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Evaluation of Biological Traits and Bioactive Compounds in Several Spinach Varieties (*Spinacia oleracea* L.) Grown in a Vertical Hydroponic System

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Abstract

The implementation of breeding programs for indoor vertical farms has been increasing to provide new varieties that have higher nutritional contents and morphological traits fitted to these sustainable production systems. Spinach (*Spinacia oleracea* L.) is one of the fastest-growing, nutrient-dense leafy vegetables to grow indoors. In Vietnam, market demand for spinach is growing together with the expansion of soilless cultivation technologies such as hydroponics and vertical farming. This research aimed to evaluate the growth parameters, initially screening the relationship between yield and biological traits, of 12 spinach varieties. In addition, this research also aimed to evaluate the lutein, vitamin C, total flavonoid, and carbohydrate contents in different varieties of spinach and their correlations with morphological traits. The research outcomes revealed significant positive correlations between plant weight and leaf area. Three spinach varieties belonging to the smooth leaf type ("AD", "CH", and "DT") were the top three yielding under low light conditions. There were significant differences in the lutein content among varieties. "Red" and "Mikado" were the two highest varieties for lutein content. Vitamin C and total flavonoid contents were statistically similar in the tested varieties, while the carbohydrate content was slightly different among them. This research firstly suggested better suited spinach varieties to be grown in vertical farming conditions, and secondly, provided breeders information on trait identifications to prepare breeding materials for vertical farms.

Keywords

Spinach, vertical farm, morphological trait, bioactive compounds

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Introduction

Vertical farming was developed as a novel solution for vegetable production in urban areas where there is a high demand for clean and fresh products but a shortage of agricultural land (Heuvelink & Marcelis, 2020). Unlike soil-based farming, which requires breeding for (a) biotic tress-resistant traits, breeding for protected agriculture systems like vertical farming aims to produce traits that minimize system operation costs, enhance product value, or produce ideal phenotypes for multilayer cultivation systems (Leong & Urano, 2018; Folta, 2019). In order to decrease operation costs, the improvement of yield per square meter and the number of cultivation cycles have been interesting challenges for breeders. Varieties that show one of these characteristics such as high-yielding, high-quality, fast-growing, or compact shape in this system could be selected to prepare materials for breeding (Butturini & Marcelis, 2020; Kozai & Niu, 2020).

Spinach (*Spinacia oleracea* L.) is a widely-grown leafy vegetable in both tropical and temperate countries, and global production has increased from 30.1 million tons in 2019 to 59.5 million tons in 2020 (FAOSTAT; http://faostat.fao.org). This vegetable is a reservoir of mineral elements, vitamins, phytochemicals, and bioactive compounds that boost health and functioning with properties such as anti-inflammatory, anti-obesity, and anti-cancer (Roberts & Moreau, 2016). Because of its high nutrition and fast-growing nature, spinach has received more attention among indoor farming communities (Vickers *et al*., 2019). Variety, light, climate in the cultivation chamber, and nutrient solutions are the main factors impacting the yield and quality of spinach in this cultivation system (den Besten, 2019). Major studies have focused on investigating LED light effects on the growth and nutritional quality of hydroponic spinach (Bian *et al*., 2015; Naznin *et al*., 2019; Bantis *et al*., 2020; Gao *et al*., 2020; Zou *et al*., 2020; Nguyen *et al*., 2021;). However, a very limited number of results for varieties specifically bred for indoor vertical farms have been published. The recent advances in spinach genomics such as the reference genome assembly (Cai *et al*., 2021), genotyping data of wild and cultivated varieties (Shi *et al*., 2017; Ribera *et al*., 2020; van Treuren *et al*., 2020; Cai *et al*., 2021), and transcriptomics data (Xu *et al*., 2017) have facilitated the investigation of many quantitative trait loci (QTL) related to phenotypic variation as well as the improvement of spinach gene annotation (Ribera *et al*., 2020; Bhattarai & Shi, 2021). This is a wealthy bibliography for ongoing research of varieties adaptable to vertical farming conditions.

Leaf types, leaf area, plant height, and slow bolting are important commercial traits and tightly linked to varieties in leafy vegetable crops like spinach (Sabaghnia *et al*., 2015; Chitwood *et al*., 2016; Liu *et al*., 2021). A good combination of the lighting schedule and suitable varieties could postpone bolting, shorten the life cycle, and enhance the speed of breeding (den Besten, 2019; SharathKumar *et al*., 2020). Based on leaf surface, spinach is mainly divided into three categories: savoy, semi-savoy, and smooth-leaf varieties. Smooth-leaf varieties are preferable for processing while savoy or semi-savoy ones are better suited for fresh products. The growth habits also vary from horizontal to semi-erect to erect (Ribera *et al*., 2020), directly impacting the way plants receive photons from artificial lights and conduct photosynthesis. In vertical farms, by growing horizontal or semi-erect varieties, the number of bed layers could be increased. In parallel, there is a need for investigations for each crop about the density, optimal distance between two vertical beds, and lighting schedule in order to gain the best photosynthesis capacity.

Human nutrient intake could be improved by consuming enriched nutrient levels of spinach instead of increasing the consumption amount. Spinach is one of the highest sources of lutein among vegetables (Niu *et al*., 2020). Lutein plays a role in protecting human eye retinas by absorbing incident high-energy blue light (Calvo, 2005). Spinach lutein modulates the expression of NF-κB-targeted genes involved in anti-inflammatory properties (Roberts & Moreau, 2016). Spinach lutein has also been specifically associated with a decreased risk of breast cancer (Freudenheim *et al*., 1996). Total carotenoids in spinach have been reported to be genetically diverse and linked with several morphological traits under vinyl house conditions (Wang *et al*., 2018). Spinach is also rich in vitamin C and flavonoid compounds, and the levels have been reported to be dependent on the growth stage, conditions, and storage method (Bergquist *et al*., 2007). A breeding program for new spinach varieties that have an optimal size and are rich in bioactive compounds could be favorable for indoor agricultural production (Folta, 2019).

In Vietnam, market demand for spinach is growing, presented by the increase of this product's import value over the last three years (FAOSTAT; http://faostat.fao.org). The expansion of soilless cultivation technologies such as hydroponics and vertical farming will boost the local cultivation of spinach, supplying the market with fresh and nutrient-rich products. Growing spinach in a vertical hydroponic system could offer many more benefits compared to traditional farming in soil. Because spinach is an annual plant normally grown in temperate regions, the growing season in an open field is only from autumn to spring to produce the best yield and quality. Meanwhile, the key benefit of the vertical hydroponic system is providing optimal conditions for growing crops in a multi-layer format year-round without the risks of unfavorable climate conditions or pathogen attacks. This allows growers to harvest the freshest and cleanest products with increases in yield of 15-20 times in comparison to open fields (Zeidler *et al*., 2013). However, spinach has still not gained much awareness from domestic researchers except in a few studies that have mentioned the effect of LED light spectrums and intensity on hydroponically grown spinach (Thi *et al*., 2020; Nguyen *et al*., 2021). Therefore, this research aimed to evaluate several growth parameters of 12 spinach varieties collected from the Vietnamese market, initially screening the relationship between yield and a selection of biological traits. Besides, this research also aimed to determine the levels of several bioactive compounds, namely lutein, vitamin C, and total flavonoids, in the different varieties of spinach. The research outcomes could suggest better suited spinach varieties to be grown in vertical farming conditions and provide breeders information on trait identifications to prepare breeding materials for vertical farms.

Materials and Methods

Panel of spinach varieties and plant growth conditions

Twelve varieties were collected from commercial seed lots from different seed companies in Vietnam. These varieties included OP and F1 lines, and were selected based on their diversity in geographical origins and leaf types. Information about these varieties, including their commercial name and code used in this experiment design, are presented in the Supplementary Table. Spinach seeds were rinsed under tap water and then soaked in an H_2O_2 0.3% solution overnight (Katzman *et al*., 2001). Seeds were sown in rockwool and kept in the dark, and they were monitored everyday by adding 1/3 of strength of the standard hydroponic solution. After germination, the seedlings were kept under LED lamps with a photoperiod of 12h per day, light intensity of 200 µmol/m^2/s, and R:B ratio of 1.2 (Gao *et al*., 2020). After 14 days, the seedings that had two expanded true leaves, were transferred to a vertical hydroponic system (**Figure 1**). Commercial hydroponic solution was provided by Hachi Co., Ltd and was maintained with a pH of 6.0 and EC of 1.2 mS/cm after transplantation. The solution formulation was provided by Hachi Co., Ltd as follows: N: 84; P_2O_5 39; K_2O: 138; Mg:15; S: 26; Ca: 99; Fe: 3; B: 0.21; Cu: 0.04; Mn: 0.23; Mo: 0.01; and Zn: 0.15 (ppm). The solution was renewed once per week. Plants were cultivated until 43 days after sowing (DAS).

Experimental design

Plants were cultivated in a four-shelf vertical hydroponic system, representing four replications. There were six tanks per shelf and 24 plots per tank. One plant was grown per plot. For each replication, a complete randomized design was used to assign the 12 varieties into the six tanks (**Figure 1, Table 1**). The experiment was conducted in an air-condition controlled chamber with a temperature of 21 ± 1ºC, humidity of 80 ± 3%, and a CO_2 concentration of 500ppm. Fans were used to improve the ventilation among the shelves.

Trait measurements

Five plants per accession were randomly selected to measure growth indexes based on the Plant descriptors of the International Union for the Protection of New Varieties of Plants (UPOV). Leaf type (LT), fruit type (FT), and petiole attitude (PA) were recorded and classified

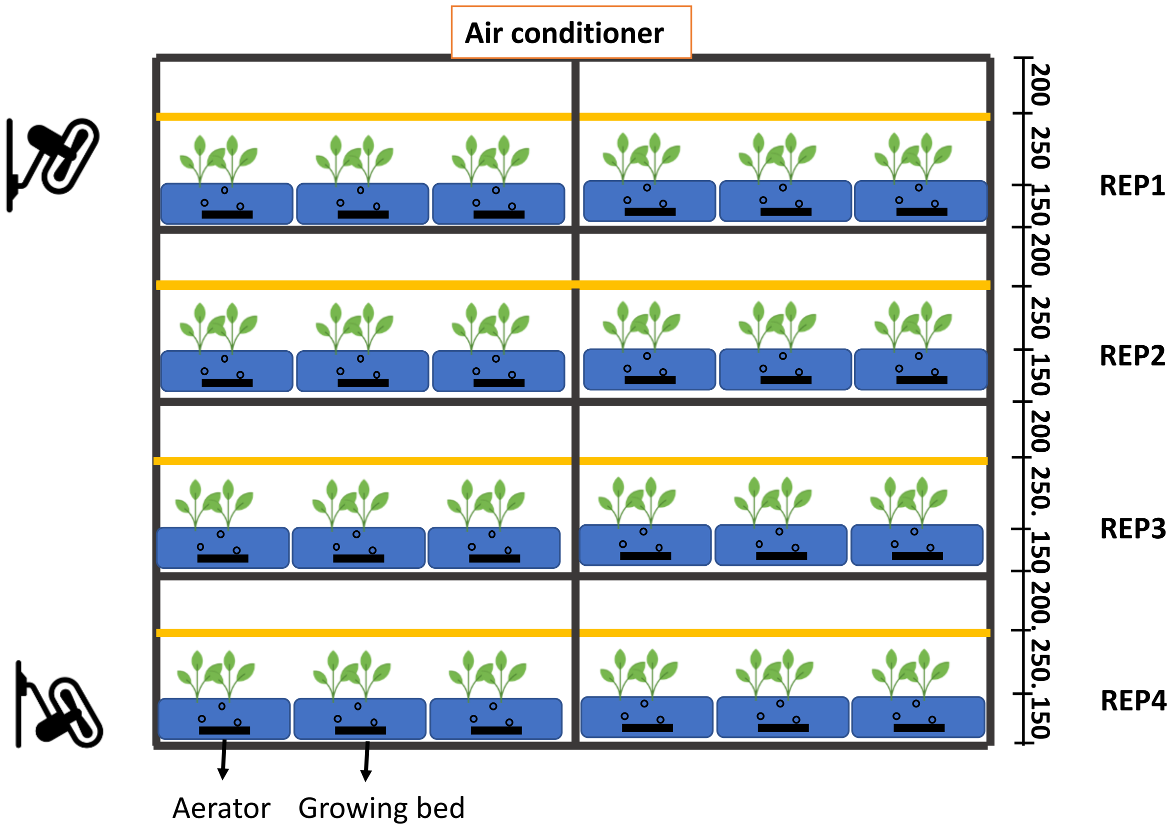

Figure 1. The vertical hydroponic system

Table 1. Placement of the 12 varieties in tanks following a complete randomized design (CRD)

	Tank 1		Tank 2		Tank 3		Tank4		Tank 5		Tank 6	
Rep 1	S8	S6	S5	S11	S7	S2	S12	S1	S4	S10	S3	S9
Rep 2	S11	S7	S9	S4	S1	S10	S12	S3	S5	S2	S8	S6
Rep 3	S9	S12	S10	S6	S3	S8	S11	S2	S1	S4	S7	S5
Rep 4	S7	S10	S12	S5	S9	S2	S4	S6	S3	S8	S11	S1

(Meng *et al*., 2017). LT was classified as smooth, semi-savoy, or savoy. PA was rated as erect, semi-erect, or horizontal (Supplementary Table). The leaf number (LN) was manually counted for every plant. Root length (RL) was measured from the end of the stem base to the deepest root tip. The longest petiole of every plant was recorded as plant height (PH). RL and PH were measured with a ruler. Leaf area (LA) was recorded using the laser leaf area meter LASER CI – 202. Three expanded leaves per plant were selected to measure chlorophyll content by using SPAD 502 Plus Chlorophyll Meters. Fresh biomass of the edible parts (FM) was determined, then placed in a forced-air oven at 80°C until it reached a constant mass, and recorded as dry mass (DM). The seven varieties that had moderate to high fresh masses were taken to evaluate their lutein contents at the laboratory of Vietnam National University of Agriculture. Five grams of fresh mixed biomass from the leaf blades of five plants per variety were used to determine the contents of bioactive compounds. Lutein content was determined by the high-performance liquid chromatographic method, according to JAS.0008. Vitamin C content was determined by TCVN 6427-2:1998. Carbohydrate content was determined by the methods of Albalasmeh *et al.* (2013). Total flavonoid content was determined according to the methods reported by Shraim *et al.* (2021).

Statistical analyses

Shapiro-Wilk tests were conducted to check the normality of data. Data were analyzed by ANOVA followed by Fisher's Least Significant Difference (LSD) tests for multiple comparison procedures in R. The results were reported as means (n = 5). Significant statistical differences were considered at $P \leq 0.05$. Pearson's correlation coefficients (r) between traits were calculated. The r values were recorded as being between -1 and 1, representing negative or positive correlations, respectively.

Results and Discussion

Biological traits evaluation

As shown in **Table 2**, strong significant differences were observed for the seven morphological traits, namely leaf number, leaf area, plant height, root length, chlorophyll content, fresh mass, and dry mass in the 12 varieties tested under indoor vertical farming conditions. Among these, FM was positively correlated with LA (r = 0.67) and PH (r= 0.65) but not statistically correlated with LN. Chlorophyll content was negatively correlated with FM (r = -0.75), LA (r = -0.63), and PH (r = -0.66). No correlations were observed for DM, RL, and LN (**Figure 2**). This suggested that to have better yielding spinach varieties, leaf extension or stem elongation should be more concentrated, instead of increasing the number of leaves. However, in vertical farming conditions, a short or moderate plant height was more interesting regarding the optimization of plant density (Masakazu *et al.*, 2019). The molecular genetic pathway that controls leaf area and petiole length as well as the effects of genetic markers and environment interactions on these two traits have been investigated in many crops such as tomato, the brassica family, soybean, and maize (Frary *et al*., 2004; Wei *et al*., 2016; Wang *et al*., 2019; Karamat *et al*., 2021). The control of leaf size is determined by tissue-specific patterns in cell proliferation and cell expansion during plant growth (Donnelly *et al*., 1999; Karamat ***et al***., 2021). In spinach, major QTLs of leaf dimension and petiole length were detected and strongly linked with the same markers on chromosome 1 (Liu *et al*., 2021).

Three smooth-leaf varieties, namely "AD", "CH", and "DT", had significantly higher biomasses while two semi-savoy varieties, namely "HP" and "Lazio", had significantly lower ones. "Red" had a slower growth rate compared with the other smooth-leaf varieties. "LAZIO", "HP", and "SC" showed less adaptive ability to the indoor vertical system due to their very low yields (**Table 2**). Analysis of variance revealed strong significant differences of leaf area, chlorophyll content, plant height, root length, and fresh mass between the semi-savoy and smooth-leaf groups in the whole panel (**Table 3 and Figure 3**). Interestingly, the smooth-leaf varieties tended to have a higher fresh mass, shorter roots, longer petiole length, bigger leaf area, and lower chlorophyll content. In contrast, the semi-savoy varieties tended to have a higher chlorophyll content and longer roots but a smaller leaf area, smaller height, and lower fresh mass. The better utilization of NO_3 with high NO_3 reductase activity was reported in smooth-leaf varieties (Barker *et al.*, 1974). This may have contributed to their higher yield than the other group. Dry mass and leaf number were not significantly different between the two groups. In general, fresh spinach had a 91% moisture content and 2.2% fiber content *(Murcia et al*., 2020). The differences in moisture content between the two groups could be an explanation for the observed correlation of leaf type and fresh mass but not dry mass, as in the case of lettuce (Mou, 2005). Among the 12 tested varieties, "G166" and "HP" had good germination rates at the beginning of the experiment.

Bioactive compounds evaluation

Seven varieties ("AD", "CH", "DT", "G176", "Mikado", "Red", and "Viking") that had moderate to higher fresh masses were included in the evaluation of bioactive compounds and carbohydrate contents in spinach blades. Lutein ranged from 3.7 to 8.34 (mg/100 g), which was consistent with previous data (Curran-Celentano *et al*., 2007; Miura *et al*., 2020), implying that the indoor vertical system

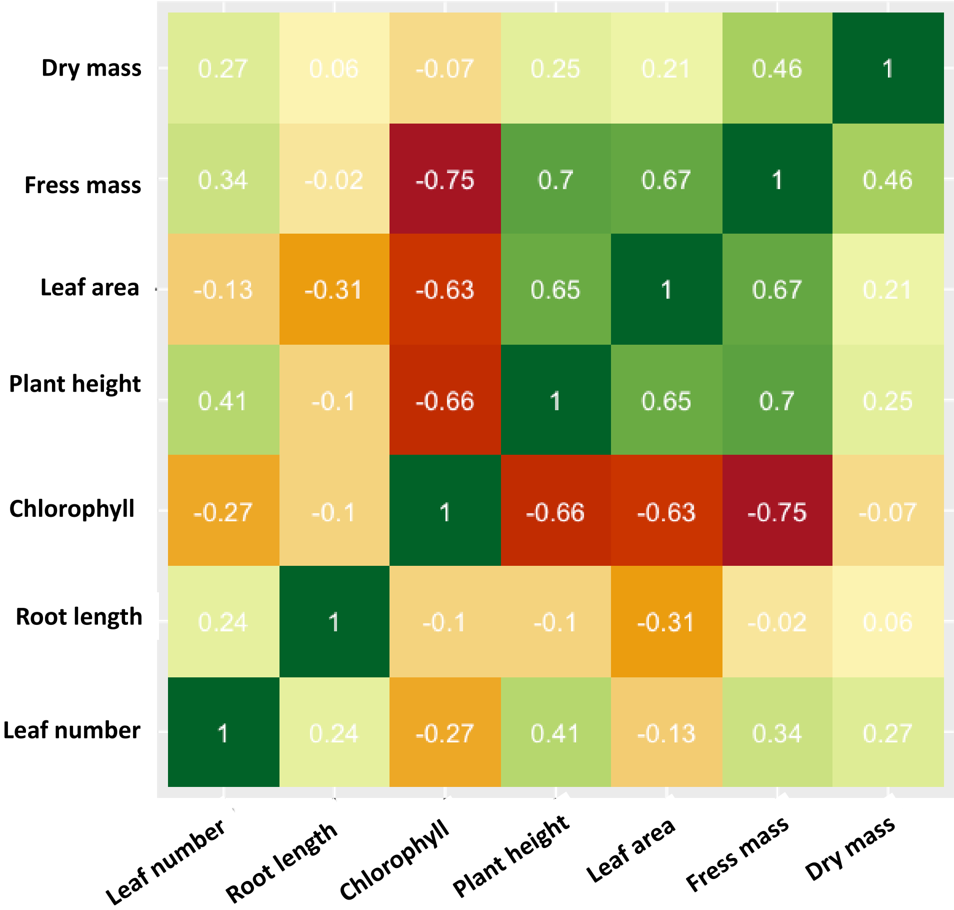

Figure 2. Pearson's correlation coefficients (r) calculated for seven morphological traits in the 12 spinach varieties

Figure 3. Phenotyping biological traits of the eight semi-savoy varieties (left) and four smooth-leaf varieties (right)

Table 2. Evaluation of morphological traits of the 12 spinach varieties at 43 DAS

Varieties	Leaf number		Leaf area (cm^2)		Chlorophyll (mg/100g)		Plant height (cm)		Root length (cm)		Fresh mass (g)		Dry mass (g)	
AD	77	a	55.7	c	38.8	f	25.8	a	34.7	bcde	28.6	ab	2.1	bc
CH	57	b	70.0	b	44.1	def	12.9	c	30.9	cde	36.8	a	2.9	ab
DT	29	ef	94.7	a	43.1	ef	20.7	b	30.9	cde	38.0	a	2.3	ab
G166	42	cd	30.2	de	57.0	ab	10.4	efg	37.7	bcd	19.0	bcd	1.9	bcd
G176	54	b	25.7	ef	55.0	ab	10.3	efg	46.8	ab	20.4	bcd	2.3	b
G904	20	fg	53.7	c	50.4	bcd	10.9	def	35.4	bcde	24.5	bc	2.3	ab
HP	41	cd	34.3	d	54.5	ab	9.1	g	38.0	bcd	12.7	d	1.7	cd
LAZIO	44	cd	18.9	f	60.2	ab	3.0	i	32.5	cde	13.6	cd	1.7	bcd
MIKADO	42	cd	33.3	de	40.7	ef	9.5	fg	54.0	a	20.8	bcd	2.0	bc
RED	19	g	69.0	b	46.0	cde	11.3	cde	24.2	e	20.1	bcd	1.3	d
SC	20	fg	31.5	de	56.7	ab	7.3	h	28.0	de	16.2	cd	2.2	bc
VIKING	34	de	53.7	c	51.0	bc	12.6	cd	41.9	abc	20.7	bcd	2.3	b
Mean $_{cv}$	40		47.5		49.8		12		36.2		22.6		2.1	
LSD $_{0.05}$	10	***	8.5	***	6.6	***	1.7	***	12.8	**	11.1	***	0.6	**

*Note: Mean $_{cv}$ in the same column followed by different letters indicate significant differences at P <0.05; *** and ** indicate the significance levels of p = 0.001 and p = 0.01, respectively.*

Table 3. Evaluation of morphological traits of two variety groups based of leaf type: semi-savoy and smooth leaves.

Leaf type	Leaf number		Leaf area (cm^2)		Chlorophyll (mg/100g)		Plant height (cm)		Root length (cm)		Fresh mass (g)		Dry mass (g)	
Semi-savoy	37.1		35.1		53.1		9.1		39.2		19.5		2.05	
Smooth	45.6	ns	72.3	***	42.9	***	17.6	***	30.1	**	32.5	***	2.18	ns

*Note: Mean $_{cv}$ in the same column followed by different letters indicate significant differences at P <0.05; *** and ** indicate the significance levels of P = 0.001 and P = 0.01, respectively; ns: non- significant difference.*

used in this research was appropriate for growing spinach in terms of nutrition content. Strongly significant differences in lutein concentration were recorded among the seven selected varieties (*P* <0.001), suggesting that this trait is highly dependent on the varieties and screening a larger spinach population might be required to further investigate the diversity of lutein content (**Table 4**). "Mikado" and "Red" contained the highest amounts of lutein while "AD" had the lowest value (**Figure 4**). This initial screening of seven varieties for lutein content revealed a slight negative correlation of lutein content and chlorophyll content (r =-0.6). However, to be more precise, this result needs to be confirmed in a larger sample size. A relationship of lutein content and the greenest leaves could be used for selective breeding. The two varieties with contrasting lutein contents could be used to study expression patterns that could be involved in the lutein synthesis pathway. Besides, lutein synthesis was shown to be stimulated by cooler temperatures, blue light, and low light intensity (Lefsrud *et al*., 2006; Li *et al*., 2009). These findings suggested that a combination of breeding programs for higher yield and lutein content, and the modification of environmental factors could be a solution to produce fresh spinach that has a good yield and is nutritional rich under specific indoor vertical farming conditions.

The vitamin C content is summarized in **Table 4**, and ranged from 45.05 to 48.65 mg/100 g. These values were similar to those published by Bergquist *et al.* (2006) in which the vitamin C contents of baby spinach leaves cultivated in an open field were between 14 and 46mg/ 100g fresh weight. However, our data was lower than the vitamin C content reported by Wang *et al.* (2018) in which the levels ranged from 51 to 130 mg/100g. In our study, there were no significant

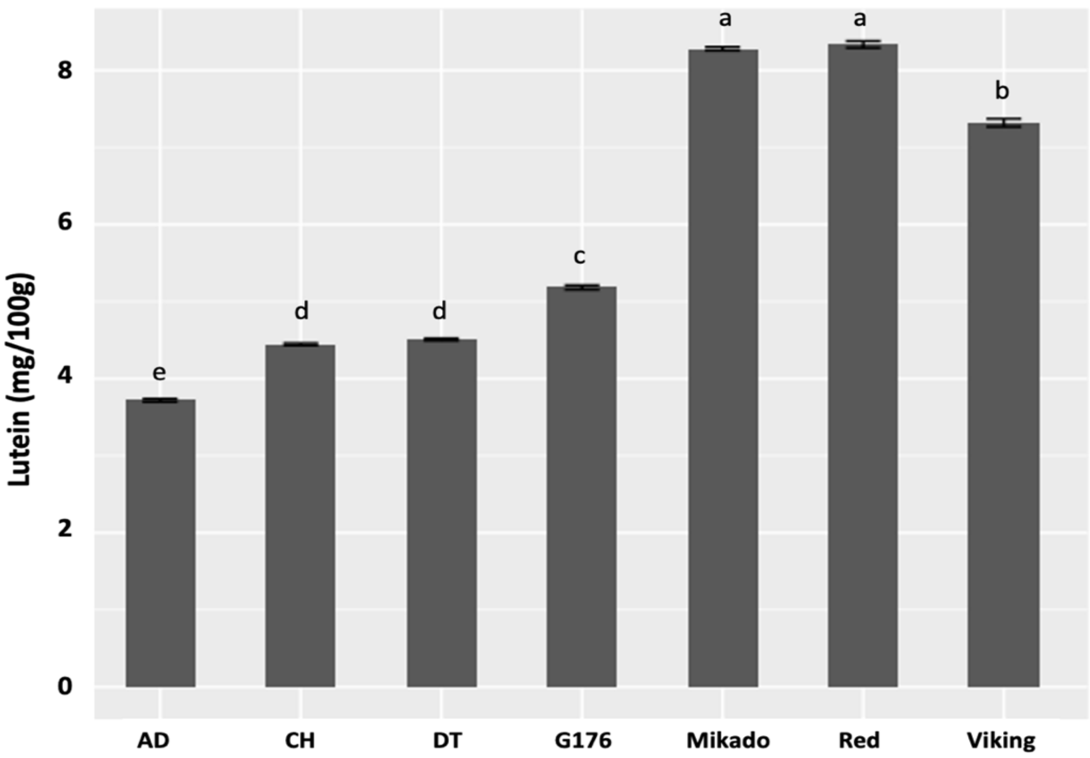

Figure 4. Evaluation of lutein content in different spinach varieties at 43 DAS

Table 4. Determination of bioactive compounds in spinach blades of different varieties at 43 DAS

Variety	Vitamin C (mg/100g)	Lutein (mg/100g)		Total Flavonoids (mgCAE/100g)	Carbohydrate (%)	
AD	45.05	3.72	e	1.58	3.6	b
CH	47.63	4.45	d	1.65	3.64	ab
DT	46.75	4.51	d	1.6	3.75	a
G176	45.97	5.18	c	1.59	3.56	b
Mikado	46.01	8.28	a	1.6	3.74	a
Red	46.13	8.34	a	1.61	3.58	b
Viking	48.65	7.32	b	1.61	3.66	ab
$Mean_{cv}$	ns	5.97		ns	3.64	
$LSD_{0.05}$		0.83	***		1.81	*

*Note: Mean $_{cv}$ in the same column followed by different letters indicate significant differences at P <0.05; *** and * indicate the significance levels of P = 0.001 and P = 0.05, respectively. ns: non-significant difference.*

differences in vitamin C content among the varieties. This might have been due to the fact that the number of tested cultivars was limited so the diversity of vitamin C content could not be seen as in the broader population studied by Wang *et al.* (2018). Besides using a high-level vitamin C variety, there are several ways to regulate the vitamin C content in spinach such as modifying the fertilizers used (Conesa *et al.*, 2008; Citak & Sonmez, 2010; Ogawa *et al.*, 2014), adjusting the air temperature (Tamura, 2004), and regulating the ratio of red/blue spectrums of LED light (Nguyen *et al.*, 2022).

The values of total flavonoid content in the seven varieties are presented in **Table 4**, with approximately 1.60 mg CAE/100 g fresh weight. All the commercial varieties presented statistically the same flavonoid concentrations. The levels of total flavonoids in spinach were highly dependent on the developmental stage and growth conditions as well as the postharvest and storage conditions (Mudau *et al.*, 2019). Total flavonoid content was extremely lower in our investigation than other studies in which spinach was cultivated in open fields (Gil *et al.*, 1999; Bergquist *et al.*, 2007).

This could partially be due to the fact that we used a low light intensity and excluded ultra-violet fixtures for the growing conditions. The research of Son *et al.* (2017) showed that under a high ratio of blue light, the total flavonoids in lettuce significantly increased in comparison to red light conditions, while plants subjected to more red light improved their accumulation of biomass. It has been suggested that the total flavonoid content of a high-yielding variety of spinach grown indoors could be improved via using a suitable lighting spectrum. From this point of view, three smooth-leaf varieties, namely “DT”, “CH”, or “AD”, could be good candidates for growers.

A small variation in the accumulation of carbohydrates was observed in the tested varieties and ranged from 3.56 to 3.75%, which is in reasonable agreement with the carbohydrate content of 2% to 10% reported previously in spinach (Ranawade *et al.*, 2017; Murcia *et al.*, 2020). The varieties “DT” and “Mikado” had the highest carbohydrate contents while “G176” and “Red” had the lowest at the harvest point. This showed that “DT” was top for fresh yield as well as carbohydrate content.

Conclusions

Environmental conditions for growing spinach in a vertical farm may vary depending on the technologies available (light, ventilation, heating, etc.) and whether or not the varieties being grown are able to adapt to the system constraints. A strong interaction of plant performance and the environment in vertical farming should steer breeding strategies away from finding an ideal variety but instead, breeders should test for multiple suitable varieties for different ranges of environmental conditions, helping growers choose good varieties for their particular cultivation systems and simplifying the producing process. Morphological traits of 12 spinach varieties were investigated and the results revealed significant positive correlations between plant weight and leaf area. The varieties “AD”, “CH”, and “DT”, which belong to the smooth-leaf type, were the top three yielding varieties under low light conditions. Moreover, there were significant differences in the lutein content among the varieties. “Red” and “Mikado” were the two varieties with the highest lutein contents. There was a weak negative correlation between the lutein content and the greenest plant leaves. The vitamin C and total flavonoid contents were statistically similar in the tested varieties while the carbohydrate content was slightly different among them. This suggested that the characterization of a larger spinach population will be required to confirm the diversity of lutein content as well as to find variation in the vitamin C and total flavonoid contents among *S. oleracea* L. commercial varieties. Based on these findings, growers should select spinach varieties that are rich in bioactive compounds and have high yields. Otherwise, it is proposed that growers should select the high-yielding variety “DT” and manipulate the environmental factors such as lighting or air temperature to enhance nutritional levels.

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