Original article

Antioxidant compounds and activity in cucumber fruit in response to foliar and soil humic acid application

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Summary

Humic acid (HA), with its hormon-like activity influences yield and quality of fruits and vegetables. This study was performed to determine the influence of foliar and soil HA fertilization on antioxidant properties of cucumber fruit. Cucumber plants were treated with soil and foliar HA at various concentrations (0 mL L^{-1}, 10 mL L^{-1}, 20 mL L^{-1}, 30 mL L^{-1} and 40 mL L^{-1}) via spraying or drenching to the plant root area. Fruits were harvested at commercial maturity and their antioxidant activities in the hydrophilic (HAA) and lipophilic (LAA) fractions, lycopene, beta carotene, xanthophylls, total phenolics and chlorogenic acid contents were determined. The results demonstrated that HA application significantly improved both HAA and LAA activities in cucumber fruit. Moreover, HA also significantly enhanced total carotenes and xanthophylls, beta carotene, lycopene and chlorogenic acid contents. The data suggested that HA could be used to significantly improve cucumber fruit quality by enhancing its content of antioxidant compounds.

Keywords
antioxidants, carotenoids, fruit, humic acid, phenolics, quality

Significance of this study

What is already known on this subject?
- Humic acid with its hormon-like activity improves the yield and quality of fruits and vegetables. Cucumber fruit has a very low level of antioxidants, and it is not known if humic acid can improve the antioxidant content of cucumber fruit.

What are the new findings?
- The application of humic acid improves cucumber fruit quality by enhancing its content of antioxidant compounds.

What is the expected impact on horticulture?
- Cucumber fruit with high contents of antioxidant compounds offer great economic and health benefits.

Introduction

Fruits and vegetables contribute significantly to human nutrition. Their effects on human health has been demonstrated through many studies (Boeing et al., 2007; Dauchet et al., 2007; Gebbers, 2007; Lee et al., 2004). Vegetables are rich in antioxidants, phenolics, vitamins, phytosterols, minerals and other phytochemicals. Among these, antioxidants have received significant attention among researchers due to their preventive effects on many human diseases. They have been shown to support human intrinsic antioxidant protection to maintain the internal oxidation status by various processes such as *in situ* regeneration of antioxidant molecules (vitamins and enzymes) or direct neutralization of oxidative compounds (Kohen and Nyska, 2002). Moreover antioxidant compounds have been implicated in the prevention of certain chronical diseases, e.g., hypertension, coronary heart diseases (Dauchet et al., 2006; He et al., 2007), stroke (Dauchet et al., 2005), cancer (Wang and Stoner, 2008), neurodegenerative diseases (Joseph et al., 2000), diabetes (Laaksonen and Sen, 2000), rheumatoid arthritis (Jawed et al., 2000), and cataracts (Taylor, 1992). Since free radicals also contribute to the process of aging, antioxidants indirectly influence aging (Kalt, 2005).

Oxidative stress causes molecular damage to many cell constituents including lipids, proteins and DNA. Mitochondrial respiration is the major contributor of oxidative stress. It has been shown that during mitochondrial electron transport, 3% to 5% of the oxygen consumed does not undergo a complete reduction and yields reactive oxygen species (Jackson, 2000). There are also external sources of free radicals that humans are exposed during their lives including cigarette smoke, pollutants, chemicals, and environmental toxins. Diets rich in vegetables with high antioxidant compounds have been very important in eliminating free radicals and thus preventing many of the resulting diseases (Boyer and Liu, 2004). The antioxidant activity in vegetables is the result of vitamins and provitamins, polyphenols, carotenoids, xanthophylls and anthocyanins (Conn et al., 1992; Di Mascio et al., 1989; Namiki, 1990; Rice-Evans et al., 1996).

Humic acid (HA) is an important source of plant nutrients and regulator of their release. Application of this organic matter-derived phytonutrient and growth regulator to vegetables including cucumber has been shown to result in a significant increase in their yield and quality characteristics (Atiyeh et al., 2002; Unlu et al., 2010). Studies have proposed that HA affects plant growth and development via enhancing nutrient uptake, serving as a source of mineral plant nutrients and regulator of their release (Atiyeh et al., 2002; Chen and Aviad, 1990; Jannin et al., 2012). Moreover, it has been suggested that HA can also directly influence plant metabolism (Nardi et al., 2002; Carletti et al., 2008; Jannin et al., 2012). On the other hand, Rubio et al. (2009) have reported the signal effect of some nutrients controls plant development by affecting hormone metabolism. This has also been confirmed in cucumber fruit by Mora et al. (2010) who have indicated that the beneficial effects of humic substances on shoot development in cucumber could be directly associated with nitrate-related effects on the shoot concentration of several active cytokinins and polyamines.

Cucumber fruit have various antioxidant compounds. Carotenoids and phenolic compounds are the major antioxidant constituents (Chu et al., 2002). However, it has been reported through screening of common vegetables that cucumber had the lowest total antioxidant activity and total phenolic contents among the vegetable species analyzed (Chu et al., 2002). Since HA significantly improves the total antioxidant activity and phenolic compounds, this study was undertaken to determine the possibilities of enhancing the antioxidant characteristics of cucumber fruit through soil and foliar HA application.

Materials and methods

Cucumber (*Cucumis sativus* L.) 'Mostar' seedlings were used as plant material in the study which was conducted in a greenhouse at the Research Farm of the College of Agriculture, Suleyman Demirel University, Isparta, Turkey, in 2008 and 2009. The seedlings were purchased from a local seedling production company (Grow Fide AS. Antalya, Turkey). The physical and chemical properties of the experimental area's soil were previously reported (Unlu et al., 2010). Before starting to the experiment, plots were fertilized with 50 tonnes ha^{-1} of cattle manure (1.7% N, 1.4% K_2O, 1.8% P_2O_5, with an EC value of 4.6 dS m^{-1}) and 400 kg ha^{-1} NPK (15:15:15). Seedlings were removed from pots and planted in rows 80 cm apart with an intra-row spacing of 50 cm. Each plot contained 10 plants. Plants were exposed to 0, 10, 20, 30 and 40 mL L^{-1} HA (Cukurova Tarim Lombrico, Adana, Turkey) solutions in order to provide 0, 300, 600, 900 and 1200 mL ha^{-1} HA respectively. Tween 20 (0.02%) was used as surfactant. Both foliar and soil HA treatments were performed three times with 15-day intervals during the vegetation period starting four weeks after planting. For foliar HA applications, a hand-held sprayer was used, and the abaxial surface of the leaves of plants were totally wetted with HA solutions since it has been reported that HA is absorbed faster and more effectively from abaxial surface (Hull et al., 1975). For soil HA treatments, the same concentrations of HA solutions were applied to the plant root area (Unlu et al., 2010). Plants sprayed both with 0.02% Tween 20 and drenched with distilled water served as the control (0 mL L^{-1} HA).

Plants were also fertilized with 100 kg ha^{-1} ammonium nitrate, 450 kg ha^{-1} potassium nitrate, 80 kg ha^{-1} potassium sulfate, 300 kg ha^{-1} calcium nitrate, 240 kg ha^{-1} mono ammonium phosphate and 150 kg ha^{-1} micronutrients with drip irrigation during the growth period. Other cultural practices (weed control, pest control, irrigation, etc.) were also applied uniformly throughout all plots. The experiment was set up in a randomized complete block design with three replications.

Fruits were harvested at commercial ripening stage (Sevgican, 2002). Ten harvested fruits from each replicate were washed with deionized water, weighed, peeled, cleaned free of seeds and cut into two halves. One half was liquefied by grinding, centrifuged and frozen at -20°C. The ten fruit extracts from the same replicate were combined; this constituted the water-soluble fraction for the antioxidant activity determinations in hydrophilic fraction (HAA). The remaining fruit pieces from the same replicate were combined, frozen at -20°C and used for the determination of carotenoids, xanthophylls, total phenolics, chlorogenic acid and antioxidant activity of the lipophilic fraction (LAA).

Total soluble phenolics

Total soluble phenolics were extracted as described by Coseteng and Lee (1987). For this purpose, cucumber fruit tissue (5 g) was grinded in liquid nitrogen with a mortar and a pestle. 20 ml of 95% ethanol was added to the grinded tissue and the slurry was boiled for 10 min. After centrifugation at 8000 rpm for 15 min, the extract was passed through GF/C filter paper. The residue was re-extracted with 20 ml of 80% ethanol and boiled for 10 min. Both supernatants were combined and used for the determination of total soluble phenolics and chlorogenic acid contents. Chlorogenic acid was determined using the method described by Coseteng and Lee (1987). Authentic chlorogenic acid was used as a Standard (Sigma). Total soluble phenolics were determined as described by Coseteng and Lee (1987) using tannic acid as a standard (Merck).

Carotenoids

Samples of cucumber fruit were homogenized using a pestle and a mortar in liquid nitrogen. One g of this homogenate was added to 16 ml of acetone-hexane (4:6) and mixed in a test-tube. Two phases separated, and an aliquot was taken from the upper solution and read in a spectrophotometer (PG Instruments) at 663, 645, 505, and 453 nm wavelengths.

Lycopene and b-carotene contents were calculated according to the Nagata and Yamashita (Nagata and Yamashita, 1992) equations: Lycopene (mg 100 ml^{-1} of extract) = $-0.0458 * A_{663} + 0.204 * A_{645} + 0.372 * A_{505} - 0.0806 * A_{453}$. b-Carotene (mg 100 ml^{-1} of extract) = $0.216 * A_{663} - 1.22 * A_{645} - 0.304 * A_{505} + 0.452 * A_{453}$. Lycopene and b-Carotene were finally expressed as µg g^{-1} FW.

Total xanthophylls were determined as described (AOAC, 1984). Results were expressed as µg per g fresh weight.

Antioxidant activity

The antioxidant capacities of the HAA and LAA of cucumber fruit were determined using the 2'2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) radical cation assay as described by Miller et al. (1993). For the preparation of $ABTS^{\cdot+}$ radical cation an excess of manganese dioxide (Sigma Chemical Co.) was added to a 5 mM aqueous stock solution of ABTS (Fluka). The solution was diluted in 5 mM phosphate-buffered saline, (pH 7.4), and pre-incubated at 30°C prior to use.

For standardization of the system, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, Sigma) was used (Miller et al., 1993, 1996). 1.0 ml of $ABTS^{\cdot+}$ solution was vortex-mixed with aliquots of trolox or the cucumber extract (extract used for carotenoids determination for LAA or cucumber juice for HAA determination) for 30 s and exactly 30 min after initiation of mixing, the absorbance at 734 nm was taken in a spectrophotometer (PG Instruments). A dose-response curve was obtained for trolox by plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation solution (blank) based on the equation:

Inhibition of A_{734} (%) = (1- the absorbance measured 30 min after the addition of antioxidant samples / the absorbance of uninhibited radical cation) × 100. Each sample was analyzed in triplicate and referenced to the trolox dose-response curve. The trolox equivalent antioxidant capacity (TEAC index) was defined as the concentration (mM) of trolox in phosphate buffer, and demonstrated as the antioxidant potential equivalent to the ml of juice utilized in the 30th reaction minute.

Statistical analyses

Statistical analyses were performed with the GLM procedure of SAS (SAS, 1985). In order to compare the effects of HA treatments, data from both years were combined and subjected to analysis of variance (ANOVA). The means were separated using Duncan's Multiple Range test at the 5% level of significance.

Results

In the study, we have investigated the influence of HA application on the antioxidant compounds of cucumber fruit. The results showed that HA significantly affected both LAA and HAA of cucumber fruit (Table 1). The highest LAA was obtained from foliar-20 HA treatment followed by foliar-30 and soil-40 HA treatments. Further increases in HA dose did not increase LAA content in the foliar treatments. Similarly, 20 mL L^{-1} HA foliar treatment also resulted in the highest HAA level. The lowest LAA and HAA levels were observed in control fruit. Application of HA to the soil also significantly increased LAA and HAA contents as compared to the control.

Total carotenoid content increased significantly in response to both soil and foliar HA treatments and the highest total carotenoid content was obtained from foliar-20 treatment followed by foliar-30, foliar-10 and soil-40 treatments (Table 1). One reason for the increase in total carotenoids was the significant increase in beta carotene content in response to HA (Table 1). The highest beta carotene content was obtained from foliar-20 HA treatment. Similarly total xanthophyll content of cucumber fruit was also significantly influenced by HA application. The highest xanthophyll content was again obtained from foliar-20 HA treatment. Another color component, lycopene, in fruits and vegetables also responded to HA treatment. Foliar-20 HA application resulted in a significant enhancement in lycopene content in cucumber fruit. However, other treatments did not affect lycopene content significantly. In general, it is evident from the data that both carotenes and xanthophylls significantly contributed to the higher antioxidative activity observed in cucumber fruit in response to HA application.

Chlorogenic acid content, a major phenolic compound, showed a significant increase in response to HA application (Table 1). The highest chlorogenic acid was obtained from foliar-30 HA application followed by foliar-20 HA treatment. However soil HA treatment did not cause a significant change in chlorogenic acid content of cucumber fruit. Similarly total soluble phenolics did not demonstrate a significant change in response to HA treatment.

Discussion

Free radicals are produced during normal metabolism of living organisms and from external sources such as pollutants, environmental toxins etc. These radicals, if not detoxified, cause significant damage to proteins, lipids and DNA and thus associating with many health problems in human (Kalt, 2005; Dauchet et al., 2005, 2006; He et al., 2007; Wang and Stoner, 2008). These radicals are effectively detoxified by antioxidative compounds. Since vegetables including cucumber fruit produce significant amounts of antioxidants, their consumption is important in the prevention of health problems caused by radical oxygen species (Boyer and Liu, 2004; Kalt, 2005; Unlu et al., 2010).

Humic acid (HA) is a phytonutrient and plant growth regulator, and it has been demonstrated to significantly promote cucumber shoot growth through nitrate-related changes and modification of root-to shoot distribution of cytokinins, polyamines and mineral nutrients (Mora et al., 2010). Moreover, foliar and soil HA application has been shown to increase the yield and quality of cucumber fruit (Ozdamar-Unlu et al., 2011). HA has also been shown to improve the antioxidant content of pepper fruit (Unlu et al., 2010). Cucumber fruit has a relatively low amount of antioxidants (Chu et al., 2002). In this study, we have investigated the influence of HA application on the antioxidant compounds of cucumber fruit. The results demonstrated

Table 1. Antioxidant activity and antioxidant compounds in cucumber fruit.

Humic acid treatment (ml/l)	TEAC Index of antioxidant activity in the lipophilic fraction (LAA)	Total carotenoids (µg/g)	Total xantophylls (µg/g)	Lycopene (µg/g)	ß-carotene (µg/g)	TEAC Index of antioxidant activity in the hydrophilic fraction (HAA)	Chlorogenic acid (µg/g)	Total soluble phenolics (µg/g)
Control	15,06 b	14,60 c	7,30 b	0,24 b	2,40 c	1,27c	0,19c	0,30
Foliar-10	16,12 b	21,59 ba	10,79 ba	0,30 b	3,71 b	2,74ab	0,28bc	0,43
Foliar-20	19,74 a	25,43 a	12,71 a	0,53 a	4,61 a	3,15a	0,32ab	0,45
Foliar-30	19,00 a	21,62 ba	10,81 ba	0,39 ba	4,29 ba	2,79ab	0,37a	0,54
Foliar-40	16,12 b	17,37 cb	8,68 b	0,29 b	3,38 b	1,97bc	0,21c	0,32
Soil-10	15,10 b	19,33 cb	9,67 ba	0,35 b	2,79 cb	2,60ab	0,21c	0,42
Soil-20	15,28 b	15,27 c	7,63 b	0,25 b	3,25 cb	2,18abc	0,22c	0,41
Soil-30	15,79 b	18,44 cb	9,22 ba	0,27 b	3,87 b	1,79bc	0,22c	0,42
Soil-40	18,48 a	20,70 b	10,35 ba	0,29 b	4,22 ba	2,22abc	0,23c	0,44

Means within each column followed by different letters are significantly different at 5% level of significance.

that HA application significantly increased both LAA and HAA of the fruit. Foliar-20 HA treatment resulted in the highest HAA and LAA content but further increases in HA dose did not cause more increase in antioxidant activity in foliar treatments possibly suggesting a toxicity effect of HA at higher doses. Similar results were also obtained from pepper fruit by Unlu et al. (2011) who have reported a significant increase in HAA and LAA of pepper fruit in response to HA treatment. These results have also been confirmed in another pepper cultivar (Aminifard et al., 2012). In all treatments and the control LAA activity was much higher than HAA activity. This could possibly result from the higher content of lipophylic antioxidant compounds of cucumber fruit (Melo et al., 2006).

There are several quality parameters contributing to the antioxidant activity in fruits and vegetables including total carotenoids and xanthophylls, lycopene, total soluble phenolics and chlorogenic acid (Unlu et al., 2010). These compounds may act as rapid donators of a hydrogen atom to peroxyl radicals, before they attack biological molecules. The influence of HA treatment on these compounds was also examined in the study. The increase in total carotenoid content observed in the study could be the results of the significant increases in beta carotene, total xanthophyll and lycopene contents in response to HA treatment. In support of our findings, Aminifard et al. (2012) have reported an increase in lycopene and beta carotene contents of pepper fruit in response to HA application. However, Unlu et al. (2010) have determined no significant change in lycopene content in pepper fruit upon exposure of the plants to HA. In general, it is evident from the data that carotenoids, xanthophylls and lycopene significantly contributed to the higher antioxidant activity observed in cucumber fruit in response to HA application.

Cucumber fruit contain high levels of other phytochemicals such as phenolics that can contribute to antioxidant activity (Ehlenfeldt and Prior, 2001; Chu et al., 2002; Unlu et al., 2010). In fact, chlorogenic acid content, a major phenolic compound, did show a significant increase in response to HA application (Table 1). On the other hand, total soluble phenolics did not demonstrate a significant change in response to HA treatment unlike the result of Unlu et al. (2010) and Aminifard et al. (2012), who have observed a significant increase in phenolic compounds in response to HA treatment and thus a significant contribution to antioxidant activity in pepper fruit. The increase in chlorogenic acid in cucumber fruit observed in our study possibly contributed to the antioxidant activity in hydrophylic fraction but overall the contribution of total soluble phenolics seems to be minimal. It has been reported that as compared to other vegetables, cucumber fruit has a very low level of phenolic compounds (Chu et al., 2002) and a negative correlation between antioxidant activity and phenolic content has been reported in cucumber (Melo et al., 2006).

In conclusion, HA application significantly increased antioxidant activity by affecting both LAA and HAA fractions. The increases in the contents of carotenoids, mainly beta carotene and xanthophylls, lycopene and chlorogenic acid in response to HA application are possibly responsible for the increase in antioxidant activity in cucumber fruit. The results suggest that humic acid application could be utilized to enhance overall fruit quality and their beneficial health effects (Shen et al., 1997) via increasing the levels of antioxidant compounds in cucumber fruit. It has been reported that humic acids enhance fruit quality via improving the availability and uptake of mineral nutrients and increases in root HATPase activity, nitrate concentration in the shoots and increases in N, C and S assimilation and several cytokinins and polyamines (Flores et al., 2004; Jannin et al., 2012; Mora et al., 2010; Mozafar, 1993). These studies have reported that N, P, K, and Ca fertilisation increased the carotenoid and phenolic contents and antioxidant activity in some fruits.

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