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Running head: Fewer crown roots improve N capture in maize

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Low crown root number enhances nitrogen acquisition from low nitrogen soils in maize (*Zea mays* L.).

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Summary: low crown root number improves nitrogen acquisition in maize by enhancing deep soil exploration in low N soils.

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Abstract

In developing nations, low soil nitrogen (N) availability is a primary limitation to crop production and food security, while in rich nations, intensive N fertilization is a primary economic, energy, and environmental cost to crop production. It has been proposed that genetic variation for root architectural and anatomical traits enhancing exploitation of deep soil strata could be deployed to develop crops with greater N acquisition. Here we provide evidence that maize (*Zea mays* L.) genotypes with few crown roots (crown root number: CN) have greater N acquisition from low N soils. Maize genotypes differed in their CN response to N limitation in greenhouse mesocosms and in the field. Low CN genotypes had 45% greater rooting depth in low N soils than high CN genotypes. Deep injection of ^{15}N-labeled nitrate showed that low CN genotypes acquired more N from deep soil strata than high CN genotypes, resulting in greater photosynthesis and total nitrogen content. Under low N, low CN genotypes had greater biomass than high CN genotypes at flowering (85% in the field study in the US and 25% in South Africa). In the field in the US, 1.8x variation in CN was associated with 1.8x variation in yield reduction by N limitation. To our knowledge, this is the first report of the utility of CN for nutrient acquisition. Our results indicate that CN deserves consideration as a potential trait for genetic improvement of nitrogen acquisition from low N soils.

Keywords: *Zea mays* L., crown root number, CN, mesocosm, nitrogen, ^{15}N

Introduction

Maize (*Zea mays* L.) is one of the world's most important crops and is a staple food in Latin America and Africa. Maize production requires a large amount of fertilizer, especially nitrogen. In the USA, N fertilizers represent the greatest economic and energy costs for maize production (Ribaudo et al., 2011). However, on-farm studies across the North-central USA revealed that more than half of applied N is not taken up by maize plants and is vulnerable to losses from volatilization, denitrification, and leaching, which pollute air and water resources (Cassman, 2002). Conversely, in developing countries suboptimal nitrogen availability is a primary limitation to crop yields and therefore food security (Azeez et al., 2006). Increasing yield in these areas is an urgent concern since chemical fertilizers are not affordable (Worku et al., 2007). Cultivars with greater nitrogen acquisition from low N soils could help alleviate food insecurity in poor nations as well as reduce environmental degradation from excessive fertilizer use in developed countries.

The two major soil N forms available to plants are ammonium and nitrate. Nitrate is the main N form in most maize production environments (Miller and Cramer, 2004). Nitrate is highly mobile in soil and the spatiotemporal availability of soil N is rather complex. In the simplest case nitrogen fertilizers applied to the soil and/or nitrogen released from mineralization of soil organic matter are rapidly converted to nitrate by soil microbes. After irrigation and precipitation events, nitrate moves with water to deeper soil strata. Leaching of nitrate from the root zone has been shown to be a significant cause of low recovery of N fertilizer in commercial agricultural systems (Cassman et al., 2002; Raun & Johnson, 1999). Differences in root depth influence the ability of plants to acquire N. Studies using ^{15}Nitrogen (^{15}N) labeled nitrate placed at different soil depths showed that only plants with deep rooting can acquire N sources from deep soil strata, which would otherwise have been lost through leaching (Kristensen & Thorup-Kristensen, 2000; Kristensen & Thorup-Kristensen, 2004). Therefore selection for root traits enhancing rapid deep soil exploration could be used as a strategy to improve crop N efficiency.

The maize root system consists of embryonic and post-embryonic components. The embryonic root system consists of two distinct root classes: a primary root and a variable

number of seminal roots formed at the scutellar node. The post-embryonic root system consists of roots that are formed at consecutive shoot nodes and lateral roots, which are initiated in the pericycle of all root classes. Shoot-borne or nodal roots that are formed below ground are called “crown roots” whereas those that are formed above ground are designated “brace roots” (Hochholdinger, 2009). While the primary root and seminal roots are essential for the establishment of seedlings after germination, nodal roots and particularly crown roots make up most of the maize root system and are primarily responsible for soil resource acquisition later in development (Hoppe et al., 1986).

Lynch (2013) proposed an ideotype for superior N and water acquisition in maize called “Steep, Cheap and Deep (SCD)”, which integrates root architectural, anatomical, and physiological traits to increase rooting depth and therefore the capture of N in leaching environments. One such trait is crown root number (CN). CN is an aggregate trait consisting of the number of belowground nodal whorls and the number of roots per whorl. The crown root system dominates resource acquisition during vegetative growth after the first few weeks and remains important during reproductive development (Hochholdinger et al., 2004). CN in maize ranges from 5 to 50 under fertile conditions (Trachsel et al., 2011). At the low end of this range, crown roots may be too spatially dispersed to sufficiently explore the soil. There is also a risk of root loss to herbivores and pathogens. If roots are lost in low N plants, there may be too few crown roots left to support the nutrient, water, and anchorage needs of the plant. At the high end, a large number of crown roots may compete with each other for water and nutrients as well as incur considerable metabolic costs for the plant (Fig 1). The SCD ideotype proposes that there is an optimal number of crown roots (CN) for N capture in maize (Lynch, 2013). Under low N conditions, resources for root growth and maintenance are limiting, and nitrate is a mobile resource that can be captured by a dispersed root system. Optimal CN should tend toward the low end of the phenotypic variation to make resources available for development of longer, deeper roots rather than more crown roots. According to the SCD ideotype, in low N soils, maize genotypes with fewer crown roots could explore soils at greater depth resulting in greater nitrogen acquisition, growth, and yield than genotypes with many crown roots.

The objective of this study was to test the hypotheses that: (i) low CN genotypes have greater rooting depth than high CN genotypes in low N soils; (ii) low CN genotypes are better at acquiring deep soil N than high CN genotypes; (iii) low CN genotypes have greater biomass and yield than high CN genotypes in low N conditions.

Results

N stress effects on CN

In mesocosms, nitrogen limitation reduced crown root number by 26% ($p<0.001$) at 28 days after planting (DAP). The CN ranged from 3 to 9 under low N conditions. The six genotypes responded differently to N limitation. OHW3, OHW74, OHW 61, and IBM133 showed significant reduction in CN whereas OHW 170 and IBM 123 maintained their CN under low N conditions (Fig 2). Nitrogen limitation reduced the average crown root whorl number from 2.75 to 2.13 ($p<0.05$; Fig 3A). Nitrogen limitation did not affect the number of roots in the first whorl but significantly reduced the number of roots of the second, third, and forth whorl, particularly low CN genotypes (Fig 3B, supplemental Fig S1).

At the field site in the USA (US2011), N limitation reduced CN by 21% at flowering. The CN ranged from 24 to 44 under low N conditions. The genotypes responded differently to N limitation. Nitrogen limitation reduced CN in genotypes NYH76, NYH57, and NYH212, but did not significantly affect CN in the three IBM lines (Fig 4A). At the field site in South Africa in 2011 (SA2011), the CN ranged from 21.5 to 35.5 under low N conditions. The six genotypes were grouped as high or low CN based on the mean difference in CN under low N conditions. The high CN genotypes consisted of IBM123, OHW3, and OHW170. The low CN genotypes consisted of IBM133, OHW61, and OHW74. Means comparison showed that no genotype had a significant decrease of CN under N limitation (Fig 4B), but ANOVA grouping genotypes into the two categories of high CN or low CN showed a significant reduction of CN by N limitation ($p<0.05$), with high CN genotypes having 10 more crown roots than low CN genotypes under low N conditions. A different set of genotypes was planted at the field site in South Africa in 2012 (SA2012). In 2012 the CN ranged from 30 to 46.5 under low N conditions. There

was no significant effect of N stress on the average CN of these genotypes. Nitrogen limitation affected CN in only one genotype, IBM165, and in this instance actually increased CN (Fig 4C).

CN effects on rooting depth and N acquisition

In mesocosms, the genotypes were grouped into high CN and low CN genotypes based on the average value of CN. The high CN genotypes consisted of OHW 170, OHW3, and IBM133; the low CN genotypes consisted of OHW61, OHW74, and IBM123. We found that most low CN genotypes had greater rooting depth than high CN genotypes under low N conditions (Fig 5A; $p<0.05$). Primary roots, seminal roots, and crown roots of low CN genotypes had greater rooting depth ($p<0.05$) than those of high CN genotypes (Fig 5B).

In SA2011 N limitation slightly increased maximum rooting depth (D_{95}) from 30.5 to 37.2 cm but the effect was not significant. Low CN genotypes had significantly greater rooting depth than high CN genotypes (Fig 5C) under low N conditions. The low CN genotypes had a D_{95} value of 34.4 cm whereas for high CN genotypes the D_{95} value was 26.7 cm ($p<0.05$, Fig 5C). In UA2011 and SA2012 Low CN genotypes again had significantly greater rooting depth than high CN genotypes (Fig 6A, supplemental Fig S2). To investigate whether low CN genotypes were better at acquiring N from deep soil strata, we injected ^{15}N-labelled nitrate in the soil at a depth of 50 cm at SA2012. One week after the ^{15}N application we found that low CN genotypes had greater ^{15}N content in shoot tissues than high CN genotypes under low N conditions (Fig 6B).

CN effects on plant growth and yield

In mesocosms N limitation reduced shoot mass by an average of 45%. Shoot biomass and leaf photosynthetic rate were affected by CN (Table I, II, supplemental table S1). ANCOVA and correlation analyses showed that under low N conditions, plants with low CN had greater leaf photosynthetic rates, canopy photosynthetic rates, tissue N content, and shoot mass, than plants with high CN (Table I,II). There was no significant relationship between these variables and CN under high N conditions (data not shown).

In the field trials N limitation reduced shoot mass by an average of 20% in SA2011 and by 24% in SA2012. ANCOVA and correlation analyses showed that under low N conditions, low CN genotypes had greater leaf photosynthetic rates, tissue N content, and shoot dry weight than plants with high CN at SA2011 (Table I,III, supplemental table S2). There was no significant relationship between these variables and CN under high N conditions (data not shown).

In US2011 N limitation reduced shoot mass by 34% at flowering (8 weeks after planting). Grain yield was reduced by 39% in low N soils. ANCOVA and correlation analyses showed that under low N conditions, low CN genotypes had greater tissue nitrogen content and shoot dry weight than high CN genotypes (Table I,III, supplemental table S2). Low CN genotypes had greater percent grain yield than high CN genotypes under low N conditions (Fig 7). Genotypic variation of 1.8x in CN was associated with 1.8x variation in yield reduction by N limitation (Fig 7).

Discussion

We demonstrate that low crown root number (CN) improves nitrogen acquisition by enhancing deep soil exploration in low N soils. Genotypes differed in their CN response to N limitation (Figs 2,4). Maize lines with low CN had greater rooting depth than high CN genotypes (Figs 5,6) and acquired more ^{15}N labeled nitrate applied in deep soil in the field (Fig 6). Low CN genotypes had greater tissue nitrogen content and shoot biomass than high CN genotypes under low N conditions in all environments tested (Fig 6,Table I). Finally, low CN genotypes had greater percent grain yield than high CN genotypes in the field under low N conditions (Fig 7).

This study is focused on the physiological utility of CN for N acquisition in low N environments. The use of monogenic mutantsis not suitable for this study, since CN is a quantitative trait controlled by several alleles in unknown ways (Burton, 2010). To date genes controlling the development of root architecture such as RTCS and RL have been identified (Jenkins, 1930; Hetz et al., 1996; Hochholdinger et al., 2004). However mutations in these genes affect the development of other root classes (*rtcs*) and plant vigor (*rl*) and thus are not desirable for our purpose. In this study, we selected near

184 isophenic lines from maize recombinant inbred lines (RILs) that vary in CN but are
185 similar in other phenotypic traits such as root angle and branching. RILs are suitable for
186 this study because they are closely related genotypes with highly similar genetic
187 backgrounds, thereby minimizing the risk of effects from genetic interactions, epistasis,
188 and pleiotropy, which may confound the interpretation of results from comparisons of
189 unrelated lines (Zhu et al., 2005; Zhu et al., 2006). In addition, each experiment consisted
190 of RILs from different populations representing high and low CN. The fact that our
191 results were consistant among different expriments with different set of RILs indicates
192 that the utility of CN for N capture is independent of the specific genotypic context.

193 In the greenhouse we used mesocosms to create nitrogen leaching environments
194 comparable to conditions in well-drained agricultural soils. The mesocosms also permit a
195 detailed investigation of root distribution by depth since entire root systems can be
196 excavated. Gaudin et al. (2011) reported that maize responded to N limitation by
197 increasing the length of individual crown roots while reducing CN (Gaudin et al., 2011).
198 These results are consistent with those of Tian et al (2008), who demonstrated that high
199 nitrate inhibits maize root elongation and is accompanied by decreasing IAA levels in the
200 roots (Tian et al., 2008). In our study, we found that not all maize genotypes reduced CN
201 in response to N limitation. For example, genotypes such as IBM133, OHW3, OHW61,
202 and OHW74 significantly reduced CN in the mesocosms under low N conditions but
203 maintained their CN in the field (Fig 2,4). These results indicate that CN response to N
204 limitation depends on genotypes and environments. In the mesocosms where CN was
205 significantly reduced by N limitation, we found that reduced CN was attributable to
206 decreased crown root whorl number and decreased number of roots per whorl (Fig 3A,
207 3B). Nitrogen stress did not affect the number of roots of the first whorl, which is the
208 earliest to emerge from the stem node, suggesting that plants may exhaust seed N
209 reserves prior to or during the development of the second whorl crown roots.

210 We found that high CN genotypes had shallower primary, seminal, and crown roots than
211 low CN genotypes under low N conditions (Fig 5). This result supports the hypothesis
212 that there exist tradeoffs between the number of crown roots and growth of different root
213 classes. These results are consistent with reports in other crop species. In wheat and

214 barley, the removal of nodal roots stimulates the growth and activity of the seminal roots
215 (Krassovsky, 1926). In common bean, increased carbon allocation to adventitious roots
216 was related to decreased allocation to tap and basal roots, which affected total root length,
217 soil exploration, and P acquisition under suboptimal P conditions (Walk et al., 2006), and
218 removal of a specific root class led to an increase in the relative proportion of the
219 remaining root classes (Rubio and Lynch, 2007). In maize the majority of axial roots in
220 the root system are crown roots. The diameter of crown roots of the third whorl and
221 subsequent nodes are much larger than that of the primary and seminal roots, and these
222 roots are thus a greater sink for photosynthates. High CN genotypes must maintain the
223 growth and development of many crown roots, which would constrain the growth and
224 elongation of crown roots and other root classes, resulting in shallower root systems
225 compared to those of low CN genotypes (Fig 5,6). In addition, competition among roots
226 within the root system for soil resources is greater in high CN genotypes, especially for a
227 mobile resource like nitrate. The effect of reduced CN on soil exploration and N
228 acquisition could result from reduced root competition for internal and external resources,
229 as proposed by Lynch (2013).

230 We investigated the ability of low CN genotypes to take up N from deep soil layers in the
231 field in SA2012 by injection of ^{15}N-labelled nitrate in the soil at 50 cm depth within a
232 planting row adjacent to the plants. We found that low CN genotypes had greater ^{15}N
233 uptake than high CN genotypes (Fig 6B). Soil nitrate analysis showed that nitrate was
234 indeed more abundant in deep soil layers than in topsoil at the time of harvest (data not
235 shown), thus, deep-rooting low CN genotypes are able to acquire N deep in the soil
236 profile better than high CN genotypes. The ability to explore soils at greater depth and
237 acquire N from N source in deep soils means that low CN plants have greater usage of N
238 and thus have better N efficiency than high CN genotypes. Low CN plants could also
239 reduce N leaching, thereby reducing environmental pollution.

240 Photosynthesis directly influences growth and yield of crop plants (Gastal and Lemaire,
241 2002). The rate of photosynthesis depends on content of N in the leaf tissue because
242 photosynthetic proteins, including Rubisco and light harvesting complex proteins,
243 represent a large proportion of total leaf N (Evans, 1983). We found that low CN

genotypes had greater tissue N content, which resulted in greater photosynthetic rates, and shoot biomass than high CN genotypes in greenhouse and field studies (Table I,II,III). In US2011, 1.8x genotypic variation in CN was associated with 1.8x variation in yield loss due to N limitation (Fig 7). This is important especially for developing countries where yield of maize is less than 10% of its yield potential (Lynch, 2007).

Considering the range of reported CN in field-grown plants of 5-50 (Trachsel et al., 2010) and 10-32 (Bayuelo-Jiménez et al., 2011), our range of CN (20-45) falls between the medium to high range of phenotypic variation observed in maize. We propose that in extremely low CN phenotypes, roots may be too spatially dispersed to sufficiently acquire soil resources and such plants may be susceptible to lodging (Hetz et al., 1996). Additionally, plants with very low CN may be at risk of root loss due to herbivores and pathogens. This is particularly important for low-input agroecosystems where root survivorship is low. In this case the optimum number of CN would be large enough to allow rapid recovery from root damage but not too large to compete for internal and external resources. The optimum range of CN is likely to be dependent upon soil type and the severity of biotic and abiotic stresses. We anticipate that the optimum range of CN is also at the low end of the range of variation under drought and is likely to be greater in low density plantings, in fine-textured soils with slow leaching, and in soils with suboptimal availability of immobile nutrients such as phosphorus (P) and potassium (K), which are abundant in the topsoil. Greater CN may be beneficial to plants in low-input systems in which N continues to be available in the topsoil as a result of mineralization of organic matter (Poudel et al., 2001). However, many low-input systems are subject to drought in addition to suboptimal N availability. In this case, low CN enhancing deep soil exploration may be preferable to high CN since low CN supports deep root system so the shallow portion of deep roots can acquire shallow N resources while the deep portion can explore deep soil for water resources.

Functional-structural modeling could be helpful in identifying optimum CN for specific environments as well as studying interactions between CN and other root traits. Recently, York et al. (2013) used the functional-structural plant model *SimRoot*, to observe interactions between CN and root cortical aerenchyma (RCA). They found that the

synergistic effects of CN and RCA on plant growth were greater than the additive effects by 32% at medium N and by 132% at medium P (York et al., 2013). In addition, an optimum number of crown roots can also interact with other traits enhancing deep soil exploration, such as steep root growth angle and few but long root branches, and may synergistically enhance resource acquisition under drought and suboptimal availability of mobile nutrients (Lynch, 2013).

The concept of optimum CN enhancing root growth and soil exploration under water and nutrient limiting conditions supports the rhizoeconomic paradigm, which considers the benefits and the costs of root traits as direct metabolic costs and as trade-offs and risks (Lynch and Ho, 2005; Nord and Lynch, 2009). We suggest that the optimum CN concept can be applied to other crop species in which nodal roots represent a major portion of the root system such as rice (*Oryza sativa*), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Krassovsky, 1926; de Dorlodot et al., 2007; Coudert et al., 2010). Our results are entirely supportive of the CN component of the SCD ideotype (Lynch 2013). The SCD ideotype applies to both water and N capture, since both of these soil resources are often localized in deep soil strata under limiting conditions. The fact that CN affects rooting depth and therefore N capture suggests that this trait should also be useful for water capture from drying soil, especially in terminal drought scenarios (Lynch 2013).

Genotypic differences in crown root number have been reported in several crop species including maize and its relatives within *Zea* (Bayuelo-Jiménez et al., 2011; Burton et al., 2013; Lynch, 2013; Trachsel et al., 2010). Moreover, CN is a heritable trait (Jenkins, 1930) and genes affecting CN expression have been identified (Jenkins, 1930; Hetz et al., 1996; Taramino et al., 2007) making CN a feasible target for plant breeding. To our knowledge, this is the first report of the utility of CN for improving nutrient acquisition. Our results support the hypothesis that CN affects rooting depth and soil N acquisition, and thus merits investigation as a potential element of more N-efficient cultivars.

Materials and Methods

Greenhouse mesocosm study

Plant materials

Based on the results of screening experiments in mesocosms in the USA and in the field in South Africa, recombinant Inbred Lines (RILs) IBM123 and IBM133 from the intermated B73 and Mo17 (IBM) population (Lee *et al.*, 2002; Sharopova *et al.*, 2002) and OHW3, OHW61, OHW74, and OHW170 from the cross between OH43 and W64a (OHW) contrasting in crown root number were selected for this study.

Experimental design

The greenhouse experiment was a randomized complete block design. The factors were two nitrogen regimes (high and low nitrogen conditions), six RILs, and four replicates. Planting was staggered one week between replicates with time of planting as a block effect.

Growth conditions

Plants were grown during October 13 to December 8, 2010 in a greenhouse 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 ^{o}C (light/darkness). Seeds were soaked for 1 h in a fungicide solution containing 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^{o}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 high-density polyethylene film to facilitate root sampling at harvest. The growth medium consisted of a mixture (volume based) of 50% medium size (0.3 to 0.5 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 were used in each mesocosm to ensure the same bulk density of the medium. One day before planting, the mesocosms were saturated with 5 liters of a nutrient solution adjusted to pH 6. The nutrient solution for the high N treatment consisted of (in μM): NO_3 (7000), NH_4 (1000), P (1000), K (3000), Ca (2000), SO_4 (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, NO_3 and NH_4 were reduced to 70 and 10 μM, respectively, and K_2SO_4 was used to replace K and SO_4. Each mesocosm received two seeds and after 4 days they were thinned to one plant per mesocosm. Plants were watered with 75 ml of deionized water every 2 days. 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 ^{o}C until processing. The concentrations of nitrate in the solutions were determined using the vanadium (III) chloride protocol according to Doane et al. (2003).

Root harvest

The plants were harvested at 28 days after planting. At harvest a polyethylene liner in each mesocosm was carefully removed and placed on a root washing station. The liners were divided into 20 cm segments starting from the base of the shoot. Media were carefully removed and the deepest layer reached by the roots was recorded for primary, seminal, and crown root classes. CN in each nodal whorl and root branching were counted. The roots were cut, separated from each segment, and preserved in 75% EtOH. Total root lengths were obtained by scanning and analyzing using the *WinRhizo* software (WinRhizo Pro, Régent Instruments, Québec City, Québec, Canada).

Shoot dry weight and plant nitrogen status

One day prior to harvest, leaf gas exchange of the first and the second youngest fully expanded 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 1200 μmol photons m^{-2} s^{-1} and constant CO_2 concentration of 400 ppm. Shoot carbon assimilation was measured with a Licor-6200 Infrared Gas Analyzer (Li-Cor Environmental Inc,

Lincoln, NE, USA). In short, a 36.5 liter (28x 28 x 46.5 cm) transparent acrylic chamber was placed around a plant. The base of the chamber was split to fit a stem of a plant. The air space around the stem and the base of the chamber was filled with modeling clay and sponges to separate the shoot from the growth media. The chamber connected to the Li-6200 with polyethylene tubing 0.03 liter in volume. Carbon dioxide exchange was measured for two minutes for each plant. Shoots were dried at 60 ^{o}C for 72h prior to dry weight determination. The shoots were ground and 2 to 3 mg of ground tissue was taken for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer).

Field studies

Field conditions, experimental design, and plant materials

Experiments were carried out during February to April in 2011 (SA2011) and 2012 (SA2012) at Alma, Limpopo province, South Africa (24°33′ 00.12 S, 28°07′25.84 E, 1235 masl) and during June - October in 2011 (US2011) at the Hancock Agricultural research station of the University of Wisconsin in Hancock, WI, USA (44°07′56″.74 N, 89°30′43″.96 W, 331 masl). The soils at the experimental sites were a Clovelly loamy sand (Typic Ustipsamment) in Alma and a Plainfield loamy sand (mixed, mesic Typic Udipsamment) in Hancock. In SA2011 and SA2012 N fertilizers were applied at the rate of 30 kg N/ha for 5 times until flowering resulting in 150 kg N ha^{-1} in total for well-fertilized plots. The low N plots received 30 kg N ha^{-1}only at the beginning of the growing season. In US2011 the well-fertilized plots were amended with 103 kg N ha^{-1} at planting and at four weeks after planting resulting in a total of 206 kg N ha^{-1} while the low N plots were amended with 34 kg N ha^{-1} at the beginning of the cropping season only. In all 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.

Plant material

The same six RILs used in the greenhouse experiment were used in SA2011. Different sets of genotypes were planted at US2011 and SA2012. These genotypes were selected

389 based on previous screening in US field (Saengwilai et al., unpublished). Seven RILs
390 consisting of IBM1, IBM9, IBM13, IBM77, IBM133, IBM165, and IBM187 were used
391 in SA2012. RILs from the IBM populations; IBM10, IBM85, IBM218 and from the cross
392 between NY821 and H99 (NyH) population; NYH76, NYH57, NYH212 were used in
393 US2011. In each location the experiment was arranged in a split-plot design replicated
394 four times with high and low N treatments. Four sections adjacent to each other in the
395 field containing both high and low N treatments were assigned as blocks. Genotypes were
396 randomly assigned to five-row plots. Each row was 4.5 m long. The distance between
397 rows was 75 cm and within a row was 23 cm, resulting in a planting density of 6 plants
398 m^{-2}. The plants were harvested at flowering, 9 weeks after planting in SA2011 and
399 SA2012 and 8 weeks after planting in US2011.

400 **Root harvest**

401 Evaluation of crown roots was carried out based on shovelomics (Trachsel et al., 2011).
402 Three representative plants were selected for excavation in each plot. The selection was
403 based on height, presence of bordering plants, and general appearance that represented
404 individuals in the plot. At harvest roots were excavated using spades. A large portion of
405 soil was removed from roots by carefully shaking. The remaining soil was removed by
406 soaking the roots in diluted commercial detergent followed by vigorously rinsing at low
407 pressure with water. Because three representative roots within a plot usually appear to be
408 homogeneous, only one root was selected for phenotyping. Crown root number (CN) was
409 measured by counting half of the root system. Assuming that the maize root system is
410 symmetrical, CN was multiplied by two to obtain the total CN prior to data analysis. Data
411 on other root traits such as root angle, diameter, and branching were also collected and
412 included in the analyses when needed.

413 **Rooting depth and ^{15}N injection**

414 Rooting depth was measured at flowering by soil coring (Giddings Machine Co.,
415 Windsor, CO, USA). Soil cores were taken within a planting row midway between two
416 plants. The diameter of soil cores was 5.1 cm. The cores were divided into 10 cm
417 segments and roots were extracted from each soil segment. Root lengths were obtained
418 by scanning and analysis using *WinRhizoPro* (Régent Instruments, Québec, Québec City

419 Canada). Percentages of root length at each depth were calculated in each soil core.
420 Depth above which 95% (D^{95}) of root length is located was calculated by linear
421 interpolation between the cumulative root lengths (Trachsel et al.,2013).

422 The ability of roots to acquire N in deep soil layers was studied by deep injection of
423 $^{15}NO_3^-$ in SA2012. PVC pipes with a length of 75 cm and a diameter of 5 cm were used
424 for $^{15}NO_3^-$ injection. Three representative plants were selected and the injections were
425 done at a midway between adjacent plants within a planting row. Each plot received two
426 injections. Prior to the injections, a soil auger was used to excavate a cylinder of soil to a
427 depth of 50 cm. A PVC pipe was inserted into the hole and the $^{15}NO_3^-$ solution was
428 poured into the hole. Each plot had 5 mL of $K^{15}NO_3^-$ solution (0.46 mg ^{15}N mL^{-1}, 98%
429 ^{15}N enriched) injected into each of two holes. Following the injection each hole was filled
430 with sand to prevent roots from growing down the hole. Seven days after $^{15}NO_3^-$
431 injection, the shoot biomass of the selected plant was harvested for ^{15}N and total N
432 analysis.

433 **Shoot dry weight and tissue nitrogen content**

434 In SA2011 and SA2012 one day prior to harvest, leaf gas exchange of the ear leaves was
435 measured with a Licor-6400 Infrared Gas Analyzer (Li-Cor Biosciences, Lincoln, NE,
436 USA) using a red-blue light at PAR intensity of 1800 μmol photons m^{-2} s^{-1} and constant
437 CO_2 concentration of 360 ppm. In all experiments, shoots were dried at 60 ^oC prior to dry
438 weight determination. The leaves and stems were ground and 2-3 mg of ground tissue
439 were analyzed for tissue nitrogen content using an elemental analyzer (SeriesII CHNS/O
440 Analyzer 2400, PerkinElmer).^{15}N in plant tissue was analyzed using a PDZ Europa
441 ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass
442 spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility, University of
443 California at Davis, USA (http://stableisotopefacility.ucdavis.edu/).

444 **Statistical analysis**

445 Statistical analyses were performed using R version 2.15.1 (R Development Core Team
446 2012). Linear mixed effect models were fit using the function lme from the package nlme
447 (Pinheiro et al., 2012) and two-way ANOVA were used for comparisons between high

and low CN groups (or individual genotypes), nitrogen levels and the interaction between these main effects. ANCOVA was performed using the lm function to test effects of CN and N treatments on response variables. The protected least significant difference post hoc (α=0.05) test and Tukey's Honest Significant Difference method (α=0.05) were used for multiple comparison tests.

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547 **Figure legends**

548 Figure 1. Visualization of maize root system of low and high crown root (CN) genotypes
549 at 40 d after germination. Crown roots are colored in blue and seminal roots are in red.
550 The number CN is 8 in the low CN genotypes and 46 in the high CN genotype (image
551 courtesy of Larry M. York).

552 Figure 2. Crown root number of maize 28 days after planting under high N and low N
553 conditions in soil mesocosms. Data shown are means of 4 replicates ± SE of the mean.
554 Means with the same letters are not significantly different ($p < 0.05$)

555 Figure 3. Crown root whorl number (3A) and crown root number per whorl (3B) of maize
556 28 days after planting under high N and low N conditions in soil mesocosms. Data shown
557 are means of six genotypes (i.e. IBM133, IBM123, OHW3, OHW61, OHW74, and
558 OHW170) with 4 replicates ± SE of the means. Means with the same letters are not
559 significantly different ($p < 0.05$)

560 Figure 4. Crown root number of maize at flowering under high N and low N conditions at
561 the fields in USA in 2011 (4A), and in South Africa in 2011 (4B) and 2012 (4C). Data
562 shown are means with 4 replicates ± SE of the means. Means with the same letters are not
563 significantly different ($p < 0.05$)

564 Figure 5 Rooting depth of six RILs at 28 DAP in soil mesocosms (5A), depth of primary,
565 seminal, and crown roots at 28 DAP under low N conditions in soil mesocosms compared
566 between high and low CN within the same root class (5B) and D_{95} of maize at 9WAP
567 under low and high N conditions at SA2011 field (5C). Data shown are means of 4
568 replicates $\pm$ SE of the mean. Different letters represent significant differences ($p<0.05$).
569 Figure 6 Correlations between 6A) crown root number and rooting depth ($R^2=0.53$,
570 $p=0.04$), 6B) ^{15}N in shoot ($R^2=0.35$, $p=0.02$), and 6C) shoot dry weight ($R^2=0.16$, $p=0.02$)
571 at flowering under low N conditions in the field in South Africa (2012).

572 Figure 7 Correlation between crown root number and grain yield (% of yield under high
573 N) ($R^2=0.19$, $p=0.02$) under low N conditions in the field in the USA.

574 Supplemental Figure S1 Crown root number per first (S1A), second (S1B), and third
575 (S1C) whorl of maize 28 days after planting under high N and low N conditions in soil
576 mesocosms. Data shown are means with 4 replicates ± SE of the means. Means with the

577 same letters are not significantly different ($p < 0.05$)

578 Supplemental Figure S2 Correlations between crown root number and rooting depth

579 (R^2=0.68, p=0.04) at flowering under low N conditions in the field in USA.

580

581 Table I. Summary of correlation analysis (correlation coefficient and significant levels)
582 between crown root number and parameter measured under low N conditions in six
583 maize genotypes in soil mesocosms at 28 days after planting and in the field in South
584 Africa and USA in 2011.
585

		Field	
Parameter	**Mesocosms**	**South Africa**	**USA**
Canopy photosynthetic rate	0.26*	-	-
Leaf photosynthetic rate	0.34*	0.31*	-
Tissue nitrogen content	0.23*	0.13*	0.13*
Shoot dry weight	0.23*	0.49**	0.22*

586 *p<0.05, **p<0.01

587 Table II. Summary of ANCOVA model (F-value and degrees of freedom) of shoot traits
588 at 28 day after planting as influenced by CN and N treatment in six maize RILs in
589 greenhouse mesocosms.

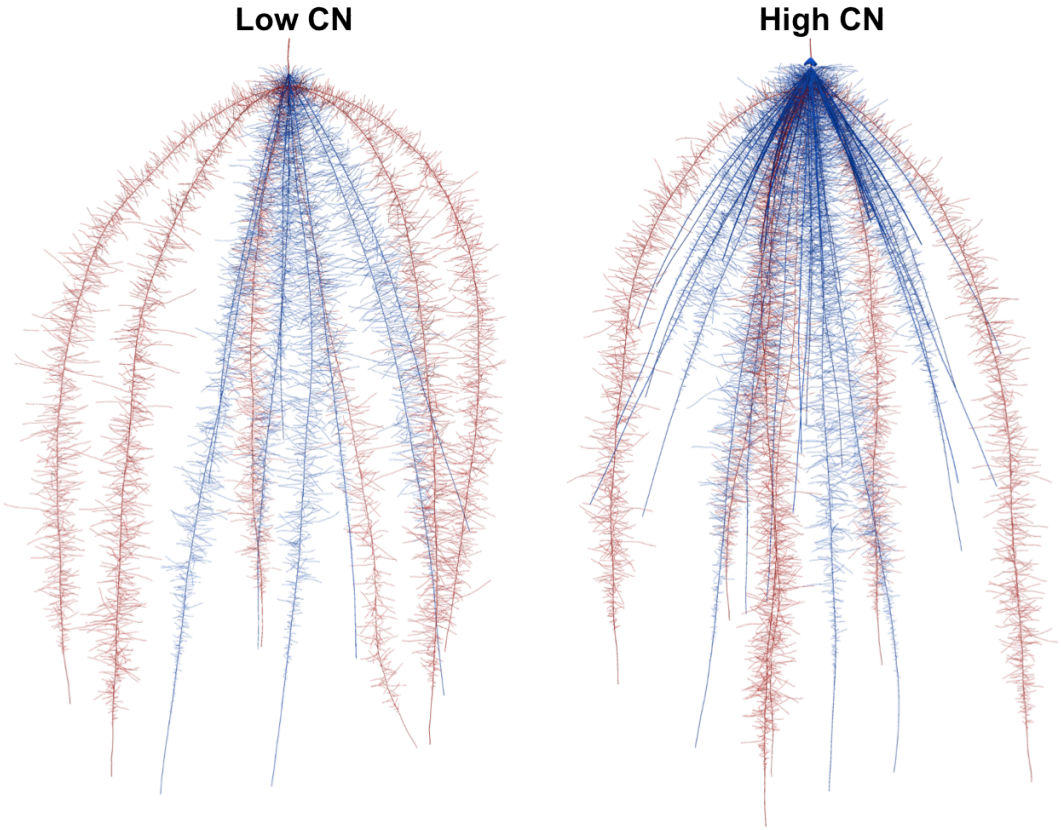
Effect	Shoot weight	Photosynthesis Rate	Carbon Assimilation	Tissue N Content
CN	22.31 (1,43)***	9.48 (1,43)**	6.51 (1,43)*	16.55 (1,44)***
N treatment	23.78 (1,43)***	31.62 (1,43)***	75.66 (1,43)***	29.15 (1,44)***
CN:N treatment	4.89 (1,43)*	14.08 (1,43)***	0.20 (1,43)	2.79 (1,44) [a]
R^2	0.66	0.53	0.63	0.49

590 [†]p<0.1, *p<0.05, **p<0.01, ***p<0.001, [a]p=0.10.Degrees of freedom shown as (numerator, denominator)

591 Table III. Summary of ANCOVA model (F-value and degrees of freedom) of shoot traits
592 at flowering as influenced by CN and N treatment in six maize RILs in SA2011 and
593 US2011 and in seven maize RILs in SA2012.
594

Effect	Shoot weight SA2011	Shoot weight SA2012	Shoot weight US2011	Yield US2011
CN	3.19 (1,44) †	0.89 (1,52)	0.84 (1,44)	21.37 (1,44)***
N treatment	63.28 (1,44)***	33.53 (1,52)***	22.39 (1,44)***	14.34 (1,44)***
CN:N treatment	1.10 (1,44)	3.05 (1,52) †	3.62 (1,44) †	2.67 (1,44) [a]
R^2	0.59	0.39	0.34	0.49

595 †p<0.1, *p<0.05, **p<0.01, **p<0.001, [a]p=0.10. Degrees of freedom shown as (numerator, denominator)
596



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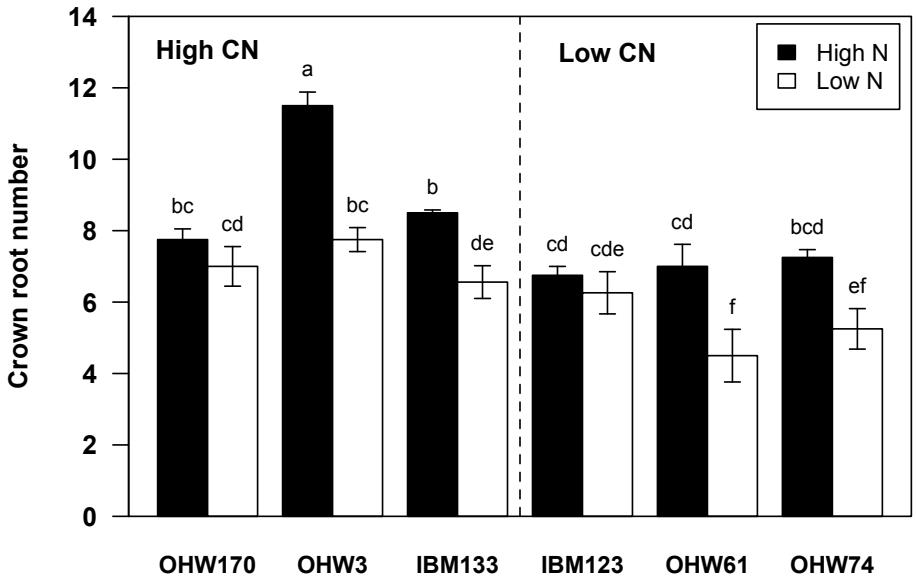

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608 Means with the same letters are not significantly different ($p < 0.05$)

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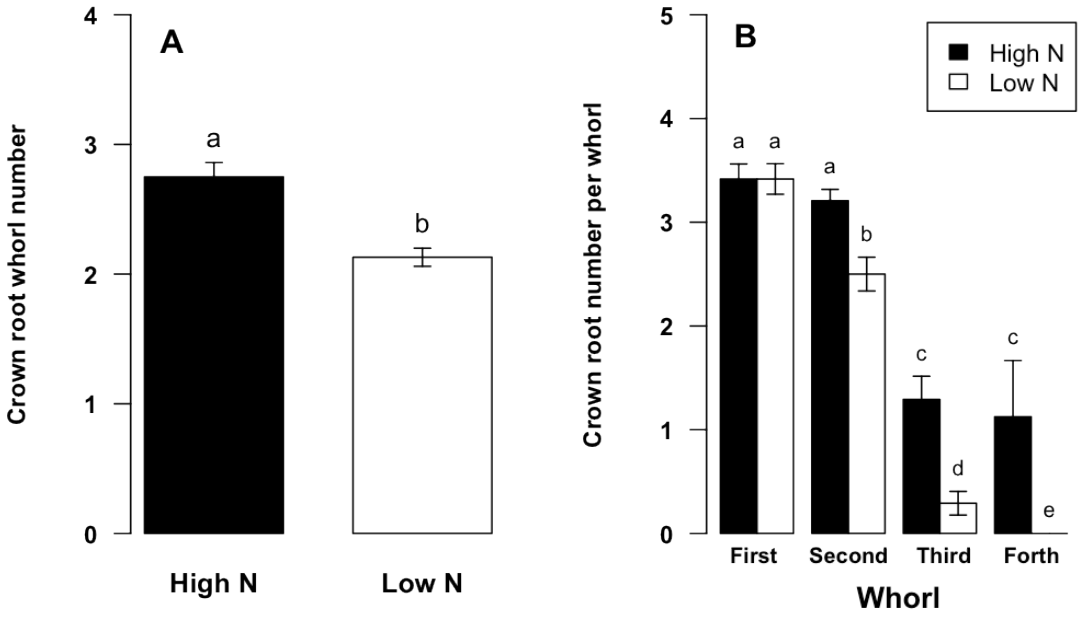

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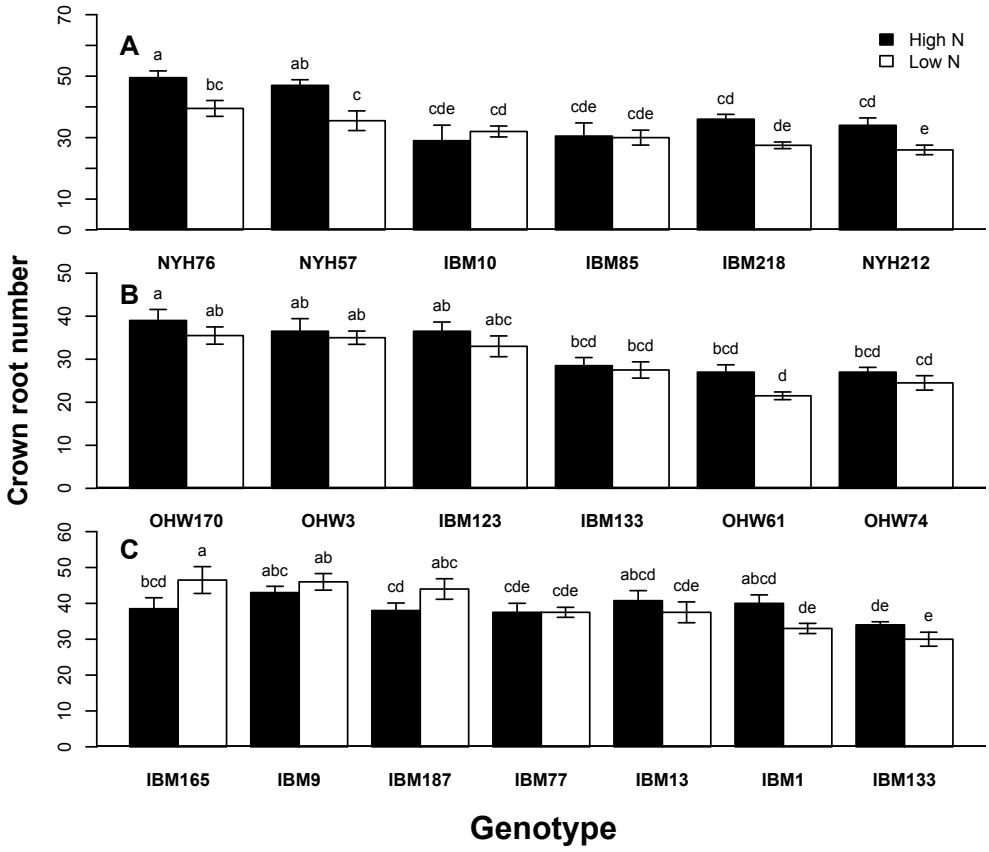

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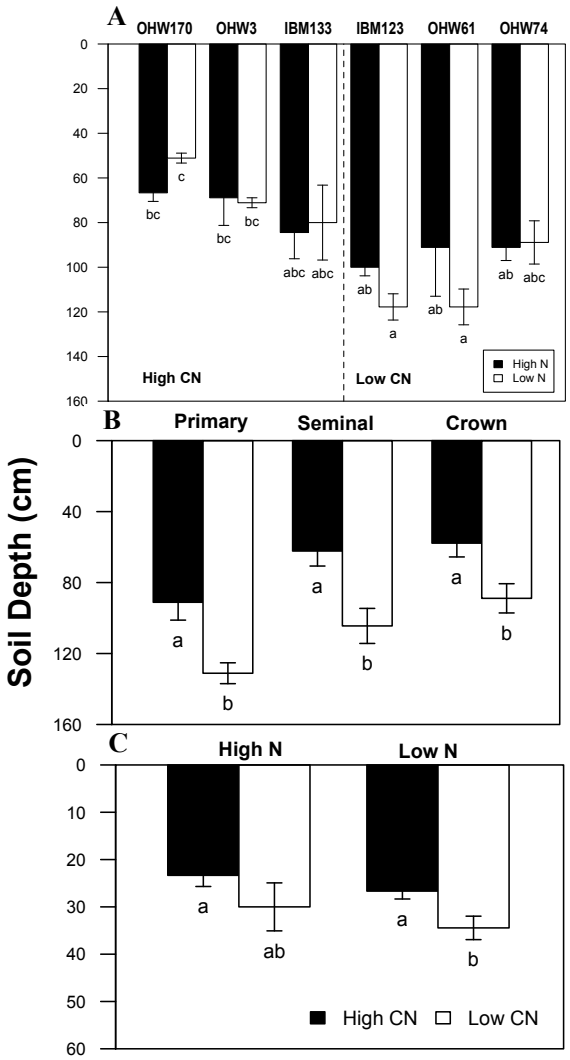

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628 of 4 replicates $\pm$ SE of the mean. Different letters represent significant differences
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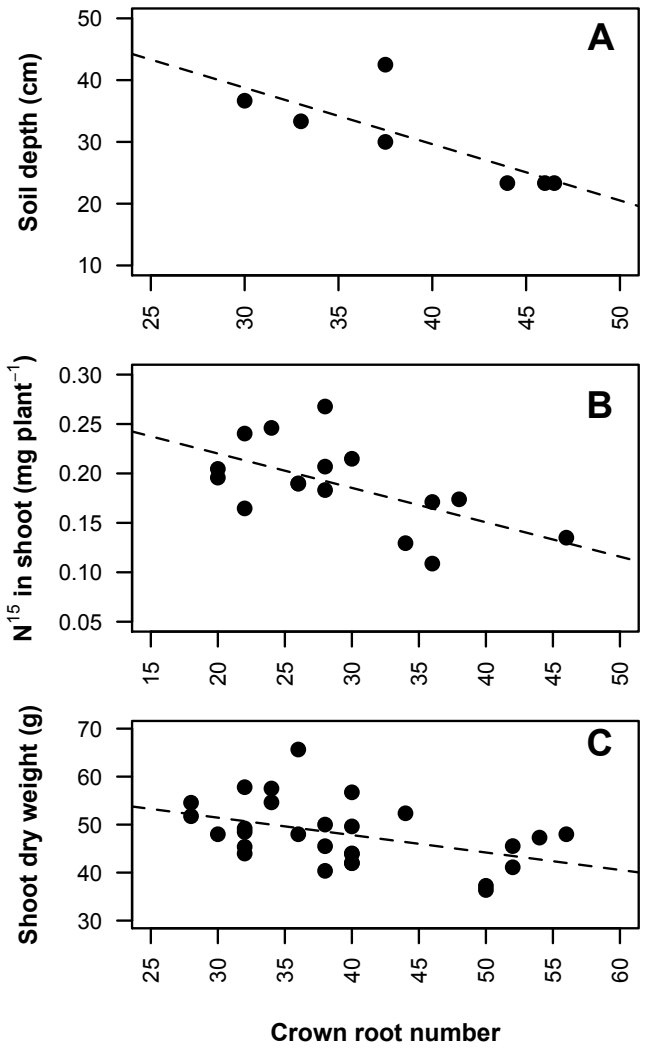

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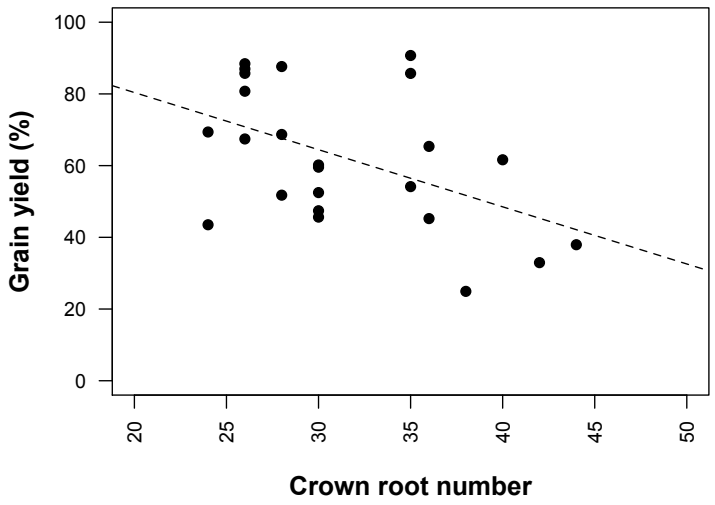

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638