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Article

Agronomic Management under Organic Farming May Affect the Bioactive Compounds of Lentil (*Lens culinaris* L.) and Grass Pea (*Lathyrus communis* L.)?

Valeria Menga †, Pasquale Codianni † and Clara Fares *

Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA)—Cereal Research Centre (CER), S.S. 673 km 25 + 200, Foggia 71122, Italy; E-Mails: valeria.menga@entecra.it (V.M.); pasquale.codianni@entecra.it (P.C.)

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: clara.fares@entecra.it; Tel.: +39-0881-742-972; Fax: +39-0881-713-150.

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Abstract: A two year field experiment was carried out to evaluate the effects of three row and eight row seeding on the total phenolic compound (TPC), total flavonoid content (TFC), hydrolyzed (HTC) and condensed tannin (CTC), antioxidant activity (ABTS assay), protein content and soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) in the extracts of lentil (*Lens culinaris* L.) and grass pea (*Lathyrus communis* L.) cultivated under organic farming. The aim of this study was to determine whether row spacing used for seeding in organic farming systems for lentil and grass pea is a suitable method to increase the accumulation of antioxidant compounds in these crops. Grass pea showed the highest mean SDF and protein while lentil varieties showed the greatest and significant content of all of the antioxidant compounds. In lentil, there were increases in TPC (52%), HTC (73%), TFC (85%) and CTC (41%), passing from three rows to eight rows, while in grass pea, the increases were lower, and only significant for TFC and CTC (37%, 13% respectively). In both lentils and grass pea, the highest correlation coefficient was between TPC and HTC, which indicates that the HTC includes the predominant phenolic compounds in lentil as well as in grass pea (r = 0.98, 0.71 $p < 0.001$, respectively). Regardless of legume species, TPC, HTC, TFC and CTC showed significant ($p < 0.001$) and linear correlations with the ABTS assay. These data confirm the key role of row spacing for the improvement of the antioxidant properties of lentil in organic farming; moreover, they hint at the major responsiveness and adaptation of lentil to environmental stimulus with respect to grass pea.

Keywords: organic farming; antioxidants; food quality; legumes; organic food

1. Introduction

Given the increasing data in support of the role of phenolic compounds in the prevention of cancer and other chronic diseases, strategies to improve the phenolic content of crops would be useful to human health. The growing popularity of organic agriculture means that an understanding of the relationships between phytochemical content and farming system is particularly relevant. Organic agriculture is an alternative farming system that involves crop production without chemicals and with differential land use. Contrary to conventional farming, the organic farming system of fertilization is supplied by crop rotation, animal manure, cover crops and/or biological preparations. While the use of pesticides and their environmental impact is avoided [1], organic farming also promotes biodiversity among species both in the soil biological activity and in the environment. All of these factors are indicated as ways in which environmental stress on plants can be increased, which in turn might induce changes in the nutrient composition of the fruit and vegetables, especially in terms of the phytochemical content and profile [2–4]. Consequently, organic agriculture might be an interesting strategy to increase the phytochemical content of crops. Moreover, statistics indicate that the attractiveness of both organic farming practices and organic products has increased in recent years in EU countries (e.g., to 10.64 million hectares in 2011; FiBL-ILFOAM survey, 2013; [5]).

Dykes *et al*. [6] reported that the concentration of flavonoids, which are phenolic compounds with antioxidant activity, has changed across cultivation practices (e.g., plant density, cropping system). In this scenario of innovation, as the second most important plant source for human and animal feeding cultivated in Europe, the Middle East, Africa and southern Asia [7], pulses represent a good source of nutrients that might be further increased by these cultivation techniques. Pulses are an important source of protein, fatty acids, carbohydrate, dietary fiber and minerals, and among the legumes, lentil (*Lens culinaris* L.) is also very important due to its phytochemical content [8]. Like many other legumes, such as soybean, broad beans, faba beans and peas, the polyphenols in lentil are located essentially in the seed coats [9]. According to Xu and Chang [10], who carried out a comparative study among different species of legumes, lentil is the legume with the highest total phenolic compounds and condensed tannin, the levels of which correlate with its high antioxidant activity. The cultivation of grass pea (*Lathyrus communis* L.) has been abandoned in the past for more common species like beans, chickpea and lentils, even though the grass pea is a legume that is very resistant to abiotic and biotic stress and is an excellent source of protein and carbohydrate. This legume is still used as a traditional foodstuff in many cultivation practices worldwide [11], and it is an annual crop that is characterized by its great adaptability under extreme conditions, and its high tolerance to drought and poor soils [12–14]. These features have raised renewed interest in the cultivation of grass pea, due to the desire to maintain traditional cultivation, as the key to the development of sustainable agriculture [15]. Like other legumes, phenolic compounds with antioxidant activity have been identified in grass pea [16].

The problem related to the consumption of this legume is due to β-oxalyl-diamino-propionic acid (ODAP), which is a neurotoxic secondary metabolite that can cause neurolathyrism if uninterrupted overconsumption of these seeds is prolonged for more than three to four months [17,18]. However, several methods have been proposed to reduce the toxicity of ODAP, such as cooking or cooking extrusion, which represent very useful ways to reduce both antinutritional factors and ODAP [18,19].

The aim of this study was to determine whether row spacing used for seeding in organic farming systems for lentils and grass pea is a suitable method to increase the accumulation of antioxidant compounds in these crops, including the total phenolics, flavonols and condensed tannins and to improve their fiber composition and protein content. This study is part of a larger study that is aimed at optimizing crop management of durum wheat and legumes (chickpea, lentil, grass pea) under organic farming systems.

2. Experimental Section

2.1. Field Experiment

The two field trials (in 2009–2010 and 2010–2011) were sown in October under wet conditions in the organic experimental field of the Cereal Research Centre of Foggia, in southern Italy. The soil, typical of the Apulian Tavoliere of south-eastern Italy, is a silty-clay Vertisol of alluvial origin, which is classified as Fine, Mesic, Typic Chromoxerert by Soil Taxonomy-USDA [20]. A complete randomized block design (three replicates) was adopted, which included plots sown with three rows (45 cm apart) and eight rows (17 cm apart) with the same number of seeds m^{-2}, as 250 seeds m^{-2} for lentil (*Lens culinaris* L.), and 150 seeds m^{-2} for grass pea (*Lathyrus communis* L.). The statistical design adopted consists of a RCBD with two factors (Row and Variety) with three replicates over two years, where year was also assumed as fixed factor. According to the concept of organic farming management, no chemical inputs were used as fertilizers or for weed control. At physiological maturity, the plots were harvested using a plot combine harvester. The four varieties of lentil used are distinguished through cotyledon color, seed coat color, and 1000-seed weight (Table 1): two have red cotyledons with brown (Pantelleria) or green (a line from Tunisia) seed coats and similar 1000-seed weights; one has medium-sized seeds and a light-brown seed coat with red cotyledons and is a line, known as San Marco dei Cavoti; the last has a large seed size and yellow cotyledons with a green seed coat and is a line from Spain. The most appreciated lentil in Italy is the small type. The two varieties of grass pea both have yellow cotyledons and a beige seed coat, although they differ greatly in 1000-seed weight, with the larger type (a line named Montefalcone) as twice that of the smaller type (a line named Salentina).

2.2. Proximate Analysis

The whole grains of the lentil and grass pea species were milled using a Tecator 1093 Cyclotec (Foss Italia, Padova, Italy) with a 1.0-mm sieve. Determination of the soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) was based on Method 991.43 [21], as proposed by Prosky *et al.* [22]. The total dietary fiber (TDF) was calculated as the sum of the SDF and the IDF. Protein content (%N × 5.70) was determined in triplicate samples using the UNI 10 274 method [23], and this is expressed according to the dry matter (d.m.). The test kernel weight (TKW) is the weight of 1000 seeds (in g), counted by hand.

Table 1. Characteristics of lentil and grass pea.

Species	Phenotype of seed coat/cotyledon	TKW (g)	Seed size
Lentils	brown/red	20.76 ± 1.56	small
	green/red	21.98 ± 0.48	small
	green/yellow	65.01 ± 5.61	large
	light brown/red	40.30 ± 4.97	medium
Grass pea	beige/yellow	314.19 ± 26.52	large
	beige/yellow	136.78 ± 8.82	small

Abbreviation: TKW, test kernel weight.

2.3. Extraction of Phenolic Compounds

Extraction of the phenolic compounds was carried out according to the method proposed by Beta *et al*. [24], with minor modifications. The samples (1 g) ground with a 1.0-mm sieve, were extracted using 8 mL methanol-water (80:20; *v*/*v*) acidified with 1% HCl, for 30 min in an ultrasonic bath. The mixtures were centrifuged at 2000 × *g* for 15 min. After centrifugation, the supernatant was used for determination of the total phenolic content (TPC).

2.4. Determination of Total Phenolic Content

Extracts (0.2 mL) were added to 1.5 mL 10-fold diluted Folin-Ciocalteau reagent [25]. The mixture was allowed to equilibrate for 5 min, and then neutralized with sodium carbonate (from a stock solution of 60 $g \cdot L^{-1}$). After incubation at room temperature for 90 min, the absorbance of the mixtures was measured at 725 nm. Acidified methanol was used as the blank. Catechin was used as the standard, and the data are expressed in mg catechin equivalents g^{-1} ($CE \cdot g^{-1}$). Determinations were performed in triplicate for each extract, and these are reported according to d.m.

2.5. Determination of Hydrolyzed Tannins

The hydrolyzed tannin content (HTC) was determined as the PVPP-bound phenolics, according to Makkar *et al*. [26]. Two milliliters of extracts were mixed with 200 mg insoluble, cross-linked PVPP. After 15 min at 4 °C, the samples were vortexed, and then centrifuged at 15,000 × *g* for 10 min. Aliquots of the supernatant (0.2 mL) were transferred into test-tubes and the non-adsorbed phenolics were determined by the same procedure used for the TPC. The content of the PVPP-bound phenolics (*i.e.*, the HTC) was calculated as the difference between the TPC and the non-adsorbed phenolics.

2.6. Determination of Condensed Tannin Content

The condensed tannin content (CTC) was determined using a butanol-HCL assay [27]. Briefly, 0.5 mL of the extracts was mixed with 3.0 mL butanol-HCL reagent (95:5; *v*/*v*) and 0.1 mL ferric reagent (2% ferric ammonium sulfate, in 2.0 M HCL). After boiling for 60 min in a water-bath, the absorbance was measured at 550 nm against a blank that contained the extraction solvent instead of a sample. The

CTC was calculated as leucocyanidin equivalents g^{-1} ($LE \cdot g^{-1}$) according to the formula: (A_{550nm} × 78.26 × dilution factor)/(dry weight), as given by Porter *et al*. [28].

2.7. Determination of Total Flavonoid Content

The total flavonoid content (TFC) was determined according to Eberhardt *et al*. [29] Briefly, 0.075 mL of 5% $NaNO_2$ was mixed with 0.5 mL sample (diluted with 1 mL water). After 6 min, 0.15 mL $AlCl_3$ (10%) solution was added, and the mixture was allowed to stand for another 5 min. Then 0.5 mL 1 M NaOH was added, and the volume was taken to 2.5 mL with distilled water. The absorbance was measured at 510 nm immediately after mixing, against a blank containing the extraction solvent without any sample.

2.8. Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacity (TEAC) was determined according to the procedure of Re *et al*. [30], with some modifications: 2,2-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid](ABTS) was dissolved in water to 7 mM ABTS radical cations ($ABTS^{\bullet+}$) were produced before use by allowing the reaction of the ABTS stock solution with potassium persulfate (final concentration, 2.45 mM) for 12–16 h in the dark and at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. The absorbance was read exactly 1 min and up to 6 min after the addition of 20 µL extract to 2 mL diluted $ABTS^{\bullet+}$. All of these tests were conducted in triplicate.

The TEAC of the extracts were calculated using a Trolox standard curve, on the basis of the percentage inhibition of the absorbance at 734 nm, and these are expressed in µmol Trolox g^{-1} d.m.

2.9. Statistical Analysis

Standard ANOVA procedures were applied to the datasets using the statistical package STATISTICA [31], the interactions between and among the main factors (Year, Row and Variety) were performed only if significance was seen. The means were separated by the last significant difference (LSD) test at a $p < 0.05$ probability level. Pearson's correlation tests were performed to determine the linear correlations among the variables.

3. Results

3.1. Proximate Composition

Table 1 gives the characteristics of the lentil and grass pea varieties. The lentils showed large variability for TKW, varying from 20.76 g for the brown/red small type, to 65.01 g for the green/yellow large type. The grass pea TKW varied from 314.19 g for the large type, to 115.78 g for the small type. Table 2 gives the mean square and the probability levels (P) obtained from the analysis of variance of the traits studied for the main factors (variety, row and year) and interactions. Significant effects of lentil variety were observed across all of the traits, except for the SDF, while for grass pea there were significant effects only for protein content, IDF, TPC and HTC. Row spacing had significant effects on the protein content and the antioxidants for the lentils, while for the grass pea, the row-spacing effects were significant for IDF and two classes of phenolics, TFC and HTC. The effects of the year were always significant for lentils, over all of the traits, while for the grass pea, the effects of the year were significant only for protein content, TPC, TFC and HTC. Regarding the interactions, only in lentils were there significant interactions of year × variety for protein content, which was due to the high protein content in the first year (data not shown) obtained for the medium and the small brown lentil types. Table 3 gives the results of the proximate composition and the phenolic compounds of the four varieties of lentils and two varieties of grass pea over the years and the row spacing. The mean protein content and TDF of the grass pea were greater than those of the lentil varieties. The variability for protein and fiber contents was particularly apparent also among varieties; e.g., the small/green and large/green lentils had the same protein content, which was significantly higher than that of the medium/light brown and small/red lentils. The SDF was not different among the lentil varieties, while the highest IDF levels were seen for the small types of both the lentils and the grass pea. Indeed, compared to the large grass pea type, the small grass pea type showed significantly higher protein content, IDF and TDF, with no significant difference only for SDF. Row spacing affected the protein content of the lentils only, while for the grass pea the protein content did not vary significantly (Table 4). In contrast, the row spacing resulted in significant differences for the IDF and TDF in the grass pea, with no differences for the lentils.

Table 2. Analysis of variance for variety, row spacing, year and interactions.

Lentil	**Variety**		**Row**		**Year**		**Year × Variety**		**Yera × Row**		**Variety × Row**		**Year × Row × Variety**	
	df	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**
Protein content	3	13.06 ***	1	4.44 *	1	25.40 ***	3	6.81 ***	1	0.48 *n.s.*	3	0.55 *n.s.*	3	0.83 *n.s.*
IDF	3	22.25 ***	1	0.01 *n.s.*	1	14.19 **	3	4.29 *	1	3.69 *n.s.*	3	1.00 *n.s.*	3	4.23 *
SDF	3	1.42 *n.s.*	1	2.04 *n.s.*	1	6.84 *	3	2.02 *n.s.*	1	0.23 *n.s.*	3	3.24 *	3	2.15 *n.s.*
TPC	3	7.28 ***	1	45.01 ***	1	0.53 *	3	0.66 **	1	14.40 ***	3	0.47 *	3	1.13 ***
HTC	3	6.84 ***	1	38.03 ***	1	4.76 ***	3	0.59 **	1	7.92 ***	3	0.55 **	3	1.07 ***
TFC	3	4.25 ***	1	19.04 ***	1	3.58 ***	3	0.20 *n.s.*	1	7.16 ***	3	0.12 *n.s.*	3	0.52 *n.s.*
CTC	3	0.82 ***	1	6.11 ***	1	5.07 ***	3	0.07 *	1	0.66 ***	3	0.08 **	3	0.06 *
Grass pea	**Variety**		**Row**		**Year**		**Year × Variety**		**Year × Row**		**Variety × Row**		**Year × Row × Variety**	
	df	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**
Protein content	1	2.34 *	1	0.52 *n.s.*	1	6.34 ***	1	0.25 *n.s.*	1	0.33 *n.s.*	1	0.07 *n.s.*	1	0.48 *n.s.*
IDF	1	16.89 **	1	30.05 ***	1	2.53 *n.s.*	1	4.33 *n.s.*	1	20.61 **	1	4.13 *n.s.*	1	10.12 *
SDF	1	0.07 *n.s.*	1	0.32 *n.s.*	1	0.05 *n.s.*	1	0.11 *n.s.*	1	0.17 *n.s.*	1	1.51 *	1	0.04 *n.s.*
TPC	1	0.66 ***	1	0.02 *n.s.*	1	1.18 ***	1	0.00 *n.s.*	1	0.02 *n.s.*	1	0.07 *n.s.*	1	0.21 **
HTC	1	0.25 **	1	0.01 *n.s.*	1	0.01 *n.s.*	1	0.01 *n.s.*	1	0.16 *	1	0.01 *n.s.*	1	0.12 *
TFC	1	0.05 *n.s.*	1	0.07 *	1	0.28 ***	1	0.08 *	1	0.00 *n.s.*	1	0.00 *n.s.*	1	0.00 *n.s.*
CTC	1	0.00 *n.s.*	1	0.02 *	1	0.06 **	1	0.00 *n.s.*	1	0.06 ***	1	0.05 **	1	0.01 *n.s.*

*, **, *** and *n.s.*: significant at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively; MS, mean square; df, degree of freedom. Abbreviations: IDF, insoluble dietary fiber; SDF, soluble dietary fiber; TPC, total phenolics content; HTC, hydrolyzed tannin content; TFC, total flavonoid content; CTC, condensed tannin content.

Table 3. Proximate composition and phenolic compounds in varieties of lentils and grass pea.

		Protein content	IDF	SDF	TDF	TPC	HTC	TFC	CTC
Species		**% d.m.**	**% d.m.**	**% d.m.**	**% d.m.**	**mg CE·g^{-1} d.m.**	**mg CE·g^{-1} d.m.**	**mg CE·g^{-1} d.m.**	**mg LE·g^{-1} d.m.**
Lentil	small (green)	22.63 ^{a}	26.05 ^{a}	5.06 ^{a}	31.11 ^{a}	4.23 ^{b}	2.95 ^{b}	1.83 ^{bc}	2.06 ^{b}
	small (brown)	20.91 ^{b}	25.33 ^{a}	4.51 ^{a}	29.85 ^{ab}	4.26 ^{b}	2.94 ^{b}	1.59 ^{d}	2.05 ^{b}
	large (green)	22.73 ^{a}	22.75 ^{c}	4.25 ^{a}	27.00 ^{c}	4.29 ^{b}	2.92 ^{b}	2.04 ^{b}	1.76 ^{c}
	medium (light brown)	20.85 ^{b}	23.86 ^{b}	4.74 ^{a}	28.59 ^{b}	5.82 ^{a}	4.45 ^{a}	2.95 ^{a}	2.40 ^{a}
	mean	21.78	24.50	4.64	29.14	4.65	3.32	2.10	2.07
Grass pea	large (beige)	22.74 ^{b}	23.69 ^{b}	6.10 ^{a}	29.80 ^{b}	1.56 ^{b}	0.28 ^{b}	0.30 ^{a}	0.40 ^{a}
	small (beige)	23.37 ^{a}	25.37 ^{a}	5.99 ^{a}	31.37 ^{a}	1.90 ^{a}	0.48 ^{a}	0.39 ^{a}	0.39 ^{a}
	mean	23.06	24.53	6.05	30.59	1.73	0.38	0.35	0.40

Different lower case in the same column, for lentil and grass pea separately, correspond to significant difference by LSD test (p = 5%). Abbreviation: IDF, insoluble dietary fiber; SDF, soluble dietary fiber; TPC, total phenolics content; HTC, hydrolyzed tannin content; TFC, total flavonoid content; CTC, condensed tannin content; d.m., dry matter.

Table 4. Proximate composition and phenolic compounds of the two species, affected by row spacing.

		Protein content	IDF	SDF	TDF	TPC	HTC	TFC	CTC
Species		**% d.m.**	**% d.m.**	**% d.m.**	**% d.m.**	**mg CE·g^{-1} d.m.**	**mg CE·g^{-1} d.m.**	**mg CE·g^{-1} d.m.**	**mg LE·g^{-1} d.m.**
Lentil	3 row	21.48 ^{b}	24.48 ^{a}	4.44 ^{a}	28.92 ^{a}	3.68 ^{b}	2.42 ^{b}	1.47 ^{b}	1.71 ^{b}
	8 row	22.09 ^{a}	24.51 ^{a}	4.85 ^{a}	29.36 ^{a}	5.62 ^{a}	4.20 ^{a}	2.73 ^{a}	2.42 ^{a}
	mean	21.78	24.49	4.64	29.14	4.65	3.31	2.10	2.06
Grass pea	3 row	23.20 ^{a}	25.65 ^{a}	5.93 ^{a}	31.81 ^{a}	1.70 ^{a}	0.37 ^{a}	0.29 ^{b}	0.37 ^{b}
	8 row	22.91 ^{a}	23.41 ^{b}	6.16 ^{a}	29.35 ^{b}	1.76 ^{a}	0.39 ^{a}	0.40 ^{a}	0.42 ^{a}
	mean	23.05	24.53	6.04	30.58	1.73	0.38	0.34	0.39

Different lower case in the same column, for lentil and grass pea separately, correspond to significant difference by LSD test (p = 5%). Abbreviation: IDF, insoluble dietary fiber; SDF, soluble dietary fiber; TPC, total phenolics content; HTC, hydrolyzed tannin content; TFC, total flavonoid content; CTC, condensed tannin content; d.m., dry matter.

3.2. Antioxidant Compounds and Antioxidant Activity

The antioxidant compounds of lentils showed higher values than those of grass pea, with the average TPC of the lentils 3-fold higher than for the grass pea, while the lentil HTC, TFC and CTC were 9-fold, 6-fold and 5-fold higher, respectively, than for the grass pea (Table 3). Among the lentils, the highest antioxidant content was for the medium type, and for the grass pea, the highest antioxidant content was seen for the small type, which confirmed its superiority for all of the studied traits. The phenolic compounds were always significantly affected by row spacing for the lentils while for the grass pea, only the TFC and CTC were affected (Table 4). Interestingly, for all of the traits and species, the 8-row sowing showed the highest antioxidant content. With regard to the interactions (Table 2), only in lentils were there significant interactions ($p < 0.001$) of year × row for all of the antioxidant compounds studied, while in grass pea only for CTC. As shown in Figure 1 the highest values of antioxidants both in lentils and grass pea were in the 8 row seedlings, although univocal results were not obtained over the two years (*i.e.*, the highest value in the second year was obtained only for the TPC). These findings confirm the key role of row spacing in the improvement of antioxidant properties of lentils in organic farming; moreover, they indicate the major responsiveness and adaptation of lentil to environmental stimuli with respect to grass pea. For the antioxidant activity (Figures 2 and 3), the 8-row sowing showed higher values than for 3 rows for both the lentils and the grass pea. In terms of the cultivar types, the highest antioxidant activities were for the medium lentils (the line named San Marco dei Cavoti), while the small grass pea had higher activity than the large grass pea. In summary here, the best lentil cultivar in relation to the antioxidant activities was the medium type, while for the grass pea it was the small type, which was also richer in protein content and TDF. Table 5 gives the correlations between the antioxidant activities and TPC, HTC, TFC and CTC: all of these phenolic compounds showed significant ($p < 0.001$) linear correlations with the antioxidant activity, as measured using ABTS. In particular, the highest correlation coefficient (r) was seen for the TPC, at 0.72, and in decreasing order for HTC, TFC and CTC, as 0.70, 0.67 and 0.59, respectively. The best indicator of the antioxidant activity was the TPC, as shown by the highest r value in both the lentils ($r = 0.45$, $p < 0.01$) and the grass pea (0.69, $p < 0.001$). For the lentils, the correlation coefficient between antioxidant activity and the CTC was not significant, and the remaining r values, for the HTC and TFC, were the lowest when all of the values were compared ($r = 0.40$, 0.44, respectively; $p < 0.01$). In contrast, for the grass pea, the HTC and TFC showed the lowest, and non-significant, r values overall. It is interesting to note that for the grass pea, the linear correlations for TPC *versus* TFC and for TPC *versus* CTC were not significant, while for the lentils when all of the values were compared, these correlations were significant ($r = 0.99$, 0.93, 0.92, and $r = 0.98$, 0.88, 0.79, respectively; $p < 0.0001$).

Figure 1. Row × Year interaction. (**A**) = TPC lentil; (**B**) = HTC lentil; (**C**) = TFC lentil; (**D**) = CTC lentil; (**E**) = CTC grass pea. Different lower case letters correspond to significant differences (LSD test, $p = 5\%$).

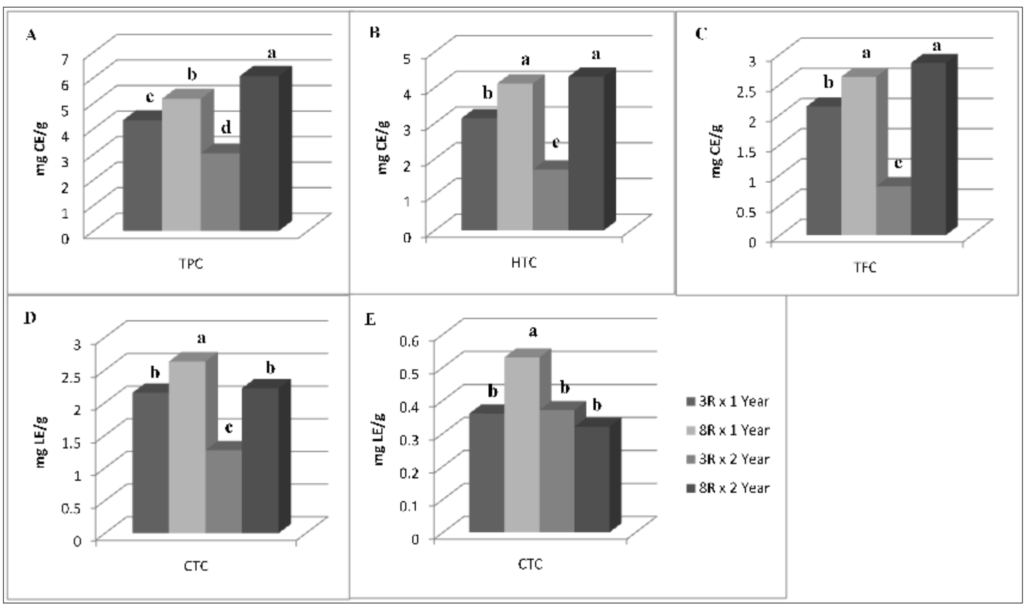

Figure 2. Antioxidant activity in lentil. Different lower case letters correspond to significant differences (LSD test p = 5%).

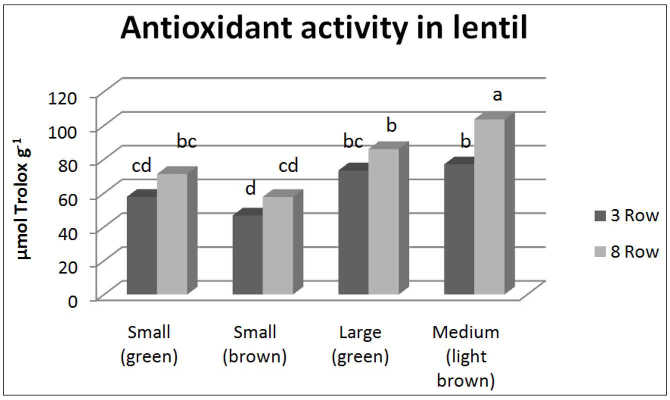

Figure 3. Antioxidant activity in grass pea. Different lower case letters correspond to significant differences (LSD test p = 5%).

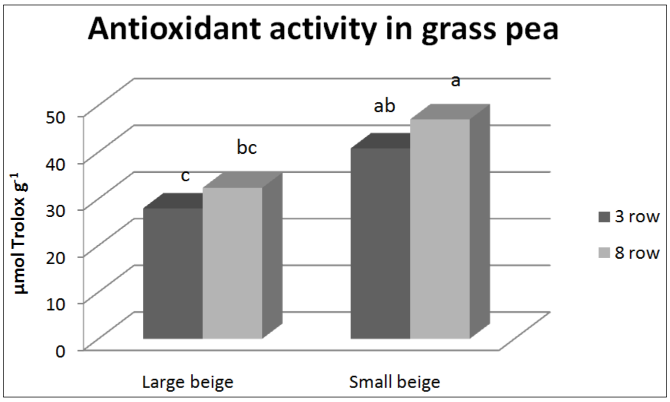

Table 5. Correlations between phenolics and antioxidant activity.

Total n = 72	**Correlation coefficient (r)**	**HTC**	**TFC**	**CTC**	**Antioxidant activity**
	TPC	0.99 ***	0.93 ***	0.92 ***	0.72 ***
	HTC		0.94 ***	0.95 ***	0.70 ***
	TFC			0.98 ***	0.67 ***
	CTC				0.59 ***
Lentils n = 48					
	TPC	0.98 ***	0.88 ***	0.79 ***	0.45 **
	HTC		0.88 ***	0.88 ***	0.40 **
	TFC			0.78 ***	0.44 **
	CTC				0.12 $^{n.s.}$
Grass pea n = 24					
	TPC	0.71 ***	−0.07 $^{n.s.}$	−0.12 $^{n.s.}$	0.69 ***
	HTC		0.46 *	0.35 $^{n.s.}$	0.15 $^{n.s.}$
	TFC			0.53 **	0.23 $^{n.s.}$
	CTC				0.47 *

*, **, *** and $^{n.s.}$: significant at p < 0.05, p < 0.01, p < 0.001 and not significant, respectively; $^{n.s.}$ not significant.abbreviations. TPC, total phenolics content; HTC, hydrolyzed tannin content; TFC, total flavonoid content; CTC, condensed tannin content; d.m., dry matter.

4. Discussion

4.1. Proximate Composition

To encourage sustainable pulse production, and hence their marketing and consumption, investigations into the nutritional value of these crops under organic farming are crucial. It was thus in this framework that our investigation was focused on lentils and grass pea. The varieties of lentil and grass pea differed in terms of TKW, seed size, and color of coat and cotyledons, as reported in Table 1. There was great variability among the lentils for these traits: the TKW varied from 20.76 g/1000 seeds for the small type, to 65.01 g/1000 seeds for the largest type. The grass pea also differed in terms of these weights, as the large type was double that of the small type. With respect to the color of the seed coat, the lentils varied from green to brown, and the cotyledons were red in the two small types and in the medium type, while it was yellow in the large type. For the grass pea, the seed coat was always beige and the cotyledons were yellow. Interestingly (Table 4), even if the fiber quality (SDF, IDF) and quantity (TDF) and protein content were slightly modified by row spacing in both the lentils and the grass pea, it was the grass pea that showed the highest values, confirming their nutritional value as a non-conventional source of SDF and protein. In a comparison of these data with those of a recent study on an Italian grass pea cultivar grown in the Valle Agricola district (Italy) in marginal areas using essentially traditional techniques [16], our protein content is slightly lower than for this other study. Significant differences (Table 3) were seen among the lentil varieties for protein content, and for fiber: in particular, the small/green and large/green lentils had the same protein content, which was higher than that of the medium/light brown and small/brown lentils. For the significant interaction of year × variety for protein content shown in Table 2, this was ascribable to the higher yield in the first year with respect to the second that was obtained for the medium/light brown and small/brown lentils (data not shown). Interestingly, the IDF was significantly higher in the small lentils, compared to the medium and large lentils, which confirms that this range of variability might be attributable to the kernel size, as the major difference between the cotyledon fibers and the hulls is in the concentration of cellulose, lignin and hemicellulose, which are typically from the cell wall [32]. Similar observations were reported by Wang *et al*. [33], who stated that large-sized seeds have a lower proportion of hull to cotyledon in terms of the surface-to-volume ratio, and therefore the larger seeds show lower IDF than the small-sized seeds. The same behavior was seen for grass pea.

4.2. Antioxidant Compounds and Antioxidant Activity

Much attention has been paid to the antioxidant contents of crops, as this appears to be involved in the prevention of many chronic diseases that are promoted by free radicals, including cancers, inflammation and ageing. Among all of the antioxidants, great attention has been given to the class of phenolic compounds, as secondary metabolites that are mainly concentrated in cereals, fruit and vegetables. Legumes are very important in human nutrition, and there is widespread awareness of their high antioxidant activity due to the presence of high amounts of the phenolic compounds. Among these phenolics, the flavonoids (here as the TFC) are the leading compound class, and they have high antioxidant activities [10,34]. The flavonoids include numerous subclasses of compounds that have a general structure characterized by two aromatic rings that are linked by three carbon bridges. These subclasses include the flavanols (catechin, epicatechin), polymeric compounds (tannins), flavanones, flavones, isoflavones, flavonols, and anthocyanins [35]. The tannins are present in their hydrolysable (HTC) and condensed (CTC) forms. The former are usually determined by a gravimetric method that is based on their binding to the insoluble PVPP. The condensed tannins are oligomers and polymers of flavan-3-ol monomer units and they have potent antioxidant activities. The condensed tannins (or proanthocyanidins) are compounds that liberate colored anthocyanidin pigments upon oxidative cleavage in hot alcohols, via acid butanol chemistry [27]. It is of note that all of the methods adopted for measuring the antioxidant compounds (TPC, PPVP, TFC and CTC) might not give the full picture of their quantities and, often, of the quality of phenolic compounds present in the extracts as interference ascribable to other chemical compounds is indeed possible, such as from sugars, tocols and carotenoids.

The role of these compounds is to protect against oxidative stress that is induced by free radicals, by increasing the capacity of the cell for the absorbing of such oxygen radicals, which prevents the generation of free radicals, stimulates the detoxification enzymes, decreases lipid peroxidation, and reduces cell proliferation [36,37]. In this context, the role of organic farming in the promotion of the accumulation of such secondary metabolites is controversial and remains to be better clarified. Many studies have demonstrated subtle differences in the bioactive phytochemicals between organically and conventionally grown crops [38,39], although no studies have been focused on crop management in an organic environment, such as we see here for varying of the row spacing in the sowing of these plants to stimulate their competitiveness *versus* weeds, in terms of light and nutrients.

Our ANOVA analysis (see Table 2) highlighted that the phenolic compounds of the lentils are significantly affected by both variety and environment (*i.e.*, row spacing and year) and the interaction between year × row ($p < 0.001$). However for grass pea, the trend was not the same, as grass pea showed significant effects of variety only for TPC and HTC, and significant effects of environment only for TFC and CTC, and for interaction between year × row only for CTC ($p < 0.001$). No reports are available in the literature related to these findings, although effects of environment on phenolics have been indicated in several studies on cereals, fruit and pulses under conventional farming [40–42]. According to the results from the present study, lentils are more receptive than grass pea to environment changes, as shown for all of the modified antioxidant compounds studied here. For lentils, as shown in Table 3, the medium type had the highest amounts of all of the phenolic compounds compared to the other varieties considered here. The TPC of our varieties, as averaged over two years and two treatments, varied from 4.23 mg CE/g for the small green lentils, to 5.82 mg CE/g for the medium type of lentils, and these are in the same ranges of values reported in other studies: Zou *et al*. [43] reported a TPC of 6.93 mg GAE/g in the lentil var. Morton, which is similar to that reported by Xu *et al*. [44] (6.96 mg GAE/g, as the average of 11 varieties). For the TFC and CTC, we found slightly lower values than Xu *et al*. [44]; however, such small differences can be attributable to both the different extraction methods used and the great variability that is known to exist among cultivars. Xu and Chang [8] reported a lot of differences in the phenolic acid profile and flavan-3-ol composition of lentils, which are probably attributable to a number of factors, such as phenotype, crop location, and environmental stress. Other studies have reported changes in tannin content that depend on the color of the seed coat, as legumes with a dark seed coat have large amounts of phenolic compounds, especially for the anthocyanins and condensed tannin [45,46]. Also, Xu *et al*. [44]

determined a correlation between phenolic compounds and color value across five types of legume, whereby species with a colored seed coat have higher TPC, TFC and CTC, in comparison to species with paler colored seed coats; within species with colored seed coats (*i.e.*, lentils and colored beans), there is a different phenolic composition in relation to the color variation. Thus, our data indicate that the phenolic profile in the lentil varieties studied can be related to the lentil pigments too.

In grass pea, we found significant differences between the two varieties only for the TPC and HTC, with the smaller grass pea being the richer, which is probably due to the greater proportion of the seed coat. The TPC ranged from 1.56 mg CE/g for the large type to 1.90 mg CE/g of the small type, and these values are in agreement with Tamburino *et al*. [16], who studied a grass pea variety grown in Valle Agricola. The highest values of TPC reported appear to be those of Pastor-Cavata *et al*. [47], who reported a range from 3.8 to 29.2 mg/g across 15 species of *Lathyrus* (grass pea) that mainly belonged to the non-cultivated wild population. According to their study, there is a negative correlation between phenolic content and seed size, such that the smaller seeds correspond to more phenolics.

As highlighted in Table 4 and supported by the row × year interaction of Figure 1A–D), significant effects were seen for row spacing, with 8-row sowing of these legumes leading to a higher content of the phenolic compounds. The lentil varieties are strongly influenced by this crop management for all of the phenolic compounds considered, showing increases of 52% for TPC, 73% for HTC, 85% for TFC and 41% for CTC, as observed going from 3 row seeding to 8 row seeding. In the grass pea (Table 4), the increases due to the variation in the row spacing were lower, and were only significant for flavonoids and condensed tannins (increases of 37% for TFC, and 13% for CTC), while being statistically non-significant for TPC and HTC, while the significant row × year interaction observed for CTC (Figure 1E), strengthens the trend previously observed for lentil. These findings confirm the key role of row spacing management in the improvement of the antioxidant properties of lentils in organic farming with respect to grass pea. The phenolic compounds act as antioxidants in plant tissues, and they are important as protectants of plants from insect pests, infestations and cell damage [48,49]. In addition, these compounds might be accumulated in plant tissues in response to cultivation in a marginal soil environment. The condensed tannins are located mainly in the testa, and they have an important role in the seed defense system, as they can act as antioxidants against oxidative damage caused by environmental factors [46]. Makoi and Ndakidemi [50] reported that the concentrations of flavonoids and anthocyanins are affected by the plant density and the cropping system. The increased competitiveness for growth factors as a consequence of increased seed-plant density might considerably stress the plants, which, in turn, can produce increased amounts of antioxidants [51]. This finding can explain the significant increases in TFC and CTC in both the lentils and grass pea considered in the present study, passing from a row spacing of 3 rows to 8 rows. The increase in TFC and CTC caused by changing the cultivation technique has considerable positive feedback for human health, as the procyanidins are well known for their anti-thrombotic properties through inhibition of platelet activation, as well as platelet-dependent inflammatory responses [52,53]. On the other hand, the effects of catechins and proanthocyanidins on blood-lipid profiles in human subjects are also well documented [54].

Figures 2 and 3 show the antioxidant activities of the lentils and the grass pea, respectively. The determination of these antioxidant activities highlight that crop management can be used to modify the properties of these legumes. In addition, for these antioxidant activities, it can be emphasized that there is an increase in passing from 3-row to 8-row sowing, for both the lentils and the grass pea. This increase was smaller and very similar in the two varieties of grass pea (15.7% and 15.3%, for large and small, respectively) and higher in the lentils (23.5% and 23.6% for the green and brown small types, respectively), with about 17% in the large lentil type, and 34.6% in the medium lentil type. These different increases in the antioxidant activities are linked to the changes in the amounts and types of the phenolics, although the rank order among the varieties and species was maintained.

For the correlation between the phenolic compounds and the ABTS-defined antioxidant activities, Table 5 reports the r values among all of these legumes, and within each species separately. Regardless of legume species, TPC, HTC, TFC and CTC showed significant ($p < 0.001$) and linear correlations with the ABTS assay (0.72, 0.70, 0.67 and 0.59, respectively). The highest r value was seen between TPC and ABTS, and this is in agreement with the current literature, as the TPC is a good descriptor of the antioxidant status of extracts [44]. Moreover, the significant linear correlations between the TPC and HTC ($r = 0.99$, $p < 0.001$), TPC and TFC ($r = 0.93$, $p < 0.001$), and the TPC and CTC ($r = 0.92$, $p < 0.001$) show that the TPC correlates well with all of the phenolic compounds tested here. In lentils, there was no correlation between antioxidant activity and CTC when all of the varieties were put together, although the correlation became significant ($r = 0.33$, $p < 0.05$) when the large lentil type (the only lentil with a yellow cotyledon) was removed. According to Xu and Chang [8], the antioxidant activities of 11 cultivars of lentil (estimated using the 2,2-diphenyl-1-picrylhydrazyl [DPPH] assay) did not correlate with CTC. Several mechanisms are involved in the determination of the antioxidant activities of extracts: their free-radical scavenging, inhibition of lipid peroxidation, and ability to chelate metals; these need to be defined by the appropriate antioxidant activity assays. Thus, there is not only one descriptor for these mechanisms that differ among these legumes. On the other hand, Xu *et al*. [44] showed that several antioxidant activity assay methods are well correlated (DPPH, *ferric reducing antioxidant power*, oxygen radical absorbance capacity). There was significant linear correlation between TPC and antioxidant activity ($r = 0.45$, $p < 0.01$) and in the decreasing rank order of TFC and antioxidant activity ($r = 0.44$, $p < 0.01$), and HTC and antioxidant activity ($r = 0.40$, $p < 0.01$). In the grass pea, a significant linear correlation was seen only between TPC and antioxidant activity ($r = 0.69$, $p < 0.001$) and between CTC and antioxidant activity ($r = 0.47$, $p < 0.5$). In both the lentils and the grass pea, the highest correlation coefficient was between TPC and HTC, and these results indicated that the HTC (hydrolyzed tannins) might be the predominant phenolic compounds in lentils as well as in grass pea ($r = 0.98$ and 0.71, $p < 0.001$, respectively). In the lentils, high correlation was seen also between TPC and TFC ($r = 0.88$, $p < 0.001$) and between TPC and CTC ($r = 0.79$, $p < 0.001$). Therefore, these data demonstrate the complexity of the phenolic profile of the lentils with respect to the grass pea, and also considering other legumes, as stated by Xu *et al*. [44].

5. Conclusions

This study demonstrates that management of the row spacing can be used to change the nutritional profile of these studied legumes, to different extents. While row spacing slightly modified the amounts of dietary fiber and the protein content in these lentils and grass pea, its effects were stronger on the phenolic profile of the lentils, rather than in the grass pea. The antioxidant activities were also modified by the row spacing, with increases passing from 3 rows to 8 rows, for both the lentils and the grass pea considered. Thus, the present study demonstrates that under an organic farming system, the choice of the varieties that are richest in antioxidant compounds and the use of alternative cultivation techniques can be very useful for the production of high-phenolic crops for human health purposes.

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Author Contributions

Clara Fares organized the field experiment and the chemical analyses, moreover has wrote the manuscript. Valeria Menga performed the chemical analyses and the statistical analysis of data set, moreover has contributed to the writing of the manuscript. Pasquale Codianni oversaw the agronomical management of the field experiment.

Conflicts of Interest

The authors declare no conflict of interest.

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