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Enhancing the growth and chemical composition of cardoon plants by fertilization with azolla, mycorrhizae, chitosan, and EDTA-Zinc

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Due to the limitations and high cost of chemical fertilizers, in addition to their negative effects on medicinal plants—especially pharmaceutical ones—two-season field experiments were conducted in clay loam soil to evaluate the efficacy of azolla, mycorrhizae, chitosan, or EDTA-Zinc on the growth, oil content, and chemical analysis of *Cynara cardunculus* plants. Azolla, mycorrhizae, and chitosan were applied alone or in combination with EDTA-Zinc. The growth traits were enhanced by foliar application of chitosan in terms of the number of leaves, heads, and seeds per head or plant, as well as chlorophyll content, while fertilization with azolla powder plus EDTA-Zinc resulted in the maximum values of leaf length, flower stem length, fresh leaf weight, and seeds per plant, and significantly improved the oil yield per plant. The highest percentage of oil (25.27% and 25.33%) was obtained from seeds of plants treated with mycorrhizae plus zinc in the two seasons. Additionally, the same treatment gave the highest levels of phenolic and flavonoid compounds in cardoon leaves. The major constituents of the fixed oil were linoleic acid, oleic acid, palmitic acid, and stearic acid. The highest amount of unsaturated fatty acids was found in azolla liquid and mycorrhizae treatments. Our findings indicated that the application of azolla, mycorrhizae, and chitosan can be used alone or as a complement to EDTA-Zinc to enhance growth, oil percentage, and oil yield in cardoon plants. Applying these biofertilizers had a significant effect by accelerating plant growth.

Keywords: Cardoon; Biofertilizers; Biomass; Medicinal plants; Oil constituents

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INTRODUCTION

The importance of cultivating medicinal and aromatic plants has increased because of their distinctive compositions. Both modern and traditional medicine can benefit greatly from their enormous potential. Raw materials for the pharmaceutical, cosmetic and fragrance industries also utilize them. The cardoon plant (*Cynara cardunculus* L.) is a herbaceous perennial species that grow in the Mediterranean basin. A highly heterozygous genetic background is promoted by the protandrous and asynchronous sexual maturity of this cross-pollinated diploid species (2n = 2x = 34) (Portis et al., 2005). It has been established that the wild cardoon plant [*C. cardunculus* var. *sylvestris* FIORI] is the parent of the cultivated cardoon (*C. cardunculus* var. *altilis* DC.) and the globe artichoke [*C. cardunculus* var. *scolymus* L. BENTH] based on the analysis of isoenzymes and molecular markers (Rottenberg et al., 1996; Sonnante et al., 2007). As a result of the species' allogamous nature, seeds generally exhibit a degree of variability. It has been used as food and medicine because it has added value as a rich source of oils, fibers, and various bioactive compounds (Barracosa et al., 2019).

The polar extracts of cardoon contain valuable polyphenolic compounds, especially caffeoylquinic acids and flavonoids, which have been isolated from the plant (Petropoulos et al., 2018). Flavonoid compounds have a crucial protective role against extreme ultraviolet radiation (Pandino et al., 2011). Flavonoids, which include luteolin, apigenin, and silymarin, are thought to be the main phytochemical components found in cardoon leaves. Silymarin is essential for managing hepatic disorders, including non-alcoholic fatty liver disease (Roy & Lyer, 2019). In addition, it has anti-inflammatory properties that help with brain and spinal cord injuries (Swaminathan et al., 2019). The natural bioactive compound known as apigenin has been shown to possess anti-cancer properties, specifically against cancers of the breast, skin, prostate, and some hematological malignancies (Shukla & Gupta, 2010). The production of dry biomass for energy production, raw material in paper pulp, and lignocellulosic biomass for lightwood panels is possible with cardoon (Fernandez et al., 2006; Gominho et al., 2009 and Martins et al., 2019). Achenes are used in the production of biofuels and as a source of oil that is safe for human consumption because of their high nutritional value (Curt et al., 2002; Raccuia & Melilli, 2007). The seed oil content varies between 14.5% and 32.4% depending on the cardoon population and on the extraction method (Carvalho et al., 2006; Fernandez et al., 2006).

The productivity of medicinal and aromatic crops is significantly correlated with the type and quantity of applied fertilizers. The significance of soil health and its contribution to agriculture sustainability has prompted many researchers, especially in studies of the medicinal and aromatic plants, to pay attention to

biological and organic fertilizers as a partial or even complete alternative to synthetic fertilizers. The application of biofertilizers to the soil creates a healthy environment for plant growth and protects plants from diseases. Such biofertilizers are used for inoculating seeds, soil, or both to enhance the availability of plant nutrients. The well-known aquatic pteridophyte genus azolla is found in tropical and semitropical areas naturally (Alalade & Iyayi, 2006). It is an aquatic fern that floats freely and grows quickly on the water's surface. Due to its symbiotic relationship with the blue-green algae (*Anabaena azollae*), which is an excellent source of biological nitrogen, it serves as a highly effective provider of biological nitrogen. Azolla demonstrated a significant increase in both dry matter yield and chlorophyll content in the sarpagandha plant (Agnihotri, 2019). When azolla was introduced to the Biduri plant at a rate of 12.5 tons per hectare, the plants' height and leaf development increased considerably in comparison to when Azolla was not applied (Sakya et al. , 2021).

Arbuscular mycorrhizal (AM) fungi can contribute significantly to the preservation of certain important medicinal plants. By altering host physiology, AM fungi can either directly or indirectly interact with other soil microorganisms, modifying their structure, function, and secretion behaviors within the mycorrhizosphere. More than 80% of terrestrial plants, comprising many important crops and tree species found in forests, form mutualistic symbiotic relationships with mycorrhizae (Smith & Zhu, 2001; Gentili & Jumpponen, 2006). The fungi receive carbohydrates from the plant roots in exchange for providing nutrients and water (Tiwari & Adholeya, 2005). When mycorrhizae were applied to dill plants, the number of seeds produced by each plant and the overall seed production increased by approximately 32% and 32.5%, respectively, compared to untreated plants (Hashemzadeh et al., 2013).

Chitosan is a natural polymer that is easily obtained from shellfish waste and is used in food processing. It is produced by deacetylating chitin and has been shown to have a beneficial impact on the growth of roots, shoots, and leaves in various crop plants (Chibu & Shibayama, 2001; Wanichpongpan et al., 2001; El-Tanahy et al., 2012; and Hemdan et al., 2023). Chitosan can be used in liquid form as a foliar spray (Abdel-Aziz et al., 2018) or applied as a powder to the soil, either as a fertilizer or as a plant defense enhancer against abiotic stresses (Walker et al., 2004; Abdalla et al., 2017; Hidangmayum et al., 2019; Drwish et al., 2023). Fertilizing *Thymus daenensis* Celak plants with chitosan as a foliar application at 400 mg L⁻¹ significantly increased biomass yields and phenolic compounds (Bistgani et al., 2021). When chitosan was added to the soil, it increased plant height, crown width, and leaf area in pepper plants (Faqir, 2021). Chitosan and nanochitosan can induce plant defense mechanisms against different pathogens including fungi and bacteria (Kanawi et al., 2021; Onaran, 2021; Imara et al., 2024).

Zinc deficiency is the most pervasive micronutrient deficiency problem, leading to significant losses in yield and nutritional quality. Zinc is an essential micronutrient with specific physiological roles, including maintaining the structural and functional integrity of biological membranes and facilitating gene expression and protein synthesis (Kobraee et al., 2011).

Previous studies have not documented the growth and chemical composition responses of *Cynara cardunculus* to treatments with azolla, mycorrhizae, and chitosan. Therefore, this study aims to evaluate the effects of these biofertilizers - applied either individually or in combination with EDTA-Zinc - on enhancing cardoon growth, oil yield, and selected chemical parameters.

MATERIALS AND METHODS

Plant material and experimental procedures

Cardoon seeds (*Cynara cardunculus* var. altilis) were obtained from Sekem Company. Dr. Ahmad Saeed, Professor of Horticulture at the Faculty of Agriculture, Moshtohor, Benha University, Egypt, authenticated the aerial parts (leaves and floral stems) collected during the flowering period. Voucher specimens (CC10) were deposited in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Zagazig University, Egypt.

During the two consecutive seasons of 2021–2022 and 2022–2023, two field experiments were carried out at the Experimental Nursery of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza (30°01'39.36"N, 31°12'36.50"E) to evaluate the impact of fertilization with biofertilizers (azolla as powder or extract and mycorrhizae) and stimulant substances (chitosan), either alone or in combination with EDTA-Zinc, on growth parameters, seed yield, oil percentage, oil yield, and chemical constituents of cardoon (*Cynara cardunculus* L.) grown as an

annual crop. Prior to fertilizer application, soil samples (0–30 cm depth) were collected, and standard analytical procedures were followed to determine soil characteristics. During the first and second seasons, the soil had a clay loam texture, with pH values of 7.65 and 7.84, EC values of 2.12 and 1.85 dS/m, organic matter content of 1.38% and 1.86%, available nitrogen (N) of 11.15 and 168.20 ppm, available phosphorus (P) of 12.50 and 16.85 ppm, and available potassium (K) of 180 and 195 ppm, respectively.

Cardoon seeds were sown on October 20th and 25th in the first and second seasons, respectively, on ridges spaced 60 cm apart with 50 cm between hills (2–3 seeds per hill). Each plot covered an area of 12 m², consisting of five 4-m-long ridges. Seed emergence occurred within 7–10 days and thinning to one plant per hill was performed four weeks after sowing. The experiment followed a randomized complete block design (RCBD) with three replicates and ten treatments per season (to be discussed in detail later).

Azolla was applied in two forms: dry powder incorporated into the soil and liquid extract as a foliar spray. The powdered form was added to soil at a rate of 5 tons/ha (equivalent to 6 kg per 12 m² plot). For the extract preparation, a 40% azolla extract was obtained from fresh plant material following the extraction technique of Wilson et al. (1997) with modifications according to Altai et al. (2019).The extraction procedure was as follows: Fresh plant material was first cleaned with tap water followed by distilled water. The cleaned material was then placed in sterilized plastic bags with distilled water at a 1:1 (w/v) ratio and stored at -20°C for at least 12 hours. After freezing, the plant material was thawed at room temperature. During thawing, cell fluids were released from the tissues and collected in the bag's corner. The mixture was then filtered through two layers of cheesecloth after 5 minutes of mixing, followed by centrifugation at 12,000 rpm for 30 minutes. The supernatant was sterilized using 0.22 µm membrane filters and stored at 5°C until use. For application, the crude extract (considered 100% concentration) was diluted to 40% using distilled water. Foliar spraying was performed three times, with the first application 40 days after planting and subsequent applications at 30-day intervals.

Mycorrhizae were added during seed sowing at a rate of 15 g/hill. To achieve the desired concentrations, chitosan was dissolved in tap water and sprayed at 2500 ppm three times: 40, 70, and 100 days after sowing (DAS). EDTA-Zinc was sprayed at 1000 ppm three times; the first application began 40 days after planting (DAP), followed by subsequent applications at 30-day intervals. The foliar application of liquid fertilizers was done before runoff. Irrigation was performed immediately after adding soil fertilizers (azolla and mycorrhizae) and seed sowing. Azolla, mycorrhizae, and chitosan were applied alone or in combination with EDTA-Zinc. Therefore, the field experiments included ten treatments: control (no fertilization), azolla powder, azolla liquid, mycorrhizae, chitosan, EDTA-Zinc, as well as the combinations between EDTA-Zinc and each of azolla either powder or liquid, mycorrhizae, and chitosan.

Data recorded

Five months after sowing, the following parameters were measured each season: leaf length, number of leaves per plant, average fresh leaf weight (g), and chlorophyll content (mg/g fresh weight). The total chlorophyll content of fresh leaves was determined using a SPAD-502 chlorophyll meter (Spectrum Technologies Inc., Plainfield, IL), where 1 SPAD unit corresponds to 10 mg chlorophyll/100g fresh weight (Netto et al., 2005).Two months later, flower stem height and number of heads per plant were measured. At harvest maturity, the following yield components were recorded: head dry weight, number of heads per plant, number of seeds per head, number of seeds per plant, seed yield per plant, oil percentage, and oil yield per plant.

Chemical Analysis

Oil Extraction: Cardoon seeds were extracted using petroleum ether in a Soxhlet extraction apparatus. The solvent was evaporated using a rotary evaporator, yielding yellow oil (AOAC, 2016). The extracted oil was stored in amber vials at 4°C until analysis.

Fatty Acids Analysis: The fatty acid composition was determined by gas chromatography-mass spectrometry (GC-MS) during the second season. Fatty acid methyl esters (FAMEs) were prepared according to Morrison and Smith (1964). Analysis was performed using a Thermo Scientific Trace GC1300-TSQ mass spectrometer (Austin, TX, USA) equipped with a TG-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness). The following was the programming for the oven temperature: 40°C for two minutes, followed by 4°C/min for two minutes at 280°C. Injections were performed in the split mode with a split flow of 1:50 and the injector temperature was 250°C. The m/z scanning range was 50–650 Da,

the ion source temperature was 250°C, and the ionization energy was 70 eV (electron impact ionization). Compound identification was achieved using WILEY 09 and NIST 11 mass spectral databases with literature verification. Analyses were conducted at the Cairo University Research Park Laboratory (CURP), Faculty of Agriculture.

Acid Value (AV): Determined according to AOAC (1993) methods. The results were expressed as mg KOH required to neutralize free fatty acids per g of oil.

Iodine Value (IV): Measured using AOAC Official Method (2005). Results reported as g I_2 absorbed per 100 g oil.

Peroxide Value (PV): Analyzed following AOAC Official Method (2005). Milliequivalents of O2 per kilogram of oxidized oil were used to express the findings.

Total Phenolic Content (TPC): Samples were taken at 5-months-old, dried cardoon leaves, and ethanol extract were quantified using the Folin-Ciocalteu method (Singleton & Rosi, 1965). Absorbance measured at 760 nm against blank. Expressed as mg gallic acid equivalents per g dry weight (mg GAE/g).

Total Flavonoids Content: Dried leaf samples from 5-month-old plants were extracted by ethanol. After that determined colorimetrically (Dewanto et al., 2002). Absorbance measured at 510 nm. Expressed as mg catechin equivalents per g dry weight (mg CE/g).

Mineral Content Analysis: Approximately 0.5 g of dried cardoon leaves from second-season samples were ground separately in a Wiley mill to pass through a 20-mesh screen, and their N, P, and K contents were measured. Nitrogen: Determined by the Kjeldahl method (Bremner, 1965). Phosphorus and Potassium: Dry ashing at 400°C for 24 hours. Ash dissolved in 1:25 HCl. Quantified using inductively coupled plasma emission spectrophotometry (Karla, 1998).

Statistical Analysis

Using the MSTAT-C computer program, replicated data of the traits under study were statistically examined using analysis of variance based on randomized complete block design (RCBD) (Gomez & Gomez, 1984; Freed et al., 1989). The least significant difference (LSD at $p \leq 0.05$) was used to compare treatment means.

RESULTS AND DISCUSSION

Growth traits and oil content

Table 1 revealed that the number of leaves, heads, and seeds per plant, as well as the average length of the leaf and flower stem, was significantly affected by various fertilization treatments during the 2021/22 and 2022/23 seasons. Results showed that all treatments exceeded the control (no fertilization) in all measured growth traits in both seasons. Growth traits varied in their responses to fertilization treatments, where the application of chitosan plus EDTA-Zinc (Fig. 1) recorded the highest number of leaves per plant (16.67 and 16.17), number of heads per plant (8.33 and 8.20), number of seeds per head (275.17 and 279.67), and number of seeds per plant (2290.50 and 2293.25) respectively. In contrast, azolla powder plus EDTA-Zinc yielded the highest average length of leaf (106.17 and 107.67 cm) and flower stem (160.23 and 170.33 cm) in the two seasons, respectively. The application of azolla, mycorrhizae, and chitosan in combination with EDTA-Zinc improved all studied growth traits compared to the control and their application alone in both seasons. Previous studies have documented that chitosan functions as a stimulant to enhance plant growth traits in various crop plants (El-Tanahy et al., 2012; Bistgani et al., 2021; and Hemdan et al., 2023). The leaves are an important part of the cardoon plant because they contain phenols and flavonoids, in addition to their use in producing high-quality paper pulp (Fernandez et al., 2006; Gominho et al., 2009; Petropoulos et al., 2018). Azolla is a highly effective source of biological nitrogen that can be used directly as a biofertilizer (Agnihotri, 2019). The positive effect of azolla on leaf enlargement has been reported by Sakya et al. (2021). Additionally, fast seed germination and subsequent increases in plant growth parameters may be attributed to the beneficial effects of mycorrhizal inoculum as demonstrated in argan seed treatments (El Rhoch et al., 2025).

Data in Table 2 presented the effect of fertilization treatments on the average weight of fresh leaf, dry head, and seeds per plant, as well as total chlorophyll content, oil percentage, and oil yield per plant of *Cynara cardunculus* var. altilis in the 2021/22 and 2022/23 seasons. Since the different fertilization treatments improved these traits compared to control plants, the results showed that all measured traits were significantly impacted in both seasons. Plants treated with azolla powder plus EDTA-Zinc achieved the heaviest fresh leaf weight (162.5 and 170.0 g), dry head weight (42.03 and 46.01 g), and seeds per plant (55.79 and 57.0 g), as well as the highest oil yield per plant (12.83 and 13.40 g) in the first and second seasons, respectively. The highest oil

Table 1. Effect of fertilization treatments on growth traits of cardoon plant during 2021/2022 and 2022/2023 seasons.

Fertilization treatments	Number of				Average length (cm)	
	Leaves/plant	Heads/plant	Seeds/head	Seeds/plant	Leaf	Flower stem
	2021/2022 season					
Control (zero)	14.83	7.00	216.67	1437.60	88.33	142.00
Azolla Powder (5ton/ha)	15.50	7.33	241.83	1773.00	103.17	157.05
Azolla Liquid (40 %)	15.00	6.50	221.33	1517.00	101.00	150.83
Chitosan (2500 ppm)	15.00	8.17	252.83	2065.00	104.17	144.00
Mycorrhizae (15 g/hill)	15.50	7.83	218.83	1712.25	102.00	143.83
EDTA-Zinc (1000 ppm)	15.50	8.00	217.50	1740.00	102.17	148.00
Azolla Powder + Zinc	16.33	8.15	257.00	2095.00	106.17	160.23
Azolla liquid+ Zinc	15.33	8.00	237.50	1898.65	102.17	154.67
Chitosan + Zinc	16.67	8.33	275.17	2290.50	103.83	156.50
Mycorrhizae + Zinc	16.33	8.00	221.00	1767.83	104.83	157.00
L.S.D. at 5%	1.38	1.30	25.42	148.00	12.01	9.18
	2022/2023 season					
Control (zero)	15.00	6.85	207.83	1424.00	85.00	136.67
Azolla Powder (5ton/ha)	15.50	7.20	253.67	1825.60	103.62	154.00
Azolla Liquid (40 %)	15.00	7.00	235.40	1648.00	101.00	140.52
Chitosan (2500 ppm)	15.17	8.00	262.33	2097.00	103.17	139.83
Mycorrhizae (15 g/hill)	15.67	7.67	210.67	1615.00	102.17	145.17
EDTA-Zinc (1000 ppm)	15.17	7.25	204.17	1480.20	102.50	141.83
Azolla Powder + Zinc	16.00	7.67	265.45	2035.00	107.67	170.33
Azolla liquid+ Zinc	15.25	7.35	243.33	1787.00	104.83	160.67
Chitosan + Zinc	16.17	8.20	279.67	2293.25	106.33	158.00
Mycorrhizae + Zinc	16.50	7.82	230.52	1803.00	105.17	161.00
L.S.D. at 5%	1.42	1.34	28.30	139.80	14.03	8.63

Data is displayed as mean values; LSD refers to the least significant difference test, significant difference at $p \leq 0.05$.

Table 2. Effect of fertilization treatments on growth traits, chlorophyll content and oil content of cardoon plant during 2021/2022 and 2022/2023 seasons.

Fertilization treatments	Average weight (g)			SPAD Chlorophyll	Oil %	Oil yield/plant(g)
	Fresh leaf	Dry head	Seeds/plant			
	2021/2022 season					
Control (zero)	122.00	25.70	34.82	41.33	20.00	6.96
Azolla Powder (5ton/ha)	152.00	36.38	46.73	47.38	23.00	10.75
Azolla Liquid (40 %)	146.50	30.54	44.17	44.85	21.60	9.53
Chitosan (2500 ppm)	139.17	28.93	41.94	42.92	21.23	8.91
Mycorrhizae (15g/hill)	141.67	29.20	42.77	43.23	23.63	10.11
EDTA-Zinc (1000ppm)	142.50	28.20	43.76	44.18	21.00	9.19
Azolla Powder + Zinc	162.50	42.03	55.79	50.05	23.00	12.83
Azolla liquid+ Zinc	152.50	33.67	48.62	48.28	22.07	10.72
Chitosan + Zinc	142.65	32.18	45.74	52.30	22.50	10.30
Mycorrhizae + Zinc	148.33	32.80	47.60	49.98	25.27	12.04
L.S.D. at 5%	13.23	4.25	5.39	5.60	1.22	1.15
	2022/2023 season					
Control (zero)	122.00	27.66	36.64	41.58	19.00	6.95
Azolla Powder (5ton/ha)	159.17	41.16	50.44	46.45	22.83	11.52
Azolla Liquid (40 %)	150.00	31.63	41.80	42.25	22.00	9.20
Chitosan (2500 ppm)	140.00	28.54	39.63	43.88	21.17	8.40
Mycorrhizae (15g/hill)	142.17	29.98	43.04	43.42	24.48	10.54
EDTA-Zinc (1000ppm)	150.83	30.08	44.99	44.03	20.50	9.22
Azolla Powder + Zinc	170.00	46.01	57.00	49.08	23.50	13.40
Azolla liquid+ Zinc	156.67	35.53	47.18	47.38	21.67	10.23
Chitosan + Zinc	152.17	32.63	49.94	49.58	22.73	11.34
Mycorrhizae + Zinc	158.50	32.83	50.10	47.92	25.33	12.70
L.S.D. at 5%	11.80	6.08	5.46	7.04	1.33	1.30

Data is displayed as mean values; LSD refers to the least significant difference test, significant difference at $p \leq 0.05$.

percentage (25.27% and 25.33%) was obtained from plants treated with mycorrhizae plus zinc in both seasons. By contrast, plants treated with chitosan plus zinc showed the highest chlorophyll content. These results align with findings showing that biofertilizers combined with nanofertilizers enhance photosynthetic pigment accumulation, as demonstrated in basil by Elbanna et al. (2024).

The growth-promoting effects of Azolla have been demonstrated by Agnihotri (2019) in sarpagandha and Sakya et al. (2021) in biduri. In our study, while plants treated with azolla powder plus zinc showed the highest oil content, the maximum oil yield was obtained from the same treatment due to the dominant effect of seed yield. These findings agree with Swaefy and Shaaban (2019), who reported a strong correlation between seed and oil yields in cardoon. Our results demonstrated that combining azolla, mycorrhizae, or chitosan with EDTA-Zinc significantly enhanced their effectiveness in improving: Growth traits, oil content and yield, and chemical composition. This synergistic effect may be attributed to zinc's physiological roles in facilitating protein synthesis and maintaining membrane integrity (Kobraee et al., 2011).

Chemical Analysis

Fatty acids composition

The main fatty acid composition of cardoon seed oil is shown in Table 3. The fatty acid profile of seed oils varied between different treatments. However, two unsaturated fatty acids (UFA), linoleic and oleic acids followed by saturated fatty acids (SFA), such as stearic and palmitic acids, were similar in all treatments. Similar fatty acid content in cardoon oils has been detected by Petropoulos et al. (2019), who investigated how the fatty acid composition is affected by the growing season and the extraction method. In the present investigation, polyunsaturated fatty acids (PUFAs) were the most prevalent ones in all treatments due to the high content of linoleic acid, which is considered very important for human health (Liu et al., 2016). Cardoon oil can be used in the food industry due to the significant amount of seed oil and linoleic acid present that may be useful in biodiesel production (Petropoulos et al., 2019). According to the present study, the highest UFA (Figure 2) were found with the treatment of azolla liquid, mycorrhizae, and chitosan plus EDTA-Zinc at 95.13, 93.86, and 89.98%, respectively. The ratio of PUFA/SFA value in plant oils is a unique advantage factor, as oils with an index higher than 1 are estimated edible oils with rich nutritional value (Angelova et al., 2019). In previous studies, PUFAs followed by SFAs and MUFAs, were the most common in cardoon seed oil collected at later stages of maturation and from various genotypes (Mandim et al., 2020). This characterization, which was based on the composition of seed oil, made it possible to identify accessions with higher quality properties and to show that cardoon oil has the right qualities to be utilized as a feedstock for the generation of biodiesel (Mancini et al., 2019).

Oil characters

The values for acid, peroxide, and iodine are presented in Table 4. The least acid value (AV) was measured in the oil of plants treated with chitosan, azolla powder, and liquid, then Mycorrhizae; nevertheless, all the investigated seed oils' AV values were below 1.0 mg KOH/g oil, elucidating that these oils were not subjected to hydrolytic processes. These oils had not undergone hydrolytic degradation (Abril et al., 2019). Furthermore, the peroxide value (PV) of cardoon oils from different treatments was less than 20 meq/kg, demonstrating that these oils were edible and unoxidized, complying with Codex Alimentarius guidelines (Alimentarius, 1999). This means that the PV for cold-pressed oil should not be more than 15 meq O_2/kg of oil. Chitosan, azolla powder, and liquid showed the least amount of PV. The amount of peroxide in the lipid is measured to determine the peroxide value, which indicates the degree of oxidation of the fat or oil. As mentioned in previous studies, the most detrimental fats to human health are saturated fats because they raise LDL cholesterol, which leads to heart disease (Whitney &Rolfes, 2011). So, we determined the iodine value (IV), which measures the average oil unsaturation level: There are more C=C double bonds when the iodine value is higher. Therefore, we determined the iodine value (IV), which measures the average degree of oil unsaturation: a higher iodine value indicates a greater number of C=C double bonds. The results from different treatments showed values of IV as 120–126 g I2/100 g, ranging from 120 to 126 g I_2/100 g, providing it with a particular resistance to oxidation, indicating a moderate resistance to oxidation.

Total phenols and flavonoids contents

Bioactive compounds in the leaves of cardoon plants were investigated due to their significance for various industrial purposes. Phenols and flavonoids are considered the principal phytochemical compounds in cardoon leaves and play a vital role in combating

many serious diseases as they act as antioxidant and anti-inflammatory agents (Shukla & Gupta, 2010; Roy &Iyengar, 2019; and Swaminathan et al., 2019). The data presented in Figure 3 show the impact of fertilization treatments on the total flavonoids and phenols in the leaves of cardoon plants as a mean for the two seasons. Fertilizing cardoon plants with azolla, mycorrhizae, and chitosan as bio-fertilizers, either alone or in combination with zinc, had a notable impact on the concentrations of total flavonoids and phenols in cardoon leaves during both seasons. The highest levels of phenols were observed in plants treated with mycorrhizae, either alone or in combination with zinc, during both seasons. Total flavonoids showed a similar pattern, where the best values were observed in plants receiving mycorrhizae, either alone or in combination with zinc. Mycorrhizae or azolla may induce a significant stress response in cardoon, which can trigger the production of secondary metabolites as a defense mechanism, in addition to their role in increasing the activity of some enzymes in secondary metabolite pathways. Increased phosphorus availability can stimulate the phenylpropanoid pathway's enzyme activity, a key pathway to produce phenols and flavonoids (Rubin & Görres, 2020). Inoculation of *aloe vera* plantlets with mycorrhizae increased flavonol and flavanone concentrations (Mota-Fernandez et al., 2011).

Chitosan, a natural biopolymer, is recognized as a promising elicitor and plant growth promoter. It can trigger various physiological interactions in plants, such as the production of secondary metabolites. Increased levels of phenolic compounds and total flavonoid contenthave been reported by Raveesha et al. (2018) in *Ruta graveolens* and *Vitex trifolia* plants, and by Chookalaii et al. (2020) in *Plantago major* plants.Regarding chitosan's application, Ahmad et al. (2019) studied its effects on cell cultures of *Linum usitatissimum* L. (flax), which showed elevated antioxidant activity and a 1.3-fold increase in total phenolic accumulation compared to the control treatment. Additionally, Ozsan & Onus (2022) demonstrated that various elicitors, including chitosan and methyl jasmonate, can increase bioactive compounds, and their concentrations vary among cultivars.Chitosan may activate enzymes in the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (PAL), flavonoid 3'-hydroxylase, and chalcone synthase. This activation leads to enhanced production of phenols and flavonoids (Xoca-Orozco et al., 2018).According to reports, propagated *Cynara cardunculus* seeds coated with *Rhizophagus intraradices*, *Funneliformis mosseae*, and *Trichoderma atroviride* showed improvements in yield, macro- and microelements, antioxidant activity, total phenolics, caffeoylquinic acids, and flavonoids (Rouphael&Colla, 2020). Because endomycorrhizal fungi enhance nutrient uptake, particularly of phosphorus and other inorganic nutrients like nitrogen, Anand et al. (2022) identified them as biostimulants. These authors also suggest that mycorrhizal fungi enhance plant performance under abiotic stress.

Mineral analysis

The data presented in Table 5 show the amounts of nitrogen (N), phosphorus (P), and potassium (K), as well as protein content in cardoon leaves, which were markedly affected by various fertilization treatments. The highest values of protein (14.69% and 14.54%) and N (2.35 and 2.33%) were found in the leaves of plants fertilized with azolla liquid and chitosan plus zinc, respectively. There is a strong correlation between N concentration and protein content in leaves. Azolla provides a continuous source of biologically fixed nitrogen to plants, which increases nitrogen content and assimilation in cardoon tissues, as well as enhances the synthesis of nitrogen-containing compounds like proteins and chlorophyll. Azolla is an excellent biological nitrogen source that can be utilized as a biofertilizer (Agnihotri, 2019).However, the application of azolla powder in combination with zinc increases the leaf's potassium content. This may be due to the improvement in plant health and root development promoted by azolla, which can indirectly enhance potassium uptake and that of other nutrients. It may also improve potassium utilization in various physiological processes, such as water regulation and enzyme activity. Chitosan can improve nutrient uptake, especially nitrogen and phosphorus, which are essential for various metabolic processes, including secondary metabolite biosynthesis (Rojas-Pirela et al., 2024).

The application of mycorrhizae increases phosphorus content, which can promote root development, flowering, and seed production in cardoon plants. In low-phosphorus soils, mycorrhizae enhance the host plant's P nutrition by improving phosphorus uptake through their extensive hyphal surface area and specialized phosphorus uptake mechanisms, resulting in plant growth promotion (Heijden et al., 2006). Some mycorrhizal fungi produce phosphatase enzymes that convert insoluble phosphorus forms into plant-available forms.

Table 3. Effect of different fertilization treatments on fatty acid composition of cardoon seed oil in 2022/2023 season.

Fatty acid (%)	Control	Azolla Powder	Azolla Liquid	Chitosan	Mycorrhizae	EDTA-Zinc	Azolla Powder + Zinc	Azolla liquid+ Zinc	Chitosan + Zinc	Mycorrhizae + Zinc
Palmitic acid	24.44	15.00		19.30	5.35	8.43	7.84	9.50	6.82	20.01
Linoleic acid	14.01	55.37	67.1	32.85	52.69	53.17	47.83	49.52	48.42	47.78
Oleic acid	38.28	29.63		21.52	22.39	33.73	40.99	39.77	26.97	26.13
Myrestic acid	2.33									
Stearic acid	20.94					4.67	3.34	1.22	0.62	3.96
Hexadecanoic acid, methyl ester			4.87						2.58	
11,14-Octadecadienoic acid, methyl ester			19.7							
13-Octadecenoic acid, methyl ester			8.36		6.43				5.29	
Hexadecanoic acid, cyclohexyl ester				26.33						
Methyl 11,12-octadecadienoate					12.35				9.31	
Methyl 16-hydroxy-hexadecanoate					0.79					

Table 4. Effect of fertilization treatments on Oil characters in cardoon oil during 2021/2022 and 2022/2023 seasons.

Fertilization treatments	Value of		
	Acid (mg KOH/g oil)	Peroxide(meq O2/kg oil)	Iodine (g I_2/100 g oil)
	2021/2022 season		
Control (zero)	0.60	14.31	124.00
Azolla Powder (5 ton/ha)	0.31	9.98	122.00
Azolla Liquid (40 %)	0.41	9.53	122.00
Chitosan (2500 ppm)	0.22	8.40	122.00
Mycorrhizae (15 g/hill)	0.46	12.23	124.00
EDTA-Zinc (1000 ppm)	0.45	11.65	125.00
Azolla Powder + Zinc	0.61	14.80	126.00
Azolla liquid+ Zinc	0.57	14.13	125.00
Chitosan + Zinc	0.30	8.47	122.00
Mycorrhizae + Zinc	0.48	13.28	125.00
L.S.D. at 5%	0.02	0.24	1.32
2022/2023 season			
Control (zero)	0.58	14.00	122.00
Control (zero)	0.30	9.50	121.00
Azolla Powder (5 tons/ha)	0.40	9.30	121.00
Azolla Liquid (40 %)	0.23	8.53	123.00
Chitosan (2500 ppm)	0.45	12.07	125.00
Mycorrhizae (15 g/hill)	0.44	11.41	125.00
EDTA-Zinc (1000 ppm)	0.60	14.08	126.00
Azolla Powder + Zinc	0.55	14.07	124.00
Azolla liquid+ Zinc	0.29	8.60	121.00
Chitosan + Zinc	0.47	12.90	124.00
L.S.D. at 5%	0.03	0.45	1.71

Data is displayed as mean values; LSD refers to the least significant difference test, significant difference at $p \leq 0.05$.

Table 5. Effect of fertilization treatments on protein and mineral content of cardoon leaves in 2022/2023 season.

Fertilization treatments	Protein (%)	N (%)	P (%)	K (%)
Control (zero)	12.80	2.05	0.32	1.47
Azolla Powder (5 ton/ha)	13.96	2.23	0.37	1.64
Azolla Liquid (40 %)	14.69	2.35	0.31	1.52
Chitosan (2500 ppm)	11.31	1.81	0.36	1.55
Mycorrhizae (15 g/hill)	9.39	1.50	0.48	1.95
EDTA-Zinc (1000 ppm)	13.96	2.23	0.13	2.44
Azolla Powder + Zinc	12.60	2.02	0.29	2.75
Azolla liquid+ Zinc	14.09	2.26	0.47	1.64
Chitosan + Zinc	14.54	2.33	0.47	2.09
Mycorrhizae + Zinc	10.40	1.67	0.22	2.53

Figure 1. The application of chitosan in combination with EDTA-Zinc recorded the highest number of leaves and number of seeds per plant.

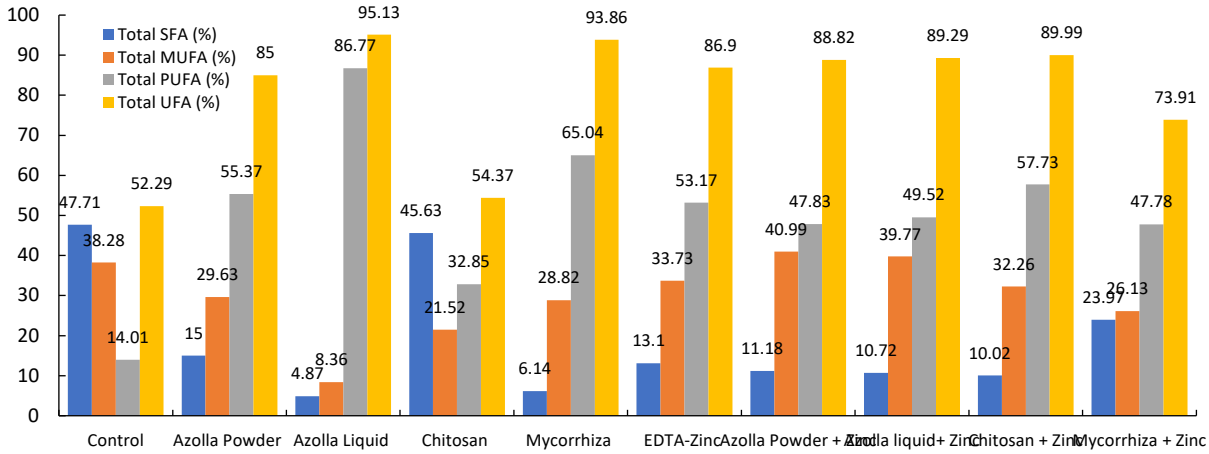

Figure 2. Total saturated fatty acids (SFA %), total monounsaturated fatty acids (MUFA %), total polyunsaturated fatty acids (PUFA %), and total unsaturated fatty acids (UFA %) in cardoon seeds oil as affected by different treatments.

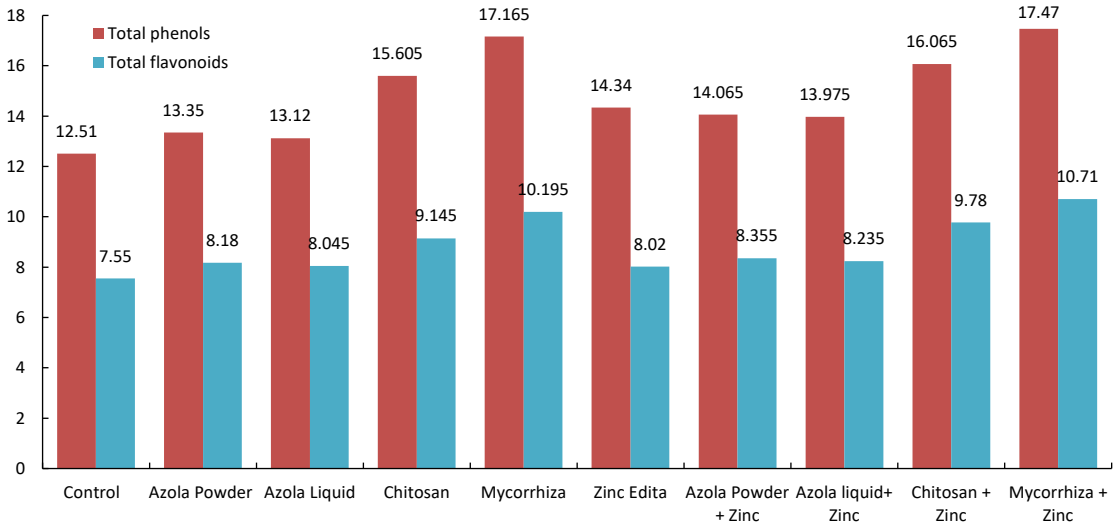

Figure 3. Total phenols and flavonoids in cardoon leave as affected by different fertilization treatments (mean of two seasons).

Aguégué et al. (2021) suggest that enhanced strain accommodation and mineral element absorption contributed to the observed increases in growth and yield variables. Similarly, Abadi (2020) found that the aerial parts of pistachio tree cultivars had higher concentrations of iron, zinc, and manganese after AMF application. The higher biomass production and grain yield observed in mycorrhized plants may result from the high mycorrhization frequencies and intensities reported (Agbodjato et al., 2022).

CONCLUSION

The application of azolla, mycorrhizae, and chitosan as biofertilizers, either alone or in combination with EDTA-Zinc, significantly enhanced the growth, biomass, oil percentage, and oil yield of cardoon plants compared to untreated plants. Plants fertilized with azolla powder plus zinc achieved the highest biomass and oil yields. However, the highest oil percentage was obtained from seeds of plants treated with mycorrhizae plus zinc. Spraying plants with azolla liquid or chitosan plus zinc increases the nitrogen and protein content in leaves, enhancing the nutritional value of cardoon leaves. Saturated and unsaturated fatty acids are abundant in cardoon seed oil. The treatments yielding the highest levels of total unsaturated fatty acids were azolla liquid and mycorrhizae. The use of azolla liquid and mycorrhizae increased the PUFA/SFA ratios in cardoon oils to values higher than one, indicating that cardoon oil has a high potential as a nutritionally rich edible oil. Using biofertilizers can help maximize their benefits for cardoon plant production and nutrient content, potentially reducing the need for synthetic fertilizers and promoting environmentally friendly agriculture. However, to facilitate the adoption of biofertilizer-based products for efficient, safe, and scalable production systems, further research is required to address the practical challenges of large-scale production.

AUTHOR CONTRIBUTIONS

A. M. Heikal designed the research, collected plant material and analyzed the data; RS Yousef performed chemical analysis; A. A. M. Heikal and RS Yousef wrote the manuscript. The manuscript was reviewed by all authors prior to submission.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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