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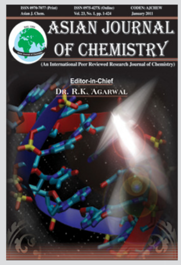

Determination of Phenolic Compounds and Chlorophyll Content of Spinach (*Spinacia oleracea* L.) at Different Growth Stages

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The aim of this studies is to determine variations in total phenolic compound and chlorophyll contents in spinach (*Spinacia oleracea* var. *matador*), which was grown in unheated glasshouse growth chamber facilities, occurred in three different growth stages like cotyledon, 5 true leaves and harvest maturity. According to the findings, greenhouse ambient yielded the highest mean in early spring spinach production with respect to different growth environment according to the criteria under consideration (119.88 mg/100 g) and was followed by open field and growth chamber. According to comparison of growth stages of spinach grown in three different growth environments, amounts of total phenolic compound (121.25 mg/100 g), total chlorophyll (877.93 mg/L), chlorophyll a (13.89 mg/L) and chlorophyll b (1.12 mg/L) increased towards the harvest phase in which the most matured and aged plant was achieved. In other words, these matters were accumulated depending on the plant's growth.

Key Words: Spinach, Growth environments, Growth stages, Total phenolic compounds, Chlorophyll content.

INTRODUCTION

Phenolic compounds are used in taxonomic studies for describing and classifying plant species and considered as inhibitors in the regulatory matters' class. It was claimed that phenols in spinach link to lipoproteins to inhibit oxidation. Most frequently eaten vegetables include antioxidants in the form of phenolic compounds like ascorbic acid, tocopherols, carotenoids, flavonols and phenolic acids. However, it is known that vegetables generally contain antioxidant compounds in lower amount compared with fruits[1]. Lipid oxidation degrades quality of foods. In other words, it causes loss in their important features like colour, odour, taste, appearance and nutritive value. Antioxidants are the matters, which preserve quality of foods preventing oxidative decomposition of lipids. Synthetic antioxidants are used in food industry for preventing oxidative decomposition. Although these antioxidants are very effective, stable and inexpensive, there are doubts relating to their potential side effects. Therefore, consumers' preferences have forced the industry to seek natural antioxidant resources. Natural antioxidants are phenolic compounds, which are formed as secondary metabolite in plants, like especially flavonoids, acid derivatives, tocopherols and organic acids[2].

Plant polyphenols have attracted interest of scientists for years due to their contribution to plant morphology (pigmentation), their relation with growth and reproduction, maturation of seeds, prevention of seeding and other reasons. Polyphenolic profile in plants varies between varieties of the same species. Therefore, polyphenols have been investigated for taxonomic purposes also. Furthermore, polyphenols have been studied for determining decomposition of foods. Polyphenols are used in industry in coating, paper and cosmetic manufacturing, in leather manufacturing as well as in food industry as natural dyers and preservatives. Also, some phenolic compounds, especially flavonoids, are used as antibiotic and in preventing diarrhea, as antipyretic against ulcer, in hypertension treatment, in preventing vascular cracking, in treatment of allergies and against high cholesterol. Phenolic compounds exist in each organ of a plant prevalently and account for an integrated part of human diet. Until recent times, effects of polyphenolic compounds were associated with macromolecules making them settle and inhibition of digestive enzymes. However, recently, the interest has focused on antioxidant and free radical catching features of phenolic compounds in foods and related to explaining their potential effects on human health[3].

Spinach is prevalently grown in gardens and fields because it is an easy-grown plant and can be harvested in a short time. It takes place in crop rotation and consequently, allows more efficient soil usage. Spinach is one of the plant species in restricted number satisfying green vegetable demands of people in winters. Vegetable consumption is increasing very rapidly because world population is growing rapidly, animal source foods fail to satisfy the demand properly and finally, people have become aware of the importance of the role of vegetables for human health and human diet[4].

Due to the aforementioned reasons, the present study is conducted to determine variation in phenolic compound and chlorophyll contents, which might occur in certain phases of spinach growth.

EXPERIMENTAL

In this study *Matador* species spinach which is grown widely was used as material (*Spinacia oleracea* var. *matador*). *Matador* is a kind of dark green coloured spinach whose leaves are large, oval and blistered and has short stems. It runs to seed late and shows fast growing. Its seeds are quite big, slightly flat and smooth[5].

Setting of experiment: The experiment was carried out according to random block design as 4 replications and 3 different growth environments (in open field, unheated glasshouse and growth chamber) and 3 different growth stages (stages of cotyledon, 5th true-leaf and harvest) were used in each replication. In all the experiments; 36 parcels in total and 20 plants for each parcel were used.

Growing plants: It was cultivated being based on the method of soil cultivation applied in open field and in glasshouse and then basic fertilization was carried out giving 10 kg nitrogen (N) 10 kg phosphor (P_2O_5) 11 kg potassium (K_2O) and 10 kg calcium (Ca) per decare[6].

After fertilization was completed, parcels were prepared and evened and then the soil was made ready for sowing. Sowing was planted at a spacing of 30 × 5 cm. Sowing depth was 2-3 cm in both open field and unheated glasshouse. Classical growing and care terms were applied until harvest time[7]. In growing spinach in glasshouse and growth chamber, plastic pots in the volume of 1.5 L that were filled with garden soil were used. In the growth chamber, plants were grown under controlled conditions in the temperature between +40 and -20 ºC and in here they were kept in (day/night) temperature, 10/14 h (light/dark) photo periodical order, 70 % humidity and 400 μmol m^{-2} s^{-1} light intensity during growth stages[8].

General information about experiment area: The experiment was carried out in the experimental field of the Horticulture Department, Faculty of Agriculture, Namik Kemal University, Tekirdag, Turkey (40°59' N, 27°29' E) in 2009. Climate data and soil analysis results of experiment area are shown in Tables 1 and 2.

Determination of total phenolic compound (mg/100 g): The amount of phenolic compounds existing in spinach extracts was determined by using Folin Ciocalteu colorimetric method. 0.5 g of plant material was collected from the plant's leaf tissues and it was homogenized in 5 mL of 0.1M phosphate buffer. The homogenate was centrifuged for 10 min at 12.800 rpm. Then, 2 mL was drawn from the solution and 3 % sodium carbonate and 0.3 N Folin-Ciocalteau were added to the sample until the final volume of 4 mL. Then, the solution was kept for 1 h at room temperature. Measurements with in spectrophotometer were conducted at a wavelength of 765 nm. The results were calculated by using the concentrations in the gallic acid standard[9].

Determination of chlorophyll content (mg/L): Leaf samples were collected from the plants grown in three different growth environments (open field, unheated greenhouse and growth chamber) during three different growth stages (cotyledon, 5 true leaves and harvest). The samples were rinsed with tap water and dried. They were weighed on a precious scale. Then, they were ground with the help of a pestle and a mortar after 0.5 g of $CaCO_3$ and 80 % acetone were added into the mortar and the leaves were completely broken into pieces. The obtained extract was eluted with 80 % acetone and filtered into 100 mL flasks through a common filter paper. The samples collected from them were centrifuged for 10 min at 3000 cycles per minute in Jec Model HN-SU make centrifuge machine. Then, volume of the rest extract was measured. Then, the raw chlorophyll extract was analyzed in Hitachi U 2000 UV model 121-002 make spectrophotometer at wavelengths of 645 and 663 nm. The obtained absorption values were used in the following Arnon (1949) formula to calculate mg total chlorophyll/l extract.

$$\text{Total chlorophyll } (C_T) = 20.2xA_{645} + 8.02 \text{ X } A_{663}$$

This equation yielded total chlorophyll content existing in per liter of the extract. Then, this result was divided into leaf weight to find chlorophyll content per gram of leaf. The values obtained through the same way were used in the following formulae to determine chlorophyll a and chlorophyll b contents[10,11].

TABLE-1
2009 CLIMATE DATA OF EXPERIMENT AREA*

	Meteorological values	Average temperature (ºC)	Max. temperature (ºC)	Min. temperature (ºC)	Average relative humidity (%)
March	Open field	7.9	11.2	2.9	86.6
	Unheated glasshouse	10.0	22.1	4.1	90.0
	Growth chamber	18.0	20.0	15.0	80.0
April	Open field	11.5	15.3	6.0	82.7
	Unheated glasshouse	12.9	23.7	6.5	84.7
	Growth chamber	18.0	20.0	15.0	80.0
May	Open field	17.5	21.4	13.0	81.0
	Unheated glasshouse	19.1	27.1	8.9	83.3
	Growth chamber	18.0	20.0	15.0	80.0
June	Open field	22.0	26.1	17.2	78.0
	Unheated glasshouse	26.3	31.4	18.2	79.6
	Growth chamber	18.0	20.0	15.0	80.0

*Resource: Tekirdag directorate of meteorology (2009).

TABLE-2
SOIL ANALYSIS RESULTS OF EXPERIMENT AREA*

Parameter	Unit	Result	Method
pH	–	7.65	Saturation
Salt	(%)	0.07	Saturation
Lime	(%)	0.71	Calcimetric
Structure	(%)	44.00	Saturation
Organic matter	(%)	1.93	Walkey-Black
Total nitrogen	(%)	0.10	Kjeldahl
Phosphorus	(ppm)	25.00	Olsen-ICP
Potassium	(ppm)	247.00	A. Asetat-ICP
Calcium	(ppm)	4.116.00	A. Asetat-ICP
Magnesium	(ppm)	290.00	A. Asetat-ICP
Iron	(ppm)	4.90	DTPA-ICP
Copper	(ppm)	1.03	DTPA-ICP
Zinc	(ppm)	0.72	DTPA-ICP
Manganese	(ppm)	16.00	DTPA-ICP

*Resource: Agricultural soil analysis report of T.R. Tekirdag commercial exchange.

Chlorophyll a (C_a) = $(0.0127xA_{663}) - (0.0026x45.6xA_{645})$

Chlorophyll b (C_b) = $(0.0229 \text{ x } A_{663}) - (0.00468xA_{645})$

Evaluation of data: The experiment was established as a factorial randomized block design with four replications. Data were subjected to analysis of variance, with factorial comparisons of main effects and interactions. Means were tested by protected LSD (at the 0.01 level). MSTAT 3.51 was used for analysis of variance.

RESULTS AND DISCUSSION

Total phenolic compound (mg/100 g): Table-3 and Fig. 1 show the means, interactions and L.S.D. test groups relating to total phenolic compound contents of spinach grown in different growth environment during certain phases of growth.

The spinach variety, matador, in present study produced highest total phenolic compound content (119.88 mg/100 g) under greenhouse conditions and this was followed by open field conditions (85.51 mg/100 g). The lowest phenolic compound amounts (40.97 mg/100 g) were produced under growth chamber's conditions.

Total phenolic compound amounts increased in 3 different growth stages of the spinach as the plant grew. Thus, the highest total phenolic compound accumulation was achieved in the harvest period (121.25 mg/100 g).

It was reported that some phenolic compounds had a role in taste formation in fruits and vegetables. They were responsible for especially two significant taste factors like bitterness and acerbity[1,12] and they could exist in plants' fruits, seeds, flowers, leaves, branches and stems[13-15]. Spinach is an important dietary vegetable for human health also and contains plenty of phenolic compounds[16].

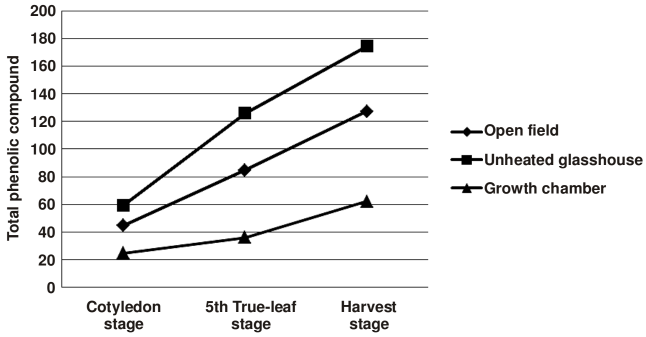

Fig. 1. Variations in total phenolic compound content of matador spinach variety grown in different growth environment and during different growth stages (mg/100 g)*

According to Table-3, total phenolic compound amounts obtained in present study range between 24.63 and 174.40 mg/100 g. First of all, these data obtained by us are correlated with the results of many studies conducted by many scientists on spinach's phenolic compound content[3,17]. Some phenolic content results found in these studies exceed the results obtained by us in present study[18,19] because different spinach varieties were investigated in these studies and phenolic compound synthesis and amount in plant tissues varies depending on many factors[20-22].

According to the obtained data in present study, total phenolic compound results are ordered as greenhouse, open field and growth chamber with respect to main effect of growth environment as it is done for other criteria. It was believed that natural sunlight and soil temperature has an effect in this order. It is also believed that position of the plant and the environment in which the plant took place as well as sucrose, nitrate and hormone contents of plant tissues in the growth environment and operations like irrigation caused variations in synthesis of phenolic compounds[23,24].

Considering the variations, which occurred in phenolic compound amounts in spinach depending at different growth stages as seen in Table-3, total phenolic compound amount increased as the plant grew. According to Delgado *et al.*[25], phenolic compound concentration in the plant varies within a year depending on growth stages. Similarly, Navarro *et al.*[26], reported that phenolic compound concentration is not constant during a year and varies according to growth stages. In fact, it was reported that polyphenols, tannins and anthocyanins increased depending on maturity in grapes harvested in June, July and August and this also varies depending on varieties. Pirie and Mullins[27] determined lower total phenolic compound amounts in grapes in early stages of maturation while they increased significantly in 28-35 days after the decrease and re-decreased toward the harvest. In present study, it was

TABLE-3
EFFECTS OF DIFFERENT GROWTH ENVIRONMENTS AND GROWTH STAGES ON TOTAL PHENOLIC COMPOUND CONTENT OF MATADOR SPINACH VARIETY (mg/100 g)*

	Growth stages			
Growth environments	Cotyledon	5th true-leaf	Harvest	Growth environment main effect
Open field	44.63 e	84.73 c	127.18 b	85.51 b
Unheated glasshouse	59.28 d	125.98 b	174.40 a	119.88 a
Growth chamber	24.63 f	36.10 ef	62.18 d	40.97 c
Growth stages main effect	42.84 c	82.27 b	121.25 a	–

*There is no difference in the level of 0.001 among averages that have the same letter.

determined that total phenolic compound content of the plant achieved the highest ratio in harvest stage in which the plant became suitable for eating.

Chlorophyll a, chlorophyll b and total chlorophyll contents (mg/L): Table-4 and Fig. 2 show chlorophyll a, chlorophyll b and total chlorophyll contents including chlorophyll a, chlorophyll b and all other chlorophylls of the matador spinach grown in different growth environments during different growth stages.

Considering the results of evaluation of the means, spinach produces similar responses in different growth environment and during growth stages with respect to chlorophyll a, chlorophyll b and total chlorophyll contents.

Chlorophyll a amount vary between 0.27-19.64 mg/g, chlorophyll b between 0.44-1.31 mg/g and total chlorophyll between 288.02 and 1032.95 mg/g for the matador spinach variety, which was under our study.

Considering chlorophyll a, chlorophyll b and total chlorophyll contents in spinach depending on growth stages, it was determined that chlorophyll content increased as leaves grew from cotyledon stage and it reached the highest level in harvest stage. Chlorophyll naturally increases as leaf surface area of a plant increases as a result of increase in mesophyllic cells including these pigments acting in photosynthesis[28]. Furthermore, Watanabe *et al.*[29] determined that total chlorophyll content increased as spinach's leaves maturated and this supports our findings.

Considering effect of different growth environments on chlorophyll a and b as well as total chlorophyll content in matador spinach variety, the highest contents were achieved in spinach leaves grown in greenhouse as seen in all three tables. Spinach leaves grown under natural open field conditions yielded lower chlorophyll a and b as well as total chlorophyll contents compared with the greenhouse media because we could not keep root and ambient temperature under control and chlorophyll contents get lower under open field conditions having lower temperatures compared with greenhouse. It is known that temperature has an effect on photosynthesis as it has on chlorophyll. Although especially photochemical phase of photosynthesis is not dependent on temperature, biochemical phase, which is controlled through enzyme activity, is completely dependent on temperature[30].

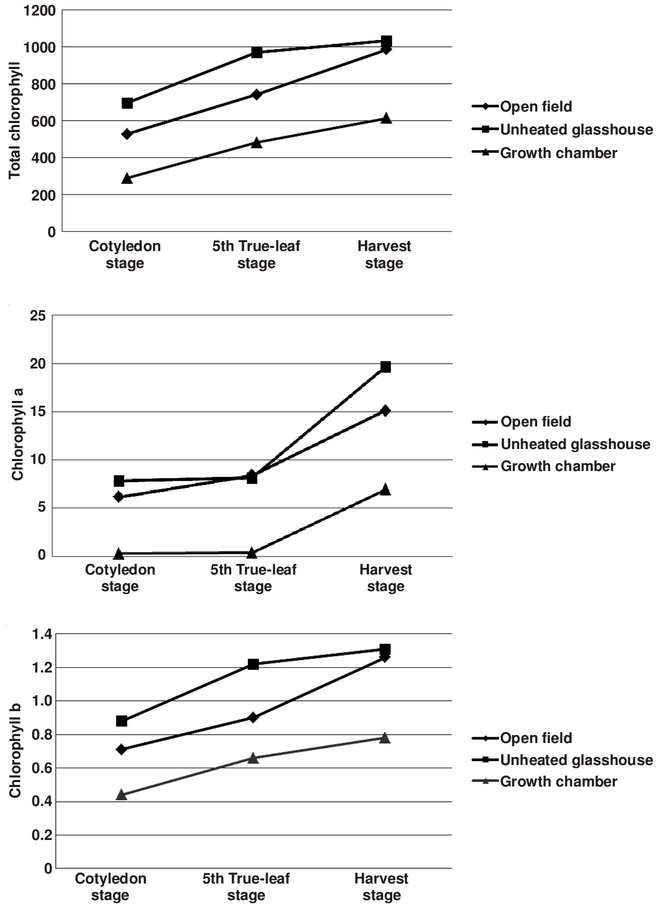

Fig. 2. Variations in chlorophyll a, chlorophyll b and total chlorophyll contents of matador spinach variety grown in different growth environment and during different growth stages (mg/g)

Growth chamber's conditions produced the lowest chlorophyll contents among different growing environments. Our plants grown under controlled conditions yielded the lowest chlorophyll contents despite optimum temperature, moisture, soil humidity and photoperiodic conditions. The reason is believed insufficient artificial lightening, in other words, insufficient light intensity.

Light is an important factor in growth of plants. It is especially important from the point of view of energy supply

TABLE-4
EFFECT OF DIFFERENT GROWTH ENVIRONMENT AND GROWTH STAGES ON CHLOROPHYLL a. CHLOROPHYLL b AND TOTAL CHLOROPHYLL CONTENTS OF MATADOR SPINACH VARIETY (mg/g)*

	Growth environments	Growth stages			Growth environment main effect
		Cotyledon stage	5 true-leaf stage	Harvest stage	
Total chlorophyll	Open field	527.19 f	741.01 c	985.48 b	751.22 b
	Unheated glasshouse	696.34 d	969.50 b	1032.95 a	899.60 a
	Growth chamber	288.02 h	482.27 g	612.92 e	461.07 c
	Growth stages main effect	503.85 c	730.93 b	877.93 a	–
Chlorophyll a	Open field	6.12 f	8.41 c	15.12 b	9.88 b
	Unheated glasshouse	7.80 d	8.16 cd	19.64 a	11.86 a
	Growth chamber	0.27 g	0.34 g	6.91 e	2.51 c
	Growth stages main effect	4.73 c	5.63 b	13.89 a	–
Chlorophyll b	Open field	0.71 e	0.90 c	1.26 ab	0.96 b
	Unheated glasshouse	0.88 c	1.22 b	1.31 a	1.13 a
	Growth chamber	0.44 f	0.66 e	0.78 d	0.63 c
	Growth stages main effect	0.67 c	0.93 b	1.12 a	–

*There is no difference in the level of 0.001 among averages that have the same letter.

for photosynthesis. In addition, it has an effect on growth rate, differentiation event in tissues and cells and organ formation in above ground parts of a plant.

As a result, spinach variety, matador was studied by many researchers, was grown in present study in three different growth environments like non-heated greenhouse, open field and growth chamber. The objective of present study is to determine variations, which occurred in total phenolic compound and chlorophyll contents during three different growth stages like cotyledon, 5 true leaves and harvest maturity. According to the obtained results, greenhouse conditions yielded the highest means relating to the criteria under study in early spring spinach production and it was followed by open field and growth chamber. Considering comparison of growth stages of spinaches grown under different growth conditions in three different environments, amounts of total phenolic compound, total chlorophyll, chlorophyll a and chlorophyll b increased towards the harvest phase in which the most maturated and aged plant was achieved. In other words, these matters were accumulated depending on the plant's growth. As a result, we recommend that spinaches produced under greenhouse conditions having the highest means with respect to phenolic compound having antioxidant effect are used in pharmacology and baby food while spinaches produced in open fields are sold in the market.

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