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Characterization of phenolics in two cultivars of broccoli and fennel grown under organic and bio-organic fertilization by high performance liquid chromatography (HPLC)

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ABSTRACT

A field experiment was carried out to studythe effect of organic and bio-organic fertilizers on the content of phenolics compound in broccoli and fennel. The resultsindicated the presence of phenolic acids and flavonoids in variable levels. Organic treatments were oriented to increase the accumulation of t phenolic compounds in broccoli and sweet fennel cultivars. Gallic acid, chlorogenic acid, 3,5-dimethoxybenzyl, *P*-cumaric, quercetin, kampferol and eugenol were found as most abundant consistuents in Calabrese and Southern star cultivars of broccoli. Whereas, Pinostrobin, Pyrogallic acid, Chlorogenic and Protocatechuic acid, were the main phenolic compounds detected in Dolce and *Zefafino* cultivars treated with organic fertilizer. These results indicated that there is a good margin for enhancing phenolic compounds of broccoli and fennel for economic production using organic fertilization. This study is alsoillustratedthe potential application of broccoli and fennel as a potent natural source of phenolic compoundsas antioxidants andnutraceuticals.

KEYWORDS

Broccoli;
Fennel;
Organic fertilizer;
Bio-organic fertilizer;
Phenolics;
HPLC.

INTRODUCTION

Brassica vegetables are known to play an important role in human nutrition due to their phytochemicals, such as vitamins, minerals, glucosinolates, and phenolic compounds[12]. In particular, it has been shown that *Brassica* species potentially exert inhibitory activity against chronic diseases like cancer (s).Much attention has been focused on broccoli as a source of bioactive compounds such as phenolics,flavonoids and glucosinolateswhich posse's antioxidant and anticancer effects[10,21,29,31]. Additionally, anthocyanins (acyloglycosides of cyanidin), are responsible for red and purple colours in a common red variety of kale, cabbage, broccoli and others Brassica vegetables[20].

Faller and Fialho[8], investigated the quality and quantity of soluble polyphenols which extracted with 50% methanol from fresh conventional and organic broccoli vegetables. They found that the soluble polyphenols content of broccoli grown using organic agriculture showed higher value (1.237 mg Gallic acid equivalent /ml), when compared with the conventionally grown samples (1.173 mg Gallic acid equivalent / ml).

Fennel (*Foeniculumvulgare*Mill. *Apiaceae*) is known as a medicinal aromatic herb. Its fruit is used in

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the remedy against digestive disorders while bitter fennel is used as food flavour, in liqueurs and in this perfumery industry[25]. The major volatile (essential) oil of this plant is anethole and fenchone[23]. Fennel extracts proved to have anti-inflammatory, antispasmodic, carminative, diuretic, expectorant, laxative, analgesic, stimulant of gastrointestinal mobility and are used in treatment of nervous disturbances[6]. Pereira et al.[19] reported the anticancer activity of fennel seed anthenol and suggested the use of a diet rich in phytochemicals to prevent and treat cardiovascular diseases and cancer.

In general, salicylic acid was the major phenolic compound in the fennel methanolic extract (449.54 mg/100 g DW), followed by phenol (61.17 mg/100 g DW). The least phenolic constituent was protocatchuic acid (0.41 mg/100 g DW)[13].

Among the studied flavonoids (aglicones), the quercetin, myricetin, kaempferol present antioxidant activity. The gallic and rosmarinic acids were the most potent antioxidants among the simple phenolic acids[11]. Nutrients and antioxidants may act together to reduce reactive oxygen species level more effectively than single dietary antioxidant, because they can function as synergists[26].

It has been claimed that due to the higher amounts of secondary phenolic metabolites in these plants, organically produced plant foods could be expected to be more health-promoting than conventional foods. Organically grown cabbage, spinach, Welsh onion, green pepper generally had higher levels of flavonoids and antioxidant activity[2]. In recent times, consumers are demanding higher quality and safer food and highly interested in organic products. Sousa et al.[24] concluded that organic fertilization has a stimulatory effect on accumulation of phenolics in broccoli florets and fennel. The higher concentrations of phenolics in the edible parts of broccoli and fennel can be explained by the role of organic fertilizers in the biosynthesis which induces the acetate shikimate pathway, resulting in higher production of flavonoids and phenolics. Also, because of the higher photo–pathogenic stress in organic farming, this in turn may have abiotic stress, and causes an increase of phenolics, grown organically[30].

The importance of phenolic compounds as antioxidant in the maintenance of health and protection against coronary heart disease and cancer is also of raising interest among scientists, food manufacturers and consumers. This trend leads the future moving toward functional food with specific health effects[16]. Serafini et al.[22] and Carbonneau et al.[5] suggested that flavonoids and other phenolics have a preventive role in the development of cancer and heart disease. Consumption of controlled high fruits and vegetables diets increased significantly the antioxidant capacity of human plasma[3]. Moreover, epidemiological studies showed that significant negative correlation between the intake of fruits and vegetables and heart disease mortality[14]. Carbanaro et al.[4] and Floridi et al.[9] showed that the presence of phenolics in food is particularly important for their oxidative stability and antimicrobial protection.

The identification of phenolics in broccoli and fennel plants will help in optimizing and increase the application and benefit of these plants as a source of therapeutic compounds.

Thus, the aim of the present study is to characterize the phenolic compounds in two cultivars of broccoli and fennel as affected by organic and bio-organic fertilizers.

RESULTS AND DISCUSSION

Identification of phenolic compounds in broccoli cultivars

The HPLC profile of crude methanolic extracts of broccoli cultivars grown with organic and bioorganic fertilizers is illustrated in TABLE 1.

Application of organic fertilizer enhanced phenolic compounds in both cultivars of broccoli compared to bio-organic fertilizers. In *Calabrace*cultivars organic treatment showed higher content in most phenolic compounds especially Quercetin (391.56 mg/ 100g DW), chlorogenic acid (117.38 mg/100g DW), trans-ciannamic acid (77.13 mg/100g DW), myristine (47.52mg/100g DW), genistin (25.30mg/100g DW), p- cumaric acid (19.19mg/100g DW) and rutin (12.27 mg/100g DW). Whereas, in *Southern star* cultivars organic treatment showed higher content in phenolic compounds especially in pyrogallic acid (106.95mg/100gdw), chlorogenic acid (113.20 mg/100g DW), 3,5-dimethoxybenzyl alcohol (103.67mg/100g DW), p-cumaric acid anhydride (17.65mg / 100g DW),genistin (12.31 mg/100gDW),trans –cinnamic acid (43.56 mg/ 100gDW) kampferol (44.27 mg/100g DW) and agacetin (1.99mg/100g DW). Gallic acid, chlorogenic acid, salicylic acid, 3,5-dimethoxybenzyl alcohol, quercetin, kampferol and eugenolwere the most abundant constituents compared to bioorganic treatment.

TABLE 1 : Phenolic compounds of two cultivars of broccoli as affected by organic and bio-organic fertilizers (mg/ 100gD.W)

Phenolic compounds	*Calabrese*		*Southern star*	
	Organic	Bioorganic	Organic	Bioorganic
Pyrogallic acid	186.72	188.47	106.95	87.80
Gallic acid	192.43	183.78	104.51	106.10
Resorcinol	59.82	56.80	24.79	37.48
Protocatechuic acid	-	-	9.23	8.31
Para-hydroxy benzoic	20.09	17.91	-	-
Chlorogenic acid	117.38	84.89	113.20	87.24
Catechines	-	18.35	-	21.80
Caffeic acid	6.26	8.13	2.79	8.76
Vanillin	5.08	4.68	4.29	6.32
Salicylic acid	186.54	175.91	127.40	136.17
Ferulic acid	9.96	11.41	39.48	41.51
3,5-Dimethoxybenzyl alcohol	262.37	255.91	103.67	77.84
Rutin	12.27	9.60	3.30	1.32
P-Coumaric acid anhydride	19.19	10.88	17.65	11.34
Genistin	25.30	12.69	12.31	4.97
Myricetine	47.52	33.13	51.83	46.39
Trans-Cinnamic acid	77.13	58.06	43.56	19.17
Quercetin	391.56	326.41	312.21	281.76
Luteolin	10.18	8.07	9.96	9.72
Kampferol	55.40	31.31	44.27	21.09
phenolphathalein	3.17	1.60	-	-
Eugenol	114.39	108.81	74.41	71.77
Acacetin	2.16	1.34	1.99	0.80
Pinostrobin	-	-	116.93	107.26

In general, quercetin and 3,5-dimethoxybenzyl alcohol were the major phenolic compounds in *Calabrase* and *Southern star* cultivars followed by gallic, pyrogallic, salicylic acid, chlorogenic acid and Eugenol. Quercetin ranged between 391.56 mg/100 g DW in *Calabrase* to 312.21 mg/100 g DW in Southern star cultivar.

The least phenolic constituent was acacetin, which was detected in *Calebrace* and *Southern star* in presence of organic fertilizer. Whereas, catechines was found in extracts of broccoli treated with bioorganic fertilizer (18.35 and 21.80mg/100 g DW). In this concern, Nakatani[18], mentioned that phenolic compounds ranged between plants according to their genus species, varieties, cultivars, and types of fertilizer. Flavonoids, as they represent the most important fraction of the phenolics were mostly represented by quercetin and kampferolderivates as also found in broccoli[21].

The two cultivars of broccoli presented significant differences in terms of constituent phenolics content and induced accumulation of phenolics in responses to organic fertilizer. A great variability of phenolic compounds levels respect to cultivars and different agronomic techniques has been reported by Vallejo et al.[27], they found that, the level of sinapic and ferulicderivates were 23 and 251 mg / Kg F.W in broccoli plant. This indicates a quit strong diversity, within the same species, in terms of metabolic responses to environmental factors and cultural practices.

The results of the present study are in agreement with Martinez et al.[17], who found that polyphenol composition of members of the *brassica*vegetables contain flavonoids, and especially flavonols. A large number of flavonoid glycosides have been found; among them, glycosides of kaempferol and quercetin, their derivatives in combination with hydroxycinnamic acids as well as sinapic acid derivatives have been found to be the most important phenolic compounds in *brassica* species.

Also, Price et al.[21], detected the individual flavonol glycosides by HPLC in *Marathon* cultivars of broccoli which was grown under commercial conditions. It was found that two main flavonol glycosides present in broccoli florets were identified as quercetin 3-*O*-sophroside and kampferol 3-*O*-sophroside. Three minor glucosides of quercetin and kampferol were also detected, namely iso-quercetin, kampferol 3-glucoside and kampferoldiglucoside.

In addition, Faller and Fialho (2009), and Zhao et al. (2009), found that the soluble polyphenols content of broccoli grown organically showed higher values when compared with the conventionally ones.

Identification of phenolic compounds in sweet fennel cultivars

The influence of organic and bio-organic fertilizers treatments on the HPLC profile of crude 80% methanolic extracts of two sweet fennel cultivars was presented in TABLE 2. Organic treatments were found to increase the content of most phenolic compounds in both sweet fennel cultivars compared with bioorganic treatment.

TABLE 2 : Phenolic compounds of sweet fennel cultivars as affected by organic and bio-organic fertilizers (mg/ 100 g D.W)

Phenolic compounds	*Dolce*		*Zefafino*	
	Organic	Bioorganic	Organic	Bioorganic
Pyrogallic acid	197.79	163.83	99.75	90.05
Gallic acid	2.21	1.52	2.12	1.18
Resorcinol	9.03	9.59	17.38	5.38
Protocatechuic acid	97.28	78.40	90.80	70.24
Para-hydroxy benzoic	0.33	0.07	0.08	0.26
Phenol	4.59	4.60	6.93	5.28
Chlorogenic acid	122.76	112.92	108.44	92.46
Caffeic acid	15.99	8.82	12.48	4.44
Vanillin	0.66	0.42	0.34	0.51
Salicylic acid	51.65	42.36	34.04	17.76
Ferulic acid	1.63	1.13	0.46	7.99
3,5-Dimethoxybenzyl alcohol	11.62	9.62	14.38	7.54
Rutin	37.01	30.25	36.47	16.65
P-Coumaric acid anhydride	0.89	0.61	-	-
Genistin	11.87	9.05	15.62	11.10
Myricetine	9.71	7.01	12.37	5.59
Trans-Cinnamic acid	0.01	0.02	0.22	-
Quercetin	2.63	2.07	0.70	2.13
Apigenine (Genistein)	4.66	2.95	3.60	2.29
Pinocembrin	17.95	15.47	15.22	11.59
Chrysin	0.86	0.86	0.75	-
Galangin	2.28	2.42	-	-
Acacetin	-	-	11.97	11.15
3,5-dihydroxy isoflavone	2.37	2.44	-	-
Pinostrobin	305.62	291.33	304.25	278.06

Dolce cultivar organic treatment showed, higher content in most phenolic compounds especially pyrogallic (197.79 mg/DW), protocatechuic acid (97.28 mg/100g DW), Chlorogenic acid (122.76 mg/100gDW),Salicylic acid (51.65 mg/100gDW) and Pinostrobin (305.62 mg/100gDW).

Whereas, in *Zefafino*organic treatment, higher content of phenolics especially in Pyrogalliic (99.75 mg/100gDW), Chlorogenic (108.44 mg/100g DW),Protocatechuic acid (90.80 mg/100gDW), caffeic and (12.48 mg/100g DW),salicylic acid (34.04 mg/100g DW,Rutin (36.47 mg/100gDW) and Pinostrobin (304.25mg/100g DW).

In contrast, bio-organic treatment showed relatively lower content in phenolic compounds in both cultivars of sweet fennel compared to organic treatment (TABLE 2). It demonstrates pyrogallic (163.83 mg/100gDW), protocatechuic acid (78.40 mg/100g DW),caffeic and (8.82 mg/100g DW), and vanillin (0.42 mg/100gDW). Whereas, *zefafina* bioorganic treatment showed lower content of phenolics especially in resorcinol (5.38mg/100gDW), protocatechuic acid (70.24 mg/100g DW),caffeic (4.44 mg/100g DW), salicylic acid (17.76 mg/100g DW),3,5 dimethoxybenzyl alcohol (7.54 mg/100g DW),rutin (16.65 mg/100gDW) and myricetine (5.59 mg/100gDW).

These results are in concomitant with Khalil et al.[13], who found that,fourteen phenolic compounds were identified in fennel methanolic extract grown under organic farming condition,. These constituents were cinnamic acid, chlorogenic acid, coumarin, ferulic acid hydroquinone, hydroxylbenzoic, *O*-coumaric acid, *p*-coumaric acid, phenol, protocatchuic acid, pyrogallic acid, resorcinol, salicylic acid and vanillin. They added that salicylic acid was the major phenolic compound in the fennel methanolic extract and the least phenolic constituent was protocatchuic acid.

Also, Krizman et al.[17], found that the major phenolic compounds present in fennel plant materials were chlorogenic acid, 4-O-caffeoylquinic acid (4-CQA), eriocitrin, rutin, miquelianin, 1,3-O-dicaffeoylquinic acid (1,3-diCQA), 1,5-O-dicaffeoylquinic acid (1,5-diCQA), 1,4-O-dicaffeoylquinic acid (1,4-diCQA) and rosmarinic acid. The content of individual analyses in fennel plant material ranged from below 10 to over 1700 mg/100 g of dry weight. Eriocitrin and rosmarinic acid were not found in any of the samples analyzed, while miquelianin content was substantially higher. A feasible explanation for such differences among fennel samples is due to thedifferences in growing conditions in different regions and also to the differences in the plant genotypes.

Organic treatment showed higher content in most phenolic compounds compared to bioorganic treatment in both cultivars of broccoli and fennel. This may explained by the findings of Young et al.[30], as they, concluded that the higher photopathogenic stress in organic farming may have abiotic stress and causes the increase of phenolics in broccoli and fennel grown organically or might be due to the dilution effect.

Therefore, organic fertilization could be concerned in elevating the production value of the plant as a source of phenolic compounds for the use in medical research against certain diseases. In addition both broccoli and fennel should be considered as a source of bioactive compounds such as phenolics and flavonoids which posses antioxidant and anticancer effects.

EXPERIMENTAL

Chemicals

All chemicals and solvents used were HPLC spectral grade, and obtained from Sigma (St. louis, USA) from Merck – Shcuchrdt (Munich, Germany).

Plant materials

Two cultivars of broccoli (*Brassica olaracea*, var. *Italica*) belong to family *Brassicacea* (*Cruciferous*). *Calabrese* was obtained from GSN semences Co., France. Southern star was obtained from Takii Co., Japan, family. While two cultivars of sweet fennel (*Foeniculumvulgar*, Mill.) belong to family *Apiaceae* (*Umbelliferae*) were *Dulce* and *Zefafino*and were btained from Topstar Co., Holland.

Field experiment

A field experiment was carried out in newly reclaimed sandy soil at El-Saff, Hellwan Governorate, Egypt. Seeds of broccoli and fennel were sown in the foam trays filled with a mixture of peat moss and vermiculite (1:1 volume). Seedlings at 45 days old were transplanted in the open field on the center of raw and spacing of 50 cm between plants. Ditches of 20 cm depth and 40 cm width were prepared in the sites of drip irrigation lines; chicken manure, rocks phosphate, calcium super phosphate and agricultural sulpher were mixed and were added and covered with soil before transplanting. Plot area was 3.5 lengths and 3.0 width in three raw which equals 10.5 m^2 (1/400 fed).

Fertilizer treatments

Organic fertilizer

Complementary between 50 % organic (chicken manure) at the rate of 100 N_2 unit/fed (5.850 m^3/fed) and 50% mineral (NPK) was used. NPK is formed from ammonium sulphate (21.5 % N) as a source of nitrogen, at the rate of 100 N_2 Kg/fed; calcium super phosphate (15.5 % P_2O_5) and rock phosphate (30% P) were used at the rate of 60 P_2O_5 Kg /fed; Potassium sulphate (48% K_2O) was used at the rate of 40 K_2O Kg/fed. In addition, agricultural sulphur was used at the rate of (200 Kg/fed) based on the recommended fertilizers requirements according to Ministry of Agriculture. Ammonium and potassium sulphate were splinted into three equal doses and applied as dressing (0, 30 and 60 day after transplanting) beside plants.

Bio-organic fertilizer

Experimental

Bio-organic fertilizer is composed of organic treatment in addition to bio-fertilizers (50% *Azotobacter chrococcum* and 50% *Bacillus megaterium*). Bio-organic fertilizer was applied to the plant once after 2, 5 and 8 weeks of transplanting at the level rate of 10 ml / plant and concentration of 10^9 cell/ml. The mixture was injected near the root plants.

The bio- organic fertilizer was kindly provided by the Agriculture Microbiology Department, National Research Center. It contained a mixture of N_2-fixing bacteria *Azotobacter chrococcum* and phosphate dissolving bacteria *Bacillus megaterium* (1:1 v/v).

In all treatments, Drip irrigation line was spread over theditches and soil was irrigated continuously three days before transplanting.

Each treatment included 3 replicates as the combination of two cultivars of broccoli or sweet fennel and three fertilizer treatments. The split plot design with three replicates was used. The main plots were assigned for cultivars, whereas the sub plots assigned for the fertilizer treatments.

Preparation of fresh plant methanolic extract

The edible parts (broccoli florets), and bulbs of sweet fennel were extracted by liquid nitrogen. Ten grams of frozen tissues were ground in mortar with pestles and soaked in 100 ml of 80% methanol for (48 h).

Identification of phenolic compounds by HPLC

Phenolic compounds ofbroccoli and sweet fennel extracts were identified according to Ben-Hammouda et al.[1].

Identification of individual phenolic compounds in methanolic extract of plant samples (Broccoli and Sweet fennel), was performed using JASCO HPLC, with a hypersil C18 reversed-phase column (250 × 4.6 mm) and 5 urn particle sizes.

Ten grams of fresh samples were soaked in 100 ml of methanol (80%) and filtered through a 0.2 μm filter sterilized membrane prior to HPLC analysis. Injection by means of a Rheodyne injection valve (Model 7125) with 50 pJ fixed loop was used. A constant flow rate of 1 ml /min was used with two mobile phases: (A) 0.5% acetic acid in hypersil C18 reversed-phase column (250 × 4.6 mm) with 5 μm particle size. Injection by means of a Rheodyne injection valve (Model 7125) with 50 pJ fixed loop was used. A constant flow rate of 1 ml / min was used with two mobile phases: (A) 0.5% acetic acid in distilled water at pH 2.65; and solvent (B) 0.5% acetic acid in 99.5% acetonitrile. The elution gradient was linear starting with (A) and ending with (B) over 50 min, using an UV detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standard mixture chromatogram. The concentration of an individual compound was calculated on the basis of peak area measurements, and then converted to μg phenolic / g dry weight.

CONCLUSION

It could be concluded that organic fertilization might be concerned in elevating the production value of the plant as a source of phenolic compounds for the use in medical research against certain diseases. In addition suggested the potential application of broccoli and fennel as a potent natural source of phenolic compounds as antioxidants and nutraceuticals.

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