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MARYAM ASLAM

BUSHRA SULTANA

FAROOQ ANWAR

HASSAN MUNIR

TÜBİTAK

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Foliar spray of selected plant growth regulators affected the biochemical and antioxidant attributes of spinach in a field experiment

Maryam ASLAM[1,2], Bushra SULTANA[1,*], Farooq ANWAR[3,4], Hassan MUNIR[5]

[1]Department of Chemistry, University of Agriculture Faisalabad, Faisalabad, Pakistan
[2]Department of Chemistry, Government College Women University, Faisalabad, Pakistan
[3]Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam bin Abdulaziz University, Kharj, Saudi Arabia
[4]Department of Chemistry, University of Sargodha, Sargodha, Pakistan
[5]Department of Crop Physiology, University of Agriculture Faisalabad, Faisalabad, Pakistan

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Abstract: This study explored the effects of foliar spray of selected plant growth regulators (PGRs) on the biochemical and phenolic antioxidant attributes of spinach (*Spinacia oleracea*) leaves. Following foliar spray at three growth stages (40, 50, and 60 days) with selected PGRs, namely humic acid (HA), *Moringa oleifera* leaf extract (MOLE), and 6-benzylamino purine (6-BAP), and their mixed form, the samples of spinach leaves were harvested. The analysis of foliar-treated leaves revealed the content of proline, malondialdehyde (MDA), total soluble proteins (TSPs), total chlorophyll, and total carotenoids to be 22.45–72.32 µg/g FW, 2.41–29.13 ng/g FW, 3.21–22.45 µg/g FW, 4.173–19.700 mg/g FW, and 0.500–1.260 mg/g FW, respectively. The total phenolic content (TPC), reducing power, and DPPH° scavenging activity (IC_{50} values) varied from 6.59 to 14.49 mg GAE/g DM, from 0.426 to 1.944 (10.0 mg/mL extract concentration), and from 0.506 to 1.073 µg/mL, respectively. HPLC analysis showed that foliar application of PGRs also improved the content of individual phenolic acids in the leaves as compared with the controls. Overall, it was concluded that, although the use of the PGRs acted as an elicitor with respect to each growth phase and exhibited significant differences ($P < 0.05$) among treatments, the leading effect was exhibited by the MOLE treatment, proving it to be a better enhancer of phenolic antioxidants and other biochemicals.

Key words: Spinach, humic acid, *Moringa* leaf extract, chlorophyll, antioxidant activity, HPLC, phenolic acids

1. Introduction

The production of safer and healthy foods using sustainable and environmentally friendly agricultural practices plays a vital role in determining their market value and nutritional benefits. In this context, due to continuously changing environmental attributes, the risk factors related to sustainable agriculture and product safety are being increasingly investigated (Arshad and Shafqat, 2012). In recent years, through utilizing effective strategies such as valid farm management practices and mechanized agriculture, efforts have been made to enhance the growth and productivity of various crops without compromising food quality standards (Chaudhry et al., 2006). Plant growth regulators (PGRs), due to their multiple growth and physiological functions, are gaining recognition as an emerging agricultural practice. However, some risk factors associated with the applications of PGRs have also been observed in certain agricultural practices in the form of volatilization, chemical degradation, leaching, oxidation, etc. Therefore, in order to gain increased efficiency in targeted crops, the effective use of appropriate doses of PGRs is recommended (Aslam et al., 2013).

Spinach (*Spinacia oleracea* L.), a member of the family Amaranthaceae, and, like other leafy green vegetables, is a valuable source of essential minerals and important bioactives required for proper growth and bodily functioning. In Pakistan, this leafy green vegetable is locally known as "palak". It is now widely recognized that dietary fiber and phytochemicals such as flavonoids, vitamins, carotenoids, phenolic acids, and phytosterols, which are inherent formulations of spinach and other leafy green vegetables, are associated with lowering the risk of certain diseases such as cataracts, diabetes, high blood pressure, obesity, coronary heart disorders, and certain cancers (Djousse et al., 2004; Hung et al., 2004). Such high-value compounds have been reported to exhibit multiple biological effects, including cytotoxic, antimutagenic, antioxidant, antiviral, and antifungal activities (Hounsome et al., 2008).

* Correspondence: bushrasultana2005@yahoo.com

Foliar application of essential nutrients is valuable to minimize nutrient deficiencies in plants. However, plants usually respond to foliar application to a different extent, depending upon the nature of the crop and agroclimatic and biochemical factors. A number of positive effects on the growth and productivity of some plants through foliar supplementation have been registered by earlier research (Wahba and Ezz El-Din, 2002; Ezz El-Din and Khalil, 2003).

In extension to our previous study, in which the concentration of PGRs was optimized for improved antioxidant attributes in spinach (Aslam et al., 2013), the current study aimed to evaluate whether or not the foliar application of selected PGRs contributes towards improving the biochemical and antioxidant attributes of spinach in a field experiment, so as to explore their practical and sustainable agricultural applications.

2. Materials and methods

2.1. Sampling

The seeds of spinach (*Spinacia oleracea* L. (curly leaf/wrinkled leaf variety)) were grown at the Agriculture Research Field of the University of Agriculture, Faisalabad, Pakistan. The selected PGRs, namely 10% humic acid (HA), fresh *Moringa oleifera* leaf extract (MOLE; 1:30 M:L ratio in distilled water), and 75 ppm 6-benzyl amino purine (6-BAP) were exogenously applied (through foliar spray) alone and in mixed form. All the plant extracts were prepared in double distilled water. To solubilize 6-BAP, a few drops of diluted NaOH solution were added. Foliar spray of water as a positive control (at the same concentration as the PGR solution) at all growth phases was also performed separately. All treatment effects were compared with negative control samples in which no exogenous application of PGRs was involved. The leaves were exposed to three foliar conducts and the samples were harvested and analyzed after each growth phase. Foliar application was applied after 10–15 days from 25–30 days after emergence (DAE) at the rate of 160 L/ha correspondingly 40, 50, and 60 days following germination. Samples were collected within 1 week of each of the foliar applications.

2.2. Chemicals and reagents

Gallic acid, ninhydrin, thiobarbituric acid, HA, 6-BAP, sulfosalicylic acid, glacial acetic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) reagent, and Folin–Ciocalteu reagent were purchased from Sigma Chemicals (St. Louis, MO, USA), while the other chemicals/reagents were procured from Merck (Darmstadt, Germany).

2.3. Proline contents

Proline contents were estimated using the well-reported protocol of Bates et al. (1973). Freshly chopped spinach leaf samples (0.5 g each) were extracted with sulfosalicylic acid (10 mL, 3%) and the extract was filtered (Whatman No. 1) to separate the residue. All the filtrates (2 mL each) were mixed with acidic ninhydrin (2 mL, 1.25 g ninhydrin per 30 mL of glacial acetic acid), orthophosphoric acid (20 mL, 6 M), and glacial acetic acid (2 mL) and incubated (at 100 °C for 60 min). The mixtures were cooled, incorporated in toluene (about 4 mL), and vortexed. Absorbance was recorded at λ520 nm with a spectrophotometer (IRMECO U2020, Geesthacht, Germany). Finally, proline concentrations were estimated using a standard calibration curve.

2.4. Malondialdehyde (MDA) contents

The MDA contents of the leafy samples of spinach were calculated using the protocol based on thiobarbituric acid (TBA) (Cakmak and Horst, 1991). The freshly chopped leaves (0.5 g) were homogenized and mixed with trichloroacetic acid (TCA) (w/v, 5 mL, 1.0%). The obtained homogenates were centrifuged (at 980 × *g*, for 12–15 min) and the supernatants (about 500 µL) were mixed with TBA in trichloroacetic acid (20%). Later on, all the samples were sequentially placed in hot water bath (at 100 °C for 60 min), cooled and centrifuged (at 10,000 × *g* for 10 min). The absorbance of the filtrates was measured at 532 nm and 600 nm with a spectrophotometer.

2.5. Total soluble proteins (TSPs) contents

The fresh leafy samples (0.5 g each) were homogenized in the presence of potassium phosphate buffer (10 mL of 50 mM, pH 7.8, Merck) and placed in an ice bath. Each aliquot was centrifuged (at 980 × *g* for 15–20 min at 4 °C). Protein contents were estimated for each extract (Bradford, 1976). A dye stock solution was added to the earlier centrifuged samples, followed by vortexing and incubation at room temperature for 25–30 min. The absorbance of the reaction mixture was recorded at 595 nm. Bovine serum albumin served as the standard.

2.6. Chlorophyll (Chl) and carotenoid contents

The determination of Chl and carotenoid pigments was made according to the method reported by Nagata and Yamashita (1992). Each freshly chopped spinach leaf sample was soaked in aqueous acetone solution (80%) in the dark. The extracted samples were centrifuged, and finally the absorbance was recorded at varying wavelengths (i.e. 453, 480, 645, and 663 nm) using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan).

2.7. Sample preparation for antioxidant assays

Sun-dried spinach leaves (10 g each) were chopped and extracted with aqueous methanol (80% v/v) in an orbital shaker (Gallenkamp, Loughborough, UK) for 10 h at room temperature. The extracts were subjected to filtration with Whatman No. 1 filter paper and then concentrated in a rotary evaporator under reduced pressure at 40–45 °C. The crude concentrated extracts obtained were preserved

(at –4 °C) until analyzed for further assays (Sultana et al., 2007).

2.8. Total phenolic content (TPC)

A colorimetric method previously described by Chaovanalikit and Wrolstad (2004) was used to determine the TPCs using Folin–Ciocalteu reagent (FCR). For this, FCR (0.5 mL) and double deionized water (7.5 mL) were mixed with methanolic leaf extract (50 mg). After 10 min, sodium carbonate (1.5 mL, 20% w/v) was added. The reaction mixture was subjected to heating (at 40 °C for 15–20 min) and then cooled to room temperature. The absorbance was measured at 755 nm using a spectrophotometer. The TPCs were calculated based upon the standard calibration curve (10–100 ppm, $R^2 = 0.9986$), and the data were reported as gallic acid equivalents (mg GAE /g DM).

2.9. Reducing power assay

The reducing power of the leaf extracts was determined as reported in Yen et al. (2000). The leaf extracts with varying concentrations (2.5–10.0 mg/mL) were mixed with sodium phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (1.0%, 0.5 mL), and incubated (at 50 °C) for 20 min. Later on, trichloroacetic acid (10%, 5 mL) was added and the resulting mixtures were centrifuged (for 8–10 min at 980 × *g*). The upper layers (5.0 mL) from the mixtures were separated, diluted with distilled water (5.0 mL), and then ferric chloride (0.1%, 1 mL) was added. The absorbance of the final reaction mixture was recorded at 700 nm by a spectrophotometer.

2.10. DPPH free radical scavenging assay

A procedure reported by Bozin et al. (2006) was followed to evaluate DPPH° scavenging capacity of spinach leaf extracts. Briefly, DPPH solution (90 µM, 1 mL) was added to the extracts and the volume was made up to 4.0 mL with methanol (95%). The mixtures obtained were placed at ambient temperature (for 30 min) and then absorbance was recorded at 515 nm using a spectrophotometer. The DPPH° (%) inhibition/scavenging potentials of the extracts were calculated and the data were reported as IC_{50} values.

2.11. HPLC analysis of phenolics

2.11.1. Sample preparation for HPLC analysis

Acid-catalyzed hydrolysis of the extract samples was performed to convert the glycoside form of phenolics into their respective aglycones following the procedure described in Tokuşoğlu et al. (2003) with slight modifications. Briefly, acidified methanol (25 mL) containing 1% (v/v) HCl and 0.5 ppm TBHQ was added to each extract (5 g) and the resultant mixture was stirred at 90 °C under reflux for 2 h to accomplish the hydrolysis. The hydrolyzed mixture was cooled to room temperature and centrifuged at 1500 × *g* for 10 min. The upper layer was collected and sonicated for 5 min to remove air bubbles. The final sample extract was filtered through a 0.45-µm (Millipore) filter membrane before injection into the HPLC column.

2.11.2. HPLC separation

An HPLC system (model LC-10A, Shimadzu, Kyoto, Japan) equipped with two LC-10 AS pumps, SCL-10A system control unit, Rheodyne injector, CTO-10A column oven, SPD-10A UV–Vis detector, and data acquisition class LC-10 software was used. A 20-µL volume of the filtered sample was injected into an analytical Supelco (Supelco Inc., Supelco Park, Bellefonte, PA, USA) ODS reverse phase (C18) column (250 × 4.6 mm; 5 µm particle size). A two solvent system ((A (H_2O):acetic acid = 94:6, pH 2.27), B (acetonitrile 100%)) gradient elution (0–15 min = 15% B, 15–30 min = 50% B, 30–45 min = 100% B, flow rate: 1 mL/min) was employed for chromatographic separation. Detection was noted at 280 nm. Identification of the phenolic acids was carried out by comparing their retention times with those of the authentic standards (Sigma Chemicals).

3. Results and discussion

Foliar applications of PGRs in agriculture crops are reported to be useful in controlling multiple physiological processes, including flower initiation, shoot elongation, and fruit abscission. In the present study the effects of exogenous applications of selected PGRs on the biochemical and antioxidant attributes of spinach were evaluated in field experiments.

3.1. Proline contents

The proline contents of the spinach leaves analyzed after foliar treatment with selected PGRs are shown in Table 1. The amount of proline varied between 22.45 and 72.32 µg/g FW. As a result of the PGR treatments, proline content decreased in all treatments and at all growth stages as compared with the controls. Among the treatments, MOLE showed the most pronounced effect, while somewhat comparable effects were shown by the 6-BAP and the mixture treatments. A decrease in proline contents in comparison with the controls was reported earlier by Farahat et al. (2012) during an investigation of exogenous applications of humic acid on seedlings of *Khaya senegalensis*.

Proline, as one of the conspicuous osmopromoters among various organic solutes, shields the proteinaceous enzymes from ion inhibitory effects (Heidari and Mesri, 2008), and hence provides defensive antioxidant lines (Ejaz et al., 2012). It further stabilizes the subcellular machinery and supports the appropriate performance of the metabolic machinery carbon (C) and nitrogen (N), in addition to scavenging active oxygen species (AOS) (Nawaz et al., 2010).

Table 1. Proline (µg/g FW), MDA (ng/g FW), and TSPs (µg/g FW) contents of spinach.

Treatment	Proline (µg/g FW)			MDA (ng/g FW)			TSP (µg/g FW)		
	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd s pray (60 days)	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)
Negative control	72.32 ± 0.81^{d}	64.21 ± 0.82^{d}	54.21 ± 0.86^{d}	29.13 ± 0.13^{e}	17.05 ± 0.11^{d}	10.15 ± 0.12^{cd}	3.21 ± 0.09^{d}	3.91 ± 0.11^{d}	6.08 ± 0.12^{d}
Water (positive control)	65.23 ± 0.71^{c}	56.21 ± 0.77^{c}	41.45 ± 0.75^{bc}	27.31 ± 0.15^{e}	16.87 ± 0.14^{d}	10.06 ± 0.13^{cd}	4.41 ± 0.03^{d}	6.4 ± 0.06^{d}	8.57 ± 0.13^{d}
HA	54.46 ± 0.52^{b}	43.76 ± 0.53^{b}	37.76 ± 0.58^{b}	10.92 ± 0.07^{b}	9.35 ± 0.09^{b}	7.96 ± 0.06^{bc}	8.52 ± 0.12^{c}	10.51 ± 0.09^{c}	12.68 ± 0.19^{c}
MOLE	41.09 ± 1.83^{a}	32.24 ± 1.82^{a}	22.45 ± 1.89^{a}	5.32 ± 0.21^{a}	3.76 ± 0.27^{a}	2.41 ± 0.24^{a}	17.31 ± 0.11^{a}	20.28 ± 0.07^{a}	22.45 ± 0.17^{a}
6-BAP	48.23 ± 0.87^{ab}	37.21 ± 0.87^{b}	27.59 ± 0.83^{a}	11.23 ± 0.15^{b}	9.23 ± 0.13^{b}	5.05 ± 0.14^{b}	15.42 ± 0.13^{a}	16.41 ± 0.06^{b}	18.58 ± 0.19^{b}
Mixture	46.3 ± 0.57^{a}	35.11 ± 0.53^{a}	27.02 ± 0.51^{a}	10.05 ± 0.14^{b}	9.67 ± 0.14^{b}	6.89 ± 0.18^{b}	16.71 ± 0.29^{a}	18.68 ± 0.04^{a}	20.85 ± 0.33^{a}

Values are means ± SD, samples of each plant material were analyzed individually in triplicate ($P < 0.05$). HA = Humic acid; MOLE = *Moringa oleifera* leaf extract; 6-BAP = 6-Benzyl amino purine. Lower case letters in superscripts within the column show significant differences among treatments.

PGR application is appreciated as an alternative way to reverse deleterious effects caused by different abiotic stresses (Nair et al., 2002). The decreased level of proline in plants in response to foliar application of PGRs might be linked with certain factors (Devi et al., 2012) such as enzymatic activity of proline oxidase as a result of which proline is catalytically changed into glutamine. This further assists in the biosynthesis of other proteins (Niakan et al., 2012). In contrast, elevated proline levels offer constraints towards plant growth and increase the corresponding survival cost in rapidly fluctuating environmental conditions. About a 21% decrease was reported in proline levels through PGR applications in different cultivars of beet (Masoumzadeh et al., 2012).

The decrease in proline contents of spinach leaves after the application of PGRs might be correlated with their beneficial effects, comparable with humic substances that increase the uptake of micronutrients, but reduce the entry of toxic elements in the cell. In addition, they also protect plants against salinity tolerance and other abiotic stresses (Mahmoudi et al., 2013). Cytokinin derivatives, including 6-BAP (at effective concentrations), are also reported to enhance the possible tolerance of plants towards stress (Nair et al., 2002). Since MOLE is blessed with cytokinin derivatives (zeatin), polyphenols, humic substances, and other nutritional assemblies, the remarkable reduction in proline levels by MOLE might be linked to the presence of these compounds (Basra et al., 2011). It can also be revealed that proline catabolism is triggered through the beneficial medicinal makeup of MOLE (Szabados and Savoure, 2010).

3.2. MDA contents

Lipid peroxidation represents one of the major causes of oxidative stress related disorders (Ceconi et al., 2003). The varying contents of MDA due to foliar treatment are presented in Table 1. The observed range for MDA contents in spinach leaves was 2.41–29.13 ng/g FW. Although all PGRs decreased MDA levels against the controls, MOLE treatment was found to be the most effective. Moreover, an appreciable decrease was seen in the case of both the 6-BAP and the mixture treatments. The results of the present study are in line with the findings reported by Asghari and Aghdam (2010).

Lipid peroxidation promotes the aging process by generating a chain of harmful reactive oxygen species (ROS) and secondary products comprising mainly aldehydes and ketones (liable for intensive oxidative disorders) (Ceconi et al., 2003). The high reactivity along with the corresponding higher longevity can distribute these oxidation products in and outside the cells. Consequently, various biomolecules are targeted and cell functionality is destroyed. Moreover, the level of peroxidation in cell membranes is manifested during oxidative stress (Del-Rio et al., 2005).

Exogenous application of PGRs progressively affects the accumulation of MDA as compared with the controls. PGRs play an effective role by protecting the fluidity and integrity of plant cell membranes. They properly mediate enzymatic (SOD, APX, and CAT) and nonenzymatic machinery with the result of preventing cell membrane damage by ROS (Jungklang and Songklanakarin, 2012). Additionally, applied PGRs reduce moisture loss through the transpiration rate and protect the plants from drought stress (Ouzounidou et al., 2011). Hence, lower MDA content is considered an enhancement of antioxidant potential, providing improved tolerance in oxidative stress conditions (Keramat et al., 2009).

The addition of cytokinin derivatives (6-BAP) delays the process of senescence in plant parts. This, in turn, retards

the decomposition rate of macromolecules, especially of those involved in the photosynthetic process (Stopari and Maksimovi, 2008). Similarly, humic substances offer effective tolerance in plant stress through hormone level regulation (Cimrin et al., 2010). This is reflected by notable biochemical effects at the cell level, preserving the status of higher permeability in the membrane, enhancing protein synthesis, and activating the elongation of root cells (Saruhan et al., 2011). This viewpoint can be supported by the literature, revealing that herbicides are linked to elevated MDA levels in plants by causing structural modifications of biomolecules (proteins, phospholipids, etc.), resulting in changes in the surface of lipids. Plants become less tolerant to a thermal sort of denaturation and show premature wilting because of decreased availability of sulfhydryl groups (Ekmekci and Terzioglu, 2005).

Since MOLE is recognized for its antioxidant potential, vibrant hormonal action, and valuable nutritional attributes, it delays leaf senescence, and hence lowers the MDA level, stabilizing the corresponding membrane integrity through enzymatic and nonenzymatic antioxidants. Consequently, MOLE is proved to be the most effective PGR in reducing plant exposure to certain stress (Rajanandh et al., 2012).

3.3. TSPs

The measurement of TSPs shows the plant's limit to manage itself during exposure to stress conditions. The observed variations in TSPs as influenced by the application of selected PGRs at varying growth stages are shown in Table 1. The calculated TSP range for spinach leaves was 3.21–22.45 µg/g FW. The highest TSPs were recorded for the MOLE treatment, followed by the mixture and the 6-BAP treatment. Water application offered better results than the controls, but lower ones than those obtained with the applied PGRs. El-Shraiy and Hegazi (2009) earlier reported a decrease in TSPs in pea seeds on exposure to GA_3 treatment.

A higher amount of TSPs exhibited by the MOLE treatment might be linked with high endogenous profile comprising hormones (cytokinins, HA, etc.), minerals, and vitamins, along with other bioactives. Such attributes promote enzymatic actions, which, in turn, are linked with increased production of protein in increased number of cells produced as a result of exogenous application of PGRs (Abdalla, 2013). Proteins play an active role in plant functions like signal transduction, translation, redox homeostasis, photorespiration, and photosynthesis, and the metabolism of carbon (C), nitrogen (N), sulfur (S), and energy (Yan et al., 2006).

3.4. Chl and carotenoid contents

Chl contents, as affected by selected PGRs in foliar mode at varying growth stages, are depicted in Table 2. The observed variations in total Chl contents of spinach due to foliar application of PGRs ranged from 4.173 to 19.700 mg/g FW (Table 2). Chl contents were further enhanced with the growth phases. The control samples showed lower values of Chl against all PGR treatments. Among the PGRs, the highest values were recorded for the MOLE-treated samples, followed by the mixture-, the 6-BAP-, and the HA-treated samples. Likewise, as a result of foliar application of GA_3, Lim et al. (2003) reported an increase in the amount of total Chl content in apple leaves. Xu et al. (2011) suggested that the exogenous application of alanine (at a low dose) proved to be a photosynthetic rate promoter.

Chl denotes an important pigment of the chloroplast and performs photosynthesis (Avenson et al., 2005).

Table 2. Chlorophyll (Chl) and carotenoid contents of spinach (mg/g FW).

Treatment	Total chlorophyll content			Carotenoids		
	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)
Negative control	4.173 ± 0.114^{de}	4.302 ± 0.161^{d}	4.611 ± 0.098^{d}	0.500 ± 0.013^{de}	0.524 ± 0.014^{d}	0.516 ± 0.019^{d}
Water (positive control)	4.443 ± 0.133^{d}	5.118 ± 0.176^{d}	5.937 ± 0.105^{d}	0.533 ± 0.016^{d}	0.595 ± 0.016^{d}	0.614 ± 0.021^{d}
HA	6.660 ± 0.272^{b}	7.598 ± 0.165^{c}	9.306 ± 0.314^{c}	0.799 ± 0.032^{b}	0.910 ± 0.029^{c}	0.911 ± 0.019^{c}
MOLE	9.621 ± 0.315^{a}	13.508 ± 0.154^{a}	19.700 ± 0.288^{a}	1.154 ± 0.037^{a}	1.192 ± 0.030^{a}	1.260 ± 0.018^{a}
6-BAP	6.836 ± 0.145^{b}	8.539 ± 0.192^{b}	10.652 ± 0.273^{b}	0.820 ± 0.017^{b}	1.033 ± 0.027^{b}	1.024 ± 0.023^{b}
Mixture	9.256 ± 0.294^{b}	10.269 ± 0.179^{b}	18.301 ± 0.342^{b}	0.834 ± 0.035^{b}	1.077 ± 0.035^{b}	1.043 ± 0.021^{b}

Values are means ± SD, samples of each plant material were analyzed individually in triplicate ($P < 0.05$). HA = Humic acid; MOLE = *Moringa oleifera* leaf extract; 6-BAP = 6-Benzyl amino purine. Lower case letters in superscripts within the column show significant differences among treatments.

Increased Chl contents show greater production yield in crops as photosynthesis and Chl contents are positively correlated (Wang et al., 2008). Chl also exhibits antioxidant activity when exposed to light. This could possibly be described by a transfer of energy from singlet excited Chl to oxygen forming ROS (Lanfer-Marquez et al., 2005). The higher level of Chl might also be connected with enhanced quantum efficiency and increased flavonoid and anthocyanin concentrations (Cheng et al., 2001). In the current study, the greater Chl contents from two treatments, i.e. the MOLE and mixture treatments, might be linked with an increased photosynthetic activity due to the activation of an internal hormonal status. The results observed in the present study are also in line with those reported by Abdalla (2013), who showed increased Chl contents due to foliar application of selected formulations of MOLE (2% and 3%).

The quantitative effects of selected PGR applications on the carotenoid contents of spinach leaves are presented in Table 2. The observed trends in the case of carotenoids remained the same as for Chl, showing dominating results by the MOLE treatment. Carotenoids are antenna pigments. They capture light and facilitate its provision for the reaction centers wherever the process of photosynthesis is likely to occur. The overall improvement in carotenoids shown in the MOLE-treated samples can be attributed to the inherited hormonal actions besides the minerals and vitamins, which collectively delay the process of leaf senescence.

3.5. Extraction yield

The yield of methanol extractable components (MECs) from spinach leaves due to PGR application ranged from 22.89 to 36.72 g/100 g DW (Table 3). Increased yield was observed at all growth phases and in all treatments. Within all growth stages, the MOLE treatment offered the highest value of extraction yield, followed by the 6-BAP and mixture treatment; samples treated with HA, however, showed smaller extract yield. Earlier, maximum extraction yield was noted for *Capsicum annum* after foliar application of GA_3 (Ouzounidou et al., 2010).

Although the foliar conducts increased the extract yield at each growth phase, MOLE offered a noticeably higher extraction yield. The derivatives of cytokinins (including 6-BAP) are widely used as exogenous PGRs as they elevate the endogenous cytokinin levels. Interestingly, they encourage certain multidevelopmental mechanisms occurring in the presence of light (Merillon et al., 1991).

3.6. TPCs

In the present study, the TPCs calculated by the FCR method in spinach leaf extracts were between 6.59 and 14.49 mg GAE/g DM (Table 3). During evaluation, lower TPCs were seen in the control sample at all growth phases as compared with those subjected to PGR application. More pronounced effects of PGRs on TPCs were noted at the peak vegetative phase rather than at the earlier ones. The MOLE treatment showed higher TPCs as compared with the other treatments after each spray. The 6-BAP and mixture treatments were next, also with high TPCs; however, the HA-treated samples contained lower TPCs.

TPC variations within plants are reported to be dependent on plant growth factor (Dumas et al., 2003). Large discrepancies have been stated at different maturity stages in leafy vegetables (Ellnain-Wojtaszek et al., 2001). Manach et al. (2004) observed that during plant maturity, the level of phenolic acid was decreased, but an increase in flavonoids was noted. Compositional variations in phenolics were also seen during the maturation of tomato fruits (Raffo et al., 2002). The greater TPCs obtained from the MOLE-treated samples with respect to each maturity stage can be attributed to bioactives, especially kaempferol, quercetin, and flavonoids of *Moringa*, which

Table 3. Percentage extract yield (g/100 g DM) and total phenolic contents (mg GAE/g DM) of spinach extracts.

Treatment	Percentage extract yield (g/100 g DM)			Total phenolic contents (mg GAE/g DM)		
	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)
Negative control	22.89 ± 0.37^{cd}	25.91 ± 0.35^{cd}	26.44 ± 0.39^{cd}	6.59 ± 0.21^{cd}	7.48 ± 0.09^{c}	9.59 ± 0.17^{cd}
Water (positive control)	23.00 ± 0.46^{cd}	26.83 ± 0.26^{c}	29.33 ± 0.51^{c}	6.87 ± 0.22c	8.75 ± 0.09^{bc}	9.97 ± 0.06^{c}
HA	24.78 ± 0.35^{c}	27.86 ± 0.23^{c}	31.37 ± 0.39^{b}	6.97 ± 0.25^{c}	9.24 ± 0.08^{b}	12.88 ± 0.13^{b}
MOLE	32.56 ± 0.48^{a}	33.35 ± 0.28^{a}	36.72 ± 0.53^{a}	9.84 ± 0.23^{a}	11.88 ± 0.14^{a}	14.49 ± 0.16^{a}
6-BAP	31.11 ± 0.51^{a}	32.66 ± 0.29^{a}	32.34 ± 0.38^{b}	9.24 ± 0.28^{ab}	10.02 ± 0.15^{a}	13.44 ± 0.17^{ab}
Mixture	29.11 ± 0.44^{b}	30.53 ± 0.25^{ab}	31.89 ± 0.39^{b}	8.99 ± 0.27^{b}	9.79 ± 0.12^{a}	13.62 ± 0.14^{ab}

Values are means ± SD, samples of each plant material were analyzed individually in triplicate ($P < 0.05$). HA = Humic acid; MOLE = *Moringa oleifera* leaf extract; 6-BAP = 6-Benzyl amino purine. Lower case letters in superscripts within the column show significant differences among treatments.

might have contributed to the enhanced antioxidant potential of MOLE. Therefore, the significance of MOLE as an effective PGR is further highlighted (Chumark et al., 2008). Similarly, cytokinin derivatives (6-BAP) are known to perceive signaling actions and generate higher phenolics and other plant secondary metabolites in variable environmental conditions. The higher TPCs recorded for HA applied samples as compared with the controls might be because of the HA's active role in enhanced plant growth due to increased respiration and improved nutrient uptake. It can be assumed that the nutrients supplied in the form of PGRs have exerted stimulatory effects on the production of TPCs (Clapp et al., 2010).

3.7. Reducing power assay

The tested spinach leaf extracts (10.0 mg/mL extract concentration) showed reducing power in the range of 0.426 to 1.944 (Table 4). Overall, the reducing power of the control sample was lower than that of the foliar applied samples. The most effective treatment was observed to be with MOLE, followed by the 6-BAP, mixture, and HA treatments. The samples harvested at the peak vegetative stage showed overall higher reducing abilities.

Overall, a higher reducing potential recorded for the MOLE-treated spinach samples can be linked to the higher contents of phenolic substances that might have been responsible for the greater reducing power.

3.8. DPPH free radical scavenging assay

Like reducing power, the scavenging activity of the PGR-treated samples was increased in a concentration dependent manner. The IC_{50} values were observed in the range of 0.506 to 1.073 µg/mL. The lowest values were recorded for the MOLE-treated samples, followed by the 6-BAP-, mixture-, and HA-treated samples at all growth phases. PGR applications effectively enhanced the free radical scavenging potential of spinach. Similar findings were also reported by El-Shabasi et al. (2005) during foliar applications of amino acids on leafy vegetables. Although all PGR-treated samples had lower IC_{50} values than the controls, the MOLE-treated samples showed the least value, and hence the highest antioxidant activity.

3.9. HPLC characterization of phenolic acids

Phenolic acids and their derivatives have attracted a great deal of scientific interest in the medical, biological, and agricultural fields. Recent studies were focused on their contribution as natural compounds with antioxidant potential (Saxena et al., 2012). The analyzed spinach samples contained considerable amounts of phenolic acids (Table 5). The detected amounts of phenolic acids in relation to foliar applications of PGRs varied in the following ranges: 28.31–191.15 ppm of gallic acid, 19.57–114.93 ppm of vanillic acid, 271.12–1180.19 ppm of ferulic acid, 25.93–445.55 ppm of chlorogenic acid, 29.34–108.03 ppm of syringic acid, 45.23–129.19 ppm of caffeic acid, 31.99–91.82 ppm of m-coumaric acid, and 23.67–160.86 ppm of p-coumaric acid. Overall, the highest amount of phenolic acids was detected for the MOLE-treated samples.

In conclusion, the present study evaluated the effects of foliar application of selected PGRs on the biochemical and antioxidant attributes of spinach leaves harvested at different vegetative phases in field experiments. The results revealed that the foliar applications of selected PGRs acted as elicitors in each growth phase and exhibited variable

Table 4. Reducing power (absorbance at λ_{max} = 700) and DPPH radical scavenging action (IC_{50} concentration, µg/mL) of spinach extracts.

Treatment	Reducing power (absorbance at λ_{max} = 700)			DPPH radical scavenging action (IC_{50} concentration, µg/mL)		
	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)	After 1st spray (40 days)	After 2nd spray (50 days)	After 3rd spray (60 days)
Negative control	0.426 ± 0.009^{c}	0.621 ± 0.011^{d}	0.727 ± 0.023^{cd}	1.073 ± 0.017^{d}	0.927 ± 0.014^{cd}	0.726 ± 0.019^{c}
Water (positive control)	0.442 ± 0.023^{c}	0.663 ± 0.027^{d}	0.891 ± 0.029^{c}	1.022 ± 0.015^{cd}	0.874 ± 0.013^{c}	0.706 ± 0.008^{c}
HA	0.935 ± 0.021^{ab}	1.064 ± 0.026^{bc}	1.709 ± 0.014^{a}	0.929 ± 0.016^{c}	0.723 ± 0.012^{b}	0.669 ± 0.007^{bc}
MOLE	0.975 ± 0.023^{a}	1.643 ± 0.028^{a}	1.944 ± 0.027^{a}	0.804 ± 0.016^{a}	0.621 ± 0.011^{a}	0.506 ± 0.006^{a}
6-BAP	0.953 ± 0.027^{a}	1.237 ± 0.031^{b}	1.925 ± 0.016^{a}	0.816 ± 0.017^{a}	0.674 ± 0.013^{a}	0.638 ± 0.006^{b}
Mixture	0.911±0.019^{ab}	0.979 ± 0.023^{c}	1.781 ± 0.08^{a}	0.834 ± 0.017^{ab}	0.744 ± 0.012^{b}	0.658 ± 0.007^{b}

Values are means ± SD, samples of each plant material were analyzed individually in triplicate ($P < 0.05$). HA = Humic acid; MOLE = *Moringa oleifera* leaf extract; 6-BAP = 6-Benzyl amino purine. Lower case letters in superscripts within the column show significant differences among treatments.

Table 5. Phenolic acid quantification of spinach leaves by HPLC (ppm).

Treatment	Gallic acid	Chlorogenic acid	Vanillic acid	Caffeic acid	Syringic acid	m-Coumaric acid	p-Coumaric acid	Ferulic acid	Total phenolic acid
Negative control	104.40 ± 0.03	182.71 ± 0.03	19.57 ± 0.03	17.42 ± 0.03	29.34 ± 0.03	55.65 ± 0.03	137.03 ± 0.03	308.34 ± 0.03	854.46 ± 0.03
Water (positive control)	124.23 ± 0.03	89.82 ± 0.03	46.19 ± 0.03	22.65 ± 0.03	43.01 ± 0.03	45.23 ± 0.03	120.98 ± 0.03	467.87 ± 0.03	959.98 ± 0.03
HA	184.02 ± 0.03	186.24 ± 0.03	114.93 ± 0.03	26.32 ± 0.03	108.03 ± 0.03	43.12 ± 0.03	138.02 ± 0.03	318.97 ± 0.03	1119.65 ± 0.03
MOLE	28.31 ± 0.03	25.93 ± 0.03	69.16 ± 0.03	30.63 ± 0.03	78.21 ± 0.03	31.99 ± 0.03	140.08 ± 0.03	1180.19 ± 0.03	1584.50 ± 0.03
6-BAP	191.15 ± 0.03	200.38 ± 0.03	107.91 ± 0.03	45.23 ± 0.03	60.63 ± 0.03	91.82 ± 0.03	23.67 ± 0.03	414.66 ± 0.03	1135.45 ± 0.03
Mixture	176.94 ± 0.03	445.55 ± 0.03	70.47 ± 0.03	18.15 ± 0.03	44.01 ± 0.03	48.69 ± 0.03	160.86 ± 0.03	271.12 ± 0.03	1235.79 ± 0.03

Values are means ± SD, samples of each plant material were analyzed individually in triplicate ($P < 0.05$). HA = Humic acid; MOLE = *Moringa oleifera* leaf extract; 6-BAP = 6-Benzyl amino purine. Lower case letters in superscripts within the column show significant differences among treatments.

effects among treatments. A considerable improvement in biochemical and antioxidant attributes of spinach was recorded due to the foliar application of the selected PGRs, and a remarkable effect was exhibited by MOLE. The present data support the potential uses of the selected PGRs, and especially of MOLE, as effective and safer alternatives to synthetic PGRs for improving the production of valuable nutrients and phenolic antioxidants in spinach and other leafy green vegetables in order to explore their functional food and nutraceutical potential for value addition.

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