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## Rooting depth and root depth distribution of *Trifolium repens* × *T. uniflorum* interspecific hybrids

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- **Background and aims** Traits related to root depth distribution were examined in *Trifolium repens* × *T. uniflorum* backcross 1 (BC<sub>1</sub>) hybrids to determine whether root characteristics of white clover could be improved by interspecific hybridization.
- **Methods** Two white clover cultivars, two *T. uniflorum* accessions and two BC<sub>1</sub> populations were grown in 1-m deep tubes of sand culture. Maximum rooting depth and root mass distribution were measured at four harvests over time, and root distribution data were fitted with a regression model to provide measures of root system shape. Morphological traits were measured at two depths at harvest 3.
- **Key Results** Root system shape of the hybrids was more similar to *T. uniflorum* than to white clover. The hybrids and *T. uniflorum* had a higher rate of decrease in root mass with depth than white clover, which would result in higher proportions of root mass in the upper profile. Percentage total root mass at 100–200 mm depth was higher for *T. uniflorum* than white clover, and for Crusader BC<sub>1</sub> than ‘Crusader’. Roots of the hybrids and *T. uniflorum* also penetrated deeper than those of white clover. *T. uniflorum* had thicker roots at 50–100 mm deep than the other entries, and more of its fine root mass at 400–500 mm. The hybrids and white clover had more of their fine root mass higher in the profile. Consequently, *T. uniflorum* had a higher root length density at 400–500 mm than most entries, and a smaller decrease in root length density with depth.
- **Conclusions** These results demonstrate that rooting characteristics of white clover can be altered by hybridization with *T. uniflorum*, potentially improving water and nutrient acquisition and drought resistance. Root traits of *T. uniflorum* are likely to be adaptations to soil moisture and fertility in its natural environment.

**Key words:** Interspecific hybrids, roots, rooting depth, root distribution, *Trifolium repens*, *Trifolium uniflorum*, white clover.

### INTRODUCTION

White clover (*Trifolium repens*) ( $2n = 4x = 32$ ) is an important perennial legume component of temperate pasture systems but its desirable characteristics are offset by a number of limitations. These include a relatively small, shallow root system, poor drought resistance and poor competitiveness for soil phosphorus in mixed swards (Mouat and Walker, 1959; Jackman and Mouat, 1970; Thomas, 1984; Knowles *et al.*, 2003). Interspecific hybridization with other *Trifolium* species has potential to improve white clover through the introduction of traits outside its existing genetic variation (Widdup *et al.*, 2003; Abberton, 2007; Williams and Nichols, 2011). Recurrent backcrossing to white clover results in plants whose genes are predominantly from white clover, with some genetic material introgressed from the second parent (Williams *et al.*, 2008; Williams and Hussain, 2008). As a result, it is possible to maintain the agronomically and economically important characteristics of white clover while introducing new traits from related species.

Rooting depth, root morphology and root architecture all influence the ability of plants to access water and nutrients. Higher proportions of root mass in deeper soil layers, or a greater maximum rooting depth, could increase access to sub-soil water (Grieu *et al.*, 2001). It could also increase interception of mobile nutrients such as nitrate, which leach down the profile (Dunbabin *et al.*, 2003). Studies on the root depth distribution of white clover are limited, but have found up to 70 % of the total root mass occurs in the top 100–150 mm of the profile (Caradus, 1981; Nichols *et al.*, 2007). Uptake of water and nutrients is also influenced by root diameter, with greater efficiency of uptake in fine roots due to a higher ratio of root surface area to soil volume (Eissenstat, 1992; Jungk, 1996).

*Trifolium uniflorum* ( $2n = 32$ ) is a perennial, wild species found in Greece, Turkey, southern France, southern Italy and Libya (Zohary and Heller, 1984), occurring in naturally dry and nutrient-poor environments (Snogerup and Snogerup, 2004; Tela Botanica, <http://www.tela-botanica.org/bdtfx-nn-69461-synthese>). Using molecular techniques, Ellison *et al.* (2006) classified *T. uniflorum* in the ‘white clover complex’. This

group comprises close relatives of white clover, considered to provide the greatest potential for successful interspecific hybridization. There are relatively few published studies on *T. uniflorum*, but various authors have noted its thick and deep roots, woody tap root and a relatively large seed (Chen and Gibson, 1971; Pandey and Petterson, 1978; Pandey et al., 1987). Introgression of these root characteristics could improve white clover performance under drought stress. Pandey et al. (1987) reported that some *T. repens* × *T. uniflorum* hybrid plants did show transgressive segregation, with strong, deep nodal roots. More recently, Hussain et al. (2012) described new *T. repens* × *T. uniflorum*  $F_1$  and  $BC_1$  hybrid combinations and particularly noted potential for superior root characteristics, with more thick roots in  $BC_1$  hybrids than in white clover plants. In a rainshelter experiment, Nichols et al. (2014b) also observed a greater overall root cross-sectional area (i.e. basal diameter) for the largest nodal root of plants in a *T. repens* × *T. uniflorum*  $BC_1$  family, compared with a related  $BC_2$  family and their white clover cultivar parent. Such tap root-like nodal roots are considered to penetrate to greater depths than finer roots (Caradus, 1977; Caradus and Woodfield, 1998), and this may also increase the hybrid's access to deep soil water.

If the root distribution and rooting depth of white clover could be altered, it is possible that access to water and nutrients could be improved. Although *T. uniflorum* is reported to have deep roots (Pandey et al., 1987), there are no data on the rooting depth or root distribution of this species compared with white clover. The effects of hybridization with white clover on these traits are also unknown. This study reports, for the first time, a comparison of the rooting depth, root depth distribution, root diameter and root length density of *T. uniflorum*, white clover and *T. repens* × *T. uniflorum* interspecific hybrids. The functional implications of observed differences are discussed.

## MATERIALS AND METHODS

### Experimental design

Plants were grown in 1-m deep × 0.15-m diameter tubes of sand culture, irrigated with a low ionic strength nutrient solution based on the typical soil solution of New Zealand pasture topsoils (Edmeades et al., 1985; Blamey et al., 1991). Full details of the experimental system are given in Nichols et al. (2014c), where plant growth results are reported. The design was based on a split-plot arrangement with ten replicates and four blocks within each replicate. Blocks corresponded to four harvests over time, and each contained one plant from each clover entry described in the following section.

### Plant material

The six clover entries included two *Trifolium uniflorum* L. accessions, two white clover cultivars and two  $BC_1$  populations generated by backcrossing  $F_1$  hybrids to each of the white clover cultivar parents (Table 1). For *T. uniflorum* accession 4382, sufficient seed germinated for five replicates only, and the remaining replicates were planted with extra seed of accession 4383. Each  $BC_1$  entry contained two family bulks derived using two  $F_1$  hybrids (80-2 and 900-4), which each had a different *T.*

*uniflorum* accession as the male parent. These family bulks will be referred to as the 80-2 and 900-4 families.

### Plant harvests

Plants were harvested 70 (harvest 1), 119 (harvest 2), 170 (harvest 3) and 237 (harvest 4) d after sowing (DAS). Harvests were conducted following the methods of Crush et al. (2010) to determine root dry weight (DW) in 50-mm depth increments to 200 mm, and then in 100-mm increments down to 1 m. At harvest 3, the roots between 50–100 and 400–500 mm were also scanned on a flatbed scanner and analysed for total root length and root diameter distribution using WinRhizo™ image analysis software (Regent Instruments Inc., Quebec, Canada). In combination with the dry weight data, root length values were used to calculate root length density (RLD,  $\text{km m}^{-3}$ ) and specific root length (SRL,  $\text{m g}^{-1}$  root DW). Root volume and surface area were calculated in root diameter classes of 0.5-mm increments. Due to the small amount of root that was >2.0 mm in diameter, all root volume and area data above this value were combined into one class >2.0 mm. The maximum diameter class measured was >4.75 mm. Root samples at 50–100 mm depth were also divided into the primary tap root and nodal roots to determine whether diameter class distribution differed between the two root types.

### Statistical analysis

Statistical comparisons were based on clover entries or families. Prior to root depth distribution analysis, root DW data in the four 50-mm deep sections at the top of each tube were combined into two 100-mm deep sections at 0–100 and 100–200 mm. All depth sections were then of equal size. At each harvest, root depth distribution (root DW by depth) was analysed using an exponential model (1) in SAS version 9.1 (SAS Institute):

$$\text{Root mass} = \beta_j R_j^{\text{depth}} \text{ for } j = 1, 2, \dots, 6$$

where  $j$  denotes the six clover entries,  $\beta_j = \text{DW at } 0\text{--}100 \text{ mm}$  and  $R_j = \text{the rate at which root DW decreases with depth}$ .

To estimate the values of  $\beta_j$  and  $R_j$  in the exponential model (1), this equation was transformed to an equivalent regression model (2) by taking natural logarithms using the general linear model (GLM) procedure:

$$\ln(\text{root mass}) = \ln \beta_j + \text{depth} \times \ln R_j = C_{1j} + C_{2j} \times \text{depth}$$

where  $C_{1j} = \ln \beta_j$  and  $C_{2j} = \ln R_j$

The results for  $C_{1j}$  and  $C_{2j}$  were then back-transformed to  $\beta_j$  and  $R_j$  in the exponential model (1), using  $\beta_j = \exp(C_{1j})$  and  $R_j = \exp(C_{2j})$ .

Differences between clover entries were determined by pairwise comparisons of the resulting values for  $\beta_j$  and  $R_j$  using Tukey's pairwise comparison method to account for unbalanced data and multiple comparisons (Milliken and Johnson, 2009).

TABLE 1. The white clover, *T. uniflorum* and *T. repens* × *T. uniflorum* BC<sub>1</sub> entries used in the experiment. Each BC<sub>1</sub> entry was made up of two family bulks with different F<sub>1</sub> parents (80-2 and 900-4). The background of the backcross and F<sub>1</sub> parents is shown

	Notes	Backcross parent	F <sub>1</sub> parentage	
			♀	♂
Grasslands 'Kopu II' <sup>1</sup>	Large leaf type			
'Crusader' <sup>1</sup>	Medium leaf type			
AZ4382 <sup>2</sup>	Greek origin			
AZ4383 <sup>2</sup>	Turkish origin			
Kopu II BC <sub>1</sub>	Kopu II × 80-2	'Kopu II'	Kopu II-2 <sup>3</sup>	T66-6 <sup>4</sup>
	Kopu II × 900-4	'Kopu II'	Kopu II-2 <sup>3</sup>	AZ4383-11 <sup>5</sup>
Crusader BC <sub>1</sub>	Crusader × 80-2	'Crusader'	Kopu II-2 <sup>3</sup>	T66-6 <sup>4</sup>
	Crusader × 900-4	'Crusader'	Kopu II-2 <sup>3</sup>	AZ4383-11 <sup>5</sup>

<sup>1</sup>White clover cultivar.

<sup>2</sup>*T. uniflorum* accession number (Margot Forde Forage Germplasm Center, New Zealand), seed from open pollinated plants.

<sup>3</sup>Specific genotype of 'Kopu II' used as maternal F<sub>1</sub> parent.

<sup>4</sup>Specific genotype of AZ4382 used as paternal F<sub>1</sub> parent.

<sup>5</sup>Specific genotype of AZ4383 used as paternal F<sub>1</sub> parent.

In addition to the model, root distribution was also assessed at harvest 4 by comparing the percentage of total root mass at 0–100, 100–200 and 400–500 mm. Maximum rooting depth, based on the deepest section in which roots were present, was compared among clover entries at each harvest.

Maximum rooting depth, percentage root mass, RLD, SRL and diameter class data were analysed using analysis of variance (ANOVA) in Minitab version 15 or 16 (Minitab Inc.). The ANOVA of maximum rooting depth consisted of clover entry and harvest and their interaction. Values at harvest 4 were excluded from analysis since roots of some plants had reached the bottom of the tubes, but the mean and standard error of the mean are presented. All other ANOVAs consisted of only the clover entry factor. Differences between clover entries were determined using Tukey's pairwise comparison method as above. Changes in RLD and SRL with depth were calculated on a comparative basis as  $RLD_{400-500}/RLD_{50-100}$  and  $SRL_{400-500}/SRL_{50-100}$ , and data were analysed using the two-sample Wilcoxon test (Conover, 1980) for non-normally distributed data.

Differences in variability among clover entries for RLD and SRL were also analysed using a test for equal variances in Minitab version 15. This compares two variances using the *F*-test or Levene's test, depending on the normality distribution of the data. Standard deviations are presented to indicate the relative size of the variance for each entry.

Within the BC<sub>1</sub> populations, differences in root distribution between the 80-2 and 900-4 families at harvest 4 were assessed by comparing  $\beta_j$  and  $R_j$  using *t*-tests. Differences in RLD and SRL between the two families were also examined using ANOVA in Minitab version 15. We combined RLD and SRL data for each family across populations (e.g. Kopu II × 80-2 plus Crusader × 80-2) to increase replication for ANOVA.

## RESULTS

### Root system shape

Changes over time in differences among clover entries for total shoot and root DW, which were reported by Nichols *et al.* (2014c), suggested that the entries differed in growth rate,

particularly at the earlier harvests. However, the presence or absence of differences among entries were similar at harvests 3 and 4, indicating they had stabilized by this time. Root dry weight distribution with depth at harvest 4 is presented in Fig. 1. Differences in root system shape occurred either through differences in root mass in the top 100 mm ( $\beta_j$ ) or differences in the rate at which root mass decreased with depth ( $R_j$ ) (Table 2). Significant differences among clover entries changed from harvest to harvest. In well-established plants at harvest 4, there was no difference in the root system shape of *T. uniflorum* accession 4382, accession 4383, Crusader BC<sub>1</sub> and Kopu II BC<sub>1</sub>, but these entries were all different from both the white clover cultivars (Table 2). For 'Kopu II' this was due to a lower  $\beta_j$  value ( $P < 0.001$ ), but  $R_j$  did not differ from that of *T. uniflorum* accessions 4382 or 4383, Crusader BC<sub>1</sub> ( $P < 0.05$ ) or Kopu BC<sub>1</sub> ( $P = 0.056$ ). In contrast, 'Crusader' had both a lower  $\beta_j$  value ( $P < 0.001$ ) and a higher  $R_j$  value (i.e. slower rate of decrease in root mass with depth) ( $P < 0.05$ ) than the *T. uniflorum* and hybrid entries. The rate of decrease did not differ between the white clover cultivars, but  $\beta_j$  (root mass at 0–100 mm) was lower for 'Crusader' ( $P < 0.001$ ).

There were no significant differences among entries in the percentage of roots at 0–100 mm at harvest 4 (Table 3), despite the white clover cultivars having significantly lower  $\beta_j$  values than *T. uniflorum* and the hybrids. However, the *T. uniflorum* parents had ~10 % more of their total root mass at 100–200 mm than the white clover cultivars ( $P < 0.015$ ) (Table 3). Crusader BC<sub>1</sub> also had a higher percentage of roots at 100–200 mm than the 'Crusader' parent ( $P = 0.044$ ). Percentages of total root mass at 400–500 mm did not differ significantly among *T. uniflorum* and the hybrids or between the hybrids and their respective white clover parents (Table 3).

### Root depth penetration

Roots of some individual *T. uniflorum* plants first reached 1 m by harvest 2, compared with harvest 3 for the hybrid and white clover plants. At harvest 3, 35 % of hybrid plants had reached 1 m, compared with 15 % for the white clover cultivars. There were no significant differences in mean maximum

rooting depth among entries for very young plants (harvest 1), or for established plants (harvest 4) when roots were accumulating at the bottom of the tubes (Fig. 2).

Rooting depth differed among both clover entries and harvests ( $P < 0.001$ ) but there was no significant interaction. The roots of the two *T. uniflorum* accessions were generally the deepest out of all the clover entries, and roots of the two hybrid entries were also deeper than those of the white clover cultivars (Fig. 2). Means taken over all harvests were consistent

with these general patterns. Overall, the mean maximum rooting depths of 'Kopu II' (0.52 m) and 'Crusader' (0.46 m) were shallower ( $P < 0.001$ ) than both *T. uniflorum* accession 4382 (0.72 m) and 4383 (0.75 m). 'Kopu II' also penetrated less ( $P < 0.03$ ) than Kopu II BC<sub>1</sub> (0.63 m), and the roots of 'Crusader' were shallower ( $P < 0.001$ ) than Crusader BC<sub>1</sub> (0.61 m). Rooting depth of the hybrid entries did not differ to those of *T. uniflorum* accession 4382, but both were shallower than that of accession 4383 ( $P = 0.005$  and  $P < 0.001$ ).

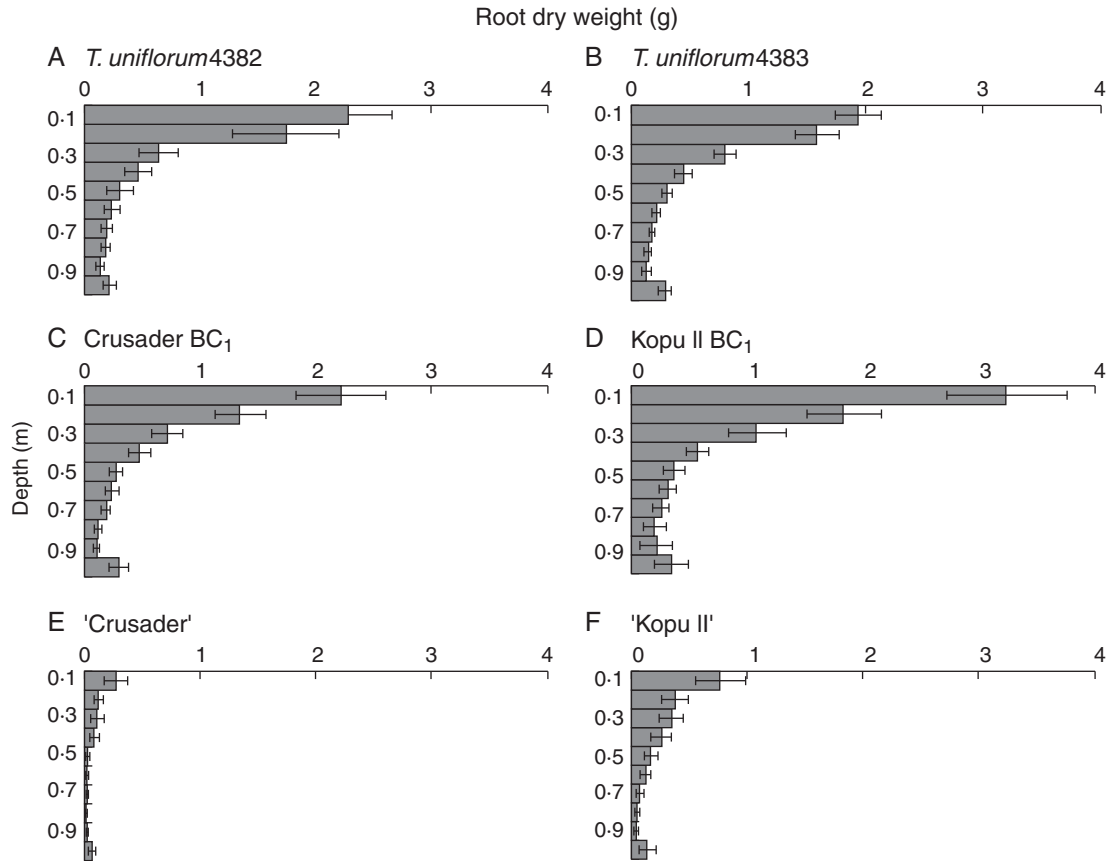


Fig. 1. Root depth distribution (g root dry weight by depth) ( $\pm$  s.e.m.) at harvest 4 (237 days after sowing) for (A) *T. uniflorum* 4382, (B) *T. uniflorum* 4383, (C) Crusader BC<sub>1</sub>, (D), Kopu II BC<sub>1</sub>, (E) 'Crusader' and (F) 'Kopu II'.

TABLE 2. Model values for root shape parameters of the six clover entries at each harvest

Harvest	Parameter	<i>T. uniflorum</i> 4382	<i>T. uniflorum</i> 4383	Crusader BC <sub>1</sub>	Kopu II BC <sub>1</sub>	'Crusader'	'Kopu II'
1	$\beta_j$	0.042 <sup>a</sup>	0.044 <sup>a</sup>	0.048 <sup>a</sup>	0.026 <sup>a</sup>	0.036 <sup>a</sup>	0.034 <sup>a</sup>
2	$\beta_j$	0.068 <sup>a</sup>	0.077 <sup>a</sup>	0.110 <sup>a</sup>	0.067 <sup>a</sup>	0.049 <sup>a</sup>	0.066 <sup>a</sup>
3	$\beta_j$	0.483 <sup>a</sup>	0.373 <sup>ab</sup>	0.115 <sup>c</sup>	0.211 <sup>bc</sup>	0.037 <sup>d</sup>	0.047 <sup>d</sup>
4	$\beta_j$	1.894 <sup>a</sup>	1.759 <sup>a</sup>	1.480 <sup>a</sup>	2.620 <sup>a</sup>	0.065 <sup>b</sup>	0.274 <sup>c</sup>
1	$R_j$	2.8E-05 <sup>ab</sup>	0.00047 <sup>b</sup>	1.4E-07 <sup>a</sup>	2.7E-05 <sup>ab</sup>	2.0E-09 <sup>a</sup>	4.1E-06 <sup>ab</sup>
2	$R_j$	0.00463 <sup>ab</sup>	0.00986 <sup>b</sup>	0.00004 <sup>c</sup>	0.00169 <sup>a</sup>	0.00030 <sup>ac</sup>	0.00004 <sup>c</sup>
3	$R_j$	0.0145 <sup>ab</sup>	0.0105 <sup>ab</sup>	0.0172 <sup>ab</sup>	0.0077 <sup>a</sup>	0.0314 <sup>bc</sup>	0.0647 <sup>c</sup>
4	$R_j$	0.0415 <sup>a</sup>	0.0435 <sup>a</sup>	0.0432 <sup>a</sup>	0.0293 <sup>a</sup>	0.1777 <sup>b</sup>	0.0935 <sup>ab</sup>

$\beta_j$ , root mass at 0–100 mm;  $R_j$ , rate of decrease with depth.

Harvest 1, 70 d after sowing (DAS); harvest 2, 119 DAS; harvest 3, 170 DAS; harvest 4, 237 DAS.

At each harvest, values with the same letter were not significantly different at the 5 % level.

### Root length density

At harvest 3 there was no significant difference in RLD ( $\text{km m}^{-3}$ ) at 50–100 mm depth for Kopu II BC<sub>1</sub> compared with ‘Kopu II’ ( $P=0.061$ ) or for ‘Crusader’ compared with Crusader BC<sub>1</sub> ( $P=0.557$ ) (Table 4). The *T. uniflorum* accessions also did not differ in RLD, nor did the two hybrid populations or the white clover cultivars (Table 4). As measured by the variance test, the RLD of Crusader BC<sub>1</sub> at 50–100 mm was more variable than that of the ‘Crusader’ cultivar ( $P=0.001$ ), but the variability of Kopu II BC<sub>1</sub> did not differ from the ‘Kopu II’ parent (Table 5). Root length density of Kopu II BC<sub>1</sub> was more variable than that of Crusader BC<sub>1</sub> ( $P=0.024$ ), and ‘Kopu II’ was also more variable than ‘Crusader’ ( $P < 0.001$ ).

At 400–500 mm depth, there were no significant differences in RLD between the hybrids and their respective parental cultivars. However, RLD of the *T. uniflorum* accessions was generally higher than that of the other clover entries (Table 4). For example, RLD of *T. uniflorum* accession 4383 was 3.2–3.7

times higher than those of Crusader BC<sub>1</sub> ( $P=0.04$ ), Kopu II BC<sub>1</sub> ( $P=0.039$ ) and ‘Kopu II’ ( $P=0.026$ ), and 9.1 times higher than that of ‘Crusader’ ( $P=0.005$ ). Root length density of *T. uniflorum* accession 4382 at 400–500 mm was 10.4 times higher than that of the ‘Crusader’ cultivar ( $P=0.019$ ), and there was also a pattern for higher RLD compared with ‘Kopu II’ ( $P=0.065$ ), Crusader BC<sub>1</sub> ( $P=0.083$ ) and Kopu II BC<sub>1</sub> ( $P=0.085$ ) (Table 4). The latter may have been influenced by the standard error of the mean of accession 4382 at this depth, which was very large relative to the other clover entries (Table 4).

Root length density decreased with depth for all entries, but the change was not as pronounced for the *T. uniflorum* accessions (Table 4). As a consequence the  $\text{RLD}_{400-500}/\text{RLD}_{50-100}$  for *T. uniflorum* accession 4383 was higher than those for Crusader BC<sub>1</sub>, Kopu II BC<sub>1</sub>, ‘Crusader’ and ‘Kopu II’ ( $P < 0.005$ ). For *T. uniflorum* accession 4382, it was also higher than for Crusader BC<sub>1</sub>, Kopu II BC<sub>1</sub> and ‘Kopu II’ ( $P < 0.017$ ), but not ‘Crusader’ ( $P=0.061$ ).

TABLE 3. Percentage ( $\pm$  s.e.) of total root mass at 0–100, 100–200 and 400–500 mm depth at harvest 4 (237 d after sowing) for the six clover entries

	0–100 mm	100–200 mm	400–500 mm
<i>T. uniflorum</i> 4382	37.5 <sup>a</sup> $\pm$ 3.81	26.4 <sup>a</sup> $\pm$ 1.4	4.4 <sup>ab</sup> $\pm$ 0.85
<i>T. uniflorum</i> 4383	33.4 <sup>a</sup> $\pm$ 1.82	26.3 <sup>a</sup> $\pm$ 1.27	4.8 <sup>ab</sup> $\pm$ 0.36
Crusader BC <sub>1</sub>	38.6 <sup>a</sup> $\pm$ 1.54	23.1 <sup>ab</sup> $\pm$ 1.52	4.3 <sup>a</sup> $\pm$ 0.41
Kopu II BC <sub>1</sub>	40.7 <sup>a</sup> $\pm$ 2.14	21.9 <sup>abc</sup> $\pm$ 1.15	4.4 <sup>ab</sup> $\pm$ 0.3
‘Crusader’	40.3 <sup>a</sup> $\pm$ 6.33	16.2 <sup>c</sup> $\pm$ 2.37	3.0 <sup>a</sup> $\pm$ 0.71
‘Kopu II’	39.7 <sup>a</sup> $\pm$ 3.76	16.8 <sup>bc</sup> $\pm$ 1.48	6.5 <sup>b</sup> $\pm$ 0.75

Within sampling depths, means with the same letter were not significantly different at the 5 % level.

### Specific root length

At 50–100 mm, the SRLs of the two *T. uniflorum* accessions were significantly lower than those of most other clover entries (Table 4). Variability of SRL at 50–100 mm did not differ among entries (Table 5). At 400–500 mm there were no differences in SRL (Table 4).

Values of  $\text{SRL}_{400-500}/\text{SRL}_{50-100}$  over 100 % indicated increases in SRL with depth, and values less than 100 % indicated decreases with depth. Data showed that SRL increased with depth for the *T. uniflorum* accessions and decreased for white clover and the hybrids (Table 4). The  $\text{SRL}_{400-500}/\text{SRL}_{50-100}$  of *T. uniflorum*

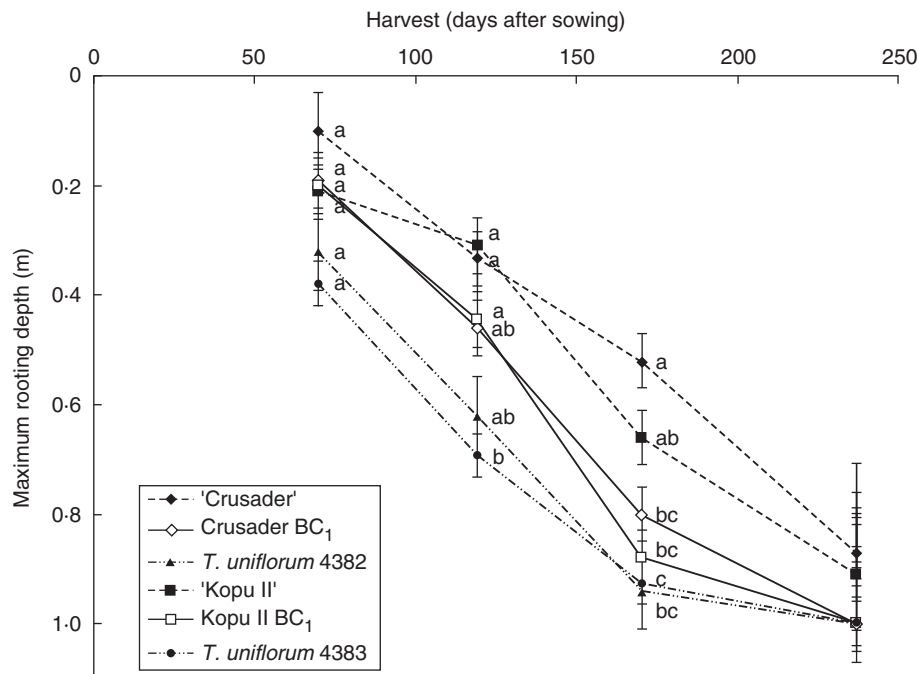


FIG. 2. Root penetration (mean maximum rooting depth  $\pm$  s.e.m.) over time for the six clover entries. At each harvest, means with the same letter were not significantly different at the 5 % level.

TABLE 4. Mean ( $\pm$  s.e.) specific root length (SRL) and root length density (RLD) at 50–100 and 400–500 mm, plus RLD and SRL at 400–500 mm deep relative to 50–100 mm deep, for the six clover entries. Measurements were made at harvest 3 (170 d after sowing)

	RLD 50–100 mm ( $\text{km m}^{-3}$ )	RLD 400–500 mm ( $\text{km m}^{-3}$ )	SRL 50–100 mm ( $\text{m g}^{-1}$ )	SRL 400–500 mm ( $\text{m g}^{-1}$ )	RLD <sub>400–500/50–100</sub> (%)	SRL <sub>400–500/50–100</sub> (%)
<i>T. uniflorum</i> 4382	16.1 <sup>ab</sup> $\pm$ 5.8	2.0 <sup>ab</sup> $\pm$ 0.8	53.9 <sup>a</sup> $\pm$ 10.2	83.0 <sup>a</sup> $\pm$ 9.9	22.1 <sup>ab</sup> $\pm$ 10.5	208 <sup>ab</sup> $\pm$ 76
<i>T. uniflorum</i> 4383	11.9 <sup>a</sup> $\pm$ 1.9	1.7 <sup>a</sup> $\pm$ 0.4	65.7 <sup>a</sup> $\pm$ 5.6	96.9 <sup>a</sup> $\pm$ 9.8	17.3 <sup>a</sup> $\pm$ 3.8	163 <sup>a</sup> $\pm$ 20
Crusader BC <sub>1</sub>	17.3 <sup>ab</sup> $\pm$ 4.8	0.5 <sup>bc</sup> $\pm$ 0.1	115.2 <sup>bc</sup> $\pm$ 12.6	96.1 <sup>a</sup> $\pm$ 17.4	3.7 <sup>c</sup> $\pm$ 1.0	81 <sup>c</sup> $\pm$ 15
Kopu II BC <sub>1</sub>	37.5 <sup>b</sup> $\pm$ 10.8	0.5 <sup>bc</sup> $\pm$ 0.1	144.7 <sup>c</sup> $\pm$ 7.5	107.8 <sup>a</sup> $\pm$ 12.7	2.4 <sup>c</sup> $\pm$ 0.7	77 <sup>c</sup> $\pm$ 10
'Crusader'	13.0 <sup>a</sup> $\pm$ 1.3	0.2 <sup>c</sup> $\pm$ 0.1	82.5 <sup>ab</sup> $\pm$ 11.8	57.3 <sup>a</sup> $\pm$ 20.3	4.3 <sup>bc</sup> $\pm$ 1.4	66 <sup>bc</sup> $\pm$ 22
'Kopu II'	14.3 <sup>ab</sup> $\pm$ 6.1	0.5 <sup>bc</sup> $\pm$ 0.2	125.4 <sup>c</sup> $\pm$ 12.2	82.5 $\pm$ 17.7	2.7 <sup>c</sup> $\pm$ 0.7	62 <sup>c</sup> $\pm$ 14

Within parameters, means with the same letter were not significantly different at the 5 % level.

TABLE 5. Standard deviations for root morphological parameters at 50–100 mm depth for the six clover entries at harvest 3 (170 d after sowing)

	Root length density ( $\text{km m}^{-3}$ )	Specific root length ( $\text{m g}^{-1}$ )
<i>T. uniflorum</i> 4382	12.90 <sup>abc</sup>	22.80 <sup>a</sup>
<i>T. uniflorum</i> 4383	7.72 <sup>a</sup>	22.39 <sup>a</sup>
Crusader BC <sub>1</sub>	14.44 <sup>b</sup>	37.67 <sup>a</sup>
Kopu II BC <sub>1</sub>	34.12 <sup>c</sup>	23.70 <sup>a</sup>
'Crusader'	3.77 <sup>d</sup>	35.50 <sup>a</sup>
'Kopu II'	19.27 <sup>bc</sup>	28.66 <sup>a</sup>

Within parameters, variability of entries with the same letter was not significantly different at the 5 % level.

TABLE 6. Model values for  $\beta_j$  (root mass at 0–100 mm) and  $R_j$  (decrease in root mass with depth) for families within BC<sub>1</sub> populations at harvest 4 (237 d after sowing)

Model value	Kopu II BC <sub>1</sub>			Crusader BC <sub>1</sub>		
	80-2	900-4	<i>P</i> value	80-2	900-4	<i>P</i> value
$\beta_j$	3.289	2.086	0.010	2.588	0.847	<0.001
$R_j$	0.0293	0.0293	1.000	0.0432	0.0432	1.000

accession 4382 was higher than those of Crusader BC<sub>1</sub> ( $P = 0.046$ ), Kopu II BC<sub>1</sub> ( $P = 0.032$ ) and 'Kopu II' ( $P = 0.012$ ), but not 'Crusader' ( $P = 0.061$ ) (Table 4). It was higher for *T. uniflorum* accession 4383 than for the hybrid populations and white clover cultivars ( $P < 0.015$ ).

#### Differences among families

There was a significant difference in root shape within both hybrid populations, with family 80-2 having higher  $\beta_j$  values (root mass at 0–100 mm) (Table 6). Based on the combined data of the Crusader BC<sub>1</sub> and Kopu II BC<sub>1</sub> populations, RLD and SRL at 50–100 mm deep were also higher for the 80-2 family than for the 900-4 family (Table 7). There were no differences between the families for RLD or SRL at 400–500 mm.

#### Diameter class distribution

At the 50–100 mm depth the 0–0.5 mm diameter class accounted for 34–39 % of total root volume in the *T. uniflorum*

TABLE 7. Mean root length density (RLD) and specific root length (SRL) ( $\pm$  s.e.) for the 80-2 and 900-4 families at 50–100 and 400–500 mm depths. Measurements were made at harvest 3 (170 d after sowing)

	Depth (mm)			<i>P</i> value
		80-2	900-4	
RLD ( $\text{km m}^{-3}$ )	50–100	41.04 $\pm$ 8.526	16.09 $\pm$ 8.088	0.049
	400–500	0.48 $\pm$ 0.125	0.54 $\pm$ 0.119	0.696
SRL ( $\text{m g}^{-1}$ )	50–100	155.5 $\pm$ 8.10	108.4 $\pm$ 7.68	0.001
	400–500	105.6 $\pm$ 15.52	99.2 $\pm$ 14.72	0.767

accessions compared with 69–77 % for white clover. In contrast, total root volume in diameter classes  $>1$  mm decreased from *T. uniflorum* to white clover (Fig. 3A). This was particularly pronounced for root volume  $>2$  mm in diameter, which accounted for 21–33 % of total volume for *T. uniflorum*, compared with 2 % for the two hybrid populations and  $<1$  % for the white clover cultivars. Statistically, the *T. uniflorum* accessions had a lower ( $P \leq 0.006$ ) percentage of root volume in the 0–0.5-mm diameter class and a higher ( $P < 0.001$ ) percentage  $>2$  mm in diameter, compared with the hybrid and white clover entries (Fig. 3A).

Relative differences among clover entries for the diameter class distribution of total root surface area at 50–100 mm followed very similar patterns to root volume, although the contribution of finer roots (0–0.5 mm) increased while thicker classes decreased (data not shown). For example, roots in the 0–0.5-mm diameter class represented 34 % of root volume but 65 % of root surface area for *T. uniflorum* accession 4382, while roots  $> 2.0$  mm diameter were 33 % of root volume but only 9 % of root surface area. Any differences between the diameter class distribution of surface area and volume usually involved the *T. uniflorum* accessions and the white clover cultivars, which had the most contrasting mean values.

Generally, there were no roots  $>2$  mm in diameter at 400–500 mm depth. However, the *T. uniflorum* accessions still had 8–14 % of root volume  $>1$  mm diameter compared with just 1 % for the hybrid populations and 'Kopu II' and 0 % for 'Crusader' (Fig. 3B). For the highest root diameter classes present at this depth, *T. uniflorum* accessions 4382 and 4383 had higher ( $P = 0.008$  and  $P < 0.001$ , respectively) percentages of root volume in the 1.0–1.5-mm diameter class than 'Crusader', and *T. uniflorum* accession 4382 also had a higher ( $P = 0.034$ ) percentage of root volume in the 1.5–2.0-mm class than the hybrids and white clover cultivars.

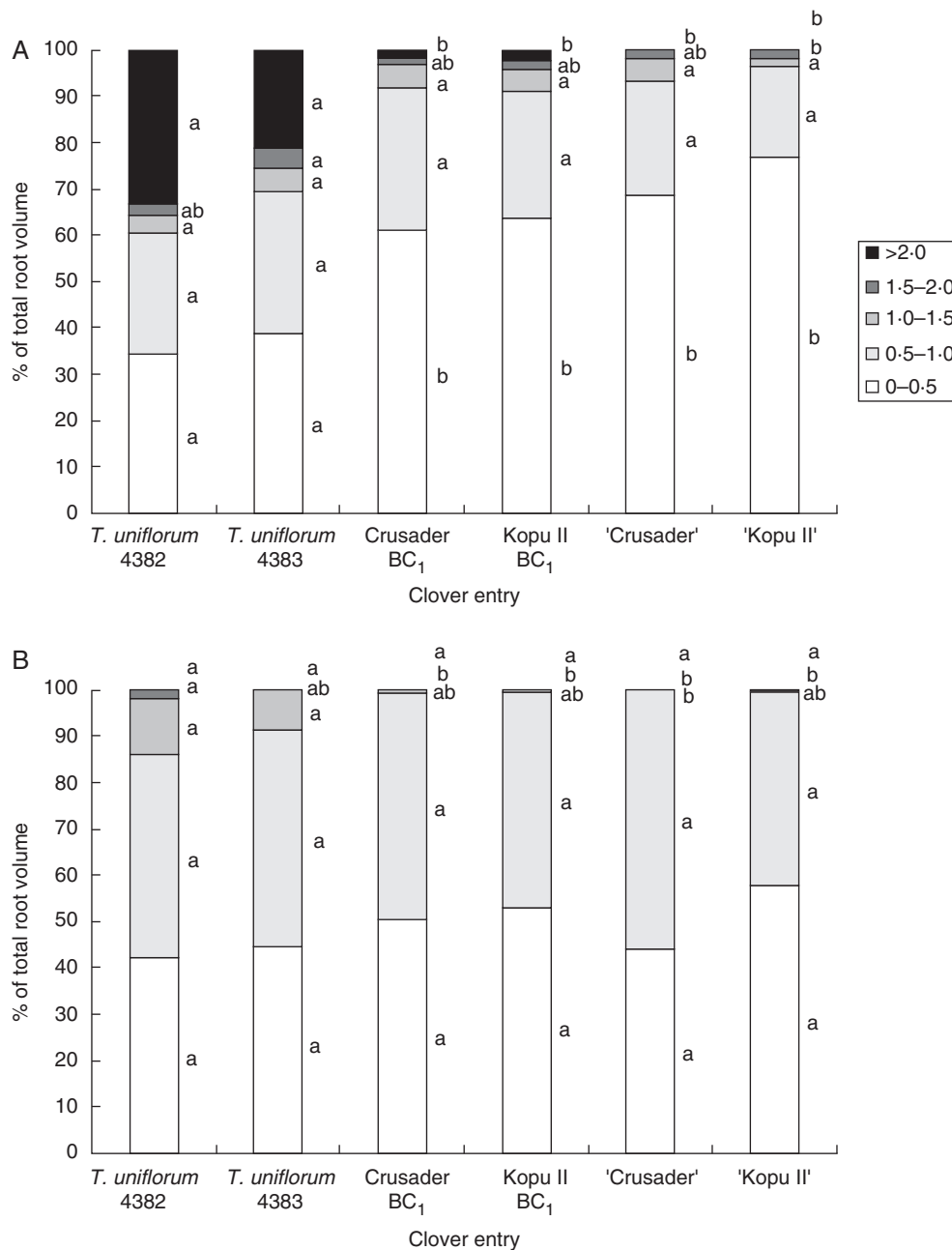


FIG. 3. Mean percentage of total root volume in root diameter classes from 0–0.5 to >2.0 mm at harvest 3 (170 d after sowing). (A) 50–100 mm depth. (B) 400–500 mm depth. Within diameter classes, means with the same letter were not significantly different at the 5% level.

Between the 50–100 and 400–500 mm depths, the pattern of changes in volume in the lower diameter classes mirrored the observed changes in SRL (Fig. 3). At 400–500 mm the absolute percentage of root volume in the 0–0.5-mm class was 6–8% higher than at 50–100 mm for the *T. uniflorum* accessions, compared with 10% lower for the hybrid populations, 25% lower for 'Crusader' and 19% lower for 'Kopu II'.

When divided into tap root and nodal roots, the diameter class distribution of root volume of both root types at 50–100 mm depth was very similar to the pattern seen with the overall data (Fig. 4). Compared with the other clover entries,

both the *T. uniflorum* accessions had a lower ( $P \leq 0.04$ ) percentage of tap root volume in the 0–0.5-mm diameter class and a higher ( $P < 0.001$ ) percentage in the >2.0-mm diameter class. For nodal roots, the pattern was also similar in *T. uniflorum* accession 4383, which had a lower ( $P \leq 0.041$ ) percentage of root volume in the 0–0.5-mm diameter class than the hybrid and white clover entries. It also had a higher percentage of root volume in all diameter classes >1.0 mm compared with 'Kopu II' ( $P \leq 0.048$ ), in the 0.5–1.0-mm class ( $P = 0.003$ ) compared with 'Crusader', and in the >2.0-mm class ( $P = 0.011$ ) compared with 'Crusader' and Crusader BC<sub>1</sub>.

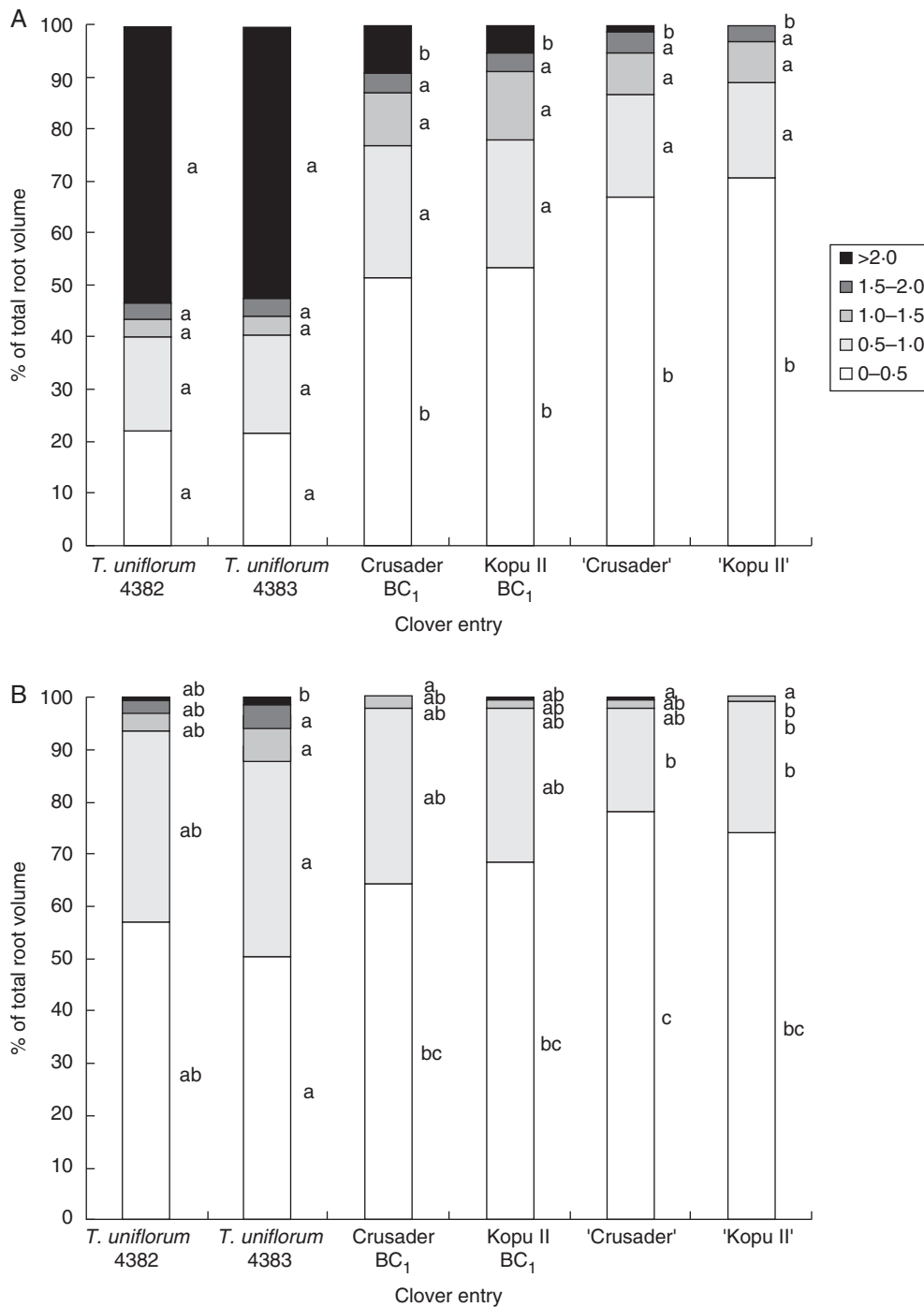


Fig. 4. Mean percentage of root volume at 50–100 mm depth in root diameter classes from 0–0.5 to >2.0 mm at harvest 3 (170 d after sowing). (A) Tap root. (B) Nodal roots. Within diameter classes, means with the same letter were not significantly different at the 5% level.

## DISCUSSION

### Root distribution

Root system shape and root depth distribution characteristics of the hybrids were more similar to the *T. uniflorum* parent than to white clover, despite the BC<sub>1</sub> hybrid genomes being, on average, 75% white clover. In particular, *T. uniflorum* and the

hybrids had a higher proportion of their total root mass high in the profile, shown by lower  $R_j$  values than white clover and, in the case of *T. uniflorum* and Crusader BC<sub>1</sub>, by a higher percentage of root mass at 100–200 mm. van Wijk (2011) modelled the effects of rooting strategies, soil type and rainfall on transpiration and suggested that as rainfall decreases, evaporation is a more important source of water loss than drainage. As a result,



roots are distributed higher in the profile to maximize water uptake. Shallower roots may also provide some adaptation for acquisition of water from intermittent rain or dew under dry conditions. This suggests that adaptation of *T. uniflorum* to low soil moisture may be a determining factor in root depth distribution of *T. repens* × *T. uniflorum* hybrids. *Trifolium uniflorum* occurs naturally in dry environments (Tela Botanica, 2015), and hybrids with white clover have been found to have increased drought resistance compared with white clover cultivars (Nichols et al., 2014b, 2015a).

Shallow rooting is also advantageous for phosphorus (P) acquisition (Ge et al., 2000; Lynch and Brown, 2001) as most soil P is concentrated near the soil surface (Haynes and Williams, 1992). The root distribution of *T. uniflorum* and the *T. repens* × *T. uniflorum* hybrids may therefore also reflect the soil fertility status in *T. uniflorum*'s natural environment. The degree of root system plasticity in response to drying conditions is unknown for *T. repens* × *T. uniflorum* hybrids, and could alter the root distribution observed here (Pang et al., 2011). Given the positive effects of P on drought stress in white clover (Singh et al., 1997; Singh and Sale, 1998) and evidence for drought resistance and low P tolerance in the hybrids (Nichols et al., 2014a, b, c, 2015a), the potential for drought stress to be ameliorated by low soil P tolerance in this material requires further investigation.

Differences in total DM production among the clover entries in the current experiment, reported by Nichols et al. (2014c), were likely to reflect differences in tolerance to low external P supply. Shoot P concentrations of the entries tested were well below reported critical internal levels for white clover growth. The impacts of nutrient availability and potential differences in critical internal P levels on the observed patterns of root shape and distribution in the current experiment are unknown. Differences in the values of root mass at 0–100 mm, derived from the model, reflect these differences in plant size and were likely to have been influenced by the effects of nutrient supply on growth. Although the percentage of roots at 0–100 mm did not differ among entries, the higher absolute root mass of *T. uniflorum* and the hybrids in the upper profile of this growth system would increase their ability to capture P in the field. Similarly, while the higher RLDs at 50–100 mm for the hybrid populations were not significant they may still have a functional effect on the hybrids' ability to acquire P in the topsoil compared with white clover. However, the degree to which root traits must change in order to produce these functional effects is unknown in white clover.

#### Rooting depth

The deeper mean maximum rooting depths of the hybrid populations appear to have been inherited from the *T. uniflorum* parent, which had the greatest maximum rooting depth. To determine whether this results in deeper rooting and improved access to deep soil moisture in the field requires further testing under those conditions. This would remove the barrier to rooting depth posed by a limited pot size, while incorporating the physical effects of field soil on root penetration. The faster penetration by roots of *T. uniflorum* and the hybrids, compared with the white clover parents, could aid establishment under

dry soil conditions or enable rapid early growth to access deeper water before seasonal decreases in soil moisture occur. The larger seed of *T. uniflorum*, compared with white clover (Gibson et al., 1971), may also aid establishment as this is a trait that has been associated with larger, more vigorous seedlings in temperate pasture species (Moot et al., 2000).

Root mass in the current study was predominantly from primary tap root systems, although nodal roots were also present, particularly at later harvests. Pederson (1989) found that cultivar differences for root characteristics were similar in primary roots and nodal roots of white clover. Therefore, it is likely that root traits observed in tap root systems in the current study would also be present in clonal plants reliant solely on nodal root systems following the loss of the tap root. This was confirmed to some extent by the diameter class distribution data, which showed similar patterns among entries in the relative proportion of finer and thicker roots for both the tap root and nodal roots. For example, *T. uniflorum* had a higher percentage of thick roots and a lower percentage of fine roots than white clover, in both root types.

#### Root morphology

Generally, higher SRL values indicate thinner roots, assuming equal tissue densities (Eissenstat, 1992). Specific root length data in this experiment indicate that the *T. uniflorum* accessions had thicker roots at 50–100 mm than white clover and the hybrids. The diameter class distribution of root volume also showed that *T. uniflorum* had more root volume in thicker diameter classes and less volume in the finest diameter class, compared with white clover and the hybrid populations. Therefore, it is unlikely that tissue density has confounded the interpretation of SRL data. The changes in SRL at 400–500 mm (increasing for the *T. uniflorum* accessions and decreasing for the hybrids and white clover) suggest that *T. uniflorum* had more of its fine root mass at depth, while the hybrids and white clover had more of their fine root mass higher in the profile. Again, the diameter class distribution data showed changes with depth in the percentage of root volume in the 0–0.5-mm diameter class that reflect changes in SRL.

Specific root length data did not indicate any differences in root diameter between the hybrids and their white clover parents. However, SRL is strongly influenced by finer roots (Boot, 1989), which accounted for a considerable proportion of both root volume and surface area in the current experiment. The diameter class data may therefore provide a more useful indication of the contribution of thicker roots to the root mass of white clover compared with the hybrids. Although the proportion of total root volume in the thickest diameter class at 50–100 mm depth was not significantly higher for the hybrid entries (2.0–2.4 %) than for white clover (0.2–0.3 %), this variation may still be sufficient, functionally, to impact drought resistance. For instance, Kirkegaard et al. (2007) found that small increases in availability of subsoil water could have considerable impacts on grain yield in wheat. In hybrids this could operate through increasing the plant's access to deeper subsoil moisture, via greater penetration of thicker roots to depth, rather than by experiencing an increase in soil water *per se*. Thicker roots were not present in *T. repens* × *T. uniflorum* hybrids at

400–500 mm deep, but as diameter distribution was not assessed between the 100- and 400-mm depths we cannot comment on where the relative depth limits may be for thicker roots of hybrids versus white clover cultivars.

Ekanayake *et al.* (1985) found significant correlations between root characteristics (such as thickness, length and density) and drought stress symptoms and recovery in rice. Babu *et al.* (2003) also reported positive correlations between quantitative trait loci for root traits and rice yield under drought. In contrast, Annicchiarico and Piano (2004) found no correlation between root traits and drought tolerance in white clover, but suggested this was due to its poor physiological adaptation to water stress. Improved physiological tolerance of *T. repens* × *T. uniflorum* BC<sub>1</sub> hybrids (Nichols *et al.*, 2015a) may enable the beneficial effects of root characteristics on drought resistance to be expressed. Annicchiarico and Piano (2004) also speculated on the possible importance of fibrous roots in white clover during drought, and it seems likely that both fine roots and thicker, deeply penetrating roots play a role in drought resistance. It is worth noting that a combination of fine and thick roots may be particularly important in the presence of multiple soil limiting factors, such as drought and low soil fertility. For example, thicker roots for access to deeper soil moisture in addition to finer roots with high surface area for absorption of both water and nutrients.

While potentially advantageous under water stress, the thicker roots of *T. uniflorum* at 50–100 mm could be a disadvantage for nutrient uptake due to a lower ratio of root surface area to soil volume (Eissenstat, 1992). This was also reflected by the differences in the relative contributions of thicker versus finer roots for surface area and volume. However, other factors could increase the capture of subsoil water and leaching nutrients in the field, such as the increase in finer roots (higher root surface area:soil or root volume) and greater RLD [increased exploration of the growth media (Dunbabin *et al.*, 2003)] at 400–500 mm. At 50–100 mm depth, higher RLD and SRL in the 80-2 population could also have increased the nutrient uptake of these plants, which may have contributed to their larger root and shoot DWs relative to the 900-4 population (Nichols *et al.*, 2014c). Further study is required on the functional impacts of hybridization on the root morphological traits that affect nutrient interception, water uptake and responses to drought stress.

#### Selection for root traits

Despite moderate heritability for responses to P application under controlled conditions (Caradus *et al.*, 1992), white clover genotypes selected for improved phosphorus use efficiency (PUE) demonstrated no advantage under field conditions (Caradus, 1994; Caradus and Dunn, 2000), possibly due to a large genotype × environment interaction on the expression of PUE traits in the field. Therefore, root characteristics may be a more achievable target for improvement of white clover growth at low soil P levels. Our data show that changes to root traits are possible through interspecific hybridization, and the limited literature on the heritability of root traits in white clover suggests there should be a good response to selection (Caradus and Woodfield, 1998). Assessment of a wider range of hybrid

populations and white clover cultivars would be useful to confirm the differences in root characteristics observed in the current experiment and help inform the potential advantages of these traits for water and nutrient interception.

As different hybrid genotypes contain different combinations of *T. uniflorum* genes, it is expected that the hybrids will be very variable for some traits. For example, the variance of RLD at 50–100 mm was significantly higher for Crusader BC<sub>1</sub> compared with ‘Crusader’ and for Kopu II BC<sub>1</sub> compared with Crusader BC<sub>1</sub>. This may be responsible for the absence of statistically significant differences in RLD between these entries. We have also observed variation among hybrid families for growth at low soil P levels, and for root traits such as branching frequency, root:shoot ratio and nodal root thickness (Nichols *et al.*, 2014a, b). The variability present within and among hybrid families provides a valuable genetic resource for selection. Wide screening of hybrid families and genotypes is therefore necessary in order to incorporate the best hybrid material into breeding populations.

#### Conclusions

Root system shape, root distribution and maximum rooting depth of *T. repens* × *T. uniflorum* interspecific hybrids were strongly influenced by the *T. uniflorum* parent. These results confirm a previous observation, under field conditions, that some root characteristics of white clover can be altered by hybridization with *T. uniflorum* (Nichols *et al.*, 2015b). In contrast, the hybrids were more similar to white clover than to *T. uniflorum* for the limited number of root morphological traits that were measured. Differences in the root traits of *T. uniflorum* and the BC<sub>1</sub> hybrids compared with those of white clover may reflect soil moisture and soil fertility conditions in the natural environment of *T. uniflorum*. These traits may influence factors such as access to deep soil water, drought resistance, acquisition of water and nutrients, and seedling establishment in the hybrids compared with white clover, but further work is required to confirm these functional and ecological links. The use of a wider range of germplasm in future studies would incorporate a greater extent of the potential genetic variation, and help to confirm both the observed results and their functional implications, as well as determining the potential for selection within or among hybrid populations.

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