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Influence of agricultural management on phytochemicals of colored corn genotypes (*Zea mays* L.) – Part II: Sowing time

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ABSTRACT

Among the agronomic practices carried out in corn cultivation, the early sowing time is increasingly used by farmers of temperate regions to improve yield and reduce mycotoxin contamination of corn grains. The present study determined the influence of sowing time on the phytochemical content of grains of ten colored genotypes of corn. There was a significant improvement of both grain yield (+26%), thousand kernel weight (+3%) and test weight (+2%) in plots sown early. The early sowing influenced significantly also the chemical composition of corn grains, with an increase in the concentration of cell wall-bound phenolic acids (+5%) and β-cryptoxanthin (+23%) and a decrease in the concentration of lutein (-18%) and total anthocyanins (-21%). Environmental conditions that occurred during grain development influenced significantly the phytochemical content of corn grain, and early spring sowing could impart advantages in terms of both productivity and content of some antioxidants of whole-meal corn flour.

KEYWORDS

INTRODUCTION

Corn (*Zea mays* L.) is the most produced cereal in the world, with a global production of 1037 million metric tonnes in 2014, followed by rice (741 Mt) and wheat (729 Mt).[1] This cereal represents not only part of the staple diet of millions of people, but also a valued ingredient in the production of gluten-free foods, whose consumption is rising. Moreover, corn grains are key sources of antioxidant compounds, such as phenolic compounds, and of both provitamin A carotenoids, such as β-carotene, and non-provitamin A carotenoids, mainly represented by lutein and zeaxanthin.[2-4]

Different agricultural practices can be adopted in corn cultivation. In Part I of the present study[5], the effect of different nitrogen (N) fertilization rates on content of bioactive compounds of whole-meal corn flour was investigated. The results showed a significant effect of N fertilization not only on the grain yield, but also on total cell wall-bound phenolics and on the two main xanthophylls of corn grains. In addition to N fertilization, another key factor that play a pivotal role in corn cultivation is the sowing time.

Several studies showed that proper selection of the sowing time can increase corn yield. In temperate areas, early sowing improves grain yield because of an increase in both kernel weight and kernel number per ear as a consequence of a higher radiation use efficiency during the ripening compared to the later sowing time.[6,7] Consequently, the optimization of the length of the growing season by means of earlier sowing of corn has been one of the main factors that has improved corn productivity in these growing areas in the last years.[8,9] Moreover, early sowing has also been proposed as a strategy in order to reduce mycotoxin contamination of corn kernel.[10,11]

Given that in temperate regions sowing time determines the environmental conditions to which corn grain is exposed during its ripening, the advance or the delay of the sowing may have a remarkable influence on the bioactive compound content of corn grains. Previous

studies performed on different cereal crops showed that the environmental conditions can affect the concentration of different groups of phytochemicals.[4,12-15] Nevertheless, no study has ever been published about the effect of sowing time on content of bioactive compounds of corn whole-meal flour. Therefore, the aim of the present study, Part II of the work, was to evaluate over a two-year period the influence of sowing time on the antioxidant capacity and content of phenolic acids, carotenoids and anthocyanins of grains of irrigated, well-fertilized corn crops taking into account also the yield and the main grain physical parameters. Given the growing interest shown by the food industry in the development of functional foods, the experiments were performed on both conventional (yellow- and white-grained types) and colored corn genotypes characterized by dark red, red and blue kernels, which are considered a valuable source of phytochemicals.[16-18]

MATERIALS AND METHODS

Experimental design

The effect of the sowing time was evaluated by means of a two-year experimental design performed on 5 open-pollinated varieties and 5 corn hybrids characterized by different kernel traits (for more information see Giordano et al. [5]). The corn open-pollinated varieties, Italian landraces provided by CREA-CI (Bergamo, Italy), included:

- Rostrato vinato: dark red-grained corn characterized by small kernels;

- Ottofile rosso: red-grained corn characterized by large kernels;

- Pignoletto rosso: light red-grained corn characterized by small kernels;

- Pignoletto giallo: yellow-grained corn characterized by small kernels;

- Ostenga: white-grained corn characterized by large kernels.

The corn hybrids included:

- Indigo Blue: blue-grained corn characterized by medium-sized kernels (provided by Clarkson grain, Illinois [US]);

- P1208: yellow-grained corn characterized by small kernels (provided by Pioneer Hi-Bred Italia, Cremona [Italy]);

- SNH48.02: light yellow-grained corn characterized by medium-sized kernels (provided by Planta Research and Seeds, Vicenza [Italy]);

- DKC6815: light yellow-grained corn characterized by medium-sized kernels (provided by Monsanto Agricoltura Italia S.p.A., Milano [Italy]);

- PR32B10: white-grained corn characterized by medium-sized kernels (provided by Pioneer Hi-Bred Italia, Cremona [Italy]).

All the corn genotypes were sown in North West Italy in a completely randomized block design with three replications. In the 2014 growing year, the experimental trial was carried out in Buriasco (Piedmont, 44°51'39"N, 7°26'10"E), while in the 2015 growing year, the

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experimental trial was carried out in Chivasso (Piedmont; 45°12'42''N, 7°55'96''E). The plot size was 6 x 3 m, and each plot consisted of four rows of 36 plants (open-pollinated varieties) or 45 plants (hybrids). Two sowing times were compared: early sowing and late sowing. In particular, the early sowing was performed on 3 April 2014 and 3 April 2015, while the late sowing was performed on 21 May 2014 and 11 May 2015. The sowing was carried out after an autumn ploughing (30 cm) and disk harrowing to prepare a proper seedbed. All the plots received 100 kg/ha of P_2O_5 and K_2O before sowing and 300 kg N/ha at the end of leaf development stage (GS 19) with urea (granular, 46%). Irrigation was carried out by employing the furrow method, according to the conventional farm management system in force in the experimental area, to avoid any drought stress until the end of the dough stage (growth stage [GS] 87). All the plots were sprayed at GS 75 with pyrethroid lambda-cyhalothrin insecticide (Karate Zeon, Syngenta Crop Protection, Milan, Italy) at 0.019 kg of active ingredient/ha, to minimize the ear injuries caused by the activity of *Ostrinia nubilalis* Hübner.

The plots sown early were harvested on 24 September 2014 and 7 September 2015, while plots sown later on 17 October 2014 and 23 September 2015. Ears were collected by hand from 4.5 m^2 of each plot to quantify grain yield and to obtain a representative sample. Ears were shelled with an electric sheller and kernels from each plot were mixed thoroughly to obtain a random distribution. Each sample was then processed in two different ways before analyses. A first subsample, designated for the assessment of grain physical parameters, was dried at 60°C for three days until reaching a kernel moisture of 14%. On the contrary, a second subsample, designated for chemical analyses, was immediately frozen and freeze-dried to avoid changes in the phytochemical content. Freeze-dried kernels were then ground to a fine powder (particle size <300µm) with a Cyclotec 1093 sample mill (Foss, Padova, Italy), and stored at -25°C until analyses were performed.

Grain physical parameters

Thousand kernel weight (TKW) and test weight (TW) were determined by means of an electronic balance and a Dickey-John GAC2000 grain analyses meter (Dickey-John Corp., Auburn, IL), respectively.

Chemical analyses

<u>Chemicals</u>

Dichloromethane (CHROMASOLV®, ≥99.9%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol (CHROMASOLV®, 99.8%), ethylacetate (CHROMASOLV®, 99.8%), hexane (CHROMASOLV®, 97.0%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), hydrochloric acid (HCl, 37.0%), methanol (CHROMASOLV®, 99.9%), potassium hydroxide (KOH, 90.0%), sodium hydroxide (NaOH, ≥98.0%), and phenolic acid stardards (*p*-coumaric acid ≥98%, sinapic acid ≥98% and *t*-ferulic acid ≥99%) were purchased from Sigma-Aldrich (St. Louis, Missouri, US). Carotenoids standards (β-carotene ≥98%, β-cryptoxanthin ≥97%, lutein ≥95% and zeaxanthin ≥98%) and cyanidin 3-*O*-glucoside chloride were purchased from Extrasynthese (Lyon, France).

<u>Extraction of phenolic acids and quantifications by means of RP-HPLC/DAD</u>

Cell wall-bound phenolics were extracted as reported by Urias-Peraldì et al.[17] with few modifications. The pellet remaining after the extraction with 80:20 (v/v) methanol water mixture was hydrolyzed for 30 min in a hot water bath (80°C), by adding 400 µL NaOH 6 M. After acidification to pH 2 with HCl 12 M, the bound phenolics were extracted with 300 µL of

ethyl acetate. The extraction was repeated three times. The combined supernatants were evaporated to dryness, and then reconstituted in 200 µL of 80:20 (v/v) methanol water mixture. The content of total cell wall-bound phenolics (TCWBPs) was determined by the Folin-Ciocalteu method as reported in Giordano et al.[19] The quantification of cell wall-bound phenolic acids was performed on filtered extracts using an HPLC system (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1200 Series diode array detector. Separations were carried out using a 150 x 4.6 mm, 5 µm, Gemini RP-18 column (Phenomenex, Torrance, CA, US) as reported by Shao et al.[20] Hydroxycinnamic acids (ferulic acid, *p*-coumaric acid and sinapic acid) were identified using the retention times and UV/Vis spectra of their respective standards. Their quantification was performed by means of external calibration curves.

Determination of the antioxidant capacity

The antioxidant capacity was determined by means of the QUENCHER-DPPH assay (direct measurement on solid samples[21]) as previously described in Giordano et al.[22] The final results were expressed as mmol of Trolox equivalents (TE)/kg of sample on a dry weight (dw) basis.

Extraction of carotenoids and quantification by means of RP-HPLC/DAD

Corn carotenoids were extracted as reported by Burt et al.[23] with few modifications. Each sample (0.3 g) was initially extracted for 6 min at 85°C with 95% ethanol, and then hydrolyzed with 125 µL of KOH (80% w/v) at 85°C for 10 min. After adding 3 mL of cold deionized water, carotenoids were extracted with 3 mL of hexane. The extraction was repeated four times, and the combined supernatants were reduced to dryness under a

nitrogen stream, and then reconstituted in 200 µL of dichloromethane. The filtered extracts were analyzed using the previously described chromatographic system as reported by Moros et al.[24] Carotenoids (β-carotene, β-cryptoxanthin, lutein and zeaxanthin) were identified using the retention times and UV/Vis spectra of their respective standards. Their quantification was performed at 450 nm by means of external calibration curves.

Extraction of anthocyanins and determination of total anthocyanin content

The total anthocyanin content (TAC) of red- and blue-grained corn samples (Rostrato vinato, Ottofile rosso, Pignoletto rosso, Indigo blue) was determined using a spectrophotometric method. Each sample (1g) was extracted using 8 mL of ethanol acidified with HCl 1 M (85:15, v/v). After adjusting the pH to 1, the mixture was shaken for 30 min and then centrifuged at 20800 g for 10 min. The supernatant was used for the determination of TAC by means of the pH differential method.[25] Results were expressed as mg of cyanidin 3-O-glucoside equivalents (Cy 3-glc eq)/kg of sample dw.

Color analyses

The chromatic analyses were performed using a Minolta Chroma Meter reflectance spectrophotometer (Model CR-400, Minolta Co., Osaka, Japan). A 45 mm diameter Petri dish was loosely filled with a ground subsample, and the dish was then tapped gently until the sample was levelled and no gaps were apparent through the base of the dish. The L^*, a^* and b^* color values were determined directly by the instrument.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) to evaluate the effect of the genotype, sowing time and environment on the content of bioactive compounds of corn grains as well as on yield and grain physical parameters. A 0.05 threshold was used to reject the null hypothesis. The Ryan/Einot and Gabriel/Welsch (REGW-Q) test was performed for multiple comparisons. Principal component analysis (PCA) was carried out to investigate the relationship among the bioactive compounds and the kernel color. Data were preliminary standardized by subtracting the means and dividing by the standard deviations within each variable. Additionally, samples were clustered on the basis of their chemical composition and color by means of a Hierarchical Clustering Analysis (HCA), performed with Euclidean squared distance and Ward's method.

Statistical analyses were carried out by means of SPSS for Windows statistical package, Version 24.0 (SPSS Inc., Chicago, Illinois).

RESULTS AND DISCUSSION

Grain yield and physical parameters

The three main factors (genotype, sowing time and environment) analyzed in the present study showed significant effects on the yield, TKW and test weight TW (Table 1). As far as the yield of corn crops was concerned, the open-pollinated varieties were significantly lower in productivity than the hybrids (Table 2). The lowest yield (5.0 t/ha) was observed for the red-grained variety Ottofile rosso, characterized by small ears (on average 166 g) with eight ranks. On the contrary, the yield of the white-grained hybrid exceeded 17 t/ha. A comparison among the open-pollinated varieties and corn hybrids indicated the highest TKW for the white- and red-grained varieties (Ostenga and Ottofile rosso) and the white- and blue-grained hybrids (PR32B10 and Indigo blue). Significant differences were observed among genotypes also for their TW in accordance with their hardness, with a high TW indicating a more corneous endosperm.

The yield and the grain physical parameters were significantly influenced by the environment. Higher yields (+17%), TKW (+6%) and TW (+2% increase) were observed in 2014 compared to 2015. As shown also in Part I of the present study,[5] the 2014 year was characterized by lower temperature and higher rainfall levels, which led to a prolonged ripening period than 2015 (Table 3 and Figure 1). In the present study, differences were observed not only between years but also between early- and late-sown cycles within the same year. A far as the reproductive stage is concerned, the average daily temperatures measured from the flowering to harvest maturity were on average higher in early- than in late-sown cycles (Figure 1). This gap in the temperature resulted in a higher corn yield (+26%), TKW (+3%) and TW (+2% increase) in early planting than in later sowing (Table 2). The results were in accordance with previous studies,[6,7] which demonstrated that late

sowings affected grain yield due to a decrease in the crop growth rate during grain filling caused by a low RUE (radiation use efficiency) and a low incident radiation.

Cell wall-bound phenolic acids and antioxidant capacity

The three-way ANOVA (Table 1) showed several significant effects of the three sources of variation and of their interactions on total cell wall-bound phenolic content, on the main cell wall-bound phenolic acids (ferulic, *p*-coumaric and sinapic acid), and on the DPPH· scavenging activity of corn whole-meal flours. Compared to the effects of both the sowing time and the environment, the influence of the genotype on phenolic compounds and antioxidant capacity was even larger. This observation was likely due to the high genetic variability among the corn genotypes analyzed in the present study. Averaged across growing seasons and sowing time (Table 2 and S1), TCWBPs was highest in the dark red variety Rostrato vinato (5946 mg FAE/kg dw), which also had the highest DPPH· scavenging activity (31.6 mmol TE/kg dw). On the contrary, even though significant differences were not observed among the blue-, yellow- and white-grained hybrids in terms of TCWBPs (4105, 4424 and 4487 mg FAE/kg dw), Indigo blue showed a significantly higher antioxidant activity (19.4 mmol TE/kg dw). These findings are in agreement with literature data,[16,26] leading to the conclusion that dark-colored corns have high radical scavenging activity.

The environment had a significant effect on the content of both TCWBPs and single cell wall-bound phenolic acids. They were all significantly higher in the 2015 growth year, which was characterized by higher daily temperatures and lower rainfall. Similarly, phenolic compounds were significantly higher in grains of early-sown corn plants, grown on average at higher temperatures than late-sown ones. Several studies demonstrated that growing conditions might significantly alter the antioxidant properties and the total phenolic content of wheat flour,[27] wheat bran[28,29] and whole-meal wheat flour.[12-30] It is widely reported that

the biosynthesis of phenolic compounds is regulated by enzymes such as phenylalanine ammonia-lyase (PAL), which catalyzes the biotransformation of L-phenylalanine to *trans*-cinnamic acid. Previous studies reported that high temperatures could increase the PAL activity in tomato, and that the phenolic compounds increase under high temperature stress.[31] Similarly, Wu et al.[32] suggested that the phenolic changes in grains of sorghum grown under high temperatures may be caused by PAL activity change, while for individual phenols, their changes might be related to changes in activity of some specific enzymes. Based on the current findings, it is likely that the phenolic changes observed between sowing times and environments may be caused by different climatic conditions, particularly different temperatures during grain filling. As shown in Figure 2, the influence of the sowing time on cell wall-bound phenolic acids is greater in the 2015 year. In fact, during that year, differences in daily temperatures during grain filling were heightened between the two growing cycles. Thus, the data suggest that early-sown cycles can significantly improve the concentration of cell wall-bound phenolics above all in dryer and warmer years. On the contrary, in wetter and cooler years (as the 2014 year), no significant difference was observed for TCWBPs, ferulic and *p*-coumaric acids between early- and late-sown clycles. The only exception was sinapic acid, which was significantly higher in early-sown cycles of both years.

As far as single genotypes are concerned, the phenolic content of the dark red variety Rostrato vinato was more influenced by the sowing time (Figure 3). In fact, early sowing improved significantly the content of TCWBPs (+14%), ferulic acid (+15%), *p*-coumaric acid (+30%) and sinapic acid (+24% increase) for this genotype. As expected, the DPPH· scavenging activity of this variety was also significantly higher (+28%, P<0.001) in grains of early-sown plants. Another genotype which was highly influenced by the sowing time was the white variety Ostenga. Even though only TCWBPs and *p*-coumaric acid increased

significantly in early-sown plants (+14% and +21%, respectively), an improvement in content of ferulic acid (+7%), sinapic acid (+17%) and antioxidant capacity (+7%) was also observed. The light yellow- and blue-grained hybrids (SNH48.02 and Indigo blue) differed from all the other genotypes in fact they showed a decrease in TCWBPs, ferulic and *p*-coumaric acid in early sowings compared to late planting. However, the only significant reduction observed was that of ferulic acid in the SNH40.02 hybrid (P<0.05).

Carotenoids

Yellow-endosperm corn genotypes have been recognized as sources of both provitamin A carotenoids and xanthophylls, which are known to be important for eye health.[33] In the present study, the open-pollinated varieties and hybrids were characterized by quantitative and qualitative differences in their carotenoid composition (Table 2 and S1). In accordance with the high multiplicity of corn genotypes analyzed, the genotype accounted on average for 88% of variation observed in the carotenoid content and a great variability was observed among genotypes. According to previous studies,[3,4,34] lutein and zeaxanthin were the main carotenoids detected, and their relative abundance was dependent on the hardness of the grain.[33,35] In regards to the levels of the provitamin A carotenoids, the open-pollinated varieties showed higher concentrations than hybrids. The yellow- and light red-grained varieties in particular, were characterized by the highest content of both β-cryptoxanthin and β-carotene.

The sowing time influenced significantly the concentration of both lutein and β-cryptoxanthin but in an opposite way (Table 2). Lutein, zeaxanthin and β-carotene were lower in grains of early-sown plants by 18, 6 and 5%, but the concentration of lutein was the only one significantly affected. On the contrary, early sowing increased significantly the content of β-cryptoxanthin by 23% compared to the late one. Similarly, the 2015 year resulted in a

significant lower concentration of all the carotenoids quantified, which averaged a 14% decrease than 2014 values. These observations supported the conclusion that carotenoid content in corn grains was higher the cooler the growing conditions were during ripening stages. The results were in accordance with a previous study performed on einkorn[36] in which in a 6-year period, an exceptionally high lutein content was observed in the growing season characterized by the coolest and wettest environment. Similarly, in studies performed on different einkorn accessions, the highest lutein content was recorded in the wettest years.[14,37] Previous studies showed that in corn, the carotenoid content of the endosperm increased sharply between 13 and 22 days after pollination.[34] In the present study, the medium temperature that occurred between 13 and 22 days after pollination (Figure 1), was lower by 2.1°C in late-sown cycles than early ones. The medium temperature was also lower by 4.5°C in the 2014 growing season than in the 2015 year. Moreover, given that the environmental differences between early and late growing cycles were considerably heightened in the warmer year, the effect of the sowing time on carotenoid content was considerably more important during 2015 (Figure 2). In fact, in 2014, lutein content decreased significantly by 13% in grains of early-sown plants, while β-cryptoxantin increased by 8%, whereas in 2015 changes in the concentrations were a 22% decrease and a 41% increase, respectively. Thus, similar to phenolic acids it was possible to postulate that the warmer the year the higher the effect of the sowing date on xanthophyll contents. β-carotene was the only compound which was not significantly influenced by the sowing time.

Finally, the present study illustrated that the extent of influence of the sowing time on carotenoid concentration was dependent on the genotype considered (Figure 4). In fact, the only genotype which showed significant changes in the content of all the carotenoids quantified was the light yellow-grained hybrid SNH48.02. A reduction of concentration was

observed for lutein (-25%), zeaxanthin (-15%) and β-carotene (-36%) in grains of early-sown plants. On the contrary, the concentration of β-cryptoxanthin increased significantly (+63%) as seen for all the other genotypes. The blue-grained hybrid Indigo Blue was the only genotype which showed a significant increase in the concentration of both lutein and zeaxathin after early sowing, but it is worth noting that this hybrid was characterized by only small quantities of both xanthophylls (<1 mg/kg dw).

Total anthocyanin content

Indigo Blue was characterized on average by a TAC of 722 mg Cy 3-glc eq/kg dw while the dark red, red and light red genotypes showed on average a TAC lower than 15 mg Cy 3-glc eq/kg dw. The results were in agreement with previous studies performed on blue and red pigmented genotypes.[17,38]

As far as the Indigo Blue was concerned, the two-way ANOVA showed a significant effect of the sowing time (P<0.01) on TAC. In particular, the highest TAC was observed in late-sown plants (808 mg Cy 3-glc eq/kg dw) while a decreaseof 21% was observed after early sowing (636 mg Cy 3-glc eq/kg dw). The environment did not influence significantly the TAC (P=0.230), but the interaction between the sowing time and the environment had a significant effect (P<0.05). In fact, in the 2014 growing season, a significantly higher TAC was observed in the late sowing time, while in 2015, this difference was not significant. Previous studies performed on purple genotypes[15] have shown that environmental factors, such as the temperature and the rainfall level influence the anthocyanin accumulation in corn grains. The highest anthocyanin content was observed in purple corn grains harvested from the location characterized by both low temperatures and high rainfall level. Similarly, in the present study the highest TAC was observed in grains of Indigo Blue grown in presence of lowest average temperatures and highest rainfall levels (Table 3 and Figure 1). Therefore,

conditions that favor anthocyanin accumulation in corn grains may be different from those that favor high cell wall-bound phenolic acids.

Multivariate analyses of bioactive compounds in colored corn genotypes

The results of the PCA based on the cell wall-bound phenolic acids, DPPH$^{\cdot}$ scavenging activity, carotenoids and color values of the ten corn genotypes analyzed (n=120) were used to produce a loading plot in which the two first principal components explained 34% and 32% of the total variation of the samples (Figure 5). It can be assumed that the PC1 differentiated the samples according to their carotenoid content and to their blu-yellow index (*b**). On the other hand, the PC2 differentiated the samples mainly according to their phenolic content, antioxidant capacity, lightness (*L**) and red-green index (*a**). As expected, loading plots showed a positive correlation between the content of carotenoids and the yellowness of the whole-meal flour. Similarly, a positive correlation was observed between phenolics and the redness of the whole-meal flour, while a negative relation was observed with the lightness. The only exception was ferulic acid. The score plot showed a characteristic clustering of data, similar to the one obtained by means of a HCA (Figure S1). In the upper side were grouped genotypes characterized by red and blue kernels, while yellow- and white-grained genotypes were all placed on the lower side. Blue- and white grained genotypes were placed on the left side of the score plot because of their low concentrations of carotenoids. White-grained genotypes (Ostenga and PR32B10) were located in close proximity as well as light yellow-grained hybrids (DKC6815 and SNH48.02), which implied a strong similarity between each other in terms of color and phytochemical composition. As far as the yellow- and red-grained open-pollinated varieties are concerned, their characteristic distribution in the score plot highlighted that the higher the content of

carotenoids (PC1 coordinate), the lower the content of cell wall-bound phenolics (PC2 coordinate).

In summary, to the best authors' knowledge, this study is the first to document the influence of the sowing time on bioactive compounds of corn grains. Results clearly show that the environmental conditions significantly affected the content of both phenolic compounds and carotenoids of corn grain, with an increase in the concentration of TCWBPs (+5%) and β-cryptoxanthin (+23%) and a decrease in the concentration of lutein (-18%) and total anthocyanins (-21%) in grains of plants sown early The implications of this study can be explained as follows: rainfall levels and daily temperatures which occur during grain filling can differ not only between environments, but also between growing cycles in the same year, hence the choice of the sowing time can play an important role in phytochemical accumulation particularly in warm and dry years. The increasing employment of earlier spring sowing of corn by farmers of temperate regions in order to improve the yield and the quality traits of corn grain, while reducing the contamination by mycotoxins, could also result in a significant increase in the content of both cell wall-bound phenolic acid and β-cryptoxanthin. At the same time this agronomic practice could significantly reduce the concentration of lutein and total anthocyanins.

Further studies will be necessary for the development of supply chains based on colored corn genotypes for the production of new food products and ingredients with added value for consumer health. It will be necessary to improve the knowledge not only in terms of agronomic management during cultivation, but also in terms of post-harvest processing, given the uneven distribution of phytochemicals in the corn kernel.[39]

ABBREVIATIONS USED

ANOVA: analysis of variance; BHT: 2,6-di-*tert*-butyl-4-methylphenol; Cy 3-glc eq: cyanidin 3-*O*-glucoside equivalents; DPPH: 2,2-diphenyl-1-picrylhydrazyl; DW: dry weight; FAE: ferulic acid equivalents; GDD: growing degree days; GS: growth stage; N: nitrogen; PAL: phenylalanine ammonia-lyase; PCA: principal component analysis; REGW-Q test: Ryan/Einot and Gabriel/Welsch test; RUE: radiation use efficiency; TCWBPs: total cell wall-bound phenolics; TE: Trolox equivalents; TKW: thousand kernel weight; TW: test weight.

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TABLES

Table 1. P values and R^2 values of the three-way ANOVA analyses performed to evaluate the effect of the genotype, the sowing time and the environment on grain yield, grain physical parameters, phenolic acid content, antioxidant capacity and carotenoids of whole-meal corn flour. Significant values are reported in bold style.

	Genotype	Sowing time	Environment	Sowing time x Genotype	Sowing time x Environment	R^2
Variable			P (F)			
Grain yield and physical traits						
Yield	**<0.001**	**<0.001**	**<0.001**	**0.004**	**0.014**	0.941
TKW	**<0.001**	**0.002**	**<0.001**	**0.003**	**0.001**	0.903
TW	**<0.001**	**<0.001**	**<0.001**	0.927	0.060	0.818
Phenolic acids and antioxidant capacity						
TCWBPs	**<0.001**	**0.001**	**<0.001**	**0.043**	**<0.001**	0.780
p-Coumaric acid	**<0.001**	**<0.001**	**0.003**	**<0.001**	**<0.001**	0.918
Ferulic acid	**<0.001**	0.140	**<0.001**	**0.038**	**<0.001**	0.826
Sinapic acid	**<0.001**	**<0.001**	**<0.001**	0.087	0.237	0.837
AC	**<0.001**	0.108	0.511	**<0.001**	**<0.001**	0.924
Carotenoids						
Lutein	**<0.001**	**<0.001**	**<0.001**	**0.001**	0.129	0.950
Zeaxanthin	**<0.001**	0462	**<0.001**	0.827	**0.040**	0.941
β-cryptoxanthin	**<0.001**	**<0.001**	**0.015**	0.541	**0.009**	0.914
β-carotene	**<0.001**	0.381	**0.002**	0.952	0.174	0.929

TKW: thousand kernel weight; TW: test weight; TCWBPs: total cell wall-bound phenolics. AC: antioxidant capacity determined by means of the QUENCHER-DPPH$\cdot$ scavenging assay.

Table 2. Effect of the genotype, the sowing time and the environment on grain yield, grain physical parameters, phenolic acid content, antioxidant capacity and carotenoids of whole-meal corn flour.

	Yield	TKW	TW	TCWBPs	*p*-Coumaric acid	Ferulic acid	Sinapic acid	AC	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene
	(t/ha)	(g)	(kg/hL)	(mg FAE/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mmol TE/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Genotype (n=12)												
Rostrato vinato	5.3^{d}	320.7^{c}	79.8^{cd}	5946.4^{a}	297.9^{a}	1696.5^{c}	23.3^{cd}	31.6^{a}	5.75^{d}	8.82^{d}	1.92^{c}	0.79^{d}
Ottofile rosso	5.0^{d}	411.8^{a}	75.9^{e}	4675.4^{cd}	248.6^{b}	1451.4^{d}	31.3^{a}	14.3^{c}	5.31^{d}	18.11^{b}	2.29^{b}	1.35^{c}
Pignoletto rosso	6.7^{d}	267.7^{d}	82.3^{ab}	4914.1^{bc}	234.2^{b}	1832.9^{c}	21.3^{d}	13.5^{c}	7.79^{c}	16.39^{b}	2.97^{a}	1.66^{b}
Pignoletto giallo	5.6^{d}	261.7^{d}	82.9^{a}	4692.6^{cd}	205.4^{c}	2073.9^{b}	20.7^{de}	8.9^{d}	9.01^{b}	21.85^{a}	2.82^{a}	3.01^{a}
Ostenga	5.2^{d}	414.5^{a}	78.7^{d}	4753.2^{cd}	145.0^{e}	2154.2^{b}	23.3^{cd}	8.9^{d}	<0.1	<0.1	<0.3	<0.3
Indigo blue	10.4^{c}	392.9^{a}	79.3^{cd}	4105.2^{e}	117.0^{f}	1732.5^{c}	31.6^{a}	19.4^{b}	0.73^{e}	0.81^{e}	<0.3	<0.3
P1208	13.0^{b}	333.9^{bc}	84.0^{a}	4424.3^{de}	177.1^{d}	1753.7^{c}	25.0^{bc}	9.0^{d}	8.18^{bc}	13.88^{c}	1.47^{d}	0.57^{de}
SNH48.02	13.3^{b}	344.9^{b}	80.7^{bc}	4174.2^{e}	130.4^{ef}	1750.8^{c}	18.5^{e}	9.8^{d}	12.09^{a}	8.48^{d}	0.55^{e}	0.44^{e}
DKC6815	16.3^{a}	346.7^{b}	74.7^{e}	5323.7^{b}	196.5^{cd}	2372.8^{a}	26.3^{b}	9.9^{d}	8.36^{bc}	6.92^{d}	0.54^{e}	0.50^{de}
PR32B10	17.3^{a}	401.1^{a}	82.4^{ab}	4487.3^{cde}	149.2^{e}	2176.2^{b}	18.4^{e}	8.9^{d}	<0.1	<0.1	<0.3	<0.3
SEM[1]	0.4	5.8	0.5	107.5	5.6	43.8	0.7	0.6	0.24	0.54	0.09	0.08
	***	***	***	***	***	***	***	***	***	***	***	***
Sowing time (n=60)												
Early	11.1	354.2	81.0	4859.3	199.9	1920.2	25.2	13.7	6.41	11.62	1.99	1.16
Late	8.8	344.0	79.2	4634.7	182.2	1873.8	22.8	13.1	7.80	12.30	1.62	1.22
SEM	0.2	2.6	0.2	48.1	2.5	19.6	0.3	0.3	0.12	0.27	0.05	0.04
	***	***	**	***	***		***		***		***	
Environment (n=60)												
Buriasco, 2014	10.8	359.2	81.0	4504.9	185.3	1795.0	23.1	13.3	7.69	13.19	1.91	1.28
Chivasso, 2015	9.2	339.1	79.1	4989.1	197.6	2001.0	24.9	13.5	6.58	10.82	1.71	1.10
SEM	0.2	2.6	0.2	48.1	2.5	19.6	0.3	0.3	0.12	0.27	0.05	0.04
	***	***	***	***	**	***	***		***	***	*	**

TKW: thousand kernel weight; TW: test weight; TCWBPs: total cell wall-bound phenolics. AC: antioxidant capacity determined by means of the QUENCHER-DPPH radical scavenging assay.

[1]SEM: Standard Error of the Mean.

The results are expressed on a dw basis. Means followed by different letters are significantly different, according to the REGW-Q test (* P (F)≤0.05; ** P (F)≤0.01; ***P (F)≤0.001; the ANOVA level of significance is shown in Table 1).

Table 3. Cumulative rainfall, rainy days and GDDs (growing degree days) measured in the experimental trials from the sowing to physiological maturity.

Experimental trial	Period	Cumulative rainfall (mm)	GDDs[a]	Experimental trial	Cumulative rainfall (mm)	GDDs
Early sowing, Buriasco, 2014	Sowing - Silking stage	299	750	Late sowing, Buriasco, 2014	315	743
	Silking stage- Dough stage	150	343		50	345
	Dough stage - Physiological maturity	19	287		121	190
	Sowing - Physiological maturity	468	1380		555	1278
Early sowing, Chivasso, 2015	Sowing - Silking stage	211	679	Late sowing, Chivasso, 2015	122	767
	Silking stage- Dough stage	4	424		109	416
	Dough stage - Physiological maturity	105	301		58	235
	Sowing - Physiological maturity	320	1404		289	1418

[a] Accumulated growing degree days for each month using a 10°C base. Source: Rete Agrometeorologica del Piemonte - Regione Piemonte - Assessorato Agricoltura - Settore Fitosanitario, sezione di Agrometeorologia.

Table S1. 95% confidence interval for the means of bioactive compounds of the corn genotypes analyzed.

Genotype	TCWBPs mg FAE/kg	p-Coumaric acid mg/kg	Ferulic acid mg/kg	Sinapic acid mg/kg	Lutein mg/kg	Zeaxanthin mg/kg	β-cryptoxanthin mg/kg	β-carotene mg/kg
Rostrato vinato	5515.6-6377.2	262.2-333.5	1541.1-1852.0	20.9-25.8	4.93-6.57	7.57-10.07	1.52-2.31	0.68-0.90
Ottofile rosso	4194.7-5156.1	228.0-269.2	1327.7-1575.1	29.1-33.6	4.81-5.80	15.13-21.09	2.00-2.57	1.14-1.56
Pignoletto rosso	4648.1-5180.2	216.4-252.1	1739.9-1925.9	20.1-22.6	6.85-8.73	15.23-17.55	2.69-3.26	1.49-1.83
Pignoletto giallo	4481.6-4903.7	190.2-220.6	1997.6-2150.2	18.9-22.5	8.44-9.57	19.76-23.94	2.58-3.06	2.68-3.34
Ostenga	4350.2-5156.3	129.8-160.3	1985.4-2322.9	21.1-25.4	-	-	-	-
Indigo blue	3939.6-4270.8	105.4-128.6	1642.5-1822.5	30.4-32.7	0.48-0.99	0.56-1.07	-	-
P1208	4107.0-4741.7	163.1-191.0	1664.9-1842.4	23.2-26.9	7.39-8.97	13.25-14.50	1.25-1.69	0.48-0.67
SNH48.02	3937.8-4410.7	122.6-138.3	1649.0-1852.6	17.1-19.9	10.54-13.64	7.88-9.09	0.42-0.68	0.37-0.52
DKC6815	5051.4-5595.9	184.0-209.0	2219.6-2526.1	24.9-27.8	7.39-9.34	6.27-7.57	0.43-0.65	0.42-0.59
PR32B10	4128.1-4846.6	134.5-163.9	1989.8-2362.6	16.3-20.5	-	-	-	-

FIGURES

Figure 1. Average daily temperatures measured from the silking stage to harvest maturity.
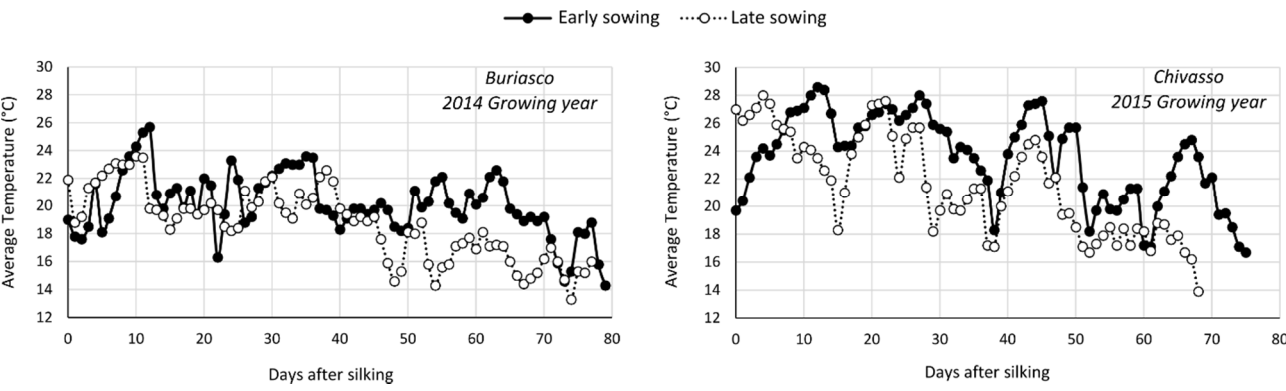
Figure 2. Effect of sowing time on total cell wall-bound phenolics (TCWBPs), *p*-coumaric acid, lutein and β-cryptoxanthin contents in the two different environments.
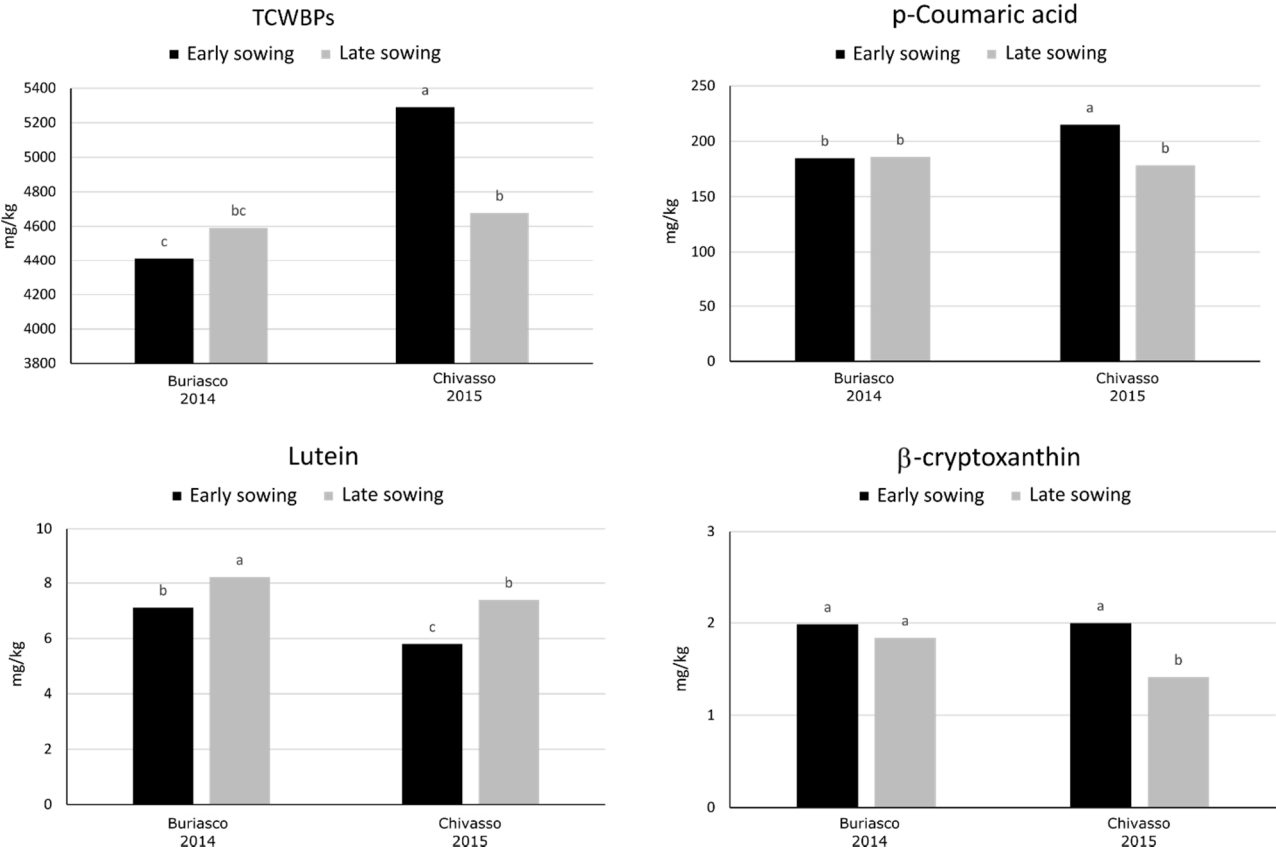
The results are expressed on a dw basis (n=30; 10 genotypes x 3 plots). Bars overlooked by different letters are significantly different, according to the REGW-Q test.

Figure 3. Total cell wall-bound phenolics (TCWBPs), ferulic, *p*-coumaric and sinapic acid

contents of the mature grains of the corn genotypes early- and late-sown.
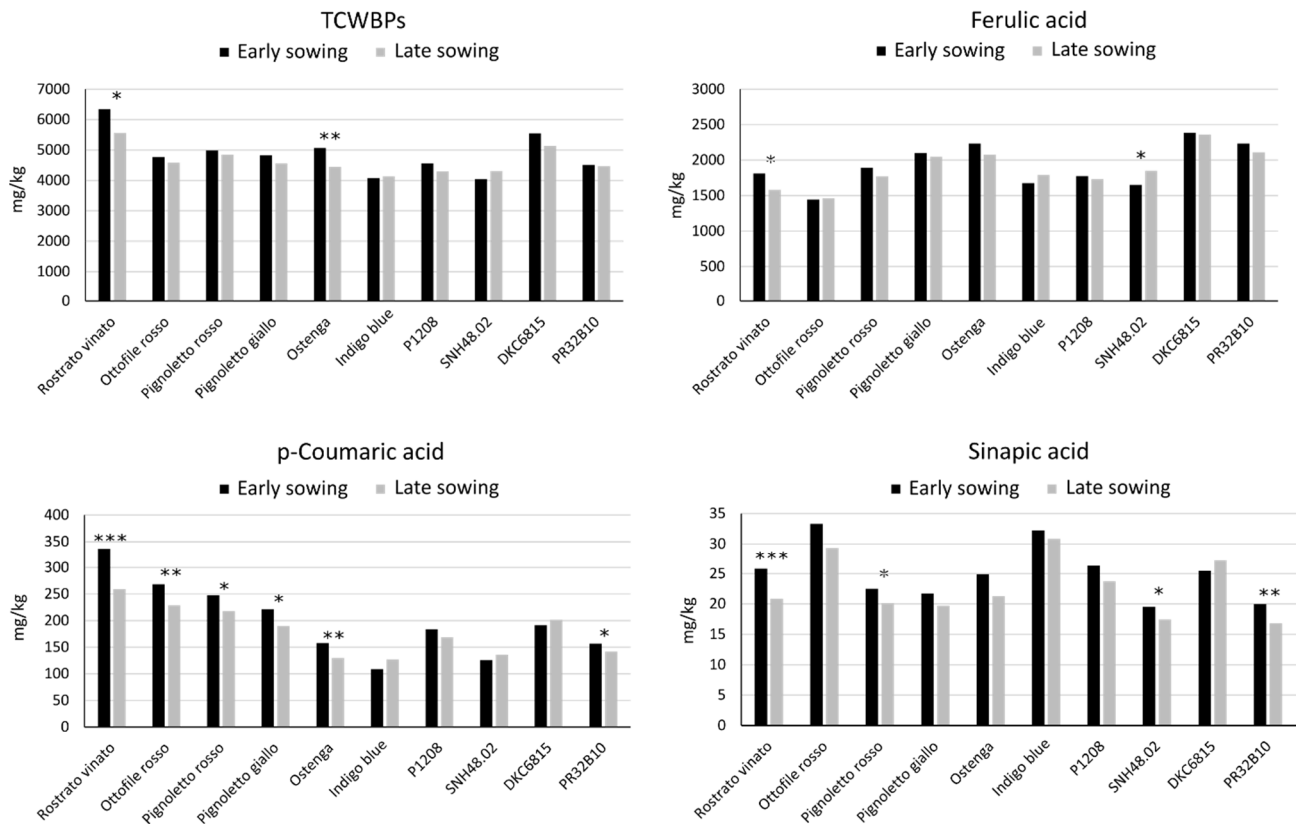
The results are expressed on a dw basis (n=6; 2 environments x 3 plots). Bars within a corn genotypes overlooked by asterisks are significantly different, according to the REGW-Q test (* P (F)≤0.05; ** P (F)≤0.01; ***P (F)≤0.001).

Figure 4. Lutein, zeaxanthin, β-cryptoxanthin and β-carotene contents of the mature grains of the corn genotypes early- and late-sown.
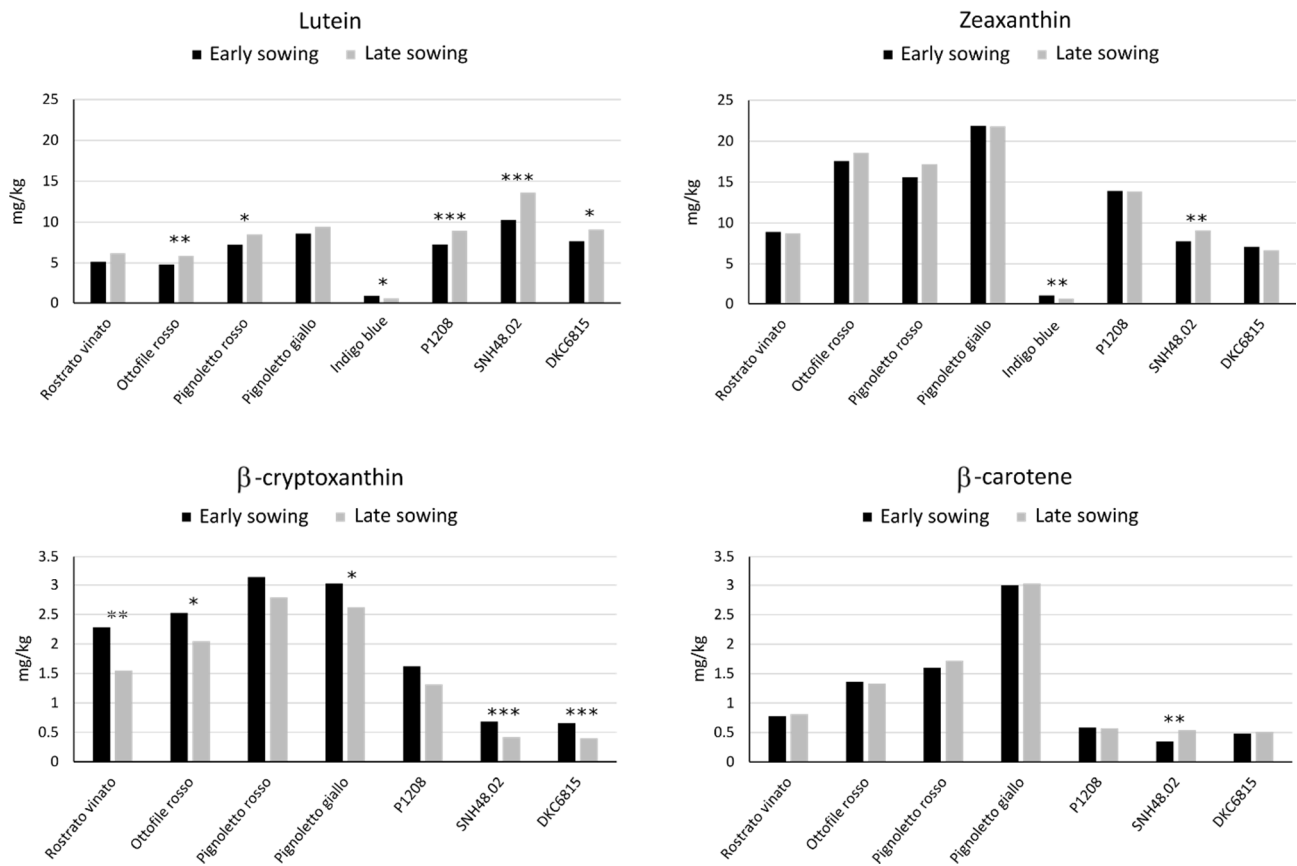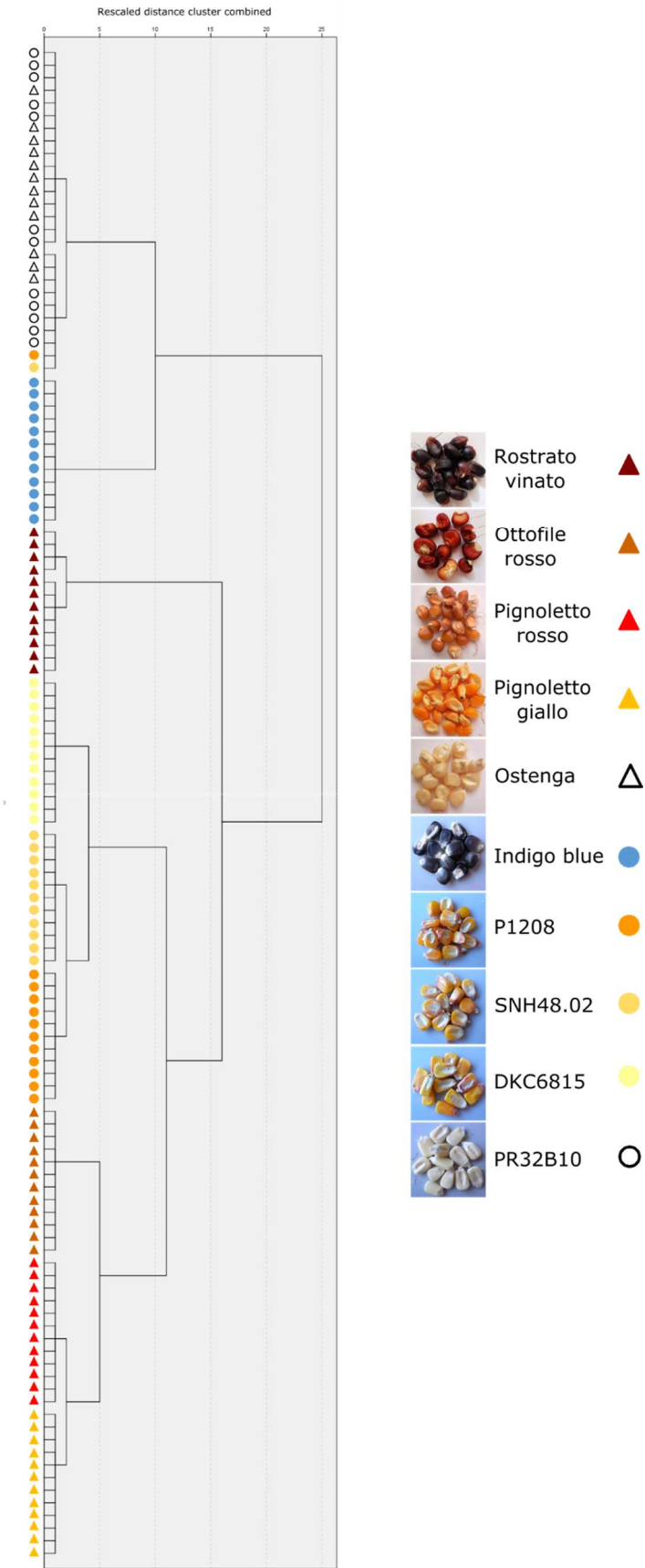

The results are expressed on a dw basis (n=6; 2 environments x 3 biological plots). Bars within a corn genotypes overlooked by asterisks are significantly different, according to the REGW-Q test (* P (F)≤0.05; ** P (F)≤0.01; ***P (F)≤0.001).

Figure 5. Principal component analysis scores plot of corn samples analyzed (n=120). The table in the right side shows the loadings of the variables.
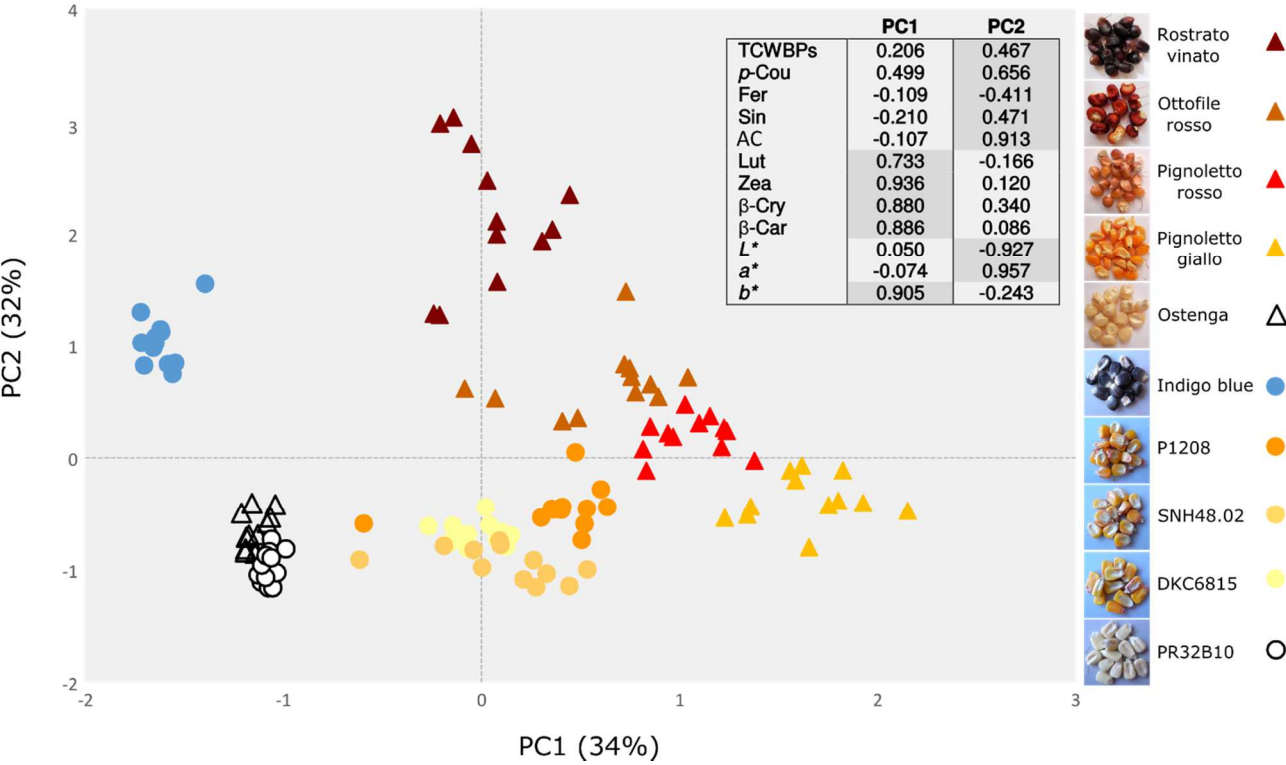
In the Figure the triangles represent open-pollinated varieties while the circles represent the hybrids. TCWBPs: total cell wall-bound phenolics; *p*-Cou: *p*-coumaric acid; Fer: ferulic acid; Sin: sinapic acid; AC: antioxidant capacity determined by means of the QUENCHER-DPPH· scavenging assay; Lut: lutein; Zea: zeaxanthin; β-cry: β-cryptoxanthin; β-car: β-carotene; *L**: lightness; *a**: red-green index; *b**: yellow-blue index.

Figure S1. Hierarchical cluster analysis dendrogram (Ward's method) based on chemical composition and color of corn samples analyzed (n=120).

In the Figure the triangles represent open-pollinated varieties while the circles represent the hybrids.