Advanced Research Journal of Biochemistry Sciences Vol. 1 (1), pp. 018-028, September, 2013. Available online at www.advancedscholarsjournals.org © Advanced Scholars Journals

Full Length Research Paper

Changes in some biochemical characteristics in response to foliar applications of chelator and micronutrients in green pungent pepper

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Accepted 22 August, 2013

In order to study the changes in some biochemical characteristics in response to foliar applications of chelator [humic acid: HA_1 (0%) and HA_2 (0.05%)] and micronutrients [manganese: Mn_1 (0%); Mn_2 (0.05%) and molybdenum: Mo_1 (0%); Mo_2 (0.01%)] in green pungent pepper cv. Bullet (*Capsicum annum* L.), a pot experiment in factorial randomized block design with three replications was conducted in the net house of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal and India. In this experiment, we analyzed some carbohydrate constituents, non enzymatic antioxidants, enzymatic antioxidants and total antioxidant activity. Obtained results show that the highest values of reducing sugar, total sugar and starch were exhibited by applications of HA_2Mn_2, HA_1Mn_2 and HA_2Mo_2, respectively. The ascorbic acid contents significantly increased in treatments of HA_2 and Mn_2. The highest value of total phenol content was obtained by application of HA_2Mo_1, while there was significant increase in the application of HA_2, HA_2Mn_2 and HA_2Mo_2 in the case of free phenol. The highest value of carotene content was obtained by foliar application of HA_2Mn_2, whereas in the case of capsaicin content, there was significant increase in all treatments, except Mo application alone. The highest activity of peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) were exhibited by the treatments HA_2Mo_2, HA_2Mo_1 and HA_2Mo_2, respectively. The total antioxidant activity was expressed as molybdate reducing antioxidant power (MRAP). The MRAP values were increased significantly by the foliar application of HA_2. Total phenol, free phenol, capsaicin, polyphenol oxidase and total antioxidant activity had a significant positive association with ascorbic acid. Based on principal component analysis and average values, foliar application of HA_2Mn_2 and HA_2Mo_2 had good performers with respect to all variables, which may bring about the proper value addition in quality of green pungent pepper by enhancing the carbohydrate constituents, antioxidant constituents and antioxidant activities.

Key words: *Capsicum annum* L., chelator, micronutrients, carbohydrates, antioxidants, enzymes, molybdate reducing antioxidant power.

INTRODUCTION

Nutritional value of vegetables depends, apart from others, on the contents of biochemical compounds such as: ascorbic acid, phenolic compounds (Mareczek and Leja, 2005; Leja et al., 2005), carotenoids (Unlu et al., 2010), total sugar, reducing sugar and starch (Unlu et al., 2011; Mousavi et al., 2007). Concentrations of these compounds in plant may be modified by various factors including foliar nutrition with organic component. Some studies estimates indicate that a large number of diverse materials can serve as sources of plant nutrients. The

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majority of nutrient input to agriculture comes from commercial mineral fertilizers. Organic manures are considered to play a significant but lesser role in nutrient contribution, leaving aside their beneficial effects on soil physicochemical and biological properties. Foliar feeding is a relatively new and controversial technique of feeding plants by applying liquid fertilizer directly to their leaves.

Most of the used organic-mineral fertilizers are humic substances, since humic acid is one of the major components of humic substances. Humic acids are termed polydisperse because of their variable chemical features. From a three dimensional aspects these complex carbon containing compounds are considered to be flexible linear polymers that exist as random coils with cross-linked bonds. On the average, 35% of the humic acid molecules are aromatic (carbon rings), while the remaining compounds are in the form of aliphatic (carbon chains) molecules. Directly humic acid compounds may have various biochemical effects either at cell wall, membrane level or in the cytoplasm, including increased photosynthesis and respiration rates in plants, enhanced protein synthesis and plant hormone like activity (Chen and Aviad, 1990). Humic substances may possibly enhance the uptake of some macro (K, Ca and P) and microelements (Fe, Zn and Mn) (Nardi et al., 2002; Eyheraguibel et al., 2008). The main enzymes involved in the antioxidative defense, such as SOD, catalase, ascorbate peroxidase and peroxidase, were also monitored by humic acid (Kesba and EI-Beltagi, 2012).

Divalent manganese ions (Mn^{2+}) is converted to Mn^{3+} or Mn^{4+} easily, therefore in the plant, manganese plays an important role in oxidation and reduction processes, as electron transport in photosynthesis. Moreover, manganese acts as an activator of several enzymes (more than 35 different enzymes), which involve oxidation reactions, carboxylation, carbohydrates metabolism, phosphorus reactions and citric acid cycle. As important as these enzymes, protein-manganese in Photosystem II and superoxide dismutase can be pointed. There are more than 90% of superoxide dismutase in chloroplasts with about 4 to 5% of it in the mitochondria (Millaleo et al., 2010; Mukhapadhyay and Sharma, 1991; Jackson et al., 1978; Uehara et al., 1974). Manganese also activates several enzymes of the shikimic acids pathway and subsequent pathways, leading to the biosynthesis of aromatic amino acids, such as tyrosine, various secondary products, such as lignin, flavonoids, as well as IAA (Burnell, 1988; Hughes and Williams, 1988). For example, Mn^{2+} affects phenylalanine ammonia-lyase (PAL) and stimulates peroxidases required for lignin biosynthesis.

It has long been known that the rare transition element molybdenum (Mo) is an essential micronutrient for plants (Arnon and Stout, 1939). However, Mo seems to be catalytically inactive in biological systems until it is combined with a special cofactor. More than 40 Mo-enzymes catalyzing diverse redox reactions were found in all organisms, however only four of them have been found in plants. These Mo-enzymes participate in essential redox reactions in the global C-, N- and S-cycles (Mendel and Hansch, 2002). Moreover Mo acts as an activator of several antioxidative enzymes such as superoxide dismutase, peroxidase and catalase, while Mo deficiency decreased the CAT activity in papaya (Agarwala et al., 1986), and increased the POD activity in mustard (Chatterjee et al., 1985) plants. Mo deficiency also decreased the concentration of ascorbic acid in several crops, such as cauliflower (Agarwala and Hewitt, 1954), leguminous plants (Avdomin and Arens, 1966) and potato tubers (Munshi and Mondy, 1988). Munshi and Mondy (1988) also reported that Mo application especially at higher rates (sodium molybdate at 10.1 $kg.ha^{-1}$) was shown to decrease the activity of polyphenol (catechol) oxidase and the contents of total phenolic compounds in potato (*Solanum tuberosum* L.) tubers. This element also has a crucial role in plant nitrogen metabolism being involved in the processes of N_2 fixation, nitrate reduction and the transport of nitrogen in plants (Marschner, 1995; Hamlin, 2006). An adequate supply of Mo leads to complex changes in the concentrations of total sugars and reducing sugars in the leaves and stems of cauliflower plants (Agarwala and Hewitt, 1955). In the presence of Mo deficiency, low sugar concentrations are related to failure of photosynthesis and non-utilization of carbohydrates for plant growth.

Pungent pepper is an important agricultural crop, not only because of its economic importance, but also for the nutritional value of its fruits, mainly due to its colour, test, pungency, flavor, aroma and excellent source of antioxidant compounds. Apart from providing basic nutrition, pepper fruit is well known for its health benefits; antioxidative potential powers in terms of antioxidant constituents and antioxidant activities have been reported in green pungent pepper (Bhattacharyay et al., 2010; Biswas et al., 2011). Foods rich in antioxidants play a role in prevention of cardiovascular diseases and cancers (Garber et al., 2002) and neurodegenerative diseases (Di-Matteo and Esposito, 2003), as well as prevention of inflammation and problems causes by cell and cutaneous aging (Ames et al., 1993).

Aerobic organisms are constantly exposed to one or more systems that generate reactive oxygen species (ROS), including the superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), various peroxyl radicals ($ROO^{\bullet}$) and singlet oxygen ($^1O_2$). These ROS are highly reactive and can damage living cells if formed in significant amounts. To avoid cellular damage by ROS, most biological systems have developed defense mechanisms, antioxidants that convert ROS to unreactive derivatives. Recently, Biswas et al. (2011) and Bhattacharya et al. (2010) reported that green pepper, is rich in antioxidants. The authors also concluded that the green pungent pepper contains a few chain breaking antioxidants (vitamin C, carotene and phenolics etc. that

Table 1. Detail doses of treatments used as foliar spray for green pungent pepper.

Treatment		Humic acid (HA) (%)	Manganese (Mn) (%)	Molybdenum (Mo) (%)
Control	$HA_1Mn_1Mo_1$	0.0	0.0	0.0
HA	$HA_2Mn_1Mo_1$	0.05	0.0	0.0
Mn	$HA_1Mn_2Mo_1$	0.0	0.05	0.0
Mo	$HA_1Mn_1Mo_2$	0.0	0.0	0.01
HA×Mn	$HA_2Mn_2Mo_1$	0.05	0.05	0.0
HA×Mo	$HA_2Mn_1Mo_2$	0.05	0.0	0.01

Where, the source of chelator was humic acid (granular form), that of manganese (Mn) was $MnSO_4 \cdot H_2O$ and molybdenum (Mo) from $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Each spraying was done four times with sticker starting from 25 days after transplanting and subsequent ones at an interval of 10 days during vegetative stage.

scavenge oxygen radicals and thereby break radical chain sequences) and few preventive antioxidants (POD and CAT, etc. which prevent or inhibit the formation of ROS). However, pepper fruit contains desirable quantity of some carbohydrate constituents (reducing sugar, total sugar and starch) (Phulari, 2012) that provides energy in the body for general activities. Therefore, in the present work, we have studied this response in green pungent pepper grown at different levels of foliar application of chelator (HA) and micronutrients (Mn and Mo), as well as the biochemical changes that take place during fruit formation, in order to improve the quality of this fruits and obtain fruits of a higher nutritional value.

MATERIALS AND METHODS

Field experiment

The seedling was gown in nursery beds prepared in a sandy loam soil and were 12 cm tall and 1.0 m wide. Weathered cow dung manure 4 $kg \cdot m^{-2}$, was mixed into the beds. Beds were drenched with formaldehyde (4.0%) and covered with polythene sheets for one week to avoid damping off disease. Seedling was treated with Dithane M-45 (2.5 $g \cdot kg^{-1}$ of seed) (Hindustan Pulverizing Mills Industrial Growth Centre, Sumba, Jammu, India) prior to sowing. Fresh seeds of pungent pepper cv. bullet (*Capsicum annuum* L.) collected from AICRP on vegetable crops were sown at 10 mm and 5 cm apart. After sowing, beds were covered with straw until seed germination and hand watering was done regularly. Seedlings were hardened by withholding water four days before transplanting.

Pot experiment

A pot experiment was conducted in the net house of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India. The 40 days old seedlings were transplanted in earthen pot of 15 cm size (one plant pot^{-1}) having a central drainage hole. Soil was prepared by mixing appropriate amount of well rotted cow dung and manures (soil 700 g pot^{-1}; cow manure 100 g pot^{-1}; urea 5 g pot^{-1}; single superphosphate 20 g pot^{-1} and muriate of potash 6 g pot^{-1}) (Department of Spices and Plantation Crops, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India). Additional fertilizer 5 g pot^{-1} was applied 21 days after transplanting. The experiment consisting of six treatments including control (only tap water sprayed) were arranged in a factorial randomized block design with three replications. The detail treatments are summarized in Table 1.

To prevent blossom and fruit drop, supplementary irrigation was required (hand water was applied at an interval of one day with the first immediately after transplanting). No weeding was required. The insecticide Rogor at 2.5 ml L^{-1} (Rallis India Ltd., Mumbai, Maharashtra, India) was applied three times beginning from flowering stage and at 15 days intervals to control aphids. About 400 g edible green pungent pepper from each replication was collected. Fresh materials were chopped with a sharp knife into small pieces before analysis of non enzymatic antioxidants (ascorbic acid, phenols and carotene), two enzymatic antioxidants (POD and CAT), one oxidative enzyme (PPO) and total antioxidant activity. Samples were shredded and dried at 40°C for 96 h. This material was prepared for reducing sugar, total sugar, starch and capsaicin analyses by grinding to a fine powder using an electric grinder. A subsample of the dried shredded material was further dried at 100°C to constant dry weight to determine percent moisture.

Chemical analysis

Analysis of reducing sugar

Reducing sugar were extracted in 10 ml of 80% alcohol by boiling 0.1 g dry powdered sample for 30 min at 80 to 90°C followed by centrifugation at 5,000 g for 10 min and subsequent analysis was followed using the Nelson-Somogyi's method (Sadasivam and Manickam, 1992). The amount of reducing sugar was determined against a glucose solution (0 to 500 µg) as standard curve.

Analysis of total sugar and starch

Sugar and starch were extracted in 10 ml of 80% anhydrous alcohol by boiling 0.1 g dry powdered sample for 30 min at 80°C followed by centrifugation at 5,000 g for 10 min and subsequent procedures was followed using the Anthrone reagent method (Sen et al., 2005). The amount of concentration was determined against a glucose solution (0 to 500 µg) as standard curve.

Analysis of ascorbic acid

One gram of finely chopped green pungent pepper was extracted with 20 ml of 4% oxalic acid following maceration in a pestle and mortar and the material was centrifuged for 30 min at 10,000 *g*.

Ascorbic acid content was determined using the dichlorophenol indophenol titration procedure (Casanas et al., 2001).

Analysis of total phenol and free phenol

Total phenol was extracted in 50% methanolic 1.2 N HCl and free phenol was extracted in 50% aqueous methanol by boiling 1 g of finely chopped tissue for 1.5 h at 80 to 90°C following the method of Vinson et al. (1995) and subsequent analysis was with the Folin-Ciocalteau reagent using gallic acid as standard.

Analysis of carotene

Carotene was extracted in 50% alcohol, 10% KOH by boiling 2 g of finely chopped green pungent pepper tissue for 1.5 h at 80 to 90°C followed by centrifugation at 10,000 g for 30 min and subsequent analysis was monitored according to the method of Singh and Bradbury (1988). Total carotene was analyzed spectrophotometrically using β-carotene as the standard.

Analysis of capsaicin

Pungency of green pepper was determined as capsaicin (phenolic compound) was extracted in 10 ml aqueous ethanol by shaking 0.5 g powdered sample for 1 h at mechanical shaker and material centrifuged for 10 min at 10,000 g. Tubes containing 1 ml extract, 5 ml of 0.4% NaOH and 3 ml of 3% phosphomolybdic acid were incubated at room temperature for 60 min. Absorbance at 650 nm was recorded according to Sadasivam and Manickam (1992). The values were converted from dry to fresh weight using the moisture content of green pungent pepper.

Analysis of POD activity

One gram of fresh tissue was macerated in a pre-chilled pestle and mortar and extracted with 10 ml tris-HCL buffer (pH 7.6) to determine POD activity. Triturated samples were centrifuged at 10,000 g for 30 min at 4°C and supernatants were assessed for enzyme activity. The POD was estimated by mixing 0.1 ml chilled enzyme extract with 2.8 ml reaction mixture (0.5% *o*-dianisidine dissolved in methanol, 0.28 ml Na-acetate buffer, 2.4 ml water). The reaction was initiated by adding 0.1 ml H_2O_2 (30%). Changes in absorbance at 430 nm were monitored at 1 min interval for up to 3 min. POD activity was measured using *o*-dianisidine as the substrate (Bhattacharya et al., 2010) and expressed as mM *o*-dianisidine oxidized•min^{-1} •g^{-1} of fresh tissue using the extinction coefficient 1.13×10^4•M^{-1}• cm^{-1}.

Analysis of PPO activity

One gram of fresh tissue from each treatment was macerated in a pre-chilled pestle and mortar and extracted with 10 ml phosphate buffer (pH 6.0) to determine PPO activity. Triturated samples were centrifuged at 10,000 g for 30 min at 0°C and supernatants were assessed for enzyme activity. Estimation of polyphenol oxidase was made by adding 2 ml phosphate buffer and 0.5 ml (0.01 M) catechol solution with 0.5 ml extract. Changes in activities were measured following the procedure of Matto and Diamond (1963).

Analysis of CAT activity

Two grams of fresh tissue was macerated in a pre-chilled pestle and mortar and extracted with 10 ml phosphate buffer (pH 7.0) to determine CAT activity. Triturated samples were centrifuged at 10,000 g for 30 min at 4°C and supernatants were assessed for enzyme activity. An assay mixture of 3 ml of phosphate buffer, 2 ml of H_2O_2, and 1 ml of enzyme source were pipette into a porcelain crucible and incubated for 1 min at 20°C to estimate CAT activity. The reaction was stopped with addition of 10 ml of 0.7 N H_2SO_4 and the reaction mixture titrated against 0.01 N $KMnO_4$ to determine residual H_2O_2 until there was a faint pink color that persisted for at least 15 s. One unit of CAT activity was defined as amount of enzyme that destroyed 1 µmol of H_2O_2•min^{-1}•g^{-1} of fresh tissue. Changes in activity were measured using the method of Kar and Mishra (1976).

Analysis of total antioxidant activity

The measurement of antioxidant activity is useful for estimating the total content and activity of the antioxidant compounds in fruits. A 0.5 g freshly chopped green pungent pepper sample was extracted by macerating with 10 ml distilled water for estimation of total antioxidant activity. Tubes containing extract and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C for 90 min. After incubation, the mixture was cooled to room temperature, absorbance of each solution was measured at 695 nm against a blank (Prieto et al., 1999). Antioxidant capacity was expressed as ascorbic acid equivalent and gallic acid equivalent.

Statistical analysis

Data were subjected to ANOVA of a factorial randomized block design, simple correlation were calculated and tested for significance and means were separated by Duncan's multiple range test. Principal Component Analysis (PCA), as the method of identifying the factor dimension of the data, was used to summarize the treatment in forming in a reduced number of factors for selection of the best performing treatment. Statistical analyses were done using SPSS Professional Statistics ver. 7.5 (SPSS Inc., Irvine, California).

RESULTS AND DISCUSSION

Reducing sugar, total sugar and starch

The effects of foliar application of HA, Mn and Mo on the content of reducing sugar, total sugar and starch are presented in Table 2. The data showed that, foliar application of HA_2 alone (~1.73 fold) significantly increased the concentration of reducing sugar as compared to that of control (HA_1) treatment. However, Mn_2, HA_2Mn_2 and HA_2Mo_2 applications also increased the concentration of reducing sugar but not significantly. The highest concentration of reducing sugar was observed from HA_2Mn_2 spray.

In case of the total sugar content, combination treatments such as HA×Mn (HA_2Mn_2) and HA×Mo (HA_2Mo_2) showed significant effect on content of total sugar. Whereas the rate of this increase was higher, 1.21 fold in HA_2Mn_2 treatment and decrease was lower, 1.04

fold in HA_2Mo_2 treatment as compared to that of their corresponding values of control (HA_1Mn_1 and HA_1Mo_1) treatments. However, HA_2 and Mn_2 applications also increased the concentration of total sugar than the control (HA_1 and Mn_1) treatments, though not significantly. The highest value was obtained from HA_1Mn_2 application.

Regarding starch content, all the foliar applications except HA×Mn (HA_2Mn_2) significantly increased as compared to that of the control treatment, whereas the rate of this increase was higher: 1.34 fold in HA_2, 1.05 fold in Mn_2, 1.07 fold in Mo_2 and 1.22 fold in HA_2Mo_2 treatments, respectively, than that of their corresponding values of control treatments. While HA_2Mn_2 spray also increased the starch content but not significantly.

Many researchers have shown that humic acid, manganese and molybdenum, influence the utilization of carbohydrates in crop plants. In recent years, Unlu et al. (2011) reported the total sugar and reducing sugar contents were significantly influenced by foliar spray of humic acid as compared to that of control in cucumber fruit, and humic acid also caused a significant increase in soluble sugars in lilium (Parandian and Samavat, 2012). Mousavi et al. (2007) reported significant increase in the starch content as compared to the control in potato, and foliar application of Mn also increased the concentration of reducing and non reducing sugar as compared with the control in sweet orange (Tariq et al., 2007). An adequate supply of Mo leads to complex changes in the concentrations of total sugars and reducing sugars in the leaves and stems of cauliflower plants (Agarwala and Hewitt, 1955). In the presence of Mo deficiency, low sugar concentrations are related to failure of photosynthesis and non-utilization of carbohydrates for plant growth.

Ascorbic acid, total phenol, free phenol, carotene and capsaicin

Ascorbic acid is one of the most important hydrophilic antioxidants that scavenge harmful free radicals and other ROS and also regenerate other antioxidants like tocopherol to its functional state. Our present experiment (Table 2) reveals that significant effects were observed in application of individual treatments (HA_2, Mn_2 and Mo_2), whereas significant increase in HA_2 (~1.21 fold) and Mn_2 (~1.13 fold) treatments and significant decrease in Mo treatment as compared to that of their control (HA_1, Mn_1 and Mo_1) treatments. Results were found to be in agreement with that of Tariq et al. (2007), who reported that foliar application of Mn increased the concentration of ascorbic acid of juice of sweet orange as compared to the control and Mehdi et al. (2008) also reported increase in ascorbic acid content of barberry plant due to foliar application of Mn. Moreover, Mo deficiency sharply decreases the contents of ascorbic acid (vitamin C) in several crops such as cauliflower (Agarwala and Hewitt, 1954), leguminous plants (Avdomin and Arens, 1966) and potato (Munshi and Mondy, 1988) tubers. The higher concentration of ascorbic acid was obtained from HA_2Mo_1 followed by HA_2Mn_2 treatment.

Pepper fruit contain high levels of phytochemicals that can contribute to antioxidant activity, such as phenolic acids; important components that may decrease the risk of degenerative diseases (Hasler, 1998; Larson, 1988). Unlu et al. (2010) reported that the total soluble phenolics content significantly increased in response to HA application in pepper fruit. Our present experiment (Table 2) also found that HA_2 application alone significantly increased content of total phenol as compared to that of the control treatment. While Mo_2 application alone signify-cantly decreased as compared to the control treatment. Moreover, decreasing concentration of total phenolic compounds in potato tubers under Mo concentration was reported by Munshi and Mondy (1988). However, Mn_2 ap-plication also increased the concentration of total phenol but not significantly.

In the case of free phenol content, there was significant increase by foliar sprays of HA_2, HA $_2Mn_2$ and HA_2Mo_2 as compared with the control treat-ment; while Mn_2 application also increased the concen-tration of free phenol, though not significantly. The higher values of total phenol and free phenol were exhibited by application of A_2Mo_1 and HA_2Mn_2, respectively.

Carotenoids are a widespread group of naturally occurring fat-soluble colorants, β-carotene which is the most abundant carotenoid in human diet, its ability and degradation products undergoes single electron transfer-based reactions (SET). Unlu et al. (2010) reported that the foliar application of HA significantly increased in content of total carotenoids and β-carotene in pepper fruit, our results also observed that the foliar application of HA significantly increased in the content of carotene in green pungent pepper (Table 2). Zhang et al. (2012) reported that the application of Mo in Chinese cabbage under salt stress significantly increased the content of carotene; we showed that the carotene content was also significantly increased in application of Mo (Mo_2). However, the Mo supply also influences the contents of carotenoids in the leaves of sugar beets (*Beta vulgaris* L.) (Godnev and Lipskaya, 1995) and grapevines (*Vitis vinifera* L.) (Lakiza, 1980).

Our present experiment reveals that with respect to carotene content, there was significant increase in all treatments except HA×Mn only, in which there was also increase, though not significant. The higher value was obtained from spray of HA_2Mn_2 followed by HA_2Mo_2.

Capsaicin, the phenolic pungent principle of pepper competes with phenol synthesis (Sukrasno and Yeoman, 1993). In our present study (Table 2), data revealed that the capsaicin content significantly increased in all treatments, except Mo, in which (Mo_2) capsaicin concentration decreased as compared to their corresponding control (Mo_1) treatment. The highest value

Table 2. Changes in some biochemical characteristics in response to foliar applications of chelator and micronutrients in green pungent pepper fruits.

Main effect	Reducing sugar (mg g^{-1})	Total Sugar (mg g^{-1})	Starch (mg g^{-1})	AAC (mg g^{-1})	TPC (mg g^{-1})	FPC (mg g^{-1})	Carotene (mg g^{-1})	Capsaicin (mg g^{-1})	POD (mM *o*-dianisidine oxidized•min^{-1} g^{-1})	PP (ΔA•^{1}g
Chelator										
HA_1	4.882^{bz}	30.298	249.595^{b}	1.078^{b}	1.612^{b}	1.198^{b}	0.322^{b}	5.125^{b}	0.119^{b}	0.9
HA_2	8.422^{a}	31.588	283.683^{a}	1.300^{a}	2.073^{a}	1.408^{a}	0.435^{a}	7.048^{a}	0.380^{a}	1.0
Micronutrients										
Mn_1	6.333	29.092	259.554^{b}	1.118^{b}	1.799	1.293	0.353^{b}	5.870^{b}	0.282	0.8
Mn_2	6.971	32.795	273.724^{a}	1.260^{a}	1.886	1.313	0.404^{a}	6.303^{a}	0.296	1.1
Mo_1	6.726	32.167	257.165^{b}	1.293^{a}	1.943^{a}	1.322	0.338^{b}	6.220	0.214^{b}	1.1
Mo_2	6.578	29.720	276.113^{a}	1.086^{b}	1.742^{b}	1.285	0.419^{a}	5.953	0.365^{a}	0.8
Chelator × Micronutrients										
HA_1										
Mn_1	5.033	24.292	242.108	1.038	1.627	1.262	0.303	5.158	0.239	0.6
Mn_2	4.730	36.305	257.082	1.118	1.597	1.135	0.340	5.092	0.159	1.3
HA_2										
Mn_1	7.632	33.892	277.00	1.198	1.972	1.325	0.402	6.582	0.326	0.9
Mn_2	9.212	29.285	290.367	1.402	2.175	1.492	0.468	7.515	0.434	1.0
HA_1										
Mo_1	5.582	28.727	243.780	1.140	1.662	1.267	0.258	5.703	0.182	0.9
Mo_2	4.182	31.870	255.410	1.017	1.562	1.130	0.385	4.547	0.216	1.0
HA_2										
Mo_1	7.870	35.607	270.550	1.445	2.225	1.377	0.417	6.737	0.246	1.3
Mo_2	8.973	27.570	296.817	1.155	1.922	1.440	0.453	7.360	0.513	0.6
Analysis of variance (F values)										
HA	5.483*	NS	244.014**	22.659**	60.070**	76.819**	34.000**	129.494**	17.515**	N
Mn	NS	NS	42.164**	9.255**	NS	NS	7.066*	6.573*	NS	7.7
Mo	NS	NS	75.295**	19.696**	11.462**	NS	17.654**	NS	12.127**	5.3
HA×Mn	NS	18.728**	NS	NS	NS	37.471**	NS	8.751*	4.775*	6.7
HA×Mo	NS	8.474*	11.247**	NS	NS	17.419**	5.360*	27.718**	7.304*	8.3

*Significant at 5%, **significant at 1%; NS, non significant. ^{z}Values followed by the same letter in a column are not significant at $P < 0.05$; HA_1, control of humic acid (0.0%); HA_2, dose of humic acid (0.05%); Mn_1, control of manganese (0.0%); Mn_2, dose of manganese (0.05%); Mo dose of molybdenum (0.01%).

of capsaicin content was observed by foliar application of HA_2Mn_2.

POD, PPO and CAT

Peroxidase (POD) activity plays a crucial role in defense mechanisms against oxidative stress, possibly in co-operation with other antioxidant concentrations. In our present study (Table 2), it was shown that the POD activity significantly increased in all treatments, except Mn, whereas the rate of this increase was higher: 3.19 fold in HA_2 treatment, 1.17 fold in Mo_2 treatment, 1.81 fold in HA_2Mn_2 treatment and 2.81 fold in HA_2Mo_2 treatments, respectively, than that of their control (HA_1, Mo_1, HA_1Mn_1 and HA_1Mo_1) treatments.

While foliar application of Mn_2 also increased POD activity in green pungent pepper, through not significantly. However, reduced POD activity by application of Mn in apple trees (El-Shazly and Dris, 2004) and in grapefruit trees (Bar-Akiva et al., 1976; Carpena et al., 1976b) were reported. The highest activity was obtained by application of HA_2Mo_2 treatment.

Polyphenol oxidase (PPO) proteins containing copper that catalyzed oxidation of hydroxyphenols to their quinine derivatives, are spontaneously polymerized. Enzymatic browning in fruits and vegetables are due to presence of PPO and is often responsible for unpleasant sensory qualities and losses in nutrient quality (Sanchez-Amat and Salano, 1997). Munshi and Mondy (1988) reported that under Mo application, declined activity of PPO (catechol) in potato tubers was observed. In our present experiment (Table 2), all the treatments showed significant effect on PPO activity in green pungent pepper, whereas Mn_2 (2.19 fold) and HA $_2Mn_2$ (1.65 fold) applications was significantly increased and Mo_2 (1.36 fold) and HA_2Mo_2 (1.50 fold) applications was significantly decreased as compared to their corresponding control (HA_1, HA_1Mn_1, Mo_1 and HA_1Mo_1) treatments.

Moreover, as per literature survey, many researchers reported that Mn toxicity induced the activity of PPO in cotton plants (Sirkar and Amin, 1974), in tobacco leaves (Nable et al., 1988) and in grape (Mou et al., 2011). High polyphenol oxidase activity in general, is a typical feature in tissues with high manganese contents (Horst, 1988). However, our study reveals that lower HA_2 treatment also increased activity of PPO, though not significantly, than that of control (HA_1) treatment. While Kesba and El-Beltagi (2012) reported that HA application induced PPO activity in nematode infected grape roots. The highest activity of PPO was found in treatment HA_2Mo_1 followed by HA_1Mn_2.

Catalase (CAT) catalyses the dismutation of H_2O_2 generated in peroxisomes by β-oxidation of fatty acids and the glycolate cycle into water and O_2 (Mckersie et al., 1996). Molybdenum deficiency decreased the activity of CAT in papaya (Agarwala et al., 1986).

Sun et al. (2006) reported that adequate supply of Mo significantly increased the activity of CAT in wheat at normal temperature. Kesba and El- Beltagi (2012) reported that HA application significantly enhanced CAT activity in nematode infected grape roots. Our results (Table 2) revealed that application of HA_2 and Mo_2 significantly increased CAT activity as compared to that of control (HA_1 and Mo_1) treatments. Whereas, rate of this increase was higher, 1.43 fold in HA_2 treatment and 1.29 fold in Mo_2 treatment than that of their control (HA_1 and Mo_1) treatments. Moreover, other treatments such as Mn_2, HA_2Mn_2 and HA_2Mo_2 application also increased the CAT activity, though not significantly. The highest activity was obtained by application of HA_2Mo_2 followed by HA_2Mn_2.

Total antioxidant activity

The antioxidant activity of fruits and vegetables is important for assessing their nutritional value (Rice-Evans et al., 1996) and this measurement allows the evaluation of this nutritional variable without analysis of each antioxidant compound (Pellegrini et al., 1999; Scalfi et al., 2000). Antioxidant activity was analyzed under different systems of assay.

In our present experiment (Table 2), antioxidant activity was expressed as molybdate reducing antioxidant power (MRAP). The MRAP values of green pungent pepper was significantly increased in application of HA (HA_2) as compared with that of control (HA_1) treatment, this result is in good agreement with the results of Unlu et al. (2010), who also found that the antioxidant activity (TEAC) increased significantly with HA application in pepper fruit.

Moreover, Mn (Mn_2) and HA×Mn (HA $_2Mn_2$) application dramatically increased as compared to the control treatment. While Mo (Mo_2) application alone produced decreasing results as compared to the control treatment. The higher activity was observed in application of HA_2Mo_1 followed by HA_2Mn_2 application.

Correlation among variables

There are correlations between pairs of variables (Table 3). Most hydrophilic antioxidant like ascorbic acid had significant positive correlations with phenolic compounds (total phenol free phenol and capsaicin) and oxidative enzyme (PPO).

There were also positively significant correlations between carotene with capsaicin, carotene with peroxidase, and carotene with catalase. There were significant positive correlations of total sugar with ascorbic acid, total phenol, polyphenol oxidase and molybdate reducing antioxidant power. Here also, significantly positive correlations of starch with phenolic

Table 3. Pearson's correlation matrix of all variables.

Variables	RS[a]	TS[a]	Starch	AA[a]	TP[a]	FP[a]	CAR[a]	CAP[a]	POD[a]	PPO[a]	CAT[a]	MRAP (AAE)[a]
RS[a]												
TS[a]	0.142											
Starch	0.503*	0.377										
AA[a]	0.389	0.506*	0.321									
TP[a]	0.332	0.592**	0.528*	0.823**								
FP[a]	0.569*	0.083	0.550*	0.595**	0.641**							
CAR[a]	0.211	0.413	0.785**	0.370	0.692**	0.403						
CAP[a]	0.420	0.440	0.735**	0.605**	0.770**	0.785**	0.555*					
POD[a]	0.331	-0.084	0.632**	-0.119	0.147	0.415	0.537*	0.391				
PPO[a]	0.215	0.636**	0.220	0.710**	0.554*	0.082	0.242	0.306	-0.421			
CAT[a]	0.275	0.091	0.745**	0.228	0.311	0.419	0.567*	0.516*	0.698**	-0.090		
MRAP (AAE)[a]	0.425	0.654**	0.497*	0.702**	0.830**	0.458	0.662**	0.526*	0.021	0.625**	0.085	
MRAP (GAE)[a]	0.396	0.684**	0.487*	0.663**	0.812**	0.399	0.648**	0.495*	-0.016	0.643**	0.040	0.991**

**Significant at 1%, *significant at 5%, Student's t-test. [a]RS, Reducing sugar; TS, total sugar; AA, ascorbic acid; TP, total phenol; FP, free phenol; CAR, carotene; CAP, capsaicin; POD, peroxidase; PPO=polyphenol oxidase; CAT, catalase; MRAP(AAE)(GAE), molybdate reducing antioxidant power (ascorbic acid equivalent) (gallic acid equivalent).

compounds, carotene, POD, CAT and MRAP was also seen. There were also significant positive correlations between POD and CAT, total phenol and MRAP. These relationships indicate that improving the total sugar and starch to enhance phenolics concentration in green pungent pepper could accompany improvement of fruit quality as well as improve total antioxidant activity.

However, the significant positive correlations between total phenolics with antiradical power (Hanson et al., 2004), total phenolic content with antioxidant activity (Connor et al., 2002; Prior et al., 1998) and antioxidant activity with total phenolics (Maisuthisakul et al., 2008) were reported, respectively.

Principal component analysis (PCA)

In this experiment, PCA was used to summarize the treatment information in a reduced number of components, where a total of three components were chosen (PC1, PC2 and PC3) due to their Eigen value being greater than 1.0 and they together explained 94.46% of total variance (Table 4).

The first principal component (PC1) explained 61.57% of total variance, in which all the variables were positively loaded (Table 4).

Therefore, on the basis of the first principal component, the treatments such as HA_2Mn_2 followed by HA_2 and HA_2Mo_2 can be selected as performers.

The second principal component (PC2) explained an additional 24.66% of total variance, in which an increase in reducing sugar, starch, free phenol [gallic acid equivalent; (GAE)], carotene, capsaicin, POD and CAT was associated with a decrease in total sugar, ascorbic acid, total phenol (GAE), polyphenol oxidase (PPO), molybdate reducing antioxidant power (ascorbic acid equivalent; [MRAP (AAE)] and MRAP (GAE) (Table 4). Based on PC1 vs. PC2, the performing treatments such as HA_2Mo_2 followed by Mo_2 can be selected as having all desirable trails (Figure 1).

The third principal component (PC3) explained another 8.23% of total variance, in which reducing sugar, ascorbic acid, GAE, free phenol (GAE), and capsaicin were positively loaded in contrast to total sugar, starch, carotene, POD, PPO, CAT, MRAP (AAE) and MRAP (GAE), which were negatively loaded (Table 4). According to the plot of regression factor scores due to PC1 vs. PC3 (Figure 2), the treatment HA_2Mn_2 can be selected as performers.

Conclusions

Based on principal component analysis and average values, foliar application of HA_2Mn_2 and HA_2Mo_2 had good performers with respect to all variables, which may bring about the proper value addition in green pungent pepper fruits by enhancing the carbohydrate constituents, antioxidant constituents and antioxidant activities.

ACKNOWLEDGEMENT

We would like to thank the Bidhan Chandra Krishi Viswavidyalaya for supporting this work.

Table 4. Results of principal component analysis (PCA) for changes in some biochemical characteristics in response to foliar applications of chelator and micronutrients in green pungent pepper fruits.

Principal component	Eigen value	Variance (%)	Cumulative variance (%)
Eigen values and variance accounted for (%) by PCA based on correlation matrix			
1	8.00	61.57	61.57
2	3.20	24.66	86.23
3	1.07	8.23	94.46
Variables	**PC1**	**PC2**	**PC3**
Factor loadings due to PCs with Eigen values greater than 1			
Reducing sugar	0.924	0.275	0.193
Total sugar	0.643	-0.547	-0.403
Starch	0.826	0.451	-0.307
Ascorbic acid	0.806	-0.489	0.309
Total phenol (GAE)	0.936	-0.228	0.204
Free phenol (GAE)	0.765	0.296	0.566
Carotene	0.818	0.256	-0.364
Capsaicin	0.882	0.222	0.277
POD	0.399	0.907	-0.108
PPO	0.604	-0.744	-0.106
CAT	0.670	0.681	-0.227
MRAP (AAE)	0.885	-0.368	-0.100
MRAP (GAE)	0.862	-0.397	-0.158

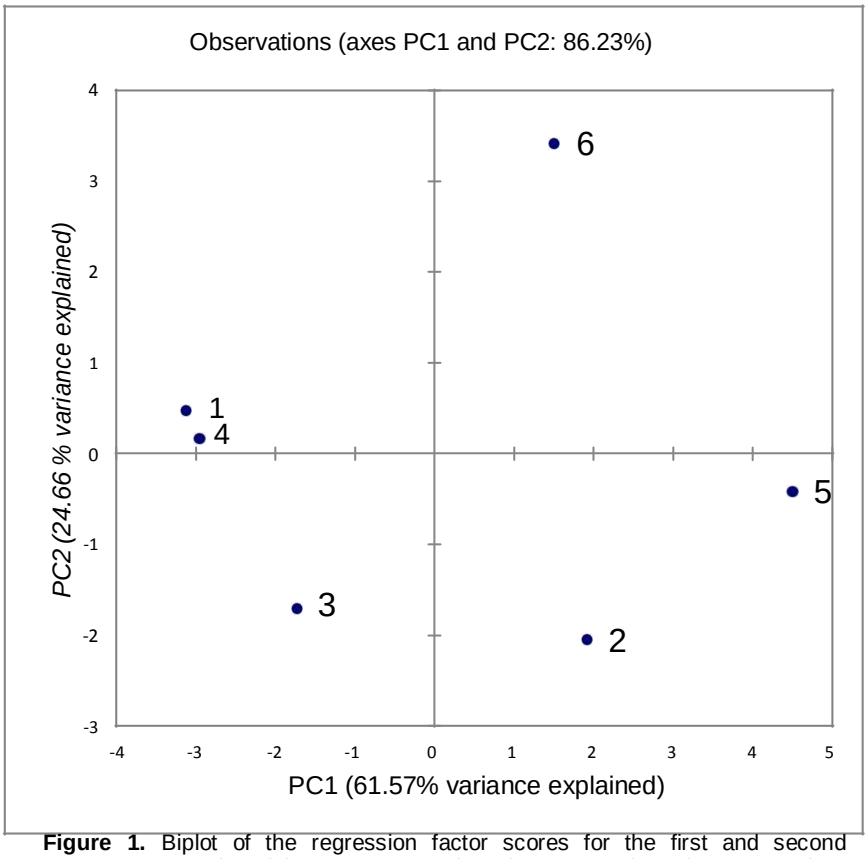

Figure 1. Biplot of the regression factor scores for the first and second components produced by PCA. Legend: 1 ($HA_1Mn_1Mo_1$); 2 ($HA_2Mn_1Mo_1$); 3 ($HA_1Mn_2Mo_1$); 4 ($HA_1Mn_1Mo_2$); 5 ($HA_2Mn_2Mo_1$); 6 ($HA_2Mn_1Mo_2$).

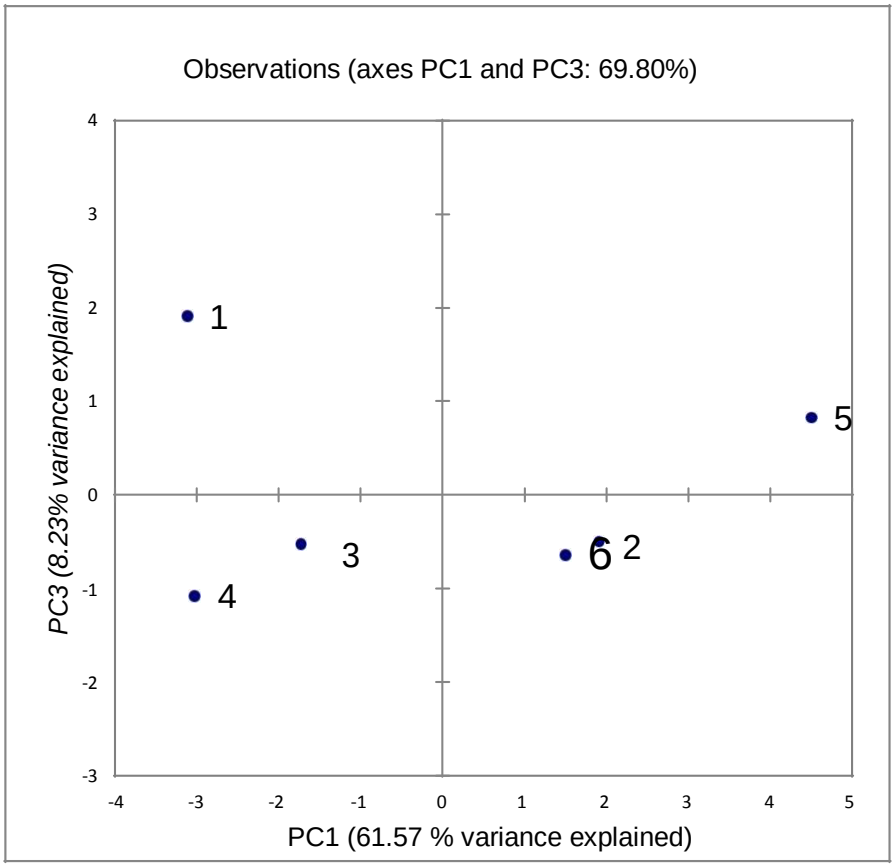

Figure 2. Biplot of the regression factor scores for the first and third components produced by PCA. Legend: 1 ($HA_1Mn_1Mo_1$); 2 ($HA_2Mn_1Mo_1$); 3 ($HA_1Mn_2Mo_1$); 4 ($HA_1Mn_1Mo_2$); 5 ($HA_2Mn_2Mo_1$); 6 ($HA_2Mn_1Mo_2$).

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