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Metabolomic diversity for biochemical traits of *Triticum* sub-species

Agata Rascio [a, *], Romina Beleggia [a], Cristiano Platani [b], Franca Nigro [a], Pasquale Codianni [a], Giuditta De Santis [a], Michele Rinaldi [a], Mariagiovanna Fragasso [a]

[a] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria – Centro di Ricerca per la Cerealicoltura (CREA-CER), SS 673 Km 25.200, 71122, Foggia, Italy

[b] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria – Unità di Ricerca per l'Orticoltura (CREA-ORA), Via Salaria 1, 63030, Monsampolo del Tronto, AP, Italy

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ABSTRACT

Whole grains were characterised using a metabolite profiling approach for 11 genotypes that belonged to five modern and ancient *Triticum* sub-species, briefly distinguished as: *monococcum*, *dicoccum*, *spelta*, *durum* and *aestivum*. Hydrophilic and lipophilic metabolites were extracted separately, and about 51 compounds of different classes were analysed: sugars, alcohols, amino acids, organic acids, plant sterols, phenolic lipids, tocopherols and fatty acids. Differences in the metabolite content among sub-species were statistically significant for 22 compounds out of 51. On the basis of multivariate analysis, the metabolites that provide the greatest contributions to discrimination among these five sub-species were identified. Galacturonic acid (precursor of ascorbic acid) was detected only in *dicoccum*. Alkylresorcinol homologues AR17 and AR19 were greatly accumulated by *aestivum* and *spelta*. Aspartic acid and asparagine were highest in *monococcum*. Asparagine was not detected in *durum* and *aestivum*, but these instead had the highest levels of the correlated essential amino acid tryptophan. The alkylresorcinol homologue composition, and in particular the AR17/AR23 ratio, can be used to distinguish between the *monococcum*, hexaploid and tetraploid species. Vice versa, the distinctions (in terms of these metabolites), between the two tetraploid species and between the hexaploid sub-species were difficult to clearly sort out.

1. Introduction

Over the last 40 years, several comparative analyses of the contents of primary and secondary metabolites have been performed to investigate wheat genetic diversity, to identify important traits for plant adaptation to biotic and abiotic stresses (Rascio et al., 1994; Mishra et al., 2010), and to determine technological and nutritional quality (Zhao et al., 2009; Troccoli et al., 2010; Chatzav et al., 2010; Lachman et al., 2013). As an example, small sugars and amino acids, osmolytes, and osmoprotectants are involved in acclimation processes to environmental stresses (Setter, 2012). Also, phytosterols (e.g., sitosterols, campesterol, stigmasterol), antioxidants (e.g., tocopherols), and phenolics (e.g., alkylresorcinols) can have beneficial effects on human health and/or in the protection of plants against herbivores and pathogens (Melake-Berhan et al., 1996; Kozubek and Tyman, 1999; Zarnowski et al., 1999; Pandey and Rizvi, 2009; Serpen, 2008; Ciccoritti et al., 2014).

Nowadays, the interest towards phenotyping for biochemical traits has the aim to recover important alleles that are responsible for the variability of different compounds in wheat grain. Indeed, from the 1960s, the diffusion of relatively few modern varieties, obtained by anthropogenic selection, resulted in the loss of important sources of cultivated biodiversity in terms of primary nutrients and secondary metabolites. Moreover, the availability of methods for simultaneous targeted or untargeted analysis of several chemical compounds greatly improves the efficiency of biochemical phenotyping.

A recent comparison of metabolite profiling of four *durum* wheat genotypes grown in the field over three years showed large effects of Year and significant Genotype × Year interactions (Beleggia et al., 2013). In the present study, the biodiversity exploitation was extended to bread wheat and wild relatives, to find clusters of correlated metabolites that might be genome dependent. Five species were analysed, and they are briefly distinguished and described here as: *monococcum* (*Triticum monococcum* L. ssp. *monococcum*), which is known as einkorn, and is a

* Corresponding author. Tel.: +39 881 742972.
E-mail address: agata.rascio@crea.gov.it (A. Rascio).

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diploid species with a tough rachis (AA genome); *dicoccum* (*Triticum turgidum* ssp. *dicoccum*), or emmer, which is also known as 'farro' in Italy, and is the cultivated allotetraploid progenitor of *durum* (AABB genome); *durum* (*Triticum turgidum* L. ssp. *durum*) a tetraploid wheat that is widely cultivated in southern Italy (AABB genome); *aestivum* (*Triticum aestivum* L.), or bread wheat, an allohexaploid species (AABBDD genome); and *spelta* (*Triticum aestivum* ssp. *spelta*) which represents a morphologically different group within *T. aestivum* (AABBDD genome) (Kimber and Sears, 1983; Dvorak et al., 1992; Rodríguez-Quijano et al., 1997).

2. Experimental

In 2011, a field trial was performed at the Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria – Centro di Ricerca per la Cerealicoltura (CREA-CER), in Foggia (southern Italy), using: 11 varieties of *durum* wheat ('Trinakria', 'Arcangelo', 'Claudio', 'Parsifal', 'Gianni', 'Aureo', 'Ofanto', 'Cappelli', 'Aldura', 'Saragolla', 'Messapia'); one variety of bread wheat ('Mec'); a *dicoccum* variety ('Giovanni Paolo') and a *dicoccum* line selected at CREA-CER from a Molise landrace (Perrino and Hammer, 1982); one Appulo-Sannitica landrace of *monococcum* (Perrino and Hammer, 1982); and two *spelta* varieties ('Altgold Rotkorn' and 'Giuseppe'). The agronomical practices were the usual ones used in this cultivation area. 400 seeds/m^2 of each genotype were sown on medium-texture soil, following a randomised complete block design with two replicates. The plots were 1.75 m^2. The plants were fertilised at stem elongation with 300 kg/ha ammonium nitrate, and were grown without irrigation. The grain were collected at maturity from each plot separately, and kept at 4 °C until analysis.

2.1. Gas chromatography–mass spectrometry analysis of grain metabolites

After collection, the whole grain samples were freeze dried, milled (Pulverisette® 7 Planetary Micro Mill; Classic Line, Fritsch) with an agate jar and balls, and stored at −25 °C until analysis.

The extraction, derivatisation and analysis of these samples for the profiling of the polar and non-polar metabolites were performed using a gas chromatography and mass spectrometry (GC-MS) based approach, following protocols described previously (Beleggia et al., 2013). All of the analyses were performed for three technical replicates, for each of two biological replicates. Briefly, 100 mg dry weight of each sample was extracted using a mixture of methanol (1 mL), ultrapure water (1 mL), and trichloromethane (3 mL), added sequentially. The samples were stored at 4 °C for 30 min and then centrifuged at 4000*g* for 10 min at 4 °C.

Aliquots (50 μL) of the polar and the trichloromethane phases (1 mL) were dried in a Speedvac for further analysis. The polar fraction was redissolved and derivatised for 90 min at 37 °C in methoxyamine hydrochloride in pyridine (70 μL; 20 mg/mL), followed by incubation with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (120 μL) at 37 °C for 30 min.

The polar metabolites were analysed using GC (Agilent 6890N, Agilent Technologies, USA) coupled to quadrupole MS (Agilent 5973, Agilent Technologies, USA). Samples (1 μL) were injected in splitless mode, with GC separation on an HP-5 ms capillary column (60 m; 0.25 mm i.d.; 0.25 mm film thickness). Helium was used as the carrier gas, at a constant flow rate of 1 mL/min. For the analysis of polar metabolites, the injection temperature, transfer line, and ion source were set at 280 °C, and the quadrupole was adjusted to 180 °C. The oven was kept at 70 °C for 1 min, then increased at a rate of 5 °C/min to 310 °C, where it was held for 15 min. Subsequently, the temperature was increased to 340 °C, and held for 1 min.

The spectrometer was operated in electron-impact mode at 70 eV, the scan range was from 30 amu to 700 amu, and the mass spectra were recorded at 2.21 scan/s. The non-polar metabolites were analysed as above, with minor modifications: the injection temperature and the transfer line were set at 250 °C; the oven was kept at 70 °C for 5 min, then increased at a rate of 5 °C/min to 310 °C, and held for 1 min. Subsequently, the system was equilibrated for 6 min at 70 °C before sample injection. The mass spectra were recorded at 2.28 scans/s, and the scan range was from 50 amu to 700 amu.

The metabolites were identified through comparisons of the MS data with those of 2008 database of the National Institute of Standards and Technology (NIST) and with a custom library obtained with reference compounds. The GC-MS quantification was performed using the Chemstation software, by comparison with standard calibration curves obtained in the range of 0.04 ng–2 ng. The standards and all of the chemicals used were of HPLC grade and were from Sigma-Aldrich Chemical Co. (Deisenhofen, Germany). The *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide was from Fluka.

2.2. Statistics

Univariate and multivariate statistical analyses were run with STATISTICA (StatSoft Inc.). The significances of the differences for the individual metabolite contents of the five *Triticum* sub-species were analysed by one-way ANOVA, using the genotype as the sample unit, and as two replicates. The linear correlation coefficients among the metabolites were also calculated. The biochemical datasets were then subjected to stepwise discriminant analysis, to ascertain which pool of compounds best differentiated between the five subspecies examined. The straightforward calculation was evaluated on the basis of the F-test of significance of the partial regression coefficient of the last variable entering the model, and using Wilks' lambda. Mahalanobis distances were also calculated, to compare the relative distances among subspecies. Canonical multiple discriminant analysis was used for graphical representation of the group distributions, by plotting the individual scores for the two principal functions obtained from the model. For each variable, the respective contribution to the discrimination was examined, using the standardised b coefficients.

3. Results and discussion

The dataset is composed of 32 polar and 19 non-polar compounds of different classes (Table 1), including: sugars, alcohols, amino acids, organic acids, plant sterols, tocopherols, phenolic lipids, and fatty acids. Univariate statistical analysis showed large variability, and for 22 compounds out of 51 (43%), the differences among the sub-species for their metabolite contents showing statistical significance ($P < 0.05$). Among these, there were hydrophilic compounds, like sugars, organic acids, and amino acids, and hydrophobic metabolites, such as fatty acids, tocopherols, tricosane, and three out of the four alkylresorcinols: AR17 (5-*n*-heptadecylresorcinol), AR19 (5-*n*-nonadecylresorcinol), and AR23 (5-*n*-tricosylresorcinol).

The linear correlation coefficients were examined, and most of the metabolites were unrelated or positively related. On the other hand, inverse correlations were less frequent. Then, stepwise forward discriminant analysis was run only with the metabolites that differed significantly among the genotypes, considering that the aim of this study was to identify genetic sources of metabolic variability. A tolerance of 0.01 was used to eliminate the variables that provided superfluous information, along with those previously included in the model. The stepwise procedure accepted 16 of these variables (Table 2) and excluded: tryptophan, glycerol, phosphate,