Plant Soil (2013) 369:269–282
DOI 10.1007/s11104-012-1571-2

REGULAR ARTICLE

Root hairs explain P uptake efficiency of soybean genotypes grown in a P-deficient Ferralsol

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Received: 12 October 2012 / Accepted: 18 December 2012 / Published online: 3 January 2013

Abstract
Background and aims Incorporating soybean (*Glycine max*) genotypes with a high nitrogen fixation potential into cropping systems can sustainably improve the livelihoods of smallholder farmers in Western Kenya. Nitrogen fixation is, however, often constrained by low phosphorus (P) availability. The selection of soybean genotypes for increased P efficiency could help to overcome this problem. This study investigated the contribution of different root traits to variation in P efficiency among soybean genotypes.
Methods Eight genotypes were grown in a Ferralsol amended with suboptimal (low P) and optimal (high P) amounts of soluble P. Root hair growth was visualized by growing plants in a novel agar system where P intensity was buffered by Al_2O_3 nanoparticles.
Results In the pot trial, P uptake was unaffected among the genotypes at high P but differed about 2-fold at low P. The genotypes differed in P uptake efficiency but not in P utilization efficiency. Regression analysis and mechanistic modeling indicated that P uptake efficiencies were to a large extent related to root hair development (length and density) and, to a lower extent, to colonization by mycorrhizal fungi.
Conclusion Breeding for improved root hair development is a promising way to increase P uptake efficiency in soybean.

Keywords Mycorrhizal fungi · Modeling P uptake · Plasticity · Phosphorus efficiency · Root hairs · Soybean genotypes

Abbreviations

AMF	Arbuscular mycorrhizal fungi
DAP	Days after planting
IITA	International Institute of Tropical Agriculture
MP	Maturity period
TSBF-CIAT	Tropical Soil Biology and Fertility Institute of the International Center for Tropical Agriculture

Responsible Editor: Tim Simon George.

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Introduction

Soil fertility decrease in low-input cropping systems is a serious threat to the livelihoods of smallholder farmers in Western Kenya. Soils in this region are generally strongly weathered and characterized by low pH, low

nitrogen (N) content, high content of aluminum and iron oxides and low phosphorus (P) availability. Large amounts of fertilizers are needed to increase production levels, but these are often beyond the reach of resource-poor farmers. In this view, the cultivation of N-fixing legumes such as soybean (*Glycine max*) offers considerable potential to increase soil fertility. By incorporating soybean into cropping systems, system productivity can be increased at low cost and soil fertility decrease can be counteracted. However, the process of N fixation needs large amounts of P (Zahran 1999; Sanginga 2003), and low P availability therefore strongly undermines possible positive effects of incorporating soybean into the rotation cycle. Breeding for increased P efficiency, i.e., the capacity to maintain production levels under low P conditions, in combination with judicious use of reduced amounts of P fertilizers, could help to overcome this problem. This strategy fits into the broader concept of Integrated Soil Fertility Management, in which the development of improved and efficient germplasm plays an essential part (Vanlauwe et al. 2010).

Several studies have reported on differences in P efficiency between species (Föhse 1988; Sanginga et al. 1996; Rao et al. 1997), and genotypes (Bonser et al. 1996; Osborne and Rengel 2002; Gahoonia and Nielsen 2004a). For soybean, genotypic variation in P efficiency has been demonstrated by Pan et al. (2008). P efficiency can be obtained either through (i) increased P uptake efficiency, defined as the ability to maintain levels of P uptake at suboptimal P availability relative to P uptake at optimal P availability, or (ii) increased P utilization efficiency, defined as the ability to utilize P taken up for the production of biomass. Among both, breeding for increased P uptake efficiency is considered most promising (Lynch 2007). Increased P uptake efficiency is related to a range of root characteristics, i.e., (i) root growth and architecture (root to shoot ratio, distribution among root types, root angles), (ii) root morphology (root diameter, root hair development, aerenchyma), (iii) rhizosphere processes (acidification, phosphatase activity, exudation of organic acids) and (iv) symbiosis with mycorrhizal fungi (reviewed by Gahoonia and Nielsen 2004b; Lambers et al. 2006; Lynch 2007; Ramaekers et al. 2010). These root characteristics can be the result of either features that are expressed irrespective of environmental conditions, or genotype-specific environmental responses, the latter being called plasticity. Plasticity, in turn, can be regulated by both localized and systemic mechanisms, whereby the first responds to the external nutrient concentration, and the second to the internal nutrient status of the plant (Forde and Lorenzo 2001). The ability to respond in a plastic way to the environment is a particularly beneficial trait when trade-offs between root characteristics exist. For instance, a reduction in root diameter enhances P uptake but also brings along increased vulnerability to root herbivores and pathogens and a reduced ability to penetrate hard soil (Lynch 2007).

The study of P efficiency can be assisted by an understanding of the mechanisms underlying P uptake efficiency. Nutrient uptake models provide insights in the factors affecting P uptake and hence P efficiency, and have proven useful tools in studies addressing P efficiency (Föhse et al. 1991; Kirk 2002; Dechassa et al. 2003). Mechanistic models allow quantifying the effect of specific root characteristics on P uptake and hence estimating the relative contribution of different traits to eventual P efficiency. This may be particularly important when traits of focus need to be selected in breeding programs. Phosphorus efficiency of soybean genotypes in China has been linked to shallow root angles, improved root hair development, enhanced root exudation and improved root symbiosis (reviewed by Wang et al. 2010). In East Africa, little is known about genotypic variation in P efficiency of soybean. In this study, we explored the variation in P efficiency among 8 soybean genotypes which are currently grown in farmer and project fields across East Africa, and subsequently tried to elucidate the relation between P efficiency and several root characteristics by regression analysis and mechanistic modeling. The specific objectives of this study were (i) to evaluate genotypic variation in P efficiency among selected soybean genotypes, (ii) to evaluate genotypic variation in root characteristics that are important for P uptake, and (iii) to assess which root characteristics contribute most to genotypic variation in P uptake efficiency and can therefore be targeted in breeding programs, in a representative Ferralsol from Western Kenya characterised by low P availability. A novel system was developed to measure root hairs on plants exposed in agar to low P intensity buffered by a solid phase, thereby overcoming drawbacks in sampling roots from

soil or exposing plants to solutions with unbuffered P supply, unlike in soils.

Materials and methods

Plant materials

Eight soybean genotypes from the seed collection of the Tropical Soil Biology and Fertility Institute of the International Center for Tropical Agriculture (TSBF-CIAT) were selected. The selection included 3 genotypes originating from the breeding program of the International Institute of Tropical Agriculture (IITA) (TGx 1740-2F, TGx 1448-2E and TGx 1903-1F), 2 genotypes originating from the USA (TGm 1360 and Ogden), a genotype from Uganda related to an IITA genotype (Namsoy 4m), and 2 genotypes from Zimbabwe (Nyala and Pan 872). TGx 1740-2F, Nyala and Pan 872 are early maturing genotypes with a maturity period (MP) around 95 days. Namsoy 4m, TGx 1903-1F, TGm 1360 and Ogden are medium maturing genotypes (MP around 110 days) and TGx 1448-2E is a late maturing genotype (MP around 120 days).

Greenhouse experiment

The experiment was carried out in a greenhouse at TSBF-CIAT in Nairobi, Kenya. Topsoil (0–20 cm) was sampled from a field in the village of Nyabeda (Siaya district, Kenya), air-dried and passed through a 5 mm sieve. The soil was classified as a Ferralsol (FAO-ISRIC-ISSS 1998) and represented a typical 'acid, red soil' characterized by low P availability. Selected soil properties are presented in Table 1.

The soil was amended with nutrient solutions containing $MgSO_4.7H_2O$, $MgCl_2.6H_2O$, KCl, $CaCl_2.2H_2O$, $ZnCl_2$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $Na_2B_4O_7.10H_2O$ and $Na_2MoO_4.2H_2O$ at rates of 40 mg Mg kg^{-1}, 22 mg S kg^{-1}, 505 mg K kg^{-1}, 65 mg Ca kg^{-1}, 0.36 mg Zn kg^{-1}, 0.14 mg Cu kg^{-1}, 0.036 mg Co kg^{-1}, 0.216 mg B kg^{-1}, and 0.036 mg Mo kg^{-1} soil. We aimed at assessing P efficiency of soybean genotypes under N-fixing conditions and therefore no N was applied. Phosphorus was added with a KH_2PO_4 solution at 2 rates: 160 mg P kg^{-1} ('low P') and 400 mg P kg^{-1} ('high P'). A reduced P rate instead of a control treatment without P was used as we intended to compare genotypes under moderate rather than extreme P-deficiency. For each treatment, a soil sample was kept separate and stored in a cold room. Per treatment combination, 8 replicate pots with an inner volume of 3 L were filled with an equivalent of 2.5 kg of dry soil, and moisture content was increased to 23 % w/w. The next day, soybean seeds were inoculated with a peat-based rhizobium inoculant and 3 seeds were planted per pot. Pots were placed on greenhouse benches in a randomized complete block design. The moisture content was kept constant by watering each day and water consumption by each plant was determined by weighing the pots before watering. Three pots without plants were included to determine evaporation. Five days after emergence, plants were thinned to 1 plant per pot. Average minimum and maximum temperatures in the greenhouse were 20 °C and 34 °C respectively.

Table 1 Selected soil properties of the topsoil sampled in Nyabeda

pH[a]		4.8
clay[b]	%	40
silt[b]	%	12
sand[b]	%	48
Total N[c]	%	0.16
Total C[c]	%	2.08
CEC[d]	($cmol_c$ kg^{-1})	10.9
exch Ca^{2+}	($cmol_c$ kg^{-1})	4.8
exch Mg^{2+}	($cmol_c$ kg^{-1})	1.2
exch K^+	($cmol_c$ kg^{-1})	0.1
P_{AEM}[e]	(mg kg^{-1})	2.2
P_{ox}[f]	(mg kg^{-1})	74
Fe_{ox}[f]	(mg kg^{-1})	2373
Al_{ox}[f]	(mg kg^{-1})	1275
Mn_{ox}[f]	(mg kg^{-1})	3369
PBC[g]	(mg kg^{-1})	24

[a] pH determined in 0.01M $CaCl_2$ (1:5)

[b] Particle size analysis by the hydrometer method (Day 1965)

[c] Total nitrogen and carbon determined by elemental analysis after dry combustion (ANCA-GSL Preparation Module 20e20 Stable Isotope Analyser; Europa Scientific, Crewe, Cheshire, UK)

[d] Effective cation exchange capacity determined by the silver-thiourea method (Chhabra et al. 1975)

[e] Anion exchange membrane extractable P (Sibbesen 1983)

[f] Ammonium oxalate extractable P, Fe, Al and Mn (Schwertmann 1964)

[g] P buffering capacity by the method of Ozanne and Shaw (1968)

The pore water of the amended soil samples was sampled by centrifugation and P concentrations were determined. Three days after planting (DAP), the moisture content of an equivalent of 60 g dry soil was increased to 41 % w/w and thoroughly mixed. The soil was placed in a centrifuge tube and left to equilibrate. The remaining soil of the amended soil samples was air-dried for analysis of available P (see further). After 24 h, the tubes were centrifuged for 20 min at 2070 g, and the standing pore water was filtered over a 0.45 μm membrane filter (Millipore). The P concentrations in the filtrate were determined by the method of Van Veldhoven and Mannaerts (1987), and equaled 0.20 mg P L^{-1} for the low P treatment and 2.5 mg P L^{-1} for the high P treatment. Anion exchange membrane (AEM) extractable P was determined on the air-dried soil samples by shaking 0.5 g of soil with 30 ml of analytical reagent water (Milli-Q) containing 2 AEM strips in HCO_3^--form (6×1 cm, product 55164 2S; BDH Laboratory supplies, Poole, England) for 16 h, and subsequent extraction of P adsorbed on the strips by shaking the strips in 0.5 M HCl for another 16 h. The P concentrations in the extracts were determined by the method of Van Veldhoven and Mannaerts (1987).

Plants were harvested at 28 (4 replicates) and 42 (4 replicates) DAP respectively. Shoots were cut and dried at 65 °C. Roots were gently washed out by soaking the pots in water. Nodules were separated from the roots and dried at 65 °C. A sub sample of the lateral roots of about 10 to 20 % of the total root weight was scanned on a scanner with a dual-lighting system and an optical resolution of 4800 dpi (STD4800, Régent Instruments, Quebec, Canada), and subsequently dried at 65 °C. The root length, root area and root diameter on these scans was determined with WinRhizo Pro software (Régent Instruments). About 1 g of fine roots from the roots of the 'low P' treatment, sampled at the second harvest, was sub sampled for assessment of colonization by arbuscular mycorrhizal fungi (AMF) and stored in 70 % ethanol in a cold room. The remaining roots were dried at 65 °C and the taproot and lateral roots were separated. Root length measured on the sub samples was extrapolated to the whole root taking into account the dry weight of the sub sample and the dry weight of the lateral roots. The percentage of the roots colonized by AMF was determined after clearing and staining, following a modified method based on the procedures described by Phillips and Hayman (1970) and Brundrett et al. (1984). The roots were cleared in 2.5 % KOH at 70 °C for 1 h, rinsed with water, and alkaline hydrogen peroxide was added (1.5 % NH_4OH and 2.7 % H_2O_2). After 20 min at 70 °C, the roots were rinsed and neutralized in 1 % HCl at room temperature for 30 min, and rinsed again. The roots were subsequently stained for 1 h at 70 °C in a glycerol solution composed of 50 % glycerol, 0.05 % HCl and 0.05 % trypan blue. Finally, the roots were destained for at least 24 h in the same glycerol solution (without trypan blue) at room temperature. The percentage of the root colonized by AMF (AMF frequency) was determined by randomly spreading out 1 cm root pieces on a glass slide (30 root pieces per sample) and determining the number of pieces colonized by AMF under a microscope.

Shoots and roots (including taproot, lateral roots and nodules) were ground and digested in hot HNO_3. P concentrations in the digests were determined with ICP-OES (Perkin-Elmer Optima 3300 DV). Total P uptake was determined as the sum of the shoot and root P content. Hence, the contribution of P relocated from the seed was ignored. Seed P contents of soybean seeds usually vary between 0.5 and 1.0 mg P $seed^{-1}$, depending on the genotype and seed lot, while plant P contents varied between 4 and 20 mg P $plant^{-1}$. P utilization efficiency was calculated as gram biomass produced per milligram P taken up, for each individual plant. P uptake efficiency was calculated as P taken up at low P as a percentage of P taken up at high P, based on the means of 3 replicates.

Root hair screening

Since root hairs of soil-grown plants are easily damaged while washing roots from soil, root hair growth was evaluated in a separate experiment. Plants were grown on an agar medium in which the P concentration in solution was buffered by Al_2O_3 particles at 2 different levels ('low P' and 'high P'). The P concentrations in solution corresponded to the P concentrations measured in the pore water of the amended soil samples used for the plant growth experiment. For each soybean genotype, seeds with similar weight (< 10 % variation) were selected, sterilized in 8 % $Ca(ClO)_2$ for 10 min, and pregerminated for 3 days in sterile perlite at 35 °C. Al_2O_3 nanopowder (American Elements) was suspended in analytical reagent water

and the suspensions were sterilized by autoclaving for 20 min at 105 °C. Different amounts of a filter-sterilized KH_2PO_4 solution were added and the suspensions were shaken end-over-end for 16 h. Subsequently, the Al_2O_3 suspensions were mixed with an autoclaved nutrient agar. The final nutrient medium contained 2 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM $MgSO_4$, 2.4 mM KNO_3, 25 μM NaCl, 10 μM FeNaEDTA, 15 μM H_3BO_3, 5 μM $MnSO_4$, 2 μM $CuSO_4$, 0.07 μM $(NH_4)_6Mo_7O_{24}$, 2 μM $ZnCl_2$, μM EDTA, 2 mM MES buffer, 10 g agar L^{-1} (bacteriological agar, No. 2, Lab M limited) and 0.22 g Al_2O_3 L^{-1}. Total initial P concentrations in the medium were 3.7 mg P L^{-1} and 7.7 mg P L^{-1} for the low and the high P treatment respectively. Equilibrium initial P concentrations in solution were 0.2 mg P L^{-1} and 2.5 mg P L^{-1} for the low and the high P treatment respectively (determined on samples treated likewise but without addition of agar). The agar medium was cooled down to 40 °C and poured in square petri dishes (12 × 12 cm, 150 ml of agar in each dish) in which an opening had been made previously at one side of the dish. The seedlings were positioned in the opening with the seedling root submerged in the agar and the hypocotyl facing outwards, and the agar was left to solidify. The dishes were closed, the sides of the dishes sealed with micropore tape and sterile silicon grease was used to close the remaining opening around the seedling. The experiment was carried out in 3 replicates. Plants were placed upright in a growth chamber with a 12 h/12 h day/night cycle (25 °C, 20 °C) and relative air humidity of 70 %. After 7 days, the plates were placed under a microscope (Nikon AZ zoom fluorescence macroscope) at 7.5 × magnification and per plate 2–7 root fragments with clearly present root hairs were photographed. The photographs were opened in the imaging software program ImageJ, and the length (L_{RH}) of 10 focused root hairs, randomly selected but evenly distributed across the picture, was measured. The mean half distance (r_s) between root hairs on the root surface was determined by counting the number of focused root hairs on a 1 mm root fragment. Root hair density (D_{RH}) was calculated as $D_{RH}=(\pi\, r_s^2)^{-1}$.

Statistical analysis

Significance of treatment effects was evaluated using the MIXED procedure in SAS software (SAS Institute Inc 2012), where genotype and P level were added as fixed factors, and replicate as random factor. The LSMEANS statement was used to test differences between the means for significance ($P<0.05$) by calculating the standard errors of the difference (SED). Contrasts between (i) the difference in log transformed P uptake between P treatments for one genotype and (ii) the same difference for another genotype, were tested for significance with the CONTRAST statement. Given the logarithm of P uptake efficiency, as defined above, equals the difference in the logarithms of P uptake between low and high P availability, this procedure allowed for pair wise comparison of P uptake efficiency between genotypes. Contrasts were adjusted for multiplicity according to the Holm-Bonferroni method. Correlations between root characteristics and P uptake efficiency were tested for significance using PROC CORR in SAS. P uptake efficiency was statistically related to root traits observed at low P supply with a stepwise regression model using PROC REG in SAS with the option 'stepwise'. Linear and quadratic terms of root to shoot ratio, average diameter, frequency of AMF colonization, root hair density and root hair length were inserted in the model and retained if inclusion increased the R^2 of the model at a significance level of $P<0.10$. The regression model was tested based on data of individual plants, whereby P uptake efficiency was calculated as the ratio of a plant's total P uptake (at low P) to mean total P uptake at high P. Root hair data were added as means of root hair length and density observed in the agar system.

Mechanistic modeling of P uptake

P uptake at low P availability was modeled using the mechanistic nutrient uptake model NST 3.0 (Claassen and Steingrobe 1999). This model calculates P uptake by an exponentially growing root using the transport equation of Nye and Mariott (1969), which is based on transport by mass flow and diffusion, and uptake according to Michaelis Menten kinetics. The transport equation was extended with a sink term by Claassen (1990) to take into account uptake by root hairs. The model takes into account inter-root competition and competition between root hairs by considering the half mean distance between the root cylinders and the half mean distance between root hairs in cylindrical compartments around the root. Effects of root exudates and symbiosis with arbuscular mycorrhizal fungi on P

uptake are not considered by the model. The estimates of plant and soil parameters required by the model were determined as follows. The initial P concentration in solution (C_i) was estimated as the P concentration measured in the pore water (0.2×10^{-3} mg cm^{-3}). The effective diffusion coefficient of P in the soil, D_e (cm^2 s^{-1}), was determined as $D_e = D_L \theta f B^{-1}$, with D_L the diffusion coefficient in water, i.e., 8.9×10^{-6} cm^2 s^{-1} (at 25 °C; Edwards and Huffman 1959), θ the volumetric water content, which was 0.2 cm^3 cm^{-3}, f the tortuosity factor, calculated as $f = 1.58\theta - 0.17$ (Barraclough and Tinker 1981), and B the buffer power of the soil, which equaled 92 cm^3 g^{-1}. B was calculated as dE/dC, determined by fitting a Freundlich isotherm of the form $E = bC^a$, with E the pool of plant-available P in the soil estimated by extraction with AEM (mg g^{-1}), and C the P concentration in the pore water (mg cm^{-3}). The maximum P influx, I_{max} (mg cm^{-2} s^{-1}), was determined as 1.1 times the observed influx at high P availability. I was calculated according to (Williams 1948) as:

$$I = \frac{U_2 - U_1}{RA_1 - RA_1} \frac{\ln(RA_2/RA_1)}{t_2 - t_1},$$

where U = total P uptake (mg), RA = total root area (cm^2) and t = the growth period from planting to harvest (s), with subscript 1 for the first harvest (28 DAP) and subscript 2 for the second (42 DAP).

The K_m, i.e., the concentration in solution at which the influx rate is half of the maximum influx rate, was estimated as 1.55×10^{-5} mg cm^{-3} according to Santner et al. (2012). The C_{min}, i.e., the P concentration in solution at which the influx rate equals 0, was assumed to equal 3.1×10^{-7} mg cm^{-3}, as determined by Jungk et al. (1990) for soybean grown at a low P concentration. Water influx, v_0 (cm^3 cm^{-2} s^{-1}) was calculated as

$$v_0 = \frac{W_2 - W_1}{RA_1 - RA_1} \frac{\ln(RA_2/RA_1)}{t_2 - t_1}$$

where W = total amount of water consumed as measured by daily water loss corrected for evaporation (cm^3).

The root radius, r_0 (cm) was estimated as the average root radius measured on scans of roots sampled at the second harvest. The mean half distance between roots, r_1 (cm), was calculated as

$$r_1 = \sqrt{\frac{V_s}{\pi RL_2}}$$

where V_s = the soil volume (cm^3) and RL_2 = total root length at the second harvest (cm).

The model assumes both r_0 and r_1 to be constant throughout the growth period. Root length was measured at 2 time points (28 DAP and 42 DAP). In addition, we assumed that during the first 10 DAP no P uptake occurred and the root length at 10 DAP equaled 1500 cm (negligible compared to final root lengths). To determine the root growth constant k (s^{-1}) and the initial root length RL_0 (cm), a root growth curve of the form $RL = RL_0 e^{kt}$ was fitted, with RL = root length (cm) and t = growth period (s). Evidently, RL_0 was close to 1500 cm. The root hair radius, r_{0RH}, was assumed to equal 5×10^{-4} cm (Barber 1984). The mean half distance between root hairs (r_{1RH}) in a cylindrical compartment at a given distance around the root, was calculated as

$$r_{1RHn} = \sqrt{\frac{V_n}{\pi TL_{RHn}}}$$

where TL_{RHn} = total root hair length in the nth cylindrical compartment.

Six compartments around the root each with a thickness of 70 μm were considered. For calculation of r_{1RHn}, root hair densities (measured on root sections with clearly present root hairs) were reduced with 50 %, to account for the fact that root hairs are usually present on only a part of the root system. The direct contribution of root hairs to P uptake (uptake at the root hair level) as opposed to the indirect contribution (additional P uptake by root hairs leading to better root growth, i.e., an increased root growth constant k, in turn leading to increased P uptake) was evaluated by sensitivity analysis. Conditions in which an increasing part of the total root length was covered with root hairs were simulated by varying root hair density from 0 to 100 % of the root hair density which was observed on root sections with clearly present root hairs. The concomitant increase in P uptake was examined for (i) root hair growth (length and density) as observed for Namsoy 4m (superior root hair growth), (ii) root hair growth as observed for Pan 872 (inferior root hair growth), and (iii) average observed root hair growth (intermediate root hair growth). All other plant parameters, including the root growth constant k, were averaged across genotypes and remained constant during the analysis.

Results

Growth, phosphorus uptake and phosphorus efficiency

Total biomass, total P uptake and shoot biomass after 6 weeks of growth were affected by P supply ($P<0.001$) for all genotypes (Table 2). Root biomass, however, remained unaffected by P supply for Namsoy 4m, Nyala and TGx 1740-2E. Significant differences in shoot and root biomass among genotypes were observed at both P levels, but at low P supply differences were much larger. At low P, Namsoy 4m produced twice as much biomass compared to Pan 872, while at high P biomass for both genotypes was similar. A significant interaction effect between genotype and P supply on total P uptake was observed ($P<0.001$). At low P, differences in total P uptake between genotypes were large ($P<0.001$), while at high P no significant differences were observed. Hence, the selected genotypes differed in P uptake efficiency (Table 2). In general, biomass accumulation and total P uptake at low P was highest for Namsoy 4m and Nyala, and lowest for Pan 872. P utilization efficiency was lower at high P for all genotypes ($P<0.001$), but remained unaffected by genotype (Fig. 1). All genotypes were infected with rhizobium at both P levels, but P supply largely affected the degree of nodulation ($P<0.001$). Nodules biomass was 5 to 40 times higher at high P compared to that at low P. At low P, large (about 10-fold) differences in nodules biomass among genotypes were observed, and nodules biomass was highest for Namsoy 4 m and TGx 1740-2F. Nodules biomass was positively correlated with total P uptake (Fig. 2).

Root characteristics

Root length was affected by P supply ($P<0.001$), but differed among genotypes only at low P ($P=0.003$) (Fig. 3a). At low P, a nearly 2-fold difference in root length between the genotype with the lowest (Pan 872) and the genotype with the highest root length (Nyala) was observed. Total P uptake per unit of root length was evaluated but no differences between genotypes were observed (data not shown). The ratio of root to shoot biomass was highest at low P for all genotypes (Fig. 3b), but for TGx 1448-2E, TGx 1903-1F, Pan 872 the difference was small. The largest change in root to shoot ratio was observed for TGx 1740-2F,

Table 2 Shoot and root dry weight, total P uptake, P uptake efficiency, and nodules dry weight of selected soybean genotypes after 6 weeks of growth at low and high P availability

	Shoot dry weight (g)		Root dry weight (g)		Total P uptake (mg)		P uptake efficiency[a] (%)	Nodules dry weight (mg)	
	Low P	High P	Low P	High P	Low P	High P		Low P	High P
Namsoy 4 m	3.0	4.5	1.1	1.2	7.4	18.8	40[a]	35.0	219
Nyala	2.7	4.4	1.1	1.2	6.6	17.9	37[ab]	15.8	193
TGm 1360	2.3	4.9	0.8	1.2	6.1	18.9	32[ab]	26.5	169
TGx 1903-1F	2.2	4.7	0.7	1.2	5.7	18.0	32[ab]	8.4	145
TGx 1740-2F	2.1	4.2	0.9	1.2	6.1	19.4	31[ab]	35.3	175
Ogden	2.2	4.5	0.8	1.3	5.1	18.1	28[ab]	8.6	155
TGx 1448-2E	1.8	4.5	0.7	1.4	4.3	17.2	25[bc]	9.9	143
Pan 872	1.6	4.6	0.5	1.4	3.9	20.9	18[c]	3.5	138
Probability of F-statistics									
P rate	<0.001		<0.001		<0.001			<0.001	
Genotype	0.003		0.004		<0.001			<0.001	
P rate x genotype	0.001		<0.001		<0.001			<0.001	
Genotype	0.001	0.048	<0.001	0.001	<0.001	ns		0.001	0.049
SED	0.29	0.18	0.11	0.06	0.7	1.43		nd[b]	nd[b]

[a] P uptake efficiencies followed by the same letter are not different at $P<0.05$

[b] data were log transformed before estimation of variance components

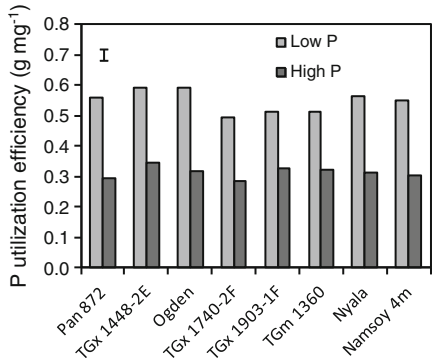

Fig. 1 P utilization efficiency (calculated as gram biomass produced per milligram P taken up) for the selected soybean genotypes after 6 weeks of growth at low and high P supply. The error bar represents the standard error of the difference between genotype x P rate means

from 0.28 at high P to 0.45 at low P. Average root diameter was not affected by P supply but differed among genotypes (P=0.006), with TGx 1740-2F and Pan 872 showing a slightly lower root diameter. However, no differences in specific root length among genotypes were observed (data not shown). The percentage of the root colonized by AMF (AMF frequency) was not determined for plants grown at high P. The lowest AMF frequency was observed for TGx 1448-2E (40 %) while Nyala showed highest AMF frequency (80 %) (Fig. 3d). AMF frequency for other genotypes was intermediate (60–68 %).

Root hair development was evaluated in a separate experiment by growing plants on agar in which P intensity was buffered by Al_2O_3 nanoparticles. After 1 week of growth, seedlings had established well on the agar and root hairs were visible for all genotypes. Root hair density (Fig. 3e) and length (Fig. 3f), were not affected by P supply, except for Namsoy 4m, which strongly increased its root hair length at low P (P<0.001). Pan 872 clearly differed from other genotypes because of lower root hair density. Namsoy 4m thus excelled in root hair development as opposed to Pan 872 which was characterized by inferior root hair development (Fig. 4).

A stepwise regression procedure selected D_{RH}^2 (P=0.03), D_{RH} (P=0.007), AMF (P=0.02), and L_{RH}^2 (P=0.04) to be jointly included in a significant descriptive model (R^2=0.58, P<0.001, Fig. 5) relating P uptake efficiency to root characteristics as follows:

$$\text{P uptake efficiency } (\%) = 0.52D_{RH} - 0.0025D_{RH}^2 + 0.16\text{AMF} + 0.00018L_{RH}^2 - 13.0$$

where D_{RH} = root hair density (mm^{-1}), AMF = the frequency of AMF colonization (%) and L_{RH} = average root hair length (μm), all observed at low P supply. No correlations were found between the selected root variables. The results indicate that variation in P uptake efficiency between genotypes was strongly related to root hair development and colonization by mycorrhiza. Linear or quadratic terms of root to shoot ratio and average diameter were not retained in the model, as their inclusion did not significantly increase the R^2 of the model. The regression relation strongly depended on the two most extreme genotypes (Namsoy 4m and Pan 872).

Mechanistic modeling of P uptake

The mechanistic model was only partly able to predict differences in P uptake at low P among genotypes (Fig. 6a). The model generally underpredicted observed P uptake but succeeded to predict about 84 % of the variation in P uptake (a 1.6-fold range compared to a 1.9-fold range). When ignoring root hair development (Fig. 6b), the model further underpredicted P uptake and predicted only 78 % of the variation in P uptake. The ranking of P uptake for genotypes with superior and inferior root hair development (i.e., Namsoy 4m and Pan 872) was correctly predicted by the model. The model was not able to adequately predict ranking for the genotype with the lowest percentage of colonization by AMF (TGx 1448-2E), and the ratio of observed to predicted P uptake was smallest (1.08) for this genotype. Sensitivity analysis predicted that at 50 % of maximum root hair density, root hairs directly increase P uptake with 4, 12 and 20 %

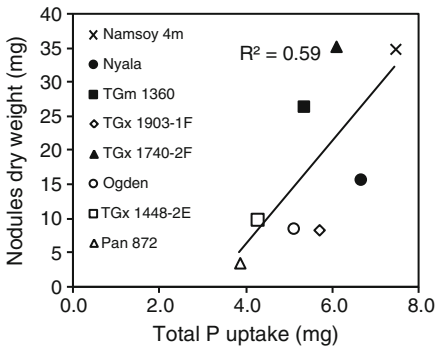

Fig. 2 Relationship between total P uptake and nodules dry weight at low P supply. Each point represents one genotype

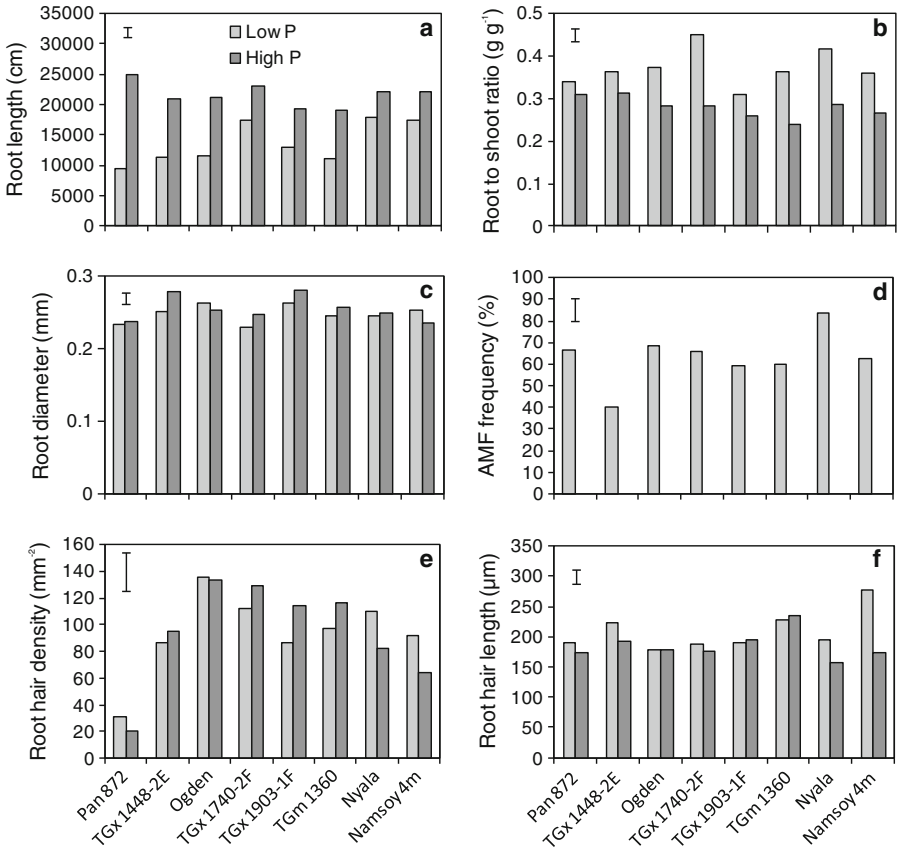

Fig. 3 Root characteristics of selected soybean genotypes at low and high P supply: **a** root length, **b** root to shoot ratio, **c** root diameter, **d** percentage of the root colonized by AMF (not measured for plants grown at high P availability), **e** root hair density and **f** average root hair length. Genotypes are ranked according to P uptake efficiency (increasing P uptake efficiency from left to right). Error bars represent standard errors of the difference between genotype x P rate means (**a**, **b**, **c**, **e** and **f**) or between genotype means within P rate (**d**)

for inferior, intermediate and superior root hair growth respectively. When root hair density was increased to its maximum (100 % of the root length covered with root hairs), a direct increase in P uptake of 8, 21 and 36 % was predicted for these three respective conditions (Fig. 7).

Discussion

Our results demonstrate that genotypic variation in P efficiency exists among soybean genotypes. Although the set of soybean genotypes was small, it still spanned a nearly 2-fold variation in P uptake efficiency. In contrast, genotypes did not differ in P utilization efficiency. Our results are in agreement with Pan et al. (2008), who observed a 2-fold variation in shoot biomass at low P relative to high P (ranging from 46 % to 95 %) among 96 soybean genotypes, and showed that this variation was not related to P utilization efficiency but rather to factors affecting P uptake.

The contribution of different root traits to variations in P uptake efficiency was investigated by regression analysis and mechanistic modelling. In a first step, genotypic variation in root characteristics that possibly affect P uptake was evaluated. Some root traits were

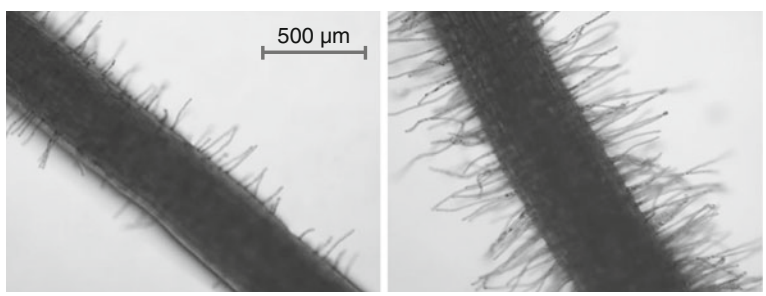

Fig. 4 Root hair development of Pan 872 (left) and Namsoy 4m (right) at low P supply

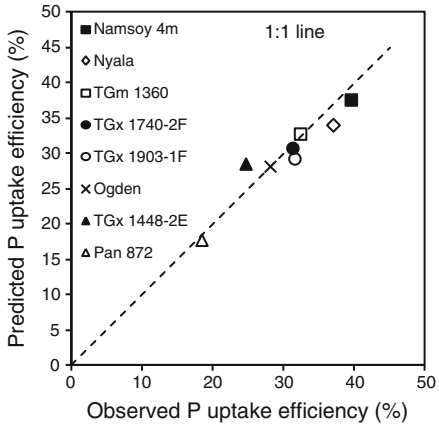

Fig. 5 Relationship between observed P uptake efficiency and P uptake efficiency predicted from root hair density, AMF colonization and root hair length (P uptake = $0.52D_{RH} - 0.0025D_{RH}^2 + 0.16AMF + 0.00018L_{RH}^2 - 13$), at low P supply. The stepwise regression was based on data from individual plants. In this figure, means for each genotype are presented

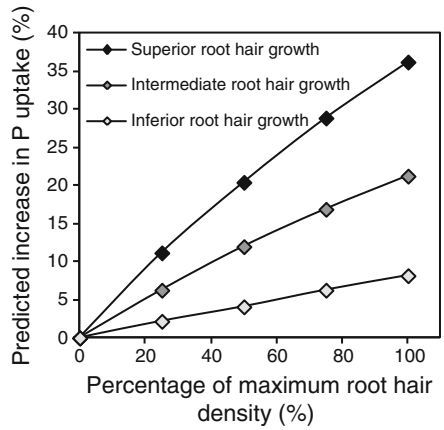

Fig. 7 Increase in P uptake with increasing percentage of maximum root hair density for 3 different scenario's: superior root hair growth (as observed for Namsoy 4 m), intermediate root hair growth (average root hair growth across genotypes), and inferior root hair growth (as observed for Pan 872). Simulation made at low P supply using the mechanistic model explained in the text

affected by genotype and P supply while other were only affected by P supply or remained unaffected. Both plastic responses and differences in features irrespective of environmental conditions were observed. An increase in root to shoot ratio with reducing P availability was observed for all genotypes, but the magnitude of this increase differed largely among genotypes, with TGx 1740-2F and Nyala exhibiting superior plasticity for this trait. Increased allocation of biomass to roots as a response to a low P environment is well known. This phenomenon can in most cases however not solely be attributed to plasticity, but is partly related to ontogenic differences (Lambers et al. 2006; Lynch 2007). Further, the percentage of the root system colonized by AMF varied between 40 and 80 % among genotypes. Similarly, Nwoko and Sanginga (1999) previously showed that mycorrhizal colonization among 10 soybean varieties grown in a low P soil varied 2-fold (between 16 and 33 %). Average root diameter was similar across genotypes and although two genotypes (TGx 1740-2F and Pan 872) exhibited a slightly lower root diameter, no significant differences in specific root length among genotypes were observed, suggesting that intra-species variation for root diameter is small for soybean. In addition, no plasticity for root diameter was observed. Plasticity for root diameter and specific root length in response to P availability has been reported in some studies (Rao et al. 1996; Fernández et al. 2009) but other studies have indicated that this response does not

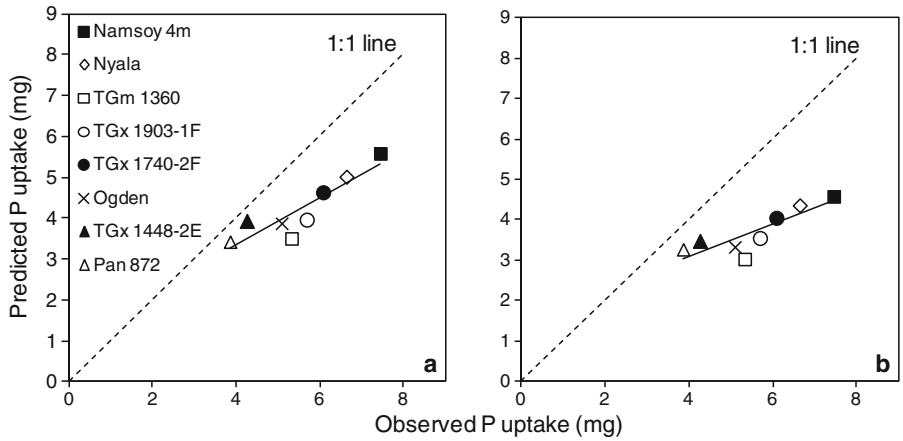

Fig. 6 Relationship between observed P uptake and P uptake predicted by a mechanistic model at low P supply for selected soybean genotypes, with (**a**) or without (**b**) accounting for root hairs

occur for all species (Schroeder and Janos 2005; Pang et al. 2010). Rao et al. (1996) found that the specific root length of a grass and a legume species increased with decreasing P supply in both a sandy and a clay soil, but for the legume grown in the clay soil a marked increase only occurred at the lowest level of P availability.

Root hair length and density was measured on plants grown for 1 week on an agar medium where P intensity was buffered at low and high P availability. The measurement of root hairs on soil-grown plants is difficult as root hairs are easily damaged while roots are washed out from soil, especially for clayey soils. Growth pouches or hydroponics have been used to study root hair development, but in these systems P supply is not spatially buffered. Here, we opted for a system where diffusion limitations to P supply mimicked those encountered in tropical soils. The use of Al_2O_3 nanoparticles as P buffer ensured clarity of the agar, allowing for in-situ measurements of root hair length and density. Both root hair density and root hair length varied among soybean genotypes. Several studies have shown that substantial genotypic variation in root hair development exists for several species including soybean (Gahoonia and Nielsen 2004a; Wang et al. 2004; Yan et al. 2004). In this study, root hair density was more variable among genotypes than root hair length and was not affected by the level of P availability, suggesting that root hair density of soybean genotypes is a feature that is expressed irrespective of environmental conditions. In contrast, strong plasticity was observed in terms of root hair length for one genotype (Namsoy 4m). Root hair length has been shown to respond plastically to low P conditions whereby root hairs become longer under low P conditions (Föhse and Jungk 1983; Bates and Lynch 1996; Gahoonia et al. 1999), but for some species this effect was not observed (Dechassa et al. 2003; Pypers 2006). It thus appears that plasticity for root hair length is not a universal trait but rather species- and genotype-specific. However, the experimental conditions used here to measure root hair development only allowed for testing plasticity regulated by localized mechanisms, i.e., in response to the external concentration. The short period of seedling growth on the agar whereby plants could largely rely on the seed P reserve for their P supply might not have affected internal P concentrations. Contradicting examples exist in literature as to which mechanisms (localized or systemic) regulate root hair development. Bates and Lynch (1996) provided evidence for root hair elongation in *Arabidopsis thaliana* responding to the external concentration of P and thus the existence of localized mechanisms. On the other hand, Föhse and Jungk (1983) demonstrated with a split-root system that root hair development of spinach (*Spinacia oleracea*) responds to the internal P concentration, and is thus regulated by systemic mechanisms.

Highest and lowest P uptake efficiencies were observed for the genotypes with the largest and smallest root length respectively. It is however difficult to thereby distinguish cause and result (Gahoonia and Nielsen 2004b). Increased P uptake efficiency and, consequently, improved growth under low P conditions, evidently results in increased root biomass and therefore root length. Any adaptation mechanism leading to improved P uptake, e.g., improved root hair development or root symbiosis, is expected to indirectly further enhance P uptake through its positive effect on root length. Wissuwa (2003) previously showed with a simulation model that a 33 % increase in external root efficiency (defined as P uptake per unit of root surface area per day) is sufficient to triple P uptake. Such a positive effect on root length might also explain why no differences in total P uptake per unit root length among genotypes were observed, although genotypes differed in root hair development and colonization by AMF.

A stepwise regression procedure suggested that P uptake efficiency was related to root hair development and symbiosis with AMF. This indication was corroborated by the mechanistic model, which took P uptake by the root and root hairs into account while ignoring P uptake by mycorrhiza. The model was able to predict the ranking of the best and worst performing genotypes in terms of root hair development, but the ranking of the genotype with the lowest percentage of colonization by mycorrhiza was not maintained. Moreover, underprediction of P uptake by the model was lowest for this genotype, indicating that AMF contributed to some extend to P uptake. When root hairs were excluded from the model, variation in predicted P uptake among genotypes decreased. Hence, variation in P uptake efficiency could at least partly be attributed to differences in root hair development. Itoh and Barber (1983) previously showed that a mechanistic nutrient uptake model could not adequately predict differences in P uptake between species unless uptake by root hairs was included. Gahoonia et al. (1997) observed a strong correlation between the surface area of root hairs of wheat and barley cultivars

grown in a solution culture and the quantity of P depleted from low-P rhizosphere soil. In another study, Gahoonia and Nielsen (1998) provided direct evidence for the participation of root hairs in P uptake. The regression model indicated that the contribution of root hairs to P uptake efficiency was large, as illustrated by a 2-fold difference in predicted P uptake between Namsoy 4m and Pan 872 (Fig. 5; colonization by AMF was similar for these genotypes). It is expected that root hair growth contributes both directly (through P uptake at the root hair level) and indirectly (through improved P nutrition stimulating root growth resulting in increased root length) to P uptake. When ignoring root hairs in the mechanistic model, and hence eliminating a direct contribution of root hairs to P uptake, Namsoy 4m and Pan 872 still differed considerably in predicted P uptake, indicating there was a significant indirect contribution of root hairs to P uptake. Sensitivity analysis showed that without an indirect effect on the root growth constant, root hairs can increase P uptake by a maximum of around 36 % (under conditions of superior root hair development and 100 % root coverage), with realistic values rather in the range of around 20 %. Indirect contribution of root hairs to P uptake thus seemed to exceed direct contribution. Compared with the large contribution of root hairs to P uptake efficiency, the contribution of arbuscular mycorrhizal fungi appeared to be small (an increase in AMF frequency from 40 to 80 % is predicted with the regression model to increase P uptake efficiency with 6 %) and more variable among genotypes. Although the hyphae of arbuscular mycorrhizal fungi may largely extend the depletion zone and hence increase efficiency of P uptake, it has been shown that P uptake by hyphae may lead to the inhibition of direct P uptake by the root epidermis including root hairs (Smith et al. 2011). The effect of colonization by AMF may however increase under field conditions because of a larger volume of soil to be explored by hyphae and a larger time-frame for the mycorrhizal symbiosis to establish and affect P uptake. On the other hand, mycorrhizal colonization may also be strongly site-specific because of diversity in mycorrhizal strains and cropping history. Importantly, colonization by AMF might have affected other factors besides P uptake. Ferralsols such as the soil used in this experiment are typically characterized by high levels of free manganese and aluminium often leading to toxicities. It has been reported that mycorrhiza can in some cases alleviate toxic effects of Mn, Al and Fe (Cardoso and Kuyper 2006). The soil used for this experiment contained high levels of free Mn, as indicated by Mn concentrations in the shoot tissue of about 700–1200 $mg\,kg^{-1}$ in the low P treatment, likely exceeding toxicity thresholds, with the highest concentration recorded for TGx 1448-2E (data not shown).

The genotype with the lowest P uptake efficiency (Pan 872) showed poor root hair density while its root hair length was similar to that of other genotypes. In contrast, the genotype performing superiorly in terms of P uptake efficiency (Namsoy 4 m) excelled in root hair length at low P conditions, whereas its root hair density did not differ from that of other genotypes. Hence, the trait which positively affected P uptake efficiency compared to most other genotypes was increased root hair length. This indicates that, for enhancing P uptake efficiency of soybean through breeding, increased root hair length may be the preferable target trait rather than increased root hair density.

The positive correlation between nodule biomass and total P uptake at low P indicates that increased P uptake improved nodulation. While it is known that N fixation strongly depends on the plant's P nutrition (Zahran 1999; Sanginga 2003), improved N nutrition may also significantly improve root growth and consequently P uptake. For plants relying on N fixation for N nutrition, synergistic effects of P uptake efficiency and improved N fixation are thus expected. Our results show that soybean genotypes differ in P uptake efficiency, and hence, production levels and N fixation on low P soils may be enhanced by the selection of P uptake-efficient genotypes. Differences in P uptake efficiency among genotypes were largely related to differences in root hair development and to a lower extent to colonization by mycorrhiza. The situation under field conditions is however likely to be more complex with other root traits such as root angles and branching possibly affecting P uptake. Nevertheless, breeding for more extensive root hair development is suggested as a promising way to increase P uptake efficiency in soybean.

Acknowledgments The authors wish to thank Philip Malala, Magdalene Mutia and Purity Nduku for their valuable help during greenhouse work and Nyawira Lukey for carrying out analyses on mycorrhizal colonization. We are also grateful to the team of the TSBF-CIAT Maseno for their assistance with soil sampling, and to Jan Diels for his expert advice on statistical analysis. Marian Renkens acknowledges a travel grant from the

University Development Cooperation of the Flemish Interuniversity Council (VLIR-UDC). Elke Vandamme acknowledges a VLIR-UDC PhD scholarship.

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