin, E., Karsavuran, Y., Tezcan, S. & Aksoy, S. 1999. Faunistic *Studies on Lygaeidae (Heteroptera) of Western Black Sea, Central Anatolia and Mediterranean Regions of Turkey.* Ege University, İzmir, ix + 58 pp.

Kitapta Bölüm: Yazarın soyadı, adının baş harfi basıldığı yıl. Bölüm adı, sayfa numaraları. Parantez içinde: Kitabın editörü/editörleri, *kitabın adı*, yayınlayan şirket veya kurum, yayınlandığı yer, toplam sayfa sayısı.

Örnek:

Soyadı, A., Yıl. Bölüm adı, sayfa aralığı. In: (editör/editörler). *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Jansson, A. 1995. Family Corixidae Leach, 1815—The water boatmen. Pp. 26–56. In: Aukema, B. & Rieger, Ch. (eds) Catalogue of the Heteroptera of the Palaearctic Region. Vol. 1. Enicocephalomorpha, Dipsocoromorpha, Nepomorpha, Gerromorpha and Leptopodomorpha. The Netherlands Entomological Society, Amsterdam, xxvi + 222 pp.

Kongre, Sempozyum: Yazarlar, Yıl. "Bildirinin adı (Sözcüklerin ilk harfi küçük), sayfa aralığı". Kongre/Sempozyum Adı, Tarihi (gün aralığı ve ay), Yayınlayan Kurum, Yayınlanma Yeri.

Örnek:

Bracko, G., Kiran, K., & Karaman, C. 2015. The ant fauna of Greek Thrace. 6th Central European Workshop of Myrmecology, 33-34. July, Debrecen-Hungary.

Internet: Eğer bir bilgi herhangi bir internet sayfasından alınmış ise (*internetten alınan ve dergilerde yayınlanan yazılar hariç*), kaynaklar bölümüne internet sitesinin ismi tam olarak yazılmalı, siteye erişim tarihi verilmelidir.

Soyadı**,** A. Yıl. Çalışmanın adı. (Sözcüklerin ilk harfi küçük). (web sayfası) **http://www……),** (Erişim tarihi: Mayıs 2009).

Hatch, S., 2001. Studentsperception of online education. Multimedia CBT Systems. http://www.scu.edu.au/schools/sawd/moconf/papers2001/hatch.pdf (Erişim: May 2009).

Kaynaklara metin içinde numara verilmemeli ve aşağıdaki örneklerde olduğu gibi belirtilmelidir.

Örnekler:
... x maddesi atmosferde kirliliğe neden olmaktadır (Landen 2002). Landen (2002) x maddesinin atmosferde kirliliğe neden olduğunu belirtmiştir. İki yazarlı bir çalışma kaynak olarak verilecekse, (Landen & Bruce 2002) veya Landen & Bruce (2002)'ye göre. ... şeklinde olmuştur; diye verilmelidir. Üç veya daha fazla yazar söz konusu ise, (Landen *et al.* 2002) veya Landen *et al.* (2002)'ye göre .... olduğu gösterilmiştir; diye yazılmalıdır.

Şekil ve Tablolar: Tablo dışında kalan fotoğraf, resim, çizim ve grafik gibi göstermeler "Şekil" olarak verilmelidir. Resim, şekil ve grafikler, net ve ofset baskı tekniğine uygun olmalıdır. Her tablo ve şeklin metin içindeki yerlerine konmalıdır. Tüm tablo ve şekiller yazı boyunca sırayla numaralandırılmalı (Tablo 1., Şekil. 1), başlık ve açıklamalar içermelidir. Şekillerin sıra numaraları ve başlıkları, alta, tabloların ki ise üstlerine yazılır.

Şekiller (tablo dışında kalan fotoğraf, resim, çizim ve grafik gibi) tek tek dosyalar halinde en az **300 dpi** çözünürlükte ve **tif** dosyası olarak şekil numaraları dosya isminde belirtilmiş şekilde ayrıca sisteme ek dosya olarak yüklenmelidir.

Sunulan yazılar, öncelikle Dergi Yayın Kurulu tarafından ön incelemeye tabii tutulur. **Dergi Yayın Kurulu, yayınlanabilecek nitelikte bulmadığı veya yazım kurallarına uygun hazırlanmayan yazıları hakemlere göndermeden red kararı verme hakkına sahiptir.** Değerlendirmeye alınabilecek olan yazılar, incelenmek üzere iki ayrı hakeme gönderilir. Dergi Yayın Kurulu, hakem raporlarını dikkate alarak yazıların yayınlanmak üzere kabul edilip edilmemesine karar verir.

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Example:

Articles with single author

Surname, N. Year. Article title (First letter of all words small). *Whole name of journal*, Volume (Issue)**:** page range.

Aybeke, M. 2016. The detection of appropriate organic fertilizer and mycorrhizal method enhancing salt stress tolerance in rice (*Oryza sativa* L.) under field conditions. *Trakya University Journal of Natural Sciences,* 17(1): 17-27.

Articles with two or more authors

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Dursun, A. & Fent, M. 2016. Contributions to The Cicadomorpha and Fulgoromorpha (Hemiptera) fauna of Turkish Thrace region. *Trakya University Journal of Natural Sciences,* 17(2): 123-128.

Surname1, N1., Surname2**,** N2. & Surname3, N. Year. Article title (First letter of words small). *Whole name of journal*, Volume (Issue)**:** page range.

Becenen, N., Uluçam, G. & Altun, Ö. 2017. Synthesis and antimicrobial activity of iron cyclohexanedicarboxylic acid and examination of pH effect on extraction in water and organic phases.*Trakya University Journal of Natural Sciences,* 18(1): 1-7.

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Czechowski, W., Radchenko, A., Czechowska, W. & Vepsäläinen, K. 2012. *The ants of Poland with reference to the myrmecofauna of Europe*. Museum and Institute of Zoology PAS, Warsaw, 496 pp.

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