Modification of Phytochemical Production and Antioxidant Activity of Dracocephalum Kotschyi Cells by Exposure to Static Magnetic Field and Magnetite Nanoparticles

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Abstract

Dracocephlum kotschyi Boiss is a genus in Lamiaceae family and a medicinal herb native to Iran. The cell suspension cultures were treated by static magnetic field (SMF) and Fe_3O_4 magnetite nanoparticles (MNP) to understand the production yield of secondary metabolites. The treatment procedure was done by cultivating the cells either with 100 ppm MNP, SMFs, or simultaneous exposure to both MNP and SMFs. The SMF at 30 mT was uniformly applied to the cells either for 3 or 4 days with a 3 h per day or a 5 h per day intervals, respectively. The contents of phenolics and phytochemicals were then examined by high performance liquid chromatography and UV-Vis spectrophotometer. These treatments imposed oxidative stress and induced polyphenol oxidase and phenylalanine ammonia lyase, accompanied by enhanced production of phenolics, anthocyanins, flavonoids, and lignin. The highest membrane embrittlement and elicitation was found upon simultaneous application of the MNPs and SMFs, followed by the MNP and SMFs. The contents of naringin, rosmarinic acid, quercetin, thymol, carvacrol, apigenin, and rutin increased in the intracellular biomass of all treated cells and extracellular culture media. These findings propose the potential of these elicitors in simultaneous production and secretion of these phytochemicals into culture media.

1. Introduction

Dracocephalum is a genus in the Lamiaceae family which has been used traditionally in herbal medicine. *Dracocephalum kotschyi* Boiss is a perennial plant endemic to Iran (known locally as ‘Zarrin-giah’ or ‘Badrandjbooie Denaie) growing in high mountainous areas (Fattahi et al. 2016; Salehi et al. 2015). *D. kotschyi* was recognized as a source of phytochemicals belonging to terpenoids, phenolics, flavonoids, and alkaloids (Fattahi et al. 2016; Moghaddam et al. 2012). The production of these secondary metabolites can be promoted by biotechnological tools such as plant tissue and cell suspension cultures. These techniques provide a promising bio-production platform for desired natural products and have been applied for increasing the quantity and quality of drugs (Dias et al. 2016; Khvatkov et al. 2015; Yue et al. 2016).

Production of secondary metabolites such as phenolic compounds can be affected by a variety of biotic and abiotic stresses (Ainsworth and Gillespie 2007; Dias et al. 2016). Phenolics serve as a means of counteracting oxidative stresses. These compounds contribute to the transfer of hydrogen to free radicals and the consequent production of a stabilized form of phenoxide radical (Akkol et al. 2008). The phenolics are capable of acting as Fenton reaction inhibitors. Besides, they can directly act as free radical scavengers and reduce oxidative damage, thereby protecting cells against oxidative stress (Heidarabadi et al. 2011).

PAL is an important enzyme in the metabolism of phenolic compounds. It is responsible for catalyzing the first step of the phenyl propanoids pathway, converts phenylalanine to cinnamic acid. Subsequent reactions in the pathway result in the production of a wide variety of phenolic metabolites such as simple phenolics, lignins, flavonoids, and anthocyanins (Dixon and Paira 1995). Moreover, its activity is believed

to be as a main regulation point between primary and secondary metabolism. The PAL activity depends on some factors, such as genotype, plant development stage, and organ. There are reports on its induction by some environmental stresses (Dixon and Paira 1995; Heidarabadi et al. 2011; Safari et al. 2013; Tahsili et al. 2014). In the oxidative degradation of phenolic compounds, polyphenol oxidase (PPO) catalyzes O_2-dependent oxidation of phenolic compounds to highly reactive quinines (Araji et al. 2014). To enhance the production of phenolics, physic-chemical elicitors such as static magnetic field (SMF) and nanoparticle (NP) for plant growth stimulation and phytochemical production have been widely applied as environmentally benign techniques in recent years (Aladjadjiyan 2010). The response of plant cells to magnetic field exposure was different depending on plant species, magnetic field intensity, and exposure time. As an example, the PPO activity and phenolic contents were strongly induced in hazel cell suspension cultures upon treatment by static magnetic field (SMF) as well as by SMF combined with salicylic acid (Rezaei et al. 2010). Shang et al. (2004) reported that taxol production was improved in *Taxuschinensis* cell suspension cultures by the SMF elicitation.

The influence of magnetic field (MF) on living organisms is through induction of oxidative stress and increase in the concentration, activity, and lifetime of free radicals. It also affects ionic currents in the cell membrane and other cellular transport processes, increases cell membrane permeability, and alters the electromagnetic properties of cell biological molecules (Çelik et al. 2009; Galland and Pazur 2005; Nagy and Fischl 2004; Payez et al. 2013; Radhakrishnan et al. 2012b; Sahebjamei et al. 2007; Tenforde 1996). Alterations of the enzymatic activities, such as catalase (CAT), superoxide dismutase (SOD), and peroxidase, by external SMF suggest their possible implication in magnetoreception. However, the exact mechanism of SMF on living organisms is still unclear and continuous research in this regard is of high demand (Payez et al. 2013; Sahebjamei et al. 2007).

The NPs have been applied for agricultural and horticultural purposes (Dimkpa et al. 2012). The positive or negative effects of metal nanoparticles on higher plants strongly depend on plant species and the physic-chemical properties of NPs. There are several reports on the enhanced production of reactive oxygen species (ROS) and oxidative stress during the application of NPs to plant culture media (Dhoke et al. 2013; Lee et al. 2012; Li et al. 2013; Ma et al. 2010; Wang et al. 2006). Li et al. (2013) demonstrated that watermelon treatment with Fe_3O_4 NPs caused physiological changes in the activities of CAT, PPO, SOD, and ferric reductase as well as in the contents of chlorophylls and malonyldialdehyde (MDA). The levels of total phenolicsin plants and the activities of antioxidant enzymes SOD and CAT were also increased upon treatment with chitosan NPs (Chandra et al. 2015). In *Taxus chinensis* cell suspension culture, AgNPs (5 ppm) significantly increased taxol production in comparison to the control cells (Jamshidi et al. 2016).

In the present research, two SMFs as physical and Fe_3O_4 magnetite NPs as chemical elicitors were applied separately and simultaneously to the suspension-cultured *D. kotschyi* cells. This study was aimed to find out the underlying physiological responses upon these treatments as well as the quantity and quality of phytochemicals. This understanding could lead us to an effective strategy towards improved yield of beneficial secondary metabolites from undifferentiated plant cells.

2. Materials And Methods

2.1. Cell Growth Conditions

D. kotschyi seeds were collected from wild-growing plant populations from Fereydonshahr mountainous area located 2700 m above sea level in Isfahan, Iran. For callus induction, the explants of *D. kotschyi* were obtained from *in vitro* grown plants on the Murashige and Skoog (MS) medium. Plant growth regulators of 1-naphthaleneacetic acid (NAA) (1 mg L^{-1}) and 6-benzylaminopurine (BAP) (6 mgL^{-1}) were added to the MS medium, which was supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agar, at 24 ± 2 ºC under dark condition. The suspension cultures were established from calli of *D. kotschyi* cells. The suspensions were grown in a modified B5 medium (Gamborg et al. 1976) containing 2% sucrose with NAA (1 mg L^{-1}) and BAP (2.5 mg L^{-1}) at 24 ºC in darkness on an orbital shaker at 120 rpm and were sub-cultured every 10 days at their logarithmic growth phase.

2.2. Elicitation Treatments with Static Magnetic Field

The *D. kotcshyi* cells were exposed to uniform SMF (30 mT) by a MF generator device designed and built in-house. The electrical power of the system was provided by a 220-V AC power supply equipped with variable transformer as well as a single-phase and full-wave rectifier (Model RD 4025, Rahavand, Tehran, Iran). The maximum power and flowing current were measured 1 kW and 50 A DC, respectively. This system was clearly described by Shokrollahi et al. (2018) and did not discuss herein for brevity.

For elicitation purposes, the SMF intensity was commonly ranged from 10 to 50 mT (Wang et al. 2008) and the field intensity of 30 mT was frequently applied to plant cell models (Haghighat et al. 2014; Sahebjamei et al. 2007; Shokrollahi et al. 2018). The elicitation period was chosen based on some preliminary experimental works and literature review (Payez et al. 2013; Sahebjamei et al. 2007; Shokrollahi et al. 2018). The elicitation group was put in the SMF producing system and the control groups was placed far away from the MF generating device and any other laboratory instruments, *e.g.* NMR, to avoid unwanted exposure to MF other than the local earth MF of 60 μT, as determined by the Geophysics Institute, University of Tehran (Tehran, Iran). The treated cells were exposed to uniform and homogenous 30 mT SMF in their exponential growth phase (from day 7 to 10 after sub-cultures) for 3 days at 3 h per day interval (so called SMF1) and for 4 days at a 5 h per day interval (so called SMF2). The elicitation duration was chosen slightly less than the previous works because there is high possibility of cellular damage upon simultaneous application of SMFs and MNPs. The presence of a pulsation frequency of 50 Hz with a range of voltage variation about ± 1 V in the current from rectifier into the SMF generating apparatus was detected possibly due to the shortcoming of the applied single-phase and full-wave rectifier, providing a ripple voltage of ~ 5%. This small ripple voltage confirms the homogeneity of the generated magnetic field, the measurement by a Teslameter (13610.93, PHYWE, Gottingen, Germany) approved its accuracy and uniformity. A precise indication of the simulated field levels in Computer Simulation Technology was presented as the test of homogeneity of SMF in previous paper (Payez et al. 2013).

2.3. Characterization and Application of Magnetite Nanoparticles

The MNPs were supplied from the US Research Nanomaterial (Houston, TX). The diameters of MNPs were ranged from 10–20 nm with 99.99% purity, according to the manufacturer protocol. The nanoparticles were dispersed by suspension in 0.05% dimethyl sulfoxide (DMSO) prepared with deionized water (Pandya and Singh, 2015). The DMSO was selected because it is a significant polar aprotic solvent dissolving both polar and nonpolar compounds. Moreover, it is miscible in many organic solvents as well as in water. The MNP in DMSO was then kept in water in an ultrasonic bath for 25 min. Seven days after cell subculture, a 100 ppm MNP was added to the liquid culture media of the cells. Two control groups were used in the present research including the cells grown in the culture medium with no MNP and the ones grown in the culture medium with 0.05% DMSO without MNP in darkness. The concentration of MNP (100 ppm) was selected based on some preliminary experimental works and literature studies (Jalali et al. 2017; Li et al. 2016; Zadeh et al. 2019).

2.4. Application of SMF Combined with Magnetite Nanoparticles

The cells in their exponential growth phase (7 day after cell subculture) were treated with 100 ppm MNPs. These samples were then divided into two groups: One group were placed in the SMF generating apparatus under 30 mT SMF for 3 days at 3 h per day interval (SMF1 + MNP) and the second group was exposed to the same SMF for 4 days at 5 h per day intervals (SMF2 + MNP), from day 7 to 10 after subculture. The control cells consists of two groups, the first group grown in the culture medium without MNP, and the second one grown in the culture medium with 0.05% DMSO, then were kept far from the SMF producing system.

The suspension cultures were harvested at the end of all elicitations by filtration using a Buchner funnel with a nylon mesh under vacuum. The harvested biomass was frozen in liquid N_2 and kept at -80°C for further phytochemical and physiological analysis.

2.5. Contents of Total Phenolics, Flavonoids, Flavonols, and Anthocyanins

Total phenolic contents were determined using the Folin-Ciocalteau method as discussed in Chua et al. (2011). A 500 µL methanolic extract of fresh cell mass (200 mg) was used for determination of phenolic content by measuring absorbance at 765 nm using a UV/Vis spectrophotometer (Cintra6, GBC, Dandenong, Australia). Total flavonoid and flavonol contents were estimated according to the protocol of Miliauskas and Venskutonis (2004). The methanolic extract of 200 mg fresh cell mass was used for UV-Vis spectrophotometer determination of total flavonoids and flavonols at 445 and 415 nm, respectively. The contents of total flavonoids and flavonols were expressed as mg rutin equivalents per gram of fresh mass (mg RU g^{-1} FW).

To measure anthocyanin contents, the acidic methanol (99/1, methanol/acetic acid) extract of a 200 mg fresh cell mass was used according to the protocol of (Hara et al. 2003). The total anthocyanin contents was determined by measuring the absorbance at 511 nm using the spectrophotometer and quantified based on the extinction coefficient of 33,000 M^{-1} cm^{-1}.

2.6. Lignin Content Assay

The dried powdered cell walls of each sample (6 mg) were extracted with 25 vol.% acetyl bromide (in glacial acetic acid) and 100 µL of 70% $HClO_4$, according to Ghanati et al. (2005). The lignin content was determined by measuring spectrophotometeric absorbance at 280 nm and quantified using the specific absorption coefficient value of 20.0 L $g^{-1}cm^{-1}$ (Iiyama et al. 1990).

2.7. Activity Assay of Phenylalanine Ammonia Lyase and Polyphenol Oxidase

The phenylalanine ammonia lyase (PAL) activity was determined by the method of Ochoa-Alejo and Gómez-Peralta (1993) through measurement of cinnamic acid production by the spectrophotometer at 290 nm. To this end, a 200 mg cell mass of each sample was extracted by β-mercaptoethanol (15 mM) in Tris-HCl (pH 8.2, 100 mM) buffer. One unit of PAL activity was expressed as amounts of cinnamic acid produced within one hour per mg of protein.

The PPO activity was assayed according to Kahn et al. (1975). A 200 mg fresh cell mass was used for enzyme extraction by 3 mL of 20 mM sodium phosphate buffer (pH 6.8) containing 0.1 mM EDTA.

2.8. Measurement of Phytochemical Products

Phytochemical products of the cells and culture media were extracted according to Barreca et al. (2016) with slight modifications. For this purpose, the lyophilized culture medium with no cells (15 mL) and the dry cell mass (100 mg) was homogenized by 2 mL acidic methanol (99 methanol / 1 acetic acid), extracted with ultrasonic for 20 min, and incubated overnight at room temperature. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was gathered and allowed to evaporate at room temperature. The residues were dissolved in 300 µL methanol and filtered (0.22 µm pore size) into clean vials.

For quantitative and qualitative determination of phytochemical products in methanol, a High Performance Liquid Chromatography (HPLC) program equipped with a UV-Vis photodiode-array detector (DAD-HPLC Waters e 2695, alliance, Milford, MA, 2489 UV- Vis detector) was used. For the analysis of phytochemical products, a 20 µL aliquot of the filtered sample was injected into the HPLC. The chromatographic separation was obtained by a C18 column (Perfectsil Target ODS-3 (5 mm), 250 4.6 mm; MZ Analysentechnik, Mainz, Germany) with solvent A (water / acetic acid, 97/3, v/v) and solvent B (methanol) under the following gradient conditions: 0–3 min, 0% B; 3–9 min, 3% B; 9–24 min, 12% B, 24–33 min, 20% B, 33–43 min, 30% B; 43–63 min, 50% B; 63–66 min, 50% B; 66–81 min, 60% B; 81–86 min, 0%, B and equilibrated 4 min for a total run time of 90 min. The flow rate was 1.0 mL min^{-1}, injection

volume was 20 µL, and the column thermostat was held constant at 26°C. The UV spectra of phytochemical products were recorded at 280 and 325 nm (Barreca et al. 2016). To confirm the peak identity, their retention times and absorption spectra were compared with those of pure (> 99%) standards of apigenin, naringin, rosmarinic acid, rutin, quercetin, thymol, and carvacrol.

2.9. Hydrogen Peroxide Contents and Lipid Peroxidation Assay

The peroxides were first extracted from the fresh biomass (200 mg) by grinding to homogenization in 5 mL of 0.1 % (v/v) trichloroacetic acid using a pestle and mortar. The homogenate was centrifuged at 10000 rpm for 20 min, then 0.5 mL of supernatant was mixed with 500 µL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of 1 M KI. Absorbance of the mixture solution was read at 390 nm by a double beam spectrophotometer (Jamshidi et al. 2016). To determine the amount of lipid peroxidation, the MDA of the cells were measured as the product of peroxidation of membrane lipids, according to the method of Heath and Packer (1968).

2.10. Data Presentation and Statistical Analyses

Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) version No 19.0, Chicago, IL, USA. The experiments followed a completely randomized design and the values were presented as means ± SE (standard error). All the experiments and observations were replicated three times each with at least three samples. The significant differences between treatments were evaluated using the Duncan's test at $p \leq 0.05$.

3. Results

3.1. Peroxide Contents and Lipid Peroxidation

The contents of peroxide and MDA increased remarkably in all elicited cells compared to those of control (Fig. 1A). The highest increases of 52% and 35% in the peroxide and MDA were observed upon treatment with SMF1 + MNP and SMF2 + MNP compared to that of the control, respectively. The cells treated with the magnetic field in combination with the nanoparticles (SMF1 + MNP and SMF2 + MNP) were of significant differences in the peroxide and MDA levels; while there were no significant differences between the levels of lipid peroxidation in the cells treated with the SMF1 and SMF2 (Fig. 1A). However, the peroxide contents of the cells treated with SMF2 was significantly higher than those treated with SMF1. The MNP application caused a significant increase of 12% in the MDA compared to that of control; while two control groups were of no significant differences in the peroxidation levels.

3.2. Total Phenolic, Flavonoids, Flavonol, Anthocyanin, and Lignin Contents

The bioaccumulation of total phenolics in the cells undergone the SMF and MNP treatments significantly increased compared to that of control (Fig. 1B). The highest effect of elicitation on total phenolic contents was observed under SMF2 + MNP treatment, amounting to 7.7 mg gallic acid g^{-1} FW that is 2.1 times higher than that of control (Fig. 1B). The MNP was of higher elicitation effect on total phenolics than treatment with the SMF. The lowest elicitation was observed upon treatment of the cells with the SMF1 (4.64 mg g^{-1} FW).

The contents of total flavonoids and flavonols were increased in the treated cells compared with those of control (Fig. 1C). The greatest amount of total flavonoids were measured 312.76 µg Rutin g^{-1} FW and 310.93µg Rutin g^{-1} FW in the cells treated with SMF2 + MNPs and SMF1 + MNPs, respectively. It was increased by about 1.25 fold compared to that of control. There were no significant differences between the levels of total flavonoids in the cells treated with the MNPs, SMF1 and SMF2 (Fig. 1C). The results indicated that the flavonol contents increased significantly from 101.21 µg Rutin g^{-1} FW in the control cells to 144.76 µg Rutin g^{-1} FW in the cells treated with the SMF1 + MNP (Fig. 1C). The increase in the flavonol contents was about 1.43 fold in comparison to that of the control samples. There were no significant differences between the levels of flavonol in the cells treated with the SMF1 and SMF2 + MNP. It was found that the flavonol contents were decreased in the cells treated with SMF2 + MNP compared to those treated with MNP, SMF2 and SMF1 + MNPs.

Anthocyanin content, as a subgroup of flavonoids, significantly increased in the cells treated with SMF2, SMF1 + MNP, and SMF2 + MNP (Fig. 1C). The highest content of anthocyanins (29.57 $nmolg^{-1}$ FW) was measured in the cells treated with SMF2 + MNP. This increase is about 1.64 times higher than that of control cells. Other elicitors showed no significant difference regarding the contents of anthocyanins (Fig. 1C).

The lignin content showed a significant increase in all treatments compared to those of control (Fig. 1D). The highest amount of lignin was observed in the SMF2 + MNP treatment (19.04 % of cell wall DW) that was three times higher than that of the control cells (6.32 % of cell wall DW). The next highest content of lignin was measured in the cells treated with MNP and SMF1 + MNP. It was found that the MNP treatment combined with the SMF1 has no significant elicitation effect than the sole MNP treatment. Among the physicochemical treatments, the SMF1 was of the lowest elicitation effect on the cells that is nearly identical to that of control (Fig. 1D). Furthermore, the MNP was of higher elicitation effect than treatment with the SMF.

3.3. Enzymatic Activities

The activity of PAL enzyme increased significantly in all treated cells (Fig. 2). The highest PAL activity was observed in the cells treated with SMF2 + MNP, amounting to 1539.49 µg Cinamicacid mg^{-1} h^{-1}. This elicitor resulted in 2.06 fold higher PAL activity than that of the control cells (Fig. 2). The cells treated with MNPs were of higher PAL activity than the ones treated with the SMFs. The combination of two

treatments more significantly affected the cells' enzymatic activity than the separate application of either one of treatments.

Similarly, the highest PPO activity was observed upon treatment with the SMF2 + MNP (Fig. 2). It was measured 16.55 Abs 410 mg^{-1} min^{-1} under the control conditions and 44.37 and 41.58 Abs 410 mg^{-1} min^{-1} upon the SMF2 + MNP and SMF2 elicitations, respectively. However, the SMF2 was of higher elicitation effect than treatment with the MNPs. The highest increase in the PPO activity was 2.54 fold and 2.39 fold higher than that of control for the cells treated with SMF2 + MNP and SMF2, respectively (Fig. 2). Therefore, all treatments were significantly enhanced the PPO and PAL activities under elicitation conditions in comparison to control cells.

3.4. The Contents of Phytochemical Products

This study showed that the contents of phytochemical products such as rutin, apigenin, naringin, thymol, quercetin, carvacrol, and rosmarinic acid in all treatments remarkably increased compared with those of the control cells (Table 1). The highest amounts of rosmarinic acid and carvacrol were observed in the cells treated with SMF1 + MNP and SMF2 + MNP, respectively. The cells treated with SMF2 and SMF2 + MNP caused the highest value of rutin. The apigenin, quercetin, and thymul measured highest in the cells treated with SMF1. Furthermore, the greatest amount of naringin was observed in the cells treated with the MNP (Table 1). As the contents of phytochemical compounds increased in the cells under all treatments, the amounts of these compounds were considerably increased in all treated culture media in comparison with the control culture media. The SMF1 induced higher contents of all phytochemical products except rutin than the SMF2 treatment (Table 1). It was of higher elicitation effect than the MNP as well. However, these two SMF treatments did not significantly different in the excreted phytochemical products into the culture media (Table 2). Except for rutin and quercetin, the extracellular contents of the cells treated with SMF2 were 2.5 and 3.0 times higher than those treated with the SMF1, respectively. The combined application of the SMF2 + MNP resulted in the highest and the SMF1 led to the lowest secretion of the phytochemicals (Table 2).

Table 1

Effects of MNP (100 ppm), static magnetic field (30 mT), and their combination on the amount of phytochemical products (µg g^{-1} DW) in the *D. kotschyi* cells measured by HPLC. MNP: Fe_3O_4 magnetic nanoparticles, SMF1: Static magnetic field for 3 days, 3h a day, SMF2: Static magnetic field for 4 days, 5 h a day, MNP + SMF1: Static magnetic field for 3 days, 3 h a day with Fe_3O_4 MNP, MNP + SMF2: Static magnetic field for 4 days, 5 h a day with Fe_3O_4 MNP.

Phytochemical products (µg g^{-1} DW)							
Treatments	**Naringin**	**Rutin**	**Quercetin**	**Apigenin**	**Rosmarinic acid**	**Thymol**	**Carvacrol**
Control	8.45 ± 4.3 ^{d}	1.63 ± 1.88^{e}	22.38 ± 3.6^{c}	23.33 ± 2.11^{f}	9.76 ± 1.8^{d}	5.17 ± 0.97^{c}	ND
MNP	73.25 ± 5.6^{a}	5.41 ± 50.78^{cd}	86 ± 1.02^{b}	103.6 ± 4.2^{c}	17.53 ± 5.8^{c}	12.34 ± 1.2^{ab}	2.29 ± 0.93^{bc}
SMF1	23.72 ± 1.4^{b}	8.062 ± 0.41^{c}	105 ± 1.12^{a}	143.3 ± 8.16^{a}	16.23 ± 9.89^{c}	13.98 ± 2.11^{a}	2.53 ± 0.72^{b}
SMF2	16.53 ± 0.92^{c}	20.69 ± 0.85^{a}	12.35 ± 2.09^{f}	121.6 ± 7. 3^{b}	8.92 ± 2.25^{d}	11.71 ± 0.5^{ab}	2.68 ± 1.57^{b}
MNP + SMF1	15.05 ± 1.48^{c}	18.42 ± 1.23^{ab}	20.78 ± 0.91^{cd}	43.01 ± 2.52^{d}	53.57 ± 17.83^{a}	9.11 ± 2.04^{b}	2.03 ± 1.1^{c}
MNP + SMF2	15.21 ± 0.23^{c}	20.98 ± 1.54^{a}	16.36 ± 0.82^{d}	31.16 ± 4.11^{e}	21.62 ± 6.01^{b}	14.9 ± 1.37^{a}	3.25 ± 2.1^{a}

Data were shown in mean ± SE. Different lowercase letters show a significant difference at $P \leq 0.05$.

Table 2
Effects of MNP (100 ppm), static magnetic field (30 mT), and their combination on the amount of phytochemical products (µg mL^{-1} culture media) in the culture media of *D. kotschyi* cells measured by HPLC. MNP: Fe_3O_4 magnetic nanoparticles, SMF1: Static magnetic field for 3 days, 3h a day, SMF2: Static magnetic field for 4 days, 5 h a day, MNP + SMF1: Static magnetic field for 3 days, 3 h a day with Fe_3O_4 MNP, MNP + SMF2: Static magnetic field for 4 days, 5 h a day with Fe_3O_4 MNP, ND: Not detected.

Phytochemical products (µg mL^{-1} culture media)							
Treatments	**Naringin**	**Rutin**	**Quercetin**	**Apigenin**	**Rosmarinic acid**	**Thymol**	**Carvacrol**
Control	ND	1.6 ± 0.01^{f}	ND	ND	ND	ND	ND
MNP	2.34 ± 0.73^{c}	4.5 ± 1.01^{d}	2.56 ± 0.18^{d}	3.53 ± 0.4^{b}	17.54 ± 1.25^{c}	2.98 ± 0.41^{b}	ND
SMF1	2.98 ± 0.7^{b}	2.58 ± 1.45^{e}	1.9 ± 3.46^{e}	2.7 ± 1.02^{c}	4.12 ± 0.88^{d}	2.68 ± 0.15^{b}	ND
SMF2	3.02 ± 0.3^{b}	6.46 ± 1.3^{c}	5.79 ± 1.25^{b}	2.59 ± 1.06^{c}	4.53 ± 3^{d}	2.57 ± 0.13^{b}	ND
MNP + SMF1	2.68 ± 0.96^{bc}	9.76 ± 0.53^{b}	5.03 ± 0.99^{c}	2.77 ± 0.05^{c}	27.75 ± 5.1^{b}	3.04 ± 0.13^{b}	ND
MNP + SMF2	15.26 ± 2.01^{a}	19.76 ± 4.37^{a}	7.74 ± 1.2^{a}	4.76 ± 0.07^{a}	84.97 ± 9.98^{a}	5.27 ± 0.23^{a}	ND

Data were shown in mean ± SE. Different lowercase letters show a significant difference at $P \leq 0.05$.

4. Discussion

4.1. Effect of the SMFs and MNPs on the Antioxidant Defense Mechanism

This study analyses the production of phytochemicals in suspension-cultured *D. kotschyi* cells upon exposure to SMF and MNP. The cell suspension cultures of higher plants were suggested as useful models for studying the regulation and production of secondary metabolites (Tahsili et al. 2014).

The SMFs alter the electrical behaviors and permeability of cellular membranes through magnetic induction (Wang et al. 2008). The magnetic field affects on the orientation of the membrane phospholipids and then causes deformation of membrane channels (Radhakrishnan et al. 2012a). The SMFs influence the spins of cellular paramagnetic molecules and interrupt the associated chemical reactions as well. All these occurrences accompanied by oxidative stress and accumulation of peroxide contents (Fig. 1A). The response of plant cells to SMF exposure is dependent on many factors such as the plant species, SMF intensity, and exposure period (Çelik et al. 2009; Maffei 2014; Payez et al. 2013).

The higher peroxide contents of the cells upon exposure to SMF2 than SMF1 can be attributed to higher exposure period.

The treatment of the plant cells with MNP supply the cells with excess iron nutrients (Zia-ur-Rehman 2018). As a consequence it could stimulate Fenton reaction and generates hydrogen peroxide, hydroxyl radicals, and the superoxide anion. All these molecules result in oxidative stress (Li et al. 2013). The attachment of MNP to the cell membranes can disturb permeability due to adverse effects on membrane transport processes resulting in the generation of reactive oxygen species (Jalali et al. 2017; Jamshidi et al. 2016), a possible reason for higher lipid peroxidation than the SMFs (Fig. 1A). However, the lower peroxide contents of the cells treated with the MNPs than the SMFs might be related to the supplementation of the plants with iron oxide NPs that stimulates the activity of antioxidant enzymes and scavenges reactive oxygen species (Zia-ur-Rehman et al. 2018).

Iron is a ferromagnetic element and act as a magneto-receptor in cells under magnetic field treatment (Rajabbeigi et al. 2013). These direct effects of MNPs on cellular function and structures could be a possible reason for imposing higher elicitation than the SMFs (Fig. 1A, 1B, and 1D). The MNPs combined with magnetic fields led to higher ROS accumulation in the cells than the separate application of either one of elicitors (Ghanati et al. 2007). Ions such as iron in the cell have the ability to absorb magnetic energy; therefore the SMF cause the torque generation of magnetite nanoparticles (Aladjadjiyan 2010) and migration into the cells and culture media. Accordingly, the MNP may get new feature under magnetic field and impact more the cell function and induce more oxidative stress. It is a possible reason for higher MDA contents of the cells treated simultaneously with both magnetic field and MNPs (Fig. 1A). The cells treated with SMF2 + MNP are also exposed more time to MNPs than the ones treated with SMF1 + MNP that is one other possible reason for their higher MDA contents. The MDA is a cytotoxic product of lipid peroxidation and an indicator of free-radical accumulation (Coşkun et al. 2009; Rajabbeigi et al. 2013). The oxidative stress was induced possibly due to disruption of the membrane integrity and enhanced membrane permeability (Abdolmaleki et al. 2007). Accordingly, the cells were not able to prevent peroxidation of their membrane lipids (Rajabbeigi et al. 2013). These observations suggest that antioxidant system is significantly stimulated by increasing the duration of exposure to magnetic field and MNPs. The present findings also suggest that the SMF amplified the effect of MNP on cell physiological responses. The contents of total phenolics, flavonoids, anthocyanins, and lignin as well as the activities of PAL and PPO under combined elicitation of SMF and MNP were significantly higher than those of cells treated with either MNP or SMF alone.

The highest to lowest increase in the MDA contents of the cells was observed respectively upon treatments with SMFs + MNP, MNPs, and SMFs that is consistent with the enhancements in PAL activities (Fig. 2), total phenolics (Fig. 1B), and lignin contents (Fig. 1D). The peroxides are signaling molecules activating defensive pathways under stress conditions (Rezaei et al. 2010). To counteract the oxidative stress damages, the plant cells developed various enzymatic and non-enzymatic antioxidant defense mechanisms. The PAL is considered as an important enzyme in the phenylpropanoid pathway and involved with the plant cells defense response to ROS. The PAL activity was also considered as an

efficient marker for identifying the intensity of environmental stresses in plant species (Golkar et al. 2019b). The phenolics are secondary metabolites with protective effects against oxidative stress (Manquián-Cerda et al. 2016; Maqsood et al. 2014). Various studies have highlighted the strong relationship between plant secondary metabolism and defense responses (Çelik et al. 2009; Maffei 2014; Sahebjamei et al. 2007; Tahsili et al. 2014). The increase in the phenolics of the cells under different elicitations can also be attributed to their role in the stimulation of enzymatic activities and the expression of the genes contributing to the shikimate pathway (Golkar and Taghizadeh 2018). Safari et al. (2013) demonstrated that the phenolic content of hazel cells increased when the cells were exposed to ultrasound treatment. Also, it has been reported that in *Taxus* cell culture, the increase in the PAL activity caused by Taxol production after exposure to magnetic fields (Shang et al. 2004). Jamshidi et al. (2016) showed an increase in PAL activity and phenolic contents during the AgNPs treatment of hazel cell suspension culture. Total polyphenol increases the antioxidant potential in plants. These compounds might act as iron chelators, Fenton reaction inhibitor, or directly remove free radicals and decrease the oxidative damage (Ghanati et al. 2007). In fact, the higher phenolic compounds may protect plant cells from the SMF- and MNP-induced stresses and it will interfere with the signaling cascades involved in plant responses. In agreement with our findings, the treatment of suspension-cultured plant cells with magnetic fields and MNPs led to oxidative stress and accompanied by a change in the activities of scavenging enzymes such as catalase, peroxidase, superoxide dismutase, and polyphenol oxidase (Çelik et al. 2009; Haghighat et al. 2014; Jalali et al. 2017; Lee et al. 2012; Li et al. 2013; Maffei 2014; Payez et al. 2013; Poinapen et al. 2013; Radhakrishnan et al. 2012b; Selim and El-Nady 2011; Trebbi et al. 2007). Similar to the PAL activity and phenolic compounds, the lignin contents were increased in the treated cells as a defense response (Fig. 1D). Lignin, as a defensive layer of plants, tightens the cell walls and plays a defensive role against ROS generation in plants in response to biotic and abiotic stresses (Morita et al. 2006). Consistently Abdolmaleki et al. (2007) reported that the lignin level in tobacco cells was significantly increased upon exposure to 10 and 30 mT SMFs.

Similar to the increase in total phenolics, our results demonstrated an increase in flavonoid, anthocyanin, and flavonol contents of the treated cells (Fig. 1C). Flavonoids are key components of the antioxidant system with such subgroups as flavonols and anthocyanins. They belong to a class of low molecular weight phenolic compounds with diverse range of functions (Falcone Ferreyra 2012). The trend of changes in the contents of flavonols and anthocyanins upon each treatment are different with each other possibly because of their parallel biosynthetic pathways from flavonoids. For example, the highest flavonol content was observed in the cells treated with SMF1 + MNP; while the highest amount of anthocyanins was measured in the cells treated with SMF2 + MNP. The other possible reason is because the oxidative processes degrade these compounds. Their induction by peroxide signaling on one hand and their oxidative degradation on the other hand caused different increments of these compounds compared to control upon different treatments (Khatami et al. 2020). This increase can also be attributed to different elicitation of their biosynthetic pathways, the enhanced enzymatic activity, and the expression of pertinent genes (Di Ferdinando et al. 2012).

The increase in the PPO activity is a common physiological response upon exposure to oxidative stress. A similar observation was reported for hazel cell suspension cultures treated with the SMF and SMF combined with salicylic acid. Both treatments led to an increase in the PPO activity (Rezaei et al. 2010). This enzyme catalyzes the O_2-dependent oxidation of phenolic compounds into highly reactive quinines. It plays a key role in lignin biosynthesis (Tahsili et al. 2014) and also in binding phenols to each other (Araji et al. 2014; Tahsili et al. 2014).

4.2. Effect of the SMF and MNPs on the Phytochemical Compounds

Phytochemical compounds are the main secondary metabolites with valuable pharmaceutical and medicinal properties (Dias et al. 2016). They play significant contributions on the antioxidant activity of plants. The phytochemical products of *D. kotschyi* cells were identified as apigenin, carvacrol, naringin, quercetin, rosmarinic acid, rutin, and thymol. The metabolism and accumulation of these phytochemical compounds can significantly be affected by different genetic and environmental factors (Golkar et al. 2019a). These metabolites significantly changed in cells and culture media of the elicited samples (Tables 1 and 2).

Thymol and carvacrol were shown nearly similar production patterns (Tables 1 and 2) because they are both monoterpenic phenol isomers, derived from the methyl erythritol phosphate (MEP) pathway (Majdi et al. 2017). The geranyl diphosphate as universal precursor of monoterpenes is synthesized by head-to-tail condensation of dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) as the final products of the MEP pathway. The difference between biosynthesis of thymol and carvacrolis is related to differences in the activity of cytochrome p450 family enzymes which are involved in the modification of terpinene to thymol and carvacrol. Consistent with other findings (Majdi et al. 2017), the biosynthesis of these products was shown to be up regulated by abiotic elicitors.

The rosmarinic acid is an important phenolic compound which is synthesized by phenylpropanoid pathway. Rosmarinic acid synthase catalyzes the transesterification reaction of 4-coumaroyl-CoA with 4-hydroxyphenyllactic acid to rosmarinic acid (Petersen et al. 2009). The rosmaric acid production was induced consistent with the enhancement in the contents of MDA, total phenolics, lignin, flavanoids, and PAL activities that is possibly because of the similar biosynthetic steps. In general, the MNP were found more effective than the SMFs in inducing the rosmarinic acid biosynthesis.

Apigenin, naringin, quercetin, and rutin belong to the six major subclasses of flavonoids (Panche et al. 2016). The first step in the biosynthesis of flavonoids is catalyzed by chalcone synthase which led to the production of chalcones by the condensation of three molecules of malonyl-CoA and a single molecule of 4-coumaryl-CoA (Panche et al. 2016). The chalcone isomerase then closes the pyrone ring of chalcone and makes naringenin. A flavanone synthase enzyme oxidizes naringenin to apigenin. Quercetin is derived from naringenin through three consecutive steps catalyzed by naringenin 3-dioxygenase, flavonoid 3’-hydroxylase, and flavonol synthase. Rutin (quercetin-3-rhamnosyl glucoside) is synthesized from quercetin as a glycone, via the addition of a rhamnoside (glucose-rhamnose) by flavonoid-3-o-

glucosyltransferase (Yang et al. 2008). The different contents of these phytochemicals under different treatments could possibly because of their competition in synthesis from the common intermediate molecules. The 4- coumaroyl-CoA, chalcone, and naringinin are used as primary intermediate molecules in the biosynthetic pathways of these compounds. Also, the type and intensity of treatments can affect on the activity and expression of genes of the specific enzymes in each compound biosynthesis, which ultimately leads to different elicitation responses.

In general, the contents of these phytochemicals were significantly different from one treatment to the other due to possible reasons including: (1) intracellular accumulation such as quercetin upon SMF1 treatment, (2) secretion into culture media such as rutin upon SMF2 + MNP treatment, (3) more biosynthetic yield such as rosmaric acid, (4) oxidative degradation, and (5) sensitivity to iron supplementation. Accordingly, further research is in high demand to scrutinize the physiological responses. All these products may protect plant cells against treatments with the SMFs and MNPs. A number of different biotic and abiotic stresses are capable of inducing the metabolism and accumulation of phytochemical compounds in plants (Dong et al. 2011; Peñuelas and Llusia 1997). These observations might be attributed to the effect of SMFs and MNPs on the structures and functions of cellular membranes. In addition, the cell membrane permeability is indirectly increased through disruption of the membrane integrity by the oxidative stress induction. It consequently disturbs normal transport processes across cell and leads to leakage of the phytochemicals into the culture media (Table 2). The higher lipid peroxidation of the treated cells is consistent with these observations (Fig. 1A). Similar observations were also reported for *Viciafaba* cells treated with 10–100 mT magnetic fields (Dhawi et al. 2009).

5. Conclusion

The elicitation effects of the MNPs on phenolic compounds and antioxidant activities in the suspension-cultured *D. kotschyi* cells were higher than that of the SMFs. The physiological responses of the cells exposed to the SMFs depend significantly on the magnetic field intensity and treatment duration. The cells experienced higher stress upon simultaneous treatment by the SMF and MNP. These physical and chemical abiotic elicitors activated the Shikimate pathway and enhanced phenolic and lignin contents. The phytochemical products (rosmarinic acid, naringin, thymol, carvacrol, apigenin, rutin, and quercetin) increased in the treated *D. kotschyi* cells and secreted to the culture media. These elicitors can be applied as a useful strategy in improving the synthesis of phytochemical products under controlled conditions.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

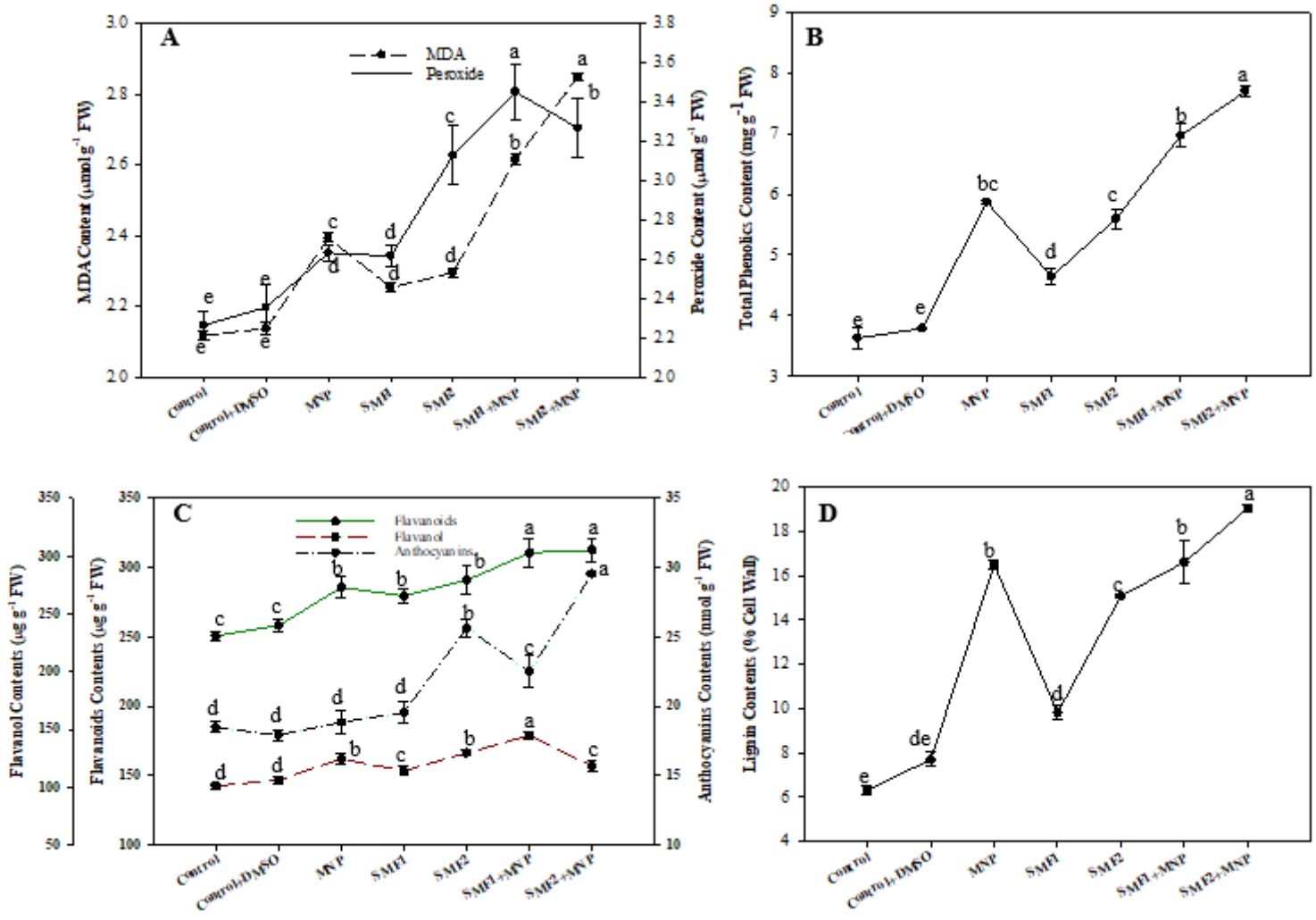

Figure 1

Effects of static magnetic field at 30 mT, 100 ppm MNP, and their combination on the contents of peroxides and MDA (A), total phenolics (B), flavonoids, flavonols, and anthocyanins (C), and lignin (D) in the suspension cultured D. kotschyi cells. MNP: Fe3O4 MNP; SMF1: Static magnetic field for 3 days, 3 h per day; SMF2: Static magnetic field for 4 days, 5 h per day; SMF1 + MNP: Static magnetic field for 3 days, 3 h per day with Fe3O4 MNP; and SMF2 + MNP: Static magnetic field for 4 days, 5 h per day with Fe3O4 MNP. Vertical bars indicate mean values ± SE of three replicates. The mean values followed by the same letter are not significantly different at $P \leq 0.05$ according to the Duncan's test.

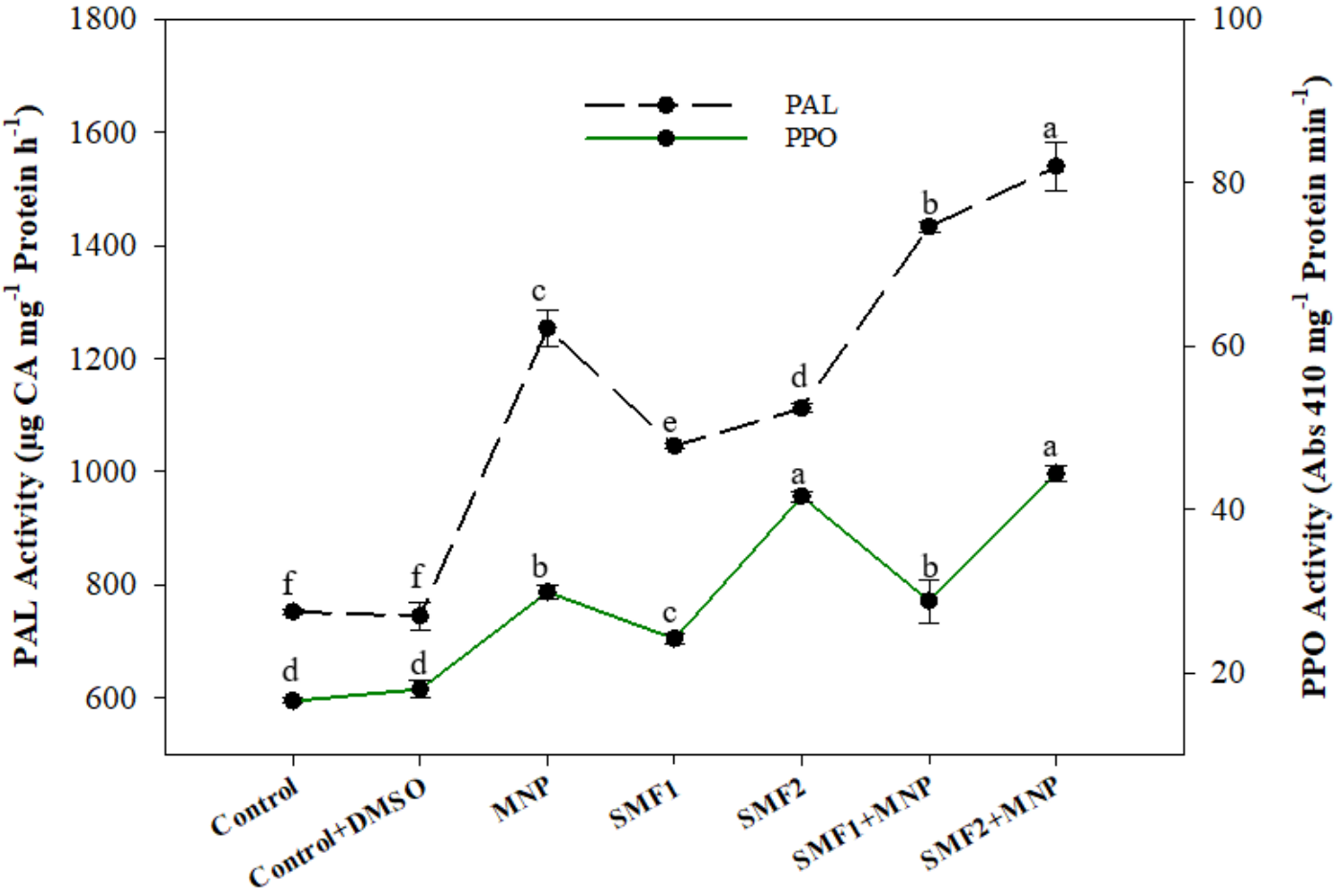

Figure 2

Effects of static magnetic field at 30 mT, 100 ppm MNP, and their combination on the activities of PAL and PPO in the suspension cultured D. kotschyi cells. MNP: Fe3O4 magnetic nanoparticles, SMF1: Static magnetic field for 3 days, 3 h a day, SMF2: Static magnetic field for 4 days, 5 h a day, MNP+SMF1: Static magnetic field for 3 days, 3 h a day with Fe3O4 MNP, MNP+SMF2: Static magnetic field for 4 days, 5 h a day with Fe3O4 MNP. Vertical bars indicate means ± SE of three replicates. Different lowercase letters show a significant difference at $P \leq 0.05$ according to Duncan's test.