plant, Algae, and Environment

Vol. 8, Issue 1, 2024

Table of Contents

Effect of Co_2 on Growth Parameters and Lipid Production in *Dunaliella* sp. ABRIINW-I_1 (Chlorophyceae) Isolated from Urmia Lake (West Azerbaijan, Iran)

Jamileh Panahy Mirzahasanlou[1]* and Mohammad Amin Hejazi[2]*

Received: 2023-12-17 Accepted: 2024-02-17

Abstract

Today, using CO_2 in microalgae cultures has been increasing for different purposes. Microalgae have the potential to produce high-value products along with CO_2 fixation. *Dunaliella* is a two-flagellate green microalga. The relatively good quality protein and fatty acid, besides lacking an indigestible cell wall make this alga an exceptional food in aquaculture and poultry fostering. In addition, there are many indigenous strains of algae with the advantage of adaptation to the regional climate condition. The main objective of this study was to evaluate the CO_2 effect on the growth pattern and biochemical composition of *Dunaliella* sp. ABRIINW-I_1 is native to Urmia Lake. Results showed that using CO_2 in the culture not only affects the biomass concentration (1.06 g/l AFDW vs 0.54 g/l in the control experiment) and growth period (reaching the stationary phase in 7 days rather than 14 days in the control experiment); but also influences the chemical composition. It seems that during the cultivation time, the lipid content increased in the cost of carbohydrates (33.1%DW). Fatty acid analysis revealed an optimal combination of saturated and unsaturated acids with the dominance of C16 and C18 fatty acids. It seems that CO_2 injection had no significant effect on the type of FA. The nutritional values of the studied strain were validated in this study, particularly when treated with CO_2. The results demonstrated that utilizing CO_2 in an algal culture could lead to decreased cost and energy requirements.

Keywords: Biomass, Fatty acid, Green alga, Growth period, Photobioreactor

Introduction

Dunaliella (Chlorophyceae, Chlorophyta), is a two-flagellate green microalga which morphologically characterized by lacking a rigid cell wall (Ben-Amotz and Avron, 1987). Various species of this genus can be isolated from marine and Salt Lake environments, ranging in salinity from 0.5% to the saturation extent (approximately 35 %). This remarkable adaptability to high salt concentration makes it the most tolerant eukaryote known (Avron,

1-Department of Biology, Faculty of Basic Science, Gonbad Kavous University, Gonbad Kavous, Iran.
2-Department of Food Biotechnology, Branch for Northwest and West Region, Agricultural Education and Extension Organization (AREEO), Agricultural Biotechnology Research Institute of Iran, Tabriz, Iran.
* Corresponding authors' email address: panahi@gonbad.ac.ir; aminhejazi@abrii.ac.ir
Doi: 10.48308/jpr.2024.235319.1074

1992; Shariati and Lilai, 1994). In addition to the use of *Dunaliella* in mass production of pigment, factors like the relatively good quality of protein and fatty acid, lack of an indigestible cell wall, presence of high levels of β carotene (vitamin A), and being healthy, making it an exceptional food in aquaculture and poultry fostering (Hoseini Tafreshi and Shariati, 2009).

CO_2 is the major atmospheric gas that contributes to Global warming (Bilanovic et al., 2009), which is emitted from anthropogenic activities. Several technologies have been employed to remove the additional CO_2 from the atmosphere to decrease the troubles of CO_2 acquisition. Abiotic techniques such as physiochemical absorbents, and injection into deep oceans and geological formations expose critical challenges; whereas, biological mitigation proves to be both economically feasible and environmentally sustainable in the long term (Kumar et al. 2010). Studies confirmed the microalgal potential for CO_2 sequestration. (Bilanovic et al., 2009; Kassim and Meng, 2017; Nayak et al., 2016; Raeesossadati et al., 2014; Razzak et al., 2013; Wang et al., 2008; Zeng et al., 2011).

Microalgae have the potential to produce high-value products such as biofuels along with CO_2 fixation. The utilization of CO_2 in microalgae cultures for efficiency enhancement has been increased (Anjos et al., 2013; Bilanovic et al., 2009; De Morais and Costa, 2007; Knudsen et al., 2009; Lam et al., 2012; Li et al., 2011; Nakanishi et al., 2014; Rinanti et al., 2014; Yeh and Chang, 2011; You et al., 2010). Furthermore, carbon dioxide (CO_2) can be utilized to effectively regulate and maintain the pH levels in the culture, eliminating the requirement for costly buffers (Qiu et al., 2017). In this regard, studies on CO_2 utilization in *Dunaliella* cultures showed that CO_2 application influences the quantity along with the quality of various products (Moghimifam et al., 2020a). On the other hand, various strains of *Dunaliella* exhibited varying capabilities in product accumulation (Hosseinzadeh Gharajeh et al., 2020; Xu et al., 2018); their responses to the CO_2 level were also different (Moghimifam et al., 2020b). Numerous indigenous strains exist as a result of diverse salt lakes and marshes. These native strains possess the advantage of adaptation to the local climate condition; nevertheless, the growth potential and yield production of the majority of them remain uncertain.

Due to the high lipid content, microalgae are one of the possible sources for biodiesel production (Benhima et al., 2018). Lipid production in microalgae is affected by growth conditions (Chavoshi and Shariati, 2019; Schenk et al., 2009) like carbon sources (De Swaaf et al., 2003) and salt concentrations (Hopkins et al., 2019; Rizwan et al., 2017; Takagi et al., 2006). Studies conducted on *Dunaliella* have revealed a relatively high growth rate and a substantial lipid content, making it a promising candidate for the production of biofuel (Rasoul-Amini et al., 2014; Tang et al., 2011). Shuping et al. (2010) represented the bio-oil product of *D. tertiolecta* as a possible eco-friendly green biofuel. Furthermore, *Dunaliella* spp. possesses the advantage of growing in brackish to saltwater and wastewater environments, exhibiting remarkable salt

tolerance and effortless lipid extraction (Tang et al., 2011). In their study, Rismani and Shariati (2017) found that the optimum growth rate of *D. salina* occurred at a 1.5 M concentration of NaCl. They suggested this species could be utilized for biodiesel and Omega-3 productions, as they observed an increase in total lipid and omega-3 fatty acids production under salt stress conditions. In comparison with the other two indigenous isolates, Hosseinzadeh Gharajeh et al. (2020) noted that *Dunaliella* sp. ABRIINW-I_1 exhibited a significant increase in a uniform culture containing 1M NaCl.

In this research, the growth pattern and lipid production of *Dunaliella* sp. ABRIINW-I_1, native to Urmia Lake (northwest Iran) was investigated. This study aimed to investigate the effects of CO_2 on biomass yield, pigment content, and fatty acid profile of native *Dunaliella* strain at 0.5 M NaCl.

Material and methods

Isolate and growth conditions

The native isolate, *Dunaliella* sp. ABRIINW-I_1 was obtained from the Agricultural Biotechnology Research Institute of Iran (ABRII), Northwest Branch. This isolate was originally derived from Urmia Lake, in northwest Iran. Urmia Lake is the world's second hypersaline lake, with a salinity range between 140-220 g/L, exceeding 380 g/L in recent years due to the drastic reduction of surface area (Sharifi et al., 2018).

Flask cultivation

Dunaliella sp. ABRIINW-I_1 was cultured in 250 ml Erlenmeyer flasks using a sterile medium. Modified Johnson medium with 1.5 M NaCl (Hejazi and Wijffels, 2003) was used for culture. The cultures were kept in

Fig. 1. A photobioreactor, with a volume of 10 liters, illuminated by four artificial light sources and continuously aerated with filtered air

a phytotron at a temperature of 25 °C, 100 µmol photons/s/m, and a 16:8 h light: dark photoperiod.

PBR Cultivation

Dunaliella sp. ABRIINW-I_1 which has been cultured for 15 days, inoculated into a water jacket glass vessel photobioreactor (PBR) (Fermentor bioreactor- BioFlo 110) with a working volume of 10 L. The vessel was illuminated by four artificial light sources that remained constant. The culture is continuously aerated by filtered air (Castilla Casadiego et al., 2016). CO_2 was injected discontinuously using a mass flow controller (MFC) to regulate the flow rate of CO_2 and controlled by a controller to maintain the culture pH at 7.5. The experimental setup is shown in Figure 1. The initial optical density (OD) was approximately 0.2 at 730 nm and the culture growth was monitored daily. Optical density and pigments were measured throughout the cultivation period while sampling for additional biochemical analysis was conducted every week.

A control experiment was also run where CO_2 injection was not performed. The pH level was maintained at 7.5 by initially adding of Tris-Base (11.12 $g.L^{-1}$).

Biomass concentration

The optical density was measured at 730 nm using a UV/VIS spectrophotometer daily (Perkin Elmer, Lambda 35). The relationship between OD_{730} and biomass was determined experimentally. The biomass concentration was measured in dry weight (DW) and as ash-free dry weight (AFDW) according to Hosseinzadeh Gharajeh et al. (2020) and calculated through the following equations:

$$DW(g.L^{-1}) = OD_{730} \times 0.8, R^2 = 0.95 \quad (1)$$

$$AFDW(g.L^{-1}) = OD_{730} \times 0.41, R^2 = 0.95 \quad (2)$$

Pigment contents

Chlorophylls and carotenoid content of the culture were measured by spectrophotometer every day. The algal sample (3 to 5 ml) was centrifuged at 5000 rpm for 5 minutes. After removal of the supernatant, 3 to 5 ml of 100% pure acetone was added and homogenized using a vortex mixer. Then, the mixer recentrifuged at 5000 rpm for 5 minutes. The supernatant was used to scan the absorbance by spectrophotometer (Perkin Elmer, Lambda 35-UV-VIS), and the content of the pigment was calculated using the following formula (Lichtenthaler and Buschmann, 2001):

$$Chl_a\ (\mu g.mL^{-1}) = 11.24 \times A_{662} - 2.04 \times A_{645}$$

$$Chl_b\ (\mu g.mL^{-1}) = 20.13 \times A_{645} - 4.19 \times A_{662}$$

$$Cart\ (\mu g.mL^{-1}) = (1000 \times A_{470} - 1.9 \times Chl_a - 63.14 \times Chl_b) / 214$$

$$TChl\ (\mu g.mL^{-1}) = Chl_a + Chl_b$$

where A is the absorption value in the specific wavelength, Chl_a, Chl_b, and Cart are chlorophyll a, chlorophyll b, and carotenoids respectively. Pigment contents were expressed as %DW.

Carbohydrate content

Carbohydrates were determined by the phenol-sulphuric acid method (Hosseinzadeh Gharajeh et al., 2020). Measurements were carried out by spectrophotometer (Perkin Elmer, Lambda 35-UV-VIS) at 485 nm using glucose as standard.

Lipid content and fatty acids profile

The modified method of Bligh and Dyer (Yang et al., 2014) was used for the extraction of lipid content. The lipid fatty acid methyl esters (FAME) were prepared by the esterification method to analyze the

fatty acid composition (Cheng et al., 2015). The preparation containing FAME and hexane was analyzed using GC analysis. The analysis was conducted on a Bruker capillary gas chromatograph (model Scion 456 GC). For the analysis, an Rt-2330 polar fused silica capillary column (with dimensions of 105 m X 0.250 mm and a film thickness of 0.20 µm) was utilized. The temperature profile was as follows: the oven temperature was set at 100°C and then raised to 140°C at a rate of 3°C per minute. Subsequently, it further increased to 170°C at a rate of 0.5°C per minute. Finally, it was raised to 220°C at a rate of 4°C per minute for 30 minutes. The carrier gas used was helium, flowing at a rate of 1 ml/m. The injector temperature was set at 250°C, and the detector which was an FID was also set at 250°C.

Statistical Analysis

Statistical analyses were performed using SPSS 16. Drawing graphs was performed by Microsoft Excel 2016.

Results

Biomass concentration

The influence of CO_2 on the biomass concentration and growth rate of native *Dunaliella* sp. ABRIINW-I_1 was studied. Higher dry weight (2.07 g/l) accounted for treatment with CO_2 after 6- days of cultivation, whereas in the control experiment, maximum dry weight was observed after 11 days of cultivation and the value was also low (1.05 g/l). The maximum biomass concentration (AFDW: 1.06 g/l) was also obtained in CO_2 treatment on the 6th day, while in the control experiment the maximum biomass concentration was 0.54 g/l (AFDW), which was obtained on the 11th day (Fig. 2). Biomass Productivity in treatment with CO_2 was calculated 0.17 g/l/day while in the control experiment was 0.04 g/l/day.

Pigments content

the content of chlorophylls (a, b, total) and total carotenoids as % DW are

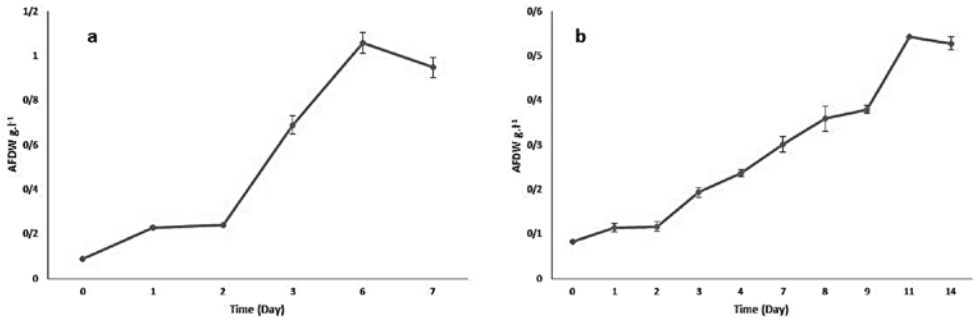


Fig. 2. Biomass concentration of Dunaliella sp. ABRIINW-I1 as AFDW measured in cultures with CO_2 injection (a) and the control experiment (b).

Table 1. Pigment contents (% DW) of *Dunaliella* sp. ABRIINW-I1 cultured in medium with and without CO_2 injection

	Chl_a	Chl_b	T Chl	Cart	T Pig	Chl_a/Chl_b	$Cart/Chl_a$	$Cart/Chl_b$
CO_2 treatment	1.45	0.4	1.86	0.43	2.29	0.19	0.015	0.056
Control experiment	1.33	1.3	2.63	0.34	2.97	0.09	0.025	0.025

shown in Table 1. In total, the maximum pigment content (sum of chlorophylls and carotenoids) was obtained at day 14 in the control experiment (2.97 % DW), whereas, in the presence of CO_2, this amount was 2.29 % DW at the end of the growth period (7^{th} day). This difference is primarily due to the higher biomass concentration in CO_2 treatment. Results of the t-test showed significant differences in chlorophyll a and total chlorophyll in biomass between two treatments ($p< 0.05$) and the amounts of chlorophyll a and total chlorophyll in biomass were significantly higher in CO_2 treatment. However, chlorophyll b and carotenoid content in biomass showed no significant difference between the two experiments ($p> 0.05$).

Lipid and carbohydrate contents

The *Dunaliella* sp. ABRIINW-I_1 biomass was harvested at weekly intervals to determine the total lipid and carbohydrate contents. The growth rate in the CO_2 treatment decreased after 6 days, leading to the termination of the experiment in the first week. On the other hand, the control experiment continued for two weeks. After 7 days of cultivation in the CO_2 treatment, the lipid and carbohydrate contents were 33.1% and 1.4% (DW) respectively, while in the control experiment, the lipid content was 21.45% (DW) and carbohydrate was 2.6% (DW) after 14 days of cultivation (Figure 3). The carbohydrate content showed a significant difference between the two treatments ($p < 0.05$), with the control experiment having a higher content due to the longer growth period.

Fatty acid profile

Table 2 shows the fatty acid composition of *Dunaliella* sp. ABRIINW-I_1 in two experiments. Fatty acid profile mainly composed of palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2), and Alpha linolenic acid (C18:3), which were dominant in both experiments.

Discussion

In this study, the effects of CO_2 on growth parameters, pigment and carbohydrate contents, lipid content, and FA profile of *Dunaliella* sp. ABRIINW-I_1 native to Urmia

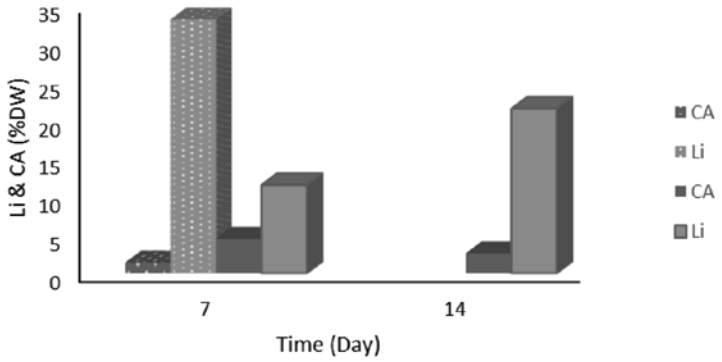


Fig. 3. Biochemical composition of *Dunaliella* sp. ABRIINW-I1 in two treatments. The dotted columns are the results of CO_2 treatment. Li: lipid, CA: Carbohydrate

Lake was evaluated. Results revealed that CO_2 injection could increase the growth and biomass of the studied isolate in addition to decreasing the growth duration. The biomass production obtained in CO_2 treatment was 4.25 fold as much for the control experiment. Other researchers that had studied the CO_2 effect achieved optimum growth of *Dunaliella* taxa in the concentration of 1-6% CO_2 (Kim et al., 2012; Suzuki et al., 1995; Tang et al., 2011).

Algae are characterized by a wide range of pigments such as chlorophylls, and carotenoids. Chlorophyll a; type of chlorophyll, plays a key role in photosynthesis. Other pigments are accessory pigments, acting as photoprotective agents and preventing harmful radicals. In total, chlorophyll amount was reported approximately 0.5-1.5% dry weight in microalgae (Becker, 1994). In both experiments, it was anticipated that the amount of chlorophyll a would be higher than that of chlorophyll b, and the results confirmed this expectation. Hosseinzadeh Gharajeh et al. (2020) reported similar results. One of the significant characteristics of *Dunaliella salina* is the high potential to accumulate enhanced levels of carotenoids particularly β carotene when subjected to specific stressors such as nutrient limitation, high salinity, and light stress (Saha et al., 2018). However, in our experiments, where these stressors were absent, the carotenoid content did not exhibit a significant increase. Carbohydrates are the primary

Table 2. Fatty acid profile of *Dunaliella* sp. ABRIINW-I1 (DW%) in two treatments

	FA	CO_2 treatment Day 7	Control experiment Day 7	Control experiment Day 14
1	14:0 Myristatic acid	0.83 ± 0.091	1.32±0.61	0.79 ± 0.049
2	16:0 Palmitic acid	26.28 ± 0.55	29.82±2.15	23.95±1.25
3	16:1 9C Palmitoleic acid	1.27 ± 0.14	2.64±0.26	3.04±0.21
4	18:0 Stearic acid	15.38 ± 2.46	22.13±4.58	13.37±1.9
5	18:1 9C Oleic acid	4.43± 0.42	4.5±0.55	4.15±0.1
6	18:1 11C Vaccenic acid	1.65± 0.15	1.77±0.16	2.35±0.12
7	19:0 Nanodecanoic acid	0.54± 0.042	0.3±0.43	0.67±0.07
8	18:2 9C,12C Linoleic acid (n6)	15.6 ±0.75	13.1±2.22	14.92±0.5
9	18:3 6C,9C,12C Gamma Linolenic acid (n6)	3.86±0.14	3.12±0.6	4.59±0.26
10	18:3 9C,12C,15C Alpha linolenic acid (n3)	29.63±0.17	20.6 ±4.37	31.75±1.85
11	20:0 Arachidic Acid	0.39±0.17	0.56±0	0.38±0.041
12	ΣSFA	43.53± 1.83	54.25± 7.07	39.17±3.05
13	ΣMUFA	7.35± 0.72	8.92± 0.12	9.55±0.44
14	ΣPUFA	49.11± 1.09	41.24±13.46	51.26±2.62
15	Σn3 PUFA	29.63±0.17	20.6 ±4.37	31.75±1.85
16	Σn6 PUFA	19.46± 0.89	16.22± 2.83	19.51±0.77
17	n3:n6	1.52	1.27	1.62
18	n6:n3	0.65	0.78	0.61
19	UFA: SFA	1.29	0.84	1.55

organic products during the process of photosynthesis. It appears that during the cultivation progresses, there is an increase in lipid content at the expense of carbohydrates. According to Hosseinzadeh Gharajeh et al. (2020), after comparing three isolates of *Dunaliella* it was determined that the cells tend to reserve the carbon and energy in the form of lipids rather than carbohydrates. On the other hand, the utilization of CO_2 resulted in the high lipid content within a brief period. In this regard, the daily lipid productivity exhibited approximately a five-fold surge when treated with CO_2. Some researchers have linked the heightened lipid content in the presence of CO_2 to the mechanism of lipid synthesis, thereby enhancing the lipid content of microalgae in the presence of inorganic carbon (Kassim and Meng 2017; White et al. 2013).

Analysis of the FA showed that the *Dunaliella* sp. ABRIINW-I_1 mostly accumulated the C16 and C18 fatty acids, which generally are dominant in algal cells (Elsey et al. 2007; Lang et al. 2011; Talebi et al. 2013). There was no significant difference in the type of FAs between the two treatments. The main components of the FA profile were n3-ALA, saturated FAs (C16:0 and C18:0), and n6- linoleic acid. Similar results were observed by Hosseinzadeh Gharajeh et al. (2020). Herrero and colleagues (2006) identified palmitic acid and ALA as the primary fatty acids in *D. salina*. In contrast to their findings, oleic acid was not found in high concentrations in this particular study. Previous studies have linked the increase of oleic acid in various algal cells to the deficiency in N and P in culture medium (Ho et al., 2010; Hu and Gao, 2006; Ji et al., 2013; Msanne et al., 2012). In our work, these nutrients were sufficiently added to the culture as KNO_3 and KH_2PO_4, and the nutrient defect was not the case.

Yecong and colleagues (2011) revealed that the growth phase could also affect the lipid content and FAMEs. The findings from the control experiment indicated that during the initial week, the amount of the SAFAs was high. However, as the growth progressed, the SAFAs decreased, while the amount of the USFAs increased. Consequently, at the stationary phase, the fatty acid profiles in both treatment groups exhibited no significant difference. Additionally, lipid content altered throughout the growth phase. Among the PUFAs, n3 and n6 fatty acids are essential for humans and all other mammals. The n6/n3 balance plays a crucial role in decreasing the risk of coronary heart disease (Simopoulos, 2008). Although in our study this ratio did not match the international standards (Ma et al., 2016), the n3/n6 ratio, which is a valuable indicator for assessing exceeded nutritional value of oily foods, exceeded certain fish species. This was supported by Cakmak et al. (2014) in their work on the biochemical composition of *Dunaliella salina*, where they found that the extracted oils had a higher nutritional value compared to some fishes. Studies on FA composition in some fishes characterized the ratio of 1.03-2.8 for the n3/n6 ratio (Cakmak et al., 2012; Donmez, 2009; Guler et al., 2007).

In total, various parameters including salinity, light intensity, duration, temperature, medium type, and nitrate

concentration could influence the growth, pigment, lipid, and carbohydrate contents of algae (Al-Adali et al., 2012). The halotolerant *Dunaliella salina* is the known commercial microalgae with various strains of *Dunaliella* growing in saltwater ecosystems of Iran. The main aim of this work was to investigate the effects of CO_2 injection on the growth and biochemistry of native *Dunaliella* sp. ABRIINW-I_1. Results revealed that utilizing CO_2 in a culture not only affects the biomass concentration and growth period but also affects the chemical composition. Furthermore, fatty acid analysis revealed an optimal combination of saturated and unsaturated acids with the dominance of C16 and C18 fatty acids. Fatty acid methyl esters play a crucial role in the biodiesel properties of algal biomass. In addition, lipids have great importance at various growth stages of many aquaculture animals. Our results confirmed the nutritional values of the studied strain particularly when treated with CO_2. The introduction of CO_2 had no significant impact on the type of fatty acids. The results demonstrated that throughout the microalgae cultivation of microalgae, CO_2 sequestration, biofuel, and food production could be combined. Additionally, the utilization of CO_2 in algal cultivation could lead to a decrease in costs and energy requirements.

References

Anjos M, Fernandes BD, Vicente AA, Teixeira JA, Dragone G. (2013). Optimization of CO_2 bio-mitigation by *Chlorella vulgaris*. Bioresource Technology. 139: 149-154. Doi:10.1016/j.biortech.2013.04.032.

Al-Adali K, Ahmed E, Kumar P, Ayaril N. (2012). Effect of salinity, temperature, nutrients and CO_2 on growth of two species of microalgae from Red Sea, Saudi Arabia. Journal of King Abdulaziz University: Marine Sciences. 23 (1): 57-73. Doi: 10.4197/Mar. 23-1.5.

Avron M. (1992). Osmoregulation In: Dunaliella: Physiology, Biochemistry, and Biotechnology. (Ed. by M. Avron, A. Ben- Amotz A), CRC Press, Boca Raton. pp. 135–159.

Becker EW. (1994). Microalgae: Biotechnology and microbiology. Cambridge University Press, Cambridge.

Ben-Amotz A and Avron M. (1987). On the mechanism of osmoregulation in *Dunaliella* In Energetic and Structure of Halophilic Microorganism, Caplan SR and Gizburg M (Ed). Elsevier, Amsterdam, North-Holland. pp. 529–541.

Benhima R, El Arroussi H, Kadmiri IM, El Mernissi N, Bennis I, Smouni AA, Bendaou N. (2018). Nitrate Reductase Inhibition Induces Lipid Enhancement of *Dunaliella tertiolecta* for Biodiesel Production. The Scientific World Journal. 2018: 1-8. Doi:10.1155/2018/6834725.

Bilanovic D, Andargatchew A, Kroeger T, Shelef G. (2009). Freshwater and marine microalgae sequestering of CO_2 at different C and N concentrations – Response surface methodology analysis. Energy Conservation and Management. 50: 262-267. Doi:10.1016/j.enconman.2008.09.024.

Castilla Casadiego D, Albis Arrieta A, Angulo Mercado E, Cervera Cahuana S, Baquero

Noriega, K, Suárez Escobar A, Moralez Avendano, ED. (2016). Evaluation of Culture Conditions to Obtain Fatty Acids from Saline Microalgae Species: *Dunaliella salina*, Sinecosyfis sp. and Chroomonas sp. BioMed Res Int. 2016: 7 pp. Doi:10.1155/2016/5081653.

Cakmak YS, Kaya M, Asan-Ozusaglam M. (2014). Biochemical composition and bioactivity screening of various extracts from *Dunaliella salina*, a green microalga. EXCLI Journal. 13: 679-690. PMID: 26417292; PMCID: PMC4464408.

Cakmak YS, Zengin G, Guler GO, Aktusek A, Ozparlak H. (2012). Fatty acid composition and ω3/ω6 ratios of muscle lipids of six fish species in Sugla Lake, Turkey. Archives of Biological Sciences. 64: 471-477. Doi: 10.2298/ABS1202471C.

Chavoshi ZZ and Shariati M. (2019). Lipid production in *Dunaliella bardawil* under autotrophic, heterotrophic, and mixotrophic conditions. Brazilian Journal of Oceanography. 67: 1-8. Doi: 10.1590/s1679-87592019024906709.

Cheng YS, Labavitch J, Vander Gheynst J. (2015). Elevated CO_2 concentration impacts the cell wall polysaccharide composition of green microalgae of the genus *Chlorella*. Letters in Applied Microbiology. 60: 1-7. Doi:10.1111/lam.12320.

De Morais MG and Costa JAV. (2007). Biofixation of carbon dioxide by *Spirulina* sp. and *Scenedesmus obliquus* cultivated in a three-stage serial tubular photobioreactor. Journal of Biotechnology. 129 (3): 439-445. Doi: 10.1016/j.jbiotec.2007.01.009.

De Swaaf ME, Sijtsma L, Pronk JT. (2003). Highcell-density fed-batch cultivation of the docosahexaenoic acid-producing marine alga *Crypthecodinium cohnii*. Biotechnology and Bioengineering. 81: 666-672. Doi: 10.1002/bit.10513.

Donmez M. (2009). Determination of fatty acid compositions and cholesterol levels of some freshwater fish living in Porsuk Dam, Turkey. Chemistry of Natural Compounds. 45: 14-7. Doi: 10.1007/s10600-009-9219-z.

Elsey D, Jameson D, Raleigh B, Cooney MJ. (2007). Fluorescent measurement of microalgal neutral lipids. Journal of Microbiological Methods. 68: 639-642. Doi: 10.1016/j.mimet.2006.11.008.

Guler GO, Aktumsek A, Citil OB, Arslan A, Torlak E. (2007). Seasonal variations on the total fatty acid composition of fillets of zander (Sander lucioperca) in Beysehir Lake (Turkey). Food Chemistry. 103: 1241-1246. Doi: 10.1016/j.foodchem.2006.10.029.

Hejazi M and Wijffels R. (2003). Effect of light intensity on β-carotene production and extraction by *Dunaliella salina* in two-phase bioreactors. Biomolecules Engineering. 20: 171-175. Doi: 10.1016/S1389-0344(03)00046-7.

Herrero M, Ibanez E, Cifuentes A, Reglero G, Santoyo S. (2006). *Dunaliella salina* microalga pressurized liquid extracts as potential antimicrobials. Journal of Food Protection. 69: 2471-2477. Doi: 10.4315/0362-028X-69.10.2471.

Ho SH, Chen CY, Chang JS. (2010).

Scenedesmus obliquus CNW-N is a potential candidate for CO_2 mitigation and biodiesel production. Bioresource Technology. 101: 8725–8730. Doi:10.1016/j.biortech.2010.06.112.

Hosseieni Tafreshi A and Shariati M. (2009). *Dunaliella* biotechnology: methods and applications. Journal of Applied Microbiology. 107: 14–35. DOI: 10.1111/j.1365-2672.2009.04153.x.

Hopkins TC, Sullivan Graham EJ, Schuler AJ. (2019). Biomass and lipid productivity of *Dunaliella tertiolecta* in a produced water-based medium over a range of salinities. Journal of Applied Phycology. 31: 3349-3358. Doi: 10.1007/s10811-019-01836-3.

Hosseinzadeh Gharajeh N, Valizadeh M, Dorani E, Hejazi MA. (2020). Biochemical profiling of three indigenous *Dunaliella* isolates with a main focus on fatty acid composition toward potential biotechnological application. Biotechnology Reports. 26: 1-9. Doi: 10.1016/j.btre.2020.e00479.

Hu H and Gao K. (2006). Response of growth and fatty acid compositions of *Nannochloropsis* sp. to environmental factors under elevated CO_2 concentration. Biotechnology Letters. 28: 987–992. Doi: 10.1007/s10529-006-9026-6.

Kassim MA and Meng TK. (2017). Carbon dioxide (CO_2) biofixation by microalgae and its potential for biorefinery and biofuel production. Science of the Total Environment. 584-585: 1121-1129. Doi: 10.1016/j.scitotenv.2017.01.172.

Kim W, Park LM, Gim GH, Jeong SH, Kang CM, Kim DJ, Kim SW. (2012). Optimization of culture conditions and comparison of biomass productivity of three green algae. Bioprocess and Biosystem Engineering. 35: 10-27. Doi: 10.1007/s00449-011-0612-1.

Knudsen JN, Jensen JN, Vilhelmsen PJ, Biede O. (2009). Experience with CO_2 capture from coal flue gas in pilot-scale: Testing of different amine solvents. Energy Procedia. 1 (1): 783-790. Doi: 10.1016/j.egypro.2009.01.104.

Kumar A, Ergas S, Yuan X, Sahu A, Zhang Q, Dewulf J, Xavier Malcata F, Van Langenhove H. (2010). Enhanced CO_2 fixation and biofuel production via microalgae: recent developments and future directions. Trends in Biotechnology. 28 (7): 371-380. Doi: 10.1016/j.tibtech.2010.04.004.

Lam MK, Lee KT, Mohamed AR. (2012). Current status and challenges on microalgae-based carbon capture. International Journal of Greenhouse Gas Control. 10: 456-469. Doi: 10.1016/j.ijggc.2012.07.010.

Lang I, Hodac L, Friedl T, Feussner I. (2011). Fatty acids profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biology. 11: 124. Doi: 10.1186/1471-2229-11-124.

Li F-F, Yang Z-H, Zeng R, Yang G, Chang X, Yan J-B, Hou Y-L. (2011). Microalgae capture of CO_2 from actual flue gas discharged from a combustion chamber. Industrial & Engineering Chemistry Research. 50 (10): 6496-6502. Doi: 10.1021/ie200040q.

Lichtenhaler HK and Buschmann C. (2001). Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Current Protocol in Food Analytical Chemistry. 1: F4. 3.1-F4. 3.8. Doi: 10.1002/0471142913.faf0403s01.

Ma X, Jiang Z, Lai C. (2016). Significance of increasing n-3 PUFA content in Pork on human health. Critical Reviews in Food Science and Nutrition. 56:1-13. Doi 10.1080/10408398.2013.850059.

Moghimifam R, Niknam V, Ebrahimzadeh H, Hejazi MA. (2020a). CO_2 biofixation and fatty acid composition of two Indigenous *Dunaliella* sp. isolates (ABRIINW-CH2 and ABRIINW-SH33) in response to extremely high CO_2 levels. Bioprocess and Biosystems Engineering. 43: 1587-1597. Doi: 10.1007/s00449-020-02350-4.

Moghimifam R, Niknam V, Ebrahimzadeh H, Hejazi MA. (2020b). The influence of different CO_2 concentrations on the biochemical and molecular response of two isolates of *Dunaliella* sp. (ABRIINW-CH2 and ABRIINW-SH33). Journal of Applied Phycology. 32: 175-187. Doi: 10.1007/s10811-019-01914-6.

Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Cerutti H. (2012). Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. Phytochemistry. 75: 50–59. Doi: 10.1016/j.phytochem.2011.12.007.

Nakanishi A, Aikawa S, Ho S, Chen C, Chang J, Hasunuma T, Kondo A. (2014). Development of lipid productivities under different CO_2 conditions of marine microalgae *Chlamydomonas* sp. JSC4. Bioresource Technology. 152: 247-252. Doi: 10.1016/j.biortech.2013.11.009.

Nayak M, Karemore A, Sen R. (2016). Performance evaluation of microalgae for concomitant wastewater bioremediation, CO_2 biofixation, and lipid biosynthesis for biodiesel application. Algal Research. 16: 216-223. Doi: 10.1016/j.algal.2016.03.020.

Qiu R, Gao S, Lopez PA, Ogden KL. (2017). Effects of pH on cell growth, lipid production and CO_2 addition of microalgae *Chlorella sorokiniana*. Algal Research. 28: 192-199. Doi: 10.1016/j.algal.2017.11.004.

Raeesossadati MJ, Ahmadzadeh H, McHenry MP, Moheimani NR. (2014). CO_2 bioremediation by microalgae in photobioreactors: Impacts of biomass and CO_2 concentrations, light, and temperature. Algal Research. 6: 78-85. Doi: 10.1016/j.algal.2014.09.007.

Rasoul-Amini S, Mousavi P, Montazeri-Najafabady N, Mobasher MA, Mousavi SB, Vosough F, Dabbagh F, Ghasemi Y. (2014). Biodiesel Properties of Native Strain of *Dunaliella Salina*. International Journal of Renewable Energy Research. 4(1): 39-41. Doi: 10.20508/ijrer.v4i1.989.g6241.

Razzak SA, Hossain MM, Lucky RA, Bassi AS, Lasa, H. (2013). Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing—A review.

Renewable and Sustainable Energy Review. 27: 622-653. Doi: 10.1016/j.rser.2013.05.063.

Rinanti A, Dewi K, Kardena E, Astuti DI. (2014). Biotechnology Carbon Capture and Storage (CCS) by Mix-culture Green Microalgae to Enhancing Carbon Uptake Rate and Carbon Dioxide Removal Efficiency with Variation Aeration Rates in Closed System Photobioreactor. Journal Teknology. 69 (6): 105-109. Doi: 10.11113/jt.v69.3317.

Rissmani S and Shariati M. (2017). Changes of the total lipid and Omega-3 fatty acid contents in two microalgae Dunaliella salina and *Chlorella vulgaris* under salt stress. Brazilian Archives of Biology and Technology. 60: 1-11. Doi: 10.1590/1678-4324-2017160555.

Rizwan M, Mujtaba J, Rashid N, Lee K. (2017). Enhancing Lipid Production of Dunaliella tertiolecta by Manipulating the Interactive Effect of Salinity and Nitrogen. Chemistry and Biochemistry Engineering. Q 31 (3): 199–207. Doi: 10.15255/CABEQ.2017.1092.

Saha SK, Kazipet N, Murray P. (2018). The carotenogenic *Dunaliella salina* CCAP 19/20 produces enhanced levels of carotenoid under specific nutrient limitations. Biomed Research International. 2018: 7532897. 11pp. Doi: 10.1155/2018/7532897.

Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C, Olaf K, Hankmer B. (2009). Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Research. 1: 20-43. Doi: 10.1007/s12155-008-9008-8.

Shariati M. Lilley RMC. (1994). Loss of intracellular glycerol from *Dunaliella* by electroporation at constant osmotic pressure: subsequent restoration of glycerol content and associated volume changes. Plant Cell & Environment. 17: 1295–1304. Doi: 10.1111/j.1365-3040.1994.tb00531.x.

Sharifi A, Shah-Hosseini M, Pourmand A, Esfahaninejad M, Haeri-Ardakani O. (2018). The Vanishing of Urmia Lake: A Geolimnological Perspective on the Hydrological Imbalance of the World's Second Largest Hypersaline Lake In Nooran et al. (eds.), Lake Urmia: A Hypersaline waterbody in a drying climate. The Handbook of Environmental Chemistry. Springer Nature Switzerland AG. Doi: 10.1007/698_2018_359.

Shuping Z, Yulong W, Mingde Y, Kaleem I, Chun L, Tong J. (2010). Production and characterization of bio-oil from hydrothermal liquefaction of microalgae *Dunaliella tertiolecta* cake. Energy. 35: 5406-5411. Doi: 10.1016/j.energy.2010.07.013.

Simopoulos AP. (2008). The Importance of the Omega-6/Omega-3 Fatty Acid Ratio in Cardiovascular disease and other chronic diseases. Experimental Biology and Medicine. 233: 674-688. Doi: 10.3181/0711-MR-311.

Suzuki T, Matsuo T, Ohtaguchi K, Koide K. (1995). Gas-sparged bioreactors for CO_2 fixation by *Dunaliella tertiolecta*. Journal of Chemical Technology & Biotechnology. 62: 351-358. Doi: 10.1016/j.jcou.2017.09.013.

Takagi M, Karseno Y, Yoshida T. (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. Journal of Bioscience and Bioengineering. 101: 223-226. Doi: 10.1263/jbb.101.223.

Talebi AF, Mohtashami SK, Tabatabaei M, Tohidfar M, Bagheri A, Zeinalabedini M, Mirzajanzadeh M, Shafaroudi SM, Bakhtiari S. (2013). Fatty acids profiling: a selective criterion for screening microalgae strains for biodiesel production. Algal Research. 2: 258-267. Doi: 10.1016/j.algal.2013.04.003.

Wang B, Li Y, Wu N, Lan CQ. (2008). CO_2 bio-mitigation using microalgae. Applied Microbiology and Biotechnology. 79:707-718. Doi: 10.1007/s00253-008-1518-y.

White DA, Pagarette A, Rooks P, Ali ST. (2013). The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures. Journal of Applied Phycology. 25: 153-165. Doi: 10.1007/s10811-012-9849-6.

Xu Y, Ibrahim IM, Wosu CI, Ben-Amotz A, Harvey PJ. (2018). Potential of New Isolates of *Dunaliella Salina* for Natural Carotene Production. Biology. 7 (14): 1-18. Doi: 10.3390/biology7010014.

Yang F, Xiang W, Sun X, Wu H, Li T, Long L. (2014). A novel lipid extraction method from wet microalga *Picochlorum* sp. at room temperature. Marine Drugs. 12: 1258–1270. Doi: 10.3390/md12031258.

Yecong L, Yi-Feng C, Paul C, Min M, Wenguang Z, Blanca M, Zhu J, Ruan R. (2011). Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresource Technology. 102: 5138-5144. Doi: 10.1016/j.biortech.2011.01.091.

Yeh K-L, and Chang J-S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implication for biofuel. Biotechnology Journal. 6: 1358-1366. Doi: 10.1002/biot.201000433.

Yoo C, Jun S-Y, Lee J-Y, Ahn C-Y, Oh H-M. (2010). Selection of microalgae for lipid production under high levels of carbon dioxide. Bioresource Technology. 101 (1): 71-74. Doi: 10.1016/j.biortech.2009.03.030.

Zeng X, Danquah MK, Dong Chen X, Lu Y. (2011). Microalgae bioengineering from CO_2 fixation to biofuel production. Renewable and Sustainable Energy Review. 15: 3252-3260. Doi: 10.1016/j.rser.2011.04.014.

Algal Diversity in Biological Soil Crusts of Qom Province (Qom, Iran): Insights into Microflora Composition and Environmental Impact

Zahara Bagheri Bafian[1], Forough Salehipour_Bavarsad[1], Hossein Riahi[1], Zeinab Shariatmadari[1*]

Received: 2024-01-10 Accepted: 2024-03-03

Abstract

Biological soil crusts (BSCs) result from the close association between soil particles and various microorganisms, including cyanobacteria, green microalgae, and free-living fungi. In this study, we investigated the BSC microflora in soil samples collected from four sites in Qom Province, a semi-arid region in Iran. The study stations included agricultural areas, farms irrigated with saline water, and the edge of Hoz-e-Soltan Lake, a saline water lake. To study algal communities, all soil samples were cultivated in four different culture media including BG11, BG11 with 1.5 M NaCl, nitrate-free BG11 medium (BG110), and BG110 with 1.5 M NaCl. We isolated and identified 37 morphospecies of algae. Cyanobacteria, such as *Phormidium* and *Pseudanabaena*, were dominant genera at most stations. Additionally, green algae and diatoms were present in the studied sites. Our results revealed that algal diversity in BSCs depends on soil physio-chemical characteristics of the soil. For example, *Phormidium* was the dominant genus in all agricultural soils, while in saline soils, the genus *Dunaliella* was reported as the dominant taxon. Therefore, salinity plays a crucial role in shaping species distribution and diversity in these ecosystems.

Keywords: Biological soil crusts, Biodiversity, Salinity, Arid land, *Phormidium*, Qom Province

Introduction

Biological soil crusts (BSCs), which develop in the upper few centimeters of the soil, are a diverse community of photoautotrophic and heterotrophic organisms (García-Carmona et al., 2023). These BSCs can be considered as the result of a close association between soil constituent particles and various organisms, including cyanobacteria, microalgae, micro-fungi, lichens, and bryophytes. The biological soil crust and its various components have the ability to greatly impact the physical and

1-Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, G.C., Tehran, Iran
*Corresponding author email address: z_shariat@sbu.ac.ir
Doi: 10.48308/jpr.2024.235399.1076

chemical properties of soil. In addition, BSCs play a vital role in maintaining soil stability and exert a notable impact on multiple soil factors, particularly in dryland regions (Eldridge et al., 2020). Microorganisms within BSCs are key factors in binding soil particles together, forming a living layer on the ground surface through their presence, interactions, and biological activities (Belnap et al., 2001). Thus, changes in this crucial soil layer can profoundly affect overall environmental health and may contribute to desertification (Nguyen et al., 2022).

Previous studies have demonstrated that some microorganisms found in the soil's upper layer exhibit remarkable resistance to harsh environmental conditions such as dryness, salinity, and high temperature (Li et al. 2021). These groups of microorganisms, called extremophiles, have developed unique adaptations to survive extreme environmental conditions. Among these organisms, extremophilic microalgae play a significant role in soil stabilization and hold potential for applications in biotechnological industries (Varshney et al., 2015).

Cyanobacteria and green algae are pivotal in the formation of soil biological communities among the diverse group of microalgae. Cyanobacteria, as pioneer group of photosynthetic microorganisms, are found worldwide in various environments, including freshwater, saltwater, and arid regions (Rahim et al., 2023; Willis and Woodhouse, 2020). Furthermore, their versatility allows them to adapt to a wide range of conditions (Rahim et al., 2023). Cyanobacteria cells are typically protected by a polysaccharide sheath (EPS), which effectively safeguards them from damage that prevent desiccation. This sheath possesses a great ability to store water and prevent the cells from drying out (Malam Issa et al., 2007). Therefore, EPS plays an important role in connecting soil particles, promoting the cohesion of biocrust inhabitants, and acting as a water-holding biomolecule under drought (Sommer et al. 2020).

The study of microalgae in BSCs, particularly in arid lands, has consistently been a notable and interesting field within algal and environmental research over the past few decades (Chamizo et al., 2016; Miralles et al., 2020; Li et al., 2021). BSCs improve soil stability, regulate soil moisture, and contribute to carbon and nitrogen cycles in arid environments (Li et al., 2021). They impact soil properties such as organic carbon (OC) and pH through their physiological activities (Miralles et al., 2020). Additionally, BSCs enhance soil quality, promote vegetation recovery in semi-arid regions (Gao et al., 2016), and reduce water evaporation from the soil (Chamizo et al., 2016).

Extreme environments, such as drylands, are characterized by severe conditions associated with non-optimal values of various physical and geochemical factors, including temperature, radiation, salinity, and pH (Rothschild & Mancinelli, 2001). Although these areas were once believed to be lifeless, they are now recognized as rich ecosystems supporting highly adapted species (Malavasi et al., 2020). Extremophile microalgae have been the focus of numerous studies worldwide due to their economic advantages (Varshney et al. 2015; Malavasi et al., 2020). Moreover, researchers in Iran have explored extreme habitats extensively, including saline lakes, hot springs, and areas with high radiation backgrounds (Safarpour et al., 2018; Heidari et al., 2020; Salehipour-Bavarsad et al., 2021). Several studies have been carried out on the algal flora of terrestrial ecosystems in Iran; however, these reports are considered insufficient, particularly in high-stress areas. In 2009, Moghtaderi et al. conducted a study on cyanobacteria in the soil desert crust of Chadormalu in the Bafgh region (Yazd province, Iran). Hokmollahi et al. (2016) also described the cyanobacteria found in the soils of Yazd province, Iran, using morphological, molecular, and physiological characteristics. Additionally, in recent years, there have been reports on the morphological and molecular studies conducted on the algal flora of Kavir National Park (Etemadi-Khah et al., 2017). In another study, Irankhahi et al. (2022) studied the diversity and distribution of heterocystous cyanobacteria across solar radiation gradient in terrestrial habitats of Iran. The findings indicate that ecological factors, including solar radiation, relative humidity, and soil salinity, have an impact on the diversity and distribution of cyanobacteria in terrestrial ecosystems. Nostoc was reported to be the prevailing genus across all stations among the identified taxa, particularly in areas with elevated solar radiation, such as Qom Province.

Recent research has explored microalgae inhabiting terrestrial habitats in Qom Province. Studies have documented nitrogen-fixing cyanobacteria such as different species of *Nostoc* (Davari et al. 2018; Irankhahi et al., 2022.), and *Trichoromus* (Davari et al. 2018). In addition, some species of *Oscillatoria* as non-nitrogen-fixing cyanobacteria was reported from this region (Davari et al. 2018). Furthermore, halophile taxa such as *Dunaliella* species have been identified in the arid lands of this province (Salehipour-Bavarsad et al., 2022).

In this study, we identified extremophilic microalgae that form BSCs in semi-arid and saline soils. The study also aims to assess the biodiversity of microalgae at the sampling sites, and determine

their distribution based on the prevailing environmental conditions.

Material and methods

Study area and sampling sites

Qom Province, covering an area of 11238000 square kilometers, is located in the central plateau of Iran (Rahmati Zadeh et al., 2014). This Province is in proximity to Iran's central desert, and boasts several salt lakes at its entrance, including the Salt Lake and the Hoz Sultan Lake (Ebrahimivand et al., 2023). This province is affected by the natural factors that dominate the desert areas. Studies show that over 70% of the province consists of desert and semi-desert areas, with approximately 91% of the region experiencing an ultra-arid and dry desert climate (Rahmati Zadeh et al., 2014). This province is affected by the natural factors that dominate the desert areas. Studies show that over 70% of the province consists of desert and semi-desert areas, with approximately 91% of the region experiencing an ultra-arid and dry desert climate (Rahmati Zadeh et al., 2014), and these conditions have made many areas of this province unsuitable for agricultural activities.

In August 2023, soil samples were collected from four distinct locations within Qom Province, Iran, following the methodology outlined by Rangaswamy (1996). The first sampling site was an operational agricultural plot cultivating *Medicago sativa* (alfalfa). The next two sampling sites were barren lands that were previously used as farmlands, but were irrigated with highly saline water for many years and became unsuitable for agricultural purposes (Fig. 2). The fourth site was the dried lakebed of Hoz-e-Soltan Lake, and soil samples were taken from the playa area. In addition to the soil samples, various vegetation samples were also collected from this site for further examination to explore potential coexistence patterns (Figure 1, Table 1).

Fig. 1. Location of Qom Province, the area which the samples were collected.

Table 1. Geographical details of the sampling sites and some physiochemical parameters recorded at each site

No.	Location	Latitude Longitude	pH	EC (dSm^{-1})	Total Nitrogen (%)	Total Organic Carbon (%)
1	Qom Province; Central part, Medicago Farmland	34°79'29.19"N 50°85'39.24"E	8.08	22.8	0.09	0.71
2	Qom Province; Mahmoud-Abad	34°80'87.85"N 50°61'22.52"E	7.74	43.1	0.20	2.20
3	Qom Province; Pachian	34°80'04.56"N 50°58'31.22"E	7.67	30.4	0.18	1.67
4	Qom Province; Hoz-e-Soltan Lake	34°96'60.99"N 50°89'73.75"E	7.73	>160	0.08	0.58

Isolation and cultivation of algae

Approximately four grams of each soil sample were individually cultivated in glass containers using four different culture media: BG11, BG11 supplemented with a 1.5 M NaCl concentration (BG11: 1.5 M), nitrate-free BG11 medium (BG110), and BG110 supplemented with a 1.5 M NaCl concentration (BG110: 1.5 M). Employing diverse algal culture conditions can provides deeper insights into the biodiversity of algae in the region and their environmental requirements. BG11 serves as a common culture medium, facilitating the growth of a diverse group of algal species. Cultivating soil samples in BG110 assists in the identifying taxa that exhibit resistance to nitrogen deficiency, particularly in favor of heterocystous cyanobacteria. BG11: 1.5 M enables the identification of algal species thriving in saline conditions. Lastly, the essential role of BG110: 1.5 M lies in distinguishing algae that are resilient to both nitrogen deficiency and saline conditions. Anderson's methodology (2005) was used to isolate species capable of thriving in saline environments. The cool white fluorescent lamps emitting about 74 µmol photons m^{-2} s^{-1} were used to provide a photoperiod consisting of 16 hours of light and 8 hours of darkness for the algal cultures, which were kept under controlled conditions at a temperature of 25 ± 2°C. After approximately two weeks, the presence of algal colonies

Identification of microalgal taxa

To identify different algal taxa, a morphometric study was conducted using light microscopy (Olympus, BH-2 model). Semipermanent slides of the colonies were prepared for identification of taxa. Various references were used to identify algae, including Prescott (1970) for identify green algae species; Komárek and Anagnostidis (1986, 2005) and Komárek (2014) for identification of cyanobacteria; and Eileen J. Cox (1996) for identification of diatoms.

Various morphological characteristics, including color, shape, size of colonies and thallus, presence or absence of mucilaginous sheath, and the shape of apical cells, vegetative cells, and special cells in filamentous taxa, were utilized for identification.

Identification of vegetations

The book Flora Iranica (Rechinger, 2005) was used to identify the collected vegetations.

Soil analysis

Various techniques were utilized to measure physicochemical properties of the soil samples including concentration of organic carbon, nitrogen, pH, and electrical conductivity (EC). The soil samples were directly measured for pH using water extracts (1:5 w/v) by the electrometric method. EC was measured using platinum electrodes. Furthermore, the nitrogen concentration was measured using the Macro Kjeldahl (Juo, 1978), and the organic carbon concentration was determined using the Walkley-Black method (Sikora and Moore, 2014).

Statistical analysis

To investigate the linear relationship between two variables, i.e. EC as a soil physicochemical parameter and the number of algae identified from collected soil crusts, Pearson's correlation coefficient was calculated using IBM SPSS Statistics (version 26). In addition, Microsoft Excel 2019 was used to draw the graphs.

Results

Climate and vegetation of the study area

This study focused on investigating algal communities in BSCs of Qom province, particularly the uncultivable

Fig. 2. Pictures of sampling sites: (a) Site 1. agricultural land, (b) Site 2. inactive farmland, (c) Site 3. uncultivated land, (d) Site 4. Hoz-e-Soltan

areas which are irrigated with salt water. Qom Province encounters a semi-arid climate, characterized by very hot summers and extremely cold winters. The average annual precipitation in Qom province is 151.6 mm, with the highest average monthly and annual precipitation recorded at 49.6 mm and 7.99 mm, respectively. The annual mean temperatures of 19.89 °C, with the highest mean monthly temperature reaching 39.5 °C, and the lowest mean monthly temperature dropping to -1°C. The vegetation in the studied sites is described as follows: in Site 1, cultivated *Medicago sativa* L. was predominant vegetation, the main vegetation of site 2 was *Alhagi maurorum* Medik and *Prosopis farcta* (Banks & Sol.) J. F. Macbr, while Site 3 was predominantly covered by *Alhagi*. Moreover, Site 4 was a barren land without any vegetation coverage (Fig 2).

Physicochemical parameters of soil

The physicochemical properties of collected soil samples were measured and represents in Table 1. According to the Table, it can be perceived that the pH of almost all of the samples fell within the normal range of 6.5-8. However, the treated soil in Site 1 exhibited slightly alkaline properties. In addition, all collected soil samples showed high salinity levels based on their EC, which is an indicator of salt concentrations and soil mineralization (Fig 3). The EC

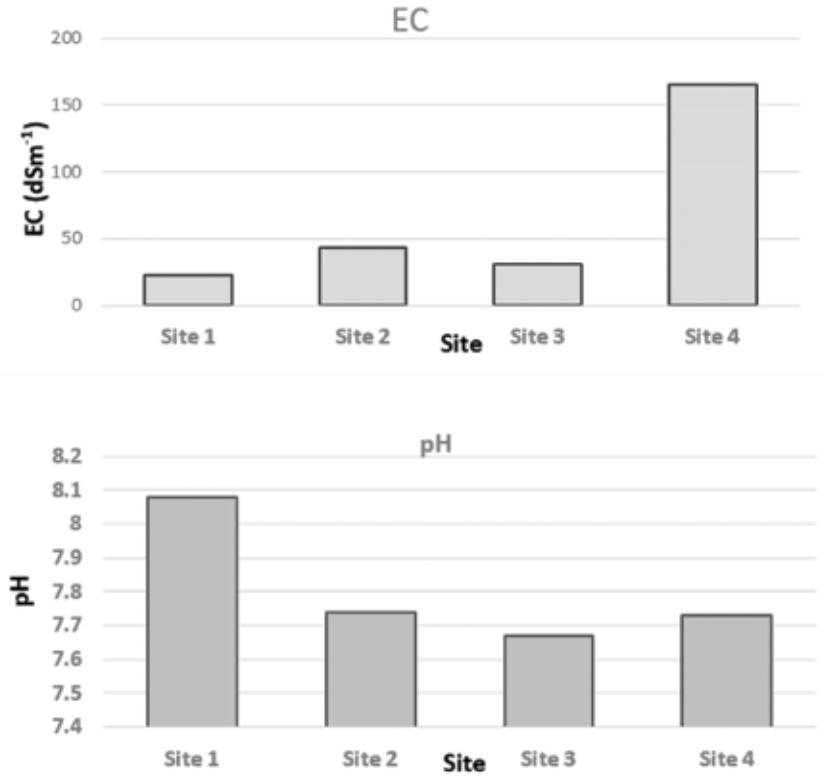


Fig. 3. Comparison of electrical conductivity (EC) and pH levels in sampling sites

values for all soil samples exceeded 4 dS/m. The lowest amount of EC was observed in Site 1 at 22.8 dS/m, while the highest EC value was recorded in Sites 2 and 4 with 43.1 and 160 dS/m. Furthermore, the total nitrogen and total organic carbon (TOC) of soil samples were measured, and the range of these parameters was about 0.08 – 0.20 % for total nitrogen, and 0.58-2.20% for TOC, respectively. The lowest amount of nitrogen and TOC were recorded in Site 4 (0.08%) for nitrogen and 0.58% for TOC), and the highest amount was seen in Site 2 with 0.20 percentage of total nitrogen content and 2.20 percentage in TOC (Fig 4).

Algal diversity

In the present study, a total of 37 microalgal taxa were identified (Table 2, Fig. 5). These taxa included 33 cyanobacterial taxa, three taxa from Chlorophyta, and one diatom species. It should be noted that the genus *Phormidium* appeared most frequently and species diversity among the identified taxa, with a presence rate of 43% (Fig 6). *Pseudanabaena* ranked second with seven species. Further, two species of the genus *Planktolyngbya* were observed among identified taxa. Additionally, some genera were present with only one species among the identified taxa including *Jaaginema pseudogeminatum (G.Schmid)* Anagnostidis and Komárek, *Kamptonema animale* (Gomont) Strunecký, Komárek and J. Smarda, Leptolyngbya foveolarum (Gomont) Anagnostidis and Komárek, *Oscillatoria rupicola* (Hansgirg) Hansgirg ex Forti, *Potamolinea aerugineocaerulea* (Gomont) M.D.Martins and L.H.Z.Branco, *Schizothrix lenormandiana* Gomont, and *Stenomitos frigidus* (F.E.Fritsch) Miscoe and J.R.Johansen. Despite the variety of cyanobacteria taxa, the only nitrogen-fixing cyanobacteria identified in this study was *Nodularia harveyana* Thuret ex Bornet and

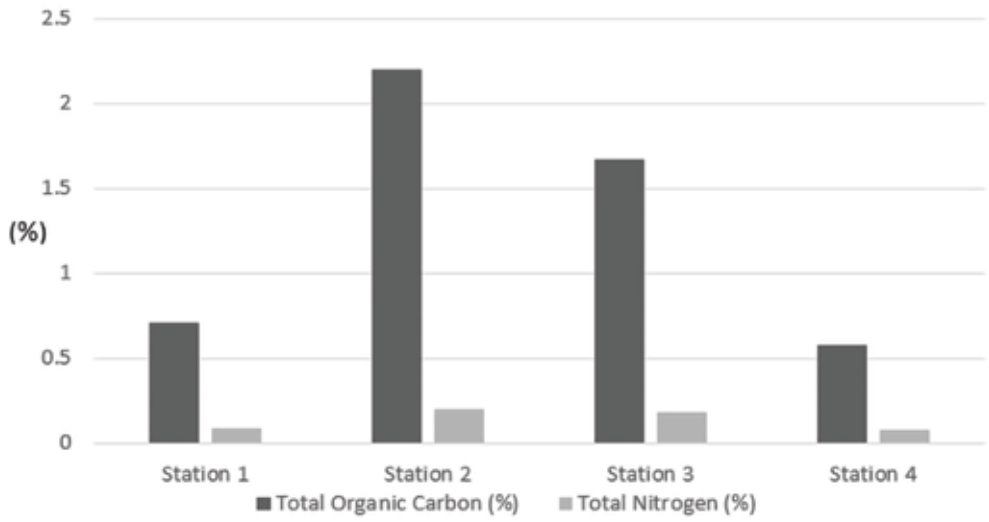


Fig. 4. Comparison of total organic carbon and total nitrogen in sampling sites

Flahault.

Site 1 showed the greatest number of *Phormidium* species totaling nine species. Additionally, Sites 2 and 3 recorded five and six *Phormidium* species, respectively. Conversely, in the final site, there were not any *Phormidium* species (Table 2).

Additionally, three genera of green microalgae were observed from one class, one order, and three families. These microalgae included *Dunaliella* Teodoresco, *Limnomonas* Tesson & Pröschold, and several *Chlorococcum* species.

Accurately classifying different species of *Chlorococcum* genus presents a challenge, thus requiring the use of molecular investigations. Moreover, *Hantzschia* sp. was the only diatom identified in this study; the presence of this species was notably observed in areas irrigated with saline water, particularly at Sites 2 and 3.

In the present study, BSCs samples collected from agricultural sites showed the highest algal diversity. This phenomenon could be attributed to agricultural practices and the utilization of varied water sources for irrigation over time. In Sites 1, 2, and 3, the number of observed microalgae genera was 20, 16 and 18, respectively. However, in the playa collected from Site 4, only two species were identified, including *Dunaliella* sp. and *Pseudanabaena minima* (G.S.An) Anagnostidis.

Data analysis

The correlation between soil properties and the number of algal species in each site was calculated using the Pearson correlation coefficient. The findings indicate a significant correlation between EC and the number of algal species at the 0.01 level. However, no significant correlation was observed between pH level and the number of algae, considering that the range of the mentioned parameter fell between 7.67 and 8.08.

Discussion

The diversity of microalgae within BSCs is intricately influenced by several environmental factors, encompassing both natural processes and human-induced activities across diverse temporal and spatial scales (Büdel et al., 2016; Hakkoum et al., 2021). Among these factors, soil physicochemical properties are decisive determinants that shape the composition and distribution of microalgal communities within terrestrial ecosystems (Hakkoum et al., 2021). Accordingly, our findings suggest that the relationship between ecological parameters and algal diversity is notably complex; nonetheless, certain generalizations can be inferred regarding pH and EC levels.

Cyanobacteria exhibit a remarkable capacity to thrive in extreme environments, including habitats

Table 2. Microalgal diversity in biological soil crusts at different sites of Qom Province

Taxon	Site 1	Site 2	Site 3	Site 4
Bacillariophyta				
Hantzschia sp.		●	●	
Chlorophyta				
Chlorococcum spp.	●	●	●	
Dunaliella sp.		●	●	●
Limnomonas sp.		●		
Cyanophyta				
Jaaginema pseudogeminatum	●		●	
Kamptonema animale		●		
Leptolyngbya foveolarum	●		●	
Nodularia harveyana	●		●	
Oscillatoria rupicola		●	●	
Phormidium breve	●	●	●	
Phormidium caerulescens			●	
Phormidium fragmentosum	●			
Phormidium grunowianum	●			
Phormidium hamelii	●			
Phormidium holdenii	●			
Phormidium incrustatum	●			
Phormidium interruptum		●		
Phormidium kuetzingianum	●			
Phormidium lusitanicum	●	●		
Phormidium nigrum			●	
Phormidium sp.1	●			
Phormidium sp.2		●		
Phormidium sp.3			●	
Phormidium sp.4			●	
Phormidium uncinatum		●	●	
Planktolyngbya holsatica		●		
Planktolyngbya limnetica		●		
Potamolinea aerugineocaerulea		●	●	
Pseudanabaena balatonica	●			
Pseudanabaena minima	●	●	●	●
Pseudanabaena sp.1	●			
Pseudanabaena sp.2	●			
Pseudanabaena sp.3			●	
Pseudanabaena sp.4			●	
Pseudanabaena starmachii	●	●		
Schizothrix lenormandiana	●			
Stenomitos frigidus	●		●	

Site 1 (agricultural land), Site 2 (inactive farmland), Site 3 (uncultivated land), Site 4 (Hoz-e-Soltan)

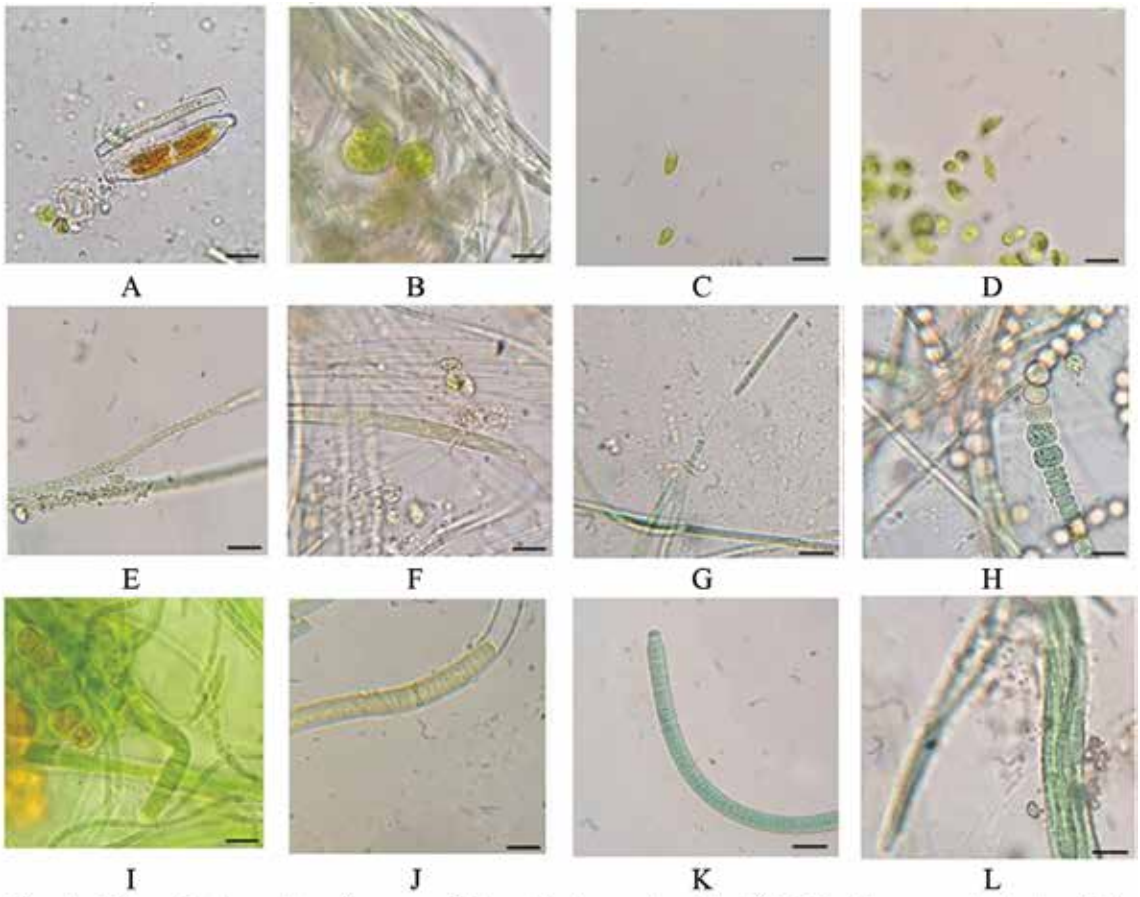


Fig. 5. Microphotographs of some of the algal species identified in the present study: (A) *Hantzschia* sp., (B) *Chlorococcum* sp., (C) *Dunaliella* sp., (D) *Limnomonas* sp., (E) *Jaaginema pseudogeminatum* (F) *Kamptonema animale* (G) *Leptolyngbya foveolarum* (H) *Nodularia harveyana* (I) *Oscillatoria rupicola* (J) *Phormidium interruptum* (K) *Phormidium uncinatum* (L) *Schizothrix lenormandiana* (Bars = 10 µm).

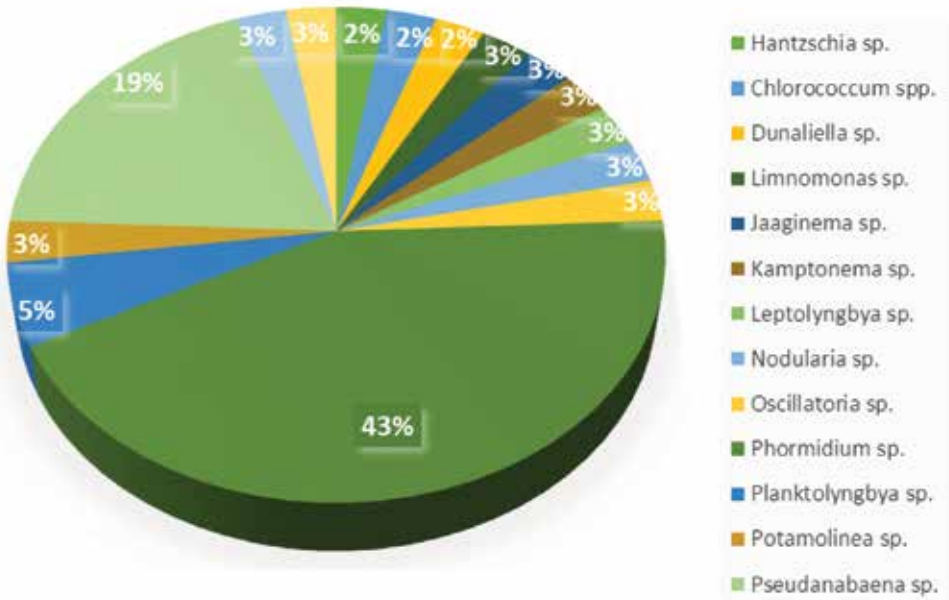


Fig. 6. The species diversity of algal communities which were identified in the studied area in Qom Province

with high radiation, low water availability, and elevated temperatures - characteristics that set them apart from many other biological species. Their autotrophic nature and ability to maintain high metabolic functions with limited moisture requirements enhance their capacity to colonize a diverse range of ecosystems, particularly arid environments (Perera et al., 2018). Moreover, not only do the mentioned features help these microorganisms to survive in saline environments, but also they use mechanisms to survive in ionic stress (Belnap, 2003). Additionally, some cyanobacteria species show specific alternations in their morphological and physiological features while adapting to harsh conditions (Perera et al., 2018).

Site 1 (cultivated land) emerged as the area with the highest algal diversity among the sampling sites. It displayed an elevated pH level (8.08) and lower EC value (22.8) in comparison to the other sites, indicating distinctive environmental conditions that likely exert an influence on the microalgal community structure in that specific area. In addition, in alignment with the observations of Kirkwood and Henley (2006), our study highlights a significant relationship between salinity levels and the diversity of algal species. Higher salinity concentrations (indicated here by high EC values) were associated with a reduced presence of algal species, revealing that excessive salinity could potentially restrict the diversity of microalgae in soil ecosystems. This emphasizes the direct influence of salinity on the overall diversity of microalgae in these environments. Therefore, the research findings enrich our understanding of how environmental factors shape microalgal diversity in soil ecosystems and emphasize the critical role of soil physicochemical properties in driving these ecological dynamics.

As stated, Site 1 is a cultivated area for *Medicago sativa* with the highest algal diversity. However, it is worth noting that *Dunaliella*, as a halotolerant microalga, is not present in this location. The absence of *Dunaliella* in Site 1, and its presence in those three uncultivated sites indicates unfavorable conditions of the other three stations for plant cultivation, as the studied strains of *Dunaliella* showed robust growth in BG11:1.5 M NaCl. Soil salinity is a critical factor that limits plant productivity and quality. Various growth parameters and yield components decline under salinity stress across numerous plant species (Shahbaz and Ashraf, 2013; Ansari et al., 2019). Salinity stress exerts negative effects on photosynthesis, gas exchange, and overall plant growth, attributed to osmotic and ionic stress (Chaves et al., 2009; Ansari at al., 2019). Similar to many other plants, the yield of *M. sativa* decreases at salinity levels exceeding 2 dS m^{-1}, although it

is considered moderately saline-tolerant among leguminous crops (Maas and Hoffman, 1977). *M. sativa*, a perennial plant extensively cultivated as forage in large irrigated areas worldwide, holds significant economic and agronomic value due to its high-quality forage and nitrogen-fixing capabilities (Anower et al., 2013).

The presence and wide distribution of *Phormidium* species in collected BSCs suggest that this genus plays a significant role in agricultural soils (Alghanmi et al., 2019). Research has shown that certain *Phormidium* species, such *as P. breve*, exhibit tolerance to salinity, as demonstrated by Tashlykova and Afonina (2022). Similarly, Sepehr et al. (2019) have reported that *P. uncinatum* is capable of surviving and adapting to saline conditions. Other similar reports also emphasize the resistance of this genus to salinity and their presence in harsh environments (Etemadi-Khah et al., 2017; Zafar et al., 2022). Overall, the presence of *Phormidium* in agricultural soils can be beneficial for soil fertility, plant growth, and potentially for disease control (Rezaee et al., 2019; Younesi et al., 2019; Neyshabouri et al., 2021; Righini et al., 2022). There are also reports on the efficient role of this genus in desalination processes (Zafar et al., 2022).

Our findings also show that *Pseudanabaena minima* was the dominant species among the other taxa, suggesting its potential as a saline-resistant species. Notably, previous research by Ahlesaadat et al. in 2017 also showed the presence of *P. minima* across a range of EC levels in agricultural fields. It should be noted that there is limited information on *Pseudanabaena* spp. in the scientific literature, including their physiology, molecular, and metabolic characteristics (Foster et al., 2020). Although these cyanobacteria are associated with blooming events in various environments and are widely present in aquatic and terrestrial habitats, this genus is frequently disregarded. Interestingly, one of the reports shed light on bioremediation potential of the *Pseudanabaena galeata* strain. Ouhsassi et al. (2020) demonstrated that these filamentous cyanobacteria may effectively remove contaminants from industrial effluents during wastewater treatment processes.

While there is currently no documented evidence of *Oscillatoria rupicola* existing in saline water or being able to tolerate saline environments, we have observed its presence in soils with high salinity levels at Sites 2 and 3. The finding indicates that this species of *Oscillatoria* survives in saline environments. Plus, the genus *Planktolyngbya* which was reported from Site 2 showed a high salinity tolerance compared to other taxa isolated from agricultural fields. This resilience

aligns with findings by Andreote et al. in 2014, who also noted the salinity tolerance of this genus.

Furthermore, *Leptolyngbya* is known as a halophile genus (Verma and Bhattacharjee, 2015), which serves as supporting evidence for the presence of its species in our research. In addition to the widespread observation of *L. foveolarum* in Sites 1 and 3, it was also found to be the predominant species cultured in BG11 medium with a 1.5 M saline concentration.

Previous studies have reported *Kamptonema* as a genus with saline tolerance (Hokmolahi, et al., 2017). In our research, one species of this genus was identified, with *Kamptonema animale* being found in the highest salinity soil among the agricultural fields.

Despite the presence of nitrogen-fixing species *Nodularia harveyana* in sites 1 and 3, the nitrogen content in the first site was only 0.09, which was one of the lowest among the soil samples studied; this can be the result for the method used in the tillage system (Szostek et al., 2022). Based on the findings of Samylina et al. (2024), *N. harveyana*, when observed in saline soils, i.e., Site 3 in the current investigation, has exhibited the capacity to withstand certain degrees of salinity. Consequently, it can be regarded as a species possessing a degree of salt tolerance.

Moreover, *Hantzschia* species exhibit a broad distribution encompassing both freshwater and marine environments, as well as terrestrial habitats (Joh, 2014). Notably, in the present study, *Hantzschia* was the sole diatom observed in saline agricultural soils at Sites 2 and 3. Given that these regions had experienced past associations with saline water, it can be inferred that this particular diatom has persisted since those earlier periods.

Similar to the mentioned genus, *Dunaliella* was also transferred to the agricultural fields while they were aggregating with saline water (Sites 2 and 3); more over this microalga was the dominant species in the soil samples collected from Site 4. Our findings were also proved by other studies representing the presence of *Dunaliella* species in saline soils (Salehipour -Bavarsad et al., 2022).

Even though the higher number of microorganisms results in higher amounts of TOC in soils (Roncero-Ramos et al., 2020; Hakkoum et al., 2021), it has been proved that different method of tillage systems in agriculture has effects on the TOC of the soils (Szostek et al., 2022). Therefore, the low amounts of TOC in the BSCs collected from site 1 can be the result of the tillage system before planting. Thus, compared to site 4 with the weakest BSC, sites 2 and 3 have more TOC concentrations; so that we can argue the BSCs in site 4 are the weak soil crusts, based on the amounts of TOC, EC, and the fewer number of algal species in the soil. Moreover, it can be mentioned that the high amounts of organic carbon in sites 2 and 3 compared with site 1, is the result of being unused through past years.

The comparison of four different BSCs that were affected by natural stress factors such as salinity and drought, as well as human-made factors like tillage methods for preparing agricultural lands and irrigating soil with high saline water, showed us that

these factors have a noteworthy effect on the biological soil crusts; also, previous research by Lan et al. (2010) showed the harmful effects of salinity and dry conditions on BSCs.

In conclusion, this study has identified numerous algae species that are tolerant to high salinity levels. However, further research is needed to explore the salinity resistance and other attributes of these microorganisms in order to determine their potential for restoring biocrusts in arid and saline environments.

Acknowledgments

The authors express their gratitude to Shahid Beheshti University's Department of Biological Science and Technology for providing us with this invaluable scientific work opportunity and the facilities needed to conduct research.

Reference

Ahlesaadat M, Riahi H, Shariatmadari Z, Hakimi Meybodi MH. (2017). A taxonomic study of cyanobacteria in wheat fields adjacent to industrial areas in Yazd province (Iran). Rostaniha. 18 (2): 107-121. Doi: http://doi.org/10.22092/BOTANY.2018.115930.

Alghanmi HA and Jawad HM. (2019). Effect of environmental factors on cyanobacteria richness in some agricultural soils. Geomicrobiology Journal. 36 (1): 75-84. Doi: https://doi.org/10.1080/01490451.2018.1517196.

Andersen, R. A. (Ed.). (2005). Algal culturing techniques. Elsevier.

Andreote APD, Vaz MGMV, Genuário DB, Barbiero L, Rezende-Filho AT, and Fiore MF. (2014). Nonheterocytous cyanobacteria from Brazilian saline-alkaline lakes. Journal of phycology. 50: 675–684. Doi: https://doi.org/10.1111/jpy.12192.

Anower MR, Mott IW, Peel MD, Wu Y. (2013). Characterization of physiological responses of two alfalfa half-sib families with improved salt tolerance. Plant Physiology and Biochemistry, 71: 103-111. Doi: https://doi.org/10.1016/j.plaphy.2013.06.026.

Ansari M, Shekari F, Mohammadi MH, Juhos K, Végvári G, Biró B. (2019). Salt-tolerant plant growth-promoting bacteria enhanced salinity tolerance of salt-tolerant alfalfa (Medicago sativa L.) cultivars at high salinity. Acta physiologiae plantarum. 41: 1-13. Doi: https://doi.org/10.1007/s11738-019-2988-5.

Belnap J, Büdel B, Lange OL. (2001). Biological soil crusts: characteristics and distribution. Biological soil crusts: structure, function, and management. Springer International Publishing. 150: 3-30. Doi: https://doi.org/10.1007/978-3-642-56475-8_1.

Belnap J. (2003). The world at your feet: desert biological soil crusts. Frontiers in Ecology and the Environment. 1 (4): 181-189. Doi: https://doi.org/10.1890/1540-9295(2003)001[0181:TWAYFD]2.0.CO;2.

Büdel B, Dulić T, Darienko T, Rybalka N, Friedl T. (2016). Cyanobacteria and algae of biological soil crusts. Biological soil crusts: an organizing principle in drylands. 226: 55-80. Doi: https://doi.

org/10.1007/978-3-319-30214-0_4.

Chamizo S, Belnap J, Eldridge DJ, Cantón Y, Malam Issa O. (2016). The role of biocrusts in arid land hydrology. Biological soil crusts: An organizing principle in drylands. 226: 321-346. Doi: https://doi.org/10.1007/978-3-319-30214-0_17.

Chaves MM, Flexas J, & Pinheiro C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of botany. 103(4): 551-560. Doi: https://doi.org/10.1093/aob/mcn125.

Davari M, Modaressi Z, Aghashariatmadari Z. (2018). Taxonomic Study on Cyanobacteria Species in Natural Habitats of Tanacetum parthenium Emphasising on Wollea and Cylindrospermum Morphological Characters. Plant, Algae, and Environment. 2 (2): 237-253.

Ebrahimivand A, Hooshyaripor F, Rajabi H. (2023). Assessment of development trend and land use change in Qom province using remote sensing technology. Water Resources. 16(57): 91-102. Doi: http://doi.org/10.30495/wej.2023.5831.

Eldridge DJ, Reed S, Travers SK, Bowker MA, Maestre FT, Ding J, Havrilla C, Rodriguez-Caballero E, Barger N, Weber B, Antoninka A, Belnap J, Chaudhary B, Faist A, Ferrenberg S, Huber-Sannwald E, Malam Issa O, Zhao Y. (2020). The pervasive and multifaceted influence of biocrusts on water in the world's drylands. Global Change Biology. 26 (10): 6003-6014. Doi: https://doi.org/10.1111/gcb.1523.

Etemadi-Khah A, Pourbabaee AA, Alikhani HA, Noroozi M, Bruno L. (2017). Biodiversity of Isolated Cyanobacteria from Desert Soils in Iran. Geomicrobiology Journal. 34 (9):784-794. Doi: https://doi.org/10.1080/01490451.2016.1271064.

Foster L, Muhamadali H, Boothman C, Sigee D, Pittman JK, Goodacre R, Morris K and Lloyd JR (2020). Radiation tolerance of Pseudanabaena catenata, a cyanobacterium relevant to the first generation magnox storage pond. Frontiers in Microbiology. 11: 515. 486666. Doi: https://doi.org/10.3389/fmicb.2020.00515

Gao X, Xu H, Ye S, Liang W. (2016). A proposal on the restoration of Nostoc flagelliforme for sustainable improvement in the ecology of arid steppes in China. Environments. 3 (2): 14. Doi: https://doi.org/10.3390/environments3020014.

García-Carmona M, García-Orenes F, Arcenegui V, Mataix-Solera J. (2023). The Recovery of Mediterranean Soils After Post-Fire Management: The Role of Biocrusts and Soil Microbial Communities. Spanish Journal of Soil Science. 13: 11388. Doi: https://doi.org/10.3389/sjss.2023.11388.

Hakkoum Z, Minaoui F, Douma M, Mouhri K, Loudiki M. (2021). Impact of human disturbances on soil cyanobacteria diversity and distribution in suburban arid area of Marrakesh, Morocco. Ecological Processes. 10(1), 42. Doi: https://doi.org/10.1186/s13717-021-00303-7.

Heidari F, Shariatmadari Z, Riahi H. (2020). Screening of Extremophile Microalgae Isolated from High Background Radiation Areas as Source of Bioactive Materials. Current Bioactive Compounds. 16 (4): 407-414. Doi: https://doi.org/10.2

174/157340721566618121910451 8.

Hokmolahi F, Riahi H, Soltani N, Shariatmadari Z, Hakimi Meibodi MH. (2017). A taxonomic study on non-heterocystous filamentous Cyanoprokaryotes from soil of yazd province, Iran. The Iranian Journal of Botany. 23 (1): 60-71. Doi: http://doi.org/10.22092/ijb.2017.109312.1147. Irankhahi P, Riahi H, Shariatmadari Z, Aghashariatmadari Z. (2022). Diversity and distribution of heterocystous cyanobacteria across solar radiation gradient in terrestrial habitats of Iran. Rostanih. 23 (2): 264-281. Doi: 10.22092/BOT.J.IRAN.2023.360711.1336.

Joh G. (2014). The diverse species of the genus Hantzschia (Bacillariophyta) in sand flats of the Nakdong River estuary in Korea. Journal of Ecology and Environment. 37 (4): 245-255. Doi: https://doi.org/10.5141/ecoenv.2014.029.

Juo ASR. (1978). Selected methods for soil and plant analysis: IITA manual series, No. 1. Doi: https://hdl.handle.net/10568/97963.

Kirkwood AE and Henley WJ. (2006). Algal community dynamics and halotolerance in a terrestrial, hypersaline environment 1. Journal of phycology. 42 (3): 537-547. Doi: https://doi.org/10.1111/j.1529-8817.2006.00227.x.

Lan S, Wu L, Zhang D, Hu C, Liu Y. (2010). Effects of drought and salt stresses on man-made cyanobacterial crusts. European Journal of Soil Biology. 46 (6): 381-386. https://doi.org/10.1016/j.ejsobi.2010.08.002.

Li X, Hui R, Tan H, Zhao Y, Liu R, Song N. (2021). Biocrust research in China: recent progress and application in land degradation control. Frontiers in Plant Science. 12: 751521. Doi: https://doi.org/10.3389/fpls.2021.751521.

Maas EV and Hoffman GJ. (1977). Crop salt tolerance—current assessment. Journal of the irrigation and drainage division. 103(2): 115-134. Doi: https://doi.org/10.1061/JRCEA4.0001137.

Malam Issa O, Défarge C, Le Bissonnais Y, Marin B, Duval O, Bruand A, d'Acqui LP, Nordenberg S Annerman M. (2007). Effects of the inoculation of cyanobacteria on the microstructure and the structural stability of a tropical soil. Plant and soil. 290: 209-219. Doi: https://doi.org/10.1007/s11104-006-9153-9.

Malavasi V, Soru S, Cao G. (2020). Extremophile microalgae: the potential for biotechnological application. Journal of phycology. 56(3): 559-573. Doi: https://doi.org/10.1111/jpy.12965.

Miralles I, Soria R, Lucas-Borja ME, Soriano M, Ortega R. (2020). Effect of biocrusts on bacterial community composition at different soil depths in Mediterranean semi-arid ecosystems. Science of the Total Environment. 733: 138613. Doi: https://doi.org/10.1016/j.scitotenv.2020.138613

Moghtaderi A, Taghavi M, Rezaei R. (2009). Cyanobacteria in biological soil crust of Chadormalu area, Bafq region in central Iran. Pakistan Journal of Nutrition. 8(7): 1083-1092.

Nguyen TCT, Khudzari JM, Imamura F, Sethi S, Iwamoto K. (2022, November). BSC (biological soil crust) Method for soil erosion control and vegetation recovery: Case studies in Japan. In IOP Conference

Series: Earth and Environmental Science. 1091(1). Doi: https://doi.org/10.1088/1755-1315/1091/1/012033.

Ouhsassi M, Khay EO, Bouyahya A, El Ouahrani A, Harsal AE, Abrini J. (2020). Evaluation of self-purifying power of cyanobacteria Pseudanabaena galeata: case of dairy factory effluents. Applied Water Science. 10: 181. Doi: https://doi.org/10.1007/s13201-020-01270-8.

Perera I, Subashchandrabose SR, Venkateswarlu K, Naidu R, Megharaj M. (2018). Consortia of cyanobacteria/microalgae and bacteria in desert soils: an underexplored microbiota. Applied microbiology and biotechnology. 102: 7351-7363. Doi: https://doi.org/10.1007/s00253-018-9192-1.

Rahim NAA, Merican FMMS, Radzi R, Omar WMW, Nor SAM, Broady P, Convey P. (2023). Unveiling the diversity of periphytic cyanobacteria (Cyanophyceae) from tropical mangroves in Penang, Malaysia. Tropical Life Sciences Research. 34 (3): 57. Doi: https://doi.org/10.21315/tlsr2023.34.3.4.

Rahmati Zadeh A, Jafari M, Karimian Eghbal M. (2014). Identifying saline lands and halophytes of Qom province. Iranian journal of range and desert research. 21 (4): 580-590. Doi: https://doi.org/10.22092/ijrdr.2016.13055.

Rangaswamy G. (1996). Agricultural microbiology. Asia Publishing House, Bombay. pages: 54–76.

Rechinger KH. (2005). Flora Iranica, vol. 1-176. Akademische Druck-u. Verlagsanstalt. Graz. Austria.

Righini H, Francioso O, Martel Quintana A, Roberti R. (2022). Cyanobacteria: a natural source for controlling agricultural plant diseases caused by fungi and oomycetes and improving plant growth. Horticulturae. 8 (1): 58. Doi: https://doi.org/10.3390/horticulturae8010058.

Roncero-Ramos B, Muñoz-Martín MA, Cantón Y, Chamizo S, Rodríguez-Caballero E, Mateo P. (2020). Land degradation effects on composition of pioneering soil communities: An alternative successional sequence for dryland cyanobacterial biocrusts. Soil Biology and Biochemistry. 146: 107824. Doi: https://doi.org/10.1016/j.soilbio.2020.107824

Rothschild LJ, and Mancinelli RL. (2001). Life in extreme environments. Nature. 409(6823): 1092-1101. Doi: https://doi.org/10.1038/35059215.

Safarpour A, Amoozegar M A, Ventosa A. (2018). Hypersaline environments of Iran: Prokaryotic biodiversity and their potentials in microbial biotechnology. Extremophiles in Eurasian ecosystems: ecology, diversity, and applications. 8: 265-298. Doi: https://doi.org/10.1007/978-981-13-0329-6_9.

Salehipour-Bavarsad F, Riahi H, Hejazi M A, Shariatmadari Z. (2022). Screening terrestrial and aquatic strains of Dunaliella from the eco-taxonomical perspective: a comparative study on fatty acid compositions as habitat indicators. Journal of Applied Phycology. 34: 461–474. Doi: https://doi.org/10.1007/s10811-021-02645-3.

Salehipour-Bavarsad F, Riahi H, Hejazi MA, Shariatmadari Z. (2021). Optimization of

β-carotene production by an indigenous isolate of Dunaliella salina under salinity gradient stress. Iranian Journal of Fisheries Sciences. 21 (1): 235-246. Doi: 10.22092/ijfs.2022.125929.

Samylina OS, Kosyakova AI, Krylov AA, Sorokin DY, Pimenov NV. (2024). Salinity-induced succession of phototrophic communities in a southwestern Siberian soda lake during the solar activity cycle. Heliyon. 10(4). e26120. Doi: https://doi.org/10.1016/j.heliyon.2024.e26120.

Sepehr A, Hassanzadeh M, Rodriguez-Caballero E. (2019). The protective role of cyanobacteria on soil stability in two Aridisols in northeastern Iran. Geoderma Regional. 16: e00201. Doi: https://doi.org/10.1016/j.geodrs.2018.e00201.

Shahbaz M and Ashraf M. (2013). Improving salinity tolerance in cereals. Critical reviews in plant sciences. 32 (4): 237-249. Doi: https://doi.org/10.1080/07352689.2013.758544.

Sikora FJ and Moore KP (2014). Soil test methods from the southeastern United States. Southern cooperative series bulletin. 419: 54-58.

Sommer V, Mikhailyuk T, Glaser K, Karsten U. Uncovering Unique Green Algae and Cyanobacteria Isolated from Biocrusts in Highly Saline Potash Tailing Pile Habitats, Using an Integrative Approach. Microorganisms. 8 (11): 1667. Doi: https://doi.org/10.3390/microorganisms8111667.

Szostek M, Szpunar-Krok E, Pawlak R, Stanek-Tarkowska J, Ilek A. (2022). Effect of different tillage systems on soil organic carbon and enzymatic activity. Agronomy. 12 (1): 208. Doi: https://doi.org/10.3390/agronomy12010208.

Tashlykova NA and Afonina E Y. (2022). Diversity of plankton communities of chloride lakes of Southeastern Transbaikalia. In IOP Conference Series: Earth and Environmental Science. 1112 (1): 012108. IOP Publishing. Doi: http://doi:10.1088/1755-1315/1112/1/012108.

Varshney P, Mikulic P, Vonshak A, Beardall J, Wangikar PP. (2015). Extremophilic micro-algae and their potential contribution in biotechnology. Bioresource technology. 184: 363-372. Doi: https://doi.org/10.1016/j.biortech.2014.11.040.

Verma V and Bhattacharjee MB. (2015). Growth and nitrate metabolism in the Thermohalophillic Cyanobacterium Leptolyngbya. Journal of Algal Biomass Util. 6: 68-75. ISSN: 2229 – 6905.

Willis A and Woodhouse JN. (2020). Defining cyanobacterial species: diversity and description through genomics. Critical Reviews in Plant Sciences. 39 (2): 101-124. Doi: https://doi.org/10.1080/07352689.2020.1763541.

Zafar AM, Javed MA, Hassan AA, Sahle-Demessie, E, Harmon S. (2022). Biodesalination using halophytic cyanobacterium Phormidium keutzingianum from brackish to the hypersaline water. Chemosphere. 307: 136082. Doi: https://doi.org/10.1016/j.chemosphere.2022.136082.

Hormonal and Enzymatic Responses and Seed Vigor of Coated Maize (*Zea Mays* L.) Seeds with Calcium Alginate in Diesel-Contaminated Soils

Mahboobe Dehyadegari[1], Mohamad Reza Ghalamboran[1*], Francois Bernard[1]

Received: 2024-01-07 Accepted: 2024-03-10

Abstract

The most important problem of oil industries and refineries is the leakage and spraying of oil compounds and products through transportation systems on agricultural soils. Penetration of petroleum compounds in the soil causes the fertility of the agricultural soil to decrease drastically. The seeds of agricultural and garden plants either do not grow at all or do not grow optimally in soils contaminated with petroleum compounds. Seed coating is one of the ways to enhance the plants' seed germination in contaminated soils with diesel. The use of natural materials is preferable to chemicals to prevent the aggravation of environmental pollutants. The main aim of this study was to evaluate the effect of calcium alginate as a coating layer on maize seed for germination in contaminated soil with diesel. This experiment was organized in a two-way ANOVA (4 × 4) in which factor A consisted of four levels of diesel-contaminated soil (0, 2, 4, 6%), and factor B consisted of calcium alginate concentrations (0, 1, 2, 3%). Experimental treatments' effects were studied in a completely randomized design with four replications. Experimental variables included some components of vegetative growth, hormones, and enzymes. The most important results showed that seeds coated with calcium alginate concentrations (1, 2, 3%) under diesel-contaminated soils increased germination percentage, shoot and root lengths, and shoot fresh weight while decreasing the activity of antioxidant enzymes including catalase, superoxide dismutase, as well as malondialdehyde production. Also, the results showed that 2 and 3% of calcium alginate concentrations produced the highest amount of Gibberellin A3 hormone, while these concentrations produced the lowest amount of abscisic acid hormone in the coated seeds under diesel-contaminated soils.

Keywords: Maize, Enzymes, Diesel, Phytohormones, Seed Coating

Introduction

Oil industries and refineries in many countries and oil-rich regions of the world are responsible for the creation and development of oil pollutants in ecosystems. Fertile agricultural soils in oil-rich areas are at risk of oil product leakage and pollution due to the construction of refineries and oil industries, and in case of oil compound leakage and penetration into agricultural

1- Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.
* Corresponding authors' email address: m_ghalamboran@sbu.ac.ir
Doi: 10.48308/jpr.2024.234741.1064

soils, they lose the ability to produce and grow plants. The relatively high hydrophobicity of petroleum hydrocarbons causes the accumulation of these pollutants in the soil and their deposition relative to the aquatic environment, Koshlaf and Ball (2017). Therefore, it is necessary to create appropriate solutions to eliminate or reduce the effects of soil oil pollutants.

Diesel is one of the reserves in nature with very poor solubility in water, the presence of which in the soil leads to pollution and toxicity. Under natural conditions, the downward movement of diesel is prevented due to adsorption by organic matter on the soil surface, so contamination remains superficially in the soil and root system of most plant species (Yakovleva et al., 2017).

The technique of seed coating dates back to 1928 and was first used by Allen and Stevenson to increase 1000-seed weight and uniform seed distribution in aerial seeding. However the general concept of the word seed coating was first used by Sweet Anion, and the technique developed very rapidly in Western countries, including the United States and Germany (Song et al., 2014).

The British company Germains was established in the 1930s to cover grain seeds and began commercial use in the 1960s for precision arable farming in Europe (Gorim, 2014). This technology involves the application of a very thin layer permeable to the seed, which increases seed yield, improves germination, and ultimately increases yield without harming the environment. This layer can contain fungicides, insecticides plant growth regulators, and fertilizers, Rocha, Ma et al. (2019). Then the use of bacteria in seed coats as biological coatings that were first used in China on rice, wheat, maize, and other grains was developed in other countries (Song et al., 2014; Kimmelshue et al., 2019).

The results of previous research show that if the seed coating is of hydrophilic polymers, it can protect the seed against drought stress and increase the rate of water absorption (Su et al., 2017).

The seeds of some plants are naturally round and large, which makes them easy to grow, but the seeds of some other plants are shapeless, very small, or have different sizes, which disrupts their cultivation in mechanized agriculture the use of seed coating technology solves this problem, while hydrophobic polymers reduce water absorption (Afzal et al., 2020).

On the other hand, several areas around most oil refineries have been left unused due to contamination with petroleum compounds, while these areas can be cultivated through biological treatment of contaminated soils and the use of resistant plants such as maize. Indeed, the economic importance of maize products and their physiological capabilities and resistance to environmental stresses such as stresses caused by oil pollutants can be used as a suitable candidate in soils contaminated with petroleum compounds (Ghalamboran et al., 2020).

One of the most important problems of planting plants in soils contaminated with petroleum compounds (such as diesel) is a sharp decrease in seed vigor rate and percentage. That is, due to the inhibitory effects of petroleum compounds such as toxicity, and hypoxia in the seed growth

medium, the seed vigor process is reduced and may be stopped (Dib and Sadoudi, (2020).

In recent decades, the seed coating technique has been used to increase seed resistance under stress conditions. However, according to reports from companies and seed industries, different coatings are used for maize seeds, that are not of natural origin (e.g., polyacrylamide + Carboxymethyl cellulose (CMC) for film former, ethylene glycol for antifreeze, gelatin for thickener, and Lauryl Alcohol Ethoxylate (LEA-9) as a nonionic surfactant in the composition of seed coatings) and the entry of these compounds into the soil, in the long run, can cause damage to the environment and shoot growth (Pedrini et al., 2017). Therefore, it is necessary to use biocompatible, degradable, available, and affordable compounds for seed coating. In addition, the material used in the seed coating must have high water absorption and retention power so that the increase in the thickness of the seed shell by the coating layer does not reduce the absorption and penetration of water inside the seed.

Calcium alginate is one of the biocompatible polymer compounds, which has the above advantages. The biggest advantage of alginates is their liquid–gel behavior in aqueous solutions. When monovalent ions (e.g., sodium in sodium alginate) are exchanged for divalent ions (particularly calcium), the reaction proceeds almost immediately, changing from a viscosity low-viscosity solution to a gel structure.

Previous results showed that the use of calcium alginate enhanced the activities of several enzymes beneficial for germination like proteases (cysteine proteases (CPs), followed by serine proteases (SPs) and aspartic proteases (APs)), and the germination process improves (Hu et al., 2004). Furthermore, the use of calcium alginate as a biodegradable compound to coat maize seeds, consequently, the coated seeds easily germinated, and if remaining residual layer in the soil would be used by microorganisms (de Castro et al., 2020).

The current research is based on the assumption that the use of calcium alginate biopolymer due to its advantages such as having high water absorption, the possibility of creating a moisture layer around and attached to the surface of the seed, the possibility of accelerating and facilitating the absorption of water by the seed in the germination process, preventing The direct contact of pollutants with the surface of the seed, the reduction of the concentration of pollutants and the possibility of their penetration into the seed, through increasing the thickness of the seed coat, which ultimately improves and increases the germination power of maize seeds under the conditions of soil stress contaminated with diesel substances. Also, the main goal of this research was whether the use of calcium alginate as a layer on the surface of maize seeds protects and improves the processes of maize seed germination and growth under the stress of soil-contaminated soils with diesel. Furthermore, other objectives such as the physiological and metabolic responses of coated and uncoated maize seeds under the stress of diesel pollution were investigated and evaluated.

Material and methods

The commercial diesel provided by the National Iranian Oil Company (NIOC) was used. The diesel components were determined in the lab of NIOC and the details are given in Tables 1 and 2.

Preparation of contaminated soil with diesel

The contaminated soil with diesel was prepared using a modified method according to Ghalamboran et al. (2020). First, the sandy loam (sand 26%, silt 68%, and clay 6% (Table 3)) was washed in a container with tap water and then immersed in 0.5% HCl for 1–2 days. The acid was drained off and then the soil was washed with at least 6–8 times of sterile water. If needed, 1 M of KOH was used to adjust the pH. To prevent the interference of the bacteria in reducing the adverse effects of oil pollutants in the growth environment of the tested plants, the soil was autoclaved for 4–6 hours at 121 °C and 131 kPa. After cooling, first, the soil was mixed with sterilized coco-peat at the rate of 5% by weight of the total soil used, and then, the soil was mixed with different concentrations of diesel (no diesel as the control and 2, 4, and 6 %). Next, 150 g of the contaminated soil was placed in each seedling tray (70 cells).

Coating method of the seeds

The seeds were coated according to the modified method of Kikowska et al. (2011).

Table 1. Details of analysis commercial diesel (light – medium) from National Iranian Oil Company

Description	Result	Method of standard
Color	3	
Density / Kg L^{-1}	0.820-0.860	ASTM D-1298
Distillation		ASTM D-86
Recovery at 375 °C/(minimum) vol%	90	
Final boiling point / (maximum) °C	385	
Flashpoint (minimum)/°C	54	ASTM D-93
Pour point (maximum)/°C	-4	ASTM D-97
Sulphur total (maximum)/mass %	1	ASTM D-1552
Viscosity kinematic at 37.8 °C/ mm^2/s	2.0 – 5.5	ASTM D-445
Cloud point (maximum)	2	ASTM D-2500
Cetan index (minimum)	50	ASTM D-976
Ash/mass%	0.01	ASTM D-482
Water and sediments/vol%	0.05	ASTM D-2709
Carbon residue/mass%	0.1	ASTM D-189
Aliphatic compounds total/%	45-55	
Aromatic compounds total/%	12-15	

The sodium alginic acid was used as a bio-polymer coating, along with calcium chloride (100 mM) with a purity of 95% and a molecular weight of 110.99. To prepare different concentrations of sodium alginic acid (1, 2, 3, 4, 5% by weight), first, different concentrations of sodium alginic powder were weighed (Figure 1). Each weighed sample was then dissolved in 100 ml of distilled water at 40-50 ° C and then the seeds (20 to 30) were mixed manually with this solution in a 500 ml beaker. Then, coated seeds were placed in 100 mM calcium chloride solution by drop-by-drop immersion method for 20 to 30 minutes to perform cation exchanges to harden the calcium alginate coating. Finally, the seeds coated with calcium alginate were placed in the open air for 2 days to dry (Kikowska and Thiem, 2011).

Plant cultivation

The seeds of Maize (*Zea mays* L.) cultivar KSC 260 were obtained from the Seed and Plant Improvement Institute, Iran. The germination seeds test was carried out based on the modified method of Lizárraga-Paulín et al. (2013). The planting of coated seeds and measuring of shoot length, root length, dry weight, and fresh weight of shoots and roots were according to the method of Ghalamboran (2011). The coated seeds were planted in the shoot trays (which were

Table 2. Characteristics of compounds in commercial diesel

Compound	Component	Carbone number	Structure
Aliphatic Hydrocarbons	n-Pentane	5	Straight chain
	Iso-Pentane	5	Branched chain
	n-Hexane	6	Straight chain
	n-Heptane	7	Straight chain
	n-Octane	8	Straight chain
	Iso-Octane	8	Branched chain
	n-Nonane	9	Straight chain
Aromatic Hydrocarbons	Benzene	6	Monocyclic
	Methyl cyclohexane	7	Monocyclic
	Toluene	7	Monocyclic
	Ethyl benzene	8	Monocyclic
	p-Xylene	8	Monocyclic
	o-Xylene	8	Monocyclic
	m-Xylene	8	Monocyclic

Table 3. Characteristics of the soil used in this experiment

Soil Texture	$CaCO_3$ (%)	Na (mg/kg)	K (mg/kg)	P (mg/kg)	N (mg/kg)	EC (µSiemens/cm)	pH	Organic matter
Sandy-Loam	7.43 ± 0.1	3.2 ± 1	63 ± 1	16.5 ± 2	0.19 ± 0.02	143.2	6.8±0.2	3.5 ± 0.5

filled with contaminated soils). Then they were placed in the greenhouse for 22–25 °C day/15–18 °C night cycles under ambient light. Each shoot tray was irrigated with 1500 ml of tap water every day. Seed vigor percentage was determined, 8 days after sowing. The vegetative components of the shoots grown in the greenhouse such as the root and shoot length were determined using a ruler (cm) at 21 days after the planting. Also fresh shoots were weighed.

Extraction of proteins

The sample preparation of protein extraction was carried out according to the modified method of Laing and Christeller et al. (2004). At 21 days after planting the maize in the greenhouse, 0.1 g of each shoot tissue was homogenized over an ice bath with 1500 µL Tris–HCl 10 nM, 0.0186 g of EDTA, and 5.47 g sucrose. The homogenized tissue was then centrifuged for 30 min at 4 °C (13,600 rpm), and the supernatant was used immediately to determine enzymatic activity (Laing and Christeller, 2004).

Quantification of enzyme activity

The activity of Catalase was determined following the adapted protocol by Shi (2005), using 50 mg of fresh shoot tissue grounded for 2 minutes on ice with 2 ml of sodium phosphate buffer (pH 6.8, 0.1 M). The resulting extracts were then centrifuged at 15000 rpm for 10 minutes at 4 °C and the supernatant was utilized to measure the enzymatic activity and the soluble protein content. Subsequently, 200 µl of the supernatant and 100 µl hydrogen peroxidase 0.2 M were added to 1.5 ml of phosphate buffer (pH 6.8, 50 mM). The absorption was determined at 240 nm to assess the activity change, and the enzyme activity was expressed as fresh weight in mg of protein/ min. Guaiacol peroxidase activity was evaluated following the method described by Ruley et al. (2004). Where 100 mg of fresh shoot sample was grounded for 4 minutes on ice with 1 ml of phosphate buffer at pH 7, 0.1M, containing 1 mM of EDTA and 1 mM of PVP 0.1%. The extract was

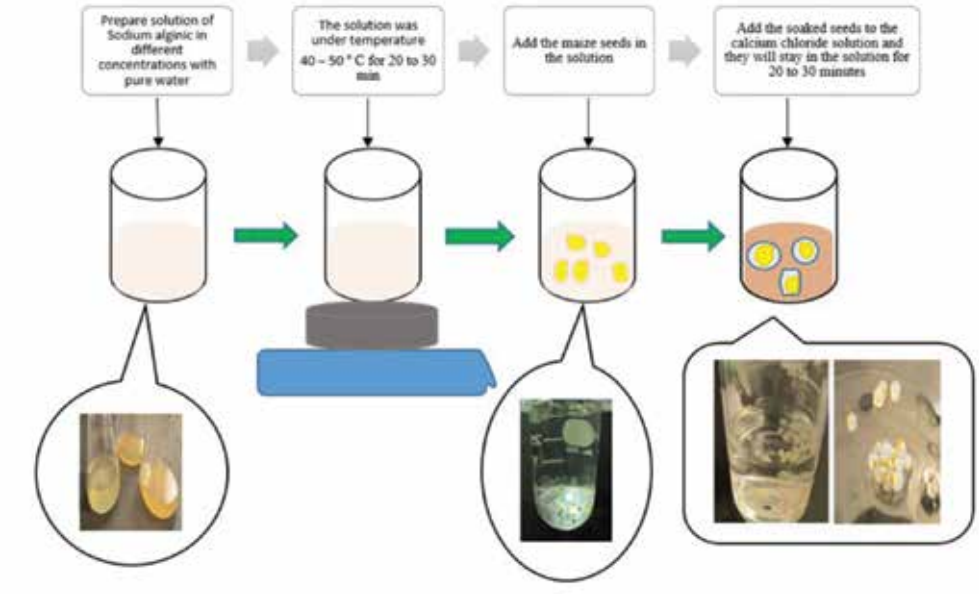


Fig. 1. Schematic of the coating process of maize seeds

then centrifuged at 4000 rpm for 10 minutes at 4 °C, then the supernatant was used to measure enzymatic activity. 100 µl of the supernatant with 450 µl of H_2O_2 (20 mM) and 450 µl of guaiacol (2%) were mixed and the adsorption changes were recorded for 3 min at 510 nm (Ruley, Sharma et al. 2004). Ascorbate peroxidase enzyme activity was measured following the protocol described by Koushesh Saba et al. (2012) with minor adjustments. The procedure were similar to the determination of Guaiacol peroxidase activity, with the exception that 100 µl of the supernatant with 60 µl of H_2O_2 (20 mM) and 850 µl of phosphate buffer at pH 7, 0.1M, containing 1 mM of EDTA and 1 mM of PVP 0.1% were mixed and the adsorption rates recorded for 1 min at 290 nm (Arzani et al., 2012). The SOD activity was measured as recommended by Du et al. (2015) with slight modification using 0.2 g of frozen sample in 3 ml HEPES–KOH at pH 7.8 containing 0.15 EDTA for absorbance at 560 nm. The SOD activity was defined as enzyme content that resulted in 50% nitroblue tetrazolium in 560 nm Du, Liu et al. (2015).

Determination of malondialdehyde content

The malondialdehyde (MDA) concentration as a biomarker for lipid peroxidation was measured according to the modified method of Du et al. (2015). 50 mg of the shoot tissue was frozen with 2.5 ml of 10 % thiobarbituric acid, and the homogenate was centrifuged at 10,000 rpm for 10 minutes. Then, 1000 µl of the extracted supernatant was mixed with 2000 µl of 10% trichloroacetic acid, and it was placed in a Bain-marie with a temperature of 80-90 °C for 30 minutes. Next, the reaction mixture was quickly cooled in an iced bath, and after, it was re-centrifuged at 1000 rpm for 10 minutes. The absorbance of the supernatant was recorded at 450 nm, 532 nm, and 600 nm. The MDA content was calculated using a constant extinction coefficient (ε = 155 mM^{-1} cm^{-1}).

Quantification of Gibberellin A3 (GA3) and abscisic acid (ABA)

The phytohormones GA3 and ABA were determined following the modified method of Delavari, et al. (2017) and Razifah et al. (2014). The coated seeds were germinated up to the emergence of the cotyledonary leaf, and then from each treatment, the emerging seeds were used to measure the hormones at 4 days after planting. This time (4 days after planting) was selected as a stage of the seed vigor duration, the emerged seeds' hormones reached maximum. First, 0.5 g of the seed tissue was homogenized on ice with 10 ml of pure methanol (99%) for 3 minutes. Next, the homogenate was placed in a sonication bath for 2 hours and then was left overnight at 0 °C. The resulting extract was centrifuged at 16,000×g and then filtered through Whatman filter paper No. 42, concentrated by evaporation under dark conditions. Subsequently, it was filtered through 0.22-mM BioFil filters, and 20 mL of the filtrate was analyzed by high-performance liquid chromatography (HPLC) (Knauer, Germany). The system was equipped with an HPLC pump K1001 and a C18 column (5 mm, 250 × 4.6 mm). Phytohormones were eluted at a flow rate of 0.6 mL min¡1 with a concave gradient of methanol acidic water (deionized water containing 0.67% acetic acid, pH 3.0). The gradient was as follows: starting from

80:20 is shifted to 70:30 within 6 minutes, and then further to 60:40 after 15 minutes. Gibberellic acid (GA3) and abscisic acid (ABA) were detected at 220 nm using an ultraviolet detector (PDA, Berlin, Germany) and were quantified by comparison of their retention times and peak area with genuine standards (Sigma) (Razifah et al., 2014; Delavar et al., 2017).

Statistical analysis

The experiment was organized in a two-way ANOVA (4 × 4) in which factor A consisted of four levels of diesel-contaminated soil (0, 2, 4, 6% diesel concentrations), and factor B consisted of sodium alginate concentrations (0, 1, 2, 3%). Experimental treatments' effects were studied in a completely randomized design with ten replications. Experimental variables included some indices of physiological growth such as germination percentage, shoot length, root length, fresh weight of shoots and roots, hormones such as gibberellic acid and abscisic acid, enzymes such as catalase, superoxide dismutase, as well as malondialdehyde activity. Experimental data were first normalized by the Kolmogorov-Smirnov test; the data were then analyzed statistically by using SPSS software version 20. Comparisons of treatment averages were performed with Duncan's test at 99% and 95% probability levels, and also the data were plotted using Excel.

Results

The seed vigor response

The results of the analysis of variance (Table 4) showed that the use of calcium alginate in the production of seed coating could be effective in preventing the negative effects of diesel compound stress on seed vigor percentage and increasing germination percentage in coated seeds. The results showed that firstly, the coating factor had no inhibitory effect on seed vigor compared to control treatment. Secondly, under contaminated soil with diesel, the germination percentage of coated seeds increased compared to the control (uncoated seeds). Among the concentrations (calcium alginate) used in this study, the highest germination percentage was obtained at concentrations of 2 and 3% calcium alginate (Figure 2-A).

The vegetative components of shoot responses

The vegetative components of the shoots, including shoot length, root length, and fresh weight of shoots, were affected by the seed coating factor (Table 4). The vegetative

Table 4. Mean squares of CAT, APX, GPX, SOD, MDA; phytohormones (GA3 and ABA); seed vigor (S.V.), and components of vegetative shoot growth under experimental treatments

Treatments	df	Mean squares										
		CAT	APX	GPX	SOD	MDA	GA3	ABA	S.V.	Shoot Weight	Shoot length	Root length
Coating (C)	3	0.205**	0.639*	0.04308**	0.0801**	64098**	98844.5**	1359264**	2970.2**	8.8**	1633.6**	167**
Diesel (D)	3	0.075**	0.555*	0.05080**	0.03031*	5992.1*	55688.45**	976766.1**	1735.1**	10.5**	878.2**	354.3**
C×D	9	0.078**	0.501 n.s	0.04778**	0.0351**	5822.2*	27683**	805331.8**	1182.5**	6.1**	301.6**	92.5**
Error	64	0.003	0.2	0.00454	0.0075	2095.2	9886.77	130999.4	411.03	1.99	56.45	20.22

* and ** *F* test significance at $P < 0.05$ and $P < 0.01$, respectively; n.s. nonsignificant result

components of the shoot increased compared to the control treatment under diesel-contaminated soil. The highest growth of vegetative components of shoots was related to coated seeds 2 and 3% calcium alginate concentration was used in the production of seed coat (Figures 2b, c and d).

The enzymatic responses to diesel-contaminated soil

According to variance analysis (Table 3), the effect of seed coating could significantly prevent the increase of enzyme activity in the coated seeds (Figure 3a, b, c, and d). By covering the seeds with calcium alginate, the negative effects of contaminants in diesel-contaminated soils on seed growth and consequently shoot growth were prevented. It means that the activity of enzymes such as the CAT, the GPX, the APX, and the SOD did not increase compared to the control under the contamination levels. Also, based on the results, it seems that the coating layer added to the surface of the maize seeds probably prevents the penetration of pollutants into the seed, because the activity of enzymes in the coated seeds was not stimulated and this response is opposite to the reaction of the control treatment (seeds without coating), therefore the difference in enzyme activity in coated and uncoated seeds can be a reason for the possibility of no penetration of pollutants. Among the concentrations of

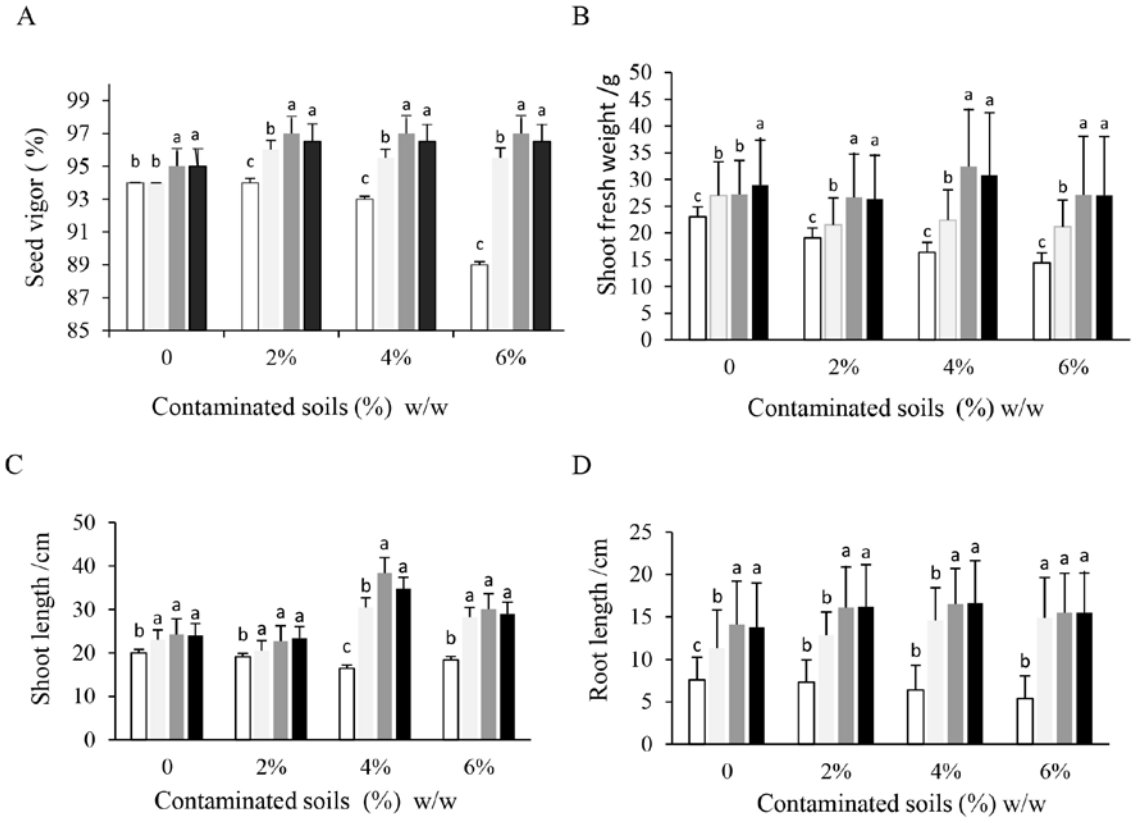


Fig. 2. Response of average seed vigor(A), shoot fresh weight (B), shoot length (C) and root length, and (D) by contamination levels and alginate calcium concentrations (white as uncoated seed (control), light gray as 1%, dark gray as 2% and black as 3% of alginate calcium). Lowercase letters denote a significant difference at $p < 0.01$. Error bars denote standard error

calcium alginate used for the production of the coating layer, 2 and 3% calcium alginate concentrations had the greatest effect in preventing the increase in the activity of antioxidant enzymes.

The malondialdehyde response to diesel-contaminated soil

At each level of gasoline pollution in this experiment, the production of malondialdehyde (MDA) was significantly increased only in the control treatment compared to other treatments (Table 4), and also the results showed that the effect of using calcium alginate as a layer on maize seeds could reduce MDA levels in coated seeds. In other words, by covering the seeds, lipid peroxidation can be reduced under diesel contamination (Figure 4a).

The phytohormone's responses to diesel-contaminated soil

Analysis of variance (ANOVA: Table 4) was used to determine the influence of seed coating on the produced phytohormones in germinated maize seeds under diesel-contaminated soil. The production of GA3 in the coated seeds increased compared to the control treatment in the diesel-contaminated soils (Figure 4b). Also, the use of 2% and 3% calcium alginate in the preparation of a coating on maize seeds, increased the production of GA3 in seeds compared to other treatments. Also, the results showed that the amount of produced ABA in the coated seeds was less than the control

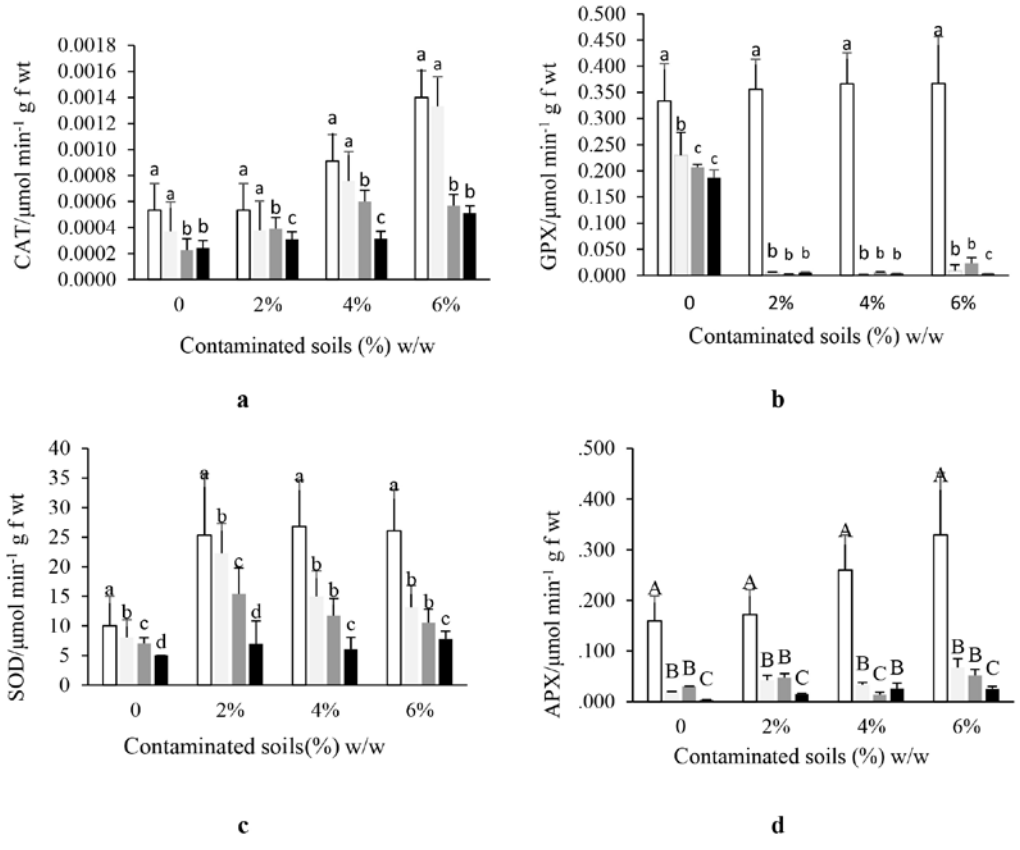


Fig. 3. Response of average CAT (a), GPX (b), SOD (c), and APX (d) by contamination levels and alginate calcium concentrations (white as uncoated seed (control), light gray as 1%, dark gray as 2% and black as 3% of alginate calcium). Capital letters denote a significant difference at $p < 0.05$, and lowercase letters denote a significant difference at $p < 0.01$. Error bars denote standard error

treatment (Figure 4c).

Discussion

Based on the results of the present study, the seed coating technique could create many benefits for the development of plant cultivation in contaminated soils with diesel. However, the results of previous research have mentioned the benefits of coated seeds,

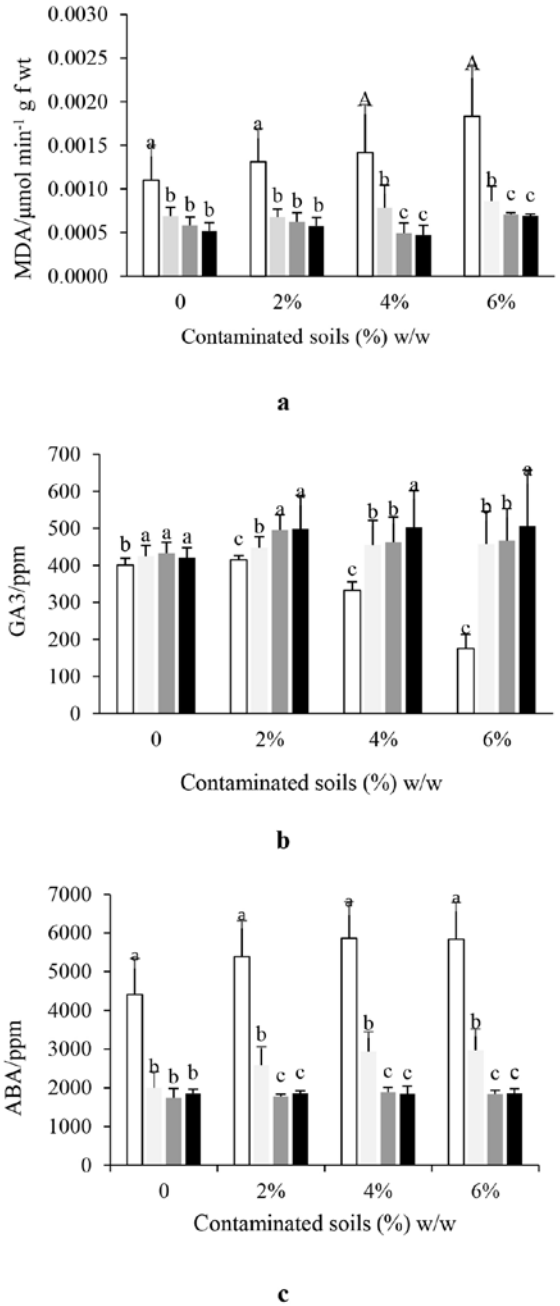


Fig. 4. Response of average MDA (a), GA3 (b) and ABA (c) by contamination levels and alginate calcium concentrations (white as uncoated seeds (control), light gray as 1%, dark gray as 2% and black as 3% of alginate calcium). Capital letters denote a significant difference at $p < 0.05$, and lowercase letters denote a significant difference at $p < 0.01$. Error bars denote standard error.

and these benefits are mainly to create uniformity of seed dimensions for ease of use in seeders' machines, and as fungicides, soil pesticides, and nutrients, all of which increase germination percentage and seed vigor, especially for small seeds Ehsanfar and Modarres-Sanavy (2005).

However, the point to consider in this study was the use of seed-coating techniques to counteract the inhibitory effects of diesel compounds on seed growth in contaminated soils. In addition, one of the most important reasons for choosing calcium alginate compound to use as a coating on the maize seeds surface was, first, the ability to store and absorb water for seed use in the germination process, to avoid contact with pollutants on the surface of maize seeds, and third, firming up the oxygen needed to continue metabolic and hormonal activities (He et al., 2018; de Castro et al., 2020). Because the molecular structure of calcium alginate can absorb water, it can play a positive role in solving the lack of water and oxygen needed for germination and metabolic processes of seeds under the stress of diesel pollution, and as a result, the coated seeds will have a better growth and germination process (Khan et al., 2011). Therefore, two important roles can be considered for the coating layer of calcium alginate: firstly, it prevents the penetration of pollutants into the seed, secondly, it delays the entry of pollutants, and these roles depend on the molecular structure of the pollutants.

The entry of diesel compounds into the soil and then into the seeds and/or roots of plants causes a series of problems in an environment of plant growth. For example, the penetration of diesel compounds into the soil, via sticking and covering the surface of soil particles and penetrating the soil pores, causes dehydration and decreases the flow of water and oxygen in the seed bed and root growth environment. In other words, a decrease in soil aeration, a decrease in the percentage of soil pores, an increase in the apparent density of the soil, a decrease in the flow of oxygen in the rhizosphere, the process of seed germination and seed vigor disturbed. In addition, when the diesel pollutant comes into contact with the surface of the seeds, the possibility of absorption and penetration of the pollutant inside the seed increases, which causes toxicity in the seeds and aerial organs (Han et al., 2016).

However, there are several ways to overcome the problems caused by petroleum contaminants in soils: first, cleaning and removal of contaminants in the environment through chemical and biological methods, second, the use of resistant cultivars to stresses caused by oil pollutants, and third, the use of seed coating technique for planting in contaminated soils. The results obtained from this study could confirm the third strategy that the use of calcium alginate, reduced part of the negative effects of diesel contaminants in the soil on the seed vigor process and shoot growth of maize. Therefore, according to the obtained results, coating maize seeds and then planting them in diesel-contaminated soils was an effective action to prevent the penetration of contaminants into the seeds and shoots. As the results showed, the reduction of seed germination in the control treatment compared to the coated seeds indicated

the ability of the calcium alginate layer to protect the seeds and reduce the inhibitory effects of contaminated soil on the growth and germination of maize seeds.

Among other noteworthy points based on the results of this research was the difference in the effect of calcium alginate used in making the coating layer of the seeds. In fact, the use of optimal concentration is an important factor in the emergence of the ability to protect corn seeds against factors that prevent germination and plant growth in soils contaminated with diesel compounds. Furthermore, an effective concentration of calcium alginate in the construction of the coating layer can have a positive effect on the ability of the coating layer against factors that inhibit seed germination and vigor. In fact, the optimal concentration means creating a sufficient thickness around the seed so that the amount of water retention meets the water requirement of the seed in the condition of soil pollution and also prevents the possible penetration of diesel pollutants by preventing the direct contact of pollutants with the surface of the seed. Therefore, the results obtained in this study showed that almost the most positive results were obtained using concentrations of 2 and 3%, and these coating layers did not allow for an increase in the activity of antioxidant enzymes as well as the production of ABA hormone. On the contrary, the use of these coatings (2 and 3% of alginate calcium) could increase the production of GA3 as an effective factor in seed growth and seed vigor under diesel-contaminated soil.

In conclusion, the use of calcium alginate for coating maize seeds was able to improve the germination percentage and vigor potential under diesel contamination soils. Thus, the approach of coating seeds can be used for other pollutants in contaminated soils. Of course, before using this approach there needs to survey of interaction responses between the type of pollutant and soil, and also properties of morphological plant seed and desired coating materials.

Acknowledgment

This research was carried out in a protected greenhouse and the analysis biomaterials center of the plant sciences department, at Shahid Beheshti University, is sincerely acknowledged. Dr. Khosrow Delavari is also appreciated for his guidance and hormonal analysis.

References

Afzal I, Javed T, Amirkhani M, Taylor A G. (2020). Modern seed technology: Seed coating delivery systems for enhancing seed and crop performance. Agriculture. 10 (11): 526. Doi: https://doi.org/10.3390/agriculture10110526.

de Castro VA, Duarte VGO, Nobre DAC, Silva GH, Constantino VRL, Pinto FG, Macedo WR, Tronto J. (2020). Plant growth regulation by seed coating with films of alginate and auxin-intercalated layered double hydroxides. Beilstein journal of nanotechnology. 11 (1): 1082-1091. Doi: https://doi.org/10.3762/bjnano.11.93.

Delavar K., Ghanati F, Zare-Maivan H, Behmanesh M. (2017). Effects of silicon on the growth of maize seedlings under normal, aluminum, and salinity stress

conditions. Journal of Plant Nutrition 40 (10): 1475-1484. Doi: https://doi.org/10.1080/01904167.2016.1269344

Dib D, Djamila. Sadoudi A A. (2020). Influence of diesel fuel contamination on *Xanthium strumarium* L. germination and growth. International Journal of Phytoremediation. 22 (3): 236-240. Doi: https://doi.org/10.1080/15226514.2019.1658705.

Du S T, Liu Y, Zhang P, Liu HJ, Zhang XQ, Zhang RR. (2015). Atmospheric application of trace amounts of nitric oxide enhances tolerance to salt stress and improves nutritional quality in spinach (*Spinacia oleracea* L.). Food chemistry 173: 905-911. Doi: https://doi.org/10.1016/j.foodchem.2014.10.115.

Ehsanfar S, Modarres-Sanavy SAM. (2005). Crop protection by seed coating. Communications in Agricultural and Applied Biological Sciences. 70 (3): 225-229. PMID: 16637182.

Ghalamboran MR, Hosseni Kordkheli SS, Bernard F. (2020). Enzymatic response and metal ion content in roots of corn and broad beans planted in soil contaminated with gasoline. International Journal of Environmental Science and Technology. 17 (2): 973-982. Doi: https://doi.org/10.1007/s13762-019-02406-3.

Gorim LY. (2014). Effects of seed coating on germination and early seedling growth in cereals. Dissertation of Doctoral (PhD) in Agricultural Sciences, University of Hohenheim. Doi: http://opus.uni-hohenheim.de/volltexte/2014/998/.

Han G, Cui BX, Zhan XX, Li KR. (2016). The effects of petroleum-contaminated soil on photosynthesis of *Amorpha fruticosa* seedlings. International Journal of Environmental Science and Technology. 13 (10): 2383-2392. Doi: https://doi.org/10.1007/s13762-016-1071-7.

He J, Li R, Sun X, Wang W, Hu J, Xie H, Yin H. (2018). Effects of calcium alginate sub microparticles on seed germination and seedling growth of wheat (*Triticum aestivum* L.). Polymers. 10 (10): 1154. Doi: https://doi.org/10.3390/polym10101154.

Hu X, Jiang X, Hwang H, Liu S, Guan H. (2004). Promotive effects of alginate-derived oligosaccharide on maize seed germination. Journal of Applied Phycology. 16 (1): 73-76. Doi: https://doi.org/10.1023/B:JAPH.0000019139.35046.0c.

Khan N, Mishra A, Chauhan CS, Nautiyal CS. (2011). Induction of Paenibacillus lentimorbus biofilm by sodium alginate and CaCl2 alleviates drought stress in chickpea. Annals of applied biology. 159 (3): 372-386. Doi: https://doi.org/10.1111/j.1744-7348.2011.00502.x.

Kikowska M and Thiem B. (2011). Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review. Herba Polonica. 57 (4). Doi: https://www.researchgate.net/publication/234168032.

Kimmelshue C, Goggi A S, Cadematiri R. (2019). The use of biological seed coatings based on bacteriophages and polymers against *Clavibacter michiganensis* subsp. *nebraskensis* in maize seeds. Scientific Reports. 9 (1): 1-11. Doi: https://doi.org/10.1038/s41598-019-54068-3.

Koshlaf E, Ball A S. (2017). Soil bioremediation approaches for petroleum hydrocarbon polluted environments. AIMS Microbiology. 3 (1): 25. Doi: https://doi.org/10.3934/microbiol.2017.1.25.

Koushesh saba M, Arzani K, Barzegar M. (2012). Postharvest polyamine application alleviates chilling injury and affects apricot storage ability. Journal of agricultural and food chemistry. 60 (36): 8947-8953. Doi: https://doi.org/10.1021/jf302088e.

Laing W, Christeller J. (2004). Extraction of proteins from plant tissues. Current protocols in protein science. 38 (1): 4.7. 1-4.7. 7. Doi: https://doi.org/10.1002/0471140864.ps0407s38

Pedrini S, Merritt D J, Stevens J, Dixon K. (2017). Seed coating: science or marketing spin? Trends in plant science 22(2): 106-116. Doi: https://doi.org/10.1016/j.tplants.2016.11.002

Razifah M, Mamat A S, Nor Shariah S. (2014). Effect of seed cluster and gibberelic acid (GA3) on the gender development of *Salacca edulis* REINW. (snake fruit) grown on BRIS soil. Journal of Biology, Agriculture and Healthcare. 4 (9): 15-20. Doi: http://www.iiste.org/Journals/index.php/JBAH/article/download/12645/12945/.

Rocha I D S, Ma Y, Souza-Alonso P, Vosatka M, Freitas H, Oliveira R S. (2019). Seed coating: A tool for delivering beneficial microbes to agricultural crops. Frontiers in Plant Science. 10: 1357. Doi: https://doi.org/10.3389/fpls.2019.01357.

Ruley AT, Sharma NC, Sahi SV. (2004). Antioxidant defense in a lead accumulating plant, Sesbania drummondii. Plant Physiology and Biochemistry. 42 (11): 899-906. Doi: https://doi.org 10.1016/j.plaphy.2004.12.001.

Shi S, Wang G, Wang Y, Zhang L, Zhang L. (2005). Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. Nitric Oxide. 13 (1): 1-9. Doi: https://doi.org/10.1016/j.niox.2005.04.006.

Song Y, Huang Y, Wang X, Shang X, Yang H. (2014). Preparation and control efficiency of seed coating agent by antagonistic actinomycetes against clubroot. Journal of Agricultural Science. 6 (3): 132. doi: https://pdfs.semanticscholar.org/d4ec/b1dd1287eeccd7d57b44d9d0f24ec0b1cbae.pdf.

Su LQ, Li JG, Wang XF. (2017). Super absorbent polymer seed coatings promote seed germination and seedling growth of *Caragana korshinskii* in drought. Journal of Zhejiang University-Science. B. 18 (8): 696-706. Doi: https://doi.org/10.1631/jzus.B1600350.

Yakovleva EV, Gabov DN, Beznosikov VA, Kondratenok BM, Dubrovskiy YA. (2017). Accumulation of PAHs in tundra plants and soils under the influence of coal mining. Polycyclic Aromatic Compounds. 37 (2-3): 203-218. Doi: https://doi.org/10.1080/10406638.2016.1244089.

Elicitor-Induced Defense Responses in Tomato Plants Against *Xanthomonas gardneri*

Reza Maleki-nejad [1], Hamid Najafi-Zarrini[1], Ghoalamali Ranjbar[1]*, Ali Dehestani[2], Heshmatollah Rahimian[3]

Received: 2024-01-18 Accepted: 2024-03-13

Abstract

Resistance inducers have been focused on promising environment-friendly options to chemical pesticides. In the present study, eleven potential resistance inducers were investigated to evaluate their efficacy in mitigating the adverse impacts of bacterial spot disease in tomato plants. Results revealed that catalase activity and hydrogen peroxide content were sustantially different in inoculated and non-inoculated plants irrespective of the resistance inducer used. The mean concentration of H_2O_2 in the inoculated plants was also increased by 25% compared to the control group. The mean catalase enzyme activity in plants treated by resistance inducers was 0.054 U/mL, while it was 0.111 U/mL in plants solely inoculated with bacteria. The highest SOD activity was recorded in potassium phosphite-treated plants inoculated with the pathogen. Mean catalase activity in inducer-treated plants was 0.052 U/ml, while it was 0.111 U/ml in these plants when inoculated with the pathogen. Malondialdehyde, as a reliable indicator of plant damage upon pathogen attack, exhibited the lowest content in succinic acid-treated plants 8 days after inoculation. This reduction was directly correlated with decreased bacterial spots on the leaves of plants treated with succinic acid. Our results show that potassium phosphite and succinic acid-induced effective defense responses in tomato plants against *X. gardneri* leading to reduced disease severity effect. According to the results, potassium phosphite and succinic acid may be used as potential resistance inducers in tomato plants against *X. gardneri.*

Keywords: Bacterial spot, Catalase, Malondialdehyde, Resistance inducer

Introduction

The growth and development of plants can adversely be affected by several biotic and abiotic stressors (Nawaz et al., 2023). Conversely, as sessile organisms, plants have evolved several strategies to overcome harsh environmental conditions and cope with the different challenges they may face. This adaptation and successful response to these stresses are important for plants to survive and reproduce in an ever-changing climate (Janse van Rensburg et al., 2020)

1- Department of Plant Breeding, Sari Agricultural Sciences and Natural Resources University, Sari, Iran
2- Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University, Sari, Iran
3- Department of Plant Protection, Sari Agricultural Sciences and Natural Resources University, Sari, Iran
* Corresponding author's email address: ali.ranjbar@sanru.ac.ir
Doi: 10.48308/jpr.2024.235152.1071

Several *Xanthomonas* species are considered the causal agent of bacterial spots in tomatoes. The disease affects all foliar parts of tomato plants, including leaves, stems, and fruits. Several strategies are used to control this disease including exploitation of resistant cultivars, agricultural practices, and bactericide application (Ritchie, 2000). Synthetic bactericides are widely used throughout the world, although their application has been limited owing to resistance development in bacterial populations, as well as, environmental and health concerns (Soto-Caro et al., 2023).

Plants possess multidisciplinary permanent and induced defense systems to deal with stresses. Permanent defense systems include various prefabricated barriers such as polysaccharide-enforced cell walls, as well as, multiple epidermal layers including cuticles and wax. These defensive structures protect the plants from both biotic and abiotic stresses by creating isolating internal tissues (Ullah et al., 2018). Pathogens also have several approaches to defeat plant defense passes through plants' physical barriers and penetrate the internal cells. Pathogen penetration, in turn, activates plant immune responses to inhibit its expansion and further disease proliferation (Hu et al., 2018).

Induced resistance is a plant defense mechanism that triggers physiological, biochemical, molecular, and metabolic changes upon pathogen attack. This particular defense mechanism is highly effective as it is activated solely when required, guaranteeing the most effective allocation of resources. (Jain et al., 2016). Upon recognition of the external stimuli, a multifaceted signaling cascade is triggered and subsequently, several defense responses are activated. Several external stimuli can trigger defense responses including the molecules of pathogen cell walls, degrading plant cell wall metabolites, and other physical and chemical factors (dos Santos and Franco, 2023). The recognition of inducers is usually accompanied by the rapid and transient development of reactive oxygen species (ROS) in apoplast. These ROS act as both antimicrobial agents and signaling molecules and are crucial for downstream signaling pathways against pest and pathogen attacks. These pathways serve as alarms for plant metabolic responses leading to plant protection and damage limitation (Sahu et al., 2022).

ROS are primarily produced in chloroplasts, mitochondria, and peroxisomes. However, under stress conditions, they can also be generated in other plant cell parts such as the cell membrane, cell wall, endoplasmic reticulum, and apoplast (Hasanuzzaman et al., 2021). Stress-induced signaling pathways will be resulted in the over-production of ROS, which subsequently harm plant cells by disrupting the redox equilibrium. This disruption results in lipid peroxidation and deteriorationgradation of chlorophyll, nucleic acids, and proteins (Hasanuzzaman et al., 2021; Schieber and Chandel, 2014) The harmful impacts of excessive ROS are mitigated by plant antioxidant mechanisms (Mittler, 2002).

High concentration accumulation of ROS in plant tissues is known as oxidative burst (Heller and Tudzynski, 2011). According to Zorbrigen et al. (2010)the oxidative burst in plants may induce a hypersensitive response

(HR), which prevents the spread of pathogens to adjacent tissues. HR can also serve as a crucial signal to activate various pathways that regulate plant defense responses and the synthesis of plant hormones (Beers and McDowell, 2001).

Plants typically enhance their antioxidant capacity in response to stress, enabling them to restore the balance between oxidation and reduction (Sahu et al., 2022). ROS also functions as a signaling molecule that activates multiple pathways to suppress pathogens and induce biochemical and physiological changes. This activation helps in overcoming stress (Atkinson and Urwin, 2012; Lamers et al., 2020).

Reactivated oxygen species (ROS) are believed to have a dual role in plants. They are required for several important signaling reactions. In plants, ROS is found to regulate growth, differentiation, redox surfaces, stress signaling, interplay with other organisms, systemic responses, cellular death, as well as toxic by -by-products of aerobic metabolism. This is because they can cause damage to DNA, lipids and proteins (Mittler, 2017).

Plants have numerous antioxidant systems that protect them from potential cytotoxic effects. Among these systems, antioxidant enzymes play a vital role in inhibiting reactive oxygen species (ROS) The main non-enzymatic antioxidants include ascorbic acid (ASA), glutathione (GSH), phenolic compounds such as flavonoids, alkaloids, non-protein amino acids, and α-tocopherol. On the other hand, the enzymatic antioxidants include peroxidase ascorbate (APX), superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and peroxidase (POX) (Berwal and Ram, 2018). Among the enzymatic systems, superoxide dismutase (SOD) is considered the first line of defense against the oxidative damage caused by ROS in almost all living cells. Catalase (CAT) also plays a crucial role in various plant physiological reactions throughout both the vegetative and reproductive stages (Zheng Yang et al., 2019; Yu Zhang et al., 2020). The successive induction of these pathways ultimalely leads to expression alteration of defense-related genes, development of phytoalexins, and callose deposition (Forman et al., 2010).

It has been revealed that similar to external stimuli, certain natural and synthetic chemicals, frequently referred to as resistance inducers, can activate the plant's immune system (Rabiei et al., 2022). Resistance inducers enhance the general resistance of plants to biotic and abiotic stresses by activating various defense mechanisms enabling plants to respond more effectively to subsequent and/or concurrent biological stresses.

During the past decades several compounds such as potassium phosphite, chitosan, seaweed extract, and salicylic acid have been commercially used as resistance inducers in modern agriculture, and the research is focused on the identification of new compounds with inducing impact on plant defense systems (Reglinski et al., 2023). Potassium phosphite is known to induce defense responses against potato late blight, passion fruit scab, coffee rust, and downy mildew of cucumber (Bonfim et al., 2023; de Fatima Pereira Silva et al., 2023; Liljeroth et al.,

2016; Ramezani et al., 2017). It has been used as fertilizer and fungicide worldwide for several decades.

Azelaic acid, a dicarboxylic acid that naturally occurs in many organisms, has been observed to confer resistance against *Pseudomonas syringae* in Arabidopsis plants when applied to the roots, leading to systemic immunity in aerial tissues (Cecchini et al., 2019).

The current study investigates the efficiency of some potential plant resistance inducers in terms of inhibiting bacterial spot development in tomato plants owing to X. gardneri infection. Different physio-biochemical responses of pathogen-infected tomato plants, as well as, controls were assessed. The results of this study probably aid in understanding the exact defense response of tomato plants treated with each resistance inducer which paves the way for their practical applications for bacterial spot disease control.

Material and methods

Plant growth and treatment

The seeds of the tomato cultivar called CH, were prepared from the company of Falat (Falat Co., Iran). These seeds were sterilized using 1 % sodium hypochlorite solution, followed by rinsing with distilled water. The pots used in the experiment were 21 cm in diameter and contained a combination of peat moss, cocopeat, and perlite in a ratio of 1:2:1. The plants were grown in a greenhouse with controlled conditions with a photoperiod of 12 hours (light/dark), 70% humidity, temperature range of 24-27°C (day/night), and fertilized weekly using the half-strength Hoagland solution (Hoagland and Arnon, 1950). The leaves were sprayed with different resistance inducers (Table 1) when reached to true-4 leave stage.

Pathogen culture and plant inoculation

The bacterium *Xanthomonas gardneri* (IBSF 1782 from the Culture Collection of the Instituto Biológico in Campinas, Bra-

Table 1. List of the potential resistance inductors used to induce resistance against *Xanthomonas gardneri* in tomato plants

Inducer	abbreviation
Potassium phosphite	Kphi
Succinic acid	Suc
Sodium sulfite	NS
Methanol	Met
Beta-aminobutyric acid	BABA
Potassium phosphite and Azelaic acid	Kphi + Az
Potassium phosphite and Azelaic acid and chitosan	Kphi + Az + chit
Potassium phosphite, Azelaic acid, and Methanol	Kphi + Az + Met
Potassium phosphite, Azelaic acid and Salicylic acid	Kphi + Az + SA
Potassium phosphite, Azelaic acid and Sodium sulfite	Kphi + Az + NS
Potassium phosphite and Beta-aminobutyric acid	Kphi + BABA
Control	CTR

zil) was prepared at the Department of Plant Protection, Sari Agriculture and Natural Resources University. The bacteria were grown on NAS medium at a temperature of 27 °C, and then it was suspended in sterile distilled water. The suspension concentration was adjusted to an optical density of 0.05 at 600 ηm, which is approximately 7×106 colony-forming units per milliliter (CFU/mL). Two days after treating tomato plants with inducers they were inoculated with bacterial suspension and the non-inoculated plants were utilized as controls. The plants were kept in the greenhouse environment, under the the same conditions as described previously. By collecting the samples at 2, 4, and 8 days after inoculation and induction, the experiment aimed to analyze the changes in various parameters over time and gain insights into the plant's response to the treatments and pathogen infection.

PAL activity assay

PAL enzyme activity was measured by determinig the quantity of cinnamic acid produced during the reaction between enzyme extract and phenylalanine used to (Barnaby et al., 2008). In each reaction, 0.1 mL of enzyme extract, 500 µL of 500 mM Tris-HCl (pH 7), and 60 µL of 10 mM phenylalanine were combined and incubated at 37°C for one hour. To stop the reaction, 250 microliters of 4 N HCl were used, followed by the addition of one milliliter of toluene to the mixture. After vortexing and a short centrifugation (1000 g for 2 minutes), the absorbance of the upper layer was measured at 290 ηm. The enzyme activity was estimated using a cinnamic acid standard (0 to 15 µg/ml) with an R2 value of 0.987.

Catalase activity assay

A mixture of 25 µL of enzyme extract and a 3 mL of a phosphate buffer (50 mM) containing H_2O_2 (5 mM) was prepared. A spectrophotometer (Aebi, 1984) was used to measure the changes in absorbance at wavelength of 240 ηm..

SOD activity assay

To coduct the SOD activity assay, a mixture of 1000 µL of 50 mM phosphate buffer, containing 1.5 mM EDTA, 10 mM methionine, and 75 µM nitrotetrazolium chloride were prepared. Additionalyy, 100 µL of 1 µM riboflavin and 100 µL of enzyme extract were added to the mixture. The test tubes were then incubated for 10 minutes under a15-watt fluorescent lampplaced 35 cm above them. After turning off the lamp, the absorption changes of the reaction mixture were measured using a spectrophotometer apparatus at wavelength of 560 ηm (Beyer and Fridovich, 1987).

MDA content analysis

An analysis of MDA content was conducted to assess the peroxidation of membrane lipids. The concentration of malondialdehyde, a byproduct of unsaturated fatty acid peroxidation, was determined using the metod outlined by Ohkawa et al. (1979) . Frozen plant tissue (leaf) weighting 0.2 g was homogenized with 5 mL of 0.1% trichloroacetic acid (TCA)and then centrifuged at 10,000 g for 5 minutes. Following this, a mixture of 4 mL of a 20% TCA solution with 0.5% thiobarbituric acid (TBA) was added on 1 mL of the supernatant solution obtained from centrifugation. The resulting solution was heated at 95°C for 30 minutes, cooled on ice and centrifuged again at 10,000 g for 10

minutes. The absorbance of the solution was measured at 532 ηm using a spectrophotometer. with the absorbance of non-specific pigments at 600 ηm subtracted. The. concentration of MDA was calculated using an extinction coefficient of 155 mM-1 cm-1, and the results were expressed in nanomoles per gram of weight.

H_2O_2 accumulation assay

The hydrogen peroxide accumulation was measured using the H_2O_2 accumulation assay as described by Velikova et al. (2000). Plant leaves were gently rubbed in an ice bath containing 0.1% trichloroacetic acid, and the resulting extract was then centrifuged at 10,000 g for 15 minutes using a refrigerated centrifuge. Next, 0.5 mL of the supernatant solution was added to 0.5 mL of a 10 mM potassium phosphate buffer with a pH of 7, along with 1 mL of a 1 M potassium iodide solution. The absorbance was measured at a wavelength of 390 ηm. The amount of hydrogen peroxide in each sample was calculated using a standard curve and reported as micromoles per gram of fresh weight.

Measurement of flavonoids

The total flavonoids content was analyzed using the method described by Chang et al. (2002). To do this, 1 mL of a 2% aluminum chloride solution was added to 1 mL of a 2% methanolic extract and left at room temperature for one hour. Afterward, the absorbance was measured at 420 ηm. Quercetin was used as the standard, ranging from zero to 200 mg (Y = 0.00227X + 0.0175, R2 = 0.97).

Disease severity assay

Assessment of disease severity was conducted following the method of Slopek (1989) with light modifications. Twelve days after inoculating the tomato plants, the disease index (on a scale of 1 to 5) was recorded for each plant based on bacterial spots, and the mean value was calculated as the disease severity. At least 5 infected plants and at least two branches from each plant were selected and photographed using a scanner. The ImageJ software was used to estimate the percentage of symptoms observed on the leaf tissue, using a scale.

where;

1 = no symptoms or spots 2 = 1–25, 3 = 26–50, 4 = 51–75 and 5 = more than 75% disease spots, respectively.

Statistical analysis

All experiments, including plant culture, inoculation, sampling, and measurements were conducted as a split-plot experiment with three replications based on a completely randomized design. The least significant difference (LSD) test was performed at a 1% probability level ($P < 0.01$) for the analysis of mean comparison of growth characters. Data were organized by Excel software and the statistical calculations were performed using SAS 9.1 software.

Results

PAL activity measurement

The findings indicated that the control groups displayed the highest PAL activity on days 4 and 8 following inoculation (Table 2). Succinic acid, on the other hand, demonstrated the lowest PAL activity 2 days post-treatmentand also exhibited the lowest PAL activity inplants infected with pathogens 4 days after inoculation. In contrast, the control plants showed the lowest PAL activ-

ity 2 days after pathogen inoculation, while displayed the highest PAL activity on days following inoculation (Figure 1).

Flavonoids content

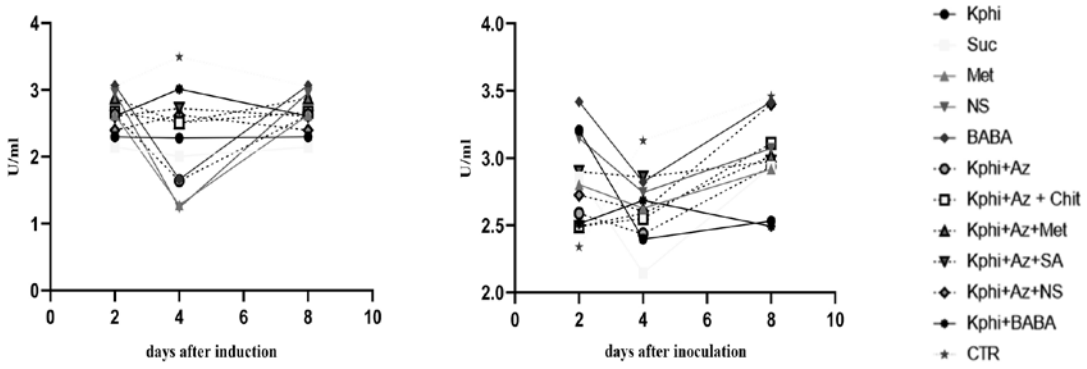
In both experiment conditions, the flavonoid contents observed 8 days post inoculation were higher compared to those observed 2 days after inoculation The average in flavonoid content was measured to be 62.33 mg/f Fw in plants inoculated with the pathogen (Figure 2). Interestingly, this average was found to be 13% higher than the average observed in plants treated solely with the resistance inducers (Table 3). Among the different treatments, the Succinic acid treatment showed the lowest flavonoid concentration after 8 days. However, when plants were incubated with bacteria, this treatment surprisigly showedthe highest flavonoids content. In plants inoculated with the pathogen, flavonoid concentration increased on the 4th days compared to the 2nd days after inoculation. Afterward, the flavonoid content remained relatively constant and did not show any significantly changes on the 8th day after inoculation.

H_2O_2 *accumulation*

The amount of H_2O_2 measured at different time points following bacterial inoculation in the presence of different inducers indicat-

Table 2. Alteration ranges and means of physio-biochemcial characteristics of traits in inducer-treated tomato palnts inoculated or non-inoculated with *Xanthomonas gardneri*

Traits	Average		Maximum		Minimum		Change in
	inoculation	inducer	inoculation	inducer	inoculation	inducer	percentage
PAL (U/ml)	2.76	2.45	4.367	3.275	2.513	2.078	11.25
CAT (U/ml)	0.111	0.052	0.299	0.178	0.009	0.008	53.51
Flavonoid (mg/g FW)	62.33	54.09	71.931	58.620	57.181	51.711	13.23
H_2O_2 (ηmol/g FW)	0.52	0.39	0.634	0.500	0.401	0.235	25.24
MDA (ηmol/g FW)	1.60	1.53	2.258	1.866	1.252	1.196	4.36
SOD (U/mg)	0.30	0.29	0.377	0.449	0.197	0.213	0.04



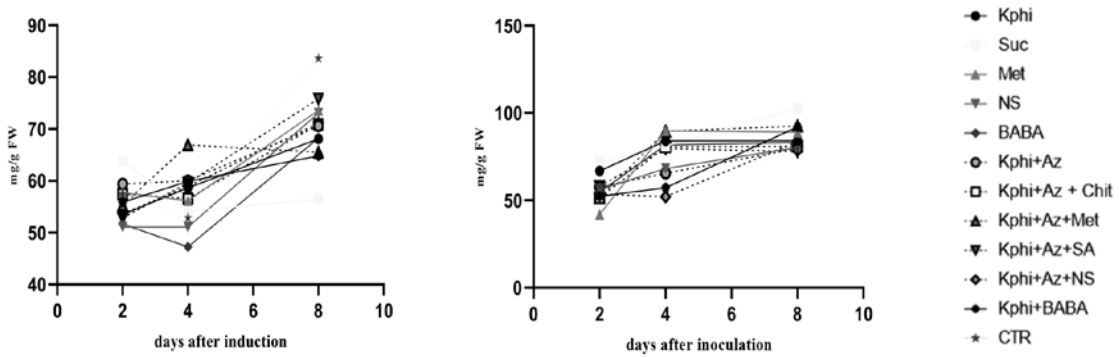
(**a**) (**b**)

Fig. 1. a) Alterations in PAL activity in tomato plants following the application of a resistance inducer in the absence of pathogen inoculation, b) Variations in PAL concentration in resistance inducer-treated tomato plants infected by *X. gardneri*

ed that succinic acid and BABA treatments exhibited a significant increase compared to other treatments after 2 days after(Figure 3). Moreover, the combination treatment of potassium phosphite+azelaic acid+salicylic acid resulted in the lowest level of H2O2. By the 4th day post-inoculation, after bacterial inoculation, the H_2O_2 levels decreased due to the inducers, only to rise again by the 8th day. The average H_2O_2 concentration in plants treated with inducers was 0.39 ηmol. In plants infected inoculated with the pathogen, there was a 25% increase in , H2O2 levelindicating a significant difference. H2O2 plays a crucial role ingene expression, cell death, and various physiological processes contribute to plant defense machanisms. Particulary in tomato plants, H_2O_2 is essential for combating a variety of disease-causing agents.

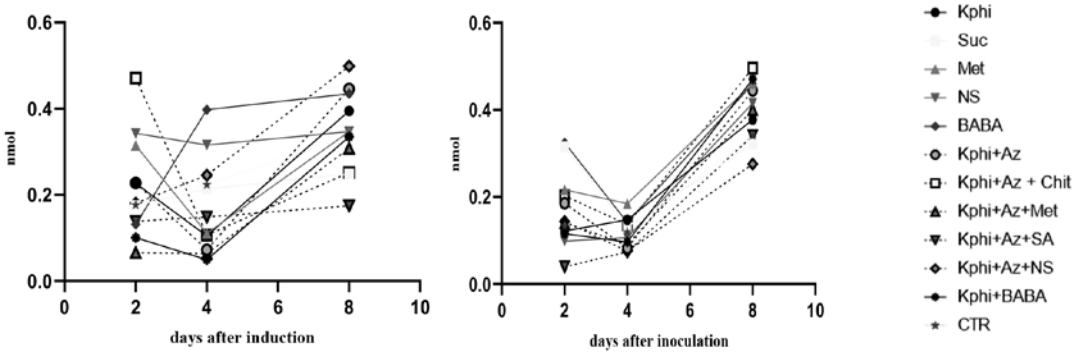
SOD activity

In this study, the SOD enzyme's activityshwed anincreaseover time (Figure 4). Notably, a significant difference in average activity was observed between the control group and treatment group after pathogeninoculation . Conversly, there is no significant difference between the treatments in the two



(a) (b)

Fig. 2. a). Changes in flavonoid contents in tomato plants treated by various inducers, b) Flavonoid contents alteration in resistance inducer-treated tomato plants infected by *X. gardneri*



(a) (b)

Fig. 3. a) Variations in H_2O_2 content in tomato plants treated by resistance inducers, b) Alterations in H_2O_2 concentration in tomato plants infected by *X. gardneri*

environments (Figure 5). This demonstrates the effect of the inducer on altering the of SOD activity levels, indicating that the inducer led to an increase in SOD levels. As a result, it can be inferred that pathogen inoculation does not influence the rise in SOD activity, suggesting that the increase in SOD levels was primarily due to the effect of inducers. However, there is a difference in the inoculated treatments at different sampling times. The lowest SOD activity level, observed 2 days post-inoculation in the treatment group receiving potassium phosphite+ azelaic acid+ salicylic acid, while hand, the highest SOD activity is linked to the potassium phosphite treatment.

MDA content

In current research, it has been observed that , the activity of the MDA enzyme has gradually increased under conditions of disease stress. This increase was evident in all cases except for t the succinic acid treatment, where the level of MDA showed gradually decreased. Furthermore, the increase in activity was more prominent at 8 days aftert inoculation compared to earlier days. The peak activity was observed at 8 days post inoculation for the combined treatment of potassium phosphite+azelaic acid+salicylic acid, as well as, for the potassium phosphite+

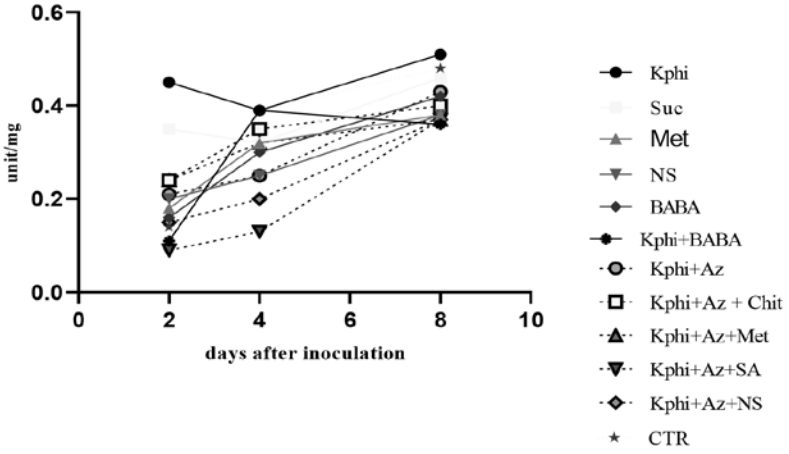


Fig. 4. Variations in SOD activity in tomato plants inoculated by *X.gardneri* bacteria

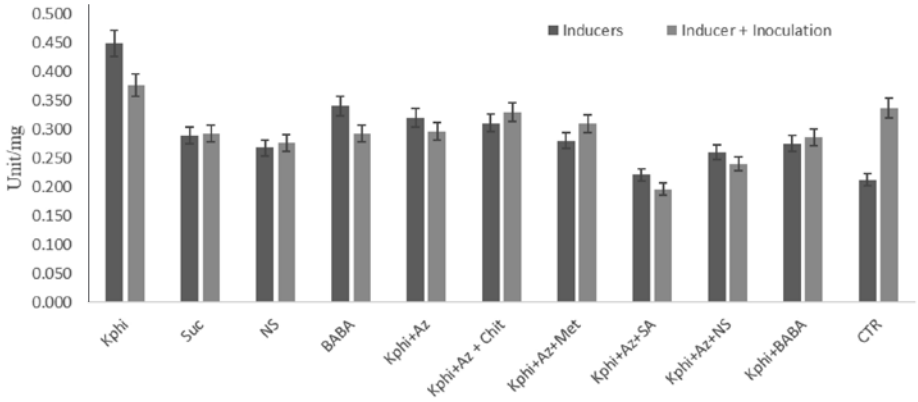


Fig. 5. Mean concentration of SOD enzyme activity in tomato plants treated with resistance inducers and inoculated by *X. gardneri*

azelaic acid+ sodium sulfite treatment (Figure 6). Conversly, the lowest activity was arecorded in the succinic acid treatment.

CAT activity

The mean catalase enzyme activity in plants exposed to resistance inducers was 0.054 U/mL, wherease it measured 0.111 U/mL in plants that were only inoculated with bacteria (Table 3). After Eight days of catalase activity decreased in certain inducers when compared to the control group. Conversly, some treatments showed an increase in catalase activity, with the most significant rise observed in plants treated by BABA. Additionally, across all inducers, catalase enzyme activity was lower in plants inoculated with bacteria than in the control treatment (Figure 7).

Disease severity

According to the results, the plants treated with control and BABA exhibited highest

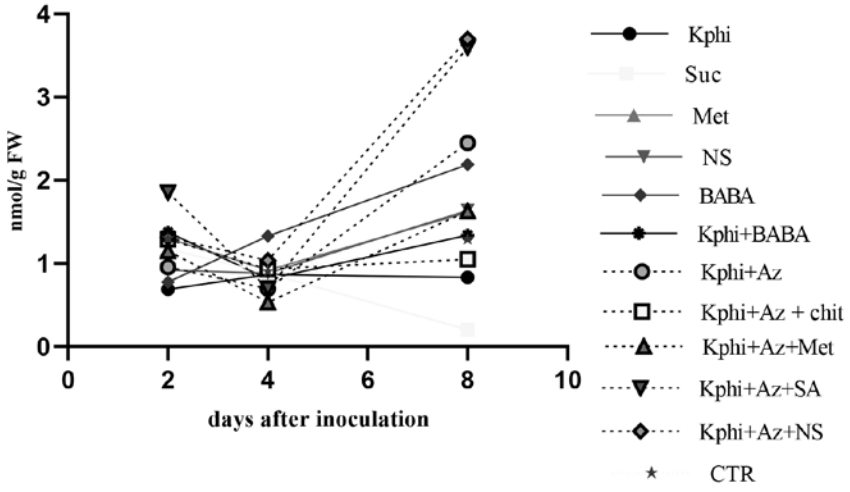
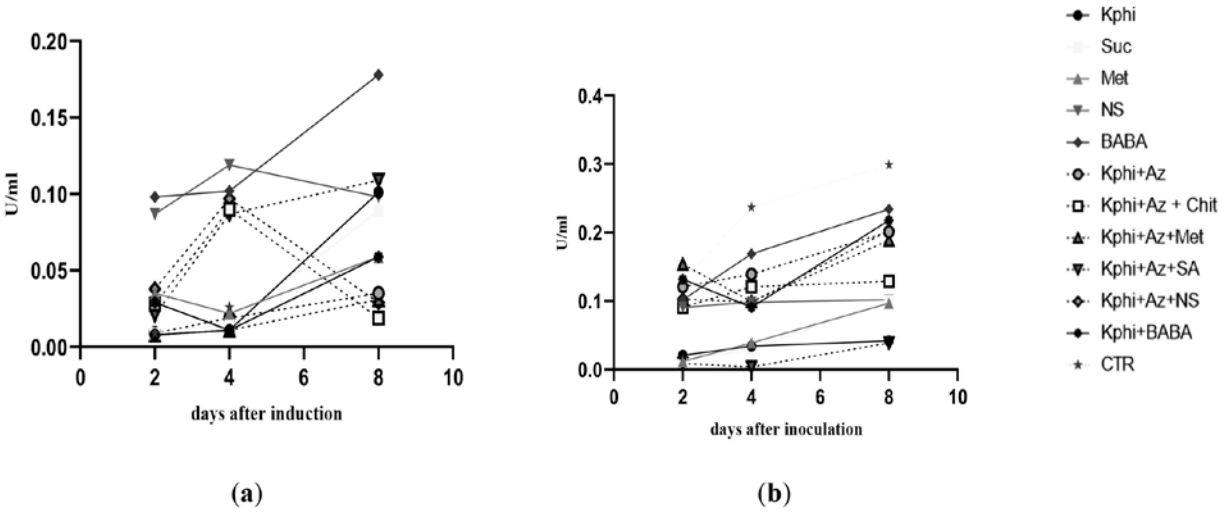


Fig. 6. MDA enzyme activity in resistance inducer-treated tomato plants inoculated by *X. gardneri*



(a) **(b)**

Fig. 7. a) Alterations in catalase activity in tomato plants treated by resistance inducers, b) Variations in catalase levels in tomato plants inoculated by *X. gardneri*

levels of necrosis on their leaves. Conversly, the plants that were pre-treated with succinic acid + potassium phosphite and then inoculated with bacteria, displayed the lowest number of bacterial sympton spots on their leaves (Figure 8).

Discussion

PAL is actively involved in the synthesis of defense compounds in tomato plants, which are vital for their ability to combat diseases. In a recent investigation conducted by (Debnath et al., 2019), the researchers examined the activity of the PAL enzyme in different tomato genotypes with varying levels of resistance and susceptibility to bacterial wilt. The findings of this study demonstrated that resistant tomato plants exhibited higher PAL activity compared to susceptible plants, indicating a positive correlation between PAL activity and tomato disease resistance. Moreover, a study conducted by Vanitha et al., (2009) revealed that the defense enzymes PAL and PPO play an active role in tomato's plants ability to resist bacterial wilt. In this experiment, Following

Table 3. Mean of physio-biochemcial characteristics inducer-treated tomato palnts inoculated or non-inoculated by *X. gardneri*

	Flavonoid		SOD		H_2O_2		MDA		Catalase		PAL	
Elicitors	Inoculation	inducer	Inoculation	inducer	Inoculation	inducer	Inoculation	inducer	Inoculation	inducer	Inoculation	inducer
Kphi	68.528	53.238	0.377	0.449	0.501	0.378	1.269	1.417	0.032	0.040	2.513	2.290
Suc	71.931	52.338	0.293	0.289	0.401	0.357	1.252	1.196	0.048	0.038	2.542	2.078
Met	73.615	62.512	0.293	0.327	0.288	0.26	1.27	0.93	0.049	0.039	2.78	2.18
NS	59.596	51.711	0.277	0.268	0.502	0.433	1.599	1.584	0.095	0.101	2.682	2.097
BABA	61.740	52.992	0.293	0.341	0.528	0.500	1.873	1.601	0.168	0.126	2.662	2.368
Kphi + Az	58.814	56.869	0.297	0.320	0.555	0.410	1.684	1.253	0.154	0.021	2.546	2.136
Kphi + Az + chit	62.981	54.559	0.330	0.311	0.561	0.427	1.585	1.659	0.114	0.046	2.730	2.590
Kphi + Az + Met	66.083	54.938	0.310	0.280	0.432	0.289	1.268	1.384	0.147	0.017	2.624	2.696
Kphi + Az + SA	61.325	56.685	0.197	0.221	0.413	0.235	2.258	1.316	0.017	0.072	2.879	2.667
Kphi+ Az + NS	57.181	54.207	0.240	0.261	0.419	0.457	2.111	1.615	0.135	0.055	2.759	2.513
Kphi + BABA	61.373	54.416	0.287	0.276	0.558	0.400	1.506	1.866	0.147	0.033	2.638	2.812
CTR	57.548	58.620	0.337	0.213	0.634	0.500	1.586	1.481	0.222	0.031	3.092	3.275
LSD 5%	1.488	1.562	0.932	0.851	0.018	0.021	0.245	0.175	0.908	0.569	0.617	0.186

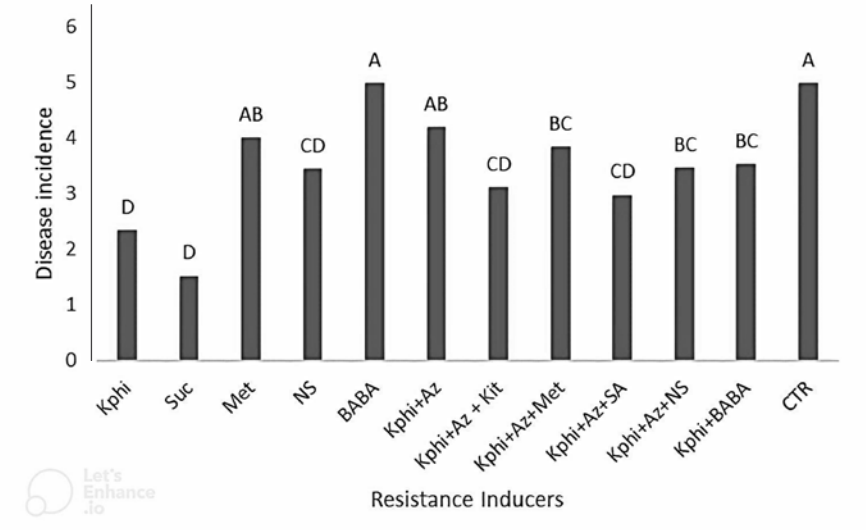


Fig. 8. The disease severity rate in tomato plants treated with a resistance inducer-and inoculated by *X. gardneri*. Leaf necrosis data were collected recorded 12 days after inoculated with *X. gardneri*. The columns represent the mean disease rating on a 1–5 scale method

inoculation, the potassium phosphite treatment showed a higher level of PALactivity compared to the control treatment after two days. This boost in PAL contributed to the plant's defense against the pathogen This enhanced PAL activity is crucial for bolstering the plant's resistance to pathogens. Potassium phosphite is recognized for its ability to promote plant health and vigor by increasing resistance against a variety of plant pathogens, such as oomycetes, soil-borne diseases like Phytophthora and Pythium species, as well aspathogenic bacteria, fungi, and nematodes. Studies have shown that treatments involving potassium phosphite can stimulate the plant immune system, leading to a significant decrease in disease incidence and improving plant resistance to pathogens.

Inducers have been shown to have a significant impact on PAL activity in tomato plants. Studies have demonstrated that these inducers can increase PAL activity, resulting in increased lignin deposition in cell walls, accumulation of phenolics, and enhanced activity of other enzymes, like peroxidase (POD), polyphenol oxidase, and cinnamyl alcohol dehydrogenase (Meena et al., 2022). These changes can contribute to enhancing resistance against pathogens and improve the overall health of the plant. For example, a study showed that water-activated plasma-inducing agents did not directly combat *Xanthomonas vesicatoria*, but bolstered the tomato plant's defenses, enhancing its disease resistance. (Perez et al., 2019). Similarly, the application of silicon in tomato plants has been foumd to elevate POD and PAL activities, along with SA contents, thereby improving disease resistance (Sun et al., 2023). in current study, it was observed that BABA and Kphi treatments increased t PAL levels in tomato plants, indicating a potential impact on the defense responses. The upregulation of PAL indicates that BABA and Kphi treatments could avtivate the plant's defense mechanisms, potentially leading to enhanced resistance against pathogens, pests, or other forms of stress.

Further research is necessary to comprehend the specific mechanisms by which BABA and Kphi treatments induce PAL expression and how this affects plant defense in the long rum. Nonetheless, these findings offer valuable insight into potential strategies for eboosting plant resilience and developing sustainable approaches for plant protection in agriculture.

Flavonoids are secondary metabolites found in plants that play an important role in protecting and defending plants against various biotic and abiotic stresses (Shah and Smith, 2020). Research has shown that flavonoids can induce systemic resistance in tomato plants against bacterial spot, thereby reducing disease severity and limiting the spread of the pathogen (Luiz et al., 2015). In the current experiment, it was observed that succinic acid treatmentresulted in the highest flavonoids levels after bacterial inoculation compared to other treatments. Furthermore, plants treated with succinic acid exhibited the lowest level of disease severity. This indicates that succinic acid not only boosts flavonoid production of flavonoids, which aids in plant defense, but also effectively reduces disease.

The findings from this experiment suggest that succinic acid treatment can effective-

ly enhance the plant's defense responses and provide protection against bacterial infections. By increasing the flavonoids production succinic acid-treated plants exhibit improved antimicrobial and antioxidant properties, contributing to their reduced disease severity.

Furthermore, flavonoids have been linked to reinforcingcell walls and modulating of plant hormone signaling pathways. These effects can assist in controlling pathogens and triggering defense mechanisms. The higher level of flavonoids in succinic acid-treated plants is believed to enhance their capacity-to withstand bacterial infections and exhibit reduced disease symptoms.

Further research is needed to fully comprehend the protective effects of succinic acid on tomato plants. It is essential to elucidate the signaling pathways and gene that are activated by succinic acidto defend the plants. Furthermore, gaining a better understanding of the interaction between succinic acid and the plant's immune system will provide valuable insights into the potential application of succinic acid as a plant defense activator.

Overall, the results highlight the potential of succinic acid as a effective treatment for boosting plant defense against bacterial pathogens. By stimulating flavonoid production and reducing disease severity, succinic acid treatment coul;ld present a sustainable and environmentally approach to disease control in tomato plants and potentially other crops as well.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) that plays a crucial role in plant defense mechanisms, particularly in combating bacterial diseases. One of the initial and most effective plant responses to pathogen invasion is referred to as oxidative burst. A high level of ROS, including H_2O_2, is produced in the plasma membrane near the pathogen. Although ROS are naturally produced during normal metabolic processes like photosynthesis and respiration, their levels can rise significantly and temporarily overpower the plant's natural antioxidant defenses. This elevated ROS concentration of can also be toxic to invading pathogens. In this experiment, the average H2O2 level in inoculated plants surpassed that of non-inoculated plants. Additionally succinic acid exhibited the highest H2O2 concentration 2 days after inoculation, indicating its ability to inhibit pathogen growth.

The experiment revealed that the average H2O2 level (hydrogen peroxide) in the inoculated plants was higher compared to non-inoculated plants. This observation suggests that bacterial inoculation triggers an oxidative burst in the plants, leading to an increase in H_2O_2 production. The plant's defense response often involves the generation of reactive oxygen species, including H_2O_2, as a part of the oxidative defense mechanism against pathogens.

Interestingly, the application of succinic acid resulted in the highest concentration of H_2O_2 two days after inoculation. This finding suggests that succinic acid treatment enhances the plant's ability to generate H_2O_2, which in turn inhibits the growth and development of the pathogen. H_2O_2 acts as a signaling molecule and has antimicrobial properties, potentially contributing to the suppression of pathogen growth and spread.

The inhibition of pathogen growth by suc-

cinic acid could be attributed to its ability to activate defense-related mechanisms in the plant. Succinic acid might trigger signal transduction pathways, leading to the activation of defense genes and subsequent production of H2O2. The higher concentration of H2O2 in the succinic acid-treated plants indicates its effectiveness in enhancing the oxidative defense response against the bacterial pathogen.

Overall, the results suggest that succinic acid treatment increases the concentration of H2O2 in tomato plants after bacterial inoculation, suggesting its potential to inhibit pathogen growth. This finding highlights succinic acid as a promising candidate for enhancing plant defense mechanisms and managing bacterial infections in agricultural settings.

Research shows that H_2O_2 plays a critical role in plant defense mechanisms when facing biological stress. Evidence suggests that H2O2 is able to effectively inhibits the proliferation and viability of plant pathogens, thereby limiting the spread of infection (Yergaliyev et al., 2016). In cases of bacterial infection such as *Xanthomonas*, plants produces H_2O_2 as a defense machanism to restrict bacterial growth (Kumar et al., 2011).

Superoxide dismutases (SODs: EC 1.15.1.1) are metalloenzymes found widely in nature and serve as the primary defense agent against reactive oxygen species (ROS). They are also highly efficient components of the antioxidant defense system in plant cells, protecting against the harmful effects of ROS (Berwal and Ram, 2018). Within living cells, SODs facilitate the conversion of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2)playing a vital role in protecting cells from damaging effects generated in various cellular compartments (del Río et al., 2003).

In this study, the treatments led to an increase in SOD levels compared to the control plants. Prticularly, the treatment involving succinic acid and potassium phosphite exhibited the highest SOD levels of 48 hours after inoculation. Several studies have investigated the relationship between SOD activity and Xanthomonas gardneri infection in tomato plants. For instance, a study by Włodarczyk et al., (2023) demonstrated that SOD activity increased in tomato plants treated with nanoparticles combined with conventional fertilizer, resulting in reduced disease severity (Włodarczyk et al., 2023). Another study by Shukla et al., (2018) frevealed that susceptible tomato cultivars responded to nematode infestation by increasing SOD activity, indicating the potential involvement of SOD in plant defense against nematodes. These findings suggest that SOD activity may play an important role in plant defense against *Xanthomonas gardneri* infection.

The experiment showed that the treatments led to an increase in SOD level (superoxide dismutase) compared to the inoculated plants. Superoxide dismutase plays a significant roleas an enzyme in protecting plants from oxidative stress (del Río et al., 2003).

succinic acid and potassium phosphite exhibited the highest significant SOD activity level two days after inoculation. This indicates that these treatments are capable of boosting the plant's antioxidant defense mechanism against *Xanthomonas gardneri*

infection in tomato plants.

Increased SOD activity level triggered by *Xanthomonas gardneri* infection can help mitigate the harmful effects of ROS accumulation. By converting superoxide radicals into H_2O_2 and O_2, SOD aids in preserving redox balance and minimize oxidative damage to plant tissues.

The higher SOD activity observed in plants treated with succinic acid and potassium phosphite-treated plants suggests that these treatments might enhance the plant's ability to scavenge ROS and mitigate oxidative stress caused by Xanthomonas gardneri infection. Consequently, this could contribute to reduced disease severity and improved plant health.

In the current study, the plant treatment with succinic acid resulted in the lowest level of malondialdehyde after inoculation. Additionally, the disease severity index was also low in this treatment, confirming that less damage was inflicted on the plant. . Malondialdehyde is a marker of lipid peroxidation and oxidative stress in plants. The low level of malondialdehyde in the succinic acid-treated plants indicates reduced oxidative damage and stress. This suggests that succinic acid treatment not only reduced oxidative stress but also effectively protected the plants from the harmful effects of the pathogen, resulting in lower disease severity. Researchers studying enzyme activity in tomato plants infected with Septoria lycopersici, foumd that MDA levels of increased significantly with disease severity. Moreover, they observed a simultaneous rise in both MDA and H_2O_2 concentration in other pathosystems, indicating a potential link between oxidative stress and the diseases progression (Silva et al., 2022). Plants produce ROS under abiotic or biotic stress conditions, disrupting the production of biomolecules such as lipids, proteins, and nucleic acids. This disruption leads to an increase in MDA content and plasma membrane permeability, resulting in cell efflux (Zhang et al., 2021). In a study on tomato plants infected with Xanthomonas perforans, an increase in ascorbic acid (ASA) was found to decrease lipid peroxidation and MDA levels. Ultimately, leading to a reduction in disease severity (Alfaro-Quezada et al., 2023).

Catalase plays a role in eliminating reactive oxygen species (ROS) and is a crucial component of plant stress response (Rotich & Mmbaga, 2023). The primary enzyme that responsible for the removal of hydrogen peroxide (H_2O_2) in plants is catalase, predominantly found in peroxisomes/glyoxysomes and in occasionally in mitochondria. The key role of CAT activity in plants is to protectt tissues from damage in the event of a significant rise in H_2O_2 concentration (Zandi and Schnug, 2022).

The average efficiency of plants that were inoculated with bacteria in this research was found to be higher compared to plants that were not inoculated. One potential reason for this enhanced efficiency in tomato plants inoculated with bacteriais the activation of the plants' defense mechanisms. When tomato plants are exposed to pathogens like *X. gardneri*, they might trigger a heightened state of alert, bolstering their overall physiological and immune responses. This activation could improve the plants' efficiency in nutrient uptake, photosynthesis, and resis-

tance to other stressors.

Catalase is also involved in tomato disease resistance, specifically early blight and leaf spot (Shoaib et al., 2019). Studies have demonstrated that tomato plants with enhanced resistance to fungal diseases have higher levels of catalase, indicating its role in mediating disease resistance (Moghaieb et al., 2021). The exact mechanism by which catalase enhances disease resistance in tomatoes is not fully understood, but it is believed to involve the breakdown of H2O2 produced during an attack by pathogens.

Recent research has elucidated the mechanisms by which catalase confers disease resistance in tomatoes. For instance, a study demonstrated that a compound known as alpha-momorcharin (αMMC) can enhance the resistance of tomato plants to Cucumber mosaic virus (CMV) infection. This is achieved by triggering autophagy and suppressing virus replication. Another study revealed that catalase activity plays a role in regulating other enzymes, including peroxidase and phenylalanine ammonia lyase. These enzymes are crucial for plant defense against pathogens (Yang et al., 2023). These findings indicate that catalase plays a mutifaced role in the resistance of tomatoes to diseases, through multiple pathways and mechanisms.

We investigated the efficiency of 11 potential resistance inducers in tomato plants *(Solanum lycopersicum)* challenged by *Xanthomonas gardneri*. The findings revealed that Kphi and Suc were particularly successful in triggering defense responses against *X. gardneri*. The reduced disease incidence in tomato by Khphi and Suc may be a result of increased flavonoids content as well as increased activity of SOD and induction of defense enzymes, while the accumulation of MDA in plant tissues was reduced. Based on the results, Khphi and Suc show promise as potential resistance inducers for protecting tomato plants against the bacterial pathogen *X. gardneri*.

Acknowledgments

The Authors thank to Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) and

The Central Laboratory of Sari Agriculture and Natural Resource University.

References

Aebi H. (1984). Catalase in vitro. Methods in Enzymology. 105: 121–126. Doi: https://doi.org/10.1016/s0076-6879(84)05016-3

Alché JD. (2019). A concise appraisal of lipid oxidation and lipoxidation in higher plants. Redox Biology. 23: 101136. Doi: 10.1016/j.redox.2019.101136.

Alfaro-Quezada JF, Martínez JP, Molinett S, Valenzuela M, Montenegro I, Ramírez I, Dorta F, Ávila-Valdés A, Gharbi E, Zhou M, Dailly H, Quinet M, Lutts S, Seeger M. (2023). Rootstock increases the physiological defence of tomato plants against *Pseudomonas syringae* pv. tomato infection. Journal of Experimental Botany. 74 (9): 2891–2911. Doi: https://doi.org/10.1093/jxb/erad040.

Atkinson NJ and Urwin PE. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. Journal of Experimental Botany. 63 (10): 3523–3543.

Barnaby A, Lam M, Scaman C, Clemens

S, Kermode A. (2008). Screening of phenylalanine ammonia lyase in plant tissues, and retention of activity during dehydration. Journal of the Science of Food and Agriculture. 88: 619–625. Doi: https://doi.org/10.1002/jsfa.3126.

Beers EP and McDowell JM. (2001). Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. Current Opinion in Plant Biology. 4 (6): 561–567.

Berwal MK and Ram C. (2018). Superoxide Dismutase: A Stable Biochemical Marker for Abiotic Stress Tolerance in Higher Plants (AB. de Oliveira (ed.); p. Ch. 7). Intech Open. Doi: https://doi.org/10.5772/intechopen.82079.

Beyer Jr WF and Fridovich I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Analytical Biochemistry. 161 (2): 559–566. Doi: 10.1016/0003-2697(87)90489-1.

Bonfim MF, Denez L, Innocenti MD, Petry HB, Bruna, ED. (2023). Doses and intervals of application of potassium phosphite for the control of passion fruit scab. Revista Brasileira de Fruticultura. Doi: https://api.semanticscholar.org/CorpusID:261143281.

Cecchini, NM, Roychoudhry S, Speed DJ, Steffes K, Tambe A, Zodrow K, Konstantinoff K, Jung HW, Engle NL, Tschaplinski TJ, Greenberg JT. (2019). Underground Azelaic Acid-Conferred Resistance to *Pseudomonas syringae* in Arabidopsis. Molecular Plant-Microbe Interactions : MPMI, 32 (1); 86–94. Doi: https://doi.org/10.1094/MPMI-07-18-0185-R

Chang CC, Yang MH, Wen HM, Chern JC. (2002). Estimation of total flavonoid content in propolis by two complementary colometric methods. Journal of Food and Drug Analysis. 10 (3): 3.

de Fátima Pereira Silva P, de Resende MLV, Reichel T, de Lima Santos M, Dos Santos Botelho D M, Ferreira EB, Freitas NC. (2023). Potassium phosphite activates components associated with constitutive defense responses in coffea arabica cultivars. Molecular Biotechnology. 65 (11): 1777–1795. Doi: https://doi.org/10.1007/s12033-023-00683-5.

Debnath D, Divya M, Golder S, Jamir I, Bhattacharjee T. (2019). Screening of different genotypes/crosses of tomato (*Lycopersicon esculentum*) for resistance to bacterial wilt (*Ralstonia solanacearum*) under field condition. Journal of Pharmacognosy and Phytochemistry. 8 (5): 174–177.

del Río LA, Sandalio LM, Altomare DA, Zilinskas BA. (2003). Mitochondrial and peroxisomal manganese superoxide dismutase: differential expression during leaf senescence. Journal of Experimental Botany. 54 (384), 923–933. Doi: https://doi.org/10.1093/jxb/erg091.

dos Santos C and Franco OL. (2023). Pathogenesis-related proteins (PRs) with enzyme activity activating plant defense responses. In Plants. 12 (11). Doi: https://doi.org/10.3390/plants12112226.

Ferrer JL, Austin MB, Stewart Jr, C, Noel JP. (2008). Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiology

and Biochemistry. 46 (3): 356–370. Doi: https://doi.org/10.1016/j.plaphy.2007.12.009.

Forman HJ, Maiorino M, Ursini F. (2010). Signaling functions of reactive oxygen species. Biochemistry. 49 (5), 835–842. Doi: https://doi.org/10.1021/bi9020378.

Hasanuzzaman M, Raihan MRH, Masud AAC, Rahman K, Nowroz F, Rahman M, Nahar K, Fujita M. (2021). Regulation of reactive oxygen species and antioxidant defense in plants under salinity. International Journal of Molecular Sciences. 22 (17). Doi: https://doi.org/10.3390/ijms22179326

Hoagland DR and Arnon DI. (1950). The water-culture method for growing plants without soil. Circular. California Agricultural Experiment Station. 347 (2nd edit).

Hu Z, Shao S, Zheng C, Sun Z, Shi J, Yu J, Qi Z, Shi K. (2018). Induction of systemic resistance in tomato against Botrytis cinerea by N-decanoyl-homoserine lactone via jasmonic acid signaling. Planta. 247 (5): 1217–1227. Doi: https://doi.org/10.1007/s00425-018-2860-7.

Jain S, Varma A, Tuteja NK, Choudhary DK. (2016). Plant growth-promoting microbial-mediated induced systemic resistance in plants: induction, mechanism, and expression. Doi: https://api.semanticscholar.org/CorpusID:87185327.

Janse van Rensburg HC, Takács Z, Freynschlag F, Toksoy Öner E, Jonak C, Van den Ende W. (2020). Fructans prime ROS dynamics and Botrytis cinerea resistance in Arabidopsis. Antioxidants. 9 (9): 805. Doi: https://doi.org/10.3390/antiox9090805.

Kumar N, Ebel RC, Roberts PD. (2011). H2O2 degradation is suppressed in kumquat leaves infected with *Xanthomonas axonopodis* pv. citri. Scientia Horticulturae. 130 (1): 241–247.

Lamers J, Van Der Meer T, Testerink C. (2020). How plants sense and respond to stressful environments. Plant Physiology. 182 (4): 1624–1635. Doi: https://doi.org/10.1104/pp.19.01464.

Liljeroth E, Lankinen Å, Wiik L, Burra DD, Alexandersson E, Andreasson E. (2016). Potassium phosphite combined with reduced doses of fungicides provides efficient protection against potato late blight in large-scale field trials. Crop Protection, 86: 42–55. Doi: https://doi.org/10.1016/j.cropro.2016.04.003

Luiz C, Neto ACR., Di Piero RM. (2015). Resistance to *Xanthomonas gardneri* in tomato leaves induced by polysaccharides from plant or microbial origin. Journal of Plant Pathology. 119–127.

Meena M, Yadav G, Sonigra P, Nagda A, Mehta T, Swapnil P, Marwal A. (2022). Role of elicitors to initiate the induction of systemic resistance in plants to biotic stress. Plant Stress. 5: 100103.

Mierziak J, Kostyn K, Kulma A. (2014). Flavonoids as important molecules of plant interactions with the environment. Molecules. 19 (10): 16240–16265.

Mittler R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science, 7 (9), 405–410.

Mittler R. (2017). ROS are good. Trends in Plant Science, 22 (1), 11–19. Doi:

10.1016/j.tplants.2016.08.002.

Moghaieb REA, Ahmed DS, Gaber A, Abdelhadi AA. (2021). Overexpression of bacterial katE gene improves the resistance of modified tomato plant against Fusarium oxysporum f. sp. lycopersici. GM Crops & Food. 12 (1): 315–327. Doi: https://doi.org/10.1080/21645698.2021.1903374.

Morales M and Munné-Bosch S. (2019). Malondialdehyde: Facts and Artifacts. Plant Physiology. 180 (3): 1246–1250. Doi: https://doi.org/10.1104/pp.19.00405.

Nawaz M, Sun J, Shabbir S, Khattak WA, Ren G, Nie X, Bo Y, Javed Q, Du D, Sonne C. (2023). A review of plants strategies to resist biotic and abiotic environmental stressors. Science of the Total Environment. 165832. Doi: https://doi.org/10.1016/j.scitotenv.2023.165832.

Ohkawa H, Ohishi N, Yagi K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 95 (2): 351–358.

Payyavula RS, Navarre DA, Kuhl JC, Pantoja A, Pillai SS. (2012). Differential effects of environment on potato phenylpropanoid and carotenoid expression. BMC Plant Biology. 12 (1): 1–17.

Perez SM, Biondi E, Laurita R, Proto M, Sarti F, Gherardi M, Bertaccini A, Colombo V. (2019). Plasma activated water as resistance inducer against bacterial leaf spot of tomato. PLOS One. 14 (5): e0217788.

Rabiei Z, Hosseini S, Dehestani A, Pirdashti H, Beiki F. (2022). Exogenous hexanoic acid induced primary defense responses in tomato (*Solanum lycopersicum* L.) plants infected with *Alternaria solani*. Scientia Horticulturae. 295: 110841. Doi: https://doi.org/https://doi.org/10.1016/j.scienta.2021.110841.

Rajput VD, Harish Singh RK., Verma KK., Sharma L, Quiroz-Figueroa, FR, Meena M, Gour VS, Minkina T, Sushkova S, Mandzhieva S. (2021). Recent developments in enzymatic antioxidant defence mechanism in plants with special reference to abiotic stress. Biology. 10 (4):267. Doi: https://doi.org/10.3390/biology10040267.

Ramezani M, Rahmani F, Dehestani A. (2017). Study of physio-biochemical responses elicited by potassium phosphite in downy mildew-infected cucumber plants. Archives of Phytopathology and Plant Protection. 50: 540–554. Doi: https://api.semanticscholar.org/CorpusID:90791338.

Reglinski T, Havis N, Rees HJ, de Jong H. (2023). The practical role of induced resistance for crop protection. Phytopathology. 113 (4): 719–731. Doi: https://doi.org/10.1094/PHYTO-10-22-0400-IA.

Ritchie DF. (2000). Bacterial spot of pepper and tomato. The Plant Health Instructor, 10.

Rotich E and Mmbaga MT. (2023). Data on plant defense enzyme activity associated with three endophytes against Cornus florida *Erysiphe pulchra* powdery mildew. Data in Brief. 48: 109220. Doi: https://doi.org/https://doi.org/10.1016/j.dib.2023.109220.

Sahu PK, Jayalakshmi K, Tilgam J, Gupta

A, Nagaraju Y, Kumar A, Hamid S, Singh HV, Minkina T, Rajput VD, Rajawat MVS. (2022). ROS generated from biotic stress: Effects on plants and alleviation by endophytic microbes. In Frontiers in Plant Science. 13: 1042936. Doi: https://www.frontiersin.org/articles/10.3389/fpls.2022.1042936.

Schieber M and Chandel NS. (2014). ROS function in redox signaling and oxidative stress. Current Biology, CB. 24 (10): R453-62. Doi: https://doi.org/10.1016/j.cub.2014.03.034.

Shah A and Smith DL. (2020). Flavonoids in agriculture: Chemistry and roles in biotic and abiotic stress responses, and microbial associations. Agronomy. 10 (8): 1209. Doi: https://doi.org/10.3390/agronomy10081209.

Shoaib A, Awan ZA, Khan KA. (2019). Intervention of antagonistic bacteria as a potential inducer of disease resistance in tomato to mitigate early blight. Scientia Horticulturae. 252: 20–28. Doi: https://doi.org/https://doi.org/10.1016/j.scienta.2019.02.073.

Shukla N, Yadav R, Kaur P, Rasmussen S, Goel S, Agarwal M, Jagannath A, Gupta R, Kumar A. (2018). Transcriptome analysis of root-knot nematode (*Meloidogyne incognita*)-infected tomato (*Solanum lycopersicum*) roots reveals complex gene expression profiles and metabolic networks of both host and nematode during susceptible and resistance responses. Molecular Plant Pathology. 19 (3): 615-633. Doi: https://doi.org/10.1111/mpp.12547.

Silva BN, Picanço BBM, Hawerroth C, Silva LC, Rodrigues FÁ. (2022). Physiological and biochemical insights into induced resistance on tomato against septoria leaf spot by a phosphite combined with free amino acids. Physiological and Molecular Plant Pathology. 120: 101854. Doi: https://doi.org/10.1016/j.pmpp.2022.101854.

Slopek SW. (1989). An improved method of estimating percent leaf area diseased using a 1 to 5 disease assessment scale. Canadian Journal of Plant Pathology. 11 (4): 381–387.

Soto-Caro A, Vallad GE, Xavier KV, Abrahamian P, Wu F, Guan Z. (2023). Managing bacterial spot of tomato: Do chemical controls pay off? Agronomy. 13 (4): 972. Doi: https://doi.org/10.3390/agronomy13040972.

Sun Y, Gui Z, Yan N, Wang Q, Zhang Z, Zhang H, Sun F, Han X, Du Y. (2023). Roles and preliminary mechanism of tobacco cis-abienol in inducing tomato resistance against bacterial wilt. International Journal of Molecular Sciences. 24 (15): 12226. Doi: https://doi.org/10.3390/ijms241512226.

Ullah A, Hussain A, Shaban M, Khan AH, Alariqi M, Gul S, Jun Z, Lin S, Li J, Jin S. (2018). Osmotin: a plant defense tool against biotic and abiotic stresses. Plant Physiology and Biochemistry. 123: 149–159.

Vanitha SC, Niranjana SR, Umesha S. (2009). Role of phenylalanine ammonia lyase and polyphenol oxidase in host resistance to bacterial wilt of tomato. Journal of Phytopathology. 157 (9): 552–557. Doi: https://doi.org/10.1111/j.1439-

0434.2008.01526.x

Velikova V, Yordanov I, Edreva A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Science. 151 (1): 59–66.

Vogt T. (2010). Phenylpropanoid biosynthesis. Molecular Plant. 3 (1): 2–20. Doi: 10.1093/mp/ssp106.

Włodarczyk K, Smolińska B, Majak I. (2023). The antioxidant potential of tomato plants (*Solanum lycopersicum* L.) under nano-ZnO treatment. International Journal of Molecular Sciences. 24 (14): 11833. Doi: https://doi.org/10.3390/ijms241411833.

Yang T, Peng Q, Lin H, Xi D. (2023). Alpha-momorcharin preserves catalase activity to inhibit viral infection by disrupting the 2b-CAT interaction in *Solanum lycopersicum*. Molecular Plant Pathology. 24 (2): 107–122. Doi: https://doi.org/10.1111/mpp.13279.

Yang Z, Mhamdi A, Noctor G. (2019). Analysis of catalase mutants underscores the essential role of CATALASE2 for plant growth and day length-dependent oxidative signalling. Plant, Cell & Environment. 42 (2): 688–700. Doi: https://doi.org/10.1111/pce.13453.

Yergaliyev TM, Nurbekova Z, Mukiyanova G, Akbassova A, Sutula M, Zhangazin S, Bari A, Tleukulova Z, Shamekova M, Masalimov ZK. (2016). The involvement of ROS producing aldehyde oxidase in plant response to Tombusvirus infection. Plant Physiology and Biochemistry. 109: 36–44. Doi: https://doi.org/10.1016/j.plaphy.2016.09.001.

Zandi, P and Schnug E. (2022). Reactive Oxygen Species, Antioxidant Responses and Implications from a Microbial Modulation Perspective. Biology. 11 (2): 155. Doi: https://doi.org/10.3390/biology11020155.

Zhang Y, Ji TT, Li TT, Tian YY, Wang LF, Liu WC. (2020). Jasmonic acid promotes leaf senescence through MYC2-mediated repression of CATALASE2 expression in Arabidopsis. Plant Science. 299: 110604. Doi: https://doi.org/10.1016/j.plantsci.2020.110604.

Zhang Y., Luan Q, Jiang J, Li Y. (2021). Prediction and utilization of malondialdehyde in exotic pine under drought stress using near-infrared spectroscopy. Frontiers in Plant Science. 12. Doi: https://www.frontiersin.org/articles/10.3389/fpls.2021.735275.

Zurbriggen MD, Carrillo N, Hajirezaei MR. (2010). ROS signaling in the hypersensitive response: when, where and what for? Plant Signaling & Behavior. 5 (4): 393–396. Doi: https://doi.org/10.4161/psb.5.4.10793.

Investigation of viruses infecting *Lycopersicum esculentum* in Iran and Molecular Analysis of Cucumber Mosaic Virus

Mehdi Safaeizadeh[1]*, Abbas Saidi[1]

Received: 2024-01-25 Accepted: 2024-03-18

Abstract

Viral diseases cause significant economic losses in tomatoes worldwide. This detect conducted a comprehensive survey in tomato (*Lycopersicum esculentum* L.) fields in Hamedan and Tehran provinces in Iran to detect and determine the incidence of tomato-infecting viruses. Using specific antibodies, collected symptomatic samples (348) were analyzed by Double antibody sandwich (DAS)-ELISA. According to the DAS-ELISA experiment, we found that 26.14% of collected samples were infected Arabis mosaic virus (ArMV), 36.78 % with Cucumber mosaic virus (CMV), 10.63% with Potato virus Y (PVY) 3.44 % with Tomato bushy stunt virus (TBSV), 7.18 % with Tomato spotted wilt virus (TSWV), and 2.87% with Tomato yellow leaf curl virus (TYLCV). Furthermore, double and triple infections were also observed in 15.08 and 6.03% of samples, respectively. CMV was the most prevalent among other tested viruses. Moreover, our findings showed that CMV was present in multiple infections of different samples. Serological diagnoses were confirmed by reverse transcription-polymerase chain reaction tests (RT-PCR) using a pair of primers that are specific for the detection of CMV and resulted in a DNA fragment of the expected size (540 bp). These results confirmed the DAS-ELISA experiment. Furthermore, in this study, we introduced a rapid method that facilitates the diagnosis of CMV in infected samples. Our findings can be used for control strategies and rapid diagnosis of viral infection in plants. Moreover, the outcome of this research can be used for the preparation of resistant cultivars against important viruses in tomatoes.

Keywords: CMV, DAS-ELISA, RT-PCR, Tomato, Viral diseases

Introduction

Infection to viral diseases has caused significant economic losses in tomato (*Lycopersicon esculentum*). Approximately more than 25 viruses from tomato were isolated globally (García-Estrada et al., 2022; Hanson 2022; Osundare, et al., 2023). Iran is a major producer of tomatoes, ranking seventh in the world according to the Food and Agriculture Organization (FAO) with a total production of 3,392,153.48 tons in 2021. Tomatoes are cultivated on over five million hectares worldwide. Because of the nutritional value of tomatoes, the

1-Department of Cellular and Molecular Biology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
*Corresponding author's email address: Ma_Safaei@sbu.ac.ir
Doi: 10.48308/jpr.2024.234970.1069

cultivation of this crop is increasing year by year especially in the Middle-East and Mediterranean regions (García-Estrada, et al., 2022; Hanson, 2022; Rivarez et al., 2021). The tomato was domesticated by indigenous peoples in Latin America, due to its high nutritional value, has become a significant vegetable crop globally (Hanson, 2022). viral infections pose a serious threat totomato plants, with viral diseases being the primary factor responsible for reducing tomato yield (García-Estrada, et al., 2022; Hanson, 2022; Osundare, et al., 2023; Scholthof, et al., 2011; Zhang et al., 2022). Among the top ten plant viruses that infect plants six can affect tomatoes, including Tobacco mosaic virus, Tomato spotted wilt virus, Tomato yellow leaf curl virus, Cucumber mosaic virus, Potato virus Y, Cauliflower mosaic virus, African cassava mosaic virus, Plum pox virus, Brome mosaic virus, and Potato virus X (Scholthof, et al., 2011). Recent studies showed that viral diseases are the major limiting factor in the world's in tomato production worldwide, leading to significant losses (García-Estrada et al., 2022; Hanson 2022; Safaeizadeh et al., 2015). Most viruses that infect tomatoes exhibit symptoms such as mosaic, stunting, leaf distortion, leaf discoloration, dwarfing, spots, discoloration, and abnormality on fruit. The transmission of primarily occurs through aphids, insect vectors, most especially arthropods, and mechanical means (Hanson 2022; Osundare et al., 2023; Scholthof et al., 2011).

Hamedan and Tehran provinces situated in different agroecological conditions in Iran, are the main vegetable-growing-areas and there are ample opportunities for increasing tomato production in these areas.

Recently, virus diseases have been causing issues for tomato production in Iran (Masumi et al., 2009; Safaeizadeh, et al., 2015). The symptoms of these diseases can vary depending on the host, environmental conditions, and individual virus infection. However, some of commonly observed symptoms include leaf mosaics, mottling, crinkling, vein clearing, bushy growth, leaf rolling, leaf and plant shrinkage, vein purpling, shoe-string, chlorosis and necrosis, reduction in fruit size, and abnormal fruit color and shape, and plant death.

To date, numerous viruses infecting tomato plants have been reported from Iran, including Alfalfa mosaic virus (AMV; Alipour al., 2021), Arabis mosaic virus (ArMV; Massumi, et al., 2009), Beet curly top virus (BCTV; Kiumarsi and Karimie Rozbahani, 1995), Cucumber mosaic virus (CMV; Saidi, A., Safaeizadeh, 2012), Eggplant mottle dwarf virus (EMDV; Babaie and Izadpanah, 2003), Potato chlorotic stunt virus (PCSV; Danesh et al., 1989), *Potato virus Y* (PVY; Massumi, et al., 2009), Tomato bushy stunt virus (TBSV; Massumi, et al., 2009), Tomato mosaic virus (ToMV; Aghamohammadi, et al., 2013), Tomato spotted wilt virus (TSWV; Abadkhah et al., 2018), Tomato vein yellowing virus (TVYV; Ghorbani, 1993), and Tomato yellow leaf curl virus (TYLCV; Shirazi, et al., 2014). A destructive virus known as Tomato brown rugose fruit virus (ToBRFV) has recently been identified in the greenhouse tomato growing region of Isfahan province, Iran (Ghorbani, et al., 2021). ToBRFV

severely affect tomato production of the main greenhouse growing cultivars in Isfahan (Ghorbani, et al., 2021). Therefore, conducting a thorough investigation of the viruses infecting tomatoes can help in evaluating strategies against viruses. ToBRFV is previously reported by Salem et al., (2016) in Jordan, who classified it as a tobamovirus (Salem, et al., 2016). Given the significant economic losses caused by viral infections in tomatoes, particularly in light of high prices of the tomatoes in Iran in recent years, it is essential to identify the viruses associated with tomato yield losses and to study the occurrence of different viruses, whether as single or mixed infections in tomato fields. Molecular analysis of the most frequently found virus and the determination of phylogenetic status of several isolates, are crucial steps in elucidating virus epidemiology and controlling of plant viral diseases to introduce the appropriate varieties of tomato to farmers.

Material and methods

Sample Collection

A total of 348 leaf samples were collected from tomato fields in nine regions of Hamedan and eight regions of Tehran province. Each sample was labeled based on its geographical origin (Table 1). Symptoms of the diseases were recorded and the samples were stored in a portable fridge at 4°C before being sent to the laboratory for testing.

Serological studies and DAS-ELISA experiment

Tomato viruses were detected using highly specific polyclonal antibodies obtained from Bioreba (CH-4153 Reinach BL1, Switzerland) through double-antibody sandwich enzyme-linked immunosorbent assay (DAS)-ELISA following the protocol by Clark and Adams (1977) and manufacturer's instruction. All buffers, reagents, and positive and negative controls were obtained commercially sourced from Bioreba. Samples were ground in a sterile mortar and pestle with extraction buffer (PBST: 0.13 M NaCl, 0.014 M KH_2PO_4, 0.08 M Na_2HPO_4, 0.002 M KCl, pH: 7.4) containing 0.05% Tween 20 and 0.1% nonfat dry milk. The extract was added to the wells of the ELISA plate (Nunc Microwell, Roskilde, Denmark) pre-coated with IgG of ArMV, CMV, PVY, TBSV, TSWV, and TYCLV diluted in carbonate buffer (pH: 9.6). Following an incubation at 4°C the plates were washed four times with PBST-Tween 20 buffer (pH: 7.4). Subsequently, the plates were coated with alkaline phosphatase conjugated antibody diluted in the extraction buffer and incubated for 5 h at 30 °C. After washing, p-nitrophenyl phosphate in diethanolamine substrate buffer (0.5 µg/mL, pH: 9.8) was added to each well and incubated in dark condition at room temperature (18-25°C) for 30 to 120 min. The absorbance was measured at 405 nm using a microplate reader (ELx 800; Bio-Tek Instruments, USA). ELISA values were considered positive when the absorbance at 405 nm (A_{405}) was at least three times higher than the average of the negative controls (Massumi et al., 2009).

In order to confirm the identity of the DAS-ELISA results, sap taken from the positive samples for ArMV, CMV, PVY, TBSV, and

TSWV were inoculated in their propagative hosts, including; *Nicotiana clevelandii* Rose ex Vasey & Rose*, Nicotiana tabacum* cv. Samsun*, Datura stramonium* L., and *N. rustica* L.*,* respectively. The mechanical inoculation tests involved using leaf sap of the positive samples at DAS-ELISA prepared by macerating in 0.1 M potassium phosphate buffer, pH 7.2 (supplemented with 0.01 M ethylenediaminetetraacetic acid and 0.1% sodium sulfite) at a ratio of 1:10 (w/v) using a sterile mortar and pestle. The extract was then rubbed on carborundum-dusted test plants, with at least three plants used for each positive sample. Subsequently, the inoculated plants were maintained in an insect-proof greenhouse under standard conditions (15-25°C). Three weeks post-inoculation, the presence of the virus in both inoculated and uninoculated upper leaves of the test plants was assessed by DAS-ELISA. The inoculated test plants were kept in an insect-proof greenhouse and observed for 6 weeks for symptom development.

RNA extraction and reverse transcription-polymerase chain reaction tests

For molecular investigation and evaluation of CMV in positive samples using DAS-ELISA, reverse transcription-polymerase chain reaction tests (RT-PCR) were used. A total of 150 mg of the symptomatic areas of the leaves were used to extract RNA, following the protocol for manufacturers' protocol supernatant extraction technique with TRI-Reagent (Sigma, Chemical, St Louis, MO, USA) according to the extraction technique. Finally, the total RNA was re-suspended in 100 µl diethylpirocarbonate (DEPC)-treated H_2O. As the control, healthy tomato extracts were used. RNA quality and concentration were determined by gel electrophoresis through 1% agarose gels and nanodrop. The highly specific oligonucleotide primers, as detailed by Safaeizadeh et al. (2015); were used to amplify the conserved sequences of CMV RNA 3 resulting in540 bp fragment. The reverse primer (5-GCGCGAAACAAGCTTCTTATC -3) corresponds to nucleotides 633 to 653 in the non-coding intergenic region, while the forward primer (5-GTAATACGACTCACTATAGGTTTTGTT TG-3) is complementary to position 114 to 132 in the coat protein gene. The specific primers utilized in this study were synthesized by CinaClone. Co. based in Tehran, Iran. The cDNAs were generated with RevertAid^{TM}M-MuLV reverse transcriptase (M-MuLV, Fermentas, Germany), at 42°C for one hour, following the manufacturer's instructions with the addition of 0.5 µl RNAse inhibitor. Subsequently, 2.5 µl of the RT reactions were used for PCR in a total volume of 25 µl. The PCR procedure was performed using a thermal Cycler (T100 Bio-Rad, USA), starting with an initial denaturation step of 95°C for 5 min followed by 35 cycles of 60 s at 95°C, 60 s at 42°C annealing temperature (AT) and 60 s at 72°C. A final step of 8 min at 72°C ended the cycle. The resulting products and DNA ladder (GeneRuler, DNA Ladder Plus, CinaClone, Co. Tehran, Iran) were analyzed by electrophoresis on 1% agarose gels in the presence of 1 µg mL^{-1} ethidium bromide using 1 X Tris-Borate EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA, pH: 8.3; Green and Sambrook, 2014).

Results and Discussion

According to the DAS-ELISA data, it was observed that samples collected from Hamedan and Tehran provinces were infected with ArMV, CMV, PVY, TBSV, TSWV, and TYLCV. CMV was the most widespread virus infecting 36.78% of the total samples tested followed by ArMV (26.14%), PVY (10.63%), TSWV (7.18%), TBSV (3.44%), and TYLCV (2.87%). Double infections were detected in 14.01% and 15.08%, of samples obtained from Hamedan, and Tehran provinces, respectively (Table 1, and Figure 1). While triple infections were found in 3.18%, and 8.9% in Hamedan and Tehran provinces, in the same order (Table 1 and Figure 1).

In the current study, CMV was identified as the most prevalent viruses, affecting 35.81% and 39.26% of the samples collected from Hamedan and Tehran provinces, respectively. Notably, CMV infection was more widespread in the Bahar area of Hamedan province, where tomatoes are widely cultivated. In Tehran province, CMV infection was more common in Varamin region, known for its significant tomato

Table 1. Detailed distribution and occurrence of viruses collected samples in Hamedan and Tehran provinces

Province	Region	No. of samples Collected and tested	ArMV	CMV	PVY	TBSV	TSWV	TYLV	Double infections	Triple infections
Hamedan	Asad abad	22	2	9	1	0	1	0	3	0
	Bahar	28	2	13	7	0	0	2	4	2
	Lalehjin	10	0	5	1	0	0	0	1	0
	Famenin	24	5	8	1	2	0	0	3	0
	Razan	16	0	7	3	0	0	0	5	1
	Malayer	18	2	2	3	0	6	0	2	0
	Maryanaj	14	3	1	1	0	3	0	1	1
	Nahavand	18	11	6	4	2	0	1	1	1
	Tooyserkan	7	1	2	0	1	0	0	2	0
	Sum	157	26	53	21	5	10	3	22	4
	Percent of infection	--	16.56	35.81	13.37	3.18	6.36	1.91	14.01	3.18
Tehran	Karaj	35	11	13	5	0	0	2	7	2
	Varamin	42	12	16	7	5	6	3	5	4
	Robat karim	15	7	9	0	0	0	0	4	2
	Shahriar	23	12	12	1	1	1	1	6	3
	Mardabad	18	5	6	2	0	0	1	3	2
	Pakdasht	22	8	8	0	1	4	0	3	2
	Rey	19	4	5	1	0	2	0	2	1
	Eslamshhar	17	6	6	0	0	2	0	3	1
	Sum	191	65	75	16	7	15	7	33	17
	Percent of infection	--	34.03	39.26	8.37	3.66	7.85	3.66	17.27	8.9
	Total	348	91	128	37	12	25	10	55	21
	Percent of infection in two provinces	--	26.14	36.78	10.63	3.44	7.18	2.87	15.08	6.03

production. The data presented in Table 1 and Figure 1, indicated a higher incidence of ArMV in Tehran province compared to in Hamedan province.

According to Belval et al., (2021) that previously reported field's infection to *Xiphinema index* as the vector of nepoviruses could be one of the most important reasons of infection to ArMV in the studied regions. PVY is found to be prevalent in Hamedan province at 13.37%, while in Tehran province, the prevalence was 8.37%. These result could be attributed to the fact that Hamedan province's status as a major potato cultivation area in Iran, serving as a potential source of PYV transmission to other host plants. Plants with triple infections showed more severe leaf and fruit symptoms in this study compared to those infected with just one or two viruses. Moreover, a notable

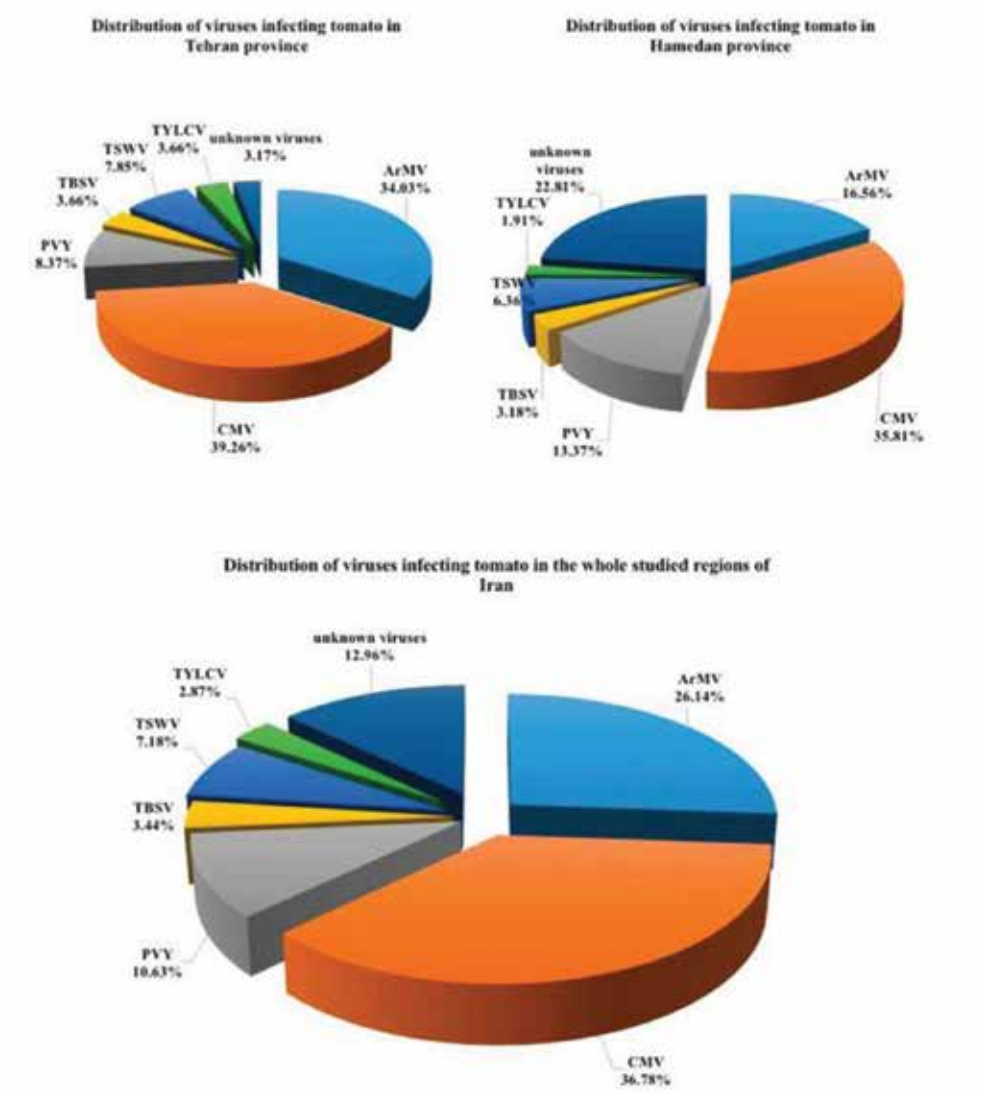


Fig. 1. Distribution of viruses infecting tomato (Lycopersicum esculentum) in Tehran and Hamedan provinces of Iran. Abbreviation: Arabis mosaic virus (ArMV), Cucumber mosaic virus (CMV), Potato virus Y (PVY), Tomato bushy stunt virus (TBSV), Tomato spotted wilt virus (TSWV), and Tomato yellow leaf curl virus (TYLCV)

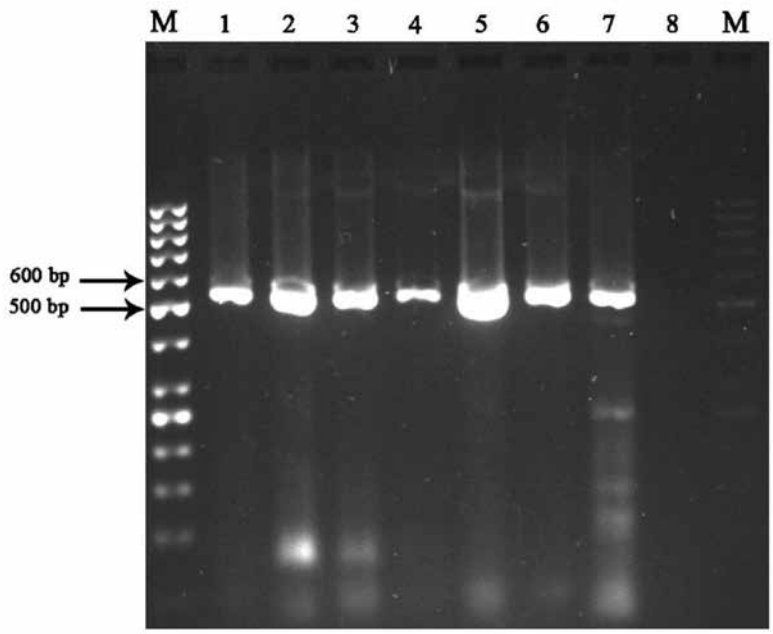


Fig. 2. Agarose gel of RT-PCR products for specific detection of Cucumber mosaic virus (CMV) infected tomato. M: molecular weight marker (DNA ladder). Numbers 1 to 8 indicate individual plants for each region corresponding as the confirmatory test to a positive sample in DAS-ELISA; three samples from Hamedan province (Bahar, Famenin, and Malayer; line number 1-3) and four samples from Tehran province (Karaj, Varamin, Mardabad and Pakdasht; line number 4-7). Line number 8: control (total RNA extracted from healthy *Lycopersicum esculentum*)

percentage of symptomatic samples did not show reactivity to any of the antibodies tested in this study, with rates of 22.89% and 3.17% observed in Hamedan and Tehran provinces, respectively.

The RT-PCR experiment was conducted as a confirmatory test for the positive samples in the DAS-ELISA analysis. Several samples from the studied area were tested, including three samples from Hamedan province (Bahar, Famenin, and Malayer) and four samples from Tehran province (Karaj, Varamin, Mardabad, and Pakdasht (Figure 2). The results of the RT-PCR analyses, using specific primers to detect CMV, were consistent with DAS-ELISA results. A PCR product of expected size (540 bp) was yielded from the infected samples, while no amplicon was generated from healthy plant extracts (Figure 2).

The presence of CMV was successfully identified using the specific primers (Figure 2). This finding is in line with the DAS-ELISA results confirming the accuracy of the DAS-ELISA experiment. The RT-PCR analysis produced a 540bp product, consistent with our evaluations. Therefore, this approach proves to be effective for prompt diagnosis of CMV in infected samples.

The results of the current study suggest that CMV strains affecting tomatoes in the surveyed area exhibited higher virulence levels and were in complete agreement with the elevated prevalence of CMV in those regions, shedding light on why CMV stands as the most widespread virus. CMV,

which leads to substantial economic losses in tomato crops, is primarily transmitted by aphids in a non-persistent manner on a global scale (Arinaitwe et al., 2022; Atarashi et al., 2020).

One crucial aspect that warrants further investigation in future research is the presence of symptomatic samples in the studied regions, that did not exhibit reactivity with any of the antibodies utilized in this study. The prevalence of such samples was 22.81% and 3.17% in Hamedan and Tehran provinces, respectively. These samples may potentially harbor other viruses such as Pepino mosaic virus (PepMV), Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), or even viroids that capable of infecting tomatoes (Anastassiadou et al., 2021; Choi, et al., 2020; Rivarez et al., 2021). A significant discovery is that the number of unidentified viruses in Hamedan province exceeded the number in Tehran province. It could be hypothesized that other unidentified viruses have been introduced from neighboring countries like Iraq and Turkey to the Hamedan province, given its proximity of these countries compared to the Tehran province. The unknown samples could include Tomato brown rugose fruit virus (ToBRFV) an emerging an RNA virus that is rapidly spreading (Zhang et al., 2022). This virus has been documented in numerous countries globally, and controlling its spread is crucial for tomato production (Zhang et al., 2022). Recent reports, have confirmed the prescence of ToBRFV in Turkey (Fidan et al. 2019); Ghorbani et al. (2021) in greenhouse complexes in Iran. Furthermore, Abou Kubaa et al., (2022), have reported this destructive virus from the Mediterranean region. Therefore, a thorough investigation into the identification of these unknown viruses is essential to develop an effective depth to formulate an adequate resistance program strategy.

In another research conducted in southeast and central regions of Iran to determine the prevalence of viruses infecting tomatoes, Massumi et al., (2009) identified ArMV as the most common viruses at 25.6%. They also found that CMV was the second most frequent virus affecting 23.4% of the studied samples. Although there are some discrepancies between their findings and our own research, this could be attributed to the significant differences in climate conditions between Hamedan and Tehran provinces (characterized by cold winters) and the southeastern and central regions of Iran, which can have a substantial impact on virus epidemiology. While TBSV has been previously reported in pelargonium and tomatoes from the Varamin region (Farzadfar et al. 2000). In the research conducted by Massumi et al., 2009 could TBSV did not detect in tomatoes, whereas we detected TBSV in 3.44% of collected samples.

In our study, numerous symptomatic samples did not exhibit reactivity towards any of the studied viruses. They may have been infected with alternative tomato-infecting viruses such as Tomato mosaic virus and Beet curly top virus previously reported by Massumi et al., (2009) with 4.8% and 6.1%, respectively. Also, they might be infected with other tomato-infecting viruses such as Pepino mosaic virus (PepMV) one of the

most important viral pathogens of tomatoes recently reported from other countries (Cho et al., 2022; He et al., 2020; Song et al., 2017). Moreover, they may be infected with Tomato leaf curl Palampur virus, a member of begomoviruses, with a rapid mutation rate and frequent recombination events that are a significant threat to tomato production worldwide and recently isolated from other hosts from Iran, neighboring countries, and many countries worldwide (Cai et al., 2023; Cao et al., 2024; Heydarnejad et al., 2009; Naganur 2023; Nayaka et al., 2024).

It is worth noting that the prevalence of CMV is attributed to its ability to infect various hosts including crop plants, vegetables, ornamental plants, and even weeds. Recently Safaeizadeh (2021), identified *Dianthus hybridus* as a new host for CMV in Hamedan province. In this study, the presence of CMV was confirmed using DAS-ELISA and RT-PCR techniques (Figures 1 and 2). Additionally, *Ibicella lutea* was previously reported as a new weed host for CMV (Safaeizadeh and Saidi, 2012). The prevalence of CMV in ornamental plants highlights the need for control strategies in greenhouses. Furthermore, Saidi and Safaeizadeh (2012) reported a severe strain of CMV from *Canna indica* that was cultivated in greenhouses in the Varamin region of Tehran province. These findings should be taken into consideration when developing strategies to control CMV in tomatoes.

The data presented in this study will be valuable in formulating future control strategies against viruses affecting tomatoes. However, the extent of crop and yield losses still needs to be determined and other properties of the studied viruses must be more studied. A comprehensive survey is also needed to investigate the occurrence of these viruses in other tomato fields across Iran and to evaluate the damage they are causing in terms of yield. It is advisable to conduct a mechanical transfer of the study on these viruses to the model genetic plant A. thaliana in order to assess the symptoms in this plant. Subsequently, it is essential to analyze the hormonal changes in comparison to the wild type A. thaliana (Safaeizadeh and Ghotbi-ravandi, 2023).

Acknowledgments

The authors express their gratitude to the researchers at the Iranian Research Institute of Plant Protection (IRIPP), specifically the Department of Plant Virology for their great help and advice during this research. Additionally, the authors wish to thank the Shahid Beheshti university research fund for their contribution to this project (research number: S/600/1153-99.10.21 and S/600/121-99.03.25). The authors also extend their thanks to their colleagues at the department of Cellular and Molecular Biology.

References

Abadkhah M, Koolivand D, Eini O. (2018). A new distinct clade for Iranian tomato spotted wilt virus isolates based on the polymerase, nucleocapsid, and nonstructural genes. Plant Pathology Journal. 34(6):514-531. Doi: 10.5423/PPJ.OA.04.2018.0062.

Abou Kubaa R, Choueiri E, Heinoun K,

Cillo F, Saponari M. (2022). First report of tomato brown rugose fruit virus infecting sweet pepper in Syria and Lebanon. Journal of Plant Pathology. 104: 425. Doi: https://doi.org/10.1007/s42161-021-00987-y

Aghamohammadi VF, Rakhshandehroo M, Shams-bakhsh and Palukaitis P. (2013). Distribution and genetic diversity of tomato mosaic virus isolates in Iran. Journal of Plant Pathology. 95 (2): 339–47. Doi: http://www.jstor.org/stable/23721524.

Alipour F, Massumi H, Heydarnejad J, Hosseinipour A, Maddahian M. (2021) Molecular characterization of Iranian isolates of Alfalfa mosaic virus based on movement protein gene. Agricultural Biotechnology Journal 13 (4), 81-100. Doi: 10.22103/JAB.2021.17934.1328.

Anastassiadou M, Arena M, Auteri D, Brancato A, Bura L, Carrasco Cabrera L, Chaideftou E, Chiusolo A, Crivellente F, De Lentdecker C, Egsmose M, Fait G, Greco L, Ippolito A, Istace F, Jarrah S, Kardassi D, Leuschner R, Lostia A, Lythgo C, Magrans O, Mangas I, Miron I, Molnar T, Padovani L, Parra Morte JM, Pedersen R, Reich H, Santos M, Sharp R, Szentes C, Terron A, Tiramani M, Vagenende B, Villamar-Bouza L. (2021). Peer review of the pesticide risk assessment of the active substances Pepino Mosaic Virus,EU strain, mild isolate Abp1 and Pepino Mosaic Virus,CH2 strain, mild isolate Abp2. European Food Safety Authority (EFSA); J. 12; 19 (1): e06388. Doi: https://doi.org/10.2903%2Fj.efsa.2021.6388

Arinaitwe W, Guyon A, Tungadi TD, Cunniffe NJ, Rhee SJ, Khalaf A, Mhlanga NM, Pate AE, Murphy AM, Carr JP. (2022). The Effects of Cucumber Mosaic Virus and Its 2a and 2b Proteins on Interactions of Tomato Plants with the Aphid Vectors Myzus persicae and *Macrosiphum euphorbiae*. Viruses. 1;14(8):1703. Doi: 10.3390/v14081703.

Atarashi H, Jayasinghe WH, Kwon J, Kim H, Taninaka Y, Igarashi M, Ito K, Yamada T, Masuta C, Nakahara KS. (2020). Artificially edited alleles of the eukaryotic translation initiation factor 4E1 gene differentially reduce susceptibility to cucumber mosaic virus and potato virus Y in tomato. Front Microbiology. 11: 564310. Doi: 10.3389/fmicb.2020.564310.

Babaie G and Izadpanah K. (2003). Vector transmission of eggplant mottled dwarf virus in Iran. Journal of Phytopathology. 151 (11-12): 679-682. Doi: https://doi.org/10.1046/j.1439-0434.2003.00788.x

Belval L, Marmonier A, Schmitt-Keichinger C, Gersch S, Andret-Link P, Komar V, Vigne E, Lemaire O, Ritzenthaler C, Demangeat G. (2019). From a movement-deficient Grapevine fanleaf virus to the identification of a new viral determinant of nematode transmission. Viruses. 11 (12): 1146. Doi: 10.3390/v11121146.

Cai L, Mei Y, Ye R, Deng Y, Zhang X, Hu Z, Zhou X, Zhang M, Yang J. (2023). Tomato leaf curl New Delhi virus: an emerging plant begomovirus threatening cucurbit production. Abiotech. 25; 4 (3): 257-266. Doi: 10.1007/s42994-023-00118-4.

Cao X, Huang M, Wang S, Li T, Huang Y. (2024). Tomato yellow leaf curl virus: Characteristics, influence, and regulation mechanism. Plant Physiology and Biochemistry. 213:108812. Doi: 10.1016/j.plaphy.2024.108812.

Cho IS, Chung BN, Yoon JY, Hammond J, Lim HS. (2022). First report of Pepino mosaic virus infecting tomatoes in South Korea. Plant Diseases. Doi: 10.1094/PDIS-02-22-0380-PDN.

Choi H, Jo Y, Cho WK, Yu J, Tran PT, Salaipeth L, Kwak HR, Choi HS, Kim KH. (2020). Identification of viruses and viroids infecting tomato and pepper plants in vietnam by metatranscriptomics. International Journal of Molecular Sciences. 13; 21 (20): 7565. Doi: 10.3390/ijms21207565.

Clark MF and Adams AN (1977): Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology. 34: 475–483. Doi: http://dx.doi.org/10.1099/0022-1317-34-3-475.

Danesh D, Bahar M, Ahoonmanesh A, Ghobadi C. (1989). Some new hosts of potato chlorotic stunt virus in Isfahan. Page 168. In: Proceedings 9th Plant Protection Congress, Mashhad, Iran.

F.A.O. (2021). F.A.O. Statistical Databases: Agricultural data. http://faostat.fao.org.

García-Estrada RS, Diaz-Lara A, Aguilar-Molina VH, Tovar-Pedraza JM. (2022). Viruses of Economic Impact on Tomato Crops in Mexico: From Diagnosis to Management-A Review. Viruses. 14 (6): 1251. Doi: 10.3390/v14061251.

Fidan H, Sarikaya P, Calis O. (2018). First report of Tomato brown rugose fruit virus on tomato in Turkey. New Disease Reports. 39 (1): 18. Doi: https://doi.org/10.5197/j.2044-0588.2019.039.018

Ghorbani S. (1993). Identification of tomato vein yellowing virus (TVYV) in Tehran province. Page 158. In: Proceedings 11th Plant Protection Congress, Rashat, Iran.

Ghorbani A, Rostami M, Seifi S, Izadpanah K. (2021). First report of Tomato brown rugose fruit virus in greenhouse tomato in Iran. New Disease Reports. 44: e12040. Doi: https://doi.org/10.1002/ndr2.12040.

Hanson SF. (2022). Viral diseases of tomato origins, impact, and future prospects with a focus on tomato spotted wilt virus and tomato yellow leaf curl virus. Tomato - from cultivation to processing technology. IntechOpen. 1-17. Doi: 10.5772/intechopen.108608.

He WQ, Wu JY, Ren YY, Zhou XP, Zhang SB, Qian YJ, Li FF, Wu JX. (2020). Highly sensitive serological approaches for Pepino mosaic virus detection. Journal of Zhejian University Science B. 21 (10): 811-822. Doi: 10.1631/jzus.B2000255.

Heydarnejad J, Mozaffari A, Massumi Fazeli HR, Alistair J, Gray A, Meredith S, Lakay F, Shepherd DN, Martin DP, Varsani A (2009). Complete sequences of tomato leaf curl Palampur virus isolates infecting cucurbits in Iran. Archives of Virology. 154:1015–1018. Doi: https://doi.org/10.1007/s00705-009-0389-6.

Massumi H, Shaabanian M, Hosseini Pour A, Heydarnejad J, Rahimian H. (2009). Incidence of viruses infecting tomato and

their natural hosts in the southeast and central regions of Iran. Plant Disease. 93 (1): 67-72. Doi: https://doi.org/10.1094/pdis-93-1-0067.

Naganur P, Shankarappa KS, Mesta RK, Rao CD, Venkataravanappa V, Maruthi MN, Reddy LRCN. (2023). Detecting Tomato leaf curl New Delhi virus causing ridge gourd yellow mosaic disease, and other Begomoviruses by antibody-based methods. Plants. 12 (3): 490. Doi: 10.3390/plants12030490.

Nayaka SN, Singh OW, Kumar P, Roy A, Mandal B. Geographical distribution of tomato-infecting begomoviruses in major cucurbits in India: a diagnostic analysis using begomovirus species specific PCR. Virus Disease. 34 (3): 421-430. Doi: 10.1007/s13337-023-00837-8.

Osundare OT, Fajinmi AA, Adelu AR. (2023). Effects of natural virus infection on field-grown eight tomato genotypes (*Lycopersicon esculentum*). Jordan Journal of Agricultural Sciences. 19 (3): 213-222. Doi: https://doi.org/10.35516/jjas.v19i3.203.

Rivarez MPS, Vučurović A, Mehle N, Ravnikar M, Kutnjak D. (2021). Global advances in tomato virome research: current status and the impact of high-throughput sequencing. Frontiers in Microbiology. 12: 671925. Doi: 10.3389/fmicb.2021.671925.

Safaeizadeh M. (2022). First report of cucumber mosaic virus infecting *Dianthus hybridus* in Iran. Journal of Plant Pathology. 104 (1): 397-397. Doi: https://doi.org/10.1007/s42161-021-00958-3.

Safaeizadeh M, Saidi A, Palukaitis P. (2015). Molecular characterization of cucumber mosaic virus (CMV) isolates infecting tomatoes in Hamedan and Tehran provinces of Iran. Acta Virologica. 59: 174-178. Doi: https://doi.org/10.4149/av_2015_02_174

Safaeizadeh M, and Saidi A. (2012). First report of Cucumber mosaic virus on Ibicella lutea in Iran. Journal of Plant Pathology. 94 (4): pS4.95. Doi: http://www.jstor.org/stable/45156331.

Saidi A and Safaeizadeh M. (2012). First report of Cucumber mosaic virus on Canna indica in Iran. Australasian Plant Disease Notes. 7: 119–121. Doi: https://doi.org/10.1007/s13314-012-0062-x.

Green MR, and Sambrook S. (2014). Molecular cloning: A laboratory manual, 4nd edition, Cold Spring Harbor Laboratory Press; 978-1-936113-42-2; New York, USA.

Scholthof KB, Adkins S, Czosnek H, Palukaitis P, Jacquot E, Hohn T, Hohn B, Saunders K, Candresse T, Ahlquist P, Hemenway C, Foster GD. (2022). Top 10 plant viruses in molecular plant pathology. Molecular Plant Pathology. 12 (9): 938-54. Doi: 10.1111/j.1364-3703.2011.00752.x.

Shirazi M, Mozafari J, Rakhshandehroo F, Shams-Bakhsh M. (2014). Genetic diversity, host range, and distribution of tomato yellow leaf curl virus in Iran. Acta Virology. 58 (2): 128-36. Doi: 10.4149/av_2014_02_128.

Zhang S, Griffiths JS, Marchand G, Bernards MA, Wang A. (2022). Tomato brown rugose fruit virus: An emerging and

rapidly spreading plant RNA virus that threatens tomato production worldwide. Molecular Plant Pathology. 23 (9): 1262-1277. Doi: 10.1111/mpp.13229.

Farzadfar Sh, Pourrahim R, Shahriari D, Golnaraghi A, Izadpanah K. (2000). Occurrence of Tomato bushy stunt virus (TBSV) in tomato fields of Varamin area. Proceeding of the second Iranian Horticultural Sciences Congress. Sept. 19-21, Karaj, Iran, p16-18.

Safaeizadeh M and Ghotbi Ravandi AA. (2023). Evaluation of the hormonal changes in the model plant Arabidopsis thaliana as the consequence of Pseudomonas aeruginosa infection. Plant, Algae and Environment. 7 (2): 1213-1227. Doi: 10.48308/jpr.2024.235141.1070.

The Genus *Ganoderma* in Iran: A Comprehensive Survey of Taxonomic Studies and Its Impact on Forest Trees

Somayeh Keypour[1]*, Mohammad Reza Asef [2], Hossein Riahi [3]

Received: 2024-01-01 Accepted: 2024-03-05

Abstract

Ganoderma Karst. is widely acknowledged within the Ganodermataceae for its exceptional healing properties for humans, but it also can cause diseases in plants. This study aims to compile all original articles and texts published on the morphological identification, phylogeny, host relationships, and dispersal of the *Ganoderma* species in Iran until 2024. The species predominantly inhabit Angiosperm trees, with occasional occurrences on gymnosperms. Recent studies showed that more trees have been infected with these species in Iran. According to the literature, nine *Ganoderma* species were reported in various provinces of Iran, including East Azerbaijan, Guilan, Mazandaran, Golestan, Khuzestan, and others. However, recent studies have revealed discrepancies in the previously reported species. One species (*G. australe* (Fr.) Pat.) was misidentified, while two other species (*G. manoutchehrii* Steyaert and *G. kosteri* Steyaert) have not been encountered during recent field studies conducted by recent Iranian mycologists. Although a herbarium sample of *G. kosteri* was found in the Meise Herbarium, no recent field collections have mentioned the existence of this species in Iran. Furthermore, this article discusses the presence of another reported species in Iran, *Ganoderma vanheurnii* Steyaert. Recent morphological and molecular studies in Iran have confirmed the existence of five species in recent years, which have had significant implications for trees and the ecosystem. This review assists environmental researchers in comprehending the forest destruction caused by the *Ganoderma* species. Additionally, it can assist taxonomists in precisely distinguishing similar species and properly introducing them to scientists engaged in pharmaceutical research on *Ganoderma*.

Keywords: *Ganoderma*, Taxonomy, Host relationship, Pharmaceutical aspects, Iran

Introduction

The genus *Ganoderma* is categorized under Polyporales, Ganodermataceae (Upadhyay, 2014). Over time, the species of *Ganoderma* have gained recognition for their valuable therapeutic properties.

1-*Department of Biology Education, Farhangian University, P.O. Box 14665-889 Tehran, Iran.
2-Department of Botany, Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.
3-Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.
*Corresponding author email address: s.keypour@cfu.ac.ir
Doi: 10.48308/jpr.2024.234963.1067

The earliest documentation of their healing abilities can be traced back to the "Classic of Materia Medica" authored by Shen Nong (Bodeker, 2012). Among these species, *G. lingzhi* previously known as *G. lucidum*, has been extensively utilized in medicine (Cao et al., 2012). Apart from their medicinal value, the species within this genus are known to act as parasitic or saprophytic agents on various trees (Tchoumi et al., 2018). Given the significance of these species, research on their taxonomy, healing properties, and plant pathogenicity has been conducted since the late twentieth century (Steyaert, 1972; Ryvarden, 1991; El-Mekkawy et al., 1998; Moncalvo et al., 2000; Ooi et al., 2002; Lee et al., 2006; Wasser et al., 2006; Saltarelli et al., 2009; Cao et al., 2012; Simone & Annesi, 2012; Bishop et al., 2015; Zeng et al., 2017; Huang et al., 2019).

It is estimated that there are approximately 250-400 *Ganoderma* species worldwide (Moncalvo et al., 1995; Richter et al., 2015), while only a maximum of nine species have been reported in different provinces of Iran, inhabiting both angiosperm and gymnosperm trees (Moradali et al., 2007; Ghobad-Nejhad & Hallenberg, 2012; Keypour et al., 2014a; Keypour et al., 2014b; Keypour & Asef, 2020). Considering the significance of the genus and the uncertainty of the species' presence in Iran, this paper aims to discuss the taxonomic status, accuracy of species availability reports, host relationships, and diversities of species of the genus *Ganoderma* in the country.

Methods

The present study aimed to compile a comprehensive collection of published articles and texts pertaining to the genus *Ganoderma* in Iran. To achieve this, a thorough search was conducted across multiple scholarly databases including Google Scholar, PubMed, ScienceDirect, Civilica, and SID. Additionally, original texts from libraries, materials provided by previous researchers, and conference papers were meticulously examined, without imposing any restrictions on the publication dates, up until 2024. Furthermore, the "Herbarium IRAN" was chosen as the main source for investigating *Ganoderma* samples that had been collected by eminent scientists. Communication with other Herbaria was also established to obtain information regarding samples from Iran that were deposited in their respective collections. Through a rigorous evaluation process, only authentic and reliable articles and texts were selected for analysis, thereby ensuring the accuracy and robustness of the collected references used in the preparation of this article.

Results

Taxonomy

Ganoderma is classified taxonomically within Basidiomycota, Agaricomycetes, Polyporales, Ganodermataceae, as documented by Keypour et al. (2019). This cosmopolitan genus has been the subject of multiple studies conducted by various scientists in Iran, including Steyaert (1972), Moradali et al. (2007), Ghobad-Nejhad & Hallenberg (2012), and Amoopour et al. (2016) (Table 1). Among the nine species reported in Iran, *Ganoderma colossus* has

been reclassified under the scientific name *Tomophagus colossus*. Additionally, it has been verified that *Ganoderma adspersum* is the accurate name for the species previously identified as *Ganoderma australe* in Iran. The morphological resemblance between *G. adspersum* and *G. australe* often leads to misidentification of the former in Iran. Furthermore, field studies conducted by mycologists in recent years have not yielded any evidence of *Ganoderma manoutchehri* and *Ganoderma kosteri*, two other species previously reported from Iran.

History of morphological identifications and numerical taxonomy

Various researchers, including Steyaert (1972), Adaskaveg and Gilbertson (1988), and Ryvarden (2000), have employed morphological characteristics to discriminate between different *Ganoderma* species. It should be noted that taxonomic confusion can arise due to the extensive morphological variability exhibited by these species (Ryvarden, 2000; Hapuarachchi et al., 2015). Despite this challenge, morphological studies serve as an initial step in species identification. Notably, *G. lucidum* Karst. was first documented in northern Iran by Buhse (1860), and subsequent reports on this species were provided by Khabiri (1968) and Saber (1987) (Table 2). Similarly, *G. applanatum* (Pers.) Pat. was first reported by Khabiri (1968) and Steyaert (1975) in Iran, followed by Saber and Minassian (2000). Saber and Minassian (2000) and Saber and Esmaeili Taheri (2004) documented the first occurrence of *G. australe* (Fr.) Pat. in Iran. The specimens of *G. kosteri* Steyaert. was collected by Ershad and Izadyar, while the sole sample of *G. manoutchehrii* Steyaert. was reported by Steyaert from Iran (Steyaert, 1972; Moradali et al., 2007; Ghobad Nejhad & Hallenberg, 2012). *G. resinaceum* Boud. was reported by Steyaert (1980), Saber (1987), and Saber and Minassian (2000), followed by the report of *G. tsugae* Murrill. from Iran by Saber & Minassian (2000). Steyaert (1972) briefly mentioned *G. vanheurnii* Steyaert. in Iran as a new taxon in the abstract of an article; however, in the description, the examined species was attributed to Indonesia, and no further information regarding its habitat in Iran was provided. In 2007, Moradali et al. conducted a morphological study on *Ganoderma* species in Iran, presenting an identification key with seven species categorized into two subgenera: *Elfivingia* subgenus (non-laccate species), including *G. applanatum, G. adspersum, G. colossus,* and *Ganoderma* subgenus (laccate species), comprising *G. lucidum, G. resinaceum, G. tsugae, and G. manoutchehrii*. Micro- and macro-morphological characteristics, as well as host associations, were the primary criteria employed to differentiate these species in their investigation. They presented a species identification key for *Ganoderma* in Iran, comprising seven species. However, their fieldwork efforts resulted in the collection of only four species, namely *G. applanatum, G. adspersum, G. lucidum,* and *G. resinaceum* from the northern regions of Iran (Fig 1.). The remaining three samples were either previously reported by other researchers or obtained from specimens deposited in the IRAN Herbarium (Steyaert, 1972; Saber and Minassian, 2000; Saber,

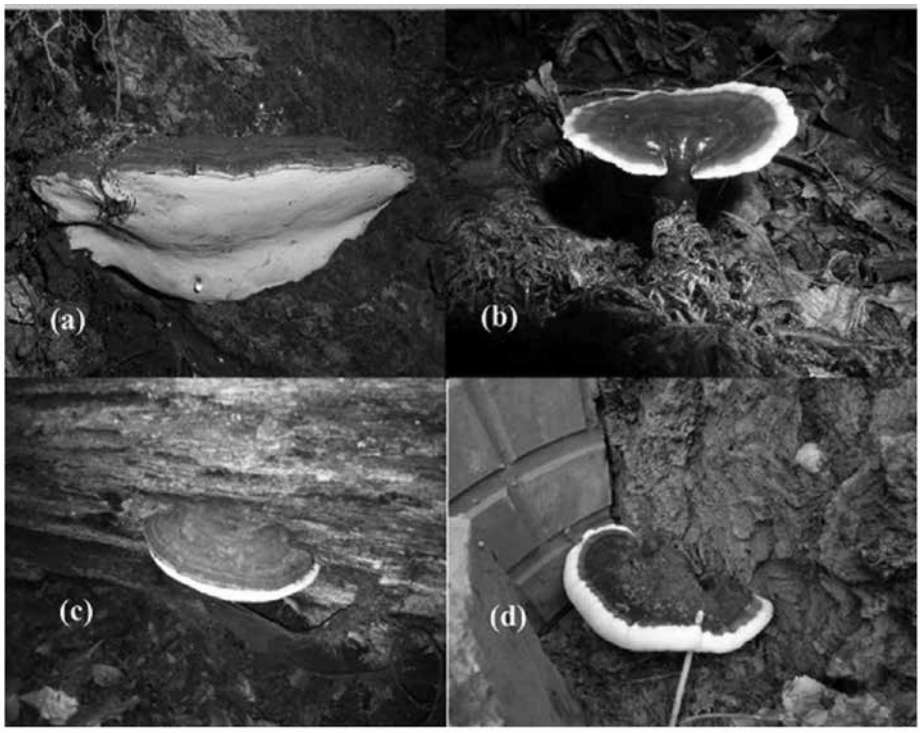


Fig 1. Four *Ganoderma* species collected from Iran; a) *G. applanatum*, b) *G. lucidum*, c) *G. adspersum*, d) *G. resinaceum*

2000). Recent investigations conducted by other scientists in the Hyrcanian forests of northern Iran led to report of five out of the nine previously documented *Ganoderma* species in Iran (Keypour et al., 2014a; Badalyan and Borhani, 2019). Figure one illustrates the macromorphological features of four species collected from the Hyrcanian forests. Among these species, *G. adspersum* which often misidentified as *G. australe* and *G. lucidum* have been frequently reported in all parts of Hyrcanian forests and mostly in Guilan Province respectively (Keypour et al., 2014; Badalyan & Borhani, 2019). According to Moncalvo and Buchanan (2008), *G. australe* is exclusively found in the Southern hemisphere, while *G. adspersum* is distributed in the northern hemisphere. Although there is a report of *G. australe* in the Kew Botanical Garden which this occurrence can be attributed to horticultural activities involving the introduction of the fungus's host from the Southern hemisphere. Thus, the appropriate scientific name for the species in Iran is *G. adspersum*. In contrast to the previous findings, *G. applanatum, G. tsugae*, and *G. resinaceum* were either absent or rarely encountered in Guilan Province (Keypour et al., 2014a; Amoopour et al., 2016). Subsequent investigations employing numerical taxonomy and other morphological studies on *Ganoderma* species in Iran revealed that these species could be classified into two distinct clades: non-laccate and laccate *Ganoderma*, based on their morphological characteristics (Keypour et al., 2014b). However, these studies demonstrated that host relationships and morphological traits were inadequate for reliable species identification (Keypour et al., 2014a; Keypour et al., 2014b). Notably, a novel host, *Pinus taeda* L., was reported for

a collected sample in Iran, which exhibited distinct morphological and cultural features. Subsequent investigations confirmed the identification of this sample as *G. lucidum* but the species has been separated from other collected *G. lucidum* specimens in the UPGMA dendrogram (Keypour et al., 2014b; Keypour et al., 2020).

Molecular identification, phylogeny and genetic diversity

The genus *Ganoderma* is known for its complex nature, with many species exhibiting similar morphological characteristics. Relying solely on morphological traits and host relationships for species identification can lead to erroneous conclusions in studies (Ryvarden, 2000). To resolve taxonomic discrepancies, Pires and Marinoni (2010) emphasized the utilization of molecular characteristics for accurate identification. Molecular markers such as the internal transcribed spacer (ITS) and mitochondrial small subunit (mtSSU rDNA) have proven valuable in elucidating phylogenetic relationships among *Ganoderma* taxa (Cao et al., 2012; Kinge et al., 2012). In a survey conducted by Badalyan et al. (2015) on genetic resources and mycelial characteristics of various Polypores from different countries using ITS sequencing, it was revealed that the Iranian strain (Gl-1093), previously identified as *G. lucidum* based on morphological features, is *G. resinaceum*.

Furthermore, investigations focusing on the ITS of a single *Ganoderma* sample confirmed the effective confirmation of the previous morphological identification of a collected sample from North Iran as *G. lucidum* (Heydarian and Hatamian-zarmi, 2017). Phylogenetic studies conducted on *Ganoderma* species in Iran utilizing ITS and mtSSU rDNA markers indicated that, despite Iran's geographical location in the Middle East (Southwest Asia), Iranian *Ganoderma* species are sister taxa to European *Ganoderma* species (Keypour et al., 2020). Additionally, molecular sequence analysis of *G. lucidum, G. resinaceum, G. adspersum*, and *G. applanatum* from European, trans-Caucasian, and Iranian regions demonstrated their close relationship while being distinct from East Asian strains (Badalyan et al., 2015; Keypour et al., 2020). In 2021, another experiment conducted using ITS sequencing of some polypores collected from Alamdardeh Forest in Mazandaran, Iran, showed that the *G. adspersum* (STF113) in their study is similar to the sample (HSBU200894) that was collected by Keypour et al. (2020), from Guilan, Iran (Keypour et al., 2020; Bari et al., 2021).

Genetic diversity plays a crucial role in shaping species morphology and their ability to endure various environmental changes and diseases. Consequently, the investigation of genetic diversity holds significant potential for comprehending species adaptation within a specific geographic region. Notably, a study conducted in 2019 by Keypour et al. revealed a substantial level of genetic diversity among laccate *Ganoderma* species in Iran (Keypour et al., 2019). Further investigations focusing on the *G. lucidum* complex collected from the Hyrcanian forests in northern Iran, utilizing the RAPD-PCR technique, unveiled a wide

Table 1. *Ganoderma* species reported from Iran since 1968 and their descriptions. **Tomophagus colossus* previously known as *Ganoderma colossus*, has been mentioned in the table due to its report as a genus of *Ganoderma* by some authors and not as a confirmed scientific name by the authors of this article

Species name	Morphological description
Ganoderma lucidum (Curtis) P. Karst.	Basidiomata sessile or centrally to laterally stipitate, laccate, 15 × 12.2 × 3.2 Cm. margin acute, yellow, or white in the active growing specimens turning to orange-red to dark reddish-brown. Stipe lateral, vertical, and cylindrical up to 3.5 Cm thick and 9.3 Cm long. Cutis is thin, shiny, and hymenodermis type. Context 1 Cm thick; creamy white becoming dark orange. The tube layer is dark brown up to 1.3 Cm thick. Pores circular to angular; 3–6 per millimeter. Basidiospores Ovid, double-walled, echinulate, ellipsoid, and truncate at the apex, 7–14 × 5–10 µm. Hyphal system trimitic; generative hyphae thin-walled, skeletal hyphae aseptate and binding hyphae.
Ganoderma resinaceum Boud.	Basidiomata stipitate, reddish-brown, laccate, 21.9 × 14.3 × 4.6 Cm. pileus irregularly rugose; margin rounded, light ochraceous; stipe absent or short, lateral, 3–5 × 2–3 Cm. Hymenial surface creamy white, later brown white, pores circular to angular, 4-6 per mm. context pale brown, up to 15 Cm thick at the base. Basidiospores truncated ellipsoid, light yellowish, 5–12 × 5–9 µm. The hyphal system was trimitic; generative hyphae thin-walled, skeletal hyphae aseptate, and binding hyphae.
Ganoderma adspersum (Schulzer) Donk.	Basidiomata sessile, non-laccate dark brown, up to 15.7 × 13.4 × 5.6 Cm. Context dark brown and 4 Cm thick. The tube layer is whitish, without distinct separating context zones between each tube layer and tubes, up to 1.9 Cm. 4 or 5 pores per millimeter. Basidiospores ovoid, with a rounded or truncate apex, spinulose, 6–12 × 4–9 µm in size. The cutis is 0.9 mm thick and characodermis type. The hyphal system is trimitic; the skeletal hyphae are thick-walled, binding hyphae branched, and generative hyphae.
Ganoderma applanatum (Pers.) Pat.	Basidiomata perennial, non-laccate, light brown to dark brown, sessile, fan-shaped to slightly convex, 27.5 × 20 × 9 Cm. The margin is rounded and white in the early stage. The context cinnamon brown to dark brown 5–20 mm thick. 4 or 5 pores per millimeter. Tube layer up to 2.4 mm thick. Basidiospores 5–12 × 4–8 µm. The crust of the characodermis type. The hyphal system; trimitic, and skeletal hyphae are thick-walled, binding hyphae branched, and thin-walled generative hyphae.
Ganoderma australe (Fr.) Pat.	in Iran, *Ganoderma adspersum* is mistaken with this species.
Ganoderma tsugae Murrill.	Basidiomata sessile, stipitate, laccate brown to orange, up to 20 ×30 ×5 Cm. Context white to brown and up to 3.5 Cm thick. The tube layer, ochraceous buff, multi-layered, up to 0.8 Cm. 4-6 pores per millimeter. Basidiospores ovoid, with a rounded apex, 9–11 × 6–8 µm in size. The hymenodermis type. The hyphal system is trimitic; the skeletal hyphae are thick-walled, binding hyphae branched, and generative hyphae.
Ganoderma colossus (Fr.) C.F. Baker.	Basidiomata sessile, laccate turning non-laccate when older, up to 35 x 9 Cm. Context cream to pale buff, up to 10 Cm thick. Chlamydospores are scattered through context and rare in the trama. The tube layer is pale brown, up to 3 Cm thick. 2-4 pores per millimeter. Basidiospores ellipsoid, 13-19.5 x 8-12.5 µm in size. Cutis hymenodermis. Hyphal system dimitic, generative hyphae thin-walled, skeletal hyphae thick-walled.
Ganoderma manoutchehrii Steyaert.	Basidiomata flat, subungulate, laccate, blackish brown. Context dark Kaiser brown and 4-5 Cm thick. The tube layer Cacao brown, without intervening layers of context tissue, up to 1.6-2 Cm. Pores round. Basidiospores ovoid, truncate at maturity, dark brown, 9.5–12 × 6.5-7 µm in size. The cutis is 0.24 mm thick and mummy brown with a central zone, close to the characodermis type. The hyphal system is trimitic; the skeletal hyphae are thick-walled, binding hyphae branched, and generative hyphae.
Ganoderma kosteri Steyaert.	Basidiomata sessile, non-laccate, blackish brown, up to 1.8 × 5 Cm. Context bay and 0.3-0.5 Cm thick. The tube layer up to 0.5 Cm, bay, generally striated by white lines. Pores are round and often irregular. Basidiospores are ovoid or subspherical, slightly fuliginous, 9-10.5 × 6.5-8 µm in size. The cutis is 0.15 mm thick and anamixoderm type.

Table 2. Biodiversity and host relationships of reported species

Species	Locality (province)	Hosts	References
G. lucidum	Mazandaran, Guilan, Golestan (northern Iran)	*Carpinus betulus, Pterocarya fraxinifolia, Populus caspica, Gleditschia caspica, Pinus taede, Fagus sp., Quercus castaneifolia*	(Buhse, 1960; Khabiri 1968; Saber, 1987; Saber & Minassian, 2000; Moradali et al., 2007; Keypour et al.,2008; Borhani et al.,2010; Borhani, Mousazadeh & Badalyan, 2013; Keypour et al., 2014a; Keypour et al., 2014b; Amoopour, Ghobad-Nejhad & Khodaparast, 2016)
G. applanatum	Mazandaran, Guilan, Golestan (northern Iran), Khuzestan (south of Iran)	*Citrus* spp., *Parrotia persica, Quercus* spp., *Pinus pinea, Fagus orientalis, Carpinus betulus, Quercus castaneifolia, Pinus* sp., *Acer* Sp.	(Khabiri 1968; Steyaert, 1975; Saber & Minassian, 2000; Moradali et al., 2007; Borhani et al., 2010; Borhani, Mousazadeh & Badalyan, 2013; Keypour et al., 2014a; Sefidi & Etemad, 2015; Amoopour, Ghobad-Nejhad & Khodaparast, 2016)
G. adspersum / G. australe	Mazandaran, Guilan, Golestan (northern Iran), Tehran	*Zelkova carpinifolia, Pterocarya fraxinifolia, Gleditschia caspica, Pinus taede, Albizzia julibrissin, Laurus* sp., *Carpinus betulus, Quercus* sp., hardwood trunk	(Saber & Minassian, 2000; Saber & Esmaeili, 2004; Moradali et al., 2007; Keypour et al., 2014a; Keypour et al., 2014b; Sefidi & Etemad, 2015; Amoopour, Ghobad-Nejhad & Khodaparast, 2016)
G. resinaceum	Mazandaran, Guilan, Golestan (northern Iran), Khuzestan (south of Iran)	*Prunus persica, Acer velatinum, Carpinus betulus, Gleditschia caspica, Populus deltoids, Morus alba, Manilkara zapota*	(Steyaert, 1980; Saber, 1987; Saber & Minassian, 2000; Moradali et al., 2007; Keypour et al., 2014a; Keypour et al., 2014b; Amoopour, Ghobad-Nejhad & Khodaparast, 2016; Keypour & Asef 2020)
G. manoutchehrii	Mazandaran (northern Iran)	*Acacia* sp.	(Steyaert, 1972)
G. tsugae	Guilan (northern - Iran)	Conifer	(Moradali et al., 2007; Amoopour, Ghobad-Nejhad & Khodaparast, 2016; Saber & Minassian, 2000)
G. colossus	South of Iran	*Ziziphus spina-christi, Ficus benghalensis*	(Saber, 2000)
G. kosteri	Mazandaran (northern Iran)	*Carpinus betulus, Parrotia persica*	(Ghobad-Nejhad & Hallenberg, 2012)

Fig. 2. *Ganoderma* species dispersal in Iran

spectrum of diversity, with inter-specific diversities of 61.48 and 40.16 observed for the laccate species *G. lucidum* and *G. resinaceum*, respectively (Keypour et al., 2019). Ariffin et al. (2000) suggested that the heightened genetic diversity observed in *Ganoderma* species may be attributed to their adaptation to a broad range of host organisms, a characteristic that is also evident in *Ganoderma* species collected from Iran (Table 1 and 2). The extensive genetic diversity observed in laccate *Ganoderma* species collected from Iran, as reported by Keypour et al. (2019), confirms the finding that a diverse array of tree species across various regions of Iran are susceptible to laccate *Ganoderma* infections. This underscores the significance of early-stage disease management in promoting forest survival and preservation.

Biodiversity and host relationship

Numerous studies have been conducted to explore the taxonomy, biodiversity, and host relationships of *Ganoderma* species in Iran. Noteworthy contributions include the works of Khabiri (1968), Saber (1972), Soleimani (1976), Hallenberg (1979), Moradali et al. (2007), Keypour et al. (2008), Ghobad-Nejad and Hallenberg (2012), Rostamian et al. (2013), Borhani et al. (2013), Keypour et al. (2014a, 2014b), and Keypour and Asef (2020) (Table 2). These investigations have significantly enhanced our understanding of *Ganoderma* species in Iran.

The initial comprehensive survey on the taxonomy and biodiversity of *Ganoderma* species in Iran was conducted by Moradali et al. (2007), which paved the way for subsequent studies focusing on various aspects of *Ganoderma* (Keypour et al., 2008; Ershad, 2009; Keypour et al., 2014a, 2014b; Sefidi and Etemad, 2015; Amoopour et al., 2016; Badalyan and Borhani, 2019). Recent research findings indicate a higher incidence of *Ganoderma* species infecting trees compared to earlier reports by Ershad

in 2009 (Keypour et al., 2014a, 2014b). Further investigations have shed light on the influence of field slope on fungal sporocarp abundance. Bari et al. (2019) revealed that *G. adspersum* and *G. lucidum* were found on slopes of various orientations in the forests of northern Iran, while *G. tsugae* was observed exclusively on the northwest slope and in *Fagus* forests, contrary to previous beliefs associating it solely with coniferous trees. Additionally, *G. applanatum* was documented on both northern and southern slopes (Borhani et al., 2013; Badalyan and Borhani, 2019).

Bari et al. (2019) attributed the high diversity of macro-fungi in the forests of northern Iran to the increased moisture content, altitude, and management and logging practices. Among the *Ganoderma* species, *G. adspersum* and *G. lucidum* exhibited the highest frequency of occurrence, while *G. tsugae*, *G. resinaceum*, and *G. applanatum* were less commonly found in the forests of northern Iran (Amoopour et al., 2016). Notably, Keypour and Asef (2020) provided an updated account of the occurrence of *G. resinaceum* in Iran, confirming its presence in the Khuzestan province (southern Iran) and on two previously unreported host species. Figure two shows *Ganoderma* species biodiversity in Iran.

Discussion

In contrast to several other Middle Eastern countries, Iran exhibits a diverse array of vegetation due to its distinctive environmental characteristics, which have a significant impact on plant diversity and abundance (Heshmati, 2007). Although scattered trees, forests, and favorable climatic conditions in other parts of the country also facilitate the occurrence of *Ganoderma* species, the northern region of Iran stands out in terms of plant richness, (Keypour and Asef, 2020). Keypour and Asef (2020) published a report documenting the presence of *G. resinaceum* in southern Iran, representing the first record of this species in that region. It is worth noting that while the majority of *Ganoderma* species are commonly found in the Hyrcanian forests of the Caspian Sea region due to the favorable climatic conditions supporting their growth (Badalyan and Borhani, 2019), certain species can be encountered in other parts of Iran where the climate may not be as conducive to their development. This recent report indicates a higher incidence of *Ganoderma* species infection among trees within the country, surpassing initial expectations. As a result, there is an augmented risk of the development of *Ganoderma*-induced diseases, such as root rot or butt rot, in affected trees.

This article provides a comprehensive collection and analysis of various studies conducted since 1860 on the morphology, taxonomy, phylogeny, and biodiversity of *Ganoderma* species isolated from Iran.

In general, different *Ganoderma* species have been reported in Iran over the years (Steyaert, 1972; Saber & Minassian, 2000; Moradali et al., 2007). However, recent studies have confirmed the presence of only five species, namely *G. lucidum, G. resinaceum, G. tsugae, G. applanatum*, and *G. adspersum*, in forests of Iran (Keypour et al., 2014a; Keypour et al., 2014b; Badalyan

and Borhani, 2019). Despite repeated visits to the type locality of *Ganoderma manoutchehri* in different years, Moradali and Keypour were unable to locate the species. Furthermore, an investigation of the IRAN Herbarium to examine the collected samples identified as *Ganoderma kosteri* and *Ganoderma manoutchehrii* yielded no results. As for the species *Ganoderma kosteri*, two samples collected from Iran were found to have been deposited in the Meise Herbarium, although information regarding the identifier(s) was incomplete.

This study contributes to the understanding of *Ganoderma* species, causing rot root in trees, in Iran by investigating their morphological taxonomy, phylogeny, host relationships, and biodiversity. It is important to note that the use of molecular tools for accurate species identification has gained prominence among scientists. By employing molecular techniques, it becomes possible to discern the susceptibility of different tree species to specific *Ganoderma* species. Molecular techniques enable the development of rapid and accurate diagnostic tools for detecting *Ganoderma* infections. These tools can detect the presence of the fungus in tree tissues or soil samples, allowing for early detection and timely implementation of control measures. Moreover, the morphological variations observed among species within the *Ganoderma lucidum* complex posed challenges for taxonomical identification and pharmaceutical companies. However, scientists have successfully overcome these challenges by employing molecular analysis techniques. In recent years, molecular markers have been utilized to assess the genetic diversity and authenticate the identification of *Ganoderma* species collected from Iran. It is worth mentioning that further studies incorporating specific molecular markers, in addition to ITS and mtSSUrDNA, hold the potential to yield new insights into the taxonomy of *Ganoderma* species in Iran in the future.

Acknowledgment

The authors express their deepest gratitude to Dr. Ghorbani Nohooji, Institute of Medicinal Plants, ACECR, for his valuable comments on the manuscript and Dr. Moradali University of Louisville, for providing us with valuable references.

References

Adaskaveg, JE and Gilbertson RL. (1988). Basidiospores, pilocystidia, and other basidiocarp characters in several species of the *Ganoderma lucidum* complex. Mycologia. 80 (4): 493-507. https://doi.org/10.1080/00275514.1988.12025571

Amoopour M, Ghobad-Nejhad M, Khodaparast SA. (2016). New records of polypores from Iran, with a checklist of polypores for Gilan Province. Czech Mycology. 68 (2): 139-148.

Ariffin D, Idris AS, Singh G. (2000). Status of *Ganoderma* in oil palm. In *Ganoderma* diseases of perennial crop. pp. 49-68. Wallingford UK: CABI.

Badalyan SM., and Borhani A. (2019). Morphological and growth characteristics of mycelial collections of medicinal xylotrophic mushrooms (Agaricomycetes) distributed in forests of Northern Iran. Proceedings of the YSU

B: Chemical and Biological Sciences. 53 (2): 249. 97-106. Doi: https://doi.org/10.46991/PYSU:B/2019.53.2.097.

Badalyan SM, Shnyreva AV, Iotti M, Zambonelli A. (2015). Genetic resources and mycelial characteristics of several medicinal polypore mushrooms (Polyporales, Basidiomycetes). International journal of medicinal mushrooms. 17 (4). Doi: 10.1615/intjmedmushrooms.v17.i4.60.

Bari E, Karimi K, Aghajani H, Schmidt O, Zaheri S, Tajick-Ghanbary MA, Juybari HZ. (2021). Characterizations of tree-decay fungi by molecular and morphological investigationsin aniranian alamdardeh forest. Maderas. Ciencia y Tecnología, 23. Doi: http://dx.doi.org/10.4067/s0718-221x2021000100433.

Bari E, Aghajani H, Ohno KM, Shahi R., Hale MD, Bahmani M. (2019). Ecology of wood-inhabiting fungi in northern forests of Iran. Forest Pathology, 49(2), e12501. Doi: https://doi.org/10.1111/efp.12501.

Bishop KS, Kao CH, Xu Y, Glucina MP, Paterson RRM, Ferguson LR. (2015). From 2000 years of *Ganoderma lucidum* to recent developments in nutraceuticals. Phytochemistry, 114, 56-65. https://doi.org/10.1016/j.phytochem.2015.02.015.

Bodeker G. (2012). Integrative oncology meets immunotherapy: new prospects for combination therapy grounded in Eastern medical knowledge. Chinese journal of integrative medicine, 18(9), 652-662. Doi: https://doi.org/10.1007/s11655-012-1201-0.

Borhani A, Badalyan SM, Garibyan NN, Mosazadeh SA. (2010). Diversity and distribution of macro fungi associated with beech forests of Northern Iran (case study Mazandaran Province). World Applied Sciences Journal. 11 (2): 151-158.

Borhani A, Mousazadeh SA, Badalyan S. (2013). Introducing macrofungi at beech stands in Mazandaran province. Journal of Wood & Forest Science and Technology, 20, 23-40. (In Persian). Doi: 20.1001.1.23222077.1392.20.3.2.4.

Buhse F. (1860). Aufzählung der auf einer Reise durch Transkaukasien und Persien gesammelten Pflanzen in Gemeinschaft mit Dr. E. Boissier in Genf (travel report with several indices, one map and plant illustrations). Nouveaux Mémoires de la Société Impériale des Naturalistes de Moscou 12: i–lxvii + 1–246.

Cao Y, Wu SH, Dai YC. (2012). Species clarification of the prize medicinal Ganoderma mushroom “Lingzhi”. Fungal Diversity, 56, 49-62. https://doi.org/10.1007/s13225-012-0178-5.

De Simone D and Annesi T. (2012). Occurrence of *Ganoderma adspersum* on *Pinus pinea*. Phytopathologia Mediterranea. 374-382.

El-Mekkawy S, Meselhy MR, Nakamura N, Tezuka Y, Hattori M, Kakiuchi N, Shimotohno K, Kawahata T, Otake T. (1998). Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. Phytochemistry. 49 (6): 1651-1657. Doi: https://doi.org/10.1016/S0031-9422(98)00254-4.

Ershad J. (2009). Fungi of Iran. 3th edition.

Tehran: Iranian Research Institute of Plant Protection, Iran.

Ghobad-Nejhad M and Hallenberg NILS. (2012). Checklist of Iranian non-gilled/non-gasteroid hymenomycetes (Agaricomycotina). Mycotaxon. 119 (494): 1-41. Doi: 10.22043/mi.2021.123456.

Hallenberg N. (1979). Wood-fungi (Polyporaceae, Ganodermataceae, Hymenochaetaceae, Cyphellaceae, Clavariaceae, Auriculariaceae, Tremellaceae, Dacrymycetaceae) in N Iran 2. Iranian Journal of Plant Pathology. 15: 11-31.

Hapuarachchi KK, Wen TC, Deng CY, Kang, JC, Hyde KD. (2015). Mycosphere essays 1: Taxonomic confusion in the *Ganoderma lucidum* species complex. Mycosphere. Doi: 10.5943/mycosphere/6/5/4.

Heshmati GA. (2007). Vegetation characteristics of four ecological zones of Iran. International Journal of Plant Production. 1 (2): 25-224. Doi: 10.22069/ijpp.2012.538.

Heydarian M and Hatamian-Zarmi, A. (2017). Molecular identification of Ganoderma lucidum from Iran. Rostaniha 17, 188-192. Doi: http://dx.doi.org/10.22092/botany.2017.109434.

Huang WC, Chang MS, Huang SY, Tsai C. J, Kuo PH, Chang HW, Huang ST, Kuo CL, Lee SL, Kao MC. (2019). Chinese herbal medicine Ganoderma tsugae displays potential anti-cancer efficacy on metastatic prostate cancer cells. International Journal of Molecular Sciences, 20(18), 4418. Doi: https://doi.org/10.3390/ijms20184418.

Keypour S and Asef MR. (2020). New reports on locality and host relationship of *Ganoderma resinaceum* from Iran. Australasian Plant Pathology. 49(2), 175-178. Doi: https://doi.org/10.1007/s13313-020-00688-7.

Keypour S, Riahi H, Asef MR, Abdollahzadeh J, Borhani A, Safaie N. (2020). The true nature of Ganoderma in Iran: Taxonomy based on ITS and mtSSU rDNA. Forest Pathology. 50 (4): e12605. Doi: https://doi.org/10.1111/efp.1260.

Keypour S, Riahi H, Ebadi M, Borhani A, Asef Shayan MR, Safaie N. (2019). The Study of the genetic diversity of two laccate species of *Ganoderma lucidum* and *Ganoderma resinaceum* using RAPD marker. Nova Biologica Reperta. 5 (4): 379-388. Doi:10.29252/nbr.5.4.379.

Keypour S, Riahi H, Safaie N, Borhani A. (2014a). Mycelial growth rate and macro- and micromorphological characteristics of medicinal species of genus *Ganoderma* (Higher Basidiomycetes) from Iran. International journal of medicinal mushrooms, 16 (4). Doi: 10.1615/IntJMedMushrooms.v16.i4.70

Keypour S, Riahi,H, Borhani A, Shayan MRA, Safaie N. (2014b). Survey on wood decay fungi *Ganoderma* species (Ganodermataceae; Polyporales) from Guilan and Mazandaran, Iran. International Journal of Agriculture and Biosciences. 3 (3): 132-135. Doi: http://www.ijagbio.com/pdf-files/volume-3-no-3-2014/132-135.pdf.

Keypour S, Riahi H, Moradali M F, Rafati H. (2008). Investigation of the antibacterial

activity of a chloroform extract of Ling Zhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae), from Iran. International journal of medicinal mushrooms, 10 (4). Doi: 10.1615/IntJMedMushr.v10.i4.70.

Khabiri E. (1968). Plant diseases: parasitic fungi, 5. Tehran University Press, Tehran. [In Farsi].

Kinge TR, Mih AM. Coetzee MPA. (2012). Phylogenetic relationships among species of *Ganoderma* (Ganodermataceae, Basidiomycota) from Cameroon. Australian Journal of Botany. 60 (6): 526-538. Doi: https://doi.org/10.1071/BT12011.

Lee JS, Lim MO, Cho KY, Cho JH, Chang SY, Nam DH. (2006). Identification of medicinal mushroom species based on nuclear large subunit rDNA sequences. Journal of microbiology-Seoul. 44 (1): 29. Doi:10.1007/0-387-26336-5_163.

Moncalvo JM and Buchanan PK. (2008). Molecular evidence for long distance dispersal across the Southern Hemisphere in the *Ganoderma applanatum-australe* species complex (Basidiomycota). Mycological Research. 112 (4): 425-436. Doi: https://doi.org/10.1016/j.mycres.2007.12.001.

Moncalvo JM, Lutzoni FM, Rehner SA, Johnson J, Vilgalys R. (2000). Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. Systematic Biology. 49 (2): 278-305. Doi: https://doi.org/10.1093/sysbio/49.2.278.

Moncalvo JM, Wang HH, Hseu RS. (1995). Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. Mycologia. 87 (2): 223-238. Doi: https://doi.org/10.1080/00275514.1995.12026524.

Moradali MF, Hedjaroude GA, Mostafavi H, Abbasi M, Ghods S, Sharifi-Tehrani, A. (2007). The genus *Ganoderma* (Basidiomycota) in Iran. Mycotaxon. 99: 251-270.

Ooi LS, Ooi VEC, Fung MC. (2002). Induction of gene expression of immunomodulatory cytokines in the mouse by a polysaccharide from Ganoderma lucidum (Curt.: Fr.) P. Karst. (Aphyllophoromycetideae). International Journal of Medicinal Mushrooms, 4 (1): Doi:10.1615/IntJMedMushr.v4.i1.30.

Pires AC and Marinoni L. (2010). DNA barcodin and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. Biota Neotropica. 10: 339-346. Doi: https://doi.org/10.1590/S1676-06032010000200035

Richter C, Wittstein K, Kirk PM, Stadler M. (2015). An assessment of the taxonomy and chemotaxonomy of *Ganoderma*. Fungal Diversity. 71: 1-15. Doi: https://doi.org/10.1007/s13225-014-0313-6.

Rostamian M, Kavosi MR, Shataee S, Mohammad Alipour Malekshah AA. (2013). Relationship of trees decaying whit wood fungi and some of habitat factors in the Shastkolateh forest of Gorgan. Journal of Wood and Forest Science and Technology. 20 (3): 167-181. Doi: 20.1001.1.23222077.1392.20.

3.11.3.

Ryvarden L. (2000). Studies in neotropical polypores 2: a preliminary key to neotropical species of Ganoderma with a laccate pileus. Mycologia, 92 (1): 180-191. Doi: https://doi.org/10.1080/00275514.2000.12061142.

Ryvarden L. (1991). Genera of polypores. Nomenclature and taxonomy. Synopsis Fungorum. 5: 1-363.

Saber M and Esmaeili Taheri A. (2004). A report on macromycete fungi from different parts of Iran. Proceedings of 16th Iranian Plant Protection Congress, Tabriz. Abstract book, p. 464.

Saber M. and Minassian V. (2000). Two Ganoderma species new to Iran. Proceedings of 14th Iranian Plant Protection Congress, Isfahan. Abstract book, p. 379.

Saber M. (1987). Contribution to the knowledge of Aphyllophorales collected in Iran. Iranian Journal of Plant Pathology, 23, 63–101 [In Farsi, with a summary in English on pp. 21–36].

Saber M. (1972). Identification of Homobasidiomycetes collected in Iran. Iranian Journal of Plant Pathology.

Saltarelli R, Ceccaroli P, Iotti M, Zambonelli A, Buffalini M, Casadei L, Stocchi, V. (2009). Biochemical characterisation and antioxidant activity of mycelium of *Ganoderma lucidum* from Central Italy. Food Chemistry, 116(1), 143-151. Doi: https://doi.org/10.1016/j.foodchem.2009.02.023.

Sefidi K and Etemad V. (2015). Dead wood characteristics influencing macrofungi species abundance and diversity in Caspian natural beech (*Fagus orientalis* Lipsky) forests. Forest Systems. 24 (2): 15. Doi: 10.5424/fs

Soleimani P. (1976). Wood destroying fungi in Iran. European Journal of Forest Pathology. 6 (2): 75-79. Doi: https://doi.org/10.1111/j.1439-0329.1976.tb00510.x.

Steyaet RL. (1980). Study of some *Ganoderma* species. Bulletin du Jardin botanique national de Belgique/ Bulletin van de Nationale Plantentuin van België. 135-186. Doi: https://doi.org/10.2307/3667780.

Steyaert RL. (1975). CMI descriptions of pathogenic fungi and bacteria. 443-448.

Steyaert RL. (1972). Species of *Ganoderma* and related genera mainly of the Bogor and Leiden Herbaria. Persoonia-Molecular Phylogeny and Evolution of Fungi. 7 (1): 55-118.

Tchotet Tchoumi JM, Coetzee MPA, Rajchenberg M, Wingfield MJ, Roux, J. (2018). Three *Ganoderma* species, including *Ganoderma dunense* sp. nov., associated with dying Acacia cyclops trees in South Africa. Australasian Plant Pathology. 47: 431-447. Doi: https://doi.org/10.1007/s13313-018-0575-7.

Wasser SP, Zmitrovich IV, Didukh MY, Spirin WA, Malysheva VF. (2006). Morphological traits of *Ganoderma lucidum* complex highlighting *G. tsugae* var. *jannieae*: the current generalization. ARA Gantner Verlag K.-G.

Zeng Q, Zhou F, Lei L, Chen J, Lu J, Zhou J, Cao K, Gao L, Xia F, Ding S, Huang L, Xiang H, Wang J, Xiao Y, Xiao R, Huang J. (2017). *Ganoderma lucidum*

polysaccharides protect fibroblasts against UVB-induced photoaging. Molecular Medicine Reports. 15 (1): 111-116. Doi: https://doi.org/10.3892/mmr.2016.6026