BIOFORTIFICATION: A SUSTAINABLE AGRONOMIC STRATEGY TO INCREASE SELENIUM CONTENT AND ANTIOXIDANT ACTIVITY IN GARLIC

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Abstract. The process of breeding to enhance nutrients, such as vitamins and minerals, in food crops through biofortification is a sustainable, eco-friendly and powerful strategy to overcome micronutrient deficiency. Therefore, the aim of the present investigation was to increase selenium content in edible parts of garlic (*Allium sativum* L.) supplied with either 20g/ha or 50g/ha aqueous solution of anhydrous sodium selenate. This was done either through foliar spray or soil flood application under open-field conditions. Results indicated that the 50g/ha concentration of sodium selenate application in the form of foliar spray significantly enhanced the selenium content in garlic bulb (3.23±0.16mgSe/Kg) and vegetative part (15.46±0.71mgSe/Kg) that is, a 12.52 and 7.8 fold increase was observed respectively, as compared to control. A significant increase in total phenolic content (4.72±1.79GAE/100g), total flavonoid content (18.50±1.82 mgQE/100g) and total antioxidant capacity (IC_{50} of 0.81mg/ml determined through DPPH radical scavenging assay) was also observed in the bulbous part of garlic. The results suggested that the consumption of 16g of dried garlic bulb, biofortified with 50g Se/ha, could cover the daily recommended dose of selenium for human beings. Selenium biofortified garlic crop can hold a market value as selenium functional food and can be used as an alternative to synthetic selenium supplements to overcome selenium deficiency.
Keywords: *Allium sativum; antioxidant activity; flavonoid content; malnutrition; polyphenols*

Introduction

Many people, particularly those living in developing countries are facing a silent crisis of malnutrition or hidden hunger (Swaminathan, 2012, Muthayya et al., 2013). About half of the world's inhabitants suffer in macronutrients and micronutrients deficiency which is responsible for 20 million adult deaths and more than five million childhood mortalities annually (Zhao and McGrath, 2009, Bouis and Welch, 2010) . In developing countries many food systems cannot provide adequate micronutrients to meet the demands of their citizens, especially families having fewer income resources (Bouis and Welch, 2010, El-Ramady et al., 2015). It is reported that among the six billion people in the world more than 15% are suffering from selenium deficiency (Fordyce, 2013). A range of chronic diseases have been associated with selenium deficiency including Keshan (an endemic congestive cardiomyopathy in China, with high rate of fatality) (Yang et al., 2008), cardiovascular diseases (Kardinaal et al., 1997, Oropeza-Moe et al., 2015), cancer, viral diseases (Clark et al., 1996, Beck et al., 2003), inflammatory conditions, diabetes mellitus, hepatopathies and HIV infection (Holben and Smith, 1999, Burk et al., 2015). Under this scenario, boifortification offers a cost

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effective and sustainable strategy in modern agriculture to allow the access of more nutritious and healthy food to large population (Bouis et al., 2011, Carvalho and Vasconcelos, 2013).

Biofortification is the process of increasing the bioavailable micronutrient density of staple crops through conventional plant breeding and modern biotechnology to achieve a measureable and positive impact on human health (Pfeiffer and McClafferty, 2007). Such an intervention can be used to enhance the uptake and accumulation of specific nutrients in edible part of plants (Rouached, 2013). It is attained through genetic engineering, conventional breeding and manipulation of agricultural practices such as rhizosphere fertilization, soil and crop management strategies (Zuo and Zhang, 2011) etc. Selenium is a vital micronutrient for human beings and animals (Hartikainen, 2005, Lobanov et al., 2008). Plant based foods are significant nutritional sources of selenium (Se) supply for both human beings and livestock to meet their daily requirement for selenium. For adults, daily consumption of 40 to 50μg selenium for women to 75 μg for men and 8.7-10 μg for infants is recommended and an intake exceeding 400μg/day is assumed to be toxic (Burk, 2002, Fordyce, 2013). Selenium poisoning, referred to as selenosis is related to dietary intake of approximately 5 mg of selenium per day. In 1960, an outbreak of endemic human selenosis was reported in China, associated with the consumption of food containing more than 300mg/Kg selenium (Fordyce, 2013). In almost all European countries, the selenium fortified foods and the use of dietary selenium supplements are quite popular to overcome the Se deficiency (Yadav et al., 2007).

To overcome the animal Se deficiency, different practices are commonly employed, e.g., the use of dietary supplements, injections, salt licks and drenches (Yadav et al., 2007). Alternatively, consumption of selenium enriched plants and their products is beneficial because selenium present in organic form is more bioavailable than in inorganic form (Terry et al., 2000, Li et al., 2017). The process of selenium accumulation in agricultural plants varies according to the plant species, soil properties and the chemical nature of selenium (Mikkelsen et al., 1989).Vegetable crops belonging to the Allium family (*Allium sativum, Allium cepa,* etc) are important part of the human daily diet. Biofortification of the vegetables, known as seleniferous plants, can contribute to the alleviation of selenium deficiency. Among these alliaceous species, garlic is one of the most popular vegetables around the globe (Ghasemi et al., 2015). In 2007 according to FAO (United Nations Food and Agriculture Organization), approximately 1.01million hectare of land was used to produce about 10 million metric tons of garlic annually in the world. China is the largest producer of garlic, accounting more than 75% of the global production (Chen et al., 2013). *Allium sativum* has the ability to uptake the inorganic Se from the soil through the roots and is able to convert it into organic forms that are accumulated in its edible parts (Yadav et al., 2007). The chemistry of selenium in seleniferous plants simply relates to sulfur chemistry because selenium share great likeness in its chemical properties with sulfur and exists in oxidation states as elemental selenium (Se^0), selenide (Se^{2-}), selenite (Se^{4+}) and selenate (Se^{6+}). Within biological systems, selenium is incorporated as a constituent of selenocysteine (SeCys) and selenomethionine (SeMet) amino acids during translation of primary structure that comprise selenoprotiens. They are stored in the form of selenium methylselenocysteine (SeMSC) in seleniferous plants including garlic, Indian mustard (*Brassica juncea* L.), onion *(Allium cepa*), broccoli (*Brassica oleraceae* L.), sugar beet (*Beta vulgaris* L.)etc (Zayed et al., 1998, Fordyce, 2013). The main available form of

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selenium to plants is sodium selenate which is actively taken up by seleniferous plants through sulfate transporter and assimilated as organic form (SeMet and SeCys) with the help of enzymes, including ATP reductase, ATP sulfurylase, SeCys-lyase and O-acetyl serine transferase (*Figure 1A*) (Adhikari, 2012).

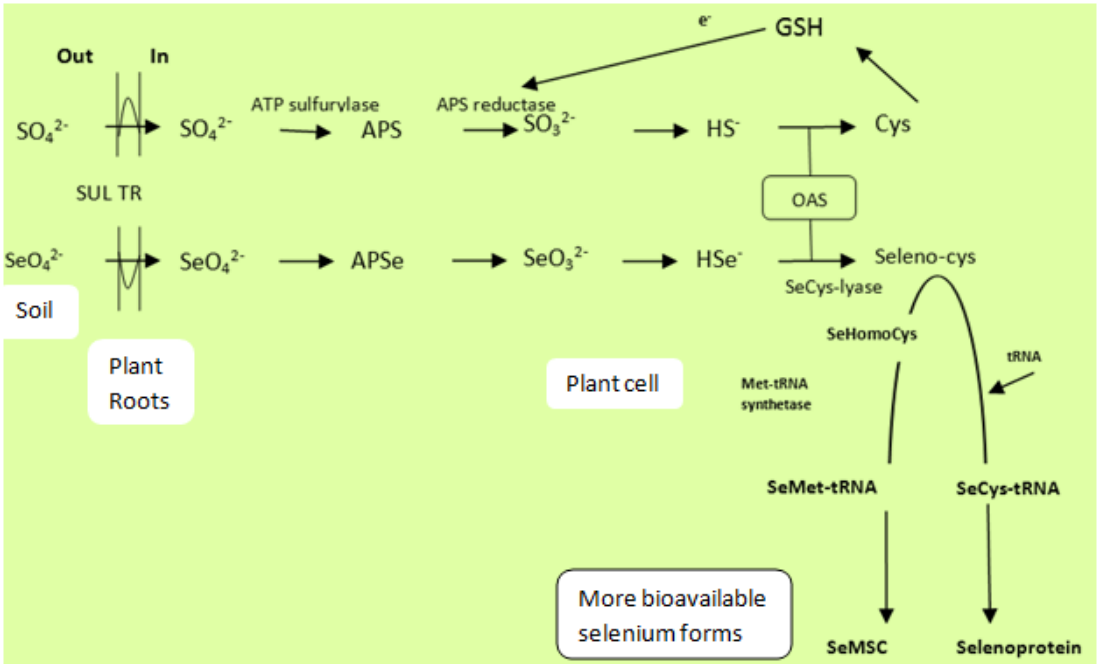

Figure 1A. *Flow diagram showing sulfur and selenate uptake and assimilation pathway in seleniferous plants. SeCys-lyase is the enzyme that is highly specific to selenium substrate. Sulfate transporter (SULTR), 5' adenylylsulfate (APS), 5' adenylylselenate (APS), O-acetylserine (Rehse et al., 2016)*

In the past few decades, interest of scientists in naturally occurring compounds that act as antioxidants and regarding particularly dietary selenoenzymes has been increasing. Selenoenzymes play a vital role in protecting the body from oxidative damage/harmful effects of reactive oxygen species (ROS) and contain one or more unpaired electrons (Birringer et al., 2002). ROS are produced either from external sources, such as chemicals/pollution, or from internal sources e.g. aerobic respiration. They react quickly with other compounds and a chain reaction starts as the other molecule loses electrons and becomes a free radical. The result is the oxidation of vital cellular parts like DNA and proteins, the disintegration of cell membrane that lead to diseases (Kaur and Kapoor, 2002). In human beings, important selenoproteins(also known as selenoenzymes) are catalase, glutathione peroxidase (GPx) and superoxide dismutase which act as antioxidant and protect cells from ROS (Steinbrenner and Sies, 2009). *Allium sativum* is a natural source of various bioactive phytomolecules, including selenoproteins, allyl thiosulfinates, flavonoids, organosulfur compounds, phenolic acids and vitamins (Choi et al., 2014). Previous studies have reported on the health-promoting benefits of garlic because of its biologically active phenolic compounds with interesting medicinal properties (González-Morales et al., 2017). The extract of garlic has a remarkable antioxidant capacity and provides protection from oxidative DNA damage (Park et al., 2009), decreases the risk of chronic diseases,

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mitigates atherosclerosis and cancer (Morihara et al., 2010) etc. The objective of this study is to enrich garlic with selenium through biofortification, to analyze its impact on garlic quality parameters including phytochemical content and biological potential as free radical scavenging property. Additionally, there is possibility to introduce the selected garlic species for selenium phytoextraction in to selenium laden soils of Punjab Pakistan.

Materials and Methods

Site description and Experimental design

A field experiment was conducted at botanical garden of PCSIR Labs Complex, Lahore, Pakistan. Local garlic variety was sown season in January 2016 and harvested in May 2016. The experimental site is situated between 31.52º North latitude, 74.33º East longitude at the altitude of 217 m above sea level. A randomized complete block (RCB) design with three replicates was used with two factors (conc. of selenium salt applied and way of applications). Garlic sets were planted in the field divided into five plots. Treatments were control (no selenium application), selenium foliar spray and selenium soil flooding and two selenium salt concentrations (20g/ha and 50g/ha). Standard agronomic practices were used. The size of each individual plot was 2 m length x 3.66m width= 29.28 m^2, with a density of 15 plants per square meter. Each plot was consisted of 11 rows, with 6 plants in each row and the distance between rows was 0.65 m. A basal dose of N-P-K in ratio of 11-5-18 kg/ha was applied prior to planting. Melathion was sprayed as a herbicide after 8 weeks of sowing.

Chemicals

All chemicals were analytical grade. sodium selenate, Folin Ciocaltaeu reagent, ascorbic acid, butylated hydroxyl Toluene (BHT), sodium carbonate, aluminium chloride potassium acetate, quercetin and gallic acid were purchased from sigma aldrich chemical Co (St.Louis, MO, USA). DPPH dye was purchased from Alfa Aesar, Germany. Ethanol, hydrochloric acid, dimethylsulphoxide (DMSO), methanol were obtained from Merck (Darmstadt, Germany).

Selenium treatments

Selenium was applied as sodium selenate (Na_2SeO_4) in the experimental field. Aqueous sodium selenate solution (1.0 g/Liter) was applied at the concentration of 20g/ha) and 50g/ha in two ways i.e. by foliar spray and through water flooding in selected plots. First application was carried out on day 9 and afterward 30 days interval from sowing, during whole growing season (*Figure 1B*). Garlic crop was harvested in May after120 ± 2 days of growth.

Preparation of sample

Finely ground sample 0.5 g was accurately weighed in a china crucible. The sample was kept in the muffle furnace at 500ºC for 4-6 hours or until white ash is formed. The ash was dissolved in 5 ml of 6 N HCl by heating on a hot plate, filtered through Whatman no.1 filter paper and the final volume was made up to 100 ml with double distilled water (AOAC, 1990).

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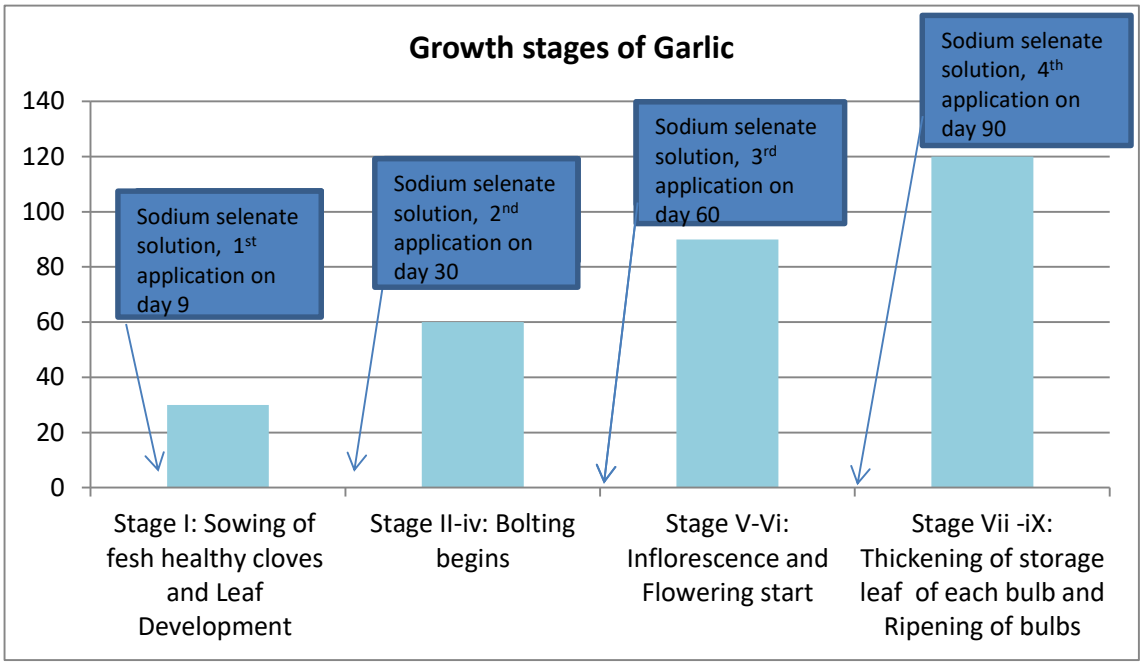

Figure 1B. *General symbolic representation of growth stages of garlic (I to ix) along with selenium application at various time periods. Information related to garlic growth stages was obtained from Meier et al. (2009)*

Determination of selenium by inductively coupled plasma-mass spectrometer using dynamic reaction cell

Shimadzu Sequential type plasma Emission Spectrometer model ICPS-1000 111 and JY 24 spectroanalyzer (ICP-MS) was used for the determination of Se. A glass Meinhard nebulizer and a glass cyclonic spray chamber were used to introduce the sample. Experimental Instrument Conditions were RF Power 1200 watts, plasma gas flow 15 L min^{-1},auxiliary gas flow 1.2 $Lmin^{-1}$, RPq 0.5, cell gas (O_2)flow rate(DRC) 0.4 $Lmin^{-1}$. A series of standards containing selenium (0.01 -0.5 mg/L) were prepared from standard stock solution of Se (1000 ± 2mg/L, Merck, Darmstadt, Germany), and used to calibrate instrument. Standard solutions of Selenium with 1.0 to 50 mg L^{-1} were used for quantification. Method validation was performed by analyzing three replicates of artificially spiked garlic powder with a final concentration of 5mg/kg of selenium. A standard deviation of 0.05 mg/kg and coefficient of variation of 2.71% was obtained with a recovery of 97%. The limit of detection (LOD=3SD) as calculated by the Eurachem Guide (Guide, 1998) was 0.15mg/kg. Measurement of uncertainty of the method (K=2) was 0.03.

Phytochemical analysis

An appropriate amount (20g) of garlic foliar mass and bulb powder were separately extracted in 80% ethanol by stirring at 25℃ for 24h in closed vessel system, according to the method described by Peschel et al. (2006) with minor modification. After solvent evaporation under vacuum, extracts were resuspended in DMSO and stored at 4 °C.

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Estimation of total phenolic content (TPC)

Total phenolic content of each treatment (garlic bulb & foliar mass) were determined by using Folin Ciocalteu reagent for color development along with sodium carbonate, by following the method with slight modifications reported by Singleton and Rossi (1965). Absorbance of developed blue coloured complex was taken at 755 nm with a spectrophotometer (Nicolet, Evlution-300, Germany). TPC of extracts was quantified through the standard curve of gallic acid (r^2 = 0.9972). The results are given in mg gallic acid equivalent (GAE)/ 100 g of dry wt.

Estimation of total flavonoid content (TFC)

Total flavonoid content was estimated by using aluminum chloride colorimetric method (Chang et al., 2002). Appropriate quantity (100µl) of each sample extract was taken and mixed with suitable amount of methanol, 10% aluminum chloride and 1 M potassium acetate for development of coloured complex. After 30 minutes of incubation period, absorbance of the developed colour was taken at 415 nm with a spectrophotometer. Quercetin standard curve (r^2 = 0.9985) was used for the quantification of TFC of experimental samples and expressed in mg quercetin equivalent (QE) /100g of dry wt.

In vitro 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity

In current study the hydrogen atoms donating capacity of garlic leaves and bulb extracts were determined through DPPH free radical assay (Brand-Williams et al., 1995). Ethanolic solutions of each extract were prepared in the range of 0.02 mg/ml to 0.1 mg/ml, following the mixing of ethanolic dilutions of extract (100 µl each) with DPPH (0.1mM) solution. BHT (Butylated hydroxytoluene) and Ascorbic Acid were used as positive controls. After an incubation period of 30 minutes in the dark at ambient temperature, absorbance of reaction mixtures were taken at 517 nm through UV-spectrophotometer. Finally, duplicate measurements were taken and percentage DPPH radical scavenging ability was calculated by using *Equation 1*.

$$DPPH\ scavenging\ activity(\%) = \left\{ (Abs._{(control)} - Abs._{(sample)} / Abs._{(control)} \right\} \times 100 \quad \text{(Eq. 1)}$$

where Abs. (control) was absorbance of DPPH radical + ethanol and Abs. (sample) was absorbance of DPPH radical + sample.

Statistical analysis

All data are presented as mean ± SD. The calculated mean values were based on the data obtained from at least three independent experiments. Two ways Analysis of Variance (Webb et al., 2012) was performed by Graph pad Prism 5 at a confidence interval of 95% to see the significant difference among results (GraphPad Software). Results showing probability value of < 0.05 were considered to be statistically significant.

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Results

Fresh weight yield m^{-2}, dry matter and climatic conditions

Observations were taken for the fresh weight of plantlets both in the case of control set as well as those treated after harvest, which showed that maximum fresh wt. yield of garlic plants was obtained in treatment III (1723 ± 32.12 g/m^2) and minimum in treatment II (1653 ± 25.86 g/m^2) in comparison to control plants (1650 ± 33.82 g/m^2). Present results were non significantly different (P > 0.05) in total fresh weight yield per square meter as shown in *Figure 2*. Dry matter of garlic plants enhanced as the concentration of selenium salt was increased. The highest amount of dry matter was obtained in treatment III (13.49 ± 0.71%) and treatment I (13.01 ± 2.46%) which were non significantly different (P > 0.05) from control (12.12 ± 1.57%) (*Figure 3* and *Table 1*).

Table 1. *ANOVA for fresh weight yield of garlic*

Source of Variation	**% of total variation**		**P value**	
Interaction	0.00		1.0000	
t	75.13		0.0045	
p	0.00		1.0000	
Source of Variation	**P value summary**		**Significant?**	
Interaction	ns		No	
t	**		Yes	
p	ns		No	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	0.0000	0.0000	0.0000
t	4	5203	1301	7.553
p	1	0.0000	0.0000	0.0000
Residual	10	1722	172.2	
Number of missing values	0			
Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1696	31.50	-13.74 to 76.74
fresh wt.yeild g/m2	1664	1696	31.50	-13.74 to 76.74
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	31.50	2.400	P > 0.05	ns
fresh wt.yeild g/m2	31.50	2.400	P > 0.05	ns
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1661	-3.000	-48.24 to 42.24
fresh wt.yeild g/m2	1664	1661	-3.000	-48.24 to 42.24
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	-3.000	0.2286	P > 0.05	ns
fresh wt.yeild g/m2	-3.000	0.2286	P > 0.05	ns
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1700	36.00	-9.243 to 81.24
fresh wt.yeild g/m2	1664	1700	36.00	-9.243 to 81.24
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	36.00	2.743	P < 0.05	*
fresh wt.yeild g/m2	36.00	2.743	P < 0.05	*
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1688	23.50	-21.74 to 68.74
fresh wt.yeild g/m2	1664	1688	23.50	-21.74 to 68.74
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	23.50	1.791	P > 0.05	ns
fresh wt.yeild g/m2	23.50	1.791	P > 0.05	ns

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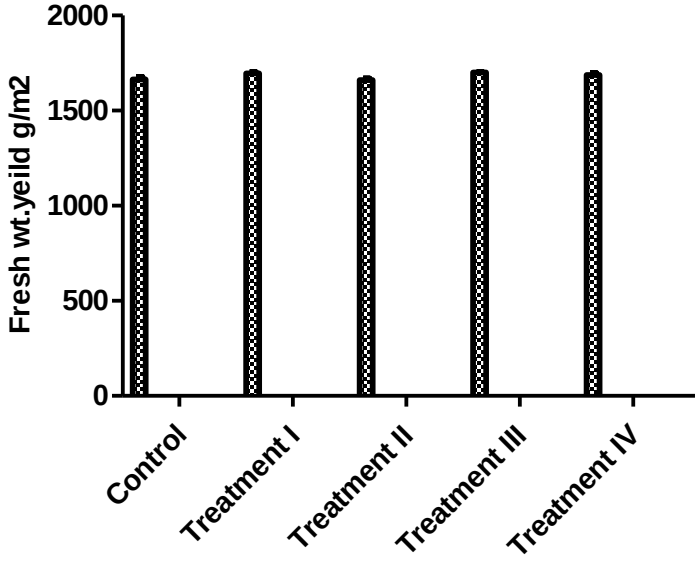

Figure 2. *Total fresh wt. yield of variously treated garlic plants. Error bars indicate standard error of the mean*

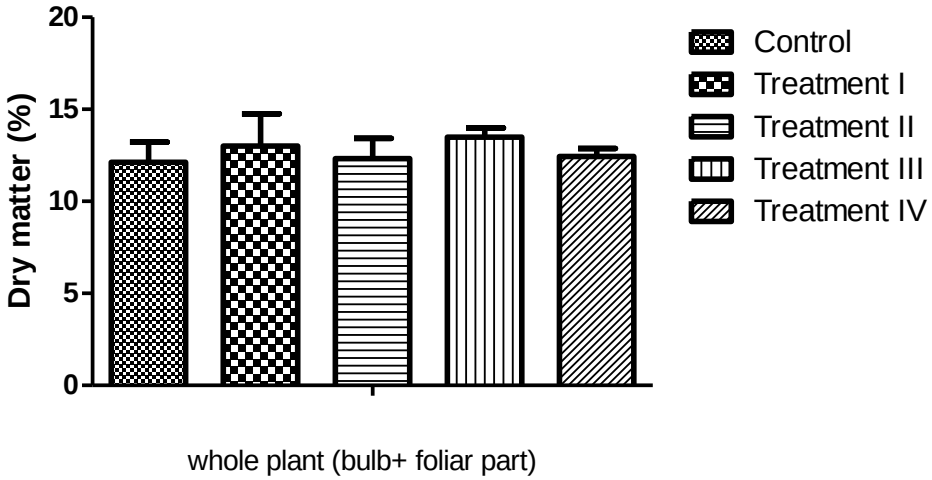

Figure 3. *Dry matter content in percentage for all four treatments and control garlic plant. Error bars indicate standard error of the mean*

Data related to monthly mean temperature (℃) and mean rainfall (mm) from the period of transplanting to harvest was collected from Pakistan Meteorological department (PMD), Lahore, Pakistan. The monthly average rainfall, maximum and minimum temperature for the garlic field location during the whole growing season were 0.62 mm, 29.05℃ and 16.13℃ respectively (*Figure 4*).

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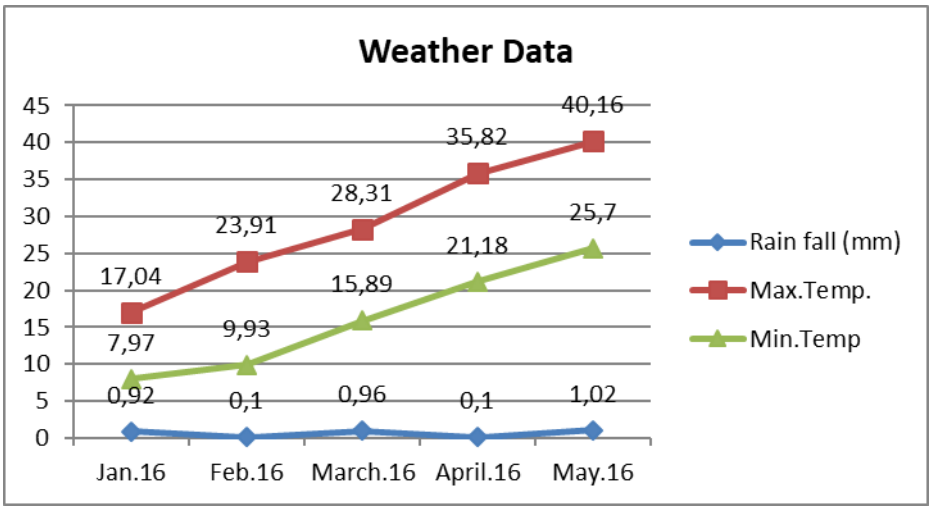

Figure 4. *Monthly average rainfall, maximum and minimum temperature recorded at local weather station during growing season*

Selenium content

Total selenium content of plant samples was determined by ICP-MS. Current results depicted that Se concentration was enhanced with increasing fertilization for all treatments. However, foliar application was found to be most effective in garlic selenium enrichment as compared to soil application (*Figure 5*). The highest average selenium concentration 3.23±0.16mgSeKg^{-1} in bulbs and 15.46±0.71 mgSeKg-^{1} in garlic vegetative part were observed in treatment III in comparison to control plant exhibiting 0.369±0.078 mgSeKg^{-1} in bulbous part and 4.96±0.49 mgSeKg^{-1} in vegetative part, respectively (*Table 2*).

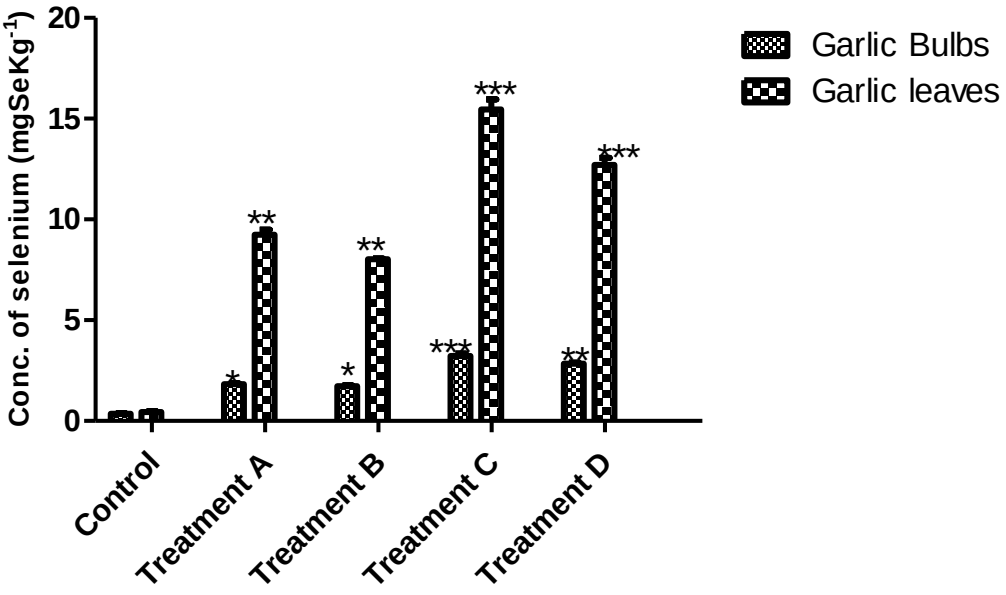

Figure 5. *Selenium concentration in foliar part and bulbs of Allium sativum subjected to four various Se treatments. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

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Table 2. *ANOVA for selenium estimation in garlic*

Source of Variation	% of total variation		P value	
Interaction	15.89		< 0.0001	
t	48.81		< 0.0001	
p	35.12		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	***		Yes	
t	***		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	83.83	20.96	229.1
t	1	257.5	257.5	2815
p	4	185.3	46.32	506.4
Residual	10	0.9146	0.09146	
Number of missing values	30			
Bonferroni posttests				
Garlic Bulbs vs Garlic leaves				
t	**Garlic Bulbs**	**Garlic leaves**	**Difference**	**95% CI of diff.**
Control	0.3550	0.4450	0.0900	-1.121 to 1.301
Treatment A	1.828	9.240	7.413	6.201 to 8.624
Treatment B	1.732	8.020	6.289	5.077 to 7.500
Treatment C	3.225	15.46	12.24	11.02 to 13.45
Treatment D	2.850	12.71	9.855	8.644 to 11.07
t	**Difference**	**t**	**P value**	**Summary**
Control	0.0900	0.2976	P > 0.05	ns
Treatment A	7.413	24.51	P<0.001	***
Treatment B	6.289	20.79	P<0.001	***
Treatment C	12.24	40.46	P<0.001	***
Treatment D	9.855	32.59	P<0.001	***

Total phenolic content (TPC)

Total phenolic content of all treated and control garlic samples exhibited significant results (*Figure 6*). The results showed that higher phenolic content are present in treatment III vegetative part (23.46±2.12 mg GAE/100g dry wt) followed by treatment IV (21.71±0.51mg GAE/100g dry wt), treatment I (19.24±0.35mgGAE/100g dry wt) and treatment II (18.82±1.17 mgGAE/100g dry wt) vegetative part, respectively. Treatment III was more effective for enhancing TPC of garlic bulbs i.e. 4.72±1.79 mgGAE/100g dry wt (1.82 fold increase) in comparison to control containing total phenolic content of 2.59±0.707mgGAE/100g dry wt which are significantly different from each other (*Table 3*).

Table 3. *ANOVA for total phenolic contents in garlic*

Source of Variation	% of total variation		P value	
Interaction	0.39		0.3750	
t	2.53		0.0044	
p	96.26		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	ns		No	
t	**		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	5.872	1.468	1.184
t	4	37.85	9.462	7.635
p	1	1442	1442	1164
Residual	10	12.39	1.239	
Number of missing values	0			

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Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	2.940	0.3500	-3.488 to 4.188
Garlic leaves	18.54	19.24	0.7050	-3.133 to 4.543
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	0.3500	0.3144	P > 0.05	ns
Garlic leaves	0.7050	0.6333	P > 0.05	ns
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	2.640	0.05000	-3.788 to 3.888
Garlic leaves	18.54	18.82	0.2850	-3.553 to 4.123
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	0.05000	0.04491	P > 0.05	ns
Garlic leaves	0.2850	0.2560	P > 0.05	ns
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	4.720	2.130	-1.708 to 5.968
Garlic leaves	18.54	23.46	4.925	1.087 to 8.763
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	2.130	1.913	P > 0.05	ns
Garlic leaves	4.925	4.424	P<0.01	**
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	3.950	1.360	-2.478 to 5.198
Garlic leaves	18.54	21.71	3.170	-0.6682 to 7.008
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	1.360	1.222	P > 0.05	ns
Garlic leaves	3.170	2.848	P < 0.05	*

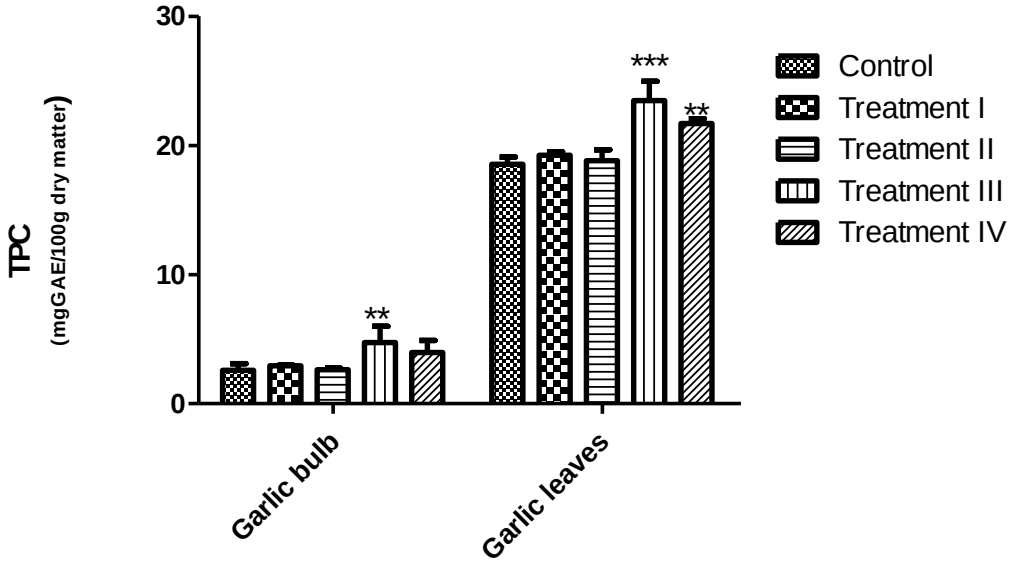

Figure 6. *Estimation of total phenolic content in foliar part and bulbs of Allium sativum subjected to four various Se treatments. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

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Total flavonoid content (TFC)

Significantly (P > 0.05) elevated amount of total flavonoid content were observed in *Allium sativum* leaves and bulb (*Figure 7*). Current results depicted that total Flavonoid content of treated *A. sativum* bulbs and leaves were in the range of 18.19 ± 1.21 to 18.50 ± 1.82 mgQE/100g dry wt and 34.13 ± 1.36 to 34.99 ± 1.54 mgQE/100g dry wt, respectively in comparison to control bulb (11.32 ± 0.95 mgQE/100g dry) and leaves (13.53 ± 0.76 mgQE/100g dry wt. as in *Table 4*).

Table 4. *ANOVA for total flavonoid contents in garlic*

Source of Variation	**% of total variation**		**P value**	
Interaction	9.20		< 0.0001	
t	37.09		< 0.0001	
p	52.77		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	***		Yes	
t	***		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	155.9	38.99	24.46
t	4	628.3	157.1	98.56
p	1	894.1	894.1	561.0
Residual	10	15.94	1.594	
Number of missing values	0			
Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.43	7.115	2.762 to 11.47
Garlic leaves	13.53	34.24	20.71	16.36 to 25.06
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	7.115	5.636	P<0.001	***
Garlic leaves	20.71	16.40	P<0.001	***
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.20	6.880	2.527 to 11.23
Garlic leaves	13.53	34.13	20.60	16.25 to 24.95
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	6.880	5.450	P<0.001	***
Garlic leaves	20.60	16.32	P<0.001	***
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.50	7.185	2.832 to 11.54
Garlic leaves	13.53	34.99	21.46	17.11 to 25.81
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	7.185	5.691	P<0.001	***
Garlic leaves	21.46	17.00	P<0.001	***
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.26	6.945	2.592 to 11.30
Garlic leaves	13.53	34.67	21.14	16.79 to 25.49
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	6.945	5.501	P<0.001	***
Garlic leaves	21.14	16.75	P<0.001	***

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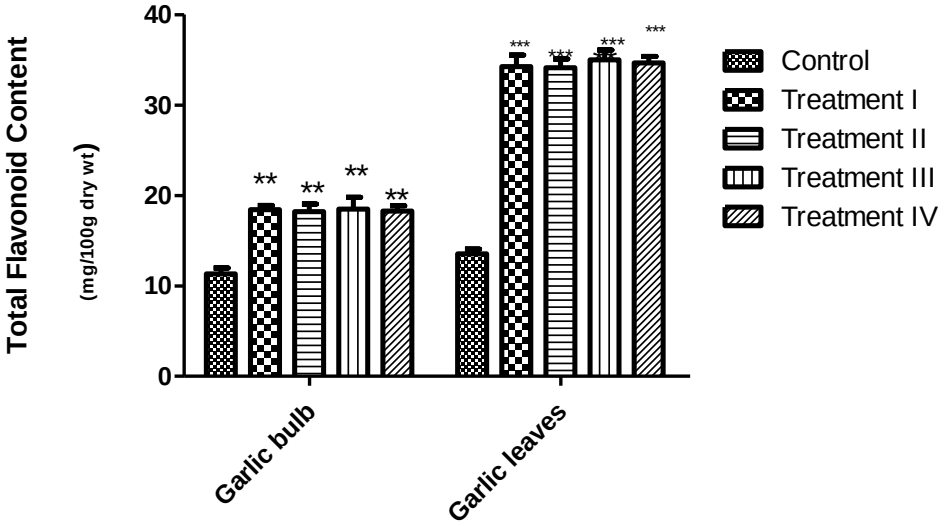

Figure 7. *Total flavonoid content in garlic bulbs and leaves subjected to four various treatments. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

In vitro antioxidant activity

The percentage DPPH radical scavenging capacity of garlic extracts with different treatments as well as controls is depicted in *Figure 8*. The IC_{50} values (sample concentration needed to scavenge 50% of DPPH dye) of all garlic extracts as well as both standards (Vitamin C and BHT) were calculated by linear regression of plots. The lowest IC_{50} indicates the higher antiradical activity of extract. Garlic extract of treatment III had an IC_{50} of 0.81 mg/ml, followed by treatment IV (0.82 mg/ml), treatment I (0.84 mg/ml) and treatment II (0.898 mg/ml). The IC_{50} value of non treated (control) garlic extract was 0.97 mg/ml. Of the two positive controls, BHT had the lowest IC_{50} (0.50 mg/ml) than vitamin C (0.51 mg/ml) but both showed significantly ($P< 0.05$) higher DPPH scavenging activity than treated garlic extracts (*Table 5*).

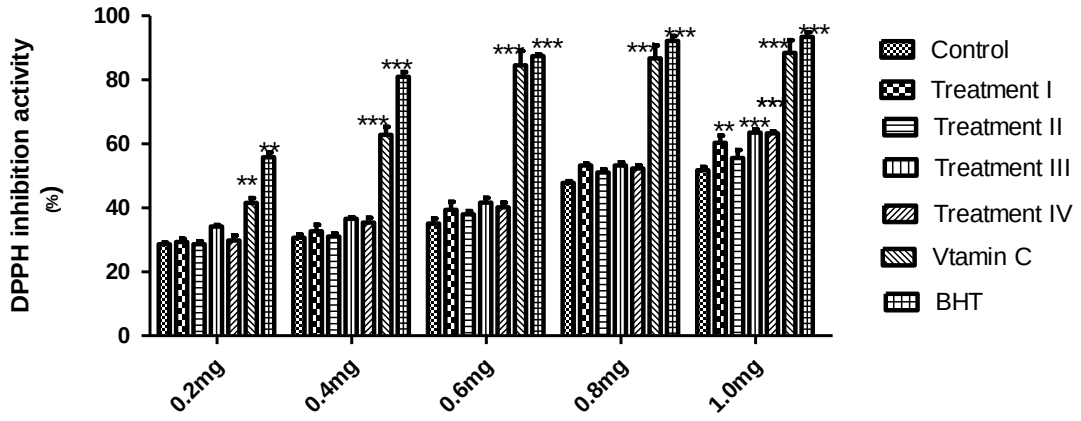

Figure 8. *DPPH radical scavenging activity of treated Allium Sativum bulbous part extracts, synthetic antioxidant BHT and Vitamin C in various concentrations. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

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Table 5. *ANOVA for antioxidant activity of garlic*

Source of Variation	**% of total variation**		**P value**	
Interaction	5.22		< 0.0001	
t	60.99		< 0.0001	
p	33.02		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	***		Yes	
t	***		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	24	1547	64.46	9.849
t	6	18080	3013	460.3
p	4	9786	2447	373.8
Residual	35	229.1	6.545	
Number of missing values	0			
Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
0.2mg	28.59	29.32	0.7300	-7.986 to 9.446
0.4mg	30.64	32.66	2.020	-6.696 to 10.74
0.6mg	35.11	39.39	4.280	-4.436 to 13.00
0.8mg	47.81	53.27	5.460	-3.256 to 14.18
1.0mg	51.74	60.30	8.560	-0.1557 to 17.28
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	0.7300	0.2853	P > 0.05	ns
0.4mg	2.020	0.7896	P > 0.05	ns
0.6mg	4.280	1.673	P > 0.05	ns
0.8mg	5.460	2.134	P > 0.05	ns
1.0mg	8.560	3.346	P<0.01	**
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
0.2mg	28.59	28.59	0.0000	-8.716 to 8.716
0.4mg	30.64	31.05	0.4100	-8.306 to 9.126
0.6mg	35.11	37.99	2.880	-5.836 to 11.60
0.8mg	47.81	51.07	3.260	-5.456 to 11.98
1.0mg	51.74	55.58	3.840	-4.876 to 12.56
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	0.0000	0.0000	P > 0.05	ns
0.4mg	0.4100	0.1603	P > 0.05	ns
0.6mg	2.880	1.126	P > 0.05	ns
0.8mg	3.260	1.274	P > 0.05	ns
1.0mg	3.840	1.501	P > 0.05	ns
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
0.2mg	28.59	34.14	5.550	-3.166 to 14.27
0.4mg	30.64	36.57	5.930	-2.786 to 14.65
0.6mg	35.11	41.63	6.520	-2.196 to 15.24
0.8mg	47.81	53.22	5.410	-3.306 to 14.13
1.0mg	51.74	63.55	11.81	3.094 to 20.53
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	5.550	2.169	P > 0.05	ns
0.4mg	5.930	2.318	P > 0.05	ns
0.6mg	6.520	2.549	P > 0.05	ns
0.8mg	5.410	2.115	P > 0.05	ns
1.0mg	11.81	4.616	P<0.001	***
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
0.2mg	28.59	29.82	1.230	-7.486 to 9.946
0.4mg	30.64	35.41	4.770	-3.946 to 13.49
0.6mg	35.11	40.16	5.050	-3.666 to 13.77
0.8mg	47.81	52.32	4.510	-4.206 to 13.23
1.0mg	51.74	63.31	11.57	2.854 to 20.29

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p	Difference	t	P value	Summary
0.2mg	1.230	0.4808	P > 0.05	ns
0.4mg	4.770	1.864	P > 0.05	ns
0.6mg	5.050	1.974	P > 0.05	ns
0.8mg	4.510	1.763	P > 0.05	ns
1.0mg	11.57	4.522	P<0.001	***
Control vs Vtamin C				
p	**Control**	**Vtamin C**	**Difference**	**95% CI of diff.**
0.2mg	28.59	41.52	12.93	4.214 to 21.65
0.4mg	30.64	62.84	32.20	23.48 to 40.92
0.6mg	35.11	84.55	49.44	40.72 to 58.16
0.8mg	47.81	86.76	38.95	30.23 to 47.67
1.0mg	51.74	88.43	36.69	27.97 to 45.41
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	12.93	5.054	P<0.001	***
0.4mg	32.20	12.59	P<0.001	***
0.6mg	49.44	19.32	P<0.001	***
0.8mg	38.95	15.22	P<0.001	***
1.0mg	36.69	14.34	P<0.001	***
Control vs BHT				
p	**Control**	**BHT**	**Difference**	**95% CI of diff.**
0.2mg	28.59	55.80	27.21	18.49 to 35.93
0.4mg	30.64	80.92	50.28	41.56 to 59.00
0.6mg	35.11	87.40	52.29	43.57 to 61.01
0.8mg	47.81	92.20	44.39	35.67 to 53.11
1.0mg	51.74	93.40	41.66	32.94 to 50.38
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	27.21	10.64	P<0.001	***
0.4mg	50.28	19.65	P<0.001	***
0.6mg	52.29	20.44	P<0.001	***
0.8mg	44.39	17.35	P<0.001	***
1.0mg	41.66	16.28	P<0.001	***

Discussion

Micronutrients malnutrition is the insufficient availability of essential dietary microminerals to the population that will negatively impact the health of people and increase the risk of diseases (El-Ramady et al., 2015). Improvement of selected nutrients such as selenium in plants edible part through the process of biofortification will increase the nutritional value of food (Hirschi, 2008) which is proved through findings of current results that selenium concentration and polyphenolic content of selected garlic cultivar was enhanced through biofortification. Present results indicated that there is no considerable difference in fresh wt yield of treated and control sets of garlic plants. These results are in consistent with the findings of Põldma et al. (2013), who reported that effects of selenium treatment on yield of onion bulb (*Allium cepa* L.) was not significant and at Se50 (50µg/ml) there was no reduction in bulb size as compared to Se100 (100µg/ml). However, these observations are contradictory to Yadav et al. (2007) who reported that leaves and bulbs of *Allium cepa* were reduced in size at high concentration of 50µg/g Se spiked soil. High concentration of selenium (50g/ha) foliar application enhances the dry matter content of the whole plant. Current findings showed that on increasing the concentration of available selenium salt (Na_2SeO_4), accumulation of selenium content was increased in garlic plants. Foliar application of $20mgSem^{-2}$ and $50mgSem^{-2}$ to garlic plants resulted in 7.8 and 12.52 fold increase of selenium content in garlic bulbs as compared to control. Similarly, 3.52 fold increase of selenium content was observed in vegetative part of garlic plants on $50mgSem^{-2}$ foliar spray (*Figure 5*), which could be used as fodder for animals to

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improve their nutritional value regarding selenium content.Hegedűsová et al. (2017) reported that foliar application of selenium salt to Ambassdor pea variety at two concentrations i.e. 5mgSe/m^2 and 10mgSe/m^2 resulted in 25.4 and 49.1 fold enhancement of selenium content, depending on applied doses. Similar observations were reported by Yadav et al. (2007) that selenium accumulation in tissues of *Allium cepa* was improved from 278 to 1248.8 µg/g along with increasing Se concentration from 25µg/g to 50µg/g of soil, respectively. Whanger et al. (2000) was also reported that selenium uptake of *Allium tricoccum* was enhanced with increasing concentration of available Selenium, despite the nature of experimental media including peatmoss (I), vermiculite and hydroponics (III). Seleniferous plants has the potential to mobilize inorganic form of selenium from soil, and to accumulate it in the biomass in organic form making it more bioavailable to animals and human beings, which is proved by the study of Yan and Johnson (2011). Due to this inherited ability of the crop plants belonging to Allium family, they can be grown in those geographical areas that naturally enriched with selenium loaded soil, to do the work of phytoremediation. Hasanuzzaman et al. (2010) reported that selenium accumulators have the ability to accumulate 4000mg/kg selenium without exhibiting signs of toxicity in comparison to non seleniferous plants like rice, which showed 10% yield reduction on selenium threshold level of 2mg/kg in shoot tissues. Thus biofortification can be indirectly linked with phytoremediation (Yadav et al., 2007). These selenium biofortified garlic can be exported as food commodity in those specific areas of the world such as China (Tan et al., 2002) that naturally deficient for selenium. Daily intake portion (80g) of selenium biofortified rice for 20days can significantly increase the serum selenium level, which is confirmed by Giacosa et al. (2014). Based on the results shown in *Figure 5*, it can be assumed that daily intake of 16g of dried garlic bulb procured in treatment III can cover the daily recommended dose (40ug to 50ug for adults) of selenium (Burk et al., 2003).

Food enriched with polyphenolic compounds such as phenolics, flavonols and flavonoids have been reported to exhibit strong antioxidant activities which protects the cells from damaging effects of free radicals and reduces the risk of chronic diseases (Otunola and Afolayan, 2013).In this study, selenium accumulation in garlic biomass enhances the nutritional value and antioxidant capacity of garlic plant. Significant value of phenolic content that is 4.72 mgGAE/100 g dry wt of garlic was observed in the present study. Beato et al. (2011) had reported that the total phenolic content in four garlic cultivars varied from 3.4 mg GAE/100 g dry wt to 10.8 mg GAE/100 g dry wt with a mean value of 6.5 mg GAE/100 g dry wt grown at Andalusia, Spain. They reported ferulic acid and caffeic acid were the major polyphenols present in garlic with mean values of 2.6 and 2.9 mg/kg of dry matter, respectively. In the present study, considerable amount of total flavonoid content (TFC) were observed in garlic extracts of all treatments depending on the amount of available selenium in comparison to control plant. Higher value of TFC of garlic extract (18.50 ± 1.82 mgQE/100 g dry wt) in treatment III could be related to increased concentration of available selenium salt (50g/ha). Stable DPPH free radical scavenging assay is a commonly used method for the estimation of free radical scavenging ability of various compounds (Ghasemi et al., 2015). In the current study, results showed that there was a significant ($P< 0.05$) increase in the scavenging ability of DPPH-radical as dose of garlic extract increased (*Figure 8*). This trend is similar to results of Park et al. (2009) who accounted that garlic extracts exhibited remarkable scavenging properties by reducing stable radical DPPH to yellow colored diphenyl picrylhydrazine. This could be due to the hydrogen donating

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ability of various vegetable extracts from their phenolic hydroxyl groups. Previously, Velioglu et al. (1998) reported a considerable association between phenolic content and antioxidant activity of various fruits, cereals and vegetable extracts. Kavalcová et al. (2014) reported statistically considerable value of antioxidant activity (4.05% to 5.07%) in association with polyphenolic content (260 t0 279 mg/Kg) in garlic samples collected from Pruzina, Strazov. Similarly, experimental garlic bulb obtained in treatment III exhibited a significant relation between higher value of polyphenolic content (2.59 to 4.72mg/100g) and total antioxidant capacity (93.75±1.54%).

Conclusion

The present study revealed that garlic selenium content was increased through biofortification process in field conditions. The process of selenium fertilization through foliar spray was more effective than soil irrigation and positive for all biochemical parameters analyzed. High polyphenolic content and antioxidant properties were observed in biofortified garlic in concordant with high selenium content which could be used as a powerful source of natural antioxidants along with selenium to combat hidden hunger of micronutrient. Further field experiments conducted in the present study will shade a new light to improve selenium content in other seleniferous crops which could be valuable considering agronomic and human health benefits.

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Conflict of interests. Authors declare no conflict of interests.

Geographic information. Field experiments were conducted at botanical garden of PCSIR Labs Complex, Lahore, Pakistan. The experimental site is situated between 31.52º North latitude, 74.33º East longitude and altitude of 217 m above the sea level.

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