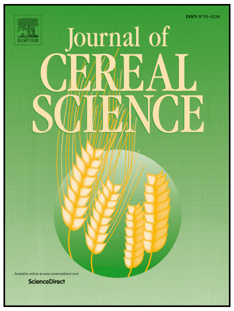

Nitrogen fertilisation effects on technological parameters and carotenoid, tocol and phenolic acid content of einkorn (*Triticum monococcum* L. subsp. *monococcum*): A two-year evaluation

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Nitrogen fertilisation effects on technological parameters and carotenoid, tocol and phenolic acid content of einkorn (*Triticum monococcum* L. subsp. *monococcum*): a two-year evaluation.

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ABSTRACT

Recent studies on einkorn wheat, an underutilised relative of durum and bread wheat, demonstrated its outstanding nutritional characteristics and fostered a renewed interest for its cultivation. Einkorn is a disease-resistant and thrifty crop, supplying flour with optimal composition even with minimal agronomic management. To understand the role of nitrogen fertilisation on its composition and nutritional quality, a two-year study comparing five different nitrogen treatments (0 kg/ha, 40 and 80 kg/ha at tillering, 40 and 80 kg/ha at heading) was performed on three einkorn accessions.

The two years had similar temperatures but very different rainfall profiles, so the climate had a strong effect on most traits, including thousand kernels weight, Falling number, viscoamylographic parameters, carotenoid and phenolic acid concentration. On the other hand, nitrogen fertilisation improved protein content, SDS sedimentation volume and phenolic acids concentration. Carotenoids synthesis was slightly limited with increasing fertilisation; a similar, but less evident, effect was present for tocols. The results demonstrate that einkorn wheat does not require abundant nitrogen fertilization to provide flour with good nutritional and technological characteristics.

Keywords: Antioxidants; Falling number; Protein; Viscosity.

1. Introduction

Einkorn wheat (*Triticum monococcum* L. ssp. *monococcum*) is a diploid wheat which has played a key role in the birth and spread of agriculture, but has since been replaced by other more productive wheats. After a long period of neglect, it has lately been re-evaluated and re-proposed as an interesting crop for modern agriculture, especially because of its outstanding nutritional characteristics (Hidalgo and Brandolini, 2014; Løje et al., 2003). Einkorn is well known for the high content of proteins (15-18%), antioxidants (carotenoids, tocols and conjugated phenolic acids), lipids (with a high percentage of unsaturated fatty acids) and microelements (Hidalgo and Brandolini, 2014). Its flour is excellent for the production of pasta and biscuits, but accessions suited for breadmaking are also available.

The renewed interest in this crop is motivated also by its low environmental impact, as even with reduced fertilisation (40-80 kg/ha vs. 180-200 kg/ha N for bread and durum wheat) gives flour with optimal composition. Nevertheless, scant information is available on the influence of agronomic management, and particularly of fertilisation, on the composition and the nutritional quality of einkorn flour. Some inferences can be drawn from studies performed on other *Triticum* (e.g. emmer, spelt, durum and bread wheats), but the distinctive characteristics of einkorn advise against a straightforward transfer of results. For example, Castagna et al. (1996), studying one einkorn line cropped with growing nitrogen doses (0, 50 and 100 kg/ha of nitrogen), recorded a significant increase in protein content and SDS sedimentation values from 0 to 50 kg/ha, but minimal changes afterwards, that is at a fertilisation level largely inferior to those normally applied to bread and durum wheats (Makowska et al., 2008; Shewry et al., 2013).

Therefore the aim of this study was to evaluate the effect of nitrogen fertilization on some technological characteristics, as well as on the content of protein, carotenoids, tocols, conjugated and bound phenolic acids of whole meal flours of einkorn. For a more precise assessment, the analysis was conducted for a two-year period, in order to embrace also the influence of the cropping year.

2. Materials and methods

2.1. Materials

Three einkorn accessions (Monlis, Monarca and SAL98-32) were cropped during the 2011-12 and 2012-13 growing seasons: the breadmaking-suitable cv. Monlis, the early-maturing advanced line Monarca and the free-threshing advanced line SAL98-32.

2.2. Field management

The effect of the different nitrogen treatments was tested using a strip plot design with 10 m^2 plots and three replications. Untreated strips of bread wheat separated the treatments strips to avoid cross-fertilisation. The trials were carried out in sandy-loamy soils; the preceding crop was always maize. The planting dates were 10 November 2011 and 16 November 2012, while the harvesting dates were 27 July 2012 and 23 July 2013; in both years Monarca, the early-ripening line, was harvested two weeks in advance. Mean temperature and total rainfall during the crucial flowering and seed-setting months (April, May and June, 2012 and 2013) are depicted in Supplementary Fig. 1. For weed control, the herbicide Ariane II (Clopiralid 1.8% + Fluroxypyr 3.6% + MCPA 18.2%; Dow AgroSciences, Milan, Italy) was applied at heading.

Five different nitrogen (N) treatments were adopted: 0 kg/ha (N0), 40 kg/ha at tillering (N40T), 40 kg/ha at heading (N40H), 80 kg/ha at tillering (N80T) and 80 kg/ha at heading (N80H). The nitrogen was supplied as ammonium nitrate (26% N; Yara Italia, Milan, Italy). At maturity the plots were machine-harvested with a Nurserymaster Expert combine (Wintersteiger AG, Ried, Austria).

2.3. Grain and flour characteristics

The thousand kernels weight (TKW) was determined by weighting two 100 kernels samples, sizing the results to 1000 and correcting to 15% humidity. Afterwards, about 500 g of each sample were ground with a Cyclotec 1093 lab mill (Foss Tecator, Hillerød, Denmark), obtaining a whole meal flour with particle size < 200 μm. The samples were stored under vacuum at -20 °C until analysis.

The following determinations were performed: dry matter (AACC method 44-15.02, AACC International); protein (N x 5.7; AACC method 46-10.01, AACC International), ash content (AACC method 08-03.01, AACC International), Falling number (AACC method 56-81.03, AACC International) with a Falling Number 1550 (Perten Instruments AB, Huddinge, Sweden), SDS sedimentation test (a breadmaking attitude predictor; Preston et al., 1982), flour viscosity with a Rapid Visco Analyzer (Newport Scientific Pty, Ltd., Warriewood, NSW, Australia).

Carotenoid extraction and quantification by NP-HPLC was carried out as described by Hidalgo et al. (2010). The following system and operating conditions were used: column Alltima Si column, 250 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); Alltima SI guard column 7.5 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); column oven at 20 °C L-2300 Elite LaChrom (Hitachi, Tokyo, Japan); mobile phase, hexane:isopropyl alcohol (5%); flow rate, 1.5 mL/min; pump L-2130 Elite LaChrom (Hitachi, Tokyo, Japan). The carotenoids were detected at 450 nm by Diode Array Detector L2450 Elite LaChrom (Hitachi, Tokyo, Japan) set in the range of 200-650 nm. The HPLC system was controlled by the software EZChrom Client/Server version 3.1.7. For peak quantification, calibration curves were built using seven different concentrations (between 0.3 and 3.0 mg/L) of the lutein standard (Fluka, St. Louis, MO, USA), seven different concentrations (between 0.15 and 1.5 mg/L) of the β-carotene standard (Sigma, St. Louis, MO, USA), ten different concentrations (between 0.05 and 1.03 mg/L) of the zeaxanthin standard (Extrasynthese, Genay, France), and seven different concentrations (between 0.02 and 0.13mg/L) of the β-cryptoxanthin standard (Extrasynthese, Genay, France), diluted with isopropyl alcohol (10%) in hexane. The calibration curves were linear ($r^2 = 1.00$; $p \leq 0.001$) in the concentration intervals assessed. Lutein, zeaxanthin, (α+β)-carotene and β-cryptoxanthin showed detection limits of 0.06, 0.01, 0.05 and 0.04 mg/L in the standard solutions. The total carotenoids were computed as the sum of the different compounds. All measurements were performed twice; the results are expressed as mg/kg on a dry matter basis (DM).

Tocols extraction and quantification were performed by NP-HPLC as detailed by Hidalgo and Brandolini (2010). The following system and operating conditions were used: Alltima SI column, 250 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); Alltima SI guard column 7.5 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); mobile phase, hexane:ethyl acetate:acetic acid (97.3:1.8:0.9, v/v/v); flow rate, 1.6 mL/min; pump L-2130 Elite LaChrom (Hitachi, Tokyo, Japan); fluorimetric detector Jasco 821 FP Intelligent Spectrofluorometer (Jasco Inc., Easton, MD, USA) at excitation-emission wavelengths of 290 nm and 330 nm, respectively; connected to a Hitachi D-7500 integrator (Hitachi, Tokyo, Japan). The tocol standard curves were constructed using eleven different concentrations (between 0.4 and 109.7 mg/L) of α-tocopherol standard (Fluka BioChemika, Buchs, Switzerland) and thirteen different concentrations (between 0.4 and 72.2 mg/L) of β-tocopherol standard (Supelco, Bellefonte, PA, USA), in hexane:propane-2-ol (90:10, v/v). The tocotrienols were quantified using the standard curves of their corresponding tocopherol. The calibration curves were linear ($r^2 = 1.00$; $p \leq 0.001$) in the concentration intervals assessed. The detection limits of α-tocopherol and β-tocopherol were 0.39 mg/L and 0.8 mg/L in the standard solutions. The total tocols were computed as the sum of α- and β- tocopherol, and α- and β- tocotrienols. All measurements were performed twice; the results are expressed as mg/kg DM.

Soluble conjugated and insoluble bound phenolic acids extractions and analysis were performed by RP-HPLC as described by Brandolini et al. (2013). The following operating conditions were adopted: column Alltima C18 5 μm 4.6 mm x 250 mm (Grace Davison Discovery Sciences, Deerfield, IL, USA), precolumn Alltima C18 5 μm 4.6 mm x 10 mm (Grace Davison Discovery Sciences, Deerfield, IL, USA) thermostated at 30 °C; pump L-2130 Elite LaChrom (Hitachi, Tokyo, Japan), column oven L-2300 Elite LaChrom (Hitachi, Tokyo, Japan); mobile phase: A) 1% (v/v) acetic acid in water, B) methanol; flow rate 1.5 mL/min. The gradient, in terms of eluent B, was: at time 0, 15%; at 10 min, 20%; at 16 min, 23%; at 24-28, 27%; at 30-34, 15%. The HPLC system was controlled by the software EZChrom Client/Server version 3.1.7. The compounds were detected at 280 nm with a Diode Array Detector L2450 Elite LaChrom (Hitachi, Tokyo, Japan). For peak quantification, calibration curves of the following compounds using standards from Sigma-Aldrich (St. Louis, MO, USA) were constructed: caffeic acid (between 0 and 7.29 mg/L), ferulic acid (0 - 200.38 mg/L), *p*-coumaric acid (0 - 9.93 mg/L), *p*-hydroxybenzoic acid (0 - 26.48 mg/L), syringaldehyde (0 - 11.44 mg/L), syringic acid (0 - 19.62 mg/L), vanillic acid (0 - 19.58 mg/L). The calibration curves were linear ($r^2 = 1.00$; $p \leq 0.001$) in the concentration intervals assessed. On the basis of the calibration curves, the detection limits in the standard solutions were 0.05, 1.18, 0.09, 0.14, 0.09, 0.11, 0.16 mg/L, respectively. The analyses were performed twice; the results are expressed as mg/kg DM.

2.4. Statistical analysis

For each trait a combined analysis of variance (ANOVA) of two-year data was performed according to a strip plot design using the software STATGRAPHICS plus version 4 (Statpoint Technologies, Inc., Warrenton, VA, USA); when the differences were significant, the means were compared following the LSD test. Means and standard errors were computed with Office Excel 2003 (Microsoft, Redmond, WA, USA).

3. Results and discussion

3.1. Technological parameters and protein content

The average protein concentration and ash content of the three tested einkorns (Table 1) are similar to those described by several authors (e.g. Hidalgo and Brandolini, 2014; Løje et al., 2003). The sedimentation volume in SDS, which indicates the suitability of flour for bread production, varied from poor to good between accessions (Table 1). The Falling number and viscosity results showed a broad range of variation that will be discussed in detail below.

The two cropping years showed similar thermal trends, but were extremely different in precipitation (Supplementary Fig. 1). The 2012 spring was characterised by mild and irregular rains, often accompanied by strong winds, throughout the kernel filling and maturation period; the 2013 spring, instead, was characterised by heavy rains until the last days of May, followed by a prolonged rain absence until early July. This sharp climatic difference has a decisive influence on many qualitative characteristics and suggested to treat the year as a fixed effect, where the two years represent typical “wet ripening” and “dry ripening” environments. The ANOVA (Supplementary Table 1) performed on the technological parameters considering as sources of variation year (Y), levels of nitrogen fertilization (N) and genotypes (G), showed that Y, N and G, as well as their interactions, were always significant, and that in general Y was the most influential trait. The year effect was particularly intense on the Falling number, which measures flours pre-germination, and the viscosity parameters. In fact the einkorn Monarca, an early-maturing genotype, was already ripe in late May, so in 2012 the repeated cycles of rain favoured pre-sprouting phenomena and α-amylase enzyme activation, leading to a partial degradation of starch and low Falling number results (Table 1 and Fig. 1). The other two accessions, later-maturing, showed a decrease of quality, but their Falling number was anyway well above the threshold of normal values (> 220 s). The genotype effect was strongest on TKW, protein content and SDS sedimentation (Supplementary Table 1), probably because the three genotypes tested are characterised by different seed size, protein content and breadmaking attitude (Table 1 and Fig. 1). The nitrogen fertilisation influence was more evident for protein and SDS sedimentation (Supplementary Table 1).

Fig. 1 shows the mean values (± standard error) of the technological parameters of the three accessions in both years. The TKW decreased slightly between 2012 and 2013 (27.3 ± 0.43 vs. 25.4 ± 0.32 g, respectively; Table 1) because the protracted rain in 2012 favoured the development of heavy kernels, while the late drought of 2013 led to healthier but lighter seeds. The fertilisation prompted minimum and irregular weight changes. Among varieties, Monarca produced the heaviest kernels. Similarly, Andruszczak et al. (2011) and Piekarczyk et al. (2011) did not find variation in TKW of wheat and spelt after increasing nitrogen additions. On the other hand, some authors associated to fertilisation a weight increase in other wheat species. For example, Daniel and Triboi (2000) in bread wheat observed that low temperatures during the filling period, coupled with nitrogen addition before or during anthesis, increased kernel weight. This result is in agreement with Makowska et al. (2008) that, analyzing the effect of increasing nitrogen doses on durum wheat, observed maximum TKW with 100 kg/ha of nitrogen; nevertheless, Kumar et al. (1995) found that the TKW increased only at low nitrogen (N) + phosphorus (P) + potash (K) levels (40 kg N, 20 kg P_2O_5 and 13.3 kg K_2O/ha) but was stable at higher fertilisations.

Overall, ash content increased slightly in 2013 (2.51 ±.0.009 g/100 g DM) compared to 2012 (2.45 ± 0.014 g/100 g DM), but the fertilisation did not modify its concentration, as observed by Fares et al. (1993) in durum wheat.

The protein content, in general more abundant in 2012 (17. 3 ± 0.30 g/100 g DM) compared to 2013 (16.0 ± 0.38 g/100 g DM) possibly because of fertiliser leaching as a consequence of the concentrated heavy rain of May 2013, increased gradually in conjunction with the enhanced nitrogen availability, particularly when the fertiliser was supplied at heading. Among the three genotypes analyzed, Monarca had the highest protein content in both years (18.8 ± 0.45 and 17. 3 ± 0.40 g/100 g DM). Working with other *Triticum* species, Souza et al. (2004) reported that environmental and genetic effects outweighed fertilization in soft wheat, but on the contrary Shewry et al. (2013) highlighted a greater importance of fertilization compared to year and genotype. Similarly, Daniel and Triboi (2000) described significant effects of year, temperature and levels of nitrogen (but not of their interactions) on proteins. Many other researchers (e.g. Al-Eid, 2006; Fares et al., 1993; Kumar et al., 1995; Makowska et al., 2008; Novoa and Loomis, 1981) observed an increase in protein content by increasing nitrogen fertilization. Furthermore, nitrogen supplied at the heading stage induced a higher increase than when supplied at tillering, as it mainly contributes to raise the protein content of kernels (Novoa and Loomis, 1981).

The sedimentation volume in SDS was higher in 2012 (39.9 ± 2.87 mL) than in 2013 (25.7 ± 1.85 mL); the three accessions confirmed their different breadmaking attitude, which is good for Monlis (on average, 46.4 ± 23.3 mL), intermediate for SAL98-32 (35.2 ± 1.78 mL) and poor for Monarca (16.8 ± 12:49 mL). As such, the effect of increasing the doses of nitrogen was particularly evident in the first two genotypes (Fig. 1); additionally, as mentioned above, the nitrogen administered at the heading stage increased the protein content of the kernels and contributed to the improvement of the breadmaking attitude of the flours. The influence of fertilisation on sedimentation volume was observed also by Fares et al. (1993) in durum wheat.

Falling number values were significantly lower in 2012 than in 2013 because of the protracted rainfall during the ripening period. As mentioned previously, the early-maturing Monarca presented the widest difference between the two years. Fertilisation showed variable effects on this parameter as the slight increase at higher N doses observed in SAL98-32 was not detected in Monlis and Monarca. Our results are thus similar to those by Makowska et al. (2008), that did not find significant changes with increasing nitrogen addition; however, Ellman (2011) and Piekarczyk et al. (2011) reported a significant increase in Falling number as a result of the intensification of nitrogen fertilization.

Peak viscosity and final viscosity, which measure starch-related attributes, showed a strong difference between years, as in 2012 the values of the three accessions were lower than in 2013, particularly in the case of Monarca; notwithstanding the broad variation in 2012 linked to pre-sprouting, nitrogen addition induced some viscosity reduction, possibly because the already mentioned increase in protein content conversely led to a decline in starch content.

3.2. Carotenoids

As shown in Table 2, lutein was by far the most abundant pigment (85.2% of total); carotenoid composition and content were similar to the results reported in the literature (Abdel-Aal et al., 2007; Hidalgo et al., 2006). The ANOVA of carotenoids (Supplementary Table 2) showed significant effects of all the three main factors and of most of the interactions. Year and genotype, *per se* or in interaction, accounted for most of the variation observed. Fertilisation was limited to a minor role; nevertheless, the concentration of most abundant carotenoid (lutein) was slightly modified by the different nitrogen concentrations.

Fig. 2 shows the mean values (± standard error) of the total carotenoid content of the three accessions of einkorn for five different nitrogen fertilization profiles for each of the two years. The 2012 spring, characterised by persistent rain throughout the heading and ripening period, led to higher levels of lutein and zeaxanthin, as well of total carotenoids, than the 2013 spring. In 2012 a reduction in carotenoid content with increasing nitrogen fertilizers is also evident, but the trend was not always confirmed in 2013; among the accessions tested, Sal98-32 had the highest concentration of lutein (8.69 ± 0.47 mg/kg DM), zeaxanthin (0.91 ± 0.05 mg/kg DM) and total carotenoids (10.29 ± 0.52 mg/kg DM). Hidalgo et al. (2009) identified the growing year as the main factor for carotenoids content in einkorn, even though the genotype plays an important role. In fact, significant changes in einkorn lutein content were reported by Abdel-Aal et al. (2007) and Hidalgo et al. (2009), with the highest values recorded in the wettest years. However, Lachman et al. (2013) observed the carotenoid content of emmer, spelt and einkorn changed between years and associated the lower concentrations of β-carotene, zeaxanthin and lutein to abundant precipitation and higher temperatures. Concerning the effect of fertilisation, Kumar et al. (1995) observed that β-carotene was not affected by increasing levels of nitrogen, potash and phosphorus.

3.3. Tocols

The α- and β- homologues of tocopherol and tocotrienol were identified (Table 2); β-tocotrienol was the most abundant compound (59.6% of total), in agreement with the results of Hidalgo et al. (2006). The average total tocol content was within the range of variation described by Hidalgo et al. (2006) and Hidalgo et al. (2009). The ANOVA of tocols (Supplementary Table 3) showed significant effects of all the three main factors and of most of the interactions; only for total tocol the year *per se* was not significant (but the interaction YxG was very strong). Year and genotype, *per se* or in interaction, accounted for most of the variation observed. Fertilisation had a minor role; nevertheless, the most abundant tocol (β-tocotrienol) was influenced by the different nitrogen concentrations.

Fig. 2 shows the mean values (± standard error) of total tocol concentration in the three accessions of einkorn cropped for two years with five different nitrogen fertilization profiles. In SAL98-32 and Monarca, the total tocol content was lower in 2012 than in 2013 (Table 2), while the increase in nitrogen fertilisation led to a minimal reduction of these compounds (Fig. 2). Among accessions, SAL98-32 showed the highest total tocols content (70.5 ± 1.00 mg/kg DM), followed by Monlis (64.6 ± 0.99 mg/kg DM) and Monarca (61.7 ± 0.65 mg/kg DM). Hidalgo et al. (2009) also reported significant effects of year and genotype on tocotrienols and total tocols, as well as a limited genotypic influence on tocopherols; on the other hand, no studies are available on the influence of nitrogen fertilisation. Not many reports are available on the effect of the environment (i.e. year and/or location) on tocol composition and content. Shewry et al. (2013), analyzing 26 wheats across six locations, showed a broad variation due to genotype and environment, and remarked that the content in total tocols was highly heritable (i.e. with a much greater effect of the genotype than of the environment). Similarly, Lampi and Piironen (2010), studying wheat cultivars grown in four different locations, observed strong environment and genotype effects on tocols content, but concluded that some genotypes were very sensitive to the impact of the environment while others were relatively stable. The comparative evaluation of the results obtained for carotenoids and tocols led Fratianni et al. (2013) to conclude that the typical Mediterranean water shortages induce an increase of lipophilic antioxidants in wheat. On the other hand, Hidalgo et al. (2009), observing the behaviour of lutein and tocotrienols in function of climate, suggested that their antithetical behaviour was due to the synthesis pathways of the two groups of compounds: tocotrienols are synthesized by the condensation of homogentistic acid and geranylgeranyl-PP, while tocopherols derive from the condensation of homogentistic acid and phytyl-P-P. As geranylgeranyl-PP is also a precursor of carotenoids, the environmental conditions that stimulate the synthesis of lutein may thus interfere with the synthesis of tocols, and the other way round.

3.4. Phenolic acids

In the conjugated fraction ferulic, vanillic, syringic, *p*-coumaric, *p*-hydroxybenzoic acids and syringaldehyde were identified, while in the bound fraction ferulic, *p*-coumaric, vanillic, syringic and *p*-hydroxybenzoic acids were recognised (Table 3). Ferulic acid was the most abundant compound in both the conjugated (65.1%) and the bound (92.8%) fractions; the conjugated phenolic acids represented a small fraction (7.7%) of the total phenolic acids, as already highlighted by Brandolini et al. (2013).

The ANOVA of the conjugated phenolic acids (Supplementary Table 4) and of the bound phenolic acids (Supplementary Table 5) showed significant effects of the three sources of variation (and of their interactions) in almost all the cases. The only exceptions were year for *p*-hydroxybenzoic acid and genotype for syringic acid (conjugated phenols), as well as year and fertilisation for *p*-hydroxybenzoic and vanillic acid (bound phenols). Year and its interactions explained the majority of the variation in nearly all cases. As evidenced in Table 3, total conjugated and total bound phenolic acids were higher in 2013 compared to 2012; the increase was particularly sharp in Monarca, while the difference was minor in the other two einkorns. Monlis and Monarca showed concentrations of total phenolic acids above SAL98-32. Brandolini et al. (2013) studied in detail the composition and content of conjugated and bound phenolic acids in thirty-nine wheat samples (13 einkorns) belonging to different species and observed a significant year effect on conjugated but not on bound phenolic acids. A strong influence of the year on the content of phenolic acids was also reported by Heimler et al. (2010) for bread wheat, as well as by Lachman et al. (2011) for *T. dicoccum*, *T. monococcum* and *T. aestivum*. Accordingly, Stracke et al. (2009), studying the effects of two different production methods (traditional and organic) for three years stated that the cropping year effect was the most important, while the two cropping systems did not lead to different results. Martini et al. (2014) studied the impact of genetic and environmental factors (year and location) on the profile and the content of free, conjugated and bound phenolic acids using three genotypes of durum wheat, and found highly significant effects of genotype, location and year for all the compounds; the content of conjugated and bound phenolic acids was determined largely by the interaction between the three factors.

Fig. 2 shows an increase of total conjugated and total bound phenolic acids for each of the three einkorns in response to nitrogen addition. In particular, conjugated syringic acid, syringaldehyde, *p*-coumaric acid and ferulic acid, as well as bound *p*-coumaric and ferulic acids reached the maximum levels with the highest intakes of nitrogen (data not shown). Phenolic acids are derived by the nonoxidative deamination of the aminoacids phenylalanine and tyrosine to form cinnamic acid and *p*-coumaric acid; ferulic acid, syringaldehyde and syringic acid are all sequentially derived from *p*-coumaric acid (Collins, 2011). Hence, higher nitrogen doses increase the content of protein, and consequently aminoacids, including those precursors of phenolic acids.

Stracke et al. (2009), analyzing the content of conjugated phenolic acids in fertilized bread wheat samples concluded that the fertilization method did not induce statistically significant differences, emphasizing that the climate has a greater influence on their concentration. Konopka et al. (2012), analyzing wheat treated with different types of fertilizers (NPK mineral, and organic as compost, manure and meat and bone meal), observed a certain variation in the content of total phenolic acids.

4. Conclusions

The two years of cultivation had similar thermal profiles but were extremely different for precipitation. The climatic differences had a significant impact on Falling number, viscosity, lutein, α-tocotrienol, conjugated syringic, syringaldehyde and *p*-coumaric as well as bound syringic and ferulic acid. Genotype was predominant for TKW, SDS, (α+β)-carotene, cryptoxanthin, zeaxanthin, β-tocotrienol, conjugated vanillic, bound vanillic and *p*-coumaric. Nitrogen fertilisation generally had minor influence, with the exception of protein content and, consequently, of SDS. Carotenoids synthesis was slightly limited with increasing fertilisation in 2012, a similar, but less evident, effect was present for tocols. Phenolic acids instead showed a certain increase after fertilisation. In conclusion einkorn, even with low nitrogen additions, is able to supply flours with good nutritional and technological characteristics.

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Captions to figures

Fig. 1. Thousand kernel weight (TKW), ash and protein content, SDS sedimentation volume, Falling number, peak viscosity and final viscosity of einkorns SAL98-32, Monlis and Monarca, cropped in 2011-12 and 2012-13 under five different nitrogen fertilisation treatments: 0 kg/ha (N0), 40 kg/ha at tillering (N40T), 40 kg/ha at heading (N40H), 80 kg/ha at tillering (N80T) and 80 kg/ha at heading (N80H). Error bars represent standard errors.

Fig. 2. Content of total carotenoids, total tocols, total conjugated and total bound phenolic acids of einkorns SAL98-32, Monlis and Monarca, cropped in 2011-12 and 2012-13 under five different nitrogen fertilisation treatments: 0 kg/ha (N0), 40 kg/ha at tillering (N40T), 40 kg/ha at heading (N40H), 80 kg/ha at tillering (N80T) and 80 kg/ha at heading (N80H). Error bars represent standard errors.

Supplementary Fig. 1. Weekly mean temperature and rainfall at Sant'Angelo Lodigiano (Italy) from February to July in 2012 and 2013.

Table 1. Mean content (± standard error) of 1000 kernels weight (TKW), protein and ash content, SDS sedimentation volume (SDS), Falling number and viscosity of the three einkorn accessions tested.

	Sal98-32		Monlis		Monarca	
	2012	2013	2012	2013	2012	2013
TKW (g)	27.2±0.13	26.8±0.27	24.3±0.14	23.4±0.45	30.5±0.21	26.1±0.29
Protein (g/100g DM)	16.6±0.40	16.6±0.45	16.6±0.47	14.0±0.66	18.8±0.45	17.3±0.40
Ash (g/100 g/DM)	2.5±0.02	2.6±0.01	2.5±0.01	2.5±0.01	2.4±0.04	2.5±0.01
SDS (mL)	42.5±1.04	27.8±1.53	58.5±2.08	34.3±3.55	18.8±0.59	14.9±0.08
Falling number (s)	265.9±2.86	394.8±6.9	280.9±4.12	391.1±3.55	151.9±2.82	405.3±5.44
Peak viscosity (cP)	1759±35.4	3153±21.6	1739±57.5	3525±32.4	597±16.1	2959±72.7
Final viscosity (cP)	1862±36.2	2928±20.9	1823±61.8	3357±34.5	299±12.4	3415±71.2

Table 2. Mean content (± standard error) of carotene and tocol content (mg/kg DM) of the three einkorn accessions tested.

	Sal98-32		Monlis		Monarca	
	2012	2013	2012	2013	2012	2013
(α+β)-carotene	0.61±0.013	0.60±0.023	0.77±0.017	0.50±0.010	0.37±0.006	0.47±0.009
β-cryptoxanthin	0.09±0.003	0.07±0.002	0.7±0.001	0.03±0.001	0.04±0.001	0.05±0.001
Lutein	10.6±0.49	6.8±0.15	9.8±0.37	5.7±0.07	7.0±0.17	4.4±0.06
Zeaxanthin	1.09±0.068	0.73±0.029	0.76±0.026	0.41±0.011	0.53±0.019	0.49±0.011
Total carotenoid	12.4±0.56	8.19±0.17	11.4±0.40	6.6±0.81	7.9±0.19	5.4±0.67
α-tocopherol	8.4±0.43	13.1±0.15	11.4±0.14	9.5±0.17	10.4±0.14	9.2±0.21
α-tocotrienol	15.4±0.24	11.7±0.07	15.5±0.32	11.1±0.17	11.0±0.11	11.2±0.16
β-tocopherol	2.5±0.15	4.2±0.06	4.1±0.07	4.1±0.12	4.0±0.10	3.4±0.08
β-tocotrienol	40.4±0.56	45.4±0.77	37.2±0.69	36.3±0.67	34.2±0.48	40.3±0.50
Total tocol	66.7±0.87	74.4±0.84	68.3±1.08	61.0±0.73	59.5±0.76	64.0±0.55

Table 3. Mean content (± standard error) of conjugated and bound phenolic acids content (mg/kg DM) of the three einkorn accessions tested.

	Sal98-32		Monlis		Monarca	
	2012	2013	2012	2013	2012	2013
Conjugated						
p-hydroxybenzoic	2.8±0.11	2.0±0.14	1.9±0.07	2.5±0.12	1.3±0.06	2.2±0.17
Vanillic	4.5±0.27	5.4±0.12	7.3±0.14	7.1±0.23	3.7±0.13	6.4±0.18
Syringic	3.4±0.25	4.8±0.11	3.8±0.09	4.5±0.15	3.3±0.13	4.6±0.19
Syringaldehide	0.6±0.06	1.2±0.06	0.7±0.05	1.2±0.11	0.5±0.06	1.9±0.12
p-coumaric	2.7±0.15	3.6±0.07	2.2±0.05	2.4±0.06	1.9±0.13	3.4±0.18
Ferulic	24.6±1.61	26.9±0.55	33.9±0.61	29.9±1.05	23.2±1.04	36.5±1.05
Total conjugated	38.5±2.29	43.8±0.70	49.8±0.75	47.7±1.42	33.9±1.34	54.9±1.39
Bound						
p-hyroxybenzoic	1.1±0.05	1.1±0.03	0.8±0.03	1.0±0.04	1.1±0.06	0.9±0.04
Vanillic	3.0±0.10	3.0±0.09	4.3±0.17	3.9±0.22	4.2±0.12	4.5±0.12
Syringic	3.3±0.13	1.4±0.04	3.5±0.17	2.7±0.20	4.4±0.14	2.4±0.14
p-coumaric	28.9±0.85	36.3±0.93	25.1±0.51	26.2±1.16	31.6±0.79	36.8±1.14
Ferulic	449.2±5.42	492.5±6.50	450.6±9.67	545.8±11.68	478.1±11.99	526.7±4.15
Total bound	485.5±6.14	534.1±7.08	484.3±10.18	579.5±13.01	519.4±12.73	571.3±4.66

Fig. 1

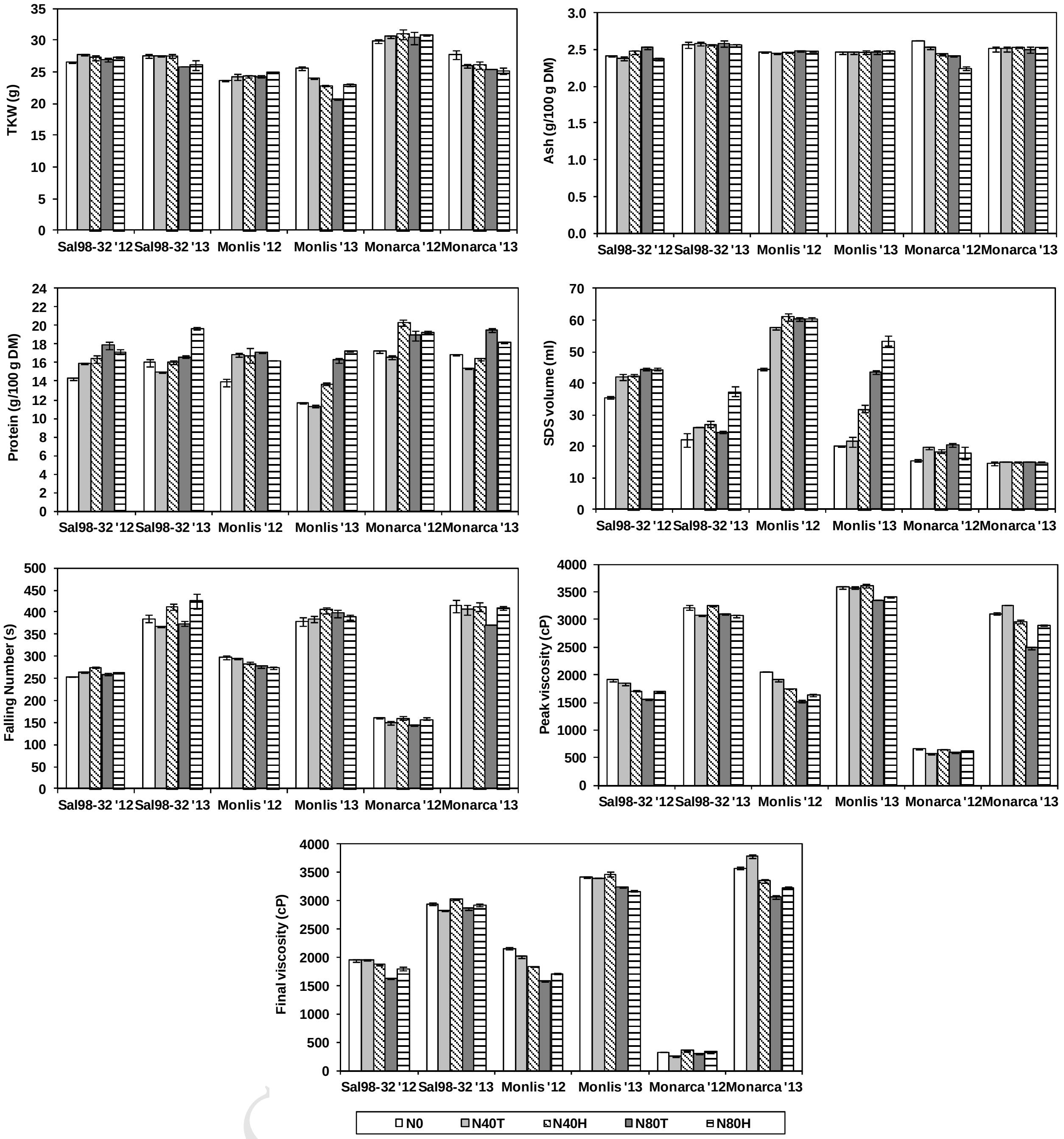
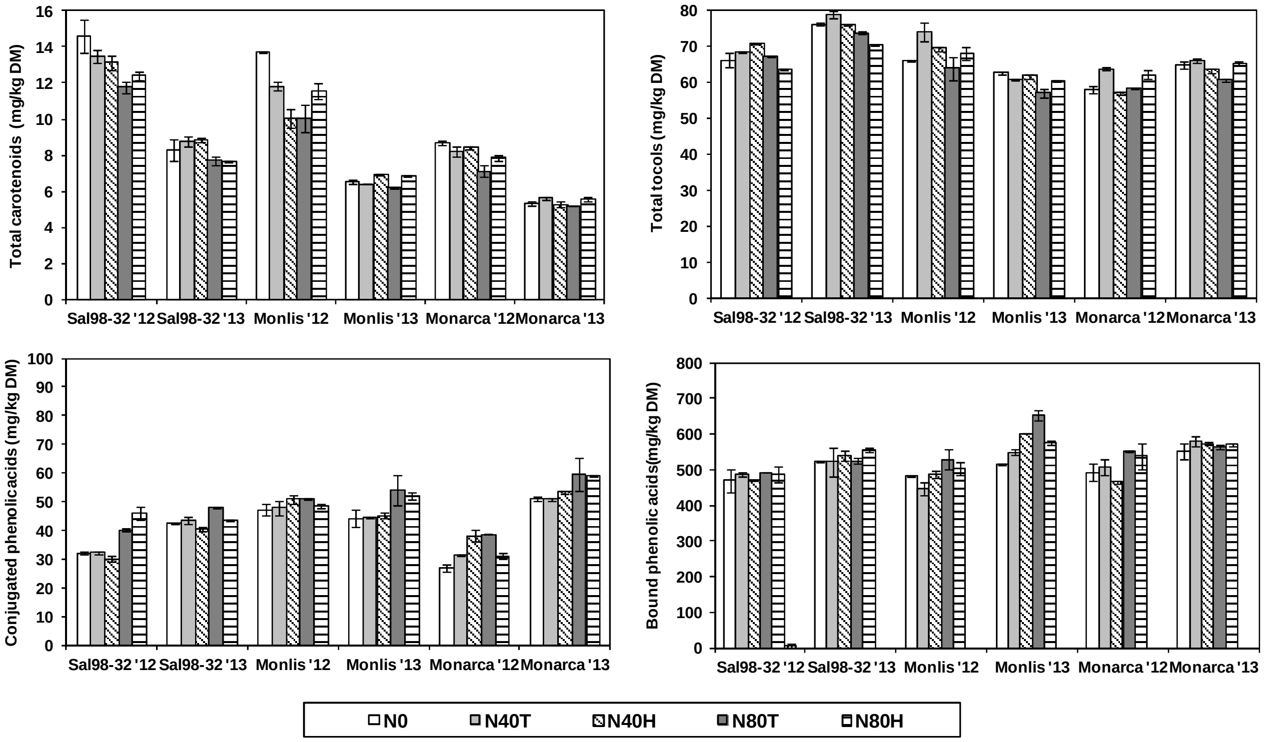

TKW (g)
0
5
10
15
20
25
30
35
Ash (g/100 g DM)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Protein (g/100 g DM)
0
2
4
6
8
10
12
14
16
18
20
22
24
SDS volume (ml)
0
10
20
30
40
50
60
70
Falling Number (s)
0
50
100
150
200
250
300
350
400
450
500
Peak viscosity (cP)
0
500
1000
1500
2000
2500
3000
3500
4000
Final viscosity (cP)
0
500
1000
1500
2000
2500
3000
3500
4000
Sal98-32 '12
Sal98-32 '13
Monlis '12
Monlis '13
Monarca '12
Monarca '13
□ N0
▨ N40T
▧ N40H
■ N80T
☰ N80H

Fig. 2


Total carotenoids (mg/kg DM)
0
2
4
6
8
10
12
14
16
Total tocols (mg/kg DM)
0
10
20
30
40
50
60
70
80
Conjugated phenolic acids (mg/kg DM)
0
10
20
30
40
50
60
70
80
90
100
Bound phenolic acids(mg/kg DM)
0
100
200
300
400
500
600
700
800
Sal98-32 '12
Sal98-32 '13
Monlis '12
Monlis '13
Monarca '12
Monarca '13
□N0
▨N40T
▧N40H
■N80T
▤N80H

Highlights:

- Three einkorns were cropped for two years under different fertilisation treatments.
- The year influenced RVA parameters, carotenoid and phenolic acid concentration.
- Fertilisation improved SDS sedimentation, protein and phenolic concentration.
- Fertilisation slightly limited carotenoids and tocols synthesis.
- Einkorn needs limited nitrogen fertilisation to give flour with optimal quality.