

## ORIGINAL ARTICLE

# Assessing genetic variation of maize (*Zea mays*) root DNA density under contrasting water supply

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## Abstract

Optimizing root systems is one crucial point in drought tolerance breeding of plants. Introducing root-related traits to breeding programmes is time-consuming and laborious. Most of the commonly used methods are not suitable to be applied in a larger amount of plants. Here we present a study applying a DNA-based root phenotyping method (root DNA density; RDD) for phenotyping the root system of maize. Twenty-one maize inbred lines were investigated in a rain-out shelter experiment and 19 maize inbred lines in a greenhouse experiment under well-watered and drought conditions. Beside other commonly used root traits, agronomic traits of the plants were recorded and compared to RDD. Within root traits, RDD showed high significant genotypic variation and the highest repeatabilities of up to 72.4%. In contrast to most agronomic traits, repeatabilities increased under drought conditions. Values showed also good correlations between rain-out shelter and greenhouse trial, indicating the potential of this method for obtaining comparable results across different environments.

## KEYWORDS

DNA-based, qPCR, real-time PCR, root DNA density (RDD), root phenotyping

## 1 | INTRODUCTION

Root characteristics have been recognized as promising and comprehensive traits to improve crop cultivars (Lynch, 2007a; Lynch & Wojciechowski, 2015; Rogers & Benfey, 2014). Roots are responsible for the uptake of water and nutrients (Gruber, Giehl, Friedel, & von Wiren, 2013; Zarebanadkouki, Kroener, Kaestner, & Carminati, 2014) and often show large exploitable genetic variation (Lynch, 2013; White et al., 2013). There is a critical mass of research demonstrating improved crop performance due to specific root phenotypes. In wheat, a more compact root system, steeper root angle and greater root length in the subsoil conferred increased drought tolerance (Christopher, Manschadi, Hammer, & Borrell, 2008; Maccaferri et al., 2016; Manschadi, Christopher, deVoil, & Hammer, 2006). Also in maize (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.), a considerable number of

studies demonstrated the potential of roots for increased resource-use efficiency (Ho, McCannon, & Lynch, 2004; Landi et al., 2010; Lynch, 2007b; Lynch, Chimungu, & Brown, 2014; Zhu, Brown, & Lynch, 2010).

Breeding approaches employing root traits to improve crop germplasm are only modest (Lynch & Brown, 2012; Zhu, Ingram, Benfey, & Elich, 2011), mainly because suitable phenotyping methods in field environments are lacking (Comas, Becker, Cruz, Byrne, & Dierig, 2013; Meister, Rajani, Ruzicka, & Schachtman, 2014; Tuberosa, 2012). Field-based phenotyping of roots, for example trenching, is accurate but destructive and laborious (Böhm, 1979). In recent years, advanced techniques such as ground-penetrating radar, X-ray (Fiorani & Schurr, 2013 and references therein) or magnet resonance tomography (van Dusschoten et al., 2016) were applied. Although some techniques can give adequate resolution of roots in soil and allow non-destructive and real-time assessment of root growth, most are still stationary or

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not ready for routine field usage. Predominant trends at the moment are hence excavation of root crowns using the *Shovelomics* method (Trachsel, Kaeppler, Brown, & Lynch, 2010) and soil coring approaches (Wasson et al., 2012). Coring-based approaches are often used, where root length density (RLD) and root mass density (RMD) were utilized to describe root proliferation (Böhm, 1979; Gregory, 2006).

Since Jackson, Moore, Hoffmann, Pockman, and Linder (1999) first used DNA to distinguish roots of different tree species in soil samples, the literature has indicated considerable potential for DNA-based root phenotyping. Mommer, Wagemaker, Kroon, and Ouborg (2008) exchanged the PCR in the first approach from Jackson and colleagues by quantitative PCR (qPCR) and were able to show that this step allowed a robust and quantitative assessment of species in mixed root samples from an artificial grassland community consisting of two grasses and two forbs. With qPCR, roots get quantified on the basis of the live cell number (Riley, Wiebkin, Hartley, & McKay, 2009), while reducing the drawback of quantifying non-functional dead roots. After treatment with herbicide or defoliation, DNA decay from ryegrass roots has shown to be reasonably quick (80% in 10 days), while root mass was not significantly reduced within the same time (McKay et al., 2008). Developing species-specific DNA oligonucleotides and probes, it enables quantification without capturing non-target species like weeds growing in the field or remnant roots from crops of previous growing seasons (Watt, Magee, & McCully, 2008). The calculation of the absolute root DNA amount in a given soil volume led to the trait called root DNA density (RDD). It has been shown for subterranean clover (*Trifolium subterraneum* L.) and ryegrass (*Lolium perenne* L.) that RDD accurately describes different masses of root tissue added to a given amount of soil (Riley et al., 2009). For mango (*Mangifera indica* L.), it was shown that using RDD is suitable to distinguish between different soil depths (Bithell et al., 2015). Considering utilization of RDD in research and breeding, Huang et al. (2013) showed significant genotypic variation for RDD in wheat (*Triticum aestivum* L.) and heritability for RDD between 50% and 90% across several field locations in Australia. In a study focused to effects of phosphorus uptake by different varieties, genotypic variation for RDD in wheat and barley was found (McDonald, McKay, Huang, & Bovill, 2017) and Pierre et al. (2018) used RDD in sugarcane (*Saccharum officinarum* L.) for quantifying live root mass.

In this study, suitability of RDD for addressing genetic variation of maize root distribution in soil was investigated. Different maize genotypes were grown in a rain-out shelter (ROS) as well as in the greenhouse (GH) under contrasting water supply, and RDD was determined in both environments. A comparison to other commonly applied methods of root phenotyping and impact on above-ground plant performance was investigated.

## 2 | MATERIALS AND METHODS

### 2.1 | Rain-out shelter and greenhouse experiments

A semi-controlled field trial using a ROS with 19 maize (*Zea mays* L.) inbred lines (Table S1) selected from previous drought stress experiments

was conducted at Freising, Germany (N 48°24'41.04", E 11°43'23.10"). Maize was sown on 9 May 2014 in single-row plots (10 plants per plot) with a plant density of 11 plants/m<sup>2</sup>. The experimental design was a randomized complete block design with six blocks per treatment and two treatments, a well-watered control (ROS\_WW) and a drought stress treatment (ROS\_D). Three blocks each were used for determining common agronomic traits; the other three were used for soil coring. Watering was carried out every second day (8 mm/m<sup>2</sup>) with a linear move irrigation system. At 35 days after sowing, watering was ceased for the drought treatment and stress was induced to peak slightly before flowering time. At 105 days after sowing, watering was resumed (for time course of soil water potential see Figure S1). Agronomic measures were carried out according to good agricultural practice.

A GH experiment comprising 21 maize inbred lines (15 shared with ROS experiment; Table S1) was conducted at the Phenodyn platform of INRA Lépse in Montpellier, France, from 13 February to 23 March 2014 (Figure S2). The experimental design was a randomized complete block with two blocks and two treatments, a well-watered control (GH\_WW) and a drought stress treatment (GH\_D). The pots had a diameter of 15 cm, a height of 40 cm, filled with 6,550 g of standard potting mix with complete fertilizer and contained four plants per pot. Until 20 days after sowing, soil water potential was kept close to field capacity in both treatments. From 21 days after sowing on, one plant per pot was retained until harvest, and drought stress was induced within -5 bar and -3 bar according to the calculated soil water potential.

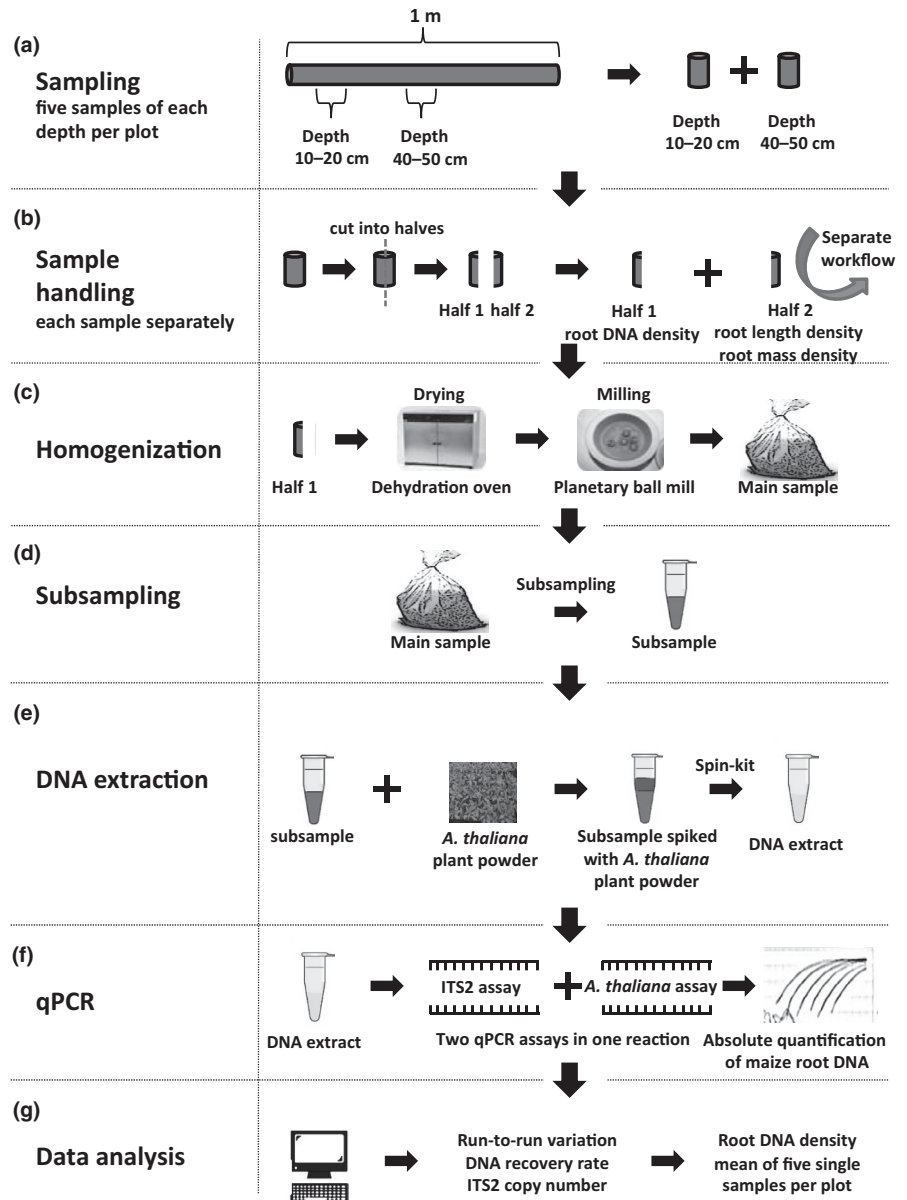
### 2.2 | Extraction of soil samples

In order to investigate RDD, RLD and RMD, each field plot in the rain-out shelter was sampled with five cores during flowering time (87–95 days after sowing) with a percussion drilling set (04.19.SE, Eijkelkamp, Giesbeek, Netherlands). The core sampler (1 m length and 4.4 cm diameter) was drilled into the soil using a percussion hammer and pulled out manually. The integrated polyethylene sleeve insertion set (04194302, Eijkelkamp) allowed to process samples in a consistent and undisturbed way. Five 2.5 to 3 kg cores per plot were taken within rows, each core between two plants. Soil horizons in 10–20 cm (top) and 40–50 cm (sub) were sampled. The mean soil bulk density of the sampled horizons was 1.45 and 1.57 g/cm<sup>3</sup>, respectively. The soil cores were cut in pieces of 10 cm length and halved lengthwise with a knife. One half was used for measuring RDD, and the other one was frozen at -20°C until root washing (Figure 1a+b).

Soil samples from GH experiment were taken using a manually operated gouge auger with 2 cm diameter at 36 DAS. Each pot was sampled four times at three depths, increment 0–13 cm (top), 14–26 cm (mid) and 27–40 cm (sub). The four samples were pooled for determination of RDD.

### 2.3 | DNA extraction from soil samples

All soil samples were dried in a drying oven (Memmert, Schwabach, Germany) at 50°C for 24 hr. Milling was done in a planetary ball mill

**FIGURE 1** Workflow for DNA-based root quantification from soil samples

(P5, Retsch, Haan, Germany) using zirconium oxide beakers for 6 min at 350 rpm (Figure 1c). From the highly homogenized soil samples of up to 400 g, 350 mg were sub-sampled (Figure 1d) and spiked with exactly 25 mg *Arabidopsis* plant powder to determine DNA recovery rate. For DNA extraction, the Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, USA) was used in combination with the homogenization device FastPrep-24 (MP Biomedicals) according to manufacturer's guide (Figure 1e).

## 2.4 | Quantitative PCR analysis of soil DNA samples

Environmental Master Mix 2.0 (Life Technologies, Carlsbad, USA) in onefold concentration was used for all qPCR reactions, which were carried out in 20  $\mu$ l volume and with three technical replicates on a Step One Plus real-time PCR system (Life Technologies). A multiplex PCR reaction was set up targeting a transgenic *Arabidopsis* construct for determining DNA recovery from extraction and the

*internal transcribed spacer 2 (ITS2; Buckler & Holtsford, 1996)* region for absolute quantification of maize root DNA (for oligonucleotide and probe information see Table S2). Each reaction contained 2  $\mu$ l of DNA sample while total DNA concentration of the samples ranged from 10 to 100 ng/ $\mu$ l. For all runs, a two-step cycling protocol was initiated by 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C (Figure 1f).

## 2.5 | Classical root phenotyping

For investigation of RLD, the soil samples were soaked in water and roots were separated from soil by using a combination of different sieves, with a maximum mesh size of 0.25 mm and a hand-held sprayer. Then, live roots were picked out of the residues using forceps, and visually distinguishing them from roots of weeds and other debris. The criteria were bright colour of live roots as compared to dark colour of dead roots from the last season (Watt et

al., 2008), and maize specific root topology and thickness. Washed roots were stored in 30% ethanol (v/v) at 4°C until scanning. The scanned roots were analysed with WinRHIZO Pro software (v2009, Regent Instruments, Quebec, Canada). After scanning, the five RLD samples per plot and depth were pooled and dried at 70°C for 48 hr to determine RMD.

For root phenotyping with the *Shovelomics* method at flowering time, root crowns of three representative plants per plot were dugged out and washed free from soil and debris. Then, number (CN) and angle (CA) of crown roots, as well as number and length of lateral roots (CB; CL) emerging from crown roots, were determined using the maize *Shovelomics* scoreboard provided by Penn State University, USA (Anonymus, 2013).

## 2.6 | Phenotyping agronomic traits

The time-point of male (Mflow) and female (Fflow) flowering was assessed as number of days from sowing to the day when 50% of the plants in a plot showed pollen shed or silking, respectively. Anthesis silking interval (ASI) was calculated as the difference of days between Fflow and Mflow. At maturity, five representative plants per plot were harvested for determination of biomass yield (BY) and dried at 50°C for 7 days. Kernel yield was determined considering all plants of a plot (KY), which were dried at 50°C for 7 days. A subsample was used to determine thousand-kernel weight (TKW), and number of kernels per ear (KPE) was calculated as total number of kernels divided by number of ears. Harvest index (HI) was calculated as the ratio of harvested grain weight to total shoot biomass at maturity.

## 2.7 | Calculations and statistical data analysis

For calculating absolute DNA quantities from adjusted quantification cycle ( $C_q$ ) values ( $C_{qX\text{corr}}$ ), a standard curve with seven 10-fold dilution steps was measured. The resulting regression of DNA quantities on  $C_q$  values was used to calculate RDD (Figure 1g):

$$\text{RDD}_X = 10^{\frac{C_{qX\text{corr}} - b}{m}}$$

$\text{RDD}_X$  is the amount of maize root DNA in qPCR reaction of the soil sample X,  $C_{qX\text{corr}}$  the  $C_q$  value of the maize root DNA in soil sample X corrected for run-to-run variation and relative DNA recovery,  $m$  the slope and  $b$  the intercept of the standard curve. Afterwards, RDD values were adjusted with a genotype-specific factor for *ITS2* copy number (see Table S3). A detailed description of the calculation workflow accounting for run-to-run variation and DNA recovery from soil is given in ESM (pp. 4–7).

Analyses of variance of phenotypic data were performed using PLABSTAT software version 3A (Utz, 1997) and the ASReml package (Butler, Cullis, Gilmour, & Gogel, 2009) implemented in R version 3.1.3 (R Core Team, 2014). Adjusted means were calculated according to the following model with the parameter  $\alpha_i$  treated as fixed effect:

$$P_{ij} = \mu + \alpha_i + \gamma_j + \varepsilon_{ij}$$

where  $P_{ij}$  denotes the phenotypic observation of the  $i$ th genotype in the  $j$ th block,  $\mu$  the grand mean,  $\alpha_i$  the effect of the  $i$ th genotype,  $\gamma_j$  the effect of the  $j$ th block and  $\varepsilon_{ij}$  a random error term. Variance components were calculated assuming all effects in the model as random. Trait repeatability was calculated as the proportion of genotypic variance to the phenotypic variance:

$$\text{Rep} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma^2}$$

where  $\sigma_g^2$  and  $\sigma^2$  denote the genotypic and residual variance components, respectively. Phenotypic correlations were calculated as Pearson's correlation coefficient using the R function *cor.test*.

## 3 | RESULTS

### 3.1 | Assessing root DNA density with quantitative PCR

In the present study, 1,392 soil samples from ROS and GH were analysed with qPCR to determine RDD. Reaction efficiency was at 99.7% with a  $R^2$  of 0.998. According to the established standard curve for the *ITS2* assay, detection limit was 500 fg of maize root DNA. The mean reaction efficiency (per cent of template that was amplified in each cycle) of the *ITS2* assay was 100.7%, with a  $R^2$  (coefficient of determination obtained for the standard curve) of 0.998 in a  $C_q$  range between 17 and 34. DNA recovery from soil samples estimated with an *Arabidopsis* assay was close to 100%, with a  $R^2$  of 0.996, in a  $C_q$  range from 20 to 33. Mean RDD values in the ROS experiment ranged from 4.8 to 71.9 ng/g and 836 to 3,786 ng/g in the GH experiment. Values for different soil depths and water availability are given in Table 1.

In both treatments and all three soil depths in GH, RDD showed significant genotypic variation (Table 2). Repeatabilities for the three soil depths ranged in the same scale as in ROS (Table 3), but were higher under drought conditions (66.9%–78.5%) than in the well-watered treatment (43.7%–47.2%). Coefficient of variance for RDD was high in GH\_WW (61.7% in subsoil) and approximately reduced to the half in GH\_D.

Root DNA density values from ROS were correlated with RDD values from GH using 15 maize genotypes phenotyped on both platforms. Across depths, the RDD values showed significant correlations (Figure 2) between both environments in well-watered ( $r = 0.81$ ) and drought treatments ( $r = 0.73$ ).

### 3.2 | Comparison of root DNA density to other root and shoot traits in maize

In order to evaluate RDD as a trait to describe the maize root system, the root coring traits RLD and RMD as well as root crown traits and shoot traits related to flowering, BY and KY were compared to RDD (Table 3). The coefficient of variation indicated similar exploitable variation for RDD, RLD and RMD, while the coefficient of

**TABLE 1** Mean root DNA density (RDD) values of 19 (rain-out shelter; ROS) and 21 (greenhouse; GH) maize genotypes, standard error (s.e.), minimum (Min.) and maximum (Max.) [ng/g] in rain-out shelter and greenhouse experiment

Environment	Treatment	Depth [cm]	Mean	s.e.	Min.	Max.
ROS	WW	10–20	71.9	7.3	29.3	153.2
ROS	D	10–20	47.9	6.5	12.1	134.6
ROS	WW	40–50	13.5	1.6	6.2	30.5
ROS	D	40–50	4.7	0.8	1.1	14.6
GH	WW	0–13	1,411	162	411	3,169
GH	D	0–13	3,145	295	1,560	6,064
GH	WW	14–26	987	120	147	2,573
GH	D	14–26	3,786	299	1,755	6,255
GH	WW	27–40	836	130	85	2,486
GH	D	27–40	2,227	227	1,096	5,111

Abbreviations: D, drought treatment; WW, well-watered.

**TABLE 2** Estimates of genotypic ( $\sigma_g^2$ ) and residual ( $\sigma^2$ ) variance components, significance of the genotypic variance (sig), repeatabilities (rep) and coefficients of variation (CV) for root DNA density (RDD) in three soil layers of 21 maize inbred lines in the greenhouse experiment

Trait	Well-watered						Drought				
	Depth [cm]	$\sigma_g^2$	$\sigma^2$	Sig	Rep [%]	CV [%]	$\sigma_g^2$	$\sigma^2$	Sig	Rep [%]	CV [%]
RDD	0–13	3,922	4,390	*	47.2	45.2	11,051	3,368	**	76.6	19.5
RDD	14–26	2,064	2,660	*	43.7	50.0	14,735	4,033	**	78.5	16.8
RDD	27–40	2,243	2,852	*	44.0	61.7	8,722	4,318	**	66.9	29.1

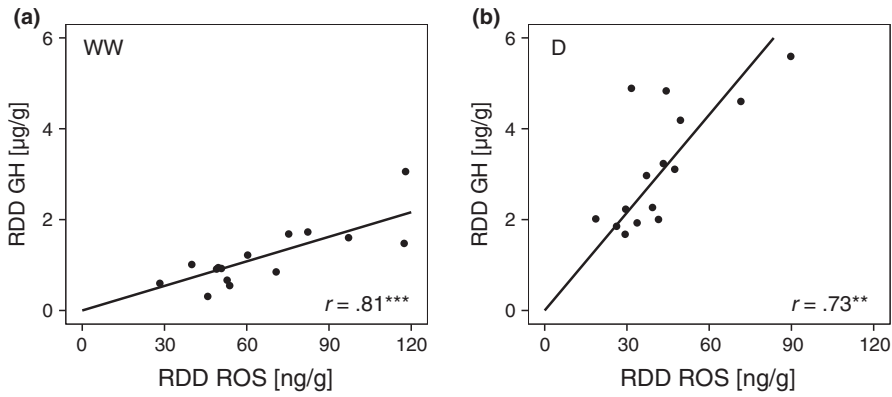
Note: Significance \*, \*\*: at  $p < .05, .01$ .

**TABLE 3** Estimates of genotypic ( $\sigma_g^2$ ) and residual ( $\sigma^2$ ) variance components, significance of the genotypic variance (sig), repeatability (rep) and coefficients of variation (CV) for root and shoot traits in 19 maize inbred lines in the rain-out shelter experiment

Trait	Well-watered						Drought				
	Depth [cm]	$\sigma_g^2$	$\sigma^2$	Sig	Rep [%]	CV [%]	$\sigma_g^2$	$\sigma^2$	Sig	Rep [%]	CV [%]
RDD	10–20	1,647.1	878.3	**	65.2	28.8	1,016.0	387.4	**	72.4	28.8
RDD	40–50	72.8	86.0	**	45.9	42.2	8.0	46.1	ns	–	82.6
RLD	10–20	0.2	1.0	ns	–	32.8	0.2	0.5	*	30.7	33.9
RLD	40–50	0.0	0.2	ns	–	45.1	0.0	0.1	ns	–	41.2
RMD	10–20	201.3	198.9	**	50.3	28.0	50.4	156.4	*	24.4	41.6
RMD	40–50	1.3	9.3	ns	–	49.2	0.9	6.3	ns	–	54.8
CN		21.5	49.1	*	30.5	23.1	14.3	18.9	**	43.2	17.8
CA		17.2	31.0	**	35.7	9.6	0.0	49.8	ns	–	11.0
CB		0.3	0.5	**	34.3	16.2	0.2	0.4	*	31.4	19.4
CL		0.1	0.3	*	30.5	31.6	0.0	0.5	ns	–	40.2
Fflow		22.6	10.2	**	69.2	3.1	17.5	12.1	**	59.2	3.9
Mflow		17.3	7.7	**	68.9	3.5	16.3	12.3	**	57.0	3.8
ASI		1.7	2.2	**	44.2	94.2	1.9	2.3	**	45.0	46.2
KPE		2,894.0	1,316.1	**	68.7	24.6	648.8	975.9	**	39.9	26.8
TKW		648.7	578.9	**	52.8	10.2	994.0	772.4	**	56.3	11.1
KY		21,986.0	9,327.5	**	70.2	28.6	1,591.4	11,082.9	ns	–	36.9
BY		10,774.0	5,771.8	**	65.1	13.0	926.4	4,953.2	ns	–	24.3
HI		1.5	0.6	**	70.0	22.7	0.4	1.0	*	27.1	20.4

Note: Significance \*, \*\*: at  $p < .05, .01$ .

Abbreviations ASI, anthesis silking interval; BY, biomass yield; CA, crown root angle; CB, number of lateral roots; CL, length of lateral roots; CN, number of crown roots; Fflow, time-point of female flowering; HI, harvest index; KPE, kernels per ear; KY, kernel yield; Mflow, time-point of male flowering; ns, not significant; RDD, root DNA density; RLD, root length density; RMD, root mass density; TKW, thousand kernel weight.



**FIGURE 2** Regression of RDD in GH on RDD in ROS. For calculations, 15 overlapping maize inbred lines between ROS and GH were used, and two and three sampled depths for ROS and GH, respectively, were pooled to result in one RDD value per treatment well-watered (a) or drought (b). The correlation coefficients ( $r$ ) are given (\*\*, \*\*\*:  $p < .01$ ,  $.001$ )

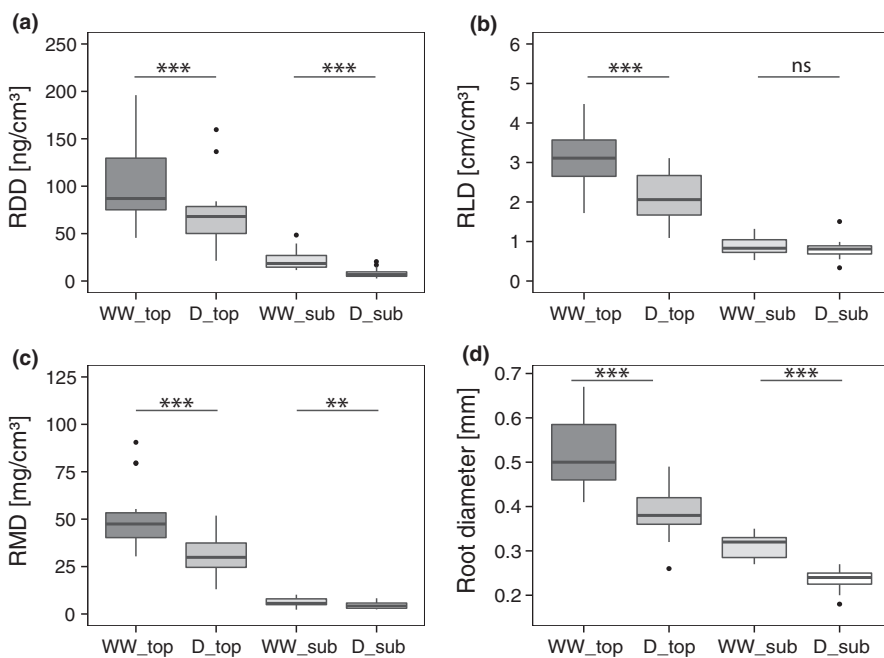
variation of these coring-based root traits was often higher than for the *Shovelomics* root traits.

In ROS\_WW, RDD showed significant genotypic variation in topsoil and subsoil, while genotypic variance component for RLD was not significant in both depths. Under well-watered conditions, repeatability of RDD was 65.2% in topsoil, comparable to shoot traits like FFlow (69.2%), KPE (68.7%) and biomass (65.1%) as well as kernel yield (70.2%). In ROS\_D, all three coring-based root traits demonstrated significant genotypic variation in topsoil, but none in subsoil. Under drought conditions, RDD in topsoil showed the highest repeatability of all traits (72.4%), whereas the shoot-related traits showed reduced repeatabilities for FFlow (59.2%) and KPE (39.9%) as well as no significant genotypic variation for kernel and biomass yield. *Shovelomics* traits CN and CB showed repeatabilities of 43.2% and 31.4%, respectively, whereas CA and CL had no significant genotypic variation in ROS\_D. In ROS\_WW, all four *Shovelomics* traits reached repeatabilities between 30.5% and 35.7%. Influence of water availability and sampling depth on RDD was significantly reduced in subsoil compared with topsoil

and under drought conditions compared with the well-watered treatment (Figure 3). Similar observations were made for RMD (both soil layers) and RLD (only topsoil).

In topsoil, RDD was correlated with RLD under well-watered and drought conditions ( $r = .56$  and  $.59$ ;  $p < .05$ ). Genotypic variation for RMD was significant in topsoil but not significant in subsoil. RDD was correlated with RMD only under drought conditions ( $r = .58$ ;  $p < .05$ ), but not in the well-watered variant ( $r = .3$ ). In subsoil, RDD showed a positive correlation with RMD under drought conditions ( $r = .67$ ;  $p < .01$ ), but not under well-watered conditions. Independent from depth or treatment, RLD and RMD were always significantly correlated (topsoil: ROS\_WW  $r = .76$ ; ROS\_D  $r = .63$ , subsoil: ROS\_WW  $r = .50$ ; ROS\_D  $r = .87$ ;  $p < .01$  each).

Root DNA density in GH experiment showed significant genotypic variation under optimal water supply across all three soil depths with repeatabilities between 43.7% and 47.2%. Under drought conditions, genotypic variance was highly significant and repeatabilities increased to a range between 66.9% and 78.5%.



**FIGURE 3** Effect of water availability and soil depth on root traits RDD (a), RLD (b), RMD (c) and root diameter (d) in ROS. Adjusted means ( $n = 19$ ) are plotted for treatment combinations ROS\_WW/topsoil (WW\_top), ROS\_D/topsoil (D\_top), ROS\_WW/subsoil (WW\_sub) and ROS\_D/subsoil (D\_sub). Significant differences between water treatments within one soil depth are indicated by means of a  $t$  test (\*\*, \*\*\*:  $p < .01$ ,  $.001$ , not significant)

## 4 | DISCUSSION

DNA-based root phenotyping holds promise to widen the phenotyping bottleneck for the characterization of root growth in field environments (Furbank & Tester, 2011; McKay et al., 2008). In the presented study, it could be shown that in maize RDD enables root phenotyping of plants in field environments. RDD showed intermediate to high repeatabilities in field and greenhouse environments, representing basic prerequisites for the use of RDD in breeding programmes (Kuijken, van Eeuwijk, Marcelis, & Bouwmeester, 2015).

### 4.1 | Comparing RDD with root traits and agronomic traits

Concerning the reaction of RDD, RLD and RMD on contrasting water supply and different soil depths in the ROS, RDD and RMD showed a decrease under drought conditions with increasing soil depth (Figure 3a+c). For RLD in subsoil, no significant reaction to drought stress was observed (Figure 3b). Due to a decrease in average root diameter and therefore increase in specific root length (Figure 3d), RLD stayed constant although the other parameters decreased. Root length and root mass often decrease with increasing soil depth (Nicoulaud, King, & Tardieu, 1994; Liedgens, 1998; Trachsel, Kaeppler, Brown, & Lynch, 2013; Cai et al., 2014; Grieder, Trachsel, & Hund, 2014; Ning, Li, White & Li, 2015; Pierre et al., 2018). Many studies investigating annual cereals indicated impaired root growth upon water limitation (Davies & Bacon, 2003; Sharp et al., 2004; Wu & Cisgrove, 2000).

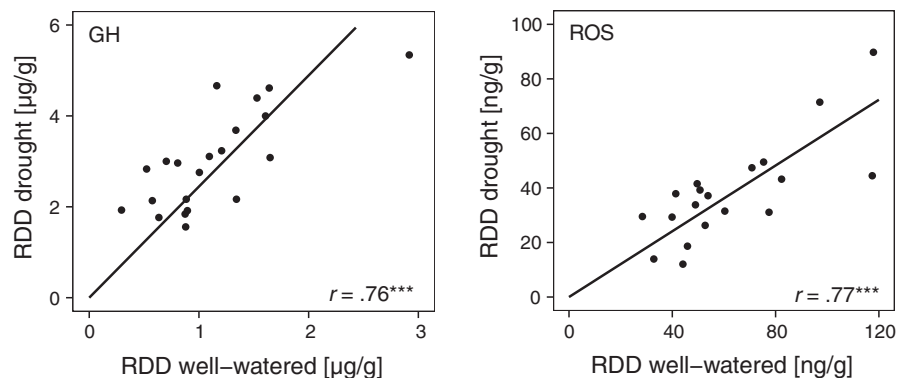
Root DNA density consistently showed highly significant genotypic variation in ROS\_WW with 65.2% repeatability in topsoil and 45.9% in subsoil, as well as in topsoil of ROS\_D (repeatability 72.4%). The only study investigating genotypic variation for RDD so far was conducted in wheat (Huang et al., 2013). The authors reported significant genotypic variation for RDD in the topsoil increment (0–10 cm) at several field locations with heritabilities always above 50%. RLD showed only significant genotypic variation in topsoil of ROS\_D with a low repeatability (30.7%). Wasson et al. (2014) were even not successful to distinguish 40 wheat genotypes in field trials using total RLD from soil cores, which points out the pitfalls of this trait. Repeatabilities for the *Shovelomics* traits CN, CA, CB and CL also remained low (30.5% to 43.2%) for ROS\_WW and failed for CA

and CL in ROS\_D, demonstrating the error proneness of direct root phenotyping. As expected, repeatabilities for agronomic and yield-related traits declined under drought conditions. Values for flowering time-point traits were slightly reduced, but all yield-related traits (KPE, KY, BY and HI) showed a severe reduction of repeatabilities under drought conditions, as described for many drought stress experiments. In contrast, RDD in topsoil showed an increase in repeatability from 65.2% to 72.4%. A similar effect was shown within the GH experiment for all three soil depths.

Correlations for RDD across depths between well-watered and drought treatment were high for ROS and GH ( $r = .77$  and  $r = .76$ , respectively; Figure 4). Correlations for RDD between soil depths were high in ROS experiment ( $r = .86$  for well-watered and  $r = .84$  for drought, respectively). RDD in topsoil was correlated with RLD in topsoil but not with RMD under both well-watered and drought. RDD in the subsoil is correlated with any of the other root traits. In the topsoil, the average root diameter was significantly higher than in subsoil (Figure 3d,  $p < .001$ ,  $n = 19$ ). Thus, conditions affecting root diameter distribution or the proportion of fine roots will influence the correlation of RDD with RLD, because higher proportions of fine roots are inevitably lost for RLD, but captured by RDD. Further, there are reports that abiotic stress triggers root aerenchyma, which leads to less cells per root cross-section and eventually to an altered association between RDD and RLD. Given these large differences in the expression of the traits RDD and RLD, both cannot be seen as interchangeable but rather complementary. Regarding factors influencing the correlation of RDD and RMD, Haling, Simpson, Culvenor, Lambers, and Richardson (2012) pointed out that the relationship of both changes with variation in species, soil sampling depth and harvest date of the roots with the need of calibration. Also the results in the presented study showed that the correlation changes with different soil sampling depths.

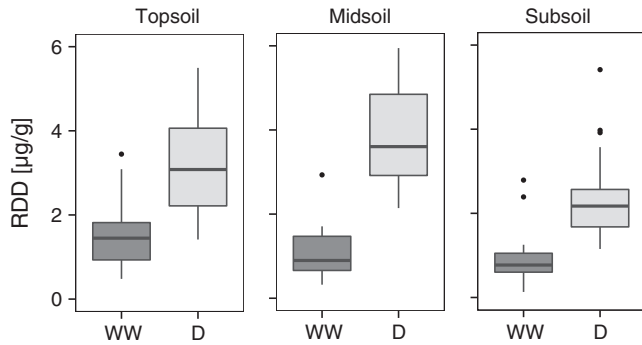
Except for the correlation of RMD and CN in ROS\_WW and topsoil ( $r = .61$ ), coring-based traits were not correlated with the *Shovelomics* traits. However, the result showed that the number of crown roots in the very topsoil, where roots are thick and have large parts of their mass, was correlated with root mass determined from the topsoil cores.

Under ROS\_WW conditions, no correlation with agronomic traits was observed. Under drought conditions, RDD in the topsoil was correlated with HI ( $r = -.53$ ) and KPE ( $r = -.50$ ). Resource input to



**FIGURE 4** Regression of root DNA density (RDD) under drought treatment on RDD under well-watered conditions in greenhouse (GH; 21 genotypes) and rain-out shelter (ROS; 19 genotypes).

\*\*\*  $p < .001$



**FIGURE 5** Effect of water availability and soil depth on root DNA density (RDD) in the greenhouse experiment for well-watered (WW) and drought (D) treatment in three soil horizons (topsoil: 0–13 cm; midsoil: 14–26 cm; subsoil: 27–40 cm). Drought treatment effect was significant in all soil depths by means of a *t* test ( $p < .001$ )

roots has to be justified with reasonable excess profit in terms of resource acquisition to keep plant growth economical (Lynch, 2014). A higher RDD and thus more root material in shallow soil, where no or little water was available for the plants during drought, could have contributed to a reduction in KY and HI. Passioura (1983) stated that an increased root growth could negatively impact HI since less assimilates would be available for grain formation. Average RLD in depth 10 under drought was 2.1 cm/cm<sup>3</sup>, indicating a potential metabolic burden (Lynch, 2014), as plant material was not preselected for root traits and resource efficiency.

## 4.2 | Comparing root DNA density in field and greenhouse environments

Significant correlations were observed between well-watered and drought treatments in GH and ROS (Figure 4). The top five ranking genotypes for RDD under well-watered conditions were identified in both environments, but the rank order was different. Under drought conditions, four genotypes could be retrieved with additional rank order changes. Thus, it is likely that significant genotype  $\times$  environment interaction effects influenced phenotypic expression for RDD. Yet, regarding the correlation of RDD between ROS and GH under well-watered ( $r = .84$ ) and drought ( $r = .73$ ) conditions (Figure 2), the correlation coefficients were high.

Root DNA density values in the ROS experiment ranged from 4.8 to 71.9 ng/cm<sup>3</sup>, comparable to values reported for wheat (60–240 ng/g soil), barley (*Hordeum vulgare* L.; 0.2–20 ng/g soil) or canarygrass (*Phalaris* spp.; 1–80 ng/cm<sup>3</sup> soil; Haling et al., 2012; Huang et al., 2013; McKay et al., 2008). As expected, there were differences between ROS and GH in terms of absolute RDD values, such as the RDD values were much higher in GH than in ROS (well-watered: 17-fold; drought: 78-fold; Figure 2). There are several reasons that likely have contributed to higher RDD values in the pot experiment. First the physiological age of plants differed between flowering stage in ROS and vegetative growth in GH. Root proliferation in cereals rapidly ceases at begin of the generative growth phase, with often

remarkable reduction of root growth in favour of carbon investment in grain towards generative growth (Gregory, 2006; Trachsel et al., 2013). Ryegrass and subterranean clover showed a decline in RDD with increased root age (Riley et al., 2009), and canarygrass plants reduced RDD by 90% from growth days 28 to 70 (Haling et al., 2011). Differences may also occur from soil bulk density as it was negatively correlated with root proliferation (Bingham & Bengough, 2003; Whitmore & Whalley, 2009). Bulk density of less than 1 g/cm<sup>3</sup> in the GH pots allowed more vigorous root growth compared to ROS with bulk densities between 1.45 and 1.57 g/cm<sup>3</sup>. A third point of influence was plant density, which affected the occurrence and properties of roots in the soil and thus RDD (Hecht, Temperton, Nagel, Rascher, & Postma, 2016; Liu, Song, Liu, Zhu, & Xu, 2012; Riley et al., 2009). Plant density in GH pots resulted in 224 plants/m<sup>2</sup> as opposed to ROS with 11 plants/m<sup>2</sup>.

Contrary to ROS and other studies where RDD in drought treated plants was reduced or showed no effect (Huang et al., 2013; Steinemann et al., 2015), the plants in GH showed an increase in RDD in response to drought (Figure 5). Despite the fact that drought was mild to intermediate (–5 bar to –3 bar soil water potential), and plants were young, an adaptation to drought in terms of root growth promotion seems possible (Sharp et al., 2004).

## 5 | CONCLUSION

The underlying data pointed out that the application of RDD could be a valuable tool throughout research and breeding. The presented method can be used to investigate other root-related traits in maize (e.g. nutrient uptake efficiency). By designing species-specific oligonucleotides, it can be adapted for research on root-related traits in any other crop species.

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## CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## AUTHOR CONTRIBUTIONS

PW conceived the study, SSt conducted the experiments and performed the analysis and interpretation of the data, PW assisted in setting up the experiment and data analysis, and SSt and PW drafted the manuscript.

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## SUPPORTING INFORMATION

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

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