IMPROVEMENT OF MILK THISTLE (*SILYBUM MARIANUM* L.) SEED YIELD AND QUALITY WITH FOLIAR FERTILIZATION AND GROWTH EFFECTOR MD 148/II

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Summary. The effects of foliar or soil fertilization and MD 48/II (3-methylphenylamide 5-tert-butylpyrazine-2-carboxylic acid) on the vegetative and reproductive growth, some physiological parameters, seed yield and silymarin content of field grown milk thistle (*Silybum marianum* L. Gaertn.) plants were studied. Foliar fertilizer Agroleaf^R was applied at different plant developmental stages, with different proportions of N, P, K. Combined application of the foliar fertilizer with MD 148/II at a concentration of 1.10^{-3} M affected most positively growth, number of plant lateral shoots and flower heads per plant. These changes were associated with altered flowering rate, enhanced seed ripening and increased yield. The accumulation of flavonoids and silymarin compounds in the seeds was also positively influenced.

Key words: 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide, foliar fertilization, milk thistle (*Silybum marianum* L. Gaertn.), seed yield, silymarin.

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INTRODUCTION

Silymarin, derived from the seeds of milk thistle plant has been used widely for the treatment of toxic liver damage (Dewick, 1998). Silymarin primarily consists of an isomeric mixture of six phenolic compounds: silydianin, silychristin, diastereoisomers of silybin (silybin A and B), and diastereoisomers of isosilybin (isosilybin A and B) (Lee et al., 2007). Geneva et al. (2008) found that the treatment of milk thistle with thidiazuron and foliar fertilizer was beneficial for plant vegetative and reproductive growth and resulted in higher silymarin content in the seeds. The efficiency of foliar fertilization on plant growth and improvement of fruit quantity and quality have been reported by Stancheva et al. (2004). The application of mineral nutrition and plant growth regulators usually results in the improvement of vegetative development due to primary metabolism acceleration (Chernyadev, 1994) but their applicability as plant secondary metabolite effectors is still unknown.

Recently Dolezal et al., (2002) synthesized and tested biological activity of amides derived from substituted pyrazinecarboxylic acid. It was found that some of these compounds possessed 1growth regulating activities in plants. Tumova et al, (2005) found that the newly synthesized compound 3-methylphenylamide 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide (**MD148/II**) increased flavonolignan production milk thistle suspension culture. On the basis of these results we carried out experiments aiming to evaluate the combined effects of abiotic elicitor MD 148/II and foliar fertilization on seed yield, flowering rate and silymarin content in the seeds of milk thistle grown in field experiments.

MATERIALS AND METHODS

The study was conducted over two years (2006-2007) at an experimental field, on a leached cinnamonic meadow soil. Milk thistle (*Silybum marianum* L. Gaertn.) seeds were sowed at a plant density of 5 plants m^{-2}. Three formulations of foliar fertilizers (Agroleaf®, Scotts Company, Ohio, USA) were applied at different developmental stages: (1) Agroleaf® total - N:P:K=20:20:20 + microelements, was applied twice during the vegetative growth stage on 20 days interval until the rosette phase; (2) Agroleaf® with high P - N:P:K=12:52:5 + microelements, was applied before the blooming stage; (3) Agroleaf® with high K - N:P:K=15:10:31 + microelements, was applied after the blooming stage. Microelements in chelated form are presented in concentrations: 0.1 % Fe, 0.06 % Mn, 0.06 % Cu, 0.06 % Zn, 0.02 % B.

MD148/II was applied in the beginning of milk thistle flowering stage by spraying. Applied concentrations of MD148/II during the first experimental year (2006) were 1.10^{-5}, 1.10^{-4} and 1.10^{-3} M. During 2007 we applied 1.10^{-3} mol concentration only. The following treatments were tested: (1) control plants, without application of fertilizers and MD148/II (control); (2) foliar fertilized plants (FF); (3) foliar fertilization plus MD148/II (FF+ MD148/II). The assessment included 4 experimental time-points: early flowering stage, flowering stage, immature flower heads, and mature flower heads. Diameter of flower head, shoot height, number of lateral stems per plant were measured at the flowering stage. The individual silymarin compounds were analyzed by a Shimadzu LC-2010 HPLC

Comparison of means from 4 replicates for the differing measurements was performed by the Fisher LSD test (P= 0.05) after performing ANOVA analysis. The STASTICA (version 6.0) package was used for statistical analysis.

RESULTS

Foliar fertilization resulted in increased DW of the seeds and seed number per flower head, total number of flower heads and eventually seeds yield in comparison with the control (Table 1). This favourable effect is intensified due to the combined application of **MD148/II** and foliar fertilization. The highest content of silymarin in the milk thistle seeds and the highest seed yield as a result mainly of the highest number of flower heads per unit area was obtained in the treatments with foliar fertilization and concentration of MD148/II - 1.10^{-3} M. According to the results obtained during 2006 we concluded that the most favourable concentration of MD148/II for the aims of our study is 1.10^{-3} M. That is why we continued the experiments with this concentration of the compound during the next year. The number of lateral shoots also increased significantly due to the application of MD148/II (Table 2), but differences regarding plant height and flower head diameter were not found.

The highest silymarin level was assayed in the seeds of control plants (Table 3). This maximal level resulted from increased silybin B, silycristin and taxifolin content mainly. Foliar fertilization negatively influenced total content of silymarin and all its components in mature seeds, a decrease with about 20 % was observed as compared to the control.

Application of MD148/II enhanced the proportion of overblown flowers since the beginning of the flower stage until the seed maturity in comparison with the other treatments (Figure 1). Therefore it can be supposed that MD148/II application influenced the dynamics of the flowering process.

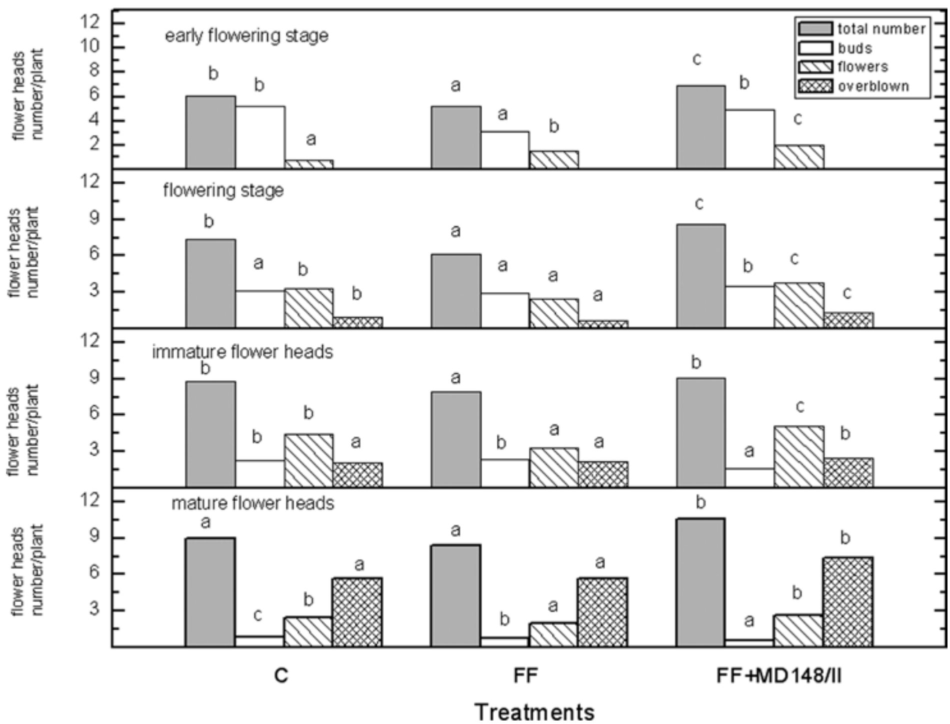

Fig. 1. Changes in the flowering dynamics of milk thistle plants grown at foliar fertilization and MD148/II application. Treatments: C - control plants, FF – foliar fertilization, FF+MD148/II – foliar fertilization + MD148/II.

Table 1. Milk thistle yield characteristics, field experiment, 2006, 2007.

Data are presented as means of 4 replications. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Variants	DW of seeds per flower head	Seeds number	Total number of fl. heads	Yield of seeds	Silimarin	Diameter of flower head	Number of lateral shoots	Plant height
	(g)	flower head^{-1}	m^2	g/m^2	%	cm	plant^{-1}	cm
2006								
Control	2.35^a	85.8^a	37^a	86.95^a	1.8	-	-	-
FF – foliar fertilized	2.70^b	105.9^{bc}	56^b	151.2^b	1.87	-	-	-
FF +1.10^{-3}M **MD148/II**	3.11^c	113.1^c	79^d	245.69^d	2.17	-	-	-
FF+1.10^{-4} M **MD148/II**	3.10^c	104.9^{bc}	67^c	207.70^c	1.48	-	-	-
FF+1.10^{-5} M **MD148/II**	2.30^a	98.1^b	60^b	138.00^b	0.6	-	-	-
LSD ($P \leq 0.05$)	0,256	9,28	4.62	15,67		-	-	-
2007								
Control	3.28^a	119.4^a	45^a	147.60^a	5.88	3.46^a	4.37^a	108.89^a
FF – foliar fertilized	3.94^b	149.4^b	42^a	177.49^b	4.76	3.39^a	4.27^a	105.66^a
FF +1.10^{-3}M **MD148/II**	4.92^c	185.5^c	53^b	260.76^c	5.62	3.38^a	5.64^b	114.40^a
LSD ($P \leq 0.05$)	0.413	15.37	6.43	20,09		0.340	0.477	11.125

Table 2. Content of taxifolin and silymarin components (% of dry matter of seeds).

Data are presented as means of 4 replications. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Treatments	Total Silymarin content	Taxifolin	Sily-cristin	Sily-dianin	Silybin A	Silybin B	Iso-Silybin A	Iso-Silybin B
Control	5.876	1.132	1.493	0.101	1.005	1.597	0.416	0.132
FF-foliar fertilized	4.755	0.923	1.193	0.065	0.812	1.295	0.350	0.117
FF + **MD148/II**	5.616	0.934	1.408	0.163	1.029	1.621	0.368	0.093

DISCUSSION

Application of liquid fertilizers on plants usually results in fast accumulation of nutrients in leaves, although in some cases they can also influence nutrient mobilization to roots (Wojcik 2004). The early application of NPK balanced foliar fertilizer enhanced plant vegetative growth. The late application of liquid fertilizer with higher potassium content can be regarded as a reason for the activation of carbohydrate metabolism as well (Kuepper, 2003). As Omer et al. (1993) suggested that N and K fertilization and plant spacing affected seed yield and seed content of flavonolignans silybin, silydianin, and silychristin of *Silybum marianum* (L.) Gaertn. Some authors (Andrzejewska and Skinder, 2007) showed that soil fertilization of milk thistle combined with the procedure of altered sowing date had a positive effect on the milk thistle seed yield, silymarin content and proportion of unsaturated fatty acids. In our previous study (Geneva et al., 2008) it was found that combined treatments with foliar fertilizer and thidiazuron (a growth regulator with high cytokinin activity) had a positive effect on the milk thistle growth, flowering rate, seed yield and silymarin content. The favorable effect of the substituted amides of pyrazine-2-carboxylic acids on the flavonolignan accumulation in callus and suspension culture of milk thistle is shown by Tumova et al. (2005). This treatment might cause increased accumulation of secondary metabolites such as phenolics resulting from some shift of the primary cell metabolism. It could suggest that late treatment with the MD 148/II at concentration 10^{-3} M might exert some retardation effect on the plant growth which, was beneficial for the flavonolignan metabolism and accumulation. From the other side the increased growth of lateral shoots bearing newly formed flower heads coincided with some plant growth delay, which appeared to be a result of the apical dominance overcoming, caused by excessive accumulation of some hormones in the shoot apex. This process can contribute also to accumulation of flavonolignans in the seeds. Application of MD148/II (5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide) in concentration 1.10^{-3} M, on the background of foliar fertilization improved growth, enhanced flowering rate, seed yield, the content of active substances in the milk thistle plants. Increased seed yield is due to the high number of flower heads per area unit as a consequence of the increased lateral stem branching of milk thistle.

Acknowledgements: This study was financially supported by the project PISA-INI №14/01.09.2005 and project № CC- 1504, Bulgarian Ministry of Education and Sciences.

References:

Andrzejewska, J., Z. Skinder, 2007. Yield and quality of milk thistle (*Silybum marianum* (L.) Gaertn.) raw material grown in monoculture and crop rotation. Part. 2. Milk thistle reaction to potassium fertilization. Herba Polon., 53, 5-9.

Chernyadev, I.I., 1994. Effect of 6-benzylaminopurine and thidiazuron on photosynthesis in crop plants. Photosynthetica, 30, 287-292.

Dewick, D.M., 1998. Medicinal Natural Products. A Biosynthetic Approach, John Wiley and Sons, Canada, 465 pp

Dolezal, M., M. Miletin, J. Kunes, K. Kralova, 2002. Substituted amides of pyrazine-2-carboxylic acids: Synthesis and biological activity. Molecules, 7, 363-373.

Geneva, M., G. Zehirov, I. Stancheva, L. Iliev, G. Georgiev, 2008. Effect of soil fertilizer, foliar fertilizer and growth regulator application on milk thistle (*Silybum marianum* L.) development, seed yield, and silymarin content. Commun. Soil Sci Plant Anal., 39, 17-24.

Kuepper, G., 2003. Foliar Fertilization, Current Topic ATTRA-National Sustainable Agriculture Information Service NCAT Agric. Spec., 10 pp

Lea, I.L., M. Narayan, J. S. Barrett, 2007. Analysis and comparison of active constituents in commercial standardizes silymarin extract by liquid chromatography-electrospray ionization mass spectrometry. J. Chrom. B, 845, 95-103.

Omer, E.A., M.E. Ibrahim, 1995. Effect of spacing nitrogen and potassium fertilization of *Silybum marianum*. Horiculture Abstr., 66, 985-989.

Stancheva, I., I. Mitova, Z. Petkova, 2004. Effects of different nitrogen fertilizer sources on the yield, nitrate content and other physiological parameters in garden beans. Envir. Exp. Bot., 52, 277-282.

Tumova, L., K. Gallova, J. Rimakova, M. Dolezal, J. Tuma, 2005. The effect of substituted amides of pyrazine – 2- carboxylic acids on flavolignan production in *Silybum marianum* culture *in vitro*. Acta Physiol. Plant., 27, 357-362.

Wojcik, P, 2004. Uptake of mineral nutrients from foliar fertilization. J. Fruit Ornam. Plant Res., 12, 201-218.