Root architecture and leaf photosynthesis traits and associations with nitrogen-use efficiency in landrace-derived lines in wheat

Shadia H.S. Kareem[ab], Malcolm J. Hawkesford[c], Jayalath DeSilva[a], Minuka Weerasinghe[a], Darren M Wells[a], Michael P. Pound[d], Jonathan A Atkinson[a], Michael J. Foulkes[a]

[a] Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Leicestershire, LE12 5RD, UK

[b] Department of Biotechnology and Crop Science, College of Agricultural Engineering Sciences, University of Sulaimani, Sulaimani, Iraq

[c] Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

[d] Computer Vision Laboratory, School of Computer Science, University of Nottingham, NG8 1BB

*** Corresponding author:** John Foulkes
John.Foulkes@nottingham.ac.uk (M.J. Foulkes).

Keywords: N-use efficiency, root traits, landraces, leaf photosynthesis, wheat

Abstract

Root system architecture (RSA) is important in optimizing the use of nitrogen. High-throughput phenotyping techniques may be used to study root system architecture traits under controlled environments. A root phenotyping platform, consisting of germination paper-based pouch and wick coupled with image analysis, was used to characterize root seedling traits in 31 wheat genotypes including landrace-derived lines under hydroponic conditions. In addition, two glasshouse experiments under high N (HN) and low N (LN) conditions were carried out to measure whole plant performance including flag-leaf photosynthetic rate, N uptake, biomass, per plant for 10 of the 31 genotypes. There were significant differences in RSA traits between genotypes for seminal root number, lateral root number and root length per plant and root angle, with transgressive segregation for landrace-derived lines above the elite parental cultivar Paragon under HN and LN conditions. Genetic variation in flag-leaf photosynthesis rate was found in landrace-derived genotypes in the range 25.9-33.3 µmol m^{-2} s^{-1} under HN and in N uptake in the range 0.37-0.48 g N $plant^{-1}$and 0.21-0.30 g $plant^{-1}$ under HN and LN conditions respectively ($P< 0.05$) with transgressive segregation above Paragon. Seminal root length per

plant in the hydroponic screen was positively correlated with biomass per plant in the glasshouse experiments under HN conditions, and seminal root angle was positively correlated with biomass per plant under LN conditions. Results from this study demonstrate genetic variation for seedling RSA traits in landrace-derived lines above the elite parental cultivar Paragon, which potentially could be utilized to improve N-use efficiency in breeding programmes.

Key words: root system architecture, leaf photosynthetic, wheat, landraces, Triticum aestivum, yield

1. Introduction

New wheat cultivars with increased Nitrogen (N)-use efficiency will be of economic benefit to growers and will help to reduce environmental impacts related to excessive N fertilizer inputs. N fertilizer inputs may be associated with nitrate leaching leading to groundwater contamination and eutrophication of rivers and lakes. Additionally, global warming may be favoured, due to emission of N_2O derived from denitrification of nitrate by soil bacteria (Foulkes *et al.*, 2009). Nitrogen-use efficiency (NUE) can be defined as grain dry matter yield divided by total N available (available N from the soil or N applied as fertilizer) (Moll *et al.*, 1982); and can be further sub-divided into: N-uptake efficiency (NUpE; aboveground crop N at harvest /available N from soil and fertilizer N) and N-utilization efficiency (NUtE; grain yield dry matter / above-ground N at harvest).

Breeding for higher NUE will require improved understanding of the physiological traits determining NUE and responses to N limitation. Genetic variation has been reported for promising traits to increase NUE in wheat including higher leaf photosynthetic rate (Gaju *et al.*, 2016; Carmo-Silva *et al.*, 2017), stay-green traits related with improved post-anthesis N remobilization (Gaju *et al.*, 2011; Hawkesford, 2014) and deeper roots for increased N uptake (Foulkes *et al.*, 2011). Under low N conditions, a correlation between the onset of flag-leaf senescence and grain yield was reported amongst 16 winter wheat cultivars grown at sites in the UK and France (Gaju *et al.*, 2011). The genetic variation in grain yield under HN conditions was associated with flag-leaf photosynthesis rate in 15 genotypes (landraces, synthetic-derived lines and UK modern cultivars) in field experiments reported by Gaju *et al.* (2016). Therefore, improvements in flag leaf photosynthesis can make a significant contribution towards genetic gains in grain yield and NUE.

Wheat breeding in the last decades has led to a decline in genetic diversity (Hoisington *et al.*, 1999). In the breeding of high yielding varieties especially in response to changing abiotic stress, this lack of genetic diversity is generally recognized as a limiting factor (Allen *et al.*, 2017). Landraces are pure hybridized ancestral varieties, which are adapted to local environment conditions. They have been developed by traditional agriculture practices and local cropping systems in semi-arid environments. Landraces are therefore an important source of genes and traits for improving wheat adaptability to abiotic stress conditions (Lopes *et al.*, 2015). Generally, landrace collections show a much higher level of genetic diversity than modern elite varieties which breeding programmes can exploit (Moore, 2015) as a source of traits for abiotic stress tolerance (Villa *et al.*, 2005). Under low N availability, wheat landraces and old varieties with a taller growth habit and lower harvest index were shown to absorb and translocate more nitrogen into the grain than modern cultivars, probably due to greater pre-anthesis uptake (Jaradat, 2013). There is evidence that the root biomass of landraces is larger compared to that of modern semi dwarf cultivars (Waines and Ehdaie, 2007). Therefore, landraces with well-developed root systems could be a source of variation for N uptake and improvement of grain yield under low N availability (Jaradat, 2013).

Field phenotyping for root traits has been conducted using different techniques such as rhizotrons, mini-rhizotrons and assessments of root parameters from soil cores (root washing and root counts/image analysis), but these methods are time-consuming and labour intensive and generally cannot be applied in experiments with large numbers of lines (Atkinson *et al.*, 2018). Screening techniques for evaluating roots have been developed recently that have potential to overcome these limitations focusing on seedling root growth in germination paper pouch and wick systems (Atkinson *et al.*, 2015; Xie *et al.*, 2017; Adeleke *et al.*, 2019; Khokhar *et al.*, 2019; Griffiths *et al.*, 2020). The seedling root phenotyping pipeline described in Atkinson *et al.* (2015) revealed wheat seedling root traits that were positively linked to mature plant traits, such as plant height and grain yield in a Savannah × Rialto DH winter wheat population. This seedling root phenotyping pipeline is used in the present study combined with a new image analysis approach RootNav 2.0 (Yasrab *et al.*, 2019). RootNav 2.0 replaces previously manual and semi-automatic feature extraction with a deep-learned, multi-task convolutional neural network architecture, automating the image analysis process.

The present experiments aimed to: (i) quantify genetic variation in 30 bread wheat landrace-derived lines and the parental spring bread wheat cultivar Paragon for root system architecture traits under high N and low N conditions in hydroponic conditions and (ii) quantify

correlations with genetic variation in leaf photosynthesis rate and NUE traits in glasshouse experiments under high and low N conditions.

2. Materials and methods

2.1. Plant materials

A Nested Association Mapping (NAM) population was developed using the maize technique (McMullen *et al.*, 2009). The NAM population consists of 23 biparental crosses using the UK spring bread wheat parent Paragon and 23 bread wheat founder lines. Paragon is a UK spring bread wheat cultivar bred by RAGT Seeds Ltd (CSW 1742/19/6/68 x (Axona x Tonic)) first listed on the UK Recommended List in 1999. The NAM lines were developed by single seed descent (SSD) by the John Innes Centre, UK. The NAM population comprised crosses between Paragon and each of 19 hexaploid landrace wheats from the AE Watkins collection, three Mexican spring wheat cultivar/advanced lines (Pfau, CIMCOG47 and CIMCOG49) and one Australian spring wheat cultivar (Wyalkatchem). After F_1, each line was self-pollinated for four generations by single seed descent (SSD). The lines were at F_4 in 2017 and F_5 in 2018. The number of lines for each sub-population in the NAM population ranged between 11-27. In the present experiments a subset of 31 NAM lines was used including the spring wheat parent Paragon (Table 1) selected based on biomass and anthesis date data from a previous field experiment (Foulkes unpublished; anthesis ranging from x to x). Lines were selected to be representative of the range of above-ground biomass in the whole NAM population with a restricted range of anthesis date.

2.2. Hydroponic 2D root phenotyping experiment

2.2.1. Experimental design and growing conditions

A germination paper-based pouch and wick system, combined with digital image analysis, was used to measure root architectural traits in 2016 at University of Nottingham, Sutton Bonington Campus (Atkinson *et al.*, 2015). Thirty wheat landrace-derived lines from six sub-populations of the NAM population and the parental genotype Paragon were used (Table 1). The experiment used a split-plot design where two N treatments were randomized at the ‘main plot’ level and genotypes were randomized at the ‘sub-plot’ level with five replicates. Within each ‘main plot’ N treatment, there were three plants per genotype as technical replicates, with a total of 15 plants per genotype (5 biological replicates × 3 technical replicates) in each of the two N treatments, and 30 plants per genotype in the experiment.

The growth system consisted of growth pouches and hydroponic tanks. Each pouch consisted of a sheet of blue germination paper (24 × 30 cm) covered with a strong black polythene film of equal area (75 μm thick), an acrylic rod (316 × 15 × 5 mm) and two 18 mm foldback clips. The germination paper and polythene film were fixed to an acrylic rod using two 18 mm fold back clips. Seeds were surface sterilized by cleaning them in 70% (v/v) ethanol for 30 s, followed by transfer to 5% (v/v) sodium hypochlorite solution for 10 min. Sterilized seeds were placed onto moistened germination paper crease-side down and kept for five days in a dark room at 4°C for synchronized germination. After cold treatment, seeds were moved to a light-impermeable box in the controlled-environment room for 48 h to complete germination. Germinated seeds with ~5 mm in length of radicle were transferred to the growth pouches, one per pouch. A single seedling was placed in each growth pouch centered 2 cm from the top edge, with the embryo facing the bottom of the paper and held in place by the adhesion of the polyethene sheet to the wet blue germination paper.

Growth pouches were fitted into five aluminum and polypropylene frame assemblies (hydroponic tanks; 104 × 62 × 102 cm) in a controlled-environment chamber. Black polypropylene side panels maintained the pouches in darkness. The base of each hydroponic tank held a black polypropylene tray (99 × 61 × 10 cm) containing 18 L modified one-quarter Hoagland's solution with HEDTA as the iron chelator. The composition (mg l^{-1}) of the nutrient solution for HN and LN treatments is given in Supplementary Table S1. The solutions were adjusted to pH 6 using KOH. The volume of nutrient solution in each tray was maintained automatically via a float valve system and header tank containing deionized water. In the controlled-environment room, the PAR was 400 μmol m^{-2} s^{-1} and the photoperiod of the growth room was 12 h. The temperature was set to 20°C during light phase and 15°C during dark phase.

"In the controlled-environment room, the PAR was 400 μmol m-2 s-1 and the photoperiod of the growth room was 12 h. The temperature was set to 20°C during light phase and 15°C during dark phase."

The vertically grown root system images were taken nine days after emergence using a Nikon D600 DSLR camera controlled using NKRemote software. All images were cropped using ImageJ software. Cropped root images were processed and analysed to quantify the different seedling root traits using the automated software RootNav 2.0 (Yasrab *et al.*, 2019). The root system architecture traits quantified included seminal roots plant^{-1}, lateral roots plant^{-}

163 [1], total length seminal roots plant^{-1} and seminal root tip angle (the average angle of all seminal
164 root tips relative to the vertical axis) (Xie *et al.*, 2017), maximum depth, and width to depth
165 ratio (Xie *et al.*, 2017). The definitions of the root system architecture traits are shown in Table
166 S2. Out of the 31 genotypes used in the hydroponic experiment, 13 genotypes were also grown
167 in the glasshouse experiments under both HN and LN conditions (Table 1).
168

169 Table 1. NAM genotypes used in hydroponic experiments and glasshouse
170 experiments under HN and LN conditions

Code		Genotypes in	Genotypes in Glasshouse	Country of origin
1	Paragon	√	√	UK
2	PxW223 – 01	√		Burma
3	PxW223 – 03	√		Burma
4	PxW223 – 85	√		Burma
5	PxW223 – 89	√	√	Burma
6	PxW223 – 94	√		Burma
7	PxW264 – 10	√	√	Canary Islands
8	PxW264 – 16	√		Canary Islands
9	PxW264 – 17	√		Canary Islands
10	PxW264 – 31	√		Canary Islands
11	PxW264 – 52	√	√	Canary Islands
12	PxW420 – 03	√		India
13	PxW420 – 21	√		India
14	PxW420 – 22		√	India
15	PxW420 – 31	√		India
16	PxW420 – 32	√	√	India
17	PxW420 – 94	√		India
18	PxW546 – 03	√	√	Spain
19	PxW546 – 08		√	Spain
20	PxW546 – 15	√		Spain
21	PxW546 – 20	√		Spain
22	PxW546 – 32	√		Spain
23	PxW546 – 47	√		Spain
24	PxW566 – 12	√	√	Greece
25	PxW566 – 14	√		Greece
26	PxW566 – 24	√		Greece
27	PxW566 – 50	√		Greece
28	PxW566 – 72	√		Greece
29	PxW685 – 01	√		Spain
30	PxW685 – 09	√		Spain
31	PxW685 – 16	√		Spain
32	PxW685 – 36	√	√	Spain
33	PxW685 – 44	√		Spain
34	PxPfau-03		√	Mexico

35	PxPfau-59	√	Mexico
36	PxPfau-86	√	Mexico

2.3. Glasshouse experiments

2.3.1. Experimental design and treatments

Two glasshouse experiments were carried out in 2017 and 2018. In each experiment, the design used was a split-plot with two levels of N (High N and Low N) as the main treatment and genotypes as the sub-treatment. There were 13 genotypes used in the experiments. Nine lines were derived from crosses between Paragon and bread wheat landrace lines, three lines were derived from a cross between Paragon and a Mexican spring wheat Pfau and also the bread wheat parent (Paragon) was included in the two experiments (Table1). The experiments were sown on 14 Feb 2017 and 7 Feb 2018 and harvested on 22 June 2017 and 24 June 2018. Two levels of fertilizer N were applied, equivalent to 120 kg N ha^{-1} (High N, HN) and 20 kg N ha^{-1} (Low N, LN) in 2017; and 200 kg ha^{-1} (HN) and 50 kg ha^{-1} (LN) in 2018 (based on pot soil surface area).

Seeds were sown in a plastic modular tray filled with soil medium compost (Levington Advance Seed & Modular F2+S). After seed germination (6 days after sowing) seedlings were transferred to a cold room for vernalisation for 2 weeks at 6°C. Two weeks after germination, seedlings were transplanted into 2 l pots, one seed per pot, filled with low N peat compost (Klasmann Medium peat 818). The amounts of P and K were 125 g m^{-3} and K was 300 g m^{-3}, respectively. Border pots (cv. Paragon) were placed around the experimental pots in each experiment to prevent border effects.

Nitrogen was applied manually as ammonium nitrate (NH_4NO_3, 34% N) dissolved in water. For low N, N was applied as one dose and for high N as three doses of 40 kg N ha^{-1}, 60 kg ha^{-1} and 20 kg ha^{-1} equivalents. The actual total amount of N fertilizer applied was 0.19 g and 0.76 g of NH_4NO_3 per pot under LN and HN conditions, respectively. In 2018, low N application was split into two doses of 30 kg N ha^{-1} and 20 kg N ha^{-1} and for high N three doses of 50 kg N ha^{-1}, 50 kg ha^{-1} and 100 kg ha^{-1} equivalents. The actual total amount of N fertilizer applied was 0.32 g and 1.27 g of NH_4NO_3 per pot under LN and HN conditions, respectively. The first application was applied immediately after transplanting of seedlings in pots and the second at GS31 for both treatments. The last application for the high N treatment was at flag-leaf emergence (GS39). In both years, plants were sprayed with fungicide and insecticide as required to minimize effects of diseases and pests. Plants were irrigated with a complete nutrient solution (minus N) regularly with a manual irrigation system to keep plants free from drought stress.

2.3.2. Glasshouse environmental conditions

Plants were grown in the glasshouse from the date of transplanting to 26 March with an extended light cycle of 16 h photoperiod through supplementary light. After that, plants were grown

with a natural light cycle. The glasshouse was maintained frost free and ventilated to maintain the temperature below 25°C. Daily minimum and maximum air temperature were measured using a tiny tag temperature data logger and are presented in supplementary Fig S1. In 2017, average mean temperature from transplanting to harvest was 23.6°C while in 2018 it was 21.9°C.

2.3.3. Plant measurements

Regular monitoring of plant development stages of the main shoot was done according to Zadoks growth stages (Zadoks *et al.*, 1974). Anthesis date (AD) was taken as when the ear showed visible anthers (GS61); and physiological maturity (GS89) as when the peduncle was 100% senesced. Plant height from soil level to the tip of the ear was measured on the main shoot at harvest.

At physiological maturity plants were cut at ground level in each pot and separated into the main shoot, remaining fertile shoots (those with ear) and infertile shoots. Plant components from each fertile shoot category were divided into: i) ear, ii) flag-leaf lamina and iii) stem and leaf sheath and remaining lamina, and each component weighed after oven drying at 70°C for 48 h. Dried ears were threshed and grains from the sample were counted. After drying at 70°C for 48 h, the grain was weighed, and the thousand grain weight (TGW) calculated.

Plant N% of: i) main-shoot grain, ii) main-shoot straw (leaf lamina + stem + leaf sheath), iii) remaining fertile shoots grain, and iv) remaining fertile shoots straw (leaf lamina + stem and + leaf sheath) was determined using the Dumas method (Dumas, 1831). The N-use efficiency (NUE), nitrogen-uptake efficiency (NUpE) and N-utilization efficiency (NUtE) at the plant level were calculated as in equations 2.1-2.3:

$$\text{NUE} = \text{Grain Yield DM } (g\ plant^{-1}) \,/\, \text{available N } (g\ plant^{-1}) \qquad \text{Equation 2.1}$$

$$\text{NUpE} = \text{AGN}_{\text{H}}\ (g\ N\ plant^{-1}) \,/\, \text{available N } (g\ plant^{-1}) \qquad \text{Equation 2.2}$$

$$\text{NUtE} = \text{Grain Yield DM } (g\ plant^{-1}) \,/\, \text{AGN}_{\text{H}}\ (g\ \ N\ plant^{-1}) \qquad \text{Equation 2.3}$$

where available N = soil N + fertilizer N supply, AGDM = above-ground dry matter and AGN = above-ground N uptake.

Flag-leaf photosynthesis measurements were taken on five dates (30 March, 06 April, 11 April, 19 April, and 10 May) in 2017 and four dates (20 April, 30 April, 03 May, and 11 May) in 2018 under HN conditions. These dates were from flag-leaf emergence (GS 39) to mid grain filling (GS 85) in each year. Light-saturated photosynthetic rate (A_{max}) and stomatal conductance (g_s) of the flag-leaf were measured using a LI-Cor LI-6400XT Portable Photosynthesis System (Licor Biosciences, Lincoln, NE, USA). For each plant, three readings were taken on the flag leaf between 10.00 and 15.00 h. The instrument settings were adjusted to flow rate 500 μmol s^{-1} and block temperature 22°C with ambient

relative humidity. The sample (cuvette) CO_2 concentration was set to 400 µmol mol^{-1} and photo synthetically active radiation (PAR) to 2,000 µmol m^{-2} s^{-1} (10% blue). The gas-exchange parameters were analysed by using the average values per plant during each of the pre-anthesis and post-anthesis periods. The relative chlorophyll content of the flag-leaf of the main shoot was measured every 7 days from GS39 to flag-leaf 100% senesced using a hand-held SPAD meter (SPAD 502, Minolta, Japan). Three measurements were made per flag leaf at basal, middle, and apical leaf positions.

2.4. Statistical analysis

In the hydroponic and glasshouse experiments, analysis of variance (ANOVA) procedures for a split-plot design was used to analyze N and genotype effects and their interaction and the three-way interaction with year using GenStat 19 (www.genstat.com; VSN International Ltd, Hemel Hempsted, UK), where replicates and years were regarded as random effects and genotypes and N treatments as fixed effects. Pearson's correlation coefficient and linear regressions were calculated using genotype means to quantify associations between traits among genotypes using GenStat 19. Principal component analysis (PCA) procedures to test associations between traits were carried out using XLSTAT software version 2019. Box plots was drawn in the XLSTAT software.

3. Results

3.1 Hydroponic 2-D root phenotyping experiment

3.1.1. Phenotypic variation in seedling root traits

The landrace-derived lines showed differences in root system architecture traits compared to the parental wheat cultivar Paragon (Figure 1; Supplementary Table S3). Twelve landrace-derived lines under HN and 19 lines under LN conditions) ($P<0.05$) had more total roots $plant^{-1}$ than Paragon. For lateral roots $plant^{-1}$, genotypes ranged from 0.8 to 30.2 under HN conditions and 7.7 to 30.5 under LN conditions ($P<0.001$). Two landrace-derived lines under HN and three lines under LN had more lateral roots $plant^{-1}$ than Paragon ($P<0.05$).

For seminal root angle genotypes ranged from 17.0 to 32.6° under HN and 16.9 to 35.4° under LN conditions ($P<0.001$). A smaller root angle (steeper angle) is hypothesized to favour deeper rooting; therefore with regard to transgressive segregation a smaller angle should be considered compared to Paragon. Twelve lines under HN and 10 lines under LN had smaller seminal root angle than Paragon ($P<0.05$). Similarly positive transgressive segregation for seminal root length $plant^{-1}$($P<0.05$) and maximum root depth ranged amongst genotypes ($P<0.001$) was observed under HN and LN conditions. For width to depth ratio genotypes ranged from 0.29 to 0.61 under HN and from 0.24 to 0.75 under LN conditions ($P<0.001$). Under HN conditions, four lines had lower WDR while under LN conditions, three lines had lower WDR than Paragon. Lines with narrower width to depth ratio were negatively correlated with maximum depth.

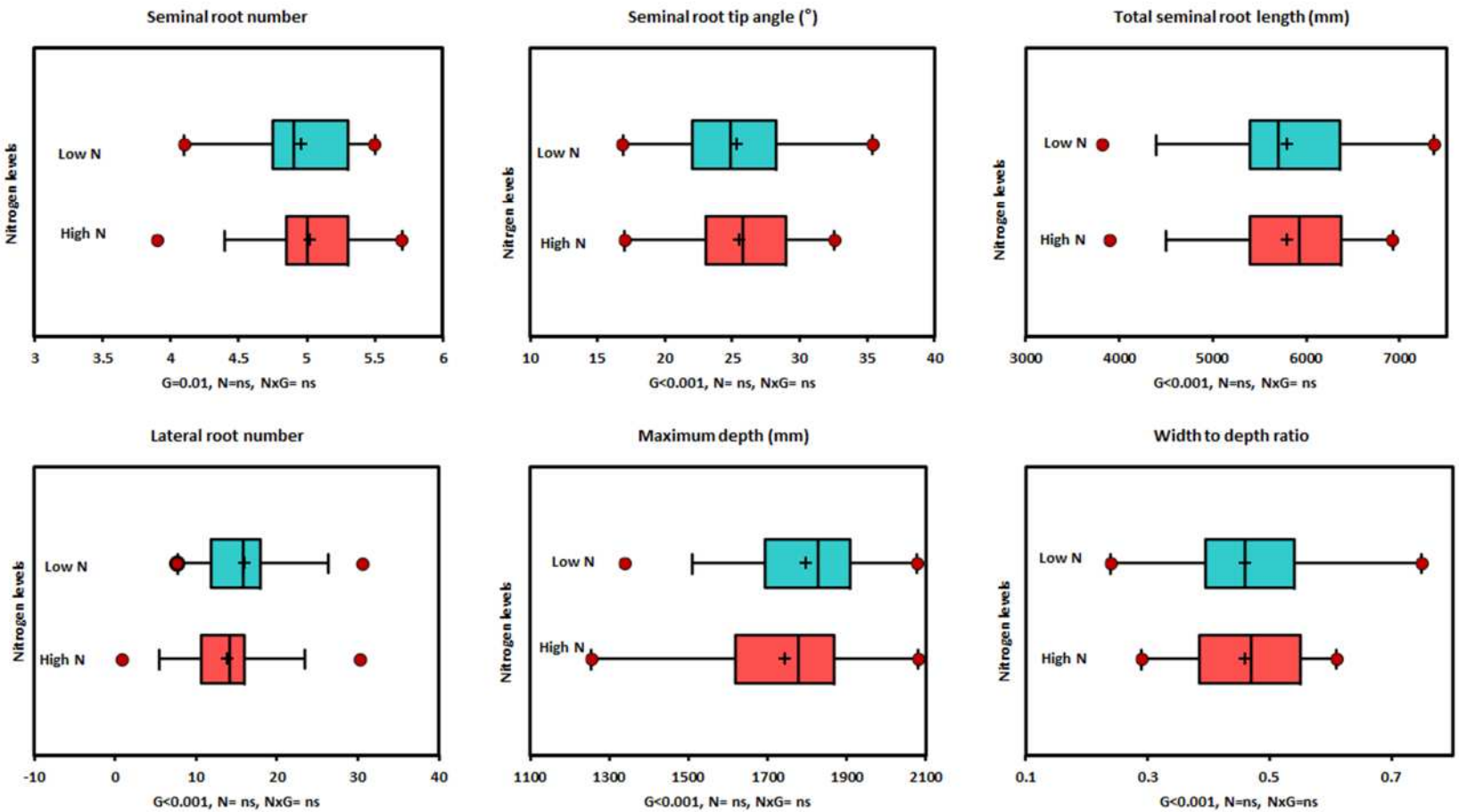

Figure 1. Boxplots for root traits for the high N and low N treatments. The central Horizontal lines splitting the boxes indicate the median values; the black crosses correspond to the means; the lower and upper limits of the box are the first and third quartiles, respectively. Points in black are minimum and maximum for each genotype. Genotype mean values are in Table S3.

3.1.2 Phenotypic correlations between seedling root traits

Significant correlations amongst genotypes between RSA traits were found (Table 2). Under high N conditions, maximum depth was positively correlated with both seminal root length and lateral root number per plant. Width to depth ratio was strongly positively associated with seminal root angle (r=0.66, P<0.001). Total seminal root length was positively associated with the lateral roots plant^{-1}. Under LN conditions, seminal root length was positively correlated with seminal roots plant^{-1}; and the width to depth ratio was strongly positively associated with seminal root tip angle and lateral roots plant^{-1}.

Table 2. Pearson's phenotypic correlations between the seedling root traits for the 30 landrace-derived lines and Paragon. Values are for HN (unshaded) and under LN (shaded)

	SRN	SRTA	TSRL	LRN	MD	WDR
SRN		-0.15	0.49*	-0.05	0.06	0.20
STA	0.09		-0.07	0.34*	0.09	0.80***
TSL	0.28	-0.17		0.50**	0.83***	0.20
LRN	-0.19	-0.04	0.64***		0.66***	0.38*
MD	-0.22	-0.18	0.74***	0.83***		0.17
WDR	0.29	0.66***	0.16	0.07	-0.09	

Trait abbreviations: SRN, seminal root number plant^{-1}; SRTA, seminal root tip angle; TSRL, total seminal root length; LRN, lateral root number plant^{-1}; MD, maximum depth; WDR, width to depth ratio.

* P< 0.05; ** P< 0.01; *** P< 0.001.

3.2 Glasshouse experiments

3.2.1. Anthesis date, plant height and physiological maturity

Averaging across years, anthesis date did not differ between the HN and LN treatments; and ranged amongst genotypes from 69-88 DAS under HN and 70-90 DAS under LN conditions (Table 3; P<0.001). Plant height ranged amongst genotypes from 66-111 cm under HN and 70-109 cm under LN conditions (P<0.001).

Table 3. Anthesis date, plant height (PH) and physiological maturity date (days after sowing, DAS) and ears per plant for 12 NAM lines and Paragon under HN and LN conditions (mean of 2017 and 2018)

Genotypes	Anthesis date (DAS)		Plant height (cm)		Physiological maturity (DAS)		Ears plant [1]	
	HN	LN	HN	LN	HN	LN	HN	LN
Paragon	78	79	85.6	84.4	119	119	7	5
ParxPfau-03	72	70	74.9	75.2	109	107	8	5
ParxPfau-59	69	70	82.8	86.9	113	111	8	5
ParxPfau-86	79	77	65.9	70.4	119	119	9	6
PxW223-89	73	72	88.5	89.0	109	110	10	6
PxW264-10	79	80	87.3	93.2	113	114	8	5
PxW264-52	85	86	92.7	96.9	121	121	8	7
PxW420-22	79	74	88.9	90.2	118	118	7	4
PxW420-32	75	76	90.3	97.6	114	117	7	5
PxW546-03	79	79	89.1	93.8	114	115	10	7
PxW546-08	88	90	110.9	94.3	120	123	11	9
PxW566-12	79	77	95.9	100.8	117	118	6	4
PxW685-36	84	82	108.4	109.1	118	117	7	5
Mean	78	78	89.3	90.9	116	116	8	6
LSD (5%) N	3.1 ns		3.1 ns		1.5 ns		0.8***	
LSD (5%) G	2.1***		7.1***		2.4***		0.9***	
LSD (5%) N*G	3.8 ns		9.9 ns		3.5 ns		1.4*	

* P< 0.05; ** P< 0.01; *** P< 0.001.

3.2.2. Grain yield and above-ground dry matter

Averaged across years, grain yield plant^{-1} (GY) reduced by 37% under LN (8.45 g plant^{-1}) compared to HN (13.48 g plant^{-1}) conditions (P<0.001; Fig. 2). Genotypes ranged from 11.0 -16.7 g plant^{-1} under HN and 6.3-10.0 g plant^{-1} under LN conditions (P<0.001). Above-ground dry matter (AGDM) was reduced by 34% under LN (22.0 g plant^{-1}) compared to HN (33.1 g plant^{-1}) conditions (P<0.001). There was genetic variation (P<0.001) and a N × G interaction (P<0.10). There was a positive association between AGDM and GY plant^{-1} under HN (R^2=0.43, P=0.01) and LN (R^2=0.31, P=0.04; Fig 3) conditions. There was also a positive association between AGDM plant^{-1} and AD under HN (R^2=0.58, P=0.002) and LN (R^2=0.60, P=0.002) conditions (data not shown).

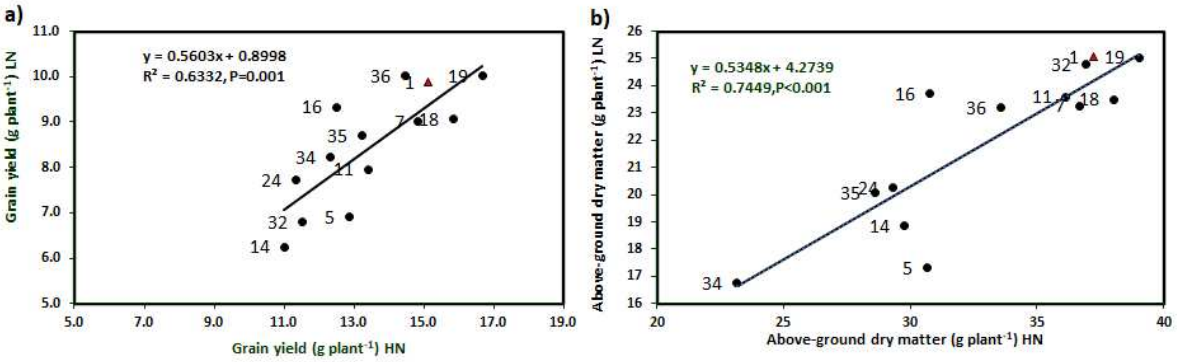

Figure 2. a) Grain yield (100% DM) and b) above-ground dry matter of 12 wheat landrace lines (codes 1-13 (Paragon code (red triangle) is 1), in high (HN) and low N (LN) treatment. Values represent means of 2017 and 2018. (see Table 1 for NAM line names)

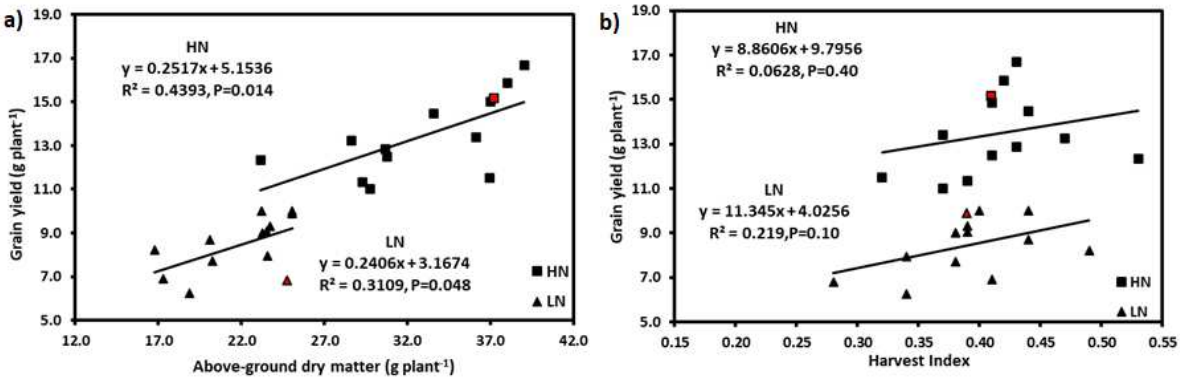

Figure 3. Linear regression of grain yield plant^{-1} (100% DM) on a) above-ground dry matter plant^{-1} and b) harvest index under high N (HN) and low N (LN) conditions for 12 NAM lines and Paragon. Values are mean of 2017 and 2018).

3.2.3. N-uptake efficiency and N-utilization efficiency

Overall, above-ground N uptake per plant at harvest (AGN_H) was reduced from 0.42 g N under HN to 0.25 g N under LN conditions (P<0.001; Fig. 4a). Genotypes ranged from 0.37-0.48 g N plant^{-1}under HN and from 0.21-0.30 g N plant^{-1} under LN conditions (P=0.003). There was a positive linear association amongst the genotypes between GY and N-uptake per plant in the LN treatment (R^2=0.35, P=0.03; Fig 4a). One genotype (PxW264-10) showed positive transgressive over Paragon for N uptake plant^{-1}. The genotypes differed in NUtE in the range 27.8-39.7 g DM g^{-1} N under HN and 28.0-40.5 g DM g^{-1} N under LN conditions (P<0.001). There was a trend for a N x G interaction (P<0.10). NUtE was positively associated amongst genotypes with GY plant^{-1} under both HN (R^2=0.51, P=0.006) and LN (R^2 =0.54, P =0.004; Fig 5b) conditions.

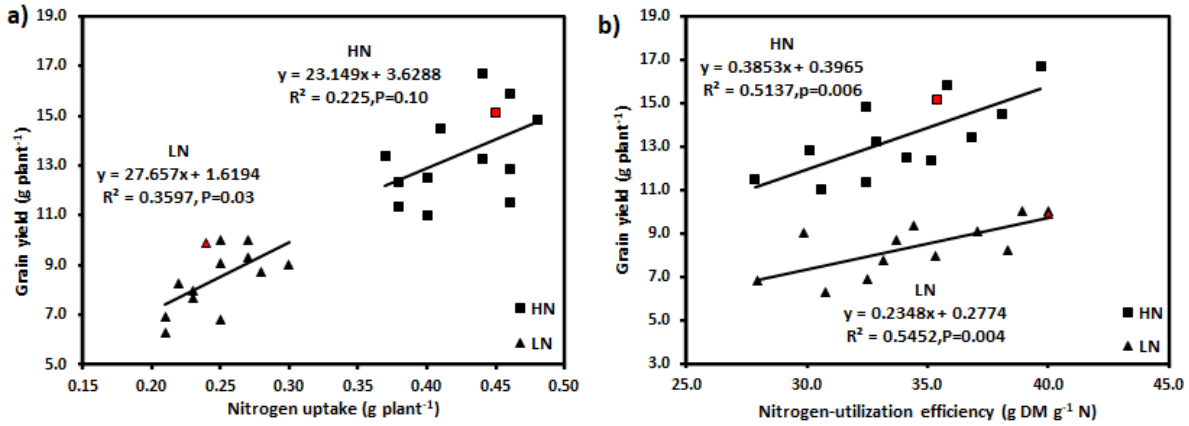

Figure 4. Linear regression of a) grain yield (100% DM) plant^{-1} on N uptake plant^{-1} at harvest and b) N-utilization efficiency (NUtE) under high N (HN) and low N (LN) conditions for 12 NAM lines and Paragon (red squares). Values are means of 2017 and 2018).

3.2.4. Flag-leaf photosynthesis rate, stomatal conductance, and chlorophyll content under HN conditions

Light saturated flag-leaf photosynthesis rate (A_{max}) and stomatal conductance (g_s) were measured pre- and post-anthesis under HN conditions. Flag-leaf A_{max} pre-anthesis ranged from 25.9 (PxW546-8) to 33.3 (PxW566-12) µmol m^{-2} s^{-1} (P<0.001, Fig. 5). No line showed transgressive segregation above Paragon for pre-anthesis A_{max}. Flag-leaf A_{max} post-anthesis ranged from 17.4 (PxW685-36) to 27.8 (ParxPfau-59) µmol m^{-2} s^{-1} (P<0.001). Three genotypes (PxW264-10, ParxPfau-3, and ParxPfau-59) showed positive transgressive segregation above Paragon (P< 0.05). Flag-leaf SPAD at anthesis ($SPAD_A$) was lower under LN (42.5) than under HN conditions (46.9) (P=0.002). Genotypes ranged from 43.0 (PxW685-36) to 51.5 (ParxPfau-3) under HN and from 36.2 (PxW566-12) to 47.0 (ParxPfau-3) under LN conditions (P<0.001). There was a strong positive association between flag-leaf SPAD at anthesis and flag-leaf post-anthesis A_{max} (R^2=0.56, P=0.003; Fig 6 a).

For flag-leaf pre-anthesis stomatal conductance (g_s) genotypes ranged from 0.349 (PxW546-8) to 0.538 (PxW566-12) mol m^{-2} s^{-1} (P<0.001). PxW566-12 showed transgressive segregation above Paragon (P< 0.05). For flag-leaf g_s post-anthesis genotypes ranged from 0.190 (PxW685-36) to 0.440 (ParxPfau-59) mol m^{-2} s^{-1} (P<0.001). Five lines (PxW264-10, PxW264-52, PxW223-89, ParxPfau-3, and ParxPfau-59) showed transgressive segregation above Paragon (P<0.05). There was a positive association between flag-leaf SPAD at anthesis and post-anthesis g_s (R^2=0.44, P=0.013; Fig 6b). Genetic variation in flag-leaf A_{max} and g_s either pre- or post- anthesis was not associated with biomass of grain yield per main shoot or per plant.

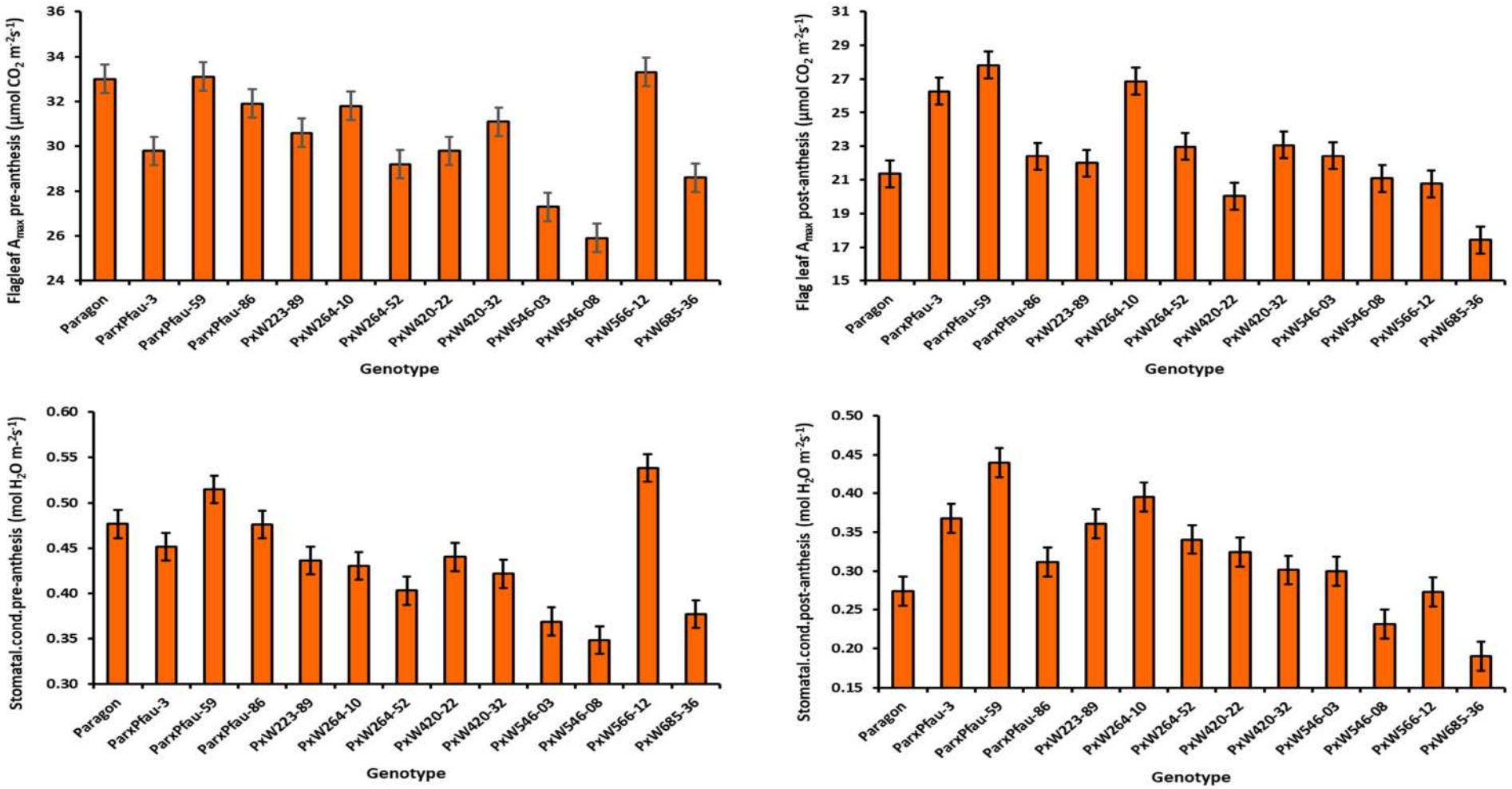

Figure 5. Genetic variation of Flag leaf A_{max} pre-anthesis, Flag leaf A_{max} post-anthesis, stomatal conductance pre-anthesis and post-anthesis of wheat landrace derived lines and Paragon. Overall mean value of landrace lines. Values represent means of 2017 and 2018.

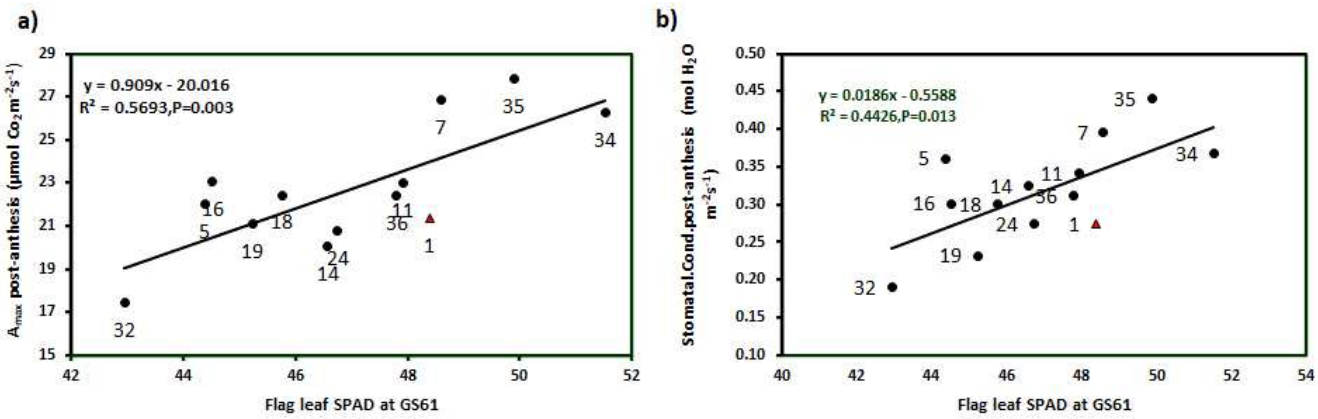

Figure 6. Linear regression of (a) post-anthesis flag leaf photosynthetic rate (A_{max}) and (b) post-anthesis flag leaf stomatal conductance on flag leaf chlorophyll content (SPAD) at anthesis (GS61) under high N (HN) for 12 landrace derived lines and Paragon. Values represent means of (2017 and 2018).

3.3 Phenotypic correlations between hydroponic root traits and physiological traits in the glasshouse experiments

Associations between seedling root traits and whole-plant traits among genotypes in the glasshouse experiments are shown in the biplots in Fig 7. Under HN conditions (Fig. 8a), seminal roots plant^{-1} was negatively correlated with above-ground N plant^{-1}. In addition, seminal root angle had a positive correlation with AGDM plant^{-1} (r=0.75, P=0.03). Under LN conditions (Fig 7b), AGDM plant^{-1} had a positive correlation with seminal root angle (r=0.73, P=0.04), maximum root depth (r=0.90, P=0.002) and lateral roots plant^{-1} (r=0.73, P=0.04). Seminal roots plant was also negatively correlated with grain yield plant^{-1} and N uptake plant^{-1}.

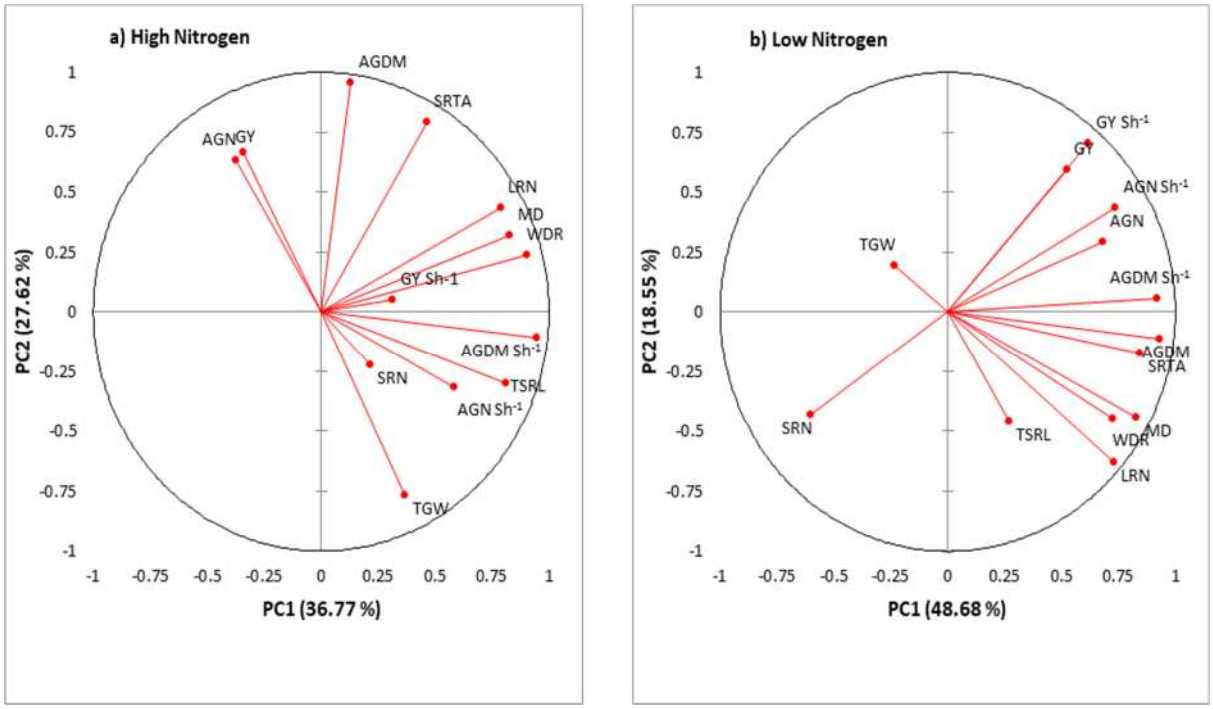

Figure 7. Biplots showing associations between seedling root traits in the 'pouch and wick' system with traits in the glasshouse based on mean of 2016-17-2017-18 for 7 NAM lines and Paragon under a) High N and b) LN conditions. **Trait abbreviations:** SRN, seminal root number plant^{-1}; SRTA, seminal root tip angle; TSRL, total seminal root length plant^{-1}; LRN, lateral root number plant^{-1}; MD, maximum depth; W/D, width to depth ratio; GY, grain yield plant^{-1}; GY Sh^{-1}, grain yield per shoot; AGDM, above-ground dry matter plant^{-1}; AGDM Sh^{-1}, above-ground dry matter per shoot; AGN, above-ground N at harvest plant^{-1}; AGN Sh^{-1}, above-ground N at harvest per shoot and TGW, thousand grain weight.

4. Discussion

4.1 Genetic diversity in the landrace-derived lines for physiological traits in wheat

In this study, significant variation in root system architecture traits was identified in the landrace-derived lines compared to the elite cultivar Paragon. Beneficial transgressive segregation for seminal roots plant^{-1}, seminal root tip angle (narrower angle) and total seminal root length plant^{-1} was observed under N limitation. For example, seminal roots plant^{-1} for PxW566-14 was 31% greater and seminal root length plant^{-1} for PxW546-03 was 33% longer than for Paragon under LN conditions. Wheat landrace collections contain wider genetic diversity than represented in most breeding programmes with potential for introgressing traits for adaptation to abiotic stress conditions and yield stability under low input systems (Zeven, 1998). The present results support previous evidence that the root size system of landraces is larger than modern cultivars (Waines and Ehdaie, 2007) implying that N uptake may be improved compared with modern cultivars particularly under low N conditions.

Significant genetic variation in pre-anthesis flag-leaf photosynthesis rate was identified in the landrace-derived lines in the glasshouse experiments under HN conditions, although no landrace-derived line was higher than Paragon. This suggested that wheat breeding has improved flag-leaf A_{max} relative to landraces and that continued improvement in A_{max} is therefore an important breeding target for raising grain yield in elite wheat cultivars. This is in agreement with Gaju *et al*. (2016) who reported pre-anthesis flag-leaf A_{max} of five modern UK cultivars at 25.2 µmol m^{-2} s^{-1} was higher than for the mean for five bread wheat landraces at 20.1 µmol m^{-2} s^{-1} in field experiments. With regard to post-anthesis flag-leaf A_{max}, our results showed one landrace-derived line had significantly higher flag-leaf A_{max} than Paragon. Improving leaf photosynthetic rate has the potential to increase grain yield and/or reduce N inputs and enhance NUE (Hawkesford, 2014). In the present study, however, there was no positive association between either pre- or post-anthesis A_{max} and grain yield under HN conditions in the glasshouse experiments. This may have reflected that grain growth of the landrace-derived lines was predominantly sink-limited. The harvest index of the landrace-derived lines was relatively low in the range 0.28-0.43, as would be expected as the landraces parents of the NAM lines were not selected intensively for HI in plant breeding. There was a strong linear positive association amongst the genotypes between flag-leaf SPAD at anthesis and post-anthesis A_{max}, indicating flag-leaf chrlophyll content could be a useful proxy to deploy to select for improved flag-leaf photosynthesis rate in breeding programs. High leaf chlorophyll content may correlate with more Rubisco per unit area. Previous

studies have also shown association between flag-leaf chlorophyll content and A_{max} in wheat genotypes, e.g. Gaju *et al.* (2016).

Overall, plant height was taller in the landrace-derived lines and Paragon than the CIMMYT spring wheat Pfau-derived lines in the NAM population subset in the glasshouse experiments. Lower plant height for the CIMMYT Pfau-derived lines was likely due to the presence of the semi-dwarfing gene *Rht-B1b* present in most modern CIMMYT spring wheat releases (Feng *et al.*, 2018). It is well established that wheat breeders introduced reduced height (*Rht*) semi-dwarf genes which increased HI, especially under high N inputs during the Green Revolution (Borojevic and Borojevic, 2005; Gooding *et al.*, 2012). The shorter plant height for Paragon x Pfau-3 and Paragon x Pfau-86 compared to the landrace-derived NAM lines was associated as expected with higher harvest index and grain yield per plant. In the glasshouse study, grain yield was also higher for the elite spring wheat cultivar Paragon than the landrace-derived lines under both HN and LN conditions as expected due to higher HI (Soriano *et al.*, 2018).

4.2 Correlation of RSA traits with physiological traits in glasshouse experiments

We found several significant correlations amongst genotypes between the seedling RSA traits and whole-plant traits in the glasshouse experiments. Under HN and LN conditions, shallower seminal root angle was correlated with higher plant biomass in the glasshouse experiments. Under HN conditions, higher width to depth ratio indicative of shallower root angle.was also positively associated with biomass per shoot. Atkinson *et al.* (2015) reported a trend for a positive correlation between width to depth ratio and GY in field experiments in a Rialto × Savannah DH population under HN conditions. The landrace-derived lines showed high expression under HN conditions of maximum depth and lateral roots plant^{-1}, although these traits were not positively associated with biomass plant^{-1}. Under LN conditions, however, increased maximum root depth and lateral roots plant^{-1} were associated with increased biomass plant^{-1}. In the present study, wider angle (shallower roots) was correlated with increased biomass and N-uptake efficiency whereas narrower root angle and steeper roots were hypothesised to increase root depth and N uptake. It may be that applying ammonium nitrate in the irrigation water to soil surface from above in the pots favoured N capture with shallower roots more than would be the case under low N environments in the field where a high proportion of available N is located in the deeper soil layers.

In our results seedling root-length traits were not correlated with the whole plant traits in the glasshouse experiments. In previous work, the lack of consistent correlation between seedling root traits and N-uptake in the field was partly due to a strong genotype × N × site × year effect, reflecting that the N uptake has a relatively low heritability (Atkinson *et al.*, 2015). Khokhar *et al.* (2019) also reported no associations between length-related root traits measured in a high-throughput seedling platform using germination paper and grain yield and yield component traits of elite bread wheat and durum genotypes at six field sites in India. Factors responsible for non-correlation between whole-plant

performance and seedling root screens may also include seedling root traits not translating to trait expression later in development at physiological maturity.In the field, root growth may also be affected by such factors as mechanical impedance of root elongation, moisture content and nutrient availability (Strock *et al.*, 2019). The lack of nodal roots at the seedling stage which are critical for nutrient and water uptake during grain filling (Boatwright and Ferguson, 1967) may also affect correlation with performance at physiological maturity. In the present study, we found no relationship between seminal root number and angle. Similarly using gel-filled root observation chambers, no relationship was reported between seminal root number and angle in the SeriM82 x Hartog DH population by Christopher *et al.* (2013). Nevertheless, the associations between maximum root depth and lateral root number plant^{-1} in the seedling root screen and biomass plant^{-1} under low N conditions indicated that these seedling root traits may be indicative of whole plant performance under LN conditions, although further studies are required to confirm the present results at the field scale.

4.3 Implications for breeders

In wheat breeding programs cultivars are selected mainly under optimal resource levels, and it would not be cost-effective to select traits for improved NUE under both LN and HN conditions at multi-location trials (Brancourt-Hulmel *et al.*, 2003). The challenges of field conditions such as difficulty of extracting intact roots and imaging roots *in situ* makes phenotyping RSA traits difficult. This limitation may have led to selection of cultivars which are not optimized for N uptake under moderate to low N availability. Present results demonstrated genetic variation for seedling RSA traits in landrace-derived lines above the elite cultivar Paragon which potentially could be utilized in breeding programs, for example, variation in seedling RSA traits was associated with biomass at maturity under LN conditions in the glasshouse experiments.

The high-throughput root phenotyping method presented here was used to image seedling root systems at 14 days after emergence. Setting up the hydroponic screen required two person days (making pouches, transferring seeds etc) and approximately one day was required for the image acquisition. The new image analysis method Root Nav. 2.0 simultaneously located seeds and first and second-order root tips to drive a search algorithm seeking optimal paths throughout the image. This was faster than semi-automatic approaches, with processing of one image using RootNAV 2.0 taking between 5 and 15 seconds with no user interaction. The throughput of the system is still restricted to hundreds of lines rather than 1000s. Nevertheless, the present high-throughput platform for screening root traits could be of potential benefit to wheat scientists and breeders. For example, it could be deployed in breeding for phenotyping progeny of targeted crosses or for screening parental material in crossing blocks to design synergistic crosses in trait-based breeding. Moreover, this tool can also be transferred to new image types and species. Present results suggested that seedling root architectural traits offer scope for use as selection criteria for selecting genotypes for higher biomass and NUE. Further genetic studies should

be carried out on the whole NAM landrace-derived panel to identify SNP markers and candidate genes for these RSA traits with potential for application in plant breeding.

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Table S1. Composition of ¼ Hoagland's nutrient solution used in wheat hydroponic experiment. For low N treatment, $Ca(NO_3)_2 \cdot 4H_2O$ and KNO_3 were removed and replaced with 101.1 mg $CaSo_4 \cdot ½H_2O$ and 112.1 mg KCl.

Macronutrients	mg	Micronutrients	mg
$(NH_4)_3PO_4$	29	$CuSO_4 \cdot 5H_2O$	0.75
$Ca(NO_3)_2 \cdot 4H_2O$	165	$MnCl_2 \cdot 4H_2O$	10.1
$MgSO_4 \cdot 7H_2O$	252.8	MoO_3	0.2
KNO_3	151.8	$ZnSO_4 \cdot 7H_2O$	2.3
H_3BO_3	28.55	FeHEDTA	25.5

Table S2. Definitions of root traits measured in the hydroponic experiment

Abbreviation	Definition	Units
SRN	The number of seminal roots in each plant	Dimensionless (Count)
ASTA	Average seminal tip angle, the average angle of all seminal root tips relative to the vertical axis	Degrees (°)
TSRL	Total length of seminal roots in each plant	mm
LRC	The number of lateral roots in each plant.	Dimensionless (Count)
MD	Maximum depth, the vertical distance from the base to the tip of the deepest seminal root	mm
WDR	Width to depth ratio, the ratio of maximum width to maximum depth	Dimensionless (Ratio)

Table S3. Seminal root number, seminal root tip angle, total seminal root length, lateral root number, maximum depth and width to depth ratio for 31 genotypes (30 Watkins landrace-derived lines and spring wheat parent (cv. Paragon) in the 2D hydroponic experiment.

Genotypes	Primary root No.		Primary root tip angle°		Total primary root length(mm)		Lateral root No		Max-Depth (mm)		Width-depth ratio	
	HN	LN	HN	LN	HN	LN	HN	LN	HN	LN	HN	LN
Paragon	4.4	4.2	29.3	28.8	5086	5539	16.2	18.6	1764	1949	0.49	0.45
PxW223 - 01	4.6	4.7	32.3	26.9	5332	6103	10.0	14.3	1585	1888	0.60	0.52
PxW223 - 03	4.7	4.8	20.5	21.5	6557	5700	30.2	16.7	2081	1834	0.44	0.35
PxW223 - 85	5.3	5.2	22.4	22.3	6142	6507	10.9	14.0	1783	1878	0.36	0.42
PxW223 - 89	4.9	5.4	19.3	19.7	5233	5432	6.5	9.4	1603	1594	0.31	0.33
PxW223 - 94	5.4	5.1	27.9	26.7	6092	5462	16.0	11.4	1776	1678	0.41	0.58
PxW264 - 10	5.1	4.5	30.6	29.9	5971	5574	14.0	16.3	1817	1842	0.54	0.51
PxW264 - 16	4.6	4.6	25.8	30.6	5853	5755	16.0	15.8	1960	1889	0.38	0.49
PxW264 - 17	4.9	4.8	26.5	28.4	6352	5718	14.1	19.3	1891	1856	0.51	0.55
PxW264 - 31	5.2	4.9	23.9	24.3	4817	5269	5.4	11.3	1415	1583	0.48	0.57
PxW264 - 52	4.7	4.8	25.8	24.3	6068	5996	17.6	17.6	1858	1932	0.55	0.41
PxW420 – 03	5.0	4.9	23.0	24.9	6560	6485	15.7	12.0	1721	1826	0.40	0.33
PxW420 – 21	5.0	5.1	30.0	27.5	5617	7029	15.2	28.7	1618	1894	0.55	0.57
PxW420 – 31	5.7	4.8	19.3	16.9	5710	5067	10.4	11.6	1630	1718	0.38	0.24
PxW420 – 32	5.3	5.3	23.0	21.6	6922	6770	15.8	17.0	1876	1921	0.55	0.34
PxW420 – 94	5.6	5.5	21.5	24.3	6821	6240	18.0	17.7	1848	1811	0.38	0.43
PxW546 – 03	5.3	5.3	24.4	24.7	5919	7365	15.2	20.5	1913	2032	0.39	0.52
PxW546 – 15	5.7	5.3	32.6	24.5	6384	7140	12.3	22.4	1741	2057	0.57	0.54
PxW546 – 20	4.8	4.7	29.0	28.2	4501	4535	6.0	7.7	1553	1570	0.40	0.46
PxW546 – 32	5.0	4.7	28.9	25.0	4852	4389	11.3	12.9	1475	1509	0.42	0.39
PxW546 – 47	4.4	4.1	24.7	21.1	4904	4606	19.6	17.5	1909	1728	0.31	0.30
PxW566 – 12	4.9	5.0	23.2	22.3	6036	5569	12.7	15.5	1782	1814	0.47	0.42
PxW566 – 14	5.4	5.5	26.4	21.7	6843	7068	15.3	16.2	1847	1921	0.58	0.40
PxW566 – 24	3.9	5.1	17.0	19.2	5723	7097	14.2	13.4	1848	2032	0.29	0.43
PxW566 – 50	5.0	4.9	21.6	21.2	5919	3814	7.5	7.9	1734	1341	0.30	0.30
PxW566 – 72	5.0	5.4	27.2	27.9	6619	5921	23.4	15.8	1885	1678	0.47	0.58
PxW685 – 01	5.0	4.4	25.2	29.6	5618	5476	11.6	26.3	1764	1794	0.47	0.54
PxW685 – 09	5.6	5.3	23.7	26.1	5520	5443	11.7	9.0	1560	1610	0.58	0.51
PxW685 – 16	5.1	4.9	26.6	29.8	3901	4956	0.8	8.3	1254	1717	0.48	0.48
PxW685 – 36	5.0	5.0	29.0	31.2	6411	6247	26.4	30.5	1968	2078	0.59	0.56
PxW685 – 44	5.3	5.4	29.8	35.4	5448	5351	6.3	18.2	1622	1706	0.61	0.75
Mean	5.0	5.0	25.5	25.4	5798	5794	13.8	15.9	1745	1796	0.46	0.46
LSD (5%) G	0.7*		5.0***		1072***		7.3***		244***		0.14***	
LSD (5%) N	0.3 ns		2.9 ns		881 ns		8.1 ns		297 ns		0.09	
LSD (5%) N*G	1.0 ns		7.2 ns		1626 ns		11.8 ns		408 ns		0.20	

Table S4. Grain yield (GY), above-ground dry matter (AGDM), N-uptake per plant, N-uptake efficiency per plant, N-utilization efficiency in 13 genotypes (12 NAM lines and Paragon) under HN and LN conditions in (12 NAM lines and Paragon for means of 2017-18.

Genotypes	GY (g plant ^{1})		AGDM (g plant ^{1})		Ears Plant^{-1}		AGN_H (g plant ^{1})		NUpE (g N g $^{-1}$ N)		NutE (g DM g^{-1} N)	
	HN	LN	HN	LN	HN	LN	HN	LN	HN	LN	HN	LN
Paragon	15.14	9.88	37.2	25.1	7	5	0.45	0.24	1.076	1.613	35.4	40.0
ParxPfau-03	12.33	8.23	23.2	16.8	8	5	0.38	0.22	0.877	1.471	35.1	38.3
ParxPfau-59	13.24	8.70	28.6	20.1	8	5	0.44	0.28	1.019	1.817	32.9	33.7
ParxPfau-86	14.48	10.01	33.6	23.2	9	6	0.41	0.25	0.986	1.654	38.1	40.5
PxW223-89	12.86	6.90	30.7	17.3	10	6	0.46	0.21	1.078	1.428	30.1	32.5
PxW264-10	14.85	9.00	36.7	23.3	8	5	0.48	0.30	1.143	1.991	32.4	29.9
PxW264-52	13.40	7.96	36.2	23.6	8	7	0.37	0.23	0.914	1.57	36.8	35.3
PxW420-22	11.00	6.25	29.7	18.9	7	4	0.40	0.21	0.945	1.426	30.6	30.8
PxW420-32	12.50	9.33	30.8	23.7	7	5	0.40	0.27	0.93	1.806	34.1	34.4
PxW546-03	15.86	9.06	38.0	23.5	10	7	0.46	0.25	1.088	1.64	35.8	37.0
PxW546-08	16.68	10.02	39.0	25.0	11	9	0.44	0.27	1.051	1.792	39.7	38.9
PxW566-12	11.34	7.72	29.3	20.3	6	4	0.38	0.23	0.886	1.532	32.5	33.1
PxW685-36	11.51	6.79	36.9	24.8	7	5	0.46	0.25	1.061	1.674	27.8	28.0
Mean	13.48	8.45	33.1	22.0	8	6	0.42	0.25	1.004	1.647	34.0	34.8
LSD (5%) G	0.41 ***		1.3***		0.8***		0.021***		0.173***		2.48 ns	
LSD (5%) N	1.59***		2.8***		0.9***		0.047**		0.193**		3.49***	
LSD (5%) N*G	2.18 ns		3.9 *		1.4 *		0.065 ns		0.292 ns		5.07 ns	

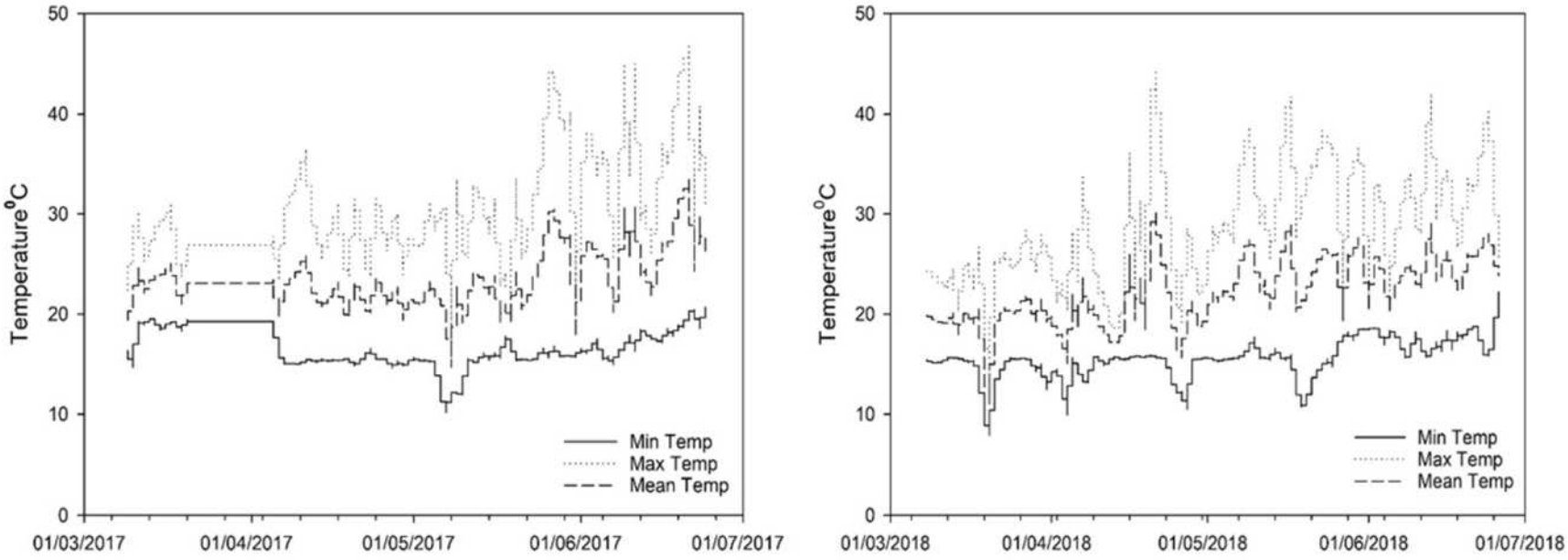


Figure S1. Minimum, maximum, and mean ambient temperature in glasshouse experiments in 2017 and 2018 after seedlings transplanted in pots (6 March 2017) and (7 March 2018).

Reviewer #1: This manuscript deals with a very relevant analysis of importance of plant root traits on adaptation of wheat genotypes on different soil N condition. It is clear that the objectives of this study is of very high importance in order to increases the capacity of crop to use endogenous soil N resources and then for minimizing the importance of N fertilizer necessary for reaching potential grain yield. The objective of this study is clearly to analyse both the two aspects of Nitrogen Use Efficiency: (i) the Nuptake Efficiency; and (ii) the N-utilization Efficiency.

NutE is analysed through flag leaf photosynthesis and correlation are made with flag-leaf SPAD... But in fact we have no information about the link between SPAD measurement and plant N nutrition status!!! So it is difficult to obtain a clear physiological interpretation of these correlation. We have only an indication that increasing SPAD should correspond to an increase in plant N nutrition status... that is very trivial. The more important thig should be to know why some genotype are able to maintain their N status higher than others in low N conditions? For that it is absolutely necessary to have a direct estimation of plant N status. Authors should then refers to the concept of critical N and Nitrogen Nutrition Index (see Lemaire et al. 2008 in EJA and more recently Lemaire and Ciampitti, 2020 in MDPI Plants). The problem in their case is a litle bit more complex because their experiment is carried on on "isolated plants" and not in a dense crop, so the well established "critical N curve" relating plant N uptake (Nup) to crop mass (W) cannot be used directly. Nevertheless, authors could use the allometric relationship between Nup and W across genotypes as a mean for segregating their data, and then to compare Nup capacity of genotype at similar W in order to eliminate the trivial effect "the higher W, the higher Nup"...

So we encourage authors to use the Nup-W curve expressed in log-Log term as a mean for analysing their data. In the same way, the use of allometry between shoot W and root W, should be also a way for eliminating the trivial "plant size effect"....

So our conclusion is that this very relevant manuscript should be highly encouraged for publication, but it require a more fundamental analysis of results implying (i) the estimation of the actual plant N status, and (ii) the elimination of the trivial "plant size" effect in order to better analysed the NupE of genotypes at similar plant size (W).... otherwise the results would be poluted by the trivial result... "the bigger plant has the higher NupE". The problem is to obtain a higher NupE at similar plant size: "intrinsic NupE".

So my recommendation is to ask author an improved version of their manuscript.

We agree that including and figures showing the allometric relationship between log Nup-W would be useful. We have added Figure x. showing

This showiws that plants of similar size have clear sifferences in Nuptake capacity

Reviewer #2: Review Manuscript „Root architecture and leaf photosynthesis traits and associations with nitrogen-use efficiency in landrace-derived lines in wheat " by Shadia H.S. Kareem, Malcolm J. Hawkesford, Jayalath DeSilva, Minuka Weerasinghe, Darren M. Wells, Michael P. Pound, Jonathan Atkinson, Michael J. Foulkes

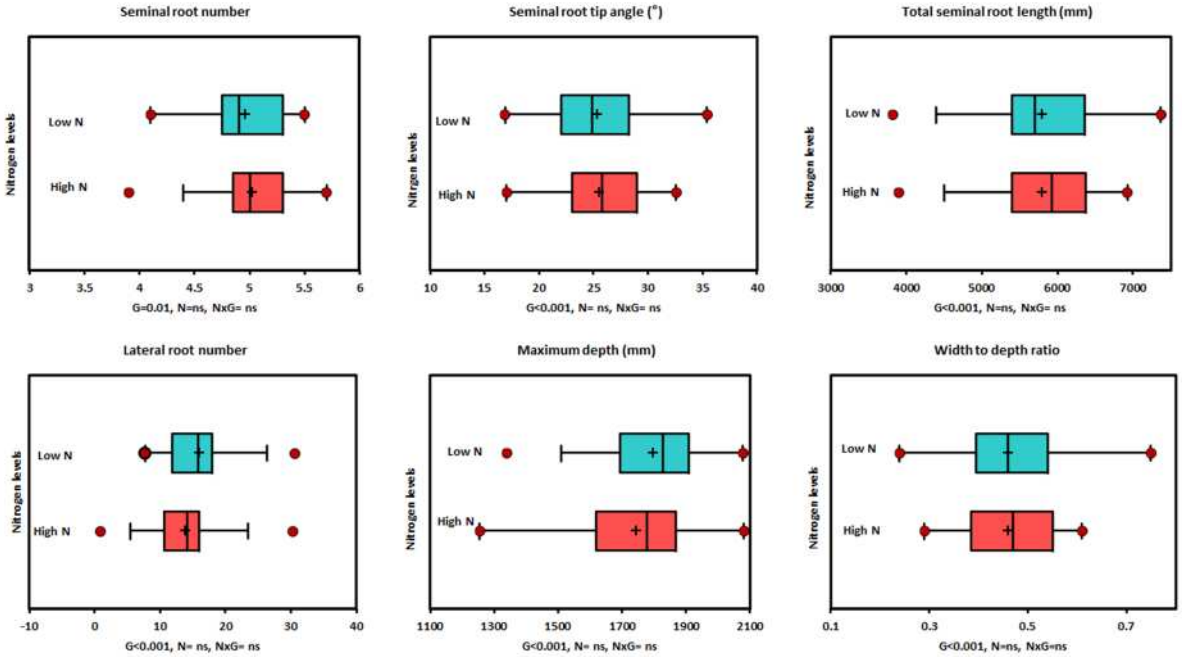
The present study analyzed root system architectural traits, nitrogen use efficiency (NUE) parameters and flag leaf photosynthesis measures in a NAM population of wheat in response to different nitrogen levels. For the NAM population, 35 landraces were crossed to the elite cultivar Paragon. First, a hydroponic screening was undertaken to determine different root architectural traits of wheat seedlings under high and low N input. In a following glasshouse experiment, about 1/3 of the lines were grown in pots until maturity and biomass and NUE measures of the whole plant as well as photosynthesis rates of the flag leaves were determined. The authors found that there was no impact of the N treatment on root system architecture of the seedlings, but it varied among genotypes. Strong associations between root architectural traits (e.g. between maximum depth and lateral root number) persisted under both N conditions. In the glasshouse experiment, high N supply promoted yield and nitrogen efficiency parameters and positive correlations were found for SPAD of the flag leaves at anthesis or post-anthesis and photosynthesis rate or stomatal conductance. Then, root system architectural traits of hydroponically-grown seedlings were correlated with yield, nitrogen efficiency parameters and photosynthesis measures from soil-grown plants. The authors found that under both N conditions the aboveground dry matter accumulation was positively associated with the seminal root angle, while the number of seminal roots was negatively correlated to aboveground N.

In general, the present manuscript describes a carefully conducted study with a comprehensive experiment description and solid data analysis providing insights into the interrelations among root system architectural traits of seedlings and grain yield and NUE measures in mature soil-grown wheat.

We thanks the reviewer for their overall positive comments on the rigour of the experiments.

However, the following major points are of some gravity:

1) In the present version Figure 1 is poorly informative as it only compares root trait variation of lines between HN and LN. However, the conclusion drawn from this figure is that there is a positive transgressive segregation for some traits. Unfortunately, this information cannot to be extracted from the figure. The box plot is less informative than would be the means of each line and its distance to Paragon to provide an idea about the transgressive segregation. In this sense, Figure 1 should be re-designed.



Need to indicate Paragon on Figure.

2) For the reader it is impossible to figure out whether lines showing transgressive segregation also perform better in terms of N uptake, NUE etc. Thus, the high performance e.g. of line PxW264-10 in the root phenotyping is not followed up in the evaluation of the agronomic traits. Thus, the full potential of the data appears not being deeply exploited.

We agree. The absolute values for root system architecture traits are given in Table 2. We have added the number so the individual genotypes can be identified in the figures 4. A and b. In addition, we have added supplementary figures x and x which show thasso between rt 1 and Nuptakeand rt2 and biomass

3) It remains unclear how the N treatments affected the nutritional status of the plants. Hence, it is unclear whether N-adequate plants from HN were compared with weakly or poorly deficient plants from LN or whether N-deficient plants were compared with severely deficient plants. Actually, the latter appears more likely in light of the similar slope for N uptake-dependent grain yield in NH and LN plants (Fig. 4). To allow the reader evaluating these correlations, he nutritional status must be clarified with new data display and discussion of the impact of the LN/HN treatments on agronomic traits.

We agree that the plant nutritional status needs to be clarified and we have added the new Figure 1 to the paper , as described above.

4) In the hydroponic experiment, the seedlings were imaged already 9 d after germination. At this stage the impact of the N supply is still quite weak as seedlings feed usually 4-6 d from the seed. Hence, the impact of the N treatment on root traits is still low. This point is a conceptual weakness and in my eyes a primary reason for the lacking differences between HN and LN root traits in Fig. 1. Unfortunately, this point is ignored.

We have added a sentence in the discussion, stating that seelding tining could paertlty explain the alck of a main treatment effect for N. We note theat there were nx genotype effect for N.

5) The study could benefit from analyzing measured parameters deeper with respect to origins of the landraces crossed to Paragon. E.g. in Figures lines from different geographical origins could be marked by same colors. This may give already a visual impression if there are origin-based patterns within the datasets, which may be analyzed and discussed later on.

We have grouped the genotypes in Supplementary into three groups with regard to the region of origin; the means for the groups as well and the P values for the significance value between groups has bee added. There was no significant effect for the groups between

6) The main goal of the study was to select early-seedling root markers to improve NUE of wheat. However, no data for NUE are shown at all and correlations between RSA traits and NUE measures are missing. Such data should be added to the manuscript (Section 3.2.3, Figure 7; see below).

The correlations between N uptake and root triats (lnfth, angle) are shown in the biplots in Fig. 7. We precdi that N upatek would be the N-related trait mots likely to assoictae with roto triats, but we have also added NUte and NUE to the varaibles in the biplots. The amin assoications are still as out;ined in the original version of the paper.

7) In section 3.2, data are presented as means over both experimental years. More robust data would be obtained by eliminating the year effect on the data by calculating best linear unbiased estimators from a linear mixed model with year as random effect.

The year effect was already included as a random effect on our ANOVA model.

8) The present version of Figure 7 does not exploit the full potential of the dataset. The whole data shown in Table 3 are completely missing in Fig. 7, although they may explain a part of the NUE of the lines. E.g., in the introduction (Lines 72-75) it is mentioned that taller landraces are more efficient in N uptake at LN. It would thus be of interest, how plant height was associated with the NupE and NUE in the NAM population as well as if early seedling root traits may be a marker for plant height.

In line with this, the authors even mention in the Discussion (Section 4.1) that crossings with Pfau lines were smaller and had a higher HI and grain yield, but there is no dataset presented which shows this correlation.

Plant height and anthesis date have also been included in the biplot and relevant text added.

9) One aim of the study is to identify the genetic variation for RSA in the NAM population. However, no quantification of the variability is given for the individual traits (such as the coefficient of phenotypic variation). Such data could give an impression on how stable or variable the expression of a feature is among lines in response to changing N conditions.

The phenotypic coefficient of variation has been added for each of the RSAT in table x.

In addition, there are a few minor points to be considered:

- There are too many phrases abbreviated. Suggestion: Either the authors reduce abbreviation number or they give abbreviations on an extra page or table. For root traits, the latter has been already done in Table S2 and could be extended for further abbreviations. In addition, please make sure that abbreviations are defined at first use in the text and used consistently.

We have reduced the number of abbreviations. Now only

- Line 42: Define N first time used. Done
- Line 43: … related to excessive N fertilizer inputs. Done
- Lines 43-46: Please rephrase sentence for better understandability to "N fertilizer inputs may be associated with nitrate leaching leading to groundwater contamination and eutrophication of rivers and lakes. Additionally, global warming may be favored due to emission of N2O derived from denitrification of nitrate by soil bacteria (Foulkes et al., 2009)." Done.
- Line 51: "Breeding for higher NUE…" Done.
- Line 52: What is "N stress"? N limitation? Done.
- Line 53: "including higher leaf photosynthetic rate" Done.
- Line 112-113/Lines 169-172/Table 1: May the authors give an explanation for the choice of the individual lines in individual experiments? E.g. why have been Line 14 and 19 and especially the crosses with the Mexican lines only used in the glasshouse experiment but not in hydroponics?

Line were chose to show variation in flag-leaf Amax according to previous measurements of Amax for these genotype, Therefore some lines not included in , to stretch the genotypes as far as possible for leaf traits and root traits ; therefore some lines not included in the root assay where involved to give a maximum variation for in addition to above ground N uptake. Text has been added for xxx.

- Line 115-116: Please define the range of anthesis date.

We have added the range of anthesis date.

- Line 150: change to "In the controlled-environment room, the PAR was 400 µmol m-2 s-1 and the photoperiod of the growth room was 12 h. The temperature was set to 20°C during light phase and 15°C during dark phase."

Done

- Lines 173-175: Please explain, why different amounts of N were applied in 2017 vs. 2018.

We were aining for a boyt a 30% reduction in yield in the experiment; in the first the yield redction although signficnat was only;, so the N stress was increased slightly in year 2 so that the relation between root triats and and the interaction with N availability would be exoamiend robustly.

- Line 193: "… from drought stress"

- Line 216: Please refer to a reference for the Dumas method.

Reference added Dumas, J.B.A., 1831. Procedes de l'analyse organique. Annales de Chimie et de Physique 2, 198–213.

- Lines 220-225: Please harmonize abbreviations used within equations with those defined below equations (refers to AGDM and AGN)
- Lines 227-229: Why have photosynthesis measures only be taken in HN plants?

Time taken to measure Amax (15 mins to calibrate in the context of the PhD student availability).

- Lines 254-259: Both, Fig. 1 and Tab. S3 do not underline the statements, that the landraces showed sign. differences in RSA compared to Paragon. Better: Mark sign. differences between each cultivar and Paragon in Tab. S3 and only refer to that table here.

Done

- Lines 268-269: "Lines with narrower width to depth ratio were negatively correlated with maximum depth." -> please refer to according correlation coefficients. From Table 2 it seems, that under both N conditions there was no sign. correlation between both traits.

We have omittedthis sentence from the revised version of the manuscript.

- Section 3.1.2: It may be interesting to mention which correlations are stable under both N conditions, e.g. MD with TSRL or LRN, respectively.

We have added text on

- Section 3.2.1: Please refer in the text also to physiological maturity and ears per plant, which are shown in Table 3.

We have referred to these two traits in the relevant sentence.

- Section 3.2.3 - Last sentence: Please refer to Fig. 4b instead of Fig. 5b.

Done.

- Section 3.2.3: Please include also NUE. An equation for calculating NUE is given in the M&Ms section, but no Figure or Table shows NUE.

- Section 3.2.3: No data for NutE are shown in that section. Please refer at least to Table S4. We have referred to Table S4.
- Section 3.3 - Second sentence: Please refer to Fig. 7a instead of Fig. 8a.

Done

- Section 4: Please refer precisely to figures and tables when discussing results.

We have adde specifi refence in paaremt to trleevat able sin the dscussions

Tables and Figures:

All Figs. and Tabs.: Please indicate if data have been produced in the hydroponic or the greenhouse experiment. Additionally, in a lot of figures and tables standard deviation is missing, which is important for data interpretation.

In tables x xnx SD has been added. In the egend of table SD has been added.

- Table 1: Please extend header of line 3 to "Genotypes in hydroponics"

Done

- Table S1: Please define concentration of salts as mg l-1

Done

- Figure 1: Please define "G" and "N" in the figure caption and give the number of replicates in each treatment group.

Done

- Table S3: Please define "G" and "N" in the figure caption and give the number of replicates in each treatment group.
- Table 2: Please check vertical oriented trait abbreviations. There, SRTA is only given as STA and TSRL is given as TSL.
- Table 3: Please remove "(PH)" from the table caption. In addition, how is the physiological maturity defined?
- Figs. 2 and 3: Please explain how grain yield (100% DM) is calculated.
- Figure 3: Harvest index appears here for the first time and has not been defined in the M&Ms section. In addition, please indicate in the caption that Paragon is colored in red.
- Figure 5: Please indicate what error bars represent in the caption and why they are of the same height in each and every treatment group. Might this be a mistake? In addition, please indicate statistical significant differences among genotypes.
- Figure 6: Please indicate in the caption that Paragon is colored in red.
- Figure 7: Why are no N efficiency parameters shown in the plots? Finding correlations between root system architectural traits and NUE would be most helpful for breeding programs. In addition, please show traits from the different growth systems in different colors.
- Figure 4a: This figure shows only N uptake, but not N uptake efficiency as indicated in the header of section 3.2.3.
- Table 3 might be better shown in the supplement.
- Please consider whether Figure 2, 3 and 4 may be better combined in one figure.

Orthography:

Line 43: Please use no abbreviation at the beginning of the sentence.

Line 49: "…aboveground crop N at harvest / available N from soil and fertilizer N)"
Line 102: "…population consisted of …"
Figs. 3 and 4 - Caption: "Values are mean of 2017 and 2018)."
Section 4.1 - First paragraph: "The present results support previous evidence that the root system size of landraces is larger than in modern cultivars"
Section 4.1 - Second paragraph: "…was higher than for the mean of five bread wheat landraces…"
Section 4.1 - Second paragraph: "…indicating flag-leaf chlorophyll content could be a useful proxy…"
Section 4.2 - First paragraph: "…indicative of shallower root angle. was also positively associated…"