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Published in:
Journal of Cereal Science

DOI:
10.1016/j.jcs.2022.103573

Publication date:
2022

Citation for published version (APA):
Girija, A., Le Bihan, V., Wang, Z., Han, J., Yadav, R., & Mur, L. A. J. (2022). A phenomic-metabolomic pipeline for assessing the seed traits in the gluten free orphan cereal, Eragrostis tef. *Journal of Cereal Science*, *108*, [103573]. https://doi.org/10.1016/j.jcs.2022.103573

Journal of Cereal Science 108 (2022) 103573

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

A phenomic-metabolomic pipeline for assessing the seed traits in the gluten free orphan cereal, *Eragrostis tef*

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ARTICLE INFO

Keywords:
Tef
Phenomics
Metabolite profiling
Seed nutrition

Orphan crops are gaining increasing attention due to their potential roles in satisfying the global food, nutritional and environmental challenges (Alaunyte et al., 2010). Tef (*Eragrostis tef*) is one of the ancient grains cultivated widely in Ethiopia (Shumoy and Raes 2017) which, as its centre of origin, Ethiopia holds the largest and most diverse germplasm collection (Assefa et al., 2015). To derive new and improved tef varieties, these resources need to be rapidly and efficiently exploited (Jifar et al., 2020). Due to their multivariate nature, 'omics' approaches may better describe germplasm diversity and facilitate its exploitation within focused breeding programmes (Girija et al., 2022).

Tef grains are gluten-free and, compared to other cereals, it is rich in protein, fibre, vitamins and minerals (Zhu 2018). Tef flour is used to prepare Ethiopian traditional flat bread 'injera', as well as other food and beverages (Assefa et al., 2015). In Ethiopia, primary selection of tef grains are based on seed coat colour which varies from white to dark brown (Abewa et al., 2019). White coloured seeds are highly priced and are mostly destined for the Western market, but red/brown coloured tef is used for local consumption (Zhang et al., 2016). However, besides seed colour, genotypes need to be identified based on nutritional value. Here we demonstrate a means to link grain imaging and 'high--throughput' metabolomics to determine the physical and nutritional traits of tef grains.

This study focused on commonly cultivated four tef accessions: two brown (Dabbi - PI 524438, Red dabi – PI 557457) and two white (Magna – PI 243908, Manyi – PI 524443) from the US-GRIN germplasm collection. Seeds were collected from tef plants grown under the controlled conditions in the National Plant Phenomics Centre (NPPC), UK at 24 °C/21 °C ± 2 °C, 14 h/10h day/night photoperiod. Three sets of images of randomly selected seeds were captured using a Jiusion HD 2 MP USB Digital Microscope 40-1000X Portable Magnification Endoscope Camera and each grain was independently analysed to determine the seed colour, height, area and width. Seed colour accumulation were calculated based on the pixels in RGB color space. The software was developed in C++ with the support of OpenCV (https://github.com/ScreenPlant/TefSeed) and mean values of the features were converted into a csv file format. Principal component analysis (PCA) of grain parameters (Table S1) showed that the white and brown seeds were distinct Fig. 1a. Brown coloured seeds showed high numbers of orange pixels whereas white seeds were higher in yellow, green and cyan pixels Fig. 1b. Within the white and brown seeded accessions there were some distinct differences, for example Magna (white) showed decrease in seed size parameters (height, area).

To assess whether these characteristics could associate with metabolite variation, we extracted the seed metabolomes using chloroform: methanol: water (1:2.5:1 v/v) and profiling was done using flow infusion electrospray ionization high resolution mass spectrometry (FIE-HRMS), coupled with Q executive plus mass analyser instrument with UHPLC system (Thermo Fisher Scientific©, Bremen, Germany). The

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https://doi.org/10.1016/j.jcs.2022.103573
Received 31 May 2022; Received in revised form 20 September 2022; Accepted 3 October 2022
Available online 6 October 2022

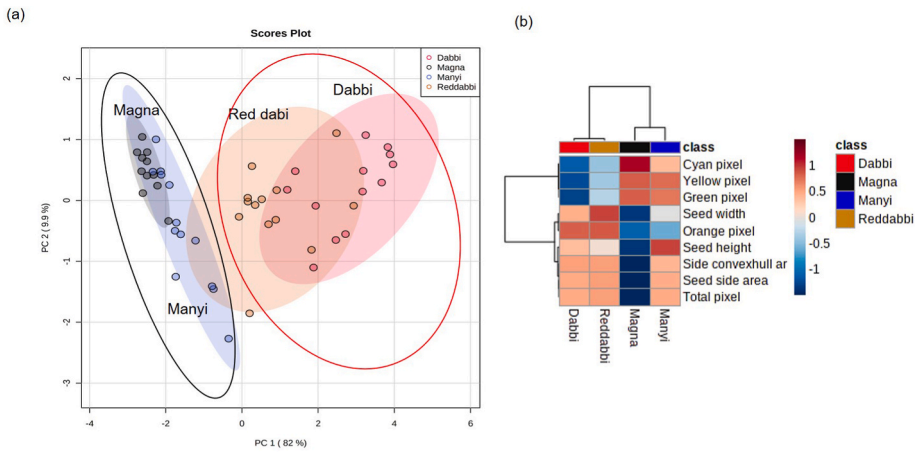

Fig. 1. (a) PCA showing the distribution of phenotypic parameters in brown and white tef seeds (b) Heatmap showing the pattern of colours, width, area and height in Dabbi, Reddabi, Magna and Manyi seed accessions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

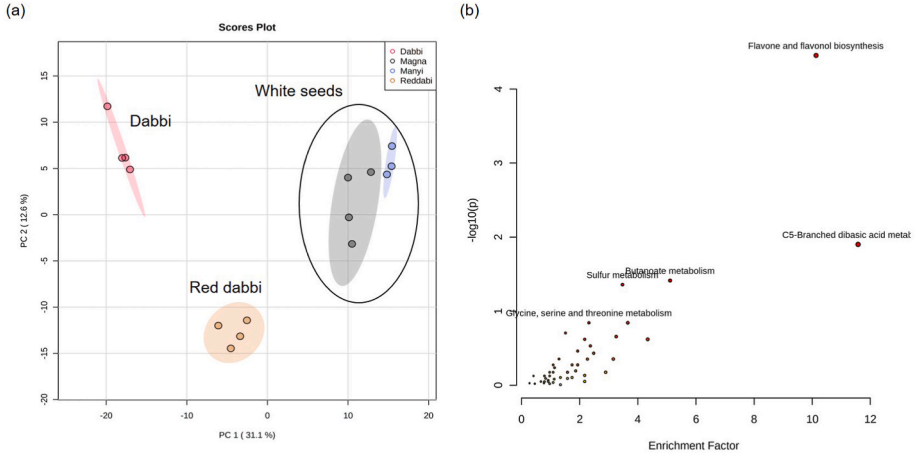

Fig. 2. (a) PCA showing distinguishing between white and brown seeds (b) Biochemical pathway enrichment analysis of significant metabolite features. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

derived mass features (*m/z)* (Table S2), were statistically assessed using unsupervised models, such as Principal Component Analysis (PCA) and ANOVA. (Bonferroni corrected for false discovery rate). The significant *m/z* features were linked to biochemical pathways generated using the R-based Metaboanalyst-5.0 platform (https://www.metaboanalyst.ca/home.xhtml). Data were $\log_{10}$ transformed followed by Pareto scaling for normality (Pang et al., 2021) as detailed in (Fisher et al., 2016). PCA showed distinct differences linked to different genotypes Fig. 2a. Our unbiased assessment indicated that the white seeded accessions, Magna and Manyi clustered closely, however the brown seeded metabolomes were distinct. The sources of variation across principal component 1 (PC1), which describes the major sources of variation were identified based on the rice reference metabolome library in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Biochemical pathway enrichment analysis indicated the prominence of flavone and flavonoid metabolites as a source of variation Fig. 2b. This aligned with reports showing tef seeds to be rich in flavones such as apigenin and luteolin, with important bioactive properties (Ravisankar et al. 2018).

ANOVA indicated 785 significant *m/z* features in the negative ionization mode. The accumulation patterns of those that could be unambiguously identified are shown as heat map Fig. 3a. In our unbiased assessment, we observed that white seeds possess high sugars (ribose, sucrose, glucose), citric acid, isopropyl malate, *cis*-aconitic acid, pantothenic acid (vitamin B5), ascorbic acid (vitamin C), glutathione, phenyalanine, tryptophan and asparagine. Dabbi and Red dabi showed similarly increased accumulation of flavonoids like rutin, quercitrin, p-coumaroyl quinic acid; chlorgenic acid (phenolics), beta-D- glucan (fibre) and heme whereas white seeds showed increased Kaempferol 3-O-rhamnoside-7-O-glucoside accumulation. These metabolites were associated with flavone and flavonoid biosynthesis, C5-branched dibasic acid, butanoate, sulfur metabolism and glycine, serine and threonine metabolism. Previous studies have shown increased iron content in brown seeds and also accumulation of phenolics and flavonoids were reported in tef seeds (Goersch et al., 2019; Girija et al., 2021). To further refine which metabolites could distinguish between brown and white seeds we employed receiver operating characteristic curves (ROC) analysis in the Metaboanalyst work package. These targeted rutin, quercitrin, kaempferol 3-O-rhamnoside-7-O-glucoside, and pantothenic acid (vitamin B5) (shown as box and whisker plots in Fig. 3b) as distinguishing between the grain types with 100% accuracies. These are of nutritional relevance to humans or could help in plant adaptation to stress.

Next, we integrated image acquired and metabolite datasets and compared them by correlation analyses. White seeds with increased yellow, green and cyan pixels showed a strong positive correlation to the key markers kaempferol 3-O-rhamnoside-7-O-glucoside and other metabolites such as aconitic acid, citric acid, pantothenic acid (Fig. 3c). However, rutin did not show any correlation with orange pixels, possibly

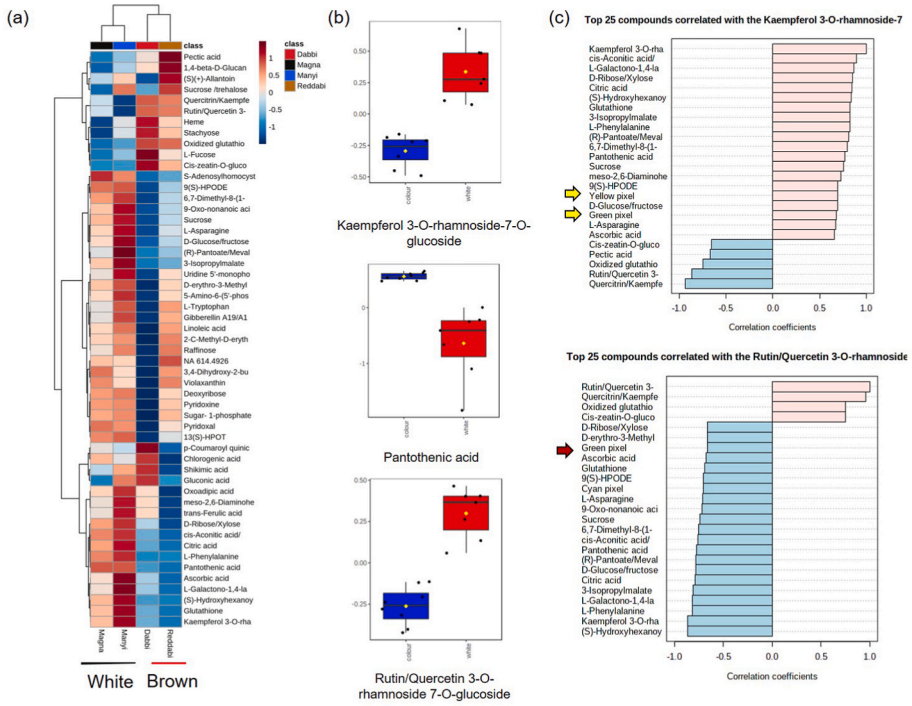

Fig. 3. Metabolites discriminating between brown and white seeds represented as (a) heatmap and (b) metabolites significantly different between white and brown seeds (c) correlation analyses of image-acquired data with metabolite kaempferol 3-O-rhamnoside-7-O-glucoside and yellow and green pixels (indicators of white seeds, [see Fig. 1]; see yellow arrow) whilst rutin/Quercetin 3-O-rhamnoside 7-O-glucoside shows an inverse relationship with green pixel (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

due to the confounding effect of genotype being greater in the brown coloured seed (Fig. 2a). However, rutin did show a significant negative correlation with green pixel numbers which were markers for white coloured seeds. These data suggests that phenotypic data has potential uses in suggesting underlying nutritional traits for rapid screening in tef varieties. This pipeline can be validated by screening the nutritional grain diversity in a wider population of tef varieties.

Funding sources

Funding for the current study was provided by the Marie Skłodowska-Curie Individual Fellowship (Horizon, 2020; Project 842118-SUPERTEFF).

Author checklist

Aiswarya Girija: Conceptualization, Methodology, Writing- Original draft preparation: Vincianne Le Bihan.: Methodology, Writing- Original draft preparation. Zhenyu Wang Methodology Jiwan Han Methodology, Writing- Original draft preparation Luis A J Mur: Supervision, Writing-Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

data are provided in supplementary files

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2022.103573.

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