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Development and validation of a DNA-based root phenotyping method in maize (*Zea mays* L.)

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Abbreviations

AMSL *height above mean sea level*
ASI *anthesis-silking interval*
B73 *reference maize inbred line*
BHQ *black hole quencher (qPCR dye)*
Biomass_flow *biomass at flowering*
Biomass_mat *biomass at maturity*
CN *crown root number*
Conc. *concentration*
Cq *quantification cycle*
CTAB *Cetrimonium bromide*
DAS *days after sowing*
df *degress of freedom*
DNA Deoxyribonucleic acid
Eff. *efficiency*
FAM *derivate of Fluorescein (qPCR dye)*
Fflow *female flowering (silking)*
GFP *green fluorescent protein*
GxE *genotype by environment interaction effect*
GxT *genotype by treatment interaction effect*
GY *grain yield*
H^2 *heritability*
HI *harvest index*
His *histidine*
HPLC *high performance liquid chromatography*
IL-DP *donor parent of introgression library*
IL-RP *recurrent parent of introgression library*
INRA *French National Institute for Agricultural Research*
IPC *internal positive control*
ITS2 *internal transcribed spacer 2*
KPE *kernels per ear*
LP *field station Lange Point*
Max *maximum*
Mflow *male flowering (anthesis)*
Min *minimum*
NaClO *sodium hypochlorite*
PCR *polymerase chain reaction*
PEG *polyethylen glycol*
PVPP *polyvinylpolypyrrolidone*
qPCR *quantitative PCR*
RCB *randomized complete block (design)*

RDD *root DNA density*
rDNA *ribosomal DNA*
Rep *repeatability*
RLD *root length density*
RMD root mass density
ROGG *field station Roggenstein*
ROS *field station rain-out shelter*
RSA *root system architecture*
s.e. *standard error*
SARDI *South Australian Research and Development Institute*
SC *stomatal conductance*
sub-IL *maize line with reduced number of donor fragments*
SWP *soil water potential*
TKW *thousand kernel weight*
TQ2 *Tide quencher (qPCR dye)*
TUM *Technische Universität München*
VSWC *volumetric soil water content*

1 Introduction

1.1 Maize and food security

1.1.1 Role of Maize for food security

Maize (*Zea mays* L. *ssp. mays*) is an annual crop species belonging to the family of the true grasses (*Poaceae)*. The crop maize is monoecious with unisexual flowering organs, has a diploid set of chromosomes (2n = 20) and is a descendant of Teosinte (*Zea mays* L. *ssp. parviglumis, huehuetenangensis, mexicana*) (Hallauer et al. 2010; Schnable et al. 2009), both originating from Mexico (Avendaño López et al. 2011).

Maize qualifies as one of the most important crop plants. In 2013, it was cultivated on 185 million ha of arable land on a world-wide scale, and yielded 1.018 billion tons, superior to wheat and rice with 741 and 716 million tons. Maize and wheat alone are responsible for half of the world's food caloric intake (FAO 2013). Maize grown in the US and Europe is mainly used for feed and also industrial purposes like fuel (FAO 2013; Foley et al. 2011). In Germany, 4.387 million tons of maize were harvested in 2013, and the major proportion was used for feed, energy production and as commodity for industrial purposes. But maize also represents an important staple food in many parts of the world, predominantly in Africa, South Asia and Latin America (Burke and Lobell 2010), where it often delivers the majority of consumed calories. The countries in those areas frequently have particular low gross incomes and large parts of the population are considered poor (Wichelns 2015). The majority of the population of poor developing countries lives in rural areas and earns their living with agriculture (Burke and Lobell 2010; Wichelns 2015). Thus maize plays a major role for food security in developing countries.

1.1.2 Food security and climate change

Maize yields are an important pillar of food security in many developing countries, as they directly influence whether people have physical, social and economic access to sufficient food that meets their dietary needs (Burke and Lobell 2010).

Population growth is one major reason that the requirements to achieve or maintain food security rise (Bruinsma 2009). The most likely models project a world population of 9 to 10 billion people in 2050 (Springer and Duchin 2014), and while there is enough food produced per capita, with produced cereals of $1 \text{ kg day}^{-1} \text{ person}^{-1}$, to feed even 11 billion

people on earth (Burke and Lobell 2010), regional food insecurity is often high for poor people.

Climate change modelling is complex and projected changes in temperature and precipitation vary widely depending on model implementation (Tebaldi and Knutti 2010). Taking only the most likely scenarios into account, central Europe will experience a slight increase in temperature (0.5-1°C) until 2050 (IPCC 2013). In the majority of regions where the population depends on agriculture, and maize is a main staple food, such as Southern and East Africa, the Sahel Zone or South Asia, temperatures are predicted to rise stronger (2-4°C) (Christensen et al. 2011; IPCC 2013; Tebaldi and Knutti 2010). Also many regions will experience changes in rainfall patterns with increased or prolonged drought during the growth period, including central Europe and many of the regions where people depend on maize yields (IPCC 2013; Tebaldi and Knutti 2010). The factor which influences worldwide crop yields to the greatest extent is drought (Peleg et al. 2012). Drought periods on worldwide scale are likely to extend or worsen due to climate change, and even more alarming, progress in drought tolerance breeding is slow or remains static for some crops and regions (Cattivelli et al. 2008; Hawkesford et al. 2013).

There are many factors that influence food security, and also manifold ways to increase it. An important way to achieve sufficient food security for poor nations are stable and high crop yields (Godfray et al. 2010). From the global perspective, food production has to increase in a range from 60 to 100 % above the level of 2005 to ensure food security in 2050 (Bruinsma 2009). Crop yield in turn can be modified by many factors. Advances in plant genetics, agronomy or biotechnology can increase crop yield (Wichelns 2015). The so called green revolution is the most prominent example where combined changes in plant genetics of wheat and rice and application of fertilizers in the framework of a more mechanized agronomic environment led to 170 % cereal yield increase over the last 50 years (Burke and Lobell 2010). However, it is estimated that nearly half of the worlds food production depends on fabricated N fertilizers (White et al. 2013), and many areas affected from food insecurity have poor access to fertilizers and petrol driven machinery (Lynch 2007).

Importantly, genetic improvement of crop varieties would contribute to a climate-smart agriculture (Campbell et al. 2014; Godfray et al. 2010). In climate-smart agriculture, reducing the dependence on high inputs and protecting the environment is as important as

high yields and yield stability (Abberton et al. 2015; Garnett et al. 2013; Ponisio et al. 2014). Thus, genetic improvement leading to yield gains would be a sufficient and sustainable way to achieve global food security (Abberton et al. 2015; Mickelbart et al. 2015; Tester and Langridge 2010; Wichelns 2015).

1.1.3   Target traits for genetic improvement of drought tolerance

Drought tolerance from agricultural perspective denotes the plants' ability to produce sufficient yield under sub-optimal water availability. Besides mechanisms conferring drought tolerance through physiological changes as for example osmotic adjustment, the two major morphological mechanisms of drought tolerance are escape and avoidance (Farooq et al. 2009).

Drought escape is governed by a shortened life cycle, enabling the plant to reach maturity before drought gets too severe and yield diminishing. Escape is a drought tolerance mechanism predominantly important under terminal drought stress scenarios, where drought evolves over the growing season. However, since yield is correlated with the length of the vegetative growth, escape can reduce yield under more favorable conditions. Drought avoidance means the maintenance of high water potential in plant tissue, leading to a decrease in experienced stress. Plants attain avoidance by more efficient regulation of transpiration, or maintained or increased water uptake. The distribution and function of the plants' root system determines its capability to access and take up water, hence the root system is denoted one key plant organ to increase drought avoidance (Comas et al. 2013; Lynch et al. 2014; Tuberosa 2012).

Although the aim is clear, genetic improvement of drought tolerance is not direct and straight forward. Breeding progress and selection for yield gain in dry environments is hampered by large genotype by environment interaction effects (GxE) and consequently low trait heritability (Araus et al. 2012; Chenu 2015; Mickelbart et al. 2015). The significant GxE often results from variation in season-to-season rainfall across different locations and years (Richards et al. 2010). With that said, as has become apparent from low rates of yield increase, so called empirical selection for the trait grain yield itself has its limits in drought environments (Araus et al. 2012; Richards et al. 2010). So called trait-based breeding has been proposed to enhance breeding success especially for these environments (Lynch 2014; Richards et al. 2002). The key rationale behind trait-based breeding is to use traits, or markers associated with traits, that confer improved plant performance or drought

tolerance in a specific set of dry target environments. Hence, such traits are correlated with yield, while simultaneously exhibiting minimal GxE and increased heritability compared to the target trait "yield under drought conditions". As a result, utilization of that secondary trait within a trait-based breeding approach allows more efficient selection (Cattivelli et al. 2008; Richards et al. 2010; Tester and Langridge 2010).

Several traits hold promise for the improvement of drought tolerance through so called trait-based breeding. Some of the most promising are transpiration efficiency, earliness, anthesis-silking interval and root traits. Increased transpiration efficiency is part of drought avoidance mechanisms and appears useful as target for genetic crop improvement for drought environments. In wheat, carbon isotope discrimination was used to develop cultivars with enhanced transpiration efficiency suited for dry target environments in Australia (Condon et al. 2004). Being successful for wheat in this particular environment, carbon isotope discrimination as secondary trait for transpiration efficiency is different in C_4 species (Farquhar 1983), and not readily accessible for genetic improvement in maize. Premature completion of the crop lifecycle, also termed earliness, is considered another solid trait to confer drought tolerance through escape, in target environments where terminal drought stress dominates (Cattivelli et al. 2008). Yet a short vegetative growth stage reduces yield potential under mild stress or favorable conditions and is thus not universally applicable (Cattivelli et al. 2008). Selection for drought tolerance over eight breeding cycles led to more drought tolerant maize cultivars in a CIMMYT breeding program, mainly due to a decrease in anthesis-silking interval (Bolanos et al. 1993). Selection for a reduced anthesis-silking interval is an efficient way to increase drought tolerance in maize when drought occurs at flowering (Bolanos and Edmeades 1993), but would have little benefit for tolerance under terminal drought.

The three traits presented before were successfully used in breeding, but as well have limitations in application in maize. Root traits have been recognized as promising and comprehensive secondary traits to improve crop cultivars in drought tolerance through drought avoidance (Lynch 2013; Lynch and Wojciechowski 2015; Rogers and Benfey 2014; White et al. 2013; Zhan et al. 2015). Better knowledge about root system architecture (RSA) traits in breeding is indeed supposed to facilitate a second green revolution (Bishopp and Lynch 2015; Herder et al. 2010; Lynch 2007; Lynch and Brown 2012). For root traits assessed so far, often large exploitable genetic variation was observed, also in maize (Lynch

2013; White et al. 2013). Incorporation of, and direct intentional selection for root traits in breeding programs is particularly promising because root traits have not been used much in the past (Lynch 2014). Hence there is also lots of unused genetic variation available in unadapted plant material which so far is not present in elite germplasm (Lynch 2014; Tester and Langridge 2010). Research indicates that breeding over the last decades, mainly taking part under optimal conditions of water and nutrient availability, led to the exclusion of germplasm harboring valuable root phenotypes, which would be useful for stress environments (Hammer et al. 2009; Lynch 2014). Utilization of unadapted plant material, including more diverse landraces, would allow the inclusion of such specific root traits. Roots are directly responsible for the uptake of water, so that changes in a specific root trait would directly influence the genotypes' ability to acquire water (Lynch 2013). Consequently, root traits were pointed out as promising candidates for trait-based breeding in drought environments (Farooq et al. 2009; Lynch 2014).

1.2   Breeding for drought tolerance using root traits

Root traits constitute a promising target for trait-based breeding approaches and drought tolerance breeding. In rice, the QTL *DRO1* was identified to modify the root growth angle while consequently modulating the rooting depth, with steeper rooting angles leading to a greater proportion of deeper roots and thus an enhanced drought tolerance (Uga et al. 2013). In wheat, the cultivar SeriM82 has a more compact root system and greater root length at depth than the wheat variety Hartog. These root characteristics confer SeriM82 increased drought tolerance under terminal drought stress because it facilitates water conservation in the early growth season and water extraction during grain filling (Christopher et al. 2008; Manschadi et al. 2006). There is further evidence that root traits have great influence on drought tolerance in other crops like Sorghum (*Sorghum bicolor* L. Moench) (Comas et al. 2013), pearl millet (*Pennisetum glaucum* L. Br) (Rostamza et al. 2013), common bean (*Phaseolus vulgaris* L.) (Ho et al. 2005) and chickpea (*Cicer arietinum* L.) (Gaur et al. 2008).

In maize, a root system with steep, deep and cheap roots was proposed as ideotype for drought tolerance (Lynch 2013) in various drought scenarios. Steep refers to the general

shape of the RSA, deep to the root proliferation at deeper soil horizons and cheap to the metabolic costs of the RSA. Many of the hypotheses stated in the original paper (Lynch 2013) were confirmed (Chimungu et al. 2014a; b; Jaramillo et al. 2013; Lynch 2014; Lynch et al. 2014; Zhan et al. 2015). Root traits conferring a lower so called metabolic burden, meaning the cost for tissue production and maintenance, improve drought tolerance in maize (Jaramillo et al. 2013; Lynch 2014; Lynch et al. 2014). Increased root cortical aerenchyma, which are cell-free spaces in the cortex of the roots initiated by cell apoptosis, improves drought tolerance since it saves metabolic costs and allows the plants to root deeper and access more water (Zhu et al. 2010). Similar relationships regarding metabolic costs and root traits were shown for the number and size of cortex cells (Chimungu et al. 2014a; b), and recently for lateral root branching density (Zhan et al. 2015), where lower number of cells, larger cortex cells and a decreased lateral root branching density improve drought tolerance due to a lower metabolic burden. There are also reports linking a plastic growth angle of crown roots with nitrogen and water uptake efficiency (Trachsel et al. 2013), and a low crown root number with increased nitrogen acquisition from low nitrogen soils (Saengwilai et al. 2014). Since nitrogen is a leaching nutrient, moving along with water in the soil, it is likely that a low to intermediate number of crown roots is also beneficial for drought tolerance (Lynch 2013). Although a considerable body of literature indicates great potential in utilizing root traits for drought tolerance breeding (Bishopp and Lynch 2015), roots are hardly used in breeding programs (Lynch 2014; Lynch and Brown 2012; Wasson et al. 2012; Zhu et al. 2011).

In one of the few breeding approaches which utilized root traits to improve drought tolerance, small metaxylem diameter in primary roots of wheat have been used to limit plant water uptake in the early growth season. The small metaxylem diameter delayed complete water depletion of the soil, and hence helped to avoid terminal drought stress during the important grain filling stage (Richards and Passioura 1989). The breeding approach in wheat resulted in higher yield in dry environments, and utilization of smaller metaxylem diameters was not accompanied by yield penalties under favorable conditions (Richards and Passioura 1989). In maize, in the frame of one ongoing breeding approach, an increased root cortical aerenchyma is used to improve drought tolerance for cropping in Malawi (Lynch 2014; Zhu et al. 2010).

The number of breeding approaches employing root traits as secondary traits to improve crop plant germplasm is only modest (Bishopp and Lynch 2015). The main reasons that roots are often ignored in breeding are their plasticity in the heterogeneous soil environment, in other words the ability to sense and respond to localized resource availability (Hodge 2004; Lynch 1995; Yu et al. 2014), and the lack of suited phenotyping methods in field environments (Meister et al. 2014; Tuberosa 2012; Zhu et al. 2011). Roots are responsible for the acquisition of many macro- and micronutrients. To increase the acquisition, the root system reacts plastic to changing environmental influences like variation in the availability of nutrients or water (Lynch 1995; Trachsel et al. 2013; Yu et al. 2015). This plasticity is genotype (Trachsel et al. 2013) or even root-type specific (Yu et al. 2015) and can lead to significant GxE and low trait heritability. It is necessary to find suitable root traits which can be reliably assessed using high throughput phenotyping. Breeding success relies on phenotyping of the target traits in the target environments (Tuberosa 2012; Watt et al. 2013; Zhu et al. 2011). Yet, there is a lack of suitable phenotyping techniques for roots in the field (Comas et al. 2013). Thus, lack in phenotyping methods is the most frequent rationale for disregard of roots in practical breeding programs (Paez-Garcia et al. 2015). Compared to shoot traits, the notorious phenotyping bottleneck is even smaller for roots (Araus et al. 2012; Fiorani and Schurr 2013; Furbank and Tester 2011).

1.3   State of the art in root phenotyping

1.3.1   Phenotyping under controlled and field conditions

Phenotyping roots is more difficult than readily accessible shoot traits, for they are growing in soil, and therefore, in contrary to the above-ground counterpart, roots are scarcely used in breeding programs. The main reason is hence the lack of appropriate root phenotyping methods in field environments (Paez-Garcia et al. 2015).

Methods for phenotyping roots under controlled conditions have advanced rapidly in the last decade (De Smet et al. 2012). These methods confer considerable throughput, but are dependent on artificial growing media and environments, like nutrient solution in hydroponics (Mathieu et al. 2015), gellan gum (Ingram et al. 2012), agarose (Bengough et al. 2004), or other substrates dissimilar to natural field soil. Experiments in the greenhouse

or climate chamber, utilizing these artificial growth media, are often conducted in growth containers restricting rooting volume (Nagel et al. 2012; Poorter et al. 2012), with mostly unpredictable influence on root growth behavior. Often, the plants get phenotyped in early developmental stages due to space requirements and to enhance throughput. All these growing conditions facilitate higher throughput under controlled conditions, but render it difficult to infer information for mature plants growing in the field, especially regarding the reproductive growth stage (Cai et al. 2012; Watt et al. 2013). Artificial growing conditions and environments combined with early developmental stage often lead to poor transferability from laboratory or growth chamber to the field (Comas et al. 2013). Thus, in order to advance in practical breeding, field based root phenotyping is required.

Field based phenotyping of roots with available methods is often laborious and low-throughput with considerable destructive influence on the field or study site (Paez-Garcia et al. 2015). Traditional methods like trenching, or exposure of whole root systems (Böhm 1979; Bucksch et al. 2014; Trachsel et al. 2010a) are accurate, but very laborious, have strong impact on the experimental site and thus cannot be used routinely in breeding. Another traditional means to assess root proliferation in the field is through the sampling of soil cores in order to measure root length density and root mass density. Both traits were the most often used field-based root traits in the past (Gregory 2006). The sampling of the soil cores can be reasonably quick, but processing of the samples poses a challenge to throughput and the methodology, comprising washing and picking of roots, introduces variation and thus error, which lowers precision (McKay et al. 2008; Wasson et al. 2014; Watt et al. 2008). In recent years, an increasing number of studies deployed more advanced techniques. These included ground penetrating radar, x-ray or magnet resonance tomography (Fiorani and Schurr 2013; Neumann et al. 2009). Although some configurations can give adequate resolution of roots in soil and allow non-destructive and real-time assessment of root growth, most are still dependent on homogeneous soil conditions, are slow in picture acquisition and not ready for routine field usage.

1.3.2 DNA-based root phenotyping

Since Jackson et al. (1999) first used DNA to distinguish roots of different tree species, the literature has indicated considerable potential for DNA-based root phenotyping. Several other studies used the same methodology to quantify the relative species abundance of mixed root samples (Fisk et al. 2010; Linder et al. 2000; Zeng et al. 2015). In these studies,

after DNA extraction from the mixed and beforehand weighed root samples, polymerase chain reaction (PCR) based on ribosomal or plastid DNA targets was carried out and clones based on these PCR were constructed. Subsequently, the sequencing of the constructed clones, and the abundance of sequences in a number of clones, allowed the quantification in relation to the beforehand recorded fresh weight of the mixed root sample. Zeng et al. (2015) reported good reproducibility using this approach, but pointed out that bias could be introduced due to different DNA extraction efficiencies of different root tissues, weighing errors of the mixed root samples and differences in PCR amplification efficiency when target gene copy number, guanine-cytosine content or amplicon size are distinct for root samples originating from varying species. Mommer et al. (2008) exchanged the sequencing step by quantitative PCR (qPCR) and were able to show that this step allowed a robust and more quantitative assessment of roots in mixed root samples. One crucial drawback of these methodologies, when aiming at the absolute quantification of roots from single species, is the essential separation of roots from the soil before further steps are carried out. The manual separation of roots from soil, in particular roots of species with high abundance of tiny fine roots like cereal crops, leads to significant losses of roots of up to 90 % of the actual root length (Amato and Pardo 1994), and is thus a potential source of error.

Using direct quantification of root DNA from soil samples without prior separation of roots from soil consequently conveys benefits in methodology. It eliminates the time effort needed for root washing and picking, and errors associated with washing, picking and also scanning procedures in determination of the traditional traits root mass density or root length density (McKay et al. 2008; Wasson et al. 2014; Watt et al. 2008). These differences in methodology hence increase throughput and precision. With this approach, namely the direct quantification of root DNA extracted from soil samples using qPCR, roots get quantified on the basis of the live cell number (McKay et al. 2008; Riley et al. 2009), also eliminating the drawback of quantifying nonfunctional dead roots. In addition, it enables species-specific quantification without capturing non-target species like weeds growing in the field or remnant roots from crops of previous growing seasons (McKay et al. 2008; Watt et al. 2008). By visual examination of washed root samples from the field using specific discrimination criteria, Watt et al. (2008) found that only 32 % of the determined total root length came from the current cereal crop, while the bigger part were remnant crop roots.

The authors also argued that, in their case, inclusion of dead root length in the total root length would lead to an underestimation of the actual water and nutrient uptake potential of the roots by approximately 70 %. Watt et al. (2008) used the cryo-scanning electron microscopy to verify that roots were alive and from the current cereal crop, which provided 80 % effectivity, but separation of roots from one field soil sample of approximately 350 cm³ took 40 min, limiting the throughput of this method significantly.

The extraction of total DNA from soil, subsequent quantification of root DNA through qPCR and the calculation of the absolute root DNA amount in the sample leads to the trait called root DNA density (RDD). In recent research, it was shown for *Trifolium subterraneum* L. and *Lolium* spp. that RDD accurately describes different masses of root tissue spiked to soil samples (Haling et al. 2011; Riley et al. 2009), which confirmed the methodological approach. However, general conversion of RDD to root mass is not possible. The relation of RDD to root mass underlies several factors like type of species or root class, sampling depth or age of the roots (Haling et al. 2011; Riley et al. 2009). Thus the conversion of RDD to root mass is not straight-forward and requires time consuming calibration experiments.

When considering utilization of RDD in field research and breeding, Huang et al. (2013) revealed significant genotypic variation for RDD in wheat and heritabilities for RDD between 0.5 and 0.9 across several field locations in Australia, depicting a crucial prerequisite for utilization in plant breeding. First results from these environments with Mediterranean climate also indicated the usefulness of RDD for selection under drought. Their report was so far the only study investigating natural variation and genetic determination of RDD in a crop plant, as well as the impact of drought on RDD.

Despite of the promising preconditions, there is no universal method available for the investigation of RDD. All studies so far dealing with direct quantification of root DNA from the total DNA of soil samples used the commercial service of the South Australian Research and Development Institute (SARDI) (Ophel-Keller et al. 2008). The molecular diagnostics service of SARDI provides sample handling, DNA extraction from soil samples and quantification through qPCR technique. Hence no information about the methodological workflow is accessible, except for the qPCR assays for the specific species used in respective studies (Haling et al. 2011; Huang et al. 2013; Riley et al. 2009). Further information about sample acquisition and handling in order to increase throughput, DNA extraction procedure

and data analysis would be valuable for future research, especially since these issues are not trivial. Acquisition of soil samples in the field and subsequent handling are crucial factors for precision and throughput in root quantification. In the literature on DNA-based root quantification, no sample pooling approach in combination with homogenization and DNA extraction is mentioned, although large main samples are an important prerequisite for proper representation of the investigated experimental unit. Needed sample sizes to guarantee representative results cannot be processed in most laboratories with standard equipment. Also, extraction of DNA from environmental samples is challenging (Mommer et al. 2008), and co-extracted inhibitors like humic substances often interfere with polymerase or binding of the primers in downstream reactions like the qPCR (Tsai and Olson 1992). Depending on the experimental design and number of samples, data analysis must contain normalization steps for qPCR device performance or DNA extraction efficiency to ensure comparability of samples across different sample batches. The challenge is to evaluate important steps and incorporate them in a way to make the whole method universally applicable. A publicly available methodology to phenotype roots based on DNA would render this approach amenable to research and breeding. That way, important crop species like maize could be investigated for RDD. If RDD shows strong genetic determination in maize, it could be a promising trait for the integration in breeding programs, with the final aim to improve drought tolerance.

1.4 Objectives of this study

The specific objectives of this study are:

  I.   Development of a DNA-based root quantification method for maize in field environments

      a. Optimization of field sampling and sample handling

      b. Development of a soil homogenization procedure

      c. Development of a robust DNA extraction method

      d. Development of specific, sensitive and robust qPCR assays

      e. Development of a robust data analysis workflow

  II.   Validation of the new method in field and greenhouse experiments

      a. Investigation of genotypic variation for RDD and correlations with commonly used root traits, root length density (RLD) and root mass density (RMD) under optimal and sub-optimal water availability in field and greenhouse experiments.

      b. Examination of the correlation of RDD across environments, depths and phenotyping platforms.

      c. Assessment of the utility of RDD to predict developmental, physiological and reproductive traits under optimal and sub-optimal water availability

      d. Comparison of time and money expenditures for RDD, RLD and RMD

In order to develop a complete workflow for DNA-based root quantification, from sample acquisition to data generation, a conclusive method to assess RDD in field environments was developed. After method development, the application of RDD in field and greenhouse experiments under optimal and sub-optimal water availability was validated, and genotypic variation of RDD in 25 maize inbred lines was examined. Several developmental, physiological and reproductive traits were assessed in the field, and the association of RDD with these traits was investigated.

2 Materials and Methods

The study has two main parts, the development of a DNA-based root quantification method in maize and the application of the method in a greenhouse and a field trial. The first part, method development, involved several steps as follows: Optimization of the field sampling procedure and sample handling, development of a soil homogenization method, investigation of suitable DNA extraction procedures, development of qPCR assays and finally the design of a robust data analysis pipeline. The development of the qPCR assay for the quantification of maize root DNA included a climate chamber experiment. The optimization of the aforementioned steps enabled the development of a new, specific, sensitive, repeatable, field-scale and DNA-based root phenotyping procedure. The second part, the validation and application of the new method, was carried out in a rain-out shelter under field conditions in Freising, Germany (field), and in a greenhouse trial (greenhouse) in the *Phenodyn* phenotyping facility at INRA in Montpellier, France, respectively.

2.1 Soil and plant material

2.1.1 Soil material

In order to develop a DNA extraction procedure for maize roots from soil samples and the qPCR assays for quantification of the maize root DNA coming from the soil samples, different soil types were used for evaluation of the methods. Soil was collected at several field locations to cover different soil types, representing differences in soil chemical and physical properties. These locations included the Technische Universität München (TUM) field station Roggenstein (Eichenau, Germany; ROGG) (N 48°11'13.24'', E 11°19'50.86'', 517 m AMSL), the TUM field station Lange Point (Freising, Germany; LP) (N 48°24'11.62'', E 11°43'21.99'', 480 m AMSL) and a rain-out shelter (Freising, Germany; ROS) (N 48°24'41.04'', E 11°43'23.10'', 466 m AMSL). Soil from the three locations ROGG, LP and ROS was characterized as humic, slightly plastic silt (pH 7.0), slightly plastic clay (pH 7.4) and slightly to highly plastic clay (pH 6.4), respectively (Blume et al. 1991). A manually operated gouge auger (Eijkelkamp, Giesbeek, Netherlands) with 2.5 cm diameter was used

to extract soil from the top 30 cm soil horizons at these field locations. Subsequently, soils were frozen and stored at -20°C until use.

2.1.2 Maize root and plant material

The maize root material used to develop the DNA extraction procedure and the qPCR assays was taken from field trials in 2011 and 2012. The root material was manually excavated using a spate and washed free from soil and debris. Subsequently the root material was frozen in aliquots and stored at -80°C until use. Maize DNA from these roots, which was used in qPCR reactions and for qPCR assay development, was extracted with a modified CTAB extraction procedure after Saghai-Maroof et al. (1984).

The maize plant material used in the three experiments climate chamber, field and greenhouse is described in Table 1. Plant material for the climate chamber experiment comprised 25 inbred lines of maize (*Zea mays* L.). Out of these lines, 21 were members of an introgression library (IL) segregating for important agronomic traits and chosen based on preliminary result regarding their root phenotype or carbon isotope discrimination. Fourteen of these lines, including the donor and the recurrent parent IL-DP and IL-RP, were used in an earlier study to conduct a genetic analysis of stable carbon isotope discrimination (Gresset et al. 2014). Seven of those 21 IL-lines (sub-ILs) were included because of a small number of donor fragments in genomic regions potentially important for carbon isotope discrimination. Another four of those 25 maize lines were part of a diversity panel, representing breeding lines of the European maize germplasm and were founder parents of half-sib panels used to investigate intraspecific recombination rate in maize (Bauer et al. 2013). In the presented study, these lines were chosen because of their drought tolerance (F353 and UH_F055) or drought susceptibility (F618 and D09), and contrasting root system architectures observed in field trials in 2013.

For method validation, a field experiment and a greenhouse experiment were conducted. Maize plant material for the field experiment comprised 19 inbred lines, fifteen members of the introgression library (IL) and the four founder lines. Plant material for the greenhouse experiment comprised 21 genotypes of the IL, the fifteen lines from the field experiment and six added lines (Table 1). The four maize founder lines from the diversity panel were not screened in the greenhouse experiment. Consequently, both experiments field and greenhouse overlapped in 15 genotypes.

Table 1 Plant material used in experiments climate chamber, field and greenhouse

Genotype	Description	Climate chamber	Field	Greenhouse	Source
10680_8	sub_IL	X	X	X	TUM, plant breeding
10687_2	sub_IL	X	X	X	TUM, plant breeding
10835_5	sub_IL	X	X	X	TUM, plant breeding
10860_2	sub_IL	X	X	X	TUM, plant breeding
10881_3	sub_IL	X	X	X	TUM, plant breeding
10913_2	sub_IL	X	X	X	TUM, plant breeding
10923_7	sub_IL	X	X	X	TUM, plant breeding
IL-DP	IL donor parent	X	X	X	Gresset et al. (2014)
IL-04	IL	X		X	Gresset et al. (2014)
IL-05	IL	X	X	X	Gresset et al. (2014)
IL-07	IL	X	X	X	Gresset et al. (2014)
IL-25	IL	X	X	X	Gresset et al. (2014)
IL-49	IL	X	X	X	Gresset et al. (2014)
IL-53	IL	X	X	X	Gresset et al. (2014)
IL-57	IL	X		X	Gresset et al. (2014)
IL-58	IL	X		X	Gresset et al. (2014)
IL-70	IL	X		X	Gresset et al. (2014)
IL-72	IL	X		X	Gresset et al. (2014)
IL-80	IL	X	X	X	Gresset et al. (2014)
IL-81	IL	X		X	Gresset et al. (2014)
IL-RP	IL recurrent parent	X	X	X	Gresset et al. (2014)
D09	Dent line	X	X		Bauer et al. (2013)
F353	Dent line	X	X		Bauer et al. (2013)
F618	Dent line	X	X		Bauer et al. (2013)
UH_F055	Flint line	X	X		Bauer et al. (2013)

2.1.3 *Arabidopsis* plant material

For the purpose of monitoring DNA extraction efficiency, transgenic *Arabidopsis (A thaliana)* plant material was used. The *Arabidopsis* plants carried a cloning vector (p6xHis-GFP), which was utilized as target for the qPCR assay to enhance specificity by reducing the risk of cross-reaction when working with soil samples from natural environments, since the cloning vector does not occur in nature. These *Arabidopsis* plants were grown in a climate chamber (VKZ PH1, Heraeus Vötsch, Balingen, Germany) in trays (30 cm x 40 cm x 5 cm) filled with standard potting mix (CL T, Einheitserde, Sinntal-Altengronau, Germany) at 23°C, 60 % humidity, 150 µmol $(m^2s)^{-1}$ and 12 h/12 h (day/night). Plants were harvested after four weeks, before senescence of leaves was visible. Subsequently, plant material was frozen in liquid nitrogen and ground with mortar and pistil before it was freeze dried. Freeze dried material was homogenized thoroughly with mortar and pistil, divided into aliquots and stored at -80°C until use.

2.2 Optimization of field sampling and sample homogenization procedure

Before maize root DNA from field experiments was quantified in the laboratory, maize root sampling in the field and subsequent processing steps were developed. The first evaluated steps were the acquisition of samples in the field and further processing of the samples until maize root DNA was extracted from soil samples.

2.2.1 Coring in the field

Development of the new method to phenotype maize roots based on DNA in field trials included evaluation of soil coring in field environments. Therefore, an electrical percussion drilling set (Eijkelkamp, Giesbeek, Netherlands) was examined for sampling quality and sample throughput. The evaluated parameters were total extractable sample weight and the ability to process these samples in a consistent and undisturbed way, which is crucial for efficient and repeatable sampling. Throughout this study, this soil coring system was used to obtain samples with the purpose to quantify maize roots. The samples acquired during this study will be termed soil samples in the following, referring to soil samples with or without maize roots.

2.2.2 Milling and drying of soil samples

Sampling field experiments with the aim to quantify roots often leads to large amounts of soil. In order to decrease the processed sample size and hence improve sample handling during the laboratory based steps DNA extraction and qPCR quantification, a sub-sampling procedure was developed. The sub-sampling procedure comprised drying and milling of the soil samples from the field.

An experiment to examine the influence of the factors milling and drying on the final extractable DNA amount and quality was conducted. The test was carried out with the DNA extraction method Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, USA), termed Spin Kit in the following, in 50 ml format, where each tested sample consisted of 15 g soil and 500 mg of maize root material. Samples were tested in three treatments, with three replicates, respectively. The treatments were (i) untreated control, where samples were extracted directly, (ii) drying the complete sample at 50°C for 24 h and (iii) drying at 50°C for 24 h with additional milling of the complete sample in a planetary ball mill for 6 min at

350 rpm. Resulting DNA extracts were then tested with qPCR for DNA yield and inhibition of the qPCR reaction as described in chapter 2.4.

2.2.3 Sub-sampling error

Also the possibly introduced error by sub-sampling large main samples of several hundred grams of soil for less than one gram was investigated with an experiment. For this purpose, a soil sample, termed main sample in the following, containing live roots, was taken from the rain-out-shelter (ROS), processed according to the final workflow, and sub-sampled 12 times for 350 mg samples. The sub-samples were processed according to the workflow described for the finally established method in the results section. In order to assess, if the sub-samples are representative of the main sample, the variation of the quantified amounts of DNA of the sub-samples was analyzed.

2.3 DNA extraction and purification procedures

To be able to quantify maize roots from soil samples based on DNA, a reliable DNA extraction method needed to be integrated in the final workflow. For the initial evaluation of DNA extraction procedures from difficult samples like diverse soil materials, modified workflows of four publications were tested (Khan et al. 2007; Miller et al. 1999; Yeates et al. 1997; Zhou et al. 1996). DNA extraction steps and buffer compositions of the four published methods are summarized in Table 2. Initially, the extraction procedures from the original publications were adopted. All extractions were carried out with three biological replicates in 2 ml tubes with 300 mg soil and 100 mg root tissue as starting material. The soil was previously dried in a dehydration oven at 50°C (Memmert, Schwabach, Germany) and ground in a planetary ball mill (P5, Retsch, Haan, Germany) using zirconium oxide beakers and grinding balls. Fresh maize roots were cut into 1-3 mm pieces, homogenized and added to the soil. For the cell lysis and homogenization step, two stainless steel bearing balls with a diameter of 2 mm (Altmann, Dachau, Germany) were added and a bead mill (Tissue Lyser 2, Qiagen, Venlo, Netherlands) was used. Additionally, for all four tested DNA extraction procedures, two different DNA precipitation methods, using either NaCl combined with PEG 8000 or isopropyl alcohol, were evaluated.

Table 2 Components and work-steps of four tested DNA extraction methods.

Designation in this study	DNA extraction buffer	Cell lysis and homogenization	Protein removal	Clearing step	Reference
Zhou	100 mM Tris-HCl 8.0, 100 mM Na-Phosphat 8.0, 100 mM Na-EDTA 8.0, 1.5 M NaCl, 1 % CTAB, 1 mg Proteinase K	Horizontal shaking, + 2 % SDS @ 65°C	2 x Chloroform-Isoamyl alcohol	-	Arbeli and Fuentes (2007); Zhou et al. (1996)
Khan	100 mM Tris-HCl 8.0, 3 % CTAB, 4 % Mercaptoethanol, 2 M NaCl, 25 mM EDTA 8.0	Bead beating @ 70°C	2 x Chloroform-Isoamyl alcohol	-	Khan et al. (2007)
Yeates	100 mM Tris-HCl 8.0, 100 mM Na-EDTA 8.0, 1.5 M NaCl	Bead beating + 1.8 % SDS @ 65°C	*Clearing step:* NaCl 1.6 M/PEG 20 mM (15 %-final), subsequent K-acetate up to 0.5 M	*Protein removal:* Phenol-Chloroform - Isoamyl alcohol + Chloroform	Yeates et al. (1997)
Miller	500 mM Tris-HCl 8.0, 50 mM Na-Phosphat 8.0, 50 mM NaCl, P-C-I	Bead beating + 5 % SDS @ 65°C	Phenol-Chloroform-Isoamyl alcohol + Chloroform	NaCl to 1,5 M and CTAB to 1 % + Chloroform Isoamyl alcohol	Miller et al. (1999); Sagova-Mareckova et al. (2008)

The resulting DNA crude extracts were examined visually in columns and through electrophoresis on a 1.5 % (w/v) agarose gel (VWR, Erlangen, Germany) stained with ethidium bromide (Carl Roth, Karlsruhe, Germany) in order to get an impression of size fractionation and grade of the extracted DNA. DNA crude extracts were also analyzed by measuring absorbance of specific wavelengths with a spectrophotometer (Epoch Take 3, Biotek, Winooski, USA). Absorbance at 260, 280 and 320 nm for DNA, protein and humic acid content, respectively, was measured (Miller et al. 1999; Sagova-Mareckova et al. 2008; Yeates et al. 1997). The amount of DNA was predicted by assuming that an optical density of 1 corresponds to 50 µg/ml dsDNA (Epoch Take 3 manual). The humic acid content was measured due to its inhibitory potential on enzymatic reactions (Tsai and Olson 1992).

Because none of the four published DNA extraction methods could consistently prevent the co-extraction of inhibitory substances, a column based approach for the purification of extracted crude DNA was tested, which is described in detail in Arbeli and Fuentes (2007). Briefly, combined spin columns were used to separate DNA from potentially inhibitory constituents such as humic acids. First, 45 mg of acid washed polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, St.Louis, USA) was added to chromatography spin columns (Mini Bio-Spin, Bio-Rad, Hercules, CA). Second, 1 ml of Sepharose 4B (Sigma-Aldrich, St.Louis, USA) was added. Then the spin columns were centrifuged once (2 min at 1100 g) to discard the residual ethanol from the Sepharose, and washed three consecutive centrifuge runs (2 min at 1100 g) with 300 µl of TE. The settled and dried columns (overnight at 4°C) were loaded with 200 µl of the crude DNA extract to be purified. By centrifuging for 4 min at 1400 g for each elution, the Sepharose matrix was used to exclude molecules of lower molecular weight than DNA molecules. Humic acids of a higher molecular weight that co-migrated with the DNA through the Sepharose, should be removed by the PVPP (Arbeli and Fuentes 2007).

An elution experiment was conducted to determine the number of elution runs resulting in the best separation of DNA and inhibitory substances. Eight biological replicates, using DNA which originated from the extraction method resulting in the crude extracts with the highest humic acid content (*Zhou*), were run in parallel and eluted in a centrifuge (5415R, Eppendorf, Hamburg, Germany) for four consecutive times. After each elution, the extracts were collected and finally tested on a spectrophotometer as described above for comparison of DNA yield and purity and humic acid content.

Subsequently, the DNA extraction method with the best performance regarding DNA yield and purity and co-extraction of humic acids, *Yeates*, was compared to the Spin Kit. As indicated from pre-evaluation and literature (Ettenauer et al. 2012; Knauth et al. 2013), DNA extraction from soil samples with the Spin Kit was competitive to the *Yeates* method. The Spin Kit incorporated the use of a homogenization device (FastPrep-24) and was used with a 50 ml falcon adapter (BigPrep) for the comparison. Consequently, the *Yeates* DNA extraction procedure was also scaled up to 50 ml format to ensure comparability. For this comparison, 300 mg of dried, ground and homogenized maize roots were added to 15 g of similar treated soils from the two locations ROGG and ROS. The two soils were used to test the consistency in extraction of maize DNA from substrates differing in chemical and

physical properties, like it would also be the case for different samples from field application of this method. Fresh maize roots extracted directly with the Spin Kit, without added soil, were used as a positive control. The comparison was carried out in two replicates. To assess DNA yield and inhibition, resulting extracts were run on agarose gels and subjected to qPCR analysis after extraction.

The samples of the climate chamber experiment, which consisted of the complete maize root systems, were manually ground in liquid nitrogen with mortar and pistil. DNA was extracted with a modified CTAB extraction procedure after Saghai-Maroof et al. (1984). Samples were adjusted to a DNA concentration of 50 ng μl^{-1} by using the Quant-it PicoGreen® dsDNA Kit (Life Technologies, Carlsbad, USA) before they were used in qPCR.

Weeds collected for specificity testing of the qPCR assays were manually ground in liquid nitrogen and DNA was extracted with a modified CTAB extraction procedure after Saghai-Maroof et al. (1984).

2.4 qPCR Assays

2.4.1 qPCR assay for maize root DNA quantification (ITS2)

The DNA crude extract after extraction from soil samples contains DNA from various organisms. In order to quantify only maize root DNA from this mixture of DNA, a specific and very sensitive qPCR assay was developed (ITS2). The ITS2 region, part of the ribosomal DNA and residing on chromosome 6 in maize (Buckler and Holtsford 1996a), was routinely used for species identification in barcoding studies in fungi (Schoch et al. 2012). High copy numbers of ITS2 gave high sensitivity of DNA quantification from soil samples, as demonstrated in wheat (*Triticum aestivum* L.) (Huang et al. 2013). The maize ITS2 sequence was obtained from NCBI based on the publication of Buckler and Holtsford (1996b). The online available NCBI primer BLAST was used to establish primers and probe for amplifying a specific DNA sequence (Table 3). The assay probe was synthesized with BHQ-1 (3') quencher and FAM (5') fluorescent dye (HPLC purified, Biomers, Ulm, Germany) for detection in qPCR. The assay was tested on maize root DNA and the resulting product was sequenced in-house through Sanger sequencing to confirm the target. PCR reaction for sequencing was carried out in 5 µl volume containing 500 nm of the primers listed in Table

3, 0.75 ng of DNA, 0.25 µl of big dye (BigDye Terminator v3.1 sequencing kit, Thermo Fisher, Waltham, USA), 1 µl of 5-fold big dye buffer and water.

Table 3 qPCR assays with respective oligonucleotide sequences, final reaction concentrations, annealing temperature, reaction efficiency, coefficient of determination, confirmed range of quantification and source.

Primer	Organism	Sequence	Conc. (mM)	T_a (°C)	Eff. (%)	R^2	Range (Cq)	Source/NCBI
ITS_F	*Z.mays*	5'-tcaagttgttgttctcggtgc-3'	0.4	60°C	100.7	0.998	17-34	Buckler and Holtsford (1996b) U46600
ITS_R		5'-gtcgctcgatgggtcctt-3'						Buckler and Holtsford (1996b) U46600
ITS_P		5'-FAM-cggccggccattcggc-BHQ1-3'						Buckler and Holtsford (1996b) U46600
HIS_F	*A.thaliana*	5'-cctgtccttttaccagacaacc-3'	0.2	60°C	100.7	0.996	23-37	U89936.1
HIS_R		5'-tcccagcagctgttacaaact-3'						U89936.1
HIS_P		5'-YakimaYellow-tccacacaatctgccctttcgaa-BHQ1-3'						U89936.1

Conc.: concentration in qPCR mastermix, T_a (°C): annealing temperature, Eff.: reaction efficiency, R^2: coefficient of determination in standard curve, Range: confirmed working range in standard curve, Cq: quantification cycle, i.e., time point when sample amplification increases exponentially while fluorescence is different from background. Identifier for primer and probe: _F: forward primer, _R: reverse primer, _P: probe, _A: amplicon.

2.4.2 qPCR assay to monitor DNA extraction efficiency (HIS)

Even under standardized conditions during DNA extraction, sample to sample fluctuations in DNA extraction efficiency can occur (Bustin et al. 2010). In order to monitor the DNA extraction efficiency of each reaction, a specific amount of *Arabidopsis* plant material got spiked extraneously into the soil sample for maize root quantification.

Table 3 continuation

Primer	Organism	Sequence	Conc. (mM)	T_a (°C)	Eff. (%)	R^2	Range (Cq)	Source/ NCBI
SPUD_F	*S.tuberosum*	5'-aacttggctttaatggacctcca-3'	0.3	60°C	99.7	0.998	17-40	Nolan et al. (2006b)
SPUD_R		5'-acattcatccttacatggcacca-3'						Nolan et al. (2006b)
SPUD_P		5'-FAM-tgcacaagctatggaacaccacgt-TQ2-3'						Nolan et al. (2006b)
SPUD_A		5'-cattcatccttacatggcaccatatgtggccgttttatgtcttacgtggtgttccatagcttgtgcacactcaaaattggaggtccattaaagccaagtt-3'	113 million copies per µl					Nolan et al. (2006b)
IVR_F	*Z.mays*	5'-ccactccatcgtggagagc-3'	0.25	60°C	103.1	0.995	24-40	Ronning et al. (2003) U16123.1
IVR_R		5'-gtgtggcgttgttgaagagg-3'						Ronning et al. (2003) U16123.1
IVR_P		5'-FAM-taccccacacgagccatctacgact-TQ2-3'						Ronning et al. (2003) U16123.1

Conc.: concentration in qPCR mastermix, T_a (°C): annealing temperature, Eff.: reaction efficiency, R^2: coefficient of determination in standard curve, Range: confirmed working range in standard curve, Cq: quantification cycle, i.e., time point when sample amplification increases exponentially while fluorescence is different from background. Identifier for primer and probe: _F: forward primer, _R: reverse primer, _P: probe, _A: amplicon.

That known amount of spiked *Arabidopsis* plant sample served as a target for qPCR quantification, in parallel to the maize root quantification, and enabled the normalization of the maize root DNA amounts to the sample-specific DNA extraction efficiency. The Taqman qPCR assay for monitoring the DNA extraction efficiency was developed based on

transgenic *Arabidopsis* plant material carrying a cloning vector (p6xHis-GFP). The cloning vector was utilized as target for the assay. The vector sequence was obtained from NCBI and primers and probe were designed using Primer 3 software (Table 3). The probe was synthesized with BHQ-1 (3') quencher and Yakima Yellow (5') fluorescent dye (HPLC purified, Biomers, Ulm, Germany). The assay was tested using DNA from transgenic *Arabidopsis* plants and the resulting product was sequenced through in-house Sanger sequencing as described above to confirm the target.

qPCR quantification of maize root DNA (ITS2) was generally carried out in duplex reactions, i.e. in the same well with the *Arabidopsis* assay (HIS) in order to monitor DNA extraction efficiency of the same sample. Both assays were run in duplex to avoid possible errors associated with two separately run reactions which would involve repeated pipetting and sample preparation.

2.4.3   qPCR assay to test inhibitory potential of DNA crude extracts (SPUD)

To be able to detect inhibition during the qPCR reaction, which could be due to inhibitory substances like humic acids predominantly residing in environmental samples like soil, a qPCR assay (SPUD; slang term for potato) was adopted from Nolan et al. (2006b). The SPUD assay is an assay including primer, probe and custom amplicon based on the potato (*Solanum tuberosum* L.) phyB gene (Table 3). A small modification in the present study was the substitution of original fluorescence source and quencher by FAM (5') and TQ2 (3'), respectively (HPLC purified, Biomers, Ulm, Germany). The SPUD assay performs stable qPCR reactions and can be used to test samples of any kind of organism except potato. By adding the sample to be tested for inhibitory potential, for example crude DNA after extraction, to the SPUD assay, and comparing this reaction to a control SPUD reaction where only water was added, the inhibitory influence of the unknown DNA crude extract sample on the SPUD qPCR reaction can be quantified. The negative influence of co-extracted inhibitors from soil samples was assessed for the different DNA extraction methods with the SPUD assay. Soil samples from the field and greenhouse experiments in the validation part of this study were tested in batches for inhibition.

2.4.4   qPCR assay for ITS2 copy number determination (IVR)

Since the ITS2 assay targets the ribosomal DNA with multiple copy numbers, an additional qPCR assay with a single-copy number genomic target, the invertase gene, was developed

(IVR). The IVR assay served as reference assay to determine the genotype specific copy number of ITS2 within the climate chamber experiment, and could further serve as alternative to ITS2 in maize root DNA quantification. Primers and probe were designed to enhance specificity of the assay to target only one genomic region. The B73 genome (Version 2) was used as reference. The probe was synthesized with TQ2 (3') quencher and FAM (5') fluorescent dye (HPLC purified, Biomers, Ulm, Germany). PCR was carried out and the product was confirmed by Sanger sequencing, as described above, for all used genotypes. By in silico analysis and using data from the Sanger sequencing, the IVR target in this assay was confirmed to be a single copy event and could therefore be used as reference assay to determine copy number variation of other genomic targets.

2.4.5 Optimization and cycler program

Design of primer and probe incorporated optimization for similar melting temperature of the duplex assays ITS2 (or IVR) and HIS, as well as the least possible interaction between oligonucleotides of all assays. For this purpose, Primer 3 (Untergasser et al. 2012), Lasergene (DNASTAR, Madison, USA) and Primer Express (Life Technologies, Carlsbad, USA) softwares were used. In order to determine the most suited oligonucleotide concentrations and annealing temperatures, temperature gradient qPCR and primer and probe concentration matrices (Nolan et al. 2006a) were run for all used qPCR assays (Table 3). The performance of the assays was confirmed with standard curves. Depending on the assay, five to eight 10-fold dilution steps were run in technical triplicates in order to calculate reaction efficiency and coefficient of determination according to Step One Plus® Software v.2.2.2. Taqman Environmental Master Mix® 2.0 (Life Technologies, Carlsbad, USA) in 1-fold concentration was used for all qPCR reactions, which were carried out in 20 µl volume and with three technical replicates on a Step One Plus® real-time PCR system (Life Technologies, Carlsbad, USA). Each reaction contained 2 µl of DNA sample. For all runs, the 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.

2.4.6 Specificity testing for ITS2 and IVR assay

In order to confirm the specificity of the ITS2 and IVR qPCR assays under field conditions, an experiment with 17 commonly found weeds in field grown maize was carried out. These weeds (Table A1, appendix) were collected in the field locations ROGG, LP and ROS. After

collection they were pooled, frozen and DNA was extracted. The resulting DNA was used to test the developed assays for specificity in qPCR reactions.

2.4.7 Assessment of quantification with the ITS2 assay

For the purpose of testing the new method for its ability to quantify maize root material in soil samples, a spiking experiment was conducted. Therein, four quantification points were set. For each respective point, 20, 40, 80 and 160 mg of lyophilized maize roots were added to 100 g of soil (ROS topsoil, free of maize roots as confirmed by qPCR). The 100 g samples were milled as described above, before sub-sampling 350 mg samples for four replicates in each root concentration, respectively. That experimental design resulted in 16 samples in total. The samples were processed according to the final quantification method, and maize root DNA was quantified using qPCR and the ITS2 assay.

2.5 Data analysis workflow for calculation of root DNA density (RDD)

For the calculation of the trait root DNA density (RDD), corresponding to root DNA mass per soil volume (pg cm^{-3}), the data analysis workflow incorporates four steps: (1) calibration for inter-run variation, i.e. variation from plate to plate, in qPCR, (2) correction for sample-specific extraction efficiency, (3) calculation of absolute amount of maize root DNA in the sample and (4) correction for genotype-specific ITS2 copy number differences. In the following, the single steps are described and corresponding calculations are given.

(1) In case samples of one experiment encompass more than one 96-well plate in qPCR, a calibration for inter-run variation is needed to make the different 96-well runs comparable. The calibration for inter-run variation is necessary due to technical variability introduced by the qPCR instrument or by chemical reagents from one 96-well plate to another (Bustin et al. 2010). To monitor this variation, an internal positive control (IPC) sample was used on every 96-well plate starting from plate 1. The IPC was always the exact same type and amount of DNA in a standard reaction with the ITS2 assay, which should (under idealized conditions) always yield the same Cq value in each run. The mean of all IPC reactions, over all included plates was used to correct each plate for the inter-run variation (adopted from Vermeulen et al. (2009)):

$$Cq_{X\ inter-run\ calibrated} = Cq_X + (Cq_{IPC\_mean} - Cq_{IPC\ X})$$

where Cq_X is the Cq of the sample used for maize root DNA quantification, Cq_{IPC_mean} the mean Cq of IPC across all plates and Cq_{IPCX} the Cq of the IPC on the same plate as Cq_X.

(2) Every sample was extracted with the same extraction kit and underwent the same handling, yet in separate columns and work-steps. From different batches of the extraction kits, or marginal differences in pipetting or handling, deviations in DNA extraction efficiency may occur from sample to sample. To monitor this influence, *Arabidopsis* plant powder was added to the soil samples, prior to the extraction. The *Arabidopsis* plants used here carried a GFP vector construct which could be reliably detected and quantified during the qPCR analysis. The DNA extraction efficiency was determined by adding a suitable amount (which was leading to quantification in the stable range of the HIS assay as determined by the standard curve, Table 3) of the *Arabidopsis* powder and evaluating the expected outcome.

Before the actual experiment started, sub-samples of a specific amount of homogenized *Arabidopsis* powder of a large batch, sufficient for the entire experiment, were extracted with the same Spin Kit method as the soil samples to be tested, and quantified in 10 biological replicates. With the information from the 10 runs, it was possible to define a relation of input plant powder and DNA to Cq in the qPCR measurement. That relationship was used to calculate the expected outcome of any *Arabidopsis* extraction. Identical results indicate an extraction efficiency of 100 %. If the results deviate, the efficiency can be calculated and used for normalization. Due to the logarithmic nature of the qPCR data, the efficiency is calculated as the ratio of expected to observed values. Since both, the soil sample and the *Arabidopsis* sample were run in the same column for DNA extraction and were analyzed in a qPCR duplex reaction, the calculated extraction efficiency of the *Arabidopsis* sample was expected to be also valid for the extraction of the soil samples. That way, all soil samples were normalized to the specific, column- and reaction-wise extraction efficiency:

a) Calculate expected Cq value of the *Arabidopsis* sample based on powder input weight, using the a priori established calibration for sample X:

$$Cq_{X\ exp} = Cq_{ref} + \log(1 + \frac{sample_{ref} - sample_X}{sample_{ref}}) * \frac{1}{\log(2)}$$

where $Cq_{X\,exp}$ is the expected Cq for a specific input amount of *Arabidopsis* powder in the DNA extraction reaction with sample X, Cq_{ref} the previously determined Cq of the reference sample *Arabidopsis* powder with the defined input weight of $sample_{ref}$, and $sample_X$ the input weight of *Arabidopsis* powder in the corresponding reaction together with the sample X.

b) Calculate extraction efficiency of the unknown *Arabidopsis*, and therefore also maize DNA sample:

$$Eff_X = \frac{2^{Cq_{X\,exp}}}{2^{Cq_{X\,obs}}}$$

where Eff_X is the DNA extraction efficiency of sample X, $Cq_{X\,exp}$ the expected Cq for the specific input amount of *Arabidopsis* powder in the DNA extraction reaction with sample X, and $Cq_{X\,obs}$ the observed Cq value of *Arabidopsis* in the extraction of sample X.

c) Use the calculated extraction efficiency to correct the Cq value of sample X:

$$Cq_{x\,corr} = Cq_x - \frac{\log(\frac{1}{Eff_X})}{\log(2)}$$

where $Cq_{x\,corr}$ is the Cq value of sample X corrected for DNA extraction efficiency Eff_x, and Cq_X the initial, uncorrected Cq value of sample X.

(3) The $Cq_{x\,corr}$ value of sample X can now be used to determine the amount of maize root DNA in the soil sample. The beforehand executed normalization steps guarantee solid data and comparability between the samples. To be able to infer absolute DNA quantities from a measured Cq value, a standard curve with seven 10-fold dilution steps was run on the first plate. The resulting regression of DNA quantities on Cq values can be used with the Cq values from the soil samples to calculate RDD.

$$RDD_X = 10^{\frac{CqX\,corr - b}{a}}$$

where RDD_X is the amount of maize root DNA in qPCR reaction X, $Cq_{x\,corr}$ the Cq value of the sample corrected for DNA extraction efficiency, a the slope and b the intercept of the standard curve.

(4) In order to correct for the genotype specific ITS2 copy number variation, the genotype specific differences were investigated in a climate chamber experiment. The resulting information was used to correct the calculated maize root DNA RDD_X for the ITS2 copy number. To do so, a reference genotype was chosen which showed the closest ITS2 copy number to the ITS2 copy number mean of all tested genotypes. To ensure comparability in one experiment, all tested genotypes were normalized to this reference genotype (modified from Huang et al. (2013)).

$$RDD_{X\ ITS\ corr} = RDD_X * \left(\frac{ITS\ CN_{ref}}{ITS\ CN_X} \right)$$

where $RDD_{X\ ITS\ corr}$ is the RDD value corrected for the genotype specific ITS2 copy number, RDD_X the uncorrected RDD value of the sample X, $ITS\ CN_{ref}$ the ITS2 copy number of the reference genotype and $ITS\ CN_X$ the ITS2 copy number of the genotype of sample X. Determination of CN_{ref} and CN_X is described in chapter 2.6.

2.6   Climate chamber experiment

ITS2 showed genotypic differences in copy number for example in wheat (Huang et al. 2013) and also in maize (Rivin et al. 1986). Therefore, for the determination of the ITS2 copy number for each of the 25 maize genotypes in this study, a climate chamber experiment was carried out at the Gewächshauslaborzentrum Dürnast of the TUM (N 48°24'19.68'', E 11°41'25.60''). The experiment was conducted in a randomized complete block design (RCB) with three replicates. Plants were grown in a climate chamber (VKZ PH1, Heraeus Vötsch, Balingen, Germany) with 25/20°C temperature, 16/8 h light, 40/70 % humidity (day/night) and adequate water supply. Light intensity was 250 µmol $(m^2s)^{-1}$ at plant level. Plant material consisted of the total set of maize lines utilized in the validation experiments in the field and greenhouse (Table 1). Tubes with a height of 60 cm and a diameter of 7 cm were used to grow the maize plants for 30 days (Figure 1A). To facilitate harvest of the maize root systems, the tubes were lined with a polyethylene sleeve which could be easily removed at harvest before washing the complete root systems (Figure 1B and C). The substrate was a mixture of hand sieved topsoil (humus, 2 mm,

45 % v/v) of a field location (Eggertshofen, Freising, Germany, N 48°21'44.87'',
E 11°43'04.92''), quartz sand (25 % v/v), vermiculite (20 % v/v) and perlite (10 % v/v). In
order to facilitate sufficient aeration and drainage of excess water, a 5 cm layer of gravel
was used at the bottom of the tubes. All tubes were watered and left for three days to
allow water drainage until field capacity was reached, and provided with 150 ml of 1 ‰
fertilizer solution (Ferty 2, Planta, Regenstauf, Germany, 16 % N, 6 % P_2O_5O, 3.4 % MgO,
0.02 % B, 0.04 % Cu, 0.1 % Fe, 0.05 % Mn, 0.01 % Mo, 0.01 % Zn) before sowing. Three
kernels per genotype were sown directly into the tubes after NaClO (2.5 %) treatment of
the seeds for 3 min and imbibing the seeds in water for 2 hours. Six days after sowing
(DAS), the maize plants were thinned to one plant per tube. All plants were watered with
60 ml water per day, which was left in the climate chamber over night to allow adjustment
to room temperature. At 30 DAS, the polyethylene sleeves were extracted and the
complete root systems were washed free of soil and debris (Figure 1C). Subsequently, the
root systems were frozen in liquid nitrogen and stored at -80°C until DNA extraction.

Figure 1 Climate chamber experiment for determination of ITS2 copy number. (A) Experimental
setup. (B) Plastic tubes were lined with foil to facilitate extraction of complete root systems at
harvest. (C) Complete maize root systems after washing from soil substrate.

The ITS2 copy number was determined in triplicate qPCR reactions. The IVR assay designed
to target a single copy gene was used as reference against the ITS2 assay. All samples were
tested for inhibition of the reaction using the SPUD assay prior to the final qPCR run.

Assuming similar reaction efficiencies of 100 % of the two qPCR assays, ITS2 and IVR, as determined by standard curve experiments (Table 3), the ITS2 copy number was calculated using the following formula (modified after Huang et al. (2013)):

$$ITS2\ copy\ number = 2^{\Delta Cq}$$

with

$$\Delta Cq = Cq_{IVR} - Cq_{ITS2}$$

where *ΔCq* corresponds to the difference in quantification cycles (Cq) between single copy number assay IVR *Cq_{IVR}*, and the ITS2 assay *Cq_{ITS2}*.

2.7 Field trial in the rain-out shelter

After development of the new method, validation was carried out in the field. The ROS location was used to conduct a semi-controlled field trial with 19 maize lines (Table 1) in 2014. The rain-out shelter is constructed on rails and automatically moves over the experimental site when sensing rain. Consequently, the entire trial could be protected from natural precipitation, and drought stress was induced as treatment.

Maize was sown May 9, 2014 in single rows of 20 kernels per genotype, which were thinned to 10 plants per row at 9 DAS. Plot length was 1.2 m, plant distance 13 cm within row and 75 cm between rows, resulting in a density of 18 plants m^{-2}. The experimental design was a RCB design with six blocks per treatment and two treatments, a well-watered control and drought stress. Since destructive harvest was necessary in order to obtain biomass data and root crowns to visually phenotype root architectural traits after Trachsel et al. (2010a) at flowering time, three blocks were harvested at flowering and three blocks were left from flowering until maturity for determination of yield parameters at maturity. Watering was carried out with a linear move irrigation system, and the experiment received 8 mm water every other day. Drought stress was induced as described in the following to imitate most common stress at regional scale. Both treatments were watered until 35 DAS. Then, in order to schedule the drought stress peak slightly before and during flowering

time, watering ceased for the drought treatment, but continued for the well-watered plants. To guarantee an exclusive watering of the control, the two treatments were separated by plots with a length of 2.4 m consisting of 20 maize plants of a maize hybrid variety. At 105 DAS, watering was resumed for the drought treatment to support grain formation. Thirty large volume soil moisture sensors (10HS, Decagon Devices, Pullman, USA) were spread across the entire field and entrenched at 20 cm and 50 cm depth to capture volumetric soil water content (VSWC) information over the entire growth season. Beforehand, a pF-curve was established to convert VSWC to soil water potential (SWP) (Figure 2). Herbicide application and fertilization was carried out according to good professional practice. Under-root fertilization with nitrogen, phosphorus and potassium at 30 Kg ha^{-1}, respectively, was used at sowing. Thirty DAS, 70 Kg ha^{-1} nitrogen were applied. During the growth season, the monthly average temperature ranged from 10.1°C to 18.1°C and average humidity ranged from 67 % to 92 %.

2.7.1 Phenotyping of root traits

Essential part of the method validation was the comparison of RDD to the commonly assessed root traits root length density (RLD) and root mass density (RMD) in the field. In order to investigate RDD, RLD and RMD, plots were sampled throughout flowering time (87-95 DAS) with a percussion drilling set (Eijkelkamp, Giesbeek, Netherlands). The core sampler (1 m length and 4.4 cm diameter) was drilled into the soil using a percussion hammer (HM1810, Makita, Anjo, Japan) and pulled out manually using a lifting jack and a lever with chain (see Figure 4, chapter 3.1.1). The core sampler was equipped with a foil insertion kit. Five cores per plot were taken in the maize plant row, each core between two plants, while avoiding border plants. The soil horizons in 10-20 cm (depth 10) and 40-50 cm (depth 40) were sampled. After extraction of the polyethylene sleeve with a diameter of 4.4 cm, the soil was cut to obtain pieces of 10 cm length. These cylindrical pieces were halved lengthwise resulting in two samples for the assessment of the traits RDD from one half and RLD and RMD from the other half. Then samples were bagged and put on ice in an icebox. The fresh weight of all samples was determined, and RLD samples were frozen at -20°C until root washing. Since development of the method to obtain RDD was part of this study, the final workflow for processing RDD samples is given in Figure 13 in the results section (chapter 3.1.5). RDD_Index was calculated as the ratio of RDD under drought to

RDD in the well-watered treatment, similar to commonly used drought tolerance indices as for example for yield (Tuberosa et al. 2002).
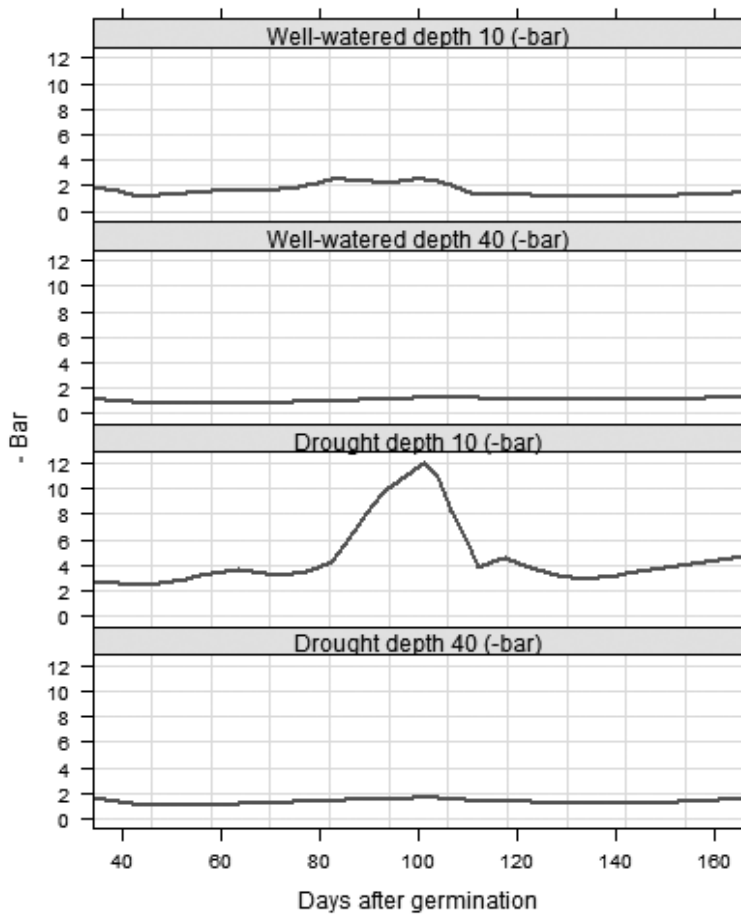
Figure 2 Soil water potential (-bar) in 10-20 cm (depth 10) and 40-50 cm (depth 40) depth throughout the growth season in the rain-out shelter.

In order to check for maize root DNA background levels at the experimental site, control samples from field patches adjacent to the field trial, where maize was grown in the previous year, were tested. These background samples were processed in the same workflow as samples tested for RDD.

The soil bulk density for the field location was determined using the dry weight of all cored samples which were taken almost undisturbed and in a fixed volume.

For determination of RLD, the thawed 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 handheld sprayer. Then, maize roots were picked from the remains using forceps. Maize roots from the ongoing season were visually distinguished from roots of weeds and other debris. The criteria were bright color of live roots as compared to dark

color of dead roots from the last season, and maize specific root topology and thickness, since most roots of present weeds in the field had very fibrous roots. The criteria to distinguish maize roots from roots of different species were inferred from Watt et al. (2008). Washed roots were stored in 30 % ethanol (v/v) at 4°C until scanning of the roots. To calculate RLD, samples were scanned on a flatbed scanner and analyzed with WinrhizoPro software v2009 (Regent Instruments, Quebec, Canada). After scanning, the RLD samples were pooled for the corresponding depths (all five cores per plot for depth 10 and for depth 40, respectively) and dried in paper bags at 70°C for 48 h. The weighing of the samples resulted in RMD.

For the visual phenotyping of maize crown root traits according to Trachsel et al. (2010a), the entire root crowns of three maize plants per plot were excavated with a spate at flowering time, soaked in water and washed free from soil and debris with a handheld sprayer where water spraying intensity could be adjusted continuously. Then crown root number (CN) was determined. The resulting mean of three plants per plot was taken for further phenotypic data analysis.

2.7.2 Phenotyping of shoot traits

Except otherwise specified, shoot traits were assessed by phenotyping three representative maize plants per plot. The mean of these three values was taken as phenotypic value for the respective plot. Male (Mflow) and female (Fflow) flowering were phenotyped as number of days from sowing to anthesis and silking, respectively. A plot was flowering when 50 % of the corresponding plants showed pollen shed (Mflow) or silking (Fflow). Anthesis silking interval (ASI) was calculated as Fflow – Mflow (days). Stomatal conductance (SC) was measured with a hand held device (SC1 leaf porometer, Decagon Devices, Pullman, USA) in the middle of the youngest, fully expanded leaf, from 11 am to 3 pm just before the flowering period. At flowering time, plant biomass at flowering (Biomass_flow) was assessed by pooling three representative plants per plot. At maturity, plants were harvested manually by cutting the shoot close to soil surface. Ears were harvested and biomass traits were assessed for the second time. Five representative plants per plot were chosen and processed to get biomass at maturity (Biomass_mat), and grain yield was determined using all plants (GY). After ears were dried at 50°C for 7 days, thousand kernel weight (TKW) was determined. 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.

2.8 Greenhouse trial

To validate the new method, a greenhouse trial was conducted using the *Phenodyn* platform of INRA Lepse in Montpellier, France (N 43°37'5.09'', E 3°51'25.47'', 44 m AMSL) from February 13 to March 23, 2014. The aim of this experiment was to obtain RDD from pots in the greenhouse. The set of 21 maize lines grown for this experiment included 15 genotypes from the maize IL which were also tested in the field experiment (Table 1). The experimental design was a RCB with two blocks for each of two treatments, well-watered control and drought stress. The pots had a diameter of 15 cm, a height of 40 cm and were filled with 6550 g of standard potting mix with complete fertilizer. Six DAS plants were thinned to four plants per pot (Figure 3). Until five DAS, pots received 70 ml of water daily to facilitate maize plant germination and early establishment. Balances were used to record the weight over time (Figure 3). The soil water content, which was derived from the pot weight as described by Welcker et al. (2007), was used to generate a pF-curve, which in turn was employed to adjust the stress level. That way, until 20 DAS, VSWC was kept close to the field capacity in both treatments using the weight recorded from balances. Subsequently, drought stress was induced as one treatment according to the measured SWP. From 21 DAS, drought treated plants were kept within -3 bar and -5 bar SWP, corresponding to a mild to intermediate drought, and three plants in each pot were cut close to the soil surface. At 36 DAS, soil samples for determination of RDD were taken using a manually operated soil corer with 2 cm diameter. After plants were cut above soil level, every pot was sampled four times at each of three depths, increment 0-13 cm (depth 1), 14-26 cm (depth 2) and 27-40 cm (depth 3). For each depth the four samples were pooled, resulting in three main samples per pot (resulting each from four cores) and a total sample number of 252. Except for the differences in soil sampling, the samples were handled and processed as described in the results section to gain RDD data.

Figure 3 Greenhouse trial conducted in the *Phenodyn* facility in Montpellier. Four maize plants were initially grown per pot. The experimental design integrates automatized weighing of pots.

2.9 Statistical data analysis and calculations

R Version 3.1.3 (R CoreTeam 2014) was used to perform statistical analyses of the phenotypic data. Data were fitted using mixed linear models and the ASReml package implemented in R (Butler et al. 2009). Analysis of variance for climate chamber (one treatment), field and greenhouse experiments was done with the model:

$$P_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where P_{ijk} denotes the phenotypic observation of the ith genotype in the kth block and j^{th} treatment, μ the grand mean, α_i the effect of the ith genotype, β_j the effect of the jth treatment, γ_k the effect of the kth block, $\alpha\beta_{ij}$ the interaction effect between the ith genotype and j^{th} treatment and ε_{ijk} the random deviation of the phenotypic observation from the fitted phenotype. Wald F-statistic was used to test for the significance of fixed effects. Adjusted means were calculated for each genotype in individual treatments with the parameter α_i, treated as fixed, and remaining parameters as random.

The calculated variance components were used to estimate broad sense heritability (H^2) according to Holland et al. (2003):

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gt}^2}{T} + \frac{\sigma^2}{R_{E1} + R_{E2}}}$$

where σ_g^2, σ_{gt}^2 and σ^2 denote the genotypic, genotype by treatment interaction and error variance components, respectively. T corresponds to the number of treatments, R_{E1} to the number of replicates in treatment one and R_{E2} to the number of replicates in treatment two.

Phenotypic correlations were calculated as Pearson's correlation coefficient using the R function *cor.test*. For comparison of DNA extraction method evaluation parameters DNA yield, DNA purity and humic acid content, and to examine the effect of qPCR plate number on DNA extraction efficiency, analysis of variance was performed using the R function *aov*. Figures were made using the *ggplot2* package.

3 Results

The aim of this study was the development and validation of a DNA-based method to quantify maize roots in field environments. The lack of high-throughput methods to phenotype root traits in field environments at low cost constitutes a major bottleneck in root research. Development included the optimization of a field sampling procedure and sample handling, development of a soil homogenization method, investigation of suitable DNA extraction procedures, development of qPCR assays and the design of a robust data analysis pipeline. With the optimization of these components, a DNA-based method to quantify maize roots in field environments was developed, resulting in the trait RDD. For validation and evaluation purpose, experiments were conducted in the field and in the greenhouse. In the field, the new trait RDD was compared to commonly assessed root traits RLD and RMD with respect to genotypic variation and heritability. Genotypic variation and heritability of RDD gained from pots in the greenhouse experiment was examined. Further, the correlation of RDD with RLD and RMD, as well as of RDD across different water regimes, soil depths and the phenotyping platforms field and greenhouse, using RDD data from pots, was assessed. Finally, RDD and other root traits were investigated for their association with 10 developmental, physiological and reproductive shoot traits in the field experiment. In addition, time and financial requirements for RDD were compared to RLD and RMD.

3.1 Development of a DNA-based method to quantify maize roots in the field

3.1.1 Optimization of field sampling and soil sample handling

Root coring in the field is often carried out manually with handheld coring devices and a hammer. In order to enable high sample-throughput with the method developed in this study (three-digit range per day), soil coring in the field had to become more automatized. For this purpose, a soil sampling system consisting of a percussion corer, a lifting jack and lever with chain and an electrical percussion hammer was used (Figure 4A, B, C). The percussion corer was equipped with a foil insertion kit, which allowed taking virtually undisturbed soil samples (Figure 4A). Every sample was extracted into the polyethylene foil where the spatial layers were conserved. The configuration allowed the precise

investigation of single, defined soil horizons, without distortion from possible compression effects through the sampling process, as it often occurs with semi-open, handheld coring devices. Therefore, in this study, soil increments of 10 cm height were obtained from the total soil cores, corresponding to a fresh weight of approximately 300 g.

The sampling scheme was adapted to the fact that the conducted field experiment consisted of one-row plots of the genotype to be investigated. According to this experimental design, the cores were taken from in-between two maize plants inside one row, while avoiding border plants. By virtue of this sampling position, sampling of neighbored plants from other plots should be avoided and the vertical maize root distribution could be explored.
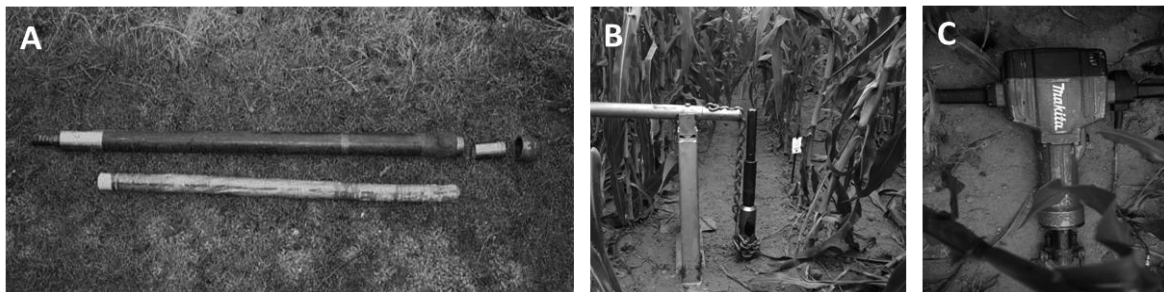
Figure 4 Field sampling tools and technique. (A) Percussion corer with sample in foil. (B) Lifting jack and lever with chain at core extraction. (C) Electrical percussion hammer.

Ultimately, five cores per plot were used in the validation. Using this soil core sampling system, a throughput in the field of several hundred samples per day was feasible. Moreover, the small configuration of the sampling devices allowed root sampling throughout the growth season without excessive disturbance of the field trial. Thus the established sampling procedure allowed the acquisition of undisturbed and representative soil cores in high-throughput. The final sampling procedure as it was carried out in the validation experiment is given in Figure 13, chapter 3.1.5.

3.1.2 Homogenization procedure

DNA degradation occurs under ambient temperatures. Since roots should be quantified using DNA, it was important to minimize DNA degradation with potential influence on the trait RDD. Prolonged episodes of temperature and humidity favorable for DNA degrading enzymes are often provided during the field work and the sample processing in the dehydration oven. In order to restrict DNA degradation in the field, polystyrene boxes filled with ice were used to store the soil samples throughout the day in the field. Subsequently, the samples were dried in a dehydration oven. Drying of samples in the oven was important

for two reasons. First, the samples got deprived of water, and water is crucial for enzymatic reactions. Thus, DNA degradation was interrupted. Second, drying prepared the samples for the next step, the milling, since the samples have to be dry for using them in most of the commonly used milling systems.

An experiment revealed that the choice of dehydration oven was important. Compared to untreated control samples, drying of samples reduced DNA yield approximately 14-fold (from Cq of 28.3 to 32.0 in qPCR reaction). The DNA-reducing effect was avoided by using drying systems with efficient removal of humidity released from the soil samples. The final workflow contains a drying step where the samples are dried for 24 h at 40°C.

A planetary ball mill was used to mill the dried soil samples. The used type of milling device exerts substantial physical force on the samples, which led to short milling runs and high throughput, allowing the processing of samples with a fresh weight of up to 400 g. Due to the milling, and the resulting flour-like consistency which ensures good homogeneity, the samples could be reduced to sub-samples of less than 1 g. The here reported reduction in sample material was key to cheaper processing with high throughput in the next step, the DNA extraction. Also the influence of milling the soil samples on DNA yield was examined. It revealed that milling does not lead to reduction in DNA yield following the drying process in qPCR reaction (Cq of 32.0 and 32.0 after drying and milling, respectively). Furthermore, the influence of the factors drying and milling on inhibition of DNA amplification in qPCR reaction was tested. Results from the SPUD assay indicated no change in extraction of inhibitory substances due to the drying and milling steps (Cq of 17.9, 17.9, 17.7 and 17.8 for SPUD reactions supplemented with water, DNA from the control, DNA from dried and DNA from dried and milled samples, respectively). Therefore, drying of the soil samples was the most critical step with regard to DNA yield, and milling with the planetary ball mill was incorporated in the final method.

In order to test if the sub-samples give a representative depiction of the main sample, and thus if the main samples have sufficient homogeneity, an experiment was conducted. Thereby, one main sample was sub-sampled 12 times. The ITS2 assay showed a low variance of measured values of the sub-samples, with a mean Cq of 20.82 and a standard error of the mean of 0.0703 (n=12). Translated to absolute DNA values, the calculated error would lead to a deviation in DNA yield of around 5.0 % for the ITS2 assay. Hence, complete homogenization of main soil samples allowed efficient sub-sampling and led to substantial

improvement of sample handling and throughput in the lab as compared to working with the larger main samples.

3.1.3 Development of a robust DNA extraction procedure

In order to ensure comparability between different samples, DNA from the samples had to be extracted in a standardized way. Moreover, the resulting DNA crude extracts had to be free of potential PCR inhibitors like humic acids (Tsai and Olson 1992). Therefore, four DNA extraction procedures from scientific literature (Table 2, chapter 2.3), were modified and tested for DNA yield, purity and potential co-extraction of inhibitors. DNA from soil samples of the two locations LP and ROGG, processed before with the optimized sample handling workflow, was extracted with each of the four tested DNA extraction methods, using two different precipitation methods, isopropyl alcohol and PEG 8000. After visual examination on agarose gels, the DNA crude extracts were measured on a spectrophotometer.

3.1.3.1 Comparison of four modified DNA extraction procedures

Visual observation of crude extracts after DNA extraction indicated stronger co-extraction of undesired substances with isopropyl alcohol than with PEG 8000. Additionally, more undesired substances were observed in the crude extracts of ROGG soil as compared to soil from LP (Figure 5). Regarding the examined methods, the extraction procedure *Zhou* led to the co-extraction of the highest amount of dark components, and *Yeates* showed the least dark crude extracts. Crude DNA extracts in columns of both methods are shown in Figure 5.

In order to examine the different DNA extraction methods for their suitability to extract sufficient amounts of good quality DNA without co-extraction of inhibitory substances, the DNA crude extracts were run on agarose gels and tested for absorbance with a spectrophotometer.

The agarose gel picture indicated no or very little extracted DNA from all samples precipitated with PEG 8000 (Figure 6). In contrast, precipitation with isopropyl alcohol yielded highest DNA amounts for *Yeates* and *Zhou*, medium amounts for *Miller* and low amounts for *Khan*. *Yeates* and *Zhou* seemed to result in DNA of the highest molecular weight. DNA extraction of soil samples from LP led to the co-extraction of less humic acids than DNA extraction of soil samples from ROGG, in particular evident by a large signal for *Zhou* extraction and isopropyl alcohol precipitation. Differences in method performance regarding crude extract colors observed beforehand were confirmed on agarose gel for the

Zhou extraction method of ROGG soil using isopropyl alcohol, which showed the darkest crude extract color and a strong signal in low molecular weight region on the agarose gel.
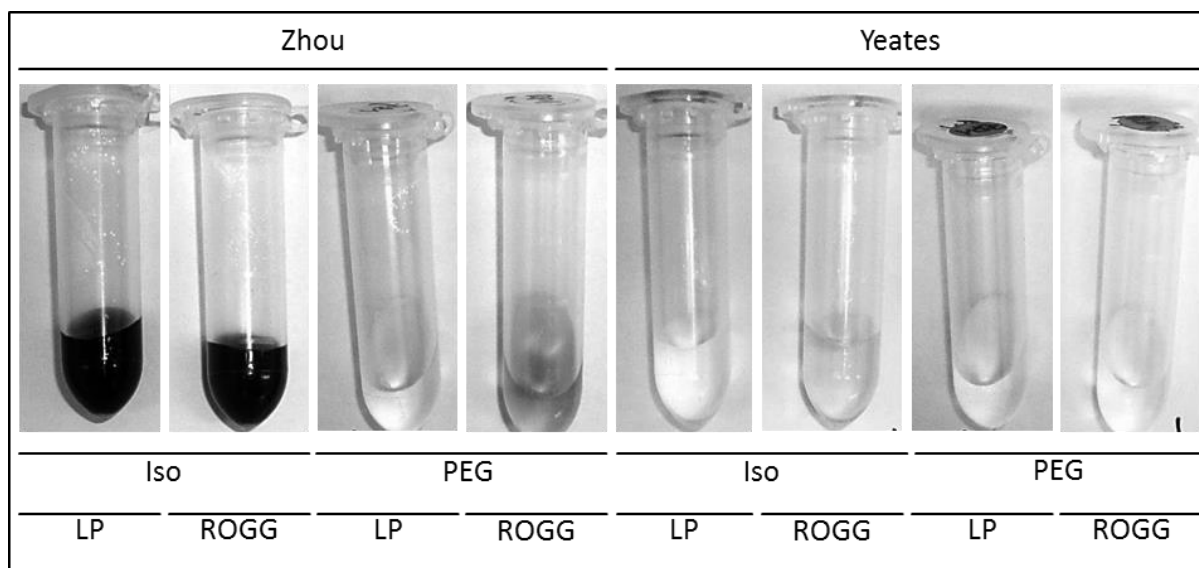
Figure 5 Visual observation of DNA crude extracts from two DNA extraction methods *Zhou* and *Yeates*. Both methods *Zhou* (Zhou et al. 1996) and *Yeates* (Yeates et al. 1997) were tested with PEG 8000 (PEG) and isopropyl alcohol (Iso) precipitation, and soils from field locations Roggenstein (ROGG) and Lange Point (LP).

After visual examination of the samples in the columns and on agarose gel, the crude DNA extracts were measured on a spectrophotometer for different wavelengths indicative of DNA yield, purity and humic acid contamination. Based on these data, an ANOVA was performed. According to the ANOVA, variation in DNA yield was significantly influenced by the factors extraction method ($p < 0.01$) and precipitation step ($p < 0.01$), but not soil type. The *Zhou* method yielded significantly higher amounts of DNA than the other three methods (Figure 7A). That was however in contrast to the agarose gel results, from which no significant difference in DNA yield between *Zhou* and *Yeates* was obvious. The precipitation with isopropyl alcohol led to more than 5-fold increased DNA yield as compared to PEG 8000 (Figure 7B). That was in agreement with the agarose gel observation. Only the factor extraction method ($p < 0.01$) had a significant influence on purity of the DNA crude extracts (Figure 7C), indicating that exchanging isopropyl alcohol by PEG 8000 did not improve DNA purity ($p = 0.105$). Regarding humic acid content of crude extracts, the factors precipitation ($p < 0.01$) and method ($p < 0.01$) showed a significant impact (Figure 7D and E). The precipitation step with PEG 8000 reduced the contamination approximately 3-fold compared to isopropyl alcohol. *Zhou* showed the strongest contamination with humic acids, the other three methods ranged on similar level at around

4 % of the contamination of the Zhou method. The factor soil had no significant impact on humic acid content (p = 0.09). That outcome was in contrast to earlier observations, because visual examination of ROGG crude extracts in columns showed darker color than LP crude extracts (Figure 5) and a strong signal on agarose emerged for *Zhou* when soil substrate changed from LP to ROGG (Figure 6).
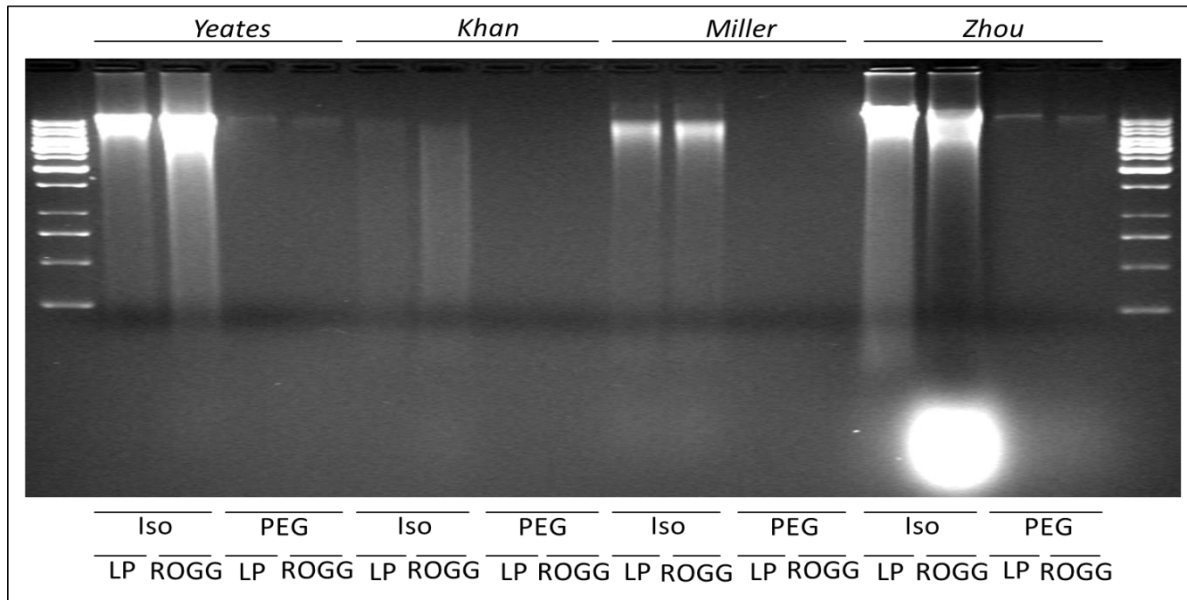
Figure 6 Comparison of DNA crude extracts on agarose gel. Four DNA extraction methods were tested with isopropyl alcohol (Iso) and PEG 8000 (PEG) precipitation and soils of two field locations Roggenstein (ROGG) and Lange Point (LP). Five µl of crude extract per line and 1 kb DNA ladder on outer lines were applied on ethidium bromide stained agarose gel (0.5 %). The 1 kb DNA ladder reaches from 10 (top band) to 0.5 kb (bottom band).

Ultimately, based on visual and spectrophotometric examination of crude extracts, *Yeates* showed high to intermediate DNA yields (Figure 6, Figure 7A) and the highest purity (Figure 7C) which was also the closest to the known 1.8 ratio for pure DNA, while having low humic acid contamination (Figure 5, Figure 7E). Therefore, *Yeates* was chosen as the best published DNA extraction method in the presented study.

3.1.3.2 *Evaluation of column based DNA purification*

In order to be able to purify DNA crude extracts contaminated with co-extracted inhibitory substances, a column based DNA purification approach after Arbeli and Fuentes (2007) was tested for its ability to clean up DNA crude extracts from difficult soil samples. Eight replicates of combined Sepharose 4B and PVPP purification columns were loaded with crude DNA extract from the *Zhou* method, which showed the strongest contamination with humic acids. Subsequently, the columns were eluted four consecutive times and the

eluents were examined on a spectrophotometer. In addition, the un-purified crude extracts were measured as reference.
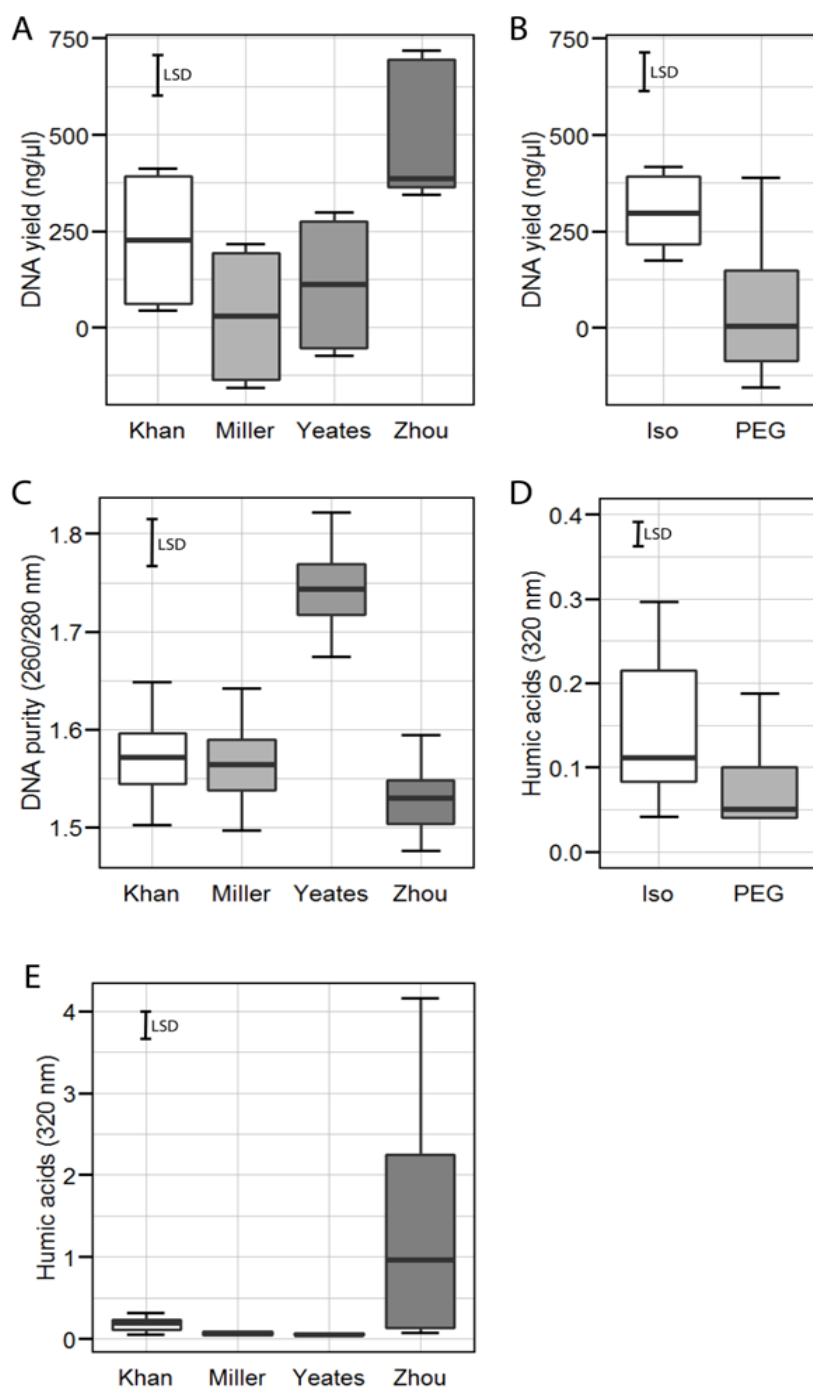
Figure 7 DNA yield, DNA purity and humic acid content of four DNA extraction methods and DNA yield and humic acid content of two precipitation procedures. Comparison of four DNA extraction methods (n=12) (A) and two precipitation procedures (n=24) (B) for yield. Comparison of four DNA extraction methods (n=12) for DNA purity (C). Comparison of two precipitation procedures (n=24) (D) and four DNA extraction methods (n=12) (E) for humic acid content. LSD line indicates the least significant difference at significance level α = 0.05. The boxplots show the median (bar), the 25th and 75th percentiles. Whiskers include all values within 1.5-fold inter-quartile range.

The influence of the elution time point was evaluated by performing an ANOVA on DNA yield, purity and contamination of crude DNA extracts with humic acids (Figure 8). In the ANOVA, the factor elution time point showed significant influence on DNA yield ($p < 0.01$), purity ($p < 0.05$) and humic acid content ($p < 0.01$). The starting material contained significantly more DNA than all subsequent elutions (Figure 8A), indicating a significant reduction in DNA from the first elution step. There was no significant difference in DNA amount between the four elutes (Figure 8A). The first elution had the highest purity of all five samples. The other four samples, the reference crude extract sample and elutes two, three and four, showed similar values for DNA purity (Figure 8B). Regarding humic acid content, the first elution of the column purification reduced the humic acid content almost 9-fold compared to the crude extract (Figure 8C). Afterwards, the humic acid content remained constant across elution steps. These results indicate that column based purification leads to substantial loss of DNA. Further, the first elution showed superior purity, and significant decrease in humic acid content compared to the un-purified crude extract. Due to the fact that DNA purity was significantly higher in the first elute, this elute could be used for further analysis. However, since the Spin kit avoided the co-extraction of inhibitory substances, this column based purification was not part of the final method workflow.

3.1.3.3 Comparison of Yeates and Spin Kit DNA extraction methods

The best performing DNA extraction method from the literature, *Yeates,* was compared to the commercially available Spin Kit, with respect to DNA yield and inhibition of the qPCR reaction. For this purpose, *Yeates* and Spin Kit DNA crude extracts were compared on an agarose gel, and the IVR and SPUD assays were used in a qPCR approach. Utilization of the qPCR approach enabled a quantitative assessment of DNA yield and inhibition, in comparison to the beforehand carried out qualitative or semi-quantitative examinations through crude extract color, agarose gel and spectrophotometer. The agarose gel indicated higher molecular weight and more yield for the crude DNA extract of the *Yeates* method (Figure 9) than for the Spin Kit. The control extraction from maize root material without soil yielded more DNA than the dried and milled soil samples with added maize roots (Figure 9). Strikingly, *Yeates* extraction of ROGG samples showed signals in low molecular weight regions, similar to the crude extracts of *Zhou* with the ROGG samples from the initial DNA extraction procedure evaluation (Figure 6).
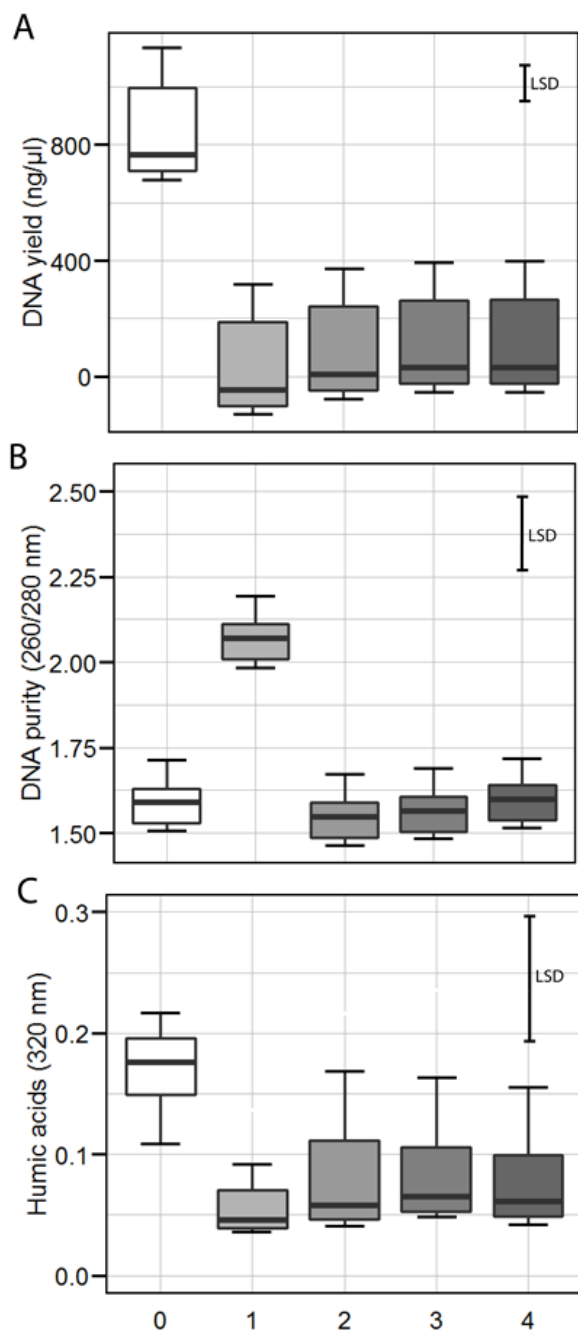
Figure 8 DNA yield, purity and humic acid content of the *Zhou* DNA crude extract (white box) and four consecutive elutions after column based purification. Comparison of crude extract (0) and elutions (1-4) for DNA yield (A), DNA purity (B) and humic acid content (C) (n=8). LSD line indicates the least significant difference at significance level $\alpha = 0.05$. The boxplots show the median (bar), the 25th and 75th percentiles. Whiskers include all values within 1.5-fold inter-quartile range.
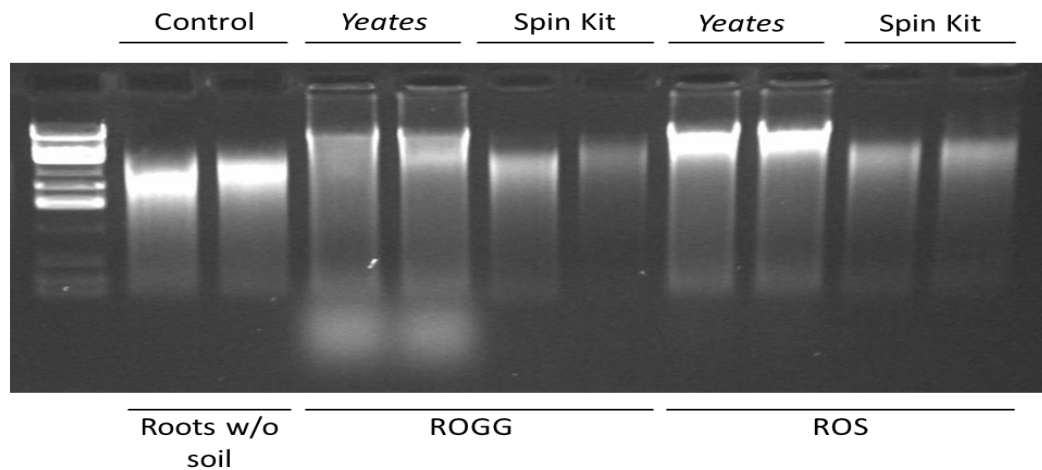
Figure 9 Comparison of *Yeates* and Spin Kit DNA crude extracts on agarose. As positive control (Control), DNA was extracted with the Spin Kit from maize roots without soil. DNA crude extracts with the Spin Kit and *Yeates* method were obtained by extracting 15 g of soil with 300 mg of added maize root material. Five μl per line of crude DNA extract and DNA ladder on outer left line were applied on an ethidium bromide stained agarose gel (0.5 %). The λ-Hind III DNA ladder reaches from 23 kb (top band) to 125 bp (bottom band).

With the quantitative qPCR approach, DNA crude extracts were tested for DNA yield and inhibition on qPCR. The qPCR showed that *Yeates* yielded 1.8-fold more maize root DNA on average than the commercial Spin Kit (mean Cq values of 32.60 and 33.43 for *Yeates* and Spin Kit, respectively), confirming the impression from the agarose gel. The only samples which deviated from the control in the SPUD inhibition assay were the *Yeates* crude extracts from the ROGG soil (mean Cq values of 24.04 and 25.11 for SPUD-control and *Yeates*-ROGG, respectively), which indicated inhibition. The other samples did not show inhibition. These results were in line with the agarose gel observations (Figure 9), where signals in the low molecular weight region of the *Yeates* extraction from the ROGG soil indicated undesirable co-extracts. Regarding earlier results, this suggests that ANOVA based on spectrophotometer measurements was not able to reveal the inhibitory influence of ROGG soil on the enzymatic reaction in the qPCR, as it was shown for the comparison of *Yeates* with the Spin Kit. Ultimately, *Yeates* yielded more DNA of higher molecular weight, but the co-extraction of inhibiting substances significantly interfered with enzymatic reaction in DNA amplification in the qPCR. The Spin Kit showed no enzymatic inhibition regardless of the tested soil type. Therefore, the Spin Kit was incorporated in the final method workflow.

3.1.3.4 Evaluation of sample-specific DNA extraction efficiencies

Despite usage of an optimized and standardized DNA extraction protocol, differences in DNA extraction efficiency between each reaction column can occur and would lead to biased results when trying to assess total maize root DNA quantity. In order to record the sample- or column-specific DNA extraction efficiency, a monitoring mechanism was integrated in the final method. Therefore, each soil sample was spiked with a defined substance with standardized DNA amount before DNA extraction. Subsequently, DNA of sample and spiking substance were extracted in the same reaction column. After DNA extraction, maize root DNA of the soil sample and the spiking substance were quantified using qPCR. Since the DNA amount of the spiking substance before and after DNA extraction was known, DNA extraction efficiency of each particular reaction column could be calculated.

Extraction efficiencies calculated from 1140 DNA samples from the validation experiment in the field are shown in Figure 10. In total, the extraction efficiency mean of all observed extractions was 1.19, with a minimum and maximum value for single samples of 0.64 and 2.70, respectively. From all qPCR runs, plate one showed a significantly higher mean (1.81, $p < 0.01$) for extraction efficiency than other plates. The enhanced efficiency was due to the correction for inter-run variation of plates using the internal positive control (IPC). The IPC was run on each 96-well plate and consisted of the exact same sample and reaction, which should always yield the same in qPCR reaction. In order to make all single plates comparable, the IPC of each single plate was related to the mean of all IPC from all plates. In the case of plate 1, the IPC performed worse than the mean, which could be related to qPCR device performance or batch quality of chemical constituents of these reactions on plate 1. Therefore, according to the data analysis workflow (chapter 2.5), the Cq values on plate 1 were corrected for this variation, leading to increased efficiencies. These results show that outliers, which would lead to fluctuations in root DNA results, do occur. The DNA extraction monitoring was part of the final method workflow. The raw data was corrected using the presented extraction efficiencies.
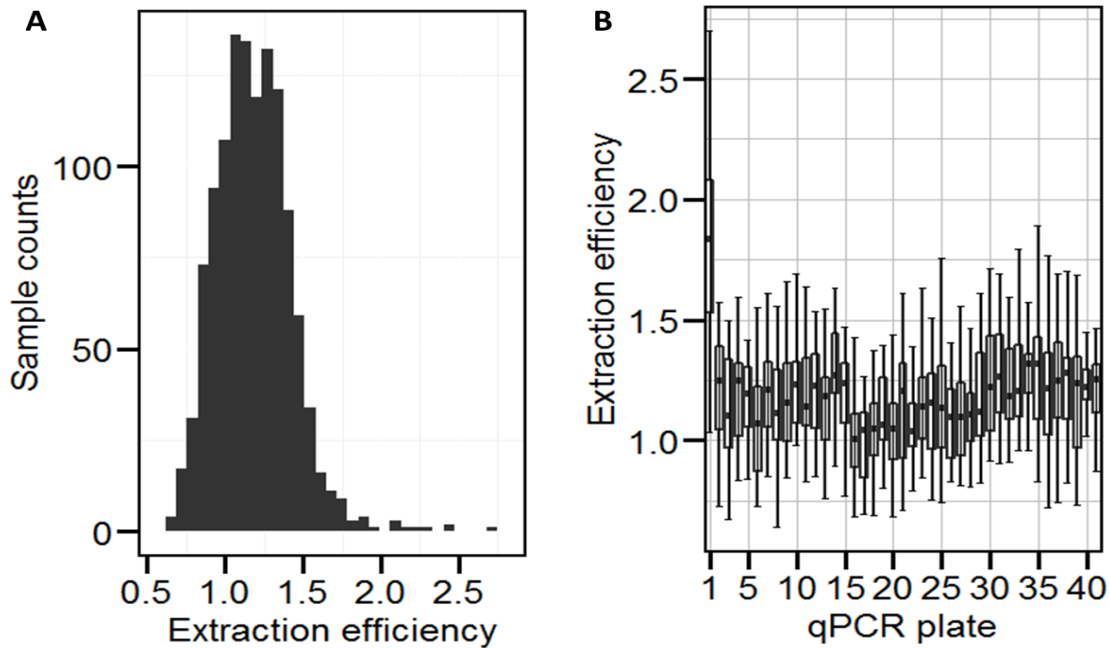
Figure 10 DNA extraction efficiency distribution and boxplots across qPCR plates. Distribution of DNA extraction efficiency from 1140 DNA samples assessed in the presented study (A) and DNA extraction efficiency of 42 qPCR runs with 96 reactions each (B). The boxplots show the median (bar), the 25th and 75th percentiles. Whiskers include all values within 1.5-fold inter-quartile range.

3.1.4 Development of specific, sensitive and robust qPCR assays

The present study involved the testing of 1140 samples from the field and 252 samples from the greenhouse with two qPCR assays, for maize root DNA quantification and extraction efficiency monitoring. With many samples to test for only one or few genomic targets, qPCR assays with Taqman probes have benefits in stability, specificity and handling as compared to assays without specific probe (Lim et al. 2011; Nolan et al. 2006a; Varkonyi-Gasic et al. 2007). Thus, to satisfy the demand for high specificity and sensitivity of the assays, the Taqman technique was utilized to design the assays.

In order to increase sensitivity for the detection of maize root DNA, the internal transcribed spacer 2 (ITS2) was selected as genomic target region. For ITS2 appears in high copy-number per genome (Buckler and Holtsford 1996a), assays utilizing this target should enable quantification of very small amounts of maize root DNA from soil samples in contrast to assays using single copy genes. According to the established standard curve for the ITS2 assay, the design enabled the detection of 0.1 pg of maize root DNA, corresponding to a fraction of the haploid maize genome (molecular weight of approximately 2.6 pg) (Arumuganathan and Earle 1991). Standard curve evaluation of the

ITS2 assay according to the qPCR cycler software showed a reaction efficiency of 100.7 and an R^2 of 0.998.

Transgenic *Arabidopsis* plants carrying a cloning vector (p6xHis-GFP) were used for the DNA extraction efficiency monitoring. With this assay, *Arabidopsis* plant powder containing the cloning vector as target construct, was quantified in a stable range of the qPCR reaction. According to the optimized standard curve, the reaction went close to 100 % efficiency, with an R^2 of 0.996, in the Cq range from 20 to 33. In order to guarantee that the reaction runs in that Cq range, and thus the measured efficiency holds true, approximately 25 mg *Arabidopsis* plant powder per reaction were added to the soil samples before DNA extraction. The optimized IVR and SPUD assays also showed high reaction efficiencies and R^2 values as indicated by standard curves (Table 3).

Moreover, qPCR specificity testing of the ITS2 and IVR assays with 17 commonly found weeds collected from maize field trials (Table A1, appendix) showed no quantification of the weed DNA in the qPCR reactions. All three assays, ITS2, HIS, SPUD were used in the final workflow (Figure 13, chapter 3.1.5), and the IVR assay was used to determine the genotype specific ITS2 copy numbers.

3.1.4.1 Determination of genotype specific ITS2 copy number

It is known from literature that ITS2 exhibits genotypic copy number variation in maize (Rivin et al. 1986). In order to enable comparisons of different maize genotypes for root DNA quantity, the ITS2 copy number of all maize genotypes included in the validation experiments field and greenhouse was determined using a climate chamber experiment (Table 1, chapter 2).

The experiment revealed significant genotypic variation for the ITS2 copy number in the tested maize lines (Figure 11). The mean was 8251 copies, minimum and maximum were 3412 and 14885 copies, respectively. Thus, in order to compare the root DNA quantity between genotypes for the trait RDD, values needed to be normalized for differences in ITS2 copy number.

Consequently, a correction factor for each genotype was calculated based on the observed ITS2 copy numbers. Maize line F618 was chosen as reference genotype, according to the normalization in Huang et al. (2013), since it was the closest to the observed mean. The ITS2 copy number of the remaining genotypes was expressed relative to the reference genotype for the RDD analysis (see chapter 2.5, step 4).
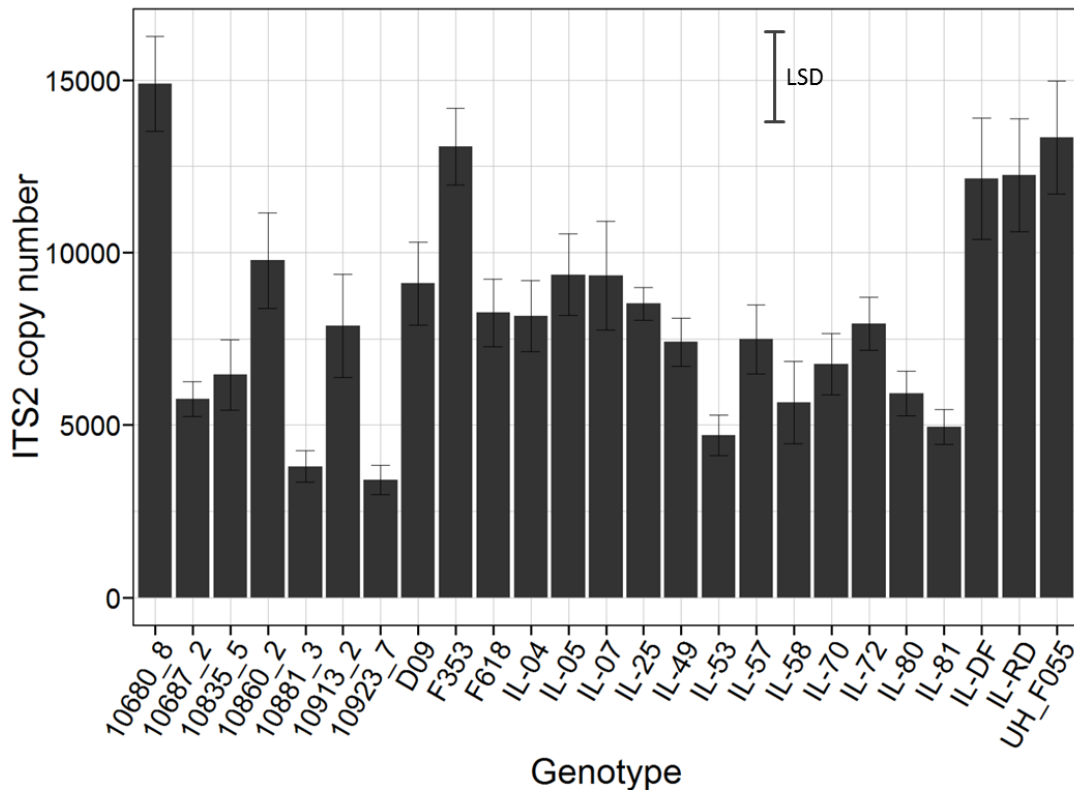
Figure 11 Genotypic variation in copy numbers of ITS2 per genome among 25 maize genotypes. LSD line indicates the least significant difference at significance level α = 0.05. Whiskers denote standard errors (n=3).

3.1.4.2 Quantification of spiked maize roots from soil samples

The ability of the maize qPCR assay ITS2 to quantify maize root DNA from spiked roots in soil from field experiments was tested with a spiking experiment. Regression of measured DNA on input root mass showed a high coefficient of determination indicating that a high proportion of the variance in DNA content from the different spiking points could be predicted by the ITS2 assay (Figure 12). According to the spiked root mass, doubling of measured DNA could be observed from 20 to 40 mg, from 40 to 80 mg and from 80 to 160 mg spiked maize root. The range of Cq values, resulting from quantification of 20, 40, 80 and 160 mg maize root, was similar to the maize root DNA concentrations observed in the field (Cq 18-22). Thus, the ITS2 assay with the new method was able to quantify maize root DNA in a range expected for field trials.
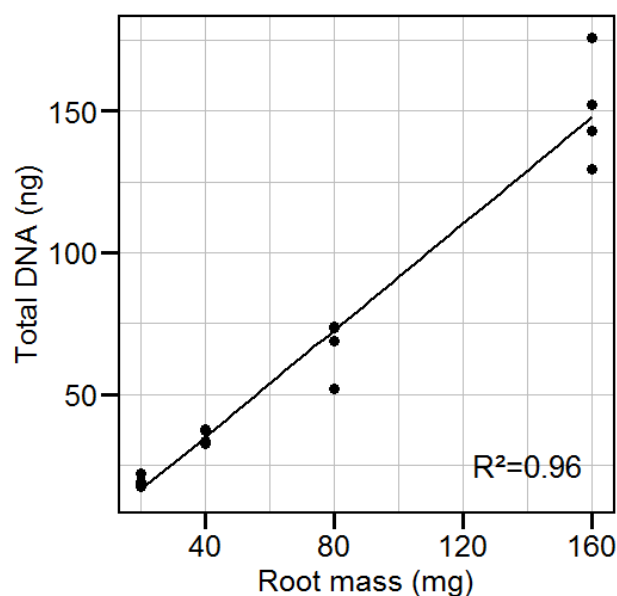
Figure 12 Relationship between the quantity of maize root added to soil (20, 40, 80, 160 mg 100 g^{-1}) and total DNA measured in soil (ng 100 g^{-1}) using the ITS2 assay.

3.1.5 Final workflow

The development part of this study resulted in the final workflow for the new method to determine RDD. Figure 13 highlights the most important steps of the new method. The aim for the sampling step was to acquire undisturbed soil samples being representative for the respective plot. Sample handling enabled the comparison of RDD to RLD or RMD from the same soil sample. The homogenization steps should prevent DNA degradation, prepare the milling step and enable robust sub-sampling from the main-sample. Sub-sampling substantially reduced the sample amount to be processed in the following steps. The DNA extraction step aimed for inhibitor free crude extracts and implemented a monitoring system to obtain the DNA extraction efficiency. The qPCR step allowed maize root DNA quantification and capturing of DNA extraction efficiency for the same sample. Finally, the data analysis rendered all samples comparable to each other by normalizing for run-to-run variation, DNA extraction efficiency and genotypic differences in ITS2 copy numbers. Finally, the workflow made it possible to calculate RDD.
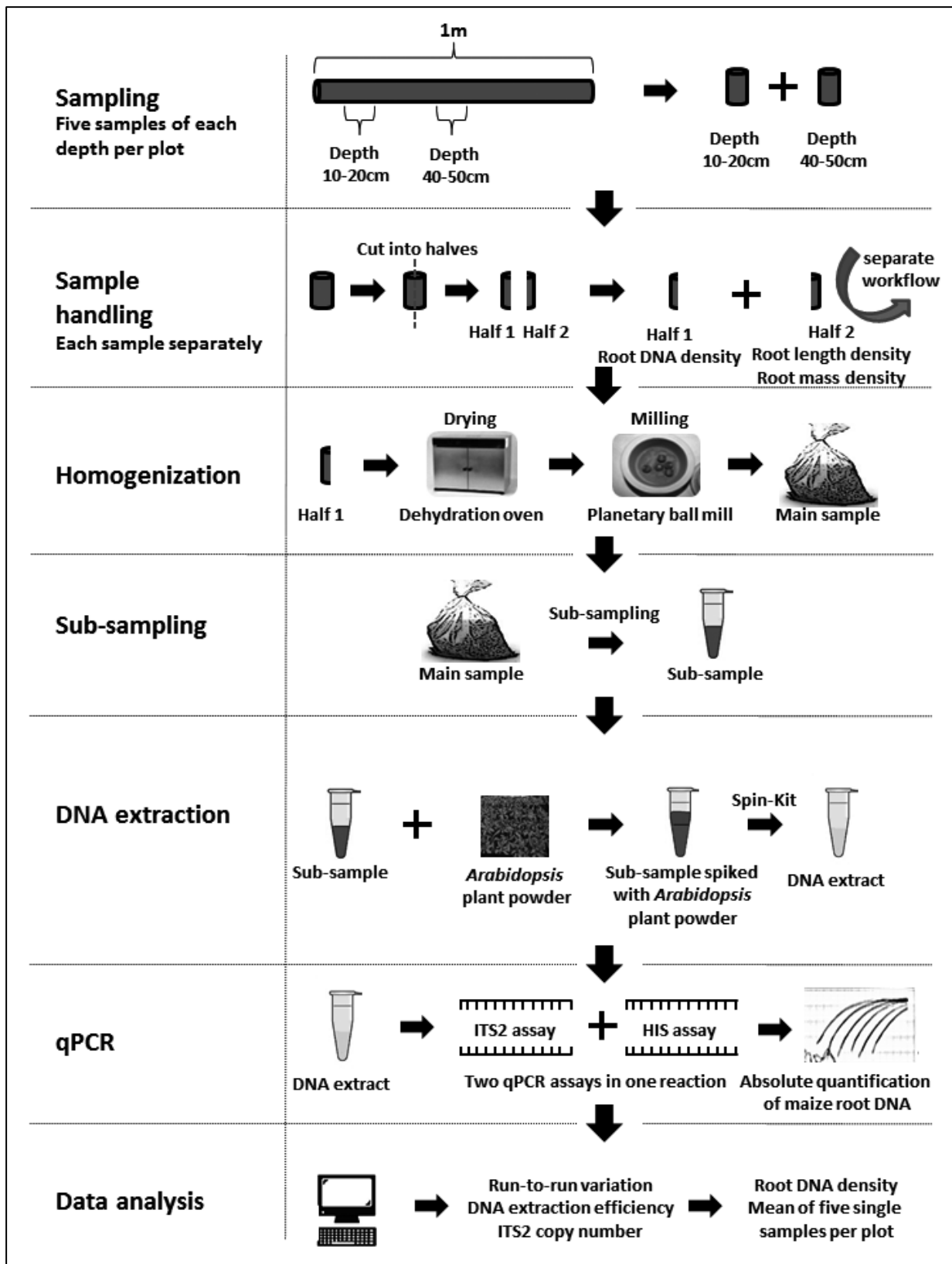
Figure 13 Final workflow for DNA-based root quantification in field environments.

3.2 Validation of a DNA-based method to quantify maize roots in the field

3.2.1 Comparison of RDD to commonly assessed root traits RMD and RLD for genotypic variation and heritability in the field

In order to validate the newly developed method, RDD was compared to RLD and RMD with respect to genotypic variation, repeatability and heritability in the field. All three traits were assessed in two treatments, well-watered and drought, and in two soil depths, depth 10 and depth 40. Background maize root DNA levels were 207 pg cm^{-3} in depth 10, and 334 pg cm^{-3} in depth 40, and the soil bulk densities for depth 10 and 40 were 1.45 and 1.57 g cm^{-3}, respectively.

Mean RDD, RLD and RMD substantially decreased from depth 10 to depth 40, both in the well-watered treatment and under drought conditions (Table 4). For RDD, a significant reduction of the mean was observed from well-watered to drought conditions within the respective depth.

Table 4 Means of 19 genotypes, associated standard errors (s.e.), minimum (Min.) and maximum (Max.) for the traits root DNA density (RDD; ng cm^{-3}), root length density (RLD; cm cm^{-3}) and root mass density (RMD; mg cm^{-3}) in well-watered and drought treatment and depths 10 and 40 in the field experiment.

Trait	Treatment	Depth	mean	(±) s.e.	Min.	Max.
RDD			104.3	10.7	42.5	222.2
RLD	Well-watered	10	3.11	0.15	1.88	4.14
RMD			0.50	0.03	0.29	0.88
RDD			69.4	9.4	17.6	195.2
RLD	Drought	10	2.12	0.16	0.91	3.26
RMD			0.30	0.02	0.12	0.51
RDD			21.2	2.5	9.8	47.9
RLD	Well-watered	40	0.86	0.05	0.50	1.25
RMD			0.06	0.005	0.02	0.10
RDD			7.4	1.2	1.8	23.0
RLD	Drought	40	0.80	0.05	0.33	1.50
RMD			0.05	0.004	0.02	0.08

In depth 40 under drought, the specific root length, i.e. the root length for a given root mass (m g^{-1}), was significantly increased compared to the well-watered control (Figure 14). In this depth, the maize plants decreased RMD, but at the same time lowered the average root diameter, resulting in similar RLD between well-watered and drought treatment. It was also found that the maximum value for RLD under drought in depth 40 was greater

than in the well-watered treatment. Based on these observations, RDD showed similar reaction to drought as RLD and RMD in depth 10, but was more similar to the behavior of RMD in depth 40 (Table 4).

In depth 10, RDD and RMD showed significant genotypic variance for the well-watered treatment and RDD and RLD for the drought treatment (Table 5). In depth 40, only RDD showed significant genotypic variance in the well-watered treatment, and none of the traits under drought. Throughout both treatments and depths, RDD showed the highest repeatabilities of all three traits (Table 5).
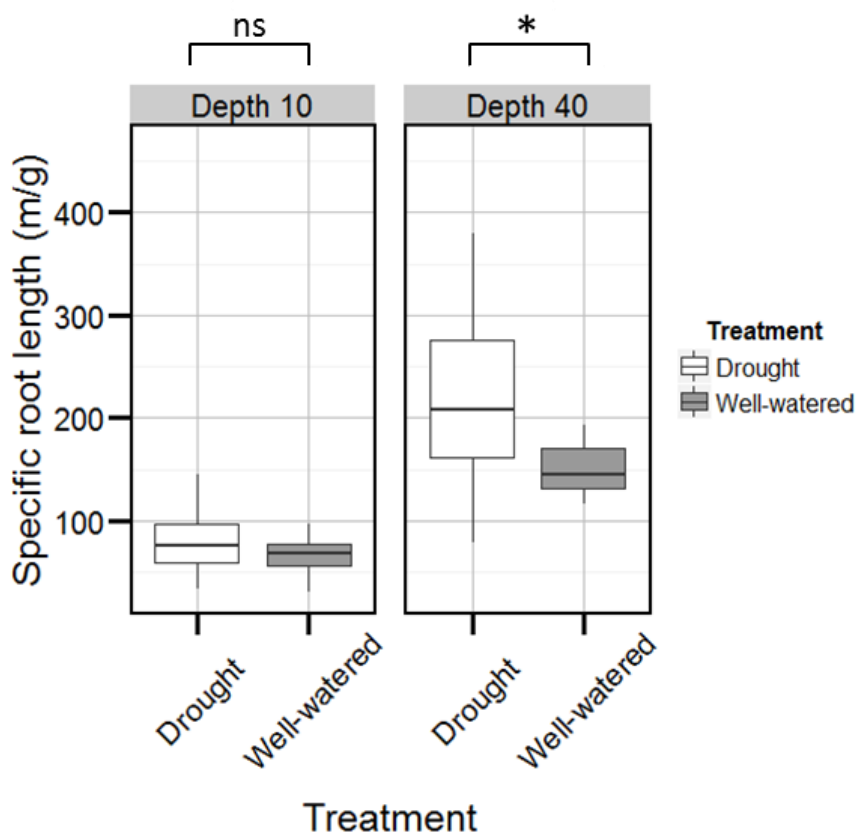
Figure 14 Specific root length of 19 maize genotypes in well-watered control and drought treatment in depth 10 and 40 in the field. Significant differences between treatments at p < 0.05 are indicated by asterisks. The boxplots show the median (bar), the 25th and 75th percentiles. Whiskers include all values within 1.5-fold inter-quartile range (n=57). ns: not significant.

RDD in both depths 10 and 40 was significantly influenced by the drought treatment (Table 4, Table 6). RDD showed no significant genotype-treatment interaction effects and high heritabilities in depth 10 and 40 (Table 6). Remarkably, RDD was the only trait with significant genotypic variance in both depths 10 and 40.

Table 5 Estimates of genotypic (σ^2_g) and error (σ^2) variance components and repeatabilities (Rep.) for the traits root DNA density (RDD; ng cm^{-3}), root length density (RLD; cm cm^{-3}) and root mass density (RMD; mg cm^{-3}) in well-watered and drought treatment and depths 10 and 40 in the field experiment.

Trait	Treatment	Depth	σ^2_g	σ^2	Rep.
RDD			1970**	1060	0.65
RLD	Well-watered	10	-[a]	-	-
RMD			0.015*	0.017	0.46
RDD			966**	398	0.71
RLD	Drought	10	0.330*	0.366	0.47
RMD			-[a]	-	-
RDD			69.9*	82.7	0.46
RLD	Well-watered	40	-[a]	-	-
RMD			-[a]	-	-
RDD			-[a]	-	-
RLD	Drought	40	-[a]	-	-
RMD			-[a]	-	-

*, **: significant at p < 0.05, 0.01, [a]: not significant at p < 0.05.

Table 6 Estimates of genotypic (σ^2_g), treatment (σ^2_t) and genotype-treatment interaction (σ^2_{gt}) variance components, heritabilities (H^2) and associated standard error (s.e.) for the trait root DNA density (RDD; ng cm^{-3}) in depths 10 and 40 in the field experiment.

Depth	σ^2_g	σ^2_t	σ^2_{gt}	H^2	($\pm$) s.e.
10	1290**	510**	30.3	0.90	0.05
40	57.8**	80.1**	0	0.88	0.05

*, **: significant at p < 0.05. 0.01

3.2.1.1 *Correlations for RDD and commonly used root traits root length density (RLD) and root mass density (RMD) under optimal and sub-optimal water availability in the field*

Phenotypic correlations of RDD with RLD and RMD were calculated (Table 7). In depth 10, RDD was significantly correlated with RLD in the well-watered and the drought treatment (Table 7). Furthermore, RLD and RMD were also significantly correlated. In depth 40 however, RDD was only correlated with RMD under drought. Again, as in depth 10, RLD and RMD were correlated in the well-watered and the drought treatment in depth 40.

Generally, these observations indicate that RDD was correlated with commonly assessed root traits RLD and RMD, but the observed correlations varied with changes in treatment from well-watered to drought (RDD and RMD in depth 40) or changes in depth from 10 to 40 (RDD and RLD).

Table 7 Phenotypic correlation coefficients between traits root DNA density (RDD), root length density (RLD) and root mass density (RMD) within and between treatments well-watered and drought for depth 10 (below diagonal) and 40 (above diagonal) in the field experiment.

	RDD_W	RLD_W	RMD_W	RDD_D	RLD_D	RMD_D
RDD_W		0.31	0.27	0.73**	0.14	0.55*
RLD_W	0.61**		0.87**	0.32	0.40	0.59**
RMD_W	0.30	0.63**		0.01	0.15	0.47*
RDD_D	0.74**	0.19	0.01		0.18	0.57*
RLD_D	0.37	0.39	0.38	0.55*		0.50*
RMD_D	0.15	0.38	0.11	0.47	0.76**	

Suffix for traits:_W: well-watered, _D: drought. *; **: significant at p < 0.05; 0.01

3.2.2 Genotypic variation and heritability of RDD in the greenhouse

In order to investigate RDD under controlled environmental conditions, a greenhouse trial was conducted. RDD levels in the greenhouse experiment were substantially higher than in the field, with at least 13-fold increase in topsoil and 39-fold increase in subsoil (Table 8). In contrast to the field, RDD in the greenhouse showed higher values under drought compared to the well-watered treatment (Table 8).

Table 8 Means of 21 genotypes, associated standard errors (s.e.), minimum (Min.) and maximum (Max.) for the trait root DNA density (RDD; ng g^{-1}) in well-watered and drought treatments and depths 1, 2 and 3 in the greenhouse experiment.

Treatment	Depth	mean	(±) s.e.	Min.	Max.
Well-watered	1	1411	162	411	3169
Drought	1	3145	295	1560	6064
Well-watered	2	987	120	147	2573
Drought	2	3786	299	1755	6255
Well-watered	3	836	130	85	2486
Drought	3	2227	227	1096	5111

Within depths 1, 2 and 3 and treatments well-watered and drought, RDD showed significant genotypic variance in the greenhouse (Table 9). The repeatabilities were higher in the drought treatment than in the well-watered control. The reason was that, although genotypic variance components decreased under drought, the associated error variance was much lower under drought than in the well-watered control.

Table 9 Estimates of genotypic (σ^2_g) and error (σ^2) variance components, and repeatabilities (Rep.) for the trait root DNA density (RDD) in well-watered and drought treatment and depths 1, 2 and 3 in the greenhouse experiment.

Treatment	Depth	σ^2_g	σ^2	Rep.
Well-watered	1	0.036*	0.044	0.45
Drought	1	0.027**	0.010	0.72
Well-watered	2	0.041*	0.033	0.55
Drought	2	0.023**	0.007	0.76
Well-watered	3	0.055*	0.093	0.37
Drought	3	0.026*	0.017	0.60

*, **: significant at $p < 0.05$, 0.01.

In all depths, the drought treatment influenced RDD significantly (Table 10). There was significant genotype-treatment interaction in depth 1, but not in depth 2 and 3. The heritability for RDD was intermediate in topsoil and high in both subsoil horizons. The observed genotype-treatment interaction led to the lower heritability in depth 1 as compared to depth 2 and 3. These observations suggest that RDD shows significant genotypic variance and intermediate to high heritabilities when measured from pots.

Table 10 Estimates of genotypic (σ^2_g), treatment (σ^2_t) and genotype-treatment interaction (σ^2_{gt}) variance components, heritabilities (H^2) and associated standard error (s.e.) for the trait root DNA density (RDD) in depths 1, 2 and 3 in the greenhouse experiment.

Depth	σ^2_g	σ^2_t	σ^2_{gt}	H^2	(±) s.e.
1	0.020*	0.058**	0.013*	0.63	0.17
2	0.023**	0.173**	0	0.82	0.09
3	0.034*	0.104**	0	0.76	0.09

*, **: significant at $p < 0.05$, 0.01.

3.2.3 Correlation of RDD across different water regimes, depths and the phenotyping platforms field and greenhouse

RDD gained from pots in the greenhouse experiment is given in DNA mass per soil weight, since the soil volume was not available for calculations due to the sampling procedure in the greenhouse. RDD from the field experiment was calculated in DNA mass per soil volume. In order to assess whether RDD given as root DNA per soil mass differs from RDD given as root DNA per soil volume, a regression analysis for depths 10 and 40 in the field was performed, since both units could be calculated from the field data. RDD given in DNA either per soil weight or soil volume was highly correlated in the field (Figure 15).
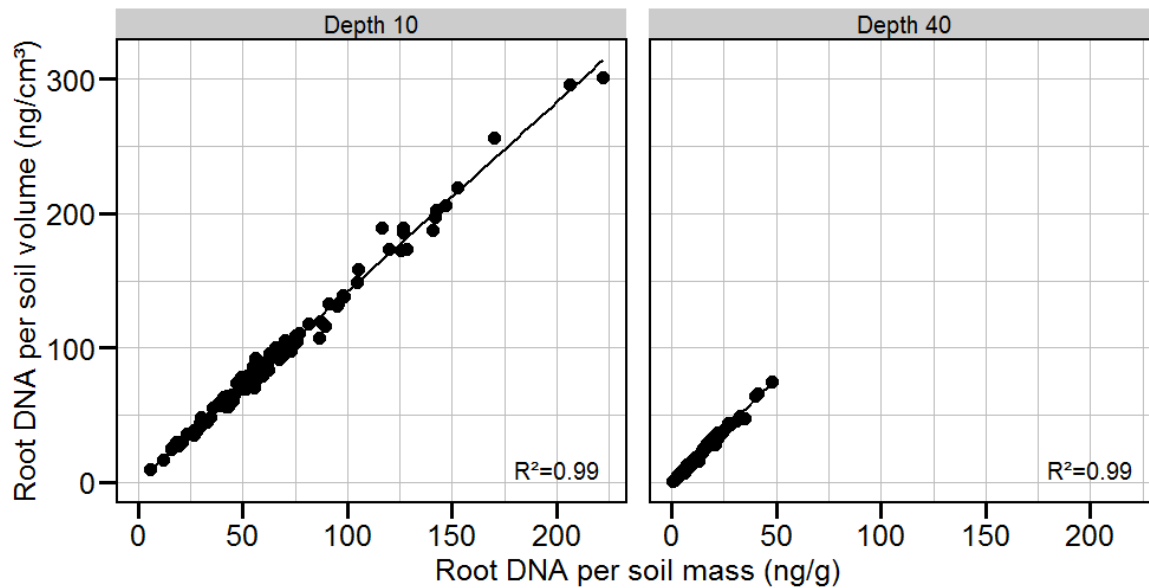
Figure 15 Regression of root DNA per soil volume on root DNA per soil mass for depths 10 and 40 in the field. For each depth, measurements of both treatments well-watered and drought are given (n=114). R^2: coefficient of determination.

In order to investigate the association of RDD across different water regimes, depths and phenotyping platforms, relevant phenotypic correlations were calculated. The relationship of RDD measurements across treatments well-watered and drought was investigated in the field experiment. In both depths 10 and 40, RDD showed a strong correlation (r = 0.74 and r = 0.73, respectively) between treatments well-watered and drought (Table 7). Also correlations of RDD between depths for each treatment well-watered and drought were calculated. RDD measurements showed strong correlations between the depths 10 and 40 within the respective treatments (Figure 16).

In order to examine the correlation of RDD between phenotyping platforms, adjusted RDD mean values from the field were correlated with adjusted RDD mean values from the greenhouse. To have the same RDD unit for both experiments, RDD values from the field were converted to the unit DNA mass per soil mass, which was found to be representative for RDD values measured in the field (Figure 15). Irrespective of treatment well-watered or drought, the RDD values showed significant correlations between the field and greenhouse (Figure 17). These results indicate that RDD is relatively constant across water regimes, depths and the phenotyping platforms field and greenhouse.
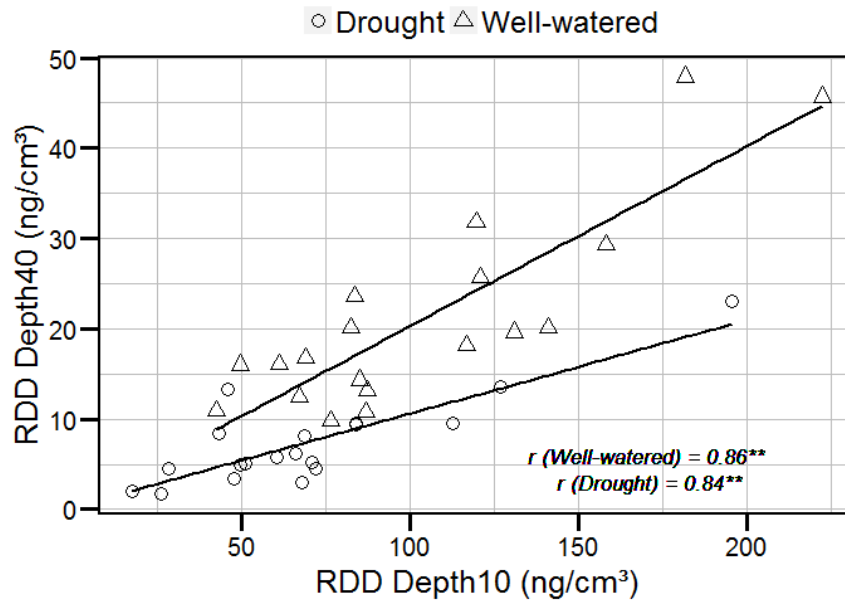
Figure 16 Regression of root DNA density (RDD) in depth 40 on RDD in depth 10. Correlation coefficients were calculated separately for treatments well-watered control (triangles) and drought (circles). **: significant at p < 0.01 (n =19).
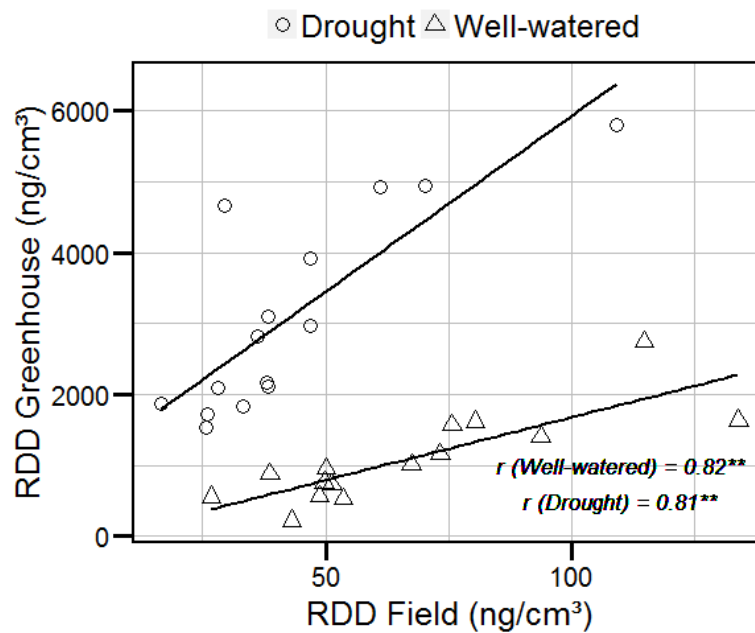
Figure 17 Regression of root DNA density (RDD) in the greenhouse on RDD in the field. RDD was calculated across depths in each treatment in the field and greenhouse. Correlation coefficients were calculated separately for treatments well-watered control (triangles) and drought (circles). **: significant at p < 0.01 (n =15).

3.2.4 Capability of RDD to predict shoot traits under optimal and sub-optimal water availability in the field

In addition to RDD, RLD and RDM, the crown root number (CN) was phenotyped in the field using excavation of whole root crowns and visual phenotyping after Trachsel et al. (2010a). Shoot traits for maize plant development, physiology and reproduction were assessed, in order to investigate the dependency of RDD and other root traits on plant development and their association with physiological and reproductive traits in well-watered and drought treatment.

3.2.4.1 Genotypic variation and heritabilities for shoot traits associated with plant development, physiology and reproduction

Except Mflow and Fflow, all traits were significantly influenced by drought (Table 11). CN, SC, Biomass_flow, Biomass_mat, GY and KPE were decreased by drought treatment, and ASI, HI and TKW were increased by drought treatment.

Table 11 Means of 19 genotypes, associated standard errors (s.e.), minimum (Min.) and maximum (Max.) for 11 traits in well-watered and drought treatment in the field experiment.

Trait	Unit/Rating	Treatment	mean	(±) s.e.	Min.	Max.
CN	nr	Well-watered	30.6	1.5	22.9	47.5
		Drought	24.4	1.0	15.6	33.8
Mflow	DAG	Well-watered	89.9	1.0	80.1	94.3
		Drought	88.8	1.1	77.9	95.2
Fflow	DAG	Well-watered	91.4	1.2	79.0	97.0
		Drought	91.7	1.0	80.9	98.0
ASI	d	Well-watered	1.6	0.36	-2.0	4.0
		Drought	3.3	0.40	0.3	6.3
SC	mmol $m^{-2}s^{-1}$	Well-watered	358.1	17.9	236.0	543.0
		Drought	187.1	11.0	122.7	275.5
Biomass_flow	g	Well-watered	71.9	3.0	38.3	98.9
		Drought	36.4	1.7	21.3	48.7
Biomass_mat	g	Well-watered	578.9	24.3	352.4	764.7
		Drought	287.6	11.1	155.3	355.3
GY	g	Well-watered	338.6	37.5	69.6	684.1
		Drought	285.0	16.7	144.6	428.9
HI	ratio	Well-watered	0.35	0.03	0.09	0.60
		Drought	0.49	0.02	0.32	0.65
TKW	g	Well-watered	236.9	6.7	172.2	295.8
		Drought	250.3	8.1	166.7	322.9
KPE	nr	Well-watered	148.2	13.3	37.0	250.7
		Drought	118.9	7.4	64.1	164.7

CN: crown root number, Mflow: male flowering, Fflow: female flowering, ASI: anthesis silking interval, SC: stomatal conductance, Biomass_flow: plant biomass at flowering, Biomass_mat: plant biomass at maturity, GY: grain yield, HI: harvest index, TKW: thousand kernel weight, KPE: kernels per ear.

Although there was no significant effect of drought on Mflow and Fflow, ASI increased significantly under drought. The significant increase of ASI was due to the fact that Mflow decreased and Fflow increased slightly under drought.

All traits except SC (well-watered), GY (drought), and HI (drought) showed significant genotypic variance components (Table 12). Mflow under both treatments well-watered and drought showed the highest repeatabilities with 0.73 and 0.78, respectively. Repeatabilities for the crown root trait CN were low to intermediate. Flowering traits Fflow and ASI showed intermediate to high repeatabilities, with no marked difference of the repeatabilities between both treatments. SC showed an intermediate repeatability under drought.

Table 12 Estimates of genotypic (σ^2_g) and error (σ^2) variance components and repeatabilities (Rep.) for 11 traits in well-watered and drought treatment in the field experiment.

Trait	Treatment	σ^2_g	σ^2	Rep.
CN	Well-watered	24.2*	41.7	0.37
	Drought	14.3*	17.0	0.46
Mflow	Well-watered	16.3**	6.1	0.73
	Drought	20.4**	5.7	0.78
Fflow	Well-watered	22.2**	8.1	0.73
	Drought	17.9**	5.6	0.76
ASI	Well-watered	1.73*	2.16	0.44
	Drought	1.87*	2.17	0.46
SC	Well-watered	-[a]	-	-
	Drought	1622*	1832	0.47
Biomass_flow	Well-watered	111.6**	79.9	0.58
	Drought	25.8*	38.8	0.40
Biomass_mat	Well-watered	9749**	4471	0.69
	Drought	1780*	1536	0.54
GY	Well-watered	23106**	7574	0.75
	Drought	-[a]	-	-
HI	Well-watered	0.0160**	0.0043	0.79
	Drought	-[a]	-	-
TKW	Well-watered	649*	579	0.53
	Drought	1013**	761	0.57
KPE	Well-watered	2862**	1286	0.69
	Drought	810*	688	0.54

CN: crown root number, Mflow: male flowering, Fflow: female flowering, ASI: anthesis silking interval, SC: stomatal conductance, Biomass_flow: plant biomass at flowering, Biomass_mat: plant biomass at maturity, GY: grain yield, HI: harvest index, TKW: thousand kernel weight, KPE: kernels per ear. *, **: significant at $p < 0.05$, 0.01, [a]: not significant at $p < 0.05$.

Biomass_flow and Biomass_mat showed intermediate repeatabilities, with slight decreases under drought conditions compared to the well-watered control. Yield parameters HI, KPE

and TKW, as well as GY in the well-watered treatment, showed intermediate to high repeatabilities, except for HI under drought.

Calculated across treatments well-watered and drought, all traits but SC showed significant genotypic variance. Only the traits Biomass_flow, Biomass_mat, GY and KPE showed significant genotype-treatment interaction. Most of the traits showed intermediate to high heritability estimates (Table 13). Biomass_mat showed the lowest heritability with 0.62, and the flowering traits Mflow and Fflow had the highest values with 0.94 and 0.92, respectively.

Table 13 Estimates of genotypic (σ^2_g), treatment (σ^2_t) and genotype-treatment interaction (σ^2_{gt}) variance components, heritabilities (H^2) and associated standard error (s.e.) for 11 traits in the field experiment.

Trait	σ^2_g	σ^2_t	σ^2_{gt}	H^2	(±) s.e.
CN	19.1**	14.7*	0	0.79	0.08
Mflow	18.9**	0.27	0	0.94	0.02
Fflow	19.5**	0	0	0.92	0.03
ASI	1.46*	1.38**	0.36	0.73	0.13
SC	-[a]	-	-	-	-
Biomass_flow	54.3*	527.7**	16.6*	0.76	0.12
Biomass_mat	2930*	41047**	2353**	0.62	0.18
GY	8962*	1220*	2564*	0.75	0.12
HI	0.0087**	0.0092**	0.0002	0.87	0.07
TKW	718**	69*	11	0.81	0.09
KPE	1474**	4641**	294*	0.82	0.09

CN: crown root number, Mflow: male flowering, Fflow: female flowering, ASI: anthesis silking interval, SC: stomatal conductance, Biomass_flow: plant biomass at flowering, Biomass_mat: plant biomass at maturity, GY: grain yield, HI: harvest index, TKW: thousand kernel weight, KPE: kernels per ear. *, **: significant at $p < 0.05$, 0.01, [a]: not significant at $p < 0.05$.

3.2.4.2 *Correlations of RDD with plant development, physiology and reproduction*

Significant correlations were found among the 17 examined traits (Table 14). Therefore only selected results are presented in the text. In the well-watered treatment, Biomass_flow and Biomass_mat were positively correlated with TKW. Biomass_mat was positively correlated with Mflow and Fflow, indicating that genotypes with prolonged vegetative growth accumulated more biomass until maturity. Biomass_mat showed also positive correlation with Biomass_flow. These data show that genotypes with more biomass had higher TKW in the well-watered control. The same observation was also made under drought treatment.

Under drought, Mflow and Fflow as well as ASI were negatively correlated with HI and GY, indicating that later flowering genotypes and genotypes with greater ASI had lower HI and GY under drought treatment. KPE showed strong positive correlations with GY and HI under drought and, in contrast to the well-watered control, a significant negative correlation with TKW. These observations show that more accumulated biomass enhanced TKW independent of the treatment, and that large TKW under drought was accompanied by low KPE.

Regarding the association of root traits with plant phenology, RDD in depth 40 showed a positive correlation with Mflow and Fflow, as well as did RMD in depth 40 with Mflow under drought. There were no correlations of RDD, RLD or RMD with flowering in the well-watered control. These data show that root traits RDD and RMD in subsoil were influenced by phenology under drought.

In the well-watered control, root traits in general showed only few significant correlations with shoot traits. RMD in depth 10 showed a positive correlation with CN, another root trait. These data show that root mass in topsoil determined by coring is associated with crown root number. CN in turn was determined by the excavation of the complete maize plant root stock.

Under drought conditions, root traits showed significant correlations with plant developmental, physiological and reproductive traits (Table 14). The trait CN showed positive correlations with Mflow, Fflow, Biomass_flow, Biomass_mat and TKW. These correlations show that genotypes with prolonged vegetative growth phase and more biomass at flowering and maturity had a higher number of crown roots.

Regarding reproductive traits, most correlations with root traits were found under drought treatment. RDD in depth 40 was negatively correlated with HI under drought, and RDD in depth 10 showed negative correlation with HI and KPE. Moreover, RLD in depth 40 showed a negative correlation with GY. These observations show that genotypes with higher RDD in topsoil as well as subsoil had lower HI. Moreover, higher RDD in topsoil was associated with lower KPE. Likewise, the trait CN showed negative correlations with HI and KPE, showing that genotypes with lower crown root number had higher HI and KPE.

Table 14 Phenotypic correlation coefficients between 17 root and shoot traits in treatments well-watered (below diagonal) and drought (above diagonal) in the field experiment.

	RDD_10	RDD_40	RLD_10	RLD_40	RMD_10	RMD_40	CN	Mflow	Fflow	ASI	SC	Bio_F	Bio_Mat	GY	HI	TKW	KPE
RDD_10		0.84**	0.55*	0.04	0.44	0.35	0.23	0.40	0.35	0.10	0.16	0.31	0.17	-0.35	-0.53*	0.32	-0.50*
RDD_40	0.86**		0.21	0.18	0.15	0.56*	0.17	0.50*	0.50*	0.22	0.40	0.16	0.20	-0.38	-0.47*	0.29	-0.44
RLD_10	0.61**	0.41		-0.07	0.76**	0.07	0.23	0.12	0.11	0.01	-0.04	0.29	0.29	-0.16	-0.36	0.37	-0.43
RLD_40	0.09	0.31	0.34		0.17	0.50*	-0.11	0.25	0.41	0.49*	0.03	-0.40	-0.09	-0.47*	-0.37	-0.25	-0.29
RMD_10	0.30	0.18	0.63**	0.17		0.07	0.38	0.31	0.31	0.01	-0.23	0.38	0.34	-0.19	-0.45	0.32	-0.37
RMD_40	0.06	0.27	0.22	0.87**	0.34		-0.07	0.46*	0.43	0.10	0.14	-0.04	0.08	-0.13	-0.20	0.11	-0.19
CN	0.01	-0.17	0.30	-0.28	0.61**	-0.14		0.56*	0.62**	0.08	0.14	0.64**	0.62**	-0.24	-0.54*	0.52*	-0.51*
Mflow	0.42	0.45	0.42	0.07	0.19	0.01	0.25		0.91**	-0.16	0.04	0.46*	0.69**	-0.19	-0.48*	0.44	-0.37
Fflow	0.39	0.39	0.37	-0.03	0.29	0.03	0.43	0.89**		0.23	0.17	0.38	0.64**	-0.45	-0.61**	0.36	-0.53*
ASI	0.27	0.18	-0.01	-0.22	0.20	-0.03	0.35	0.21	0.58**		0.40	-0.29	-0.21	-0.75**	-0.43	-0.25	-0.43
SC	-0.08	-0.24	0.12	-0.09	0.02	-0.21	0.38	0.44	0.53*	0.40		-0.21	-0.06	-0.41	-0.18	-0.04	-0.19
Bio_F	0.06	0.10	0.10	-0.24	0.15	-0.30	0.28	0.40	0.32	-0.12	-0.08		0.72**	0.11	-0.39	0.70**	-0.31
Bio_Mat	0.10	0.22	0.14	0.15	0.42	0.20	0.26	0.55*	0.54*	0.15	0.18	0.67**		-0.01	-0.40	0.55*	-0.28
GY	-0.43	-0.34	-0.42	-0.07	-0.26	-0.13	-0.19	-0.56*	-0.62**	-0.55*	-0.33	0.16	0.01		0.80**	0.09	0.71**
HI	-0.39	-0.37	-0.41	-0.14	-0.41	-0.20	-0.29	-0.71**	-0.72**	-0.47*	-0.32	-0.15	-0.37	0.91**		-0.29	0.82**
TKW	0.34	0.35	0.26	0.15	0.21	-0.03	-0.07	0.33	0.19	-0.21	-0.05	0.67**	0.64**	0.22	-0.06		-0.55*
KPE	-0.39	-0.35	-0.54*	-0.18	-0.45	-0.24	-0.25	-0.67**	-0.71**	-0.48*	-0.32	-0.05	-0.30	0.91**	0.95**	0.01	

Suffix for coring traits: _10: depth 10, _40: depth 40, RDD: root DNA density, RLD: root length density, RMD: root mass density, CN: crown root number, Mflow: male flowering, Fflow: female flowering, ASI: anthesis silking interval, SC: stomatal conductance, Biomass_flow: plant biomass at flowering, Biomass_mat: plant biomass at maturity, GY: grain yield, HI: harvest index, TKW: thousand kernel weight, KPE: kernels per ear, *; **: significant at p < 0.05; 0.01.

3.2.4.3 Correlation of RDD_Index with stomatal conductance in the field

The trait RDD predominantly captures root DNA of live cells. Therefore, by the formation of the ratio of RDD under drought treatment to RDD under well-watered conditions, impact of drought on cell viability and number of cells can be assessed. In order to test the resulting ratio RDD_Index, it was formed for both depths 10 and 40 from adjusted RDD means. According to a t-test, RDD_Index showed a significantly lower mean ($p < 0.01$, df = 32) in depth 40 than in depth 10 (Table 15). Soil water potential in depth 10 was very low before and during flowering, when soil cores were taken (Figure 2, chapter 2.7). But RDD reduction was stronger in depth 40 (-1.8 bar water potential), where the soil water potential was higher than in depth 10 (-10 bar water potential).

Table 15 Means of 19 genotypes, associated standard errors (s.e.), minimum (Min.) and maximum (Max.) for the trait root DNA density index (RDD_index) in depths 10 and 40 in the field experiment.

Depth	mean	(±) s.e.	Min.	Max.
10	0.68	0.06	0.25	1.20
40	0.36	0.04	0.11	0.78

The impact of RDD_Index on transpiration was assessed by calculating correlations with SC from the field experiment. In depth 40 and both treatments well-watered and drought, there was significant positive correlation of RDD_Index and SC (Figure 18), but not in depth 10 (data not shown).
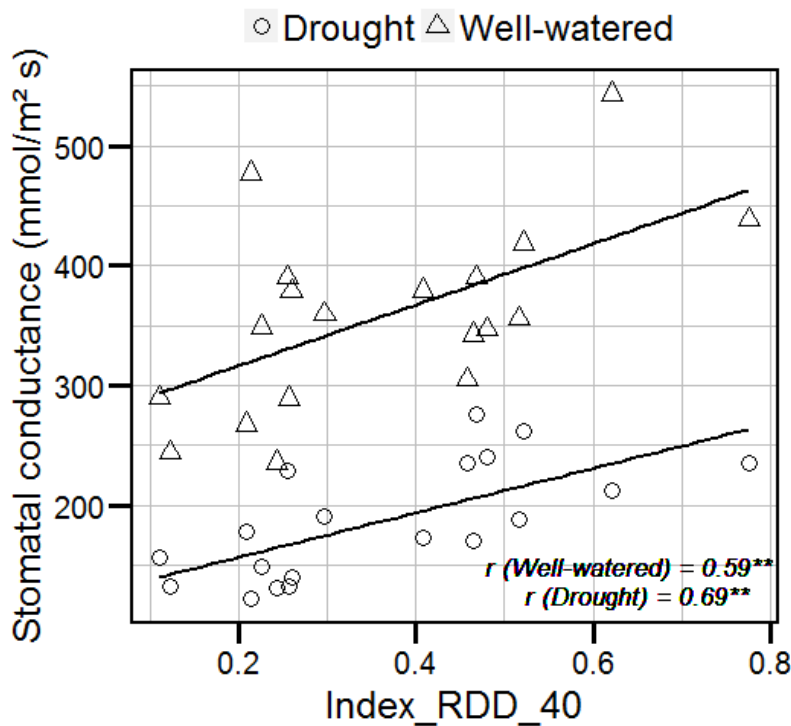
Figure 18 Correlation of root DNA density index in depth 40 and stomatal conductance based on 19 maize genotypes in well-watered and drought treatment in the field. Correlation coefficients were calculated separately for treatments well-watered control (triangles) and drought (circles). **: significant at p < 0.01

3.2.5 Comparison of RDD to commonly assessed root traits for expenditure in time and money in the field

An important aspect regarding application of traits in the field are time and financial expenses related to trait data generation. In this regard, the new trait RDD was compared to the commonly assessed root traits RLD and RMD (Table 16).

Table 16 Comparison of trait root DNA density (RDD) with root length density (RLD) and root mass density (RMD) for expenditure in time.

Work-step [min/sample]	RLD	RMD	RDD
Sampling and drying	8.8	8.8	8.8
Milling	-	-	4.3
Washing and picking	15.0	15.0	-
DNA extraction	-	-	6.9
Scanning	10.0	-	-
qPCR	-	-	1.5
Drying and weighing	-	2.0	-
Total	**33.8**	**25.8**	**21.5**

Time effort is given in minutes per sample and was calculated from 1140 samples and average batch-wise or daily sample throughput and invested working hours for each respective step.

The comparison for time expenses revealed that RDD saved 12.3 and 4.3 min per sample compared to RLD and RMD, respectively. Mainly responsible for the time savings was the fact, that washing and picking procedures became no longer necessary for RDD. Washing was carried out manually to a large extent, and required thorough handling of different sieves and precise picking of live roots which belonged to the investigated crop maize. Hence, it was the major task in terms of time investment. Additionally, regarding the comparison of RDD and RMD to RLD, omitting the scanning step, which also requires time-consuming root handling with forceps, saved further working time. Cutting of these two essential steps led to greater time savings than incorporation of the new steps milling, DNA extraction and qPCR for RDD. The comparison shows that time expenses for RDD compared to RLD and RMD were reduced.

Costs are composed of expenses for material and labor. The final configuration of the newly developed method required 7.89 Euro for material per sample. Primarily the DNA extraction Spin Kit (4.44 Euro) and the qPCR reaction chemistry (2.95 Euro) accounted for the major part. The material expenses for RLD and RMD were relatively low, with 0.5 Euro per sample for sampling and drying, provided that reusable utensils such as aluminum trays and plastic bags are available. Ultimately, financial expenses regarding consumable material were higher for RDD compared to RLD and RMD. However, when expenses for labor are also considered, RDD saves money compared to commonly assessed root traits RLD and RMD, whereby degree of the saving depends on hourly wages for workforces.

4 Discussion

In the presented study, a DNA-based root quantification method for maize was developed, enabling high-throughput phenotyping of roots on mature plants in the field. The new method was validated in field and greenhouse trials. The application allowed the assessment of root DNA density (RDD) in 19 maize inbred lines with a small sampling system during the growth period. Several hundred samples per day were taken and 1392 samples were processed in a workflow which incorporates sub-sampling and thus facilitates labor-efficient DNA-extraction and qPCR reaction. The trait RDD, which gives an estimate of living maize root cells in the soil, showed substantial genotypic variance, intermediate to high heritabilities across the treatments well-watered and drought, and a strong correlation across different phenotyping platforms. In order to examine the potential of RDD to predict developmental, physiological and reproductive traits, correlations were calculated between RDD and shoot traits. RDD in topsoil (10-20 cm depth) was negatively correlated with harvest index and kernels per ear under drought influence. Comparison for expenditure for time and money between RDD, RLD and RMD revealed that RDD leads to time savings per sample and that moderate costs for RDD allow large scale application in research and plant breeding.

4.1 Development of a new, DNA-based method to capture root proliferation of maize in field environments

Precise time- and work-efficient root phenotyping methods for field environments are in great demand, since traditional methods often lack resolution and throughput to be used in plant breeding (Zhu et al. 2011). DNA-based root phenotyping holds great promise to widen the phenotyping bottleneck for the characterization of root system architecture in field environments (Furbank and Tester 2011; Huang et al. 2013; McKay et al. 2008). However, to date, no complete workflow from sampling to the point of data acquisition and phenotypic analysis of RDD of any crop species has been published. Therefore, a DNA-based root quantification method in maize for field environments was developed.

4.1.1 Optimization of field sampling and sample handling

An important parameter for optimized field sampling of roots is the sample throughput, which is dependent on the choice of the sampling equipment, the core diameter and the core number to be taken (Böhm 1979; Gregory 2006; Zhu et al. 2011). Using the core sampling system with foil lining and electrical percussion hammer in the presented study, the new method to assess RDD enabled the acquisition of hundreds of samples per day. High-throughput has often been mentioned in literature, but seldom defined in numbers. One reason might be the dependency of this definition from the trait to be examined and from the target environment. In general, screening of thousands of single plants per year in automatized fashion under controlled conditions is considered high-throughput (Fiorani and Schurr 2013), but high-throughput, especially in field environments, often goes along with lower precision (Furbank and Tester 2011). For shoot traits in field environments, increased throughput has recently been enabled by modern techniques like spectroscopy and remote sensing (Palanichamy and Cobb 2015). Root traits in field environments are more difficult to assess, therefore phenotyping the rootstock of 80 plants per day can be regarded as high-throughput (Colombi et al. 2015). In this context, the new method to assess RDD, enabling to obtain hundreds of samples per day, while conferring high precision expressed in high heritabilities, can be regarded as high-throughput.

When investigating plots of crop plants in field trials, at least five boreholes with a 7 cm diameter auger per plot or experimental unit were recommended (Böhm 1979). As a general rule for soil core sampling, the lower the core diameter, the more samples need to be taken to reduce the sampling error (Böhm 1979), and a higher number of replicated samples lowers the variability of measurements per experimental unit (Canode et al. 1977). In case of the utilized core sampling system in the presented study, five cores per plot were taken and the core diameters were 4.4 cm which resulted in a sampled soil volume of 1520 cm³ per 1 m core. Gregory (2006) argued that approx. 15 to 20 samples of 10 cm diameter would be necessary to detect treatment or genotype differences at 10 % significance level when using traditional root coring traits like RLD or RMD. Compared to these reports, the presented method shows an improvement because five samples of 4.4 cm diameter and 10 cm length were enough to distinguish genotypes and treatments in RDD at 5 % significance level in all but one case (depth 40 under drought). The observation of reduced precision in depth 40 under drought could be expected, especially for deeper

soil horizons where greater variability in root growth, and lower rooting densities occur (Böhm 1979; Levillain et al. 2011). The optimum number of cores confers sufficient precision, but likewise reasonable core or sample number is needed to keep working and processing effort as low as possible. An increase in core and thus sample number would be beneficial in depth 40 and in deeper soil horizons, where less maize roots can be expected than in topsoil horizons. Following these criteria from literature and empirical data from this study, the recommendation for the number of cores to sample would be at least five or more cores in depth 40 under drought and in deeper soil horizons. In depth 10 however, five cores were sufficient for data generation, and the potential to use less cores when investigating topsoil horizons with more residing roots could be tested in order to facilitate greater throughput.

The utilized core sampling system in the presented study allowed sampling of undisturbed soil cylinders through an integrated polyethylene foil catching system. The spatial fixation of soil structure was a crucial prerequisite for true separation of different soil horizons and thus low introduced variation from the sampling procedure. In recent studies of Wasson et al. (2014) and Watt et al. (2013), soil samples for root assessment in the field were taken by a tractor-mounted, hydraulic system. Subsequently, the samples had to be removed from the core and soil increments were separated manually. The manual separation of unfixed soil increments, in order to gain the final samples, demands careful and more time-consuming processing of the samples, being more error prone in the end. The additional error source could contribute to the experimental error variance and consequently lead to lower repeatabilities and diminish the ability to detect real genotypic differences at an error probability of 0.05. Accordingly, Wasson et al. (2014) were not able to find significant genotypic variance for RLD in the field when comparing 40 wheat genotypes. Ultimately, the used coring system with foil lining in this study reduced the additional error source from manual sample separation.

In the presented study, due to the small configuration of the sampling equipment, the samples could be taken at the end of the vegetative growth period without entering the field trial with heavy machinery like a tractor. Also, time series would be possible to monitor dynamic processes of root growth, for example related to the impact of nutrient or water deficiency. When soil cores for root phenotyping were taken in the field with tractor-mounted systems (Wasson et al. 2014; Watt et al. 2013), they were acquired at specific

time points. It is likely that only one coring time point at relatively narrow study sites is possible, due to the strong mechanical impact of the tractor on soil and aboveground plant parts. In other studies, cores with a maximum depth of 60 cm and diameter of 25 to 58 mm were taken by using a hammer or hand driven devices (Bithell et al. 2014; Huang et al. 2013; Shashidhar et al. 2012; Watt et al. 2013). Use of manual devices allows flexibility for plant growth stage and sampling in a given experimental design without strong impact on the investigated plant population and soil structure of the location, but lowers throughput and sampling depth, especially when soil is considerably dry or stony. Moreover, these sampling procedures involve cleaning of the coring equipment following each single sample (Bithell et al. 2014), which could be avoided in the new method due to the foil lining. Consequently, the sampling system used here combines flexibility with precise and time-efficient sampling, due to its small dimensions and the foil lining system which allows cross-contamination free working without laborious cleaning steps. However, also with the new method, an impact of repeated coring throughout the growth season on plant performance is possible and has to be taken into account.

4.1.2 Homogenization procedure

Milling of the dried soil samples from up to 400 g weight in the present study enabled the generation of perfectly homogenized samples and ensured accurate sub-sampling of the whole sample. Sub-sampling was possible with a relatively small standard error (approximately 5 % of the mean) in relation to the genotypic variation of RDD observed in the field (range of 390-1200 %) and greenhouse (range of 260-2820 %). Habib (1988) demonstrated varying standard deviations from 5 to 15 % when sub-sampling of root pieces for the estimation of total RLD was carried out, and concluded this could be considered accurate in estimation of root length. Thus, sub-sampling with the presented method would allow sufficient resolution to find significant genotypic variation and to distinguish single maize genotypes.

Direct extraction of DNA from large soil samples is up to now only possible using the commercially available DNA extraction Service from SARDI in Australia, that processes maximum sample fresh weights of 500 g (Ophel-Keller et al. 2008). Handling hundreds of 500 g soil samples for DNA extraction would require specialized, custom-made equipment of industrial extent, and could not be performed by average research laboratories. That is

why the majority of published research aims at DNA extraction from only a few hundred milligrams of soil (Bainard et al. 2010; Berthelet et al. 1996; Haling et al. 2011; Miller 2001; Purdy et al. 1996; Sagova-Mareckova et al. 2008; Töwe et al. 2011) or a few grams (Arbeli and Fuentes 2007; LaMontagne et al. 2002; Zhou et al. 1996) up to 10 g (Haling et al. 2011). Processing of large main samples with the possibility of sub-sampling facilitates time-efficient handling of DNA extraction and qPCR quantification, and hence lower costs, since single replicate samples of the same kind are condensed as one main sample beforehand. The larger the main sample can be, the higher is the capacity for replicate sample pooling and thus for the reduction of spatial sample variability or random bias (Huang et al. 2013; Spencer and Spencer 2004).

Before sub-sampling was possible, the soil samples were dried in order to conserve them and prepare the adjacent milling step. Also in other studies, where the workflow was provided by SARDI, soil samples were dried prior to RDD determination (Huang et al. 2013; McKay et al. 2008; Riley et al. 2009). However, as evident from an experiment, soil samples can lose substantial amounts of DNA during the drying process. In the conducted experiment, up to 14-fold DNA loss became evident from drying soil samples in an oven with no exhaust air function, as they are often available in laboratories. In the literature, a slight degradation of DNA due to drying, evident on agarose gels, was reported from Bainard et al. (2010), although the authors stated drying only marginally affected DNA concentration after extraction from plant material. Increased exhaust air during the drying process led to a reduction in DNA degradation, showing that a dehydration oven with exhaust air function is essential to avoid DNA degradation, which is governed by nuclease enzymes (Lee et al. 2010). After the drying, milling homogenized the dried soil samples to flour-like consistency, enabling sub-sampling in the new method. The soil samples were milled without any influence on maize root DNA yield or co-extraction of inhibitory substances leading to inhibition in following qPCR reactions.

Using direct DNA extraction from 500 g soil samples, like SARDI provides, dismisses the need for the homogenization step by milling, and is supposed to save time (Haling et al. 2011). However, there is no data available describing the time effort for SARDI to extract DNA from soil samples, which would enable a comparison with the new method, developed in the presented study. In contrary, sub-sampling from the milled main samples in this

study allowed very efficient work in the laboratory. Thus, sub-sampling is an essential step to remarkably reduce the amount of sample to extract DNA from.

4.1.3 Development of a robust DNA extraction procedure

DNA extraction from soil samples is of special interest, since it should provide clean and ready-to-use DNA. Resulting DNA extracts need to be free from inhibitory substances, which could inhibit downstream enzymatic reactions in the qPCR. For soil samples, primarily humic and phenolic compounds are reported to inhibit PCR reaction (Wilson 1997). In the present study, none of the four modified and tested DNA extraction procedures from literature (Khan et al. 2007; Miller et al. 1999; Yeates et al. 1997; Zhou et al. 1996) consistently resulted in inhibitor free DNA crude extracts.

Using the data from spectrophotometer measurements, a significant influence of the used soil type on humic acid co-extraction was not detected. In the examination of extracts obtained with the *Yeates* method and the Spin Kit in qPCR however, inhibition was observed only for the ROGG soil, and not for LP soil. These observations indicate that the spectrophotometer measurements have only limited value in predicting the contamination of extracts and enzymatic inhibition in qPCR reactions. These results confirm that DNA extraction from soil is often complicated by co-extraction of inhibitory substances like humic acids, which can be dependent on soil mineral or organic components (Knauth et al. 2013). Here, the most humic soil ROGG showed the strongest co-extraction of humic acids and inhibition in qPCR reaction. Tested DNA extraction methods from literature with Isopropyl alcohol in the precipitation step often led to higher humic acid content in the crude extracts compared to PEG precipitation, and higher DNA yield. These observations confirmed literature reports, where PEG precipitation was described to reduce humic acid content in crude extracts from difficult environmental samples (Purdy et al. 1996), but led to lower DNA yield (Krsek and Wellington 1999; LaMontagne et al. 2002). Although removing significant amounts of humic acids from the DNA crude extracts, using PEG for precipitation led to substantial decrease in DNA yield, excluding this precipitation method from the incorporation in the final method.

Ultimately, the *Yeates* method (Yeates et al. 1997) provided the lowest co-extraction of humic acids, highest DNA purity and intermediate to high DNA yield, when compared to the other three tested methods. *Yeates* was therefore chosen as best manual DNA extraction

method and subsequently compared to the commercial Spin Kit from MPBio in a qPCR experiment, since the Spin Kit showed promising results in first evaluation extractions.

With this comparison it became evident that the *Yeates* extraction led to higher molecular weight DNA and higher yield compared to the Spin Kit. These differences might be due to the fact, that Phenol-Chloroform based DNA extraction methods have no limit in DNA binding capacity, as it is the case for column-based DNA binding to membranes (Knauth et al. 2013; Mülhardt 2013). Moreover, additional bead-beating with the Spin Kit method could have led to increased DNA shearing (Robe et al. 2003). Likely for these reasons, *Yeates* was superior for the parameter DNA yield and molecular weight. However, regarding co-extraction of inhibitory substances, the qPCR experiment with the SPUD inhibition testing assay from Nolan et al. (2006b) indicated the presence of humic acids for the *Yeates* extraction.

Due to the superiority of the commercially available Spin Kit in reducing co-extraction of inhibitory substances in our experiments, which is the most crucial requirement for reproducible DNA quantification, the final workflow takes hold of this option. In literature, the Spin Kit (Fast DNA Spin Kit for Soil) was also superior when compared to 12 different DNA extraction procedures (Ettenauer et al. 2012). Here, using the Spin Kit reduced the resulting DNA concentration compared to the published method *Yeates*, yet this solution was the best possible trade-off between DNA yield and co-extraction of inhibitory substances. Inhibition could be avoided, and a very sensitive qPCR assay, which was specifically developed to quantify small amounts of maize roots from environmental samples, is capable of quantifying tiny amounts of DNA.

As an alternative to the rather expensive commercial Spin Kit, also manual extraction procedures like the *Yeates* method could be used in the workflow. Depending on soil material and precipitation method, this option would need an additional column purification step to reduce enzymatic inhibition through humic acid contamination. The results of the DNA purification underlined the potential to decrease humic acid content, but also showed that this improvement occurred at the expense of DNA yield. Noticeably, the measured DNA yield decrease from the starting crude DNA sample to the following elutes was potentially exaggerated. The observed sharp decrease in DNA content was likely due to the fact that spectrophotometric measurements of dark crude extracts, such as the very first sample in this experiment, often lead to overestimation of contained DNA. Already the

first elute was much brighter in color, which led to contrasting results for the spectrophotometric measurements of this sample compared to the starting sample. Arbeli and Fuentes (2007) observed substantial reduction in humic acid content using this purification method, and stated that DNA yield was suitable for PCR amplification. Even so, in the presented study, this method and using the first elute can only be recommended if low amounts of DNA are sufficient for downstream applications. An improvement of DNA yield might be achieved by repeated loading of the columns with the same crude DNA extracts.

4.1.3.1 Evaluation of sample-specific DNA extraction efficiencies

In order to guarantee comparability of different tested soil samples, DNA within a study needs to be extracted and purified in a consistent and reproducible way. Although the DNA extraction procedure with the commercial Spin Kit is standardized per se, especially working with soil samples can lead to differences in reaction efficiency due to the varying quality of supplied chemicals in different commercial kit package units or sample specific differences in environmental compounds residing in soil, like heavy metals or large proportion of constituents like clay (Robe et al. 2003; Wilson 1997). Haling et al. (2011) tested three different methods to extract *Phalaris aquatica* L. root DNA from soil samples, including the DNA testing service from SARDI, and found that an internal standard was necessary to account for variability between different samples for both a phenol-based extraction and the MPBio Powersoil kit, but not for the SARDI procedure.

Therefore, in this study, a monitoring system (HIS) for the sample-specific DNA extraction efficiency, by means of an internal standard, was developed. The spiking of defined amounts of *Arabidopsis* plant powder into the samples prior to the extraction process enabled monitoring of the DNA extraction efficiency of any sample, which could be used to normalize the extracted maize root DNA for the inherent DNA extraction efficiency. Haling et al. (2011) used milled lucerne (*Medicago sativa* L.) seeds to account for extraction efficiency, but in literature also use of *E.coli* cells (Trochimchuk et al. 2003) or fragmented plasmid DNA (Devonshire et al. 2014) was reported. The advantage of using a cloning vector as assay target in qPCR quantification of the transgenic *Arabidopsis* plants was an increased specificity. As compared to any other system using material which could be present in natural environments, the system utilizing transgenic plants excludes the risk of amplification of non-target DNA from the total DNA mixture. Disadvantage of the system

using transgenic *Arabidopsis* can be that growing of transgenic *Arabidopsis* and seed propagation requires S1 facilities. However, this assay is interchangeable with assays targeting other genomic regions without violation of the monitoring principle. Accordingly, a range of PCR amplifiable material can be used as spike for efficiency control.

The mean observed DNA extraction efficiency for 1140 samples in the field experiment was 1.19. Since 10 *Arabidopsis* samples were used a priori to establish a reference of input *Arabidopsis* powder to output DNA yield, and this relation was used in the data processing workflow (step 2, chapter 2.5) the observed extraction efficiencies for the 1140 samples relate to this reference of 10 samples. That means the extraction of spiked *Arabidopsis* powder in the 1140 samples yielded 19 % more DNA on average as compared to the 10 *Arabidopsis* samples used for the calibration of the method. The reason for the slightly enhanced extraction efficiency in the field experiment compared to the 10 *Arabidopsis* reference samples might be differences in extraction performance of the kit or differences from qPCR run to run. Run-to-run differences could occur since the 10 *Arabidopsis* samples were run on a separate 96-well plate in a different run, which was not normalized to the field experiment by an IPC. Hence, these results emphasize the need of an IPC in order to render samples of the same experiment comparable. A correction step to normalize run to run variation using an IPC is implemented in the data analysis for the calculation of RDD for field and greenhouse experiments. Importantly, although the 10 *Arabidopsis* reference samples were not normalized to the field or greenhouse experiment, which led to extraction efficiencies greater than 1.0, the samples within both experiments are comparable to each other when related to the same reference. Efficiency values of single samples ranged from 0.64- to 2.70-fold and confirmed the need for an efficiency correction. Mumy and Findlay (2004) also found that the performance of kits with regard to DNA recovery efficiency varies on a sample by sample basis.

In order to make all single 96-well plates in the qPCR comparable, each single plate contained an IPC, an identical DNA sample analyzed in each run, which was related to the IPC mean of all plates. In future work, with this approach it could be important to consider that the IPC is based on the ITS2 assay. Variation in IPC performance is thus directly related to the ITS2 quantification, but not necessarily to the HIS assay performance, when variation is coming from ITS2 related qPCR primer and probe chemistry or fluorescence measurements. Thus, the calibration of the HIS-assay Cq values for inter-run variation in

qPCR based on the IPC, which in turn is run with the ITS2 assay, needs to be carried out with care. However, bad quality of ITS2 reaction chemistry as a cause for the observed inter-run variation between plate 1 and subsequent qPCR plates in the presented study can be excluded, since primer and probes for ITS2 from a large aliquot were used for the first and all subsequent 96-well plates.

4.1.4 Development of specific, sensitive and robust qPCR assays

A robust and stable qPCR assay is crucial for high-throughput quantification of DNA. qPCR assays utilizing Taqman probes have advantages in stability, specificity and handling compared to other real-time PCR detection methods as for example the SYBR Green approach (Lim et al. 2011; Nolan et al. 2006a; Varkonyi-Gasic et al. 2007). Hence, all qPCR assays were developed using Taqman probes.

Specificity and sensitivity of the maize root quantification assay needs to be high to avoid quantification of other organisms and to have the ability to detect even the smallest amounts of roots, like root hairs or marginal amounts of root tissue in deeper soil horizons. To ensure these two crucial factors, the assay was developed using the internal transcribed spacer region, which is part of the ribosomal DNA (Buckler and Holtsford 1996a). Ribosomal DNA shows patterns of concerted evolution, which are used to distinguish different taxa (Bilodeau et al. 2007; Buckler and Holtsford 1996a; Spencer and Spencer 2004), and occurs in multiple copies per genome (Huang et al. 2013; Prokopowich et al. 2003; Spencer and Spencer 2004). The specificity of the assay was confirmed by testing it against 17 weeds which were collected at field locations, and thus likely to appear in the soil samples tested in the presented study. The multiple copies of the ITS2 region used in this study gave a high sensitivity (Bilodeau et al. 2007), with the assay being able to quantify 0.1 pg of DNA by default, corresponding only to a fraction of the molecular weight of the haploid maize genome (~2.6 pg) (Arumuganathan and Earle 1991). The sensitivity is comparable to reported results where ITS2 was used to quantify *Trifolium subterraneum* L. and *Lolium* spp. root DNA (Riley et al. 2009). The higher sensitivity of these assays compared to single copy assays is a result of multiple target regions per single genome when using such a target. Consequently, when assuming similar DNA amount input in the qPCR reaction, the assay with the high copy number target will show an earlier quantification signal and is thus conferring higher sensitivity.

4.1.4.1 Determination of genotype specific ITS2 copy number

Huang et al. (2013) reported genotypic differences for ITS2 copy number in wheat, and used this information to correct RDD values for different wheat breeding lines. Moreover, also in maize genotypic differences for the ITS2 copy number were reported (Phillips et al. 1988). Thus, a climate chamber experiment was conducted to determine the ITS2 copy number of all used 25 maize lines in field and greenhouse experiments. The experiment revealed significant genotypic variation for the ITS2 copy number in the tested maize lines and copy numbers (3400 to 14900 copies) in a similar range as described in wheat (2900 to 15000 copies) (Huang et al. 2013) and maize (2500 to 24000 copies) (Phillips et al. 1988). Consequently, in order to correct for ITS2 copy number differences to allow genotype comparison in the field and greenhouse experiments, correction factors were calculated according to Huang et al. (2013).

Twenty one of the screened 25 maize lines were part of an introgression library including both parents DP and RP. The ITS2 copy number of 17 ILs was determined to be lower than that of the parents of the IL library. These observations were not expected assuming Mendelian inheritance. The IL was established through several backcrosses of the F_1 offspring with the DP, and six inbreeding cycles (Gresset 2014). One possible reason for the observed reduced ITS2 copy number of most ILs could be that diminishing mechanisms were acting during the crosses from parents to the offspring. The 17S, 5.8S and 26S ribosomal DNA in maize is located in the nucleolus organizer region on chromosome 6 (Buckler and Holtsford 1996a). Several mechanisms potentially increase or decrease the number of repeated rDNA elements per genome. Suggested mechanisms for quantitative modulation of repetitive DNA are unequal homologous recombination and illegitimate recombination. These mechanisms are the main reason of redundancy variation in repeated elements and responsible for a decrease of DNA content in the nucleus (Ceccarelli et al. 2011).

The IL was established over several generations, and through the initial crossing and the repeated backcrossing to the recurrent parent, progenies or final ILs and sub_ILs were exposed to differing growth conditions than present in the initial environments where RP and DP were established or bred. Changes in nuclear DNA, according to the term plant genome plasticity, are means to optimize a given genotype for seed germination and plant development at given temperatures, altitudes or latitudes (Bennett 1976; Ceccarelli et al.

2011; Smarda and Bures 2010). Often, repetitive DNA components are involved in such adaptive processes. Thus, potential changes in transcription of rDNA genes governed by a decrease in ITS2 segment copy numbers would be possible. These insights arise from the fact that genotypic changes can occur as direct response to the environment and in short time period of one generation (Ceccarelli et al. 2011). Nevertheless, until further experimental evidence, these explanations remain speculative.

In general, there is a trade-off relationship between effort (climate chamber experiment) and sensitivity to quantify tiny amounts of root DNA in soil. Taking reported RLD values as basis, root DNA concentrations in the field, especially in deep soil horizons, can be fairly low (Gao et al. 2010), yet the ability of genotypes to root in deeper soil strata was reported to be associated with enhanced drought tolerance or nitrogen acquisition (Lynch 2013; Lynch and Wojciechowski 2015; Trachsel et al. 2013). Thus, assessment of roots in deep soil is valuable, but demands sensitive quantification which could be guaranteed using ITS2 as target. As alternative, an assay using a fixed copy number gene could be used for experiments, where sufficiently high sensitivity in qPCR reaction is guaranteed due to higher root DNA concentrations in the conducted experiment. Here, the developed IVR assay could be used, for example in pot experiments with high root DNA concentrations, as indicated by the greenhouse experiment or in field trials where root-dense topsoil is investigated. That way, usage of the IVR assay would reduce effort and potential introduction of unwanted variation, but still confer sufficient sensitivity for the respective experiment. Ultimately, using a single copy genomic target could potentially further improve the observed intermediate to high heritabilities for RDD. The use of a single copy genomic target should be investigated carefully when planning an experiment for RDD determination.

Finally, the assay's ability to quantify maize roots which were spiked to soil was examined and confirmed with an experiment. Quantification of root DNA through the ITS2 assay was confirmed for the concentration range which was present in field and greenhouse.

4.2 Validation of the new, DNA-based method to capture root proliferation of maize in field environments

4.2.1 Comparison of RDD to commonly assessed root traits RMD and RLD for genotypic variation and heritability in the field

In the presented study, RDD in the field ranged from 1.8 to 222 ng cm^{-3} independent of the treatments well-watered or drought and the depths. The values observed in the field showed similar ranges as RDD from field trials reported for wheat in 0-10 cm depth (60 to 240 ng g^{-1} soil), barley in 0-60 cm depth (*Hordeum vulgare* L.; 0.2 to 20 ng g^{-1} soil), phalaris in 10-40 cm depth (1 to 80 ng cm^{-3} soil), weeping grass in 10-40 cm depth (*Microlaena stipoides* B.; 6 to 80 ng cm^{-3} soil) and tall wheatgrass in 10-40 cm depth (*Thinopyrum ponticum* (Z.W.Liu & R.C.Wang); 1 to 80 ng cm^{-3} soil) (Haling et al. 2012; Huang et al. 2013; McKay et al. 2008). Slightly enhanced values for maize RDD in this study compared to barley or weeping grass could result from differences in investigated species and therein cultivar, plant age and sampling position or watering regime or even sample storage conditions (Bainard et al. 2010; Haling et al. 2011; Huang et al. 2013; McKay et al. 2008; Riley et al. 2009).

Natural variation is one of the crucial requirements for breeding progress. The genotypic standard deviation, which is determined by the choice of the genetic material, is multiplied by values for selection intensity and the square root of the heritability to determine selection progress (Becker 2011). Thus, in order to use a trait in selection it needs a significant and possibly large genotypic variation. RDD showed significant genotypic variance in depth 10 in both treatments well-watered and drought, and in depth 40 in the well-watered control, but not under drought. The significance of the genotypic variance was similar for RMD, whereas RLD did not show significant genotypic variance in depth 40 at all. The only study investigating genotypic variation for RDD so far was conducted in wheat (Huang et al. 2013). The authors also reported significant genotypic variance for RDD in the topsoil increment (0-10 cm) in several field locations. The present study indicates that also in maize, RDD is under genetic control. Noteworthy, RDD did not show significant genotypic variance in depth 40 under drought, whereas the error variance in this depth and treatment was more than 7-fold larger than the genotypic variance. Variance which cannot

be explained by all the parameters of the statistical model is estimated as error variance. Reasons for an increased error variance can be any environmental influence for which the statistical model does not account, or variation introduced by the method itself. The utilized RCB design for example does not account for small-scale differences in soil-bulk density which could in turn lead to differences in root penetration and proliferation. As also indicated by analysis of the standard errors resulting from the corresponding number of cores taken per plot, an increase in accuracy could be achieved by taking more cores, especially for depth 40 and the drought treatment. The reason might be that root estimation gets more error prone in soil horizons where only little roots reside. Intensifying the sampling in terms of sampled volume of such horizons would most likely lead to less error variance, and thus better resolution of genotypic values and significant genotypic variance (Levillain et al. 2011; Ping et al. 2010). The present study suggests that sampling more cores for deeper soil horizons holds promise to increase resolution, and future work should test this option.

Furthermore, considerably high heritability estimates of 0.90 and 0.88 were found for RDD in depth 10 and 40, respectively, when calculated across treatments well-watered and drought in the field. Intermediate to high heritabilities for five field locations, ranging from 0.55 to 0.90, were also reported in wheat (Huang et al. 2013). Hence, the presented study shows for the first time the genetic determination of RDD in maize, as it was shown before in wheat. On the basis of the significant genotypic variance and high heritabilities, the data of this study underline the potential of the trait RDD to distinguish genotypes for their root proliferation in the field. The commonly used trait RLD is more error prone than RDD due to several reasons, including washing errors, dead roots or roots from different species (McKay et al. 2008). In a recent study, root researchers were not successful to significantly distinguish 40 wheat genotypes in field trials using total RLD from cores (Wasson et al. 2014). They stated that carryover during the washing process could have increased the experimental error. Moreover, they specified that the sensitivity of this approach, measuring roots in different soil horizons with RLD, needs to be improved in order to capture all physiologically relevant root lengths. That is a strong sign that improved phenotyping methodologies are needed, and that RDD has the potential to fill the gap.

In the presented study, RDD in the field was generally reduced in depth 40 and under drought as opposed to depth 10 and the well-watered control. Reduction under sub-

optimal water availability was also true for RMD and RLD, except for RLD in depth 40. Generally, root length and mass decreases with increasing soil depth (Gregory 2006). That was also found in numerous studies for maize (Cai et al. 2014; Grieder et al. 2014; Liedgens 1998; Nicoullaud et al. 1994; Ning et al. 2015; Trachsel et al. 2013). Most of the root biomass is located in the top 30 cm soil. Hence the distinct decrease, with at least 4.9-, 2.7- and 6-fold reduction for RDD, RLD and RMD, respectively, from depth 10 to 40, was expected. Therefore, the observations made in this study are in line with reports from the literature.

Root proliferation in most cases is reduced under drought influence (Davies and Bacon 2003). However, when common root traits RLD or RMD are considered, some studies report increased root proliferation under drought influence for cereals (Gregory 2006; Sharp and Davies 1979). Since the root system is responsible for the maintenance of water and nutrient uptake during drought episodes, it is crucial for whole plant survival and performance. That is the main reason why shoot growth of crops is much more sensitive to reduced soil water potential than root growth, and plants invest relatively more carbon in root than shoot growth, leading to larger root-to-shoot ratios under drought (Asseng et al. 1998; Kalapos et al. 1996; Sharp and Davies 1979; Sharp et al. 2004). However, the vast body of literature for annual cereals generally indicates impaired root growth upon water limitation (Sharp et al. 2004; Wu and Cisgrove 2000). One possible reason for contrasting reports seems to be that drought can have multiple and varying implications on the respective plant organs or tissues, depending on type and severity of the applied drought scenario (Tardieu 2012).

Here, in contrary to RDD in the field, RLD did not show a significant response to drought in depth 40, because of the significantly increased specific root length in the drought treatment. Specific root length under drought was 223 m g^{-1} as compared to 152 m g^{-1} in the well-watered control. These observations are in the same range as the before reported specific root length of 200 m g^{-1} for cereals (Noordwijk and Brouwer 1991). Under drought, plants decreased RMD but also lowered the average root diameter relatively stronger, leading to more root length per biomass. The enhanced proliferation of roots with small diameter under drought influence is a well-known phenomenon to increase carbon use efficiency under stress (Eissenstat 1992). Very fine roots are cheap to build and are able to penetrate smaller soil pores (Hutchings and John 2003; Robinson et al. 2003). That way the

plants can increase soil exploration and thus potential water uptake from a given mass of roots (Hutchings and John 2003).

4.2.2 Genotypic variation and heritability of RDD in the greenhouse

The maximum RDD values acquired from the greenhouse pots (85 to 6255 ng g^{-1} soil) were approximately 80-fold increased, compared to previously reported RDD values (40 to 80 ng cm^{-3}) for pot experiments in wheat (Huang et al. 2013; Steinemann et al. 2015). There are several reasons likely to contribute to higher RDD values in maize in the presented pot experiment compared to literature for wheat, including species, plant age, soil volume, soil bulk density or sowing density. In the following, these reasons, as well as the differences between RDD taken from pots in the greenhouse and in the field (1.8 to 222 ng cm^{-3}) are discussed.

The difference in species could itself contribute to an increased RDD in young maize plants as compared to older wheat plants. Work done by McKay et al. (2008) indicates a 10- to 100-fold difference in RDD between subterranean clover and lucerne in the top 20 cm soil horizon when sampled in spring. Also Haling et al. (2011) identified a more than 5-fold difference in RDD between three grass species. Consequently, using maize as species could also have led to an increase in RDD, yet there are no comparative studies for maize so far.

The sampling time point of the greenhouse experiment was 36 DAS, when five to seven leaves were fully emerged and the plants were in the midst of the vegetative growth stage. In contrary, other studies conducted so far, using pot experiments, sampled around flowering time (Huang et al. 2013; Steinemann et al. 2015). It is known that root proliferation in cereals rapidly ceases at the beginning of the generative growth phase, with often remarkable reduction of root growth in favor of carbon investment in grain towards generative growth (Gregory 2006; Trachsel et al. 2013). In other reports, RDD from *Lolium perenne* L. and *Trifolium subterraneum* L. behaved similarly, showing a decline of RDD with increased root age (Riley et al. 2009). Using phalaris plants, Haling et al. (2011) reported a reduction of RDD per unit root mass of approximately 90 % when comparing 28 day old plants to 70 day old plants. That difference in days is similar to the difference between the 36 DAS when the maize plants in this study in the greenhouse were sampled and the number of DAS (approximately 80) until wheat flowered in the pots of the studies to be compared (Huang et al. 2013; Izanloo et al. 2008; Steinemann et al. 2015). Thus, a 10-fold

increased RDD content in this study in pots could hypothetically be explained, when ignoring species boundaries, by differences in plant age at sampling. That assumption is supported by the results of the present study, where the mature plants in the field yielded 5- to 24-fold and 53- to 158-fold smaller RDD values in well-watered and drought treatment, respectively, than the 36 day old maize plants in the greenhouse experiment.

High soil bulk density is known to negatively influence root proliferation (Bingham and Bengough 2003; Haling et al. 2014; Whitmore and Whalley 2009). Generally, soil bulk density of 1.1 g cm^{-3} can be regarded non-restrictive, 1.4 g cm^{-3} indicates rather high soil bulk density with possible negative effects on root growth (Bingham and Bengough 2003). For example, an increase in soil bulk density from 1.2 to 1.7 g cm^{-3} led to a reduction of 80 % in total root length in barley (Haling et al. 2014). Manually filled pots used for greenhouse experiments can show lower soil bulk densities due to the coarse structure of most potting substrates. Thus, the relatively low soil bulk density of around 1 g cm^{-3} (deduced from pot volume and used mass of the potting mix) in pots in the presented greenhouse experiment could have allowed more vigorous root growth compared to the field. This assumption is based on the fact that root elongation rate shares an inverse relationship with soil mechanical resistance (Bengough and Mullins 1990). However, in Steinemann et al. (2015), where RDD was measured from wheat in pots after flowering, soil bulk density was comparable to the presented study, yet the investigated plant species differed. In other pot studies, information about soil bulk densities is lacking. Soil bulk densities in the field for the sampled depths 10 and 40 were 1.45 and 1.57 g cm^{-3}, respectively. Considering the relative high clay content of the field soil, these values indicate rather high soil bulk densities, which are likely to restrict root growth. Thus, differences in RDD between pots and field are probably affected by distinct differences in soil bulk densities.

Also planting densities influence the occurrence and properties of roots in the soil (Liu et al. 2012). Calculation of the hypothetical planting density in the greenhouse pots results in 224 plants m^{-2} (when taking 0.0177 m² pot-area as basis) as opposed to the field with 18 plants m^{-2}. Riley et al. (2009) reported a positive correlation of planting density and RDD. The respective studies where RDD was measured from wheat in pots both utilized one plant per pot (Huang et al. 2013; Steinemann et al. 2015). Also, a positive interaction effect of low soil bulk density with the higher planting density in the pots is possible.

Summarizing, it is most likely that the early developmental stage of the sampled roots from pots in the greenhouse experiment, as well as the extreme planting density paired with low soil bulk density, were responsible for the generally higher levels of RDD observed in pots as compared to the literature and to the results of the field experiment in the presented study.

RDD in the greenhouse also showed significant genotypic variance in both treatments well-watered and drought, as well as in all three depths. Notably, there was significant GxT in depth 1, the topsoil horizon of the pots, but not in depth 2 and 3. Similar to the field results, heritabilities calculated across treatments were intermediate to high. These results emphasize the observations from the field and suggest that RDD in maize is under genetic control.

Regarding the influence of the treatments on RDD, an increased RDD under drought, when compared to the well-watered control, was observed in the greenhouse pots. RDD under drought in depth 1 (0-13 cm), depth 2 (14-26 cm) and depth 3 (27-40 cm) was increased by 122, 284 and 166 % in comparison to the well-watered control. When considering the impact of drought on RDD in pot experiments in literature reports, at least 64 % reduction in drought treated wheat plants in relation to sufficiently watered plants was reported by Huang et al. (2013). In another pot experiment under greenhouse conditions, drought did not have a significant influence on RDD (Steinemann et al. 2015). These are so far the only reports regarding the influence of drought on RDD. It is noteworthy that drought in the study of Huang et al. (2013) was induced around flowering time, and RDD measurements were done at the end of flowering time. In the present study, drought was induced from 20 DAS, in early vegetative growth stage, and RDD was determined from samples taken 36 DAS. Due to the general contrast in root growth behavior between vegetative and generative growth stage, it can be expected that root proliferation at vegetative growth stage 20 DAS is stronger than post flowering, when plants already change the allocation patterns from biomass to grain and root senescence increases (Cai et al. 2012; Gregory 2006). Thus, it is more likely that young plants are generally more competent to adapt to environmental influences like drought stress. The soil water potential in the greenhouse experiment was kept at -3 to -5 bar, corresponding to a mild to intermediate stress level (Bengough 2003). The phenomenon of increased fine root length and biomass in response

to induced drought stress in growth containers has been reported for *Lupinus albus* L., an herbaceous annual (Rodrigues et al. 1995). But also in monocot crops like maize, increased fine root length upon mild drought stress has been reported (Trachsel et al. 2010b) for seedlings. The direction and amplitude of changes in root growth are largely dependent on duration and intensity of the sub-optimal water availability (Davies and Bacon 2003; van der Weele et al. 2000), whereby mild stress can have positive influence on root growth, but severe stress is detrimental. Thus, plant developmental stage and imposed drought stress level might have led to an adaptive response in root proliferation in the present study, as reported by Gregory (2006) and Sharp and Davies (1979). However, since RLD was not assessed in the greenhouse experiment, one can only speculate if the plants were increasing fine root length density which in turn led to higher RDD. Information from literature is often conflicting, presumably due to the many different drought scenarios applied to different plant species, in different sets of experimental designs.

Another possible reason for enhanced RDD under drought could be of technical nature, namely the DNA degradation rate in soil. DNA degradation from root cells has been investigated in several studies (Bithell et al. 2014; McKay et al. 2008; Riley et al. 2009). When root DNA degradation was examined for dead *Lolium perenne* L. and *Trifolium subterraneum* L. roots in wet soil, decrease in RDD was quick, with a 10-fold DNA reduction by 3 to 4 days, and a 50-fold reduction by 7 days (Riley et al. 2009). However, DNA degradation in dry soil was not investigated in this study. Bithell et al. (2014) explored the possibility of different DNA degradation velocities between wet and dry soil for mango (*Mangifera indica* L.) roots. The results showed that DNA decay was significantly delayed in dry soil. Thus, a possible cause for the strong DNA retention effect could be the lack of water and resulting non-functioning of DNA degrading enzymes (Lee et al. 2010). Moreover, small pots and used potting mix create artificial conditions compared to the field, regarding for example soil constitution, organic material (Passioura 2006) or abundance and diversity of microorganisms. All those factors influence the decay of organic material in soil (Hodge et al. 2001; Tisdall and Oades 1982; van Veen and Kuikman 1990). Thus, very dry soil combined with artificial substrate and environment might conserve root DNA even longer due to reduced biological activity and throughput of organic material. That hypothesis could be tested for example with an experiment encompassing soils with

different degrees of water content and active microorganisms and measuring RDD at otherwise constant conditions.

These results have to be confirmed for different species and soil water contents, also since this is the first report on enhanced RDD under drought. Notably, when assuming an influence of technical nature, all samples were affected with the same intensity, since the repeatabilities were fairly high and the phenotypic correlations between field and greenhouse were all significant and positive.

4.2.3 Effect of genotype-environment interaction on RDD

RDD in the field was calculated in the unit pg DNA per cm³, because unlike in the greenhouse experiment, the volume was fixed and measured. In order to compare both units and phenotyping platforms, the relationship of the units pg DNA per cm³ and pg DNA per g soil was examined. Results imply that RDD from both units is synonymous, with a coefficient of determination of 0.99 in the field.

Strong correlations across the phenotyping platforms field and greenhouse suggest a uniform expression of the trait RDD, even across different environments. Putative differences in growth conditions between field and greenhouse, potentially influencing root growth, were manifold. Differences were presumably present in soil organic matter content, microbiome, soil bulk density, temperatures of rooting matrix, oxygen, depth of growth column or distribution of nutrients. The correlations between field and greenhouse for RDD underline the finding of a strong genetic component, evident from significant genotypic variances, and the reduced influence of the genotype by environment interaction effect (GxE) on phenotypic expression of RDD.

Indeed, in the presented study, there was no significant genotype by treatment (GxT) interaction effect for RDD in the field. These data indicate there was no differential response of the tested maize IL lines in RDD under two treatments of varying water availability, well-watered and drought. In general, many root traits show a considerable extent of GxE (El-Soda et al. 2014; Hebert et al. 1995; MacMillan et al. 2006), have rather complex genetic architectures (Hund et al. 2011), and tend to show enhanced phenotypic plasticity (Dorlodot de et al. 2007; Zhu et al. 2005). Differential plastic responses of root traits have been shown for example for maize crown root angles in response to nitrogen deficiency (Trachsel et al. 2013). In that study, a set of 29 maize genotypes was classified into 11 steep, 11 plastic and 7 shallow rooting genotypes, according to their response in

crown root angle to low nitrogen in soil. In this study, the possibility that the IL with a common genetic background was facilitating the low observed GxE cannot be excluded. Further examinations regarding different plant material, differences in growth conditions, and therein the response of RDD would increase the knowledge of GxE in RDD.

In contrary to RDD, RMD showed significant GxT in the field. The differences in GxT between RDD and RMD indicate differences in the interaction with the environment. GxE for root mass in varying field environments was reported by Kondo et al. (2003) for rice. The authors pointed out that, especially for root mass, genotypes responded variably to soil conditions. RDD is an estimate of live root cells (McKay et al. 2008), whereas RMD also captures dead and inactive root material. Accordingly, phenotypic correlations in the presented study have shown no constant relationship between both traits RDD and RMD across treatments and depth, further arguing for the differences in phenotypic expression in relation to environmental influences. Haling et al. (2012) pointed out that the relationship of RMD and RDD changes with variation in species, soil sampling depth and harvest date of the roots. Change of that relationship was also observed in the present study with varying depth and water supply. Thus, results in the presented study are congruent with reports from literature, and underline that calibration is needed for conversion of RDD to RMD, as it was implemented in Haling et al. (2012). It can be concluded that the correlation between both traits RDD and RMD is strongly dependent on the environment.

In contrast to the field, RDD showed significant GxT in depth 1 (0-13 cm) in the greenhouse experiment. These differences in GxT between phenotyping platforms might be explained with differential soil water content and resulting water potentials (Passioura 2006) and with differences in plant developmental stage between field and pots in the greenhouse, respectively. The greenhouse pots were filled with coarse potting substrate, and the topsoil, referring to depth 1 in the present study, presumably dried more artificially and rapid than field soil or the soil below in the pots, leading to more rapid stress induction (Passioura 1983; Ray and Sinclair 1998). Depth 2 (13-26 cm) and 3 (27-40 cm) did not show any GxT. Thus, one possibility is that the combination of the specific water stress imposed in topsoil with the used soil substrate and growing conditions led to GxT in the greenhouse. Hence, it might be an effect coming from artificial conditions in pots (Passioura 2006). Nevertheless, this result needs to be confirmed in further studies. Another reason for

observed GxT only in the greenhouse and not in the field might be the differences in developmental stage of the tested maize plants. The plants response to drought is dependent on the growth stage when stress occurs. Since field and greenhouse plants were at different developmental stages when drought stress was induced, the difference in observed GxT might result from these distinct growth stages at stress, or the interaction of the artificial conditions in the pot topsoil and the plant developmental stage.

The low observed GxT in RDD in the field, and the supposedly low GxE across field and greenhouse, allowing correlation across these phenotyping platforms, could constitute a major facilitation in phenotyping root morphology. The main bottleneck for adaptation of root traits in breeding programs is the enhanced effort for phenotyping root traits in the field (Herrera et al. 2012). Traits with low GxE effect are valuable in this regard, since examined genotypes need to be screened in a reduced number of environments in order to estimate the genotypic values. Also, high phenotypic correlations between phenotyping platforms could allow utilization of controlled environments like greenhouses for pre-screening of plant material prior to field trials, leading to savings in time and money.

4.2.4 Yield components under drought and the capability of RDD to predict shoot traits in the field

4.2.4.1 Influence of drought on plant performance
Correlations were calculated for root and shoot traits in the field. The traits GY under drought and SC in the well-watered control had no significant genotypic variance. Phenotypic correlations were still calculated for these traits, assuming that the genotypic variance could be larger than zero but was not significant due to large experimental error. Therefore selection decisions should only be made after the presented correlations involving these traits are confirmed in further work.

The drought regime in the presented study significantly reduced GY by 16 % compared to the well-watered control. Yield reduction in this dimension in field trials was reported by Ne Smith and Ritchie (1990), but drought in that study was introduced at growth stage V9, corresponding to the vegetative growth stage. Thus, the relatively mild influence of drought on yield in the study of Ne Smith and Ritchie (1990) might be attributed to stress occurrence in early plant growth stages. Stronger reduction in yield (30-40 % and 60 %, respectively) was reported in Çakir (2004) or Ribaut et al. (1997), although applied at similar

developmental stages as in the presented study. Hence, drought stress level in the presented study was presumably mild to intermediate, as yield integrates the stress the plants experience throughout the whole growing season, in all soil depths, and is suited to assess the actual drought stress level. In this study, drought was induced to peak before flowering time in the field, at the end of the vegetative growth stage, and to continue until plants shifted to generative growth. Drought reduces GY in maize depending on severity and timing of water shortage (Çakir 2004; Yazar et al. 1999), with the tasseling and silking growth stages during flowering time being the most sensitive time points (Çakir 2004; Campos et al. 2006; Magorokosho et al. 2003). Reasons for this susceptibility are enhanced water usage in this growth stage (Chapman and Edmeades 1999), delayed female flowering due to reduced elongation rate of silks from the ear and kernel abortion (Fuad-Hassan et al. 2008; Otegui et al. 1995; Ribaut et al. 1997). The presented results indicate that mainly KPE was influenced as yield component under drought stress. Similar results were obtained by Ribaut et al. (1997). They pointed out, that water stress before and during flowering in maize mainly influenced KPE, but seldom TKW. Low impact of drought on TKW was also reported in Çakir (2004). These reported results are in line with the negative correlation of KPE and TKW in the presented study, which was only observed under drought conditions. These results are similar to reports of Çakir (2004), who stated, that this observation could be the consequence of a decreased set of kernel number per ear due to bad pollination or kernel abortion, and the improved grain filling of this reduced amount of kernels. Additionally, Yazar et al. (1999) pointed out that the predominant reason for yield reduction under drought was a reduction in kernel numbers, yet the kernel weight was not that strongly influenced since water availability in the grain filling stage leveled out the effect of sub-optimal water availability on kernel mass. Also in the present study, plants were supplied with water during the grain filling stages, reinforcing this assumption.

4.2.4.2 Correlation of RDD with kernels per ear and harvest index

Root system architecture is responsible to place the roots in a spatio-temporal soil environment throughout the growing season (Lynch 1995), which is directly linked to the acquisition efficiency of resources (Wasson et al. 2012).

The observation that most correlations of the root traits RDD, RLD, RMD and CN with plant developmental and reproductive traits were found under drought conditions, and not in

the well-watered control treatment, show the enhanced role of root characteristics under drought conditions and are in line with this notion.

Root proliferation at depth or increased maximum rooting depth have been reported to influence water extraction under drought and increase drought tolerance (Li et al. 2005; Uga et al. 2011; Uga et al. 2013), but the association of root traits with yield parameters is not always aiming in the same direction. Increased root proliferation or mass in the top 30 cm of soil can have positive (Sadras and Rodriguez 2007) or negative (Palta et al. 2011; Siddique et al. 1990) influence on yield under drought conditions. Generally, usefulness of a root phenotype as target trait is dependent on the underlying environment, and if the observed root phenotype is beneficial in the respective target environment (Chenu et al. 2013; Lynch and Brown 2012; Tardieu 2012).

Here, RDD in depth 10 showed a negative correlation with the yield parameters KPE and HI only under drought, but not in the well-watered control. The root system is a vegetative growth organ which needs to get established and nourished throughout the plants life cycle, while these invested resources are no longer available for shoot growth. Thus, any resource input in the root system needs to be justified with reasonable excess profit in terms of resource acquisition to keep plant growth economical (Lynch 2014). That suggests more RDD in shallow soil, where no or little water was available for the plants during drought, could have contributed to less kernel yield and hence to lower HI. Passioura (1983) stressed that a RLD of 1 cm cm^{-3} would be sufficient for the plant to extract the plant available water given enough time. He also stated, that an increased root growth, not able to offset elevated water and carbon investments due to enhanced resource acquisition, bears the risk to negatively impact HI since less assimilates would be available for grain formation (Passioura 1983). Notably, average RLD in depth 10 under drought was 2.1 cm cm^{-3}, also indicating a potential metabolic burden, in other words increased carbon costs for tissue production and maintenance (Lynch 2014). No significant correlation of RDD with KPE and HI in the well-watered control indicates that plants with more roots in topsoil under optimal water supply could still compensate their increased metabolic expenses, likely by undiminished or equivalent water uptake and maintained nutrient uptake (Rouphael et al. 2012).

Along the same line are the determined negative correlations of the trait CN with KPE and HI under drought. A high number of crown roots can constitute a metabolic burden, especially under abiotic stress, where the water and nutrient uptake function of crown roots is restricted. Lynch (2013) argued that a large CN demands many metabolic resources, which could potentially be a wasted effort when crown roots compete with each other for soil resources. A report from Saengwilai et al. (2014) shows that low CN under nitrogen deficient conditions increases rooting depth and thus nitrogen acquisition. Notably, nitrogen leaches with water through the soil and thus acquisition efficiency of both water and nitrogen is likely related to similar root traits.

Regarding these results, potential target ideotypes for the applied drought scenario in the present study, with drought mainly at anthesis, have less roots in 10 cm depth and sufficient roots in deeper soil strata. Hence, RDD could be useful as surrogate trait in selection for drought tolerance in chosen target environments. In this context, coring of deeper soil horizons for determination of RDD than in the presented study would lead to further utility of RDD for selection for drought tolerance in drought environments where root proliferation at depth is beneficial.

4.2.4.3 Correlation of RDD_Index with stomatal conductance in the field

RDD_Index is an approximation of how much RDD was retained under drought influence compared to the well-watered control. RDD_Index is similar to other reported indices, as for example the drought tolerance index (Ribaut et al. 1997), allowing to relate the performance under stressed conditions to the performance under well-watered conditions. Usually, these indices range from 0 to 1, where 1 means unimpaired performance under stress, and 0 total loss.

A significantly lower mean of RDD_Index in depth 40 than in depth 10 was observed. Since root DNA is measured from root cells, the decrease of RDD_Index indicates plants under drought influence showed a stronger reduction of live cells in depth 40 than in depth 10, although the soil was much drier in depth 10 than in depth 40.

RDD_Index in depth 40 showed a positive correlation with SC in the field. That means plants which generally show a better retention of root cells from well-watered to drought conditions have higher transpiration. Moreover, the reason that no correlation was found in depth 10 could be that, under drought, no water for uptake and transpiration was available in that soil depth.

The root cortex makes up the majority of cell bearing tissue. The functions of the root cortex include radial transport of water and nutrients, from the soil outside of the root towards the stele, where xylem and phloem reside (Steudle and Peterson 1998). In the literature, there are reports that reduced cortical cell number in turn might decrease the plant roots ability to take up water and nutrients (Lynch et al. 2014). Hence, when root cortical cells die from drought, as indicated by a low RDD_Index, the water transport from outside the root profile towards the stele might be reduced, since cortex cells are important for apoplastic, symplastic and transcellular transport of water, the three kinds of radial transport occurring in roots (Steudle and Peterson 1998). At first sight, this would explain the correlation under drought influence, but not in the well-watered control. There, the correlation might be explained with general cell organization in the root. However, information on this topic in literature is very rare (Lynch et al. 2014). Further investigation of the influence of the number of cortex cells and their organization on radial water transport in roots warrants further research.

4.2.5 Comparison of RDD to other root traits RLD, RMD and CN for expenditure in time and money

Currently available root phenotyping procedures for the characterization of root system architecture in field environments are mostly low throughput and error prone (McKay et al. 2008; Watt et al. 2008; Zhu et al. 2011). In recent years, the so called *Shovelomics* method was introduced as an elegant means to phenotype root crowns of maize plants in the field in a relatively fast and accurate way (Colombi et al. 2015; Trachsel et al. 2010a). However, phenotyping of root crowns gives limited information about total root distribution in horizontal and vertical direction, since the root crown resides in a mostly limited volume of topsoil. That is why coring based approaches were used in the majority of studies over the last decades until now, and RLD or RMD were utilized as investigated traits to describe root proliferation (Böhm 1979; Gregory 2006; Wasson et al. 2014). In the presented study, the new trait RDD was compared to the most commonly used traits RLD and RMD (Gregory 2006) and CN regarding time and money expenses.

The validation experiment in the field revealed an improvement in time effort per sample for RDD compared to RLD and RMD. In contrast to the observed 34 and 26 min for RLD and RMD, respectively, 22 min for RDD constitutes a reasonable improvement in time investment. The improvement for RDD was due to the drop out of the working steps

washing in relation to RLD and RMD, and scanning in relation to RLD. CN required approximately 5 min per root crown or sample. Screening roots for CN demands accurate rinsing of the whole maize plant crown and consequently the speed of this approach depends largely on soil properties, whereby processing of maize crowns is faster in sandy soils than in clay soils. Thus, compared to one of the fastest manual root phenotyping methods *Shovelomics*, RDD is still not competitive in throughput. However, one critical work-step in the field, the sampling, is as fast and samples can be conserved for later processing, which was one of the major bottlenecks in the initial *Shovelomics* approach (Colombi et al. 2015), where phenotyping of the excavated root crowns has to be done quickly before natural decay commences. Other studies which used coring for root phenotyping, reported similar effort to estimate RLD through washing of cores as the observed 34 min in the presented study, namely up to 40 min per sample (Watt et al. 2008). Since RMD needs the same washing and picking work-steps as RLD, the observed results for RMD were expected. The improvement in time investment for RDD as a coring related root trait, paired with other advantages of the approach, facilitates the incorporation of the trait RDD in field research programs.

Following the sample throughput, financial expenses are another important aspect, whether incorporation of a trait in research or breeding programs is feasible. Regarding money expenses per sample for material, RDD was more expensive (7.89 Euro) compared to RLD and RMD (0.5 Euro). The high financial expenses were mainly due to the costs for commercial chemicals in DNA extraction and qPCR reaction work-steps. However, when financial expenses for working hours are considered, costs for RDD were reduced since it was the trait with the least time effort per sample. Financial expenses for CN range in the region of RLD and RMD or lower, since only spate and hose with water and sprayer are necessary. With 5 min labor per sample, also financial expenses for working hours are manageable. Regarding costs for RDD, the combined solution of finally used chemicals from commercial suppliers represents arguably a rather expensive option. Yet, DNA extraction kits enable standardized working procedures, with increased repeatabilities, and in case of environmental samples, the avoidance of co-extraction of inhibitors (Nelson et al. 2010). Another advantage of working with kits is that they mostly utilize non-hazardous chemicals. DNA extraction kits for various environmental samples range from 1.1 US\$ to 6.84 US\$ (Ball and Armstrong 2008; Nelson et al. 2010) per sample. In contrary, phenol-chloroform based

extraction methods are much cheaper with only a fraction of the costs (Nelson et al. 2010; Psifidi et al. 2015). In the presented study, costs for one phenol-chloroform based extraction, carried out in the same volume as with the DNA Spin Kit for soil, were approximately 20 cents, as compared to the kit related costs of 4.44 Euro. Thus, tuning of the DNA extraction step could lead to substantial financial savings. Therefore, incorporation of a modified DNA-extraction step using cheaper chemicals could render the trait RDD more attractive for use in large scale application. Ultimately, the new approach improved the time from sampling to data acquisition compared to similar coring techniques, which might justify the higher costs.

5 Outlook

The present study showed that RDD in maize is under genetic determination, and gives intermediate to high heritabilities in field and greenhouse environments. These results constitute basic prerequisites for the use of RDD in plant breeding, where the high heritabilities would enable more rapid breeding progress. Given these results, RDD would be a promising candidate to phenotype roots in field trials. Another result emphasizing the utility of RDD for phenotyping is the significant positive correlation across the phenotyping platforms field and greenhouse, suggesting only weak genotype by environment interaction effects. The underlying data point out that the application of pre-screening steps for RDD under controlled environments could be a valuable tool to decrease time and money expenses in breeding.

An important point of technical nature is to test the possibility of slow DNA degradation under drought conditions, which could introduce unwanted variation and hamper the comparison of genotypes between different water regimes and, in case of interaction effects, inside of water regimes with sub-optimal water availability.

Taking the found correlations of RDD with kernel number per ear and harvest index into account, RDD could be used to screen genotypes for low RDD in topsoil as a secondary trait for increased yield under drought. It is important to note that drought is a very complex abiotic stress with many manifestations depending on various factors, with the most important being the timing and quantity of precipitation and soil characteristics. Low RDD in topsoil might be beneficial in the applied drought scenario, where no water was available in the upper 20 cm before and after the most critical developmental stage of flowering time, where maize is most susceptible. Under different drought scenarios, for example where the plants rely on intermittent rainfall throughout the entire growth period, it might in turn be beneficial to maintain a critical root mass in the topsoil to ensure efficient water uptake after intermittent rain.

Additionally, also deeper soil horizons could be investigated for RDD, in order to determine the genotypes intrinsic relation of topsoil to subsoil RDD, offering further information for the ability to extract water from deep soil layers and the possible implications for drought tolerance.

Along that line, correlations of the index of RDD with stomatal conductance in the field indicate the potential usefulness of RDD to predict the plants capability to take up water. These data should be further validated, which could for example be done using measurements of the isotopes of the water molecules present in the plants, and by investigating from which soil horizons the water was taken up and if the measured RDD in the respective soil horizons relate to the observed water uptake. That way RDD could be given functional implication which would enhance its value for research and breeding, especially since so far no information about the impact of RDD on root function is available. When sampling leads to largely undisturbed samples, as it was possible in the present study using a foil lining, the identical sample or even DNA crude extract can be used for determination of both RDD and pathogens. In that way, RDD could be directly related to the level of biotic stress, and give information about potential impact of pathogens on root proliferation or viability. In order to confirm these results, especially regarding the effect of the GxE, as well as implications for RDD in regard of drought tolerance breeding, field trials with more plants and more diverse plant material would be necessary.

Since with this study the whole workflow for the acquisition of RDD is available, it is also amenable to changes and modifications. In principle, every single step can be modified, optimized and adjusted to the underlying study. As the non-significant genotypic variation for RDD in depth 40 under drought indicates, an increase in sample number would be beneficial to enhance the precision of RDD in soil depths where only minute amounts of roots reside. Sample homogenization by milling was implemented in the method, followed by sub-sampling and DNA extraction. These two steps could be fused into one step, provided a custom-made solution for large sample volumes will be developed. In average, also method costs could be reduced using custom made DNA extraction by eliminating the dependency on expensive commercial kits. The throughput and monetary expenses could be optimized when building custom-made sample handling and DNA extraction procedure.

Conceivable is also the exchange of the qPCR assays in order to investigate different crop species, or single copy gene targets. The increased copy number of the ribosomal DNA ITS2 provides high sensitivity in the qPCR-based DNA quantification. But the ITS2 copy number in maize showed significant genotypic variation, making it necessary to normalize each genotype to render them comparable to each other. This is linked with more effort, since a controlled experiment needs to be carried out in order to determine the ITS2 copy numbers

of the examined plant material. Moreover, results and literature indicate that the ITS2 copy numbers are not fixed across generations and underlie diminishing or increasing mechanisms acting in short amounts of time, which indicates that non-recurrent determination of the ITS2 copy number might not be sufficient. Another issue is the transferability of the information about the copy numbers, determined under very artificial conditions, to natural field environments. Plants in different developmental stages and different root classes get sampled in the copy number determination as opposed to the actual field experiment. Huang et al. (2013) for example used primary roots to determine the ITS2 copy number in 21 wheat genotypes from a pot experiment. Yet also nodal and their lateral roots were included in the soil samples from the field to determine RDD, among which the different root classes cannot be distinguished. Thus the question arises if these results are transferable. Alternatively, in order to reduce effort and potential errors associated with ITS2 copy number determination, single copy gene assays, as for example the IVR assay for maize from the present study, or new assays with a fixed copy number target, could be utilized. This way, less labor and lower introduced variability could lead to more precise estimation of RDD. When working with mapping populations over longer time periods or a range of experiments, determination of copy number for ITS2 could be valuable to enhance sensitivity when root DNA quantification is carried out from soil depths where only minute amounts of roots reside. Regarding breeding efforts where multiple genotypes are tested however, utilization of assays targeting fixed copy number genes would be the only way to guarantee practicability. More research for suitable quantification targets is warranted.

Additionally, construction of a more automatized sampling tool would facilitate higher throughput in field trials. A higher degree of automation can be reached by equipment especially engineered for this purpose to make manual operation of a percussion hammer superfluous, for example by attaching coring system components, like a pneumatic or hydraulic drill system, to a so called *Phenomobile*. *Phenomobiles* are generally made for high-throughput field phenotyping, with the aim to enable cost efficient handling of samples and to avoid high sampling errors (Palanichamy and Cobb 2015). A mounted drill system to drive cores in and out of the soil would allow deeper sampling, comparable to tractor based sampling, but could keep the disturbance at field sites at a minimum. It could

be challenging to keep the phenomobile small and handy enough, however, phenotyping related methods are now more and more targeted by research.

6 Summary

Maize (Zea mays L. ssp. mays) plays a major role for global food security, but the availability of sufficient food to satisfy nutritional demands is restricted for large parts of the human population and the situation is likely to worsen in the future due to population growth and climate change. Limited yield increases in dry environments are further threatening food security, and suggest the complementation of yield-based breeding, the primary route in genetic crop improvement since the green revolution, with trait-based breeding approaches, that utilize beneficial secondary traits to enhance breeding progress. Research indicates that root traits have great potential to be used as secondary traits for genetic crop improvement, in particular to improve water and nutrient use. From a breeder's view, root traits constitute a resource for unused natural genotypic variation since they have not been used much in the past. Despite the huge potential root traits might have in trait-based breeding, characteristics of the root system have hardly been exploited in breeding programs. The main reason root traits have been widely neglected in breeding is the phenotyping bottleneck in natural field environments. Methods to phenotype roots under controlled conditions advanced rapidly during the last decade, but transferability of information gained in controlled experiments to natural conditions is often low. There is a lack in phenotyping methods for roots in the field, which combine throughput with precision. DNA-based root phenotyping is a promising approach for more time-efficient and precise phenotyping of roots under natural field conditions. The aim of this work was to develop a DNA-based method for maize to phenotype roots in field soil, and to demonstrate that the method is feasible and gives reproducible results. Simultaneously, this approach had the objective to elucidate the genetic determination and the extent of natural variation available for the trait root DNA density

In order to make DNA-based root phenotyping amenable to research and breeding, a new method to capture root DNA density in field environments was developed and subsequently validated. The development comprised the optimization of field sampling, sample handling and homogenization. DNA extraction methods were investigated and a qPCR assay for the quantification of maize root DNA from soil samples was established. The validation part was carried out in the field using a rain-out shelter and in the greenhouse using pots, both under optimal and sub-optimal water availability.

This is the first time a complete and universal method for the investigation of root DNA density from field trials was developed and reported. The validation trials revealed significant genotypic variation for root DNA density in maize and intermediate to high heritabilities across watering regimes. The observed low genotype by treatment interaction effect across watering regimes indicates the trait's usefulness for phenotyping. Significant, positive correlations of root DNA density from controlled conditions in pots with root DNA density from natural conditions in the field suggest low genotype by environment interaction across different environments for the investigated plant material and plant developmental stages. These are promising data for the combined use of controlled and field environments in breeding approaches. Correlations with shoot traits demonstrated the negative relationship of root DNA density in topsoil with the reproductive traits kernels per ear and harvest index under sub-optimal water availability in the field. These observations indicate increased yield and harvest index for maize inbred lines with better resource management and lower metabolic costs for the construction and maintenance of the vegetative growth organ root in topsoil. Therefore, root DNA density might be useful as a secondary trait in trait-based breeding.

For the first time, it was shown that root DNA density is under genetic control in the important crop plant maize. The developed method constitutes a novel tool for the investigation of root DNA density in field trials in maize, with the potential of specific modifications to assess other important crop plants. The new method offers a platform for the investigation of further important questions, for example permitting the determination of pathogens and their influence on the roots in the identical soil samples, also enabling research in a broader ecological context.

7 Zusammenfassung

Die Kulturpflanze Mais (Zea mays L. ssp. mays) stellt, neben der Verwendung in der Tierernährung und als Ausgangsprodukt für technische Anwendungen, ein wichtiges Grundnahrungsmittel in weiten Teilen der Welt dar. Durch natürliche Schwankungen in der Nahrungsmittelproduktion ist eine ausreichende Nahrungsmittelversorgung für große Teile der Weltbevölkerung nicht sichergestellt. Die Versorgungssituation wird zudem durch stetiges Bevölkerungswachstum und einen fortschreitenden Klimawandel erschwert. Die Sicherung und Erhöhung der Erträge, besonders unter zunehmender Häufigkeit von Dürreperioden, ist eine der großen Herausforderungen der Zukunft. Züchtungsfortschritt wurde bislang fast ausschließlich unter optimalen Feldbedingungen durch direkte Selektion auf Ertrag generiert. Da Ertragsversuche unter Trockenstress eine geringe Heritabilität aufweisen, soll die indirekte Selektion auf Hilfsmerkmale genutzt werden, um Ertragszuwachs auch unter Stressbedingungen zu sichern. Die Selektion auf Wurzelmerkmale hat hierbei ein großes Potenzial, die Wasser- und Nährstoffnutzung der Pflanzen züchterisch zu verbessern, denn Wurzelmerkmale weisen meist einen großen Anteil ungenutzter, natürlicher genetischer Variation auf. Trotz des großen Potenzials für eine züchterische Verbesserung der Trockentoleranz fanden Wurzelmerkmale aufgrund von fehlenden Phänotypisierungsmethoden unter Feldbedingungen bislang nur selten Eingang in Züchtungsprogramme. Methoden zur Erfassung von Wurzelmerkmalen unter kontrollierten Labor- oder Gewächshausbedingungen haben in den letzten Jahren eine ausgeprägte Entwicklung erfahren. Die Ergebnisse aus diesen Ansätzen sind jedoch häufig nur bedingt auf die Pflanzenleistung unter Feldbedingungen übertragbar. Deshalb besteht großer Bedarf an Phänotypisierungmethoden unter realen Anbaubedingungen, die Präzision und Durchsatz miteinander kombinieren. Ziel dieser Arbeit war, eine DNA-basierte Methode zur Quantifizierung der Durchwurzelungsdichte des Bodens unter Feldbedingungen zu entwickeln und in Validierungsexperimenten die Durchführbarkeit und Reproduzierbarkeit der Methode zu demonstrieren. Dadurch sollte nachgewiesen werden, dass die Variation für Wurzelmerkmale in der Kulturart Mais auf genetischen Faktoren basiert und damit Selektion auf dieses Merkmal im Rahmen eines Zuchtprogramms möglich ist.

Um DNA-basierte Wurzelphänotypisierung für Forschung und Züchtung zugänglich zu machen, wurde in dieser Arbeit eine neue Methode in Mais entwickelt und validiert. Die Entwicklung umfasste die Arbeitsschritte Probenahme im Feld, die Prozessierung und Homogenisierung großer Bodenprobenvolumina, die Validierung geeigneter DNA-Extraktionsverfahren sowie die Entwicklung eines qPCR Assays für die Quantifizierung von Maiswurzel-DNA aus Bodenproben. Die Validierungsexperimente wurden mit einem Sortiment von Mais-Inzuchtlinien in einem Rain-out Shelter sowie in einem Gefäßversuch im Gewächshaus unter Trockenstress und unter optimaler Wasserversorgung durchgeführt.

Mit der vorliegenden Arbeit wurde zum ersten Mal eine vollständige und universell einsetzbare Methode zur DNA-basierten Wurzelphänotypisierung in Mais entwickelt und veröffentlicht. Für das neu entwickelte Merkmal Wurzel-DNA Dichte konnten in den Validierungsexperimenten eine signifikante genotypische Variation und mittlere bis hohe Heritabilitäten über Bewässerungsregime hinweg nachgewiesen werden. Niedrige Genotyp-Behandlungs Interaktion über Bewässerungsregime deutet auf das Potenzial des Merkmals für die Phänotypisierung hin. Die konstanten, positiven Korrelationen zwischen den zwei unterschiedlichen Umwelten Rain-out Shelter und Gewächshaus weisen ebenfalls auf eine geringe Genotyp-Umwelt Interaktion für das Merkmal Wurzel-DNA-Dichte hin. Die erzielten Ergebnisse sind vielversprechend in Bezug auf die gleichzeitige Nutzung verschiedener Phänotypisierungsplattformen in Züchtungsansätzen wie Feld und Gewächshaus. Die negative Korrelation von Wurzel-DNA-Dichte im Oberboden mit der Ertragskomponente Körneranzahl je Kolben unter reduzierter Bewässerung im Feld deutet auf höhere Trockentoleranz für Maisinzuchtlinien hin, die weniger metabolische Kosten für die Etablierung und Versorgung des vegetativen Pflanzenorgans Wurzel im Oberboden haben. Aufgrund dieser Daten könnte das Merkmal Wurzel-DNA-Dichte als Sekundärmerkmal in der Züchtung für Trockentoleranz geeignet sein.

Mit dieser Studie wurde zum ersten Mal gezeigt, dass das Merkmal Wurzel-DNA-Dichte in Mais genetisch kontrolliert wird. Die neu entwickelte Methode stellt ein vielversprechendes Werkzeug für die Erfassung des Wurzelwachstums in Mais dar und hat das Potenzial auch bei der Züchtung anderer Kulturpflanzen eingesetzt zu werden. Zusätzlich eröffnet die neue Methode die Möglichkeit, gleichzeitig andere Fragestellungen zu berücksichtigen. Beispielsweise können bodenbürtige Pathogene quantifiziert, deren Einfluss auf das

Wurzelwachstum erfasst, und damit die Untersuchungen in einen breiteren ökologischen Kontext gestellt werden.

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9 Appendix

Table A1 Collected weeds for specificity testing of qPCR assays.

Species
Field bindweed (*Convolvulus arvensis* L.)
Black bindweed *(Fallopia convolvulus* L. Á.Löve)
Chamomile (*Matricaria chamomilla* L.)
Lamb's quarters *(Chenopodium album* L.)
Spear Saltbush (*Atriplex patula* L.)
Green field-speedwell *(Veronica agrestis* L.)
Catchweed *(Galium aparine* L.)
Couch grass (*Elymus repens* L. Gould)
Redroot pigweed (*Amaranthus retroflexus* L.)
Red deadnettle (*Lamium purpureum* L.)
Sun sporge (*Euphorbia helioscopia* L.)
Common knotgrass *(Polygonum aviculare* L.)
Cockspur (*Echinochloa crus-galli* L. Beauv)
Chickweed *(Stellaria media* L. Vill.)
Lady's thumb *(Persicaria maculosa* Gray)
Creeping Thistle *(Cirsium arvense* L. Scop.)
Field horsetail (*Equisetum arvense* L.)

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