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Original Research Article

IODINE AND SELENIUM BIOFORTIFICATION OF MICROGREENS (SPINACH) TO ALLEVIATE MINERAL NUTRIENT DEFICIENCY

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Abstract *Iodine (I) and Selenium (Se) are essential nutrients for the proper functioning of human and plant bodies. This study aimed to optimize the application method and dose/rate of nutrients for spinach biofortification to better serve human health and may prove more economical than adding these nutrients in supplements. The trial was conducted at the growth room, Department of Horticulture, University of Layyah, Layyah. Seed of spinach was procured from a certified agency and directly sown in the trays (2 inches deep). This study employed a total of three concentrations of two distinct nutrient treatments (I and Se) as well as a control, applied as basal and foliar spray methods. Each treatment was replicated thrice, each replication with a tray. The end product was analyzed for different attributes like morphological parameters, biochemical parameters (Total soluble solids, Vitamin C, acidity, carotenoids, chlorophyll contents, lipophilic antioxidant (LPA), starch contents, amino acids, carotenoids, flavonoids, and phenolic contents) and ionic contents (I and Se). Our results indicated that supplementation of I @ 2.5mg/L and Se @ 1mg/L concentration is optimal for maintaining the normal growth of plants and promoting the major I and Se concentration in the edible part of spinach. Thus, it could be proposed that the growth of spinach under I @ 2.5mg/L iodine can amount to 208µg and 238µg and Se ranged from 215.09µg and 186.51µg in shoots and roots, which can match the 150µg day-1 and 55 µg day-1 dietary iodine and selenium allowance recommended by WHO.*

Keywords: *Iodine; Selenium; nutrients; lipophilic antioxidant; carotenoids; flavonoids*

Introduction

Microgreens are a unique group of edible plants that can be grown for home consumption as well as extensively on a commercial scale with higher concentrations of nutrients, as compared to their respective mature growth stage (Mir et al., 2017). Being available in less period with a high nutrient profile makes them the best candidate for biofortification studies. The incorporation of microgreens into the human diet can prove to be life life-changing experience for those, who consume them regularly. Currently, people are consuming meals that are not much more diverse than 30 years ago, such diets are micronutrient deficient (Genc et al., 2009). Vegetables with increased levels of iodine can be proposed as the alternative source of this microelement in the diet. The idea of biofortification of crops concerns the introduction not only of iodine but also other elements such as Se, Fe, Zn, Mg, and Ca into the edible parts of plants (Hirschi, 2009). It has been demonstrated that the consumption of vegetables biofortified with iodine (potatoes, cherry tomatoes, carrots, and green salad) contributed to the significant increase in the level of urinary iodine excretion (UIE) which is one of the major indicator of iodine status of the population (Tonacchera et al., 2013).

Iodine is one of the important micronutrients which is required for humans and animals to perform their activities properly. Iodine is very important for endocrine glands which release some important

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hormones including thyroxin (T4), thyroid hormones, and tri-iodothyronine (MelseBoonstra and Jaiswal, 2010). It has been roughly calculated that 30-38% of the world population has inappropriate iodine consumption. In case of insufficient iodine consumption, the whole spectrum of organisms is going to be dysfunctional and causing iodine deficiency disorder (IDD) (Winger et al., 2008; White and Broadley 2009). It has been investigated that millions of people are selenium deficient and delivery of selenium in food is dependent on the availability of selenium in the soil (Winkel et al., 2012). Plants can uptake selenium with the help of their roots in the form of selenite, selenate, or organoselenium (White et al., 2004; Li et al., 2008). Foliar applications of selenium-based fertilizers can be used to prevent the disorders related to its deficiency. However, there are limited effects of selenium element on the different parts of plants like seeds, roots, and tubers because this element has complex translocation patterns but the foliar application of selenium has proven good results in many experiments and it is a good biofortification tool (Yuan et al., 2018). The main objectives of the study are to determine the influence of I and Se on the yield, and nutrient profile of spinach and to optimize the application method and dose/rate of nutrients for vegetable biofortification.

Materials and methods

In this experiment, the seed of spinach (*Spinacia oleracea* L.) was procured from a certified agency and directly sown in the trays (2 inches deep) which were lined with foil to prevent the leaching of water and mineral nutrients from the soil. Spinach was grown in a growth room with optimal growing temperature (14-18 ^{o} C) Department of Horticulture, University of Layyah, Layyah. Media used in trays were comprised of 35% sand, 28% silt, and 37% clay with a mean organic matter content. This study employed a total of three concentrations of I and Se, applied through basal and foliar spray methods as well as a control treatment. The details of these treatments are outlined in Table 1. Each treatment was replicated three times, each replication containing 1 tray. There were 36 trays (for each foliar and basal) for nutrients and 6 trays as a control in this experiment. An average of five plants from each replication were taken to analyze data. Irrigation was applied as per requirement. Following that, basal and foliar applications were applied at given stages.

- **1st** Application of nutrients (foliar and basal) was provided in germination stage
- **2nd** Application of nutrients (foliar and basal) was provided one week after germination.
- **3rd** Application of nutrients (foliar and basal) was applied one week before harvesting.

Table: 1. Treatment details of nutrients applied to spinach

Nutrient	Application method	Concentrations
No nutrient	Foliar & Basal	Control (distilled water)
I	Foliar & Basal	I_1 (2.5mg/L)
		I_2 (5mg/L)
		I_3 (10mg/L)
Se	Foliar & Basal	Se_1 (1mg/L)
		Se_2 (1.5mg/L)
		Se_3 (2mg/L)

Plant analysis of fresh samples of spinach

Shoot length and root length were measured in cm with the help of a measuring scale.

The fresh and dry weight of the spinach plant was assessed by placing the plant on a weighing balance (PA-413 manufactured by Chaus Corporation, USA). The number of leaves were counted and averaged. Leaf area was measured with the help of a measuring scale by following the formula;

Leaf area (cm^2) = length of leaf (cm) × width of leaf (cm)

Total soluble solids (TSS) were determined through a digital refractometer by placing a few drops of freshly extracted spinach leaf juice on the prism in the specimen chamber of the refractometer. The ascorbic acid concentration was measured using the titration method of 2, 6-dichlorophenol-indophenol (Horwitz, 1975; SAPRC, 1986a). Oxalic acid was used to dilute the juice and the filter aliquot was titrated with the help of indophenol dye to the light pink endpoint coloration.

$$\text{Vitamin C} = R_1 \times V \times 100/R \times W \times V_1$$

R_1= flask/burette value, V= volume of aliquot made by adding 0.4% oxalic acid, V_1= ml of aliquot used after filtration for titration, W= juice taken (ml), R= standard reading

To measure total carotenoid contents, a grounded leaf sample was centrifuged with 80% acetone (10 mL) at 12000 rpm for 5 min. The absorbance of Carotenoids was recorded in nm in UV-VIS spectrophotometer (WE6000). The aluminum chloride colorimetric method was used for the determination of the total flavonoid content of the sample with the help of UV-Vis spectrophotometer (WE6000). The concentration of total flavonoid content in the test samples was calculated from the calibration plot ($Y = 0.0162x + 0.0044$, $R2 = 0.999$). To measure total phenolic compounds, sample and standard readings were made using a spectrophotometer (WE6000) at 765 nm. Amino acids (proline contents) were determined with the help of a ninhydrin reagent. Absorbance was recorded with the help of UV-VIS spectrophotometer (WE6000).

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A sample of 0.5 g grounded leaf was centrifuged with 80% acetone (10 mL) at 12000 rpm for 5 min. The absorbance of Chlorophyll ‘‘a” and ‘‘b” was recorded at 645 nm and 663 nm in UV-VIS spectrophotometer (WE6000). The iodine contents of various plant organs (roots, stems, leaves, and fruits) were measured using an alkaline ashing technique.

Plant analysis of Dry samples of spinach

The lipophilic antioxidant activity (LAA) was measured with the 2, 20-azinobis 3-ethylbenzothiazoline-6-sulfonic acid ABTS method by UV–Vis spectrophotometry. After measuring absorbance at 734nm for 30mints, ABTS % was calculated by using the formula

$$ABTS\ \% = (AB\text{-}AA/AB) \times 100$$

- AB =absorbance of ABTS radical + methanol
- AA= absorbance of ABTS radical + sample extract

Total Se concentrations were measured in samples using 500 mg dried ground plant material from each replicate digested with HNO_3, H_2O_2, and HCl, and analyzed by spectrophotometer.

Results

Vegetative parameters

Statistically, results are significant regarding plant fresh and dry weight in response to iodine supplementation in spinach which were compared by the Tuckey test at a 1% probability level. Maximum average plant fresh weight (34.78 g), plant dry weight (5.56 g), plant longest shoot and root length (8.06 cm and 6.46 cm), number of leaves (6.66), and leaf area (15.36 cm^2) in spinach were noticed when I was applied @ 2.5mg/L concentration (Table:1.1a). The interaction effect between treatment and method is illustrated in Table 1.1b which shows that I @ 2.5mg/L concentration with basal application gave maximum results regarding all above-mentioned vegetative parameters. In the current study, a comparison of treatment means showed that Se concentration @ 1mg/L performed best among all the concentrations. Maximum average plant fresh weight (36.78 g) and maximum average plant dry weight (8.11 g), longest shoot and root length (12.48 cm and 6.53 cm), number of leaves/plant (7) and leaf area (17.11cm^2) were assessed in Se @ 1mg/L concentration (Table:1.2a). Treatment and method interaction is given in Table 1.2b. Maximum results regarding growth parameters were assessed in Se @ 1mg/L concentration with basal application method. The present findings of the current study also recorded that when a high dose of iodine was supplied to the plants they represented different symptoms like reduced plant growth and biomass, physiological disorders as chlorotic spots, and more toxicity which caused plant death but low iodine supplementation promoted the plant growth and development.

Biochemical indices

Statistically, Comparison of treatment means showed that I @ 2.5mg/L concentration performed best regarding TSS (9.66 ◦Brix), maximum ascorbic acid (13.33 mg/100 g), higher phenolic contents (42.16 mg/ g FW), LAA (26.83 µM trolox g^{-1} FW), amino acids (29.33 mmol g^{-1}), carotenoids (5.45 µg g^{-1}), chlorophyll a (0.99 mg/g/FW) and chlorophyll b (0.69 mg/g/FW) contents. It was observed that the introduction of iodine into nutrient solution led to various plant responses. Application of iodine @ 2.5mg/L concentration when compared with other concentrations and control treatment contributed to a significant increase in all the biochemical indices as well as decreased the flavonoid contents given in Table 1.1a. Interaction effect regarding TSS, phenolic contents, and lipophilic antioxidants, chlorophyll “a” and “b” was best found in I @2.5mg/L concentration with foliar application method (Fig. 1) while ascorbic acid, amino acids, flavonoids, and carotenoids were assessed in I @2.5mg/L concentration with basal application method (Fig. 1). In this study, Comparison of treatment means showed that Se @ 1mg/L concentration gave maximum TSS (13.23◦Brix), ascorbic acid (13.83 mg/100 g), phenolic contents (43.83 mg/ g FW), LAA (26.33 µM trolox g^{-1} FW), flavonoids, and carotenoids (13.66 mg, 5.54 µg g^{-1}). However, chlorophyll a (0.88 mg/g/FW) and chlorophyll b (0.59 mg/g/FW) contents were assessed in Se @1.5mg/L concentration but no effect of selenium treatments was observed on amino acids. Maximum amino acids were recorded in the control treatment (Table: 1.2a). Interaction effect regarding ascorbic acid, phenolic contents, and lipophilic antioxidants was best found in Se @1mg/L concentration with the foliar application method (Fig. 1) while TSS, flavonoids, and carotenoids were assessed in Se @1mg/L concentration with basal application method (Fig. 1 and 2). Treatment and method interaction was non-significant for amino acids (Fig. 1).

Nutrient analysis

The results demonstrated that maximum iodine concentration (208.43µg in shoots in foliar and 238.51µg in roots in basal application) was measured in I @10mg/L concentration (Fig. 1). Furthermore, Se @ 2mg/L concentration gave maximum Se contents (215.09µg in shoots and 186.80µg in roots) in spinach (Fig. 1), respectively. However, higher selenium and iodine doses depicted chlorotic symptoms and death of a few plants but lower doses reached the highest values regarding all the analyses.

Table 1.1a. Effect of Iodine supplementation on spinach vegetative and biochemical analysis

Parameters	Treatment means	Application Methods means

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(Vegetative)	(control)	T_1(1mg/L)	T_2(1.5mg/L)	T_3(2mg/L)	Foliar	Basal
Fresh weight (g)	17.86 (c)	34.78 (a)	26.50 (b)	20.68 (c)	22.64 (b)	27.27(a)
Dry weight (g)	4.50 (bc)	5.56 (a)	4.83 (ab)	3.85 (c)	4.42 (b)	4.95 (a)
Shoot length (cm)	6.76 (b)	8.06 (a)	7.05 (b)	5.55 (b)	7	6.70
Root length (cm)	4.76 (b)	6.46 (a)	4.93 (b)	4.71 (b)	5.10	5.34
No. of leaves	3.66 (c)	6.66 (a)	5.66 (b)	5 (b)	5 (b)	5.50 (a)
Leaf area (cm^2)	7.017 (c)	15.36 (a)	12.60 (b)	11.88 (b)	11.53	11.89
Bio chemical						
TSS (◦Brix)	6.73 (c)	9.66 (a)	8.16 (b)	6.58 (c)	8.07 (a)	7.49 (b)
Ascorbic acid (mg)	8.33 (c)	13.33 (a)	10.16 (b)	7.33 (d)	10	9.58
Phenols (mg /g f.w)	32.66 (b)	42.16 (a)	29.50 (b)	24.16 (c)	34.33 (a)	29.91 (b)
LAA (µM trolox g^{-1} f.w.)	21.76 (b)	26.83 (a)	22 (b)	18.16 (c)	23.10 (a)	21.27 (b)
Amino acids (nmol/g)	21.00 (b)	29.33 (a)	22.50 (b)	15.33 (c)	22.08	22
Flavonoids (mg)	9.93 (a)	9.83 (a)	8.83 (ab)	8.25 (b)	9.30	9.11
Carotenoids (µg $g^{-1)}$	4.01(c)	5.45 (a)	4.49 (b)	3.65 (d)	4.43	4.37
Chlorophyll a (mg/g/F. wt)	0.32(c)	0.99 (a)	0.49 (b)	0.23(c)	0.54 (a)	0.48 (a)
Chlorophyll b (mg/g/F. wt)	0.26(c)	0.69 (a)	0.43 (b)	0. 19 (c)	0.42(a)	0.36 (b)

Table 1.1 b. Effect of treatment and method interaction in response to iodine supplementation on spinach vegetative and biochemical analysis

Parameter	Foliar				Basal			
	T_0	T_1	T_2	T_3	T_0	T_1	T_2	T_3
Fresh weight (g)	17.86c	33.7ab	24.06c	14.9d	17.74c	35.8a	28.9bc	26.4c
Dry weight (g)	4.50	5.3	4.73	3.13	4.44	5.80	4.93	4.56
Shoot length(cm)	6.76	7.96	6.83	5.26	6.65	8.16	7.26	5.83
Root length (cm)	4.76	6.30	4.83	4.50	4.70	6.63	5.03	4.93
No. of leaves	3.66	6.33	5.33	4.66	3.33	7	6	5.33
Leaf area (cm^2)	7.01	15.03	12.5	11.6	7.01	15.7	12.7	12.16

Table 1.2a. Effect of different treatments and application methods of Selenium on spinach vegetative and biochemical analysis

Parameters (Vegetative)	Treatment means				Application Methods means	
	control)	T_1(1mg/L)	T_2(1.5mg/L)	T_3(2mg/L)	Foliar	Basal
Fresh weight (g)	17.86 (c)	36.78 (a)	27.25 (b)	17.06(a)	24.35	25.13
Dry weight (g)	4.50 (c)	8.11 (a)	5.55 (b)	4.15 (c)	5.72	5.43
Shoot length (cm)	6.76 (c)	12.48 (a)	9.30 (b)	6.90 (c)	8.74	8.98
Root length (cm)	4.76 (c)	6.53 (a)	5.80 (b)	4.26 (c)	5.21	5.46
No. of leaves/Plant	3.66 (c)	7 (a)	5.83(b)	3.33 (c)	5	4.91
Leaf area (cm^2)	7.017 (b)	17.11(a)	13.65 (b)	7 (c)	10.79	11.59
Bio chemical						
TSS (◦Brix)	6.73 (c)	13.23 (a)	10.66 (b)	6.38 (c)	8.89 (b)	9.61(a)
Ascorbic acid (mg)	8.33 (c)	13.83 (a)	11 (b)	7.33 (d)	10.66 (a)	9.58 (b)
Phenols (mg /g f.w)	32.66 (b)	43.83 (a)	32.50 (b)	24.83 (c)	36.50 (a)	30.41 (b)
LAA (µM trolox g^{-1} f.w.)	21.76 (b)	26.33 (a)	23.50 (b)	19.50 (c)	23.69 (a)	21.85 (b)
Amino acids (nmol/g)	21.00 (a)	19.50 (ab)	16.83 (b)	13.66 (c)	17.91	17.58
Flavonoids (mg)	9.93 (c)	13.66 (a)	11.46 (b)	8.73 (d)	10.95	10.94
Carotenoids (µg $g^{-1)}$	4.01(c)	5.54 (a)	4.45 (b)	3.97 (c)	4.52	4.47
Chlorophyll a	0.32(c)	0.67(b)	0.88 (a)	0.29(c)	0.51 (b)	0.56(a)

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(mg/g/F. wt)						
Chlorophyll b (mg/g/F. wt)	0.26(c)	0.59 (b)	0.59 (a)	0.22 (c)	0.37	0.37

Table 1.2b. Effect of treatment and method interaction in response to selenium supplementation on spinach vegetative and biochemical analysis

Parameter	**Foliar**				**Basal**			
	T_0	**T_1**	**T_2**	**T_3**	**T_0**	**T_1**	**T_2**	**T_3**
Fresh weight (g)		36.23	26.46	16.83	17.74	37.3	28.03	17.3
Dry weight (g)	4.50	7.9	5.40	3.93	4.44	8.33	5.70	4.36
Shoot length (cm)	6.76	12.26	9.13	6.80	6.65	12.70	9.46	7
Root length (cm)	4.76	6.36	5.60	4.13	4.70	6.70	6	4.40
No. of leaves/Plant	3.66	7	5.66	3.33	3.33	7	6	3.33
Leaf area (cm^2)	7.01	16.5	13.06	6.53	7.01	17.6	14.23	7.46

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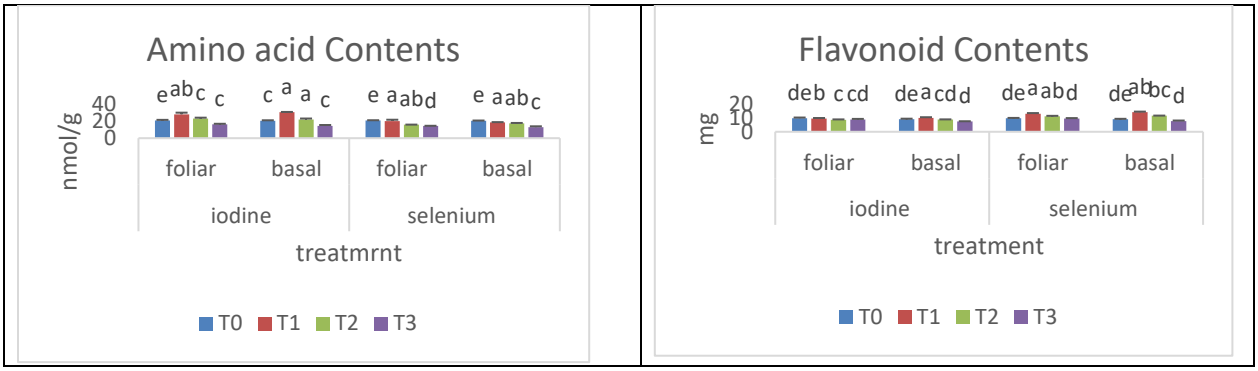

Fig. 1. Effect of treatment and method interaction of iodine (I) and selenium (Se) concentration on their concentrations in shoot and roots, TSS, Vitamin C, Phenolic Contents, LAA, Amino acid contents and Flavonoid contents of spinach plants

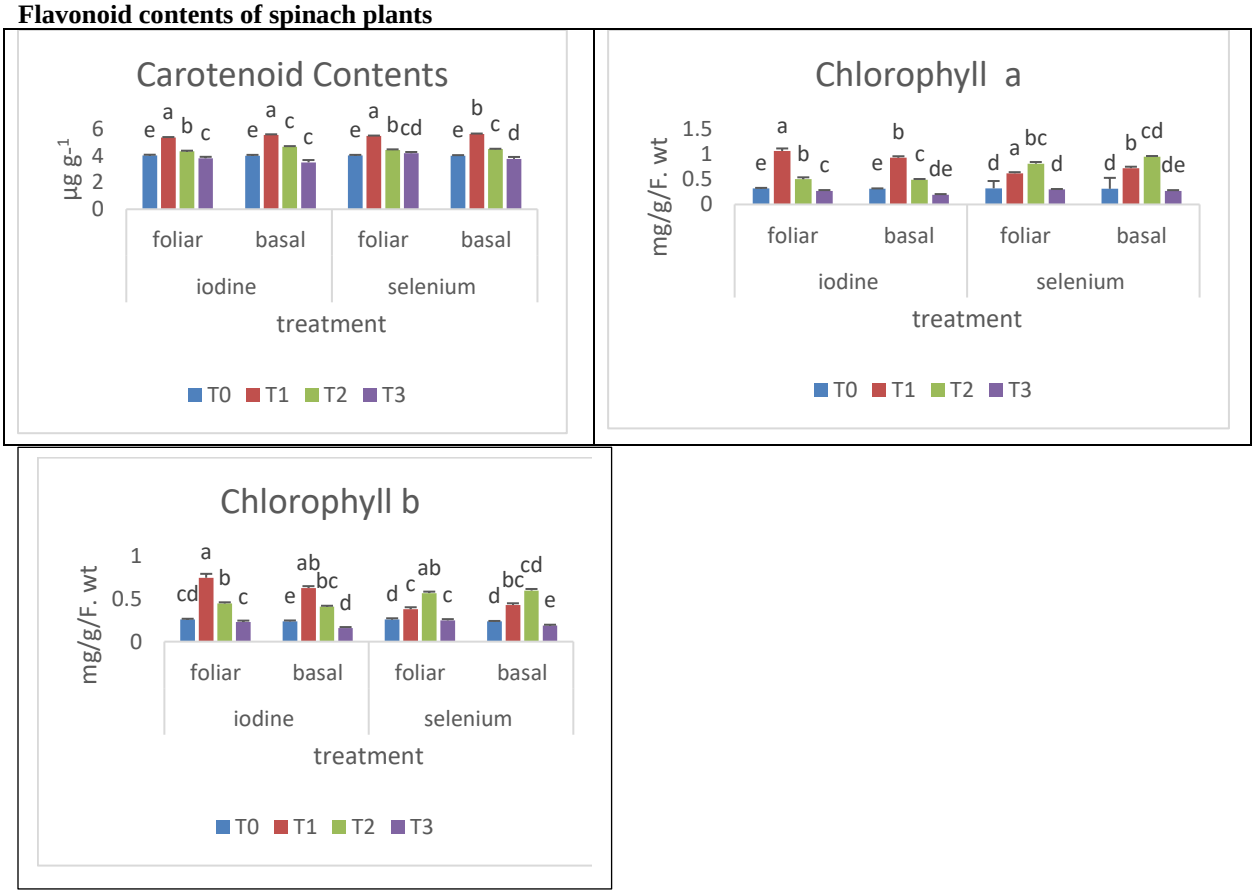

Fig. 2. Effect of treatment and method interaction of iodine (I) and selenium (Se) concentration Carotenoid contents, Chlorophyll a, and b contents of spinach plants

Discussion

In the present investigation, low iodine doses (2.5mg/L) increased the plant biomass including plant fresh weight, plant dry weight, shoot length, root length, and number of leaves while high iodine doses (10mg/L) decreased the plant biomass, biochemical parameters and showed chlorotic spots in spinach plants. The findings of the current study are also in agreement with (Mackowiak and Grossl, 1999) who discussed that when a high dose of iodine is supplied to the plants they represent different symptoms like impairment in plant growth, biomass reduction, physiological disorders such as chlorotic spots and more toxicity also caused plant death but low iodine influenced the plant growth and development because it can modulate the plant transcriptome. In the present experiment leaf green pigments (chlorophyll a and b contents) along with flavonoids, carotenoids, and antioxidants increased in the beginning but a high dose of iodine affected it adversely. Current work was also in agreement with the previous studies of Li et al., (2017) who found that, in the beginning, chlorophyll contents of ryegrass leaf enhanced with increasing the

concentration of iodine and then started to reduce. In this study, selenium low doses also enhanced the vegetative and biochemical indices of spinach plants but high concentration had deleterious impacts on vegetative and biochemical indices. This study is also following the work of Medrano-Macías et al., (2016) who found similar results in their experiment that the advantageous effect of I and Se on plants. He concluded that iodine and selenium responded more positively to biochemical and physiological indices of plants as compared to vegetative parts. Furthermore, the current study, concluded that low concentrations of iodine and selenium ($2.5mgL^{-1}$ and $1mgL^{-1}$) increased plant biomass and biochemical indices like antioxidants, amino acids proline contents and phenols as compared to high concentrations. There was no more difference in the results of foliar and basal doses of these two nutrients. Iodine and selenium both are mobile elements so there was noted a minute difference in results but overall, basal doses gave more radiant results as compared to foliar and studies are according to findings that using high exogenous doses of I (Blasco et al., 2010; Kato et al., 2013) and Se (Ríos et al., 2008; Ríos et al., 2010)

Depending on the age and sex, in humans, there are different recommendations about the level of RDA-I and RDA For example, for an adult human, RDA for I and Se amount to 150 µg I and 55 µg Se (Institute of Medicine, 2000; Andersson et al., 2007). In our study, biofortification of spinach with selenium and iodine found maximum values (215.09ug and 238.51ug) which reached the WHO recommendations. In conclusion, the applied doses of I and Se (5mg, 10mg, and 2mg) were sufficiently high for the effective intake and accumulation of these elements in the analyzed vegetative parts (shoots and roots). However, Se @1mg and I @2.5mg concentrations are effective doses.

Conclusion

The applied doses (1mg/L, 1.5mg/L and 2mg/L) of selenium (foliar and basal) both affected the spinach plants. Se @1mg uptake gave maximum results regarding the plant vegetative and biochemical analysis. However, there was no effect of selenium (foliar and basal doses) on amino acids in the spinach plant. Basal dose of selenium increased plant biomass including plant fresh weight, shoot length, root length, and leaf area while biochemical data including TSS and chlorophyll a contents while all other parameters (vegetative and biochemical) responded positively to foliar application of selenium. Successfully, a low dose of iodine (2.5mg/L) demonstrated good results in all morphological and analytical analyses of spinach plants except flavonoids. Iodine concentrations adversely affected the flavonoid contents of plants. Vegetative parameters including plant fresh and dry weight, root length, No. of leaves/plant and leaf area response was best to basal application while all the biochemical parameters and shoot length were positively subjected to the foliar application method.

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