

The effects of nutrient and macronutrient stress on certain secondary metabolite accumulations and redox regulation in callus cultures of *Bellis perennis* L.

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Abstract: The main purpose of this study was to investigate the effects of nutrient and macronutrient stress on complex variations in the phenolic profile and antioxidant status of *Bellis perennis* L. callus cultures. To obtain stress conditions, callus cultures were grown under insufficient nutrient conditions (different strengths of MS medium) or under conditions of limiting calcium (-Ca), magnesium (-Mg), or both (-Ca & -Mg). Phenolic content of callus cultures obtained from different stress treatments was detected quantitatively by LC-ESI-MS/MS analysis. The major compound was chlorogenic acid in all treatments. The accumulation of hesperidin, rutin hydrate, and chlorogenic acid was significantly induced by MS/2 while the highest quantity of caffeic acid was found in the MS/4 treatment. Nutrient and macronutrient stress resulted in a considerable increase in the activities of superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6), as well as in the content of proline, total phenolics, and flavonoids. The maximum increase of all antioxidants was obtained when calli were cultured on MS/2 medium. Our results revealed that different stress factors resulted in a complex variations in the phenolic profile with the induced antioxidant system, which could be associated with increased stress protection.

Key words: Antioxidant system, macronutrient stress, nutrient stress, phenolics

1. Introduction

Plant secondary metabolites are unique resources for pharmaceuticals, food additives, and fine chemicals. They also provide original materials used in other areas. Besides direct extraction from plants and chemical synthesis to provide those compounds or derivatives with similar uses, plant cell culture has been developed as a promising alternative for producing metabolites that are difficult to obtain by chemical synthesis or plant extraction. However, in spite of four decades of efforts, production of plant secondary metabolites by plant cell culture technology is still facing many biological and biotechnological limitations. One of the major obstacles is the low yield of plant secondary metabolites in plant cell cultures. Since the major roles of plant secondary metabolites are to protect plants from attack by insects, herbivores, and pathogens, or to survive other biotic and abiotic stresses, some strategies for culture production of the metabolites based on this principle have been developed to improve the yield of such plant secondary metabolites. These include treatment with various elicitors, signal compounds, and abiotic stresses (Ravishankar and Venkataraman, 1990; Zhao et al., 2000; Ashraf and Orooj, 2006; Cingoz et al., 2014).

Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have metal chelation potential (Rice-Evans et al., 1995). Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolic compounds are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects (Michalak, 2006). Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease.

Bellis perennis L., a medicinal plant in the family Asteraceae, have been recognized to have medicinal

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properties and beneficial impacts on health. It contains many nutritional and medicinal substances such as triterpenoid saponins (Pehlivan Karakas et al., 2014), essential oils, and phenolic compounds (Pehlivan Karakas and Ucar Turker, 2013). Aerial parts are widely used for treating rheumatism, common cold, cough, and wounds in traditional medicine (Siatka and Kasparova, 2010; Pehlivan Karakas et al., 2012; Pehlivan Karakas and Ucar Turker, 2013). In addition to healing effects, preliminary studies have revealed that it also possesses different pharmacological properties such as a biphasic effect on learning performance (Pehlivan Karakas et al., 2011) and antitumor (Pehlivan Karakas et al., 2014), antimicrobial (Kavalcioglu et al., 2010), and cytotoxic activity (Pehlivan Karakas et al., 2015). Many of these biological functions of *B. perennis* may originate from antioxidant properties based on its phenolic quantity (Yang et al., 2001).

Although *B. perennis* is a valuable medicinal plant, to the best of our knowledge, there are no reports related to its phenolic content and antioxidant activity under abiotic stress conditions. Therefore, the aim of this study was to evaluate phenolic accumulation in *B. perennis* using different MS medium (Murashige and Skoog, 1962) strengths [full (MS), half (MS/2), and quarter (MS/4) strength] and macronutrient-deprived cultures [elimination of calcium (-Ca), magnesium (-Mg), or both (-Ca & -Mg)] as well as examining changes in enzymatic (CAT, SOD) and nonenzymatic (total phenolics, total flavonoids, proline) antioxidants.

2. Materials and methods

2.1. Plant material and culture conditions

Plant materials of *Bellis perennis* L. were collected from the campus of Abant İzzet Baysal University, Bolu, Turkey, in May 2013. Fresh field-grown plant parts (pedicels) were surface-sterilized by 0.1% HgCl₂ (mercuric chloride) for 10 min and 70% EtOH (ethanol) for 1–2 min (Verma et al., 2012). Callus induced from 5–6 mm length of pedicel segments were cultured on MS medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, 0.5 mg/L thidiazuron (TDZ), and 0.5 mg/L indole-3-acetic acid (IAA) (Pehlivan Karakas and Ucar Turker, 2013). All cultures were grown under a 16-h light : 8-h dark photoperiod at 23 ± 2 °C. After 30 days of culture, calli were transferred to petri plates containing quarter-strength (MS/4) and half-strength (MS/2) hormone-free MS medium for creating nutrient stress and calcium-, magnesium-, or both calcium and magnesium-eliminated hormone-free MS medium (-Ca, -Mg, and -Ca & -Mg, respectively) for creating macronutrient stress.

2.2. Extract preparation for phenolics

Samples of 100 mg of freeze-dried callus were incubated with 2 mL of 80% methanol (MeOH) for 16 h in the dark

at room temperature. Samples were then centrifuged at 10,000 rpm for 12 min. The supernatant was filtrated and transferred to a new centrifuge tube (Pehlivan Karakas and Ucar Turker, 2013). The extract solutions were kept at -80 °C until analyses.

2.3. Assay of nonenzymatic antioxidants

2.3.1. Total phenolic assay

The total phenolic content (TPC) was determined according to a modified version of Slinkard and Singleton's method (1977). First, 20 mL of extract was placed in a reaction test tube containing 1.58 mL of water and 100 mL of Folin-Ciocalteu reagent. The test tube was allowed to stand for 5 min, and then 300 mL of 20% Na₂CO₃ was added to the tube. After 20 min at 40 °C, absorbance was measured at 750 nm. The TPC of methanolic extracts from calli was expressed as mg gallic acid equivalents (GAE)/g, dried weight (dw).

2.3.2. Total flavonoid assay

The total flavonoid content (TFC) (mg catechol (CE)/g, dw) was determined by aluminum chloride colorimetric assay (Chang et al., 2002). First, 500 µL of extract solution or standard solution of catechol (CE) was added to a test tube containing 2 mL of distilled water. Next, 150 µL of 5% sodium nitrate (NaNO₃) was added to the test tube. After 5 min, 150 µL of 10% AlCl₃ and 1000 µL of 1 M sodium hydroxide (NaOH) were added to the mixture. The resulting mixture was kept at room temperature for 30 min and then absorbance of the mixture was measured at 510 nm against standard catechol.

2.3.3. Proline analysis

Proline contents of callus extracts obtained from different stress treatments were determined spectrophotometrically and expressed as µmol/g dw against standard proline at 520 nm (Bates et al., 1973). For proline estimation callus tissues (0.5 g) were extracted with 5 mL of 3% sulfosalicylic acid and centrifuged at 5000 rpm for 20 min. Two milliliters of the sample of the supernatant was added to a test tube containing 2 mL of ninhydrin and 2 mL of glacial acetic acid. After 1 h at 100 °C, the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL of toluene and mixed by vortex for 20 s. The chromophore that contained toluene was transferred into a new test tube and warmed to room temperature. Proline content was calculated spectrophotometrically as mmol/g dw against standard proline at 520 nm.

2.4. Assay of antioxidant enzymes

Callus material was homogenized as previously described (Cingoz et al., 2014) and tissue extracts were kept at -80 °C for determination of catalase (CAT; EC 1.11.1.6) (Lartillot et al., 1988) and superoxide dismutase (SOD; EC 1.15.1.1) activities (Sun et al., 1988). The protein content was determined according to Lowry et al. (1951).

2.5. LC-ESI-MS/MS analysis

The crude MeOH extracts of callus obtained from five different nutrient stress treatments and a control group were used for LC-ESI-MS/MS analysis. The amount of 20 commercial phenolic compounds (apigenin, caffeic acid, *p*-coumaric acid, gallic acid, genistein, kaempferol, luteolin, myricetin, procyanidin-*C1*, quercetin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, chlorogenic acid, hesperidin, naringenin, rosmarinic acid, and isorhamnetin) was measured using the LC-ESI-MS/MS method. The analysis was performed by the METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey, with the Agilent 6460 Triple Quadrupole System (ESI + Agilent Jet Stream) coupled with Agilent 1200 Series HPLC. The Agilent 1200 HPLC series liquid chromatograph system, consisting of a vacuum degasser, binary pump (Agilent BinPump-SL, G1312B9), autosampler (Agilent h-ALS-SL+, G1367D), thermostated column compartment (Agilent 1200 series Ther. Col. Comp. SL G1316B), and microdegasser (Agilent 1200 Microdegasser G1379B), was used for separation of all analyses. The column (Zorbax SB-C18, 2.1 mm × 50 mm × 1.8 µm) was kept at 35 °C. The binary mobile phase consisted of 0.05% formic acid + 5 mM ammonium formate (solvent A) and methanol (solvent B) was delivered at a flow rate of 0.3 mL/min for 13 min (run time). An Agilent series 6460 triple-quadrupole mass spectrometer was used with an electrospray ionization source (ESI + Agilent Jet Stream). The injection volume for all samples was 5 µL. Gradient elution was performed. The eluate was forwarded, without flow splitting, into an ESI ion source with the following settings: nebulizer gas temperature, 350 °C; flow, 10 mL min⁻¹; nebulizer gas pressure, 45 psi; sheath gas temperature, 350 °C; flow, 10 mL/min; capillary voltage, 4000 V (+, -); nozzle voltage, 500 V (+, -). Mass Hunter optimizer software (Agilent G3793AA) was used in determination and quantification. All compounds were quantified in dynamic MRM mode (multiple reaction monitoring mode).

The MeOH extracts of calli obtained from different stress treatments used for LC-MS/MS quantification were dissolved in starting methanol (MS Grade) (solvent B) to a concentration of 10 µg/mL. All used standards were dissolved in methanol to prepare stock solutions of 1 µg/mL. The mix of stock solutions was prepared with the concentration of each compound being 25 µg/mL. The mix was subsequently serially diluted, giving working standard solutions with concentrations ranging from 10.0 to 0.01 µg/mL that were used for the construction of the calibration curves. All standard solutions and samples were kept at -20 °C throughout the lab and bench work. Concentrations of standard compounds in extracts were

determined from the peak areas by using the equation for linear regression obtained from the calibration curves (R^2 : 0.99).

2.6. Statistical analysis

Data were statistically analyzed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test at $P \leq 0.05$. Three replicates per treatment were used, each replicate involving three measurements.

3. Results and discussion

Application of biotic and abiotic elicitors in plant cultures has been considered as an alternative strategy for the promotion of secondary metabolite production (Ramakrishna and Ravishankar, 2011). In the current study, pedicel explants were cultured on MS medium containing 0.5 mg/L IAA and 0.5 mg/L TDZ for initial callus induction. Following 30 days of culture, calli were transferred to MS/2, MS/4, -Ca, -Mg, and -Ca & -Mg media for 15 days. For the control group, callus cultures were subcultured on full-strength MS medium for 15 days. Therefore, we investigated the effect of different strengths of MS medium (MS/2, MS/4) and macronutrient deficiency (-Ca, -Mg, or -Ca & -Mg) on callus cultures of *B. perennis*.

3.1. Analysis of phenolic compounds

Six different callus materials (MS/2, MS/4, -Ca, -Mg, -Ca & -Mg, and MS-control) were extracted with MeOH solvent. The amount of 20 commercial phenolic compounds in callus extracts obtained from different nutrient stress conditions was expressed as µg/g dw (Table). Among all treatments, chlorogenic acid, rutin hydrate, caffeic acid, ferulic acid, rosmarinic acid, and *p*-coumaric acid were the most widespread compounds. The highest amounts of rutin hydrate (19.793 µg/g dw), hesperidin (11.669 µg/g dw), caffeic acid (3.6780 µg/g dw), ferulic acid (0.1508 µg/g dw), rosmarinic acid (0.1283 µg/g dw), *p*-coumaric acid (0.0708 µg/g dw), and luteolin (0.0148 µg/g dw) were detected in the methanol extract of the MS/2 treatment (Table). The total phenolic content ranged from 206 µg/g dw for MS-control (nontreated calli) to 564.145 µg/g dw for MS/2 treatment. Gallic acid, myricetin, procyanidin-*C1*, vanillic acid, and ellagic acid were not detected in any of the treated callus tissues (data not shown). MS/2 treatment induced an 11-fold increase in accumulation of hesperidin (11.669 ± 0.804 µg/g), whereas it was not detected in the control group. Among all treatments, the highest chlorogenic acid level (528.64 µg/g dw) was observed when calli were cultured on MS/2 (Figure 1).

Earlier studies reported that *B. perennis* mainly contains quercetin, apigenin, kaempferol, and isorhamnetin (Nazaruk and Gudej, 2001; Pehlivan Karakas and Ucar Turker, 2013). Previous reports have also shown that the methanol extract of in vitro-grown leaves contained

Table. The contents of phenolic compounds obtained from calli of *B. perennis* cultures from different media stress treatments (MS/2, MS/4, -Ca, -Mg, -Ca & -Mg) and MS-control. Values are means \pm SD of three measurements. Nd: not detected, RT: retention time.

Phenolic compounds		RT	Amount of phenolics of different nutrient stress treatments ($\mu\text{g/g}$ dry weight)					
		(min)	MS/2	MS/4	-Ca	-Mg	-Ca & -Mg	MS-Control
1	Apigenin	6.998	Nd	0.0121 ± 0.0014	Nd	Nd	0.1775 ± 0.0185	0.0112 ± 0.0002
2	Caffeic acid	3.150	3.6780 ± 0.0251	4.3254 ± 0.0198	2.4833 ± 0.0021	1.9719 ± 0.0098	1.7804 ± 0.0007	1.6351 ± 0.0082
3	<i>p</i> -Coumaric acid	3.959	0.0708 ± 0.0006	0.0558 ± 0.0002	0.0383 ± 0.0001	0.0493 ± 0.0001	0.1596 ± 0.0006	0.0274 ± 0.0002
4	Gallic acid	0.943	Nd	Nd	Nd	Nd	Nd	Nd
5	Genistein	6.493	Nd	Nd	Nd	Nd	0.1540 ± 0.0171	Nd
6	Kaempferol	6.864	Nd	Nd	Nd	Nd	0.070 ± 0.003	Nd
7	Luteolin	6.489	0.0148 ± 0.0003	0.0229 ± 0.0004	0.0170 ± 0.0009	Nd	0.2213 ± 0.0110	0.0205 ± 0.0004
8	Myricetin	5.347	Nd	Nd	Nd	Nd	Nd	Nd
9	Procyanidin-C1	3.181	Nd	Nd	Nd	Nd	Nd	Nd
10	Quercetin	6.135	Nd	Nd	Nd	Nd	0.0513 ± 0.0051	Nd
11	Rutin hydrate	4.985	19.793 ± 0.6623	10.551 ± 0.7107	7.2462 ± 0.1171	5.3571 ± 0.0221	3.7034 ± 0.1157	4.2488 ± 0.4760
12	Vanillic acid	3.120	Nd	Nd	Nd	Nd	Nd	Nd
13	Ferulic acid	4.271	0.1508 ± 0.0007	0.1463 ± 0.0003	0.2073 ± 0.0006	0.1681 ± 0.0017	0.3025 ± 0.0066	0.1670 ± 0.0038
14	Salicylic acid	3.933	Nd	Nd	Nd	Nd	0.1894 ± 0.0126	0.0443 ± 0.0041
15	Sinapic acid	4.377	Nd	Nd	Nd	Nd	0.1207 ± 0.0010	Nd
16	Chlorogenic acid	2.807	528.64 ± 4.1489	515.30 ± 1.0374	232.61 ± 0.4425	238.27 ± 3.8597	201.95 ± 1.4036	200.07 ± 0.6049
17	Hesperidin	5.169	11.669 ± 0.8041	Nd	Nd	Nd	0.1599 ± 0.0086	Nd
18	Naringenin	6.235	Nd	Nd	Nd	Nd	0.1908 ± 0.0180	Nd
19	Rosmarinic acid	4.949	0.1283 ± 0.0016	0.0637 ± 0.0001	0.2158 ± 0.0005	0.4113 ± 0.0015	0.2800 ± 0.0136	0.2075 ± 0.0029
20	Isorhamnetin	7.003	Nd	Nd	Nd	Nd	0.2004 ± 0.0128	Nd
Total			564.145	530.477	242.818	246.228	210.167	206.432

gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid, luteolin, rutin hydrate, myricetin, kaempferol, and 3- β -D-glucopyranoside (Pehlivan Karakas and Ucar Turker, 2013). In the present study, the total phenolic content in callus tissues obtained from MS/2 stress treatment ($564.145 \mu\text{g/g dw}$) was 10-fold higher than that of in vitro-grown leaves ($65.6 \mu\text{g/g dw}$). We investigated a broad range of potential metabolites in callus cultures of *B. perennis* under abiotic stress conditions and found that chlorogenic acid, rutin hydrate, hesperidin, caffeic acid, ferulic acid, rosmarinic acid, *p*-coumaric acid, and luteolin were produced most extensively. To our knowledge, this is the first controlled study identifying as well as quantifying a complete profile of all phenolic acid metabolites in *B. perennis*. In an attempt to improve cell growth and production of secondary metabolites,

nutrient stress strategy was employed by changing the strength of the culture medium (Buitelaar and Tramper, 1992). Moreover, the salt strength of the culture medium is directly correlated with the total macro/micronutrient levels in the medium. It was reported that the synthesis of some phenolic molecules was increased when plants were grown under low nutrient conditions (Ruiz et al., 2003). In addition, Cui et al. (2010) found that MS/2 and MS/4 strengths were appropriate for phenolic and flavonoid accumulation in adventitious root cultures of *Hypericum perforatum* L. It was also reported that magnesium and calcium deficiency induced total phenolic content in different *Digitalis* species (Sahin et al., 2013). Similarly, in the present study, significant differences were found in the phenolic profiles between the callus cultures that received macronutrient/nutrient deficiency treatments and those

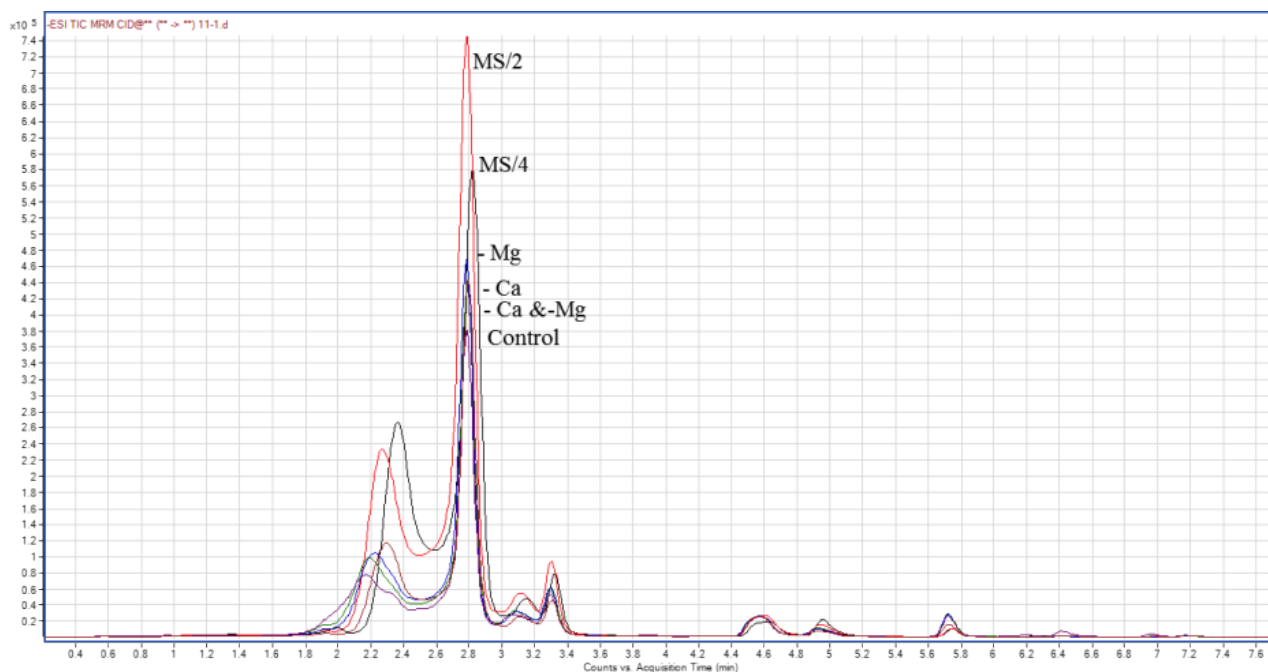


Figure 1. Chromatogram of the chlorogenic acid extracted from callus cultures treated with nutrient (MS/2, MS/4) and macronutrient (-Ca, -Mg, -Ca & -Mg) stress by LC-ESI-MS/MS analysis.

that were raised under normal growth conditions. When both Ca and Mg were eliminated from the medium, the level of total phenolic increased significantly (210.16 $\mu\text{g/g}$ dw). It was found that 206.43 $\mu\text{g/g}$ total phenolic content was produced in the nontreated group compared to calli cultured on medium lacking either Ca or Mg, which produced 242.81 $\mu\text{g/g}$ dw and 246.22 $\mu\text{g/g}$ dw, respectively.

Plant growth also depends on the availability of mineral nutrients and the macronutrients. They have numerous physiological, morphological, biochemical, and molecular mechanisms that are induced under nutrient limitation and that act to improve the acquisition and/or use of nutrients. These include changes in root architecture, the expression of transporter proteins, and numerous changes in metabolism (Vance et al., 2003). One relatively poorly understood adaptive response is that the growth rate of different plant parts is adjusted in relation to nutrient availability (Stitt and Scheible, 1998). More generally, numerous stress conditions are associated with reduced growth, a response that may have some adaptive advantage (Thomas and Sadras, 2001). In our study, the accumulation of phenolic compounds that are probably flavonols under abiotic stress conditions indicates that these compounds could protect callus cultures from the damaging effects of abiotic stress.

Considering all these results, specific metabolite accumulation related to the stress type might be attributed to the fact that expression levels of several genes encoding

key enzymes in the phenylpropanoid pathway control the flux of precursors into the phenol network and thus are critical in determining the overall composition of the phenolic pool (Winkel-Shirley, 2002).

3.2. Catalase and superoxide dismutase activity

The reactive nature of ROS makes them potentially harmful to all cellular components. Fortunately, all aerobic organisms and also plants have efficient ROS scavenging systems. However, during treatment periods of stress situations, the scavenging system may become saturated by the increased rate of radical production (May et al., 1998). Therefore, investigation of responses to different nutrient stress conditions in the same plant species may be important for understanding and improving defense strategies via the activity variations of SOD and CAT. In the present study, nutrient and macronutrient stress treatments lead to an appreciable increase in the CAT and SOD activity in callus cultures of *B. perennis* (Figures 2A and 2B). The CAT activity increased from 31.23 mmol $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein (MS-control) to 45.26 mmol $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein for MS/2 followed by 41.33 mmol $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein for MS/4, 39.28 mmol $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein for -Mg, 37.11 mmol $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein for -Ca, and 34.35 mmol $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein for -Ca & -Mg. Stress treatments increased the SOD activity from 0.56 (MS-control) up to 0.74 U/mg protein (MS/2) (Figure 2B). Among all treatments, the highest increase in the CAT and SOD activity was observed when calli were cultured

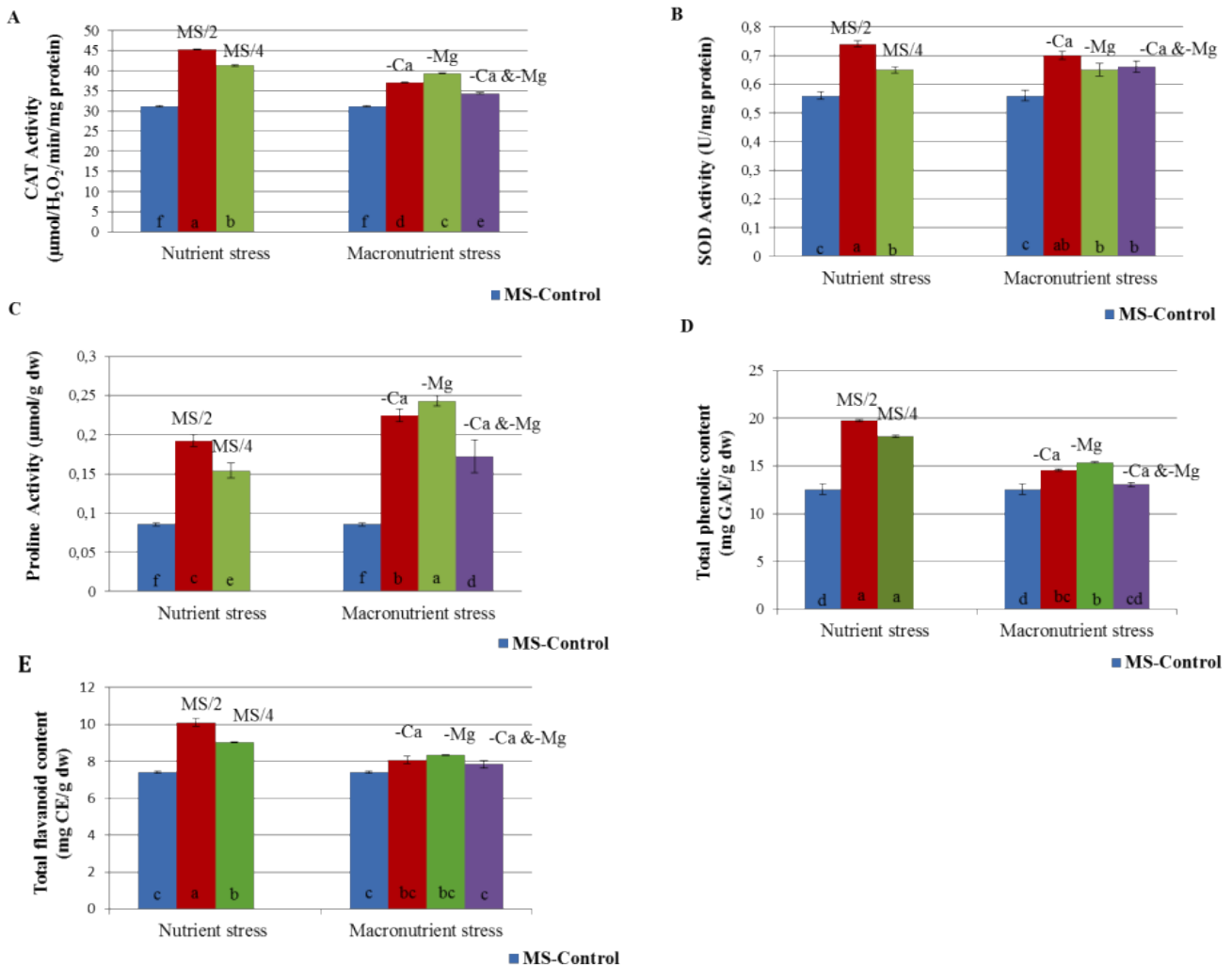


Figure 2. Results of CAT activity (A), SOD activity (B), proline content (C), total phenolic content (D), and total flavanoid content (E) of *B. perennis* callus cultures treated with nutrient (MS/2, MS/4) and macronutrient (-Ca, -Mg, -Ca & -Mg) stress. Data represented are means of three separate experiments \pm SD and different letters indicate that the mean results are statistically different.

on MS/2. It was found that the absence of Ca^{2+} , Mg^{2+} , and Zn^{2+} caused higher values of SOD and CAT in all organs of *M. pulegium* than in the control and maximum increases were obtained in the absence of Ca^{2+} , Mg^{2+} , and Zn^{2+} , respectively (Candan and Tarhan, 2003). In our study, elimination of calcium, magnesium, or both from the medium of callus cultures of *B. perennis* increased the CAT and SOD activity. The highest CAT activity was observed when callus cultures were incubated on MS medium lacking Mg while the highest SOD activity was determined when callus cultures were cultured on calcium-deprived cultures. Such an increase in both enzymes' activities with stress treatments might be related to increased stress tolerance.

3.3. Contents of total phenolics, flavonoids and proline

In the present study, contents of total phenolics, flavonoids, and proline were significantly increased when calli were elicited with nutrient and macronutrient stress. The exposure of nutrient and macronutrient stress lead to a prominent rise of the proline content ranging from 0.085 mmol/g dw (MS-control) to 0.243 mmol/g dw (-Mg) (Figure 2C). The highest increase of proline, total phenolics, and total flavonoids (10.10 ± 0.24 mg CE/g dw) in callus cultures of *B. perennis* was observed when calli were cultured on MS/2 for nutrient stress. However, among the macronutrient stress treatments, the highest amounts of proline, total phenolics, and total flavonoids were observed when calli were incubated on MS medium

lacking Mg. The total phenolic content of stress-applied calli (MS/2, MS/4, -Ca, -Mg, -Ca & -Mg, and MS-control) was 19.76, 19.01, 14.55, 15.37, 13.04, and 12.55 mg GAE/g dw, respectively.

Higher phenolic and flavonoid levels in plants under stress were accompanied by enhanced activities of phenylalanine ammonia-lyase (PAL; EC 4.1.1.5), the principal enzyme involved in the phenylpropanoid pathway (Ruiz et al., 2003). An increase of phenolics under abiotic stress conditions was correlated with the increase in enzyme activity. It is well known that different strengths of MS-treated plants induced phenolic compounds and antioxidant production (Alturki et al., 2013). In *Digitalis obscura* L. shoot-tip cultures, lower concentrations of MS major salts were found most effective for cardenolide content (Gavidia and Perez-Bermudez, 1997). In our study, it was observed that application of MS/2 to the callus cultures was more effective for producing phenolics than the other applied stress factors such as, MS/4, -Ca, -Mg, or -Ca & -Mg.

3.4. Conclusions

Our results suggested that the treatments of nutrient media stress (MS/2, MS/4) or macronutrient stress (-Ca, -Mg, or

-Ca & -Mg) may be an efficient strategy to increase some medicinally important phenolic compounds. The results of the present study indicated that when MS/2 was applied to the cultures for phenolic production, a better elicitor effect was achieved. It was also demonstrated that there was a positive correlation between antioxidant activity and phenolic accumulation. Increased phenolic accumulation and antioxidant activity could be associated with increased stress protection. In addition, each phenolic compound identified and estimated in the present work has specific health benefits. Therefore, the protocol described here could have a considerable contribution to future efforts for extensive production of some valuable and specific phenolic compounds such as chlorogenic acid, hesperidin, rutin, and caffeic acid.

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