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Root cortical aerenchyma enhances nitrogen acquisition from low nitrogen soils in maize (Zea mays L.)

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Summary: Abundant root cortical aerenchyma improves plant growth under nitrogen-limiting conditions by decreasing root metabolic costs, enhancing soil exploration in deep soil strata, thereby increasing N acquisition at greater depths.
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Abstract
Suboptimal nitrogen availability is a primary constraint for crop production in developing nations, while in rich nations intensive nitrogen fertilization carries substantial environmental and economic costs. Understanding root phenes that enhance nitrogen acquisition is therefore of considerable importance. Structural-functional modeling predicts that root cortical aerenchyma (RCA) could improve nitrogen acquisition in maize. We evaluated the utility of RCA for nitrogen acquisition by physiological comparison of maize Recombinant Inbred Lines (RILs) contrasting in RCA grown under suboptimal and adequate N availability in greenhouse mesocosms and in the field in the USA and South Africa. Nitrogen stress increased RCA formation by 200% in mesocosms and by 90-100% in the field. RCA formation substantially reduced root respiration and root N content. Under low N conditions, RCA formation increased rooting depth by 15% to 31%, increased leaf N content by 28% to 81%, increased leaf chlorophyll content by 22%, increased leaf CO₂ assimilation by 22%, increased vegetative biomass by 31% to 66%, and increased grain yield by 58%. Our results are consistent with the hypothesis that RCA improves plant growth under N limiting conditions by decreasing root metabolic costs, thereby enhancing soil exploration and N acquisition in deep soil strata. Although potential fitness tradeoffs of RCA formation are poorly understood, increased RCA formation appears be a promising breeding target for enhancing crop nitrogen acquisition.

Keywords: Zea mays L., root cortical aerenchyma, RCA, mesocosm, nitrogen
**Introduction**

Nitrogen (N) deficiency is one of the most limiting factors in maize production worldwide (Ladha et al., 2005). In developing countries such as those in sub-Saharan Africa, less than 20 Kg N ha\(^{-1}\) is applied to fields of smallholder farmers due to high fertilizer cost (Azeez et al., 2006; Worku et al., 2007). In developed countries intensive N fertilization is used to maintain satisfactory yield (Tilman et al., 2002). In the USA, N fertilizers are the greatest economic and energy cost for maize production (Ribaudo et al., 2011). However, less than half of N applied to crops is actually acquired, and most of the remaining N becomes a source of environmental pollution (Raun and Johnson, 1999; Smil, 1999; Tilman et al., 2002). For example, N and P effluents into marine systems from agriculture cause eutrophication and hypoxic zones (Diaz and Rosenberg, 2008; Robertson and Vitousek, 2009). Nitrate contamination in surface water and groundwater systems poses serious health risks such as methemoglobinemia and N-nitroso-induced cancers (UNEP and WHRC, 2007). Emission of nitrous oxides (N\(_2\)O) from agricultural activities contributes to ozone damage and global warming (Kulkarni et al., 2008; Sutton et al. 2011). Furthermore, the production of nitrogen fertilizers requires considerable energy from fossil fuels, and since energy costs have risen in recent years, farmers face economic pressure from increasing nitrogen fertilizer costs, which are linked to higher food prices. It is estimated that a 1% increase in crop nitrogen efficiency could save more than 1 billion US dollars annually worldwide (Kant et al., 2011). Therefore, even a small improvement in nitrogen efficiency would have significant positive impacts on the environment and the economy.

Soil nitrogen is heterogeneous and dynamic. The bioavailability of soil N depends on the balance between the rates of mineralization, nitrification, and denitrification. These processes are determined by several factors including soil composition, microbial activity, soil temperature, and soil water status (Miller and Cramer, 2004). The predominant form of soil nitrogen available to plants in most agricultural systems is nitrate, which is highly soluble in water and thus mobile in the soil (Barber, 1995; Marschner, 1995). Mineralization of organic matter and/or the application of nitrogen fertilizer at the beginning of the growing season followed by precipitation and irrigation...
create a pulse of nitrate which may exceed the N acquisition capacity of seedlings and
leach below the root zone. Therefore, it has been proposed that increasing the speed of
root exploration of deep soil strata could benefit nitrogen acquisition (Lynch, 2013).
However, the structural investments and metabolic expenditures of root systems are
substantial and can exceed half of daily photosynthesis (Lambers et al., 2002). Full
consideration of the costs and benefits of root systems is therefore crucial for identifying
root traits to improve crop production especially in water and nutrient deficient
environments (Lynch, 2007). Taking rhizoeconomics and the spatiotemporal availability
of soil nitrogen into account, (Lynch, 2013) proposed a root ideotype for enhanced N
acquisition in maize called “steep, cheap, and deep”, in which ‘steep’ refers to
architectural phenes and ‘cheap’ refers to phenes that reduce the metabolic cost of soil
exploration. One element of this ideotype is abundant root cortical aerenchyma.

Root Cortical Aerenchyma (RCA) consists of enlarged air spaces in the root cortex (Esau,
1977). RCA is known to form in response to hypoxia and the role of RCA in improving
oxygen transport to roots of many plant species under hypoxic conditions has been well
researched (Vartapetian and Jackson, 1997; Jackson and Armstrong, 1999; Mano and
Omori, 2007; Mano and Omori, 2013). Interestingly, RCA can also form in response to
drought and edaphic stresses such as N, P, and S deficiencies (Bouranis et al., 2003;
Drew et al., 1989; Fan et al., 2003; Zhu et al., 2010), which suggests that the benefit of
RCA extends beyond facilitating oxygen transport. Several lines of evidence suggest that
RCA enhances root metabolic efficiency under stress. Fan et al. (2003) found that RCA
formation significantly reduced root segment respiration and P content of root tissue,
which allowed greater shoot growth in soils with low phosphorus availability. Under
drought, maize (Zea mays L.) genotypes with high RCA formation had greater root
length, deeper rooting, better leaf water status, and eight times greater yield than closely
related genotypes with low RCA (Zhu et al., 2010a). Effects of RCA on root respiration
were more pronounced for large-diameter roots compared to small-diameter roots
(Jaramillo et al., 2013). Results from the functional-structural plant model SimRoot
showed that RCA formation could be an adaptive response to deficiency of N, P, and K
by decreasing the metabolic cost of soil exploration. By reducing root respiration, RCA
decreases the carbon cost of soil exploration, and by decreasing the N and P content of
root tissue, RCA permits internal reallocation of nutrients to growing root tissue, which is particularly beneficial under conditions of low N and P availability (Postma and Lynch, 2011a). Under suboptimal P availability, RCA increased growth of a simulated 40 day-old maize by 70% (Postma and Lynch, 2011b). In the case of nitrogen, RCA increased the growth of simulated maize plants up to 55% in low N conditions, and plants benefit from RCA more in high N leaching environments than low N leaching environments (Postma and Lynch, 2011a). In addition, the formation of RCA decreases critical soil nutrient levels, defined as the soil fertility below which growth is reduced, suggesting that cultivars with high RCA may require less fertilizer under non-stressed conditions. These in silico results suggest that RCA has potential utility for improving crop nutrient acquisition in both high- and low-input agroecosystems.

The overall objective of this research was to assess the utility of RCA for nitrogen acquisition in maize under nitrogen-limiting conditions. Maize ‘near isophenic’ recombinant inbred lines (RILs) sharing a common genetic background (i.e. descending from the same parents) with common root phenotypes but contrasting in RCA formation were grown under nitrogen stress to test the hypothesis that RCA formation is associated with reduced root respiration, reduced tissue nutrient content, greater rooting depth, enhanced N acquisition, and therefore greater plant growth and yield under N limitation.

Results

RCA formation and nitrogen stress

Nitrogen (N) stress substantially increased RCA of plants grown in mesocosms (GH2010) by an average of 200% at 35 d after planting (DAP). The increase in RCA was significant in all root classes: primary roots (62%, p=0.015), seminal roots (218%, p<0.001) and second whorl crown roots (74%, p=0.0454) (Figure 1). N stress did not affect root diameter, cortical cell file number, and xylem diameter of the root segments collected 20-24 cm from the base of the primary, seminal, and second whorl crown roots (Table I). The genotypes were grouped based on RCA phenotypes in the second whorl crown roots, which has been shown to be the representative position of RCA distribution in maize roots system (Burton et al., 2013b). Low RCA RILs consisted of 133, 177, and
337 and high RCA RILs consisted of 196, 199, and 345. We found that the differences among RCA phenotypes were accentuated by low N treatment. Low RCA RILs averaged 5% of the root cortical cross sectional area as RCA, while high RCA RILs averaged 18% RCA under low N conditions (Figure 2).

At the field site in South Africa (SA) N stress increased RCA of the plants by an average of 102% at flowering. Low RCA RILs (1, 157, and 177) averaged 9% RCA, while high RCA RILs (31, 34, and 338) averaged 19% RCA under N stress (Figure 2). At the field site in Pennsylvania (PA) N stress increased RCA of the plants by an average of 94% at flowering. Low RCA RILs (1, 85, 97, 157, and 165) averaged 5% RCA, while high RCA RILs (56, 82, 224, 284, and 353) averaged 16% RCA under N stress. RCA of High RCA RILs was significantly greater than that of low RCA RILs under low N conditions in all environments (p<0.05, Figure 2).

**RCA, root respiration, and root tissue N content**

RCA reduced root respiration in both mesocosm studies (GH2010 and GH2013) and in the field (Figure 3,4,5). High RCA RILs had less specific root respiration than low RCA RILs by 39% under high N conditions and 42% under low N conditions in GH2010 (Figure 4). In GH2013 N stress reduced root segment respiration of the second whorl crown roots by 1.3 fold and the N content by 5.25 fold (Figure 5A and 5B, p<0.001). Under low N conditions RCA was negatively correlated with root segment respiration \((r=-0.75, p<0.05)\) and root tissue N content \((r=-0.60, p<0.05)\). The regression equation between root segment respiration and RCA indicated that conversion of 10% and 11% of cortical area to RCA reduced root segment respiration and N content by 50% (Figure 5C).

**RCA and root growth**

In GH2010, N stress reduced the average total root length of all genotypes by 42%. High RCA RILs had 35% greater total root length than the low RCA RILs under low N conditions \((p<0.05, \text{Figure 6})\). Nitrogen stress increased rooting depth \((D_{95}; \text{the depth attained by the 95th percentile of root length})\) of all genotypes by 29%. \(D_{95}\) of high RCA
RILs was 15% greater than that of low RCA RILs under low N conditions (Figure 7). In South Africa (SA), the D_{25} of high RCA RILs was 31% greater than that of low RCA RILs at flowering under low N conditions (Figure 7).

**Photosynthesis, nitrogen acquisition, and shoot mass**

Under low N conditions in mesocosms the chlorophyll content of high RCA RILs was 22% greater than that of low RCA RILs (Figure 8A). Nitrogen stress reduced leaf photosynthetic rates on average by 8%. The high RCA RILs had 22% greater photosynthetic rates than the low RCA RILs under low N conditions (Figure 8B). In GH2010, nitrogen stress reduced the shoot biomass of all genotypes by 58%. Under N stress, high RCA RILs had 66% more shoot mass and 68% greater tissue N content at 35 DAP compared with low RCA RILs (Figure 9). In the field in SA, N stress reduced shoot mass by an average of 35% at flowering. The high RCA RILs had 52% greater shoot mass and 81% greater tissue N content than low RCA RILs at flowering under low N conditions (Figure 9). In the field in PA, N stress reduced shoot mass by an average of 36% at flowering. The high RCA RILs had 31% greater shoot mass and 28% greater tissue N content than low RCA RILs under low N conditions (Figure 9). The regression equation between grain yield and RCA indicated that grain yield of the highest RCA genotypes was 58% greater than that of genotypes with no RCA under low N conditions (Figure 10).

**Discussion**

In this study we show that N stress induces RCA expression in greenhouse and field conditions, which confirms earlier reports in solution culture (He et al., 1992). This effect was stronger in maize lines with high RCA formation under high N (Figure 2). Experiments in mesocosms revealed that RCA substantially reduced root respiration and tissue N content (Figure 3,4,5). Under suboptimal N availability, high RCA RILs had greater rooting depth than low RCA RILs in the field in South Africa (Figure 7). High RCA RILs had greater shoot biomass than low RCA RILs under low N conditions in all environments observed (Figure 9). At the field site in PA, RCA was associated with 58% increased grain yield under low N conditions (Figure 10). Our results are consistent with
the hypothesis that RCA enhances N acquisition by reducing root metabolic costs, decreasing tissue N content, permitting greater rooting depth, enhanced N acquisition, and greater plant growth under suboptimal nitrogen conditions.

In this study we evaluated the utility of RCA in RILs segregating for RCA expression but sharing a common genetic background. In studies of the effects of individual alleles, it is desirable to compare isogenic lines varying for that allele. RCA is a typical quantitative trait controlled by many alleles in unknown ways (Saengwilai 2013). Analysis of three maize RIL populations (B73xMo17, OH43xW64a, and NY821xH99) identified 5 QTL for aerenchyma area explaining from 4.7 to 9.4 % of phenotypic variation, and 6 QTL for percent aerenchyma explaining from 5.6 to 12.9 % of phenotypic variation (Burton 2010). Different QTL were observed in the three populations, and QTL observed in these maize RILs did not correspond with previously reported QTL for aerenchyma induced by hypoxia in maize x teosinte crosses (Mano et al., 2007). It is therefore not possible to generate simple isogenic lines that vary for RCA formation across maize inbreds- many allele variants and combinations would need to be generated and compared for such a study. This study is focused on the phenome, and specifically on the physiological utility of RCA. For such a study it is desirable to vary RCA while holding other aspects of the plant phenotype as constant as possible. RILs are ideal for this purpose since each RIL represents a distinct genotype combining a shared set of alleles from common parents. In these experiments, our goal was to select ‘near isophenic’ RILs with common root phenotypes other than RCA, to minimize the potential effects of variation in nodal root number, root growth angles, lateral root branching, and crown root diameter (Supplemental table S1) in root deployment and N acquisition. An alternative way to compare contrasting ‘isophenic’ lines is in silico, where every feature of the plant phenotype can be controlled, as accomplished in SimRoot (Postma and Lynch, 2011a; Postma and Lynch, 2011b). The combination of results from the field and from mesocosms is noteworthy, as the field includes variable environmental factors such as soil temperature, soil biota, and soil physical properties that may affect results, while mesocosms are simplified soil environments that permit greater environmental control and more detailed measurement of root properties. The fact that our results with
contrasting RILs in mesocosms and two field environments agree with each other as well as with previous *in silico* results is strong evidence that they are robust.

We found variation in RCA formation in maize RILs under unstressed conditions and greater RCA formation with suboptimal availability of N. These results are consistent with other studies (He et al., 1992; Zhu et al., 2010a). Interestingly not all RILs increased RCA in response to N stress, particularly low RCA RILs (Figure 2). Genetic variation for the degree of RCA formation in response to N stress suggests that breeders could select for genotypes with consistently high, low or plastic RCA. The utility of phenotypic plasticity of RCA is currently unknown, but genetic control and the utility of plastic traits such as root hair length have been documented in maize (Zhu et al., 2010).

RCA reduces root respiration (Figure 3; Fan et al., 2003; Zhu et al., 2010a). Root respiration associated with growth, maintenance, and ion uptake are major components of root metabolic costs (Lambers et al., 1996; Lynch & Ho, 2005). Without root maintenance respiration, simulated maize plants had up to 72% greater growth under nutrient limiting conditions (Postma and Lynch, 2011a; Postma and Lynch, 2011b). An additional benefit of RCA is reallocation of nutrients from cortical tissue, which is predicted by simulation modeling to be an important function in N and P deficient plants (Postma and Lynch, 2011a). In this study, we found that high RCA RILs had less root respiration than low RCA RILs under both stressed and non-stressed conditions (Figure 4). High RCA was also associated with reduced root tissue N content in low N soils (Figure 5). Nitrogen in lysed root tissue of high RCA plants could be reabsorbed and utilized to support plant growth, as evidenced by greater root and shoot growth of high RCA RILs compared to low RCA RILs in low N soils. These results are consistent with responses found under suboptimal availability of phosphorus and water (Fan et al., 2003; Zhu et al., 2010a). The results support our hypothesis that reduced root maintenance cost allows high RCA RILs to support a larger root system and have greater soil exploration than low RCA RILs.

Fan et al. (2003) showed that 20% RCA reduced root respiration by 50% in seminal root segments of maize. In our study, we found that around 30% RCA is needed to reduce root
respiration of crown root segments by half (Figure 3). Crown and seminal root anatomy
are fundamentally similar but these root classes differ in size and number of cells; crown
roots tend to have greater diameter, more cortical cell layers, and larger cortical area
(Burton et al., 2013). It has been shown that root respiration is substantially influenced by
living portions of the root segments such as living cells in the cortex (Jaramillo et al.,
2013). Since crown roots have a larger proportion of living tissue than seminal roots, we
would expect that more RCA would be required in order to significantly affect root
respiration in crown roots.

Distribution of roots in soil influences nutrient and water acquisition efficiency. For
example, shallow rooting is beneficial for acquisition of topsoil-available nutrients such
as phosphorus and potassium (Lynch and Brown, 2001), while deeper rooting allows
plants to acquire highly mobile resources such as water and nitrate before it is lost from
the root zone (Ho et al., 2005; Kristensen & Kristensen, 2000; Postma & Lynch, 2011;
Zhu et al. 2010). Under low N conditions, high RCA RILs had greater rooting depth (D95)
in the mesocosms and in the field (SA) than the low RCA RILs (Figure 7). Since the high
RCA RILs had reduced metabolic costs for root maintenance compared to the low RCA
RILs, the high RCA RILs are able to support more root growth resulting in greater
rooting depth, which could enhance nitrogen acquisition in low N soils. Enhanced
nitrogen acquisition in the deep soil profile resulted in greater leaf N content, chlorophyll
content, and photosynthesis, which benefitted overall plant growth and yield (Figure
8,9,10).

In the field, we found that the utility of RCA was greater in the loamy sand of the SA
field site than in the silt loam of PA. Although the relative reduction in shoot mass caused
by N stress was similar between sites, plants in SA were 2.5 times smaller than plants at
PA under low N conditions (Figure 9), which indicated that they suffered from greater
stress. The temperature in SA was greater than in PA, and may have been supraoptimal
for these temperate maize lines. At flowering, shoot biomass of high RCA RILs in SA
was 52% greater than that of low RCA RILs whereas shoot biomass of high RCA RILs in
PA was only 31% higher than that of low RCA genotypes. In high leaching environments
such as the loamy sand in SA, the benefit of increased rooting depth could be more
pronounced since nitrate leaching is more rapid in coarser soils. These results are consistent with simulation results (Postma and Lynch, 2011a).

Selection for high RCA may indirectly select for greater ethylene sensitivity (He et al., 1992), which may affect other adaptive root traits. In this study, we carefully selected RILs and compared root phenes such as angle, number of crown roots and root branching under high and low N conditions (data not shown). We found no significant difference for other root anatomical phenes between high and low RCA RILs grown in mesocosms (Table I). We conclude that the results observed in this study are primarily due to contrasting RCA phenotypes.

Knowledge of interactions among phenes is essential in developing ideotypes for nutrient efficient crops. Interactions among root phenes could result in synergistic or antagonistic effects on resource acquisition. As an example of an antagonistic interaction, increased adventitious rooting in common bean reduces growth of lateral roots arising from the tap and basal roots, which results in reduced P acquisition in low P soils (Walk et al. 2006). As an example of a synergistic interaction, under low P conditions, common bean gains more benefit from having long root hair length combined with shallow root angle than would be predicted from the additive benefits of each phene in isolation (Miguel, 2011). As for RCA, simulation modeling predicts synergism between RCA and lateral root branching density in maize under low P conditions (Postma and Lynch, 2011a). Under low N conditions, RCA benefits metabolically costly root phenes such as a greater number of crown roots because more crown root number allows greater volume of soil exploration at the expense of root growth and maintenance (York et al., 2013). Since RCA reduces metabolic costs for root growth in general, we propose that RCA may also be synergistic with root phenes that enhance soil exploration in different soil domains such as root angle.

Substantial genetic variation for RCA occurs in maize and its relatives in the genus *Zea* (Burton et al., 2013). This suggests that there may be costs associated with RCA. It has been shown that RCA contributed to reduced root hydraulic conductivity in maize roots under low P conditions (Fan et al., 2007). RCA formation also inhibits radial
transportation of nutrients such as phosphate and calcium (Hu et al., 2014), although the importance of these small effects in older root segments for nutrient uptake of entire root systems is unclear. In addition, RCA may affect the colonization and spread of microbes within roots. For example, in wheat, cultivars with high root cortical cell death are more susceptible to common root rot (Deacon et al., 1982). RCA may have less effect on disease susceptibility than does cortical cell death, since after RCA formation the epidermis remains intact. RCA formation may reduce mycorrhizal symbiosis, which requires living cortical tissue. RCA may also affect the mechanical strength of roots, especially in plant species that lack a structural support in the outer part of cortex, although maize was not in that category in a study of resistance to radial compression (Striker et al., 2007). The cost/benefit of RCA and its interactions with other root traits are likely to be complex and may differ in different environments. This merits research.

There is increasing evidence that RCA enhances water and nutrient capture under drought and edaphic stress (Fan et al., 2003; Zhu et al., 2010a; Postma and Lynch, 2011a; Postma and Lynch, 2011b). This report empirically demonstrates the benefit of RCA for N acquisition from low N soils. Genetic variation of RCA is present in several important agronomic species including wheat, barley, sorghum, rice, common bean, and maize (Colmer 2003; Fan et al. 2003; Haque, et al. 2012; Liljeroth 1995; Promkhambut et al. 2011; Zhu et al. 2010), making RCA amenable to plant breeding. We suggest that increased RCA formation may be a promising breeding target for enhancing nitrogen acquisition from low N soils, and for reducing the N requirement of high input agriculture.

Materials and Methods

Greenhouse mesocosm study

Plant materials

Seeds of maize RILs from the Intermated B73 and Mo17 (IBM) population were obtained from Dr Shawn Kaeppler (University of Wisconsin, Madison, USA) (Senior et al., 1996; Kaeppler et al., 2000). Previous screening indicated that RILs 337, 133, 177
had low RCA, and RILs 196, 199, 345, had high RCA under low N conditions. These
RILs were planted in greenhouse mesocosms in 2010 (GH2010). A set of six IBM RILs
(14, 111, 106, 43, 101, and 199) were planted in greenhouse mesocosms in 2013
(GH2013) to examine the effect of RCA on root tissue nitrogen content.

**Experimental design**

The experiments were arranged in randomized complete block design. The factors were
two nitrogen regimes (high and low nitrogen conditions), six RILs, and four replicates
over 4 blocks. Planting was staggered one day between replicates with time of planting as
a block effect.

**Growth conditions**

Plants were grown during October 4 to 24 November, 2010 for GH2010 and during
September 23 to October 29, 2013 for GH2013. The greenhouse is located on the campus
of The Pennsylvania State University in University Park, PA, USA (40°48′N, 77°51′W),
with a photoperiod of 14/10 h at 28/24 °C. Seeds were soaked for 1 h in a fungicide
solution consisting of benomyl (Benlate fungicide, E.I. DuPont and Company,
Wilmington, DE, USA) and 1.3 M metalaxyl (Allegiance fungicide, Bayer CropScience,
Monheim am Rhein, Germany) and then were surface-sterilized in 10% NaOCl for 1 min.
The seeds were pre-germinated in rolled germination paper (Anchor Paper Company, St.
Paul, MN, USA) soaked with 0.5 mM CaSO$_4$ and placed in darkness at 28°C in a
germination chamber for two days. At planting, the plants were transferred to mesocosms
consisting of PVC cylinders 15.7 cm in diameter and 160 cm in height. The mesocosms
were lined with transparent hi-density polyethylene film to facilitate root sampling at
harvest. The growth medium consisted of a mixture (volume based) of 50% medium size
(0.5 – 0.3 mm) commercial grade sand (Quikrete Companies Inc., Harrisburg, PA, USA),
35% horticultural vermiculite, 5% Perlite (Whittemore Companies Inc., Lawrence, MA,
USA) and 10% topsoil. The topsoil was collected from the Russell E. Larson Agricultural
Research Center in Rock Springs, PA (Fine, mixed, semiactive, mesic Typic Hapludalf,
pH ≈ 6.7, silt loam). Thirty-three liters of the mixture was used in each mesocosm to
ensure the same bulk density of the media. One day before planting the mesocosms were
saturated with 5 liters of a nutrient solution adjusted to pH 6. In GH2010, the nutrient solution for the high N treatment consisted of (in μM): NO₃ (7000), NH₄ (1000), P (1000), K (3000), Ca (2000), SO₄ (500), Mg (500), Cl (25), B (12.5), Mn (1), Zn (1), Cu (0.25), Mo (0.25) and FeDTPA (100). For the low N treatment, NO₃ and NH₄ were reduced to 70 and 10 μM, respectively. In GH2013, nitrate was used as the only nitrogen source for both high and low N treatments. Two germinated seeds were sown per mesocosm and were thinned after 4 days to one plant per mesocosm. Plants were watered every other day with 100 ml of deionized water. Environmental data were collected hourly in the greenhouse using a HOBO U10-003 data logger (Onset Corporation, Pocasset, MA, USA). Soil solutions were collected at 20 cm depth intervals weekly using a micro-sampler 2.5 mm in diameter and 9 cm in length (Soilmoisture Equipment CORP., Santa Barbara, CA, USA). The solutions were stored at -80 °C until processing. The concentrations of nitrate in the solutions were determined using vanadium (III) chloride protocol according to (Doane and Horwáth, 2003).

**Root sampling, root segment respiration and root distribution in mesocosms**

Shoots and roots were harvested at 35 d after planting. At harvest, the polyethylene liners were removed from the mesocosms and laid on a root washing station. Root segments were collected 20-24 cm from the base of the primary, seminal, and second whorl crown roots. The samples were stored in 75% EtOH at 4°C until processing and analysis. For root distribution studies, the liners were divided into 20 cm segments starting from the base of the shoot. Roots were cut and separated from each segment by carefully washing with tap water. The roots were preserved in 75% EtOH. Total root lengths were obtained by scanning and analyzing preserved root samples using WinRHIZO Pro (Régent Instruments, Québec City, Québec, Canada). Whole root respiration was measured one day before harvest in GH2010 according to (Jaramillo et al., 2013). In short, an acrylic plate was placed around a single plant on the top of the mesocosm and carefully sealed with modeling clay around the stem of the plant. The plate was connected to a Li-6200 IRGA (LI-COR, Lincoln, NE, USA) with polyethylene tubing to measure the respiration of the whole root system. Carbon dioxide concentration was monitored for 2 min for each plant. Root respiration per unit length was calculated by dividing the rate of whole root
respiration with the total root length obtained by WinRHIZO Pro as described above. Root segment respiration was measured on three 4 cm root segments of second whorl crown roots in GH2010 and on three 8 cm root segments in GH2013. The segments were excised 20 cm from the base of the root and lateral roots were removed with a Teflon-coated blade. Twenty minutes after excision, the samples were placed in a chamber connected to a Li-6200 IRGA (LI-COR, Lincoln, NE, USA) in GH2010 and to a LI-6400 IRGA (LI-COR, Lincoln, NE, USA) in GH2013. For both experiments, the temperature of the chamber was maintained at 27°C using a water bath. Carbon dioxide evolution from the root segments was recorded every 5 seconds for 180 seconds. After the respiration measurements, the root segments were stored in 75% EtOH for anatomical analysis.

**RCA measurement**

In GH2010 root cross-sections were obtained by hand-sectioning with Teflon-coated double-edged stainless steel blades (Electron Microscopy Sciences, Hatfield, PA, USA). The root sections were examined on a Diaphot inverted light microscope (Nikon, Chiyoda-ku, Japan) at 2.8x magnification. Three sections were selected as subsamples for image capture. The microscope was fitted with a black and white XC-77 CCD Video Camera Module (Hamamatsu, Iwata-City, Japan). ImageMaster 5.0 software (Photon Technology International, Birmingham, NJ, USA) was used to capture and save images. Analysis of images was performed in MatLab 7.6 2008a (The MathWorks Company, Natick, MA), using RootScan which is a program for semi-automated image analysis of anatomical traits in root-cross sections (Burton et al., 2012). RCA was expressed as percentage of the root cortical area. In GH2013 the roots were ablated using laser ablation tomography (Saengwilai, 2013). In brief, laser ablation tomography is a semi-automated system that uses a laser beam to vaporize or sublimate the root at the camera focal plane ahead of an imaging stage. The sample is incremented, vaporized or sublimated, and imaged simultaneously. The cross-section images were taken using a Canon T3i (Canon Inc. Tokyo, Japan) camera with 5X micro lens (MP-E 65 mm) on the laser-illuminated surface.
 Shoot dry weight and plant nitrogen status

For both GH2010 and GH2013, one day prior to harvest, leaf gas exchange of the second youngest fully expanded leaves was measured with a LI-6400 Infrared Gas Analyzer (LI-COR, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1200 μmol photons m⁻² s⁻¹ and constant CO₂ concentration of 400 ppm. At harvest, 6-mm diameter leaf discs were collected from the second youngest fully expanded leaves for chlorophyll measurement. Chlorophyll was extracted in 80% acetone. The concentrations of chlorophyll a and b in the extracts were determined at the wavelengths of 663.2 and 646.8 nm with a spectrophotometer (Lichtenthaler and Buschmann, 2001). Shoots and root segments were dried at 60 °C for 72h prior to dry weight determination. The shoots were ground and 2-3 mg ground tissues were used for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer, Shelton, CT, USA).

Field studies

 Field conditions, experimental design, and plant materials

Experiments were carried out during February to April in 2010 at Alma, Limpopo province, Republic of South Africa (SA) (24°33′ 00.12 S, 28° 07′25.84 E, 1235 masl) and during June to August in 2011 at the Russell Larson Research and Education Center of the Pennsylvania State University in Rock Springs, PA, USA (PA) (40°42′37″.52 N, 77°57′07″.54 W, 366 masl). The soils at the experimental sites were a Clovelly loamy sand (Typic Ustipsamment) in Alma and Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalf) in Rock Springs. Based on soil analysis at the beginning of growing season, N fertilizers were applied at the rate of 30 kg N/ha 5 times until flowering resulting in 150 kg N/ ha in total for high N plots at Alma. Low N plots received 30 kg N/ ha only at the beginning of growing season. At Rock Springs, fields were amended with 915 g/m² of sawdust to immobilize soil N. High N plots were fertilized with 150 Kg N/ha of urea while low N plots did not receive any N fertilizer. In both environments, soil nutrient levels of other macro and micronutrients were adjusted to meet the requirements for maize production as determined by soil tests. Pest control and irrigation were carried out as needed. Based on previous experiments conducted in
the field (Saengwilai et al., unpublished), six IBM RILs consisting of low RCA RILs (1, 157, and 177) and high RCA RILs (31, 34, and 338) were planted at Alma and ten IBM RILs consisting of low RCA RILs (1, 85, 97, 157, and 165) and high RCA RILs (56, 82, 224, 284, and 353) were planted at Rock Springs. The experiments were arranged in a split-plot design with the two nitrogen treatments as the whole plot factor, and genotype as the split-plot. Five-row plots of each genotype (six meters long) were randomly assigned within each whole plot. Row width was 75 cm, and distance within a row was 23 cm, resulting in a planting density of 5.80 plants m\(^{-2}\). The plants were harvested at 9 weeks after planting (flowering stage) at the SA and the PA field.

**Root sampling, root segment respiration and root distribution in the field**

At harvest, three 4 cm root segments of second whorl crown roots were excised from 8-12 cm away from the base of the root and lateral roots were removed with a Teflon-coated blade. The three root segments were placed in a tube chamber connected to a LI-6400 IRGA (LI-COR, Lincoln, NE, USA). The temperature of the chamber was maintained at 27°C using a water bath. Carbon dioxide evolution from the root segments was recorded every 5 seconds for 180 seconds. After the respiration measurements, the root segments were stored in 75% EtOH for anatomical analysis.

For root distribution, soil cores were taken within a planting row midway between two plants by soil coring equipment (Giddings Machine Co., Windsor, CO, USA). The cores were divided into 10 cm segments and roots were extracted from each soil segment.

Root length was obtained as previously described for mesocosm samples. Percentages of root length at each depth were calculated in each soil core. Depth above which 95% of root length is located (D\(_{95}\)) was calculated by linear interpolation between the cumulative root lengths (Trachsel et al., 2013).

**Shoot dry weight, chlorophyll measurements, tissue nitrogen content, and yield**

One day prior to harvest, leaf gas exchange of the ear leaves was measured with a Licor-6400 Infrared Gas Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1800 μmol photons m\(^{-2}\) s\(^{-1}\) and constant CO\(_2\) concentration of
360 ppm. At Rock Springs, 6-mm diameter leaf discs were collected from the ear leaves for chlorophyll measurement. Chlorophyll was extracted in 80% acetone. The concentrations of chlorophyll a and b in the extracts were determined at the wavelength of 663.2 and 646.8 nm with a spectrophotometer (Lichtenthaler and Buschmann, 2001). Shoots were dried at 60° C for 72 h prior to dry weight determination. The leaves and stems were ground and 2 to 3 mg ground tissues were taken for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer, Shelton, CT, USA). Yield was collected at physiological maturity in the field study in PA.

Statistical analysis

Statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012). Linear mixed effect models were fit using the function lme from the package nlme (Pinheiro et al., 2012) and a two-way ANOVA were used for comparisons between high and low RCA groups (or individual RILs), nitrogen levels and the interaction between these main effects. A protected least significant difference post hoc (\(\alpha = 0.05\)) test and Tukey’s Honest Significant Difference method (\(\alpha = 0.05\)) were used for multiple comparisons. Correlations and linear regressions were carried out between shoot and root traits with RCA and root respiration and between RCA and yield.

Acknowledgements

We thank Bob Snyder, Curtis Frederick, and Johan Prinsloo for the management of the experiments in greenhouse mesocosms and in the field in the USA and South Africa, Francis Harriman, Gina Riggio, and Michael Williams for assistance with sectioning and image analysis, and Larry M York and Johannes Postma for review of the manuscript.
Literature cited


Miguel M (2011) Functional role and synergistic effect of root traits for phosphorus
acquisition efficiency and their genetic basis in common bean (Phaseolus vulgaris L.). Pennsylvania State University, University Park


Figure legends

Figure 1. Production of root cortical aerenchyma as percent of cortical area in three root classes of maize harvested 35 days after planting (DAP) under high N and low N conditions in soil mesocosms (GH2010). Data shown are means of 4 replicates ± SE of the means. Different letters represent significant differences (p<0.05).

Figure 2. Production of root cortical aerenchyma between high RCA and low RCA maize RILs grown under high N and low N conditions and harvested at 35 DAP in soil mesocosms (GH) in 2010 and at 63 DAP in the field at South Africa (SA) and Pennsylvania (PA). The data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05) compared within each location.

Figure 3. Negative correlation of root segment respiration with RCA in soil mesocosms (GH2010; r = -0.78, p < 0.001) and in the field (r = -0.85, p < 0.001).

Figure 4. Specific root respiration (i.e. root respiration per unit root length derived from the respiration of whole intact root systems) in high and low RCA genotypes at 35 days after planting (DAP) in both high and low N conditions in the mesocosms in 2010. Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).

Figure 5. Nitrogen stress reduced root segment respiration (5A) and root nitrogen content (5B) in second whorl crown roots in soil mesocosms (GH2013). Root cortical aerenchyma is negatively correlated with root respiration (r=-0.75, p<0.05) and nitrogen content (r=-0.60, p<0.05) under low N conditions (5C).

Figure 6 Total root length of high and low RCA RILs at 35 DAP under high and low N conditions in mesocosms (GH2010). Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).

Figure 7 Rooting depth (D95) of maize lines at 35 DAP in mesocosms (GH2010) and 63 DAP in the field in South Africa under low N conditions. Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05) within the experiment.
Figure 8 Chlorophyll concentration (8A) and photosynthesis rate (8B) of high and low RCA RILs at 35 DAP in both high and low N conditions in mesocosms (GH2010). Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).

Figure 9 Relative shoot biomass under high N and low N conditions at 35 DAP in soil mesocosms (GH) in 2010 and at flowering (63 DAP) in the field at South Africa (SA) and Pennsylvania (PA). The data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05) compared within each location. Base line for shoot mass of GH=1.77g, SA=75.28g, PA=159.08g)

Figure 10 Correlation between yield and percentage of root cortical aerenchyma (% of cortex) under high (not significant) and low N (r=0.40, p=0.05) conditions in the field in PA.
Table I Root anatomical traits of different root classes at 35 days after planting in the mesocosms. Root segments were collected 20-24 cm from the base of the primary, seminal, and second whorl crown roots. Data shown are means of 4 replicates of six RILs grown under high and low N conditions. “ns” indicates that nitrogen treatment had no significant effect at p=0.05.

<table>
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<tr>
<th>Root class</th>
<th>treatment</th>
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<th>Cortical cell file number</th>
<th>Meta xylem diameter (mm)</th>
<th>p value</th>
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