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Unveiling the root–rhizosphere environment of perennial wheat: a metabolomic perspective

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Abstract

Background Perennial grain roots grow continuously, enhancing soil carbon sequestration and forming a "holobiont" with the microbiome, essential for nutrient acquisition and stress resilience. Consequently, perennial grains serve as ideal models for investigating long-term dynamics between root systems and the rhizosphere environment. Despite their potential, the rhizosphere environment of perennial grains remains underexplored. This research utilizes an untargeted metabolomic approach to characterize the root–rhizosphere molecular signals in four new perennial grain (NPGs) lines named 235a, 280b, 11,955, and OK72, across four years of growth.

Results Metabolomic analysis annotated 2,527 metabolites, most of which originated from fungi (30.3%), bacteria (23%), and plants (15.5%). Principal component analysis explained 54.8% of the variation between rhizosphere and root metabolites, with 8.7% variation separating 1st and 4th year root metabolites, while rhizosphere metabolites showed less variation between years. The comparison between the annual durum wheat variety and NPGs revealed 616 differentially abundant metabolites in roots and 15 in the rhizosphere, already at the 1st year of growth. In the 4th year, NPGs metabolomes diverged significantly from *Thinopyrum intermedium*, which stood in the soil for 11 years, with 184 root and 138 rhizosphere differentially abundant metabolites. Comparison between genotypes diversified NPGs in the 1st year, showing a higher abundance of root metabolites for OK72 compared to the other lines, including key modulators of root architecture like glutathione and serotonin, and compounds from α-linoleic acid metabolism, which are known to induce systemic resistance against pathogens and herbivore defense. Differences among NPGs also emerged in the 4th year, with OK72 separating from the other three, sharing with *Thinopyrum intermedium* a higher abundance of purine nucleosides and diazanaphthalenes.

Conclusions The metabolomic analysis revealed that starting from the 1st year, the roots of NPGs produce a set of metabolites distinct from those of the annual durum species, many of which are defense molecules against biotic and abiotic stresses (e.g., syringic acid, glutathione, and α-linoleic acid pathway compounds). The OK72 genotype, which

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exhibits below-ground traits more aligned with perennialism, differs from the other lines in the abundance of several interesting metabolites, confirming it as an ideal parental candidate for developing new perennial wheat lines.

Keywords Root metabolomics, Rhizosphere environment, Perennialism trait, Crop resilience

Background

Plants produce and secrete primary and specialized metabolites that play a critical role in a wide range of biological activities [1]. The rhizosphere environment is one of the most significant hotspots for chemical signaling and interactions between plants and soil organisms [2]. Plant roots generate metabolites to protect against biotic and abiotic stresses, many of which are exuded into the rhizosphere, where they can also influence the composition and function of microbial communities [2]. Primary metabolites, such as lipids, amino acids, nucleic acids, and carbohydrates, are universally present in the plant kingdom and form the bulk of rhizodeposits. These compounds also serve as precursors for specialized metabolites [3]. Among specialized metabolites, phenolics, terpenes, and nitrogen-containing compounds are the major groups and the most investigated [4]. Produced in trace amounts, these compounds play pivotal roles in helping plants adapt to unfavorable conditions [5]. Root-released specialized metabolites have also been demonstrated to modulate the rhizosphere microbiome selectively. Compounds like phenolic-related compounds, triterpenes, and coumarins have been shown to influence the formation and maintenance of *Arabidopsis* specific microbiota, shaping the microbial community in and around its roots [6, 7]. Benzoxazinoids, which are indole-derived defense compounds found in the roots of cereal crops, impact not only the root and bud microbial communities but also the rhizosphere microbial community [8]. The metabolite composition in the rhizosphere is also influenced by secretions from rhizobacteria, fungi, and other soil organisms [9], which are increasingly recognized for their potential to shape plant growth, development, and resilience. Rhizosphere microorganisms directly impact crop growth, development, and health by secreting a diverse array of metabolites, including phytohormones, antibiotics, vitamins, and other bioactive compounds. As well as those produced by plants, these metabolites are essential for mediating plant-microbe interactions, influencing nutrient uptake, disease resistance, and responses to environmental stress [10, 11]. The application of metabolomics, utilizing high-resolution methodologies, provides qualitative and quantitative insights into detailed metabolite profiles, facilitating the monitoring of changes in crop rhizosphere soil. This aids in decrypting the relationships that arise within this intricate and dynamic environment and holds the potential to facilitate the modeling of plant-microorganisms' reciprocal responses [12, 13]. In this context, an unbiased analysis of metabolites is essential for comprehending the complex interactions between plants and the rhizosphere inhabitants, including physiological, symbiotic, and pathological relationships [9].

Although cereals are a critical food supply, they have not received sufficient attention in studies of root and rhizosphere metabolomes. Saia et al. [14] reported that metabolomic studies on cereals account for only 17% of the total, and of these, just 30% focus on wheat, with most research directed toward the metabolomic profiling of grains rather than roots or the rhizosphere. Unlike annual wheat, perennial wheat can be cultivated for multiple seasons and harvested yearly, significantly reducing the need for tillage. Indeed, perennial wheat is gaining attention for its environmental benefits, but its adoption is limited by very low yields (approximately 460 kg/ha), which is less than a quarter of typical annual wheat yields. Nevertheless, ongoing research and breeding efforts aim to enhance the yield and agronomic traits of perennial wheat, with the goal of scaling up production to better compete with annual wheat in the future. In the 1st year of growth, perennial wheat develops root biomass comparable to that of annual wheat. However, recent studies indicate that root expansion primarily occurs during the first regrowth period [15]. From the 2nd year onward, the continuous input of carbon into the soil stimulates microbial biomass and activity, gradually shaping the composition and functions of rhizosphere microbial communities over time [16–18]. Over the years, the roots of perennial wheat continue to grow, actively contributing to soil carbon accumulation [19]. The perennial habit entails numerous benefits, including enhanced nutrient uptake, environmental resilience, weed suppression, reduced soil erosion, and minimized nutrient leaching [19]. Additionally, permanent soil cover and reduced soil disturbance foster highly structured and complex food webs, promoting functional biodiversity and increasing the biomass of soil microbial communities—key conditions for conserving soil diversity [20, 21]. For these reasons, perennial grains serve as an excellent model for studying the long-term dynamics between root system growth and the rhizosphere environment. Despite their potential, there is limited information on the rhizosphere environment of perennial grains. Recent studies have focused on characterizing the endosphere and rhizosphere microbiomes of perennial wheat grown across various European locations, as well as isolating and characterizing bacterial species with plant growth-promoting rhizobacteria (PGPR) traits and biocontrol activity

against pathogenic fungi [18, 22–24]. To date, only one study has explored the metabolome profile of roots and root exudates of the perennial wheatgrass *Thinopyrum intermedium* (Kernza®), highlighting the significant role of its metabolome in suppressing nitrification through the active exudation of multiple nitrification inhibitors [25].

We previously demonstrated that changes in the chemical properties of the rhizosphere environment over time led to shifts in the microbial community composition of four new perennial grains (NPGs) lines. These findings revealed perennialism as the primary factor influencing soil biodiversity composition and functions, although some differences were also observed among genotypes, independent of the duration of residence in the soil [18]. In this study, we aimed to define the metabolomic profile of the roots and rhizosphere environment of the same four selected NPG lines, 235a, 280b, 11,955, and OK72, from the 1 st to the 4th year of residence in the soil. For this purpose, we compared these profiles to those of an annual durum wheat variety and the 11-year-old perennial species *Thinopyrum intermedium*. We hypothesized that both the time of soil residence and the genotype could determine specific changes in root exudation that can influence the rhizosphere environment and crop resilience.

Methods

Plant materials

The field experiment was set up in Central Italy at the "Montelibretti" experimental farm station (CREA-IT, Rome) (Lat 42°08'N; Long 12°44'E; 20 m a.s.l.) in the Tiber valley. The area is characterized by a sub-humid Mediterranean climate with annual rainfall of 848 mm and a mean air temperature of 15.9 °C (historical series 2005–2020). The soil is classified as arenosol with a silty clay loam soil texture. The experimental field (30 m x 5 m) was placed in a flat and homogeneous area of the experimental farm. Before planting, the experimental site hosted common and durum wheat. The perennial wheatgrass *Thinopyrum intermedium* (Tpi) and four NPGs derived from crosses between *Triticum aestivum* and *Thinopyrum* spp. kindly provided by the Land Institute (USA) and previously selected for their good technological and nutritional quality traits [26, 27] (namely CPI-147235a, CPI-147280b, 11955, and OK7211542, hereafter 235a, 280b, 11955, and OK72, respectively) were sown in November 2020 (year 1) and in November 2017 (year 4). The annual durum wheat *cv* Ardente was sown only in November 2020, whereas Tpi was sown in November 2010. The elementary plot consisted of eight rows, 17 cm apart, sown with 400 germinating kernels/m^2. Plots were fertilized only in the first year at a rate of 150 kg/ha of N (commercial urea fertilizer), applied in three top-dressings: at sowing, at emergence, and at tillering phases, and no irrigation was used all the years of plant growth. Weeds between plots were mechanically controlled, while those within rows were removed by hand.

Rhizosphere and root sampling

Rhizosphere samples of perennial genotypes (years 1 and 4) and the annual durum wheat cultivar were collected in June 2021. Four plants for the genotypes 235a, 280b, 11,955, OK72, and *Triticum durum*, and only three plants for *Thinopyrum intermedium*, due to the smaller amount of plant material. Rhizosphere and root samples were collected from the initial 20 cm of the soil's root zone and following the procedure described by McPherson et al. [28]. Before collecting both rhizosphere and root samples, non-adherent soil was manually removed from the roots. Rhizosphere samples were obtained by detaching soil adhering to the roots using a spatula and then freeze-dried before metabolomic analysis. For root sampling, excised roots were placed in 35 mL of 50% bleach + 0.01% Tween 20 and shaken for 60 s. Half of the volume was poured off, and 35 mL of 70% EtOH was added. After 60 s of shaking, EtOH was removed, and roots were washed three times with 35 mL of sterile, ultrapure water. Root samples were stored at −80 °C until metabolite extraction.

Metabolomic analysis

Metabolites from root and rhizosphere soil samples were extracted in 0.1% HCOOH-acidified 80% methanol, centrifuged, and filtered through a 0.22 µm cellulose membrane into vials for analysis. Untargeted metabolomic analysis and metabolite identification in sample extracts were performed at oloBion Laboratory (Barcelona, Spain) using an ultra-high performance liquid chromatograph coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS) (Agilent 1290 Infinity II UHPLC; Agilent 6560 Ion Mobility Q-TOF) and an Acquity UPLC BEH C18 (1.7 µm, 2.1 mm × 100 mm, Waters) column, following the procedure described by Bonini et al. [29]. The analytical conditions and MS settings are described in Tsugawa et al. [30], with some modifications. MS/MS spectra were collected at collision energies of 10, 20, and 50 eV with an acquisition rate for MS1 of 4 spectra/s (100 ms) and an acquisition rate for MS/MS of 3 spectra/s (77 ms) with 4 precursor ions per cycle. The samples were injected in both electrospray ionization (i.e., positive and negative) modes. Feature identification, alignment, and deisotoping were performed using MS-DIAL 4.9 [31], and feature quantification used raw peak height. Sample normalization was performed using sample weight. MS-FLO [32] was used to filter common adducts. Identification was performed with accurate mass filtering, MS/MS similarity matching, as

well as retention time filtering using validated retention times from standards when available and otherwise using predicted retention times from Retip [29]. The majority of erroneous identification candidates for each feature, as well as in-source fragmentation identifications, were removed using this predicted retention time filter, thus improving the identification confidence beyond a putative annotation. The compounds detected were annotated and classified through the PlantCyc 16.0.2 database (Plant Metabolic Network, Michigan State University). From the InChIKey associated with each identified feature, chemical classification information is retrieved from ClassyFire [33] and NPClassifier [34] to enhance biological interpretation of the results. The annotation of the biological origin of metabolites as well as the handling of metabolites that are common to multiple organisms is described in: https://patents.google.com/patent/WO2024074492A1/ [35].

Data mining and processing

All data analyses and principal component analyses (PCA) visualizations were performed after log transformation and Pareto scaling of metabolite data in the MetaboAnalyst platform. To analyze the differentially abundant metabolites (DAMs), One-way ANOVA followed by *post hoc* Fisher's LSD ($p < 0.05$, FDR < 0.05) was performed on the abundance of individual metabolites. Hierarchical clustering was calculated on mean values of DAMs classes, with similarity determined by Euclidean distance and Ward's clustering. Dendrograms were represented as heatmaps generated with R Studio (version 2024.12.1) *pheatmap* package. PCA Biplots were generated using R Studio *FactoMineR* and *factoextra* packages.

Results

Metabolites classification and origin

Following the untargeted analysis of the samples, a total of 2527 unique metabolites were annotated, belonging to 17 different superclasses and 169 classes of metabolites (Suppl. Table 1). The most prevalent identified superclasses included lipid and lipid-like molecules (21.8%), organoheterocyclic compounds (20.6%), organic acids and derivatives (19.4%), benzenoids (11.4%), organic oxygen compounds (10.9%), and phenylpropanoids and polyketides (9.7%) (Fig. 1A), which were predominantly associated with fungi, followed by bacteria and plants, according to LOTUS classification (Fig. 1B).

Effects of genotype and year on root and rhizosphere metabolome

To evaluate the contribution of both plant genotypes and permanence in the soil in shaping the root- and rhizosphere-metabolome, a principal component analysis (PCA) was employed, comparing 1 st -year and 4th -year roots and rhizosphere metabolites of NPGs. The annual durum wheat *cv* Ardente and the 11-year-old perennial wheatgrass Tpi were also inserted in the comparison (Fig. 2). PCA allowed the discrimination of four distinct groups. The synthetic variable PC1 explained the largest variability (54.8%) and allowed for the separation of the root- vs. rhizosphere-metabolome. PC2 (explaining 8.7% variability) allowed efficient separation based on the permanence of plants in the soil. Furthermore, PCA emphasized that the metabolomic profile of 1 st year NPGs aligned with that of *cv* Ardente. On the other hand, the metabolome of Tpi, which is characterized by the longest residence time in the soil, did not join any groups, remaining independent for both the root- and rhizosphere-samples (Fig. 2). To summarize, these data demonstrate the significant impact that the residence time of perennial plants in the soil had on their root-rhizosphere metabolomic profiles.

The ANOVA test was used to better define the impact of time of residence and genotype in shaping the metabolome profiles of roots and rhizosphere NPGs samples. The comparisons were performed to highlight NPGs' metabolome, comparing the 1 st year of growth with *T. durum cv* Ardente at the 4th year of growth with the eleven-year old Tpi. In addition, the NPGs' genotype-specific metabolome in both root and rhizosphere was compared (Fig. 3). In root, the comparison between the NPGs at the 1 st year and durum wheat *cv* Ardente showed 616 differentially abundant metabolites (DAMs), while only 15 DAMs were identified in the rhizosphere (Fig. 3A, Suppl. Table 2). The comparison between the four NPGs genotypes showed 352 DAMs in the roots of the 1 st year plants, while a very small number of DAMs were found in the roots at the 4th year and in the rhizosphere at the 1 st and 4th year samples (Fig. 3B, Suppl. Table 3). A higher number of metabolites, 184 in the root and 138 in the rhizosphere, showed a significant variation in abundance in the comparison between the NPGs at the 4th year and the 11-year-old Tpi (Fig. 3A; Suppl. Table 4).

The root and rhizosphere metabolome of NPGs vs. the annual durum wheat cultivar

PCA analysis on root metabolites of NPGs lines and *cv* Ardente at the 1 st year allowed discrimination of the annual durum wheat cultivar from NPGs lines, with PC1 and PC2 explaining 17.4% and 15% of variability, respectively (Fig. 4A). In roots, 616 DAMs were found between the samples (Fig. 3A, Suppl. Table 2), belonging to 99 classes. Sixty-three classes showed a higher number of metabolites that were more abundant in NPGs with respect to the annual species *cv* Ardente, while 36 classes showed the opposite trend (Fig. 4B). The most represented DAMs belonged to

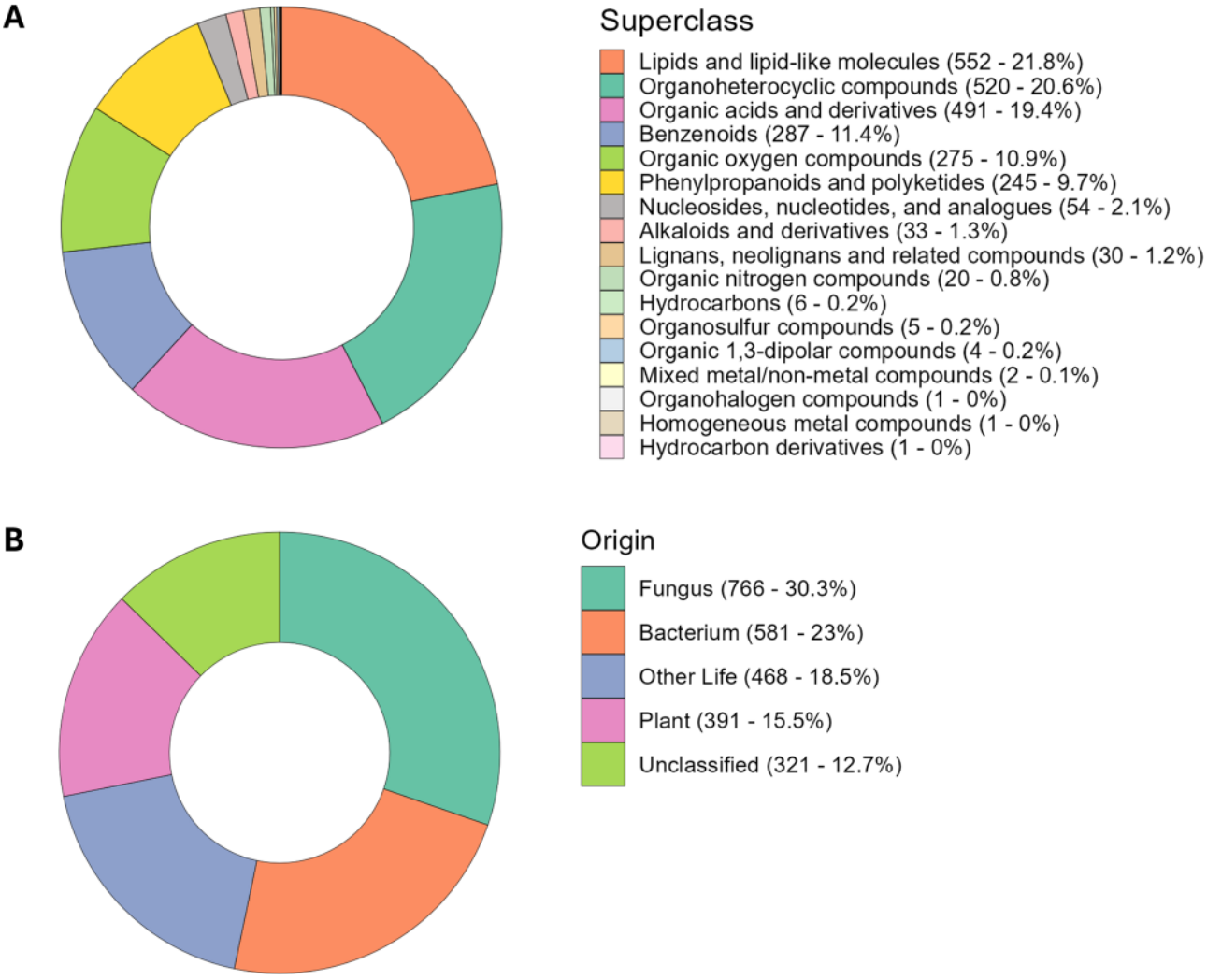

Fig. 1 Overview of the 2527 unique metabolites annotated, subdivided according to their superclass (ClassyFire classification) (**A**) and their origin according to Lotus (**B**). The values in brackets represent the number of metabolites annotated for each superclass and their relative percentage, respectively

organoheterocyclic compounds (39.4%) and phenylpropanoids and polyketides (20.2%) superclasses, followed by the benzenoids superclass (9.1%). Many interesting classes as flavonoids, lactones, benzofuranes, benzene, and substituted derivatives, were found to be more abundant in the NPGs at the 1st year with respect to the annual durum wheat. Among metabolites, pseudouridine (nucleosides, nucleotides, and analogues), adenine (organoheterocyclic compounds), syringic acid (benzenoids), and dalpatein-apiofuranosyl-glucopyranoside (phenylpropanoids and polyketides) showed a higher abundance in all NPGs lines *vs. cv* Ardente (Fig. 4C).

PCA analysis performed also on rhizosphere metabolites of NPGs and *cv* Ardente at the 1st year did not reveal a clear separation between groups (Fig. 5A). In the rhizosphere, only 15 metabolites (Fig. 3A, Suppl. Table 2), belonging to 10 classes, showed differences between durum wheat *cv* Ardente and NPGs (Fig. 5B). Nine classes showed a higher number of metabolites that were more abundant in NPGs vs. the annual durum wheat, including fatty acyls, isoflavonoids, carboxylic acid and derivates. Only the prenol lipids class showed a higher number of metabolites more abundant in *cv* Ardente than in NPGs (Fig. 5B). Molecules specifically secreted by plants like neoarctin A (furanoid lignans) and malonyldaidzin (isoflavones) were found to be more abundant in at least two perennial genotypes, 280b and 235a, and OK72 and 11,955, respectively, while, Cyclo (D-Tyr-L-Leu) (carboxylic acids and derivatives) and dechlorodehydrogriseofulvin (benzofurans) were found to be more abundant in all the NPGs with respect to durum wheat *cv* Ardente (Fig. 5C).

The 1st year root- NPGs genotype specific metabolome

PCA performed on root metabolites at the 1st year NPGs allowed a slight separation between genotypes;

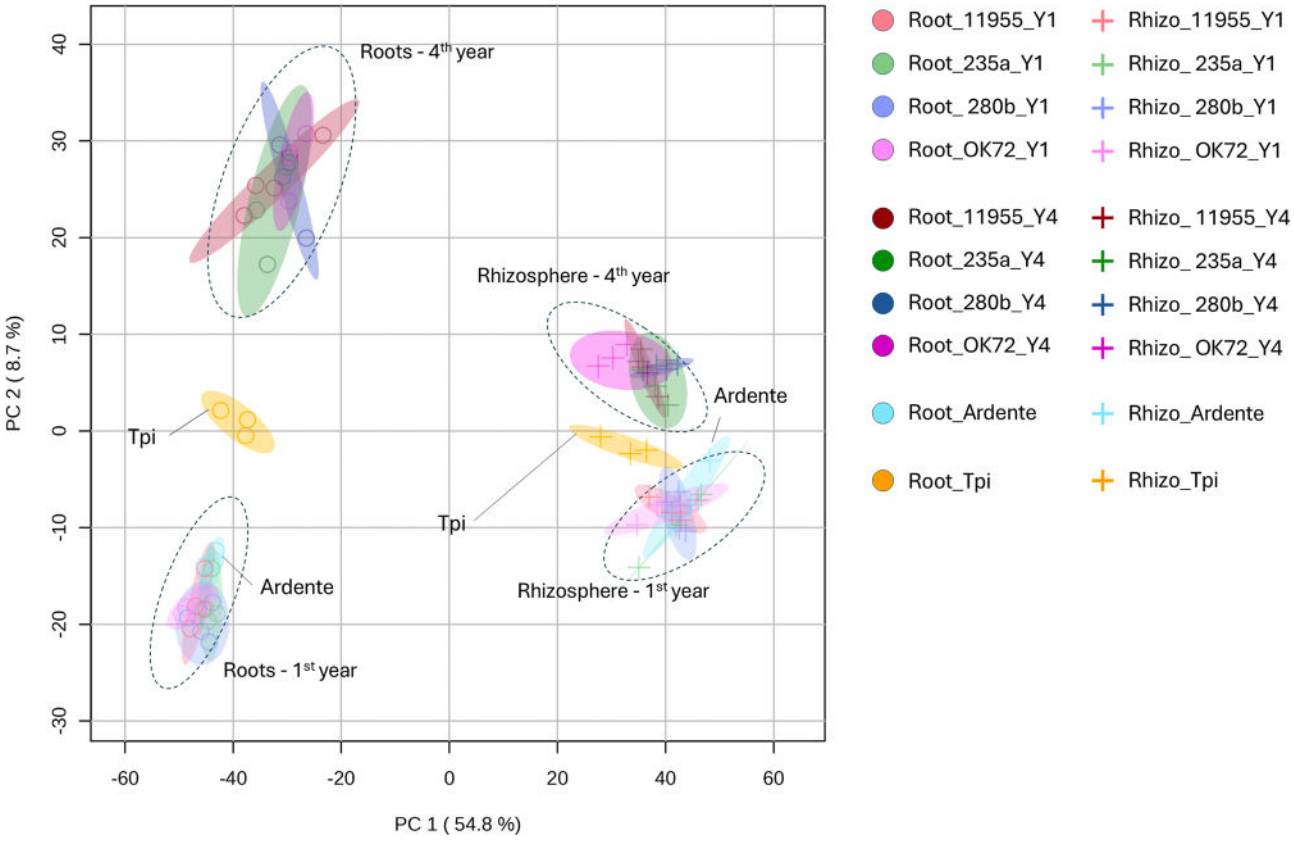

Fig. 2 Principal component plots (PCA) of metabolite profiles in 11955, 235a, 280b, and OK72 root or rhizosphere metabolome collected after one year (Y1) or four years (Y4) of growth. Metabolites in *cv* Ardente (*Triticum durum*) and Tpi (*Thinopyrum intermedium*) root and rhizosphere were collected after one and eleven years of growth, respectively. Ellipse displays 95% confidence regions for each cluster. Dotted ellipses highlight the time- and root/rhizosphere-specific clustering of NPGs

PC1 accounted for 21.5% of the total variance, while PC2 accounted for 11.1%. (Fig. 6A). Genotypes 235a and 280b appeared to overlap with each other, while the OK72 genotype separated from 235a and 280b and partially overlapped with 11,955. Three hundred and fifty-two DAMs (78 metabolite classes) were found among the root metabolome of NPGs at the 1st year of growth (Fig. 3B, Suppl. Table 3). DAMs belonged to organoheterocyclic compounds (42.3%), phenyl propanoids and polyketides (16.7%), benzenoids (10.3%), and lipid like molecules (6.4%). Among the NPGs, OK72 distinguished from the other genotypes, showing a higher number of metabolites that were significantly more abundant in 51 different classes (Fig. 6B).

A highly represented class in OK72 with respect to the other genotypes was the fatty acyl class (Fig. 6B), which includes the metabolites hydroxy-oxo-octadecenoic acid, HpOTrE, and OPDA, belonging to the a linoleic acid metabolism (Fig. 6C, Suppl. Figure 1). Other interesting classes, highly represented in OK72 with respect to the other NPGs genotypes, were the cinnamic acids and derivatives class, to which trans-Ferulic acid belongs, and the indole and derivatives class (Fig. 6C). Serotonin (indole and derivatives) was shown to be more abundant in both the OK72 and the 280b genotypes. Similarities between the OK72 and 11,955 lines were observed for two metabolites: glutathione (carboxylic acids and derivatives) and kaempferol-glucosyl-glucosyl-glucoside (flavonoids), which were respectively significantly more and less abundant with respect to the other two NPGs lines (Fig. 6C).

4th year root and rhizosphere NPGs metabolome vs. Thinopyrum intermedium

The PCA on NPGs in the 4th year and the 11-year-old Tpi metabolome showed a clear separation between NPGs and Tpi, both for root and rhizosphere (Fig. 2). As expected, the uniqueness of the metabolomic profile of Tpi in comparison to NPGs is well evident in Fig. 3A, which showed 184 and 138 DAMs in roots and rhizosphere, respectively. Metabolites belonging to 50 and 42 classes were more abundant in the root and rhizosphere of Tpi, respectively (Suppl. Table 4, Suppl. Figure 2). The opposite trend was observed for 6 classes in the root and 11 classes in the rhizosphere (Suppl. Table 4, Suppl. Figure 2). PCA performed on the DAM classes between the

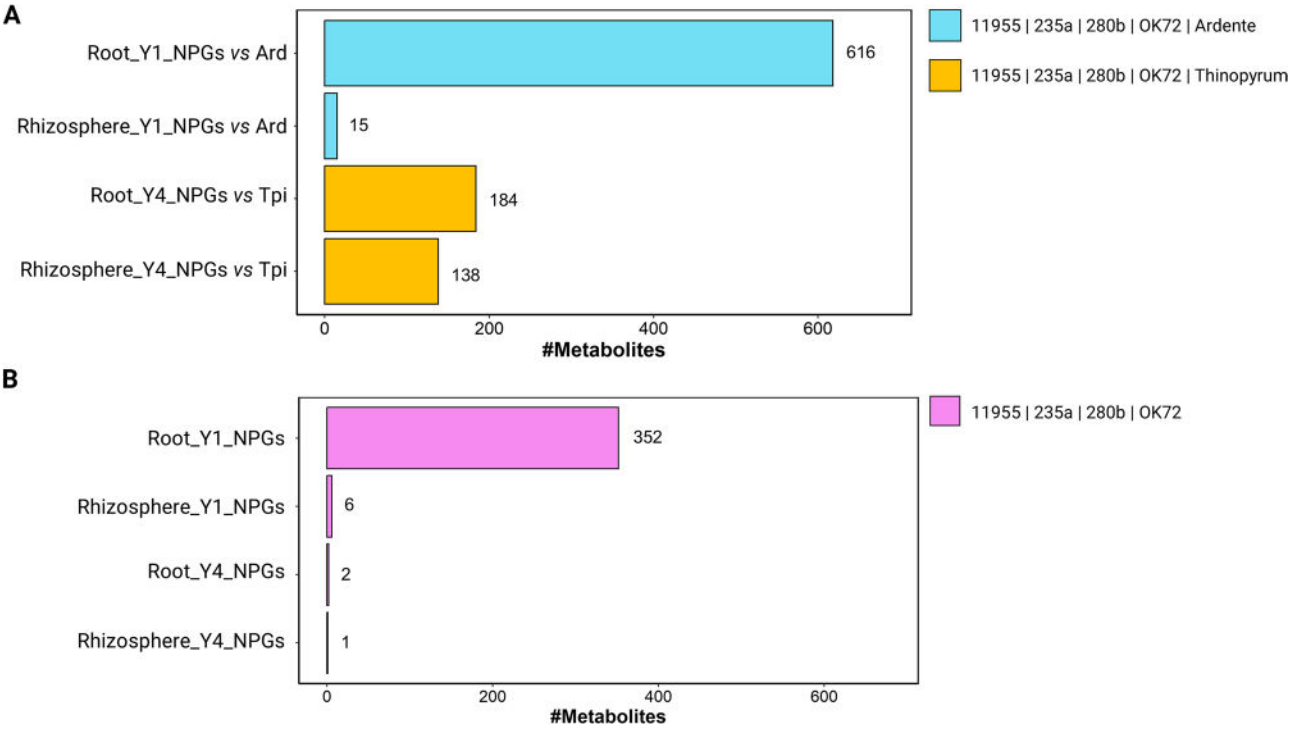

Fig. 3 Graphical representation of DAMs identified in the different comparisons (ANOVA and Fisher's LSD *post hoc* $p < 0.05$). **A** Number of DAMs showing statistical differences in at least one NPG genotype compared to *T. durum cv* Ardente (Ard) or *Thinopyrum intermedium* (Tpi) in root and rhizosphere in the 1 st year (Y1) and in the 4th year (Y4), respectively. **B** Number of metabolites showing statistical differences within the four NPG genotypes in the 1 st year of growth in root and rhizosphere and the 4th year of growth in root and rhizosphere

11-year-old Tpi and NPGs at the 4th year of cultivation showed that PCA1, explaining 66.6% of the variability, separated Tpi and NPGs in the root samples. Metabolites such as lactams, flavonoids, harmala alkaloids, quinolines, and derivatives, associated with Tpi species (Fig. 7A). In the rhizosphere samples, PCA1, explaining 50.7% of the variability, separated Tpi from NPGs lines, and metabolites belonging to classes isocoumarins and derivatives, harmala alkaloids, isoquinolines and derivatives, and pyridine nucleotides highly correlated with Tpi species. It is interesting to note that in rhizosphere samples OK72 line diverged from the other NPGs lines with the contribution of metabolites belonging to purine nucleosides and diazanaphthalenes classes, aligning more closely with the perennial parental species (Fig. 7B). The comparative analysis of NPG genotypes at the 4th year of growth revealed the variation of only 2 metabolites in roots and only one metabolite in the rhizosphere, indicating that the differences among NPG genotypes observed after the 1 st year of residence in the soil were scarce after 4-years of soil residence (Fig. 3B, Suppl. Table 3).

Discussion

The root-rhizosphere metabolome of NPGs

Perennial grains can regrow for several years, developing a root system that grows over time, making perennials a good model for studying molecular plant-microorganism interactions in the rhizosphere over time. In this work, the untargeted metabolomics approach was functional to investigate variations in the composition of the root and rhizosphere metabolome between the 1 st and 4th year of NPGs residence in the soil. The soil-root metabolomics approach has led to the identification of different classes of metabolites, which include, in order of abundance, lipid and lipid like molecules, organoheterocyclic compounds, organic acid derivatives, benzenoids, organic oxygen compounds, phenyl propanoids, and polyketides (Fig. 1). The metabolites identified vary mainly depending on the root or rhizosphere environment, the year of cultivation, and the genotype (Fig. 2). Analyzing the metabolomic profiles of both root and rhizosphere of NPGs at the 1 st and 4th years, we observed a clear separation based on the origin of the samples (root and rhizosphere) but also on their time of residence in the soil (Fig. 2). Several studies have focused on the effect of genotype, growth stage, and soil residence in shaping the rhizosphere microbiota, indicating a prevalence of the effect of soil residence [36, 37]. Furthermore, it is well established that the root system of perennial plants represents a continuous influx of C to the soil and that this can stimulate activity, biomass, and can change the microbial community over time [16, 20, 22, 38]. Since the

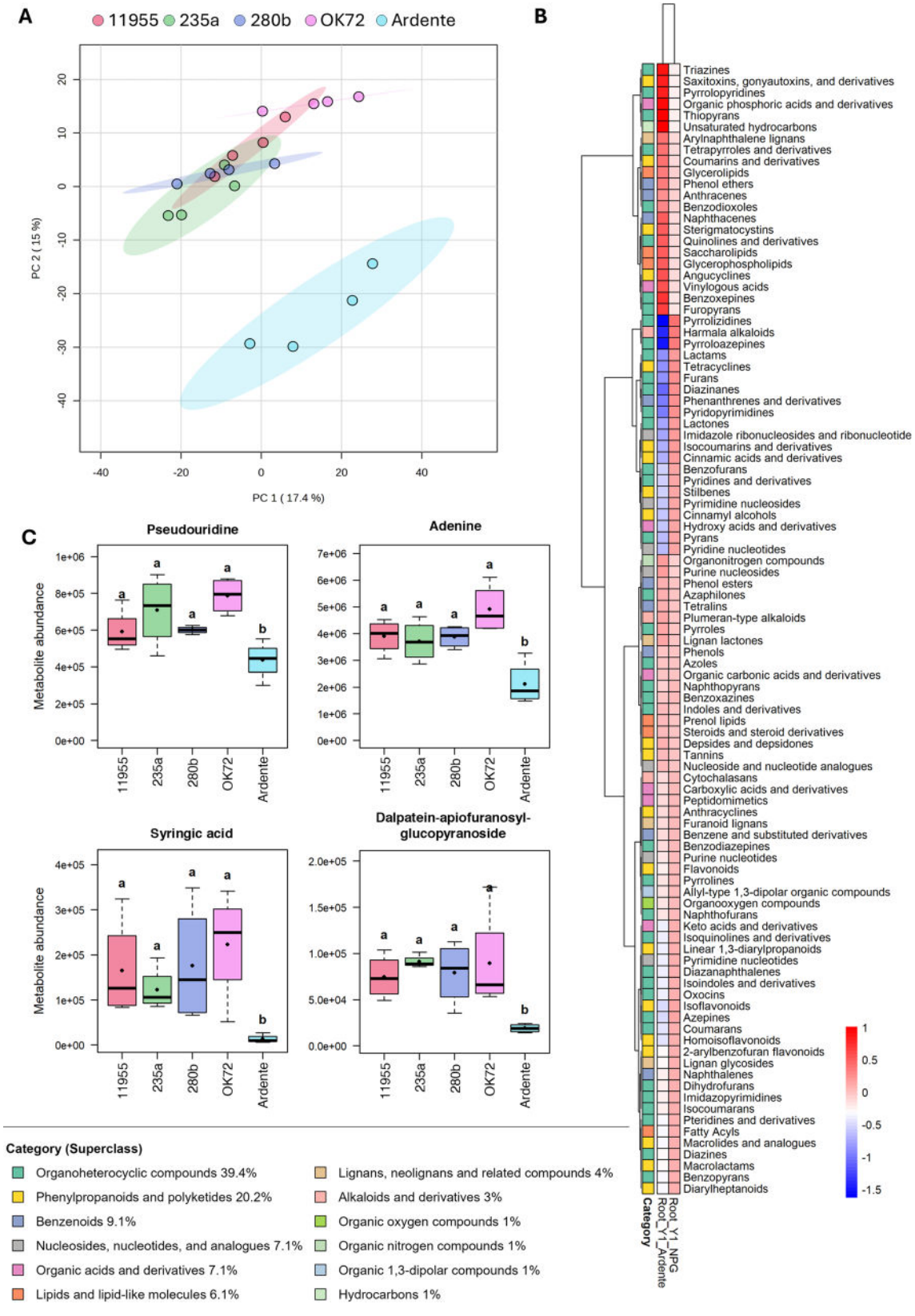

Fig. 4 (See legend on next page.)

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Fig. 4 Comparison of the root metabolomic profiles between NPGs in the 1 st year of growth and *T. durum cv* Ardente. **A** Principal component plot (PCA) of metabolite profiles from root and grouped according to their genotype (11955, 235a, 280b, OK72, and durum wheat *cv* Ardente) after one year of growth. Ellipse displays 95% confidence regions for each cluster. **B** Hierarchical clustering analysis and heatmap visualization of root metabolites grouped according to their classes (ClassyFire classification). Each class is associated with its own Superclass by a different color. Significant metabolites in the comparison of NPGs vs. durum *cv* Ardente were determined using One-way ANOVA and Fisher's *post hoc* ($p > 0.05$). Higher concentrations are shown in red, while lower concentrations are shown in blue. **C** Box plots showing abundance of root metabolites adenine, dalpatein-apiofuranosyl-glucopyranoside, pseudouridine, and syringic acid in NPGs at the1st year and *cv* Ardente. Different letters represent statistically significant differences (One-way ANOVA and *post hoc* Fisher's LSD ($p < 0.05$, FDR < 0.05)). Means and medians are represented by black dots and lines, respectively

rhizosphere metabolome is, for all intents and purposes, a combination of metabolites produced and transformed by plants and the microbial colonizers of their rhizosphere, it is not surprising that the metabolomic profiles of rhizospheres vary considerably over time. Similarly, a clear year-specific clustering was observed for root, even though a larger contribution of genotype would have been expected [39]. Thus, our results converge with the statements found in the literature, but they also indicate that time could have an impact on the metabolites produced within the root. Tpi was the only genotype to segregate from the others for both root and rhizosphere (Fig. 2), but this is likely attributable to the eleven-year stay in the soil, with the ability to continue to grow below-ground and to produce culms and spikelets over the years.

NPGs' metabolome reveals distinctive traits compared to an annual durum wheat cultivar after 1 st year of soil residence

Comparing the 1 st year NPGs root metabolome with durum wheat *cv* Ardente, a large number of DAMs were observed (Figs. 3A and 4). Among those, lactones and isoflavonoids, which are important rhizospheric signals that influence rhizobia-root interactions and modulate uptake of essential nutrients and water absorption by plants [40–42], and cinnamic acid and derivatives, involved in shaping root architecture by stimulating the formation of lateral roots [43], were found enriched in NPGs. Notably, lactones, cinnamic acids, and isoflavonoids, which include derivatives such as a glucopyranoside compound (Fig. 4C), comprise a substantial category of allelochemicals. These compounds have the potential to influence soil weed growth, microbial communities, and overall soil health [44, 45]. Isocoumarins and derivatives is another class of metabolites more abundant in NPGs vs. annual durum wheat variety; these molecules are produced by endophytic fungi and have antibacterial, antifungal, and antioxidant activities [46]. The comparative analysis of NPGs and *cv* Ardente during the first year allowed the identification of nitrogen management-related molecules, specifically adenine, pseudouridine, and syringic acid, which were found to be more prevalent in the roots of the NPGs line (Fig. 4C). Adenine and pseudouridine serve as nitrogen sources for immediate catabolism, enabling the liberation of ammonia for reassimilation [47]. Recently, Issifu and colleagues [25] found syringic acid less effective than vanillic acid, caffeic acid, vanillin, and phenylalanine in suppressing the growth of ammonia-oxidizing bacteria (AOB) and archaea (AOA). In another work, syringic acid was found to inhibit the growth of AOB and AOA, enhancing the availability of N to plants and reducing N loss via NO_3^- run-off and leaching and gaseous losses [48]. Interestingly, this observation is in line with our previous results, showing a decrease in the *Nitrososphaera* genus in NPGs rhizosphere from the 1 st to the 4th year of residence in the soil [18], and with other authors, who reported a lower abundance of ammonia-oxidizer bacteria in Kernza® than in annual durum wheat growing soils, indicating a potential of Kernza® to inhibit nitrification, an eco-physiological trait linked to the plant's perenniality and enduring root system [49]. The concurrent increase in levels of adenine, pseudouridine, and syringic acid, detected within the roots of NPGs, could contribute to improved nitrogen management - a trait commonly attributed to NPGs. This enhancement is potentially associated with the sustained development of the root system and a diminished nitrogen loss over time, thereby enabling a greater nitrogen reserve for forthcoming regrowth cycles. In addition, syringic acid is a root-derived specialized metabolite that can move to aerial parts and confer resistance to fungal pathogens [50]. The long-term exposure to pathogens imposed by perennial lifestyle probably played a role in determining the lower susceptibility to pathogens than annual wheats [51]. Indeed, for a long time, *Thinopyrum* spp. have been recognized as genetic resources for wheat improvement since their species contain numerous genes for resistance to pathogens [52, 53]. Wheatgrass shows high levels of resistance to many common wheat diseases that can be inherited by relative hybrids [51, 54].

Contrary to the root environment, fewer DAMs were observed analyzing the metabolome of the rhizosphere after one year, both in the NPG vs. *cv* Ardente comparison and in the comparison among the different NPGs lines (Fig. 3A, B). These data are consistent with our previous research, which demonstrated that one year post-sowing, the composition of bacterial and fungal microbial communities, as well as the primary metabolites in the NPG rhizosphere, did not significantly differ in comparison to *cv* Ardente [18], suggesting that the root-microbial

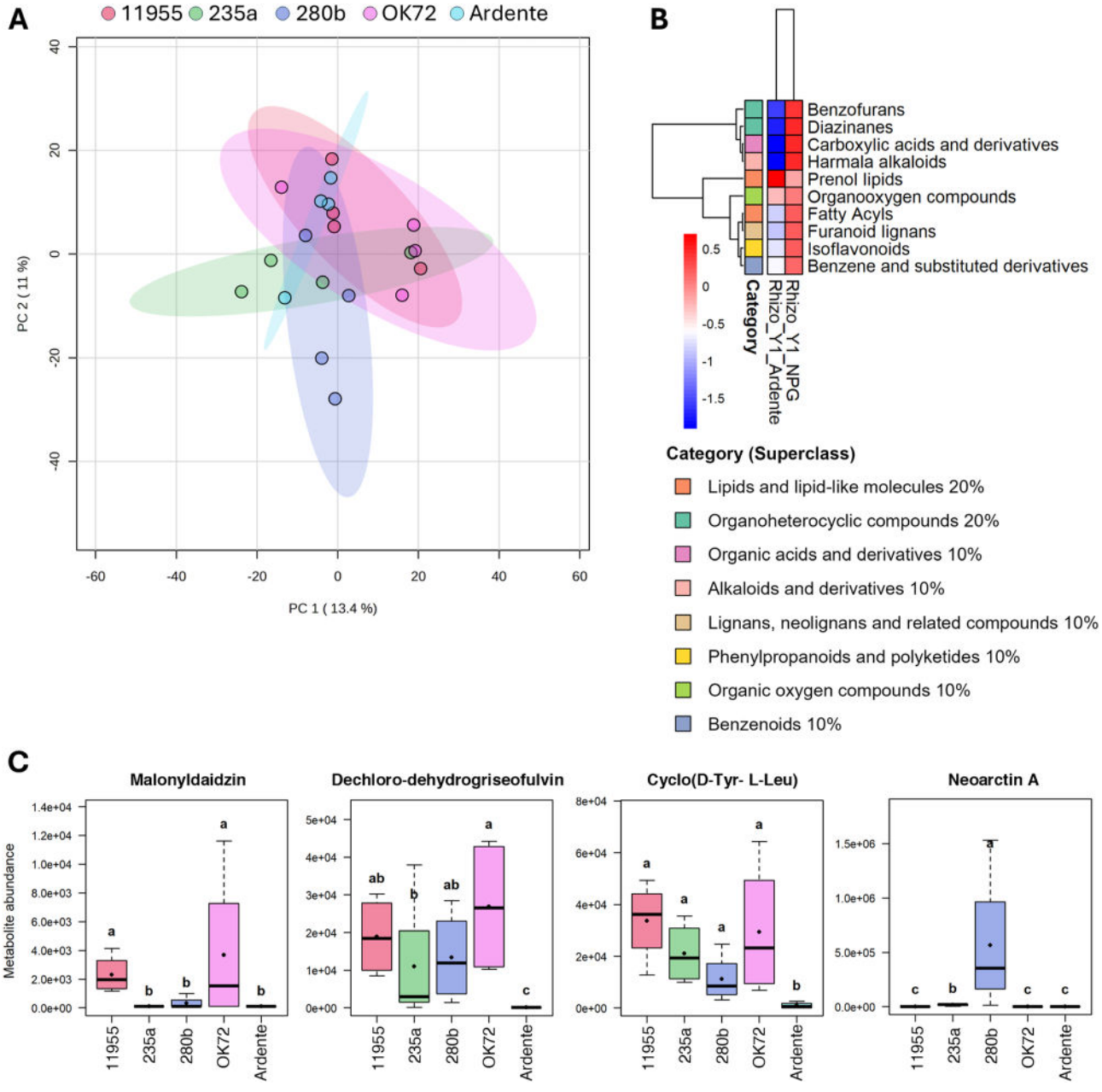

Fig. 5 Comparison of the rhizosphere metabolomic profiles between NPGs in the 1 st year of growth and *T. durum cv* Ardente. **A** Principal component plot (PCA) of metabolite profiles from rhizosphere and grouped according to their genotype (11955, 235a, 280b, OK72 and durum wheat *cv* Ardente) after one year of growth. Ellipse displays 95% confidence regions for each cluster. **B** Hierarchical clustering analysis and heatmap visualization of rhizosphere metabolites grouped according to their classes (ClassyFire classification). Each class is associated with its own Superclass by a different color. Significant metabolites in the comparison NPGs vs. cultivar Ardente were determined using One-way ANOVA and Fisher's *post hoc* ($p > 0.05$). Higher concentrations are shown in red, while lower concentrations are shown in blue. **C** Box plots showing abundance of rhizosphere metabolites malonyldaidzin, dechloro-dehydrogriseofulvin, Cyclo (D-Tyr- L-Leu) and neoarctin A, among NPGs. Different letters represent statistically significant differences (One-way ANOVA and *post hoc* Fisher's LSD ($p < 0.05$, FDR < 0.05)). Means and medians are represented by black dots and lines, respectively

processes that occur in the soil are very slow and take time to settle and fix to produce appreciable differences. In their rhizosphere, NPGs showed higher abundance of the antimicrobial molecules dechloro-dehydrogriseofulvin, belonging to organoheterocyclic compounds, and Cyclo (D-Tyr- L-Leu), belonging to carboxylic acids and derivatives classes and produced by fungi and bacteria, respectively (Fig. 5C) [55, 56]. At least two NPGs showed enrichment in malonyldaidzin (flavonoids) and neoarctin A (furanoid lignans) (Fig. 5C). Malonyldaidzin, released from root, is known to play a key role in the regulation of plant – plant and plant – microbe interactions

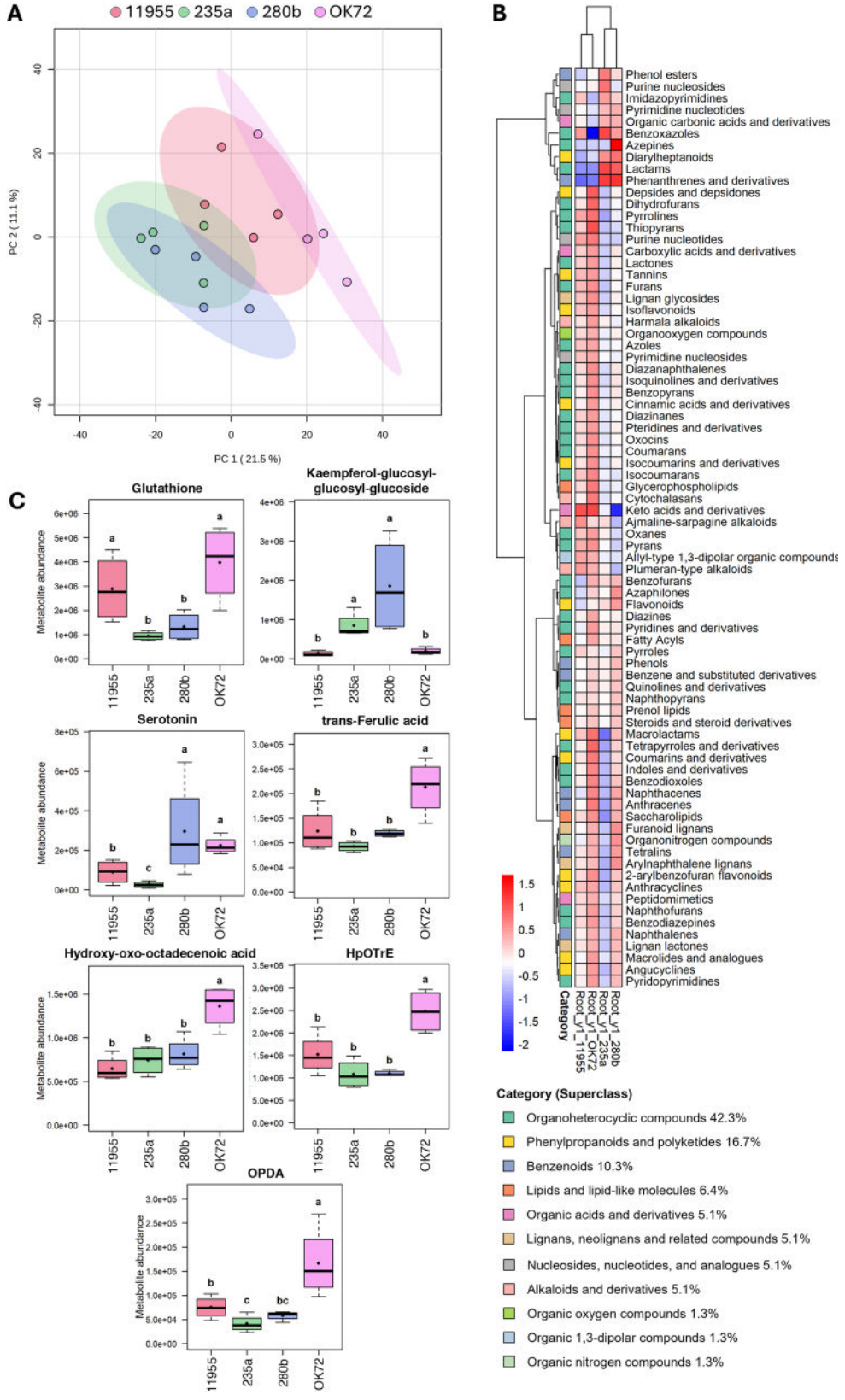

Fig. 6 (See legend on next page.)

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Fig. 6 Comparison of the root metabolomic profiles between NPGs. **A** Principal component plot (PCA) of metabolite profiles from root and grouped according to their genotype (11955, 235a, 280b, OK72) after one year of growth. Ellipse displays 95% confidence regions for each cluster. **B** Hierarchical clustering analysis and heatmap visualization of root metabolites grouped according to their classes (ClassyFire classification). Each class is associated with its own Superclass by a different color. Significant metabolites in the comparison between NPGs were determined using One-way ANOVA and Fisher's *post hoc* ($p > 0.05$). Higher concentrations are shown in red, while lower concentrations are shown in blue. **C** Box plots showing abundance of root metabolites glutathione, kaempferol-glucosyl-glucosyl-glucoside, serotonin, trans-Ferulic acid, hydroxy-oxo-octadecenoic acid, hydroperoxyoctadeca-trienoic acid (HpOTrE), and oxo-[pent-enyl]cyclopentenyl]octanoic acid (OPDA) among NPGs. Different letters represent statistically significant differences (One-way ANOVA and *post hoc* Fisher's LSD ($p < 0.05$, FDR < 0.05)). Means and medians are represented by black dots and lines, respectively

[57]. Although the biological activity of Neoarctin A has not yet been elucidated, lignans are well known for their -among others- antioxidant, antifungal, and insecticidal properties [58].

Comparing the DAMs between the four perennial hybrids at the 1st year, significant DAMs associated with root development and architecture were observed. In particular, glutathione was found to be more abundant in OK72 and 11955 genotypes than in the other two NPGs. This metabolite is primarily known as a cellular antioxidant molecule, but increased glutathione (GSH) promoted both the number and length of lateral roots in cereals [59]. In addition, the same two NPGs lines showed a significantly lower level of kaempferol-glucosyl-glucosyl-glucoside, which belongs to the flavonoid class. Kaempferol, its precursor molecule, is known to negatively modulate lateral root formation [60]. Serotonin, a member of the indoles and derivatives class, was more abundant in OK72 and 280b with respect to the other two lines. In *Arabidopsis*, Pelagio-Flores et al. [61] found that, at low concentrations, exogenous serotonin enhanced lateral root formation. In addition, it acts downstream of ABA in regulating suberization in rice and *Arabidopsis* and negatively regulates suberization in rice roots in response to salinity [62]. Interestingly, the OK72 genotype differentiated from the other NPGs by a higher abundance of several classes of metabolites (Fig. 6), such as cinnamic acids and derivatives, to which *trans*-Ferulic acid belongs (Fig. 6C). Common wheat accessions with high levels of *trans*-ferulic acid and other phenolic acids in the roots were generally strongly allelopathic to the growth of annual ryegrass [63]. In addition, *trans*-ferulic acid augments the antioxidant response of wheat, suggesting that it can improve the performance of common wheat under various environmental constraints [64]. Furthermore, a higher abundance of fatty acyls compounds was found in the OK72 rhizosphere; several molecules belonging to this class are known to be involved in rhizosphere signaling [65]. Three compounds belonging to the a linoleic acid metabolism, OPDA, hydroxy-oxo-octadecenoic acid and HpOTrE, shown to be more abundant in OK72 (Fig. 6C, Suppl. Figure 1). OPDA is a precursor of jasmonic acid, and it is involved in the trade-off between growth and defense responses to biotic and abiotic stresses in plants [66, 67]. Hydroxy-oxo-octadecenoic acid was found to be more abundant in *N. tabacum* and *P. persica* under abiotic stresses [68, 69]. Moreover, Gu et al. [70] found an enrichment of hydroxy-oxo-octadecenoic acid metabolite in *O. sativa* under pest attacks. In addition, the induction of the linoleic pathway has been associated with a defensive response against pathogenic microbes and herbivorous insects [71].

OK72 4th year metabolome shares similarity with Tpi and its perennial traits

The comparison between 4th year NPGs and Tpi highlighted significant differences in metabolite abundance both in roots and rhizosphere. Many classes of metabolites were more abundant in Tpi than NPGs lines in both root and rhizosphere (Fig. 7, Suppl. Figure 2). This could be attributable to the ability of Tpi to continue growing both at the epigeal and hypogeal levels over time, which is instead limited to a few years in NPGs. Major differences were observed at root levels (Fig. 3A, Suppl. Figure 2 A), where there was a general decrease over time of metabolite production in NPGs. Metabolites belonging to flavonoids, alkaloids, quinolines, and lactams were found to be more abundant in Tpi roots compared to NPGs (Fig. 7A, Suppl. Figure 2 A). The critical role of flavonoids in plant-rhizobiome crosstalk has been well-documented [72]. The antimicrobial activity of alkaloids, quinolines, and lactams may contribute to rhizosphere microbial community selection and pathogen control [73, 74]. In addition, the ability of β-lactams to modify root architecture, with an increase in hair roots, has been demonstrated in model plants [75]. Globally, the production of these compounds may contribute to the continuous stimulation of root growth in Tpi. Previous data showed that, in the rhizosphere, after four years, the composition of the NPGs rhizobiome was similar to the parental species *Thinopyrum intermedium*. This could probably be due to the minor environmental disturbances, which result in a saturation of the rhizospheric environment, which ultimately turns redundant [18, 76]. Despite similarity in the microbial composition, many DAMs distinguished the Tpi rhizosphere from 11,955, 280b, 235a, and OK72 genotypes (Fig. 3A, Suppl. Figure 2B). The differences between the rhizosphere of Tpi and NPGs were highlighted in Fig. 7B. OK72 separated from the other three NPGs, sharing with Tpi a higher abundance of purine nucleosides and diazanaphthalenes. The latter is a class of N-heteroaromatic compounds that includes compounds

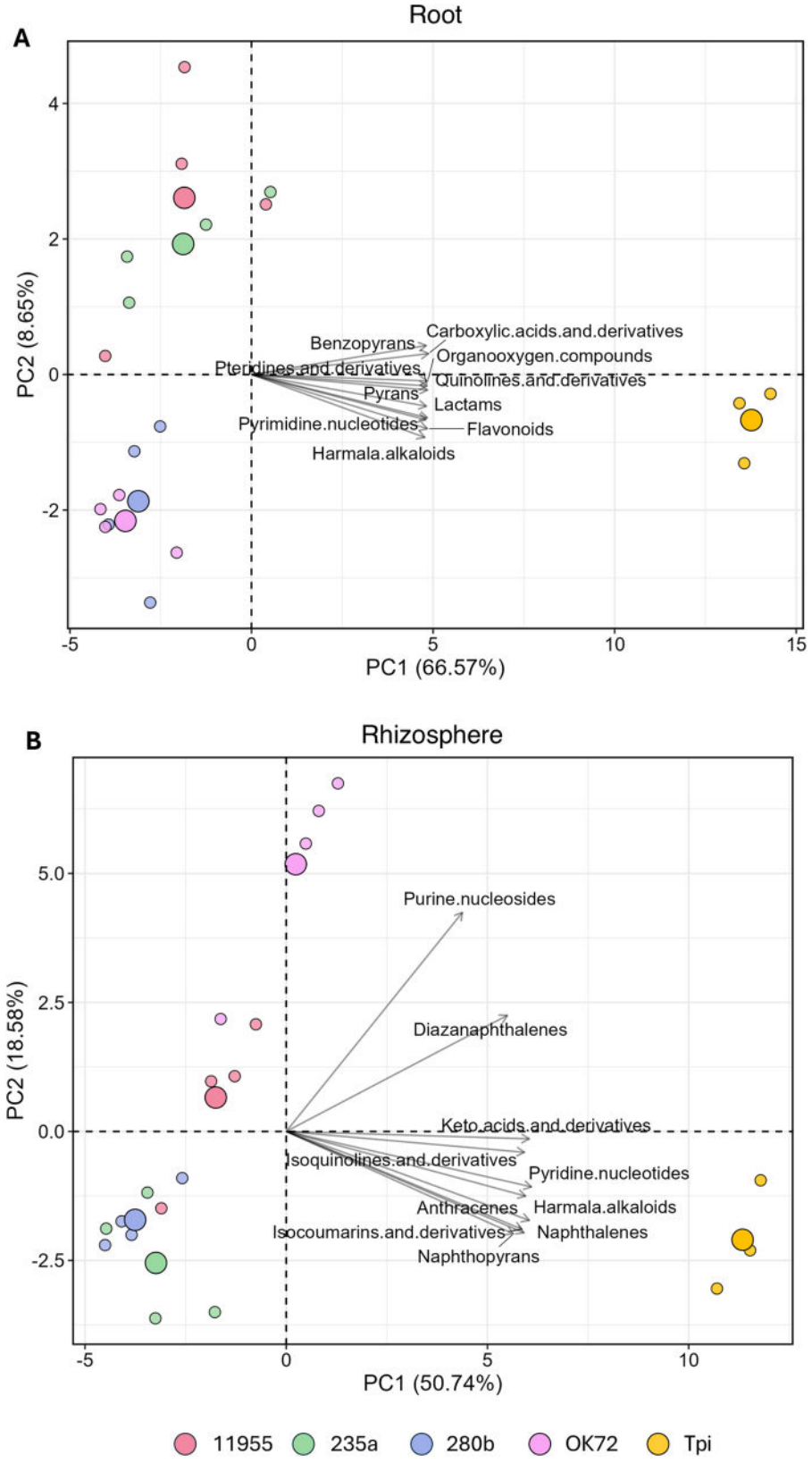

Fig. 7 Comparison of the metabolomic profiles between Tpi and NPGs at the 4th year. Principal component plot (PCA) biplot showing the loadings of the top 10 metabolite classes of DAMs annotated from the comparison between the root (**A**) and rhizosphere (**B**) of the 11-year old *Thinopyrum intermedium* (Tpi) and 4-year-old NPGs. Group centroids are represented by larger circles

with antibacterial, antiprotozoal, and antimycobacterial activity [77]. On the other hand, purine nucleosides can serve as a source of energy [78] and are involved in plant growth and development, particularly in the development of storage organs and germination. Taken together, these results suggest that OK72 may have acquired a greater share of traits associated with perenniality than the other three NPGs examined.

Conclusions

This study explored the intricate metabolome of the root-rhizosphere in four NPGs lines, comparing their profiles with those of an annual durum wheat variety and with the 11-year-old *Thinopyrum intermedium*. We demonstrated that the metabolites produced by NPG roots, even after just one year of soil residence, differed from those of the annual species. These metabolites comprised critical compounds for nitrogen catabolism, allelopathic weed suppression, and modulation of root-microbe interactions, which could contribute to shaping the specific phenotypic traits characteristic of perennial wheat genotypes, such as greater resistance to pathogens, improved utilization of soil resources, and a more developed root structure. In addition, the comparison of the 4th year NPGs with the 11-year-old Tpi revealed significantly higher metabolite concentrations in both roots and rhizosphere, likely reflecting its perennial habit. Among the NPG lines, the root metabolome of OK72 diverged from the other lines starting from the 1 st year onwards. At the same time, its rhizosphere metabolome at the 4th year aligned more closely to the 11-year old Tpi, highlighting its potential as a candidate for developing new perennial wheat hybrids.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-07008-5.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

Supplementary Material 6.

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Authors' contributions

P.B.: Data curation, Formal Analysis. G.G.: Funding acquisition, Project Administration, Resources. L.G.: Writing Review & Editing. G-Giannelli: Data curation, Formal Analysis, Investigation, Writing-Original Draft, Writing-Review & Editing. S.L.: Data curation, Formal Analysis, Investigation, Visualization, Writing-Original Draft. L.R.: Conceptualization, Data curation, Formal Analysis, Writing-Review & Editing. G.V.: Conceptualization, Investigation, Supervision, Writing-Original Draft, Writing-Review & Editing. All authors reviewed the manuscript.

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Data availability

The raw data are provided within the Supplementary Materials.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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