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Legume shovelomics: High—Throughput phenotyping of common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* subsp, *unguiculata*) root architecture in the field

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ABSTRACT

Low phosphorus (P) availability and drought are primary constraints to common bean and cowpea production in developing countries. Genetic variation of particular root architectural phenes of common bean is associated with improved acquisition of water and phosphorus. Quantitative evaluation of root architectural phenotypes of mature plants in the field is challenging Nonetheless, in situ phenotyping captures responses to environmental variation and is critical to improving crop performance in the target environment. The objective of this study was to develop flexible high-throughput root architectural phenotyping platforms for bean and cowpea, which have distinct but comparable root architectures. The bean phenotyping platform was specifically designed to scale from the lab to the field. Initial laboratory studies revealed cowpea does not have basal root whorls so the cowpea phenotypic platform was taken directly to field evaluation. Protocol development passed through several stages including comparisons of lab to field quantification systems and comparing manual and image-based phenotyping tools of field grown roots. Comparing lab-grown bean seedlings and field measurements at pod elongation stage resulted in a R^2 of 0.66 for basal root whorl number (BRWN) and 0.92 for basal root number (BRN) between lab and field observations. Visual ratings were found to agree well with manual measurements for 12 root parameters of common bean. Heritability for 51 traits ranged from zero to eighty-three, with greatest heritability for BRWN and least for disease and secondary branching traits. Heritability for cowpea traits ranged from 0.01 to 0.80 to with number of large hypocotyl roots (1.5A) being most heritable, nodule score (NS) and tap root diameter at 5 cm (TD5) being moderately heritable and tap root diameter 15 cm below the soil level (TD15) being least heritable. Two minutes per root crown were required to evaluate 12 root phene descriptors manually and image analysis required 1 h to analyze 5000 images for 39 phenes. Manual and image-based platforms can differentiate field-grown genotypes on the basis of these traits. We suggest an integrated protocol combining visual scoring, manual measurements, and image analysis. The integrated phenotyping platform presented here has utility for identifying and selecting useful root architectural phenotypes for bean and cowpea and potentially extends to other annual legume or dicotyledonous crops.

1. Introduction

Global food production must double by the year 2050 in order to meet the projected demand (Tilman et al., 2011). Common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata*) are staple foods in many areas of the tropics and sub-tropics and make significant contributions to food security, income, and soil fertility (Beebe, 2012; Ehlers and Hall, 1997). Both legumes are important crops for smallholder farmers with limited access to irrigation and fertilizers and their production is critical for human nutrition and agroecosystem productivity. Their production is challenged by increasingly marginal soils and climate instability, amplifying the need to develop high yielding cultivars under drought and low soil fertility (Lynch, 2007; Wortmann et al., 1998).

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Root architecture is an important factor for the acquisition of key soil resources including nitrogen and water (Lynch and Brown, 2001; Lynch, 2013) and is particularly important for the highly immobile and commonly limiting nutrient phosphorus (P) (Lynch, 1995). Bean exhibits considerable genetic diversity for root architectural traits related to growth in low P and water limited environments (Lynch and Beebe, 1995; Bonser et al., 1996; Miller et al., 2003; Miguel, 2004; Ochoa et al., 2006; Rubio et al., 2003; Ho et al., 2005). Greater root hair length and density increase root surface area and enhance phosphorus uptake but will not be dealt with here, as their quantification requires a different sampling procedure (Brown et al., 2013; Lynch, 2011; Miguel et al., 2015). Several quantitative trait loci (QTL) of architectural traits have been identified in common bean using simple visual evaluations of phene descriptors ('phene' is to 'phenotype' as 'gene' is to 'genotype', sensu (Lynch, 2011; Serebrovsky, 1925; York et al., 2013)) including basal root growth angle (BRGA) (Ho et al., 2005; Liao et al., 2004), basal root whorl number (BRWN) (Miguel et al., 2013), and hypocotyl root number (Ochoa et al., 2006; Walk et al., 2006). Note that some literature uses the term adventitious root rather than the more correct hypocotyl root (Zobel, 2011). The impact of genetic and phenotypic variation of cowpea root architectural traits on performance has received less attention.

Common bean and cowpea both have epigeal germination and have embryonic root systems composed of the primary root and basal roots. Following germination hypocotyl roots develop and lateral roots develop from tap root, basal and hypocotyl roots (Lynch and Beem, 1991). The number and growth angle of basal roots in bean vary and are organized in discrete whorls, each with capacity for 4 basal roots (Basu et al., 2007). In bean, hypocotyl roots typically grow horizontally from the hypocotyl (Bonser et al., 1996; Miller et al., 2003). In contrast, we have observed both hypocotyl and basal roots in cowpea to have large variation in plagiogravitropism. Another major difference with common bean is that cowpea basal roots are not arranged in discrete whorls and are not always clearly distinct from hypocotyl or primary root laterals. In common bean the primary (tap) root is orthogravitropic (Basu et al., 2007) and cowpea shows the same tendency. Variation between cowpea and common bean represents phenotypic extremes along the spectrum of root architectural phenotypes observed in grain legumes. Therefore, these two species can serve as models for other legumes including soybean (*Glycine max*), tepary bean (*Phaseolus acutifolious*), fava bean (*Vicia faba*), chickpea (*Cicer arietinum*), pigeon pea (*Cajanus cajan*), and groundnut (*Arachis hypogaea*).

Phenotyping root traits related to edaphic stress tolerance is a bottleneck that limits genetic analysis and crop improvement (Varshney et al., 2014). A low-cost, field-based phenotyping platform would enable plant breeders with limited economic resources to address local environmental constraints. Genotype by environment (GxE) and genotype by environment by management (GxExM) interactions are of particular interest to breeding programs that require phenotyping in the agroecosystem of interest. Greenhouse trials are typically reliant upon pots that generally limit soil volume and restrict root growth and development (Poorter et al., 2012). which can obscure and constrain plant responses to environmental stress. Many studies of root architecture utilize young plants in controlled environments in the interest of lower cost and higher throughput. Frequently used experimental techniques include paper germination rolls, pouches and sand or media-filled pots. These platforms facilitate physiological studies (York et al., 2013) and permit the phenotypic and genotypic characterization of large sets of genotypes (for a review see (Lynch, 2011)) but also do not represent natural soil conditions. Advances in imaging software have expanded the throughput of root phenotyping platforms (Basu and Pal, 2012; Clark et al., 2013; De Smet et al., 2012; Galkovskyi et al., 2012; Zhu et al., 2011). MRI, X-Ray, CT, and other media systems are able to extract complex 3D root architecture traits (Clark et al., 2011; Iyer-Pascuzzi et al., 2010; Mairhofer et al., 2013; Metzner et al., 2015; Rellán-Álvarez et al., 2015; Schulz et al., 2013; Topp et al., 2013). These imaging platforms implement a non-invasive measurement procedure that allows capturing times series of one individual root. However, pot size introduces root growth artifacts (Poorter et al., 2012) and to reduce these artifacts trials must be limited to timeframes of 2–3 weeks since roots that hit the side of the pot should be discarded. At that stage some root traits of bean and cowpea, e.g. hypocotyl root number and tap root diameter, have not fully developed.

In contrast, phenotyping of root architecture in real soils is challenging because soils are diverse and opaque. For that reason, 'Shovelomics' (Trachsel et al., 2011) complements laboratory platforms in that it permits phenotyping of mature roots in actual soil in the field. Shovelomics defines visual scores for 10 architectural root phenes of maize crowns at the rate of 2 min per plot. Shovelomics is a simple, robust and inexpensive tool for crop breeders to evaluate root systems and functional responses to varying environments. However, the structurally and functionally dissimilar root architecture and development of maize and bean makes a unique legume protocol necessary. Key differences that distinguish legumes and grasses include the occurrence of secondary growth and the long- term contribution of the embryonic root system in legumes. A key example is the role of legume primary roots in extracting deeply available soil water, which involves significant secondary thickening. In field grown maize the primary root is not always identifiably functional in mature plants and hypocotyl (nodal) roots become important for resource acquisition deeper in the soil profile (Saengwilai et al., 2014).

Recently, an automated image analysis platform, DIRT (Digital Imaging of Root Traits) has been validated for quantifying root architecture of field-excavated root crowns (Bucksch et al., 2014). DIRT is an automated image analysis software developed for quantifying and differentiating crop root phenotypes. Over seventy DIRT traits can be objectively and automatically extracted from thousands of images in under an hour, shifting the bottleneck to root excavation and washing. Several novel DIRT traits have no manual analog due to their mathematical definition. However, the 2D projection to a digital image inhibits DIRT (Bucksch et al., 2014) to extract traits that are observable in the third dimension. Here, manual phenotyping complements DIRT through mechanical testing of tissue flexibility and visual color assessment. Together, image-based and manual field evaluations constitute an untapped potential to confirm phene utility, responses to environmental factors and to appropriately match a phenotype to an environment.

Four development steps were taken to formulate a shovelomics protocol for common bean and cowpea. First we determined root trait scaling from laboratory to field environments in bean. Second, two different rapid field methods to evaluate root architectural phenotypes were developed for bean and cowpea. Third, we compared a rating based system and a quantitative measurement system in bean. A final analysis was carried out to compare visual trait scores, manual trait measurements and image-based trait estimation for bean and cowpea. On the basis of these results a protocol is recommended.

2. Material and methods

2.1. Common bean

2.1.1. Bean laboratory experiment

A customized panel of sixty-four genotypes was obtained from CIAT (Centro Internacional de Agricultura Tropical) were used in this study (Table S1). Genotypes were selected based on diversity

in root phenotypes, and tolerance to low P and drought conditions. Genotypes G 19833 and DOR 364 were included as checks with contrasting adaptation to low P. G 19833 is an Andean genotype tolerant to low P (Beebe et al., 1997; CIAT, 1996) and has shallow basal roots (less than 25° from horizontal) (Liao et al., 2001; Lynch, 1995), three basal root whorls (Basu et al., 2007) and many (more than 25) hypocotyl roots (Miller et al., 2003). DOR 364, from the Mesoamerican gene pool, has poor yield under P deficiency (Beebe et al., 1997), steeper (greater than 30°) basal roots (Liao et al., 2001) and two basal root whorls (Basu et al., 2007). Five bean genotypes obtained from the Agricultural Research Institute of Mozambique (IIAM): Doutor, LIC-04-1-3, Diacol Calima, Ica Pijão and one commercial variety, Bonus, were included as checks. Twenty accessions evaluated in Rock Springs were a subset of the CIAT bean core collection composed of accessions from different races and geographic regions (Colombia, El Salvador, Guatemala, Mexico, Ecuador, Peru, Brazil and Haiti), and representing Andean and Middle American gene pools (Beebe et al., 2000).

Four samples of each genotype in an individual germination paper roll with 4 replications over time were evaluated in the laboratory as a randomized complete block design (RCBD) at The Pennsylvania State University (PSU) in 2006. Seeds were surface-sterilized for 1–2 min with 10% (v/v) NaOCl, rinsed with deionized water, mechanically scarified with a razor and germinated in rolls of brown germination paper No 78 (Anchor Paper Company, St. Paul, MN, USA). The rolls were placed upright in 5 l beakers containing 1 l of 0.5 mM $CaSO_4$. Seeds were allowed to germinate in darkness at 28 °C for 3–4 days. The seedlings were then placed in a plant culture room at 26 °C for 4 days with 12 h of light (120 $\mu m\, m^{-2}\, s^{-1}$). Basal root whorl number and total number of basal roots were counted on 8 day old seedlings.

2.1.2. Bean field experiments

Field trials were conducted at the IIAM Agriculture Research Station of Chokwe, (24°31′S; 33°0′E, 40 m.a.s.l) in 2008 and 2009, the Agriculture Research Station of Umbeluzi, Mozambique (26°03′S; 32°21′E, 64 m.a.s.l) in 2008, and the Russell Larson Agricultural Research Station of The Pennsylvania State University in Rock Springs, Pennsylvania, USA (40°44′N; 77°53′W, 366 m.a.s.l.) in 2010. The soil in Chokwe is a Mollic Ustifluvent with silt-loam texture (Mollic Fluvisols, FAO, 1988), while the soil at the Umbeluzi site is a Mollic Ustifluvent with sandy-loam texture (Eutric Fluvisols, FAO, 1988). The P availability in Chokwe was 38 ppm (Olsen), with pH of 6.8 and 1.8% organic matter, and in Umbeluzi the P availability was 20 ppm (Olsen). In Rock Springs, the genotypes were grown in a Hagerstown silt loam soil (fine, mixed, semi-active, medic Typic Hapludult). The P availability at the field in Rock Springs was 10.5 ppm (P—Mehlich 3 extraction).

Thirty genotypes (Table S2) were planted in RCBD in Chokwe in 2008 and 2009, and in Umbeluzi in 2008. The experiment consisted of 4 replications and each experimental unit was composed of two rows of 5 m. Twenty-five seeds were sown in each row with spacing of 0.7 m between rows and 0.2 m between plants in a row. Nitrogen in the form of urea was applied 25 days after planting at a rate of 30 kg N/ha in trials conducted in Chokwe and Umbeluzi. Phosphorus was not applied in any trial. Irrigation, weed and pest management were applied as needed.

In 2010, twenty accessions of the bean core collection from different gene pools (Beebe et al., 2000) (Supplementary materials Table S2) were evaluated under low phosphorus in Rock Springs Pennsylvania in order to compare values of measured and visually scored root traits. The experiment was planted in a RCBD with 4 replications. Seeds of each genotype were sown in one row of 1.6 m, and the space between rows was 0.7 m and in row plant spacing was 0.2 m. Each experimental unit had 8 plants. Irrigation, weed and pest management were applied as needed.

In the Rock Springs, Pennsylvania 2014 trial 12 Recombinant Inbred Lines (RILs) from the DOR364 × BAT477 population (Blair et al., 2012) were grown under non-limiting and water limiting conditions. The trial had 4 replications, between-row spacing of 76 cm and in-row spacing of 10 cm. Irrigation was withheld from the water-limited plots 14 days after emergence using automated rainout shelters (i.e. the field was protected from precipitation by plastic greenhouse superstructures activated by precipitation sensors but in dry weather were positioned off the field) and irrigation was applied to the well watered treatment when needed to maintain soil water potential near field capacity. Weed and pest management were applied as needed.

2.2. Cowpea field experiments

Seeds of 188 lines of a cowpea diversity panel, a subset of a larger 422 entry diversity panel (Huynh et al., 2013), representing worldwide diversity were obtained from University of California—Riverside (Table S3). Cowpea field trials were conducted in 2012 and 2013 at Ukulima Root Biology Center (URBC), Limpopo South Africa (24°33′00.12 S, 28°07′25.84 E, 1235 m.a.s.l). URBC has a loamy sand (Clovelly Plinthic Entisol) and was fertilized, irrigated and had pesticides applied to provide non-limiting growing conditions. The trials were arranged in RCBD with 4 blocks and each experimental unit consisted of a single 4 m row per genotype. Seeds were planted with a jab planter 4 cm below the soil surface, 30 cm apart in row and 76 cm between rows resulting in a plant density of 100,000/ha. Two representative samples of the four excavated samples were visually evaluated for 11 roots traits and an image of the root crown with identifying tag and scale marker was taken.

2.3. Excavation and evaluation

Root crowns of bean and cowpea were excavated from all experiments between 35 and 45 days after planting (DAP) using a standard spade. Evaluation during the flowering period minimizes differences in phenology among lines that may affect root development. Also, at this stage the mature root phenotype is evident. Excavation volume was defined by a cylinder with a radius of 25 cm around the shoot and a depth of 25 cm. As much as possible of the soil cylinder with the root inside was removed from the surrounding soil and gently washed in water. The looser soils of Umbeluzi and URBC permitted only the removal of the root crown with associated soil aggregates rather than the entire soil cylinder. In the higher clay content soils of Rock Springs (30 g clay/kg soil) 0.5% v/v detergent was added and the root cylinders were soaked prior to washing. Total time required from excavation to evaluation varied from 4 min per plot in soils with sandy-loam texture to 11 min per plot in silt-loam soils with evaluation of 12 phene descriptors requiring 2 min for an experienced phenotyper in all soils (Table 1). Root crowns evaluated in Rock Springs required additional time (approximately 8.5 min) for soaking and washing the roots while root crowns evaluated in the sandier soils of South Africa and Mozambique required only a brief water rinse. Excavated roots were evaluated manually and using the image-based traits estimation of DIRT. Manual measurements were taken as described below. Images were taken and analyzed according the DIRT protocol (Bucksch et al., 2014). In brief, the additional requirements for image analysis includes taking a standard photograph of the root crown on a flat black background with a scale marker, recording the image number with plot identifier information and uploading the image to the online DIRT platform.

Table 1
Time required for field evaluation of 12 root phenes from one crown in different soil textures: Chokwe: Mollic Ustifluvent (silt-loam texture), Umbeluzi, Mollic Ustifluvents (sandy-loam texture), Ukulima, Clovelly Plinthic Entisol (unstructured young sandy texture) and Rock Springs Typic Hapludalf (silt-loam texture). "-" indicates root crowns were not washed.

Activity	Mollic Ustifluvents (Chokwe)	Mollic Ustifluvents (Umbeluzi)	Clovely Plinthic Entisol (Ukulima)	Typic Hapludalf (Rock Springs)
Crown excavation	2.0 min	2.0 min	2.0 min	2.5 min
Soaking	-	-	-	5.0 min
Washing	-	-	1.0 min	1.5 min
Evaluation	2.0 min	2.0 min	2.0 min	2.0 min
Total	4.0 min	4.0 min	5.0 min	11 min

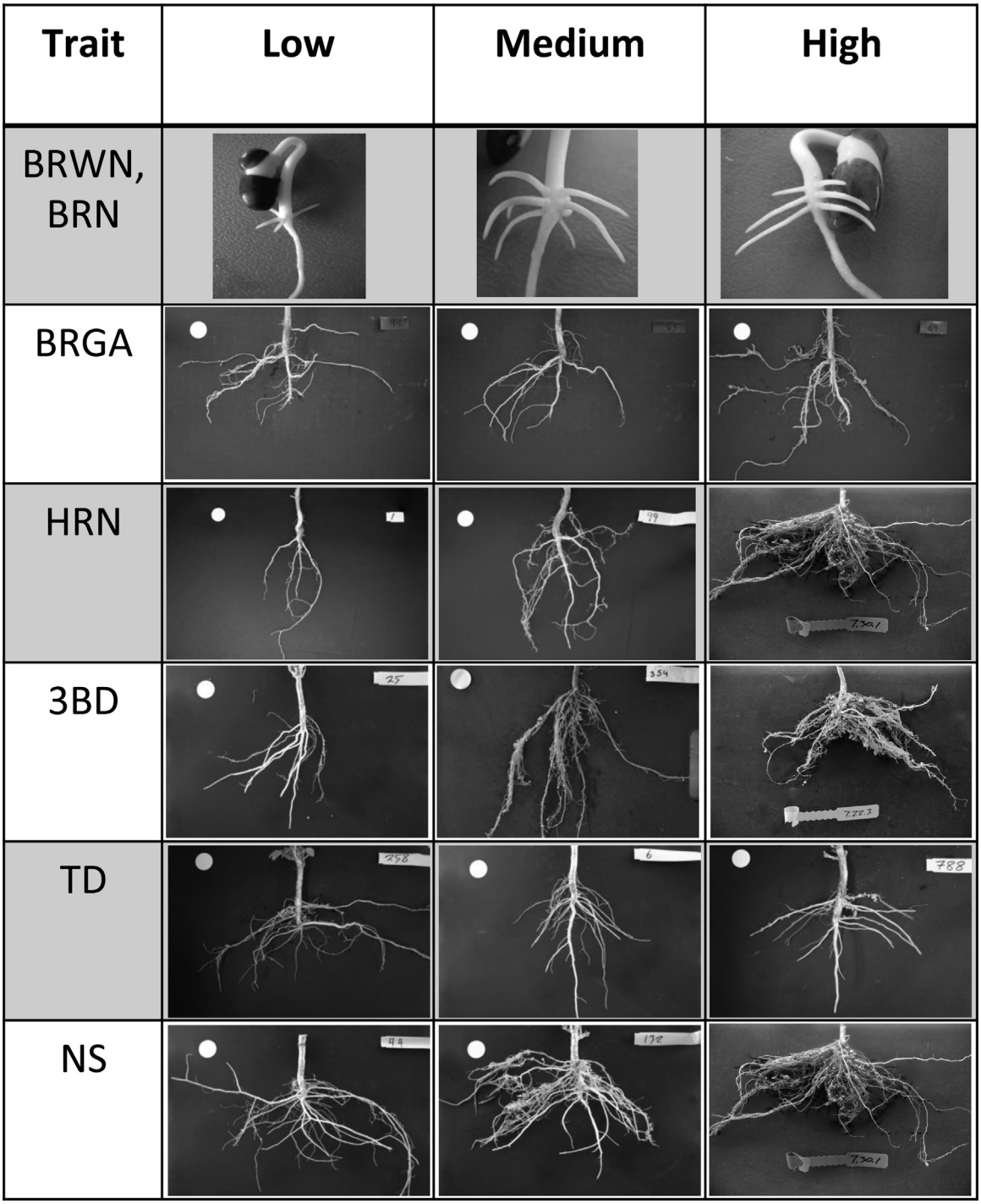

Fig. 1. Photographs of common bean root crowns showing phene states. Trait definitions not in Table 2 are listed here: tap root diameter (TD) is measured 2 cm below the basal roots; third order branching density (3BD) is a rating of overall root system branching density including 2nd and 3rd order laterals: nodulation score (NS) is a rating of visible functional nodules that takes into account both number and size—high (30 +), middle (10–30), and low (0–10), disease score D is a 1–9 rating of root disease where 1 is no visible symptoms of disease and 9 is severely infected.

Table 2
Description of common bean root measurements used in Chokwe, Umbeluzi (2008 and 2009) and Rock Springs 2010.

Trait name	Abbreviation	Method	Definition
Hypocotyl root number	HRN	Count	number of visibly functional hypocotyl roots
Hypocotyl root length	HRL	Board	1 = ≤1 cm; 3 = 4–5 cm; 5 = 8–9 cm; 7 = 12–13 cm; 9 = 15–20 cm
Hypocotyl root branching	HRB	Board	1 = no lateral branching; 5 = 2 orders of laterals; 9 = 4 orders of dense laterals
Basal root whorl number	BRWN	Count	Count sets of 4 basal root whorls, typical range is 1–4
Basal root number	BRN	Count	Number of basal roots
Basal root branching	BRB	Board	1 = no lateral branching; 5 = 2 orders of laterals; 9 = 4 orders of dense laterals
Basal root growth angle	BRGA	Board	Approximate angle (degrees in groups of 10) where basal roots intersect 10 cm arc on board when root origin placed in center. Zero is horizontal and 90° is vertical.
Basal root length	BRL	Board	1 = ≤1 cm; 3 = 4–5 cm; 5 = 8–9 cm; 7 = 12–13 cm; 9 = 15–20 cm
Primary root length	PRL	Board	1 ≤ 3 cm; 3 = 7–9 cm; 5 = 13–15 cm; 7 = 19–21 cm; 9 = 25–30 cm (depth of excavation)
Primary root branching	PRB	Board	1 = no lateral branching; 5 = 2 orders of laterals; 9 = 4 orders of dense laterals
Nodulation	NN or NS	Rating	Estimation of active nodule number 1 = > 80; 3 = 41–80; 5 = 21-40 nodules; 7 = 10-20; 9 = poor < 10
Root Rot	RR	Rating	1 = no visible symptoms; 3 = 10%, 5 = 25%; 7 = 50%; 9 = 75% or more of hypocotyl and root with severe lesions

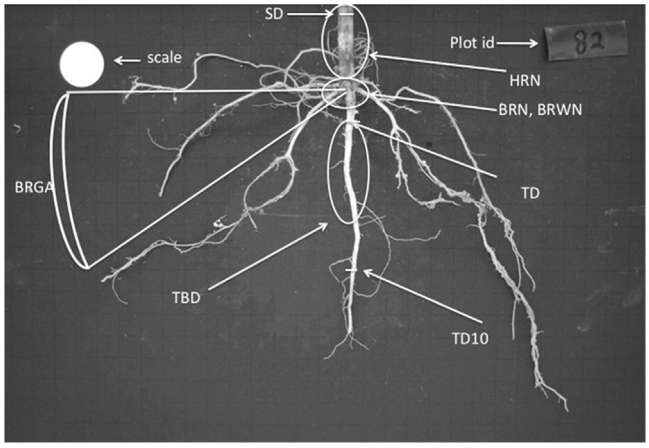

Fig. 2. Annotated image of a common bean root crown highlighting important components.

2.4. Evaluation of root traits

2.4.1. Bean

In Mozambique in 2008 and 2009 9 bean traits were visually evaluated on a linear scale from 1 to 9, where 1 denotes the minimum expression of a trait and 9 the maximum (Table 2 and Fig. 1): hypocotyl root length (HRL); hypocotyl root branching (HB); basal root growth angle (BRGA); basal root length (BRL); basal root branching density (BB); primary root length (PRL); primary root branching density (PB); number of nodules (NN); and root rots (RR). Counts were taken for the total number of hypocotyl and basal roots (HRN, BRN), and basal root whorls (BRWN). One representative sample, based upon overall size, branching density and root deployment pattern was scored for each root trait per replication after observation of 4 root crowns.

In Rock Springs 2010 root crowns of 20 bean accessions were evaluated using the 1–9 scoring system described above. A phenotyping board containing a length scale and protractor was used to obtain quantitative measures of root traits including length of hypocotyl, basal and primary roots and basal root angle. Root branching density was determined by a count of lateral roots in a representative (based upon a visual scan of the entire sample) 2 cm segment of hypocotyl, basal and primary roots (Fig. 2). Stem and tap root diameter were measured using a standard digital caliper with 0.01 mm resolution, at the soil level and 2 cm below the base of the hypocotyl, respectively.

In Rock Springs 2014 we excavated four root crowns per plot and evaluated two root crowns for 6 root traits (BRN, HRN, BRGA, 3BD, TBD, DS) using the phenotyping board technique described above. We imaged all visually evaluated root crowns to obtain stem diameter, tap root diameter, hypocotyl root number and branching density using a proprietary plugin to Image J (available here). The same images were used to estimate root traits with the DIRT platform (Bucksch et al., 2014) and made available to the plant science community at http://dirt.iplantcollaborative.org/ (Das et al., 2015).

2.4.2. Cowpea

The two most representative samples of the four roots excavated were chosen for evaluation, based upon overall root system size and branching density. Root crowns were evaluated for the following 11 parameters both years using the phenotyping board technique described above; tap root diameter 5 cm from soil surface (TD5), tap diameter 10 cm below soil surface (TD10), tap diameter 15 cm below soil surface (TD15), hypocotyl root number (HRN), basal region root number (BRN), number of tap root laterals below the basals to 10 cm from soil level (BD10), number of hypocotyl roots with diameter greater than 1.5 mm 2 cm from origin (1.5A), number of basal roots with diameter greater than 1.5 mm 2 cm from origin (1.5B), root system score for branching density (3BD), nodulation score (NS) and disease score (D) (Table 3, Figs. 3 and 4,).

In 2013, 6 additional parameters were evaluated: stem diameter at soil surface (SD), tap diameter 20 cm below soil surface (TD20), tap diameter 25 cm below soil surface (TD25), dominant hypocotyl root angle (ARGA), dominant basal region root angle (BRGA), and number of 1st order lateral roots 10–15 cm below soil surface (BD15). The root crown was placed on a non-reflective black background and an image taken with a tripod mounted digital camera for image analysis.

2.5. Statistical analysis

Data from Mozambique and from greenhouse and field experiments in Rock Springs 2010 were analyzed using Minitab 16 statistical software (Minitab Inc., State College, Pennsylvania, USA), and Statistix, version 8 (Analytical Software, Tallahassee, FL, USA). Analysis of variance was performed separately for laboratory and field experiments. Genotype and year were considered fixed effects for experiments for Chokwe 2008 and 2009, while location was a fixed effect for Umbeluzi and Chokwe 2008 and block was considered random for both. For laboratory and field experiments in Rock Springs, genotypes were considered fixed factors. Correlation analysis was performed to determine relationship among phene descriptors, and to compare laboratory versus field results as well

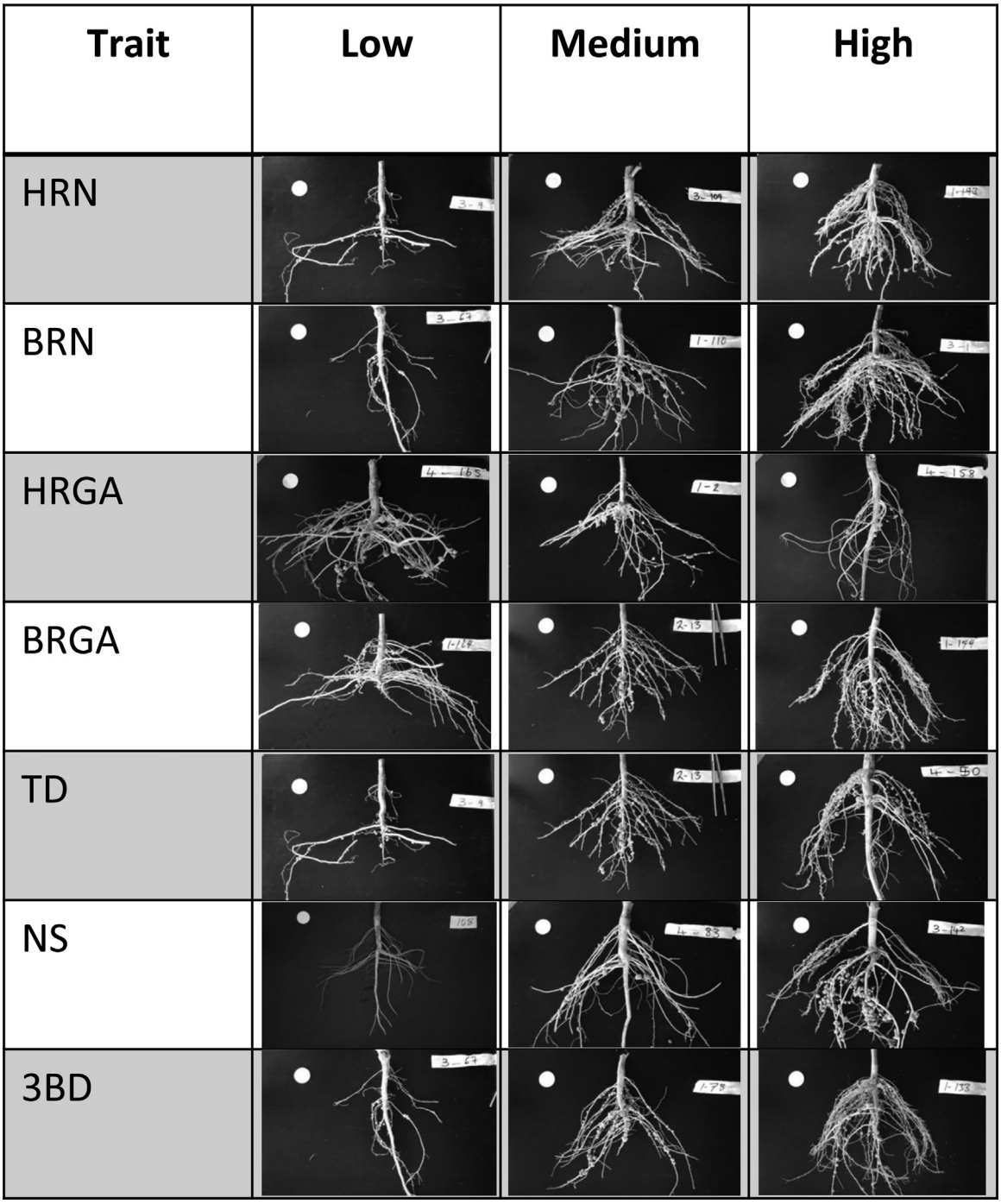

Fig. 3. Cowpea root crowns showing phene states. Trait definitions are listed in Table 3.

visual scores and measured root phenes values. Broad sense heritability was calculated using Fehr's method (see below) across year for Chokwe 2008 and 2009 and by environment (location) in Chokwe and Umbeluzi 2008.

Data from cowpea trials were analyzed using R (R Core Team, 2014). Correlation analysis was performed using the Pearson method, and analysis of variance using the analysis of variance function with genotype, year and genotype by year as fixed factors and block as a random effect. The broad sense heritability H across seasons was estimated using Fehr's method:

$$H = \frac{V_g}{V_g + \frac{V_{gv}}{\#ofreps} + \frac{V_e}{\#ofyears}}$$

where V_g is genotypic variance, V_{gv} is the genotypic by year variance and V_e is the residual variance (Fehr, 1993).

3. Results

3.1. Common bean

3.1.1. Root phenotypic variability

Common bean genotypes differed significantly for basal root whorl number (BRWN) and basal root number (BRN) in 8 d old seedlings ($p \leq 0.001$; Table S1). We observed large phenotypic variation for most root phene descriptors evaluated in the field including BRWN, which ranged from 1 to 3.75 and BRN, which

Table 3
Description of manual cowpea root architectural measurements used URBC 2012 and 2013.

Trait name	Abbreviation	Method	Definition
Hypocotyl root growth angle	HRGA	Board	approximate angle where hypocotyl roots intersect 10 cm arc on board when root origin placed in center
Basal root growth angle	BRGA	Board	approximate angle where basal roots intersect 10 cm arc on board when root origin placed in center
Stem diameter	SD	Caliper	stem diameter at soil level
Tap diameter 5 cm	TD5	Caliper	tap diameter 5 cm below soil surface or just below basal region
Tap diameter 10 cm	TD10	Caliper	tap diameter 10 below soil surface
Tap diameter 15 cm	TD15	Caliper	tap diameter 15 cm below soil surface
Basal root number	BRN	Count	number of basal region roots
Hypocotyl root number	HRN	Count	number of hypocotyl roots
Branching Density 10	BD10	Count	number of 1st order lateral roots (not counting basal region roots) between 5 and 10 cm or from just below basal region to 10 cm below soil level
Branching density 15	BD15	Count	number of 1st order lateral roots between 10 and 15 cm below soil surface
Hypocotyl roots1.5 mm or larger	1.5H	Count	number of hypocotyl roots with diameter greater than 1.5 mm 2 cm from hypocotyl
Basal roots 1.5 mm or larger	1.5B	Count	number of basal roots with diameter greater than 1.5 mm 2 cm from hypocotyl
Number of large tap root laterals	1.5BD10	Count	number of roots in region 5–10 cm below soil surface with diameter greater than 1.5 mm 2 cm from hypocotyl
3rd order branching density	3BD	Rating	3 order branching density score 1 = few, 9 = many
Nodulation	NS	Rating	nodulation score 1 = none, 9 = many large nodules
Disease	D	Rating	disease score 1 = healthy, 9 = severely affected

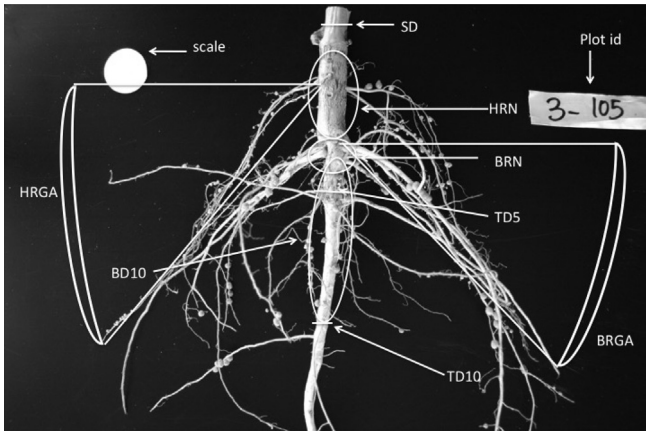

Fig. 4. Annotated image of a cowpea root crown highlighting important components.

ranged from 4 to 13.5 (Fig. S2 and S3). Significant differences among genotypes within environment and within year were observed for hypocotyl root number and branching, basal root growth angle, BRWN, BRN, and primary root length (Table 4, summary statistics in Table S4).

3.1.2. Field and laboratory evaluations were highly correlated

Bean BRWN evaluated in 8 d old seedlings in the laboratory was highly correlated with BRN evaluated in 45 d old plants in the field in Chokwe in 2009 ($R^2 = 0.803$, $p < 0.001$). BRWN and BRN evaluated both in the laboratory and in the field were also highly correlated ($R^2 = 0.93$ and $R^2 = 0.66$, $p < 0.001$, respectively, Fig. S4). BRN evaluated in 8 d old seedlings in the laboratory was greater than the BRN evaluated in 45 d old plants in the field, suggesting root loss in the field. BRWN was strongly correlated with BRN when both were evaluated in the laboratory ($R^2 = 0.949$, $p \leq 0.001$), or field ($R^2 = 0.867$, $p \leq 0.001$, Fig. S5).

3.1.3. Root phenotypes were consistent across years and environments

Effects of genotype by environment and genotype by year interactions were evaluated using ANOVA for all 12 phene descriptors. Besides HRN ($p < 0.01$) and root rot infection ($p < 0.01$) we did not observe significant interactions of genotype with year or environment on phenotypes (Table 4). Phene descriptors with moderately high heritability in 2 years in the same environment and in 2 environments in the same year include BRWN (86, 83), BRN (79, 74) and BRGA (67, 60) and one with moderately high heritability is HRN (56, 53) (Tables 5 and 6). A graphical representation of the range,

Table 4
ANOVA for root traits in two environments (Chokwe and Umbeluzi) and two years (2008 and 2009 in Chokwe) in 30 genotypes. F values and significance levels for the effect of the environment (E), year, and genotype (G) within environment and year, and interactions G by E and G by year are shown for the following traits: hypocotyl root number (HRN), hypocotyl root length (HRL), hypocotyl root branching (HRB), basal root whorl number (BRWN), basal root number (BRN), basal root length (BRL), basal root branching (BRB), basal root growth angle (BRGA), primary root length (PRL), primary root branching (PRB), number of nodules (Nod) and root rot (RRot). Level of significance: *** = significant at $p < 0.001$, ** = significant at $p < 0.01$, * - significant at $p < 0.05$, ns = not significant. G = genotype and E = environment.

	HRN	HRL	HRB	BRWN	BRN	BRL	BRB	BRGA	PRL	PRB	NN	RR
E	301.8 ***	8.58 **	14.05 ***	0.92 ns	1.48 ns	23.07 ***	0.50 ns	65.4 ***	170.47 ***	3.70 ns	4.55 *	15.39 ***
Geno	6.80 ***	1.23	1.44 ns	19.71 ***	12.04 ***	1.96 *	1.14 ns	6.18 ***	1.72 *	1.74 *	1.03 ns	1.47 ns
G*E	1.78 *	0.92 ns	1.49 ns	0.16 ns	0.86 ns	1.28 ns	0.61 ns	0.56 ns	1.06 ns	0.73 ns	0.71 ns	1.72 *
Year	10.45 **	20.52 ***	0.15 ns	0.71 ns	19.62 ***	1.84 ns	12.97 ***	0.10 ns	8.75 **	24.94 ***	1.85 ns	1.79 ns
Geno	7.26 ***	1.26 ns	2.07 **	23.87 ***	16.81 ***	1.57 *	1.43 ns	8.58 ***	1.83 **	1.36 ns	1.16 ns	1.09 ns
G*Year	1.69 *	0.72 ns	1.04 ns	0.43 ns	1.24 ns	0.91 ns	0.65 ns	0.74 ns	1.38 ns	1.18 ns	0.76 ns	1.56 *

Table 5
Estimation of broad sense heritability (h2) from two years of data from one location, Chokwe 2008 and 2009 100* $V_g/[V_g + (V_{gy}/\#\text{ of reps}) + (V_e/\#\text{ of years})]$.

		Variance component (V)			
2008 and 2009	Year	Genotype	G*Y	Error	H^2
HRN	2.282	21.748	5.401	31.36	0.56
HRL	0.3088	0.1267	−0.133	1.8716	0.12
HRB	−0.00323	0.05625	0.0047	0.4357	0.20
BRWN	0.0002	0.2758	−0.0133	0.0942	0.86
BRN	0.1979	2.5148	0.0771	1.2918	0.79
BRL	0.0093	0.0996	−0.0271	1.2004	0.14
BRB	0.0338	0.0321	−0.0285	0.3291	0.17
BRGA	−0.0078	1.4305	−0.0947	1.4601	0.67
PRL	0.0491	0.0448	0.0768	0.8006	0.10
PRB	0.1113	0.0129	0.0253	0.5621	0.04
NN	0.0029	0.0163	−0.0196	0.3248	0.09
RR	0.00029	−0.0088	0.0208	0.1488	−0.12

mean and median indicates which traits may be better suited to differentiate genotypes (Fig. 5).

3.1.4. Scored vs. measured root traits in common bean

In order to validate our field visual root scoring method values of measured phenes were compared with values from visual scoring. Significant differences were found between genotypes for all measured and visually scored root phenes, except for primary root branching (Tables S5, S6). In addition, correlations between measured and visually scored phenes of twenty genotypes evaluated in Rock Springs 2010 varied from low to high (0.31 for BRB to 0.76 for BRGA) and all were statistically significant (Table S7). High correlations were found for BRGA (0.755), HRL (0.733), PRL (0.644), and BRL (0.584).

3.2. Cowpea

ANOVA of the two cowpea seasons revealed significant variation associated with genotype and year with generally normal distributions (Fig. S6). For almost all traits both genotype and year had significant effects while genotype by year interactions were only significant for AN, BN, 1.5A, 3BD and NS (Table 7). Moderate heritability was found for TD5 (27), HRN (27), 3BD (21), NS (44) and high heritability for 1.5A (79) (Table 8). A graphical representation of coefficient of variation in cowpea phene descriptors indicates which may be better suited to differentiate genotypes and phenotypes (Fig. 6). Tap root diameter measurements deeper than 10 cm below the soil surface had very low heritability.

Table 6
Estimation of broad sense heritability (h2) for two environments, Chokwe and Umbeluzi 2008 100*$V_g/[V_g + (V_{ge}/\#\text{ of reps}) + (V_e/\#\text{ of envirn})]$.

		Variance component (V)			
Chokwe and Umbeluzi	Envirn	Genotype	G*E	Error	H^2
HRN	63.655	15.958	4.981	25.461	0.53
HRL	0.108	0.068	−0.036	1.691	0.08
HRB	0.063	−0.004	0.074	0.600	−0.01
BRWN	0.001	0.278	0.278	−0.024	0.83
BRN	0.009	2.454	−0.062	1.756	0.74
BRL	0.156	0.074	0.059	0.860	0.14
BRB	−0.001	0.035	−0.052	0.533	0.12
BRGA	0.684	0.891	−0.140	1.267	0.60
PRL	1.380	0.081	0.081	0.015	0.75
PRB	0.019	0.097	−0.051	0.761	0.21
NN	0.010	0.012	−0.021	0.297	0.08
RR	0.011	−0.003	0.018	0.098	−0.06

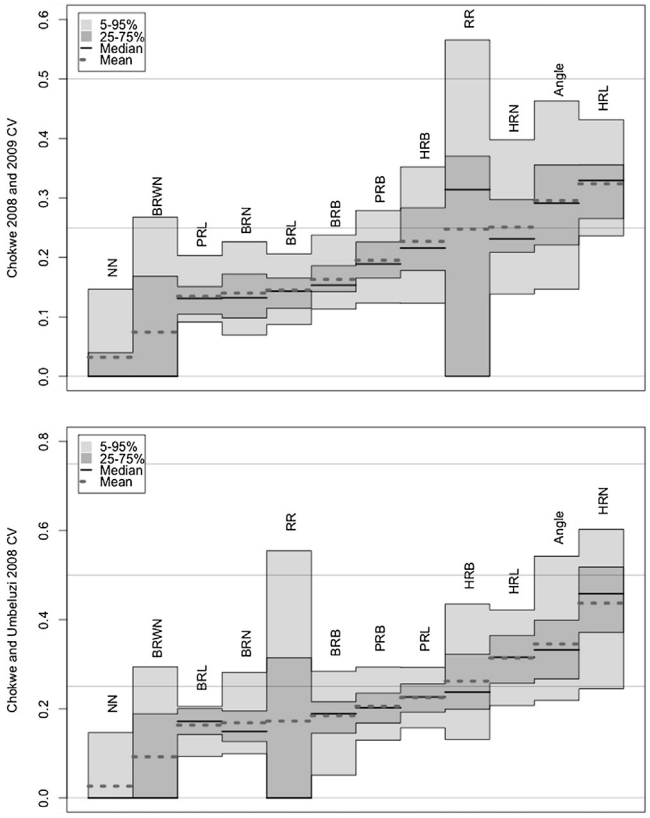

Fig. 5. Graphical representation of coefficient of variation of observations for common bean genotypes. CV was calculated by dividing the standard deviation by the mean, in this case for each genotype across blocks, environments and years. Larger values indicate greater variation and less confidence in the repeatability of the observation.

Table 8
Estimation of broad sense heritability for cowpea root traits over two seasons at URBC. 100*$V_g/[V_g + (V_{gy}/\#\text{ of reps}) + (V_e/\#\text{ of years})]$.

Trait	Genotype	Residual	Genotype x Year	Year	H^2
TD5	0.339	1.806	0.1	0.0616	0.27
TD10	0.08	1.35	0.1	0.0001	0.10
TD15	0.0074	0.954	0.026	0.0675	0.02
BRN	0.373	4.46	0.447	4.73	0.14
HRN	2.5	12.45	1.847	14.7	0.27
BD10	0.459	15.64	0.983	13.85	0.05
1.5H	6.682	2.176	2.364	2.781	0.80
1.5B	0.0893	0.8002	0.0454	0.0157	0.18
3BD	0.269	1.856	0.3368	1.063	0.21
NS	0.861	1.987	0.3114	0.2033	0.45
D	0.249	1.9634	0.0677	0.113	0.20

Table 7
ANOVA Table for cowpea root traits over two seasons showing F value and significance level. Significance values * = significant at 0.01, ** = significant at 0.001.

	TD5	TD10	TD15	HRN	BRN	BD10	1.5H	1.5B	3BD	NS	D
Genotype	2.4	1.6 **	1	4.4 **	2.1 **	2.2 **	3.4**	2.0 **	3.2 **	4.8 **	2.1
Year	51.3	3.1	49.3	756.8	662.6	551.0*	7.8**	21.6*	359.6	75.9*	32.9
Genotype x Year	1.2	1.2	1.1	1.6 **	1.3 *	1.2	1.3*	1.2	1.5 **	1.5 **	1.2

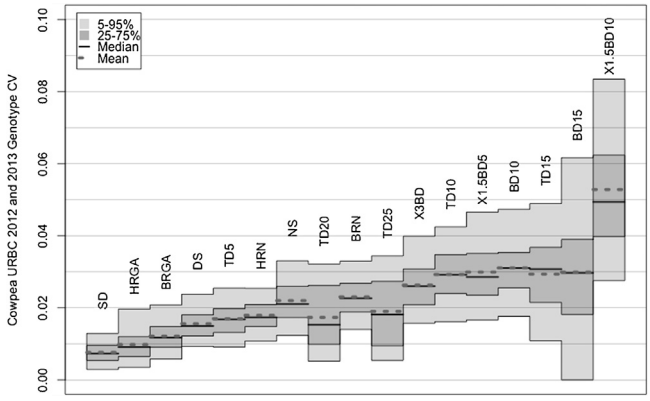

Fig. 6. Graphical representation of coefficient of variation of observations for cowpea genotypes. CV was calculated by dividing the standard deviation by the mean, in this case for each genotype across blocks, environments and years. Larger values indicate greater variation and less confidence in the repeatability of the observation.

3.3. Image based (DIRT) vs. manual phenotyping

Four minutes were required to excavate and manually evaluate 12 phenes from one root crown. Soil type and texture influenced time required for root excavation and washing. Manual evaluation requires between 1 and 2 min per crown and arranging a root for image acquisition requires approximately 30 s. Remote image analysis using DIRT allows more samples to be processed in a shorter period of time, which reduces the effects of secondary growth and environmental factors. Image J analysis requires approximately 1 min per image whereas thousands of images can be analyzed by DIRT per hour. Significant correlations exist between DIRT and manual descriptors of bean root architecture indicating which descriptors may be a reasonable substitute. Table 9 shows a comparison of substitutes such as the tap root diameter ($r = 0.71$, $P = 0.01$) or basal root number ($r = 0.51$, $P = 0.008$). Additionally, DIRT provides manually inaccessible trait definitions that, so far, have proven to be distinctive for genotypes such as D_x and DS_x values, and RTA and STA ranges. D_x and DS_x values are functions describing the rate of width accumulation over the depth, thus capturing the shape of the root hull. RTA and STA ranges describe, in relation to their respective means, variation within individual root systems.

Given the relatively small sample size per genotype we accept a pair of genotypes to be distinguishable if their respective standard error of the mean cover distinct numerical ranges. The image-based phenotyping could distinguish all possible combinations of bean genotypes. In contrast, the manually measured bean traits failed to distinguish six genotype combinations in Chokwe and all but 4 genotype combinations in Umbeluzi (for example differentiation plot see Fig. S7). For the cowpea diversity panel, we found that all 188 cowpea genotypes could be distinguished by at least one image-based measurement and the manually measured phenes distinguished all but 5 genotype combinations (Bucksch et al., 2014).

Investigation of an apparent lack of agreement between DIRT and manual measurements of BRGA and HRN in the Rock Springs 2014 experiment revealed some of the variation to be caused by root flexibility and color variation. Roots of plants with less secondary growth are more flexible, increasing the potential influence of root placement on the phenotyping board on subsequent BRGA measurements. For HRN, we compared an image-based manual count of all hypocotyl roots and then all hypocotyl roots judged to be functional to DIRT results. Initial Pearson correlation coefficients were 0.32 and 0.28 respectively and a robust regression estimator (RANSAC) was used to create inlier and outlier groups at a 99% confidence interval that were uniformly dispersed on both sides of the regression line. After the RANSAC conversion we found coefficients with p values <0.001 of 0.72 and 0.49 for all roots and functional roots, respectively. These results indicate DIRT adequately captures hypocotyl root number but disease and or drought pressure complicates root counts as roots become dysfunctional and die. Manual measurements take into account color variation that indicates health and functionality while the binary image DIRT uses cannot account for these subtle differences.

3.4. Bean and cowpea comparisons

Basal and hypocotyl roots show genetic variation for growth angle but angles of basal roots vary less in cowpea than in common bean (Fig. 7). Cowpea adjusts the number and diameter of lateral roots in a more gradual manner along the hypocotyl and radical than common bean, which has a root system architecture dominated by clearly defined basal roots. The 'herringbone' pattern of cowpea may be more typical of other annual legumes. Tap root strength, gauged by tap root diameter varies in both species but a much higher median and larger range was found in cowpea. Cowpea shoots are generally larger than bean and cross species comparisons should consider allometry. Here we use a *t*-test to compare log of biomass and log of TD for bean and cowpea and find the slopes are significantly different. Correlation coefficients between TD and plant biomass are moderate (0.36 for bean and 0.25 for cowpea) indicating genetic variation exists for TD independent of shoot biomass.

4. Discussion

We have developed a protocol combining manual and automated image-based analysis of field grown roots of common bean and cowpea that takes advantage of the strengths of each technique. Automated evaluation is objective, has higher throughput, and is able to describe root architecture with higher-order phene descriptors in terms of mathematical functions and in relation to other parameters of the individual root system. However, automated evaluation lacks the ability to quantify color, flexibility and is limited by root occlusion and root placement on the imaging board. Manual phenotyping is slower and more subjective but is better suited to gauge nodulation, disease, count fine roots and obtain information through manipulating and examining traits best viewed in three dimensions, such as angle. A weakness of the protocol is that it samples only a portion of the entire root system and may not capture fine roots. However relationships between crown measurements and root length density have been shown in maize (Trachsel et al., 2013; Zhu et al., 2010). A related weakness is the difficulty inherent with recovering small diameter roots, which make up the majority of root length. The most time consuming steps of either manual or image analysis are root excavation and washing, meaning combining both has a favorable cost to benefit ratio. This protocol can differentiate genotypes in a given environment and can quantify genotypic by environmental variation through evaluation in multiple environments. A principle advantage of this protocol is that it directly evaluates expressed phenotypes in the target environment.

Observation of root architectural phenes over multiple growing seasons and locations permitted evaluation of environmental influence on root phenes and the utility of a given observation to differentiate genotypes. Although we found differences in root phenotypes across years and environments, they did not lead to genotype by year, and genotype by environment interactions for most phenes. The exception is hypocotyl root number (Table 3), which was likely due to differences in surface soil moisture. Differences in precipitation (64.6 mm in 2008 and 109.5 in 2009) likely

reduced the number of observable hypocotyl roots as their more horizontal growth makes them susceptible to soil drying. The limited genotypic variation in root branching and length of hypocotyl, basal and primary roots is influenced by the excavation process and we do not recommend using length measurements from field-grown root crowns.

We also validated a laboratory-based 'roll-up' phenotyping of BRWN and BRN, which simplifies the evaluation for these important phenes in common bean. We observed fewer basal roots in the field compared to the laboratory, which we interpret as root loss due to biotic and abiotic stresses (Fisher, 2002). In addition to BRWN and BRN having utility in low P, drought and combined low P and drought environments, they may also have utility in environments with significant belowground biotic stress, in which more numerous basal roots may compensate for root loss from herbivores and pathogens.

4.1. Cowpea and bean strategies

Based on the relatively small diversity panels included in this study we suggest that cowpea exhibits greater phenotypic variation among genotypes for TD and BRN (Fig. 7) but less intra-genotypic variation than does bean (Fig. 6). Most cowpea phenes have lower CV values than do comparable phenes in common bean. Cowpea's greater range in TD and BRN indicates that in terms of root architecture cowpea offers greater phenotypic variation for some soil resource scavenging traits, particularly those hypothesized to be beneficial for deep water acquisition. A larger TD may enable a plant to extend deeper into the soil profile and access water resources that are inaccessible to shallower-rooted genotypes. This greater range of TD and much greater median TD indicates that cowpea root architecture is taproot dominated. Measuring tap root diameter at multiple depths may help to differentiate allocation strategies. Allometric comparisons indicate the greater median TD is not due only to allometry. The different slopes of log TD and log biomass between cowpea and common suggest bean has divergent root strategies and that cowpea is more tap root dominated. Cowpea's strong taproot paired with variable BRN may help contribute to its drought tolerance. An alternate approach to accessing more water and nutrient resources is to extend many smaller basal roots over a range of soil zones. While cowpea has a slightly greater range of BRN, common bean has greater median BRN and a greater BRGA range as well as greater median HRN. This suggests common bean has a basal root dominated system that has the ability to target shallow or deep soil exploration. Its adaptive plasticity stems from variation in BRGA, BRN and HRN. Both of these strategies seem to make sense given cowpea's purported evolution and domestication in drought-prone areas and common bean in nutrient limited, highly competitive riparian zones. The higher CV values observed for root traits in bean are suggestive of an adaptive strategy in which plasticity itself is a beneficial trait that allows a phenotype to match and respond to a given environment as has been shown in maize (Zhu et al., 2005).

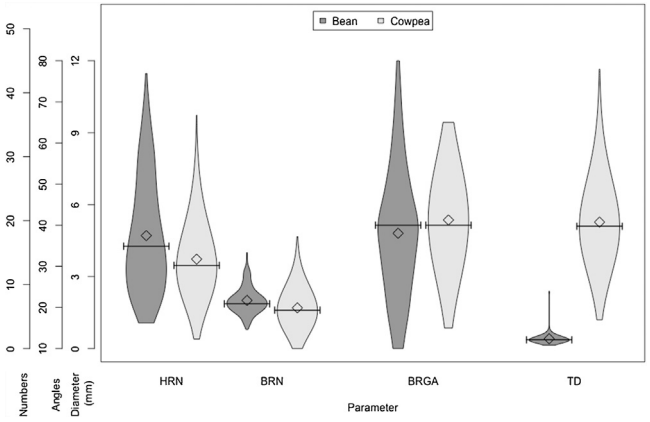

Fig. 7. Comparison of variation between relevant manually accessed common bean (Chokwe and Umbeluzi 2008, for TD ROS 2014 data used) and cowpea (URBC 2012 and 2013) root parameters. Shaded area resprsents variation, line shows median, square indicates mean. Note that common bean tap root diameter in ROS 2014 trial was generally smaller than observed in other trials where a range up to 3 mm may be expected.

Table 9
Correlations between comparable manual and DIRT observations for the PA ROS 2014 common bean data set on a per plot basis. Level of significance: *** = significant at $p < 0.001$, ** = significant at $p < 0.01$, * - significant at $p < 0.05$, ns = not significant.

DIRT	Manual	Pearson r
#Basal roots	BRN	0.51**
#Basal roots	TBD	0.78**
angBas	BRGA	0.64*
D30	BRGA	0.65*
D50	BRGA	0.66*
D60	BRGA	0.67*
D70	BRGA	0.66*
D80	Brga	0.64*
DD90 max.	TBD	0.65*
DS10	3BD	0.67*
DS20	BRGA	−0.73**
DS30	BRGA	−0.6*
DS 40	BRGA	−0.58*
Nr.RTPs	3BD	0.38 ns
projected root area	TBD	0.74**
projected root area	3BD	0.59*
STA 90%	BRGA	0.61*
STA 90%	BRN	−0.72**
Taproot dia	TBD	0.71**

4.2. Recommended protocol

We recommend the following field-based shovelomics protocol for common bean and cowpea that combines manual- and image-based phene descriptors. Tutorials are available online (http://plantscience.psu.edu/research/labs/roots/projects/usaid-crb/resources/english/shovelomics-videos). Additionally, visual evaluation of seedlings grown in roll-ups offers a rapid method to screen for BRWN, BRN and root hair length and density. At flowering or pod elongation excavate 4–6 plants per plot and select the most representative crowns based on a quick visual evaluation of health, vigor, symmetry, diameters and branching density. Select 2–4 plants for evaluation using manual and image based measurements from at least 3 replications (Table 10). For image acquisition and DIRT analysis position root crown naturally on flat finished black background approximately 40–50 cm from lens with identifying tag and circular scale marker. Do not allow roots to intersect with other objects on the board. Record image and record image number in spreadsheet with associated manual

Table 10
Recommended manual root observations for cowpea and common bean. See Tables 2 and 3 for details on how to measure.

Trait name	Abbreviation	Method
Hypocotyl root number	HRN	Count
Basal root number	BRN	Count
Basal root whorl number	BRWN	Count
Basal root growth angle	BRGA	Measure using board
Hypocotyl root growth angle	HRGA	Measure using board
Tap root branching density	TBD	Count or Image J count
Tap root diameter	TD5	Caliper or Image J measure
Nodulation score	NS	Score
Disease score	DS	Score

data. Upload images to DIRT (http://dirt.iplantcollaborative.org/), set parameters for automated analysis and submit for analysis. Additional descriptions can be made at the researcher's discretion, such as measuring basal or hypocotyl root lateral root branching density, separating the disease score into radical and stem components, counting the number of roots of a given class greater than a certain diameter threshold, or rating or counting nodules. Measuring tap root diameter of cowpea 10 cm below soil level is recommended if time permits. Scores are given on a 1–9 scale with 1 being very low and 9 being high. Angles are measured in increments of 10° from 0 to 90 with 0 being horizontal with respect to gravity.

5. Conclusion

We suggest this legume-tailored, flexible and low-cost phenotyping platform as an appropriate tool for in-field identification and selection of bean and cowpea genotypes with desirable root phenotypes. Several root phenes are highly plastic and environmental responses make field evaluation in the target environment fundamental to effective characterization. Future research should address the value of different phene descriptors to further focus and streamline the crop improvement process. These phenotyping tools should be useful to traditional yield-based breeding programs and to those employing trait-based selection. In either case the phene descriptors should lend themselves to GWAS. The demonstration of manual and image based analysis on two distinct legume root systems suggests general utility of customized protocols for phenotyping tepary bean, soybean, pigeon pea, groundnut, chickpea and other pulse or even non-pulse roots such as tomato or potato. The low cost, high-throughput and high reproducibility afforded by the combination of manual and image based analysis enables large multi-location trials that are able to track genotypic responses and phenotypic utility across environments. We anticipate that broad application of the shovelomics technique will reveal biologically relevant phenes and aid in identifying their genetic control. We expect this platform to be scaled up and automated, potentially including mechanized excavation and imaging. Integration with *SimRoot* or other heuristic computer simulations would enable projections of rooting patterns outside the crown root area and enable estimation of their physiologic contribution. Pairing *in-vivo* with *in-silico* approaches would be synergistic and would accelerate the identification of ideotypes based on actual field observations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2016.04.008.

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