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Multi-Environment Evaluation and Genetic Characterisation of Common Bean Breeding Lines for Organic Farming Systems

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Abstract: It is recognised that one of the main causes for the relative low yields under organic conditions is the use of modern cultivars which are bred for high-input management systems. The work described here aimed to study and test possible breeding strategies to produce cultivars of common bean for organic agriculture. To this purpose, crosses between a traditional Italian landrace named "Gnocchetto" and a cultivar were carried out. The F_1 plants obtained were either backcrossed or self-fertilised and the obtained materials subjected to selection for quality traits at different development stages. The resulting lines were tested under four different environmental conditions for three years in order to determine their potential performance. The resulting data were analysed using a Multi-Environment Trial Analysis (MET) approach and different visualisations of the GGE biplot were generated. Furthermore, to assess the level of genetic similarity, the lines were characterised using 25 Simple Sequence Repeat (SSR) molecular markers. Results showed that the breeding approach applied allowed to select lines with the same technological and agronomic characteristics as commercially available cultivars, but with different adaptation abilities that make them suitable for organic agriculture.

Keywords: common bean; organic agriculture; landrace germplasm; multi-environment trials; GGE biplot; SSR markers

1. Introduction

The demand for organic food is currently increasing worldwide and interest in organic agriculture has grown significantly [1]. Behind this trend, there is the consumer perception that organic products are healthier, safer and more beneficial for the environment and biodiversity when compared to those produced under conventional management systems [2].

In the past three decades, the impact of organic management on crop yields has been widely discussed and contrasting results have been highlighted. While many studies showed that organic management resulted in significantly lower yields than conventional ones [3–6], others reported comparable yields [7–11].

Recently, many reviews have used comprehensive meta-analyses to investigate the contrasting results mentioned above. Considering data from over 350 published studies, De Ponti et al. [12] concluded that organic yields are on average 20% lower than conventional ones. On the other

hand, Seufert and colleagues [13] showed that yield differences between organic and conventional agriculture do exist, but that they are highly contextual, depending on the system, site characteristics and crop types. Moreover, Ponisio et al. [14] built a hierarchical meta-analytic model to better dissect and estimate the yield gap between the two management systems. They found that organic yields are, on average, 19.2% (±3.7%) lower than conventional, while, in contrast to the previous studies, their results demonstrated a lack of significant differences in yield gaps for leguminous versus non-leguminous crops and perennials versus annuals; even more interestingly, results showed that agricultural diversification practices such as rotations or multi-cropping can reduce the yield gap between the two management systems.

It has been suggested that one of the main causes for the low yields achievable under organic conditions is the use of modern cultivars which are bred for high-input production systems [15,16]. Currently, most cultivars are specifically bred for conventional agriculture and rely on the use of pesticides and fertilisers for optimal performance [14,17,18]. Nevertheless, there is also evidence that conventionally bred cultivars do not always perform worse than those bred for organic agriculture when tested under organic conditions; for example, *genotype* by *system* interactions were not reported in studies carried out on wheat [19] or oats [20]. In comparative trials of 32 dry bean genotypes under organic versus conventional systems, Heiling and Kelly [21] reported that some genotypes were better suited for organic production than others. However, those genotypes poorly performing under organic conditions performed badly also under conventional conditions, confirming the absence of a *genotype* by *system* interaction in the studied materials. Studying soft wheat, Annicchiarico et al. [22] highlighted a high correlation between yield in organic and conventional management systems and, in particular, that *genotype* by *location* interactions were much more important than *genotype* by *system* interactions. A similar result was also obtained by Raggi et al. [23] in barley. Even if the effect of *location* is more relevant than the management system in determining yield performances, certain traits that are useful for organic farming are often lacking in modern cultivars [24].

Many consumers prefer organic products with certain physico-chemical properties in accordance with their personal values that go beyond the basic ethical criteria established by EU regulation on organic farming (EC 834/2007). In this context, landraces represent a useful genetic resource as starting material for breeding new cultivars for organic agriculture that meet both consumers' and growers' needs [25]. Landraces are variable but identifiable populations that lack "formal" improvement and have been cultivated for hundreds of years in restricted areas by small-scale farmers under marginal conditions which are often analogous to those of organic or low-input management [26,27]. For these reasons, landraces are characterised by (i) specific adaptation to certain environmental conditions and (ii) rare or even unique organoleptic properties. However, despite the above-mentioned beneficial qualities, landraces may also have agronomic disadvantages when compared to modern cultivars such as particular plant architecture or inadequate physiological attributes that make cultivation more difficult and expensive. Therefore, obtaining and testing new breeding lines that combine landraces and commercial cultivar qualities are among the main objectives for many plant breeders [15].

Common bean landraces are populations that maintain a relatively high degree of genetic diversity and are able to adapt to different environmental conditions [26]. Genetic diversity itself provides a buffer against environmental stresses caused by a multitude of factors which are generally referred to as "biotic and abiotic stresses" [15]. In a recent study, Klaedtke and colleagues also showed that the genetic diversity present in some historical common bean cultivars was sufficient for short-term local adaptation to occur in two different environmental conditions under organic management [28].

Common bean (*Phaseolus vulgaris* L.) is one of the most important grain legume species for direct human consumption, being a rich source of proteins, vitamins, minerals and fibre. This crop is also highly valued for the long storage life of its seeds and for its numerous culinary applications [29]. Due to these characteristics, common bean is considered a model food legume [30]. The total world production of dry beans is estimated at 26 million tons/year, harvested from an area covering about 30 million ha [31]. Currently, detailed information about global organic production is lacking for this

crop. However, it was recently estimated that organic certified bean production accounts for 2.4% and 1.5% of total bean production in the U.S. and Europe respectively, and these percentages are steadily rising [32,33].

As a leguminous species, common bean contributes a substantial amount of nitrogen to the soil [34]. Nitrogen is one of the most significant nutrients for plant health, and cost-effective nitrogen management practices are necessary for an efficient production. For this reason, common bean is frequently intercropped or included in rotation schemes in organic agriculture and therefore, breeding and trials for high-performing cultivars specifically bred for organic and low-input agriculture are of great importance for optimising production in these systems.

This paper reports on the yield evaluation of 17 common bean lines bred for organic agriculture that were developed from a cross between a landrace and a commercial cultivar. The lines were compared with cultivars of the same commercial class phenotype in multi-environment field trials (MET) under organic management. In order to compare the level of genetic similarity between the breeding and the parental lines, molecular characterization was also carried out using 18 "neutral" and 7 "gene derived" Simple Sequence Repeat (SSR) molecular markers.

2. Materials and Methods

2.1. The Breeding Programme and Lines

The landrace used in the breeding programme, known as "Gnocchetto" or "Fagiolo a pisello" (*Gn*), is traditionally cultivated in Colle di Tora (Rieti, Italy, 42°12′31.33″N, 12°56′49.92″E, 557 m a.s.l.) on the steep hillsides of Lake Turano in the Apennines, mainly under organic conditions [35]. *Gn* is a landrace characterised by indeterminate growth habit, late flowering, white flowers and small round white seeds, ascribable to the Navy commercial class. Due to its growth habit and climbing ability, cultivation is difficult and expensive: plants can be more than four metres tall, requiring long supports, and only flower in the upper part. The flowering of this landrace continues until late in the cropping season, shifting the harvesting period by approximately a month compared to the most common Italian late-flowering cultivars. Seeds of the *Gn* landrace have a thin external tegument which means they cook quickly. Furthermore, beyond the technological advantages of this characteristic, it has also been recently demonstrated that fast-cooking common bean cultivars have higher protein and mineral retention compared to slow-cooking ones, suggesting that they have a greater nutritive value [36]. All these characteristics together with its smooth doughy taste [26] make this landrace an interesting genetic resource for a breeding programme.

Coco nano (*Cn*) is a cultivar characterised by determinate (dwarf) growth habit, early and prolific seed production which was selected under conventional conditions. Since *Cn* is the same commercial seed type as *Gn* (i.e., Navy), resultant crosses maintain this market class seed-phenotype.

The final aim of the breeding programme was to develop a panel of dwarf common bean lines characterised by high yield performance under organic management producing fast-cooking, smooth and doughy tasting seeds. Indeed, in *P. vulgaris* the determinate growth habit has been extensively exploited in breeding programmes to decrease plant biomass and optimise allocation between vegetative and reproductive growth [37]. In fact, the determinate growth habit and the shorter cycle allow many mechanical operations, including the harvest [38].

A schematic representation of the breeding programme carried out is shown in Figure 1. In 1998, 150 manual crosses were performed using the landrace *Gn* as the recurrent and seed-bearing female parent and the cultivar *Cn* (male parent) as the donor of the "dwarf" trait. In common bean this trait has been described as controlled by a single locus [39]. The high number of initial crosses performed was necessary to ensure introgression of the existing genetic diversity of *Gn* in the new breeding lines [35,40]. The F_1 plants obtained, and those of the subsequent generations, were separated into families, corresponding to each single cross. With no exceptions, all the F_1 plants showed an indeterminate growth habit. To fulfil the breeding objectives, two different approaches were used

throughout the following years: (A) 33 F_1 plants were backcrossed to the recurrent parent (*Gn*); (B) 47 F_1 plants were self-fertilised (F_2).

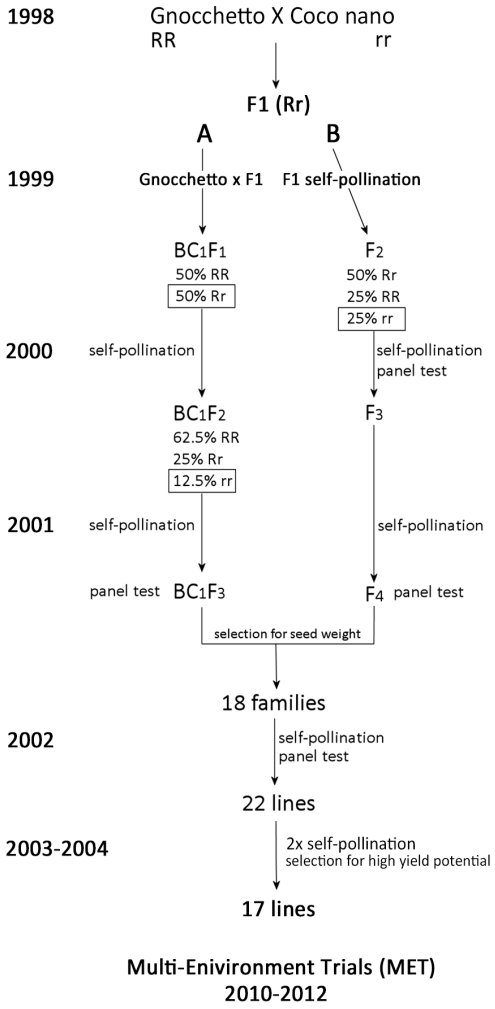

Figure 1. A schematic representation of the common bean breeding programme carried out at Dipartimento di Scienze Agrarie, Alimentari e Ambientali (DSA3). "R" and "r" indicate the dominant and recessive alleles controlling the climbing and dwarf trait, respectively.

In the year 2000, lines of the two groups were self-pollinated and those in group B were selected for the dwarf growth habit. In the same year, the seed quality was evaluated by means of a panel test. The principal aim of the panel test was to identify the above-mentioned taste characteristics which are peculiar to *Gn* together with its fast cooking feature. All the samples tested were weighed and prepared according to the experimental conditions (soaking/cooking) described by Torricelli and colleagues [40]. Only the lines with a score equal to 4 (*Gn*-like) were selected as detailed in [40]. In 2001, the same procedure was repeated and the panel test was also carried out on the lines in group A (Figure 1). At the end of the same year, the best 18 families were identified according to the results of the panel test and the seed weight (lines that produced seeds with an average weight $\leq$ 0.38 g were discarded). In the panel test, seeds from the breeding lines were scored by "blind" tasters according to their organoleptic qualities and their response to the soaking/cooking treatment. The panellists were trained according to Garruti and Bourne [41] using seed samples of *Gn* and *Cn;* therefore, the breeding lines tested were scored according to the following criteria: 1 = *Cn*-like; 2 = similar to *Cn*; 3 = similar to *Gn*; 4 = *Gn*-like.

In 2002, 78 lines derived from the 18 previously selected families were subjected to the panel test again and the best 22 lines were selected. In the following breeding step (2003–2004), plants were selected for yield potential (i.e., number of seeds per plant) and 5 low yielding lines were discarded.

2.2. Multi-Environment Field Trials

Seventeen lines derived from the above described breeding programme and two cultivars, *Cn* and *Cb* (as commercial controls), were included in multi-environment field trials [42]. *Cn* is the dwarf cultivar included in the breeding programme, while *Cb* (Coco Blanc Gautier) is a cultivar, characterised by the same growth habit and belonging to the same commercial class seed phenotype as *Cn* and *Gn*.

The *Gn* landrace was not included in the trials because it shows a vigorous vine growth, with plants that can be as tall as four metres, and consequently are usually spaced 100 × 100 cm and starts flowering late (mid-August). For all these reasons it was impossible to compare *Gn* with dwarf plants, which require less space and flower much earlier (June). Therefore, all tested materials belonged to the same commercial seed class and plant growth habit according to Voysest and Dessert [43]. The list of assessed materials, with information on their biological status and pedigree notes, is reported in Table 1.

Table 1. List of studied materials with their biological status and pedigree notes.

ID	Name	Biological Status	Pedigree Note
Line-1	Solibam-01	Breeding line	BC_1F_1 offspring
Line-2	Solibam-02	Breeding line	BC_1F_1 offspring
Line-3	Solibam-03	Breeding line	BC_1F_1 offspring
Line-4	Solibam-04	Breeding line	BC_1F_1 offspring
Line-5	Solibam-05	Breeding line	F_2 offspring
Line-6	Solibam-06	Breeding line	F_2 offspring
Line-7	Solibam-07	Breeding line	F_2 offspring
Line-8	Solibam-08	Breeding line	BC_1F_1 offspring
Line-9	Solibam-09	Breeding line	F_2 offspring
Line-10	Solibam-10	Breeding line	BC_1F_1 offspring
Line-11	Solibam-11	Breeding line	BC_1F_1 offspring
Line-12	Solibam-12	Breeding line	BC_1F_1 offspring
Line-13	Solibam-13	Breeding line	BC_1F_1 offspring
Line-14	Solibam-14	Breeding line	BC_1F_1 offspring
Line-15	Solibam-15	Breeding line	BC_1F_1 offspring
Line-16	Solibam-16	Breeding line	BC_1F_1 offspring
Line-17	Solibam-17	Breeding line	BC_1F_1 offspring
Gn *	Gnocchetto	Landrace	Parental line
Cn	Coco nano	Cultivar	Parental line
Cb	Coco blanc Gautier	Cultivar	Control

* The *Gn* landrace was not included in the MET trials.

Eight field trials were carried out in four different locations during three consecutive crop seasons between 2010 and 2012. In order to include a wide range of environmental variability, the four experimental sites covered a latitude range of about 10° (from 42°02′35.34′′N to 52°21′14.15′′N) and an altitude from 26 to 310 m a.s.l. Two experimental sites were located in Italy, in San Martino in Campo, Perugia (L1) and Rispescia, Grosseto (L2). The other two were located in Fressingfield, United Kingdom (L3) and Bellegade du Razes, France (L4).

All experiments were carried out following the principles and guidelines for organic management systems established by the International Foundation for Organic Agriculture (IFOAM, 2005). Throughout the trials, average daily temperature (°C), average daily relative humidity (%), and total rainfall precipitations (mm) (from May to August) were recorded using a weather station (Table 2).

Table 2. Weather data recorded in the different tested environments (i.e., location by year). The environments are designated by a code combining the location code and the year (e.g., L1-10 = San Martino in Campo, 2010).

	San Martino in Campo, Perugia, IT			**Rispescia, Grosseto, IT**		**Fressingfield, Suffolk, UK**		**Bellegarde du Razes Limoux, FR**
Coordinates	**43°02′35.34′′ N; 12°25′00.94′′ E**			**42°42′11.81′′N; 11°08′31.33′′E**		**52°21′14.15′′N; 1°21′16.73′′E**		**43°06′31.07′′N; 2°02′44.43′′E**
Altitude (m a.s.l.)	**174**			**26**		**50**		**310**
Environment	L1-10	L1-11	L1-12	L2-11	L2-12	L3-10	L3-11	L4-10
Year	2010	2011	2012	2011	2012	2010	2011	2010
T_{min} [a] SF [b]	13.0	15.1	13.1	15.2	14.7	11.5	9.0	14.1
T_{max} [c] SF	26.8	28.4	28.0	27.4	27.1	20.9	18.4	25.1
Rh [d] SF	62.3	59.4	56.9	67.0	44.8	68.9	70.3	68.1
Rnfl [e] SF	95.8	86.8	63.4	41.8	24.6	49.0	38.0	81.3
T_{min} FM [f]	16.8	16.7	17.6	17.3	18.6	11.1	11.6	17.2
T_{max} FM	31.9	31.5	34.1	29.6	31.5	18.7	20.0	29.2
Rh FM	51.3	51.3	41.1	66.8	42.3	77.3	75.8	59.0
Rnfl FM	87.2	63.6	53.6	116.2	32.6	111.8	82.0	65.4
Sowing date	20 May	24 May	16 May	20 May	21 May	2 June	12 May	21 May
Average dtf [g]	45	44	47	47	40	43	53	41
Average dtm [h]	112	103	107	98	107	136	121	101

[a] Minimum temperature (°C); [b] From sowing to flowering; [c] Maximum temperature (°C); [d] Relative humidity (%); [e] Rain precipitations (mm); [f] From flowering to maturity; [g] Days to flowering; [h] Days to maturity.

The entries were arranged in a completely randomised block design with three replications. For each entry and in each replication, five plants were assessed [44]. The row plots measured 300 cm in length and were spaced 70 cm apart; plants within row plots were spaced 50 cm apart. In all the environments, watering was applied according to Thung [45]. Finally, plants were manually harvested at maturity and the yield of each plant was measured as grams of seeds per plant (g plant^{-1}).

2.3. Statistical Analysis of Yield Data

In the MET, each location-year combination was considered as a different environment (hereafter the environments are designated by a code combining the location ID with the year. e.g., L1-10 = San Martino in Campo 2010). Mean yield was calculated for each environment and for each line as grams of seeds per plant. In each environment, the harvest was carried out manually at maturity. Mean yields of the tested lines in each environment were separated using Duncan's multiple range test using XLSTAT 2013 (Addinsoft).

In order to quantify the environment (E), the genotype (G) and the *genotype* by *environment* interaction (GE) effects on the yield data, a multi-environment analysis of variance (ANOVA) was performed using the same software according to the following mixed model:

$$y_{ij} = \mu + \alpha_i + \beta_j + \varphi_{ij} + \varepsilon_{ij}$$

where y_{ij} is the yield of genotype i in environment j, μ is the grand mean, α_i is the genotype effect, β_j is the environmental effect, φ_{ij} is the *genotype* by *environment* interaction and ε_{ij} is the random error.

GGE biplot analyses were carried out using GenStat 12.1 software (VSN International). Different graphical outputs were produced to: (i) identify adaptation patterns by visualising relations between the studied lines and the environments under organic management; (ii) rank the lines on the basis of the mean potential yield performance and stability; and (iii) highlight meaningful mega-environments and attempt to identify the best yielding line in each mega-environment (the so-called "which-won-where" method).

2.4. DNA Isolation, Markers Selection and Genetic Characterization

Young fresh leaves were collected from (i) six individual plants of each breeding line and (ii) a bulk of six plants of each of the parents (*Gn* and *Cn*) and of the control cultivar (*Cb*). Leaf tissues

were disrupted using the TissueLyser II (Qiagen, Hilden, Germany) and the Genomic DNA was extracted using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. DNA quality and concentration were assessed by means of UV-Vis spectrophotometry using the NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and 1.2% (*w*/*v*) agarose gel separation.

A set of 59 Simple Sequence Repeat (SSR) markers (35 genomic and 24 gene-based) covering all 11 linkage groups was initially identified [46–52] (Table S1). Seven out of the 24 gene-based SSRs were located in genes involved in flowering control in common bean [52]. Flowering related traits are strongly linked to the reproductive success and seed production of a crop in a certain environment. Microsatellite prediction was carried out using WebSat software [53] and primer sequences were designed using Primer3 software [54]. All the other SSRs were retrieved through bibliographic analysis using the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). The entire set of 59 SSRs was arranged and optimised in multiplex PCRs using MPprimer software [55] and initially tested on eight genotypes (*Cn*, *Cb* and six breeding lines randomly chosen from those available).

Amplification of SSR loci was performed by PCR using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in a volume of 12.5 µL, containing the following reagents: 20 ng of template DNA, 1× Type-it® Multiplex PCR Master 1 Mix (Qiagen, Hilden, Germany) and 0.4 µM of each primer. The reaction mixture was initially denatured at 94 °C for 4 min, followed by 12 cycles of amplification composed of: denaturation (94 °C for 30 s), annealing (temperatures are reported in Table S1) for 30 s and extension (72 °C for 30 s). Subsequently, 25 cycles of amplification were carried out as follows: 94 °C for 10 s, annealing temperature (decreased by 1 °C) for 15 s and 72 °C for 20 s. The final extension was carried out for 20 min at 72 °C.

2 µL of the amplified DNA was mixed with 3 µL of GelPilot DNA Loading Dye 5x (Qiagen, Hilden, Germany) and 10 µL of ultra-pure H_2O and then loaded into 3% (*w*/*v*) agarose gels with 1× TAE buffer. The electrophoresis was performed at 100 V for approximately 1.5 h. All gels were visualised under UV light after staining with ethidium bromide. Taking into account the observed polymorphisms, amplification quality, annealing temperature and amplification fragment sizes, 25 SSR markers were selected for genotyping. The forward primers were 5′-end-labeled with one of the fluorescent tags 6-FAM, HEX, PET and VIC (Applied Biosystems, Foster City, CA, USA). Amplicons were separated and sized according to the internal size standard GeneScan 500 LIZ (Applied Biosystems, Foster City, CA, USA) using the automatic sequencer 3130XL Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The visualisation and scoring of the amplicons were manually performed using the GENEMAPPER software (Applied Biosystems, Foster City, CA, USA).

2.5. Genetic Diversity Analysis

Number of polymorphic loci (N_{pol}), different alleles (N_a), effective alleles (N_e) [56], observed and expected homozygosity (Hom_o and Hom_e, respectively) and heterozygosity (H_o and H_e, respectively) of the tested breeding lines were assessed using the GenAlEx [57] and PowerMarker software [58]. The significance of differences among the breeding lines and among individuals within lines was tested using Analysis of Molecular Variance (AMOVA) in Arlequin v 3.5 software [59]. The significance level of the tested sources of variation was assessed using 9999 permutations.

A pairwise accession-by-accession genetic distance matrix was calculated using GenAlEx [60]. In order to obtain information on the genetic similarity/dissimilarity of the breeding lines with parental lines (*Gn*, *Cn*) and the control (*Cb*), the latter were also included in the analysis. The resulting matrix was used to perform a Principal Coordinate Analysis (PCoA) using the covariance matrix with data standardisation and to build a neighbor joining phylogenetic tree, using Mega 6 software [61].

3. Results

3.1. Multi-Environment Field Trials and Statistical Analysis

The mean yields of all the tested lines (17 breeding lines and two controls) in the eight environments (L1-10, L1-11, L1-12, L2-11, L2-12, L3-10, L3-11, L4-10) are reported in Table 3. The highest mean yield across environment was recorded for line 11 (55.66 g plant^{-1}) and the lowest for line 9 (28.71 g plant^{-1}). Among the tested environments, the highest mean yield was obtained in L3-11 (93.60 g plant^{-1}) and the lowest in L1-11 (14.27 g plant^{-1}).

Table 3. Mean yield (g plant^{-1}) of the 17 lines and the two cultivars (controls) tested at eight environments under organic management.

ID	Environment																	
	L1-10		L1-11		L1-12		L2-11		L2-12		L3-10		L3-11		L4-10		Mean	
Line-1	65.3	AB	14.6	AB	27.7	A	39.5	CD	18.8	D	72.2	BCDE	119.3	A	13.0	AB	**46.31**	ABC
Line-2	64.4	ABC	14.7	AB	15.7	AB	56.0	ABCD	28.1	ABCD	69.6	BCDE	110.6	AB	20.5	AB	**47.44**	ABC
Line-3	68.4	A	13.9	AB	6.4	B	52.5	ABCD	18.2	D	85.5	ABCD	94.8	AB	24.6	A	**45.53**	BC
Line-4	57.7	ABCD	19.6	AB	24.3	AB	65.3	ABC	41.7	ABC	106.5	A	84.9	AB	17.6	AB	**52.21**	AB
Line-5	23.8	DE	9.9	AB	11.1	AB	43.3	BCD	29.7	ABCD	61.7	CDE	82.4	B	19.2	AB	**35.12**	DEF
Line-6	11.7	E	3.2	B	15.0	AB	35.1	D	18.9	D	44.3	E	101.3	AB	20.7	AB	**31.26**	F
Line-7	15.3	E	4.6	AB	9.2	AB	55.1	ABCD	29.9	ABCD	57.8	DE	48.7	C	15.3	AB	**29.50**	F
Line-8	52.7	ABCD	11.7	AB	13.7	AB	57.6	ABCD	30.6	ABCD	79.6	ABCD	107.9	AB	20.9	AB	**46.85**	ABC
Line-9	16.3	DE	4.9	AB	7.8	AB	35.4	D	24.8	CD	48.3	E	80.3	BC	11.9	B	**28.71**	F
Line-10	48.4	ABCD	25.3	A	4.3	B	66.2	ABC	33.1	ABCD	89.6	ABCD	85.4	AB	19.5	AB	**46.48**	ABC
Line-11	59.5	ABC	21.9	AB	29.9	A	74.5	A	45.8	A	83.0	ABCD	109.4	AB	21.2	AB	**55.66**	A
Line-12	54.2	ABCD	20.3	AB	39.0	A	67.3	ABC	44.9	AB	75.2	ABCDE	103.3	AB	16.4	AB	**52.58**	AB
Line-13	41.1	BCD	9.9	AB	15.5	AB	46.2	BCD	33.8	ABCD	71.4	BCDE	78.8	BC	17.1	AB	**39.24**	CDE
Line-14	36.7	BCDE	11.8	AB	22.2	AB	46.6	ABCD	19.7	D	66.5	BCDE	100.9	AB	13.6	AB	**39.74**	CDE
Line-15	54.4	ABCD	20.5	AB	17.3	AB	53.5	ABCD	30.9	ABCD	94.1	ABC	82.0	B	17.1	AB	**46.24**	ABC
Line-16	35.5	CDE	15.0	AB	19.8	AB	53.6	ABCD	36.6	ABCD	69.7	BCDE	90.9	AB	19.1	AB	**42.53**	BCD
Line-17	35.8	BCDE	20.1	AB	42.7	A	59.0	ABCD	26.7	BCD	91.2	ABC	95.5	AB	19.0	AB	**48.75**	ABC
Cn	46.5	ABCD	17.2	AB	21.4	AB	65.9	ABC	27.9	ABCD	96.5	AB	98.3	AB	19.1	AB	**49.10**	AB
Cb	28.9	DE	11.9	AB	8.9	AB	67.9	AB	32.2	ABCD	89.8	ABC	103.6	AB	19.9	AB	**45.38**	BC
Mean	**42.98**		**14.27**		**18.53**		**54.76**		**30.12**		**76.44**		**93.60**		**18.19**		**43.61**	

The environments L3-10 and L3-11 were the best yielding ones because they showed the most favourable climatic conditions, characterized by the lowest average maximum and minimum temperatures between flowering and maturity of the pods (T_{min} FM = 11.1 and 11.6, T_{max} FM = 18.7 and 20.0 °C, respectively, Table 2).

All the sources of variation modelled in the ANOVA: E, G and GE, were significant and explained 55.5%, 4.7% and 7.1% of yield variance, respectively (Table 4). According to ANOVA results, environment was the most important source of yield variation. The relatively high value of GE compared to G suggests the probable existence of different mega-environments (ME) that need to be investigated [42]. Knowledge of MEs, namely, a group of environments defined by similar biotic and abiotic stresses and cropping system requirements, is important to direct more effectively the development of the germplasm involved in a breeding programme.

Table 4. ANOVA results.

Source of Variation	d.f. [a]	SS [b]	SS (%) [b]	F [c]	*p*-Value
E [d]	7	665,932.4	55.5	207.2	***
G [e]	18	56,264.2	4.7	6.8	***
G x E [f]	126	85,050.6	7.1	1.5	***
Error	1368	393,525.0	32.8		
Total	1519	1,200,772.1	100.0		

[a] Degree of freedom; [b] Sum of squares; [c] Fisher"s exact test; [d] Environment; [e] Genotype; [f] *Genotype* by *environment* interaction; *** Significant at $p < 0.001$.

The GGE biplot explained a high proportion of the total GGE variance (i.e., 83.71%) (Figure 2). In Figure 2a, the cosine of the angle between two vectors approximates the correlation between them: the more acute an angle is, the more positively correlated the vectors are. In the same figure,

both genotype (red) and environment (black) vectors are drawn such that the specific relations between a certain genotype and a certain environment can be visualised.

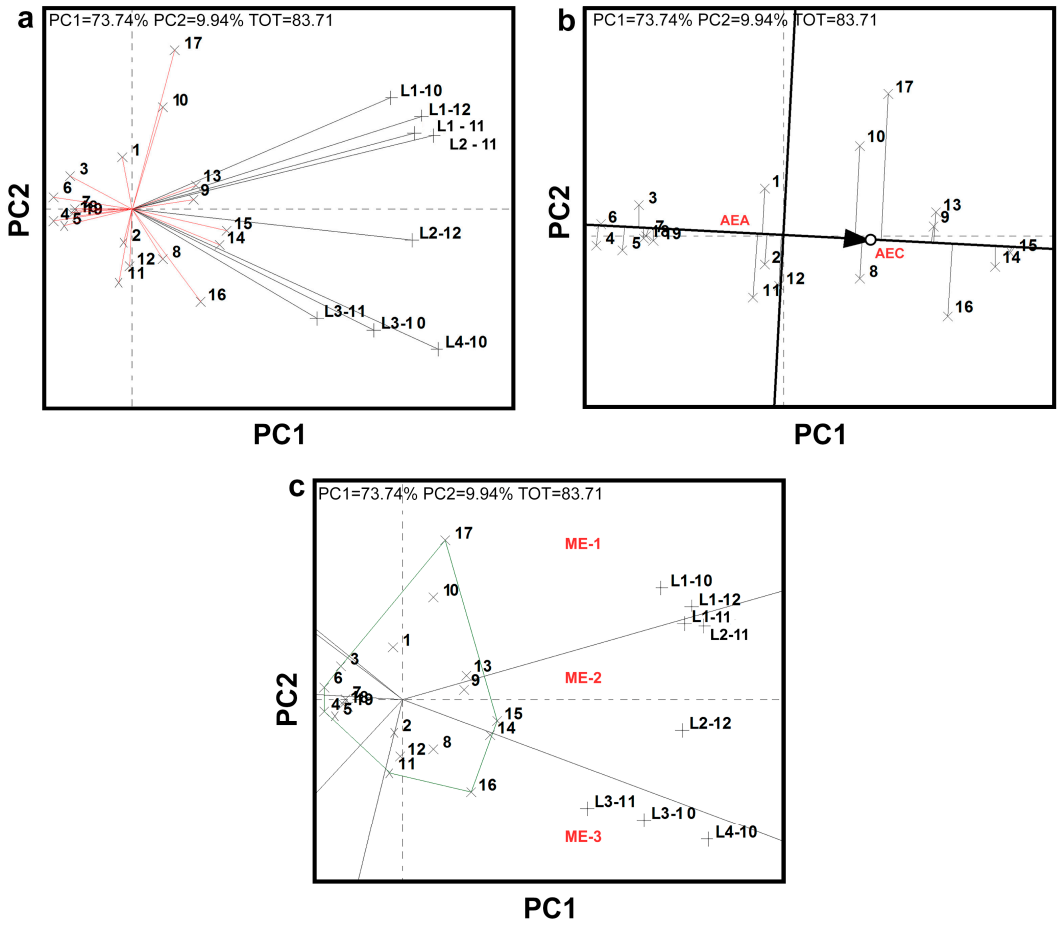

Figure 2. Different views of the GGE biplot for yield data of 17 common bean lines and two cultivars in eight environments under organic management. In the biplot, each environment is a location by year combination. Location labels are: L1, San Martino in Campo, Perugia, IT; L2, Rispescia, Grosseto, IT; L3, Fressingfield, Suffolk, UK; L4, Bellegarde du Razes, Limoux, FR. In the figure, numbers from 1 to 17 refer to the UNIPG corresponding breeding lines; 18 to Coco nano (*Cn*); and 19, to Coco blanc Gautier (*Cb*). (**a**) relationships among the tested lines (×) and test environments (+ vectors); (**b**) ranking of the lines by mean yield potential and stability. In this biplot view, the average environment axes (AEA), and average environment coordinates (AEC) are also shown; (**c**) The "which-won-where" view of the GGE biplot. ME 1, ME 2 and ME-3 indicate the three meaningful mega-environments. Biplots (**a**,**c**) are environment-centred, without scaling, and environment-metric preserving; biplot (**b**) is environment-centred, without scaling, and genotype-metric preserving.

The genotypic vectors of the breeding lines 3, 4, 5, 6, 7 and of the *Cn* (18) and *Cb* (19) cultivars, showed obtuse angles (i.e., negative interaction) with vectors of all environments: these genotypes were not able to take advantage of the conditions in any environment. On the contrary, genotypic vectors of lines 8, 9, 13, 14, 15, and 16 formed acute angles with all the environment vectors, showing a positive interaction at all the experimental sites. Breeding lines 1, 2, 10, 11, 12 and 17 showed right angles and/or large acute angles (i.e., poorer interactions) with some environment vectors and positive interaction with others.

In Figure 2b, the GGE biplot reports a ranking of the genotypes based on their mean yield performance and stability. In the figure, the average-environment axis (AEA) abscissa points to higher mean yield across environments and the AEA ordinate points to greater variability (i.e., poorer

stability or stronger interaction with a certain environment) in both directions. The average yield of the accessions on the left part of plot was generally lower than the grand mean, whilst that of the accessions on the right was higher.

The cultivars and breeding lines that were characterised by negative interaction with the environments (see above) also showed an average yield lower than the grand mean (Figure 2b). However, the other breeding lines showed mean yields higher than the grand mean. The "which-won-where" GGE biplot allowed us to identify MEs and to distinguish the best yielding line in each of them. In Figure 2c, a polygon is drawn on genotypes that are furthest from the biplot origin and perpendicular lines to each side of the polygon (or to their extension) are drawn, starting from the biplot origin. The latter line divides the biplot into different sectors that are different MEs. The eight studied environments fall into three sectors (ME-1, ME-2 and ME-3), indicating the existence of three meaningful MEs (Figure 2c). In the "which-won-where" biplot, the best yielding lines were 17, 15 and 16, that won in ME-1, ME-2 and ME-3 respectively.

3.2. Genotyping and Genetic Diversity

Among the 17 breeding lines, DNA was successfully extracted from 93 individuals and from the three bulks representing the two parents and the control. Of the 59 SSR markers amplified, 18 neutral and 7 gene-derived were found to be polymorphic and were consequently used for the genotyping (42.4% of the total). The selected SSR markers detected a total of 47 alleles; number of alleles per locus ranged from 1 to 3. Of the 47 identified alleles, 3 (6.38%) were private: two belonged to line 6 and one to line 7. The average number of alleles (N_a) ranged from 1.00 to 1.08 and the average number of effective alleles (N_e) from 1.00 to 1.06, confirming that the breeding materials developed are virtually pure lines.

At each studied locus, both *Cn* and *Cb* bulks showed the presence of a single allele (N_a = 1 and N_e = 1) which agrees with the genetic condition of common bean cultivars (i.e., pure lines). On the contrary, higher values of N_a and N_e (both 1.64) were observed for *Gn*, complying with the known within-population diversity of this landrace (Table S2) [26].

The AMOVA (Table 5) showed that 94.0% of total molecular variance of the breeding lines was due to variation among lines ($p < 0.001$) and only 3.4% among individual plants within lines; finally, 2.6% of the molecular variance was due to variation among alleles within individuals. The molecular results reported clearly show that the UNIPG lines are genetically differentiated and can be regarded as near-pure lines.

Table 5. AMOVA results.

Source of Variation	d.f. [a]	SS [b]	v.c. [c]	v [d]
Among lines	16	449.9	2.5	94.0%
Among individuals within lines	76	19.2	0.1	3.4%
Within Individuals	93	6.5	0.1	2.6%
Total	185	475.7	2.7	100.0%

[a] Degree of freedom; [b] Sum of squares; [c] Variance components; [d] Percentage of variation.

In the PCoA, the first two principal coordinates accounted for 54.86% and 14.79% of the variation, respectively (total 69.65%) (Figure 3). The first axes clearly distinguished three groups of samples: (i) *Gn* together with most of the breeding lines (i.e., 1, 2, 3, 4, 8, 10, 11, 12, 13, 14, 15, 16, 17); (ii) lines 5, 6, 7, 9 and (iii) *Cn* and *Cb*. The second axes mainly distinguished lines 6 and 9 from lines 5 and 7, underlining the existence of a higher level of diversity in the second group in comparison with the other two. Indeed, according to their position on the plot, all the samples in the first group are highly similar. Finally, the PCoA did not highlight any genetic differentiation between the two cultivars (*Cn* and *Cb*).

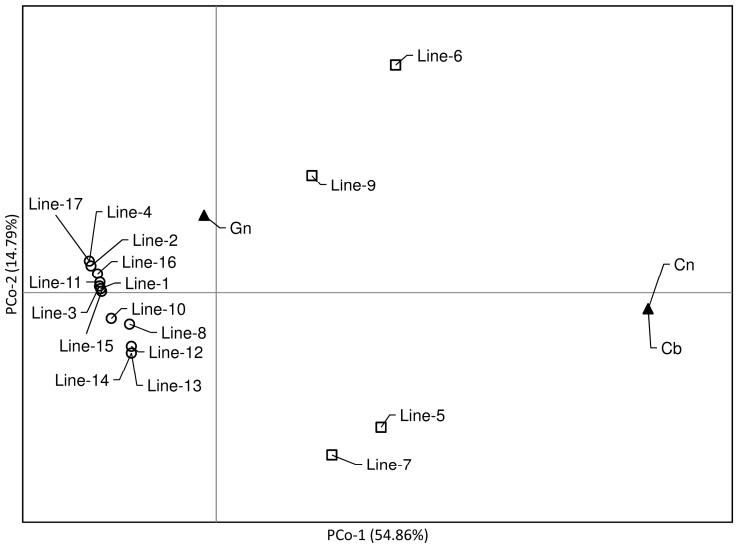

Figure 3. Plot of the first two axes of the PCoA. Open circles and open squares refer to breeding lines obtained using the breeding approach A (backcross) and B (F_1 self-pollination), respectively; filled triangles refer to the two parents (*Gn* and *Cn*). In this genetic assessment *Cb* was identical to *Cn* such that they are not distinguishable in the Figure.

The neighbor joining tree (Figure 4) confirmed that lines of the first group belong to a single cluster that also included the landrace (*Gn*). All the lines in this cluster derived from breeding strategy A, while lines 5, 6, 7, 9 from breeding strategy B. *Cn* and *Cb* are not grouped in any major cluster. Results of the genetic analyses comply with the pedigree of the lines, clearly separating those derived from the backcross (A) from those obtained by the simple cross (B).

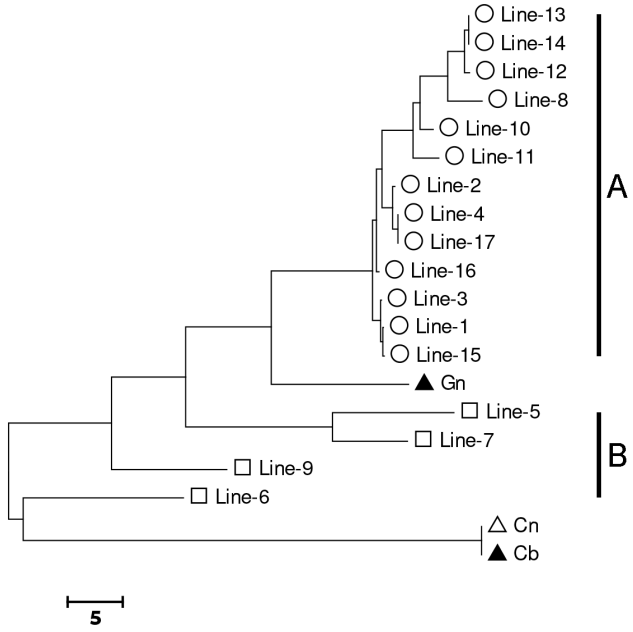

Figure 4. Neighbor joining tree based on the genetic distance matrix. Open circles and open squares refer to breeding lines obtained using the breeding approach A (backcross) and B (F_1 self-pollination) respectively; filled triangles refer to the two parents (*Gn* and *Cn*).

4. Discussion

The two principal aims of this study were: (1) to evaluate the performance of 17 breeding lines that share the same organoleptic and fast-cooking features by using a multi-environmental approach under organic conditions and (2) to understand the level of genetic diversity among these lines. The high yields recorded in Fressingfield in 2010 and 2011 were due to favourable weather conditions. Indeed, in comparison to the other environments, the two years (i.e., L3-10 and L3-11) were characterized by the lowest average maximum and minimum temperatures between flowering and maturity of the pods. It is well known that most of the crops are particularly sensitive to the temperature recorded between flowering and maturity and how heat stress can reduce yields [62]. In common bean, it has been reported that high temperature stress causes abortion of buds, flowers and pods, leading to high yield losses [63–65]. Moreover, the ANOVA showed that all the sources of variation were significant and, of these, the effect of environment (E) was the greatest. The strong differences among environments (i.e., the combination of location and year) can be considered the main cause of the predominant role of E. Similar results have been reported by studies carried out on different self-pollinated leguminous crops, such as pea [66] and soybean [67], as well as on major cereals [22,68,69]. The effect of genetic variation (G) was also significant, suggesting that the genotypic variability among the tested lines significantly affected their performance. Considering that all the best performing materials were some of the lines obtained by crossing the Gnocchetto landrace and the Coco nano cultivar (a pure line according to our data), it can be inferred that the genomic contribution of the landrace determined their good performance under organic conditions.

To better understand the potential utility of the selected common bean lines under organic and different agro-climatic conditions, genotype main effect (G) plus genotype by environment (GE) interaction biplot analyses were performed. By dissecting the GE interaction, this methodology allows the identification of promising genotypes under different environmental conditions. This method has also been, and is still largely used by many breeding companies and research institutions to evaluate and test the performance of different genotypes in certain areas using conventional agricultural practices. It has already been underlined how GE interaction (positive or negative) is of major importance when a breeding programme is intended for organic and low-input agriculture [70]. Indeed, important traits such as yield and overall quality were reported to be strongly linked to tolerance of various stresses and are complex inherited traits characterised by high GE interactions [71].

The GGE biplot analysis revealed strong differences in terms of potential performance with three of the 17 breeding lines expressing their best performance in different mega-environments. In Figure 2b, both breeding lines 16 and 17, which were shown to be the "winning lines" in ME-1 and ME-3, respectively, are the furthest lines from the AEA abscissa on the vertical axis. This distance is closely related to genotype instability (as it represents the contribution of GE to the genotype score) which, in this case, might be interpreted as a specific interaction with a narrower range of environmental conditions in a certain location. By contrast, line 15, which was the "winning line" in ME-2, together with line 14, showed a broader ability to interact positively with all the study environments since the distance of their genotype score falls very close to the AEA abscissa. It is also noteworthy that in the same figure, line 11, which showed the highest mean yield across all environments (see Table 3), was not ultimately among the "winning lines". In this case, the effect of the environment, discarded by the analysis, had a strong effect on the mean yield across environments. It is important to stress that Figure 2 explains only a portion of the total variation (i.e., G and GE) and some apparently stable genotypes may not be truly stable as the variation in their performance is not fully explained by the biplot.

The three MEs identified mainly group environments belonging to same geographical location, with very few exceptions. In fact, ME-1 includes two out of the three testing environments of San Martino in Campo, IT (L1-10, L1-12,) and ME-2 includes all the testing environments in the second Italian location (Rispescia) plus the only environment in San Martino in Campo (L1-11) that does not belong to ME-1. ME-1 and ME-2 group all the Italian test environments. In addition, it is noteworthy

that in ME-2, the environment L2-12 lies farther from the other two (L1-11, L2-11) and it is characterised by severely dry conditions. Since L2-11 (the same location but in a different year) is characterised by the highest level of total precipitation, ME-2 is definitely characterized by variable rainfall conditions and, consequently, it seems that other environmental factors may have played a stronger role in the definition of this ME. Finally, ME-3 clearly groups all the environments of the two northern European experimental sites.

According to our results, the level of diversity among the breeding lines developed allowed the best performing lines in the different MEs to be identified, which mainly corresponded to the different geographical areas considered. Therefore, the breeding programme was successful in selecting a panel of diverse dwarf common bean breeding lines sharing the organoleptic qualities of the landrace *Gn*. Moreover, according to our data, the residual genetic diversity among lines sharing the same quality and technological traits (i.e., *Gn* tasting qualities and fast-cooking features) allowed those with different adaptation ability to be identified. It should be noted that a previous study highlighted the existence of a relatively high level of genetic diversity in *Gn* [26], which is in line with our results.

As suggested by Fess and colleagues [25], our data confirm that the genetic diversity within a landrace population is likely to be a very important resource for coping with unexpected stresses and/or adapting to different environmental conditions, especially when the population is conserved on-farm for many generations. Indeed, on-farm conservation is a dynamic form of conservation that allows crops to evolve in response to both biotic and abiotic stresses. In this regard, it also important to note that all the lines suitable for organic farming (lines 15, 16, 17) belong to cluster A which also includes *Gn* (Figure 4). This grouping of the tested materials suggests that the landrace might have contributed with traits for high yields under organic conditions.

Regarding the two different breeding approaches used to develop the trailed lines, apparently, the use of a single backcross (A) did not negatively affect introgression of the quality traits of *Gn*, indicating that early selection, based on the growth habit and the panel tests, effectively increased the general performance of the breeding programme. This *modus operandi* allowed the number of backcrosses—generally a substantial investment in terms of time and development costs for a breeding company and/or institution—to be minimised.

5. Conclusions

The breeding strategy adopted in this study allowed the optimisation of time and cost in developing new common bean lines for organic agriculture. Moreover, across a panel of lines that share the same organoleptic and technological qualities, multi-environment trials enabled effective selection of those with the highest yields in the different MEs. As current cultivars for organic and low-input agriculture—characterised by environmental variability—are still limited, these results, together with careful preservation, screening and use of common bean germplasm such as landraces, which are adapted to organic agriculture per se, are an important step forward in fostering and accelerating successful breeding programmes for this growing sector.

Supplementary Materials: The following are available online at www.mdpi.com/2071-1050/10/3/777/s1, Table S1: List of SSR markers used for the genotyping; Table S2: Genetic descriptive statistics of the studied common bean lines.

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Author Contributions: Renzo Torricelli, Lorenzo Raggi and Valeria Negri conceived and designed the experiments; Leonardo Caproni, Lorenzo Raggi, Carlo Tissi and Sally Howlett performed the experiments; Leonardo Caproni, Carlo Tissi and Lorenzo Raggi analysed the data; Valeria Negri provided reagents/materials/analysis tools; all the authors contributed to the paper writing.

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