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Influence of *Nostoc entophytum* and *Tetracystis* sp. on Winter Survival of Rapeseed

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Abstract: Bioassay results proved that several microalgae strains of the Mosonmagyaróvár Algal Culture Collection (MACC) enhanced plant growth, due to their hormone content and other secondary metabolites. The aim of the current research was to improve autumn growth and winter survival of rapeseed (*Brassica napus* L.) by treatment with two microalgae strains selected by bioassay results. Experimental plots were set up in Mosonmagyaróvár in 2010 and 2013. Winter rapeseed hybrid (*B. napus* L. cv. *Orlando*) plants were treated in 4-6 leaves stage with 0.3 g/L and 1 g/L suspensions of MACC-612 *Nostoc entophytum* Bornet & Flahault and MACC-430 *Tetracystis* sp. in middle of October. After the treatments, the following parameters were recorded: chlorophyll-a and b, carotenoid, dry matter content of leaves, average amount of autumn foliage, diameter of root collar, length of shoot tips, fresh and dry weight of root, and number of plants in autumn and spring. Both microalgae treatments significantly increased pigment concentration and dry matter content of leaves, number of fully grown leaves (13%-46%) and dry root weight (16%-36%). Treatments with 0.3 g/L and 1 g/L MACC-612 suspensions increased the length of shoot apices by 14%-18% and 25%-35%, respectively. Number of overwintered control plants decreased significantly in both years (31%), but there was no decrease in parcels treated with 1 g/L of MACC-612 and MACC-430. Microalgae treatments could increase plant growth and survival, which contributed to the significant increase of thousand seed weight (18%-25%) and total yield (by 10%-24%).

Key words: Microalgae, photosynthetic pigments, winter oilseed rape, winter survival.

1. Introduction

The changing global climate has made previously secure agricultural production more difficult worldwide. Drought is typical of the Hungarian climate with the distribution of precipitation varying both in space and time [1]. In most areas of the country, rainfall has either significantly declined or has become irregular during the last half century. The largest precipitation scarcity occurs during spring [2]. The overwintering of rape highly influences the yield [3]. While delayed sowing improves plant survival during the winter months, deeper planting of the seeds may slightly decrease the successful overwintering of the plants but has no influence on the amount of oil collected from the seeds [4, 5]. Choosing the right timing for sowing can significantly increase the yield. Water and temperature have the biggest impact on growth of plants. The extent of growth and the length of the growing cycle influence the yield, although there is little information as to what kind of physiological changes can lead to the formation of biomass and produce [6]. Stress caused by cold weather also significantly affects the fertility of plants [7]. In multiple rape-cultivating areas worldwide, the frosty period determines the rape plant growth and the yields while viability of the planted seeds indicates the plant's tolerance against cold [8]. With decreasing

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temperatures, the size of developing ice crystals in the leaves of plants preparing for winter, can lead to some cell damage [7].

Different biostimulants have a positive impact on the growth of plants [9-14]. They contribute to the development of rootage [15] and nutrient absorption, increase photosynthetic activity and improve their ability to cope with environmental stress factors [16, 17]. For example, application of artificial auxin analogues, increased the content of proline and saccharose of the roots and shoot tips of rape, which led to the survival of an increased number of overwintered plants and also improved their growth indicators [18].

Seaweed-based products that contain a mixture of phytohormones enhance nutrient uptake of plants, favourably influence root formation and increase the plant's chlorophyll concentration [11]. Positive effects of *Ecklonia maxima* seaweed extract (Kelpak) on rape's physiological progression have been recorded [12]. Root formation and absorption of N-P-K-S substances were enhanced in rape with application of a biostimulant made of seaweed and black peat [10]. Similarly, beneficial effects on root formation by *Salvia splendens* L. [19] and nitrogen uptake in rape were achieved with an *Ascophyllum nodosum* extract [20]. Relative water content, chlorophyll concentration and cell membrane stability of rape were improved with a biostimulant treatment in combination with a nitrogen and sulphur-based chemical fertilizer [21]. The highest values of morphological traits of plants determined in autumn before inhibition of growth as well as plants most complete winter survival were obtained under conditions of spraying with Asahi SL biostimulator, applied at the stage Biologische Bundesanstalt für Land und Forstwirtschaft, Bundessortenamt und Chemische Industrie (BBCH) 13-15. The population cultivar overwintered better in comparison to the two heterotic cultivars, produced rosettes with a higher number of leaves (on average 8.4 leaves), a thicker root collar (on average 7.9 mm), as well as a longer taproot (on average 17.3 cm) [22]. This positively influenced growth and increased the yield, too [23]. However, no scientific study has been found that would describe the influence of freshwater cyanobacteria and microalgae on the overwintering of different plants. The aim of the present study was to determine the effect of a cyanobacterium and green algae treatment on the growth and overwintering ability of rape.

2. Materials and Methods

The test plant was *Brassica napus* L. cv. *Orlando*, a winter rapeseed hybrid. For the treatment, two strains were obtained from the Mosonmagyaróvár Algal Culture Collection (MACC): a cyanobacterium MACC-612 *Nostoc entophytum* and a green microalga MACC-430 *Tetracystis* sp. These were selected for the phytohormone content as determined in previous studies [13, 24]. The biomass of both strains was produced in the algal culture apparatus previously described by Ördög [25].

The field experiments were set up in random block layout in Mosonmagyaróvár in 2010 and 2013. The size of the parcels was 14.4 m^2 (six rows, 24 cm row distance) with four repetitions and six treatments. The plants of the two middle rows composed the sampling rows. The experimental area's genetic soil type was alluvial meadow, luvisol soil, the soil hygroscopy and the sticky point index [26] of K_A: 46, pH_{KCl}: 7-7.17. To supplement the soil's nutrient content, 45 kg/ha nitrogen, 45 kg/ha phosphor, 45 kg/ha potassium active ingredient were distributed in the form of 3 × 15 complex chemical fertilizer. The preceding crop was winter wheat for both trials. Sowing was carried out using a Sulky seeder at double row distance (24 cm) on September 11th, 2010 and September 7th, 2013, using 3.5 kg/ha of seeds. The number of seedlings produced from seeds planted with an average of 2 cm deep was 47.5 ± 2.5 plants/m^2 in 2010 and 51.5 ± 3.5 plants/m^2 in 2013.

The microalgae treatments were applied at two concentrations, i.e., 0.3 g/L and 1 g/L to 4-6 leaf plants (BBCH-14-16) on October 15th, 2010 and on October 10th, 2013 (Table 1). The effects of the two microalgae water-suspensions were compared to other formulations used in rape's pest control technology: (6/1) Route: chemical fertilizer with high zinc content; (6/2) Folicur® Solo: 250 g/L tebuconazole, fungicide and regulator. The plants were treated in both years in spring, at the beginning of stem elongation (BBCH-30) (March 25th, 2011 and March 1st, 2014) and at the beginning of flowering, green bud-state (BBCH-51) (April 13th, 2011 and April 5th, 2014). For the sixth parcel Route® and Folicur® Solo were used in the 4-6 leaf period during autumn, and Wuxal® Boron (400 L/ha, 0.5%) in the spring growing period, at green bud-state (Table 1).

The experiments were carried out and the pigment concentration and dry matter content of the leaves were measured at the times and phenological phases presented in Table 2. After the microalgae treatment through the course of five weeks in autumn, 1-1 completely developed leaves of identical ages and sizes from 10-20 plants, located in the two middle rows of each experimental parcel were removed and collected with the sparse sampling method. After the comminution and homogenization of leaves, 10 g was dried to a constant weight at 104 °C. Based on the data of the fresh and dried leaf samples, the dry matter content was calculated. The homogenized fresh leaf samples were used to determine the pigment content of rape leaves where 0.1 g was placed in a pre-cooled mortar with a pinch of $CaCO_3$. The pigments were extracted with 20 mL acetone. The absorbance of the obtained clear extract was measured against acetone at 662, 644 and 440.5 nm. The following formulas were used to calculate pigment content:

$$\text{Kl-a} = [(9.78 \times \text{E662}) - (0.99 \times \text{E644})] \times [V/(1{,}000 \times W)] \quad (1)$$

$$\text{Kl-b} = [(21.4 \times \text{E644} - 4.65 \times \text{E662})] \times [V/(1{,}000 \times W)] \quad (2)$$

$$\text{Kar} = [(4.695 \times \text{E440.5}) - (0.268 \times Z)] \times [V/(1{,}000 \times W)] \quad (3)$$

where, Z = 5.13 × E662 + 20.41 × E644; E = extinction of the solution at given wavelengths; V = final volume of extract (mL); W = fresh weight of leaf material used for extraction (g). The pigment content was obtained in mg/g fresh weight [27].

The development of root collar was examined at the end of the autumn vegetation period to coincide with the beginning of soil frosting. Plants (10-15 plants) were removed from the two middle rows of each experimental parcel after reaching the rosette phase. Soil residue was cleaned from the root system and the root removed at the collar. After separating the leaves, the shoot tip was cut into two equal parts by the center line. The length and diameter at the root collar intersection were measured with a digital mortise gauge. The plant number examinations was carried out twice in both experimental years, before the winter period (December 3rd, 2010; December 7th, 2013) and at the beginning of the spring vegetation period (March 17th, 2010; March 15th, 2014).

Table 1 The experimental treatments of rape in Mosonmagyaróvár in 2010 and 2013.

	Treatments	Dose (g/ha)	Concentration (g/L); (mL/L)	Phenological phases	Volume of spray (L/ha)
1	Control	-	-	BBCH-14-16	400
2	MACC-612	120	0.3	BBCH-14-16	400
3	MACC-612	400	1	BBCH-14-16	400
4	MACC-430	120	0.3	BBCH-14-16	400
5	MACC-430	400	1	BBCH-14-16	400
6	(1) Route®	0.8 L/ha	4	BBCH-14-16	200
	(2) Folicur® Solo	2 L/ha	1	BBCH-14-16	200

BBCH (Biologische Bundesanstalt für Land und Forstwirtschaft, Bundessortenamt und Chemische Industrie): growth stages of mono- and dicotyledonous plants.

Table 2 The investigated parameters, dates and phenological phases of rapeseed in Mosonmagyaróvár in 2010/2011 and in 2013/2014.

	Studied parameters and developmental stages	Date		Phenological phase
		2010/2011	2013/2014	
1	Sowing	11.09.2010	07.09.2013	-
2	Germination	20.09.2010-02.10.2010	15.09.2013-25.09.2013	1-2 leaves unfolded
3	4-6 leaves stage	10.10.2010-20.10.2010	05.10.2013-20.10.2013	4-6 leaves unfolded
4	Microalgal treatment	15.10.2010	10.10.2013	4-6 leaves unfolded
		20.10.2010	17.10.2013	
		27.10.2010	26.10.2013	
5	Chlorophyll-a, -b and carotenoid	03.11.2010	31.10.2013	4-10 leaves unfolded
		10.11.2010	09.11.2013	
		17.11.2010	13.11.2013	
6	Development of young plants	13.10.2010-13.12.2010	10.10.2013-07.12.2013	4-15 leaves unfolded
7	Rosette stage	02.12.2010-	06.12.2013-	9 ≤ leaves unfolded
8	Amount of foliage	13.10.2010-13.12.2010	05.10.2013-07.12.2013	9 ≤ leaves unfolded
9	Development of shoot top			
10	Development of roots	13.12.2010	07.12.2013	9 ≤ leaves unfolded
11	Thickness of root collar			
12	Examination of winter survival	17.03.2011	15.03.2014	9 ≤ leaves unfolded
13	Harvest	20.06.2011	06.06.2014	Seeds are ≤ 80% black

Upon harvest (June 20th, 2011 and June 6th, 2014), plants were removed from the 3 × 1 m^2 per parcel and representative samples were created where 30-40 pods per plant were examined. Total yield, thousand seed weight, oil- and water-content were determined under laboratory conditions.

The measured data of the two experimental years were evaluated together, in accordance with the split-plot experimental setup. The main parcel was the year and the secondary parcel the treatment. The significant treatment effects were compared by using the Duncan's multiple range test (DMRT). The results of the analysis of variance (ANOVA) and two-way analysis of variance (Two-way ANOVA) were analyzed using Microsoft Excel^R 2007 spreadsheet software, and IBM SPSS^R Statistics 19.0 for Windows, GenStat for Windows (5th edition). The correlations between spring and autumn plant numbers (dependent variable), root dry weights, the diameter of root collars, length of shoot tips and leaf numbers (independent variable) were observed by using the multiple regression analysis. The laboratory research and experiments were carried out at the Department of Plant Sciences, Faculty of Agricultural and Food Sciences, Széchenyi István University.

3. Results

Both experimental years experienced highly variable weather conditions (Figs. 1a-1c). Based on 50 years' data, the average amount of monthly precipitation of Mosonmagyaróvár varies between 32-57 mm. January is the coldest month (-1.0 °C) and December has the lowest amount of direct sunlight (1.4 h/d) reaching the ground (Table 3).

During the 2010/2011 trial, 44 mm more precipitation fell compared to the 50-year average precipitation before the sowing period. This enhanced seed germination. Following sowing (October), the amount of precipitation declined by 30% compared to the 50-year average. The average snow coverage in Mosonmagyaróvár and its surroundings is 35-45 d. In 2010/2011, the amount of solid precipitation was 32.3 mm in December and 15.3 mm in January that ensured snow coverage up to the first 10 d of February. The temperature during the sowing and shooting periods resembled the 50-year average although

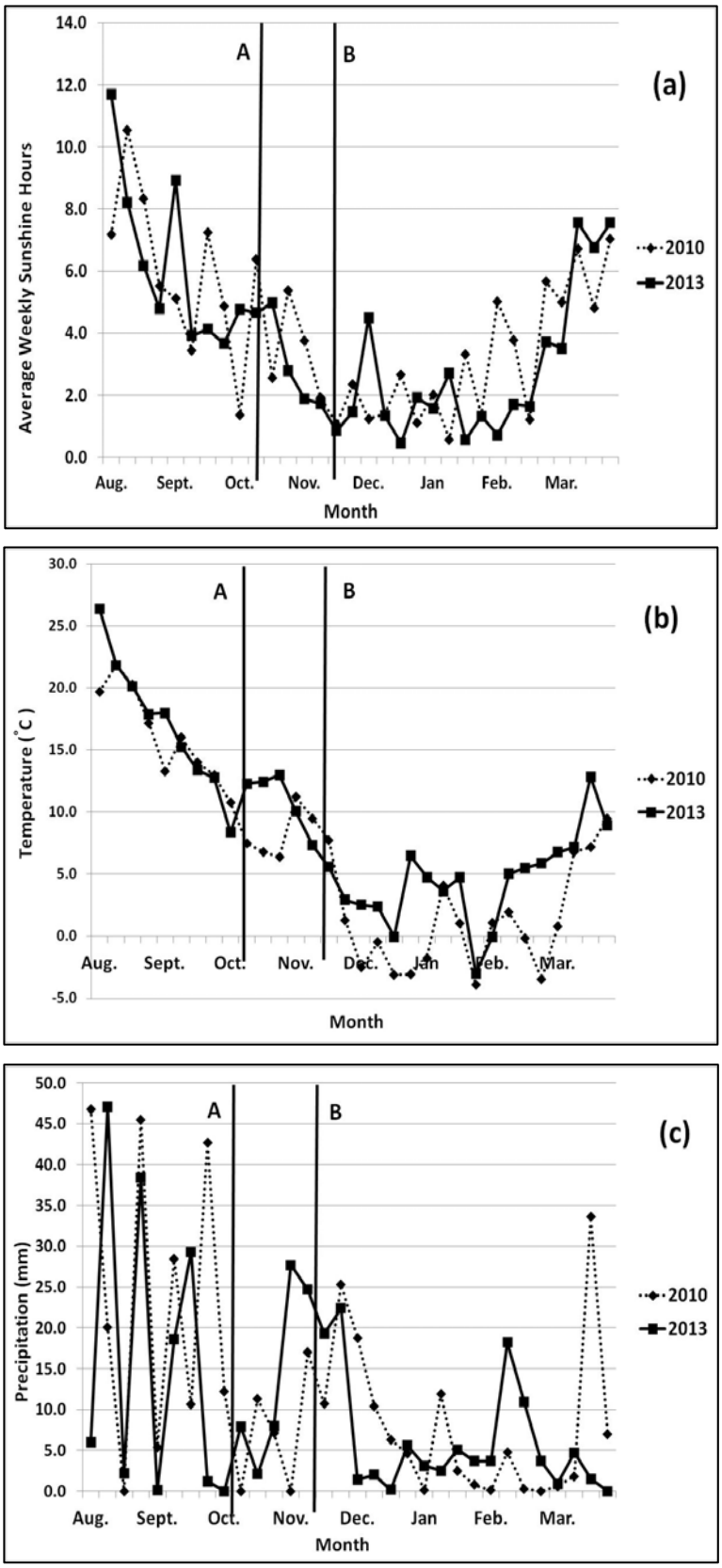

Fig. 1 The average weekly sunshine hours (a), temperatures (b) and precipitation (c) in the growing season of rape between August and March of 2010/2011 and 2013/2014 in Mosonmagyaróvár [28]. Pigment analysis between dates A and B.

Table 3 The average monthly precipitation, monthly average temperatures and daily sunny hours in Mosonmagyaróvár in the experimental years and between 1951 and 2000 [28].

Month	Weather parameters								
	Precipitation (mm)			Temperature (°C)			Sunny hours (h)		
	1951-2000	2010/2011	2013/2014	1951-2000	2010/2011	2013/2014	1951-2000	2010/2011	2013/2014
Aug.	57.4	112.4	33.7	19.9	19.7	21.4	8.2	7.9	8.6
Sept.	45.9	87.1	49.2	15.8	14.4	14.9	6.3	5.2	5.2
Oct.	42.6	30.6	18.0	10.4	7.76	11.6	4.2	4.0	4.3
Nov.	52.6	53.0	94.1	5.0	7.53	6.4	2.1	2.2	1.5
Dec.	43.6	40.1	9.2	1.0	-2.3	2.6	1.4	1.6	2.0
Jan.	33.1	15.3	14.4	-1.0	-0.07	2.5	1.8	1.8	1.5
Feb.	32.7	5.2	46.1	1.0	-0.15	4.1	2.8	3.9	1.9
Mar.	35.0	43.0	7.1	5.3	6.3	9.0	4.4	5.9	6.4
Sum	342.9	386.7	271.8	57.4	53.14	72.5	31.2	32.63	31.49

temperature in December 2010 was -3.3 °C, in February 2011 -1.15 °C lower than the 50-year average. The number of sunny hours was 5% higher than the 50-year average (Table 3).

During the 2013 trial, there was 21% less precipitation than the 50-year average. There was no snow coverage in the winter period. During the measurement period, it was 3.7 °C warmer than the 50-year average. The average number of sunny hours was 1% higher than the 50-year average (Table 3).

3.1 Effect of N. entophytum on Pigment and Dry Matter Content of Rapeseed Leaves

The chlorophyll-a content of the control plants' leaves varied between 0.47 mg/g and 0.79 mg/g (fresh rapeseed leaves (FW)) in the observed period of the first experimental year (Fig. 2a). At the first measurement there was no difference between the treatments. The MACC-612 in a 0.3 g/L concentration increased the leaves' chlorophyll-a content compared to the control by 30% (p = 5%) at the second measurement, 16% at the third, 12% at the fourth and 93% (p = 5%) at the fifth time. The MACC-612 in a 1 g/L concentration increased the leaves' chlorophyll-a content compared to the control by 28% (p = 5%) at the second measurement, 17% at the third, while 112% (p = 0.1%) at the fifth time. The conventional treatment did not have a significant influence on the chlorophyll-a content of the leaves in the first experimental year.

In the second experimental year the chlorophyll-a content of the control plants was 21% higher than in the first year, which decreased at the last measurement in both years to the same level (Fig. 2b). The MACC-612 in a 0.3 g/L concentration resulted in an 88% (p = 0.1%) and a 219% (p = 0.1%) higher pigment concentration at the fourth and fifth measurements compared to the control results. The growth of chlorophyll-a, after using a 1 g/L concentration was 58% (p = 1%) at the fourth measurement and 172% higher at the fifth one. The conventional cropping technology decreased the chlorophyll-a concentration by 40% at the third and fourth measurements compared to the control.

In the first year the chlorophyll-b content of the leaves of the control plants increased at the beginning, but in the end, compared to the first measurement, it decreased by 14% by the time of the fifth measurement (Fig. 2c). The initial growth applies to every treatment but the decrease did not occur at the time of the fifth measurement. The 0.3 g/L treatment resulted in a 26% (p = 5%) and an 81% (p = 1%) increase in the chlorophyll-b content at the second and the fifth measurements. The same values with a 1 g/L treatment showed a 20% (p = 5%) and a 99% (p = 0.1%) growth compared to the control. By using the Route-Folicur treatment a 33% (p = 1%) increase could be detected in the chlorophyll-b content of the leaves at the fifth measurement.

In the second experimental year the chlorophyll-b content of the control plants' leaves decreased by 26% by November 13th, 2013 compared to the first measurement (Fig. 2d). The MACC-612 in a 0.3 g/L concentration increased the leaves' chlorophyll-b content by 78% (p = 0.1%) at the fourth, while by 184% at the fifth measurement. The 1 g/L treatment increased the chlorophyll-b content compared to the control by 54% (p = 1%) at the fourth and 138% (p = 5%) at the fifth measurement. The conventional cropping technology decreased the chlorophyll-b concentration by 28% (p = 10%), 38% (p = 5%) and 33% at the third, fourth and fifth measurements.

In the first experimental year the carotenoid content of the control plants' leaves decreased by 26% compared to the first measurement (Fig. 2e). The MACC-612 lower concentration treatment increased the carotenoid content of the leaves compared to the control by 31% at the second measurement and 66% (p = 5%) at the fifth measurement. The 1 g/L treatment resulted in a 73% (p = 5%) growth at the fifth measurement, while the conventional treatment procedure did not influence the carotenoid content of the leaves significantly.

In the second experimental year the carotenoid content of the control plants' leaves decreased by 51% (Fig. 2f). After the treatment with MACC-612 in a 0.3 g/L concentration, the carotenoid content of the experimental plants' leaves decreased only by 17% by the end of the observed period. The carotenoid content of the treated plants' leaves did not significantly differ from the carotenoid content of the control parcel's plants at the first three measurements, while at the fourth and fifth time a 61% (p = 0.1%) and a 79% (p = 5%) increase was traceable. The MACC-612 in a 1 g/L concentration increased the concentration of carotenoids at the last two measurements by 40% and 63% (p = 5%). By following the conventional cropping technology, after the second measurement the carotenoid content decreased by 29% on average, however it reached the level of the control plants' by the last measurement.

In conclusion, it is to say that in both trials, application of MACC-612 significantly increased the chlorophyll-a content compared to the control and Route + Folicur treatments. In the 2010/2011 trial, 1 g/L MACC-612 application was the most effective while in the 2013/2014 trial, 0.3 g/L application was more effective (Figs. 2a and 2b). A similar trend was observed for chlorophyll-b (Figs. 2c and 2d) and carotenoid content (Figs. 2e and 2f).

In the first experimental year the dry matter content of the leaves by the end of the observed period dropped below the initial value by 5% after a promising increase at the beginning (Fig. 3a). The MACC-612 in a 0.3 g/L concentration increased the dry matter content of the leaves by 17% at the fourth and 37% (p = 0.1%) at the fifth measurement compared to the control. The 1 g/L dose treatment showed an increase of 16% (p = 5%) at the fourth and 23% (p = 1%) at the fifth measurement in the dry matter content of the leaves compared to the control. The MACC-612 in a 0.3 g/L concentration increased the dry matter content of the leaves by 26%, and in a 1 g/L concentration by 21% compared to the results of the first measurement. After the conventional treatment procedure the leaves' dry matter content was 17% lower at the second and the third measurements compared to the control.

In the second experimental year the MACC-612 0.3 g/L treatment significantly increased the dry matter content of the leaves, namely by 18% (p = 1%) at the fourth and 32% (p = 0.1%) at the fifth measurement. The 1 g/L treatment increased the dry matter content of the leaves by 16 % (p = 5%) at the fourth and 27% (p = 0.1%) at the fifth measurement. Following the conventional cropping technology did not result in any significant change concerning the dry matter content of the leaves in the observed period. In the observation period the MACC-612 in a 0.3 g/L concentration increased the dry matter content of the leaves by 28%, while with a 1 g/L dosage by 24%, compared to the initial figures.

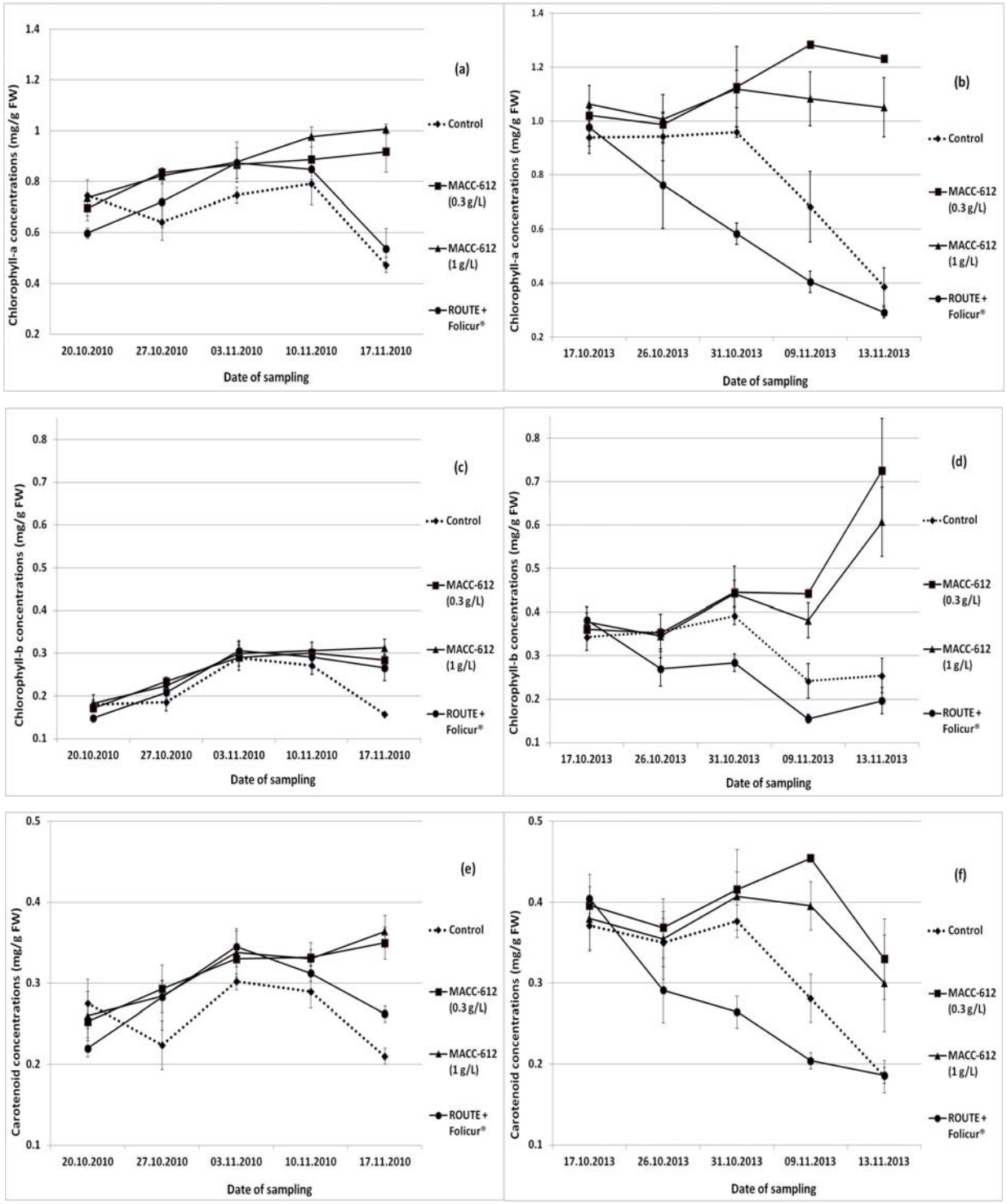

Fig. 2 The changes of chlorophyll-a concentration in fresh rapeseed leaves (FW) treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *Nostoc entophytum* in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (a) and between 17.10.2013 and 13.11.2013 (b); The changes of chlorophyll-b concentration in FW treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (c) and between 17.10.2013 and 13.11.2013 (d); The changes of carotenoid concentration in FW treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (e) and between 17.10.2013 and 13.11.2013 (f).

As a conclusion application of MACC-612 significantly increased the dry matter content compared to the control and Route + Folicur treatments with 0.3 g/L application being more effective than 1 g/L application (Figs. 3a and 3b).

3.2 Effect of Tetracystis sp. on Pigment and Dry Matter Content of Rapeseed Leaves

During the first five-week experimental period the MACC-430 0.3 g/L treatment increased the leaves' chlorophyll-a content practically as significantly as the MACC-612 0.3 g/L treatment (Fig. 4a). A notable difference can be seen with the 1 g/L treatment, as the chlorophyll content increased by 185% (p = 5%) at the fifth measurement compared to the control, while the MACC-612 showed only a 112% growth. In the second experimental year there was no remarkable difference between the influence of cyanobacteria and green algae on the chlorophyll-a content (Fig. 4b).

The effects of the MACC-430 and the MACC-612 on the leaves' chlorophyll-b content showed similar tendencies in both years, the change correlated to the control was practically identical (Figs. 4c and 4d). The biomass of the cyanobacterium and the green alga used in two different dosages had similar impact on the changes in carotenoid content during the experimental years (Figs. 4e and 4f).

In both trials, application of MACC-430 significantly increased the chlorophyll-a, chlorophyll-b and carotenoid content compared to the control and Route + Folicur

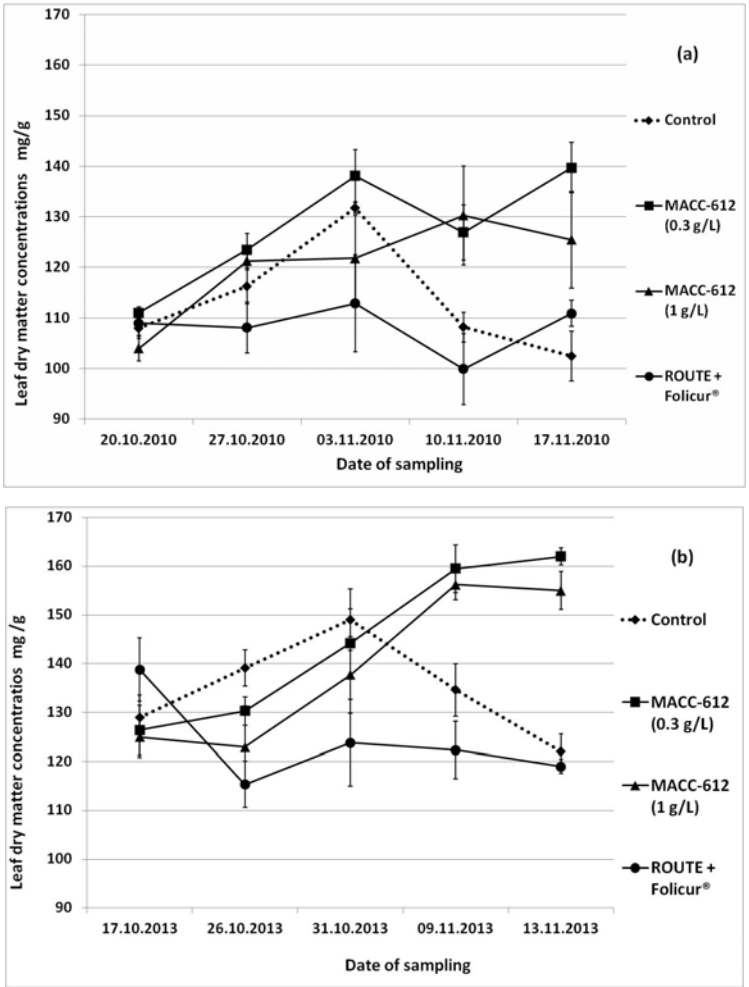

Fig. 3 The changes of dry matter concentration in FW treated on 13.10.2010 and 15.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (a) and between 17.10.2013 and 13.11.2013 (b).

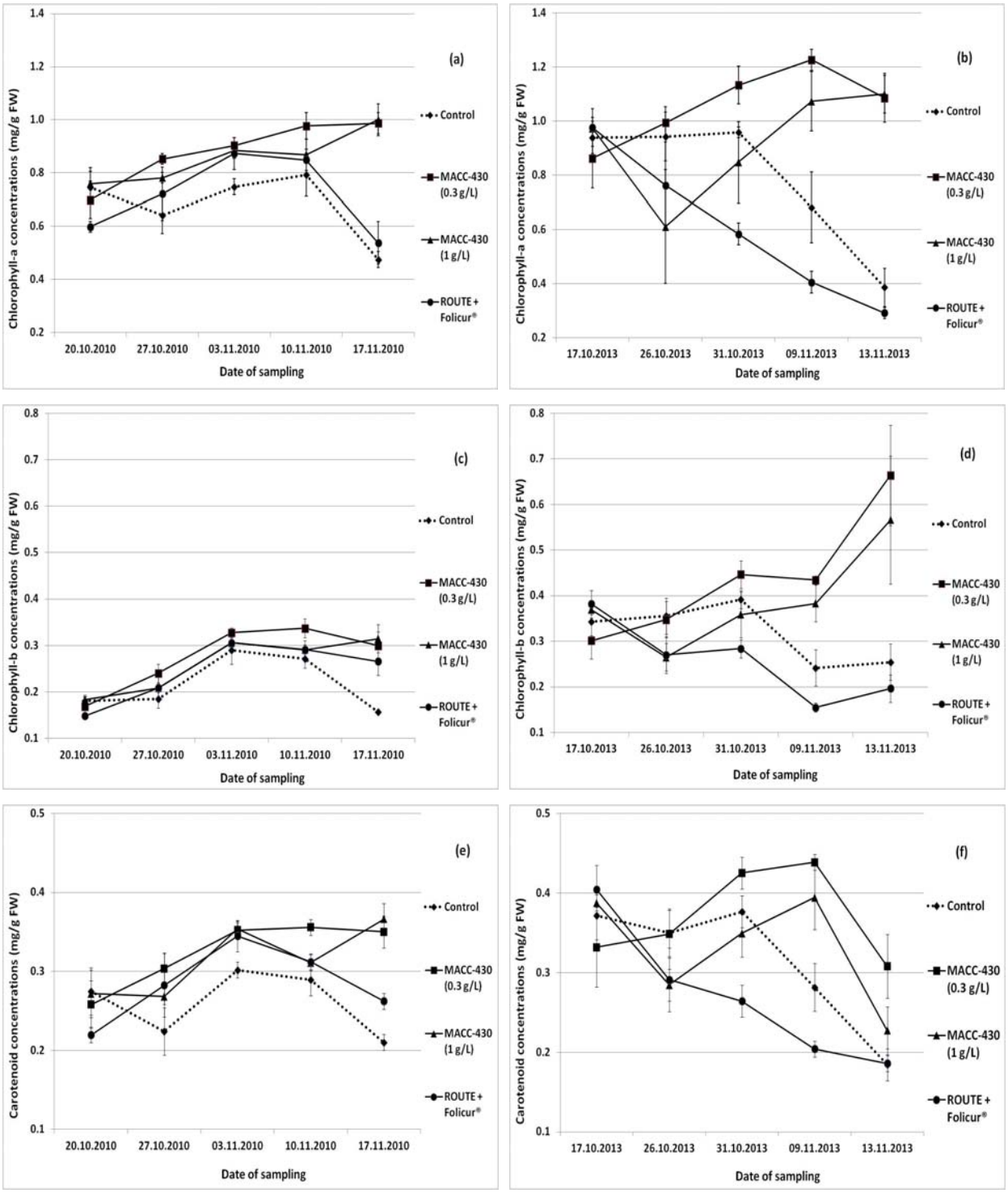

Fig. 4 The changes of chlorophyll-a concentration in FW treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-430 *Tetracystis* sp. in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (a) and between 17.10.2013 and 13.11.2013 (b); The changes of chlorophyll-b concentration in FW treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-430 *Tetracystis* sp. in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (c) and between 17.10.2013 and 13.11.2013 (d); The changes of carotenoid concentration in FW treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-430 *Tetracystis* sp. in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (e) and between 17.10.2013 and 13.11.2013 (f).

treatments with 0.3 g/L application generally being more effective than the 1 g/L application (Figs. 4a-4f).

In the first experimental year the MACC-430 0.3 g/L treatment increased the dry matter content of the leaves by 20% (p = 5%) at the fourth, while by 26% (p = 1%) at the fifth measurement (Fig. 5a). The 1 g/L treatment at the time of the last two occasions showed a significant, 22%-23% (p = 5%, p = 1%) increase.

In the second experimental year the increase of the dry matter content after a 0.3 g/L treatment reached 16% (p = 5%) at the fourth, while at the last measurement 33% (p = 0.1%) (Fig. 5b). A significant difference was detected when using the 1 g/L treatment only at the last measurement, where the increase was 34% (p = 0.1%). The change in the leaves' dry matter content showed similar tendencies in both experimental years, after using the biomass of the algae strains.

As a conclusion in both trials, application of MACC-430 significantly (p = 1% in 2010; p = 0.1% in 2013) increased the dry matter content compared to the control and Route + Folicur treatments with both 0.3 g/L and 1 g/L producing similar results (Figs. 5a and 5b).

3.3 Effect of Microalgal Suspensions on Plant Growth

Application of MACC-612 significantly increased the shoot tip length compared to the control and Route

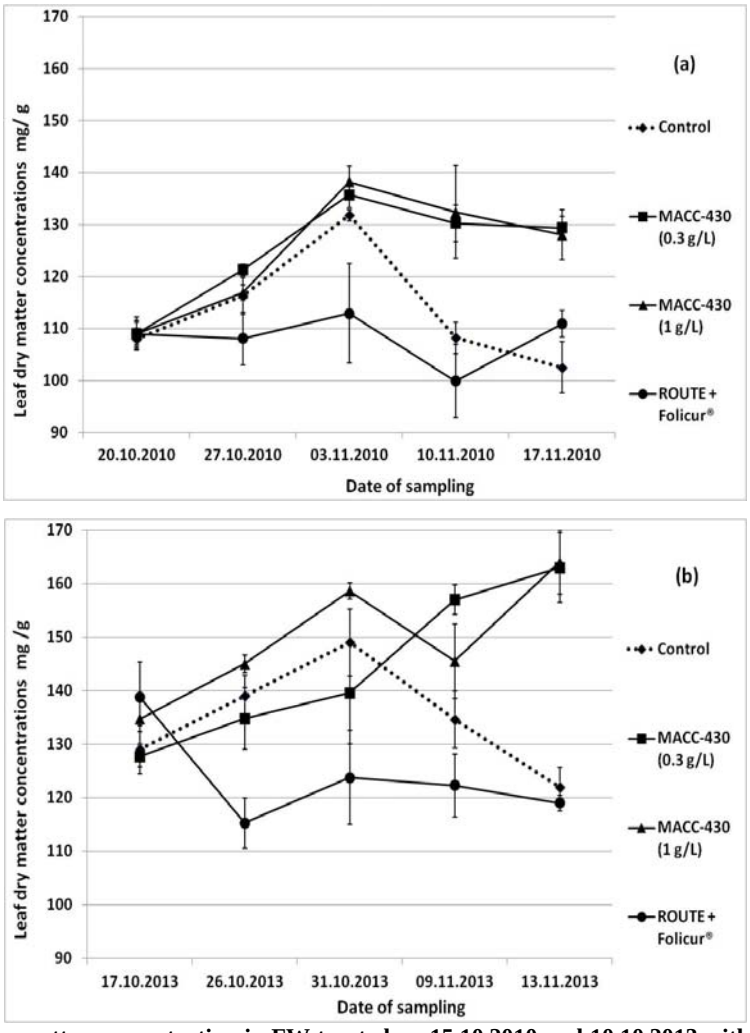

Fig. 5 The changes of dry matter concentration in FW treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-430 *Tetracystis* sp. in Mosonmagyaróvár between 20.10.2010 and 17.11.2010 (a) and between 17.10.2013 and 13.11.2013 (b).

+ Folicur treatments with MACC-612 1 g/L suspension being the most effective. By comparison, MACC-430 had no effect on the shoot tips' length (Fig. 6a). According to the Two-way ANOVA test, the microalgae treatments significantly (p = 0.1%) increased the length of shoot tips (2010/2013), however, the year and the year × treatment correlation did not prove to be significant. Based on the result of the DMRT, the treatments of MACC-612 in 0.3 g/L and 1 g/L concentrations did significantly (p = 5%) increase the length of shoot tips. Both microalgae treatments significantly increased the diameter of the root collar compared to the control and Route + Folicur treatments with 1 g/L MACC-612 being the most effective (Fig. 6b). Based on the variance chart of Two-way ANOVA, the microalgae treatments had a significant (p = 0.1%) impact on the diameter of the root collar. The year and the year × treatment correlation did not have a significant impact. Based on the DMRT, each microalgae treatment significantly (p = 5%) improved the diameter of the root collar, while the conventional Route + Folicur combined treatment did not cause a significant root collar growth compared to the control. Similarly, both microalgae treatments significantly increased the average leaf number per plant with 1 g/L MACC-612 and MACC-430 being the most effective (Fig. 6c).

Both, the microalgae-based treatments (p = 0.1%) and the year (p = 5%) had a significant influence on the average number of leaves per plant, according to the Two-way ANOVA. The year × treatment correlation was not significant. Based on the DMRT, each microalgae-based treatment showed a significant (p = 5%) impact. Those of the conventional treatments did not show a statistically verifiable difference compared to the average number of leaves of the control parcel.

Although the microalgae treatments did increase the fresh root weight, this was not significant compared to the control (Fig. 7a). According to the Two-way ANOVA, the fresh weight of the roots was significantly affected by the treatments (p = 1%) and the year (p = 5%). The year × treatment correlation did not have a significant impact. As for the DMRT, each microalgae-based treatment had a significant impact (p = 5%) on the wet weight of the roots. There had not been a statistically verifiable difference between the control and the conventional treatments. In contrast, both the MACC-612 and MACC-430 treatments resulted in a significantly increase of the dry weight of the roots with the MACC-430 treatments being the most effective (Fig. 7b). Based on the variance table of Two-way ANOVA, microalgae-based treatments had a significant (p = 0.1%) impact on the dry weight of the roots but the year and the year × treatment interference did not (Table 4). The results of the DMRT show that each microalgae treatment significantly increased the dry weight of the roots (Table 5).

3.4 Effect of Microalgae Treatments on Autumn and Spring Plant Number

The microalgae treatments had little effect on the plant number with the only significant increase observed at the beginning of the spring vegetation period for the 1 g/L MACC-612, 1 g/L MACC-430 and Route + Folicur treatments (Figs. 8a and 8b). The F test of regression analysis showed a significant (p = 0.1%) correlation between the autumn and spring plant numbers and the observed plant characteristics.

The T test of partial regression coefficients proved a significant impact on leaf number and the spring plant numbers (dependent variable), while it affected the invariant, the leaf number and spring number of the autumn plant number (dependent variable) significantly. There had been a close (p = 0.1%) correlation (r^2 = 0.739) between the autumn and spring plant numbers.

3.5 Effect of MACC-612 and MACC-430 Treatments on Yield

Both microalgae treatments significantly increased the seed weight with 0.3 g/L MACC-430 consistently

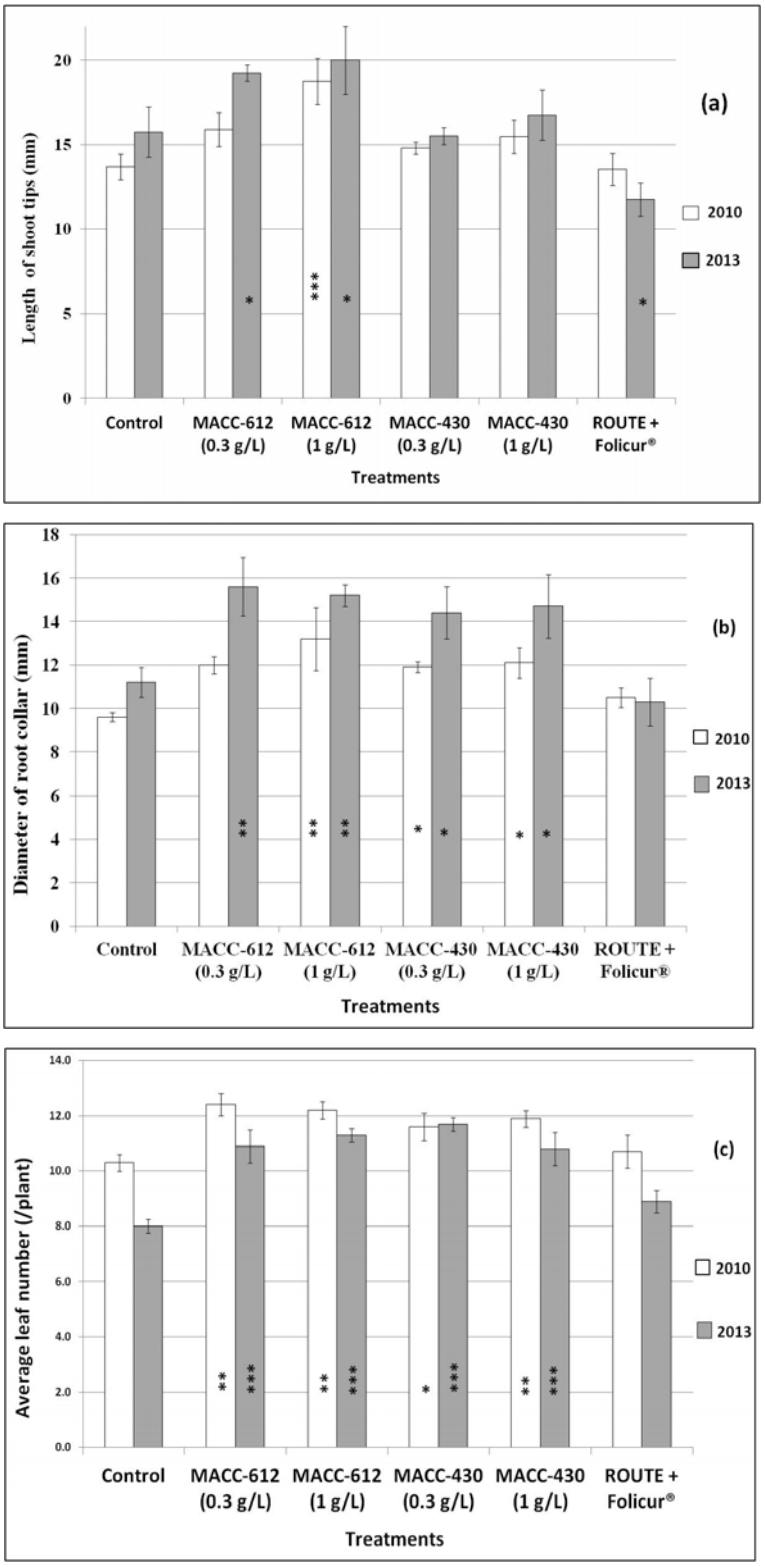

Fig. 6 Length of shoot tip (a) the diameter of root collar (b) and average leaf number (c) of rapeseed treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* and MACC-430 *Tetracystis* sp. in Mosonmagyaróvár. The results are from two trials performed on 13.12.2010 and on 07.12.2013 (p^{***} = 0.1%; p^{**} = 1%; p^{*} = 5%; shoot tip: $SzD0.1\%_{2010}$ = 5.1; $SzD5\%_{2013}$ = 3.9; diameter of root collar: $SzD1\%_{2010}$ = 3.48; $SzD5\%_{2010}$ = 2.56; $SzD1\%_{2013}$ = 4.29; $SzD5\%_{2013}$ = 3.2; leaf number: $SzD1\%_{2010}$ = 1.69; $SzD5\%_{2010}$ = 1.24; $SzD0.1\%_{2013}$ = 2.30).

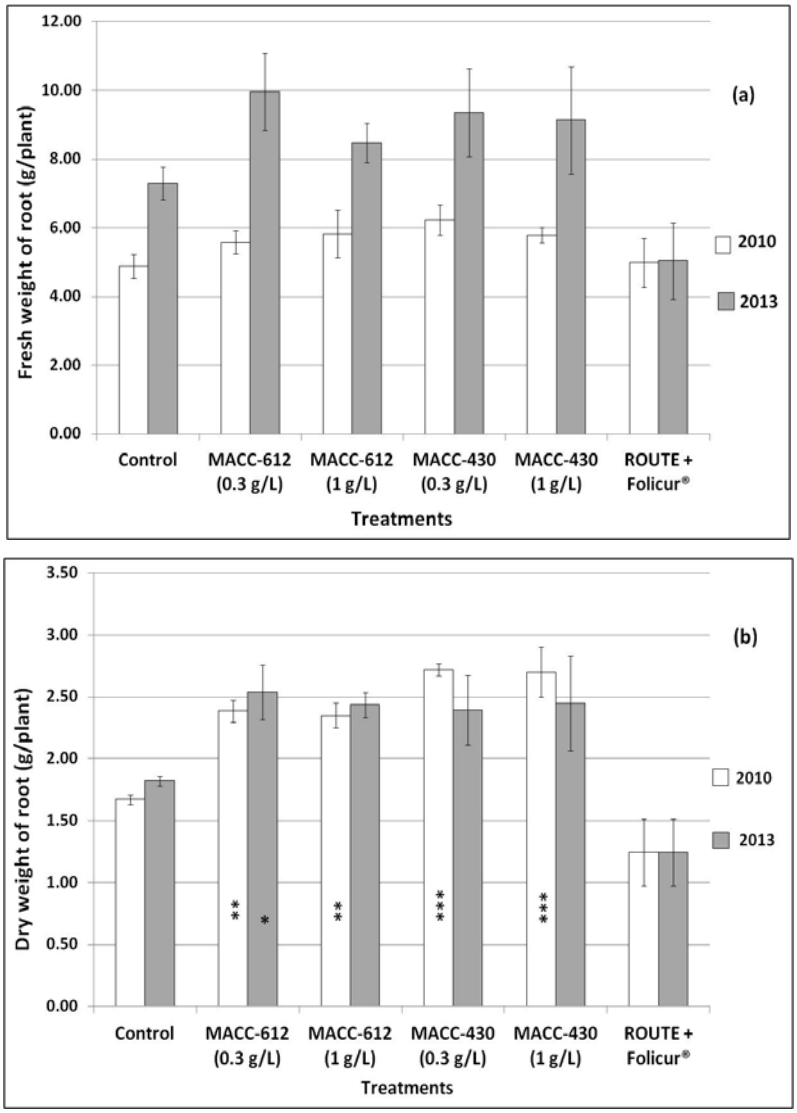

Fig. 7 Average fresh (a) and dry weight (b) of rapeseed roots treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* and MACC-430 *Tetracystis* sp. in Mosonmagyaróvár. The results are from two trials performed on 13.12.2010 and 07.12.2013 (p^{***} = 0.1%; p^{**} = 1%; p^{*} = 5%; dry weight: $SzD1\%_{2010}$ = 0.66; $SzD0.1\%_{2010}$ = 0.88; $SzD5\%_{2013}$ = 0.70).

Table 4 Variance table on the impact of microalgae treatments in 2010/2011 and 2013/2014.

Crop parameters		Root DW	Root FW	Root collar	Shoot tip	Leaf number	Moisture
Source of variation	d.f.	m.s.	m.s.	m.s.	m.s.	m.s.	m.s.
Replication	3	0.7484	8.166	2.717	6.194	0.3378	0.1635
Year	1	0.0216^{ns}	108.671^{*}	58.630^{ns}	27.440^{ns}	22.7587^{*}	0.9516^{***}
Residual	3	0.0725	6.106	10.253	3.203	1.4035	0.0021
Treatments	6	1.9968^{***}	8.439^{**}	20.057^{***}	37.303^{***}	9.2220^{***}	0.7260^{*}
Year × treatments	6	0.0675^{ns}	3.727^{ns}	2.681^{ns}	5.803^{ns}	1.0913^{ns}	0.2283^{ns}
Residual	36	0.1298	2.172	3.360	5.500	0.6979	0.2829

ns = not significant; *** significant at 0.1%; ** significant at 1%; * significant at 5%.

Table 5 Significant impact of microalgae treatments on 2010/2011 and 2013/2014 based on the Duncan's multiple range test (DMRT).

Treatments		Root DW	Root FW	Root collar	Shoot tip	Leaf number	Moisture
1	Control	1.747b*	6.08ab*	10.40a*	14.72ab	9.16a	9.287a
2	MACC-612 (0.3 g/L)	2.460c*	7.77c	13.79b	17.56d*	11.65b	9.250a
3	MACC-612 (1 g/L)	2.351c	7.15bc*	14.17b	19.36cd*	11.76b	9.188a
4	MACC-430 (0.3 g/L)	2.555c	7.82c	13.15b	15.15abc*	11.64b	9.988b
5	MACC-430 (1 g/L)	2.574c	7.46bc	13.42b	16.11bc	11.31b	9.350a
6	MACC-612 (1 g/L) Wuxal	2.531c	7.24bc	13.36b	15.04abc	11.75b	9.488ab
7	Route; Folicur, Wuxal	1.243a	5.01a	10.41a	12.65a	9.79a	9.787ab

Data followed by the same letter within a column do not differ significantly according to the DMRT at the $p \leq 0.05$ level.

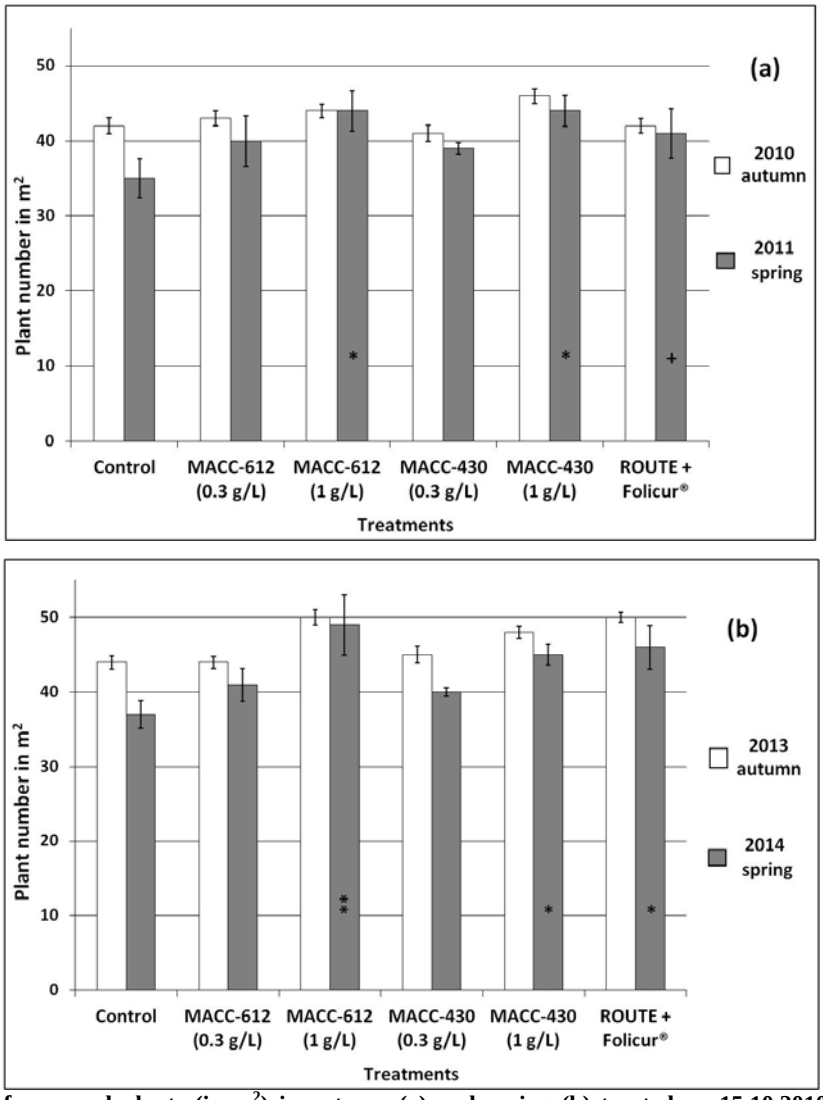

Fig. 8 The number of rapeseed plants (in m^2) in autumn (a) and spring (b) treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* and MACC-430 *Tetracystis* sp. in Mosonmagyaróvár. The results are from two trials performed on March 17th, 2011 and March 1st, 2014 (p^{**} = 1%; p^{*} = 5%; p^{+} = 10%; $SzD5\%_{2011}$ = 7.21; $SzD10\%_{2011}$ = 5.96; $SzD1\%_{2014}$ = 9.39; $SzD5\%_{2014}$ = 6.90).

giving the best results (Table 4). Both 0.3 g/L MACC-612 and 0.3 g/L MACC-430 significantly increased the yield and oil content per hectare. However, while the MACC-612 treatments had no effect on the % oil content, the MACC-430 treatment resulted in a significant decrease in the % oil content but a significant increase in the water content (Table 4).

According to multivariate analysis of variance (MANOVA), the microalgae-based treatments significantly (p = 5%) impacted the moisture content of seeds, while the year influenced it by p = 0.1%. Based on the DMRT, the treatments of MACC-430 in 0.3 g/L concentration resulted in a significantly (p = 5%) higher moisture content in the harvested seeds, compared to results of the control and every other microalgae and conventional treatment.

Within one column the treatments indicated with the same letters do not show a significant difference based on the DMRT (p = 5%).

According to the Two-way ANOVA analysis, neither the MACC-612 and the MACC-430, nor the conventional cropping technology's applied formulation treatments affected the yield, the fresh and dry oil content of the seeds.

4. Discussion

In the present study, the applied microalgae had a positive effect on the pigment and dry matter content of rapeseed leaves, which largely contributed to their successful overwintering. This is a similar response to those achieved with the application of an algae-based biostimulant treatment [10] and a tebuconazole-based regulator combined with an *E. maxima* seaweed biostimulant [29] applied to rape. Secondary metabolites produced by cyanobacteria increase the chlorophyll content of the higher plants' leaves [13]. The amount of chlorophyll-a and b is important in photosynthesis [30, 31]. As a result of higher chlorophyll content, plants are able to produce ATP and NADPH for a longer period of time. In the case of rape, this acquired energy surplus is used to propagate leaves and roots in the autumn period which increases the chances of successful overwintering. This was seen in the increase of dry matter content in the present study. Similarly, dry matter content increased in *B. napus* L. leaves when a saline treatment was applied [32] and increased the weight of shoot tips in greenhouse treated with plant growth-promoting rhizobacteria (PGPR) inoculant [15]. Seaweed and freshwater algae as growth-enhancers optimize the nutrient uptake of plants and enhance their development [33]. Both N content and nitrate uptake of the plant increased in rapeseed roots when treated with an *A. nodosum* seaweed extracts [20].

In addition, different growth regulators also improve the changes of successful overwintering (Fig. 9). These regulators which may be present is fungicide formulations. Tebuconazole-based regulators have a significant effect on the gibberellin's synthesis and its metabolic process [34], which plays a crucial part in plants' growth. The concentration of chlorophylls is influenced negatively by numerous stress factors such as drought [35, 36] while natural plant growth regulators protect the chloroplasts from damage [37]. Plant biostimulants, besides their numerous positive effects, also enhance photosynthetic and enzyme activity [38].

The MACC-612 treatments increased the length of shoot tips while the MACC-430 treatment did not have any effect in the present study. Similarly, application of *E. maxima* (Kelpak) seaweed extract increased the dry weight of rapeseed shoot tips [29] and two other algae-based biostimulants had a similar effect [9]. Shoot tips of bean (*Pisum sativa*: master b) increased with a *N. entophytum* treatment [39] and *Nostoc* sp. increased *B. rapa* var. *peruviridis* shoot tip dry weight [40].

Both microalgae suspensions used in the present study significantly increased the root collar diameter. Similarly, two growth regulators increased the diameter of rapeseed root collars [41]. Root collar diameter

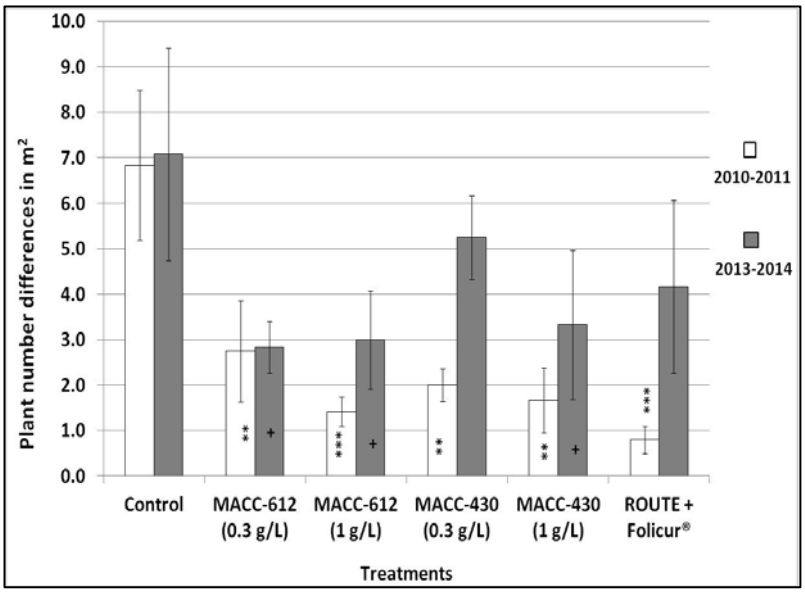

Fig. 9 The decrease of rapeseed plants from autumn to spring between 13.12.2010 and 17.03.2011 (white column), as well as between 07.12.2013 and 01.03.2014 (gray column). The plants were treated on 15.10.2010 and 10.10.2013 with various concentrations of microalgae MACC-612 *N. entophytum* and MACC-430 *Tetracystis* sp. in Mosonmagyaróvár (p^{***} = 0.1%, p^{**} = 1%, p^{*} = 5%, p^{+} = 10%; $SzD0.1\%_{2011}$ = 12.8; $SzD1\%_{2011}$ = 9.5; $SzD10\%_{2014}$ = 8.9).

in rapeseed is an important parameter as thick collars significantly increase the chances of successful overwintering [42]. Both microalgae suspensions also significantly increased rapeseed foliage. Rapeseed foliage may also be increased with growth regulators such as 3-DEC (diethylamine chloride) and 17-DMC (morpholinium chloride) [41]. Cyanobacteria have a positive effect on the growth of shoot tips [14, 39, 43, 44] and improve nutrient uptake and utilization [40]. The size of the rapeseed leaves' surface fundamentally determines the vegetative growth, while the loss of foliage during the frosty winter period vitally influences the quantity and quality of yield [14]. Macro- and micro-algae-based plant-growth influencing compounds have a favorable influence on root development, nutrient absorbance, leaf and shoot tip growth, blossoming, yield formation, yield maturation, the quantity and quality of yield and the oil content and quality of seeds [14, 45]. In the present study, MACC-612 and MACC-430 applied at 1 g/L dosage significantly improved the length of shoot tips, the diameter of root collars and the foliage of rapeseed in both experimental years.

Neither the MACC-612 nor MACC-430 treatments affected the fresh root weight of rapeseeds but increased the root dry weight. Similarly, root dry weight was increased using growth regulators [41] and algae-based biostimulants [9, 10]. Similar results were obtained using a nitrogen fixing cyanobacterium strain [46] and bean [39] and Kelpak improved root dry weight in *Medicago sativa* subsp. × *varia* [47]. Vigorous root development contributes to an increased length and weight of rapeseed roots while the thickness and maturity of roots determine the water and nutrient absorbing ability of the plant. Plants with underdeveloped roots are more prone to fungal infections and their winter hardiness is weaker [34]. Developed rapeseeds with strong roots during the overwintering period grow successfully in the spring and have increased tolerance during drier periods [34]. Plant hormones and secondary metabolites that can be found in microalgae have a positive effect on root development [39, 40, 44]. The two microalgae strains applied in the experiment favorably influenced the root development of rapeseed, consequently its water and nutrient absorbing ability

as well. The better water and nutrient absorbing ability provided a sufficient amount of nutrients during the intensive autumn development.

The applied cyanobacteria and green algae strains successfully contributed to the overwintering of rapeseed in the present study. There are many examples of auxin analogues, as well as other regulators improving the overwintering of rapeseed. For example, auxin analogues (TA-12 (2×10^{-3} M; 417 g/ha), and TA-14 (4×10^{-3} M; 369 g/ha)) (p = 5%) significantly improved the overwintering of *B. napus* L. ssp. *olifera biennis* Metzg. var. "Casino" winter rape. This was due to the higher proline and sugar concentration that accumulated in the roots and shoot tips [48]. Treatment with *Kappaphycus alvarezii* seaweed-based biostimulant increased plant number of soy (*Glycine max* L.) [49].

Countless biotic and abiotic factors play an important role in the successful overwintering of rapeseed [20, 50, 51]. The foliage is responsible for the successful overwintering of rape [50] while warmer, sunnier winter periods help the process, presumably due to the extending of photosynthetic activity [52]. Biostimulants such as gibberellins play a substantial role in vernalization and successful overwintering [53]. Endogenous abscisic acid plays an important part in the winter adaptation of plants [54], while auxin (IAA) contributes to overwintering by inducing a more powerful growth, however very little is known about the other biological mechanisms [55]. By using plant-growth promoting compounds, the plants' tolerance against cold can be increased, along with the proportion of generative organs to the yield formation elements [56-58].

Compared to other cereals, winter oilseed rape requires a higher amount of nutrients, and available nitrogen that frequently limits yield. Water is the most limiting factor for crop yield in most years [59]. Biostimulants increase productivity [10], and act on the physiology of the plant through different pathways to improve crop vigour, yield and quality. The beneficial effects of biostimulant treatments include improved seed germination, seedling development, enhanced nutrient mobilization, improved rooting, flowering, fruit setting, increased chlorophyll content, and an increase in crop and yield [45]. Ferreira and Lourens [12] significantly increased the yield of canola with foliar application (2 L/ha) of Kelpak. Rathore *et al.* [49] increased the yield of leguminous-plant soybean with different concentrations of seaweed extract prepared from *K. alvarezii.*

5. Conclusions

The application of MACC-612 significantly (p = 0.1% in both experimental year) increased the chlorophyll-a content compared to the control and Route + Folicur treatments. In the 2010/2011 trial, 1 g/L MACC-612 application was the most effective while in the 2013/2014 trial, 0.3 g/L application was more effective. A similar trend was observed for chlorophyll-b and carotenoid content.

In both experimental years, application of MACC-430 (0.3 g/L and 1 g/L) significantly (p = 1% in 2010; p = 0.1% in 2013) increased the dry matter content of the leaves compared to the control and Route + Folicur treatments.

The treatments of MACC-612 in 0.3 g/L and 1 g/L concentrations did significantly (p = 5%) increase the length of shoot tips. Both microalgae treatments significantly increased the diameter of the root collar compared to the control and Route + Folicur, treatments with 1 g/L MACC-612 being the most effective. Each microalgae treatment in both years significantly (p = 5%) improved the diameter of the root collar, while the conventional Route + Folicur combined treatment did not cause a significant root collar growth compared to the control.

Both the microalgae-based treatments (p = 0.1%) had a significant influence on the average number of leaves per plant. The MACC-612 and MACC-430 treatments resulted in a significant increase in the dry

weight of the roots with the MACC-430 treatments being the most effective.

The present study showed the two microalgae treatments had a positive physiological influence on the overwintering of rapeseed. The microalgae, presumably due to their hormone content and other secondary metabolites, increased the pigment and dry matter content of the leaves and consequently the photosynthesis of the plants. The higher organic matter appeared in the more developed plant's thicker roots, increased foliage and dry matter content which protected the plants during the dry, cold periods. Due to the favorable physiological effects, the winter hardiness of rapeseed improved.

Acknowledgments

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