Influence of agricultural management on phytochemicals of colored corn genotypes (Zea mays L.). Part 1: Nitrogen fertilization

Influence of agricultural management on phytochemicals of colored corn genotypes (*Zea mays* L.) – Part I: Nitrogen fertilization

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ABSTRACT

In this study, the influence of nitrogen (N) fertilization (170 vs 300 kg N/ha) on the content of bioactive compounds of whole-meal flour of 10 different colored corn genotypes was investigated. Considerable differences in antioxidant capacity and phytochemical concentrations were observed among genotypes. Higher N fertilization rates significantly ($P<0.05$) increased the content of both total cell-wall bound phenolics and xanthophylls (lutein and zeaxanthin). Nevertheless, the main phenolic acids (ferulic, *p*-coumaric and sinapic acids), as well as the antioxidant capacity and the content of β-cryptoxanthin, β-carotene and total anthocyanins did not show significant differences as far as the N fertilization rate is concerned. For corn cultivation, the application of high N fertilization rates, generally carried out in order to obtain higher grain yields, could positively influence the content of some bioactives particularly in years characterized by high rainfall levels responsible of a N leaching from the soil.

KEYWORDS

Zea mays, Colored corn, Nitrogen fertilization, Phenolic acids, Carotenoids, Anthocyanins.

INTRODUCTION

Corn (*Zea mays* L.) is one of the major cereal grains cultivated all over the world, accounting for part of the staple diet of millions of people in Latin America, Asia, and Africa. Corn can be utilized directly for human food; however, it can also be widely processed into various types of products, such as flour, cornmeal, grits, starch, snacks, tortillas, and breakfast cereals, and it is also used for animal feed. In the last few years, the bioactive compounds derived from corn and their health properties have become one of the major focus of studies on this cereal crop, because of the increasing attention drawn to the development of functional foods.[1] In fact, beyond being a pivotal source of micro- and macronutrients, corn is an important source of various phytochemicals,[2] such as carotenoids,[3,4] and phenolic compounds[5] that have shown numerous beneficial effects.[6,7]

The most commonly consumed corn types in developed and developing countries are the yellow- and white-grained ones, respectively.[2] Nevertheless, the nutritional value of red-, purple-, blue- and black-grained corn genotypes has drawn the attention of researches in the last few years.[8-11] At present, these corn types are produced only in small amounts for making specialty foods, but growing interest has recently been shown in the genetic development of novel colored genotypes.[12] In fact, these unconventional genotypes might be important sources of biologically active phytochemicals, and as a result, they could be valuable raw material to produce functional foods.

Previous studies showed that the agronomic practices such as nitrogen (N) fertilization and irrigation could influence grain quality in terms of both physical and nutritional characteristics (protein content, amino acid balance, etc.).[13] These practices may have an influence also on the phytochemical concentrations of the grain, as observed in cereal crops other than corn.[14-17] Nevertheless, at present no studies investigating the influence of the agricultural management (i.e. N fertilization rate) on the phytochemical concentration of whole-meal corn

flour have been published, and the distinctive characteristics of corn advise against straightforward transfer of results. Therefore, the aim of the present study was to evaluate the content of bioactive compounds (cell wall-bound phenolic acids, carotenoids and anthocyanins) as well as the antioxidant capacity of corn genotypes grown adopting different N fertilization rates (170 kg N/ha and 300 kg N/ha) taking into account also the grain yield and the main physical kernel traits. Field trials were performed on both open-pollinated varieties and hybrids of corn characterized by kernels with different color, size and hardness. Moreover, the experiments were performed over a two-year period to evaluate also the influence of the growing season.

MATERIALS AND METHODS

Experimental design

The effect of the N fertilization was evaluated by means of a two-year experimental design. Five open-pollinated varieties (Italian landraces) and five hybrids of corn (Figure 1), characterized by a wide array of kernel traits (Table 1), were sown in North West Italy (Chivasso, Piedmont; 45°12'42''N, 7°55'96''E) in a completely randomized block design with three replications. 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). The sowing was carried out on 7 April 2014 and 3 April 2015 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. 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 physiological maturity (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 ear injuries caused by the activity of *Ostrinia nubilalis* Hübner. Two N fertilization treatments were compared: low N fertilization (170 kg N/ha) and high N fertilization (300 kg N/ha). The N fertilization was performed at the end of the leaf development stage (GS 19) with urea (granular, 46%). Daily temperatures and precipitations were measured by using a meteorological station located near the experimental area.

At harvest maturity, ears were collected by hand from 4.5 m^2 of each plot to quantify grain yield and to obtain a representative sample. The harvesting was performed on 20 September 2014 and 7 September 2015, respectively. Ears were shelled with an electric sheller and kernels from each plot were mixed thoroughly to obtain a random distribution. Each sample was then split in two subsamples before the analyses. The 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, the 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) was determined on three 100-kernel sets of each sample, using an electronic balance. Test weight (TW) was determined by means of a Dickey-John GAC2000 grain analyses meter (Dickey-John Corp., Auburn, IL), using the supplied program, after validation with reference materials.

Chemical analyses

Chemicals

Dichloromethane (CHROMASOLV®, ≥99.9%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-methylphenol (BHT, ≥99.0%), ethanol (CHROMASOLV®, 99.8%), ethyl acetate (CHROMASOLV®, 99.8%), iron(III) chloride ($FeCl_3$, >97%), 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%), *tert*-butyl methyl ether (CHROMASOLV®, 99.9%), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, ≥99%) and phenolic acid standards (*p*-coumaric acid ≥98%, sinapic acid ≥98% and *t*-ferulic acid ≥99%) were purchased from Sigma-Aldrich (St. Louis, Missouri, US). Carotenoid standards (β-carotene

≥98%, β-cryptoxanthin ≥97%, lutein ≥95% and zeaxanthin ≥98%) and cyanidin 3-*O*-glucoside chloride were purchased from Extrasynthese (Lyon, France).

Extraction of cell-wall bound phenolics

The extraction of cell-wall bound phenolics was performed as reported by Urias-Peraldì et al.[11] with few modifications. Each sample (100 mg) was mixed with 1 mL of 80:20 (v/v) methanol water mixture and sonicated for 10 min in an ultrasonic bath (35 kHz, Sonorex Super RK 156 BH, Bandelin Electronic, Berlin, Germany) while maintaining the temperature at 4°C. Samples were then mixed for 10 min and centrifuged at 10600 *g* for 5 min at 4°C. The supernatant was discarded, and a second extraction was carried out. The pellet remaining after the extraction was hydrolyzed in a hot water bath set at 80°C for 30 min by adding 400 µL NaOH 6M. After acidification to pH 2 with HCl 12 M, 800 µL of hexane were added to remove lipids. After discarding the supernatant, the cell-wall bound phenolics were extracted with 300 µL of ethyl acetate. Samples were vortex-mixed and centrifuged at 10600 *g* for 5 min at 4°C. The supernatant was transferred into a new microcentrifuge tube, and the extraction was repeated three times. The combined supernatants were reduced to dryness under a nitrogen stream, and then reconstituted in 200 µL of 80:20 (v/v) methanol water mixture.

Spectrophotometric determination of total cell wall-bound phenolics

The content of total cell wall-bound phenolics (TCWBPs) was determined by means of the Folin-Ciocalteu method as reported in Giordano et al.[18] The results were expressed as mg of ferulic acid equivalents (FAE)/kg of sample on a dry weight (dw) basis through a calibration curve (linearity range: 0.08-0.8 mg/mL; r^2: 0.999).

Quantification of cell wall-bound phenolic acids by means of RP-HPLC/DAD

The extracts of cell wall-bound phenolics were filtered through a 0.2 µm filter and then analyzed by means of a high performance liquid chromatograph Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, US) 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); the column temperature was set at 35°C. The mobile phase consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B). The following operating linear gradient was used[19]: 0-11 min, 9-14% B; 11-14 min, 14-15% B; 14-17 min, 15% B; 17-24 min, 15-16.5% B; 24-28 min, 16.5-19 % B; 28-30 min, 19-25% B; 30-36 min, 25-26% B; 36-38 min, 26-28% B; 38-41 min, 28-35% B; 41-46 min, 35-40% B; 46-48 min, 40-48% B; 48-53 min, 48-53% B; 53-70 min, 53-70% B. Finally, the mobile phase was brought to 9% B in 5 min, and this was followed by 10 min of equilibration. The flow rate of the mobile phase was 0.9 mL/min, and the injection volume was 10 µL. Phenolic acids were identified using the retention times and the UV/Vis spectra of their respective standards. Phenolic acid standards were also prepared and diluted to different concentrations to obtain calibration curves for quantification purposes (linearity range: 0.25-500 µg/mL). The quantifications were performed at maximum absorption wavelengths of phenolic acids.

Determination of the antioxidant capacity

The antioxidant capacity was determined by means of the QUENCHER method (direct measurement on solid samples[20]) employing both the DPPH and the FRAP assays.

The DPPH· scavenging capacity was determined as previously described in Giordano et al.[21] Whole-meal corn flour was mixed with 700 µL of water and 700 µL of a DPPH methanolic solution (100 µM). The reaction was carried out at 20°C in the dark with shaking at 1000 rpm (PCMT Thermoshaker, Grant Instruments, Cambrige, UK) for 25 min. The samples were promptly centrifuged for 1 min at 20800 *g*, and the absorbance was measured at 515 nm after exactly 30 min from the initial start of the reaction. A control, without the ground sample, was tested under the same conditions and used to calculate the DPPH· inhibition percentage of the samples. The final results were expressed as mmol of Trolox equivalents (TE)/kg of sample dw using a calibration curve (linearity range 2-20 µM; r^2: 0.999).

The ferric reducing antioxidant power was determined as described by Serpen et al.[22] Briefly, FRAP working solution was prepared by mixing 10 mM TPTZ and 20 mM $FeCl_3$ in 300 mM sodium acetate buffer (pH 3.6) at a ratio of 1:1:10 (v:v:v). Two mg of whole-meal corn flour (preliminary diluted if necessary) was mixed with 2 mL of FRAP working solution. The reaction was carried out in the dark under stirring at 20°C and 1000 rpm for 2 hours. The samples were then centrifuged for 1 min at 20800 *g*, and the absorbance was measured at 593 nm. The results were expressed as mmol of Trolox equivalents (TE)/ kg of sample dw using a calibration curve (linearity range 0.8-31 µM; r^2: 0.995).

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

The extraction of carotenoids was performed as reported by Burt et al.[23] with few modifications. Briefly, each sample (0.3 g) was extracted for 6 min at 85°C with 95% ethanol, containing 1 g/L BHT. The extracts, including solids, were hydrolyzed with 125 µL of KOH (80% w/v) at 85°C for 10 min, chilled on ice and 3 mL of cold deionized water was then added. This was followed by the addition of 3 mL of hexane, containing 1 g/L BHT. The test

tubes were then vortex-mixed and centrifuged at 1200 *g* for 10 min. The extraction was repeated four times, and the combined supernatants were evaporated to dryness under a nitrogen stream, and then reconstituted in 200 µL of dichloromethane.

The extracts were analyzed using the previously described chromatographic system. Separations were carried out using a 250 x 4.6 mm, 3 µm, C30 carotenoid YMC column (YMC Co., Kyoto, Japan); the column temperature was set at 25°C. The mobile phase consisted of methanol:*tert*-butyl methyl ether:water [81:15:4, v/v; (solvent A)] and *tert*-butyl methyl ether:methanol [91:9, v/v; (solvent B)]. The following operating linear gradient was used[24]: 0-45 min, 0-50% B; 45-60 min, 50-100% B. Finally, the mobile phase was brought to 0% B in 10 min, and this was followed by 15 min of equilibration. The flow rate of the mobile phase was 1.0 mL/min, and the injection volume was 5 µL. Carotenoids were identified using the retention times and the UV/Vis spectra of their respective standards. Carotenoid standards were also prepared and diluted to different concentrations to obtain calibration curves for quantification purposes (linearity range: 0.1-100 µg/mL for lutein and zeaxanthin; 0.3-40 µg/mL for β-cryptoxanthin and β-carotene). The quantifications were performed at 450 nm.

Extraction of anthocyanins and determination of the total anthocyanin content by means of the pH differential method

The total anthocyanin content (TAC) was determined only for the red- and blue-grained corn samples Rostrato vinato, Ottofile rosso, Pignoletto rosso and Indigo blue. Each sample (1g) was extracted using 8 mL of ethanol acidified with HCl 1 M (85:15, v/v). The pH of the mixture was adjusted to 1. The mixture was shaken for 30 min and centrifuged at 20800 *g* for 10 min. The supernatant was then used for the determination of total anthocyanin content by means of the pH differential method.[25] Samples were diluted ten times with potassium

chloride buffer (0.03 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5) to a final volume of 2 mL, respectively. Results were expressed as mg of cyanidin 3-*O*-glucoside equivalents (Cy 3-glc eq)/kg of sample dw.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) in order to evaluate the effect of the genotype, the N fertilization and the growing year on contents of bioactive compounds of whole-meal corn flour 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.

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-way ANOVA (Table 2) showed significant effects of all the three main factors (genotype, N fertilization and growing year) on yield, TKW and TW. The broad genotype diversity accounted for more than 75% of the variation, in accordance with the high multiplicity of corn genotypes analyzed in the present study. As expected, the yield of the hybrids was higher than that of the open-pollinated varieties (13.8 vs 5.9 t/ha – Table 3), because of their higher plant density, their vigor and the phenomenon of heterosis. A great variation was observed in the TKW of open-pollinated varieties (from 240.2 to 417.9 g), while hybrids gave more uniform results for this parameter (from 325.9 to 385.0 g). Genotypes characterized by high hardness, stated as total milling energy, showed a higher TW, while the lowest TW value (74.7 kg/hL) was observed in the DKC6815 hybrid, characterized by the lowest total milling energy.

The second most important factor affecting yield and grain physical parameters was the growing year. Significantly higher yield (+14%), TKW (+5%) and TW (+1%) were observed in 2014 than in 2015. The results are in accordance with the longer ripening period observed in 2014, characterized during the growth stages by higher rainfall levels and lower daily temperatures than the 2015 growing year (Figure 2). In particular, in 2014 the average daily temperature and the rainfall level were 1°C lower and 277 mm higher than 2015, respectively.

In agreement with previous studies,[26-28] the N fertilization rate significantly affected both yield and TKW (Table 2). The application of 300 kg N/ha resulted in an increase of yield and TKW of about 8% and 3%, respectively.

Cell wall-bound phenolic acids and antioxidant capacity

Phenolics exist in free and bound forms, but in cereal grains and particularly in corn grains about 85% of the total amount of phenolics are in the cell wall-bound form.[5] A previous experiment performed on some of the genotypes analyzed in the present study[18] showed that at harvest maturity cell wall-bound phenolic acids were on average 13 folds higher than free phenolic acids, and that at this stage of kernel development ferulic acid was the main phenolic acid, followed by *p*-coumaric acid. Moreover, the slow and continuous release into the bloodstream of phenolics coming from the dietary fiber is likely one of the main mechanisms of the health benefits associated with the regular consumption of whole-grain cereals.[29] For these reasons, in the present study the analysis of the phenolic component of corn grain was focused on cell wall-bound phenolics.

The three-way ANOVA (Table 2) showed significant effects of all the three sources of variations (genotype, N fertilization and growing year) and of the interaction N fertilization x genotype and N fertilization x growing year on total cell wall-bound phenolic content. The genotype and the growing year explained most of the variation observed, 46 and 25%, respectively. TCWBP content ranged between 4166 and 6217 mg FAE/kg dw. The highest concentration of TCWBPs was observed in the dark red variety Rostrato vinato while Indigo blue hybrid had the lowest. The HPLC-DAD analysis of phenolic acids revealed that ferulic acid was the most abundant phenolic acid followed by *p*-coumaric and sinapic acid. Either way, a characteristic profile was observed depending on the genotype considered.

A strong influence of the year on the content of phenolic acids was also reported in previous studies for wheat, emmer and einkorn.[15,16,30,31] In the present study, TCWBPs were higher in 2015 (+18%) than in 2014 (Table 3). Similarly, *p*-coumaric, ferulic and sinapic acid were all significantly influenced by the growing year and resulted in higher levels in 2015 (+17%, +13% and +19% respectively) than in 2014.

N fertilization influenced significantly only the TCWBP content. In particular, an increase of 4% was observed in grains obtained from plots treated with 300 kg N/ha (Table 3). The higher content of TCWBPs associated with higher N fertilization rate was especially noticeable in the 2014 growing year (+13%), while no significant difference was observed in 2015 (Figure 3). This observation could be related to the higher N leaching that probably occurred in 2014 because of the higher rainfall level observed from April to July, which decreased N content of the soil, intensifying differences between experimental theses. Nevertheless, no significant difference was observed for ferulic, *p*-coumaric or sinapic acid between the two N fertilization rates. As far as single growing year is concerned, ferulic acid did not show any significant difference in the 2014 growing year at the two N fertilization rates, while a significant decrease of 5% was detected in 2015 in plots treated with 300 kg N/ha (Figure 3).

In accordance with the antioxidant activity related to phenolic compounds,[5] the genotype explained also most of the variation observed in the antioxidant capacity of the whole-meal corn flours (96% and 90% employing the DPPH and the FRAP assay, respectively). The red/blue-grained genotypes showed the highest $DPPH^{\cdot}$ radical scavenging activity (mean value: 20.4 mmol TE/kg dw) and ferric reducing antioxidant power (mean value: 18.1 mmol TE/kg dw), while the yellow/white-grained genotypes showed the lowest antioxidant capacity (mean values: 9.3 mmol TE/kg dw - DPPH assay, 11.7 mmol TE/kg dw - FRAP assay) and did not differ significantly among each other (Table 3). The N fertilization did not influence significantly the antioxidant capacity, while the growing year influenced significantly this parameter, however results differed depending on which type of assay was considered.

Results showed that N fertilization could positively influence the content of phenolic compounds of corn grain. Some authors postulated that plants change their metabolisms toward carbon-containing compounds, such as phenolic acids and terpenoids, when N

availability is limited for growth.[32] However, when N is readily available, plants will primarily form compounds with high N content, like proteins and N-containing secondary metabolites. Nevertheless, the accumulation of N differs between plant organs: in vegetative organs, such as leaves and roots, the N accumulation is higher than in reproductive ones like fruits and seeds.[33] Previous studies showed that high N fertilization rates significantly reduce phenolic compound content in wheat leaves.[34,35] On the contrary, N fertilization induce a little decrease[36] or no differences in the phenolic content of wheat grain.[15] Moreover, Kesarwani et al.[37] did not observe significant differences in the phenolic content of rice grains under different agronomic practices, while Hidalgo and Brandolini[17] observed an increase in the total conjugated and total bound phenolic acids of einkorn in response to N addition. The significant increase in the TCWBPs without an analogous increase in the three main cell wall-bound phenolic acids observed in the present study could be related to an increase in other phenolic compounds such as ferulic acid dehydrodimers generally detected in the bound fraction of corn grains.[18,38,39]

Carotenoids

The three-way ANOVA (Table 2) showed significant effects of all the three main factors and of the interaction of N fertilization x growing year on both lutein and zeaxanthin.

Genotype accounted for most of the variation observed for xanthophylls (>78%). As shown in Table 3, zeaxanthin was the most abundant xanthophyll detected in all corn genotypes, representing 51-65% of total xanthophylls, while lutein accounted only for 20-33% and β-cryptoxanthin for <15%. The only exceptions were the hybrids DKC6815 and SNH48.02, which showed higher concentrations of lutein (on average 50% of total xanthophylls) than zeaxanthin (44%) and β-cryptoxanthin (4%). β-carotene was lower than 3 mg/kg dw in all the genotypes and higher concentrations were observed in open-pollinated varieties than in

hybrids (1.55 vs 0.49 mg/kg dw). Kurilich and Juvik[3] reported that lutein and zeaxanthin made up 57 and 21% of the carotenoids in 44 dent and sweet corn varieties, while β-carotene and β-cryptoxanthin accounted for 6 and 5% of total carotenoids, respectively. More recently, the analyses of 22 landraces, with large variations in grain color, hardness and shape[40] showed that depending on the genotype, the main xanthophyll could be either lutein or zeaxanthin. In the present study, generally zeaxanthin was the main xanthophyll in genotypes characterized by high hardness, while lutein was the most abundant in the low hardness hybrid DKC6815, in accordance with previous studies which showed that horny genotypes generally have the highest zeaxanthin levels, whereas floury genotypes have more lutein.[2,41] The white genotypes showed very low concentration of carotenoids in agreement with previous studies.[4] Similarly, Indigo blue showed a concentration of β-cryptoxanthin and β-carotene lower than 0.3 mg/kg dw, while lutein and zeaxanthin content was lower than 1 mg/kg dw. In fact, white-grained inbreds are generally preferred in the conversion of elite field corn lines into blue corn because a yellow endosperm could cause a greenish yellow off-color.[12]

The second most important factor affecting carotenoid content was the growing year, that influenced significantly the content of lutein, zeaxanthin and β-cryptoxanthin. The lowest concentration of both lutein and zeaxanthin was observed in the 2015 growing year (Table 3). In contrast, β-cryptoxanthin showed an opposite trend. Little data have been published about the effects of environmental conditions on carotenoids in cereals. Previous studies showed that the content of lutein in einkorn varied significantly over the years.[42] In particular, the highest values have been recorded in wetter and cooler years.[17,42] Thus, the higher carotenoid concentrations observed in corn samples of the 2014 growing year could be related to the higher precipitation levels and lower temperatures that occurred during the grain growth stages.

The concentration of the most abundant carotenoids was also significantly modified by the different N rates tested. Higher N doses increased the content of both lutein and zeaxanthin by about 10% (Table 3). As for TCWBPs, the effect of N fertilization was especially accentuated in the 2014 growing year (21% increase of lutein and 17% increase of zeaxanthin), while a non-significant increase of 4% was observed for both xanthophylls in the 2015 growing year (Figure 3). β-cryptoxanthin and β-carotene were not significantly affected by increasing levels of N (Table 2) even though an increase of 6% and 13% was observed in plots fertilized with 300 kg N/ha, respectively (Table 3). Results are in accordance with previous studies performed on other crops. In particular, numerous reports show that an increased supply of N increases the concentration of carotenoids in a wide range of fruits and vegetables.[43] As far as cereal crops are concerned, different results have been obtained; for example, Abad et al.[14] observed that carotenoids of durum wheat increased on average by 11% when N increased from 0 to 200 kg/ha. N fertilization improved carotenoid content also in three durum wheat varieties tested in the North of Tunisia.[44] Other studies did not observe any correlation between N and carotenoid content of the grains.[45]

Total anthocyanin content

Indigo Blue, a blue corn hybrid developed and commercialized in the US, was characterized on average by a TAC of 740 mg Cy 3-glc eq/kg dw. The TAC of the whole-meal flour of the blue hybrid analyzed in the present study, characterized by a pH of 6.4, resulted in higher levels than previously reported for other blue corn types.[8,10,11] The red-grained genotypes (Rostrato vinato, Ottofile rosso and Pignoletto rosso) showed on average a TAC lower than 15 mg Cy 3-glc eq/kg dw. The result was in accordance with previous studies which showed that the reddish color of the kernels of these corn types is mainly ascribed to phlobaphenes

instead of monomeric anthocyanins.[46] As shown in Figure S1, the blue/red pigmentation of all these corn genotypes was located only in the outer layers of the kernels.

Even if the N fertilization did not significantly influence (P=0.061) the TAC of the Indigo Blue hybrid, grains obtained from plots fertilized with 170 kg N/ha were characterized by a higher TAC than plots fed with 300 kg N/ha (+11%). Neither the growing year (P=0.834) nor the interaction of N fertilization x growing year (P=0.198) influenced significantly the TAC.

In summary, the present study highlights that N fertilization influenced the content of the main carbon-containing secondary metabolites of whole-meal flour of different colored corn genotypes, although it played a minor role compared to the genotype and the growing year. Higher concentrations of cell-wall bound phenolics, lutein and zeaxanthin were observed after applying higher N fertilization rates. Nevertheless, the concentration of the main cell wall-bound phenolic acids as well as the antioxidant capacity and the total anthocyanin content was not significantly influenced.

Results showed that an increase in corn grain yield, resulting from the application of higher N fertilization rates, did not lead to a decrease in the content of bioactive compounds of the grain. On the contrary, a low N availability due to low N fertilization rates or to high rainfall levels responsible for N depletion from the soil, negatively influenced the nutritional quality of whole-meal corn flours in terms of both cell wall-bound phenolics and xanthophylls. Therefore, taking into consideration the antioxidant content as well as the yield and grain technological quality, the cultivation of corn for food production should be preferred in fertile areas, and the application of good agronomic practices such as the N fertilization, could prove useful under these terms.

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; FRAP: ferric reducing antioxidant power; GDD: growing degree days; GS: growth stage; N: nitrogen; REGW-Q test: Ryan/Einot and Gabriel/Welsch test; TCWBPs: total cell wall-bound phenolics; TE: Trolox equivalents; TKW: thousand kernel weight; TPTZ: 2,4,6-tris(2-tripyridyl)-*s*-triazine; TW: test weight.

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TABLES

Table 1. Colored corn genotypes compared in the study and their characteristics.

Name	Type	Maturity duration length (Relative days)	Kernel color[1]	Whole-meal flour color[2]			Kernel size[3]	Total milling energy (J)[4]
				*L**	*a**	*b**		
Rostrato vinato	Open-pollinated variety	120	Dark red	66.0	7.4	16.6	Small	1422
Ottofile rosso	Open-pollinated variety	115	Red	78.0	2.4	28.2	Large	1435
Pignoletto rosso	Open-pollinated variety	120	Light red	73.6	2.0	30.9	Small	1531
Pignoletto giallo	Open-pollinated variety	120	Yellow	82.0	0.7	36.9	Small	1319
Ostenga	Open-pollinated variety	120	White	86.6	-0.3	10.6	Large	1591
Indigo Blue	Hybrid	135	Blue	67.8	5.3	0.6	Medium	1338
P1208	Hybrid	125	Yellow	83.5	-0.1	31.6	Small	1552
SNH48.02	Hybrid	132	Light yellow	84.7	-1.1	30.2	Medium	1461
DKC6815	Hybrid	130	Light yellow	86.4	-1.2	25.9	Medium	808
PR32B10	Hybrid	132	White	89.1	-0.8	9.8	Medium	1220

The open-pollinated varieties were provided by CREA-CI (Bergamo, Italy), Indigo Blue by Clarkson grain (Illinois, US), P1208 and PR32B10 by Pioneer Hi-Bred Italia (Cremona, Italy), SNH48.02 by Planta Research and Seeds (Vicenza, Italy) and DKC6815 by Monsanto Agricoltura Italia S.p.A. (Milano, Italy).

[1] Kernel color: see Figure 1.

[2] Whole meal flour color: the color of the whole meal flour was analyzed by means of a Minolta Chroma Meter reflectance spectrophotometer (Model CR-400, Minolta Co., Osaka, Japan).

[3] Kernel size: for major information see the thousand kernel weight reported in Table 3 for each genotype.

[4] Total milling energy: the total milling energy was determined using a Polimix® PX-MCF 90 D (Kinematica AG, Luzern, Switzerland) fitted with a 2 mm sieve. The laboratory mill was equipped with a computerized data-logging system to log the instantaneous electric power consumption during the milling test, as reported by Blandino et al.[47]

Table 2. P values and R^2 values of the three-way ANOVA analyses performed to evaluate the effect of the genotype, the N fertilization and the growing year 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.

	ANOVA					
	Genotype	N fertilization	Growing year	N fertilization x Genotype	N fertilization x Growing year	R^2
Variable	P (F)					
Grain yield and physical parameters						
Yield	**<0.001**	**0.005**	**<0.001**	0.471	**0.011**	0.942
TKW	**<0.001**	**0.003**	**<0.001**	0.951	0.552	0.924
TW	**<0.001**	0.750	**0.003**	0.999	0.927	0.774
Phenolic acids and antioxidant capacity						
TCWBPs	**<0.001**	**0.009**	**<0.001**	**0.005**	**<0.001**	0.822
p-Coumaric acid	**<0.001**	0.095	**<0.001**	0.084	0.295	0.900
Ferulic acid	**<0.001**	0.522	**<0.001**	0.117	**0.001**	0.821
Sinapic acid	**<0.001**	0.396	**<0.001**	0.805	0.665	0.755
$DPPH^{\cdot}$ scavenging capacity	**<0.001**	0.062	**0.001**	0.161	**0.002**	0.969
Ferric reducing antioxidant power	**<0.001**	0.256	**<0.001**	0.415	0.411	0.924
Carotenoids						
Lutein	**<0.001**	**0.001**	**<0.001**	0.144	**0.016**	0.892
Zeaxanthin	**<0.001**	**0.003**	**0.003**	0.588	**0.035**	0.918
β-cryptoxanthin	**<0.001**	0.232	**0.017**	0.581	0.579	0.848
β-carotene	**<0.001**	0.198	0.596	0.159	0.595	0.741

TKW: thousand kernel weight; TW: test weight; TCWBPs: total cell wall-bound phenolics.

Table 3. Effect of the genotype, the N fertilization rate and the growing year on grain yield, grain physical parameters, phenolic acid content, antioxidant capacity and carotenoids of whole-meal corn flour.

	Yield (t/ha)	TKW (g)	TW (kg/hL)	TCWBPs (mg FAE/kg)	*p*-Coumaric acid (mg/kg)	Ferulic acid (mg/kg)	Sinapic acid (mg/kg)	DPPH· scavenging capacity (mmol TE/kg)	Ferric reducing antioxidant power (mmol TE/kg)	Lutein (mg/kg)	Zeaxanthin (mg/kg)	β-cryptoxanthin (mg/kg)	β-carotene (mg/kg)
Genotype (n=12)													
Rostrato vinato	5.7^{ef}	304.9^{g}	80.5^{cd}	6217.3^{a}	328.7^{a}	1904.5^{de}	26.2^{b}	34.3^{a}	27.3^{a}	5.47^{c}	8.42^{d}	1.93^{c}	0.78^{c}
Ottofile rosso	4.7^{f}	401.9^{ab}	76.5^{e}	4890.4^{d}	244.1^{b}	1433.2^{f}	31.2^{a}	14.4^{c}	14.0^{c}	5.65^{c}	18.07^{a}	2.52^{b}	1.54^{b}
Pignoletto rosso	7.2^{d}	259.3^{h}	82.8^{ab}	5320.3^{bc}	255.2^{b}	2014.7^{cd}	20.5^{cd}	13.3^{c}	14.1^{c}	7.17^{b}	15.82^{b}	3.01^{a}	1.57^{b}
Pignoletto giallo	5.4^{ef}	240.2^{h}	84.6^{a}	5010.1^{cd}	216.5^{c}	2266.9^{b}	19.9^{d}	8.7^{d}	12.5^{cd}	7.47^{b}	19.14^{a}	2.64^{ab}	2.32^{a}
Ostenga	6.6^{de}	417.9^{a}	79.3^{d}	4757.8^{d}	141.7^{e}	2193.6^{bc}	22.8^{bcd}	9.2^{d}	11.7^{d}	<0.1	<0.1	<0.3	<0.3
Indigo blue	11.2^{c}	385.0^{bc}	80.7^{cd}	4166.3^{f}	109.6^{f}	1763.5^{e}	31.2^{a}	19.5^{b}	17.1^{b}	0.89^{d}	0.99^{e}	<0.3	<0.3
P1208	12.6^{b}	325.9^{f}	84.4^{a}	4664.0^{de}	192.5^{cd}	1808.5^{e}	23.6^{bc}	9.2^{d}	10.9^{d}	7.55^{b}	13.51^{c}	1.57^{c}	0.60^{c}
SNH48.02	12.6^{b}	341.9^{ef}	81.8^{bc}	4305.7^{ef}	132.7^{ef}	1863.7^{de}	19.5^{d}	9.7^{d}	11.3^{d}	9.88^{a}	7.78^{d}	0.75^{d}	0.42^{c}
DKC6815	16.7^{a}	359.2^{de}	74.7^{e}	5466.1^{b}	199.4^{c}	2553.9^{a}	25.6^{b}	10.1^{d}	12.3^{cd}	7.27^{b}	7.04^{d}	0.58^{d}	0.46^{c}
PR32B10	16.0^{a}	375.2^{cd}	82.2^{bc}	4805.9^{d}	169.7^{d}	2318.9^{b}	20.7^{cd}	8.6^{d}	11.3^{d}	<0.1	<0.1	<0.3	<0.3
SEM[1]	0.4	5.2	0.5	112.6	7.0	51.4	0.8	0.4	0.4	0.30	0.58	0.12	0.13
	***	***	***	***	***	***	***	***	***	***	***	***	***
Nitrogen fertilization (n=60)													
170 kg N/ha	9.7	336.2	80.7	4851.2	194.4	2012.6	24.3	13.4	14.5	6.04	10.84	1.80	1.03
300 kg N/ha	10.5	346.1	80.8	5062.5	202.6	2002.6	23.8	14.0	14.1	6.80	11.91	1.91	1.16
SEM	0.2	2.3	0.2	50.3	3.1	23.0	0.4	0.2	0.2	0.15	0.29	0.06	0.07
	***	**		**						***	**		
Growing year (n=60)													
2014	10.7	349.6	81.3	4553.6	183.4	1882.3	22.0	13.2	15.0	7.16	12.03	1.75	1.12
2015	9.4	332.7	80.3	5377.6	213.7	2135.0	26.1	14.2	13.6	5.68	10.76	1.97	1.07
SEM	0.2	2.3	0.2	50.3	3.1	23.0	0.4	0.2	0.2	0.15	0.29	0.06	0.07
	***	***	**	***	***	***	***	***	***	***	**	*	

TKW: thousand kernel weight; TW: test weight; TCWBPs: total cell wall-bound phenolics.

[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 2).

FIGURES

Figure 1. Colored corn genotypes analyzed: A - Rostrato vinato; B - Ottofile rosso; C - Pignoletto rosso; D - Pignoletto giallo; E - Ostenga; F - Indigo Blue; G - P1208; H - SNH48.02; I - DKC6815; J - PR32B10.

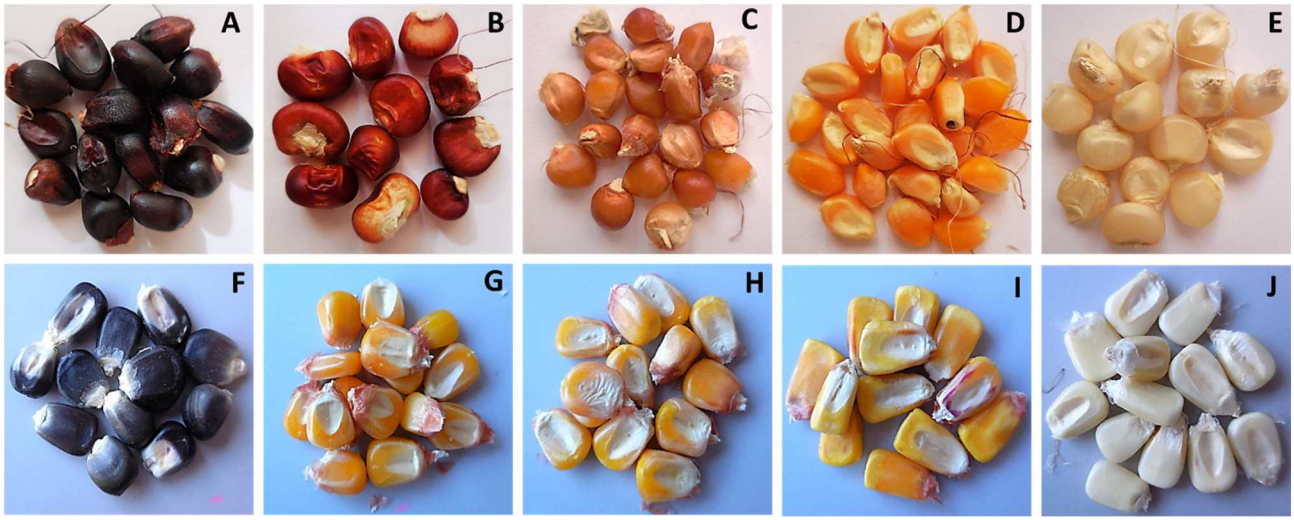


Figure 2. Cumulative rainfall, and growing degree days (GDD, using a 10°C base) measured in the experimental trials from April to September (growing year: 2014, 2015).

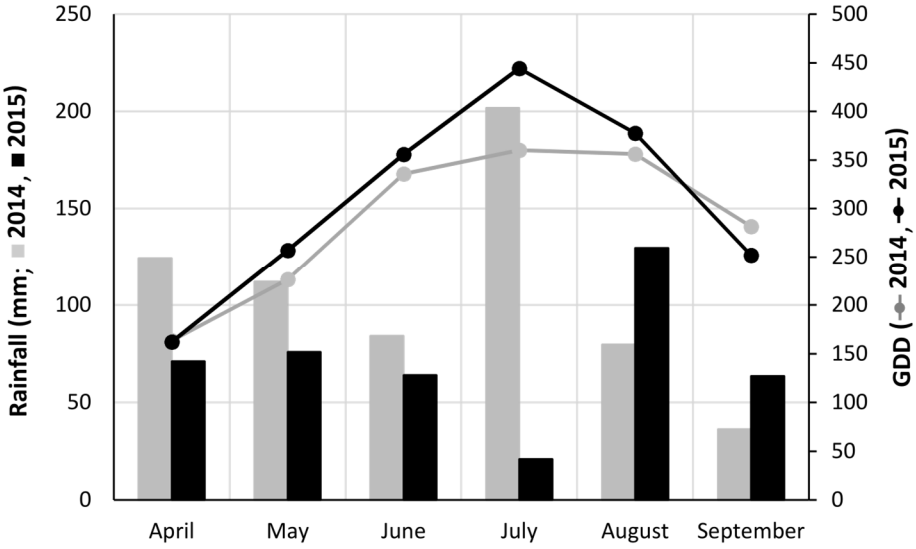
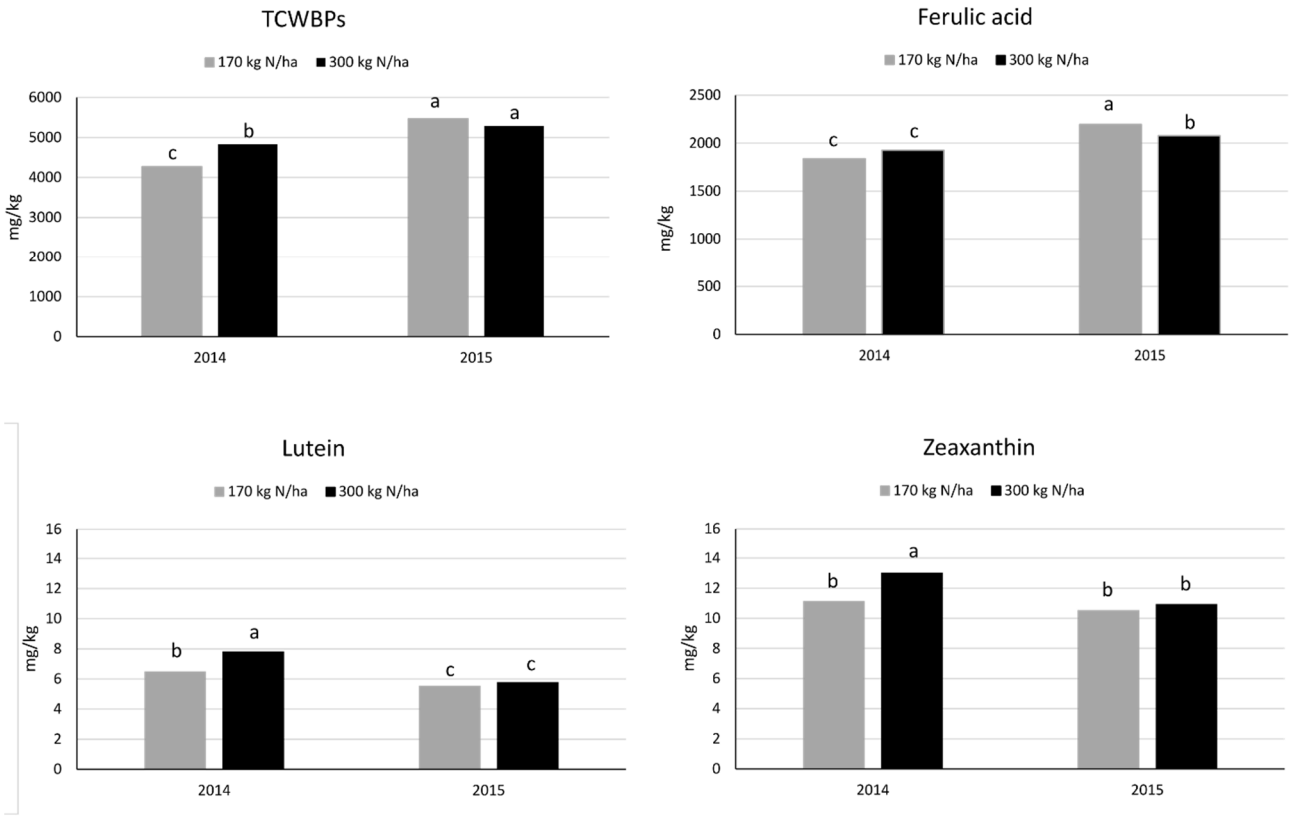
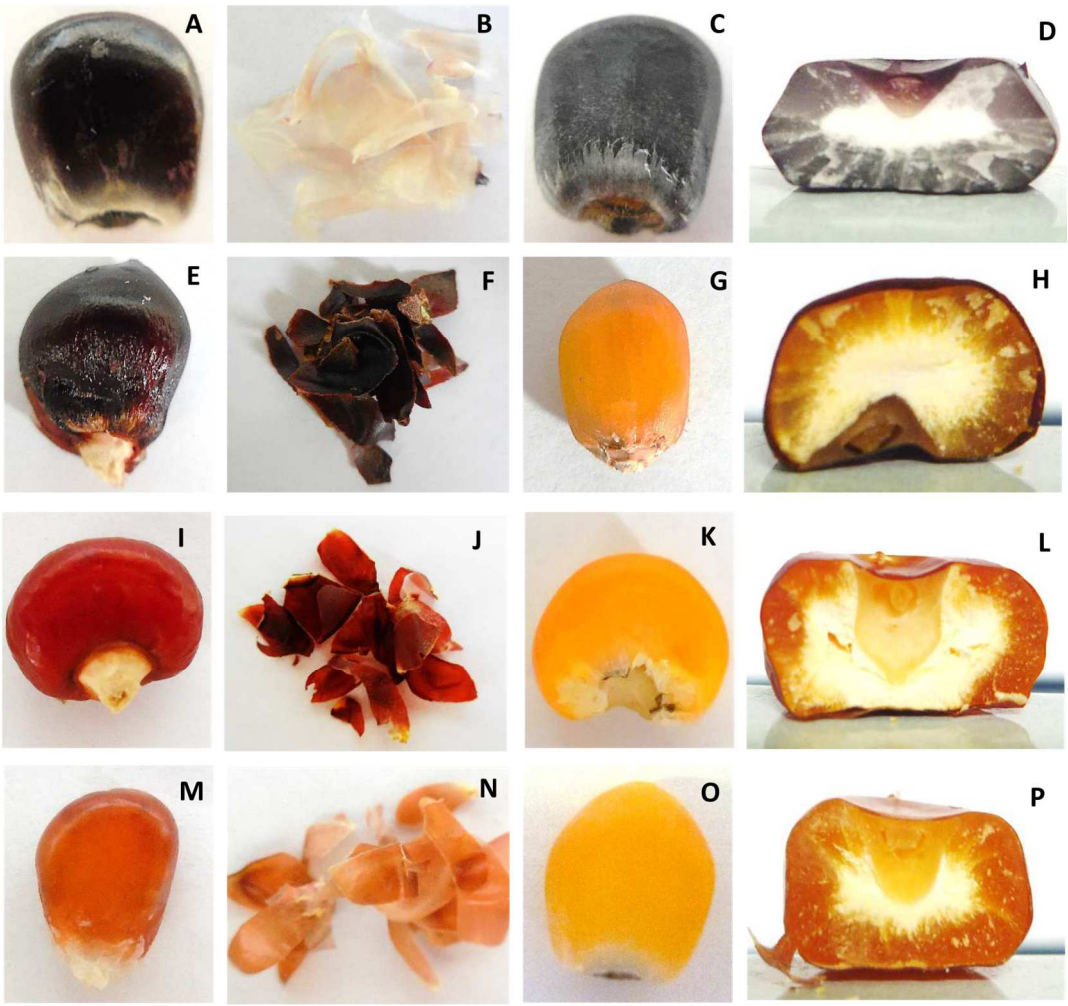


Figure 3. Effect of N fertilization on total cell wall-bound phenolics (TCWBPs), ferulic acid, lutein and zeaxanthin in the two different growing years.



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 S1. Localization of anthocyanin-type compounds in pigmented maize grains.



Starting from the first column are shown: the whole kernel, the pericarp, the kernel without the pericarp and the transverse section of the whole kernel. A-D: Indigo Blue; E-H: Rostrato vinato; I-L: Ottofile rosso; M-P: Pignoletto rosso.