Metabolic changes in tobacco cells after exposure to static magnetic field is mediated by hydrogen peroxide

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Abstract

Plant cells metabolism is known to undergo considerable reprogramming in response to static magnetic field (SMF). In the present research changes of metabolism induced by SMF and the underlying mechanism(s) were investigated in suspension-cultured tobacco (*Nicotiana tabacum* cv. Barley 21) cells. Considering the potential role of SMF in production of hydrogen peroxide and the role of Ascorbic acid (Asc.) as a strong scavenger of H2O2, tobacco cells were exposed to SMF alone, Asc. (40 µM), SMF+Asc., and control (no SMF, no Asc.) in the experiment. Sugars and amino acids were monitored by HPLC, components of redox system were measured by spectrophotometer, and expression of genes was evaluated by RT-PCR. Exposure to SMF decreased the adenosine triphosphate, glucose, fructose, and sucrose contents but increased hydrogen peroxide, nitric oxide, hydroxyl radical, proline, and reduced glutathione (GSH). SMF also increased the gene expression and activity of catalase, compared to the control group. Moreover, the exposure to SMF increased the contents of phenylalanine and tyrosine, elevated the gene expression and activity of phenylalanine ammonia-lyase, and subsequently increased soluble phenolic compounds. In the groups treated with Asc., all above-mentioned parameters reduced except for nitric oxide and hydroxyl radical contents. The rate of membrane lipid peroxidation also increased in Ascorbate-pretreated cells. The results suggested that changes in primary and secondary metabolic pathways in SMF-treated tobacco cells after were mediated by H_2O_2.

Keywords: Ascorbic acid, hydrogen peroxide, metabolism; *Nicotiana tabacum*, phenolic acid, static magnetic field

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Introduction

Although the exact mechanism(s) of the effects of static magnetic field (SMF) on plant cells are still a matter of debate, increase in the activity, concentration, and lifetime of free radicals have been introduced as potential links between SMF and its effects. Many studies have shown the

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alteration of metabolites after exposure to SMF. Changes in the activity and tertiary structure of certain iron-containing proteins, e.g. catalase (CAT) and ferritin, after exposure to SMF have been recently documented in vivo and in vitro (Shokrollahi et al., 2018). Exposure to 0.5 µT to 30 mT SMF altered the metabolism of phenolic compounds and the essential oils in sweet basil (*Ocimum basilicum*) (Ghanati et al., 2007). Coordinate increase in the activity of phenylalanine ammonialyase (PAL) and the contents of flavonoids, anthocyanins, and lignin was observed in suspension cultures of *Dracocephlum polychaetum* after exposure to SMF (Taghizadeh et. al., 2019). Stimulated production of other secondary metabolites with more complicated structure such as Taxol has also been reported in suspension-cultured *Taxus chinensis* cells (Shang et al., 2004). Despite the fact that SMF is a non-ionizing radiation, its biological effects have been attributed to uncoupling of free radical processes in membranes and enhanced ROS generation (Maffei, 2014).

Inconsistency in the function of ROS scavengers leading to oxidative burst and cell death have been observed in tobacco cells exposed to SMF (Sahebjamei et al., 2007). Treatment of broad bean (*Vicia faba* L.) with SMF showed similar results in terms of decrease in antioxidant defense system and increase in peroxidation of membrane lipids (Jouni et al., 2012). Calabro et al. (2013) reported significant increase in H_2O_2 in SH-SY5Y neuronal-like cells after 24 h exposure to 2.2 mT SMF. The same effect was y confirmed by Vergallo et al. (2014) by applying 31.7-232.0 mT SMF on the same cell line as they observed that SMF elevated superoxide anion radicals to 123% of the control.

Besides ROS, increase in reactive nitrogen species, e.g. nitric oxide (NO), has also been reported in animal models exposed to magnetic fields (Yoshikawa et al., 2000; Coballase-Urrutia et al., 2018). Little is known about the influences of static magnetic field treatment on nitric oxide in plant cells. Enhancement of nitric oxide and its signaling pathway during germination of magneto-primed maize seeds have been recently reported by Patel et al. (2017). It is well known that ROS and NO, particularly at low levels, can critically function in different signaling pathways, where they usually interact with each other and enable the cells to process and interpret multiple inputs differently (Farnese et al., 2016). In order to detoxify ROS and NO, plant cells have evolved two protective mechanisms including enzymatic (e.g., catalase and peroxidase) and non-enzymatic ones (e.g., Ascorbic acid and glutathione) (Gill and Tuteja, 2010).

The role of Ascorbic acid as a redox buffer as well as a metabolic interface to modulate the appropriate induction of acclimation responses has been widely studied. Asc. has a crucial role in detoxification of H_2O_2 through Ascorbate–glutathione cycle (Foyer and Noctor, 2005). To the best of our knowledge however, the interaction of exogenously applied Asc. with ROS under stress of SMF has not been elucidated yet. The present study was conducted to delve into the effects of SMF exposure on tobacco cells and the possible ameliorating role of Asc. More specifically, the changes in SMF-treated plant cells, both in the presence and absence of Asc., were investigated in terms of phenolic compounds, soluble sugars, free amino acids, and ATP contents as well as oxidative status (oxidative stress, enzymatic activities, and gene expression). Also, the possible role of H_2O_2 as an upstream regulator of primary and secondary metabolic responses of tobacco cells to SMF is studied.

Materials and Methods

Design and treatments of the study

The experiment was structured following a Completely Randomized Design (CRD) using suspension-cultured cells of a rapidly growing line of tobacco (*Nicotiana tabacum* L. cv. Burley 21). The cells were grown in a modified LS medium supplemented with 3 mg. mL^{-1} IAA, 3 mg. mL^{-1} NAA, and 0.1 mg. mL^{-1} Kinetin, pH 5.8. The cells were grown at 25 ±2 ℃ in the dark on a reciprocal shaker at 110 rpm. The cells were synchronized via sucrose starvation at early stages of stationary growth phase (Mohammadi et al., 2018).

The synchronized cells were randomly divided into four groups. A group of the cells were placed in an appropriate distance of 500 MHz NMR

device (Bruker Avance DRX NMR, Germany) to receive SMF by 0.2 mT (Supplementary 1). The intensity and homogeneity of the field was checked by a Teslameter (13610.93, PHYWE, Gottingen). The cells in the second group were pretreated with filter sterilized Asc. to a final concentration of 40 µM and then were exposed to SMF. The third group were only treated with 40 µM Asc. The fourth group (control) received no treatment (no SMF, no Asc.). All groups were shuffled every 1h and were harvested at 3, 6, 8, 12, 18, and 24 h intervals, and then washed thoroughly with distilled water.

The control cells and those which received only Asc., were kept under the same conditions in term of temperature and humidity with only local magnetic field of the Earth (40 µT, calculated by proton magnetometer with integrated GPS (Envi Pro Mag, Scintrex, Ontario, Canada). The cells were harvested at different intervals, immediately freeze-dried in liquid N_2, and then stored at -80 ℃ for further analysis.

Chromatographic separation of soluble sugars

Soluble sugars were extracted and analyzed according to the method described by Nemati et al. (2018). In brief, the samples were extracted with EtOH (80%) and evaporated by heating at 90 ℃. Water was added to the precipitate in a shaking water bath at 80 ℃ for 1 h followed by centrifugation at 12,000 ×g for 10 min. Glucose, fructose, and sucrose in the supernatant were detected by a HPLC system equipped with an AZURA P 6.1 L pump (Knauer, Germany), a Decade Elite electrochemical detector (Antec), and Clarity Chrom 6.1.0 software for data acquisition. The stationary phase was a Hamilton RCX-107 µM (4.6×250 mm) analytical column (Dionex Corp.), and the mobile phases were (A) water and (B) 50 mM NaOH/ water, 80/20, v/v. A gradient program was used as follows: 0-15 min 80% B, 15-15.02 min 0% B, 15.02-25 min 0% B, 25-25.02 min 80% B, and 25.02-30 min 80% B. The flow rate was 1mL min^{-1}.

Measurement of Adenosine triphosphate (ATP) Pool

The cells were extracted by trichloroacetic acid (0.1%, w/v) followed by centrifugation at 12,000 ×g, 14 min, at 4 ℃. An ATP bioluminescence assay kit (CLS II, Sigma-Aldrich) was used. The manufacturer instruction was followed and the ATP content of the cell lysate was measured by an FB12 tube luminometer (Berthold detection system, Germany) and quantified by a calibration curve (nm.mg $protein^{-1}$) (Nahidian et al., 2018).

Redox assessment

Hydrogen peroxide content was evaluated by the method of Velikova (2000) and the values were expressed as µmol g^{-1} FW.

Nitric oxide content was measured by the method of Green et al. (1982) with few modifications and was quantified by a standard calibration curve using 0-50 nM sodium nitrite. The values were expressed as nmol g^{-1} FW.

The content of hydroxyl radical (OH^{-}) was measured using 2-deoxyribose (DOR) as a scavenger of extremely short-lived hydroxyl radicals (Nemati et al., 2018).

The rates of membrane lipid peroxidation of tobacco cells were determined by measuring the complex of malondialdehyde (MDA) as the end product of the membrane lipid peroxidation as described by De Vos et al. (1991). The amount of MDA was determined from the absorbance at 532 nm followed by correction for the nonspecific absorbance at 600 nm by a spectrophotometer (GBC, Cintra 6, Australia).

Catalase activity was assayed based on the initial rate of decomposition of hydrogen peroxide at 240 nm for 1 min (Pormehr et al., 2018). Total soluble protein contents were evaluated by the method of Bradford (1976), using bovine serum albumin as standard.

The content of reduced form of glutathione (GSH) was measured in trichloroacetic cell extracts using 5, 5'-dithiobis 2-nitrobenzoic acid (Garg and Kaur, 2012), and the content of Ascorbate was estimated according to De Pinto et al. (1999).

Phenolics metabolism assay

Table 1
Sequences of primer pairs used in the RT-PCR analysis

Genes	Primer Sequences	Amplicon Size (bp)	PCR Conditions	Accession Numbers
ACT	Forward 5'- TCTGGAGATGGTGTGAGCCACAC - 3' Reverse 5'- GGAAGGTACTGAGGGAGGCCAAG -3'	600	94 °C/2 min; 94 °C/40 s 58 °C/30 s, 72 °C/1 min 35cycles; 72 °C/7 min	XM009589609.2
CAT	Forward 5'- GTCCCACATTCAGGAGAACTGG - 3' Reverse 5'- CTGGGAGCTGCAGATAGTTTGG -3'	600	94 °C/2 min; 94 °C/40 s 58 °C/30 s, 72 °C/1 min 35cycles; 72 °C/7 min	NM001325093.1
PAL	Forward 5'- CATCAGATTTGAAATCTTGGAAGC - 3' Reverse 5'- GCCATGGCGATTTCAGCTCCCTTGAA-3'	800	94 °C/2 min; 94 °C/40 s 58 °C/30 s, 72 °C/1 min 35cycles; 72 °C/7 min	NM001325017.1.

The activity of Phenylalanine ammonia-lyase (PAL) was measured based on the amount of cinnamic acid produced by the cell extract during 1h incubation in the presence of Phenylalanine (Wang et al., 2006).

Total content of soluble phenolics was measured by Folin-Ciocalteu using gallic acid as standard (Akkol et al., 2008). The quantitative determination of phenolic compounds was conducted using HPLC (Waters e2695, Detector 2489 uv/vis) as described by Mohammadi et al. (2018).

Amino acids assay

The free amino acids from cells were extracted with EtOH 80% followed by centrifugation at 13000 ×g for 15 min. The supernatant was evaporated to dryness under vacuum, dissolved in 1 mL H_2O, derivatized with *o*-phthalaldehyde, and analyzed by an HPLC system equipped with a fluorimetric detector (FLD HP 1100). The stationary phase was a Zorbax Eclipse-AAA column (4.6×150 mm, 3.5-µm particle size; Agilent Technologies, USA). The mobile phases were (A) Na-Pi buffer (25 mM, pH 7.2) containing tetrahydrofuran (5%) and (B) Na-Pi buffer (25 mM, pH 7.2) containing MeOH (35%) and acetonitrile (15%). A gradient program was used as follows: 0-0.6 min 10% B, 0.6-9.0 min 50% B, 9.0-48 min 60% B, 48.0-51.0 min 100% B, 51.0-56.0 min 100% B, 56.0-57.0 min 10% B, and 57.0-59.9 min 10% B. The constant flow rate was 0.5 mL/min. Fluorescence detection and quantification were carried out by excitation wavelength 230 nm and emission wavelength 455 nm (Zafari et al., 2016).

Proline content was estimated according to the method of Bates et al. (1973).

RNA extraction and gene expression analysis by RT-PCR

Total RNA was isolated using RNX™-Plus kit (Cinna Gen, Iran), and the first strand cDNA was synthesized. The *reverse transcription polymerase chain reaction* (*RT-PCR)* primers were designed based on their protein-conserved domain from *Nicotiana tabacum* cited in NCBI. The resulting amplicons were sequenced and compared with their sequences in NCBI (Table 1). The PCR products were separated on 1% agarose gel electrophoresis and stained with ethidium bromide. The bands intensities were quantified by ImageJ software (version 1.52b).

Data acquisition and statistical analysis

All experiments and observations were repeated three times independently, with at least three samples in each repetition. SPSS (version 16, Chicago, IL, USA) was used for statistical analysis. A Duncan test was used for multiple mean comparisons at a significance level of $p \leq 0.05$.

Results

Soluble sugars and ATP contents

The contents of glucose, fructose, and sucrose before and after exposure of tobacco cells to SMF and Asc. are shown in Fig. (I). Exposure to SMF significantly increased the glucose and fructose, but decreased sucrose in the tobacco cells at 3h by 1.9, 2.5, and 1.3 folds of their controls, respectively (Fig. I). Significant decrease was observed in glucose and fructose contents of SMF-treated cells at 12 h of the treatment, compared to the control groups (Fig. I, A and B). Conversely, the supply of SMF+Asc. increased glucose and fructose levels at 3 and 6 h compared to SMF-treated groups and then reached a steady-state rate to equal that of control and SMF-treated groups at 24 h, although the levels of sucrose did not differ significantly compared to these groups (Fig. I).

The ATP content of tobacco cells decreased by SMF up to 6 h of the treatment, afterward however, it was restored more or less to the control level (Fig. II). Treatment of tobacco cells with SMF+Asc. was identical up to 3 h of the exposure and thereafter significantly increased the content of ATP, 6 h after treatment (Fig. II).

Redox system

The amount of H_2O_2 increased significantly in response to SMF treatment, suggesting ROS signaling events and/or oxidative damage (Fig. III, A). The amount of detected H_2O_2 peaked at 6 h (a 102% increase over control) but then slowly decreased back to the control levels after 24 h. The presence of Asc. inhibitor significantly reduced the H_2O_2 content compared to SMF-treated groups (Fig. III, A). Additionally, a significant decrease in H_2O_2 content of Asc.-treated plants confirmed the role of Asc. in H_2O_2 scavenging (Fig. III. A).

H_2O_2 often acts in conjunction with NO which probably link to influence SMF tolerance. In parallel to changes in H_2O_2 content, exposure to 0.2 mT of SMF led to an increase in the amount of NO to its maximum at 3 h. The content of NO at 6 h was still significantly higher in the SMF-treated cells (150% of the control cells), but it decreased afterward and reached to the level of controls. In cells treated with SMF+Asc. no significant change of NO content was observed compared to SMF-treated groups (Fig. III, B).

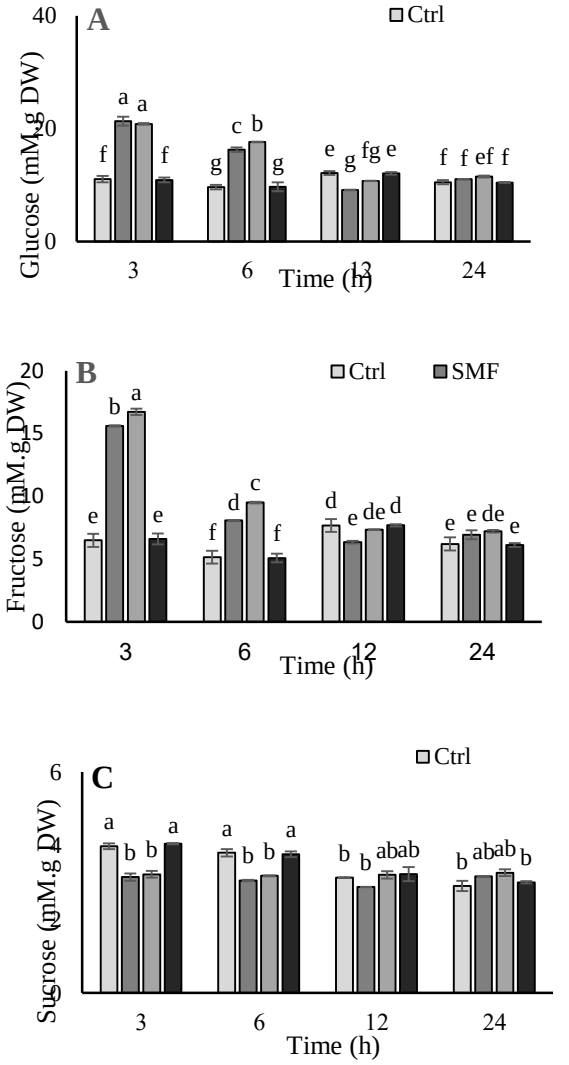

Fig. I. Effect of SMF (0.2 mT) in the absence or presence of Asc. inhibitor on the (A) glucose, (B) fructose, and (C) sucrose contents of tobacco cells; "Ctrl" refers to unexposed samples. Data are presented as the means of 3 replicates ± standard deviation (SD). Different letters indicate significant differences at P≤ 0.05.

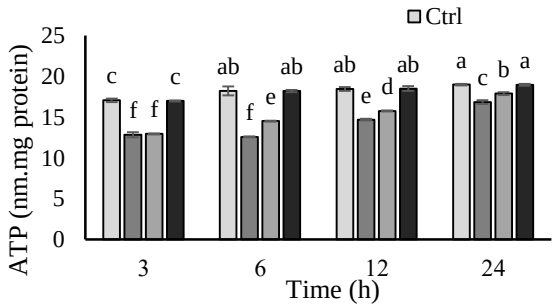

Fig. II. Effect of SMF (0.2 mT) in the absence or presence of Asc. inhibitor on the ATP content of tobacco cells; "Ctrl" refers to unexposed samples. Data are presented as the means of 3 replicates ± SD. Different letters indicate significant differences at P≤0.05.

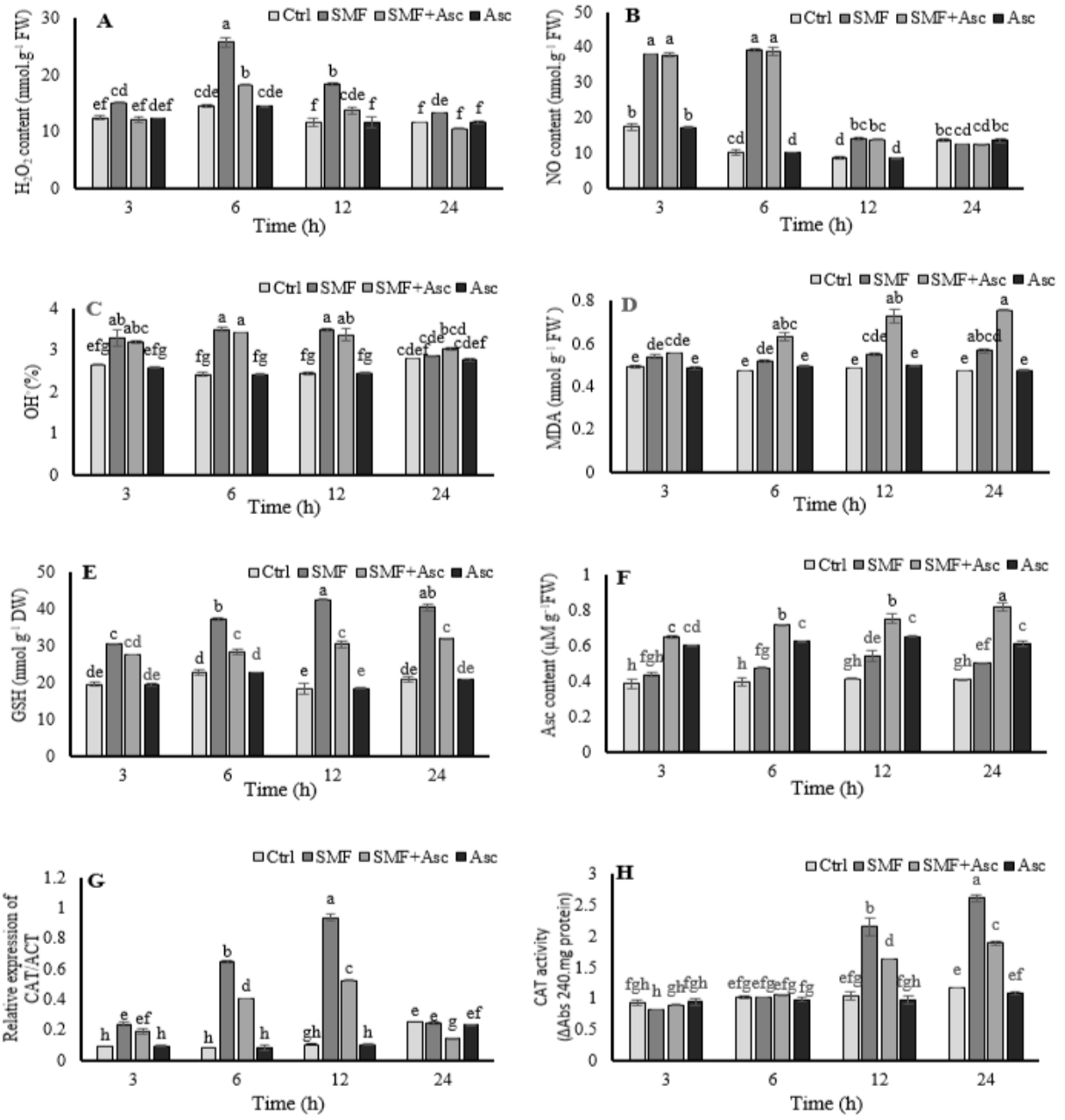

Fig. III. **.** Effect of SMF (0.2 mT) in the absence or presence of Asc inhibitor on the oxidative status of tobacco cells: (A) H_2O_2, (B) NO, (C) OH·, (D) MDA, (E) GSH, (F) Ascorbat content, (G) *CAT* gene expression, and (H) CAT activity; "Ctrl" refers to unexposed samples. Data are presented as the means of 3 replicates ± SD. Different letters indicate significant differences at P≤0.05.

Exposure to SMF significantly increased OH· radicals as the most destructive ROS for the membranes of tobacco cells, but the combined treatment with Asc. and SMF resulted in no change in OH· radicals compared to SMF exposure alone (Fig. III, C).

Besides, in SMF-treated cells the MDA level, as the sign of membrane damage, did not show significant differences with that in control (Fig. III, D). In contrast, increased MDA level in the cells treated with SMF+As was remarkable (33.9% of the control cells) up to 24 h of the treatment (Fig. III, D).

The GSH content of SMF-treated tobacco cells was significantly higher (up to 55%) than the controls (Fig. III, E). The GSH content in cells treated with SMF+Asc. was identical up to 3 h of the treatment and thereafter significantly decreased so that after

Figure 4

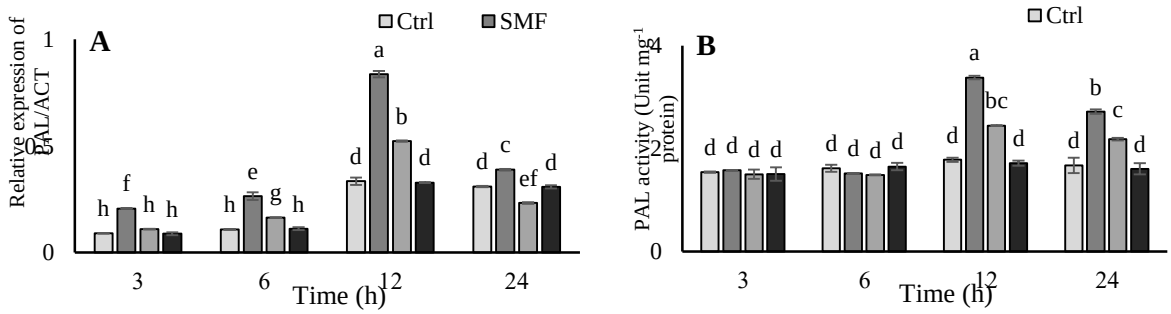

Fig. IV. Effect of SMF (0.2 mT) in the absence or presence of Asc. inhibitor on the (A) *PAL* gene expression and (B) PAL enzyme activity of tobacco cells; "Ctrl" refers to unexposed samples. Data are presented as the mean of 3 replicates ± SD. Different letters indicate significant differences at P≤0.05.

Table 2

Effect of SMF (0.2 mT) in the presence or absence of Asc. inhibitor on phenolic compounds in tobacco cells; "Ctrl" refers to unexposed samples. Data are presented as the mean of 3 replicates. Different letters indicate significant differences at P≤ 0.05. The ratio of standard errors to means was less than 5%.

Time (h)		Total phenol	Caffeic acid	Cinnamic acid	Salicylic acid	Ferulic acid	P-Coumaric acid	Benzoic acid	Vanillin	Quercetin	Catechin	Epicatechin
		mg g^{-1} DW	µg g^{-1}DW									
Ctrl	3	11.00^{f}	14.46^{f}	0.54^{f}	7.92^{g}	1.05^{bc}	1.42^{c}	0.36^{g}	6.3^{ef}	11.43^{f}	12.03^{c}	11.14^{c}
Ctrl	6	11.52^{ef}	15.96^{e}	0.75^{de}	8.10^{g}	1.70^{abc}	1.25^{cd}	0.40^{fg}	5.85^{hi}	16.02^{c}	11.94^{c}	11.05^{cd}
Ctrl	12	11.49^{ef}	12.06^{g}	0.80^{cde}	9.43^{de}	2.07^{abc}	1.43^{c}	0.46^{d}	5.74^{i}	15.70^{c}	11.90^{c}	10.12^{e}
Ctrl	24	13.02^{d}	10.00^{h}	0.87^{cd}	8.75^{efg}	2.08^{abc}	1.44^{c}	0.37^{g}	5.96^{fghi}	26.04^{a}	12.04^{c}	11.17^{c}
SMF	3	11.65^{ef}	12.38^{g}	0.50^{f}	8.20^{fg}	0.91^{c}	1.54^{c}	0.45^{de}	6.45^{e}	12.03^{e}	12.00^{c}	11.13^{c}
SMF	6	12.95^{d}	18.43^{d}	0.64^{ef}	8.35^{efg}	0.92^{c}	1.56^{c}	0.40^{fg}	6.26^{ef}	16.00^{c}	11.95^{c}	11.06^{cd}
SMF	12	15.00^{c}	24.20a	1.85^{a}	11.98^{b}	3.02^{abc}	3.24^{a}	0.66^{b}	13.13^{b}	15.53^{c}	15.35^{a}	14.45^{a}
SMF	24	19.54^{a}	22.01^{b}	1.81^{h}	13.18^{a}	3.51^{ab}	3.30^{a}	0.79^{a}	15.63^{a}	26.03^{a}	11.33^{d}	11.43^{c}
SMF +Asc	3	10.52^{f}	12.38^{g}	0.54^{f}	7.77^{g}	0.90^{c}	1.01^{c}	0.40^{fg}	6.15^{efgh}	11.05^{f}	11.32^{d}	10.99^{cd}
SMF +Asc	6	11.07^{f}	16.73^{e}	0.51^{f}	8.05^{g}	0.86^{c}	1.29^{cd}	0.39^{g}	5.99^{fghi}	14.73^{d}	11.93^{c}	11.46^{c}
SMF +Asc	12	12.34^{de}	19.87^{c}	0.98^{c}	10.68^{cd}	2.15^{abc}	2.06^{b}	0.53^{c}	11.10^{d}	14.37^{d}	13.27^{b}	12.35^{b}
SMF +Asc	24	16.44^{b}	19.88^{c}	1.21^{b}	11.73^{bc}	2.16^{abc}	1.91^{b}	0.56^{c}	12.57^{c}	23.97^{b}	10.73^{e}	10.56^{de}
Asc	3	10.87^{f}	14.39^{f}	0.51^{f}	8.02^{g}	3.95^{a}	1.38^{c}	0.40^{fg}	6.25^{ef}	11.49^{f}	12.10^{c}	11.19^{c}
Asc	6	11.32^{ef}	16.01^{e}	0.76^{de}	7.96^{g}	1.60^{abc}	1.27^{cd}	0.41^{efg}	5.88^{ghi}	15.99^{c}	11.87^{c}	10.99^{cd}
Asc	12	11.39^{ef}	12.13^{g}	0.78^{cde}	9.25^{ef}	2.10^{abc}	1.44^{c}	0.44^{def}	5.72^{i}	15.67^{c}	11.93^{c}	10.20^{e}
Asc	24	12.93^{d}	10.11^{h}	0.89^{cd}	8.79^{efg}	1.98^{abc}	1.43^{c}	0.38^{g}	6.01^{fghi}	25.95^{a}	12.09^{c}	11.13^{c}

24h the GSH content of combined SMF+Asc. treatment cells diminished to 21% of the SMF-treated cells (Fig. III, E).

To confirm the effect of exogenous Asc., application, its endogenous content in different samples was measured. Results showed that the Ascorbate levels in response to SMF application were 0.50 µmol g^{-1} fresh weight, 25% more than that in control after 24 h (Fig. III, F). While, SMF+Asc. treatments enhanced the Ascorbate level up to 64%, in comparison with SMF treatment alone (Fig. III, F).

Increase in the expression of *CAT* gene after exposure to SMF was detectable from the first hours of the treatment (Fig. III, G). However, the activity of CAT significantly increased after 12 h

exposure to SMF, compared to the control cells (Fig. III, H). Combined application of SMF+Asc. significantly decreased both gene expression and activity of CAT at all treatments compared to SMF treatment alone (Fig. III, G & H).

Phenolic metabolism

Fig. (IV) shows the rates of gene expression and activity of PAL in tobacco cells treated with or without SMF and Asc. As shown, exposure to SMF significantly induced both gene expression and activity of PAL in the cells. The higher expression was observed 12 h after exposure (2.51-folds in SMF cells over control) and the higher activity was observed 12 h after exposure (1.89-folds in SMF cells over control). In contrast, SMF+Asc. treatments significantly decreased both gene expression and activity of PAL at all treatments of the study compared to SMF treatment alone (Fig. IV, A & B).

Fluctuations were observed in phenolic contents of the control cells along with the time (Table 2). Nonetheless, at the end of the treatment period, the sum and individual contents of phenolic compounds were significantly higher in SMF-treated cells, e.g., caffeic (2.2-folds), cinnamic (2-folds), salicylic (1.6-fold), ferulic (1.75-folds), p-coumaric (2.3-folds), benzoic acid (2.1-folds), and vanillin (3-folds) increases over control cells (Table 2). With SMF treatment, a significant increase in catechin and epicatechin occurred 12 h after SMF treatment; however, quercetin levels did not differ significantly compared to controls. In addition, the phenolic compound contents of the cells treated with Asc. followed a similar trend to that of SMF alone, albeit to a lesser extent (Table 2).

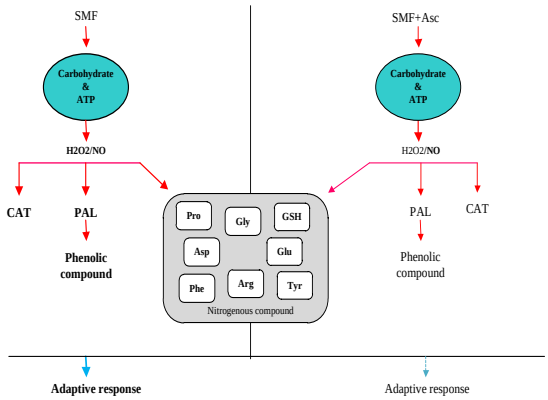

Fig. V. A Schematic representation showing a difference in signaling molecules levels in directing enzymatic and metabolic intermediates into tobacco adaptive response to SMF and SMF+Asc. treatments, respectively. The increased routs of metabolites are highlighted by bold lines and words. All metabolites, except Glu reduced under both treatments.

Free amino acids

Table 3.
The effect of SMF (0.2 mT) in the presence or absence of Asc. inhibitor on free amino acids in tobacco cells; "Ctrl" refers to unexposed samples. Data are presented the means of 3 replicates. Different letters indicate significant differences at P≤ 0.05. The ratio of standard errors to means was less than 3.5%.

Time (h)		Met	Ile	Leu	Val	Ala	Ser	Gly/Thr	Glu	Asp	Phe	Tyr	Arg	Pro
		nmol g^{-1}DW												mg g^{-1}FW
Ctrl	3	25.06^{d}	6.91^{f}	9.39^{b}	25.26^{fg}	44.86^{bc}	84.42^{cd}	9.19^{f}	69.14^{a}	42.25^{f}	19.91^{de}	8.73^{cd}	32.56^{ab}	0.93^{f}
Ctrl	6	25.37^{c}	8.03^{ef}	9.19^{b}	21.31^{h}	41.04^{de}	76.94^{fg}	11.70^{e}	65.00^{ab}	36.09^{gh}	17.22^{f}	9.04^{cd}	27.56^{cd}	0.96^{f}
Ctrl	12	29.16^{b}	11.76^{c}	11.69^{a}	35.78^{c}	40.69^{de}	71.14^{h}	10.79^{ef}	61.44^{abc}	29.18^{i}	21.36^{bc}	12.02^{b}	32.08^{ab}	1.21^{cd}
Ctrl	24	29.07^{b}	10.44^{cd}	11.41^{a}	37.80^{b}	46.74^{b}	82.76^{de}	11.41^{ef}	56.40^{bcde}	34.40^{h}	21.07^{bcd}	11.37^{b}	37.43^{a}	1.03^{ef}
SMF	3	25.51^{c}	8.08^{ef}	7.84^{b}	29.75^{e}	44.19^{bc}	75.91^{fg}	11.19^{ef}	64.72^{abc}	59.72^{d}	20.05^{bcd}	9.24^{cd}	32.23^{ab}	0.92^{f}
SMF	6	19.29^{d}	10.27^{cd}	9.29^{b}	17.94^{i}	29.04^{g}	71.02^{h}	33.37^{b}	50.90^{de}	80.60^{a}	19.94^{de}	12.05^{b}	25.76^{d}	1.01^{f}
SMF	12	31.41^{a}	17.78^{a}	12.45^{a}	46.48^{a}	42.02^{cd}	91.11^{b}	38.92^{a}	39.34^{f}	69.52^{b}	12.81^{h}	16.67^{a}	39.81^{a}	1.38^{b}
SMF	24	31.02^{a}	18.14^{a}	12.07^{a}	27.76^{f}	49.41^{a}	97.20^{a}	40.07^{a}	73.91^{a}	39.67^{fg}	23.01^{a}	10.01^{c}	41.37^{a}	2.08^{a}
SMF +Asc	3	25.17^{d}	7.08^{f}	8.17^{b}	25.08^{g}	43.86^{bc}	78.91^{fg}	10.68^{ef}	62.72^{abc}	50.05^{e}	19.68^{de}	9.04^{cd}	30.56^{ab}	0.90^{f}
SMF +Asc	6	23.62^{d}	9.17^{de}	8.96^{b}	22.02^{h}	33.04^{f}	74.43^{gh}	25.04^{d}	54.24^{bcde}	63.94^{c}	19.84^{de}	10.12^{c}	27.76^{cd}	0.97^{f}
SMF +Asc	12	28.74^{b}	13.44^{b}	11.35^{a}	35.48^{c}	38.83^{de}	83.52^{cd}	27.92^{c}	47.34^{ef}	59.52^{d}	15.81^{g}	12.01^{b}	39.80^{a}	1.08^{def}
SMF +Asc	24	29.87^{ab}	13.80^{b}	11.21^{a}	31.66^{d}	41.07^{de}	87.20^{c}	28.74^{c}	50.25^{de}	46.67^{e}	20.01^{cde}	9.65^{cd}	41.34^{a}	1.33^{bc}
Asc	3	24.73^{d}	7.01^{f}	9.26^{b}	25.92^{fg}	44.43^{bc}	84.08^{cd}	9.35^{f}	69.04^{a}	41.80^{f}	19.45^{e}	8.69^{d}	32.25^{ab}	0.92^{f}
Asc	6	25.30^{d}	7.94^{f}	8.94^{b}	21.11^{h}	39.70^{de}	77.27^{fg}	11.59^{ef}	64.93^{ab}	36.25^{gh}	16.99^{fg}	9.00^{cd}	26.89^{cd}	0.94^{f}
Asc	12	28.92^{b}	11.75^{c}	11.56^{a}	35.54^{c}	40.12^{de}	71.48^{h}	10.69^{ef}	61.58^{abc}	28.91^{i}	21.46^{b}	11.96^{b}	31.74^{ab}	1.19^{cde}
Asc	24	29.11^{b}	10.48^{cd}	11.34^{a}	38.08^{b}	46.41^{b}	82.76^{de}	11.28^{ef}	56.54^{bcde}	34.35^{h}	20.97^{bcd}	11.41^{b}	37.43^{a}	0.99^{f}

Alterations in the contents of individual free amino acids are shown in Table 3. Among detected amino acids, the most noticeable increases in response to SMF were observed in GlyThr and Asp (3.8 and 2.9 fold increases, respectively) while lower increases were observed in Val, Met, Ser, Pro, and Ile (1-2 folds) (Table 3). The Phe content exhibited a clear decrease at 12 h of treatment and then increased. Interestingly, there was a significant decrease in Phe compared with an enhancement sin Tyr (1.33 fold) at 12, and as both of these amino acids were derived from the shikimate pathway, this suggests a shift between these two aromatic amino acids (Table 3). No significant changes were observed in Ala, Arg, and Leu contents of tobacco cells after exposure to SMF (Table 3). Under SMF+Asc., free amino acids followed a similar trend to SMF alone, albeit to a lesser extent (Table 3). These data were indicative of differential amino acid metabolism at differing times following the application of SMF and SMF+Asc.

Discussion

Exposure to 0.2 mT SMF reduced the contents of soluble carbohydrates in the tobacco cells in this study, which was also accompanied by a decrease in ATP. It is possible that upon expression of Adenylyl cyclase gene in SMF-stressed tobacco cells, a part of ATP was consumed for production of cAMP and promotion of signaling pathways in the plant defense system (Mohammadi et al., 2018). Also, glucose is used in the mitochondrial Ascorbic acid synthesis via Smirnoff-Wheeler pathway (Akram et al., 2017). It is well documented that there is a high risk of ROS production during the oxidation of glucose and generation of ATP in mitochondria. It has also been shown that exposure to SMF disrupts/increases the cell respiratory activity, resulting in oxidation of large amounts of glucose, collapse of ATP production, and overproduction of ROS (Small et al., 2012; Wang and Zhang, 2017). Dose-dependent changes of cellular NO (Okano et al., 2005) and H_2O_2 (Haghighat et al., 2014) concentrations have been shown after exposure to SMF. Cell membranes are highly susceptible to injury by ROS, particularly $OH^{\cdot}$ radicals (Wang and Zhang, 2017). Coincidently, consumption of glucose by SMF-treated tobacco cells was accompanied by increase in NO, H_2O_2, and $OH^{\cdot}$ radicals. A previous study by the authors demonstrated SMF-induced NO, H_2O_2, and $OH^{\cdot}$ radical production as an early response (3 and 6 h of the treatment) of tobacco cells to SMF stress. Also, it was demonstrated that despite increases in these radicals, membranes were not damaged, and no significant change was observed in MDA content of tobacco cells during SMF exposure period, suggesting the efficiency of the damage repair system and improved innate immunity in plants (Mohammadi et al., 2018). Co-treatment with SMF+Asc. had no effect on the NO and $OH^{\cdot}$ contents but, decreased and increased the contents of H_2O_2 and MDA, respectively during treatment.

The induction of immunity occurs by the overexpression and production of defense-related products. Reactive oxygen species and NO radicals emerge as a potent bioactive signal molecule that triggers a transient metabolic reprogramming (Leon et al., 2016) and post-translationally regulate the expression of downstream genes and proteins activity such as CAT and PAL through *S*-glutathionylation and *S*-nitrosylation short after exposure to stress (Farnese et al., 2016). This hypothesis was implied by remarkable increase in gene expression and activity of CAT toward the end of SMF treatment period. The reduced gene expression and activity of CAT and PAL during the SMF+Asc. application The reduced expression and activity of CAT and PAL during the SMF+Asc. application is a consequence of the ROS scavenging activities of these enzymes to reduce the accumulated H_2O_2 and restore it to normal levels when there is no need for further scavenging.

Plants have several metabolic pathways leading to secondary products that provide the metabolic plasticity essential for responding to stress. As shown in Fig. (V), alteration of ROS and NO may also promote downstream signaling pathways resulting in the activation of key enzyme involved in the biosynthesis of secondary metabolites (Farnese et al., 2016; Zafari et al., 2017). The shikimate pathway is a critical interface between primary and secondary metabolism and

is used in the synthesis of aromatic compounds like phenylalanine (Phe). Among determined amino acids, aromatic ones serve as precursors for a variety of plant hormones, such as salicylic acid and a wide range of secondary metabolites such as phenolics (Tzin and Galili, 2010; Zafari et al., 2017). Phenolic acids are reductant agents that can quench ROS thus, diminishing stress effects in cells (Flora, 2009; Jouni et al., 2012; Taghizadeh et al., 2019). In the present study increased levels of aromatic amino acids (Phe and Tyr), phenolic compounds, and PAL gene expression and activity of tobacco cells showed similar tendencies along with the period of SMF treatment. Conversely, SMF+Asc. significantly decreased these parameters. It is likely that as a signaling molecule, H_2O_2 regulates metabolome changes in tobacco cells that result in SMF tolerance strategies while Ascorbic acid by scavenging H_2O_2 diminishes this role (Fig. V).

Large amount of evidence illustrated that detoxification of ROS is facilitated by non-enzymatic molecules, including GSH, Pro, and Asc. Glutamic acid is a key molecule in cellular metabolism and serves as the main precursor for production of Pro, Arg, Gly, and tripeptide GSH. Stimulatory effects of SMF on the production of GSH has been shown in *Saccharomyces cerevisiae* (Santos et al., 2010). Investigating the effects of SMF on Almond Seeds, Abdollahi et al. (2019) found an increasing pattern of proline content, similar to the response to other stresses. The reduction of Glu content in SMF-treated tobacco cells was accompanied by increases in Gly, Arg, Pro, and GSH. In contrast, in SMF+Asc. treatment, the change of Glu was accompanied by decreasing levels of Arg, Pro, and GSH compared to the SMF-treated group. Interestingly, Ascorbic acid is a cofactor of proline hydroxylase enzyme and causes the conversion of proline into hydroxyproline, a cell wall component in plants (Davey et al., 2000; Zhang et al., 2014). Also, amino acids contributed to the energy state of plant cells under certain physiological conditions, e.g. carbon starvation (Hildebrandt et al., 2015). Altogether, the results suggest that SMF triggered periodic oscillations in ROS and NO that changed normally designed framework of metabolites channeling into specific metabolic pathways: survival in the face of conflicting demands. It seems that a central role might be considered for H_2O_2 under SMF stress in tobacco cells (Fig. V).

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References

Abdollahi, F., H. Amiri, V. Niknam, F. Ghanati, and K. Mahdigholi. 2019. Effects of Static Magnetic Fields on the Antioxidant System of Almond Seeds. *Russian Journal of Plant Physiology,* 66: 299-307.

Akkol, EK., F. Goger, M. Koşar and KHC. Başer. 2008. Phenolic composition and biological activities of *Salvia halophila* and *Salvia virgata* from Turkey. *Food Chemistry,* 108: 942-949.

Bates, LS., R. P. Waldren, and ID Teare. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil*, 39: 205-207.

Akram, NA., F. Shafiq, and M. Ashraf. 2017. Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Frontiers in Plant Science*, 613(8):1-17.

Bradford, MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72: 248-254.

Calabro, E.,S. Condello, M. Curro, N. Ferlazzo, D. Caccamo, S. Magazu, and R. Ientile. 2013. Effects of low intensity static magnetic field on FTIR spectra and ROS production in SH-SY5Y neuronal-like cells. *Bioelectromagnetics*, 34: 618-629.

Davey, MW., MV. Managu, I. Dirk, S. Maite, K. Angelos, N. Smirnoff, IJJ Binenzir, JJ. Strain, D. Favell, and J. Fletcher.2000. Plant Ascorbic

acid: chemistry, function, metabolism, bioavailability, and effects of processing. *Journal of the Science of Food and Agriculture*, 80: 825–860.

De Gara, L., MC. de Pinto, and O. Arrigoni.1997. Asc.orbate synthesis and Ascorbate peroxidase activity during the early stage of wheat germination. *Physiologia Plantarum*, 100: 894–90.

De Vos, CHR., H. Scha,t, MAM. De Waal, R. Vooijs, and WHO. Ernst. 1991. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. *Physiologia Plantarum*, 82: 523-528.

Farnese, FS., PE. Menezes-Silva, GS. Gusman, and JA. Oliveira. 2016**.** When bad guys become good ones: the key role of reactive oxygen species and nitric oxide in the plant responses to abiotic stress. *Frontiers in Plant Science*, 7: 471.

Foyer, CH. And G. Noctor. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell,* 17:1866–1875.

Garg, N. and H. Kaur. 2012. Influence of zinc on cadmium-induced toxicity in nodules of pigeon pea *(Cajanuscajan*L. Millsp*.)* inoculated with arbuscular mycorrhizal (AM) fungi. *Acta Physiologiae Plantarum*, 34: 1363-1380.

Ghanati, F., P. Abdolmaleki, M. Vaezzadeh, E. Rajabbeigi, and M. Yazdani. 2007. Application of magnetic field and iron to change medicinal products of *Ocimum basilicum*. *Environmentalist*, 27: 429-434.

Gill, SS. and N. Tuteja. 2010**.**Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants*. Plant Physiology and Biochemistry*, 48: 909–930.

Haghighat, N.,P. Abdolmaleki, F. Ghanati, M. Behmanesh, and A. Payez, A. 2014**.** Modification of catalase and MAPK in *Vicia faba* cultivated in soil with high natural radioactivity and treated with a static magnetic field. *Journal of Plant Physiology*, 171: 99-103.

Green, LC., DA. Wagner, J. Glogowski, PL. Skipper, JS. Wishnok, and SR. Tannenbaum. 1982**.**Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Analytical Biochemistry*, 126: 131-138.

Hildebrandt, TM., AN. Nesi, WL. Araujo, and HP. Braun. 2015**.** Amino acid catabolism in plants. *Molecular plant*, 8: 1563-1579.

Jouni, FJ., P. Abdolmaleki, and F. Ghanati. 2012.Oxidative stress in broad bean *(Vicia faba* L*.)* induced by static magnetic field under natural radioactivity. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis*, 741: 116–121.

Leon, J., A. Costa, and MC. Castillo. 2016.Nitric oxide triggers a transient metabolic reprogramming in Arabidopsis. *Scientific Reports*, 6: 37945.

Maffei, ME. 2014. Magnetic field effects on plant growth, development, and evolution. *Frontiers in Plant Science,* 5: 445.

Mohammadi, F., F. Ghanati, M. Sharifi, and N. Ahmadian Chashmi. 2018. On the mechanism of the cell cycle control of suspension-cultured tobacco cells after exposure to the static magnetic field. *Plant Science*, 277: 139-144.

Nahidian, B., F. Ghanati, M. Shahbazi, and N. Soltani. 2018. Effect of nutrients on the growth and physiological features of newly isolated *Haematococcus Pluvialis* TMU1. *Bioresource Technology,* 255: 229-237.

Nemati, F., F. Ghanati, H. Ahmadi Gavlighi, and M. Sharifi. 2018. Fructan dynamics and antioxidant capacity of 4-day-old seedlings of wheat *(Triticum aestivum)* cultivars during drought stress and recovery. *Functional Plant Biology*, 45: 1000-1008.

Okano, H., H. Masuda, and C. Ohkubo. 2005. Decreased plasma levels of nitric oxide metabolites, angiotensin II, and aldosterone in spontaneously hypertensive rats exposed to 5 mT static magnetic field. *Bioelectromagnetics*, 26: 161-172.

Pormehr, M., F. Ghanati, M. Sharifi, PF. McCabe, S. Hosseinkhani, and H. Zare-Maivan. 2019. The role of SIPK signaling pathway in antioxidant activity and programmed cell death of tobacco cells after exposure to cadmium. *Plant Science*, 280: 416-423.

Sahebjamei, H.,P. Abdolmaleki, and F. Ghanati. 2007. Effects of magnetic field on the antioxidant enzyme activities of suspension-

cultured tobacco cells. *Bioelectromagnetics,* 28: 42-47.

Santos, LO., RM. Alegre, C. Garcia-Diego, and J. Cuellar. 2010.Effects of magnetic fields on biomass and glutathione production by the yeast *Saccharomyces cerevisiae*. *Process Biochemistry,* 45: 1362-1367.

Shang, GM., JC. Wu, and YJ. Yuan. 2004. Improved cell growth and Taxol production of suspension-cultured *Taxus chinensis* var. mairei in alternating and direct current magnetic fields. *Biotechnology Letters,* 26: 875-878.

Shokrollahi, S., F. Ghanati, RH. Sajedi, and M. Sharifi. 2018. Possible role of iron-containing proteins in physiological responses of soybean to the static magnetic field. *Journal of Plant Physiology*, 226: 163-171.

Smal,l DP., NP. Hüner, and W. Wan. 2012. Effect of static magnetic fields on the growth, photosynthesis, and ultrastructure of *Chlorella kessleri* microalgae. *Bioelectromagnetics,* 33: 298-308.

Taghizadeh, M., F. Nasibi, K.M. Kalantari, and F. Ghanati. 2019.Evaluation of secondary metabolites and antioxidant activity in *Dracocephalum polychaetum* Bornm. cell suspension culture under magnetite nanoparticles and static magnetic field elicitation*. Plant Cell, Tissue and Organ Culture*, 1-10.

Tzin, V. and G. Galili. 2010. The biosynthetic pathways for shikimate and aromatic amino acids in *Arabidopsis thaliana*. *The Arabidopsis Book*, 8: e0132.

Velikova, V., I. Yordanov, and A. Edreva. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science,* 15159: e66.

Vergallo, C., M. Ahmadi, H. Mobasheri, and L. Dini. 2014. Impact of inhomogeneous static magnetic field (31.7–232.0 mT) exposure on human neuroblastoma SH-SY5Y cells during cisplatin administration*. PloS One*, 9: e113530.

Wang, H. and X. Zhang. 2017. Magnetic fields and reactive oxygen species. *International Journal of Molecular Science*, 18: 2175.

Wang, JW., LP. Zheng, JY. Wu. and RX. Tan. 2006. Involvement of nitric oxide in the oxidative burst, phenylalanine ammonia-lyase activation, and Taxol production induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures. *Nitric Oxide* 15: 351-358.

Zafari, S., M. Sharifi, N. Ahmadian Chashmi, and LA. Mur. 2016. Modulation of Pb-induced stress in *Prosopis* shoots through an interconnected network of signaling molecules, phenolic compounds, and amino acids. *Plant Physiology and Biochemistry*, 99: 11-20.

Zafari, S., M. Sharifi, and LA. Mur. and NA. Chashmi. 2017. Favoring NO over H_2O_2 production will increase Pb tolerance in *Prosopis farcta* via altered primary metabolism. *Ecotoxicology and Environmental safety*, 142: 293-302.

Zhang, X., H. Ma, H. Qi, and J. Zhao. 2014. Roles of hydroxyproline-rich glycoproteins in the pollen tube and style cell growth of tobacco *(Nicotiana tabacum* L.*). Journal of Plant Physiology*, 171: 1036–1045.