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CHANGES IN PROXIMATE COMPOSITION, BIOCHEMICAL AND ANTIOXIDANT ATTRIBUTES OF BROCCOLI (*BRASSICA OLERACEA* L.) IN RELATION TO FOLIAR APPLICATION OF SELECTED PLANT GROWTH REGULATORS

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Abstract

Field experiments were conducted to investigate the effect of foliar application of selected plant growth regulators (PGRs) on the proximate composition, biochemical and antioxidants attributes of broccoli (*Brassica oleracea* L.) leaves. Different concentrations of exogenously applied PGRs such as humic acid (HA), cytokinin (CK), gibberellic acid (GA_3) and GA_3+CK exhibited variable effect on the tested parameters. The foliar spray with HA (0.6%), GA_3+CK (20+20 ppm), GA_3 (20 ppm) and CK (40 ppm) increased the contents of moisture, ash, crude fiber and crude protein in broccoli leaves. With few exceptions, the antioxidant capacity of broccoli leaves, in terms of estimation of total phenolics content (TPC), total flavonoids content (TFC), reducing power and DPPH radical scavenging activity, was also improved due to foliar spray of PGRs. The results showed that maximum contents of total chlorophyll (1.98 mg/g FW), protein (7.09 mg/g FW), and proline (0.62 µg/g FW) were exhibited by GA_3+CK (10+10 ppm), GA_3 (20 ppm) and GA_3+CK (20+20 ppm) treated samples, respectively. Meanwhile, GA_3+CK (10+10 ppm) was found to be the most effective growth promoter in lowering melondialdehyde (MDA) content (0.89 µM/g FW) of broccoli leaves. In conclusion, it can be revealed that optimized foliar spray of selected PGRs, and especially of HA and mixture of GA_3 and CK, is practically applicable towards improving biochemical and antioxidant attributes of broccoli leaves with potential nutritional benefits.

Key words: Broccoli leaves, Plant growth regulators, Foliar applications, Biochemicals, Antioxidant activity, Phenolics.

Introduction

Plant growth regulators (PGRs) are organic substances known for their function in regulating various physiological processes in plants even employed in low concentration. When plants produce these organic substances endogenously, they are termed as phytohormones or plant hormones. These plant hormones act as chemical messengers capable of regulating various physiological processes in accordance with environmental changes (Qasim *et al.,* 2009; 2010). The assessment of potential of PGRs in alteration of growth pattern and morphogenesis of plants is frequently correlated with the type and concentration of PGRs, type of species, stage of application and agro-climatic conditions of harvest etc. Among plant growth regulators, gibberellic acid (GA_3), kinetin and humic acid exhibited beneficial effect in several crops (Singh *et al.,* 2011; Shabbir *et al.,* 2015).

Humic acid formulation is an organic-mineral fertilizer containing humic acid as one of the major (60 to 70%) components. Humus contributes both micro (metal ions) as well as macronutrients (potassium, nitrate, phosphate etc.) to the soil so as to provide mineral nutrition to plants. Besides this, humic acid produces proliferation of root system and enhances the macro (P, N and S) and micro nutrient (Zn, Cu, Mn and Fe) uptake by formation of complex with these ions resulting in improved crop yield and productivity (Saruhan *et al.,* 2011).

Cytokinins (CKs), as an important class of phytohormones, are considered as abscisic acid (ABA) and auxin antagonists, and exhibit potential role in apical dominance, leaf senescence, development of chloroplast and regulation of cell division of plants (Hutchinson & Kieber, 2002). Seed priming and foliar application of CKs at optimum concentration is reported to improve the germination, growth and yield of various vegetable crops among others (Gurmani *et al.,* 2006).

Gibberellins are the group of diterpenoid acid derivatives being widely used as plant hormones regulating various developmental processes such as germination, stem elongation, breaking of dormancy in flowers, leaf senescence, fruit senescence, enzyme induction and sex expression etc. GA_3 also has effect on color development (Kim *et al.,* 2009).

Reportedly, a great deal of research work has been reported on the potential uses of plant growth regulators in different vegetable crops. Broccoli (*Brassica oleracea*), a member of *Brassicaceae* family, is an important winter season vegetable crop which resembles cauliflower. Broccoli has its origin from the Mediterranean region and is now cultivated in different parts of the world. Broccoli can grow in all the soil types; however, the encouraging conditions for well growth includes high humidity, sunlight, temp of 18-27ºC and pH about 6.0-6.5 (Chuanphongpanich *et al.,* 2006). Broccoli is a good source of valuable phytochemicals including phenolic antioxidants, vitamin C, vitamin A, vitamin B2, vitamin K1 etc., and other essential dietary minerals (Ca, Na, Mg, K, Zn, Fe, etc.). Broccoli also contains a special group of secondary metabolites called glucosinolates which have chemoprotective effect against cancer (Moreno *et al.,* 2006).

Most of the studies on the cruciferous vegetables have been carried out to evaluate the antioxidant activity of edible parts but inedible part like leaves are often discarded as an agro-waste. Moreover, little information is available related to studying the effect of PGRs on nutraceutical value of cruciferous vegetables. Hence, the present study is aimed to evaluate the effect of foliar application of selected PGRs on the proximate and biochemical composition and antioxidant status of broccoli leaves.

Materials and Methods

Collection of sample: Seeds of broccoli (*Brassica oleracea* L.) were collected from Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Field experiments were conducted in experimental farm area allocated by Directorate of Farms, University of Agriculture, Faisalabad, Pakistan during October-November, 2011 (average temperature 18.5 ˚C, 62.3 % relative humidity). Plant growth regulators (PGRs) such as humic acid (HA), kinetin "a cytokinin" (CK), gibberellic acid (GA_3) and GA_3+CK were exogenously applied 60 days after sowing (DAS) at varying concentrations of 0.2%, 0.4%, 0.6%;, 60, 40, 20 ppm;, 60, 40, 20 ppm and 30+30, 20+20, 10+10 ppm, respectively. Broccoli leaves were harvested 10 days after spray and analyzed for proximate and biochemical attributes and antioxidant potential.

Biochemical Parameters

Chlorophyll (Chl) contents: The chlorophyll pigments (Chl *a* and *b*) were determined according to method described by Lichtenthaler *et al*. (1996). The freshly harvested leaves were chopped and soaked overnight in 80% acetone solution for extraction purposes. The extracted material was centrifuged and then absorbance of the decanted solution was measured at 480, 645 and 663 nm with a spectrophotometer (IRMECO, U2020).

The formula for chl a and b
Chl. *a* = [12.7 (OD 663) -2.69 (OD 645)] * V/1000 * W
Chl. *b* = [22.9 (OD 645) -4.68 (OD 663)] * V/1000 * W
Total Chl. = Chl. *a* + Chl. *b*
V = Volume of extract used (mL)
W = Weight of fresh leaf sample used (g)

Total soluble proteins: Fresh leaf material (0.5 g) was homogenized with 10 mL of 50 m*M* potassium phosphate buffer [K_2HPO_4 + KH_2PO_4 , Merck, Germany] (pH 7.8) in an ice bath. The aliquot was centrifuged at 980 x *g* for 15 min at 4 °C. The supernatant was collected in a separate centrifuge tube and used for total soluble protein analysis. Protein contents of the extract were determined following a standard method (Bradford, 1976). Equal amount of dye stock (Biorad, USA) was added to centrifuged samples. Then mixture was vortexed and incubated for 30 min at room temperature. Absorbance of the final reaction mixture was recorded at 595 nm. Bovine serum albumin (BSA) was used as standard.

Proline content: Concentration of proline was estimated by a reported method (Bates *et al.,* 1973). Fresh leaves (0.5 g) were extracted in 10 mL of sulfo-salicylic acid (3%) and then filtered by means of Whatman No.2 filter paper. Then 2 mL of filtrate was mixed with 2 mL of acidic ninhydrin (1.25 g ninhydrine in 30 mL glacial acetic acid) and 20 mL of 6 *M* orthophosphoric acid and 2 mL of glacial acetic acid in a test tube. Then mixture was incubated at 100ºC for 60 min. After cooling, 4 mL of toluene was added in the mixture and then vortexed. Then absorbance of the final mixture was measured at 520 nm with a spectrophotometer (IRMECO U2020). Finally the concentration of proline was estimated relative to the values of standard curve.

Malondialdehyde (MDA) content: For determining the extent of lipid peroxidation in leaf samples, thiobarbituric acid-based assay was employed (Carmark & Horst, 1991). Fresh leaves (0.5 g) were homogenized in 5 mL of 1.0% (w/v) TCA. The homogenate was centrifuged at 980 x *g* for 15 min using a Sigma Model 3K30 (Germany) centrifuge machine. The supernatant (500 µL) was reacted with 2 mL of 0.5% (v/v) 2-thiobarbituric acid (TBA) [Sigma-Aldrich Chemie GmbH, Steinheim, Germany] in 20% TCA. The sample was placed in a water bath at 100ºC for 60 min. After cooling, samples were again centrifuged at 10,000 × *g* for 10 min to clarify the solution and the absorbance of the filtrate was recorded at 532 and 600 nm. The level of TBARS was calculated using the absorption co-efficient, 155 mmol cm^{-1}.

$$MDA = \Delta (OD532\text{-}OD600)/1.56\times105$$

Proximate analysis: Moisture content (%), crude fiber content (%), crude protein content (%) and ash content (%) were determined by using standard method (Anon., 1990).

Antioxidant analysis

Sample preparation: Fresh leaves of each sample (10g) were chopped and extracted with 100 mL of 80% methanol in a 250 mL conical flask in orbital shaker (Gallenkamp, UK) for 8 hours at room temperature. All the extracts were filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator under reduced pressure at 45ºC. The concentrated extracts were stored at -4ºC until analyzed (Sultana *et al.,* 2007).

Total phenolics content (TPC): A previously reported protocol was followed for the estimation of TPC (Chaovanalikit & Wrolstad, 2004). Briefly, 50mg of all methanolic extracts were separately mixed with 0.5mL of Folin-Ciocalteu reagent. Then 7.5mL of deionized water was added. After 10 min, 1.5 mL of sodium carbonate (20% w/v) was added to each mixture. All the mixtures were heated at 40°C on a water bath for 20 min and then ice cooled. Absorbance was measured at 755nm by a spectrophotometer. Amount of TPC were calculated using gallic acid calibration curve ranging from 10-100 ppm (R^2 = 0.9986) and the results were expressed as gallic acid equivalents (GAE mg/100g DW).

Total flavonoid content (TFC): A spectrophotometric method was used for TFC measurement (Sultana *et al.,* 2009). Each extract (1 mL containing 0.1 mg/mL) was diluted with distilled water (4 mL) in a 10 mL volumetric flask. Initially, 0.3 mL $NaNO_2$ solution (5%) was added to each volumetric flask, at 5 min, 0.3 mL $AlCl_3$ (10%) was added. At 6 min, 2 mL NaOH (1.0 *M*) was added followed by addition of 2.4 mL water. Absorbance of the final reaction mixture was read at 430 nm using a spectrophotometer. The results were expressed as catechin equivalents (CE mg/100g DW).

DPPH free radical scavenging assay: A previously reported method was employed for the assessment of DPPH free radical scavenging potential of the extracts (Bozin *et al.,* 2006). All the extracts (0.01 to 10 mg/mL) were analyzed for their free radical scavenging activity. To the extracts 1mL of 90 µM DPPH solution was added and diluted up to the volume of 4 mL with 95% methanol. Mixtures of the extracts were kept for 60 min at room temperature and then absorbance was recorded at 515 nm spetrophotometrically. Percent inhibition of DPPH free radical by the extracts was calculated as follow:

$$I\% = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

Determination of reducing power: The extracts were also investigated for their reducing power by following an established protocol (Yen *et al.,* 2000). To the concentrated extract (2.5-10.0 mg/mL), 0.5 mL of sodium phosphate buffer (0.2 *M*, pH 6.6) was added. The mixtures were incubated at 50^{o}C for 20 min after addition of 0.5mL of 1% potassium ferricyanide. Then 5 mL of 10% trichloroacetic acid (10%) was added to each mixture and centrifuged for 10 min at 980 x *g*. The upper layer of the solution was separated and diluted with 5 mL distilled water and 1 mL of 0.1% ferric chloride. Absorbance of the final mixture was recorded at 700 nm by using a spectrophotometer.

Statistical analysis: The results obtained were presented as mean ± S.D. of three replicates. Data was analyzed by using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test for observing difference in means using MSTAT-C software (version 1.3). Differences among values were considered statistically significant at the 5% confidence level.

Results and Discussion

Biochemical parameters: Total chlorophyll content (a, b) of broccoli leaves as affected by foliar application of PGRs is presented in the Table 1. Most of the treatments negatively affected chlorophyll content of broccoli leaves while GA_3 at 20 ppm and GA_3+CK (10+10 ppm) exhibited appreciable increase in the total chlorophyll content (1.94 mg/g FW and 1.98 mg/g FW, respectively) as compared to control.

According to some researchers GA_3 treatment causes ultrastructure modifications in plastids which is responsible for retention of chlorophyll and delays senescence (Shah, 2007). Wax apple fruit treated with GA_3 (20 ppm) exhibited highest total chlorophyll content as compared to control while GA_3 treatments at concentration of 50 ppm and 100 ppm decreased chlorophyll content (Moneruzzaman *et al.,* 2010). The presently recorded increase in total chlorophyll content as resulted by treatment with GA_3 (20 ppm) is in accordance with previous findings who reported the increase in total chlorophyll content in GA_3 treated apple leaves (Lim *et al.,* 2003).

During assessment of total soluble proteins, all the treatments markedly decreased these contents in broccoli leaves except GA_3 (20 ppm) which leads to enhance the total soluble protein content (7.09 mg/g FW) as compared to control (6.42 mg/g FW) (Table 1). These results are in agreement with a previous study which reported that GA_3 treatment increased the soluble protein content in chick-pea seeds (Thakare *et al.,* 2011).

Meanwhile, CK (40 ppm), GA_3 (20 ppm), HA (0.6%) and HA (0.2%) exhibited significant (p<0.05) decline in proline content of broccoli leaves as compared to control (0.21 µg/ g FW). However, all the remaining treatments lead to enhance proline content of broccoli leaves. Maximum proline content was observed in GA_3+CK (20+20 ppm) treated broccoli leaves (0.62 µg/g FW). Proline is an important polyamine that acts as osmoprotactant. Proline also provides energy for various developmental processes (Al-Whaibi *et al.,* 2012). In a previous investigation, GA_3 treatment at concentration of 50 and 100 ppm increased proline content in pea seeds (El-Shraiy & Hegazi, 2009). The present results are also in line with the previous findings of researchers who reported increased proline content in GA_3 treated plants (Khan *et al.,* 2010; Siddiqui *et al.,* 2011).

MDA content is the measure of lipid peroxidation of certain plant part. Most of the treatments lead to increase MDA content except GA_3+CK (30+30 ppm) and GA_3+CK (10+10 ppm) treatments which slightly decreased MDA content of broccoli leaves. In a previous study, GA_3 treatment reduced MDA content in onion and garlic plant (Ouzounidou *et al.,* 2011). Decrease in lipid peroxidation may be attributed to improved enzymatic and non-enzymatic defense system by PGR treatment (Kesba & El-Beltagi, 2012).

Proximate attributes: The effect of various treatments of PGRs on the proximate parameters of broccoli leaves is given in Table 2. Moisture content varied from 92% to 95%. Most of the treatments of plant growth regulators increased the moisture content of broccoli leaves to some extent except GA_3 (20 ppm), GA_3+CK (10+10 ppm), HA (0.6% and 0.2%) which exhibited same moisture content as control (92%). There was an optimum concentration for each growth regulator *i.e.* CK (40 ppm), GA_3 (40 ppm), GA_3+CK (20+20 ppm) and HA (0.4%) which significantly enhanced the moisture content of broccoli leaves as compared to control. In comparison among growth regulators while considering their optimum concentrations, HA (0.4%) exhibited better result towards enhancing moisture content up to 95%. High moisture content revealed that broccoli leaves are good from metabolic point of view as they have high activity of water soluble enzymes (Iheanacho & Udebuani, 2009). The determined value of moisture content in broccoli leaves corresponded to moisture content value of *Brassica oleracea* (92.5%) and *Lactuca sativa* (94.9%) reported in the literature (Caunii *et al.,* 2011).

Table 1. Total chlorophyll, total soluble protein, proline and malondialdehyde contents of broccoli (*Brassica oleracea*) leaves as affected by PGRs treatments.

Treatments	Concentrations applied	Total chlorophyll content (mg/g FW)	Total soluble proteins (mg/g FW)	Proline content (µg/g FW)	MDA content (µg/g FW)
Control (water spray)	50 mL	1.55 ± 0.04^{b}	6.60 ± 0.01^{a}	0.210 ± 0.006^{d}	2.16 ± 0.06^{cd}
	60 ppm	1.63 ± 0.03^{b}	2.62 ± 0.02^{d}	0.270 ± 0.006^{cd}	2.02 ± 0.08^{de}
Cytokinin (CK)	40 ppm	1.65 ± 0.06^{b}	3.02 ± 0.01^{d}	0.160 ± 0.006^{d}	3.63 ± 0.14^{a}
	20 ppm	1.78 ± 0.07^{a}	5.64 ± 0.01^{b}	0.220 ± 0.003^{d}	2.54 ± 0.10^{bc}
Gibberellic acid (GA_3)	60 ppm	1.66 ± 0.06^{b}	2.82 ± 0.03^{d}	0.270 ± 0.006^{cd}	3.81 ± 0.15^{a}
	40 ppm	1.39 ± 0.05^{c}	2.86 ± 0.15^{d}	0.200 ± 0.003^{d}	3.48 ± 0.14^{ab}
	20 ppm	1.94 ± 0.07^{a}	7.09 ± 0.02^{a}	0.180 ± 0.003^{d}	2.76 ± 0.11^{bc}
GA_3+CK	(30+30) ppm	1.05 ± 0.04^{d}	2.46 ± 0.02^{e}	0.290 ± 0.009^{c}	2.83 ± 0.11^{bc}
	(20+20) ppm	1.83 ± 0.07^{a}	2.87 ± 0.01^{d}	0.620 ± 0.006^{a}	2.47 ± 0.09^{c}
	(10+10) ppm	1.98 ± 0.08^{a}	2.33 ± 0.01^{e}	0.390 ± 0.009^{bc}	2.55 ± 0.10^{bc}
Humic acid (HA)	0.6%	0.95 ± 0.03^{d}	3.55 ± 0.15^{d}	0.160 ± 0.006^{d}	1.54 ± 0.06^{de}
	0.4%	1.14 ± 0.04^{cd}	2.67 ± 0.01^{de}	0.200 ± 0.006^{d}	1.84 ± 0.07^{d}
	0.2%	1.28 ± 0.05^{bc}	4.57 ± 0.01^{c}	0.140 ± 0.006^{de}	2.49 ± 0.09^{bc}

Values are mean ± SD of three replications

Different letters in each column represent significant differences ($p \leq 0.05$) of means among the treatments

Table 2. Proximate composition of broccoli (*Brassica oleracea*) leaves as affected by PGRs treatments

Treatments	Concentrations applied	Moisture content (%)	Ash content (%)	Crude fiber content (%)	Crude protein content (%)
Control (water spray)	50 mL	92.00 ± 0.05^{b}	6.60 ± 0.33^{e}	17.00 ± 0.85^{b}	4.70 ± 0.14^{c}
	60 ppm	93.00 ± 0.04 ^{ab}	17.60 ± 0.88^{b}	22.00 ± 1.1^{b}	7.60 ± 0.23^{b}
Cytokinin (CK)	40 ppm	94.00 ± 0.07^{a}	6.60 ± 0.33^{e}	24.00 ± 1.2^{b}	12.70 ± 0.38^{a}
	20 ppm	93.00 ± 0.06^{ab}	7.20 ± 0.36^{e}	18.00 ± 0.9^{c}	10.20 ± 0.30^{ab}
Gibberellic acid (GA_3)	60 ppm	93.00 ± 0.09^{ab}	16.60 ± 0.83^{b}	14.00 ± 0.7^{c}	6.90 ± 0.21^{b}
	40 ppm	94.00 ± 0.05^{a}	9.60 ± 0.48^{d}	15.00 ± 0.8^{c}	11.20 ± 0.33^{a}
	20 ppm	92.00 ± 0.09^{b}	5.50 ± 0.27^{e}	29.00 ± 1.4^{a}	8.90 ± 0.26^{b}
GA_3+CK	(30+30) ppm	93.00 ± 0.07^{ab}	7.60 ± 0.38^{e}	15.00 ± 0.7^{c}	9.50 ± 0.28^{ab}
	(20+20) ppm	92.00 ± 0.05^{b}	22.0 ± 1.10^{a}	18.00 ± 0.9^{b}	11.70 ± 0.35^{a}
	(10+10) ppm	94.00 ± 0.09^{a}	6.60 ± 0.33^{e}	24.00 ± 1.2^{b}	7.90 ± 0.23^{b}
Humic acid (HA)	0.6%	92.00 ± 0.08^{b}	11.80 ± 0.59^{c}	22.00 ± 1.1^{b}	8.70 ± 0.26^{b}
	0.4%	95.00 ± 0.0^{a}	9.00 ± 0.45^{d}	24.00 ± 1.2^{b}	10.20 ± 0.30^{a}
	0.2%	92.00 ± 0.06^{b}	10.50 ± 0.52^{c}	28.00 ± 1.4^{a}	9.80 ± 0.29^{ab}

Values are mean ± SD of three replications.

Different letters in each column represent significant differences ($p \leq 0.05$) of means among the treatments

Most of the treatments lead to enhance the ash content except GA_3 (20 ppm) which reduced the ash content (5.5%) as compared to control (6.6%). While GA_3 (40 ppm) and GA_3+CK (10+10 ppm) exhibited same ash content as control. The order of effectiveness for different treatments of growth regulators was: CK (60 ppm> 20 ppm> 40 ppm), GA_3 (60 ppm> 40 ppm> 20 ppm), GA_3+CK (20+20 ppm, 30+30 ppm, 10+10 ppm) and HA (0.6%, 0.2%, 0.4%). There is an optimum concentration for each growth regulator affecting the ash content of broccoli leaves as compared to control. The most effective treatments which enhanced ash contents were CK 60 ppm (17.6%), GA_3 60 ppm (16.6%), GA_3+CK 20+20 ppm (22%) and HA 0.6% (11.8%). A comparison among most effective treatments of each growth regulator demonstrated that GA_3+CK (20+20 ppm) has most pronounced effect on ash content (22%) of broccoli leaves as compared to control (6.6%). The ash content (22 %) of broccoli leaves was found to be higher as compared to the earlier reported ash content for *Amaranthus hybridus* (17.7 %) (Chuanphongpanich *et al.,* 2006). Ash content is the index of minerals content. During metabolic processes these minerals serves as cofactors of various reactions. Deficiency of the essential minerals may lead to impaired metabolism (Iheanacho & Udebuani, 2009).

Fiber content of broccoli leaves varied significantly among different treatments (Table 2). Most of the treatments of PGRs, except GA_3 (40, 60 ppm) and GA_3+CK (30+30 ppm), lead to enhance fiber content of broccoli leaves as compared to control . For each growth regulator there was an optimum concentration which had more pronounced effect on fiber content. These most effective concentrations of each regulator were; CK 40 ppm (24%), GA_3 20 ppm (29%), GA_3+CK 10+10 ppm (24%) and HA 0.2 % (28%). In a comparison among growth regulators with regards to their effective concentrations, GA_3 20 ppm exhibited highest fiber content (29%). The determined highest fiber content (29%) of broccoli leaves corresponded to the previous findings for *Colocasia esculenta* (26.24%) (Lewu *et al.,* 2009). High

crude fiber content may be correlated with several health benefits such as treating hypercholesterolemia and several gastrointestinal disorders (Balo *et al.,* 2002).

Interestingly, all the treatments significantly (p<0.05) enhanced the crude protein content of broccoli leaves as compared to control (Table 2). For each growth regulator there was an optimum concentration which exhibited pronounced effect. These effective concentrations were; CK 40 ppm (12.7%), GA_3 40 ppm (11.2 %), GA_3+CK 20+20 ppm (11.7%) and HA 0.4% (10.2%). CK (40 ppm) exhibited the highest crude protein content (12.7%). This high value of crude protein (12.7%) is correlated with protein content of quinoa (12.5%) (Vega-Galvez *et al.,* 2010). Leafy vegetables are cheapest source of plant protein (Lewu *et al.,* 2009). A higher crude protein content of leafy vegetable indicates that they are rich source of essential amino acids which may act as alternative source of energy through gluconeogenesis when carbohydrate metabolism is impaired (Iheanacho & Udebuani, 2009).

Antioxidant parameters: The effect of different plant growth regulators on total phenolics content (TPC) and total flavonoids contents (TFC) of broccoli leaves is presented in the Table 3. The effect of growth regulators on TPC at different concentration varied significantly (p<0.05). The order for variations of TPC in relation to different treatments of each growth regulator was: CK (`40 ppm> 20 ppm> 60 ppm), GA_3 (40 ppm> 60 ppm> 20 ppm), GA_3+CK (10+10 ppm> 20+20 ppm> 30+30 ppm) and HA (0.4%> 0.2%> 0.6%). This comparison showed that there is an optimum concentration for each growth regulator (HA 0.4%, CK 40 ppm, GA_3 40 ppm, GA_3+CK 10+10 ppm) which significantly increased TPC (390.30, 239.24, 274.07, 275.99 mg GAE/100 g, respectively) as compared to control (220.92 mg GAE/100 g). Based upon these results it can be concluded that among all optimum concentrations of different growth regulators, HA 0.4 % was the most effective to enhance TPC in broccoli leaves (390.30 mg GAE/100 g) as compared with control (220.92 mg GAE/100g). These findings are similar to those of some previous studies (Moneruzzaman *et al.,* 2010), who reported that GA_3 application increased TPC in wax apple fruits (535 mg GAE/100 g) as compared to control (311 mg GAE/ 100 g). A higher TPC (63 mg CAE/100 g) for HA (400 mg/mL) treated *Thymus vulgaris* L. plant as compared to HA (200 mg/mL) treatment was also reported (Juarez-Montiel *et al.,* 2011). A higher TPC can be directly correlated with greater antioxidant activity because phenolic compounds act as a good free radical scavengers by donating their hydrogen to free radicals (Qasim *et al.,* 2012).

As given in Table 2, most of the treatments lead to enhance TFC as compared to control (77.95 CE mg/ 100 g). However, CK (60 ppm), GA_3 (20 ppm) and GA_3+CK (30+30 ppm) decreased TFC as compared with the control that might be due to change in physiological functions resulting in response to plant growth regulators applied. Comparison among growth regulators regarding their effective concentrations depicted that HA (0.4%) offered maximum total flavonoids (188.72 CE mg/ 100 g) followed by CK 40 ppm (183.87 CE mg/100 g), GA_3 40 ppm (154.37 CE mg/100 g) and GA_3+CK (20+20 ppm) (109.48 CE mg/100 g). TFC of HA treated broccoli leaves was found to be higher as compared to previous researchers who reported TFC (51.63 µg QE/ 100 g FW) in HA treated *T. vulgaris* L. plant (Juarez-Montiel *et al.,* 2011). Moreover, it has been reported that GA_3 and CK treatment increased TFC in dandelion plant (120 to 125 µg/ g DW) compared to the control (100 µg/ g DW). The results of this study revealed that PGRs may have their potential role in biosynthesis of flavonoids hence a higher concentration of TFC in PGR treated samples was observed. So, the use of suitable PGRs can be recommended for enhancing TFC of such vegetables (Kim *et al.,* 2009). Consumption of flavonoids is correlated with anti-carcinogenic and anti-inflammatory properties as these compounds are known to be effective free radical scavengers (Sharma *et al.,* 2010).

A reliable method to evaluate the antioxidant potential of plant extract is to measure their capability to reduce Fe (III) to Fe (II). Change in color from yellow to bluish green indicates the reduction of Fe (III) to Fe (II). Change in intensity of color is taken as a direct measure of reduction ability of the extracts. Concentration of extracts is directly correlated with the reducing ability (Yang *et al.,* 2000). Reducing potential of methanolic extracts of different PGR's treated broccoli leaves was measured over the concentration range of 2.5-10 mg/mL (Table 4). All the extracts exhibited increase in reducing power as the concentration of extracts increased between 2.5-10 mg/mL. Reducing potential measured in terms of absorbance for all extracts at 10 mg/mL varied significantly (p<0.05) from 0.81 to 1.9. The order of effectiveness regarding reducing power at 10 mg/mL of extract concentration was; CK (40 ppm> 20 ppm> 60 ppm), GA_3 (40 ppm> 60 ppm> 20 ppm), GA_3+CK (10+10 ppm> 20+20 ppm> 30+30 ppm) and HA (0.4 %> 0.2 %> 0.6 %). Among the treatments maximum reducing power was exhibited by methanolic extract of HA (0.4 %) treated broccoli leaves (1.9). The results of the present study were found to be in agreement with the previous researchers who reported that methanolic extract of broccoli leaves has strong reducing power (Guo *et al.,* 2001).

Measurement of DPPH radical scavenging activity is another reliable approach which can be used to predict the antioxidant activity of plant extracts. DPPH, an organic stable free radical, is very sensitive to antioxidant compounds even at low concentration. During the reaction with phenolics/antioxidants from the test plant extract, DPPH free radical is stabilized by acquiring proton or electron from the extract and noticeable color change takes place from deep violet to yellow color. Greater the concentration of extracts higher will be the radical scavenging activity (%). So, antioxidant activity of extract under investigation can be directly correlated with their effectiveness to scavenge DPPH free radical (Sanchez-Morenzo *et al.,* 2002).

Table 3. Total phenolics content (TPC GAE mg/100g) and total flavonoids content (TFC CE mg/100g) of methanolic extracts of broccoli leaves as affected by PGRs treatments.

Treatments	Concentrations Applied	TPC (mg GAE/ 100g DW)	TFC (mg CE/ 100g DW)
Control (water spray)	50 mL	220.92±6.62^{e}	77.95±2.46^{e}
Cytokinin (CK)	60 ppm	196.29 ± 5.88^{g}	54.71 ± 1.34^{g}
	40 ppm	239.24 ± 7.17^{d}	183.87 ± 1.81^{a}
	20 ppm	214.62 ± 6.43^{f}	98.17 ± 1.78^{d}
Gibberellic acid (GA_3)	60 ppm	256.36 ± 7.69^{c}	90.21 ± 1.13^{d}
	40 ppm	274.07 ± 8.22^{b}	154.37 ± 2.04^{b}
	20 ppm	138.75 ± 4.16^{h}	66.27 ± 2.05^{f}
GA_3+CK	(30+30) ppm	187.06 ± 5.61^{g}	68.33 ± 2.01^{f}
	(20+20) ppm	221.56 ± 6.64^{e}	109.48 ± 2.87^{c}
	(10+10) ppm	275.99 ± 8.27^{b}	95.40 ± 1.43^{d}
Humic acid (HA)	0.6%	245.52 ± 7.36^{c}	149.86 ± 3.04^{b}
	0.4%	390.30 ± 9.33^{a}	188.72 ± 4.50^{a}
	0.2%	276.73 ± 8.30^{b}	107.58 ± 1.58^{c}

Values are mean ± SD of three replications

Different letters in each column represent significant differences ($p \leq 0.05$) of means among the treatments

Table 4. Reducing power and radical scavenging activity of methanolic extracts of broccoli leaves at 10 mg/mL of extracts concentration as affected by PGRs treatments.

Treatments	Concentrations applied	Reducing power	Radical scavenging activity (%)
Control (water spray)	50 mL	1.60 ± 0.05^{d}	82.00 ±2.52^{c}
Cytokinin (CK)	60 ppm	1.56 ± 0.04^{d}	80.00 ± 2.40^{c}
	40 ppm	1.80 ± 0.05^{b}	90.00 ± 2.70^{b}
	20 ppm	1.70 ± 0.05^{c}	86.00 ± 2.58^{bc}
Gibberellic acid (GA_3)	60 ppm	1.15 ± 0.03^{g}	85.00 ± 2.55^{bc}
	40 ppm	1.72 ± 0.05^{c}	88.00 ± 2.64^{bc}
	20 ppm	1.83 ± 0.05^{b}	74.00 ± 2.22^{d}
GA_3+CK	(30+30) ppm	1.40 ± 0.04^{e}	82.00 ± 2.46^{c}
	(20+20) ppm	1.63 ± 0.05^{d}	91.00 ± 2.73^{b}
	(10+10) ppm	0.81 ± 0.02^{h}	84.00 ± 2.52^{bc}
Humic acid (HA)	0.6%	1.24 ± 0.03^{f}	87.00 ± 2.61^{bc}
	0.4%	1.90 ± 0.06^{a}	96.00 ± 2.88^{a}
	0.2%	1.82 ± 0.05^{b}	90.00 ± 2.70^{b}

Values are mean ± SD of three replications

Different letters in each column represent significant differences ($p \leq 0.05$) of means among the treatments

In the present experiments, all the extracts exhibited increase in radical scavenging activity (%) following a concentration dependent manner over the range of 0.01 to 10 mg/mL. Maximum radical scavenging activity was observed at 10 mg/mL by the extracts. A comparison of the extracts at concentration 10 mg/mL revealed that the order of effectiveness for DPPH radical scavenging capacity varied significantly (p<0.05) among various treatments (80% to 96%). Most of the treatments of PGRs lead to increase the radical scavenging activity as compared to control (84%) except GA_3 20 ppm, CK 60 ppm and GA_3+CK (30+30 ppm) which slightly decreased percent radical scavenging activity. A comparison among growth regulators with respect to their effective concentration revealed that HA (0.4 %) treated broccoli leaves extract had higher radical scavenging activity (96%) followed by, GA_3+CK 20+20 ppm (91%), CK 40 ppm (90%) and GA_3 (85%). The results of the present study were found to be comparable with those of previous researchers who reported radical scavenging activity as high as 94% for methanolic extracts of broccoli (Guo *et al.,* 2001). Effectiveness of HA treatments towards enhancing DPPH radical scavenging activity of some crops such as *Thymus vulgaris* L. has already been appraised (Juarez-Montiel *et al.,* 2011).

Conclusion

The foliar spray of PGRs (at an optimum concentration) was found to be effective towards improving nutritional and antioxidant status of broccoli leaves. In this context, humic acid (HA) and mixture of gibberellic acid and cytokinin (CK) treated broccoli leaves exhibited improved antioxidant and biochemical attributes. However, the results revealed that different PGR have varied effect on broccoli leaves in relation to different parameters. The selected PGRs applied broccoli leaves can be commercially utilized as a potential source of high-value nutrients /components. Besides, broccoli leaves extract can be used as a food preservative as they are good source of antioxidants. However, further detailed investigation is needed for isolation and structural elucidation of functional bioactives in broccoli leaves and to evaluating their specific food preservation and nutraceutical uses.

References

Al-Whaibi, M.H., M.H. Siddiqui, B.M.A. Al-Munqadhi, A.M. Sakran, H.M. Ali and M.O. Basalah. 2012. Influence of plant growth regulators on growth performance and photosynthetic pigments status of *Eruca sativa* Mill. *J. Med. Plants Res.,* 6: 1948-1954.

Arnon, D.T. 1949. Copper enzyme in isolated chloropolyphenoloxidase in *Beta vulgaris. Plant Physiol*., 24: 01-15.

Anonymous. 1990. Official and recommended methods of AOAC, 15th eds., AOAC Standard 976.05. AOAC Inc.,Virginia,USA.

Bako, S.P., S.A. Luka, E.B. Bealo and J. Aula. 2002. Ethanobotany and Nutrient Content of *Gnetum africana* in Nigeria; Recent progress in medicinal plants. *Ethnomed. Pharmacog.,* 1: 79-84.

Bates, L.S., R.P. Waldren and I.D. Teare. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil*, 39: 16-18.

Bozin, B., N.M. Dukic, N. Smin and G. Anackov. 2006. Characterization of the volatile composition of essential oil of some *lamiaceae* species and the antimicrobial and antioxidant activities of the entire oils. *J. Agri. Food Chem.,* 54: 1822-1828.

Bradford, M.M. 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 72: 248-254.

Carmak, I. and J.H. Horst. 1991. Effects of aluminum on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). *Plant Physiol.,* 83: 463-468.

Caunii, A., G. Pribac and M. Butnariu. 2011. Operational marketing of vegetables having antioxidant biocomponents. *Studia Universitatis* "*Vasile Goldiş*", 21: 557-561.

Chaovanalikit and R.E. Wrolstad. 2004. Total anthyocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. *J. Food Sci.,* 69: 67-72.

Chuanphongpanich, S., S. Phanichphant, D. Bhuddasukh, M. Suttajit and B. Sirithunyalug. 2006. Bioactive glucosinolates and antioxidant properties of broccoli seeds cultivated in Thailand. *J. Sci. Technol.,* 28: 55-61.

El-Shraiy, A.M. and A.M. Hegazi. 2009. Effect of acetylsalicylic acid, indole-3- bytric acid and gibberellic acid on plant growth and yield of Pea (*Pisum sativum* L.). *Aust. J. Basic Appl. Sci.,* 3: 3514-3523.

Guo, J.T., H.L. Lee, S.H. Chiang, F.I. Lin and C.Y. Chang. 2001. Antioxidant properties of the extracts from different parts of broccoli in Taiwan. *J. Food Drug Anal.,* 9: 96-101.

Gurmani, A.R. A. Bano and M. Salim. 2006. Effect of growth regulators on growth, yield and ion accumulation of rice (*Oryza sativa* L.) under salt stress. *Pak. J. Bot.,* 38: 1415-1424.

Hutchinson, C.E. and J.J. Kieber. 2002. Cytokinin signalling in *Arabidopsis*. *Plant Cell,* 14: 47-59.

Iheanacho, K.M.E. and A.M.E. Udebuani. 2009. Nutritional composition of some leafy vegetables consumed in Imo state, Nigeria. *J. Appl. Sci. Envir. Manag.,* 13: 35-38.

Juarez-Montiel, M., S.R. de Leon, G. Chavez-Camarillo, C. Herandez-Rodriguez and L. Villa-Tanaca. 2011. *Huitlacoche* (corn smut), caused by the phytopathogenic fungus Ustilago maydis, as a functional food. *Rev Iberoam Micol.,* 28: 69-73.

Kesba, H.H. and H.E.S. El-Beltagi. 2012. Biochemical changes in grape rootstocks resulted from humic acid treatments in relation to nematode infection. *Asian Pac. J. Trop Biomed.,* 2: 287-293.

Khan, M.N., M.H. Siddiqui, F. Mohammad, M. Naeem and M.M.A. Khan, 2010. Calcium chloride and gibberellic acid protect linseed (*Linum usitatissimum* L.) from NaCl stress by inducing antioxidative defence system and osmoprotectant accumulation. *Acta Physiol. Plant,* 32: 121-132.

Kim, Y.H., M. Hamayun, A.L. Khan, I.N. Chae, S.M. Kang, H.H. Han and I.J. Lee. 2009. Exogenous application of plant growth regulators increased the total flavonoid content in *Taraxacum officinale* Wigg. *Afri. J. Biotechnol.,* 8: 5727-5732.

Lewu, M.N., P.O. Adebola and A.J. Folayan. 2009. Effect of cooking on the proximate composition of the leaves of some accessions of *Colocasia esculenta* (L.) Schott in KwaZulu-Natal province of South Africa. *Afri. J. Biotechnol.,* 8: 1619-1622.

Lichtenthaler, H.K., A. Gitelson, and M. Lang. 1996. Non-destructive determination of chlorophyll content of leaves of a green and an aurea mutant of tobacco by reflectance measurements. *J. Plant Physiol.,* 148(3): 483-493.

Lim, P.O., H.R. Woo and H.G. Nam. 2003. Molecular genetics of leaf senescence in *Arabidopsis*. *Trends Plant Sci.,* 6: 272-278.

Moneruzzaman, K.M., A.B.M.S. Hossain, O. Normaniza and A. N. Boyce. 2010. Growth, yield and quality responses to gibberellic acid (GA_3) of Wax apple *Syzygium samarangense* var. *Jambu air madu* fruits grown under field conditions. *Afri. J. Biotechnol.,* 10: 11911-11918.

Moreno, D.A., M. Carvajal, C.L. Berenguer and C.G. Viguera. 2006. Chemical and biological characterization of nutraceutical compounds of broccoli. *J. Pharm. Biomed.,* 41: 1508-1522.

Ouzounidou, G., A. Giannakoula, M. Asfi and I. Ilias. 2011. Differential responses of onion and garlic against plant growth regulators. *Pak. J. Bot.,* 43: 2051-2057.

Pila, N., N.B. Gol and T.V.R. Rao. 2010. Effect of post-harvest treatments on physicochemical characteristics and shelf life of tomato (*Lycopersicon esculentum* Mill.) fruits during storage. *Am. Eu J. Agri. Envir. Sci.,* 9: 470-479.

Qasim, A., M. Ashraf and F. Anwar, 2012. Trehalose-induced changes in seed oil composition and antioxidant potential of maize grown under drought stress. *J. Am. Oil Chem. Soc.,* 89: 1485-1493.

Qasim, A., M. Ashraf and F. Anwar. 2010. Seed composition and seed oil antioxidant activity of maize under water stress. *J. Am. Oil Chem. Soc.,* 87: 1179-1187.

Qasim, A., M. Ashraf and F. Anwar. 2009. Physico-chemical attributes of seed oil from drought stressed sunflower (*Helianthus annuus* L.) plants. *Grasas y Aceites,* 60: 475-481.

Sanchez-Moreno, C., J.A. Larrauri and F. Saura-Calixto. 2002. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. *Food Res. Int.,* 32: 407-412.

Saruhan, V., A. Kusvuran and K. Kokten. 2011. The effect of different replications of humic acid fertilization on yield performances of common vetch (*Vicia sativa* L.). *Afri. J. Biotechnol.,* 10: 5587-5592.

Shah, S.H. 2007. Effects of salt stress on mustard as affected by giberrellic acid application. *Appl. Plant Physiol.,* 3391: 97-103.

Shabbir, R.N., M.Y. Ashraf, E.A. Waraich, R. Ahmad and M. Shahbaz. 2015. Combined effects of drought stress and NPK foliar spray on growth, physiological processes and nutrient uptake in wheat. *Pak. J. Bot*., 47: 1207-1216.

Sharma, R.J., S.R. Chaphalkar and A.D. Adsool. 2010. Evaluating antioxidant potential, cytotoxicity and intestinal absorption of flavonoids extracted from medicinal plants. *Int. J. Biotechnol. Appl.,* 2: 01-05.

Siddiqui, M.H., M.H. Al-Whaibi and M.O. Basalah. 2011. Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. *Protoplasma,* 248: 503-511.

Singh, M., D.K. Rana, J.M.S. Rawat and S.S. Rawat. 2011. Effect of GA_3 and kinetin on growth, yield and quality of sprouting broccoli (*Brassica oleracea* var. italica). *J. Horti. Fores.,* 3: 282-285.

Sultana, B., F. Anwar and M. Ashraf. 2009. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules*, 14: 2167-2180.

Thakare, U., N. Patil and N. Malpathak. 2011. Performance of chick pea under the influence of gibberellic acid and oxygenated peptone during germination. *Adv Biosci. Biotechnol.,* 2: 40-45.

Vega-Galvez, A., M. Miranda, J. Vergara, E. Uribe, L. Puente and E.A. Martinez. 2010. Nutrition facts and functional potential of quinoa (*Chenopodium quinoa* Willd.), an ancient Andean grain. *J. Sci. Food Agric.,* 90: 2541-2547.

Yang, J.H., J.L. Mau, P.P. Ko and L.C. Huang. 2000. Antioxidant properties of fermented soybean broth. *Food Chem.,* 71: 249-254.

Yen, G.C., P.D. Duh and D.Y. Chuang. 2000. Antioxidant activity of anthraquinones and anthrone. *Food Chem.,* 70: 307-315.

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