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Effects of Two Varieties and Fertilization Regimes on Growth, Fruit, and Silymarin Yield of Milk Thistle Crop

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Abstract: Milk thistle is an alternative crop to winter cereals for southern Europe as this species is drought tolerant and its fruits contain silymarin. The aim of this study was to assess the impact of two varieties and fertilization regimes (sheep manure and inorganic fertilizer) on crop productivity. A two-factor experiment was conducted in a randomized split-plot design with three replicates. The varieties were Palaionterveno and Spata, while the fertilization treatments were control, sheep manure, and calcium ammonium nitrate applied at 75 and 125 kg N ha^{-1}. Variety and fertilization significantly affected plants development and productivity, as well as oil and silymarin yield. The use of manure and inorganic nitrogen fertilizer increased rosette diameter, oil and silymarin yield, above-ground biomass, and fruit yield. The influence of inorganic fertilization, regardless of the application dose, was more apparent than organic fertilization. Moreover, variety significantly affected plants growth and silymarin content, as well as silymarin composition. The variety Spata had the greatest silymarin content, reaching 4.40%, and a high silybin B concentration. In conclusion, the selection of a suitable variety is important for achieving high fruit and silymarin yields, while inorganic nitrogen fertilization can maximize the productivity of the milk thistle crop.

Keywords: flavonolignans content; inorganic fertilization; productivity; quality; *Silybum marianum*

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1. Introduction

Milk thistle (*Silybum marianum* (L.) Gaertn.) is a well-known source of silymarin, which has anticancer [1], hepatoprotective, anti-inflammatory [2], anti-collagenase [3], immunomodulatory [4], and neuroprotective properties [5]. Due to its pharmaceutical properties, this species is an important medicinal plant and it is cultivated in many European countries such as Austria, Bulgaria, Czech Republic, Germany, and Poland [6–8]. However, except for silymarin production, milk thistle can be cultivated for oil production [9,10]. This oil is edible [11] and can be used in cooking [12] or in the pharmaceutical and cosmetic industries [11,13,14].

Milk thistle crop can be included in rotation systems since it is a low input crop, while its fruits have high economic value since the demand for silymarin is high [15,16]. The optimization of cultivation practices is really important to increase crop productivity [17]. Cultivation practices such as plant density [18], irrigation, and fertilization [16] affect both plants development and productivity of this crop. The application of organic or inorganic fertilizers at appropriate doses contributes significantly to the increase in milk thistle crop yield. Nitrogen and phosphorus application led to increased fruit yield [19], while the application of manure enhanced the plants height, silymarin content [20], and fruit and silymarin yield [16]. In contrast, in Bulgaria under different climatic conditions, the use of nitrogen, phosphorus, and potassium negatively affected silymarin accumulation in the fruits; although, silymarin yield was increased [21].

Except for cultivation techniques, genetic material plays an important role in plants development and productivity [15]. For instance, Shokrpour et al. [22], assessing different milk thistle genotypes in Iran, observed that the plant height ranged from 131.87 to 160.61 cm and the fruit yield from 889 to 2416 kg ha^{-1}, revealing the importance of genetic material in increasing crop yields. Moreover, genetic material can affect both the silymarin content and its composition in flavonolignans (silybins A and B, isosilybins A and B, silychristin, and silydianin) and taxifolin [23–25]. In the literature, there is limited information about the productivity and quality (e.g., silymarin content) of genotypes originating from southern Europe, a region that is characterized by semi-arid conditions. For instance, Arampatzis et al. [25] evaluated several native genotypes originating from Greece and observed that silymarin content ranged from 2.3% to 7.7%. These genotypes varied in flavonolignans and taxifolin content and only two genotypes (Spata and Kastoria) exhibited both the highest silymarin and silybin A + B content. In Italy, a country with similar climatic conditions to Greece, twenty-six milk thistle genotypes, originating from Italy and other countries, varied both in silymarin content and composition [23]. Four of these genotypes exhibited high silybin and silychristin content, while fourteen genotypes had high silydianin content [23]. Moreover, genotypes originating from southern Europe can have high productivity since Arampatzis et al. [18] reported that the fruit yield of a genotype originating from Greece ranged from 1444 to 2222 kg ha^{-1} depending on plant density. The assessment of milk thistle genotypes adaptation to semi-arid climate conditions of this region is crucial to maximize both the crop productivity and the commercial value of the final product (fruits or silymarin extracts). Thus, the evaluation of milk thistle genotypes of Greek origin, which exhibit high productivity and quality (e.g., high silymarin content), under low input and high input conditions is important in order to select genotypes that could be included in breeding programs. In this context, the aims of this study were (1) to assess two milk thistle varieties in terms of productivity and quality (e.g., silymarin content), (2) to evaluate the impact of sheep manure and inorganic fertilizer on crop yield and quality, (3) to examine the interaction effects of variety and fertilization on fruit and silymarin yield of milk thistle crop.

2. Materials and Methods

2.1. Study Site, Growing Conditions, and Experimental Design

A two-year experiment was set up at the experimental field of the University of Thessaly in Velestino (Thessaly Region, Greece) during the growing seasons 2019–2020 and 2020–2021. The soil was sandy clay loam with a pH of 7.4. In the first experimental year, the total precipitation from November to May was 368.6 mm, while in the second experimental year was 273.5 mm. Two varieties of milk thistle originating from Greece were sown on 29 October in both years. The row spacing was 50 cm, while the density of the plants in the row was 13 plants m^{-1} (Figure 1).

A two-factor experiment was conducted in a randomized split-plot design with three replicates. Variety and fertilization were the main plot and sub-plot factors, respectively. The tested varieties were Palaionterveno and Spata, while the fertilization treatments were control without fertilization, sheep manure, and calcium ammonium nitrate applied at two doses (Table 1). The selection of the two varieties was based on the content and composition of silymarin. According to Arampatzis et al. (2019b), Spata has high silymarin (5.9–7.7%) and silybin A + B content, while Palaionterveno is characterized by lower silymarin (2.4–3.3%) and silybin A + B content compared with Spata.

Figure 1. Experimental field (plot size: 2 × 3 m, density: 26 plants m^2) of milk thistle in the second experimental period on 20 November 2020.

Table 1. Description of organic and inorganic fertilization treatments.

Fertilizers	Dose	Application Time
Sheep Manure	13 t ha^{-1}	Pre-sowing
Chemical properties: C/N ratio: 10.4, organic matter: 47.5%, pH: 7.3, total nitrogen (TN): 22,695 mg kg^{-1}, phosphorus (P): 773 mg kg^{-1}, potassium (K): 3739 mg kg^{-1}, magnesium (Mg): 3549 mg kg^{-1}, copper (Cu): 3.1 mg kg^{-1}, zinc (Zn): 24.3 mg kg^{-1}, manganese (Mn): 62.1 mg kg^{-1}, iron (Fe): 29.5 mg kg^{-1}, boron (B): 17.8 mg kg^{-1}, and sodium (Na): <100 mg kg^{-1}.		
Calcium ammonium nitrate	75 kg N ha^{-1} applied at two doses (25 and 50 kg N ha^{-1})	1st dose: 15 January 2020 and 13 January 2021 2nd dose: 3 March at both seasons
	125 Kg N ha^{-1} applied at two doses (50 and 75 kg N ha^{-1})	

2.2. Measurements

2.2.1. Agronomic Parameters

Within each sub-plot, rosette diameter and height were measured for five plants in the central rows avoiding plants at the edges of the rows. The rosette diameter was measured at 144 and 138 DAS (days after sowing) in 2020 and 2021, respectively, while the maximum height of plants was recorded at 193 and 190 DAS in 2020 and 2021, respectively. For the above-ground dry biomass determination, four consecutive plants from a central row were selected at the growth stage where the plants had the maximum height, and then after drying of samples at 60 °C for four days the dry biomass was estimated. Moreover, the number of inflorescences per plant was measured in five plants per treatment. Harvest was made manually in two central rows (1 m per row) at the end of May. After the harvest, the fruits were separated from the other parts of the inflorescences and the 1000-fruit weight was measured in three samples of 100 fruits.

2.2.2. Chemical Composition Analysis: Oil and Silymarin

Oil was extracted from powdered dry fruit samples with hexane according to the procedure described in the previous study of Arampatzis et al. [25]. After the oil extraction, firstly the defatted fruit samples were extracted with methanol using a Soxhlet extraction apparatus and then, the silymarin determination was made by a HPLC system (HP 1100 Liquid Chromatograph, Hewlett-Packard GmbH, Waldbronn, Germany) with a UV detector and coupled to a ternary-delivery system following the analytical conditions described by Arampatzis et al. [25]. The identification and quantification of silymarin compounds (flavonolignans and taxifolin) were made according to the procedure described in our previous work [17]. Finally, oil and silymarin yield (kg ha^{-1}) was calculated according to Equations (1) and (2).

$$\text{Oil yield} = \text{oil content} \times \text{fruit yield} \quad (1)$$

$$\text{Silymarin yield} = \text{silymarin content} \times \text{fruit yield} \quad (2)$$

2.3. Statistical Analysis

The results of morphological parameters (rosette diameter, height), fruit yield and its components, above-ground biomass, and quality parameters (oil, silymarin, flavonolignans, and taxifolin content) were statistically analyzed using the SigmaPlot 12 statistical package (Systat Software, San Jose, CA, USA). A two-way analysis of variance (ANOVA) was conducted to assess the effects of two factors (variety and fertilization) and their interactions on growth, yield, and quality of milk thistle, while the differences between means were separated by Fisher's least significant dereference (LSD) test at $p = 0.05$.

3. Results

3.1. Plants Growth Traits

Variety and fertilization exhibited positive effects on plants growth traits. In 2020 and 2021, the maximum rosette diameter and plant height were recorded in the variety Spata (Table 2). In general, plants growth was greater during the first year. Concerning fertilization regimes, in both growing seasons, the application of organic and inorganic fertilization significantly increased both rosette diameter and plant height. An exception was the application of sheep manure during the second season as there was no difference among this treatment and the untreated control at the plant height.

Table 2. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on rosette diameter and plant height of milk thistle.

Treatmentss	**Rosette Diameter (cm)**		**Plant Height (cm)**	
	2020	**2021**	**2020**	**2021**
Varieties				
Palaionterveno	69.0 b	52.2 b	170.3 b	159.6 b
Spata	72.9 a	60.4 a	201.3 a	177.6 a
$LSD_{5\%}$	0.74	1.85	4.51	5.22
Fertilization				
Control	65.6 d	46.0 d	169.6 d	153.7 c
Sheep manure	69.0 c	51.5 c	184.1 c	156.4 c
CAN-75 kg N ha^{-1}	72.8 b	59.7 b	190.9 b	176.3 b
CAN-125 kg N ha^{-1}	76.3 a	67.9 a	198.7 a	188.0 a
$LSD_{5\%}$	1.04	2.62	6.37	7.39
F-values and significant differences				
Variety (V)	126.603 ***	86.628 ***	212.446 ***	53.062 ***
Fertilization (F)	179.866 ***	120.464 ***	33.691 ***	44.131 ***
V × F	3.153 ^{ns}	0.366 ^{ns}	0.846 ^{ns}	1.782 ^{ns}

CAN: calcium ammonium nitrate. For each factor, means followed by different letters within the same column show significant differences according to the LSD test. *** significant at $p \leq 0.001$ and ns = not significant.

The application of calcium ammonium nitrate (CAN) regardless of the application dose enhanced plants development compared to organic fertilization. For instance, the application of CAN fertilizer increased rosette diameter and plant height by 5.2–24.2% and 3.6–16.8%, respectively, compared to the use of manure.

The greatest values of dry weight were measured in 2020, specifically in the variety Spata, while there was an interaction effect between the two factors (Table 3). During the two-year experiment, in both varieties, the minimum above-ground dry biomass was recorded in the unfertilized plots. The organic fertilization significantly influenced this trait, especially in the first growing period, increasing the above-ground biomass of the variety Palaionterveno by 29.4%. Moreover, CAN fertilizer further enhanced this trait. For instance, in the variety Spata, inorganic nitrogen fertilizer increased the dry biomass by 43.6–56.1% and 44.1–45.7% compared to the control treatment in 2020 and 2021, respectively. The results also revealed that the low inorganic fertilizer dose affected more the variety Spata compared with Palaionterveno, while the high dose similarly affected the two varieties.

Table 3. Interaction effects between variety and fertilization on aboveground dry biomass of milk thistle crop.

Varieties	Fertilization	Above-Ground Biomass (kg ha^{-1}) 2020	2021
Palaionterveno	Control	15,293.7 e	15,097.1 d
	Sheep manure	21,651.3 d	18,405.0 c
	CAN-75 kg N ha^{-1}	26,506.0 bc	22,218.2 b
	CAN-125 kg N ha^{-1}	29,340.7 b	26,808.9 a
Spata	Control	17,282.7 e	15,465.9 d
	Sheep manure	22,952.3 cd	18,221.9 c
	CAN-75 kg N ha^{-1}	30,648.0 b	27,671.5 a
	CAN-125 kg N ha^{-1}	39,347.3 a	28,475.2 a
$LSD_{5\%}$		4236.02	2419.16
F-values and significant differences			
Varieties (V)		19.041 ***	10.254 **
Fertilization (F)		61.013 ***	100.774 ***
V × F		3.916 *	4.951 *

CAN: calcium ammonium nitrate. Means followed by different letters within the same column show significant differences according to the LSD test. *, **, and *** significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

3.2. Productivity

In both years, variety and fertilization had a significant impact on the number of inflorescences (Table 4). In particular, in 2020, the variety Palaionterveno had the greatest value, while in 2021, Spata reached the maximum number. In both years, organic fertilization did not augment the number of inflorescences compared with control. However, the application of CAN fertilizer significantly increased this trait, especially in the second growing period when the high dose of the fertilizer was applied. Moreover, neither variety nor fertilization affected the 1000-fruit weight. In 2021, the 1000-fruit weight was higher as it ranged from 23.7 to 25.0 g, while in 2020, it ranged from 21.7 to 22.4 g.

Table 4. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on yield parameters (number of inflorescences, 1000-fruit number, and fruits per central inflorescence) of milk thistle.

Treatments	Number of Inflorescences (No Plant^{-1}) 2020	2021	1000-Fruit Weight (g) 2020	2021	Fruits Per Central Inflorescence (No) 2020	2021
Varieties						
Palaionterveno	6.7 a	4.9 b	21.7 a	24.3 a	147.5 a	73.6 a
Spata	5.5 b	5.7 a	22.4 a	24.3 a	99.7 b	58.9 b
$LSD_{5\%}$	1.04	0.57	-	-	6.41	4.12
Fertilization						
Control	5.3 b	3.5 c	21.8 a	23.9 a	103.7 d	49.2 d
Sheep manure	4.3 b	3.5 c	22.3 a	23.7 a	113.9 c	57.3 c
CAN-75 kg N ha^{-1}	7.2 a	6.5 b	22.4 a	25.0 a	129.3 b	72.5 b
CAN-125 kg N ha^{-1}	7.5 a	7.8 a	21.7 a	24.6 a	147.4 a	85.8 a
$LSD_{5\%}$	1.48	0.80	-	-	9.06	5.83
F-values and significant differences						
Varieties (V)	6.177 *	11.692 **	3.518 ^{ns}	0.0129 ^{ns}	249.724 ***	57.223 ***
Fertilization (F)	9.826 ***	64.289 ***	0.664 ^{ns}	1.922 ^{ns}	39.871 ***	69.863 ***
V × F	0.757 ^{ns}	0.231 ^{ns}	0.524 ^{ns}	1.532 ^{ns}	0.140 ^{ns}	1.629 ^{ns}

CAN: calcium ammonium nitrate. For each factor, means followed by different letters within the same column show significant differences according to the LSD test. *, **, and *** significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. ns = not significant.

Concerning the number of fruits on the central inflorescence, there was a significant difference between the two varieties, and Palaionterveno had the highest value. Fertilization significantly affected this parameter as the lowest number was recorded in the unfertilized control, followed by the use of manure. In 2021, inorganic fertilization increased the number of fruits on the central inflorescence by up to 42.7% and 33.2% compared with control and organic fertilization. Moreover, in 2020, the number of fruits per inflorescence in each treatment was almost doubled compared with the equal treatment in 2021. However, the impact of fertilization was more intense in the second growing season.

In the first growing season, fruit yield was significantly affected only by fertilization, as there was no significant difference among the two varieties (Table 5). Organic and inorganic fertilization enhanced fruit yield, although the use of manure affected to a lesser extent this trait. Moreover, the high rate of CAN fertilizer increased fruit yield by 19.7% (Spata) and 22.7% (Palaionterveno) compared with the application of manure. In 2021, there was an interaction effect between variety and fertilization on fruit yield. The yield ranged from 726.5 kg ha^{-1} to 1504.1 kg ha^{-1} and there were significant differences between the two varieties in the same fertilization regime, as the greatest values were recorded in Palaionterveno.

Table 5. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on fruit yield of milk thistle crop.

Fertilization	Fruit Yield (kg ha^{-1})			
	2020		2021	
	Varieties		Varieties	
	Spata	Palaionterveno	Spata	Palaionterveno
Control	963.0 Da	975.8 Da	726.5 Cb	856.7 Da
Sheep manure	1184.7 Ca	1083.5 Ca	781.6 Cb	1018.6 Ca
CAN-75 kg N ha^{-1}	1339.9 Ba	1245.9 Ba	976.7 Bb	1307.7 Ba
CAN-125 kg N ha^{-1}	1475.9 Aa	1401.5 Aa	1128.5 Ab	1504.1 Aa
$LSD_{5\%\ fertilization}$	93.50		76.99	
$LSD_{5\%\ varieties}$	-		54.43	
F-values and significant differences				
Varieties (V)	4.231 ^{ns}		109.256 ***	
Fertilization (F)	42.086 ***		84.297 ***	
V × F	0.710 ^{ns}		4.483 ^{ns}	

CAN: calcium ammonium nitrate. Small letters show significant differences between the two varieties, while capital letters show significant differences among the fertilization treatments. *** Significant at $p \leq 0.001$, respectively. ns = not significant.

Assessing each variety separately, the inorganic nitrogen fertilization led to significantly higher fruit yield compared with organic fertilization and control. The high rate of CAN fertilizer increased the fruit yield by 32.3% in Palaionterveno and 30.7% in Spata compared with the use of manure. Finally, organic fertilization had no impact on Spata as there was no difference between manure and control.

3.3. Oil Content and Yield

In 2020, Palaionterveno had significantly higher oil content compared with Spata (Table 6). However, in 2021, there was no difference between the two varieties. Moreover, in both growing seasons, neither organic nor inorganic fertilization had an impact on oil content. Concerning oil yield, in 2020, the application of manure did not enhance this trait compared with the untreated control (Table 7). However, inorganic fertilization significantly increased oil yield regardless of the fertilization rate. In particular, nitrogen fertilization increased oil yield by up to 21.3% compared with the use of manure. In 2021, there was an interaction effect between variety and fertilization on oil yield. In Palaionterveno, organic and inorganic fertilization significantly increased oil yield, while in Spata, organic fertilization had no impact. In both varieties, the application of inorganic nitrogen fertilization affected more the oil yield compared with the use of manure. Assessing the two varieties in the equal treatment, there were significant differences in all the cases as Palaionterveno had the greatest values. The highest oil yield (377.4 kg ha^{-1}) was recorded in Palaionterveno when the inorganic fertilizer was applied at a high rate. In general, fertilization affected more the variety Palaionterveno than Spata.

Table 6. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilization) on oil content in milk thistle.

Treatments	Oil Content (%)	
	2020	2021
Variety		
Palaionterveno	23.3 a	24.7 a
Spata	22.1 b	24.9 a
$LSD_{5\%}$	0.72	-
Fertilization		
Control	23.2 a	24.6 a
Sheep manure	22.8 a	24.5 a
CAN-75 kg N ha^{-1}	22.6 a	25.1 a
CAN-125 kg N ha^{-1}	22.1 a	24.9 a
F-values and significant differences		
Variety (V)	10.720 *	2.667 ^{ns}
Fertilization (F)	1.894 ^{ns}	2.822 ^{ns}
V × F	1.661 ^{ns}	1.759 ^{ns}

CAN: calcium ammonium nitrate. For each factor, means followed by different letters within the same column show significant differences according to the LSD test. * Significant at $p \leq 0.05$. ns = not significant.

Table 7. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on oil yield of milk thistle.

Fertilization	Oil Yield (kg ha^{-1})			
	2020		2021	
	Varieties		Varieties	
	Spata	Palaionterveno	Spata	Palaionterveno
Control	215.7 Ca	235.4 Ca	181.2 Cb	207.7 Da
Sheep manure	264.5 Ba	253.3 BCa	193.1 Cb	247.9 Ca
CAN-75 kg N ha^{-1}	303.8 Aa	280.9 Ba	246.2 Bb	326.0 Ba
CAN-125 kg N ha^{-1}	314.3 Aa	321.7 Aa	279.5 Ab	377.4 Aa
$LSD_{5\% \text{ fertilization}}$	28.09		19.35	
$LSD_{5\% \text{ varieties}}$	-		13.68	
F-values and significant differences				
Varieties (V)	0.035 ^{ns}		100.740 ***	
Fertilization (F)	18.407 ***		89.666 ***	
V × F	1.024 ^{ns}		5.776 ^{ns}	

CAN: calcium ammonium nitrate. Small letters show significant differences between the two varieties, while capital letters show significant differences among the fertilization treatments. *** Significant at $p \leq 0.001$, respectively. ns = not significant.

3.4. Silymarin Content and Yield

The results of the two-year experiment indicate that silymarin content is primarily influenced by genetic material (Table 8). The variety Spata had significantly higher silymarin content than Palaionterveno. Organic and inorganic fertilization had no impact on this trait. In contrast, in 2020 and 2021, inorganic nitrogen fertilization increased the silymarin yield by 21.5–30.7% and 28.1–37.8%, respectively, compared to the untreated control. The impact of manure on this trait was noticeable only in 2020. Finally, in both varieties, the concentration of silymarin was greater in the second year; although, the maximum silymarin yields were recorded in the first year owing to the higher fruit yields.

Table 8. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on silymarin content and yield of milk thistle.

Treatments	Silymarin Content (%)		Silymarin Yield (kg ha^{-1})	
	2020	2021	2020	2021
Varieties				
Palaionterveno	2.40 b	2.51 b	28.3 b	29.4 b
Spata	4.04 a	4.40 a	50.0 a	39.7 a
$LSD_{5\%}$	0.15	0.09	3.56	2.18
Fertilization				
Control	3.31 a	3.50 a	32.1 c	27.1 c
Sheep manure	3.24 a	3.44 a	37.2 b	29.9 c
CAN-75 kg N ha^{-1}	3.14 a	3.43 a	40.9 b	37.7 b
CAN-125 kg N ha^{-1}	3.20 a	3.44 a	46.3 a	43.6 a
$LSD_{5\%}$	-	-	5.04	3.09
F-values and significant differences				
Varieties (V)	536.430 ***	1858.094 ***	167.331 ***	100.694 ***
Fertilization (F)	1.059 ^{ns}	0.474 ^{ns}	12.699 ***	53.650 ***
V × F	0.664 ^{ns}	0.614 ^{ns}	0.952 ^{ns}	0.358 ^{ns}

CAN: calcium ammonium nitrate. For each factor, means followed by different letters within the same column show significant differences according to the LSD test. *** Significant at $p \leq 0.001$. ns = not significant.

3.5. Silymarin Active Constituents

In both growing seasons, variety significantly affected silymarin composition. In 2021, there were differences between the two varieties in all the evaluated compounds, while in the first year, the accumulation of isosilybin B, silydianin, and isosilychristin in the fruits of varieties was not significantly different. In both years, taxifolin, silybin A and B, silychristin, and isosilybin A content were higher in the variety Spata (Tables 9 and 10). The silybin A + B content in the variety Spata was 79.3–81.9% higher than that in Palaionterveno. In contrast, the dominant components of Palaionterveno were silydianin and isosilychristin. Finally, the organic and inorganic nitrogen fertilization did not influence the accumulation of silymarin constituents, as there were no significant differences between the fertilization regimes.

Table 9. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on the content of silymarin active constituents in fruits of milk thistle in 2020.

Treatments	Silymarin Active Constituents (mg/g)-2020						
	TXF	SCS	SDN + ISCS	SBA	SBB	ISBA	ISBB
Varieties							
Palaionterveno	2.37 b	2.20 b	10.79 a	0.79 b	2.20 b	3.56 b	2.14 a
Spata	4.86 a	5.84 a	9.28 a	4.66 a	9.77 a	4.02 a	1.97 a
$LSD_{5\%}$	0.41	0.66	-	0.46	0.97	0.20	-
Fertilization							
Control	3.87 a	3.66 a	11.29 a	2.64 a	5.69 a	3.88 a	2.10 a
Sheep manure	3.47 a	4.16 a	9.79 a	2.64 a	6.49 a	3.72 a	2.10 a
CAN-75 kg N ha^{-1}	3.42 a	4.21 a	9.48 a	2.62 a	5.86 a	3.79 a	2.00 a
CAN-125 kg N ha^{-1}	3.71 a	4.04 a	9.57 a	3.00 a	5.91 a	3.77 a	2.03 a
F-values and significant differences							
Varieties (V)	169.358 ***	137.984 ***	4.196 ^{ns}	316.275 ***	275.831 ***	23.747 ***	3.744 ^{ns}
Fertilization (F)	1.188 ^{ns}	0.633 ^{ns}	1.321 ^{ns}	0.731 ^{ns}	0.581 ^{ns}	0.493 ^{ns}	0.339 ^{ns}
V × F	0.510 ^{ns}	1.357 ^{ns}	1.247 ^{ns}	0.885 ^{ns}	0.735 ^{ns}	0.586 ^{ns}	0.692 ^{ns}

Taxifolin: TXF, silychristin: SCS, silydianin + isosilychristin: SDN + ISCS, silybin A: SBA, silybin B: SBB, isosilybin A: ISBA, isosilybin B: ISBB, and calcium ammonium nitrate: CAN. For each factor, means followed by different letters within the same column show significant differences according to the LSD test. *** Significant at $p \leq 0.001$, respectively. ns = not significant.

Table 10. Effects of two milk thistle varieties (Palaionterveno and Spata) and fertilization regimes (sheep manure and inorganic fertilizer) on the content of silymarin active constituents in fruits of milk thistle in 2021.

Treatments	Silymarin Active Constituents (mg/g)-2021						
	TXF	SCS	SDN + ISCS	SBA	SBB	ISBA	ISBB
Varieties							
Palaionterveno	2.52 b	2.27 b	10.97 a	0.91 b	2.26 b	3.81 b	2.33 a
Spata	5.04 a	6.20 a	9.12 b	6.37 a	11.08 a	4.12 a	2.09 b
$LSD_{5\%}$	0.25	0.18	0.19	0.25	0.34	0.17	0.11
Fertilization							
Control	3.86 a	4.31 a	10.16 a	3.65 a	6.73 a	4.03 a	2.26 a
Sheep manure	3.67 a	4.31 a	10.18 a	3.55 a	6.62 a	3.90 a	2.18 a
CAN-75 kg N ha^{-1}	3.82 a	4.19 a	9.83 a	3.66 a	6.61 a	3.96 a	2.26 a
CAN-125 kg N ha^{-1}	3.76 a	4.13 a	10.01 a	3.70 a	6.72 a	3.97 a	2.16 a
F-values and significant differences							
Varieties (V)	449.584 ***	2136.812 ***	409.389 ***	2163.437 ***	3015.044 ***	15.523 ***	19.612 ***
Fertilization (F)	0.509 ^{ns}	1.119 ^{ns}	3.186 ^{ns}	0.320 ^{ns}	0.157 ^{ns}	0.498 ^{ns}	0.852 ^{ns}
V × F	0.244 ^{ns}	0.557 ^{ns}	1.059 ^{ns}	0.912 ^{ns}	0.234 ^{ns}	0.187 ^{ns}	2.205 ^{ns}

Taxifolin: TXF, silychristin: SCS, silydianin + isosilychristin: SDN + ISCS, silybin A: SBA, silybin B: SBB, isosilybin A: ISBA, isosilybin B: ISBB, and calcium ammonium nitrate: CAN. For each factor, means followed by different letters within the same column show significant differences according to the LSD test. *** significant at $p \leq 0.001$, respectively. ns = not significant.

4. Discussion

4.1. Milk Thistle Varieties

Variety significantly affected plants growth, since the rosette diameter and height of plants in Spata were higher by up to 13.57% and 15.40%, respectively, compared with Palaionterveno. The impact of genetic material in plants growth parameters was observed in previous studies as Ram et al. [15], Gresta et al. [26], Shokrpour et al. [22], and Sulas et al. [27] recorded plant height from 78 cm to 207 cm in various milk thistle genotypes. Moreover, varieties significantly affected the number of fruits per inflorescence as in Palaionterveno this number was higher by up to 32.38% than that in Spata. The number of fruits per inflorescence in these two varieties of Greek origin ranged from 58.9 to 147.5 and was similar to that observed in previous studies. Stancheva et al. [28] reported values from 119.4 to 185.5, while Shokrpour et al. [22] recorded fewer fruits per inflorescence, ranging from 51.6 to 101.4. In contrast, 1000-fruit weight was not affected by variety. With regard to fruit yield, in 2021, the highest values were recorded in the variety Palaionterveno, while in 2020, there were no differences between the two varieties. In 2020, in Palaionterveno, a reduction in the seed germination was observed and as a consequence, the plant density was reduced by 20% resulting in a lower fruit yield than that in 2021. According to Arampatzis et al. [18], a high plant density can lead to the greatest seed yield. In another study, Shokrpour et al. [22] assessed various ecotypes and recorded yields from 889 to 2416 kg ha^{-1}. The above results show that the selection of a productive variety is important in order to maximize crop yield.

The oil content was ranged from 22.1 to 25.1% in the two varieties. In other studies conducted in Italy and Greece, Martinelli et al. [23] and Arampatzis et al. [25] observed that oil content in fruits of several milk thistle genotypes had higher values that ranged from 26.7 to 31.7% and from 24.7 to 31.1%, respectively. Compared with other species, the oil content of milk thistle is similar to hemp (*Cannabis sativa* L.) in which is ranging from 25.5 to 28.2% [29], but less than that in sesame (*Sesamum indicum* L.) and sunflower (*Helianthus annuus* L.) seeds that oil content is ranging from 34.2 to 36.5% and from 37.9 to 51%, respectively [30]. In 2021, the oil content in both varieties was higher compared with that in 2020, probably due to the wetter weather conditions that prevailed during April and May in 2020. Similarly, in Iran, water stress increased oil accumulation in the fruits of the plants [10].

Silymarin content in the fruits of the two varieties ranged from 2.4 to 4.4%, and variety Spata had significantly higher silymarin content than Palaionterveno. This finding is in agreement with Arampatzis et al. [25] as Spata had the highest silymarin content between thirty genotypes. In previous studies conducted in Greece, Italy, and India, the silymarin content in the fruits of several milk thistle genotypes ranged from 2.0 to 7.72% [15,23,25]. Moreover, the accumulation of silymarin active components was different, and Spata had a high content of silybin B, while silybin A and B constituted 35–39% of silymarin in Spata and 12% in Palaionterveno. In 2021, the silymarin accumulation was higher by 4.38–8.18% compared with 2020, probably due to the dryer weather conditions that prevailed during the second growing season. However, it is well documented in other studies that water stress causes an increase in silymarin accumulation in the fruits [10,18].

4.2. Fertilization Regimes

The application of sheep manure significantly increased rosette diameter and aboveground biomass in both years compared with the untreated control, while plant height was affected only in the first growing season. Similarly, Saad-Allah et al. [20], reported that the use of poultry manure increased plant height and dry biomass of milk thistle, while in rice (*Oryza sativa* L.), the application of manure increased plant height and the effect was more obvious when the manure was combined with urea [31]. However, the plant growth was greater when the calcium ammonium nitrate was applied compared to the use of sheep manure. The application of the sheep manure was not sufficient to fully meet plants nitrogen requirements due to the slow nitrogen mineralization from this organic fertilizer. This result can be explained since milk thistle is characterized by rapid growth in the period of mid-March to early May and as a consequence, the requirements are more intense during this period. Similarly, Popin et al. [32] reported that the use of urea or manure increased the height of maize (*Zea mays* L.) compared with control, and the effect of urea was more intense. It is also important to point out that the application of calcium ammonium nitrate at a high dose led to the maximum rosette diameter, aboveground biomass, and height of plants. Previously, an increase in nitrogen fertilizer dose increased plants height or biomass of different milk thistle genotypes [17,33]. Moreover, the use of sheep manure or calcium ammonium nitrate significantly influenced the number of fruits per inflorescence. Similarly, Afshar et al. [16] observed that poultry manure increased this trait. In contrast, 1000-fruit weight was not affected by fertilization.

The use of sheep manure beneficially affected fruit yield. This finding is in agreement with previous experiments as Saad-Allah et al. [20] observed that the application of chicken manure led to higher fruit yield compared to control, and an augmentation in the rate of the applied manure further increased the fruit yield. In general, the application of manure increased yield in other crops such as winter wheat (*Triticum aestivum* L.), maize, rice, and the effect was more obvious when the manure was combined with chemical fertilizers [31,34,35]. However, milk thistle plants show rapid growth and produce high aboveground biomass, and thus the nitrogen needs cannot be completely covered by sheep manure due to the slow nitrogen mineralization. As a result, the application of calcium ammonium nitrate led to a greater yield than manure. In a previous study, nitrogen fertilization significantly increased plants productivity, especially the application of a high dose [17]. In 2021, there was an interaction effect on fruit yield between the two factors, since sheep manure influenced the fruit yield only in Palaionterveno. Similarly, in rice [36] and bread wheat [37], there was an interaction between genotype and nitrogen fertilization on grain yield.

Moreover, fertilization had no impact on oil content. Similarly, Afshar et al. [16] observed that manure application had no impact on oil content in milk thistle fruits, while Li et al. [38] reported that the application of nitrogen fertilizers did not affect the oil content in sunflower seeds. These results show that the oil content mainly depends on the genetic material. Moreover, oil yield ranged from 181.2 to 377.4 kg ha^{-1} similar to previous studies that recorded 217.5–376.1 kg ha^{-1} [16] and 353–591 kg ha^{-1} [18] in different genotypes.

However, sunflower shows greater oil productivity (669 to 1210 kg ha^{-1}) [30] compared to milk thistle crop. In our study, the application of calcium ammonium nitrate led to higher oil yield compared with manure; consistent with the maximum oil yield in canola crop that was observed in the plots where the manure was applied in [39]. Moreover, the calcium ammonium nitrate increased the oil yield of both varieties, while sheep manure affected this parameter only in the variety Palaionterveno. Similarly, in milk thistle [16], canola (*Brassica napus* L.) [39], and hemp [29] crops, the application of manure increased oil yield compared to control.

Silymarin content was not influenced by sheep manure and calcium ammonium nitrate application. In other studies, Afshar et al. [16] observed that the use of manure did not affect the silymarin content, while Saad-Allah et al. [20] mentioned that manure application led to a higher silymarin accumulation than that in control. Regarding the effects of fertilization on the content of silymarin constituents, our results indicated no significant differences between sheep manure and calcium ammonium nitrate, while Geneva et al. [21] and Stancheva et al. [28] reported that the soil and foliar fertilization (NPK) decreased the content of silybins A and B, silychristin, silydianin, and taxifolin. However, both varieties showed silymarin yield (27.1–50 kg ha^{-1}) comparable to previous studies that recorded 13.3–63.3 kg ha^{-1} [6,18], while fertilization positively affected silymarin yield. Sheep manure induced an increase only in the first year of the experiment, while calcium ammonium nitrate, especially in a high dose, influenced silymarin yield of the two varieties in both years. These findings are in agreement with that of Geneva et al. [21], Afshar et al. [16], and Liava et al. [17] in different genotypes, as they observed that the application of manure or inorganic fertilizers enhanced silymarin yield owing to higher fruit yield.

5. Conclusions

The outcome indicates that variety and fertilization are important factors that can influence fruit and silymarin yield of milk thistle crop. Spata had greater silymarin content and yield, and higher accumulation of silybin A and B compared with Palaionterveno, while fertilization regimes had no impact on flavonolignans and taxifolin content in the fruits. The results of the experiment clearly show that the application of sheep manure and calcium ammonium nitrate promoted plants growth and yield. In general, the application of the inorganic fertilization significantly improved the productivity of this crop compared with sheep manure; although, further studies are needed to evaluate the effects of the combination of inorganic with organic fertilization on this crop. Finally, our results revealed that Spata can be exploited in breeding programs owing to high silymarin productivity and high silybins A and B content. The development of new varieties with the desired traits will help to further improve the productivity of this crop and the quality of the final product (fruits or silymarin extracts).

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