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Size Matters: Influence of Available Soil Volume on the Root Architecture and Plant Response at Transcriptomic and Metabolomic Levels in Barley

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ABSTRACT

Pot size is a critical factor in plant growth experiments, influencing root architecture, nutrient uptake, and overall plant development as well as sensing of stress. In controlled environments, variation in pot size can impact phenotypic and molecular outcomes and may bias experimental results. Here, we investigated how pot size affects the root system architecture and molecular responses of two barley genotypes, the landrace BERE and the modern elite CONCERTO, through assessment of shoot and root traits and by using X-ray computed tomography complemented by transcriptomic and metabolomic analyses. The two genotypes showed distinctly different adaptations to changes in pot size. The landrace showed greater stability and adaptability with consistent root traits and enhanced accumulation of osmoprotectant metabolites across different pot sizes with respect to CONCERTO. Conversely, the elite line was more sensitive to pot size variations, particularly showing altered root architecture and transcriptomic responses. Overall, this study highlights the importance of selecting an appropriate pot size for plant growth experiments, particularly when focused on root traits, and highlights the importance of considering the physiological and molecular changes due to growth environment choice in experimental design in barley.

1 | Introduction

To understand fundamental plant–soil interaction mechanisms, we need to conduct experiments in controlled conditions. In these growth experiments, pot size needs to be considered as it affects the soil volume available for growth. Unfortunately, the soil volume available for a plant in a pot is generally much smaller than that experienced in field conditions (Vetterlein et al. 2021). Selecting pot size involves balancing soil volume, growing period, resource requirement and imaging capabilities for noninvasive analysis. An important question is whether pot size influences experimental outcomes, especially regarding phenotypic plasticity, i.e., the ability of organisms to modify their phenotypes in response to environmental changes (Sultan 2003). Pot size variation alters the

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environment, affecting physiology, morphology, resource allocation or mutualistic interactions with other organisms. Previous research has mainly focused on pot size effect on above-ground biomass, given its importance in commercial plant cultivation. As a result, there is a body of available literature on the shoots of different crops (Liu and Latimer 1995; Goreta et al. 2008; Nord et al. 2011; Chen et al. 2015; Hassan et al. 2019; Obede da Silva Aragão et al. 2020). Despite the variation in species, pot sizes and cultivation types, studies consistently concluded that plants exhibit better growth in larger pots, while growth is hindered when pots become too small. Passioura (2002) coined the term 'bonsai effect' to describe how plants grown in small pots are significantly smaller than those in larger pots, even with adequate water and nutrients (Ismail and Davies 1998; Correa et al. 2019). In small pots, a significant portion of roots may become 'pot bound' causing potential negative effects (Herold and McNeil 1979; Sinclair et al. 2017).

Even if the geometry is kept similar, i.e., only increasing the diameter, maintaining height, there is (i) an exponential (with the square root of pot radius) increase in volume (V) available, amount of plant available water and nutrients, (ii) a linear increase in pot wall surface (W) and a decrease in the likelihood that roots encounter the pot wall (W/V). While resource availability generally increases with pot volume, smaller pots lead to greater temporal fluctuations in soil water content, causing more variable soil pore saturation with water or air. Additionally, soil relative humidity may be positively correlated with microbiota richness, including pathogens (Aung et al. 2018).

Root growth under field conditions is optimised for exploration of large soil volumes and local exploitation of resources (Doussan et al. 2003). In the field, large local root length densities with overlapping rhizospheres from individual root segments are commonly observed (Watt et al. 2006). They may serve to mobilise resources as is shown for proteoid roots (Lamont 2003). However, how root segments of the same plant communicate or sense each other in soil remains largely unknown (Freschet et al. 2021). Overlapping rhizospheres may result in root–root competition and local release in exudates altering the microbiome (Schenk 2006); increased local oxygen consumption (Freschet et al. 2021), pH changes (Faget et al. 2013) or elevated concentrations of plant hormones like ethylene (Jacobsen et al. 2021).

While large local root densities can benefit plants, excessive root length may have negative effects, offering no added return for carbon investment. Thus, pot size choice is not solely about optimising the trade-off between sample size and resolution (Poorter et al. 2012; Hill et al. 2024) or saving substrate; it is crucial to avoid stressing plants and biasing genotype comparisons. A meta-analysis on the pot size effect by Poorter et al. (2012), covering an extremely wide range of pot sizes and plant species, highlighted a consistent linear relationship between pot volume and shoot biomass formation. This analysis concluded that doubling pot size increased shoot biomass by 43% on average, with effects becoming evident when the biomass-to-volume ratio (BVR) exceeded $2\,g\,L^{-1}$ and particularly strong at higher BVR values. While there is a growing recognition of the importance of roots in water and nutrient uptake, and of their role in enhancing resilience to changing environments, the misconception of investigating exploration and exploitation-related traits in limiting soil volumes is often ignored. Similar to shoot response, root traits such as total length and area have been observed to increase with increasing pot size in several crops (Audet and Charest 2010; Chen et al. 2015; Dambreville et al. 2016; Obede da Silva Aragão et al. 2020), and it has been documented that influence of pot volume can be species- and genotype-dependent (Nord et al. 2011).

Moreover, even where studies focused on alteration of root phenotypes, evidence of pot size-induced effects at molecular levels in roots is very limited. Nevertheless, transcriptome- and metabolome-related root traits are now recognised as highly complementary to physiological and biometric ones (Roy and Bassham 2014; Freschet et al. 2021; Drobnitch et al. 2024). The interaction between root growth and pot size is deemed to affect plant cell wall remodelling since roots can sense and respond to mechanical and physical constraints, leading to a reduction in cell elongation and modifications in wall composition (Young et al. 1997; Monshausen and Haswell 2013).

Here, an experiment under controlled conditions was performed to investigate the influence of pot size on root traits and aboveground development in two barley genotypes, a landrace (BERE; Martin et al. 2023) and a modern elite variety (CONCERTO). Four pot volumes were considered, shoot and root parameters were measured, and roots were analysed via X-ray computed tomography (CT) for 3D architecture. Root samples were also collected for transcriptomics via RNA-seq and untargeted metabolomics (GC/MS) to assess responses at a molecular level. This experiment tested several hypotheses: (1) barley biomass will increase with pot volume; (2) the landrace BERE, being less affected by breeding bottleneck, will be more plastic to the environment; and (3) *omics* analysis will reveal root stress responses across different pot volumes.

2 | Materials and Methods

2.1 | Plant Materials

Two barley genotypes with contrasting breeding backgrounds were selected. BERE is a six-row hulled landrace, which was widely grown in the northern UK, particularly Scotland, until replaced by high-yielding genotypes in 20th century (Wallace et al. 2019; Brown et al. 2020). CONCERTO is a two-row modern elite cultivar (Brown et al. 2020) with a longer breeding history, used as a standard spring barley cultivar (Marin et al. 2021) having been developed, selected and customised for both distiling and brewing (Bringhurst 2015) and is one of the most widely used UK varieties.

2.2 | Experimental Design

The pot experiment was set up as a two-factorial randomised design with four replicates. The term 'replicate' here refers to individual soil columns. Factor one was the *Hordeum vulgare* L. genotype (2 levels: BERE and CONCERTO). Factor two was the pot diameter (4 levels: 3, 5, 7 and 10 cm). Additionally, a small sub-experiment with 5 cm columns (three replicates per genotype) was used to monitor plant transpiration and leaf area

development in high temporal detail, serving as a control to test potential X-ray radiation influence on plant development. Mean transpiration rate was determined considering the stable rates of the last 10 days of the experiment with amounts of 0.0096 (±0.0003) and 0.0070 (±0.0003) mL h^{-1} cm^{-2} in BERE and CONCERTO, respectively. Based on the mean transpiration rate and leaf area development, daily transpiration was estimated. The growth substrate consisted of 83.3% quartz sand (WF33, Quarzwerke Weferlingen, Germany) mixed with 16.7% sieved loam from the top 50 cm of a haplic Phaeozem soil profile. Details on physicochemical properties are provided by Vetterlein et al. (2021). Soil fertilisation was similar to Lippold et al. (2021), with the following changes: (1) boron concentration was reduced by 50% and (2) N-application was split in two with the first half being mixed with the soil before filling the columns and the second half being applied with the regular re-watering on Day 14. Individual soil columns were 25 cm tall acrylic glass tubes. Four different diameters (referred as 'pot size') were utilised to provide different soil volumes to the plants. A 30 μm nylon mesh was placed at the bottom of the column to retain the soil while allowing water and gas exchange. The columns were filled up to 23 cm height with the substrate, and the soil volumes available for plant growth were 142, 452, 885, and 1735 cm^3, respectively. Columns were packed to prevent particle sorting and layering by moving a 4 mm mesh sieve across the top during filling. The sand was packed to a bulk density of 1.47 g cm^3 by tamping the entire column on a flat surface.

2.3 | Plant Growth Conditions

Barley seeds were surface sterilised for 5 min in 10% H_2O_2 and rinsed with distilled water. Seeds were placed in a Petri dish with filter paper soaked in saturated $CaSO_4$ until the first root tip emerged, then sown 1 cm deep and covered with coarse quartz gravel to reduce evaporation. Columns were carefully watered from the top (one-third) and bottom (two-thirds) to an average volumetric water content of 18%. Based on the different soil volumes given by different pot sizes, 18% water content reflected different total amounts of water, i.e., 26, 81, 159, and 312 mL with increasing pot size. Intervals of re-watering were every second day at the beginning and shortened during the final week of the growth period to daily watering to cover the water demands of the plants. The growth chamber was set to 22°C day/18°C night with a 12 h photoperiod, 350 μM m^{-2} s^{-1} photosynthetically active radiation and a constant relative humidity of 65%. Harvest was performed on day 21 after planting.

2.4 | Shoot and Root Sampling, Biometric Parameters and Biochemical Analyses

During plant growth, leaf area development was recorded regularly by measuring the length and width of unfolded leaves. Leaf area was calculated with an adapted formula from Rybinski and Garczynski (2004) using the median factor of 0.81 (Table S1). At harvest, collected shoots were dried at 65°C for 48 h. C and N were analysed by combustion with a CNS analyser (vario EL cube, Elementar, Germany). After cutting the shoots, roots were removed from the columns using two sieves (mesh 0.63 and 2 mm), and the total root fresh weight was determined. Subsamples of roots for transcriptomics and metabolomics were collected from one main root with at least two laterals. Each subsample was divided into two halves in separate 2 mL tubes for metabolomic and transcriptomic analyses, frozen in liquid nitrogen, and stored at −80°C. The remaining part of the root system was stored in 50% diluted Rotisol. Roots were scanned using a flatbed scanner (EPSON Perfection V700 Photo) and analysed with WinRhizo2022 (Regent Instruments, Canada). Root length, surface, volume, mean root diameter and root length diameter distribution were determined. False-positive 'roots', primarily consisting of root hairs, were excluded from the analysis by establishing a threshold of 0.05 mm as an optimal cut-off to distinguish false positives without excluding actual roots.

2.5 | X-Ray Computed Tomography (CT) of Roots

The X-ray CT was performed with an industrial μCT (X-TEK XTH 225, Nikon Metrology). Representative scans were performed from all column sizes. Only the 5 cm columns were used for repeated scanning after 7 and 14 days of plant growth to allow assessment of early root growth dynamics and determination of seminal root angle. For the scans, settings were 150 kV, 233 μA with 2400 projections at 500 ms each, resulting in 20 min per scan (image resolution 30 μm). Three scans were performed as stacks to capture in total of 15 cm of the column height. A 0.5 mm copper filter was used to reduce beam hardening effects. A lead shield with a window was placed between the source and samples to minimise unproductive radiation dose, although barley is known to be less sensitive to X-ray radiation than other species (Blaser et al. 2018). Reconstruction was performed with Nikon's proprietary CT Pro 3D software. CT data was analysed with an adapted version of RootineV2 (Phalempin et al. 2021) to segment roots and allow for visualisation and analysis of root length and diameter distribution. To avoid impacting the transcriptomics/metabolomics analysis, additional CT scans were not performed after 21 days (Ganther et al. 2020).

2.6 | Statistical Analysis and Relative Distance Plasticity Index (RDPI)

Data of shoot and root traits were tested for normality and homoscedasticity using Shapiro–Wilk and Levene tests, respectively. To assess differences within genotypes, one-way ANOVA and Tukey's post-hoc test ($p \leq 0.05$) were used, with pot size as a main factor. Plasticity of root and shoot traits was determined using the relative distance plasticity index (RDPI) as described by Valladares et al. (2006) (Supporting Information 1). The RDPI was calculated by *Plasticity* R package (Ameztegui et al. 2017).

2.7 | Transcriptomics

2.7.1 | RNA Extraction, Library Preparation and Sequencing

At least three biological replicates for each pot size and genotype were used for transcriptomics. Total RNA was isolated by

using a CTAB-based lysis buffer (Chang et al. 1993). Library preparation and RNA sequencing were performed by IGATech (Udine, Italy). The Universal Plus mRNA-Seq kit (Tecan Genomics, Redwood City, CA) was used for library preparation. In total, 31 Paired-end 150 bp libraries were sequenced on NovaSeq 6000 system (Illumina, San Diego, CA).

2.8 | Bioinformatic Analyses and Statistical Assessment of Transcriptomic Data

The RNA-Seq reads were subjected to base calling, demultiplexing and adaptor masking with Illumina BCL Convert v3.9.31. Trimming of low-quality bases and adaptors was performed through ERNE (Del Fabbro et al. 2013). Raw reads were submitted to NCBI SRA under BioProject PRJNA1052589. Reads were aligned on *Hordeum vulgare* reference genome (cultivar Morex; Mayer et al. 2012) with STAR (Dobin et al. 2013). Assembling and quantitation of full-length transcripts representing multiple spliced variants for each gene was carried out via StringTie (Pertea et al. 2015). The software htseq-count (Anders and Huber 2010) was utilised to pre-process data and DESeq.2 (Love et al. 2014) was used to compare levels of expression of genes. The differentially expressed genes (DEGs) identification was performed after normalisation of count data and correction for multiple testing through the Wald test. A principal component analysis (PCA) on normalised read counts was performed. BERE_7CM and CONCERTO_7CM were used as reference for DEGs identification in BERE and CONCERTO samples, respectively, with a threshold of adjusted $p \leq 0.05$. Both the identified DEGs and all transcripts of the *H. vulgare* genome were annotated through Blast2GO (Conesa et al. 2005) to obtain functional annotation and to assign Gene Ontology (GO) terms, and a GO enrichment analysis was performed (false discovery rate (FDR) ≤ 0.05). The enriched GO terms were further imported into REVIGO for identification of representative subsets (Supek et al. 2011).

2.9 | Metabolomics

2.9.1 | Sample Extraction and Derivatization

Samples of frozen roots were ground in liquid nitrogen with a mortar and pestle and up to 80 mg were weighed into 2 mL tubes. Extraction, derivatization and GC-MS analyses were performed as described by Lisec et al. (2006) with the modifications proposed by Misra et al. (2020). Additionally, three quality controls and three blank solvents were prepared. The analyses were performed in four sets, including one replicate each; however, due to a failure in the facilities with the final set, only three biological replicates were analysed.

2.10 | GC-Quadrupole/MS Analysis and Data Processing

A gas chromatograph apparatus (Agilent 789 A GC) equipped with a single quadrupole mass spectrometer (Agilent 5975 C) was used to inject the derivatized extracts into a MEGA-5MS capillary column (30 m × 0.25 mm × 0.25 µm equipped with a 10 m pre-column). Details on GC-Quadrupole/MS Analysis are provided in Supporting Information 2. Data were processed using MS-DIAL for peak extraction, identification and alignment, following MSI guidelines with public libraries (Golm Metabolome Database, MassBank, MoNA) for level 2 and 3 metabolite annotation. MetaboAnalyst 5.0 was used for further statistical analysis. Data were normalised using internal standards and QC samples, cube root-transformed and Pareto-scaled. PCA was performed to visualise group discrimination, two-way ANOVA ($p \leq 0.05$) assessed the effects of genotype and pot size, and one-way ANOVA with post-hoc tests identified significant metabolite changes. Hierarchical clustering heatmaps were used to show the relative accumulation of significantly affected metabolites.

3 | Results

3.1 | Shoot Development and Transpiration

In BERE, leaf area (LA) was consistent across different pot sizes, with the largest values in 7 cm pots. While 3 cm pots started with smaller LA values, they matched growth rates of other sizes until slowing 15 days after planting (DAP; Figure S1). Conversely, CONCERTO showed limited growth in 3 and 5 cm pots after 14 DAP, with only plants in 7- and 10-cm pots continuing to expand until harvest. Final LA in 10 cm pots was almost identical for both genotypes (Figure S1). Shoot dry weight (Figure 1a) remained relatively constant across all pot sizes, with larger averages in BERE compared to CONCERTO. In BERE_10CM, the values were less than for BERE_7CM and were similar to values obtained with BERE_5CM (Figure 1a). In CONCERTO, increasing the pot size from 3 to 5 cm led to no increase in biomass production (Figure 1a). When the pot size was further increased to 7 cm, there was an increasing trend in biomass and LA, although not significant (Figure 1a). When comparing genotypes, CONCERTO exhibited slightly greater biomass in the smallest pot size. However, in 5 and 7 cm pots, BERE achieved greater biomass values. The BVR decreased as pot size increased in both genotypes (Figure 1b), with a sharper drop in CONCERTO than in BERE. In the smallest pots, CONCERTO reached the largest overall BVR value of 2.0 g L^{-1} on average, but in the next pot size, BVR dropped to only 0.43 g L^{-1}. A slower decline was observed in BERE plants growing in 5 and 7 cm pots, and at the 10 cm pot size, BVR dropped below 0.5 g L^{-1}, reaching similar values to CONCERTO. The shoot N concentration at 3 cm was significantly less than in the 7 cm pot for both genotypes (Figure 1c). Overall, BERE showed greater N concentrations compared to CONCERTO, suggesting a larger N acquisition potential. For N-uptake, in the smallest pot size both genotypes showed similar values (Figure S2). Raw data of shoot parameters are reported in Table S2.

Transpiration rate per unit LA and time was higher in BERE than in CONCERTO throughout the experiment and reached stable rates from about 11 DAP onwards (Figure S3). In both genotypes relative daily transpiration, expressed as % of soil water available per pot size, was greatest in the 3 cm pots, reaching up to 45% at 21 DAP (Figure 2). In BERE, the sequence of daily transpiration corresponded directly to the increasing

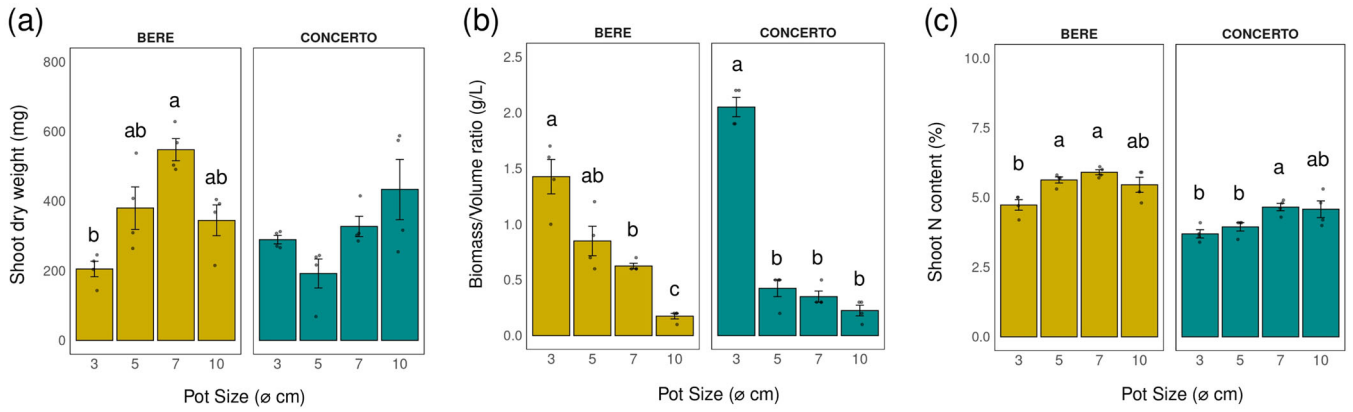

FIGURE 1 | Shoot traits at harvest (22 days after planting). (a) shoot dry weight; (b) biomass-volume-ratio BVR; (c) shoot N content. Letters are plotted according to significant differences detected by Tukey HSD post-hoc test after One-way ANOVA ($p < 0.05$), $n = 4$.

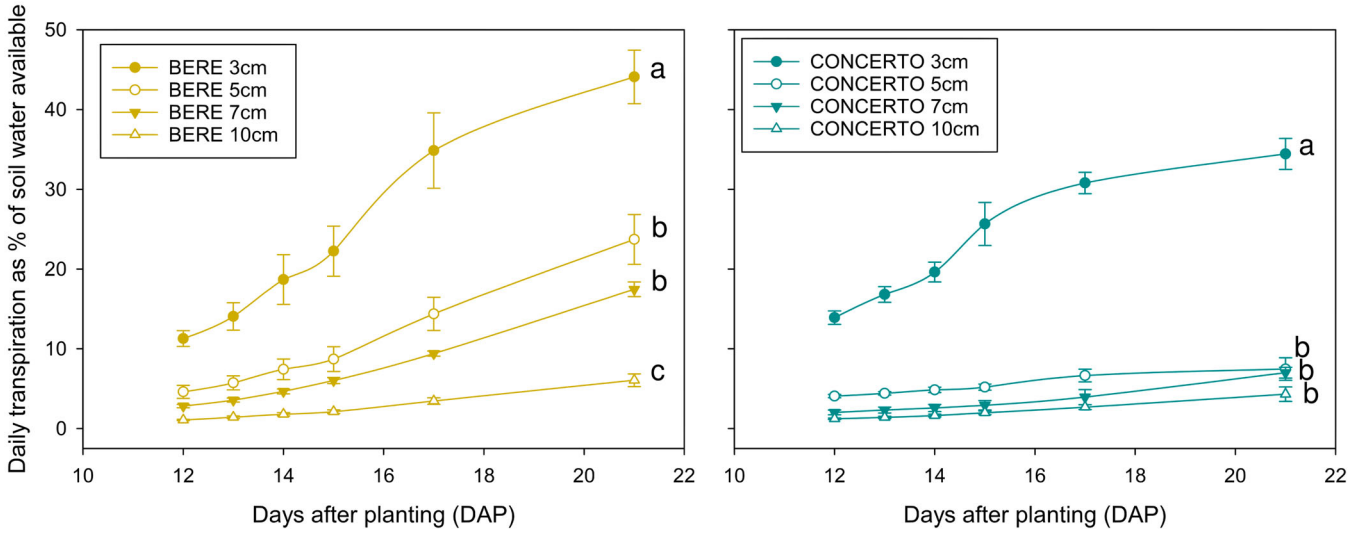

FIGURE 2 | Daily transpiration rate over time as % of soil volume, for BERE and CONCERTO. [Color figure can be viewed at wileyonlinelibrary.com]

pot size, while in CONCERTO transpiration values were less than in BERE for all pot sizes, due to smaller leaf area and lower transpiration rate. In contrast to BERE, final relative transpiration was the same for 5, 7 and 10 cm pots in CONCERTO (Figure 2).

3.2 | Root Traits

Raw data of root parameters are reported in Table S2. In summary, there was an increase in root length (Figure 3a) and root volume (Figure 3c) of BERE with increasing pot size up to 7 cm, followed by smaller values in 10 cm pots (optimum curve). CONCERTO showed decreased values when the pot size increased from 3 to 5 cm, followed by an increase with increasing available soil volume(Figure 3a–c). The root length density (Figure 3b) decreased proportionally to the BVR (Figure 1c) with increasing pot size in both genotypes. The decrease was more abrupt in CONCERTO compared to BERE. The half mean distance between roots, measuring how thoroughly space is explored by roots, was smallest in the smallest pots and increased with pot size (Figure 3d). The half mean distances were larger in CONCERTO, as this genotype showed smaller root length density for the same soil volume (Figure 3d). The mean root diameter (Figure 3e) fluctuated in all pot sizes and for both genotypes in the same range, approximately between 0.30 and 0.45 mm. Likewise, root length distribution in diameter classes revealed no consistent differences between genotypes and pot sizes (Tables S3–S5). In both genotypes, there was a tendency for decreasing root:shoot ratio with increasing pot size, and for CONCERTO this was significant (Figure 3f).

3.3 | X-Ray CT of Roots

X-ray CT analysis revealed no differences in root length between genotypes after 7 days of growth (Figures S3–S5). After 14 DAP, root length in BERE was greater (496 ± 101 cm) than in CONCERTO (271 ± 41 cm) (Figure 4). Root distribution within the column depth showed similar results for both genotypes after 7 days of growth. Differences occurred after 14 days with BERE utilising deeper soil layers for soil exploration while

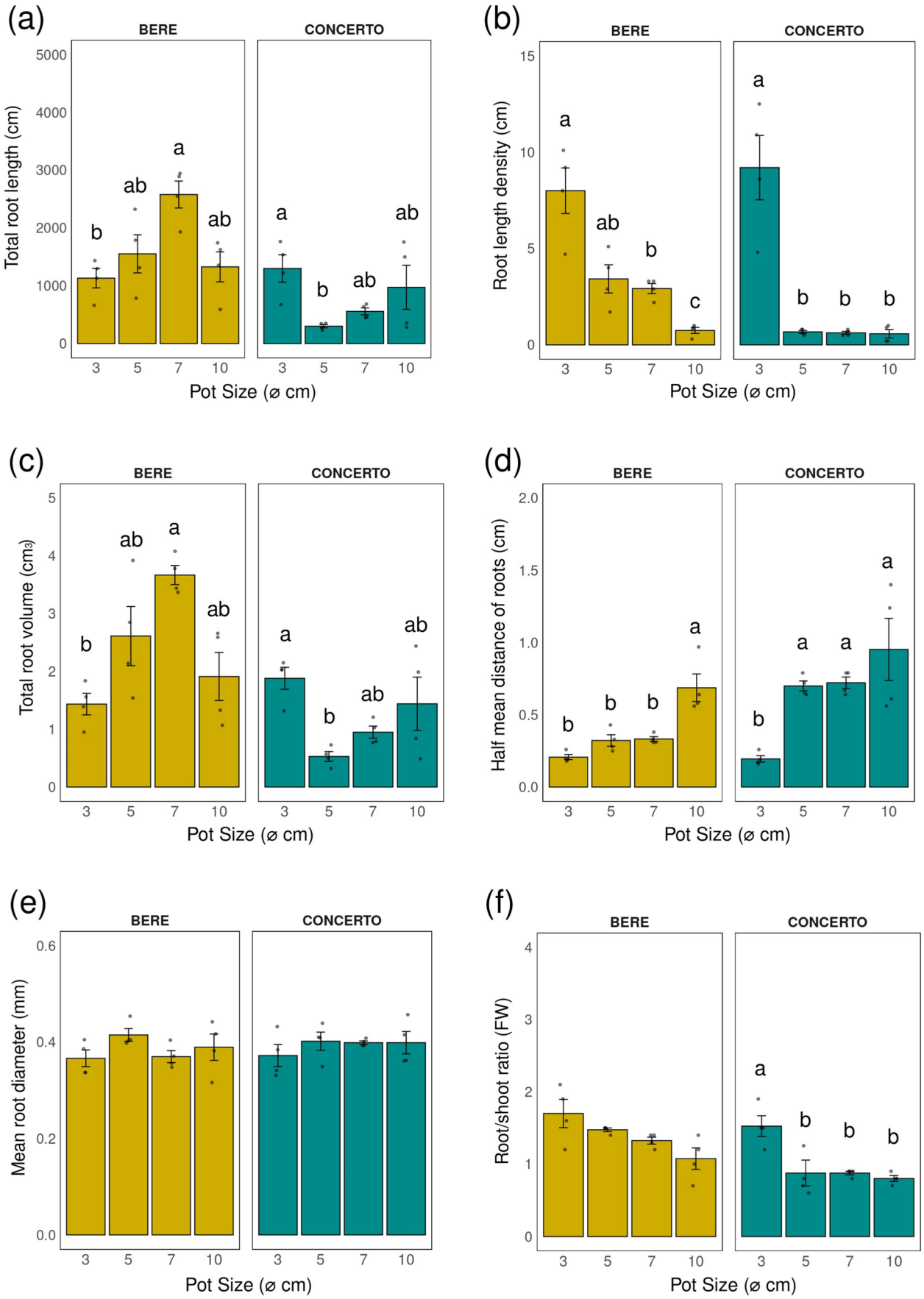

FIGURE 3 | Root traits at harvest (22 days after planting). (a) root length; (b) root length density; (c) root volume; (d) half mean distance of roots; (e) mean root diameter; (f) root/shoot ratio. Letters are plotted according to significant differences detected by Tukey HSD post-hoc test after one-way ANOVA ($p < 0.05$), $n = 4$. [Color figure can be viewed at wileyonlinelibrary.com]

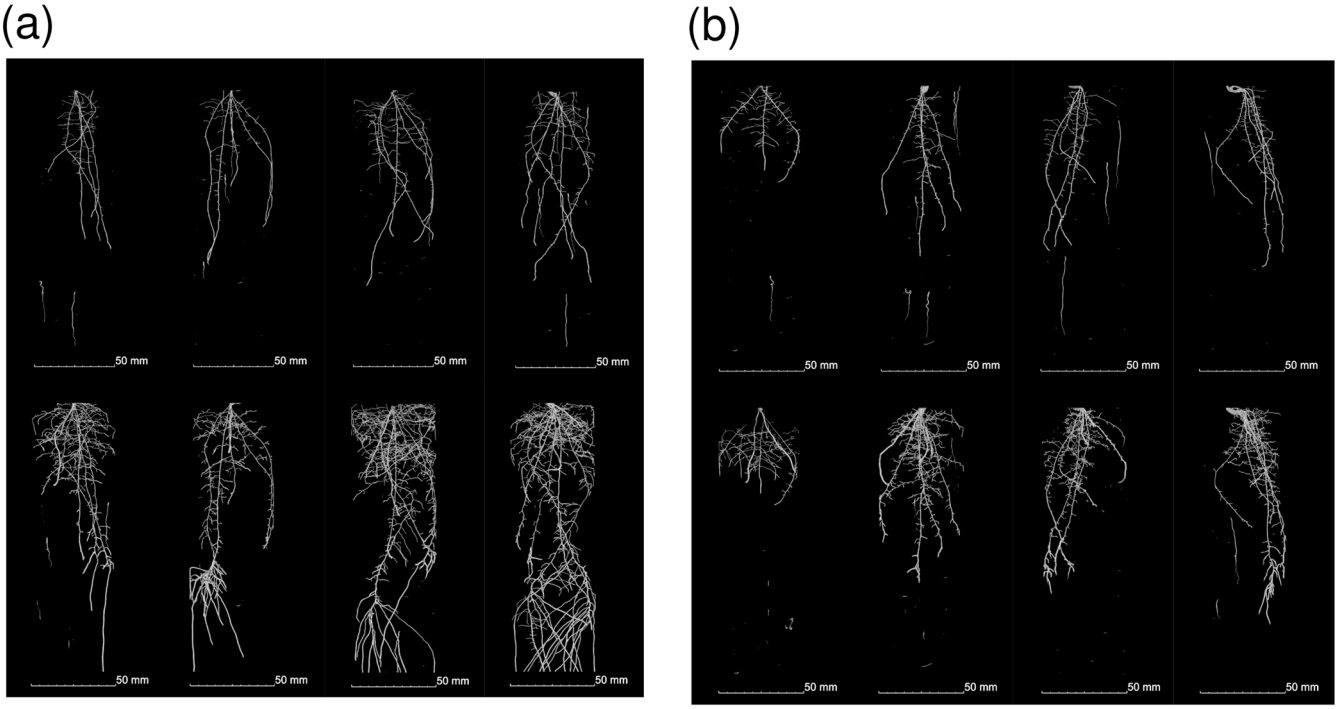

FIGURE 4 | Roots of BERE (a) and CONCERTO (b) under X-ray CT over time (7 DAP upper row, 14 DAP bottom row).

CONCERTO had greater root length in the top layer. Seminal root angle relative to the perpendicular revealed a greater range for BERE, including larger minimum and maximum values (Figure S6). Number of initiated seminals was similar for both genotypes with BERE having 5.5 ± 0.29 and CONCERTO 5.25 ± 0.25 per plant (Figure S6).

3.4 | RDPI

Figure 5 shows spider plots of the normalised shoot and root parameter RDPIs for both genotypes. When all pot sizes were included (Figure 5a), CONCERTO had larger plasticity than BERE for several traits, which included root length (RL), density (RLD), half mean distance of roots (HMD), specific root length (SRL), and ratio between root surface area and leaf area (RA:LA). No differences were found between genotypes for plasticity of LA and mean root diameter (RD). Plant N-uptake was the only trait with greater plasticity in BERE than in CONCERTO. Figure 5b–d depicts how relative plasticity indexes can change depending on the selection of pot size. While the general trends barely changed when excluding 10 cm pot size, the picture was very different when excluding both the smallest and largest pots. In this case, BERE showed greater plasticity for four traits, while two traits were 'neutral' and only two traits showed greater plasticity by CONCERTO (Figure 5d).

3.5 | Transcriptomics

3.5.1 | RNA-Seq Metrics

A total of 766 778 038 raw reads were generated from the 31 libraries. Two outlier samples of BERE_3CM and CONCERTO_3CM were excluded to avoid biased results in the DEG identification due to Type II errors. Raw metrics of read mapping to reference genome and DESeq.2 normalised counts are reported in Tables S6 and S7. The PCA showed a clear separation along PC1 suggesting a difference in expression profiles between samples from 3 cm pots and the other ones, while PC2 allowed discrimination of the two genotypes (Figure 6a). Considering BERE_7CM and CONCERTO_7CM as controls, 6483 genes (out of 35 826 *Hordeum vulgare* total gene models) were found to be differentially regulated (DEGs) across all samples. Particularly, 2326, 18 and 23 genes were found to be significantly upregulated in BERE_3CM, BERE_5CM and BERE_10CM, respectively, when compared to BERE_7CM (Figure 6b–c and Tables S8–S13). In CONCERTO_3CM, CONCERTO_5CM and CONCERTO_10CM, the number of upregulated genes compared to CONCERTO_7CM was 1,594, 296 and 40, respectively (Figure 6b,c). Downregulated genes were 2,107, 13 and 36 in BERE_3CM, BERE_5CM and BERE_10CM, respectively, while 1360, 628 and 58 were downregulated in CONCERTO_3CM, CONCERTO_5CM and CONCERTO_10CM, respectively (Figure 6b,c). A total of 1329 DEGs were shared between BERE_3CM and CONCERTO_3CM (Figure 6c) and included upregulated genes associated with heat shock proteins (21), late embryogenesis abundant (LEA) proteins (18), sucrose transport (3), and downregulated genes associated with expansins (6).

3.6 | Specific and Common Transcriptomic Responses in the Two Genotypes

The lists of DEGs for each condition are provided as Tables S8–S13, while Table S14 includes DEGs in all conditions.

In BERE_3CM, we detected upregulation of genes putatively coding ABC transporters (21 DEGs) and sugar transporters (6), auxin-

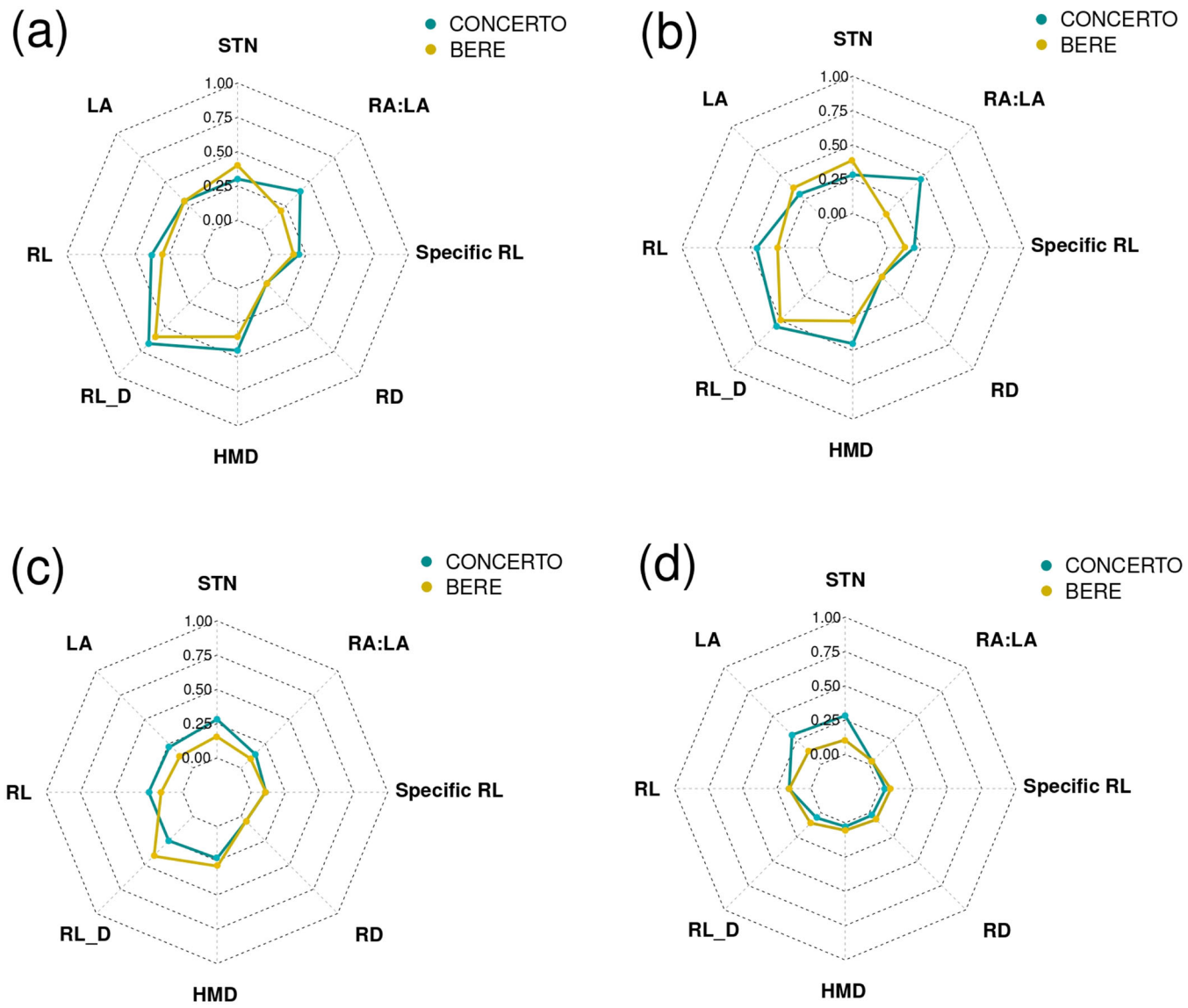

FIGURE 5 | Radar charts for RDPI values. (a) all pot sizes; (b) excluding 10 cm pot size; (c) excluding 3 cm pot size; (d) excluding 3 and 10 cm pot sizes. Root length (RL), root length density (RLD), half mean distance of roots (HMD), root diameter (RD), specific root length (Specific RL), ratio between root surface area and leaf area (RA:LA), N uptake (STN), and leaf area (LA) are reported. Plant N-uptake was determined by multiplication of N-content (%) and the evaluated dry weight. [Color figure can be viewed at wileyonlinelibrary.com]

related genes (6), ethylene-responsive genes (16), heat shock proteins (9), and other genes associated to response to stress, along with several genes associated with late embryogenesis abundant (LEA) proteins (24 DEGs, with a Log_2FC ranging from 0.76 to 12.75) and putative dehydrins (9, Log_2FC from 2.23 to 8.88) (Figure 6d and Table S8). Genes involved in nutrient transport, including putative high-affinity nitrate transporters, were highly upregulated in BERE_3CM. A gene identified as a high-affinity nitrate transporter 2.1-like was the second most upregulated gene in BERE_3CM ($Log_2FC = 14.670$). Additionally, a cytosolic glutamine synthetase-related gene ($Log_2FC = 4.581$) was also found to be upregulated. Genes involved in lipid metabolism, including three oil body-associated proteins ($Log_2FC = 10.237$, 9.207 and 8.456), showed significant upregulation, along with genes responsive to biotic stress, such as a rust resistance kinase Lr10-like ($Log_2FC = 3.532$), two pathogenesis-related proteins ($Log_2FC = 2.092$ and 3.08), and five chitinase-related genes. Among the downregulated genes, BERE_3CM showed the presence of genes related to histones (49), chitinases (7), peroxidases (36), and genes related to plant cell wall remodelling, e.g., expansins (17), xyloglucan-related genes (6). The gene HORVU.MOREX.r3.6HG0607550.1 (*HvEGT1*) showed downregulation in BERE_3CM. Diverse genes encoding enzymes involved in pectin modification, such as pectinesterases and pectinesterase inhibitors, were differentially regulated in BERE_3CM (5 up- and 9 downregulated; Table S8).

In CONCERTO_3CM, upregulated genes included genes related to carbohydrate metabolism (e.g., 6 bidirectional sugar transporters SWEET-like), detoxification processes (e.g., 6 peroxidases), and response to abiotic stress (i.e., water deficit, salinity), such as *NCED5* and LEA proteins (Table S9), with the highest upregulation observed in three LEA protein-related genes ($Log_2FC = 13.893$, 12.009 and 10.082; Table S9). Several genes correlated to plant defence were regulated in CONCERTO_3CM: genes coding for glucan endo-1,3-beta-glucosidases (10 up-, 7 downregulated), chitinases (1 up-, 7 downregulated), wall-associated receptor kinases (13

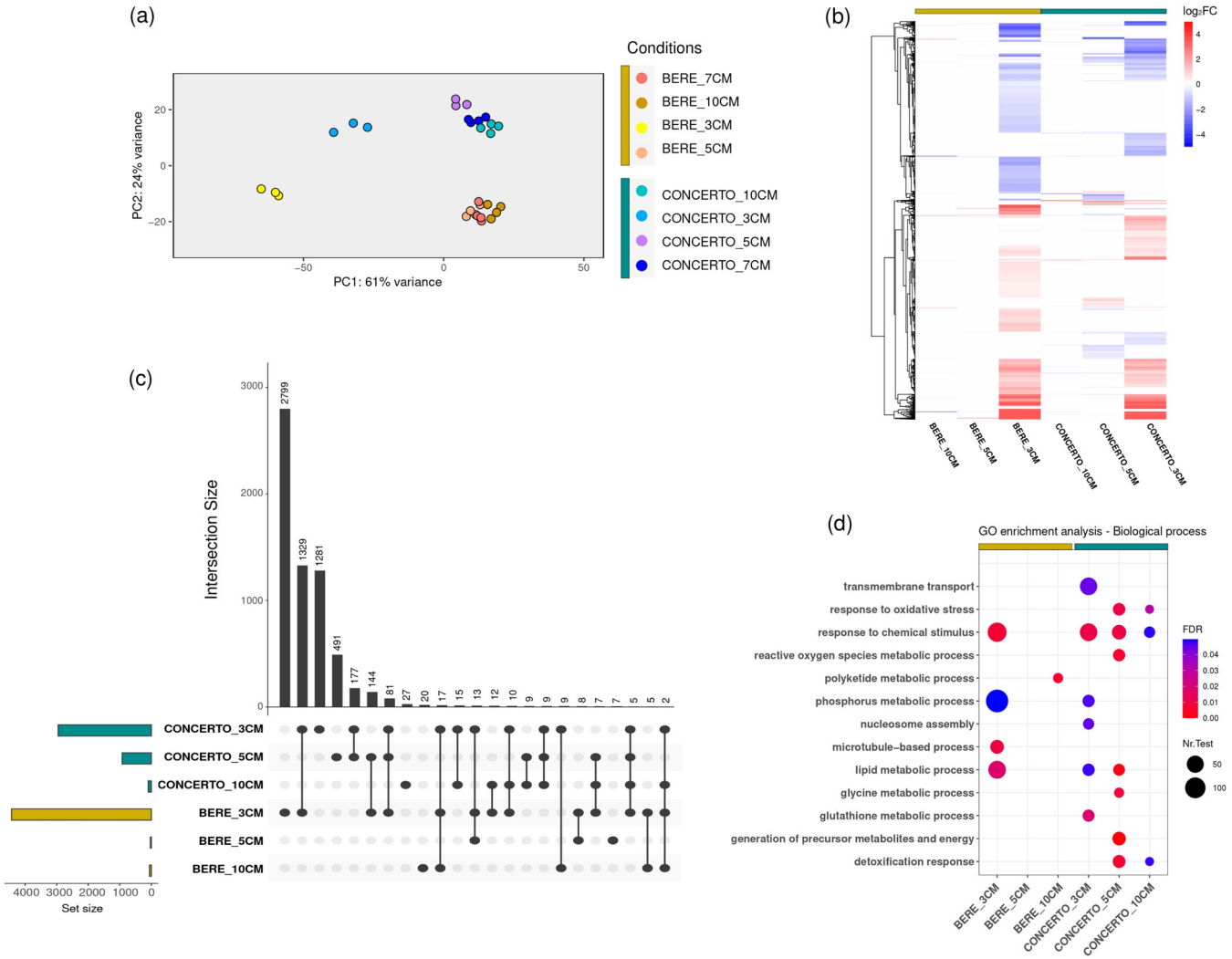

FIGURE 6 | Transcriptomic profiles of the tested conditions. In (a), PCA on raw read counts to assess differences in transcriptomic patterns across samples. In (b), heatmap depicting DEGs in the six conditions compared to their related controls (BERE_7CM and CONCERTO_7CM), and hierarchical clustering of DEGs using the McQuitty algorithm. The heatmap shows the expression patterns of DEGs across six conditions, with red levels indicating upregulated and blue representing downregulated DEGs. Different colour intensity indicates different levels of expression (log2 Fold Change). In (c), UpSet plot to summarise DEGs in all conditions. The panels summarise the DEGs overlap in each condition. Bottom left horizontal bars (cyan and yellow for CONCERTO and BERE, respectively) labelled DEG set size. Circles in each panel matrix represent unique and overlapping DEGs. Connected circles indicate an intersection of DEGs between conditions. Top bar graph in each panel summarises the number of DEGs for each unique or overlapping combination. In (d), bubble plot showing GO-enriched terms classified as Biological Process (BP). The y-axis reports the GO terms. Sizes of bubbles are proportional to the number of genes assigned to the related GO term (Nr.test), while bubble's colour indicates the significance of the enriched term (False Discovery Rate values) as calculated by the enrichment analysis by Blast2GO. [Color figure can be viewed at wileyonlinelibrary.com]

downregulated), and also genes related to rust resistance kinases Lr10-like (4 downregulated). A high-affinity nitrate transporter encoding gene was also upregulated ($Log_2FC = 5.966$), while the other two resulted to be downregulated in the same conditions ($Log_2FC = -3.253$ and -3.661; Table S9). Two ammonium transporters (AMT)-related genes were downregulated ($Log_2FC = -1.049$ and -1.81), while two alcohol dehydrogenases (ADH) encoding genes were upregulated exclusively in CONCERTO_3CM with a $Log_2FC > 5.00$ (Table S9).

In both genotypes, several transcription factors were differentially expressed (both up- and downregulated) in 3 cm pots (Tables S8 and S9). Genes coding for two auxin-responsive SAUR proteins, two auxin response factors (ARFs) and one auxin-induced protein were upregulated in BERE_3CM and CONCERTO_3CM compared to their controls (Tables S8 and S9). Three TIP aquaporin genes were upregulated in BERE_3CM, and two were also upregulated in CONCERTO_3CM (Tables S8 and S9). The gene *NCED5* was upregulated in both BERE_3CM and CONCERTO_3CM (Tables S8 and S9).

In BERE_5CM, a limited set of DEGs were identified (Table S10). In addition to the high-affinity nitrate transporter 2.1-like ($Log_2FC = 7.652$), several other genes coding for nitrate transporters were upregulated in BERE_5CM condition. Considering nitrate transporters, it is worth noting that this

function was particularly represented both in BERE_3CM (9 upregulated with average $Log_2FC = 5.21$; Table S8) and BERE_5CM (5 upregulated with average $Log_2FC = 3.66$; Table S10).

Conversely, more genes were regulated in CONCERTO_5CM (Table S11). Among the upregulated genes a gene coding for an aminocyclopropane carboxylate (ACC) oxidase ($Log_2FC = 1.963$) was found. The most upregulated genes in CONCERTO_5CM included a gene coding for a DEEPER ROOTING 1-like protein ($Log_2FC = 4.923$). The upregulation of *HvEGT1*, along with those coding for LATERAL ROOT PRIMORDIUM protein and SHORT-ROOT-like protein, was observed in CONCERTO_5CM compared to its control. Aquaporin-related genes were also downregulated in CONCERTO_5CM (Table S11). Two ammonium transporter genes were downregulated ($Log_2FC = -0.650$ and -1.82), and a NRT1/PTR gene, known to repress lateral root initiation, was slightly upregulated in CONCERTO_5CM ($Log_2FC = 1.93$).

In BERE_10CM, only a few genes were differentially regulated compared to its control (Table S12). A putative thiamine pyrophosphate-dependent dehydrogenase ($Log_2FC = 4.270$), involved in primary metabolism, was the most upregulated gene. Genes potentially involved in response to biotic stresses were also identified, including a gene coding for a pathogenesis-related protein 1-like ($Log_2FC = 3.108$), a gene coding for a rust resistance kinase ($Log_2FC = 1.221$), two genes coding for glucan endo-1,3-beta-glucosidases (Log_2FC 2.151 and 2.056, respectively), two genes coding for putative wall-associated receptor kinases ($Log_2FC = 1.737$ and 1.278, respectively), and a gene coding for a chitinase ($Log_2FC = 1.732$).

In CONCERTO_10CM, an aminoacyl-histidine dipeptidase-related gene was the most upregulated ($Log_2FC = 7.246$), followed by a gene encoding a putative ABC transporter ($Log_2FC = 7.138$; Table S13). A gene encoding a WALLS ARE THIN1 (WAT1)-related protein was upregulated ($Log_2FC = 7.063$): this is a plant-specific protein driving secondary cell wall thickness of wood fibres, playing a role as vacuolar auxin transport facilitator (Ranocha et al. 2013). Another gene coding a WAT1-related protein was upregulated in CONCERTO_10CM, while in CONCERTO_3CM the first one was upregulated and the second one downregulated. Both genes were downregulated in BERE_3CM. Several transcription factors belonging to diverse categories were also upregulated in CONCERTO_10CM (Table S13). Among the root-associated genes influenced by the different conditions, a total of 33 genes putatively coding for expansins were identified as DEGs. Most of these genes were downregulated, particularly in BERE_3CM (17), CONCERTO_3CM (15) and CONCERTO_5CM (8) (Table S14). Downregulation of a gene putatively encoding an auxin-induced-in-root-cultures protein in CONCERTO_10CM, CONCERTO_3CM, as well as in BERE_3CM, was also observed (Table S14).

GO enrichment analysis revealed distinct patterns in BERE and CONCERTO. In BERE, 3CM pots were enriched for lipid metabolism (GO:0006629), microtubule-based processes (GO:0007017), phosphorus metabolism (GO:0006793) and response to chemical stimuli (GO:0042221). The 10CM treatment showed enrichment in polyketide metabolism (GO:0030638), indicating pathways involving specific metabolites, while no significant enrichment was found in 5CM pots (Figure 6d).

3.7 | Metabolomics

3.7.1 | Identity and Accumulation of Metabolites

We annotated 130 metabolites (mainly primary metabolites) on BERE and CONCERTO roots. Unsupervised PCA score plot of the annotated metabolites revealed good separation and grouping of samples according to pot size and, to a lesser extent, to genotype, using three principal components (Figure 7). The total variance explained for PC1, PC2 and PC3 was 62.6%, 9.6% and 5.4%, respectively. The PCA loadings plot (Table S15), highlighted that PC1 was dominated by phosphoric acid, sucrose, sorbose, fructose, glucose, DL-malic acid and inositol; PC2 was dominated by glycine, hydroxylamine, quinic acid, galactinol and L-aspartic, oleic and DL-pyroglutamic acids; PC3 was dominated by L-serine, L-phenylalanine, and quinic, succinic, gluconic and dehydroascorbic acids. Correlations for all metabolites with each principal component were weak, with strongest correlations ranging between 0.19 and 0.33.

Group separation according to the pot size was more influenced in terms of PC2 (Figure 7b), and by PC3 also for BERE_5CM (Figure 7a). CONCERTO_3CM and CONCERTO_5CM showed large variability and less grouping due to one replicate each that was found separated from the other two, making these replicates the main contributors to data variability in PC1 (Figure 7b). Therefore, in this case, PC2 and PC3 were responsible for most of the data variability explaining group separation according to pot size and genotype. Two groups, CONCERTO_7CM and BERE_5CM, were clearly separated from the others.

Twenty out of 130 annotated metabolites were found differentially accumulated between genotypes and pot size after two-way ANOVA (Figure 7c and Table S16). The genotype had less effect than the pot size on metabolites. In fact, L-proline was the only metabolite significantly affected by genotype, while 16 metabolites were affected by pot size, and 7 by the interaction of both factors (Figure 7c). L-proline was affected by three factors (genotype, pot size, and the interaction of genotype and pot size).

The relative accumulation of the 20 significant metabolites (Figure 7d) allowed the identification of changes occurring in BERE and CONCERTO roots due to different pot diameters. L-proline was strongly up-accumulated in BERE_3CM, but also to a lesser extent in BERE_5CM and CONCERTO_7CM. The pot sizes significantly affected the accumulation of the annotated metabolites L-proline, oleic acid, D-penicillamine, galactinol, tyramine, tetradecane, L-threonine, DL-pyroglutamic acid, digalacturonic acid, myristic acid, malonic acid, maltose, heptadecanoic acid, pentadecanoic acid, quinic acid and hydroxylamine. Most of these metabolites showed a similar pattern of accumulation between pot sizes; e.g., roots growing in 7 cm diameter pots showed the highest accumulation and this accumulation was even higher in CONCERTO_7CM than in BERE_7CM.

The interaction genotype-pot size significantly affected the presence of L-proline, oleic acid, maltose, beta-alanine, gluconic

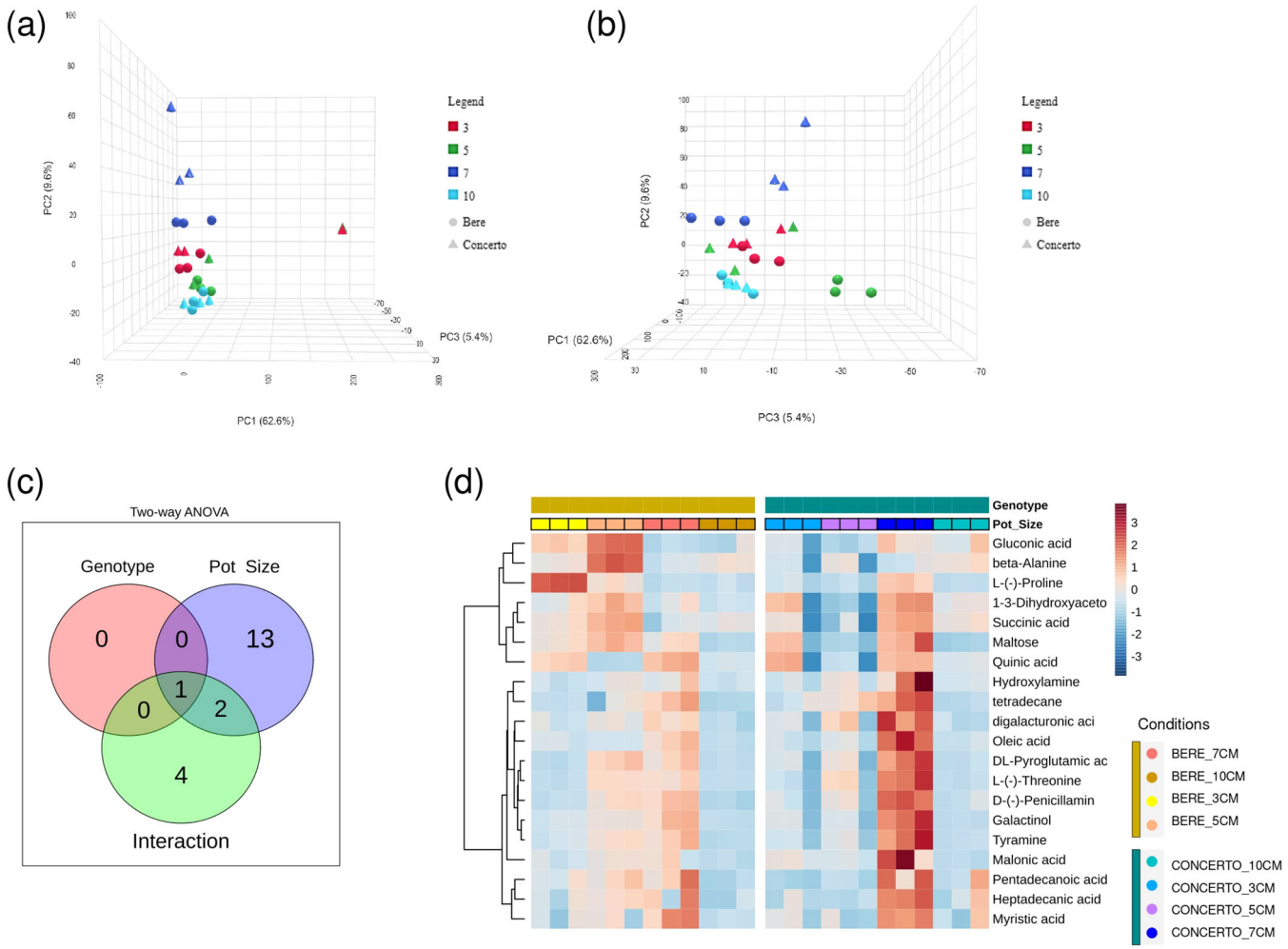

FIGURE 7 | Metabolomic profiles of the tested conditions. In (a, b), Unsupervised Principal Component Analysis (PCA) of all the annotated metabolites data from CONCERTO and BERE roots grown in pots of 3, 5, 7 and 10 cm of diameter grouping due to PC1 and PC2 (a) and due to PC2 and PC3 (b). In (c), the Venn diagram of two-way ANOVA results showing the number of significantly altered metabolites (adj.$p \leq 0.05$) due to the factor genotype, the factor pot size and the interaction of factors. In (d), heatmap and hierarchical clustering of metabolites showing the relative up-accumulation (red) or down-accumulation (blue) of the significantly altered metabolites after two-way ANOVA. [Color figure can be viewed at wileyonlinelibrary.com]

acid, 1,3-dihydroxyacetone dimer and succinic acid. The last four metabolites were exclusively affected by the interaction genotype-pot size, and all of them were found in greater quantities in BERE_5CM, particularly beta-alanine and gluconic acid, while succinic acid and 1,3-dihydroxyacetone dimer were also greater in CONCERTO_7CM.

To further determine the differences in metabolite accumulation due to different pot sizes, one-way analyses were performed in BERE and CONCERTO separately (Figure 8; Tables S17 and S18). The PCA showed good separation of groups (Figure 8a,b). However, the percentage of variance explained by the principal components was moderate for BERE, with 36% (PC1) and 19.9% (PC2), and better explained for CONCERTO with 73.6% (PC1) and 9.8% (PC2). Similarly to the PCA shown in Figure 7, most of the variability explained by PC1 in CONCERTO was due to the already mentioned deviated replicates from CONCERTO_3CM and CONCERTO_5CM.

Only eight metabolites (oleic acid, L-proline, tetradecane, galactinol, D-penicillamine, tyramine, heptadecanic acid and L-threonine) were found significantly down-accumulated in CONCERTO_3CM, CONCERTO_5CM and CONCERTO_10CM when compared to CONCERTO_7CM (Figure 8d). In contrast, a much larger number of 32 metabolites was found to be significantly altered in BERE_3CM, BERE_5CM and BERE_10CM when compared to BERE_7CM, showing a more diversified response to pot diameter, with BERE_10CM roots mainly showing down-accumulation (Figure 8c).

3.8 | Metabolites Related to Root Development and Stress Tolerance

Five metabolites related to myo-inositol were significantly affected in barley roots, with four specific to BERE. Galactinol, a myo-inositol derivative, was down-accumulated in both BERE and CONCERTO across 3, 5, and 10 cm pots compared to 7 cm. Pectin components glucuronic acid and digalacturonic acid decreased in BERE_3CM, BERE_5CM and BERE_10CM pots relative to 7 cm. Additionally, conduritol-beta-epoxide, a glucosidase inhibitor (Falshaw et al. 2000), decreased in

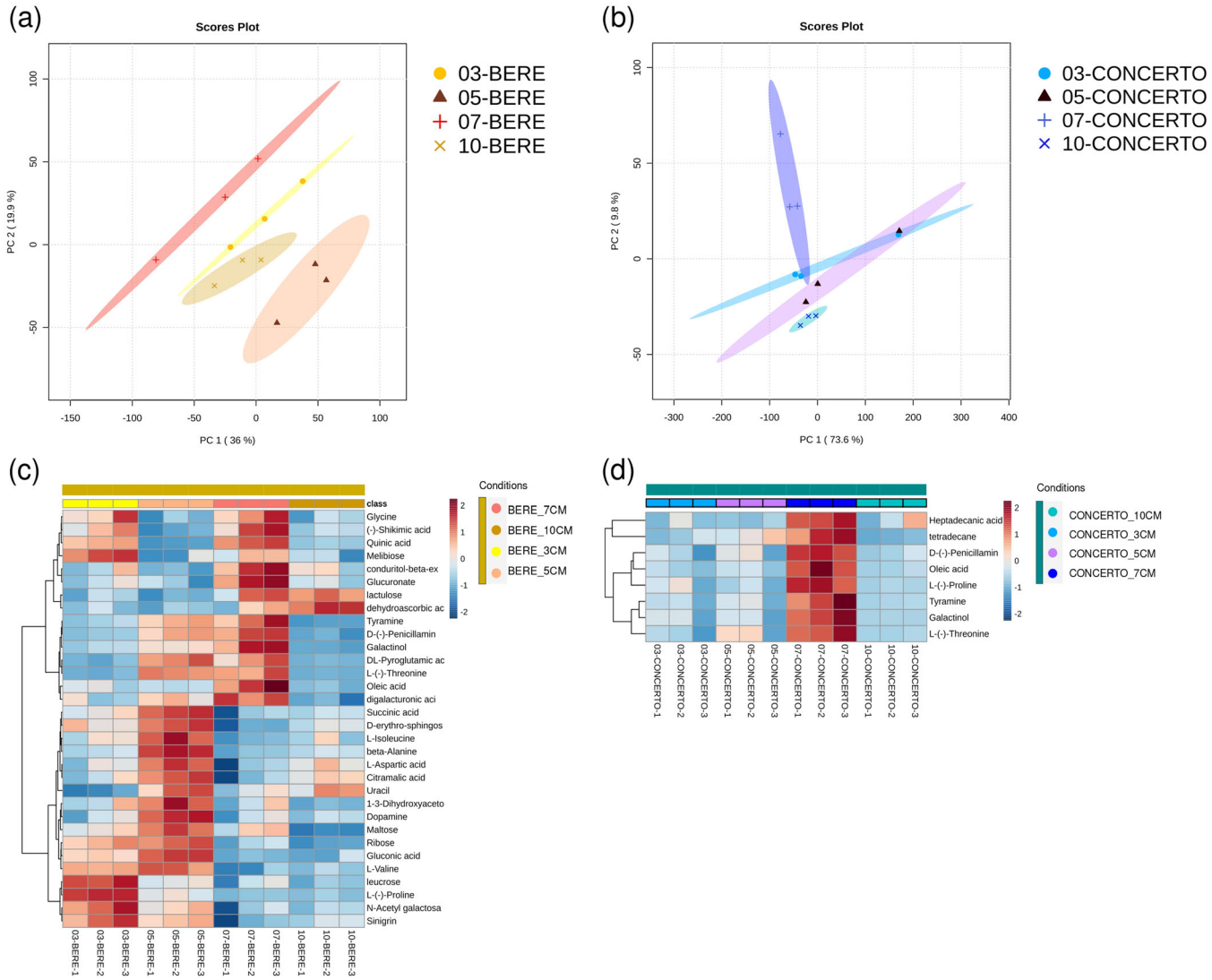

FIGURE 8 | Unsupervised Principal Component Analyses (PCA) of the annotated metabolites found in BERE (a) and CONCERTO (b) roots after grown in pots with 3, 5, 7 and 10 cm of diameter, and hierarchical clustering heatmaps of the statistically significant metabolites (after one-way ANOVA, FDR ≤ 0.05) differentially up-accumulated (red) or down-accumulated (blue) in BERE (c) and CONCERTO (d). [Color figure can be viewed at wileyonlinelibrary.com]

BERE_3CM and 5CM, while D-erythro-sphingosine increased in BERE_5CM. Carbon metabolism was also influenced by genotype and pot size interactions, with increased gluconic acid levels in BERE_3CM and BERE_5CM. Pot size affected the catecholamines dopamine and tyramine, with tyramine reduced in all pot sizes for both genotypes and dopamine increasing significantly in BERE_5CM.

Concerning amino-acids, L-proline was the only metabolite with significant differences between genotypes, showing high up-accumulation in BERE_3CM and BERE_5CM, and down-accumulation in CONCERTO across all pot sizes. Eight other amino acids were differentially accumulated across pot sizes, with all affected in BERE and only L-threonine down-accumulated in both genotypes, specifically in CONCERTO_3CM, CONCERTO_5CM and CONCERTO_10CM, and BERE_3CM and BERE_5CM. DL-pyroglutamic acid was down-accumulated in BERE_3CM and BERE_10CM roots. In contrast, L-aspartic acid, beta-alanine and L-isoleucine increased in BERE_5CM; L-valine increased in BERE_3CM and BERE_5CM; and glycine decreased in BERE_5CM and BERE_10CM.

4 | Discussion

4.1 | Increasing Pot Size Affected Shoots and Roots Differently in the Two Genotypes

According to Poorter et al. (2012), a gradual increase in shoot biomass with increasing pot size was expected. However, this trend was only partially observed. For the landrace BERE, biomass production increased with pot size up to 7 cm, aligning with previous findings of about a 40% increase per doubling of soil volume (Poorter et al. 2012), which could be attributed to an increase in available water and available nutrients (Murphy et al. 2013). Nevertheless, further increase in pot size to 10 cm resulted in a decrease in shoot weight. Poorter et al. (2012) recommended maintaining a BVR ideally below 1 g L^{-1} to avoid

pot size becoming the limiting factor for growth. In our study, the observed ratio in the smallest pot (1.0–2.2 g L^{-1}) may indicate a limiting influence of pot size on plant development. In the 5 cm pots, the BVR ranged between 0.5 and 0.8 g L^{-1}, suggesting that this pot size should no longer or only minimally induce stress due to insufficient soil volume, representing a transition to less restricted or even unrestricted growth, at least for the given young age of the plants. For the larger pot sizes, the BVR continued to decrease, with a value below 0.5 g L^{-1} in 10 cm pots. However, both genotypes can only use the available soil volume to a limited extent for their advantage, potentially indicating that their water and nutrient requirement was met in smaller pots. An alternative explanation for the unexpected lack of increasing growth in the largest pot size could be that the limited fluctuation in water content favoured the development of pathogens. Although this remains a speculation, there are some indications that could support it, such as the upregulation of genes putatively involved in plant response to biotic stresses in BERE_10CM, i.e., genes coding for a pathogenesis-related protein and a rust resistance kinase. Other reasons might be the lack of root-root signalling over larger distances—on average the distance between roots ranges from 1.6 to 2 cm in the 10 cm pots. However, our knowledge of root-root signalling is very scarce (Wang et al. 2021) beyond the recently elicited interplay of local perception and systemic response (Korenblum et al. 2020). Interestingly, none of the published papers on the interaction of root growth and pot size used a cereal like the one in the present study (Audet and Charest 2010; Nord et al. 2011; Chen et al. 2015; Dambreville et al. 2016; Obede da Silva Aragão et al. 2020).

Both genotypes were expected to adapt root length to the available soil volume, with this adaptation, or plasticity, likely differing due to their distinct breeding histories. Landraces are expected to either consistently produce a redundant root system as a safety measure for conditions with resources becoming more limiting (low plasticity) or being able to adapt their root system to adverse conditions (high plasticity) (Aziz et al. 2017). Elite genotypes are expected to produce a consistently small root system (low plasticity) as they have often been selected for yield only under well-watered and well-fertilised conditions. However, both genotypes showed similar root lengths in the smallest pot, suggesting that limited soil volume equally restricted growth through shoot-root signalling. In the smallest pots, CONCERTO showed more inhibited shoot than root growth, with root:shoot ratios differing significantly from other pot sizes. BERE, however, maintained a more balanced root-to-shoot allocation across pot sizes, likely adapting better to limited soil without compromising shoot growth as much as CONCERTO. Few studies on pot size and plant growth report on root growth, often using only two pot sizes with a much narrower range than ours (Audet and Charest 2010; Nord et al. 2011; Dambreville et al. 2016). The most comparable pot sizes were used in the studies by Obede da Silva Aragão et al. (2020) on common beans in 1-3-5 kg pots and by Chen et al. (2015) on peas in pots of 0.6-1.1-1.68 L (excluding split-root experiments). The relatively consistent changes in shoot and root weight detected in these studies align with our results (Chen et al. 2015; Obede da Silva Aragão et al. 2020).

4.2 | Small Pot Sizes Affect Water Dynamics Especially in the Modern Elite Genotype

The smaller the pot, the less absolute water reserves are available per pot. For BERE, daily transpiration rates increased with pot size, which might suggest adaptation to increasing soil volume. In contrast, transpiration values in CONCERTO indicate a plateau in its response to increasing pot sizes, suggesting a different probably less efficient response to soil volume compared to BERE. In the smaller pots, frequent fluctuations between waterlogging and drought have been observed (Poorter et al. 2012). The related drop in BVR between CONCERTO_3CM and CONCERTO_5CM suggests that this genotype experienced changes in water availability as a stress response. The fact that BERE showed a slower decline in BVR as pot size increased, further indicates that this landrace may be more effective to manage fluctuating water availability compared to the modern elite genotype. Some BERE accessions are known for greater drought resistance (Martin et al. 2023), which may explain their stable growth and transpiration rates even in limited soil volumes. On the other hand, the response of CONCERTO in 3 cm pots may be linked to the upregulation of *HvADH4* and other two ADH genes, which are known to be associated with hypoxia acclimation in roots (Ellis et al. 1999) and responses to waterlogging in barley (Luan et al. 2023). It could be hypothesised that CONCERTO genotype was affected by fluctuations in water content in the smallest pot size, supported by CT results showing this genotype explored depth less than BERE. Moreover, enriched GO terms in CONCERTO_5CM related to 'response to oxidative stress' and 'detoxification response' suggest that this genotype was affected by environmental stress also in pots of this size. The differential expression of aquaporin coding genes, especially the downregulation in 5 cm pot size, may be also indicative of a different water uptake compared to the landrace BERE. Aquaporins are known to play a role in the water uptake of barley, with most of this uptake occurring through the lateral roots (Boursiac et al. 2005), and downregulation of aquaporin coding genes in roots was linked to the perception of osmotic stresses (Knipfer et al. 2011).

4.3 | Transcriptomic Profiles Showed Root Responses to Small Pot Stress Mainly in the Modern Elite Genotype

Limited soil volume induces stress-related gene upregulation in both BERE_3CM and CONCERTO_3CM, which aligns with the proline accumulation observed in metabolomic analyses. In BERE_3CM, upregulation of a cytosolic glutamine synthetase gene supports this finding, consistent with studies showing proline increases under drought stress via GS-GOGAT cycle, enhancing cellular osmoregulation (Szabados and Savouré 2010). Additionally, downregulation of a gene for mitochondrial proline dehydrogenase in BERE_3CM suggests reduced proline breakdown under these conditions. A gene which encodes for a glutamate decarboxylase 1, homologue to GAD1 in *Arabidopsis* reported to be essential for sustaining GABA levels in roots (Bouché et al. 2004), also showed upregulation in this condition. Moreover, induction of *NCED5* in both CONCERTO and BERE in the smallest pot size suggested a clear stress response. In cereals, this gene is deemed to regulate abiotic stress tolerance through modulation of ABA biosynthesis (Huang et al. 2019). In both BERE_3CM and CONCERTO_3CM, genes

related to ethylene response were upregulated, consistent with role of ethylene in shaping root architecture, including root hair development (Zhang 2003). By suppressing root elongation and promoting radial swelling, ethylene may strengthen roots in compacted soils (Pierik et al. 2006). The upregulation of several LEA proteins-related genes also suggests a response to stress (Olvera-Carrillo et al. 2010). Moreover, upregulation of genes related to SAUR proteins and ARFs in plants from 3 cm pots suggests the activation of early responses to auxin, allowing the plants to adapt to the limited soil volume. The SAUR proteins are reported to be rapid responders to auxin and involved in cell elongation (Stortenbeker and Bemer 2019), while the ARFs are key transcription factors that translate auxin signals into modulations in gene expression (Guilfoyle and Hagen 2007). Notably, downregulation of a gene putatively encoding an auxin-induced-in-root-cultures protein in both CONCERTO_3CM and BERE_3CM may be indicative of remodelling of auxin-induced lateral root formation, potentially altering normal root development (Neuteboom et al. 1999). Changes in root length in 3 cm pots were also mirrored by the downregulation of genes coding for expansins. Expansins are a family of non-enzymatic plant cell-wall proteins, playing a crucial role in cell enlargement and other developmental processes, facilitating cell growth and morphogenesis (Marowa et al. 2016). They are supposed to drive root growth by regulating cell wall loosening and remodelling (Samalova et al. 2023). The downregulation of these genes in roots suggests alterations in cell wall remodelling, potentially impacting root growth. These genes were also downregulated in CONCERTO_5CM, likely indicating alterations in root development.

The expression of specific genes associated with root development in CONCERTO and BERE highlighted their different adaptation strategies. In CONCERTO, the upregulation of a gene coding for a DEEPER ROOTING 1-like protein (HvDRO1) in 3 and 5 cm pots suggests an active modification of root architecture aimed at enhancing water and nutrient uptake under limited soil volume. In monocots, these proteins regulate root gravitropism and growth angle (Uga et al. 2013), and genetic analyses in Japanese barley accessions showed that *HvDRO1* polymorphisms are linked to local soil adaptation (Nakano et al. 2022). The regulation of *HvEGT1* (Fusi et al. 2022), which influences root angle (upregulated in CONCERTO_5CM, downregulated in BERE_3CM), may also indicate differences in root spreading and soil exploration. The upregulation of the genes coding for LATERAL ROOT PRIMORDIUM and SHORT-ROOT-like proteins, involved in root cell expansion and vascular development (Smith et al. 1995; Kurata et al. 2005), in CONCERTO_5CM suggests a compensatory increase in root surface area in response to small pot sizes. Conversely, downregulation of these genes in BERE might indicate a different growth strategy that prioritises root depth over branching, suitable for accessing deeper water reserves, as observed in our X-ray CT analysis.

4.4 | Specific Metabolite Accumulation in the Landrace Involved in the Stress Response

Glucuronic acid, an oxidised form of *myo*-inositol, that plays structural and biochemical roles in plants, contributing to sphingolipid and raffinose family oligosaccharide (RFO) biosynthesis, and in auxin regulation and stress tolerance (Kanter et al. 2005; Sengupta et al. 2015; Mamode Cassim et al. 2020), was found to be down-accumulated in BERE roots in 3, 5, and 10 cm pots compared to BERE_7CM, while remaining unaffected in CONCERTO. Accordingly, transcriptomics revealed differential expression of genes for pectinesterases and inhibitors, primarily in BERE_3CM. In correlation to this, galactinol was found to be down-accumulated both in BERE and CONCERTO in 3, 5, and 10 cm pots compared to 7 cm. Galactinol, synthesised from UDP-galactose and myo-inositol via galactinol synthase (ElSayed et al. 2014; Sengupta et al. 2015), is a key regulator in the RFOs pathway and often accumulates under stress in the plant aerial part (De Koning et al. 2023), although in barley shoots it was shown to decrease under salt, drought, and nitrogen deficiency (Zhao et al. 2021). Recent evidence suggests that raffinose and *myo*-inositol, rather than galactinol, play a central role in drought tolerance (Li et al. 2020). The great accumulation of galactinol observed in BERE and CONCERTO in 7 cm pots may indicate a reduced need for RFO biosynthesis typically linked to stress adaptation. In this line, two other compounds structural and functionally related to *myo*-inositol were found to be affected in BERE roots in 3 and 5 cm pots, i.e., conduritol-beta-epoxide, that has been reported as a glucosidase inhibitor (Falshaw et al. 2000), and D-erythro-sphingosine, a primary part of sphingolipids, which are bounded to inositol-phosphate and sugars (Mamode Cassim et al. 2020). The alteration in the levels of metabolites related to pectin and *myo*-inositol suggests an involvement of cell wall growth and/or remodelling processes and a role of auxins in BERE roots. These results are in line with the greater root length in BERE than in CONCERTO, mainly in plants grown in 3 and 5 cm pot diameters. Additionally, proline, a protective stress metabolite (Hayat et al. 2012; Szabados and Savouré 2010) that is known to play important roles for plant development (Lehmann et al. 2010), was found highly up-accumulated in BERE_3CM and BERE_5CM, indicating activation of stress response in these roots, in agreement with the expression of proline and stress-related genes. Gluconic acid, which is used to generate energy, NAD(P)H, and biosynthetic precursors for amino acids, nucleotides, and fatty acids via the Entner–Doudoroff pathway, an alternative to glycolysis previously identified in barley (Chen et al. 2016), was strongly up-accumulated in BERE_3CM and BERE_5CM, suggesting that BERE roots invest energy in synthesising stress-related compounds, like proline and other amino acids, which aid in development, stress defence, and inorganic N assimilation. Branched-chain amino acids (leucine, isoleucine, valine) promote drought tolerance (Buffagni et al. 2020), while threonine, aspartate, isoleucine, glutamine, and tyrosine contribute to stress adaptation in various species (Ma et al. 2013). Beta-alanine also accumulates under hypoxia, waterlogging, and drought (Parthasarathy et al. 2019), and alanine aminotransferase is linked to N use efficiency (McAllister and Good 2015; Tiong et al. 2021).

On the other hand, aspartic acid plays many metabolic roles that are crucial for plant growth, cell proliferation, and survival

(Han et al. 2021). Therefore, any variation in its content is linked to stress acclimation. It also acts as a nitrogen carrier, coordinating N assimilation into amino acids, particularly under fluctuating N conditions (Han et al. 2021). Accumulation of L-aspartic acid, beta-alanine and L-isoleucine in BERE_5CM, and L-valine in BERE_3CM and BERE_5CM, might play a role in stress response and N uptake and transport. These results are aligned with shoot N content, N uptake values, and the observed upregulation of high-affinity nitrate transporter genes in BERE roots in 3 and 5 cm pots.

Catecholamines seem to play also a key role in nitrogen and carbon metabolism in BERE and CONCERTO. Plant catecholamines are involved in growth promotion under stress conditions, nitrogen detoxification, carbohydrate metabolism regulation, the interaction with various hormones (including IAA, ethylene and gibberellins), and are precursors of alkaloids (Kulma and Szopa 2007). Dopamine, highly up-accumulated in BERE_5CM enhances tolerance to drought, salt stress, and nutrient deficiency by inducing stress-related genes, including those for chlorophyll degradation, senescence, IAA oxidase, aquaporin, and nitrate transport (Li et al. 2015; Liang et al. 2018; Liu et al. 2020). The accumulation of dopamine in BERE_5CM can be related to the expression of nitrate transporter-related genes, suggesting a putative-enhanced N assimilation and transport in shoots.

Although growth in 3 and 5 cm pot diameters is per se a stressful condition for plants, our results suggest that BERE_5CM plants were able to activate mechanisms to face this stress and to enhance energy production, N uptake and assimilation, and growth promotion mainly due to the activation of genes and the increase in metabolites putatively involved in stress response. BERE_3CM reacted by increasing proline production, while minor differences were detected in amino acids and catecholamines, suggesting that these roots were not able to activate mechanisms for growth promotion, and carbon and N use under stressful conditions. Conversely, CONCERTO metabolomic responses were not as substantially affected by the pot size.

5 | Conclusions

Our study demonstrated distinct responses of the two barley genotypes to pot size, with differences in root trait adaptation. Transcriptomics and metabolomics revealed genotype- and pot size-specific gene regulation and metabolic changes. The smallest pot size caused clear stress in both genotypes and is not recommended for growth experiments. Among the other tested pot sizes, the landrace BERE showed greater stability and metabolic resilience, while the modern elite genotype CONCERTO exhibited more sensitivity, with significant changes in transcriptomic profiles but less in metabolomics, suggesting a delayed stress response. Our results highlight the greater plasticity of the landrace BERE in adapting to varying pot sizes compared to the modern elite genotype CONCERTO, likely due to distinct genetic mechanisms conferring resilience across environmental conditions. This aligns with the current vision of developing root phenotypes that enhance crop resilience to environmental stresses by exploiting landrace genetic make-up (Schneider and Lynch 2020; Amtmann et al. 2022).

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.