PROJECT FINAL REPORT

EURoot

Enhancing resource Uptake from Roots under stress in cereal crops

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I EXECUTIVE SUMMARY

European agriculture is facing climate instability and has to respond to the economic and social demands for reducing both irrigation water and chemical fertilizers. Water and nutrients for plant growth are unevenly distributed in the soil, both spatially and temporally over the crop cycle, and a drying topsoil further reduces access to these resources, notably phosphorus because of its low mobility in the soil profile. As a result, plants have to adjust the placement of their new roots to capture the resources in response to variable soil water and nutrient distribution. The amenability of the plant to explore the soil and capture its resources is largely determined by the architecture of the root system and its ability to being altered in response to environmental cues, the biogeochemical interactions occurring in the root-soil interface and root resource uptake efficiency resulting from efficient intrinsic transport and signaling.

The 20 partners of the EURoot consortium joined in a 4-year multidisciplinary research effort aiming at better understanding how cereals acquire water and nutrients through their roots and maintain growth and performance under stressful conditions.

Using diversity panels or high resolution populations in 4 cereals -maize, rice, barley and durum wheat, EURoot identified markers linked to chromosomal regions underlying important traits -such as rooting depth and nitrogen response, drought tolerance and nitrogen use efficiency- that can be readily used to assist breeding programs. The project also identified new traits that should be considered as major targets for root breeding such as root hairs, revealed as environmental sensors, root efficiency for phosphorus acquisition, amenability to mycorrhizal colonization, type of lateral root development and selective root placement under uneven distribution of nutrients. To better understand the genetic and molecular control of root traits and their conservation, EURoot has developed advanced lines and root mutant population that will be further used for gene isolation, and a specialized database EURootDB which allows bridging of syntenic regions and orthologous genes underlying root traits across cereals.

EURoot has demonstrated that deep placement of nutrients attracts maize roots to deeper soil layer which may prepare plants to better respond to a topsoil dryness progressing during the crop cycle. Deep placement of nutrients may therefore improve the performance of maize hybrids while maize was also found capable to produce more roots in response to the nutrient hotspots than anticipated. Also, soil type pH was found to have a major influence on the root trait development strategy that maize has to favor for efficiently acquiring P. Consequently, the best-adapted crop root ideotype for P-depleted soils might be rather different according to field soil pH. These results have important implications in the screening procedures in breeding programs and soil management practices. Investigating root-sourced molecular and biochemical signals in maize, EURoot identified hormone - related signatures for steep rooting, response to uneven nitrate and mycorrhization. Signal transduction pathways and cell processes are coordinated according to a spatio-temporal pattern with respect to tissue type and stress intensity and duration in maize roots subjected to an osmotic stress. We also showed that variation of root hydraulic conductance in response to high nitrate is related to a genotype-dependent regulation by nitrate of the subcellular trafficking or activity of the water channels aquaporins. These results will help developing strategies to optimize water use for plants grown under different nitrogen fertilizer regimes.

In the course of EURoot, root phenotyping platforms have been optimized for quantifying cereal root architecture responses to heterogenous N and/or P supply in cereal populations. We have shown the great complementarity of root trait data acquired in MRI and X-ray computerized tomography (CT) imaging devices for describing the complexity of root architecture through 3D reconstructions. Also, the capacity for X-ray CT imaging to deal with a high genetic resolution population in barley through scaling up and standardization has been demonstrated for the first time. Two major questions for root traits were their heritability and consistency of observations from platforms to the field. Importantly, heritability of field root traits observed in maize and barley was found generally high and sufficient to carry genetic analyses and some important root traits were positively correlated.

New pipelines were developed allowing efficient extraction of data generated by root phenotyping platforms for calibrating root architectural development models. New models and software describing the way water and nutrient flow around the root and enter the root system have been generated. These novel functional-structural models coupled to soil processes enable to simulate impacts of root system architectural trait variations on uptake processes.

II. PROJECT CONTEXT AND OBJECTIVES

Context

Food security in a context of shrinking arable land and erratic environment

World agriculture is facing the challenge of needing to produce more food, feed, fiber, and fuel on a shrinking proportion of arable land and in an increasingly erratic climatic environment. While availability of arable land and water resources are becoming limiting because of industrialization and urbanization, global capacities for food and feed production will increasingly have to compete with a growing need for energy, chemicals and the production of plants for purposes other than nutrition (e.g. clothing, housing, and biofuel).

The need to decrease inputs in European agriculture to enhance its competitiveness and reduce water use and fertilizer pollutions

Another challenge is the need for a reduction of inputs, particularly in response to legislative and public pressure in developed countries, notably in regard to N- and P- based fertilizers. These mineral forms cause pollution through leaching (nitrate) and runoff (phosphate), and greenhouse gases are emitted both during production and when used agriculturally. Production of N-based fertilizers consumes precious fossil fuel reserves. Also, it has been predicted that known global deposits of phosphate-rich rocks will become exhausted in no more than 60-100 years at current consumption levels, and with no easy alternative, mined P consumption is likely to increase due to the intensification of agriculture in rapidly developing countries.

Within the context of an uncertain climate, maintaining productivity of crops under reduced nutrient and water inputs can be brought about through a combination of waste reduction, recycling of organic matter, improved crop management, and plant breeding. Plant breeding aims at developing improved cultivars exhibiting an enhanced ability to capture water, and the main nutrients nitrogen and phosphorus, particularly under erratic water supply, and use all three resources more efficiently, to convert them into food via a higher harvest index. Resource acquisition, use efficiency and harvest index are therefore the key targets for cultivar resilience and maintenance of productivity under stressful conditions.

The capacity of roots to extract nutrients and water from a given volume of soil is heavily influenced by root system architecture (RSA), which is a combination of traits such as, root axis elongation, root number and branching angles. These traits make soil exploration more or less efficient in relation to different soil types and in different environmental conditions. Secondly, the capacity to extract resources also relies on intrinsic factors such as the production of root exudates, root hairs, specialized root anatomy, and the efficiency of protein carriers involved in resource uptake and intracelllular transport. This capacity is also largely influenced by complex root-soil interactions in the rhizosphere, such as relationships with beneficial microorganisms as mycorrhizal fungi.

The plant roots: a hidden, complex and highly adaptive system

In terms of their architecture, root systems are complex objects to study. They consist of large numbers of segments and branches, whose position and topological relationships influence the efficiency of plant resource capture from the environment. However, root systems grow through a limited number of processes, e.g. elongation, branching and senescence, dissected into clearly identified mechanisms e.g. cell division, cell expansion and lateral root initiation. The final shape of the root system therefore depends on the magnitude and frequency of these processes, which, in turn, respond to environmental signals, so that root system architecture often provides a historical record of environmental influences. To account for the variability of root system architecture in terms of mechanisms and to better understand its functional significance, we need to find ways of measuring and modeling these processes accurately at different spatial and temporal scales.

Though much progress has been made in the understanding of the genetic and molecular control of RSA and root intrinsic functioning, and of the signaling circuitry between roots and shoots in response to either water, nitrogen or phosphorus shortage, and in the analysis of isolated root-soil interactions, they have rarely been investigated together in a holistic approach and under a combination of stresses and situations that have full relevance to whole plant performance under well-defined field conditions. This complex integration requires a multidisciplinary effort involving the development of shared phenotyping methods and multi-scale and interactive modeling of developmental and environmental processes.

Soil resources and biogeochemical properties vary both spatially and temporally during crop cycles

Following germination, plant root growth is prone to a combination of bio-geo-chemical influences. Roots also face spatially and temporally heterogeneous conditions when growing in any soil. Also, the ability to acquire resource varies along the developing root system, the aging root parts becoming less efficient compared to newly formed root axis. Even with intensive agricultural management, nutrients (N, P) and water occur in patches or gradients that vary in space and time, and root system architecture need to respond to these diverse conditions to optimize acquisition of resources (schematized in **FIGURE 1A**). Proliferation of lateral roots at places where more nutrients and water are available (selective root placement) increases the surface of the root-soil interface, as do mycorrhizal interactions that are triggered by nutrient and water deficiency. Local changes of pH and release of exudates may further improve uptake efficiency, directly by dissolving tightly bound and otherwise unavailable P , or indirectly via effects on soil biota.

Whilst the necessity of a sufficient water supply for plant growth and development is incontrovertible, nutrient acquisition by roots also requires that water is available, to allow nutrient diffusion or mass flow towards the roots and from roots to shoots. However, under conditions with limited water (in either rain-fed or irrigated crops), the topsoil, where most of the nitrogen and phosphorus soil reserves reside, dries first (schematized in **FIGURE 1B**). This limits the capacity of roots to acquire and transport these essential nutrients. Thus, whilst triggering the more obvious water-stress-induced effects in plants, water shortage is also often combined with a reduction in the capacity of the plant to extract nutrients from the soil, and prolonged water stress may also result in nutrient deprivation. This is likely to cause constraints in the optimal phenotype of root system architecture under these conditions and questions the choice of the most relevant combinations of root traits that should be selected for when designing crop genotypes for low input farming systems.

Importance to specifically focus on cereal root biology and ecology

Cereals are grown over more than 60 million hectares of the available 94 million ha of the EU 27 arable land, and the yearly yield is 314 million tons of grains. Half of total EU fertilizer application is given over to cereals, and one fourth to wheat. Due to improved land management, monitoring practices and enforcement of European directives, fertilizer use has been slowly decreasing in the EU 15 over the last 15 years, where cereals occupy 36% of arable land acreage. However, due to agricultural intensification, fertilizer use has been steadily increasing in the remnant EU 12, where cereals occupy 61% of arable land acreage.

Also, much of the molecular understanding of root development and response to abiotic constraints (water deficit, nutrient limitation) has been established in the model dicotyledonous plant, *Arabidopsis*, the phylum of which diverged more than 150 million year ago from monocotyledonous plants like cereals, raising questions as to whether the same mechanisms have been conserved in cereals, which have a very contrasted root system (fasciated vs. pivotal) compared to dicot plants). It is therefore important and timely to translate this knowledge into cereal crop plants such as barley, maize, wheat and rice, for which an enormous wealth of molecular and biological resources have been accumulated over the last decade, such that they now lend themselves as models for efficient investigation.

Objectives

The long-term objective of EURoot is to assist plant breeders and farmers to enhance the cereal plant capability to acquire water and nutrients through their roots and maintain growth and performance under stress conditions. Research activities of EURoot aimed at defining an ideal cereal root system in terms of architecture, resource uptake and signaling processes (including beneficial interactions with micro-organisms) that allows soil exploration and resource acquisition under limited soil water and nutrients. Taking the socio ecological context of European agriculture detailed above, the primary, short-term objectives of EURoot were two fold:

The first objective was to enhance our current understanding and establish new concepts regarding the genetic, biochemical and molecular processes underlying the ability of the plant to alter its root system architecture, and its capacity to acquire, transport and use water and nutrients, such that whole plant performance is maintained in response to resource scarcity. To reach that goal EURoot aimed at:

- Further the understanding of the genetic and functional bases of the traits involved in cereal root system architecture and its modification in response to abiotic stress. Investigation of these traits across cereals further allows addressing the question of their conservation among different cereals,
- Identifying biochemical and molecular fingerprints corresponding to the perception of soil environmental cues by the root system, and their translation into adaptive responses.
- Identifying traits that can be manipulated for optimized capture of scarce and heterogeneously distributed water and nutrients in different biogeochemical soil scenarios
- Deciphering the impact of cultivar amenability for establishing beneficial fungal association, and/or alterations in root system architecture and signaling on water and nutrient uptake, and plant performance

The second objective was to develop innovative methods, models, tools and products for basic research and targeted breeding of new cereal cultivars that rely less on water and mineral fertilizer inputs to achieve their yield potential. To reach that goal EURoot aimed at:

- Identifying root-sourced biochemical and molecular signatures that can be used as diagnostic tools of intensity of perception of resource scarcity and success of translation into adaptive morphological and functional changes aiding in resource capture in relation to whole plant performance.
- Making use of integrative and comparative bioinformatics to bridge root QTL and functional information across sequenced cereals,

- Establishing innovative root phenotyping and imaging methods allowing the screening of cereals germplasm for response behavior under heterogeneous and reduced water, N and P and with relevance to well-defined field conditions,
- Building predictive models of root architecture integrating soil resource dynamics to construct virtual root ideotypes with appropriate response to nutrient and water distribution and availability that can be used for simulation of scenarios and as a blueprint for genetic and physiological analyses and breeding.

To reach its strategic objectives, the 4 year (2012-2015) EURoot project gathered 20 partners including 3 private companies (including 2 SMEs) and 3 extra-European countries (Japan, USA, Australia), and mobilized complementary expertise in root biology and ecology in a trans-disciplinary approach including soil science, agronomy, physiology, genetics, mathematical modeling, and chemistry engineering.

EURoot conducted experiments integrated into 3 work packages (WPs) designed to better understand and model: i. the genetic and functional bases of root traits involved in soil exploration and resource uptake (WP1); ii. the bio-geochemical properties of the soil, including beneficial association with mycorrhizal fungi influencing extraction of nutrient and water by the root system (WP2), and iii. the plant signaling processes involved in soil environment sensing and responsible for adaptive root system response enhancing soil exploration and resource acquisition (WP3). In addition, two WPs allowed the development of innovative phenotyping methods and platforms that could be compared for relevance to well-defined field conditions and crop performance (WP4) and integrate root architecture, resource dynamics in the soil and root uptake through modeling, to design root ideotypes allowing enhanced resource acquisition under stress (WP5)(**FIGURE 2**).

To facilitate comparison of results, transfer and integration of information within and across WPs, EURoot partners shared protocols and the genetic materials. Maize has been used as the core cereal crop in the project and the model for investigating the questions addressed in WP2 and WP3. A panel of hybrids, mainly derived from the related EU project DROPs, has been used to define core subsets of 5, 10, 20, 30 or more hybrids that has been used over lab, platform and field experiments. (**FIGURE 3**)

Barley, rice and durum wheat were also used in WP1 and WP4: In that aim, the EURoot teams also used common panels of genetic resources. Altogether, these 4 species spanned the diversity of root system architecture in cereals, and further allowed to investigate conserved vs. specific root trait and responses across cereals. An overview of the experiments carried out in W1 to WP3 as well as genetic materials used in these experiments, is shown in **FIGURE 3**. **FIGURE 4** summarizes the work done in the platform WP, phenotyping (WP4) and modeling (WP5).

Altogether, the EURoot project addressed the following questions:

- *What are the genes underlying root system traits that are involved in soil exploration and resource acquisition? To which extent these genes and their functions are conserved among cereals?*
- *How the root system placement occurs in a soil with uneven distribution of nutrients and a progressing topsoil drought? How this influences the soil microbial activity, including that of beneficial mycorrhizae? How the soil properties modulate these processes?*
- *What are the root- and shoot- sourced signals responding to constraints of the root bio-geo-chemical environment that modulate root architecture and resource acquisition efficiency and ultimately plant performance?*
- *How to reliably observe root system traits in artificial conditions that are somehow representative of field conditions? To which extent these traits are heritable and consistent*

with those eventually observed under field growth conditions? How to extract dynamic architectural parameters during the root system establishment from data collected on observation platforms to build models and simulate spatial and temporal change in resource acquisition efficiency of the root system along plant growth?

The next sections provide an overview of the results obtained during the EURoot project to address each of these 4 sets of key questions.

III MAIN S&T RESULTS

Genetic control of root traits involved in resource acquisition and drought tolerance

Genes for root hairs, revealed as environmental sensors: (US)

Root hairs are tip-growing tubular extensions of root epidermal cells that play an important role in the absorption of water and nutrients from the soil. It is known, that they extend root surface making this absorption more efficient. For this reason, the study of genetic mechanisms of root hair development and their role in drought stress response in barley was undertaken, with the use of two approaches.

The first approach was aimed at the characterization of transcriptomes (the products of gene transcription into RNAs) of a root hairless mutant rhl1.a and its parent cultivar 'Karat' under water-stress conditions. The objective of this analysis was to elucidate how roots of a root-hairless mutant perceive and respond to drought stress in comparison to the wild type (WT), non-mutant cultivar.

To address this question, we have identified genes that play a role in root-hair related/dependent response and adaptation to drought conditions in barley. This study was coupled with the analysis of photosynthetic efficiency under the imposed stress. Based on the global analysis of root and leaf transcriptomes, we showed that at the onset of water stress, a specific activation of genes regulating processes that are related to water stress signaling and protection against stress are observed in the roots of the WT plants, but not in roots of the root-hairless mutant. These genes are involved in the biosynthesis of abscisic acid (ABA), a key hormone triggering the stress response of the plant, and in cell wall biogenesis. High expression of genes responsible for ABA biosynthesis only in the roots of WT cultivar suggests that the plants, which produce normal root hairs respond to water stress with a **more efficient stimulation of ABA biosynthesis** than the root hairless mutant. This observation, consequently, could result in an earlier or more efficient induction of systemic response leading to the adaptation to stress conditions. Similarly, the specific activation of genes involved in **cell wall biogenesis** may increase the efficiency of the synthesis and the incorporation of newly synthesized polysaccharides into the walls of root cells that could influence the water-stress adaptation potential. On the other hand, at the onset of water stress, **the processes of advanced response to drought stress were specifically up-regulated in the leaves of the rhl1.a** but not in WT 'Karat' plants. This gene set was highly enriched in genes encoding factors involved in the response to hydrogen peroxide, heat and high light intensity, which are related to latter stages in the stress response. It has been also shown that an extended period of severe stress caused more drastic transcriptome changes in the roots and leaves of the rhl1.a mutant than in those of the WT. Likewise, the extended period of water stress caused much stronger damage to photosystem II in the rhl1.a mutant than in its parent cultivar. Moreover, the functions of selected root hair development-related genes that are expressed in a cultivar with normal root hairs suggest that they may be potentially related to sensing and signaling of water stress. Taking into account this hypothesis we suggest that root hairs may be important not only for physical improvement of water and nutrient uptake but may also **play a role as sensors of environmental conditions**.

Because the molecular basis of root hair development is highly related to the processes of cell wall biogenesis, it was interesting to find which genes of this biological process are directly responsible to the initiation and/or tip growth of the root hairs in barley. For this purpose, we used the TILLING strategy to find mutations in the candidate genes. The analysis was based on TILLING population developed at the University of Silesia after combined mutagenic treatments of spring cv. 'Sebastian' with two chemical mutagens: sodium azide and N-Nitroso-N-methylurea (MNU). The mutated population consists of ca. 9,000 M2 individuals from which DNA was isolated and seeds deposited in a seed bank. Genes with a role in root hair formation determined in other species and genes identified in our previous transcriptome profiling of barley roots, were selected as candidates for TILLING. In three analyzed genes the **mutations that caused a changed root hair phenotype have been found**: in gene HvRTH3 encoding a COBRA-like protein involved in cell expansion and cell wall biosynthesis, in gene HvEXT1 that encodes one of the components of a cell wall that takes part in cell wall assembly and in gene HvEXPB4 hat plays a role in cell wall extension during plant growth (**FIGURE 5**). The individuals bearing mutations in these genes show alterations in root hair development. The mutant in HvRTH3 gene develops roots almost completely lacking root hairs or roots with irregularly spaced root hairs. The mutants in HvEXT1 and HvEXPB4 genes have a significantly reduced root hair length, by more than 90% in the comparison to the parent cultivar. Analysis of orthologous genes revealed their conservation among monocot and dicot species. Our studies provide evidence about conservation of their function in cereals. It can be concluded that **the genetic basis of specific processes of cell wall biosynthesis, cell expansion and cell wall loosening, involved in root hair growth, is conserved among cereals**.

FIGURE 5

Additionally, using a large population of 5,000 F2 plants, the chromosome 7H interval where resides the *RHL1* gene, responsible for lack of root hairs, was further reduced to a 302 kbp segment which include seven genes, among which one promising candidate gene.

Genes for deep rooting traits and drought tolerance: (UNOTT, JHI, CIRAD)

In barley, we have combined unique genetic resources with a novel field approach to identify potential candidate genes involved in drought tolerance. The genetic resources combine a high yielding, elite barley cultivar with drought tolerant wild barley from Israel, adapted to a wide range of environments. Following rounds of crosses, we have a set of lines, which have small regions of wild barley (green) in an elite barley background (red in **FIGURE 6**). Twenty-nine of these lines were grown in a rainout shelter (**FIGURE 7**) with three different water treatments, no irrigation (drought), partial irrigation and full irrigation and scored for a range of agronomic characteristics for two years (2013 & 2014).

The lines were also genotyped using over 6,000 gene-based markers that are distributed along the barley genome. Based on all of the information we calculated a 'drought tolerance index' and identified two lines that show similar yield regardless of water stress. These lines have **unique and different gene combinations, which may be involved in yield stability under drought**. To complement this analysis we identified candidate genes associated with drought related traits, such as flowering time and yield components. All of the lines identified have been used to develop new genetic resources for future research and gene identification.

FIGURE 6 and 7

In rice, a quantitative trait locus (QTL) for root depth detected on chromosome 9 in a population derived from a cross between Azucena, a deep rooted variety, and IR64, an elite variety with shallow roots was initially fine mapped to a segment of 600 kbp carrying approximately 50 genes through the development and characterization of near isogenic lines (NILs). NILs, produced by marker-aided selection backcrosses, constitute an excellent material to assess the effect of a QTL without perturbations due to differences in the rest of the genetic background. IR64 was used as the recurrent parent. A new generation of back cross followed by marker aided selection of recombinants and phenotypic evaluation of the BC5F4 recombinants in an hydroponic system (**FIGURE 8**) helped to narrow down the segment carrying the QTL to **a zone of 30 kbp carrying only three genes**. Additional sequencing was conducted in the target area but did not enable yet to determine which gene among the three was truly responsible of the variation in root depth. New analyses based on gene expression in roots are being undertaken to go down to one gene, enabling its validation through transformation.

FIGURE 8:

Genes for field performance and root traits in response to nitrogen application: (CIRAD, UNIBO)

Nitrogen fertilization is one of the main factors influencing crop productivity and nutritional value. An inadequate nitrogen placement may negatively affect crop performance since nitrogen represents a major element for several cell components (chlorophyll, aminoacids, DNA etc.). On the other hand, unnecessary and/or excessive inputs are subjected to diminishing returns and may cause deleterious impacts on the environment. In this context, one of the challenges to face towards a more sustainable agriculture is to improve crop plant Nitrogen-Use-Efficiency (NUE). NUE measures the capability of the plant to perform in correlation with nitrogen inputs. Plant NUE is a complex trait controlled by many QTLs in the genome and it is also influenced by environmental changes. To dissect the genetic control of NUE in durum wheat, a QTL mapping analyses for N-related traits on a collection of 183 durum wheat elite cultivars were performed at the University of Bologna in collaboration with Società Produttori Sementi (SPS). Phenotypic data were obtained in 2013-2014 and 2014-2015 growing seasons by testing two different N-levels (standard-N1 and low-N2 input. **FIGURE 9**).

FIGURE 9.

Typical agronomic relevant traits (grain yield, grain test weight, protein content, protein/ha, vitrousness), as well as canopy coverage-related remote sensing traits (NDVI and Red NIR, considered good early predictors of vegetation health and plant productivity) were recorded in the field trials. Dataset of a genome-scan using a total of almost 24,000 molecular markers spread on all 14 chromosomes was already available for the cultivar collection. Statistical analysis combining the genome-scan information with the phenotypic data enabled to map the QTLs controlling NUE and N-related traits in this representative sample of durum wheat genetic diversity. The positions of the QTLs identified are reported in **FIGURE 10**.

FIGURE 10.

QTL mapping revealed the presence of QTLs for agronomic traits, which appeared nitrogen-level specific, supporting the existence of partially independent genetic control of those traits at different fertilization levels. More specifically, a cluster of QTLs for grain yield (GY) and NDVI was identified at N2 (ie. low) fertilization level only, on chr. 6A, near the centromere. Although validation is necessary, **several of the QTLs identified in this study are of potential great interest in breeding programs** based on marker-assisted selection, aimed to develop new cultivars adapted to cropping systems with reduced inputs.

In another study, in rice, the detection of QTLs for root growth in low N conditions was conducted on a collection of 168 tropical japonica lines that had been genotyped with 16,600 molecular markers. The plants were grown in a hydroponic system with three different conditions (normal nutrient solution against two nutrient solutions including one half and one eight of the normal N quantity, respectively). For the 15 root and shoot traits measured that included the rate of N in leaf tissues, indices of response to N deficiency were computed using the data obtained both under control and stressed conditions. A total of 31 QTLs were detected. Some QTLs were common across traits. For example, two QTLs were common to three traits related to root depth with effects in same direction on chromosomes 2 and 11. None of these QTLs seemed to correspond to root genes known to be effective in rice but some were in genes annotated as leucine rich repeat-receptor like kinases. **This work enabled to establish a list of markers linked to QTLs and donors of favorable alleles at these QTLs that can be used in breeding for better N use efficiency.**

FIGURE 11:

QTLs and genes for root traits for phosphorus acquisition and mycorrhizal colonization ability: (JIRCAS, ABDN)

Over the last 20 years there have been a growing number of reports from genetic mapping studies of QTLs for traits associated with root distribution, and a great proportion have been on rice. Recently, progress has been made identifying the genes underlying those QTLs, and that includes work being done in EURoot (see above). It is rare, however, for genetic mapping studies to look at the function or biology of roots. Two studies have been conducted exploiting the "Rice Diversity Panel 1", a set of 372 rice accessions from across the globe for which a dense marker set is available.

First, we have assessed the growth of the panel in low and very low phosphorus (P) soils, in comparison with phosphorus replete soils. They show that growth in the very low P soils is strongly correlated with the size of the root system and that some accessions that do well in low P have high "root efficiency" which is the amount of P acquired per unit of root size**. A genome wide association study (GWAS) revealed several QTLs for root efficiency, most notable on chromosome 11 where a rare haplotype present is a few *aus* accessions improves root efficiency (**For more details see Mori et al. (2016)).

FIGURE 12:

We studied the same panel for colonization rate with mycorrhiza, a symbiotic relationship that helps the plant take up soil-bound P forms, under greenhouse conditions. That revealed substantial variation from 21% of roots colonized to 89%. A GWAS **revealed four QTLs for this trait**, most notably on chromosome 2 at 24.5 Mbp where there is an excellent candidate gene, and ammonium transporter. After P, ammonium is considered the second most important nutrient transferred by mycorrhiza to the host plants, the expression of an ammonium transporter (not this one but a close homolog) is used a marker for mycorrhizal colonization because it is so dramatically induced by the association and this gene its self is highly induced by mycorrhiza. This research must be considered pre-breeding work because the biological and agronomic significance of genetic variation in this important and ancient symbiosis is not fully understood.

Root-soil interactions and resource acquisition

The root system selectively responds to split application of nitrogen. (ETHZ)

Phenotyping of genotype-by-environment interactions in the root-zone is of major importance for crop improvement. Yet, the development of phenotyping methods to quantify such interactions is still in its infancy. We developed a system that is able to a) monitor root and shoot growth synchronously, b) investigate their dynamic responses and c) analyze the effect of heterogeneous N distribution to parts of the root system in a split-nutrient setup with a throughput (200 individual maize plants at once) sufficient for mapping of quantitative trait loci or for screens of multiple environmental factors. In a test trial, 24 genotypes of the EURoot maize set were grown under split nitrogen conditions and the response of shoot and root growth was investigated. An almost double elongation rate of crown and lateral roots was observed under high N for all genotypes. The intensity of genotype-specific responses varied strongly. For example, elongation of crown roots differed almost two times between the fastest and slowest growing genotype. A stronger selective root placement in the high-N compartment was related to an increased shoot development **indicating that early vigor might be related to a more intense foraging behavior**.

Responses of roots to local nutrient patches can help to prevent topsoil drought stress (RUN, CRAG)

Root systems of cereals are very responsive to the conditions that they encounter in the soil. The development of new roots and the branching patterns of these roots strongly depend on the environmental signals that are perceived by the root system. This typically results in the construction of a root system that is optimally adapted to the prevalent conditions. A very clear response occurs if roots grow into a hotspot of nutrients in an otherwise nutrient-poor soil. This evokes a local change in the architecture of the root system, as it triggers stronger branching and elongation of lateral roots. This results in a rapid colonization of the nutrient patch, and the greater root abundance enables then an efficient acquisition of the nutrient resources.

In an agronomic context, such selective root placement causes roots to grow in the upper layers of the soil, where the farmer mostly applies the fertilizers during or prior to the growing season. However, under conditions where progressive drought of this topsoil is expected, like in many Mediterranean areas, such a shallow root system will lead to problems in water uptake during the drier second half of the season. We, therefore, explored the possibility to “lure” roots into deeper soil layers prior to the expected drought period, by the use of deeper placed nutrient patches. If roots find these patches early in the season and respond to them with deeper clusters of dense root biomass, this would prevent problems with water acquisition later in the year.

To test this assumption, we sowed maize plants in large and deep containers filled with nutrient-poor soil, where nutrients were either applied in the topsoil only, or in deeper layers of the soil (**FIGURE 13**). Spores of a mycorrhizal fungus were also added to improve the chance for mycorrhiza formation and to study the effects of nutrient patches and topsoil drought on this process.

FIGURE 13

Rapidly after sowing, some maize roots were already found in the deeper layers of the soil, irrespective of where nutrients were placed. But, during the progress of the growing season, we found a very strong root development in the nutrient patches compared to the remainder of the soil (**FIGURE 14**). Apparently, **maize was well capable to produce more roots in response to the nutrient hotspots, and we found higher root densities than so far published in literature**. We did see, however, that very deep patches at approximately 45 cm below the soil surface were colonized more slowly, probably because roots took longer to find them.

FIGURE 14

Then, in summer, a number of containers were subjected to drought stress in the top half of the soil column. In these containers, we found an initial slower growth of the plants, linked to slower photosynthesis and water content, although at the end of the growing season the final height of all plants was similar and plants in drought apparently caught up with the irrigated control plants. However, there were significant differences in biomass, and in drought, plants that performed best were those where nutrients were placed deep in the soil, but not in a very condense patch but rather more spread out over the entire lower soil column. These plants performed ca. 10% better than plants in drought that received their nutrients in the topsoil, whereas plants with nutrients in a deep condense patch performed worst.

The reason for better performance in drought was probably linked to both an improved water uptake by root systems with more roots in the deeper, wetter soil, and an improved uptake of nutrients from these deeper soil layers. This is caused by the fact that topsoil drought does not only limit water uptake by the plants, but we also found that local nutrients became rapidly immobilized after the drying up of the upper soil layers, further affecting growth potential.

The conclusions from this experiment and additional trials in the greenhouse were that the **manipulation of rooting depth by deeper placement of nutrients could be an important tool to improve the performance of maize genotypes** that normally are not deep rooting under topsoil drought.

The effect of different nutrient treatments on root colonization by *Rhizophagus irregularis* was examined in maize plants (B73xUH007) grown in rhizotrons under different nutrient conditions (N, P, K), with nutrients either mixed throughout the substrate or in a concentrated patch, and under two water regimes (top soil: either well irrigated, drought; botton soil: always well irrigated). Root colonization by *R. irregularis* was found to be affected by soil nutrient and water availability, the highest colonization levels being observed when nutrients were mixed throughout the substrate compared to nutrients placed in a patch. The average colonization in the whole root system is negatively affected by drought, whether the nutrients are placed in the topsoil or in the bottom soil.

Root and rhizosphere-related traits determining phosphorus acquisition efficiency in field-grown maize hybrids vary with soil properties (INRA)

Phosphorus (P) is frequently limiting crop production in low input agro-ecosystems. Large progress has been achieved in understanding root traits associated with phosphorus acquisition efficiency in the past decades. Most of former studies were however conducted in controlled environment, avoiding the complexity of soil-root interactions. Thus, we aimed at identifying the predominant root and rhizosphere-related traits determining P acquisition efficiency in field-grown genotypes of maize, in contrasting soil conditions. For this purpose, we conducted a field experiment with 23 maize hybrids, obtained from inbred dent maize lines crossed to the inbred flint line UH007 as a tester. These hybrids were grown at two contrasting P levels of a long term P-fertilizer trial in two adjacent soil types: alkaline and neutral. The low-P level had not received any P fertilizer since 1968 and thus showed extremely low soil P availability, while the other level had been over-fertilized since 1968. Bulk soil, rhizosphere and root parameters were studied in relation to plant growth and P acquisition at two stages of development: vegetative (6-8 leaf) stage and flowering stage. An important finding was that the ranking of maize hybrids in terms of field performance at low P availability was not the same at vegetative and reproductive stages, which is challenging the environmental relevance of phenotyping root traits in young plants grown in artificial conditions. At the 6-8 leaf stage, we found that shoot biomass was 80% higher and P acquisition was more than double at high versus low P availability. Yet, root biomass was not modified by the P level, suggesting a major increase of root/shoot ratio under P-limiting conditions. Although root biomass was comparable at this vegetative growth stage, roots were finer and longer under such conditions. Across the 23 maize hybrids, P acquisition in P-limiting conditions was not positively correlated to the total root length, but there was a significant, positive, but rather poor correlation with the root diameter, and hence with the whole root surface area. At the flowering stage, the shovelomics approach, i.e. the excavation and imaging of the root crowns followed by image analysis with the REST software (Colombi et al, 2015), revealed that, across the 23 maize hybrids, **there was a positive correlation between maize performance (either shoot biomass or P acquisition) and root system depth, root system opening angle, and more so with root system area for the roots sampled from the topsoil**. A major finding was that the **soil type had a robust effect on root traits and P acquisition efficiency.**

The field performances of hybrids in one soil type were hardly related to those in the other soil type. In the neutral soil, roots exhibited higher specific root length, higher root/shoot ratio, P depletion in the rhizosphere and, ultimately lower P acquisition efficiency than in the alkaline soil. The best performing maize hybrids in the neutral soil were characterized by a 'foraging strategy' i.e. large root length and topsoil foraging. In contrast, in the alkaline soil, P acquisition efficiency and foraging traits were not correlated. In such conditions, maize roots increased P availability in the rhizosphere. Therefore, while in the neutral soil the 'foraging strategy' seemed to be predominant, the 'mining strategy' seemed to be governing in the alkaline soil. Our results indicate the key role of environmental factors such as soil properties for root traits determining high P acquisition efficiency in maize. **The study highlight the need to consider soil properties when breeding for high P acquisition efficiency as various soils are likely to require different crop ideotypes**.

Mycorrhizal status, microbial biomass and activities in the rhizosphere of field-grown maize hybrids at contrasting levels of phosphorus availability (INRA, CRAG)

Phosphorus (P) acquisition is known to be only partly determined by root system architecture in crops, as arbuscular mycorrhizal (AM) fungi and rhizosphere microorganisms can contribute significantly. The impact of soil P availability on these microbial communities has been little investigated in in field-grown crops though (**FIGURE 15**).

FIGURE 15

We achieved this for a range of genotypes of maize, at two contrasting P levels of a long term P-fertilizer trial in two adjacent soil types: alkaline and neutral. The low-P level had not received any P fertilizer since 1968 and thus showed extremely low soil P availability, while the other level had been over-fertilized since 1968. The experiment compared 23 maize hybrids, obtained from inbred dent maize lines crossed to the inbred flint line UH007 as a tester. Bulk soil, rhizosphere and root segments were assessed at the vegetative (6-8 leaf) stage of maize development**. Microbial biomass, catabolic activities and phosphatase activities were stimulated in the rhizosphere compared with the bulk soil.** This rhizosphere effect was larger for acid phosphatase (enzymes that are more specific to root activities) compared to alkaline phosphatase. Microbial biomass, catabolic activities and phosphatase activities were also increased at higher soil P availability, particularly for the neutral soil. As for observations on root traits, we observed a strong effect of soil type (alkaline versus neutral) on microbial parameters and phosphatase activities: **microbial and phosphatase activities were generally higher in the alkaline compared to the neutral soil type, and they exhibited a different catabolic activity profile.** In contrast with the effect of P availability, we observed very few significant effects of maize hybrids on soil microbial parameters. The level of colonization by one or another AM fungal species in a particular maize genotype varied depending on soil P levels. For the two most abundant AM fungal species found in maize roots, *R. irregularis,* its abundance significantly decreased at high P level, as usually reported. However, the opposite was observed for the second most abundant species, *Funelliformis mosseae*. Soil type did not affect root colonization for *R. irregularis*, while *F. mosseae* exhibited 3-fold more colonization in the alkaline than in the neutral soils. The negative impact of P fertilization on root colonization by AM fungi was observed in only about half of the maize genotypes. **This finding provides evidence against the usual expectation that high soil P availability reduces root colonization by AM fungi, which has been little studied in field-grown crops so far**. The *ZmPht1;6* maize phosphate transporter gene is an AM-inducible transporter involved in P acquisition through the AM pathway in symbiotic plants, and is thus an indicator of the functionality of the AM symbiosis. In most varieties (but not all), *ZmPht1,6* expression was higher at low than high P availability. Different levels of *ZmPht1;6* expression might be indicative of a functional diversity in P uptake in the range of studied maize hybrids. While we found a significant, positive relationship between root colonization by AM fungi and field performance at low P (shoot biomass at the 6-8 leaf stage of development), this relationship was rather weak and thus only partly explained the large range of variation of P acquisition efficiency observed amongst the 23 maize hybrids.

Root signaling in stress perception and tolerance

A hormonal signature is associated with deep rooting (ULAN)

There is growing physiological and molecular evidence that hormones interplay for determining various functions such as germination, stomatal aperture, defense against pathogens or many others. In this project, and using the set of maize hybrids and the skills of the partners in hormone determination, we identified an emerging hormonal fingerprint associated with steep rooting (i.e. roots making low angle from the vertical and thus contributing to root water uptake from deep soil layers). **Such type of root architecture is consistently associated with low ABA, low auxin, high cytokinins, and low salicylic acid. This is a major step forward towards the identification of drought tolerant varieties based on hormonal balance**.

We were keen to link hormonal signatures to root morphology that might contribute to a drought resistance phenotype. This was quantified here as a maintenance of the rate of accumulation of shoot biomass under soil drying. By studying 14 maize genotypes under well-watered, mild drought and severe drought for 17 days, we found that 1) there was significant genetic variation in crown and nodal root angles, and their plasticity to drought; 2) the crown and nodal root angles were positively correlated to each other as well as their plasticity; 3) maize genotypes with steep nodal root angles tended to show more stimulation or less inhibition in the size of the root system under drought; 4) size-related root traits (e.g. total length, surface area and biomass) combined with the root angle and its plasticity might be important predictors for plant drought resistance (maintenance of biomass accumulation as soil dries); 5) root angles were correlated with plant hormone levels (particularly ABA, tZ) under well-watered conditions, especially the root ABA; 6) the root hormone level changes (especially changes in ABA, ethylene and tZ) may be involved in regulating the root angle changes under drought. Specifically, four genotypes (i.e. UH250 × UH007, F98902 × UH007, EC169 × UH007 and FV353 × UH007) with relatively higher leaf ABA concentrations and lower root tZ concentrations tended to show shallower nodal and crown root angles. By contrast, root angles were not correlated with the leaf or root ethylene level. Therefore, **the data suggest that the levels of endogenous ABA and tZ, but not the ethylene in well-watered maize might be involved in determining root angles.**

Detailed analysis of root morphological responses to soil drying revealed that maize root angle in well-watered plants was related to root system size (as indicated by the total root length, surface area and dry weight) changes under drought. Different magnitude of change of root system size was exhibited among the 14 maize genotypes under drought, including stimulation, inhibition and no change. The nodal root angle of watered plants was negatively correlated with the increase in the root surface area under drought. Similar correlation was also seen between the nodal root angle and the increase in the total root length under drought though not statistically significant.

The total root length, surface area and dry weight are important traits for plant performance under drought since they determine the root system size and thus the plant's capacity for water and nutrient uptake. Some studies report that plants with larger root systems under drought showed better drought resistance. However, some other studies report that wheat and sorghum plants with steeper root angles display greater drought-resistance, which might be attributed to the fact that that they allocate dry weight to roots in deeper soil. However, our results showed combined responses to drought in both root size and angle. This suggests that **maize genotypes with steeper root angles outperform those with shallower root angles in dry soil and that this may also relate to their capacity to maintain a larger root size (more stimulation or less inhibition) under drought.**

Adaptation to uneven nitrate is associated with molecular fingerprints (INRA)

We questioned the potential role of hormones in determining root architecture responses to even or uneven distribution of nitrate in the soil, searching for hormonal fingerprints that could be best associated with local or systemic responses. Based on fine analysis of root growth of maize genotypes presenting marked root system architecture (deep and shallow rooting genotypes) cultivated on a 2D hydroponic based culture system, we observed on homogeneous stress different adaptive strategies with shallow rooting lines dramatically stimulating expression of nitrate uptake system without changing root architecture, lines favoring root growth stimulation for better foraging with little over expression of nitrate transporters and lines combining, at varying levels, both responses.

Based on an extensive literature survey and experimental approaches, we identified a large set of molecular markers for fingerprinting by quantitative RT-PCR. Using these markers, we were able to **correlate adaptive root response with differential expression of cytokinin and auxin markers in comparison to the non-responding genotypes.**

We then initiated a similar approach to delineate the sensing and signaling mechanisms involved in response to local uneven distribution of nitrate. Interestingly, the root adaptive response where markedly different from the ones observed on homogeneous nitrate limitation with the shallow rooting genotypes colonizing the rich nutrient patch much more efficiently resulting in an enhanced shoot biomass production. It is worth to remark that the observed expression pattern of cytokinin and auxin molecular fingerprints is also markedly different from the one observed on homogeneous medium confirming at the molecular level differences in the root adaptive responses. Taken together, these results open the door to design nitrate uptake more efficient varieties by combining and optimizing the observed adaptive strategies. Interestingly, **the breeding process can be significantly speed up using the identified molecular fingerprints**.

Transcriptomics confirms implication of hormonal signals and transcription factors in root growth under drought (RFWU)

A limitation in water supply is sensed by plant roots that convert the physical stress into a biochemical response. Several multi-layered signaling pathways are triggered to induce the downstream processes that initiate the adequate response. The complex metabolic and physiologic remodeling processes are conferred by changes in gene expression.

In this part of the project, we studied early gene expression changes in the maize primary root upon drought treatment. Young maize seedlings were subjected to short (6 hours) or long-term (24 hours), mild (-0.2 MPa) or severe (-0.8 MPa) water deficit treatment. Subsequently, transcriptome changes in the primary root and its four tissues were investigated. Transcriptomes were analyzed by RNA-sequencing and gene expression changes determined by comparison of data obtained from seedlings subjected to control and water deficit conditions.

Several hundreds of genes were detected as differentially expressed in response to drought. The more intense – more severe or longer – the treatment was, the more genes were differentially regulated. Furthermore, root tissue transcriptome analyses revealed that genes were often active in additional tissues upon water deficit i.e. expanded their zone of expression. Overall, the tissues reacted remarkably distinct and most differentially expressed genes were exclusively regulated in a single tissue. Functional categorization of regulated genes **confirmed the importance of hormonal signal transduction and transcriptional regulation during the drought stress response** as genes involved in these processes were overrepresented among stress responsive genes. For instance, genes involved in biosynthesis and signaling of the hormone ABA were induced in all root tissues and stronger up-regulated the more intense the treatment was. ABA can regulate a plethora of target genes and coordinates the drought response. ABA is also involved in the regulation of transcription factors (TFs) e.g. of the bZIP-type. bZIP TFs activate downstream stress responses like expression of chaperones that help mitigate cellular damage caused by water deficit. While bZIP TFs were regulated in all root tissues, many TFs were regulated in one specific tissue. Additionally, cellular protection processes were induced upon drought treatment, including modification of the membrane system, cellular redox balance, and cell wall architecture. Cell wall composition is crucial for cell elongation and thus growth. Especially in the apical region of the root an up-regulation of expansin expression was detected. Accumulation of expansins leads to an increase in cell wall extensibility and facilitates cell elongation so that root growth can be maintained even under severe water deficit conditions.

Together these findings **revealed that signal transduction pathways and cellular processes are coordinated in a spatio-temporal pattern with respect to tissue type and stress intensity/duration in the maize primary root under drought stress**.

Variability of lateral root growth, an important component of root efficiency, depends on auxin and sugars (INRA)

In maize as in most plant species, lateral roots (LR) display huge variability in terms of length, growth rates and duration. In the frame of the project, we measured elongation rates of ~ 6000 maize LR using rhizotrons comparable to those developed within WP4 and we designed a statistical package using stochastic (ie probabilistic) models to identify common patterns within this dataset. From this analysis, we identified 3 LR types that show early or late cessation of elongation or, by contrast, sustained growth for several days after emergence. It is suspected that these roots contribute to various root functions such as mineral or water uptake or relationship with the rhizosphere. We examined the meristem of these roots and found that decelerating roots had shrinking meristems until the last stages of meristem exhaustion. This analysis required the development of a statistical package to identify patterns (and regions) within root tip in order to correctly estimate meristem length from cell length data. In order to question the contribution of auxin and sugars as signals responsible for these various developmental patterns, we used various treatments (root excision, shading), biochemical and molecular assays (measuring sugars or sugar and auxin responsive genes in root tips) as well as auxin mutants. We found that in most cases, the probabilities associated with these types were altered indicating that these two signals are involved in the determinism of the intrinsic variability of LR. **Since both sugars and auxin are at the center of responses to environmental clues, they are also likely to be responsible for the changes of the proportion of LR types under various environmental situations such as the localized presence of minerals or water.** Preliminary analysis on various maize hybrids suggests a great genetic variability of LR intrinsic variability. This opens the door to better exploration of this trait in breeding programs in order to design optimized root systems for water and nutrient uptake, in particular under poor resources conditions.

The genetic variability of permeability to water depends on nitrate and is associated with aquaporins (UCL)

Root water uptake is the result of complex interactions between the distribution of roots, their hydraulic properties and the properties of the soil. Interestingly, this process is influenced by the nitrate concentration in the soil. Understanding the mechanisms by which maize pants regulate their root hydraulic conductivity (Lpr) is therefore essential. It involves the regulation of aquaporins, channels facilitating water diffusion through cellular membranes. Maize plants express a higher number of aquaporin homologs, including twelve plasma membrane intrinsic proteins (PIPs). These water channels are highly regulated at different levels including gene transcription, protein abundance, traffic in the secretory pathway and opening/closure at the plasma membrane, providing plants with the means to rapidly and reversibly modify the cell water permeability at different developmental stages and under varying environmental conditions.

In this context, we characterized the influence of short-term modifications of the nitrate availability on the Lpr of twelve different maize genotypes and investigated the contribution of PIP aquaporins in this process. Measurement of the Lpr in these genotypes in response to short term high nitrate availability and after recovery revealed a high variability of responses between lines: some of them did not respond to changes in nitrate concentration while other showed a significant increase in Lpr in presence of high nitrate concentration. Remarkably, these changes in Lpr are correlated to variation in the membrane permeability of single root cortex cell. In addition, the Lpr behavior after recovery varied also according to the lines. The relative levels of PIP aquaporin did not vary in the same way as it did for Lpr upon high nitrate and recovery treatments suggesting that **nitrate might regulate the subcellular trafficking and/or activity of aquaporins in a genotype-dependent manner**.
To get clues on PIP trafficking dynamics, transgenic maize plants expressing a fluorescently-tagged PIP isoform were treated with a solution complemented with high nitrate concentration. Analysis of the subcellular localization of the PIP aquaporin determined by confocal microscopy showed that nitrogen favors its accumulation in the plasma membrane.
In conclusion, this work demonstrated the **contribution of nitrate in the regulation of the root and cell hydraulic conductivity** in several maize genotypes. The regulation mechanisms involve modifications in the abundance of aquaporins in the plasma membrane. **These data will be very useful for breeders to optimize water use of maize crops in varying nitrogen environment**.

Mycorrhiza influence both drought tolerance and hormone signaling (CRAG)

Most terrestrial plants in both natural and agroecosystems have the ability to establish symbiotic associations with arbuscular mycorrhizal (AM) fungi. Root colonization by AM fungi improves the uptake of water and mineral nutrients (mainly phosphorus and nitrogen) in the host plant, in exchange of photosynthetically fixed carbon (FIGURE 15), thus improving plant growth and fitness. Yet another benefit conferred by the AM symbiosis in an improved level of tolerance to abiotic stress and/or resistance to pathogen infection. Improved growth and/or mineral nutrition in the presence of AM fungi have been associated with crop cultivars and root architecture. The input of agrochemicals might also have an impact on root colonization by AM fungi and mycorrhizal functioning. Moreover, **breeding programs might have selected maize genotypes that form ineffective associations with AM fungi** and, accordingly, the benefits received by the AM symbiosis may have been lost in modern cultivated maize varieties. At the molecular and physiological levels, successful symbiosis with AM fungi relies on the fine tuning and appropriate control of host gene expression and signaling processes, including hormone signaling.

FIGURE 15

In this context, we conducted studies to assess genotypic differences in colonization by AM fungi and the impact of the AM symbiosis on tolerance to drought stress in mycorrhizal maize plants. The maize genotypes were selected on the basis of their root architectural features when grown under field conditions. Although the degree of root colonization greatly varied among genotypes, all maize genotypes were found to be susceptible to colonization by the AM fungus *R. irregularis*. Of them, the MS153/UH007 maize hybrid (hallow/branched roots) showed the highest level of mycorrhization. Additionally, the mycorrhizal MS153/UH007 maize hybrid showed better performance and tolerance to drought stress than any of the other maize hybrids assayed in this study (FIGURE 16). There was a clear mycorrhiza-mediated regulation in the expression of genes involved in ABA biosynthesis (e.g. ZmZEP, ZmNCED), **supporting that the AM symbiosis positively influences both drought tolerance and hormone signaling** in the MS153xUH007 maize hybrid.

FIGURE 16

It is also known that root systems are endowed with a strong architectural plasticity that also manifests itself during the AM symbiosis, predominantly in lateral root proliferation. Lateral roots exhibit an increased responsiveness to AM fungal signaling molecules and, accordingly, are preferentially colonized by AM fungi. When examining the expression of genes involved in root development, ZmLrp1 expression was found to be up-regulated during the AM symbiosis, its expression remaining at high level in drought-treated mycorrhizal plants compared to drought-treated non-mycorrhizal plants. As ZmLrp1 functions in lateral root formation, this finding suggest that **under drought conditions, root development is more severely affected in non-mycorrhizal maize plants compared to mycorrhizal plants.** This is also consistent with the observation that roots of mycorrhizal MS153xUH007 exhibited a denser appearance and had higher biomass.

Together, these findings suggest that **a case-by-case study is needed when evaluating the impact of the AM symbiosis on drought tolerance in maize**. As the AM fungi provide the plant with extended arms that help them exploring soil nutrients for a better uptake of mineral nutrients and water, the use of mycorrhizal plants decrease dependence on chemical fertilizers and high water supply. Thus, the identification and use of maize hybrids benefiting from the AM symbiosis and better adapted to drought stress will greatly contribute to boost maize productivity with less inputs of agrochemicals in water-limiting agro-ecosystems.

Accurate phenotyping and modelling of the root system with reference to water uptake efficiency

Adaptation of controlled platforms and imaging devices to the cereal root system: from 2D imaging to 3D volumetric reconstruction of root architecture (FJZ, UNOTT)

To date, several plant phenotyping automated platforms based on non- or minimally- invasive measurements of plant development at high-throughput (i.e., hundreds of plants for each experimental run) have been developed with the aim to provide quantitative descriptors of shoot growth, such as leaf area development, shoot branching and plant photosynthetic status. However, quantifying root architecture development to determine genetic and environmental influences on valuable germplasm collection in cereals and doing so at high-throughput in phenotyping platforms is still subject of intensive research. In particular, soil-based rooting containers as rhizoboxes with a transparent interface have been used in the last two decades for observation of root architecture responses to water and nutrient availability.

We have established capacities for phenotyping roots non-invasively in 2D in soil-filled rhizoboxes towards the simultaneous characterization of root system architecture and shoot development over time series. By far the current potential and associated limitations of such platforms has not been explored systematically. We were able to provide evidence that RGB imaging based screening of root development in 2D spatial dimension is suitable to characterize in detail genotypic differences in cereals (total root length, root length density, number of lateral roots) using maize and rice genotypes that were selected together with other EURoot partners. Specifically, **we have designed protocols for standardization of these assays to quantify root architecture responses to localized and stratified supply of nitrogen and phosphorous.** These protocols can be applied for phenotypic characterization of cereal populations and extended to other important factors that influence nutrient uptake, such as a varying soil compaction to root penetration (Pfeifer et al, 2014).

The suite of 2D spatial characterization methods of root system using transparent media or soil-based assays has great value for the rapid characterization of genotype x environment interactions. However, 3D spatial reconstruction of root architecture with non- or minimally-invasive techniques remains of great importance to quantify local root responses to varying availability of soil resources and linking root architectural changes to models of water and nutrient uptake. The EURoot consortium had the opportunity to compare state-of-the-art methods for 3D root reconstruction. Work on this section of EURoot began with an exploratory experiment in which partners (FJZ and UNOTT) used both MRI and X-ray CT scanning to study the root system architectural traits of maize plants in combination with soil parameters and carbon allocation processes. Four replications of the maize line B73 x UH007 were grown in two soil bulk density treatments. The seedlings were germinated and scanned at UNOTT before being transported to FZJ for MRI and PET analysis. They were then returned to UNOTT for a second CT scan and harvest.

Comparison of MRI and CT-based root descriptions showed the two modalities to be complementary. MRI provides a more effective way to detect root material, as in most cases roots can clearly be distinguished from the background. MRI is, however, comparatively low resolution, which along with image noise causes breaks to appear in the reported root descriptions. Its greater contrast means that it can also slightly over-estimate the size of the root segments detected. In contrast, root detection in x-ray CT is challenging, as the density values associated with root material often coincide with those found in the surrounding soil. CT scans are, however, much higher resolution and, if root material can be detected, can provide a more accurate, description of root system architectures. To demonstrate this, root sections detected in the MRI data were used to both seed segmentation of roots from soil in CT data and set the parameters controlling that segmentation; the result was a more complete, higher resolution description of the roots. The comparison demonstrates the strengths and weaknesses of the methods concerned, and suggests that **identification of an initial, lower-resolution segmentation could lead to improved high-resolution results** (Figure 17).

FIGURE 17:

The experimental phase was followed by a workshop, held at FZJ, which brought together EURoot partners engaged in the development of image-based phenotyping methods. The aims of the workshop were to broaden the comparison of methods and identify commonalities in the image analysis techniques and approaches used within the EURoot consortium, key challenges, and possible future directions.

It was found that the traits extracted are broadly the same but, more importantly, that the systems discussed during the workshop take broadly similar approach to their extraction. Following the usual noise reduction and image enhancement operations, key features are extracted. These features are typically the top of the root system and a selection of root tips, but could include other points such as crossing points and short root sections. The initial data elements are then combined by a sequential tracking or tracing process to produce a structural description. That process typically involves optimisation of some cost or energy function comprising terms in the shape of the root and its fit to the available data. Both feature detection and tracing can include user interaction, but in general more user interaction is required to identify key features. The tracing is usually performed automatically, as a number of well-established algorithms are available. This leads to the suggestion that **root feature detection is now the major bottleneck – several speakers commented that it was unexpectedly challenging – and should be the focus of future work**.

Alongside these review and agenda-setting activities, partners' individual phenotyping methods and tools were developed to meet the experimental needs of the project. The level set segmentation method employed in the original RooTrak revisited, and an alternative tracking method employing particle level sets developed, to allow finer roots to be extracted. A prototype interactive version of the system was also produced, as it was discovered that biologists sometimes find the effect of changing the two parameters that control RooTrak's level set method difficult to understand.

Finally, a new software tool, RooTh, extracting RSML format architectural descriptions and associated trait measurements from segmented images was also produced. RooTh estimates the locations of a large set of points on each root from a series of directional passes through the segment, then adopts an active contour approach to fit a smooth curve to each root segment. The resulting root descriptions show a higher correlation with standard 2D root measuring tools than the previous single-pass curve fitting algorithm. (FIGURE 18)

FIGURE 18:

Imaging maize root traits in multiple field environments reveals high heritability but limited genotype-specific response to low nitrogen. (ETH, PSU, DSP)

To explore the impact of N availability on root system architecture and to investigate the impact of the growth environment, the whole EURoot set of 36 inbred dent maize lines crossed to the inbred flint line UH007 as a tester was evaluated for N-response over two years in three environments. Root system architecture was investigated by excavating and imaging of the root crowns followed by image analysis with REST software (Colombi et al, 2015). Despite strong site and year effects, trait heritability was generally high. Root traits showing the greatest heritability ($h^2 > 0.7$) were the width of the root system, indicative of the horizontal expansion, and the fill factor, a measure of the density of the root system. Heritabilities were in a similar range under high or low N application. Under N deficiency the root system size and its horizontal expansion decreased and the root systems became less dense. The lack of correlation between the root system density and its horizontal expansion indicates that these traits may be independently selected. For example a wide, sparse root system would increase lodging resistance and, potentially minimize carbon costs. As already found in the phosphorus field experiment of INRA, **root system depth assessed with REST was weak but positively related to plant performance** (yield and shoot weight). This is remarkable given the expected comparable low heritability (h^2 o.32-o.42) of the trait and its bias by the excavation process.
For most observed root traits the genotype ranking was not strongly altered by nitrogen fertilization, indicated by low genotypes-by-nitrogen interaction. Accordingly, **selection of the observed root traits may be made either under high or low nitrogen fertilization**. These findings indicate, that there is only a limited potential to utilized genetic variation in nitrogen-responsiveness for selection. By contrast, stay green and silage yield showed not only highest heritability but also a differential response of the tested genotypes to low nitrogen. Assuming that those responses may be related to differences in nitrogen uptake from soil, the shovelomics method may miss important root traits, related to the response to low nitrogen fertilization. **In conclusion, rather a constitutive change in root system architecture across environments and fertility levels may be a valuable selection target**.

FIGURE 19:

Correlating root response to nitrogen supply at seedling stage in a controlled platform to that of adult plants under field conditions (ETHZ, DSP, PSU)

A total of 15 seedling root traits (RADIX) were correlated with 11-14 adult root traits (shovelomics REST). **A total of 210 comparisons under low nitrogen conditions in the RADIX and in the field resulted in yielded 20 (10%) significant correlations.** Thirteen of these were related to maximum and medium lateral root length. The same comparisons under high nitrogen yielded only 8 (4%) significant correlations. Three of these were related to the maximum length of lateral roots. However, assuming 5% false positive correlations, we consider that there was no significant correlation. For the response index low/high N a total of 165 comparisons yielded 25 (15 %)

significant correlations. Eleven of these were related to the maximum length of lateral roots. Figure 20 shows on of these 11 correlations. A relative strong decrease of the longest root on the low-N part of the rhizoslides was correlated with a comparably weak response of root structure under low Nitrogen conditions in the field. Though not significant also other responses of root structure traits, such as branching density or the elongation of axile roots were negatively correlated to response measured by REST. This negative correlation is unexpected as root structure measures of REST are correlated with increased root structure in the field, as shown by Colombi et al. (2015). **This indicates that seedling responses and adult-plant responses are differing.**

FIGURE 20:

When data collected on platforms meet the models: from static to dynamic data extraction (JHI, INRA)

Mathematical model help identify strategies for optimal sampling in root screening. Making measurements of roots growing in soil is time consuming, costly and often destructive. Collecting data on the roots of crops is therefore a severe bottleneck and scientists are always faced with the dilemma of either collecting a higher number of replicates to improve the power of statistical analysis, or to increase the number of treatments to get a better grasp on the underlying biological processes. A key question is therefore what to measure and when. Combining data and mathematical models from the project, **EURoot scientists have discovered how the ability to detect changes in root properties is linked to the number of replicates**. Results indicates that it is usually safer to collect more replicates since detecting small temporal changes in root systems rely on the ability to describe accurately a root system at a given time point, which is directly linked to the number of replicates (FIGURE 21).

FIGURE 21:

Software pipeline facilitate analysis of phenotyping data from EURoot platforms. The project has employed various approaches to tackle the problem of root phenotyping, and information collected from these platforms usually consist of numerous images of partial or entire root systems observed on transparent interfaces with the growing media (rhizotrons or pouch systems). When crops are grown in rhizotrons or pouch systems, it is possible to capture a lot of information on the root system while the plant is growing, and a main challenge is to convert such visual observations into quantitative metrics that describe the dynamic of the growth of root systems both quantitatively and in a way that reveal the nature of the mechanisms of growth. This was achieved in the EURoot project through the development of a chain of computer processes that transform image data into growth parameters. Since these parameters can be obtained for any genotype and at any time point during growth, it **allows a fine mapping of the dynamics of the establishment of root systems in a range of environment.** The software pipeline starts with converting the traces of the root systems observed from the images into a vectorial or pixel format through images analysis techniques. The analysis then makes use of statistical estimators to derive root density maps from the images and then use mathematical rules (mathematical model) that evolve iteratively toward mimicking the experimental density maps. The method **allows root growth parameters to be obtained in a near automatic way (**FIGURE 22) **and also to determine temporal changes in root growth parameters in response to developmental or environmental cues**. It can also operate when only a partial description the root system is available.

FIGURE 22

Mathematical models help identification of genotypic factors linked to root growth. Root data from a range of experiments were collected during the project, and they have informed root research in various ways. First, measurements on root system images have been produced using the "rhizoscope" plateform on a set of 38 rice genotypes, using data from the CIRAD partner. The fine calibration process required for the construction of root architectural models was done using two types of data: rhizoscope pictures allowed the calibration of the parameters for the emission and growth of main roots, as well as root angle and gravitropism parameters; scanned images of roots allowed the estimation of branching parameters. The parameterization of architectural model showed a large genotypic variations for RSA of rice, and this was be used to study genotypic variations of the efficiency of water capture combining various RSA traits (described below - Modelling the influence of root architectural and anatomical traits and on RSA conductance and water uptake efficiency)

Barley, rice and brassica crops were also analysed using the software pipeline described above, with data being either static (Brassica grown in pouch), dynamic (Barley grown in pouch) or incomplete (rice grown in rhizotron). The method applied on these different systems showed **automated extraction of growth parameters is possible when the model used to describe the distribution of roots in space represent well the growth processes.** Finally, it was possible to show that **both genotypic and temporal factors affect root growth parameters, and that the genotypic variations in the mapping population used in the project have potential to segregate for root traits** (FIGURE 23).

FIGURE 23:

Modeling the influence of root architectural and anatomical traits on RSA conductance and water uptake efficiency (INRA)

Mathematical models have been developed to predict root growth and water and nutrient uptake from the soil. Accessing and studying roots is difficult because measurements in soil are usually destructive. That's why mathematical models and computer simulations are now seen as an essential part of the toolbox to help discover how roots acquire resources from soils and identify new genes related to these processes. In the EURoot project, models and software have been developed to describe the way water and nutrient flow around the root and are taken up by the root system. A modeling approach developed in EURoot, called "functional structural modeling", combines an explicit geometrical representation of the plant, its biological functioning and interactions with environment. A fine 3D representation of the root system has been done with the development of root architecture models based on basic root growth rules that describe the development and deployment in space of architecture of the root system. These virtual 3 dimensional architectures are then functionalized to represent the biological processes of acquisition and transport of water into and along the roots (see HydroRS software product highlight). The functional root architecture is then immersed in a 3D soil model that simulates the water transfer in soil and to roots. Linked to the soil and plant model, a coupling algorithm has been developed and implemented to describe the transfer processes at the root-soil interface and the interactions between neighboring roots for water acquisition. In this way, morphological roots traits, but also physiological properties (e.g. root hydraulic conductance), in interaction with soil type and hydric properties can be examined to test the efficiency of water uptake of various species/genotypes.

Along with the developed models, an important part of the work also focused on the estimation of the parameters of the mathematical models. Growth models were calibrated on a range of species (barley, rice and brassica in particular) using the automated pipeline described above, but additional calibration was also carried out on rice cultivars to obtain additional refined architectural information on root systems observed in the Rhizoscope platform of CIRAD.

Computer simulations reveal how changes in root architecture affect water uptake. As root systems are extremely complex structures, it is very difficult to understand how they function as a system.

The functional-structural mathematical models developed above were used to help biologists in exploring how the genotypic variations in root system architecture affect the ability of a crop to capture soil resources. Based on a combination of root traits, derived from a range of variation observed on rice cultivars, **computer simulations have been carried to test *in silico* a range of genotypes differing in root architectures for their abilities to acquire water from the soil**. Main root emission rate proved to affect water uptake very severely and could result in stress for young root systems, even in soil with available water (FIGURE 24). Length distribution and elongation of laterals is the second main factor, and proximal basal angle of the root system were also important factors in water acquisition for rice and drought stress resistance. Axial conductance of xylem in rice appears to be a limiting factor in water transport within the root system. The project also revealed that **the possible alteration of radial conductivity by root cortex decay, a process that often occurs in cereals, could lead to a decrease of uptake in upper soil parts, while increasing the share of deeper soil to transpiration**.

EURoot product highlight :
A barley root mutant resource (UNIBO)

All plants rely on their roots to acquire the water and mineral elements necessary for their survival in nature and/or their yield production or adaptability to different environments in agriculture. Despite all this, information about the genetic control of root architecture in barley and wheat is lagging behind, where very few mutants and genes have been described and no one was cloned.

Our aim was to search for mutants for altered root morphology and architecture within a pre-existing collection of barley mutant lines available at UNIBO, in order to create the first comprehensive collection of this type. A total of 3,071 mutagenized barley lines originally developed by treating seeds of the cultivar Morex were screened twice using a simple semi-hydroponic system on filter paper and lines showing altered root morphology were identified. These lines were further checked using soil-filled transparent rhizotron boxes, which enabled to not-destructively collect root observations (images were collected using a flatbed A3 scanner) during seedling growth.

We eventually identified 64 lines altered in root morphology and/or architecture, categorized as 52 mutants for root length (subdivided in short and long), eight mutants for root morphology (coiling, geotropic, sclerified), and four mutants root hairs (hairless and shorthairs). Examples of root mutant lines are shown in FIGURE 25.

FIGURE 25 :

The herein produced collection of root mutants is **the largest resource of this type assembled so far and represents an extremely valuable resource for both the scientific community involved in the molecular genetics dissection of root development**, and for plant breeding purposes. Indeed, the availability of extreme innovative root phenotypes (eg. longer roots, altered root angle growth, etc) will enable to test innovative ideotypes for plant breeding applications and could also be directly exploited by marker-assisted selection in cultivar improvement.

EURoot product highlight:
EURoot DB : a database for the genetic and molecular controls of root traits bridging cereals (CIRAD)

To facilitate access to the results of previous studies on root genetics, an information system (IS) called EURoot DB has been constituted, accessible at http://gohelle.cirad.fr:8080/euroot/JSP/. EURoot DB is composed of four MySQL databases (one per EURoot crop) that can be queried using Java customizable interfaces automatically generated to fit the database content. The IS is connected to a CMap vizualisation tool that enables a projection of genes or quantitative trait loci (QTLs) on genetic or physical maps of a given species and establishes links between chromosomal segments of the different species based on markers conserved across species.

The aims of the IS system was to assemble data on QTLs and genes for root growth and development under stress (drought, N and P deficiency) on rice, maize, barley, and wheat. Information on all constitutive or adaptive QTLs for root features previously published in good quality papers were collected by biologist curators of EURoot project, specialists of a specific crop, and integrated in the IS. The same work was done on genes with demonstrated function on rice roots. The positions of the QTLs and genes can be projected on consensus genetics or physical (for the sequenced genomes) maps of the four species. The IS includes only published data and, for this reason, does not include EURoot data yet.

More specifically, the barley database includes 86 QTLs from 10 studies. Since there is not a unique consensus map for barley, two maps, an SSR map with 967 markers and a DArT map with 2935 markers, have also been integrated in the database. The positions are given in cM.

The maize database includes 540 QTLs from 26 studies, presently positioned on 24 genetic maps. The IBM maps (physical and genetic) were also added in the database. The physical positions are those from the B73 genome.

The rice database includes a gene module, a QTL module and a trait module. The gene module includes 208 genes with validated function relative to roots in rice, coming from 109 studies with indication of the DOI of the reference sending back to the journal article. The rice QTL module includes 767 QTLs from 32 studies. The positions are given in Mbp on a synthetic physical map that includes 590 markers. The reference for the position of genes and QTLs is the Os-Nipponbare-Reference-IRGSP-1.0 pseudomolecule assembly. The trait module gives a short description of the trait, its acronym and the ID of the trait in Gramene Trait Ontology when known.

The wheat database includes 302 QTLs from 16 studies. Two genetic maps have been included.

For rice and maize, **bridges between physical maps were established using data on syntenic regions between the two species** recovered from the CoGe comparative genomic system (https://genomevolution.org/CoGe/), enabling to identify true orthologous genes based on the conservation of gene order between genomic regions related by common descent. The bridges encompass approximately 19,000 genes in 727 syntenic blocks and cover about 98% of the genomes. The positions of the limit of the syntenic blocks in rice and maize are used in CMap to display the syntenic relationships between the two species. However the limited number of maize QTLs with a physical position makes it a more useful tool to go from rice to maize than the reverse.

FIGURE 26:

EURoot Product highlight:
RADIX (ETHZ)

Within the framework of EURoot, the RADIX phenotyping platform was developed. The platform consists of rhizoslides (FIGURE27 A), a paper-based growth system optimized for regular, image-based observation of the root system (Le Marie et al. 2014). The system is optimized to characterize the responsiveness of root systems to soil resources varying in time and space (in t´Zandt et al. 2015; FIGURE 27 03 B). The rhizoslides were assembled in the RADIX platform consisting of a slide rack holding 100 slides, an automated irrigation system to supply differential nutrient concentrations and a semi-automatic imaging unit enabling the observation of root and shoot development (FIGURE 27 C).

FIGURE 27:

EURoot product highlight:
REST (ETHZ)

To acquire reliable and reproducible measurements of field-grown root systems within the EURoot consortium the software "root estimator for shovelomics traits" (Colombi et al. 2015). The software summarizes traits related to shape and structure of imaged root stocks of maize (FIGURE 28) but may be also utilized for similar purposes in other crops. It is working best with well-illuminated root systems in front of a black or blue background. REST is publicly available. A Windows version can be downloaded from is http://sourceforge.net/projects/rest4roots/.

FIGURE 28:

EURoot product highlight:
IMPROVED SENSORS (PreSens)

The efficiency of the consortium was also shown by the development of new commercial products. Only the close cooperation of SME and Research institutes enabled a very fast development and product release of two new sensing tools. Both of them are related to the 2D Imaging system to visualize nutrient distributions and root – soil interactions (**Figure 29**).

FIGURE 29:

Low-pH sensing foil LV1R
Up to now pH sensor foils for imaging applications were restricted to the pH range of 5.5 to 7.5. During EURoot a new pH Sensor was developed which covers the important range of 2.5 to 4.5. This sensor is already brought to the market:
http://www.presens.de/fileadmin/user_upload/downloads/manuals/IM_SF-HP5R_LV1R_dv1.pdf

Adapter tube
During the EURoot project a new adapter tube was developed to increase the field of sight of the Visisens and to allow an easier alignment of the instrument to the sensor foil (90° arrangement). This novel add on the Visisense camera was developed to enable easier readings in difficult geometrical situations.

During the EURoot project:

- pH, DO and NH3 distributions were measured by using PreSens Imaging systems:

- Time series of pH, DO and NH3 distributions were recorded during different growth periods and changing experimental conditions.

- Microsensors for high spatial resolution were established by PreSens to enable the partners to measure profiles not only close to the wall of the rhizotron.

EURoot product highlight:
HYDRO RS: A software for studying hydraulic traits of root systems.

HydroRS stands for a Hydraulic Model of the Root System. This software describes water uptake and transfer within roots from the scale of the single root to the whole root system. It couples a simulator of growing 3D root systems (adapted to cereals) with laws describing the fluxes of water into and along roots, accounting for the variable distribution of root hydraulic conductance within the root system. HydroRS helps examining interactions between root system morphology and topology (length of roots, branching density, branching order, main roots emission pattern…) and physiological parameters (axial and radial conductance and their distribution within the root system) on the water uptake pattern. The software has been made more efficient in calculation speed by making using of multi-core CPUs and code parallelization during the EURoot project. In this version, if variation in soil water potential within soil can be accounted for, however, no water transfer in soil is considered as the 3D soil water simulator is not included.

The initial model description can be found in: Doussan C., Pagès L., Vercambre G., 1998, Modelling of the Hydraulic Architecture of Root Systems: An Integrated Approach to Water Absorption - Model Description. Annals of Botany. 81: 213-223.

Information about the software (computer requirements, download) can be obtained from claude.doussan@inra.paca.fr

IV POTENTIAL IMPACTS

TOWARDS NEW TARGET TRAITS FOR CEREAL ROOT BREEDING

Root hairs are environmental sensors for stress perception in cereals

By using mutants altered in the root hair formation, EURoot scientists have shown that the presence of root hairs influence the stimulation of ABA synthesis by allowing efficient water stress sensing. Conversely, absence of root hairs makes plant rather blind to stress perception and consequently more vulnerable to the detrimental effect of water stress since it does not timely induce defense and protective genes. Aside their role in nutrient and water uptake, EURoot evidenced the role of root hairs as sensors of environmental conditions. This could have a considerable impact if following a survey of diversity for root hair traits (eg density) across genetic resource a correlation can be found with the speed of plants stress responses.

Also, as EURoot scientists have shown that the function of genes involved in root hair growth are conserved among cereals, findings in one species may prove of general interest for the other cereals.

These results call for an intensification of the effort of phenotyping the long-neglected root hair traits in genetic resources.

The current status of knowledge on root hair formation in monocots is presented in a review publication written under the EURoot project entitled "Root hair development in the grasses: what we already know and what we still need to know?" (Marzec et al. 2015).

Variability of lateral root development is influenced by sugars and auxin

Cereals have a complex, fasciated root system, of mainly post-embryonic origin consisting of crown roots emerging from phytomers. Along the main root axes, several type of lateral root (LR) are produced having a determined or undetermined growth and different growth speed.

EURoot scientists have confirmed that auxin and sugars are at the centre of responses of environmental cues and have demonstrated that they are likely responsible for the changes in proportion in LR types. In the frame of the project, we also have identified sugar and auxin responsive genes using bibliographic information and experimental validation. These markers could be of great use for further analyses devoted to signaling questions in maize roots. Moreover, the various statistical pipelines developed will be made available to the community.

As a follow-up of this project, using the statistical pipelines developed with EURoot, we are currently running very similar analyses in millet and find very similar LR patterns. **This suggests that the intrinsic variability of LR is a common feature of (at least) cereals and confirms that this undermined trait is an avenue of exploration for future breeding programs.**

Root efficiency rather than root size is a key trait for phosphorus acquisition

EURoot scientists have identified root efficiency, defined as phosphorus acquired per unit of root size, as a major trait to breed for phosphorus-deficient soils such as lateritic soils, which are common notably in Africa, South America and South East Asia. Donors with high root efficiency at acquiring P have been identified in rice diversity panels in the frame of the project and will be used as donors in breeding programs. **One of the *aus* accessions is notably already used as a donor to improve adaptation to low soil fertility in Africa and efforts to identify underlying and isolating candidate genes are ongoing**.

Comparable trait, syntenic regions and orthologous genes are being explored in other cereals. Also, progresses have been made towards the cloning of genes underlying other traits important in P uptake. **The discovery of this new target trait is of immense breadth for breeding cereals for low input/organic agriculture.**

Existence of genetic variation for amenability to beneficial root colonization by mycorrhizae suggests breeding for that trait is possible

The variation for mycorrhizal colonization existing in genetic diversity panels has been examined in the frame of the project by using core panels in maize and very large panels for association mapping in rice. EURoot scientists have shown that genetic variation exists for cereal amenability to being colonized by arbuscular mycorrhizal fungi (AMF) and that chromosomal segments underlying that trait can be identified. In lab experiments, genetic variation has been found in both the diversity and density of AMF species colonizing the cereal root system. A beneficial influence of AMF on both root development and drought tolerance has been evidenced in one maize hybrid. This very promising result needs to be expanded both to a range of field conditions and of genetic materials. Also, relationships between abundance of AMF colonization and beneficial effects on nutrient uptake and drought tolerance has to be further investigated. Nevertheless, progresses brought by EURoot suggest that **breeding for amenability to colonization by AMF, conducting to beneficial consequence on nutrient acquisition, drought tolerance and root system development, can be envisaged.**

Selective crown root placement is linked to early vigor and might reflect foraging intensity

EURoot scientists have found that selective crown root placement in platform pouches with split nitrogen application is correlated to the early vigor and shoot development of maize hybrids: therefore early vigor, which is a very important trait for crop establishment on the field may be related to a more intense foraging behavior. **We propose using the differential response of crown roots to split-nutrient application to quantify foraging behavior in genome mapping or selection experiments**. Screening for that trait is achievable at rather high throughput on platforms.

NEW MARKERS FOR BREEDING AND GENE ISOLATION

Markers for Root depth and drought tolerance

Root at depth, notably influenced by root angle, has long been considered as a major trait to breed for to achieve drought tolerance in avoiding progressing topsoil drying. Root depth and drought tolerance traits have been investigated in barley where a single, small (29 lines)-sized resource of powerful genetic resolution, RCSLs derived from the cross between Harrington and a wild barley, have been extensively phenotyped in the frame of EURoot. The RCSLs have indeed been challenged for performance under stress under field conditions in two sites (tunnel and rainfed/irrigated) for 2 years, examined for root profile on the rainfed/irrigated trial, and grown in soil columns for root system scanning and reconstruction (X-ray CT). Some extreme lines have also been examined in pouches under lab conditions. This unique comparison is still yielding results (due to the time needed for data processing) but has shown a significant correlation between X-ray CT root area and root volume and field root length depth in the 0-30 cm soil depth layer. In addition, correlations were observed between X-ray CT root area and root volume and field root dry matter in the 0-30 cm soil depth layer. Chromosomal segments involved in heading date and yield components under drought have been delineated.

Lines associating a deep root system, high biomass and harvested grain have been identified that will be very useful in breeding barley lines tolerant to field drought conditions. Marker trait associations co-locating for field and X-ray-CT root traits will be investigated as well as association with field-based traits including biomass and grain yield. This will be the first time that marker-trait associations have been reported for X-ray-CT root traits.

The detailed field data on variation in the core spring barley germplasm (Harrington elite barley x Caesarea 26-24 wild barley; 29 RCSLs) in root system architecture traits, canopy development and water-use will provide crop simulation modellers with data for identifying quantitative

frameworks to predict effects of genotype-specific root parameters on water uptake underpinning improved drought tolerance in cereals.

The genetic analysis of field root system architecture traits has revealed chromosome segments associated with novel variation in root length density at depth. This information will be made available to breeders via CerealsDB providing targeted chromosomal segments to introgress into their existing elite germplasm. To allow fine mapping of these identified genomic regions we have backcroosed all 29 RCSLs to the recurrent parent via a programme of selfing developed populations for future use by breeders and reserachers. Also the material (F1s of one of the RCSLs) generated is being used as part of ClimBar which is funded under FACCE ERA-NET Plus Joint Programming Initiative on Climate Smart Agriculture (http://plen.ku.dk/english/research/plant_soil/breeding/quality/climbar/).

Also, EURoot scientists have progressed towards the isolation of a gene involved in deep rooting in Rice. The corresponding chromosomal interval is already used in breeding programs notably in India. Isolation the gene will soon allow to study its presence and allelic variation in rice genetic resources and possibly to look at orthologous genes in cereals, to breed for deep root system.

Rice knowledge generated in EURoot is being used in a FACCE-JPI project (GREENRICE) coordinated by CIRAD and associating two EURoot partner.

Markers for breeding for Nitrogen Use efficiency

A cluster of QTLs for grain yield at low fertilization level was identified in a **durum wheat association diversity panel. Added to several additional chromosomal regions identified. EURoot results considerably strengthened the potential interest of marker-assisted generation of new cultivars with enhanced nitrogen use efficiency that will be of great value for adaptation to cropping systems with reduced inputs.** This panel has been phenotyped for early root traits and effort to correlate some of these traits with field nitrogen use efficiency is underway**.** The study and identification of correlations between agronomics (eg. grain yield) and physiological traits (eg. NDVI) will support the introduction and use of indices such as NDVI and Red-nir as early predictors of the plant performance. Elite cultivars/lines providing superior nitrogen efficiency for biomass/protein content in low input practices or in standard agronomic practices have been identified. The collection of agronomic data is suitable for updating the best fertilization practices for Durum Wheat varieties.

In rice, the detection of QTLs for root growth in low N situations allowed the detection 31 chromosomal regions some being common across traits. None of these regions seemed to correspond to genes known to be involved in root development in rice. **Altogether, EURoot efforts enabled to establish a list of markers linked to chromosomal regions and donors of favorable alleles that can be used in breeding for better nitrogen use efficiency, and thereby reduce the need for inputs in agriculture.**

NOVEL RESOURCES FOR GENE DISCOVERY ACROSS CEREALS

An unique root mutant resource for gene discovery

Valuable genetic stocks that have been produced in the framework of EURoot can be made available to the scientific community. Notably of resource of 69 root mutant has been identified in Barley (see EURoot product highlight) **that is a wealth for further discovering new regulator and effector genes involved in root development**. The collection includes mutants of extremely variable architecture, including mutants altered in root length, and geo/hydrotropism response. The mutants of this collection should provide an entry point to clarify the genetic control of root development and architecture in barley and wheat. Mutants could be tested in field trial for their effect on yield and/or agronomic traits in variable environmental conditions. **It is possible that some mutants might have a direct positive effect on grain yield at least in specific environments**. Several scientists outside EURoot have already shown interest in accessing root mutants of this collection for collaborative studies. **The protocol of quick mapping and cloning of root mutants can be applied to any type of**

traits, provided that the visible mutations have a simple genetic basis. A Material Transfer Agreement provided by the resource producer will define under what conditions on a case-by-case basis (freely, collaboration, restricted access, etc).

A database to bridge discoveries across cereals

EURoot DB (http://gohelle.cirad.fr:8080/euroot/JSP/) was developed by Cirad in the framework of Work Package 1 (Genetic and functional bases of root architectural traits under abiotic stress). EURoot DB is based on MySQL databases (one per crop) that can be queried using Java customizable interfaces automatically generated to fit the database content. It is connected to a CMap vizualisation tool. EURoot DB is a component of the South Green Bioinformatics platform. This database aims at managing data on QTLs and genes for root growth and development under stress (drought, N and P deficiency) on rice, maize, barley, wheat. Information on all constitutive or adaptive QTL/genes for root features previously published in good quality papers were collected by biologist curators of EURoot project (see contributors), specialists of a specific crop, and integrated in the IS. The same work was done on genes with demonstrated function on rice roots. The positions of the QTLs and genes can be projected on consensus or physical (for the sequenced genomes) maps of the four species. For rice and maize, bridges between physical maps were established using data on syntenic regions between the two species recovered from the CoGe comparative genomic system (https://genomevolution.org/CoGe/), **enabling to identify true orthologous genes based on the conservation of gene order between genomic regions related by common descent.** The bridges encompass approximately 19,000 genes in 727 syntenic blocks and cover about 98% of the genomes. The existence and value of this tool needs to be publicized on EURoot website and in publications.

TOWARDS NEW FERTILIZATION PRACTICES

Placement of nutrients at depth help cereal preparing their root system for sustaining progressive drought

EURoot scientists have demonstrated that deep placement of nutrients attracts roots to deeper soil layer which may prepare plants to better respond to a topsoil dryness progressing during the crop cycle. **This indicates that deep placement of nutrients may improve the performance of maize hybrids**. On the other hand, as higher root density is observed in nutrient patches, notably in surface ones, it would be worthwhile to spread the nutrients at depth, which may prove difficult to implement as a field practice.

Spatial variation of soil pH favors contrasting root strategies for phosphorus acquisition in low P fields

We have demonstrated that soil type pH has a major influence on the root trait development strategy that maize has to favor for efficiently acquiring P. Whether a positive correlation between maize performance, root system depth and angle and shoot biomass and P acquisition was observed in neutral soil, no correlation between P acquisition efficiency and foraging traits was conversely observed in alkaline soil. In other words if root traits favoring an efficient foraging of the root system are desirable in neutral soil, root traits favoring a mining strategy through intense exploration per unit of soil volume by lateral roots or release of exudates are desirable in alkaline soil. **Therefore the best-adapted crop root ideotype might be rather different according to field soil pH. T**he possibility of combining these seemingly opposite ideotype behaviors has to be investigated. **These results have important implications in the screening procedures in breeding programs and soil management practices.**

EURoot scientists have also evidenced that microbial and phosphatase activities which influence immobile P mobilization are largely influenced by soil pH, in being higher in alkaline soil and are stimulated in the rhizosphere of field grown plants. However, the plant genotype seems to do not influence soil microbial parameters. Though the usual expectation is that high soil P availability reduces root colonization by AM fungi, we have demonstrated that AMF species abundance and diversity in roots of field grown maize plants may vary in opposite manner with P fertilization and maize genotype. **This has also important implications in screening for amenability to establish AMF symbiosis in a context of developing agro-ecological practices.**

TOWARDS NEW DIAGNOSTIC SIGNALS FOR ROOT ADAPTIVE POTENTIAL

Hormone related markers for steep rooting, response to uneven nitrate and mycorrhization

EURoot scientists have found that a hormonal signature is associated with steep rooting, a desirable trait for drought avoidance. This signature associates low ABA, low auxin, high cytokinin and low Salicylic acid. Altogether, this shows that ABA and the trans-zaetin cytokinin may be involved in determining root angles. Also a molecular fingerprint correlating adaptive root response to uneven nitrate involving the differential expression of cytokinin and auxin markers has been identified. Furthermore we found a clear mycorrhiza mediated regulation in the expression of genes involved in ABA synthesis

Following confirmation on a large range of genotypes and contrasting situations these signals could be used as diagnostic tools for adaptability of the root system to major environmental cues that could be further implemented in large scale screenings of breeding programs

Nitrate contributes to root hydraulic conductivity

EURoot scientists have shown that variation in response of root hydraulic conductance to high nitrate is related to a genotype dependent regulation by nitrate of the subcellular trafficking or activity of water channels, the aquaporins**. These findings linking two abiotic constraints together may help developing strategies to optimize water use for plants grown under different nitrogen fertilizer regimes.**

Transcriptional changes associated with water deficit /osmotic stress vary with tissue type

Our work has shown that signal transduction pathways and cell processes are coordinated according to a spatio-temporal pattern with respect to tissue type and stress intensity and duration. **This work could now be extended to other genotypes with contrasting root behavior and drought tolerance.**

NEW PHENOTYPING METHODS AND TOOLS WITH IMPROVED RELEVANCE TO FIELD CONDITIONS

EURoot scientists have further optimized and standardized existing automated root phenotyping platforms for 2D architecture analyses quantifying root architecture responses in soil in response to water and nutrient supply. In particular, the GROWSCREEN-Rhizo platform at FZJ has been further optimized for studies of cereals roots and their responses to localized and stratified supply of N and P. **These platforms can now be further used for the characterization of cereal populations and to study the influence of abiotic factors including other constraints such as soil compaction.**

The X-ray-CT platform at UNOTT has been developed involving scanning of soil columns and using software developed at UNOTT called RooTrak for segmenting roots from the soil background. After segmentation of a 3-dimensional root system as a volume, another new software developed at UNOTT, RooTh, has been used to skeletonize the data to determine the root system architecture including numbers, angles, and lengths of all root classes, such as primary, seminal, and lateral. Also, the capacity to deal with high genetic resolution panels (here a 29 line population of barley RCSLs) through a scaling up and standardization of procedures has been demonstrated for the first time. **This enabling technology will pioneer our understanding of the genetic control of cereal root architecture. We anticipate that many new projects will flourish and many more opportunities to**

screen for key traits will arise as a consequence of all the novel methodology developed in this project.

We have also shown the great complementarity of root trait data acquired in MRI and X-ray CT devices for describing the complexity of root architecture. Root feature detection remains a major bottleneck to be investigated in further research projects.

Collectively, these approaches have already proven very valuable for a number of national and international projects providing access to user groups in academia, namely through the EU-funded EPPN project (http://www.plant-phenotyping-network.eu/) and in industry alike via bi-lateral collaborations (for example, http://www.fz-juelich.de/ibg/ibg-2/DE/Aktuelles/Meldungen/Dokumente/kws_collaboration.html?nn=548490). It is anticipated that the demand of the plant research community and of the private sector will remain high for several years. To respond to this demand, these phenotyping systems will be used to address root-related research questions in EU-H2020 upcoming calls and will remain part of the EU Research Infrastructure program including ESFRI. Recently, the new project (EMPHASIS) dedicated to plant phenotyping infrastructure in Europe has been officially listed as part of the ESFRI roadma

A first major question addressed by EURoot scientist was to determine whether the root traits observed in platform and field experiments are heritable. This was investigated in maize and barley. In maize, the conclusion resulted from data collected in six contrasting environments (2 years x 3 locations), which **were used to valuate how heritable root responses to nitrogen are. Despite a strong site and year effect, the heritability was generally high. Root traits showing the highest heritability > 0.7 were the maximal width, indicative for the horizontal expansion of the root system and the fill factor, a measure for the density of the root system. Heritabilities were in a similar range under high or low N application. In barley, heritabilities of the field root system architecture traits (e.g. root length density) were sufficiently high to justify genetic analysis of the field root traits.**

A second major question was to determine whether root system data collected from platforms on young plants provide scientists with relevant information with what is observed at later developmental stages under field conditions. This has been investigated for the nitrogen response in maize (pouches experiment with split N application under artificial growth conditions and nitrogen fertilization regimes under field conditions: root system development is respectively observed from actual observation at a very early stage and estimated from shovelomics excavation at adult stage) and the drought response in barley (severe or mild drought imposed to plots grown on the field under rain-out tunnel or not respectively and in soil column in greenhouse : the root system development is respectively observed from field excavation and micro CT scans and 3D reconstruction respectively).

Though the wealth of information is still being processed, notably in barley at time of writing this report, results in maize show that from a total of 210 comparisons for 15 seedling root traits (RADIX) under low nitrogen conditions and 11-14 adult root traits (shovelomics, REST), **20 (10%) significant correlations were found. Thirteen of these were related to maximum and medium lateral root length. For the response index low/high N a total of 165 comparisons yielded 25 (15 %) significant correlations. Eleven of these were related to the maximum length of lateral roots. These results show that despite the fact that seedling responses and adult-plant responses are likely very different, some important root traits are positively correlated.**

INNOVATIVE TOOLS

During the course of the project EURoot scientists measured pH, DO and NH3 distributions using PreSens Imaging systems. Notably, time series of pH, DO and NH3 distributions were recorded during different growth periods and changing experimental conditions. Microsensors for high spatial

resolution were established by PreSens to enable the partners to measure profiles not only close to the wall of the rhizotron. To address specific questions, a new pH Sensor was developed which covers the important range of 2.5 to 4.5. This sensor is already brought to the market: http://www.presens.de/fileadmin/user_upload/downloads/manuals/IM_SF-HP5R_LV1R_dv1.pdf
The experimental set ups of EURoot also challenged engineers to develop and adapt new products. Notably, a special adapter was constructed, produced and delivered to Nijmegen to make measurement in the semi field rhizotrons, which are equiped with transparent tubes to gain access to soil, rhizosphere and roots. **EURoot contributed to make these tools more precise and of extended range for more applications to investigate the biogeochesmistry of the rhizosphere.**

INNOVATIVE MODELS

EURoot scientists have been working on how to better integrate information generated by root screening facilities (also termed root phenotyping platforms). The method allows root growth parameters to be obtained in a near automatic way and to detect temporal changes. It can also operate when only a partial description the root system is available. New software is available for download at www.archiroot.org.uk. **This new software pipeline allows handling of root data and information generated by geneticists: this will help identification of genes related to root growth.**

Mathematical models were used to propose a strategy for optimal sampling of root data during experiments. EURoot scientists have discovered how the ability to detect changes in root properties is linked to the number of replicates. Results indicates that **it is usually safer to collect more replicates since detecting small temporal changes in root systems rely on the ability to describe accurately a root system at a given time point**, which is directly linked to the number of replicates.

EURoot scientists have developed new models and software to describe the way water and nutrient flow around the root and enter the root system.
Computer simulations have been carried where extreme root characteristics observed in some genotypes were tested in silico for rice for their abilities to acquire water from the soil. Root emission rate and length distribution and elongation of laterals and proximal basal angle of the root system were also important factors in determining water uptake. The project also revealed the possible alteration of radial conductivity by root cortex decay could lead to a decrease of uptake in upper soil parts but these phenomena could be compensated by an increase in uptake deeper in the soil. We can now test in silico a range of genotypes differing in root architectures for their ability to acquire water from the soil. Such functional-structural RSA models coupled to soil processes enable to study impacts of RSA traits variations on uptake processes. **It can be used on specific soil/plant/climate scenarios to study RSA traits influence on tolerance to drought or designing ideotypes. Identification of realistic multi-stress resistant genotypes that can be tested in the field remains a long-term objective**.

Altogether, EURoot has generated a large amount of output. It includes 25 refereed journal articles, a database of relevant QTLs in cereal crops, reports on markers and alleles for QTLs, genetic material in the form of mutants, near isogenic lines and mapping populations, a root phenotyping platform, software for image analysis and modeling and products for improved use of optodes for assessing soil chemical processes. A great deal is available now, or on request or through purchase. Project partners will continue to promote these in their everyday work. Some output, especially journal articles, will continue to be produced, and the website will be used to ensure these are available so that they can be used

The EURoot project has increased our understanding on how roots of cereal crop plants capture heterogeneously distributed or limited resources while maintaining whole plant performance, and,

in particular, how the efficiency of resource capture and use is altered by rainfall scarcity resulting in a drying topsoil.

Gathering a wide range of expertise and innovative platforms it has contributed to our understanding of the mechanisms underlying the uptake of water and major nutrients, as well as the complex interactions between soil, water and root growth for efficient resource capture linked to whole plant performance.

The experiments undertaken in EURoot enabled to make progress in identifying genes involved in root development under various conditions of stress in cereals and in locating them with much more precision in the genome, up to the identification of one or few candidate genes.

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DISSEMINATION OF EURoot

Logo

The EURoot logo is shown in **Figure 30**

Website

The EURoot web site has been established in the first 3 months of the project and maintained since then. University of Aberdeen has committed to keep it updated with EURoot results at least till 2019 The web site home page can be seen in **Figure 30**.
http:// EURoot.eu

Leaflet

The EURoot leaflet has been prepared in the early course of the project and distributed to partners for dissemination to visitors and stakeholders and in congresses or public events.

Publications

Altogether 25 publications acknowledge EURoot contribution (either review or original papers). The partners have elaborated an impressive list of 16 manuscripts in preparation. At the end, EURoot publications may be reach about 40.

PhD theses

EURoot supported the work of 11 PhD students who have defended their thesis during the course of the project or will defend their theses shortly after.

Communications

Altogether, more than 100 communications, oral or poster have been presented in national or international symposia or congresses by EURoot partners.
Adam Price and the Society for Experimental Biology (SEB) organized a session of 2 and a half days was held at the SEB annual meeting in Prague 30th June to 2nd July 2015 called “Plant roots: New challenges in a changing world”.
Seven manuscripts acknowledging EURoot contribution have been published in the special issue of Journal of Experimental Botany published in February 2016 (**Figure 31**).

Workshops and summer schools

The DPPN/EURoot Winter School on Root Phenotyping took place from the 2nd to the 6th of November at the Institute of Bio- and Geosciences (IBG), IBG-2 Plant Sciences in Jülich. This one week course addressed mainly young researchers and covered a wide range of topics and to demonstrate methods highly relevant to root phenotyping in lectures and practical exercises related to: > Root structure and function in 2D & 3D > Shovelomics > Image based identification of root traits > Modelling > Data management. In total 16 PhD students and young Post-Docs from 10 different countries attended this course and were able to establish new important contacts. The Winter School comprised theoretical lectures as well as excursions and practical exercises, therefore the students were able to put their knowledge to practical use.

Other disseminations towards stakeholders

Fascination of Plants Day. This was a Europe wide event day on the 18th of May 2015 (http://www.plantday12.eu/home.htm) and several EURoot partners made contributions.