

Phosphorus responses of *Trifolium pallescens* and *T. occidentale*, the progenitors of white clover (*T. repens*)

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ABSTRACT

Context. Rock phosphate reserves are decreasing, and requirements to mitigate environmental impacts of farming soils with excess phosphorus (P) are increasing. **Aims.** White clover is an allotetraploid hybrid between *Trifolium pallescens* and *T. occidentale*. Understanding the P response characteristics of these progenitor species will allow selection of germplasm for development of synthetic white clovers with improved phosphorus-use efficiency (PUE). **Methods.** Shoot and root weights and P nutrition characteristics were compared for three *Trifolium pallescens*, six *T. occidentale* accessions, and a white clover cultivar in a glasshouse experiment using a low P soil amended with five rates of P. **Key results.** White clover had the highest and most P responsive shoot and root dry weights (DW), and *T. pallescens* was least responsive to P. In the highest P treatment, *T. pallescens* had the highest shoot and root %P and the lowest shoot DW:total plant P ratio. There was significant variation among progenitor accessions. Spanish accessions of *T. occidentale* had comparable shoot DW to white clover and higher PUE. Traits of *T. pallescens* indicated strong adaptation to low P but low productivity, while traits of *T. occidentale* were more aligned with the white clover cultivar. **Conclusions.** The substantial variation in P nutrition characteristics within the progenitor species of white clover could be exploited in breeding programs. **Implications.** Comparing the P responses of a wider range of progenitor accessions could inform breeding to improve white clover's low soil P tolerance through development of synthetic white clovers.

Keywords: evolution, hybridisation, phosphorus acquisition, phosphorus utilisation, progenitors, synthetic white clover, *Trifolium occidentale*, *Trifolium pallescens*, white clover.

Introduction

White clover (*Trifolium repens* L.) is a recent (15 000–28 000 years before present) allotetraploid hybrid between *T. pallescens* (Schreb.) and *T. occidentale* (Coombe; [Ellison *et al.* 2006](#); [Williams *et al.* 2012](#)) and is found globally throughout temperate grasslands. The progenitor species are currently geographically isolated. *Trifolium occidentale* (western clover) has a maritime distribution, growing within 100 m of the coast on beaches, sand dunes and cliff tops in Brittany, Spain, Portugal, Cornwall and Ireland ([Coombe 1961](#); [Coombe and Morisset 1967](#); [Akeroyd 1983](#)). *Trifolium pallescens* (pale clover) is a European alpine species, found above 1800 m altitude ([Zohary and Heller 1984](#)), particularly on glacial forelands ([Raffl *et al.* 2008](#)). The two species maintain their compatibility and can be hybridised to create synthetic white clover ([Williams *et al.* 2012](#)). The progenitor genomes within white clover reside as independent subgenomes/homoeologues and their integrity has been largely maintained, enabling retention of a 'genetic toolbox' likely to underpin the adaptability of white clover to diverse environments ([Griffiths *et al.* 2019](#)). Whereas most genes in white clover showed consistent expression of one homoeologue relative to the other across tissues, the few genes that switched between homoeologues in different tissues were associated with flavonoid synthesis, indicating an adaptive role for these transcriptional changes ([Griffiths *et al.* 2019](#)).

White clover is an important component of mixed swards in temperate pastoral agriculture, but it has a higher requirement for soil phosphorus (P) than do its companion grasses

(Dunlop and Hart 1987) and some other forage legumes e.g. *Lotus pedunculatus* (Crush 1974). This is due in part to poor competitive ability against companion grasses for P, i.e. P-acquisition efficiency (Simpson *et al.* 2014), and partly because of the internal P physiology of the plant, i.e. P-utilisation efficiency (Dunlop and Hart 1987). The root competition is driven by grasses having finer, more branching roots, with longer root hairs (Jackman and Mouat 1972a, 1972b; Evans 1977) and more rapid root-turnover rates, so that the grass roots are constantly growing into fresh soil (Reid and Crush 2013; Reid *et al.* 2015). There is increasing concern in New Zealand about the environmental impacts of farming soils with elevated P status (Monaghan *et al.* 2007), and the external environmental footprint of importing phosphate rock. Interspecific hybridisation is being investigated as a strategy for improving the tolerance of white clover to low soil P. Hybrids with the wild relative *T. uniflorum* L. have been shown to have higher growth than does white clover at low P supply under glasshouse conditions (Nichols *et al.* 2014a, 2014b; Nichols and Crush 2015).

The P responses of the two progenitor species of white clover are unknown. Here, we report on the P response characteristics of some contemporary *T. occidentale* and *T. pallescens* populations compared with a commercial white clover cultivar. Understanding the origins of the P response characteristics of white clover should allow targeted hybridisation for improved P-use efficiency, utilising selected accessions of the progenitor species.

Methods

Plant material

Seeds of six accessions of *T. occidentale* and three accessions of *T. pallescens* were obtained from the Margot Forde

Germplasm Centre (MFGC), Palmerston North, New Zealand. *T. occidentale* accessions were from three geographical regions, with two accessions per region collected from beach, cliff top, or other littoral sites (Table 1). *T. pallescens* accessions were from three sites across the geographical range of the germplasm available in the MFGC for this research. The cultivar ‘Grasslands Kopu II’ was chosen as the white clover control because it has been used in other research on P responses of *Trifolium* germplasm and as a backcross parent for interspecific hybridisation (Nichols *et al.* 2014b; Crush *et al.* 2015; Nichols and Crush 2015). This is a large-leaved cultivar bred for dairying pastures. In related white clover cultivars released over a 57-year period, including Kopu II, internal phosphorus-use efficiency (PUE) was higher in the newer cultivars but there were no differences among the cultivars in the measured root traits. This suggested that positive selection pressure for PUE had occurred in conjunction with selecting for yield (Crush *et al.* 2015). Because Kopu II was the newest cultivar from that related sequence available at the time of this experiment, we would expect it provided a relatively high PUE control compared with older cultivars.

Experimental

Subsoil of an allophanic ash Horotiu silt loam (Hewitt 2010; USDA Classification: Typic Udivitrand) collected from a former dairy farm was screened through a 1 cm sieve, analysed for Olsen P concentration and plant macronutrients. The soil had an Olsen P concentration of 7 mg L⁻¹, and the soil pH was 6.4. It was amended with CaHPO₄ to provide five soil P treatments (P1–P5) with Olsen P concentrations of 7, 8, 9, 11 or 27 mg L⁻¹. The values from 7–11 mg L⁻¹ are common on east coast New Zealand hill country sheep and beef farms (e.g. Gillingham *et al.* 2007) and 27 mg L⁻¹ is within the target range for dairying on ash soils (Roberts and Morton 2016).

Table 1. Accession numbers in the Margot Forde Germplasm Centre, Palmerston North, New Zealand, and sites of origin for the clover lines used in the experiment.

Species	Accession	ID	Country	Habitat	Collection notes
<i>T. occidentale</i>	OCD1134	OCD 1	Ireland	Beach	Forlorn Point, Wexford. Short, rocky grassland; 3 m asl
	OCD1135	OCD 2	Ireland	Cliff top	Cliff Road, Ballyhire, Wexford
	OCD1138	OCD 3	France	Beach	Lechiagat, Brittany. Sand dune behind retaining wall; 6 m asl
	OCD1140	OCD 4	France	Cliff top	Pointe de Corsen, Brittany. <i>Festuca arundinacea</i> turf
	OCD1157	OCD 5	Spain	Beach	Faro de Punta Larino, Galicia. Low grassy area on sand dunes, 50 m from sea
	OCD1169	OCD 6	Spain	Cliff top	Muella do Caido, Galicia. Semi-consolidated sandy area, 100 m inland; 15 m asl
<i>T. pallescens</i>	AZ1895	PAD 1	–		Uncertain origin, probably European alpine ^A
	AZ4856	PAD 2	Austria	Alpine	Rotmoos Valley, Austrian Tyrol. 2400 m asl
	WA75a	PAD 3	Spain	Alpine	Parc Naturel Regional des Pyrenees. Pine forest on mountain slope; 2132 m asl
<i>T. repens</i>	C25624	RET			Cultivar Kopu II

asl, above sea level; ID, line identification used in figures and tables; OCD, *T. occidentale*; PAD, *T. pallescens*; RET, *T. repens*.

^AImported seeds not accompanied by information on location. DNA sequences (nuclear ITS and a chloroplast sequence), done by NW Ellison, AgResearch, were identical to *T. pallescens* collected from the European Alps (W. M. Williams, pers. comm.).

Details of the methodology have been described in Nichols *et al.* (2014b). The treated soils were transferred into 1.8 L pots with potassium sulfate (0.8 g) and magnesium sulfate (0.53 g) added to each pot to bring concentrations of these elements up to recommended levels for dairy farms on the soil type (Roberts and Morton 2016). The potassium and magnesium were applied in 100 mL pot⁻¹ of Long Ashton trace-element solution (Hewitt 1966) containing boron (B), chlorine (Cl), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn) to avoid any micronutrient limitations to plant growth. The experimental design was generated using GenStat, 16th edition (VSN International 2011). The pots were arranged in a split-plot design across 10 glasshouse tables (replicates), with five main plots (five phosphate treatments) per table, and 10 subplots in each main plot (one plant of each clover line). On each table, the pots were laid out in a 10 row by five column grid, with the columns constituting the main plots. The soil was taken through two cycles of wetting and drying to ensure adsorption of the added P before planting.

Seeds were scarified with sandpaper and germinated on damp filter paper in Petri dishes. Germinated seeds were then transplanted to the experimental pots when the radicles were 1 cm long and inoculated with a suspension of *Rhizobium leguminosarum* bv *trifolii* strain TA1 in 10% (w/v) aqueous sucrose solution.

The experiment ran from 1 April 2013 to 1 October 2013 in a temperature-controlled glasshouse with mean day/night temperatures of 19.4°C/12.9°C. Two 400 W day extension lights were used per replicate block, from 0600–0800 hours and 1600–1800 hours, to maintain a 12 h photoperiod and the plants were watered daily. Two weeks prior to the end of the experiment, the second fully expanded leaf from one stolon on each plant was collected, scanned with an Epson Expression 1680 flatbed scanner and analysed for leaf area. Leaves were then dried at 60°C for 24 h and weighed, and specific leaf area (cm² mg⁻¹) was calculated for each leaf. At harvest, soil was washed off the roots, then the roots, shoots and flowers were separated and dried for 24 h at 60°C before being weighed. Dried root and shoot samples were ground and then analysed for %P by RJ Hill Laboratories Ltd, Hamilton, New Zealand. Dry weight (g) and %P concentrations were used to calculate the root P content, shoot P content, and total P content (mg) for each plant ((DW × 1000) × (%P / 100)). P content data was used to calculate shoot DW per unit total plant P content ratio (shoot DW:total plant P) as an alternative measure of P efficiency, shoot P content per unit root DW, and total plant P content per unit of root DW as a measure of P uptake.

Data analysis

All variables were analysed using a linear mixed model fitted using residual maximum likelihood in GenStat 16th edition (VSN International 2011). The fixed model comprised clover

line (a factor with 10 levels), P treatment (five levels) and clover line by P treatment interaction. At harvest, root tubers of parasitic broomrape (*Orobancha minor*) were found attached to roots of some plants; so, an additional binary factor was included in the fixed model to adjust for their presence. The model accounting for the randomisation structure of the split-plot design comprised the following three random terms: tables, main plots within tables, and subplots within main plots. Residual diagnostic plots were inspected for evidence of departure from the assumptions of normality, independence and constant variance. All variables were natural log-transformed prior to analysis to stabilise the variance. Back-transformed predicted means and least significant ratios were calculated. A principal-component analysis on the correlation matrix was generated in DeltaGen (Jahufer and Luo 2018) by using the back-transformed means for all traits.

Results

There were significant effects of clover line and phosphate treatment level on all the vegetative traits (shoot and root dry weight (DW), root:shoot DW ratio, leaf area, specific leaf area) and all the phosphorus-related traits (shoot and root %P, shoot and root P content, root P:shoot P content ratio, total plant P, shoot DW:total plant P; Table 2). There were significant clover line × phosphate concentration interactions for all factors except specific leaf area (Table 2).

Table 2. Significance levels for the effects of phosphorus treatment, clover line and the clover line × phosphorus treatment interaction for the measured parameters.

Item	Phosphorus	Line	Line × phosphorus
Shoot DW	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Root DW	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> = 0.003
Root:shoot DW ratio	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Leaf area	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> = 0.049
Specific leaf area	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> = 0.282
Shoot %P	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Root %P	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Shoot P content	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Root P content	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> = 0.022
Root P:shoot P ratio	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Total plant P	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Shoot DW:total plant P	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Total plant P uptake:unit root DW	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
Shoot P content:unit root DW	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001

DW, dry weight.

Shoot and root dry weight

Differences in plant dry weight were most apparent in the highest (P5) phosphate treatment (Fig. 1a). In this treatment, white clover shoot DW was heavier than for all but the largest *T. occidentale* line, and all *T. pallescens* lines were lighter than the *T. occidentale* lines (Table 3). Among the six *T. occidentale* lines, shoot DW varied by a factor of two, with differences among the heavier and lighter lines. There were no consistent effects of source location on shoot DW in the P5 treatment for *T. occidentale*. Shoot DW of *T. pallescens* differed among all three lines in the P5 treatment (Table 3), with a nine-fold variation between the lightest and heaviest lines. PAD 2 had minimal response to additional P supply (Fig. 1a, Table 3).

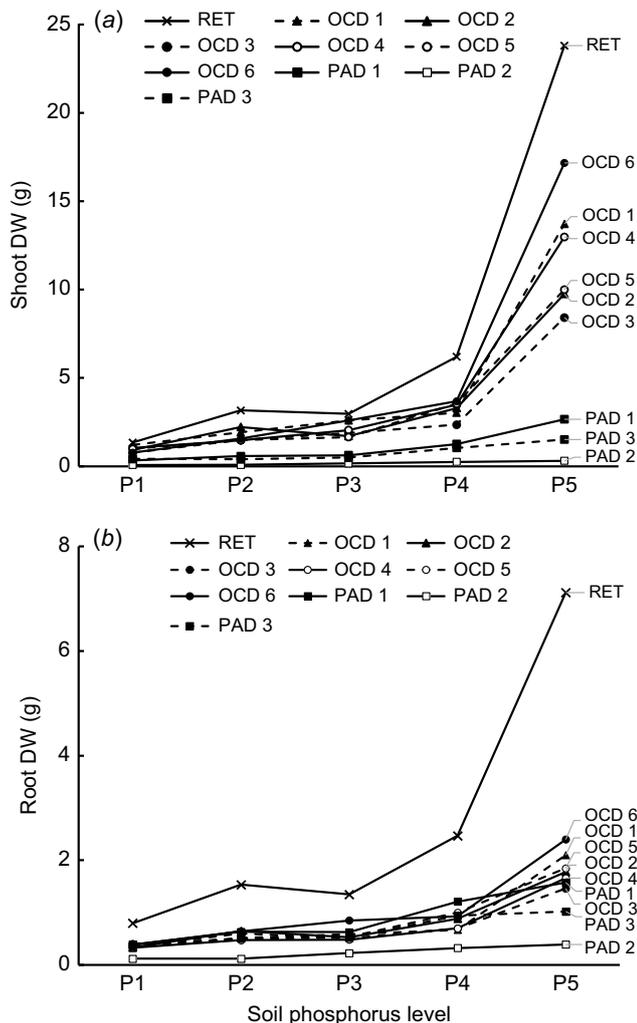


Fig. 1. Back-transformed mean (a) shoot dry weight (DW) and (b) root DW for white clover (RET), six *T. occidentale* (OCD), and three *T. pallescens* (PAD) lines grown at five soil phosphorus concentrations (equivalent to Olsen P 7, 8, 9, 11 and 27 mg L⁻¹). LSR_{0.05} shoot DW = 1.68; LSR_{0.05} root DW = 1.65; see Table 3 for pairwise comparisons between means at P5.

Root DW P-response patterns were essentially similar to those for shoot DW, with white clover having the heaviest and most P-responsive root system (Fig. 1b, Table 3), which was heavier than all the *T. occidentale* lines. *T. pallescens* had the lightest and least P-responsive root systems (Fig. 1b). Among the *T. pallescens* lines, roots of all three lines differed from each other in the P5 treatment, with PAD 2 being considerably lighter than the other two lines (Table 3). In the P5 treatment, the only difference in root DW among the *T. occidentale* lines was between the heaviest (OCD 6) and the lightest (OCD 3) lines (Table 3).

Root:shoot DW ratios decreased with increasing soil P concentrations for all three species (Fig. 2). In the P5 treatment, all the *T. pallescens* lines had higher root:shoot ratios than did white clover and *T. occidentale*, especially for PAD 2, with its particularly small root mass (Table 3). White clover also had a higher root:shoot DW ratio than all the *T. occidentale* lines in the P5 treatment (Table 3). There was a narrow range of root:shoot DW ratios (0.12–0.18) among the *T. occidentale* lines, with differences only between the lines with the highest (OCD 2 and 5) and lowest (OCD 4) values (Table 3).

Leaf area

Individual leaf area of the white clover cultivar was substantially greater than that of lines of the two progenitor species (Fig. 3a, Table 3), and leaf area increased in all the populations as soil P increased. In the P5 treatment, the Spanish *T. occidentale* lines OCD 5 and OCD 6 had larger leaf areas than did the other *T. occidentale* lines (Table 3). Among the *T. pallescens* lines, line PAD 2 had smaller leaves than did the other two lines (Fig. 3a, Table 3). Specific leaf area (cm² mg⁻¹) of five of the six *T. occidentale* lines was lower than Kopu II and *T. pallescens*, which did not differ from each other (Fig. 3b, Table 3).

Phosphorus in roots and shoots

In the P5 treatment, *T. pallescens* had higher shoot P concentrations than did *T. occidentale* and white clover (Table 4) and PAD 2 had higher shoot %P than did PAD 1. Shoot %P in both PAD 1 and PAD 3 increased strongly from the P4 treatment to the P5 treatment, unlike for PAD 2 (Fig. 4a). Among the *T. occidentale* lines, OCD 1 and 2 (from Ireland) and OCD 3 and 4 (France) did not differ for shoot %P, but their concentrations were higher than for OCD 5 and OCD 6 (Spain) (Table 4, Fig. 4a). Shoot %P of OCD 5 and OCD 6 did not differ from white clover, which was, in turn, lower than the other *T. occidentale* lines and *T. pallescens*. There was a similar pattern of results for root P concentrations, with higher values for *T. pallescens* than for *T. occidentale*, and lower values for white clover than for all the *T. occidentale* lines (Table 4, Fig. 4b). The response patterns for root P concentration in the *T. pallescens* lines were very similar to those observed for

Table 3. Back-transformed mean values for shoot and root dry weight (DW) (g), root:shoot DW ratio, leaf area (cm²) and specific leaf area (SLA, cm² mg⁻¹) for the experimental lines in the P5 treatment (Olsen P = 27).

Species	ID	Shoot DW (g)	Root DW (g)	Root:shoot ratio	Leaf area (cm ²)	SLA (cm ² mg ⁻¹)
<i>T. occidentale</i>	OCD 1	13.7ef	2.10cd	0.15abc	1.14bc	0.19a
	OCD 2	9.8de	1.78cd	0.18c	1.20bc	0.17a
	OCD 3	8.4d	1.46bc	0.17bc	1.31bc	0.20ab
	OCD 4	13.0def	1.66cd	0.12a	1.04b	0.18a
	OCD 5	10.0de	1.85cd	0.18c	1.79e	0.17a
	OCD 6	17.2fg	2.40d	0.14ab	1.69de	0.19a
<i>T. pallescens</i>	PAD 1	2.7c	1.58c	0.59e	1.12bc	0.25b
	PAD 2	0.3a	0.39a	1.24f	0.73a	0.25b
	PAD 3	1.5b	1.02b	0.67e	1.38cd	0.25b
<i>T. repens</i>	RET	23.8g	7.12e	0.30d	4.81f	0.24b
LSR _{0.05}		1.68	1.65	1.30	1.31	1.27

Within columns, populations not sharing a common letter are different on the basis of the least significant ratio (LSR) at *P* = 0.05.

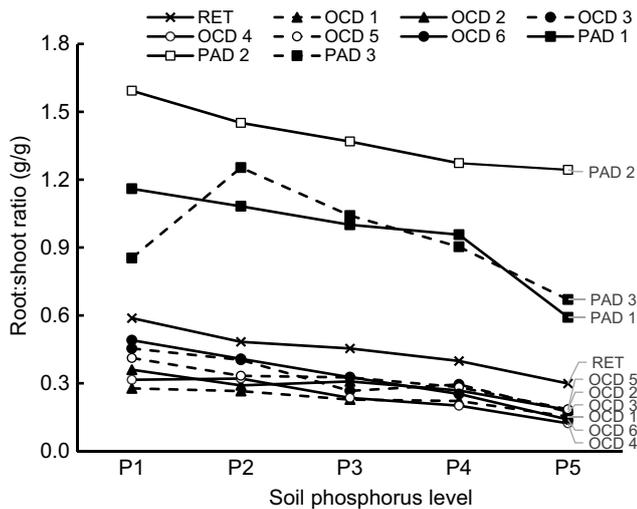


Fig. 2. Back-transformed mean root:shoot dry weight ratios for white clover (RET), six *T. occidentale* (OCD), and three *T. pallescens* (PAD) lines grown at five soil phosphorus concentrations (equivalent to Olsen P 7, 8, 9, 11 and 27 mg L⁻¹). LSR_{0.05} = 1.30; see Table 3 for pairwise comparisons between means at P5.

shoot %P. There was little variation in root %P among the *T. occidentale* lines, in contrast to shoot %P results.

Shoot P content (mg P) was generally lower in *T. pallescens* than in *T. occidentale* and white clover (Table 4). The exception was PAD 1, which had a shoot P content similar to that of OCD 3 and OCD 5, and a much higher shoot P content than that of the other two PAD lines. There was no evidence of a difference in the shoot P contents of white clover and OCD Lines 1, 4, and 6. The root P content of white clover was substantially higher than for all the other lines (Table 4). PAD 2 had a lower root P content than did four of the six OCD lines, and that of PAD 1 was higher than that of four of the OCD lines.

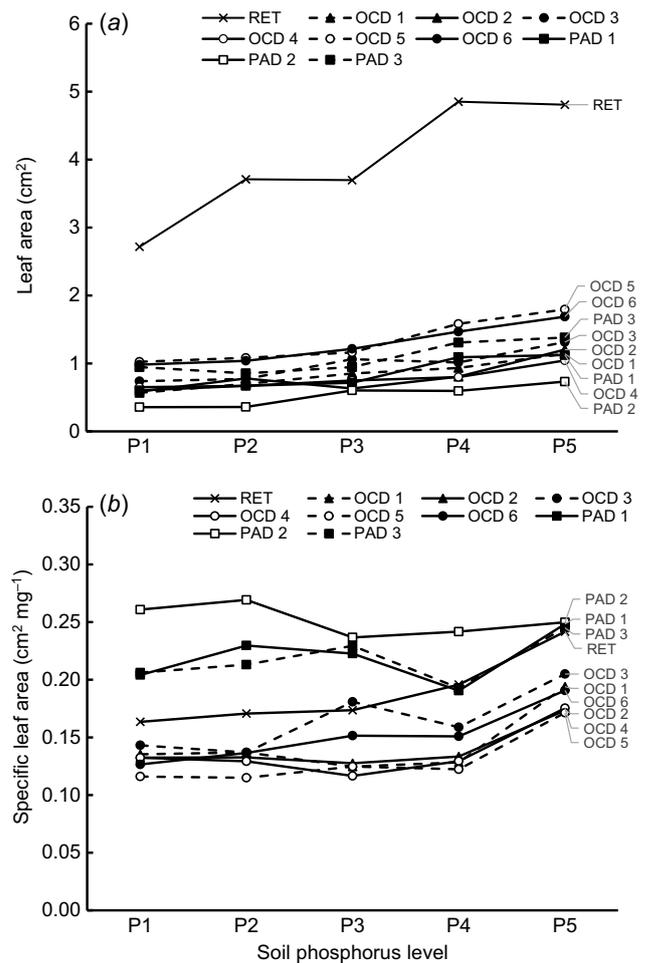


Fig. 3. Back-transformed mean (a) leaf area and (b) leaf-specific area for white clover (RET), six *T. occidentale* (OCD), and three *T. pallescens* (PAD) lines grown at five soil phosphorus concentrations (equivalent to Olsen P 7, 8, 9, 11 and 27 mg L⁻¹). LSR_{0.05} leaf area = 1.31; LSR_{0.05} SLA = 1.27; see Table 3 for pairwise comparisons between means at P5.

Table 4. Back-transformed mean values for shoot and root %P, shoot and root P content (mg), root P:shoot P content ratio (RP:SP), total plant P (mg), shoot DW:total plant P (SDW:PP), total plant P uptake per unit root DW (TP:RDW), and shoot P content per unit root DW (SP:RDW), for clover lines in the P5 treatment (Olsen P = 27); and the percentage change in TP:RDW between P4 and P5.

Species	ID	Shoot P		Root P		RP:SP	Plant P (mg)	SDW:PP (g DW mg ⁻¹ P)	TP:RDW (mg g ⁻¹)	SP:RDW (mg g ⁻¹)	Change TP:RDW (%)
		(%)	(mg)	(%)	(mg)						
<i>T. occidentale</i>	OCD 1	0.10b	14.14g	0.13c	2.79cd	0.20ab	16.97ef	0.808c	8.1d	6.7de	81
	OCD 2	0.09b	9.18def	0.13c	2.31bc	0.25c	11.52cd	0.847c	6.5c	5.1cd	63
	OCD 3	0.09b	7.46cde	0.13bc	1.84ab	0.25bc	9.33c	0.903c	6.4c	5.1c	96
	OCD 4	0.09b	11.54fg	0.14c	2.28bc	0.19a	14.33def	0.937c	8.7d	7.2e	68
	OCD 5	0.06a	6.32cd	0.11b	2.06abc	0.33d	8.45c	1.184d	4.6b	3.4b	52
	OCD 6	0.06a	10.72efg	0.12bc	2.93cd	0.27cd	13.72de	1.251d	5.7c	4.4c	71
<i>T. pallescens</i>	PAD 1	0.19c	5.10c	0.23d	3.55d	0.70f	8.89c	0.299b	5.7c	3.2b	116
	PAD 2	0.24d	0.75a	0.36e	1.39a	1.83g	2.20a	0.141a	5.7bc	1.9a	3
	PAD 3	0.21cd	3.21b	0.24d	2.47bcd	0.77f	5.74b	0.264b	5.7c	3.1b	132
<i>T. repens</i>	RET	0.06a	14.03g	0.09a	6.24e	0.45e	20.36f	1.169d	2.9a	2.0a	28
LSR _{0.05}		1.24	1.61	1.20	1.56	1.32	1.57	1.24	1.30	1.43	

Within columns, populations not sharing a common letter are different on the basis of the least significant ratio (LSR) at $P = 0.05$. DW, dry weight.

Ratios of root P content:shoot P content generally decreased as soil P concentration increased (Fig. 5a). They were highest in *T. pallescens*, particularly in PAD 2 (Fig. 5a, Table 4), which had a higher ratio than the other two *T. pallescens* lines, and higher in white clover than in all the *T. occidentale* lines. The ratio also varied among the *T. occidentale* lines (Fig. 5a, Table 4), with the French line collected from the beach (OCD 1) having a lower ratio than the line collected from the cliff-top site (OCD 2); however, this pattern was reversed in the Irish lines (OCD 3 and 4, Table 4). The two Spanish lines (OCD 5 and 6) did not differ from each other, and OCD 5 from the beach site had a higher root P:shoot P ratio than for all the Irish and French lines, whereas OCD 6 had a higher ratio than did OCD 1 and 4.

The ratio of shoot DW per unit total plant P content was higher in *T. occidentale* and white clover than in *T. pallescens* (Fig. 5b, Table 4). It was also higher in white clover and the two Spanish accessions of *T. occidentale* than in the French and Irish accessions of *T. occidentale*. The *T. occidentale* accessions further segregated into types where this ratio either increased (the Spanish lines, OCD 5 and 6) or decreased (OCD 1, 2, 3, and 4) in the P5 treatment (Fig. 5b). Shoot DW:total plant P also increased between the P4 to P5 treatments in white clover (Fig. 5b), but decreased in PAD 1 and 3. For PAD 2, the soil P treatment level had very little effect.

Individual lines had similar responses to increasing soil P for both P uptake rate (Fig. 6a) and shoot P content:unit root DW (Fig. 6b), and relative differences among lines were similar for both of these traits. The exception was PAD 2, where P uptake rate was considerably higher than shoot P content:unit root DW, reflecting the higher proportion of P retained in the roots.

P uptake rates per unit root DW generally increased with an increasing soil P, although for most lines these changes were

small across P1 to P4, with the largest responses occurring at P5 (Fig. 6). The exception again was PAD 2, which was varied little across all P concentrations. Between P4 and P5, the increase in uptake rates per unit of root dry weight was generally as follows: PAD 1 and PAD 3 > OCD > Kopu II > PAD 2 (Table 4).

At P5, the total P uptake rate per unit root DW was lower in Kopu II than in the other lines (Table 4). Among *T. occidentale* lines, OCD 5 had the lowest total P uptake rate and OCD 1 and OCD 4 had the highest. There was no evidence of a difference in total P uptake rate among the remaining *T. occidentale* or *T. pallescens* lines. Although the total P uptake rate of PAD 2 was similar to most other OCD and PAD lines, its shoot P content per unit root DW was lower, as was that of Kopu II. PAD 1 and PAD 3 also had lower shoot P contents per unit root DW than did the OCD lines, except for OCD 5. Differences among the OCD lines were similar to those for total P uptake rate, being lowest in OCD 5 and generally highest in OCD 1 and 4.

Principal-component analysis

Principal-component analysis on the means of all traits at P5 showed clear separation among the three species (Fig. 7), with overall trait combinations reflecting the relative differences among lines for individual traits (Tables 3, 4). *T. pallescens* exhibited low DWs, low shoot DW:total plant P, high %P concentrations, high partitioning of DW and P to roots, and low leaf size, but high specific leaf area. Kopu II had high DWs and shoot DW:total plant P, low %P concentrations, low P uptake rates, and higher partitioning to roots compared with *T. occidentale*. *T. occidentale* had high P uptake rates, low partitioning of DW and P to roots, and above-average

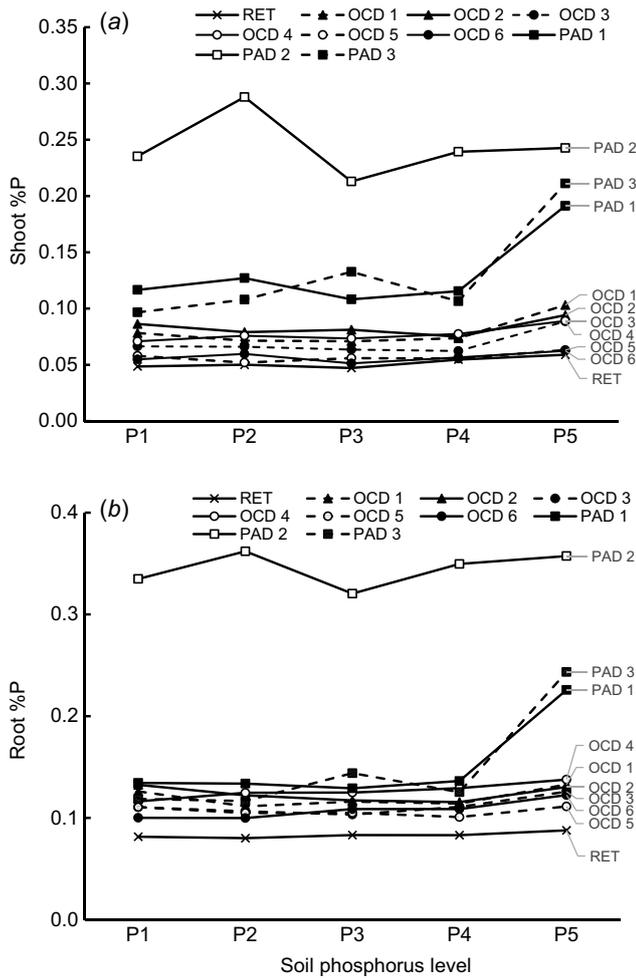


Fig. 4. Back-transformed mean (a) shoot %P and (b) root %P for white clover (RET), six *T. occidentale* (OCD), and three *T. pallescens* (PAD) lines grown at five soil phosphorus concentrations (equivalent to Olsen P 7, 8, 9, 11 and 27 mg L⁻¹). LSR_{0.05} shoot %P = 1.24; LSR_{0.05} root %P = 1.20; see Table 4 for pairwise comparisons between means at P5.

shoot DW and shoot DW:total plant P. Within *T. occidentale* and *T. pallescens*, there were some differences in the alignment of individual lines to particular traits (Fig. 7), and when leaf area and SLA were removed from the PCA, these lines formed more clusters (data not shown).

At lower P treatment levels, the position of the vectors and the plant lines relative to the vectors were very similar to the P5 treatment, including separation of the species and overall trait combinations, but there was more separation among lines within the progenitor species (Fig. 8). This reflects the differences for individual traits shown in Tables 3, 4. For example, at P4 the *T. occidentale* lines separated into two clusters, one containing OCD 1, 2 and 4, and one containing OCD 3, 5 and 6 (Fig. 8). Among *T. pallescens* lines, PAD 2 formed a separate cluster from PAD 1 and 3.

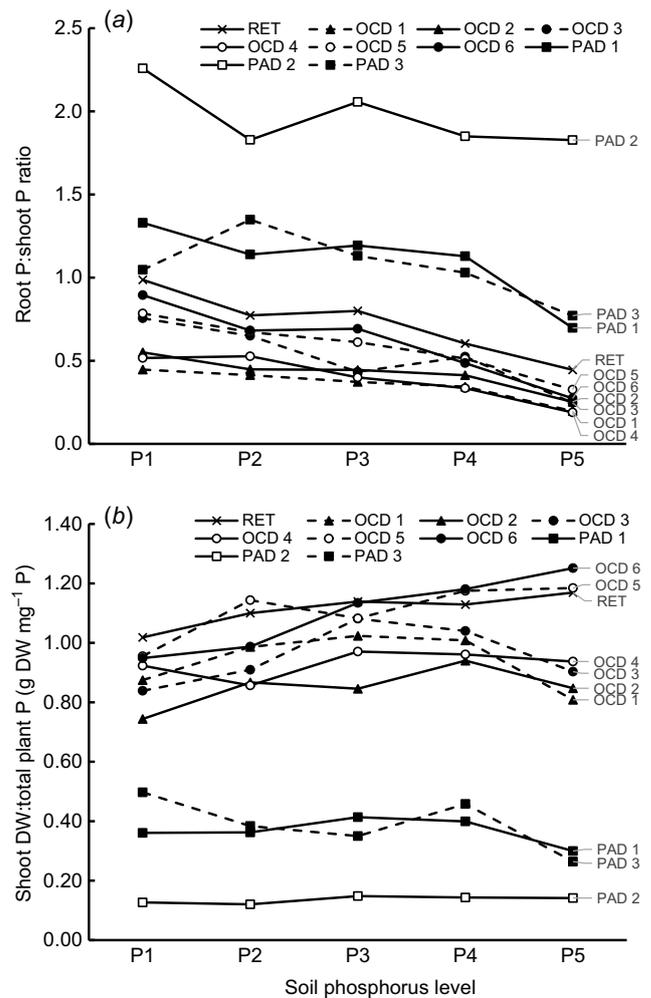


Fig. 5. Back-transformed mean (a) root P content:shoot P content ratios and (b) shoot dry weight per unit total plant P content for white clover (RET), six *T. occidentale* (OCD), and three *T. pallescens* (PAD) lines grown at five soil phosphorus concentrations (equivalent to Olsen P 7, 8, 9, 11 and 27 mg L⁻¹). LSR_{0.05} root P content:shoot P content = 1.32; LSR_{0.05} shoot DW unit total plant P = 1.24; see Table 4 for pairwise comparisons between means at P5.

Discussion

Individual traits, and combinations of traits, showed distinctive differences within and among species, which likely reflect edaphic adaptation. This included greater separation among lines within progenitor species in lower soil P treatments. Knowledge of these differences will contribute to improved understanding of the influence of the *T. occidentale* and *T. pallescens* progenitors on the soil P requirements of white clover. The variation within the progenitor species for P nutrition-related traits indicates that there is considerable potential for future development of synthetic white clover with an increased PUE.

Root:shoot DW ratios were much higher in *T. pallescens* than in white clover, and were lowest in *T. occidentale*. This suggests

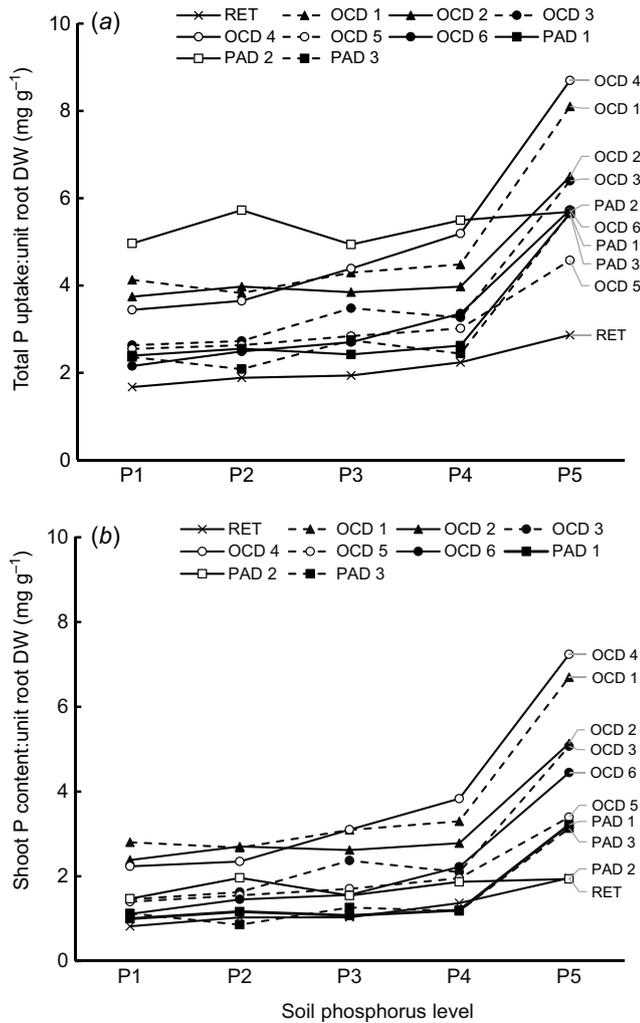


Fig. 6. Back-transformed mean (a) total P uptake per unit root dry weight and (b) shoot P content per unit root dry weight for white clover (RET), six *T. occidentale* (OCD) and three *T. pallescens* (PAD) lines grown at five soil-phosphorus concentrations (equivalent to Olsen P 7, 8, 9, 11 and 27 mg L⁻¹). LSR_{0.05} total P uptake per unit root dry weight = 1.30; LSR_{0.05} shoot P content per unit root dry weight = 1.43; see Table 4 for pairwise comparisons between means at P5.

that *T. pallescens* is adapted to survive on young, low available-P glacial outwash soils through high investment in root growth. Available-P concentrations in these alpine soils are not often recorded, but one Austrian site with *T. pallescens*, near the source of PAD 2, had 0.06 µg P g⁻¹ soil (acetate-lactate extraction; Tschirko et al. 2005). This is grossly P-deficient soil by agricultural standards, equivalent to an Olsen test value of <1 mg L⁻¹ compared with recommended phosphate test values of 20–30 for New Zealand sheep/beef farms on sedimentary soils (Morton and Roberts 2018). Another wild relative of white clover, *Trifolium uniflorum* L., has also been shown to have higher root:shoot DW ratios than white clover in low-P experimental soils (Nichols et al. 2014a, 2014b; Nichols and Crush 2015), but with values usually <0.8,

which contrasts with values in the current study of up to 1.24 in PAD 2. Root:shoot DW ratios generally declined in all three species as soil P concentration increased and this is the normal response pattern (Lynch and Brown 2008; Nichols et al. 2014b). However, in the P5 treatment the root:shoot DW ratios of the *T. pallescens* lines were still clearly greater than those for white clover or *T. occidentale*. There were also significant differences in root:shoot ratio within *T. pallescens*, with particularly high values for PAD 2. Seeds of this line were collected from an alpine site in the Austrian Tyrol (Table 1). The young alpine soils that are the typical habitat for *T. pallescens* on the glacier forelands are characterised by extremely localised variation in edaphic conditions (Burga et al. 2010) where flexible root:shoot ratios would be an adaptive advantage. There was little variation within the quite tightly clustered root:shoot ratios for *T. occidentale*, with all values being lower than in white clover but much closer to white clover than *T. pallescens*. The shallow soils of *T. occidentale*'s natural habitat are prone to rapid drying and it flowers much earlier than does white clover growing at the same location (Coombe 1961). This suggests that *T. occidentale* uses early flowering to mitigate soil-moisture deficits rather than development of large root systems, which would not necessarily be effective on shallow, drying soils. In the current study, the patterns of allocation of resources to roots or shoots under P deprivation had similar trajectories in all three species, but *T. pallescens* had much higher root:shoot ratios, suggesting it may have a stronger adaptation to low-P soils.

Shoot and root P concentrations were also much higher in *T. pallescens* than in white clover and *T. occidentale*. This may have resulted in part from growth dilution of tissue P in the faster-growing species, but also suggests lower internal P-use efficiency in *T. pallescens*, which was supported by lower values for shoot DW:total plant P. As it is an alpine species, it is possible that *T. pallescens* produces antifreeze proteins (AFP) as many species do (Gupta and Deswal 2014). If this is the case, the AFP would be responsible for some of the elevated shoot and root P concentrations, and lower shoot DW:total plant P.

The low shoot P concentrations in the Spanish accessions of *T. occidentale* (OCD 5 and OCD 6) also suggested that they had a higher shoot-level PUE than did the French and Irish accessions and similar efficiency to white clover. For the white clover cultivar, both shoot (0.047–0.059%) and root (0.08–0.088%) P concentrations were low compared with the concentrations previously reported from pot experiments, such as, for example, shoots 0.19–0.32 %P, roots 0.24–0.34 % P (Crush et al. 2015). This reflects the low soil P treatment levels that were chosen to test whether the progenitor species were adapted to infertile, non-agricultural soils, and which were representative of on-farm Olsen P concentrations in farm systems where improved PUE would be particularly valuable.

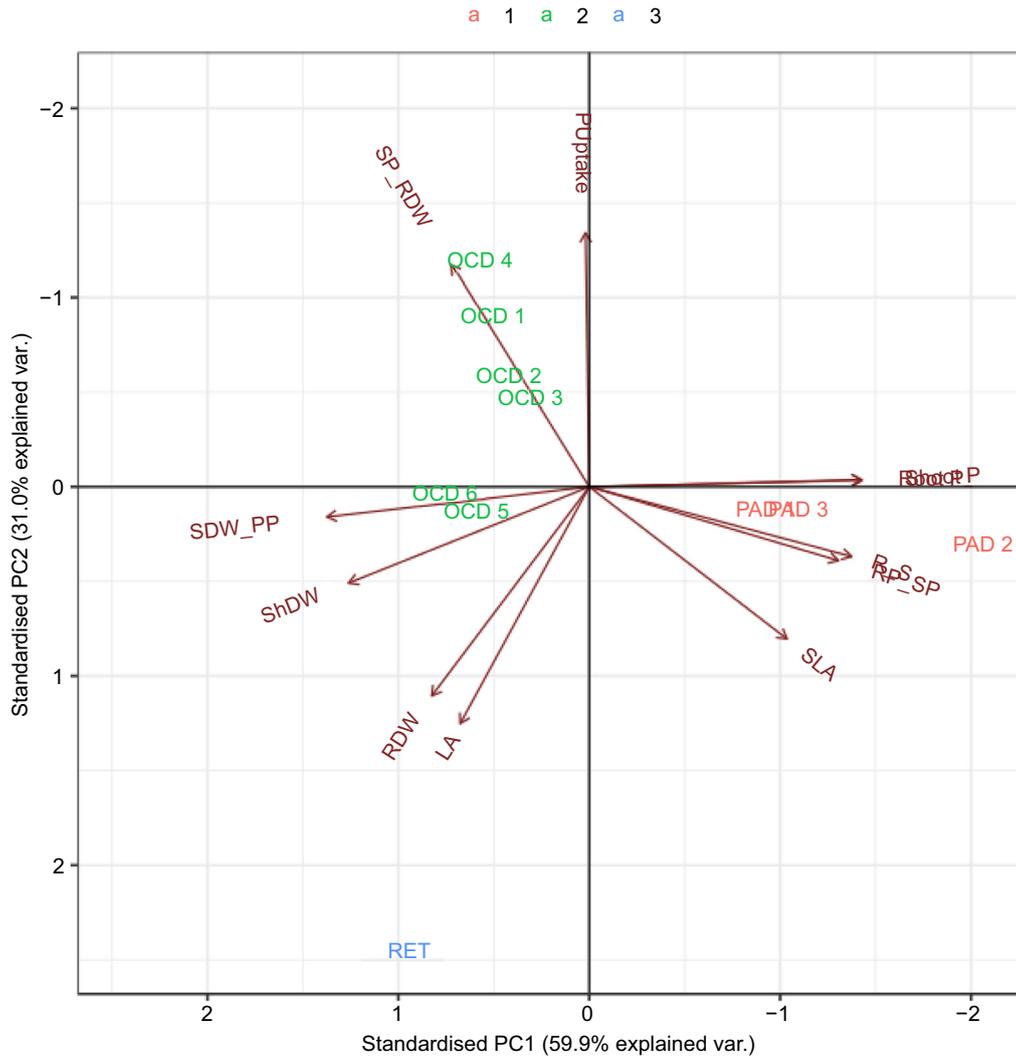


Fig. 7. Principal-component analysis of shoot dry weight (ShDW), root DW (RDW), shoot DW per unit plant P (SDW_PP), leaf area (LA), specific leaf area (SLA), shoot %P (Shoot_P), root %P (Root_P), root:shoot ratio (R_S), root P:shoot P content ratio (RP_SP), total plant P uptake per unit root DW (PUptake) and shoot P content per unit root DW (SP_RDW) at P5 (Olsen P = 27). RET, *Trifolium repens*; OCD, *T. occidentale*; PAD, *T. pallescens*.

White clover populations tolerant of low-P soils have higher shoot P concentrations than do those from high-P soils (Snaydon and Bradshaw 1962). This suggests that gene expression from *T. pallescens* may be contributing more than that from *T. occidentale* in low soil P-adapted ecotypes of white clover. In its natural environment *T. pallescens* has a thick taproot, with only a few thin lateral roots (Kuen and Erschbamer 2002). This is probably an adaptation to surviving frost-heave but would not be very effective for P capture, which is optimised with long, fine, branching roots. No information was located on root characteristics of *T. occidentale* growing in its natural habitat; however, observations from potted plants (Williams *et al.* 2011) have suggested it may have thinner roots than does *T. pallescens*. However, the current results showed that P uptake rates per unit of root DW were

higher in *T. occidentale* and *T. pallescens* than in Kopu II. The low values for Kopu II may be a result of the large root system in this cultivar bred for high yield in fertile soils. There is ecotypic variation in white clover root systems related to soil P concentrations (Caradus and Snaydon 1988). Types adapted to low-P soils have higher proportions of fine roots, which would improve the efficiency of P acquisition. A comparison of root morphology and architecture in multiple populations of both progenitor species and a range of white clover ecotypes and cultivars would help our understanding of the relative contribution of the progenitor species to P-acquisition characteristics of white clover root systems. Because there is substantial genotype-level variation in root-trait morphology in white clover (Jahufer *et al.* 2008), comparing roots of progenitor genotypes with those of

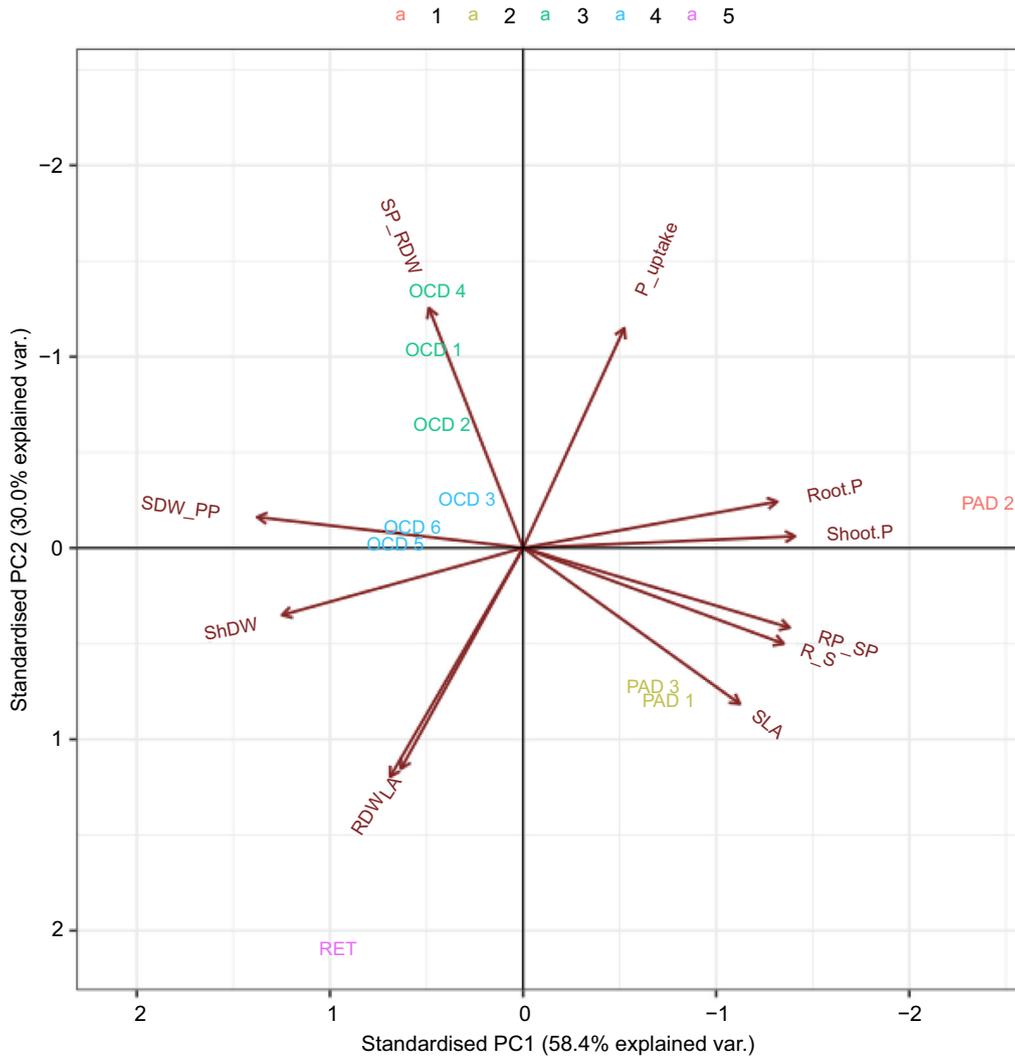


Fig. 8. Principal-component analysis of shoot dry weight (ShDW), root DW (RDW), shoot DW per unit plant P (SDW_PP), leaf area (LA), specific leaf area (SLA), shoot %P (Shoot_P), root %P (Root_P), root:shoot ratio (R_S), root:P:shoot P content ratio (RP_SP), total plant P uptake per unit root DW (PUptake) and shoot P content per unit root DW (SP_RDW) at P4 (Olsen P = 11). RET, *Trifolium repens*; OCD, *T. occidentale*; PAD, *T. pallescens*.

synthetic white clover derived from them would be an alternative research strategy.

Root P content:shoot P content ratios were higher in *T. pallescens* than in the other species, which suggests that *T. pallescens* may store P in the roots as an adaptation to low soil P. Similar adaptations have been reported in white clover ecotypes from New Zealand hill country pastures where soil P is often low (Caradus 1986), and in a small-leaved white clover cultivar bred from New Zealand hill country ecotypes (Chapman and Hay 1993), as well as in *T. uniflorum*, which also shows adaptation to low-P soils (Nichols and Crush 2015). In pastures, storage of P below grazing height enables internal P reserves to be conserved and later mobilised for shoot growth. The presence of this trait in *T. pallescens* may provide a source of genetics for improving performance of

white clover in P-deficient environments. *T. occidentale* stored proportionally less P in its roots than did *T. pallescens* and white clover, but the higher root P:shoot P in Spanish *T. occidentale* accessions than in the other lines shows that there is some variation for this trait in this species.

Shoot DW per unit plant P can indicate the potential P fertiliser costs of growing a plant for forage. Values were low in *T. pallescens*, reflecting its slow growth and high internal P concentrations compared to other lines. The highest values were in the white clover cultivar and the Spanish *T. occidentale* Lines OCD 5 and OCD 6, all of which had low shoot %P concentrations compared to other lines, and moderate to high growth rates. This Spanish germplasm could be a useful resource for hybridising with white clover to investigate P-use efficiency. Values for shoot DW:total

plant P of the other four *T. occidentale* populations were tightly clustered, with maximum values in the P3 soil treatment and lower values in the P5 treatment. The reasons for this pattern are not immediately evident but may indicate optimal soil P concentrations for P-use efficiency. The white clover result was expected because clover breeding programs have resulted in increased shoot dry weight per unit P absorbed, i.e. an increase in internal P-use efficiency (Crush *et al.* 2015). Snaydon and Bradshaw (1962) also reported lower shoot P concentrations (i.e. higher P efficiency) in white clover adapted to high-P soils. This may offset the lower P uptake rates per unit root DW for Kopu II than for most *T. occidentale* and *T. pallescens* lines. Results indicated that *T. occidentale* and *T. pallescens* had higher uptake of P per unit root DW, but used P less efficiently than did Kopu II, except for OCD 5 and OCD 6, which had a similar efficiency. In *T. pallescens*, this may be driven by the high proportion of P stored in roots, as well as possible sequestration in shoot tissues (e.g. anti-freeze proteins). In contrast, the *T. occidentale* lines had lower storage of P in roots and higher shoot P content:root DW compared with *T. pallescens* and Kopu II. Given their differences in P efficiency, this indicated that the French and Irish accessions of *T. occidentale* may be sequestering P in shoot tissues (e.g. storage as inorganic P in vacuoles), which prevents the conversion of higher P uptake into growth. Among *T. occidentale* lines, P uptake results also indicated that the Spanish accessions were taking up similar or lower amounts of P per unit root DW, but using it more efficiently than did the other lines, possibly reflecting the absence of shoot sequestration of P. Generally, OCD 2 and 3 also exhibited similar responses when compared with OCD 1 and 4, taking up less P per unit root DW, but using it equally efficiently.

Shoot and root weights and leaf size of the white clover cultivar Kopu II were larger than those of both progenitor species. This was expected because of selection for large plant and leaf size during development of Kopu II as a dairy pasture type cultivar (Woodfield *et al.* 2001), and the tetraploid nature of white clover compared with the diploid progenitors. Leaf area was similar in both wild species, and this smaller leaf size is also a common feature in white clover ecotypes adapted to infertile conditions (Caradus 1994). The Spanish accessions of *T. occidentale* (OCD 5 and OCD 6) had larger leaves than did the Irish and French accessions.

Specific leaf area (SLA, $\text{cm}^2 \text{mg}^{-1}$) was identical in Kopu II white clover and *T. pallescens*. Species with lower growth rates, such as the *T. pallescens* in this experiment, generally have a lower SLA than do faster-growing species such as white clover (Poorter *et al.* 2009), and species from highly productive habitats usually have a higher SLA than do those from sites of low productivity (Poorter and De Jong 1999). It is not at all clear why SLA was very similar in the *T. pallescens* and white clover, but there is considerable variation reported in the relationships among SLA, relative growth rate, and the

productivity of plants' natural habitat (Poorter and De Jong 1999; Poorter *et al.* 2009).

Compared with Kopu II and *T. pallescens*, SLA was much lower in *T. occidentale*, probably owing to the additional layers of leaf palisade mesophyll cells that characterise the species (Coombe 1961). Low SLA is also characteristic of many species from arid areas (Garnier *et al.* 2019). Coombe (1961) reported that leaves of *T. occidentale* were thicker than those of white clover, suggesting an adaptation to moisture-stress conditions experienced in sandy littoral soils. In contrast, Kopu II and *T. pallescens*, both with thinner leaves, would be better adapted to the moister conditions of temperate New Zealand pastures and the European Alps. The lower altitudinal limit for *T. pallescens* is determined by increased seedling mortality caused by drying of soils during the growing season (Hillgardt 1993), which is consistent with a requirement for moister conditions.

White clover has largely retained progenitor genome integrity (Griffiths *et al.* 2019) and the contribution from both parents can be seen in its vegetative characteristics. For example, SLA in the current study was similar in *T. pallescens* and white clover, and lower in *T. occidentale*, which has additional mesophyll palisade layers. Parameters related to P nutrition (e.g. shoot and root %P, shoot DW:total plant P and root P:shoot P ratio) were generally more closely aligned in the white clover cultivar and *T. occidentale* and different in *T. pallescens*, suggesting that the relevant genes from *T. occidentale* were expressed in the white clover, whereas those from *T. pallescens* were not. This may be an artefact from the use of Kopu II as the white clover control because this cultivar was bred for intensive lowland dairy pastures (Caradus and Woodfield 1997) that characteristically have high soil fertility. In contrast, small-leaved white clover ecotypes grown under low soil P conditions (Caradus 1986) show many of the P-response characteristics of *T. pallescens*, which in this experiment had many traits indicative of adaptation to low soil P (high %P, low shoot DW:total plant P, high root:shoot and high root P:shoot P), including low utilisation of acquired P. Although P uptake rates per unit of root DW of the *T. pallescens* lines were similar or lower than those of the *T. occidentale* lines at P5, those for PAD 2 were much higher at lower-P treatments more representative of low on-farm Olsen P concentrations. This may indicate a strategy of high acquisition of P under limiting soil P conditions. Although the uptake rates of this line did not respond greatly to increasing soil P overall, this may be a further indication of adaptation to low soil P. Similar rates of P uptake among the *T. pallescens* lines at P5, combined with lower shoot P content per unit root DW for PAD 2, reflect the higher proportion of total plant P sequestered to roots by this line.

White clover arose from multiple hybridisation events (Griffiths *et al.* 2019) and the ecotypes of the ancestral progenitors are unknown. There was population-level variation for P traits, and combinations of traits, in both progenitor species (e.g. Spanish accessions of *T. occidentale* and PAD 2)

and this has also been observed in white clover (Snaydon and Bradshaw 1962; Caradus and Snaydon 1986). Faville et al. (2020) found wide genomic diversity in a screen of global ecotype collections of white clover, of which New Zealand accessions represented only a small portion of diversity. Comparing the P-response traits of a range of white clover ecotypes with those of a wider range of progenitor populations would be informative and could support breeding targeted at the progenitor subgenomes of white clover for improvement of low soil P tolerance. This could be implemented either via interspecific hybridisation with existing cultivars or creation of new synthetic white clover populations. In addition to overall differences in trait combinations among species, principal-component analysis showed that separation within progenitor species differed between higher and lower P treatments, indicating the importance of screening across a range of relevant on-farm plant-available soil P concentrations.

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