

Effect of static magnetic field on growth factors, antioxidant activity and anatomical responses of *Silybum marianum* seedlings

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Abstract

Silybum marianum is an important medicinal plant with high antioxidant properties. Static magnetic field (SMF) is a kind of abiotic stresses which can affect growth and antioxidant capacity of medicinal plants. In this research, the effects of SMF on total phenolic and flavonoid contents, radical scavenging activity, and anatomical alterations were investigated in *S. marianum* seedlings. Five-day-old seedlings were treated with different intensities of SMF (0, 2, 4 and 6mT) for one hour, and then were irrigated with 1/2 Hoagland solution for four weeks. Results showed that SMF up to 4 mT increased fresh weight, dry weight, leaf area, relative water content (RWC), root length significantly and then decreased these parameters at 6 mT. At 4 mT, SMF caused an 125, 132.73 and 52.19% increase in dry weight, leaf area and root length as compared to the control, respectively. Metaxylem number and size, aerenchyma formation and stele diameter were improved under SMF. Lipid peroxidation (MDA) decreased especially at 4 mT, although H₂O₂ level increased in *S. marianum* shoots. Also, SMF induced total phenolic (93.89%) and flavonoid contents (36.43%), and decreased IC₅₀ values (43.01%) especially at 4 mT in leaves. The results suggest that SMF at proper frequency could improve antioxidant capacity of plant cells by regulation of H₂O₂ level and membrane stability.

Keywords: Flavonoid, *Silybum marianum*, Static magnetic field, Total phenol

Introduction

Silybum marianum (milk thistle, Asteraceae) is a medicinal plant which is native to the Middle East and Mediterranean region. Bioactive compounds obtained from plants play an important role in medicinal and pharmaceutical applications. Flavonoids possess antiviral, anti-inflammatory, and antioxidant properties related to their ability to scavenge free radicals, and they also modulate cell signaling pathways (Jimenez-Garcia *et al.*, 2013). The most important secondary metabolite of *S. marianum* is silymarin, which is a mixture of stereoisomeric flavonolignans including silybin, isosilybin, silydianin, silychristin and taxi-folin (Radjabian *et al.*, 2008; Khalili *et al.*, 2009). Silymarin has a strong protective effect against oxidative stress damage at the protein level which has a direct connection to its use as hepatoprotector for chronic inflammatory liver diseases and liver cirrhosis (Pilat *et al.*, 2011).

Effects of static magnetic fields (SMF) on living organisms have become considerable public concern, but have been paid less attention. In recent years,

several studies have suggested possible negative bioeffects of magnetic fields (Miyakoshi, 2005; Zhang, 2003). These fields can affect human health and lead to various types of cancer and genetic disorders. Although the exact mechanism of these effects remain unknown, one of the most recognized hypothesis is the radical pair's mechanism in which an external magnetic field, influences the kinetics of chemical reactions with radical pair intermediates. Therefore, it can be lead to increase of the concentration of free radicals and reactive oxygen species (ROS) in the cells. Accumulation of these compounds can induce an oxidative stress and change gene expression, membrane structure, the activity of antioxidative enzymes, secondary metabolites and following cell growth (Green *et al.*, 1999). There are some studies about the effects of magnetic fields on plant antioxidant systems. For example, Abdolmaleki *et al.* (2007) showed that magnetic field at 30 mT increased cell wall lignifications, peroxidase (POX) and superoxide dismutase (SOD) activities in tobacco cells, while decreased catalase (CAT) and ascorbat peroxidase (APX) activities. In *Artemisia sieberi* seedlings,

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magnetic field priming changed total phenolic content as well as antioxidant enzymatic activities (Azimian and Roshandel, 2015). There is a correlation between antioxidant properties and polyphenols in plants (Ksouri *et al.*, 2007; Gill and Tuteja, 2010). There are some information about the effect of magnetic field on Asteraceae family. Magnetic field at 0.22 mT increased germination rate (10%) in *S. marianum* compared to control (Pop *et al.*, 2015). In *Allium cepa*, SMF at 0.06 T increased mitotic activity and chromosomal aberration after 30 min (Hozayn *et al.*, 2015). We did not find any data about the effect of SMF on antioxidative responses of *S. marianum* seedlings. The hypothesis of the present study is that SMF could improve some metabolite with antioxidative properties in *S. marianum*. The aim of the present study was to investigate the quantitative changes of secondary metabolites and their possible roles in seedling growth and anatomical alteration under the magnetic field.

Materials and methods

Plant culture and EMF treatments: The seeds of *S. marianum* were purchased from Pakan Bazr, Isfahan Co. The experiment was conducted at Department of Pharmaceutical Chemistry of Tehran Medicinal Sciences, Islamic Azad University in 2017. Seeds were placed on the filter paper in 8 cm Petri dishes and submerged in the distilled water. After 5 days of germination, seedlings were treated with different intensities of static magnetic field (0, 2, 4 and 6 mT) for one hour and then were transplanted into the plastic pots (14×9×12 cm) containing perlite in the greenhouse with 16/8 hrs. (light/dark period) per 24 hrs. and 25/18°C (day/night temperature) and 40–45% humidity. Pots were irrigated with 1/2 Hoagland solution every week for four weeks. Exposure to EMF was performed using a locally designed EMF generator. The electrical power was provided by a 220V, DC (direct current) power supply with a static voltage. This system was consisted of a copper wire (1.1 mm in diameter) wrapped 1300 rounds around a polyethylene tube in 12 cm diameter and 50 cm length. After 30 days, plants with four replicates were collected for the biochemical analyses.

Growth parameters: Fresh weight (FW) and dry weight (DW) of leaves and roots were determined on six individual plants. For determination of DW, samples were dried for 72 hrs. at 40°C. RWC estimation in leaves was performed based on Weatherley, 1973. $RWC (\%) = (FW - DW) / (SW - DW) \times 100$

Saturated weight (SW) of fresh leaves were defined by immersing samples in ultra pure water for 24 hrs. in the dark at 4°C, and DW determination of leaves were dried in oven for 72 hrs. at 40°C until constant weight was reached.

Anatomical study: Stems of plants were fixed in formalin–alcohol–glacial acetic acid (1:18:1 % v/v). Cross sections were made at the middle of stems (1.5 cm above the root). Sections were cleared with sodium hypochlorite, dehydrated, and stained with Methyl

Green and Bismarck Brown colors (Noorbakhsh *et al.*, 2008). Sections were observed by microscope (Nikon, E200, Japan) and images were taken with a digital camera (Nikon, Coolpix S10 Model, Japan).

Lipid peroxidation and H₂O₂ contents: Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content with slightly modification (Heath and Packer, 1968). Fresh leaf and root samples (0.5 g) were homogenized in 0.1% trichloroacetic acid (TCA) and centrifuged at 11,000 ×g for 20 mins. using a centrifuge (Sigma 1-16K, German). The supernatant (1 ml) was mixed with 4 ml of a thiobarbituric acid (TBA) reagent in 20 % TCA. The reaction mixture was boiled at 95°C for 30 mins. in a water bath and then quickly cooled in an ice. The absorbance of the supernatant was measured at 532 nm using an UV–visible spectrophotometer (UV-160, Shimadzu, Tokyo, Japan). The content of MDA was calculated using extinction coefficient of 155 mM⁻¹ cm⁻¹.

Hydrogen peroxide levels were determined according to Velikova *et al.* (2000). Fresh leaves (1g) were homogenized with 5 ml of 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12,000×g for 15 mins. and 0.5 ml of the extract added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. Absorbance of the supernatant was measured at 390 nm, and H₂O₂ content was calculated using a standard curve.

Total phenolic and flavonoids: For extraction, 0.5 g of dried leaf powder of various SMF treatments were put in 80% (v/v) methanol for 24 hrs., and then were transferred in a ultrasonic bath for 15 mins. The extracts were centrifuged (10 mins. at 11,200×g) and the supernatant was used for quantification of total phenolics, flavonoids and DPPH activity.

Total phenolic content was measured using a modified Folin-Ciocalteu procedure (Singleton and Rossi, 1965). An aliquot of the diluted extract (100 μL) was mixed with 500 μL Folin–Ciocalteu reagent and 400 μL 0.7 M L⁻¹ sodium carbonate. Samples were incubated 90 mins. at room temperature in the dark. Absorbance was measured using an UV–visible spectrophotometer at 725 nm. A calibration curve was prepared using a standard solution of gallic acid and the total phenolic content was expressed as mg gallic acid equivalents per gram of extract (mg GAE/ g extract).

Flavonoid content was determined according to method of Hatamnia *et al.* (2014) with a minor modification. Briefly, 50 μL of sodium nitrate solution (5 %) was added to 500 μL of the extracts and allowed to react for 5 mins. Then 50 μL of 10 % aluminum chloride solution was added. Finally, 250 μL of 4 % sodium hydroxide solution was added into the mixture 5 mins. later. The absorbance of the mixture was immediately recorded by an UV–visible spectrophotometer at 518 nm. A calibration curve was prepared using a standard solution of rutin and the total flavonoid content was expressed as mg of rutin

equivalents per gram of extract (mg RU/ g extract).

DPPH activity: Radical scavenging effects of the extracts were determined based on reducing the 2, 2-diphenyl-1-picryl hydrazyl (DPPH) solution. Different concentrations of the extract were added to 2 mL of a methanol solution of DPPH. The absorbance of the solution was reported by an UV-visible spectrophotometer at 517 nm at room temperature after 30 mins. incubation. The radical scavenging activity was calculated as (%) = $100(A - B)/A$, where A and B are the absorption of the control and the corrected absorption of the sample reaction mixture.

The free radical scavenging activities of the solutions were expressed as IC₅₀ (mg ml⁻¹) which was calculated graphically using different concentrations of samples versus DPPH inhibition percentage. IC₅₀ is the antiradical dose required to cause a 50% inhibition (Patro *et al.*, 2005).

Statistical analysis: The experimental design was a completely randomized. The obtained data were analyzed as a completely randomized design with four replications by analysis of variance (ANOVA) using SPSS (version 18) software, and mean separation was performed with Duncan's multiple range test, at the level of P < 0.05.

Results

Effects of SMF on growth parameters and anatomical changes: Growth parameters of SMF-treated plants were followed by measuring plant fresh weight and dry weights, root length, leaf area and RWC. SMF treatment up to 4 mT increased markedly fresh and dry weights, leaf area and root length, but at 6 mT SMF decreased these parameters (Table 1, Figure 1). The optimum growth parameters were observed at 4 mT SMF. RWC also increased significantly under SMF treatment. The highest RWC content was identified at 4 mT, and an 26.73% increase of RWC was observed as compared to the control.

Cross section of *S. marianum* shoot under different intensities of SMF (0, 2, 4 and 6 mT) was presented in Figure 1. SMF increased stele diameter and xylem vessels as compared to control. Metaxylem size and number, and pith parenchyma increased at 2 and 4 mT, and then decreased at 6 mT as compared to the control. SMF also induced the aerenchyma formation especially at 4 mT. It was found that the possible potential of proper EMF to influence on the growth and differentiation processes of vessels.

Effects of SMF on H₂O₂ and Lipid peroxidation levels: Different intensities of SMF changed H₂O₂ contents in both leaf and root organs (Figure 2). SMF increased H₂O₂ level significantly especially at 4 mT in leaf organs as compared to the control, and a 46.42% increase of H₂O₂ level was identified as compared to the control (Figure 2a). At 6 mT SMF, H₂O₂ level decreased significantly, although its content was higher than control plants. MDA as a marker of lipid peroxidation decreased under SMF treatment up to 4 mT, and then

increased at 6 mT SMF (Figure 2b). At 4 mT, MDA showed a 31.25 and 56.16% decrease in leaf and root organs as compared to the control.

Effects of SMF on total phenolic and flavonoid contents: Total phenolic content increased significantly under different intensities of SMF in leaf and root organs (Figure 2c). The highest phenolic content was observed in leaves under 4 and 6 mT SMF that showed an 1.93 and 1.79-fold increase as compare to the control, respectively.

Total flavonoid content increased with the increase of SMF intensities, and the optimum content was observed at 6 mT (Figure 2d). Leaf and root organs showed about 1.91 and 2.16-fold increase of total flavonoids at 6 mT comparing to the control, respectively.

Effects of SMF on antioxidant activity: Antioxidant activity was determined by evaluating the effects of leaf extracts on DPPH free radical scavenging activities and calculating IC₅₀ at all SMF treatments. At 4 mT SMF, leaf extracts with the IC₅₀ value of 244.61 µg ml⁻¹ displayed the highest DPPH free radical quenching activity as compared to other treatments (Figure 3). These effects may be related to the higher total phenolic contents in *S. marianum* leaf under 4 mT SMF treatment. At 6 mT treatment, although the differences were not significant, the free radical scavenging activity of the leaf extracts decreased as compared to 4 mT treatments.

Discussion

This study was conducted to determine SMF tolerance mechanisms and the participation of ROS and some antioxidant metabolite in *S. marianum* growth and anatomical changes under SMF. Growth alterations are the visible reactions of plants under stress conditions. In the present study, fresh and dry weights, RWC, leaf area and root length increased up to 4 mT, and then decreased at higher SMF intensities. Increased growth parameters under SMF treatments has been previously observed in soybean (Radhakrishnan and Kumari, 2012; Baghel *et al.*, 2017), sunflower (Fischer *et al.*, 2004) and barley (Martinez *et al.*, 2000). Induction of growth parameters can be related to the effect of magnetic field on ions uptake and water absorption (Dhawi *et al.*, 2009), hormonal homeostasis (Dalia *et al.*, 2009) and other biochemical processes (Parola *et al.*, 2006). On the other hand, our results showed that magnetic field higher than 4 mT decreased aerial part and root dry mass in *P. alkekengi* seedlings. Similarly, Shabrangi and Majd (2009) showed that magnetic field at higher intensity of 1 mT decreased growth and shoot length in canola. Reduction in plant growth and length can be related to negative effect of magnetic field on the growth regulator such as indol-3-actinic acid (IAA) (Ros *et al.*, 1995). It seems that plant growth under SMF may be related to anatomical alterations for water transport and changes in hormone level of *P. alkekengi* seedling.

Cross section of *S. marianum* shoot showed SMF

Table 1. Effect of different intensities of SMF (0, 2, 4 and 6 mT) on fresh weight, dry weight and leaf area, root Length and relative water content (RWC) of *S. marianum* seedlings. Bars indicate \pm SE (n = 4) in each group. Different letters indicate significant differences at $P \leq 0.05$ (LSD)

SMF treatment (mT)	Parametr				
	fresh weight	dry weight	leaf area	root length	RWC
0	1.41 \pm 0.43 ^b	0.12 \pm 0.012 ^{bc}	20.71 \pm 3.92 ^c	2.51 \pm 0.94 ^c	52.36 \pm 2.13 ^b
2	3.12 \pm 0.33 ^a	0.23 \pm 0.052 ^{ab}	42.76 \pm 7.51 ^b	3.11 \pm 1.04 ^b	64.54 \pm 4.27 ^a
4	3.38 \pm 0.72 ^a	0.27 \pm 0.031 ^a	48.20 \pm 5.04 ^a	3.82 \pm 0.51 ^a	66.36 \pm 7.36 ^a
6	1.35 \pm 0.39 ^b	0.09 \pm 0.018 ^c	19.30 \pm 4.37 ^c	2.76 \pm 0.38 ^c	43.23 \pm 3.43 ^c

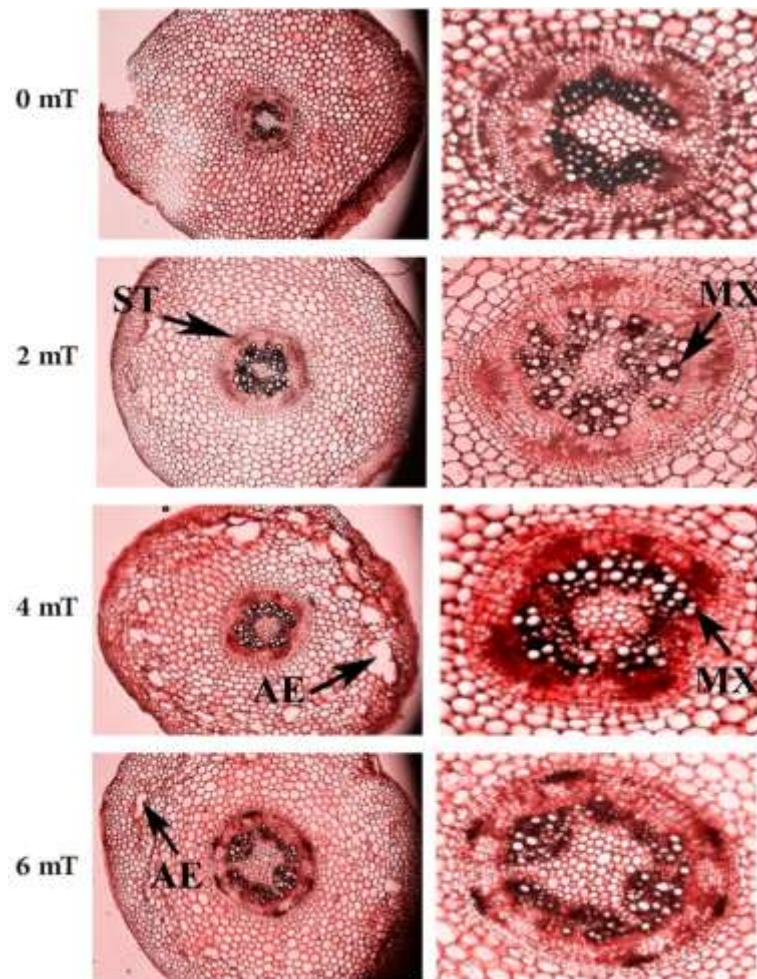


Figure 1. Cross section of *S. marianum* shoots (2 cm over the root) under different intensities of SMF (0, 2, 4 and 6 mT). AE aeranchyma, ST stele, MX metaxylem.

induced metaxylem size and number, aeranchyma formation and pith parenchyma. Similar to our results, Selim and El-Nady (2011) showed that SMF induced cambium differentiation and vascular tissues in tomato. It seems that development of metaxylem in shoot may contribute to more convenient water and sap flow in the xylem (Kozłowski, 1997). Moreover, the aeranchyma formation in plant organs is due to cortex degeneration and cell death. Ethylene as a plant hormone leads to the death and disintegration of cells in the cortex (Taiz and Zeiger, 2010). Other chemicals such as H_2O_2 could

also induce this phenomenon, and facilitate movement of oxygen, and following growth induction (Steffens *et al.*, 2011).

Lipid Peroxidation of membranes in higher plants reflects free radical-induced oxidative damage at the cellular level under oxidative stress (Demiral and Turkan, 2004). In our study, SMF decreased MDA content, whereas it increased H_2O_2 level in *S. marianum* aerial part. Similarly, Azimian and Roshandel, in the year 2015 showed that magnetic water decreased MDA content in *Artemisia sieberi*. Magnetic field also

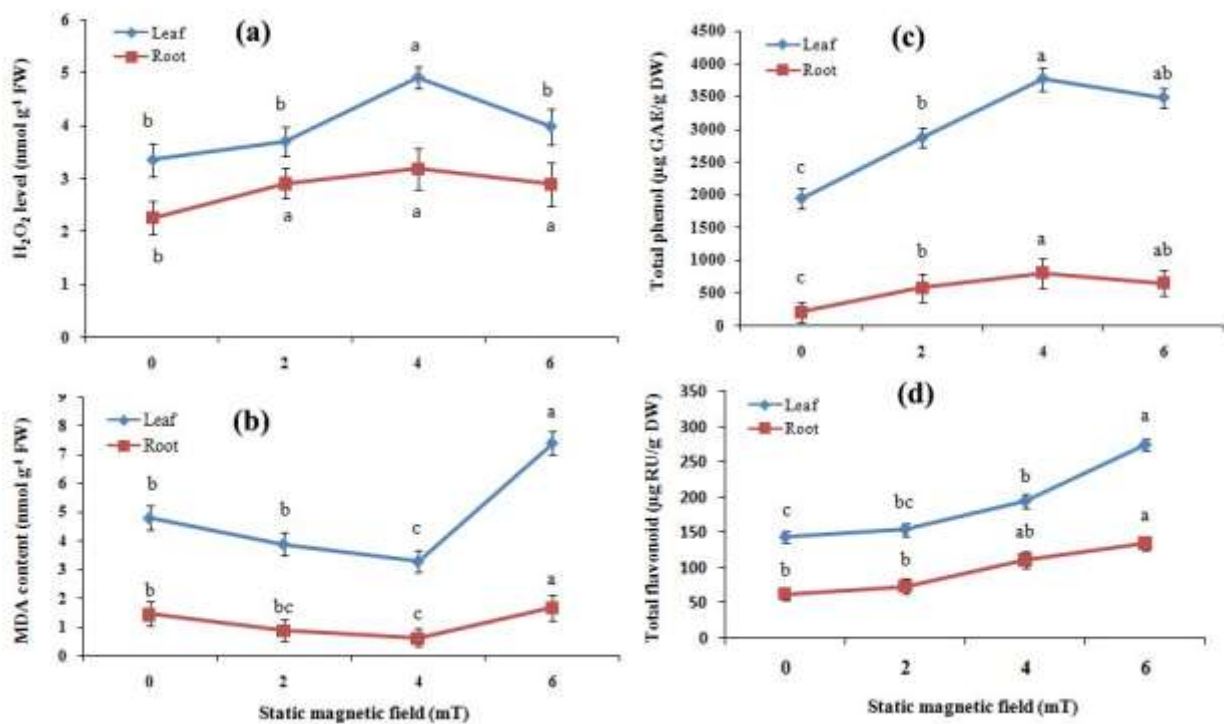


Figure 2. Effect of different intensities of SMF (0, 2, 4 and 6 mT) on the H₂O₂ level (a), MDA content (b), total phenolic (c) and total flavonoid (d) of *S. marianum* seedlings. Bars indicate ± SE (n = 4) in each group. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

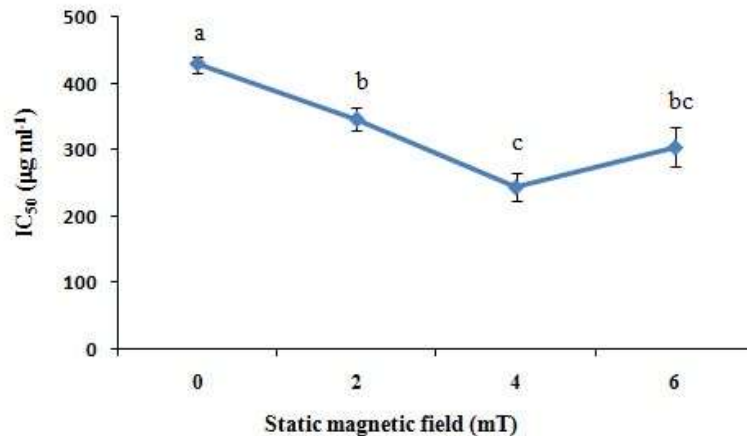


Figure 3. Effect of different intensities of SMF (0, 2, 4, and 6 mT) on IC₅₀ activity of *S. marianum* seedlings. Bars indicate ± SE (n = 4) in each group. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

increased ROS production and cell wall peroxidase activity in glycine max hypocotyls (Shine *et al.*, 2012). It seems that induction of cell membrane stability under SMF could correlate with increasing antioxidant activity in different parts of *S. marianum*.

Phenolic compounds and flavonoids act as antioxidants that their extensive conjugated p-electron systems allow ready donation of electrons or hydrogen atoms from their hydroxyl moieties to free radicals (Rice-Evans *et al.*, 1996). A highly positive relationship between total phenols and antioxidant activity has been

reported in many plant species (Rainha *et al.*, 2011). Our results showed that SMF increased markedly total phenolic and flavonoid compounds. Similarly, Azimian and Roshandel, in the year 2015 showed that magnetic field induced total phenolic content and antioxidant activity in *Artemisia sieberi*. On the other hand, the induction of cellular antioxidant machinery is critical for protecting plant cells against the adverse effects of abiotic stresses (Hameed *et al.*, 2011). Some reports indicate that MF can induce antioxidative capacity of plant cells against oxidative injuries (Li and Chow,

2001; Sobczak *et al.*, 2002; Zhang *et al.*, 2003). In this study, IC50 value decreased significantly in SMF-treated plants especially at 4 mT SMF. Decreased IC50 value is directly related to higher radical-scavenging activity. It was found that ROS produced due to SMF in *S. marianum* could act as a signal to enhance production of antioxidants and can cause a dramatic increase in radical scavenging activity.

Conclusion

The results suggested that SMF priming at proper

intensity in early growth stages (5 day-old seedlings) can improve seedling growth by increasing water transport and metaxylem number in *S. marianum*. Also, antioxidant capacity, total phenolic and flavonoid also increased under SMF, and could induce membrane stability. Overall, SMF treatment at 4 mT appears to be an effective SMF treatment for induction of growth and antioxidant capacity in *S. marianum*. However, the elaborate mechanisms in which antioxidant enzymes play such a crucial role under EMF stress remains unclear and needs to be investigated further.

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