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Contemporary Agriculture, Vol. 73, No. 3-4 (2024): 192-199
DOI: https://doi.org/10.2478/contagri-2024-0023
UDC: 581.132.1

Original scientific paper

ISSN 2466-4774
https://www.contagri.info/

EVALUATION OF *Chlorella vulgaris* S45 APPLICATION ON SWISS CHARD (*Beta vulgaris* L. subsp. *cicla*) SEED GERMINATION, PIGMENT CONTENT, TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY

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Submitted: 26.02.2024.
Reviewed: 14.05.2024.
Accepted: 20.05.2024.

SUMMARY

Contemporary agriculture faces the challenge of sustainably feeding constantly growing human population. Application of green algae in plant production is a new, relatively low-cost green technology with potential benefits for plant health. This study examines the application of Chlorella vulgaris S45 and its effect on seed germination, pigment content, total phenolic content and antioxidant capacity of Swiss chard. Treatments included 5% and 10% algal suspensions applied by spraying on plants and in substrate, respectively. C. vulgaris S45 positively affected all the investigated parameters. Seed germination percentage (GP) increased in comparison to the control treatment. The highest GP (46%) and germination index (GI) values were observed in the 10% suspension treatment. Photosyntetic pigment content also increased significantly under 10% suspension application, while both total phenol content and antioxidant capacity improved following plant spraying with 10% suspension. Correlation analysis indicated statistically significant interdependence among the tested parameters (pigment content, total phenolic content and antioxidant activity). Foliar application of Chlorella vulgaris S45 could be an alternative fertilization method in sustainable Swiss chard production.

Key words:
microalgae, Swiss chard, chlorophylls, bioactive compounds

Abbreviations:
ARP - antiradical power; CAR - Carotenoids; DPPH - 1,1 - diphenyl - 2 - picrylhydrazyl; DW - dry weight; EM - effective microorganisms; FRAP - Ferric Reducing Antioxidant Power; GA - gallic acid; GE - germination energy; GI - germination index; GP - germination percentage; TPC - Total phenolic content; TPTZ - Tris (2-pyridyl)-s-triazine

INTRODUCTION

One of the challenges of contemporary agriculture is sustainable food production for growing human population. Application of innovative technologies to minimize the adverse effects of agrochemicals on plants and soil could be one sustainable solution. Formulations that contain different effective microorganisms (EM) with plant growth-promoting properties may be an alternative to chemicals used in conventional agriculture. Different bacteria, fungi and algae are already marketed and applied worldwide, especially in organic plant production. EM have demonstrated positive effects on plant growth (Stamenov et al., 2021), plant quality enhancement (Žunić et al., 2024) and soil properties improvement (De Jong et al., 2008). In recent years, microalgae have received increased attention

due to their multifunctionality, particularly their capacity to stimulate plant growth. These growth-promoting effects of microalgae are related to excretion of hormones (especially cytokinins), vitamins, polysaccharides, amino and organic acids (Hajimahmoodi et al., 2010; Mohamed, 2008). Application of green microalgae in plant production, known as algalization, is well-documented in previous studies (Hajnal Jafari et al., 2016; Kim et al., 2018). Typically, live algal cells or cell extracts are applied directly to the soil, but they can also be administered through seed coating (seed priming) and by spraying of plants (foliar application). In the latter method, a thin biofilm of algae forms on the plant surface, facilitating faster nutrients uptake, reducing evapotranspiration and creating an additional protective layer against different pathogens (Ortiz-Moreno et al., 2019).

Swiss chard (*Beta vulgaris* L. subsp. *cicla*) is considered one of the healthiest leafy vegetables. There are two main varieties commonly grown (white and red). This leafy vegetable is often labeled a functional food due to its numerous health benefits (Khoo et al., 2017; Upadhyay, 2018). It is rich in vitamins (vitamin A, C and K), but also in lipids, sugars, pigments, folic acid, and secondary metabolites (Gao et al., 2009). Swiss chard also contains significant amounts of strong phenolic antioxidants, known for their ability to fight free radicals (Pyo et al., 2004). Owing to its low calorie count and high concentration of health-promoting phytochemicals, Swiss chard should be included in a regular diet.

Although some studies have explored the application of EM in the production of Swiss chard (Mouhamad et al., 2017; Daiss et al., 2008) or similar leafy vegetables (Khalid et al., 2017), the data on utilization of microalgae to enhance yield and/or improve quality remain very limited. Therefore, the aim of this study was to investigate the effects of *Chlorella vulgaris* S45 application on photosynthetic pigment (chlorophylls and carotenoids) content in Swiss chard leaves. Additionally, the experiment sought to determine whether algalization could alter the total phenolic content and antioxidant capacity in Swiss chard leaves under controlled conditions.

MATERIAL AND METHODS

Algae cultivation

Soil microalga *Chlorella vulgaris* S45 (Accession number: OR506256) from Algae Collection, Laboratory of Microbiology, Faculty of Agriculture, University of Novi Sad, was used in this study (Fig. 1). The microalgae strain was grown in BG11 medium (Waterbury & Stanier, 1981) at room temperature (23±2°C) under white light (2x950 Lm) and 14:10 (day/night) photoperiod for two weeks. An orbital shaker (110 rpm) was used to agitate algal culture in a vessel.

Cell number was calculated using Neubauer chamber and light microscope (Motic, B1-252). The experiment used microalgae suspension containing $14x10^6$ CFU·mL^{-1}. Algal suspensions (5% and 10%) and BG11 medium (10% conc.) were diluted with distilled water (v/v). Microalgal suspensions were applied foliarly (5 and 10%) and in substrate (10%) to test its effect on seed germination and accumulation of bioactive compounds in Swiss chard leaves. BG11 medium was used as a control treatment (applied foliarly).

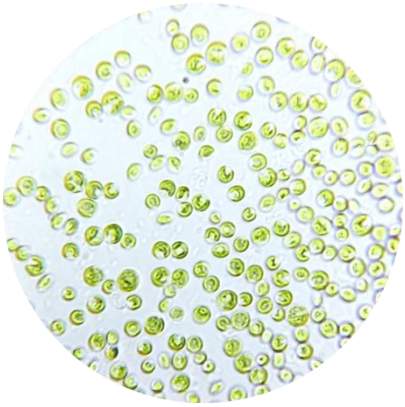

Figure 1. *Chlorella vulgaris* S45 (magnification 1000×)

Seed germination experiment

Seeds of the Swiss chard cultivar "Blitva srebrnolisna" (NSSeme, Serbia) were selected for this experiment. Seeds were surface-sterilized using 70% ethanol and rinsed three times with sterile water. Subsequently, 50 seeds were placed on a filter paper in a sterilized Petri plate and inoculated by 10 mL of 5% and 10% concentrated microalgae suspension, while BG11 medium (10%) served as the control. Each treatment was performed in triplicate. The dishes

were then placed in a thermostat (28°C) for incubation. Distilled water was added when needed to maintain moisture. Seed germination was monitored daily from the fourth day, continuing for a total of eight days.
Percent of germinated seeds was calculated as:

$$GP = (\text{number of germinated seeds/total number of treated seeds}) \times 100$$

Germination energy (GE) was determined according to Hernández-Herrera et al. (2013), using the formula:

$$GE = (\text{number of germinated seeds/number of total seeds per treatment after germination for 4 days}) \times 100$$

Germination index (GI) was calculated following the equation:

$$GI = \Sigma(Gt/Tt)$$

where Gt is the total number of germinated seeds on day t, Tt – the number of days (AOSA, 2005).

Plant fertilization experiment
Swiss chard seeds of the cultivar "Blitva srebrnolisna" (NSSeme, Serbia) were used for the study. Seedlings were initially grown for two weeks on Klasmann Potground H substrate (Klasmann®, Germany) in a growing chamber at temperature 24±2 °C with a natural day length. After this period, the seedlings (3 per pot) were transplanted into 25cm pots containing the same growing substrate. A complete randomized block design was applied with four replicates for each treatment. The first application of microalgae was conducted one week after seedlings transplantation. The second application was performed after four weeks. Each time, the algal inoculum (15 mL) was either applied foliarly (the substrate was covered to prevent runoff of inoculum to the substrate) or added to the growing substrate. The control treatment was also sprayed but with distilled water. Plant material was collected seven days after the second application. Swiss chard leaves were freeze-dried (Lyophilizer, model Alpha 1-2 LD plus) and stored for later analyses of pigment and total phenol content as well as antioxidant capacity measurements.

Pigment content
The contents of chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and total carotenoids in Swiss chard leaves were calculated following the method of Von Wettstein (1957). Lyophilized samples (0.1g) were grinded with 25 mL (80%) acetone. To prevent chlorophyllase activities, 0.1% $CaCO_3$ was added in the mortar. After grinding, the samples were centrifuged at 3500 rpm for 5 minutes, and the final volume (25 mL) was transmitted to centrifuge tubes. The absorbance was read at 662, 644 and 440 nm (Unicam SP600 spectrophotometer Series 2, Cambridge, England). According to Wettstein's formula, pigment concentrations were calculated as follows:

$$\text{Chl } a = 9.784 \times \text{A662} - 0.99 \times \text{A644}$$

$$\text{Chl } b = 21.426 \times \text{A644} - 4.65 \times \text{A662}$$

$$\text{Carotenoids} = 4.695 \times \text{A440} - 0.268 \times (\text{Chl } a + b)$$

Pigment concentration was expressed in mg/g of dry leaves weight (DW).

Total phenolic content (TPC)
Total phenolic content (TPC) was determined by extracting dried, powdered Swiss chard leaves (0.2 g) with 10 mL of 80% ethanol in an ultrasonic bath for 30 minutes. The mixture was then centrifuged at 4500rpm for 10 min (U-320 R, Boeco, Germany) and the supernatant was separated by pipetting.
Total polyphenols were determined using Folin & Ciocalteu reagent (Singleton & Rossi, 1965). Ethanolic extract (50 µL) was mixed with 0.8 mL Na_2CO_3 solution (700 Mm) and 1 mL Folin & Ciocalteu reagent (5%). After two hours in the dark, the mixtures were measured spectrophotometrically at 720 nm. TPC values were calculated using the standard gallic acid (GA) calibration curve and expressed as mg GA equivalents per one gram of dry weight.

Antioxidant capacity determination (DPPH and FRAP test)
The potential antioxidant activity of the investigated leaf extracts was assessed based on their scavenging of 1,1-diphenyl- 2- picrylhydrazyl (DPPH) free radicals, following the method by Frezzini et al. (2019) with minor

modifications. Ethanol extracts were dissolved in the 80% ethanol to obtain various concentrations (0.33 – 1.25 $mg \cdot mL^{-1}$) and 0.1 mL of the obtained diluted extracts were mixed with 1.5 mL of 0.1 mM DPPH in ethanol. The mixtures were kept at room temperature in the dark for 1 h, after which absorbance was measured spectrophotometrically (Evolution 220, Thermo scientific, USA) at 517 nm. The control used 80% ethanol instead of the sample solution, and the 80% ethanol was used as the blank. All measurements were conducted in triplicate. The DPPH radical scavenging capacity of the Swiss chard leaf extracts was expressed as antiradical power (ARP) and it was calculated by the following equation:

$$ARP = 1/IC_{50} \times 100$$

where IC_{50} is determined as the concentration of extract required to reduce the initial DPPH concentration by 50% (Brand-Williams et al., 1995).

The Ferric Reducing Antioxidant Power (FRAP) analysis was performed following the method of Benzie & Strain (1998). Fresh FRAP reagent was prepared by adding 10 mM of 2,4,6- Tris (2-pyridyl)-s-triazine (TPTZ) (dissolved in 40 mM of HCl), 20 mM of $FeCl_3$ in water and 300 mM of acetate buffer (pH 3.6) in the ratio of 1:1:10. FRAP solution (3 mL) was mixed with 20 µL of the extracts. The solution in the test tubes was mixed using a vortex mixer. After 10 minutes, the mixture was measured using a spectrophotometer at 593 nm absorbance. FRAP values were expressed as mg ascorbic acid equivalents (EAscA) per 1 g DW, calculated based on the standard calibration curve.

Statistical analysis

All the experiments were performed in triplicate. Statistical analyses were conducted using Statistical Package ver. 10.0 Software (Hamburg, Germany). Spearman correlation analysis and LSD (least significant difference) test were performed to identify significant differences among treatments ($p<0.05$).

RESULTS AND DISCUSSION

Effect of *Chlorella vulgaris* on Swiss chard germination

Swiss chard seed germinated in all investigated treatments after four days. The number of germinated seeds increased daily until the end of experiment in all treatments. The application of *C. vulgaris* S45 suspension improved GP compared to the control. The highest average GP (39.73%) was observed in the treatment with 10% algal inoculum, whereas the control treatment reached only 24.53% GP. These findings align with those reported by Seman et al. (2021) on the germination in kohlrabi and radish.

GE, as an indicator of a seed's potential to germinate, also increased in seeds treated with *C. vulgaris* S45 (31.33% and 34%, respectively) compared to the control (Tab. 1). A similar trend was observed with GI, which considers both the percentage and speed of germination. The highest GI value was calculated in the treatment with 10% algal suspension. Odgerel & Tserendulam (2016) also documented positive effects of *Chlorella* sp. 56 applications on seed germination in wheat and barley.

Table 1. Effect of *Chlorella vulgaris* S45 on germination percentage (GP), germination energy (GE), germination index (GI) of Swiss chard seeds (average values)

Treatments	GP %	GE %	GI
Control	24.53±2.083	18.67±2.403	2.089±0.187
5% suspension	38.93±7.737	31.33±7.688	3.34±0.679
10% suspension	39.73±10.313	34±9.866	3.42±0.896

Effect of *Chlorella vulgaris* on pigment, TPC and antioxidant activity

The application of 10% concentration of *C. vulgaris* S45 via foliar spray led to the highest content of Chl *a* and Chl *b* (7.88 $mg \cdot g^{-1}$ and 3.11 $mg \cdot g^{-1}$, respectively), as presented in Table 2. However, this treatment did not produce a significant increase in carotenoid content compared to the control.

In leafy green vegetables, pigments associated with color are green chlorophylls (fat soluble) and yellow, orange, and red carotenoids. Accumulation of these photosynthetic pigments, as stated by Barickman et al. (2016), is influenced by plant attributes such as morphology, physiology and biochemistry, as well as by growth environmental factors like light intensity, water availability, temperature fluctuations and level of fertilization. Algalization of Swiss chard led to increased content of Chl *a*, Chl *b* and carotenoids, consistent with similar findings by Đurić et al. (2014) on application of biostimulants on tomato seedling. Coppens et al. (2016) found that dry biomass of *Nannochloropsis* spp., *Ulothrix* spp. and *Klebsormidium* spp increased carotenoid concentrations in tomato. Moreover, seed priming

and plant treatments with various microbial consortia containing algae also stimulated pigment accumulation in plants (Ellora Malakar & Kalita, 2012; Dineshkumar et al., 2018). The study by Faheed and Fattah (2008) documented that lettuce treated with *Chlorella vulgaris* showed stimulated biosynthesis of both chlorophylls and carotenoids. Higher pigment content usually means that the photosynthesis is increased which could influence plant growth and yield development.

Foliar application of the 10% algal suspension resulted in a statistically significant increase of TPC, DPPH and FRAP values in relation to the control. The 5% algal suspension slightly increased TPC and FRAP values and statistically significantly reduced DPPH ARP value compared to the control. Soil treatment with *C. vulgaris* S45 led to a decrease in DPPH ARP value of extract; however, in this extract no statistically significant changes were observed in Chl *a*, Chl *b* and TPC content or FRAP value compared to the control.

Table 2. Effect of *Chlorella vulgaris* S45 on pigment content, TPC and antioxidant activity

Treatments	Chl *a*	Chl *b*	CAR	TPC	DPPH	FRAP
Control	7.42bc*	2.98b	2.34a	9.32b	128.21b	2.78b
5% foliar	7.18c	2.93b	2.15b	9.65b	102.04c	3.04b
10% foliar	7.88a	3.11a	2.42a	12.69a	151.51a	4.28a
10% soil	7.54b	2.93b	2.24b	10.36b	88.49d	3.00b

Legend: *Different letters in subscripts indicate statistically significant difference according to Fisher LSD test ($p < 0.05$); Chl a – Chlorophyll a (mg g^{-1} DW); Chl b – Chlorophyll b (mg g^{-1} DW); CAR – Carotenoids (mg g^{-1} DW); TPC – total phenolic content (mg GAE·g^{-1} DW); DPPH –diphenyl- 2-picrylhydrazyl (ARP); FRAP – Ferric Reducing Antioxidant Power (mg ·EAscA·g^{-1} DW)

Consumption of polyphenol-rich food may markedly reduce oxidative stress, a process closely associated with the development of different chronic and degenerative disorders (Pyo et al., 2004). Since Swiss chard is a valuable functional food (Ninfali & Angelino, 2013; Mzoughi et al., 2019), this study aimed to assess whether the application of *Chlorella vulgaris* could affect the antioxidant capacity of the treated plants. The results showed a significant increase of the total phenolic content (12.69 mg·g^{-1} DW) and antioxidant activity (both DPPH and FRAP) in Swiss chard when sprayed with 10% algal suspension. These findings align with those of Khalid et al. (2017), who reported significant increases in the content of phenol compounds and DPPH in spinach treated with *Bacillus* sp. and *Glomus* sp. compared to the untreated control. Positive effects of AMF inoculation (*Glomus intraradices, G. mosseae, G. etunicatum*) on total phenolic content and antioxidant activity (DPPH) were also observed in artichoke and tomato (Ordookhani et al., 2010; Ceccarelli et al., 2010). Avio et al. (2017) demonstrated the improvement of nutritional properties of greenhouse-grown vegetables by biostimulant application, finding that lettuce inoculated with AMF (*Rhizoglomus irregulare*, *Funneliformis mosseae)* had increased antioxidant activity, phenolic and anthocyanin content in relation to non-inoculated plants, which highlighted the importance of mycorrhizal fungi in phytochemical production. Fan et al. (2011) reported that roots treated with extract of brown algae *Ascophyllum nodosum* (0.1, 1.0 or 5.0 g·L^{-1}) stimulated flavonoids synthesis and improved nutritional quality in spinach.

Correlation analysis

Correlation analysis indicated statistically significant relationships among the investigated parameters of pigments, total phenolic content and antioxidant capacity (Tab. 3). Chl *a* content showed a strong positive correlation with Chl *b* and carotenoid content (r = 0.8513 and r = 0.8586, respectively). High positive correlations were observed between Chl *b* content and FRAP (r = 0.9009), and Chl *b* content and DPPH ARP value (r = 0.9304). Additionally, there was a positive correlation between carotenoid content and DPPH (r = 0.8679), as well as between TPC and FRAP (r = 0.9755).

Table 3. Correlation coefficients between investigated parameters

Treatments	Chl *a*	Chl *b*	CAR	TPC	DPPH	FRAP
Chl a	-					
Chl b	0.8513	-				
CAR	0.8586	0.8830	-			
TPC	0.8978	0.8619	0.6561	-		
DPPH	0.6477	0.9304	0.8679	0.6191	-	
FRAP	0.8175	0.9009	0.6363	0.9755	0.7092	-

Legend: All values are significant correlations ($p < 0.05$)

In this study, TPC had a positive correlation with DPPH ARP value (r = 0.6191) and statistically significant positive correlation with FRAP value (r = 0.9755). These results indicate that phenolic compounds are not the sole contributors to the DPPH antioxidant activity of Swiss chard, but that other metabolic constituents, such as carotenoids, vitamin C, enzymes and other compounds, may also play a role (Gorinstein et al., 2007). Pyo et al. (2004) also found a significant positive linear correlation between TPC and radical scavenging activity in Swiss chard extract. Significantly positive linear correlation between TPC and antioxidant activity in red beet was confirmed by Bavec et al. (2010), who also observed that different farming systems influenced the antioxidant activity, TPC, sugar and organic acid content.

CONCLUSION

Foliar application of 10% algal suspension positively affected the content of both photosynthetic pigments in Swiss chard leaves and improved antioxidant properties. The findings indicate that *Chlorella vulgaris* S45, when used as a foliar fertilizer, could contribute to enhanced growth and improved nutritional properties of Swiss chard. Application of appropriate microalgae formulation could be an alternative method in achieving a more sustainable and eco-friendly production of Swiss chard.

***Acknowledgements*:** This study was financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia (No. 451-03-47/2023-01/200117).

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Conflict of interest: The authors declare that they have no conflict of interest.

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