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Isoflavonoids, Flavonoids, Phenolic Acids Profiles and Antioxidant Activity of Soybean Seeds as Affected by Organic and Bioorganic Fertilization

[1]Hanan A.A. Taie, [2]R. El-Mergawi and [3]S. Radwan

[1]Plant Biochemistry Department, National Research Centre, Cairo, Egypt
[2]Botany Department, National Research Centre, Cairo, Egypt
[3]Agricultural Microbiology Department, National Research Centre, Cairo, Egypt

Abstract: Soybean intake is inversely correlated with risks of several chronic diseases in human. Phytochemicals and in particular phenolic acids, flavonoids and isoflavonoids compounds present in soybean seeds may be partially responsible for these health benefits through their antioxidants activity. In a pot experiment, soybean plants was grown in sandy media and irrigated with Hoagland solution as inorganic treatment or grown under one of the three levels of compost i.e. 25, 50 and 75% alone or in a mixture with multi-bioorganic. Individual phenolic acids, quercetin, genistein and daidzein in seeds were quantified by HPLC. Changes in the levels of individual phenolics and less value of quercetin under various compost levels resulting in insignificant lower values of total phenolics and total flavonoids as compared with inorganic grown seeds. Adding multi-bioorganic to 50 or 75% compost treatments produce great enhancement effects on total phenolics, total flavonoids, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, quercetin, genistein and daidzein contents as compared with other treatments. The most enhancement effect was exhibited phenolic acids, quercetin and genistein. Addition of multi-bioorganic to 50% compost treatments resulted in 68 and 40% increases in quercetin and genistein, respectively, as compare with inorganic treatment, as well as 90 and 47% increases, respectively, as compare with 50% compost alone. The highest values of antioxidant activity was determined in soybean seeds grown under 50 and 75% compost, especially when mixed with bioorganic. Using either 50 or 75%, compost in a mixture with bioorganic caused increases in antioxidant activity ranged between 25 and 60% when compared with other inorganic and compost alone treatments. These results indicate that bioorganic fertilizers play a role in determining antioxidant activity and phenolic metabolites of soybean seeds.

Key wards: Soybean · Compost · Bioorganic · Isoflavonoids · Phenolics · Quercetin · Antioxidant

INTRODUCTION

Soybean has received considerable attention of late decade for their potential role in reducing the formation and progression of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer.s disease, and osteoporosis [1-5]. Several anti-carcinogens, including phenolics such as phenolic acids, flavonoids and isoflavonoids have been identified in soybeans. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activity of these phenolic compounds [6,7]. As with other phenolic and flavonoid antioxidants, soybean isoflavones may exhibit their cancer preventive function through their antioxidant properties.

Genistein, the major component of soybean isoflavones, has been demonstrated to inhibit ultraviolet-B (UVB)-induced skin tumorigenesis in hairless mice. The antioxidant properties of genistein may explain the mechanisms of its anti-photocarcinogenic action because through either direct quenching of reactive oxygen species or indirect anti-inflammatory effects, genistein was found to substantially inhibit a series of oxidative events elicited by UVB irradiation, including hydrogen peroxide production, lipid peroxidation, and 8-hydroxy-2.-deoxyguanosine formation [8].

Over the last few decades, consumers demand for healthier food and governments policies focused on environmentally sustainable agricultural systems have both promoted a rapid expansion of organic farming [9,10]. Organic food production is characterized by the absence

Corresponding Author: Dr. Hanan A.A. Taie, Plant Biochemistry Department, National Research Centre, Cairo, Egypt

of synthetic compound (herbicides, pesticides) and readily soluble fertilizers and relies more on natural mechanisms of controlling the growth, yield and diseases. Application of compost to sandy soil increased organic matter, cation exchange capacity, available nutrients and biological properties [11].

Manach *et al.* [12] reported that nowadays, emphasis on multi-strain biofertilizer has already been tidied. Biofertilizers are biological preparations embodying, essentially, sufficient densities of potent strains of microorganisms, having a tangible beneficial role in fitting a proper rhizosphere for plant growth [13].

Some researchers suggest that, of all the measures of nutrient quality, concentrations of phytochemicals have the potential to differ the most between organic and conventional foods [14]. It has been claimed that due to their higher amounts of secondary phenolic metabolites, organically produced plant foods could be expected to be more health-promoting than conventional foods [15, 16]. Organically grown cabbage, spinach, Welsh onion, green pepper generally had higher levels of flavonoids and antioxidant activity [17]. Also, Asami *et al.* [18] mentioned that phenolics and ascorbic acid are presented in higher levels in organics corn, strawberry and Marino berry than in conventional. Comparing antioxidant levels in conventionally and organically-cultivated crops are variable and subject to numerous confounding factors. Studies have reported a range of results including higher levels [18, 19] or no effect [20] or lower levels [11] of specific antioxidants in some organic fruits and vegetables. Profiling soybean isoflavones, flavonoids and phenolic acids could help to elucidate how these phenolic constituents are regulated under organic and bioorganic fertilization to produce soybean crops and soy foods with greater health benefits. The aim of this study was to investigate the difference effect between organic fertilization using various levels of compost alone or in mixture with biofertilizers and inorganic fertilization on isoflavonoids, quercetin, phenolic acids contents and antioxidant activity of soybean seeds.

MATERIALS AND METHODS

A pot experiment was conducted in the green house of National Research Center, Giza, Egypt. The experiment was conducted in a complete randomized block design with 8 replicates and included 7 fertilization treatments using washed sand, compost and multi-bioorganic fertilizer, as follows:

Table 1: Chemical analysis of composted organic wastes

Character	Values
pH	7.32
EC (mmhos/cm)	2.85
O.C. (%)	35.11
Total N (%)	1.78
Total P (%)	0.15
C/N	19.72

- 100% Sand (Inorganic fertilization treatment)
- 25% Compost +75% sand (v/v)
- 50% Compost + 50% sand (v/v)
- 75% Compost + 25% sand (v/v)
- 25% Compost + 75% sand (v/v) + multi- bioorganic fertilizer
- 50% Compost +50% sand (v/v) + multi- bioorganic fertilizer
- 75% Compost + 25% sand (v/v) + multi- bioorganic fertilizer

Preparation and Application of Compost: The plant residues were air-dried, sieved to pass through 0.2 mm sieve and moistened to 70% of their water holding capacity and mixed with a chemical accelerator (7 kg superphosphate, 40 kg ammonium sulphate and 35 kg calcium carbonate and 100 kg fertile soil per ton dry matter). Treated plant residues were packed plastic bags capacity of 20 kg, and were turned off every two weeks. The moisture content during the composting course, 120 days, was kept at a proper level throughout irrigation [21]. The chemical analysis of composted organic wastes was determined (Table 1). Composted organic wastes were added and mixed with sand before sowing in composted treatments pots.

Preparation and Application of Bioorganic Fertilizer: Highly efficient strains of *Bradyrhizopium japonicum*; phosphate dissolving bacteria (*Bacillus megaterium* var. *phosphaticum*), *Azospirillum* spp. and *Pseudomonas* spp. were independently grown in nutrient broth [22] for 48 hours at 30°C in a rotary shaking incubator. Liquid broth cultures initially containing 7×10^{8}, 5×10^{7} and 3×10^{7} viable /ml, respectively. In biofertilization treatments, 100 ml of either tested microorganisms suspension were added to the soil in each biofertilized pot just after sowing.

Soybean Cultivation: Soybean seeds obtained from Agricultural Research Center were sown in plastic pots

30× 40 cm at the rate of 3 seeds per pot. The seedlings were thinned to two after 15 days of sowing. Pots of first treatment irrigated with Hoagland solution as inorganic fertilization treatment, while pots of other treatments were irrigated routinely with tap water. The moisture content was kept in the pots at a proper level by eventual irrigation. At harvest time, (after 180 days). Seeds were collected and subjected to analysis.

Determination of Total Phenolics: The total phenolic content of ethanol extract of powdered soybean seeds was determined according to the method described by Makkar *et al*. [23]. Aliquots of the extract were taken in a test tube and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The amount of total phenolics was calculated as pyrogallol equivalents from a calibration curve.

Determination of Total Flavonoids: Total flavonoids were estimated using the method of Ordoñez *et al.* [24]. To 0.5 ml of ethanolic extract, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature filtered, then the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin equivalent from a calibration curve.

Antioxidant Activity (DPPH Assay): The free radical scavenging activity using the 1.1-diphenyl-2-picryl-hydrazil (DPPH) reagent was determined according to Brand-Williams *et al.* [25] The powdered seeds(1 g) was extracted with 50% methanol: water. To 0.75 ml of the extract sample 1.5 ml of freshly prepared methanolic DPPH solution (20 μg ml^{-1}) was added and stirred. The decolourizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control.

Antioxidant activity = [(control absorbance - sample absorbance) / control absorbance] × 100%

Determination of Phenolic Acids: Phenolic acids were extracted with ethyl acetate after alkaline hydrolysis and subjected to HPLC analysis as described by Mckeehen *et al*. [26]. Ethyl acetate residue was resolubilized in methanol, filtered through a 0.2 μmPTFE filter prior to subjected to analysis using Shimadzu HPLC. Separations were achieved on a 250×4.6 mm C18 ODS column with a 30 × 4.6mm precolumn of the same material. Phenolic acids were separated by gradient elution using methanol and phosphate buffer and detected at 280 nm.

Determination of Quercetin, Genistein and Daidzein: flavonoids and isoflavonoids in soybean seeds were hydrolyzed and determined according Ewald *et al*. [27] as follows: 0.5 g of powder was hydrolyzed in 40 ml of 62.5% aqueous methanol with 2 mg /ml of BHT and 10 ml of 6 M HCl at 90°C with reflux for 2 h. Methanol was added to all samples to 100ml after hydrolysis, and the samples were finally sonicated for 5 min to remove oxygen before subjected to analysis using HPLC. The samples were separated using HPLC with reversed phase column manufactured by Shimadzu, Japan. The mobile phase consisted of 30% of the acetonitrile in 0. 025 M potassium dihydrogen phosphate (pH 2.4) with a flow rate 1.3 ml/min. The compounds were detected by a UV detector set at 330 nm.

RESULTS

Total Phenolics: Seeds of soybean grown organically using 25 or 50 or 75% compost had non-significant lower phenolics than those of inorganic fertilization treatments. Additions of bioorganic to compost at 50 or 75% resulted in significant effect on total phenolic contents as compared with other treatments. These two treatments increase phenolics by 10 and 9% compared with inorganic fertilization treatment, respectively and with more than 20% when compared with the corresponded organic fertilization treatments.

Total Flavonoids: There were no significant differences in total flavonoids of soybean seeds between inorganic, organic and bioorganic fertilization treatments (Table 2). Seeds of soybean grown using mixture of compost and biofertilizer exhibited insignificant higher flavonoid concentrations relative to those of inorganic or organic fertilization treatments.

Antioxidant Activity: As shown in Table 2 organic and bioorganic treatments tended to increase antioxidant activity of soybean seeds. All organic and bioorganic treatments produced significant effect on antioxidant activity as compared with inorganic treatments, except antioxidant activity of 25% compost alone or with biofertilizer. Soybean grown using compost at 50 and

Table 2: Effect of inorganic, organic and bioorganic fertilizers on phenolics, flavonoids and antioxidant capacity of soybean seeds

Treatments	Total phenolics (mg/g)	Total flavonoids (mg/g)	Antioxidant Capacity
Inorganic fertilizers	10.65	6.81	14.0
25% Compost	10.06	6.96	13.1
50% Compost	9.51	6.58	14.7
75% Compost	9.41	6.91	15.3
25% Compost+ Bioorganic	9.73	7.50	11.4
50% Compost +Bioorganic	11.67	7.31	19.1
75% Compost +Bioorganic	11.58	7.10	21.1
LSD 5%	1.80	NS	0.5

Table 3: Effect of inorganic, organic and bioorganic fertilizers on phenolic acids (µg/g) of soybean seeds

Treatments	Protoc.	pHB	Chlorog.	Caffeic	Coumaric	Ferulic
Inorganic fertilizers	1.00	19.46	57.46	57.45	25.68	29.44
25% Compost	0.96	18.33	54.34	52.54	24.16	20.37
50% Compost	0.33	20.93	62.50	58.77	22.57	28.66
75% Compost	0.34	21.07	76.15	84.57	14.82	25.70
25% Compost +Bio.	0.56	16.00	69.37	66.44	12.98	28.48
50% Compost + Bio.	1.75	26.18	85.16	85.32	27.45	20.07
75% Compost + Bio.	6.36	26.19	67.89	69.66	31.99	21.62

Protoc. = Protocatechuic acid; PHB=*p*- Hydroxybenzoic acid; Chlorog.= Chlorogenic acid

Table 4: Effect of inorganic, organic and bioorganic fertilizers on quercetin flavonoid and isoflavonoids genistein & daidzein

Treatments	Quercetin (µg/g)	Genistein (µg/g)	Daidzein (µg/g)	Genistein + Daidzein
Inorganic fertilizers	152	136	79	215
25% Compost	148	142	73	215
50% Compost	135	129	84	213
75% Compost	136	139	75	214
25% Compost + Bioorganic	148	113	99	212
50% Compost +Bioorganic	256	190	87	277
75% Compost+ Bioorganic	266	145	92	237

75% had 5 and 9% higher antioxidant activity than the inorganic fertilization treatment, respectively. Moreover, addition of biofertilizer to compost at 50 or 75% resulted in the highest antioxidant activity that reached 136 and 150% as relative to those of inorganic treatment, respectively.

Phenolic Acids: Table 3 shows the concentration of individual phenolic acids in the soybean seeds as affected by different fertilization treatments. Application of 25% compost alone decreased the concentration of all phenolic acids in the seeds relative to inorganic fertilizer. Phenolic acids varied in their response to increasing the amount of compost to 50 or to 75% in the soil media. These treatments caused decreases in the concentration of protocatechuic, coumaric and ferulic acids and increases in the concentration of *p*-Hydroxybenzoic, chlorogenic and caffeic acids as compare with inorganic fertilization treatment. Meanwhile, addition of biofertilizer to compost at 50 and 75% resulted in a pronounced increase on the concentration of protocatechuic, *p*-hydroxybenzoic, chlorogenic and caffeic acids.

Quercetin: Quercetin is the most abundant flavonoids present in soybean seeds. As shown in Table 4 lower quercetin concentrations was determined for organic fertilization using different levels of compost as compared with other treatments. Addition of biofertilizer to compost, especially at 50 and 75% levels resulted in a great enhancement effect in quercetin contents. These two treatments increased quercetin contents with 90 and 96% as compared with those of corresponded compost alone and with 68 and 75% increases when compared with inorganic fertilization treatment, respectively.

Isoflavonoids: Two major isoflavonoids compounds (i.e. daidzein and genistein) in soybean seeds were determined using HPLC. Data in Table 4 indicated that organic fertilization using different levels of compost did not produce any great affect on daidzein, genistein and the sum of two compounds as compared with inorganic fertilization treatment. Addition biofertilizer to 50 or 75% compost caused dramatic increase in the concentration of daidzein, genistein compared with other treatments. The highest value of the sum of two isoflavonoid compounds was determined when mixing biofertilizer with 50% compost, that reached about 130% relative to those of either 50% compost alone or those of inorganic treatment.

DISCUSSION

Organically grown seeds using various compost levels had insignificant lower values of total phenolics and total flavonoids as compared with inorganic treatment. Variability in the levels of individual phenolic acids and less values of quercetin under organic condition appeared to be major factors in the failure to find statistical significance. These results are in line to those obtained by Haakkinen and Torronen [28] and Young *et al*. [20]. They reported that organic production method alone did not enhance biosynthesis of phytochemicals as phenolics in strawberries, lettuce and collards. On the contrary, Asami *et al.* [18], Caronaro *et al.* [19] and Mitchell *et al.* [29] used agricultural production techniques to examine phenolic levels in organic and conventional plant foods and found a higher level of certain phenolics on organic samples than in those conventionally grown. They attributed these results to the possibility of higher pathogenic pressures in organic methods of growing, in which no pesticides were used, which in turn may have produced biotic and abiotic stresses and caused an increase in the levels of phenolics produced by the plants. In this concern, Young *et al.* [30] reported that increases of total phenolics in organically grown pac choi was attributed to insect attack.

Biofertilizers play major role in determining the levels of phenolics, flavonoids and isoflavonoids of soybean seeds organically grown. Adding multi-biofertilizers, including *Bacillus megaterium* var. *phosphaticum*, *Azospirillum* spp. *Pseudomonas* spp and *Bradyrhizobium japonicum*, to 50 or 75% compost resulted in great enhancement effect on total phenolics, total flavonoids, protocatechuic acid, p-hydroxybezoic acids, chlorogenic acid caffeic acid, quercetin, genistein and daidzein contents compared with those of inorganic fertilizer or organic fertilized with various compost levels alone. The most enhancement effect was exhibited in case of phenolic acids, quercetin and genistein. Soybean grown under 50% compost in mixture with muti-biofertilizers had about 68 and 40% higher quercetin and genistein, respectively as compare with those of inorganically seeds. Moreover, increases in these compounds reached 90 and 47% as compared with those of plants grown under 50% compost alone. Similar enhancement effect was reported for seeds after addition biofertilizers to 75% compost. In line of these results, Sheteawi and Tawfik, [31] noticed that mixing compost with commercial biofertilizers biogin or nitrobin resulted in an increase in genistein content of mung bean seeds. Multi-biofertilizers used in this experiment are usually referred to as plant growth promoting rhizobacteria. These microorganisms can fix atmospheric nitrogen and supply it to plants, they synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant s, they synthesize several different phytohormones that can act to enhance various stages of plant growth, they may have mechanisms for the solubilization of minerals, such as phosphorus that then become more readily available for plant growth and they may synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development [32]. In the present study, as a result of the slow release of nutrients from at least 50% compost, due to microorganisms activity, soybean photosynthetate could allocated for synthesis secondary compounds as phenolics, flavonoids and isoflavonoids [33].

The antioxidant activity was significantly higher in soybean seeds grown using 50 or 75% compost alone or with biofertilizers. The highest values of antioxidant activity was produced when addition biofertilizers to either 50 or 75% compost. These two treatments accelerated antioxidant activity between 25 and 60% as compared with other inorganic and organic treatments respectively. These high values of antioxidant activity were associated with high values of phenolics, individual phenolic acids, flavonoids and isoflavonoids. A study of five vegetables common in the Japanese diet, Ren *et al.* [17] demonstrated that organically grown spinach contained 120 percent higher antioxidant activity while Welsh onion, Chinese cabbage and qing-gen-cai contained 20-50 percent higher antioxidant activity compared to their conventionally grown counterparts.

In conclusion, fertilizers sources can have a significant effect on the antioxidant activity and phenolic metabolites. The results indicated that addition multi-bioorganic to compost at 50 or 75% produced soybean seeds with higher antioxidant activity and antioxidant phenolic metabolites including phenolic acids, quercetin.

flavonoids as well as isoflavonoids; daidzein and genistein. Soybean grown under mixture of organic and bioorganic fertilizers may benefit human health more than inorganically or organically produce.

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