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EFFECT OF NITROGEN FERTILIZATION ON YIELD AND QUALITY OF MILK THISTLE [*SILYBUM MARIANUM* L. (GAERTN.)] ACHENES*

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Abstract

Milk thistle is cultivated especially for the medical substance called the silymarin complex, which can be used in human medicine and in animal nutrition. The composition of this complex depends on many factors and one of these factors is fertilization. This aim of this study was to determine the effect of nitrogen fertilization on the yield and quality of milk thistle achenes. A pot experiment was established and the milk thistle cultivar Mirel was used as a model crop. The effect of four variants of nitrogen fertilization (lower and higher single doses, lower and higher split doses) was observed. The observing parameters were the content of macronutrients in aerial vegetative mass, height, weight and dry matter weight of milk thistle plants, number of inflorescences and achenes per plant, 1000 achenes weight, yield of achenes per plant and content of the silymarin complex in achenes. The variant with a higher single dose of N showed the significantly highest number of achenes (254.5) and yield of achenes (7.9 g per plant). The significantly highest number of mature inflorescences (2.5 per plant) was found in variants with single doses of N. Nitrogen fertilization slightly modified the content of silymarin complex constituents in achenes. The highest content of silybin was in the variant with a higher split dose of N, while the significantly highest content of silychristin was in the variant with a lower single dose. Plants from the variant with a higher single dose had a significantly higher content of silychristin and isosilybin than the control variant. The total content of the silymarin complex in achenes was increased only in the variant with a higher split dose of nitrogen (higher by about 6% in comparison with the control).

Keywords: milk thistle, nitrogen fertilization, yield of achenes, number of inflorescences, silymarin complex.

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INTRODUCTION

Milk thistle [*Silybum marianum* L. (Gaernt.)] belongs to popular herbal plants. This plant is not regularly cultivated plant in the Czech Republic, but it is the most often cultivated crop among the group of medicinal and aromatic plants and spices. The Czech Statistical Office does not monitor the area cropped with milk thistle, but the estimated cultivation area is approximately 5 000 hectares and the average yield of achenes is 0.7 t ha^{-1} (Přibylová 2014).

Milk thistle is cultivated especially for the medical substance called the silymarin complex. This complex is contained in achenes (Pradhan, Girish 2006) and it is a mixture of flavonolignans (silybin A and B, isosilybin A and B, silychristin and silydianin) and also flavonolignan taxifolin (Abbasi et al. 2010, Abenavoli et al. 2010, Elwekeel et al. 2013). There are many factors, like the sowing depth, raw spacing, fertilization, harvesting and post harvesting treatment, which affect the silymarin complex content and composition in achenes (Omer et al. 1993, Karkanis et al. 2011). The silymarin complex is used in human medicine, especially as treatment of liver and spleen diseases (Cardile, Mbuy 2013) or to reduce the risk for developing cancer (Deep et al. 2008). Milk thistle is also used in animal nutrition. It could be applied as mixtures of herbs with whole milk thistle fruits, silymarin concentrate or green matter in a form of silage (Tedesco et al. 2004).

Mineral nutrition plays a huge role in plant cultivation; especially nitrogen (N) has important impact on the production of milk thistle, as it affects the growth and the duration of the vegetative growth of this plant (Omidbaigi, Nobakht 2001, Estaji et al. 2016), inflorescence development (Stancheva et al. 2008) and yield of achenes (Omer et al. 1993, Warren, Sams 2011, Cwalina-Ambroziak et al. 2012, Estaji et al. 2016). The aim of this study was to assess the effect of nitrogen fertilization on milk thistle growth, inflorescence development, achene production and content of the silymarin complex in achenes.

MATERIAL AND METHODS

The effect of nitrogen fertilization on the growth of milk thistle was observed in a pot experiment. The experiment was established in a greenhouse at the Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of AgriSciences, Mendel University in Brno in 2016. Pots were filled with 6 kg of silt soil, the content of available nutrients in the soil was analyzed prior to the establishment of the experiment and proved to be satisfactory (content of P – 113.3 mg kg^{-1}, content of K – 305.5 mg kg^{-1}, content of Ca – 1766 mg kg^{-1} and content of Mg – 132.3 mg kg^{-1}). The soil reaction was slightly acid (pH/CaCl = 5.7). P, K, Ca and Mg were estimated by the method of Jones (1990), in the soil extracted with a solution Mehlich III.

Five seeds of the milk thistle cultivar Mirel were sown on April 22, 2016. Ammonium nitrate (27% N) was used as nitrogen fertilizer. Fertilizer was applied in single doses (on the day of sowing) and split doses (on the day of sowing and at the beginning of the elongation growth during the elongation and branching of plants – BBCH 21 according to Martinelli et al. (2015), which was on June 9, 2016, according to the scheme in Table 1. All variants were conducted in four repetitions. N_0 variant without fertilization was considered as the control variant. Two plants per pot were left after fertilizer application on June 9, 2016. On June 17, 2016, at stage BBCH 37 according to Martinelli et al. (2015), one plant was left in a pot and one plant from each variant was taken for the determination of the content of macronutrients in aerial vegetative mass. The samples of plant mass were dried at a temp. of 60°C, then crushed in a grinder and homogenized. The resultant crushed plant mass was mineralized using a mixture of H_2SO_4 and H_2O_2 (Zbíral et al. 2005). For determination of the nitrogen content the Kjeldahl method was applied (Persson et al. 1995). Phosphorus was determined colorimetrically using a Unicam 8625 UV/Vis spectrometer (ATI Unicam, Cambridge, UK), while the content of potassium, magnesium and calcium was assayed by Atomic Absorption Spectrophotometry (AAS) in the mineralized samples with a ContrAA 700 instrument (Analytik Jena AG, Jena, Germany) according to Zbíral et al. (2005). The samples of plant mass for determination of sulphur were also dried, crushed and homogenized and after that the samples were mineralized using a mixture of H_2O_2 and HNO_3 in a microwave system (MILESTONE MLS 1200 MEGA, Bergamo, Italy). The determination of sulphur was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a JY-24 instrument (JOBIN-YVON, France) according to Zbíral et al. (2005). The content of the silymarin complex in achenes was analyzed by the means of HPLC (high-performance liquid chromatography). The samples of achenes were ground to fine powder. After that, 20 mg of each sample were homogenized in a mortar and pestle, adding sea sand and isooctane (0.5 cm^3) and methanol (0.5 cm^3). Methanol (1.5 cm^3) and isooctane (1 cm^3) were added after homogenization and an amount of 2 cm^3

Table 1

Scheme of the pot experiment

Variant of fertilization	Dose of N (mg pot^{-1})	Fertilizer	Date of application
N_0 (control)	0	–	–
N_1 (single dose)	157	AN	22. 04. 2016
N_2 (single dose)	314	AN	22. 04. 2016
N_3 (split dose)	78.5 + 78.5	AN	22. 04. + 9. 06. 2016
N_4 (split dose)	157 + 157	AN	22. 04. + 9. 06. 2016

AN – ammonium nitrate

of the aliquot was centrifuged for 5 min at 14.000 rpm. Then, 0.75 cm^3 of lower phase was taken from each sample and analyzed in a Dionex Ultimate 3000 instrument (Thermo Fisher Scientific, Waltham, USA). The silymarin complex was separated using a Hypersil GOLD column 150 × 4.6 mm set at 30°C, with 0.005 cm^3 of the sample injected to the column. The analysis was performed at the mobile phase flow rate of 1 cm^3 min^{-1} and the UV detection at 288 nm. The analytes were evaluated under isocratic conditions with 65% of mobile phase A (0.1% formic acid) and 35% of mobile phase B (100% methanol). One analysis lasted 45 min (Snyder et al. 1997).

In the phase of the maturity of terminal flower heads (July 2, 2016), the height and weight of plants, number of inflorescence (buds, with immature achenes, with mature achenes), number and yield of mature achenes per plant and 1000 achenes weight were determined using 4 plants in each variant. The effect of nitrogen fertilization on all characteristics was evaluated with the Statistica CZ 12 programme. Analysis of variance (ANOVA) was used and the results are expressed as a mean ± standard error (SE). The differences among the treatments were evaluated by follow-up tests according to Fisher (LSD test) at a 95% ($P < 0.05$) level of significance.

RESULTS AND DISCUSSION

Nitrogen fertilization had a positive effect on the number of achenes (Figure 1), as all fertilized variants had more achenes than the control variant. It is also obvious that the yield of achenes per plant in the individual variants was affected by the number of achenes (yield = 0.079 + 0.031 num-

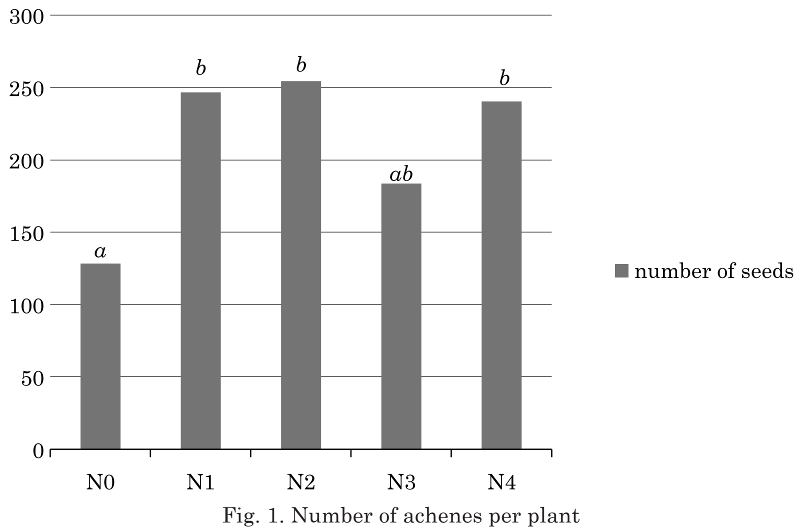

Fig. 1. Number of achenes per plant
(variants with same letter are not significantly different at $P < 0.05$)

ber of achenes; r = 0.985; P < 0.000) but the yield of achenes was not affected by the 1000 achenes weight (yield = 6.546 - 0.013weight of 1000 achenes; r = -0.013; P < 0.950). Similarly to OMER et al. (1993), we noticed that nitrogen application had a significant effect on the yield of achenes in contrast to the variant without N application (Figure 2). OMER et al. (1993) indicated that the yield of achenes rises proportionally to the increasing dose of nitro-

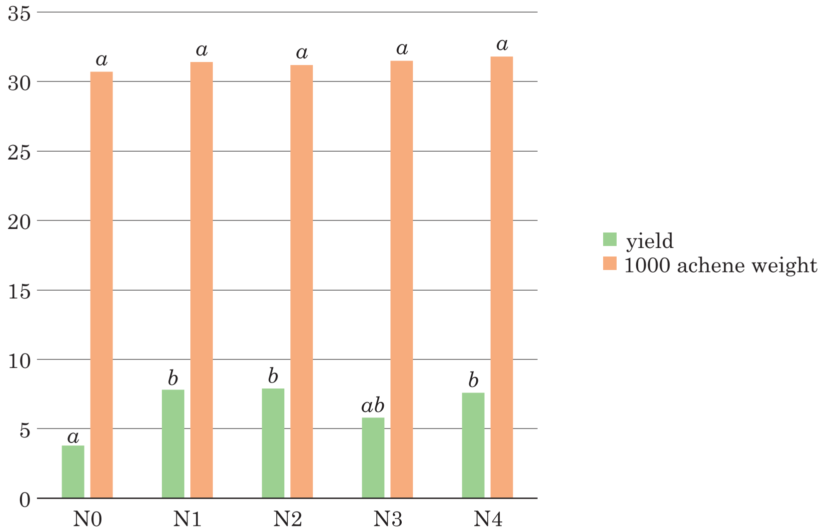

Fig. 2. Yield of achenes (g per plant) and 1000 achenes weight
(variants with same letter are not significantly different at $P < 0.05$)

gen, and the highest number and yield of achenes was on plants with the higher single dose from N_2 variant in this experiment. Like OMIDBAIGI, NOBAKHT (2001), we did not find a significant effect of nitrogen fertilization on the 1000 achenes weight, as this parameter was only slightly enhanced in fertilized variants in comparison with the control. In a study of WIERZBOWSKA et al. (2012), application of nitrogen also caused an increase in the number of achenes, yield of achenes and 1000 achenes weight in contrast to unfertilized treatments. The effect of split doses on the observed parameters was not as strong as the effect of single doses, but all parameters were increased in comparison with the control.

Accordning to the results shown in Figure 3, the highest number of buds was determined in variant N_4 (higher split dose of N). The number of buds in this variant was three times higher than the number of buds in the control, thus plants with a later application (split doses) of nitrogen tended to prolong the growing season. This corresponds with results obtained by DROSSOPOULOS et al. (1997), who reported that nitrogen fertilization delays senescence. The number of mature inflorescences per plant was increased owing to N fertilization, and the significantly highest number of mature inflorescences was found in variants with single doses of N (N_1 and N_2).

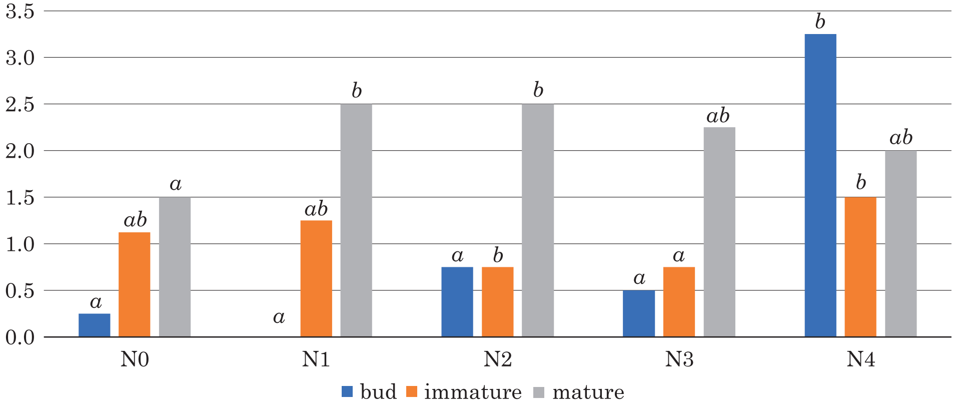

Fig. 3. Number of inflorescences per plant
(variants with same letter are not significantly different at $P < 0.05$)

Table 2

Height, weight and dry matter weight of milk thistle

Variant	Height of plant (cm)	Weight of whole plant (g)	Dry matter weight (g plant^{-1})
N_0	$77.00^{c*} \pm 1.690$	$21.13^{a} \pm 1.093$	$7.159^{a} \pm 0.312$
N_1	$75.00^{c} \pm 5.115$	$36.25^{bc} \pm 2.394$	$10.40^{ab} \pm 1.584$
N_2	$59.75^{a} \pm 4.230$	$34.50^{bc} \pm 3.069$	$12.00^{ab} \pm 1.454$
N_3	$69.50^{bc} \pm 1.555$	$28.25^{ab} \pm 1.652$	$10.27^{b} \pm 0.565$
N_4	$62.00^{ab} \pm 2.380$	$40.75^{c} \pm 5.779$	$8.622^{ab} \pm 2.825$

Values are expressed as means ± SE, * different superscription letters in the rows indicate significant ($p \leq 0.05$) differences between means.

The results in Table 2 did not show an increase in the plants' height after single or split nitrogen application, and likewise OMIDBAIGI, NOBAKHT (2001) did not note a higher height of milk thistle plants after split doses of nitrogen. N fertilization had a big effect on the weight of whole plants, plants from N_4 variant with a split dose showed the significantly highest increase in contrast to control plants. Dry matter weight was also increased in all variants with fertilizer in contrast to the control, with the highest dry matter weight achieved by plants from N_2 variant.

The content of nitrogen in dry matter (DM) was significantly increased in all fertilized variants in contrast to the control variant, and the significantly highest content of N was determined in N_3 variant with the lower split dose of N (Table 3). The significantly highest level of P and K content was determined in N_4 variant (higher split dose), where the content of P increased by about 33% and the content of K rose by about 47% in comparison to the control variant. WIERZBOWSKA (2013) reported that the content of Mg in milk thistle achenes were not significantly influenced by different levels of N fertilization and, according to the results in Table 3, N fertilization

Table 3

Content of macronutrients in aerial vegetative mass of milk thistle

Variant	N	P	K	Ca	Mg	S
	(g kg $^{-1}$ DM)					
N_0	$11.45^{a*} \pm 0.580$	$3.320^{a} \pm 0.250$	$35.96^{a} \pm 3.440$	$38.09^{a} \pm 1.740$	$2.690^{a} \pm 0.110$	$0.840^{a} \pm 0.000$
N_1	$22.64^{c} \pm 0.600$	$3.820^{ab} \pm 0.150$	$49.01^{b} \pm 3.270$	$40.13^{a} \pm 2.190$	$2.760^{a} \pm 0.080$	$1.290^{c} \pm 0.000$
N_2	$16.99^{b} \pm 1.280$	$3.700^{ab} \pm 0.040$	$47.36^{b} \pm 2.140$	$39.32^{a} \pm 1.530$	$2.720^{a} \pm 0.070$	$1.160^{b} \pm 0.020$
N_3	$31.14^{d} \pm 1.990$	$4.200^{b} \pm 0.200$	$49.90^{b} \pm 2.840$	$39.19^{a} \pm 1.140$	$2.990^{a} \pm 0.040$	$1.310^{c} \pm 0.050$
N_4	$30.08^{d} \pm 1.910$	$4.400^{b} \pm 0.260$	$52.90^{b} \pm 0.650$	$37.88^{a} \pm 1.080$	$2.870^{a} \pm 0.180$	$1.270^{c} \pm 0.040$

Values are expressed as means ± SE, * different superscription letters in the rows indicate significant ($p \leq 0.05$) differences between means.

in our experiment did not have a significant effect on the content of Mg in aerial vegetative mass. In all fertilized variants, the content of sulphur was significantly higher than in the control variant. The content of sulphur reached an almost identical level in all fertilized variants, thus the level of sulphur content was not influenced by different doses of nitrogen.

The content of the silymarin complex depends on many factors and the contribution of individual constituents could vary. According to SERSEN et al. (2004), the silymarin complex contains 36.3% of silybin, 15.7% of silychristin, 5.9% of silydianin and 5.1% of isosilybin. Others have reported that the silymarin complex consists of 50 to 60% of silybinin, around 20% of silychristin, around 10% of silydianin and around 5% of isosilybinin (ABENAVOLI et al. 2010). In our experiment, the percentages of these substances were slightly different, for example the control variant contained 58% of silybin, 25.1% of silychristin, 5.4% of silydianin and 11.5% of isosilybin. It is reported that the composition of the silymarin complex is strongly genetically dependent (MARTIN et al. 2006), and SMITH et al. (2005) mentioned that the proportions of flavolignans inthe silymarin complex could be highly diverse in different seed lines of milk thistle. The composition of the silymarin complex is also strongly affected by factors like the sowing depth, fertilizers, harvesting and post harvesting treatment (OMER et al. 1993, KARKANIS et al. 2011). SADOWSKA et al. (2011) reported that the chemical composition of milk thistle fruits depends on the weather conditions during the plant growing season, which have a stronger effect on the silymarin complex than agronomical factors.

The results in Table 4 show slight enhancement of each constituent after fertilization. The highest content of silybin was in N_4 variant with the split dose of N, while the significantly highest content of silychristin was in N_1 variant. Plant from N_2 variant had the significantly highest content of silydianin and isosilybin. However, the total content of silymarin complex in achenes was enhanced only in N_4 variant in which was about 6% higher in comparison with control. The silymarin complex content was not increased in the other fertilized variants. OMIDBAIGI, NOBAKHT (2001) and STANCHEVA

Table 4

Percentages of individual constituents of the silymarin complex and total content of silymarin

Variant	Silybin	Silychristin	Silydianin	Isosilybin	Total silymarin complex
	(%)				(mg g^{-1})
N_0	57.97^{abc*} ± 0.180	25.07^{b} ± 0.136	5.413^{b} ± 0.023	11.55^{a} ± 0.095	33.55^{bc} ± 0.661
N_1	57.53^{a} ± 0.027	25.53^{c} ± 0.097	5.140^{a} ± 0.098	11.81^{ab} ± 0.056	28.47^{a} ± 1.637
N_2	58.01^{bc} ± 0,140	24.31^{a} ± 0.105	5.717^{c} ± 0.084	11.96^{b} ± 0.080	31.60^{abc} ± 1.221
N_3	57.78^{ab} ± 0,132	24.95^{b} ± 0.046	5.572^{bc} ± 0.125	11.70^{ab} ± 0.065	30.40^{ab} ± 1.377
N_4	58.34^{c} ± 0.252	24.51^{a} ± 0.106	5.390^{ab} ± 0.065	11.76^{ab} ± 0.219	35.56^{c} ± 1.696

Values are expressed as means ± SE, * different superscription letters in the rows indicate significant ($p \leq 0.05$) differences between means.

et al. (2008) did not notice any positive correlation between a dose of nitrogen fertilization supplied to field grown milk thistle plants and the silymarin content in achenes.

CONCLUSIONS

Nitrogen fertilization increased the whole plant weight and dry matter weight. The significantly highest weight of a whole plant was found in N_4 variant with a split dose of nitrogen. A high positive effect of split doses of N (N_4 variant) on the number of buds was found and all variants with nitrogen application had an enhanced number of mature inflorescences per plant. This improvement contributed to an increasing number of achenes per plant and yield of achenes per plant, which were higher in all fertilized variants than in the control variant. The significantly highest number of achenes and yield of achenes per plant were demonstrated by plants from N_2 variant with a single dose of N. Nevertheless, 1000 achenes weight was not significantly affected by N fertilization. The content of silymarin complex constituents in achenes of fertilized milk thistle varied but the total content of the silymarin complex in achenes was increased only in N_4 variant with a split dose of nitrogen.

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