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Nitrogen and Potassium Fertilisation Influences Growth, Rhizosphere Carboxylate Exudation and Mycorrhizal Colonisation in Temperate Perennial Pasture Grasses

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Abstract: Optimisation of potassium (K) use efficiency in pastures on sandy soil is challenging. We characterised growth response, root carboxylate exudation and mycorrhizal colonisation in three perennial pasture grasses: tall fescue (*Festuca arundinacea* L.), veldt grass (*Ehrharta calycina* Sm.) and tall wheatgrass (*Thinopyrum ponticum* L.) in two glasshouse experiments with: (1) four K rates (0, 40, 80 and 120 mg K kg⁻¹ soil), and (2) four N and K treatments (no N and K (–N–K), 81 mg N kg⁻¹ soil but no K, 80 mg K kg⁻¹ soil but no N, and N at 81 and K at 80 mg kg⁻¹ soil (+N+K)) in low-K sandy soil. Veldt grass had the highest shoot dry weight and shoot P content, but the lowest mycorrhizal colonisation. Potassium fertilisation had no significant impact on exudation of citrate and oxalate. The K0 plants had significantly lower exudation of acetate and total carboxylates than K40 plants. The +N+K plants had maximum shoot growth at both harvests (30 and 60 days after sowing (DAS)) and highest N and K shoot contents at 60 DAS. The –N–K plants exuded maximum amounts of citrate and malate at 30 DAS, but at 60 DAS tall fescue had the highest rhizosphere concentrations of citrate and malate in the +N+K treatment. At 60 DAS, mycorrhizal colonisation was significantly lower with than without N and K fertilisation. We concluded that pasture grasses could yield well even in inherently low-K soil without external K fertilisation and mycorrhizal symbiosis. However, the +N+K plants had the highest yield and root carboxylate exudation.

Keywords: citrate; malate; nutrient uptake; rhizosphere; root exudates

1. Introduction

Potassium (K) regulates various biochemical and physiological processes, including protein synthesis, energy metabolism and photosynthesis [1]. Most Australian soils had sufficient availability of K to support plant growth [2]. However, as the cereals and dairy production intensified, K deficiency became widespread due to removal of K in the silage and agricultural products [2,3]. About 2/3 of wheat belt soils in southern Australia are now considered low in available K [1].

In south-western Australia, almost 75% of the soils are sandy and have low K availability [4]. In these soils, K is prone to leaching [5]. Even though K application to pastures is advocated (either as split and/or at a later stage when plants have developed adequate root systems) [6], optimising K fertiliser

efficiency continues to be a major problem in sandy soils [7]. Despite this challenge, inorganic K fertilisation has become inevitable [2] as most sandy soils in Western Australia are now low in K [3].

Understanding the fertiliser K requirement of pasture species is vital as it significantly determines the productivity and quality of pastures. Apart from its direct effect on the growth of individual species, K can influence botanical composition [8], with growing pastures of different botanical composition being an established system used by dairy farmers in south-western Australia. Given the higher K-use efficiency of grasses compared with legumes, in a mixed pasture sward of subterranean clover (*Trifolium subterraneum* L.) and ryegrasses (annual: *Lolium rigidum* Gaud. and Italian: *L. multiflorum* Lam.), K deficiency led to lower seed production in clover and its disappearance from the system [9]. Therefore, attempts were made to define the K fertiliser requirement based on the critical soil test values. Some of these critical values are (bicarbonate Colwell soil test in mg K kg⁻¹): <50 in wheat [10], <60 in canola [11] and <100 in clover [6]. In general, sandy soils with <50 mg K kg⁻¹ of bicarbonate Colwell are considered low in K, thus requiring fertiliser application [3]. However, information on the K requirements of temperate perennial pasture grasses is limited.

Rhizosphere carboxylate (organic acid anion) exudation is a plant mechanism for mobilising nutrients, and is particularly important in enhancing the acquisition and availability of immobile nutrient phosphorus (P) [12–15], with the extensive relevant literature [16–18]. A potential exists for carboxylates to mobilise K that is often unavailable due to fixation as non-exchangeable K [19,20]. However, the exudates data are mostly non-existent for temperate perennial pasture grasses in spite of usefulness of having species/varieties with a strong K mobilisation potential via root exudates [21].

The available literature indicated that citrate and oxalate [22] as well as malate [23] are essential in K dissolution and release. Species/varieties with high K uptake efficiency generally release more carboxylates than the in-efficient ones [24]. In maize (*Zea mays* L.), the low K supply was associated with significantly higher citrate and oxalate exudation compared with the K-sufficient treatments [25]. The increased mobilisation of non-exchangeable K by efficient potato (*Solanum tuberosum* L.) cultivars was also attributed to root exudation [26], with the K-mobilising efficiency of carboxylates dependent on the amount exuded [27,28].

The efficiency of K utilisation by plants is governed by nitrogen (N) availability and vice versa. Plants capacity to utilise N was restricted by K deficiency [21], and similarly K uptake was enhanced by N supply [29]. The N and K supply also influenced accumulation of organic acid anions in grass shoots, with aconitate concentration decreasing with applied N but increasing with applied K in green panic grass (*Panicum maximum* var. *trichoglume*) [30]. In contrast, oxalate concentration in shoots of green panic grass increased with applied N but declined with applied K [30]. In cocksfoot (*Dactylis glomerata* L.) and brome grass (*Bromus inermis* L.), N fertilization increased malate and *trans*-aconitate concentrations in shoots, and K decreased them [31]. However, such information is not available for tall fescue and tall wheatgrass.

The main objectives of the current study were to characterise the effect of different K and N rates on growth parameters and concentrations of a wide range of carboxylates in the rhizosphere of three temperate perennial pasture grasses in various growth stages. The significance of these three pasture grass species in Australian farming system was described previously [32]. We also measured arbuscular mycorrhizal (AM) colonisation because root exudation is positively influenced by colonisation [33].

2. Materials and Methods

2.1. Experimental Design

2.1.1. Experiment 1 (Growth and Rhizosphere Carboxylates at Different K Rates)

Three temperate perennial pasture grasses—tall fescue (*Festuca arundinacea* L. cv. Prosper), veldt grass (*Ehrharta calycina* Sm. cv. Mission) and tall wheatgrass (*Thinopyrum ponticum* L. cv. Dundas) were chosen. Previous experiments [32] with these species have shown differences in growth and rhizosphere carboxylates as influenced by species and phosphorus applications.

Sandy soil was collected (0–10 cm) from The University of Western Australia Shenton Park Field Station (31°94'69'' S, 115°79'53'' E); and it had the following chemical properties: 4.8 pH (CaCl₂) [34], 2 mg nitrate N kg⁻¹ [35], 3 mg P kg⁻¹ and 20 mg K kg⁻¹ [36]. The sandy soil with <50 mg K kg⁻¹ soil is considered to be low in K [3].

Plants were grown in pots (200 mm diameter and 180 mm deep) lined with a polyethylene bag and containing 5 kg of sieved air-dry soil. Soil was supplemented with essential elements (mg kg⁻¹ soil): N (33.3), P (20.5), S (34), Ca (41), Mg (3.95), Mn (3), Zn (2), Cu (0.5), B (0.1), Co (0.1) and Mo (0.08). Potassium was applied as potassium chloride (fertiliser used in pastures in south-western Australia, [6]) at four rates (0 (K0), 40 (K40), 80 (K80) and 120 (K120) mg K kg⁻¹ soil). The K rates and basal nutrients were applied following our published study [37]. Nitrogen was top-dressed at 33.3 mg N kg⁻¹ soil 4 weeks after sowing to ensure plant growth was not limited by N supply.

There were eight plants sown per pot, and the moisture content was maintained at 70% field capacity by frequent weighing and watering. Plants were grown in a temperature-controlled glasshouse at The University of Western Australia, Perth (31°58' S, 115°49' E) with the mean day and night temperatures of ~20 and 15 °C, respectively. The study used a randomised complete block design with four replications, and pots were re-randomised within the block periodically to minimise environmental influence. Plants were harvested 7 weeks after sowing at tillering (Biologische Bundesantalt Bundessortenamt and Chemische Industrie, BBCH 27 [38]).

2.1.2. Experiment 2 (Growth and Rhizosphere Carboxylates under N and K Fertilisation)

Two species, tall fescue and tall wheatgrass, were selected from experiment 1 (based on the highest and lowest, respectively, rhizosphere concentration of malate, with no difference in citrate exudation) to assess their growth and root exudation under N and K fertilisation.

The soil used was the same sandy soil as in experiment 1. Air-dried soil (3.5 kg) was placed in pots (150 mm diameter and 170 mm deep). All pots received the same basal fertilisation as described above, but without N. There were four treatments: no N and K (–N–K), 81 mg N kg⁻¹ soil but no K (+N–K), 80 mg K kg⁻¹ soil but no N (+K–N), and 81 mg N kg⁻¹ and 80 mg K kg⁻¹ soil (+N+K). Nitrogen was applied as ammonium nitrate and K as KCl. The N rate was adapted from the published study [39], and the K rate was selected from experiment 1.

Each pot had five plants. The same experimental design was used, and similar growth conditions were maintained as in experiment 1. There were two harvests, 30 (BBCH 22) and 60 (BBCH 27) days after sowing.

2.2. Carboxylate Extraction

In each harvest, the shoots were cut above the soil surface. The protocol for carboxylate extraction was the same as routinely followed [17,40,41]. Briefly, plants with intact roots were lifted cautiously, soil loosened and bulk soil removed through gentle shaking. Roots with the attached rhizosphere soil [42] were then immersed into a 500 mL beaker containing 20 to 90 mL of 0.2 mM CaCl₂ depending on root size. Immersion lasted for about 1 min with frequent shaking to increase the amount of rhizosphere soil removed. Roots were then taken out, and a subsample of the suspension was filtered (0.45 µm) into a 1 mL HPLC vial. Each vial was acidified with 20 µL of concentrated orthophosphoric acid. The filled vials were placed on ice during extraction and stored at –20 °C until analysis.

2.3. Plant Nutrient Measurements

The roots were washed in running water to remove the remaining soil particles and were subsampled for assessment of AM colonisation. The shoots and remaining roots were oven-dried at 65 °C for 3 days and weighed.

Dried shoots (about 25 mg) were digested in a mixture of concentrated HNO₃ and HClO₄, and nutrient concentrations were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (Optima 5300 DV; Perkin-Elmer Inc., Waltham, MA, USA) as described elsewhere [37].

The shoot N concentration was measured by a Vario Macro CNS Analyser (Hanau, Germany) through dry total combustion and thermal conductivity [42]. Nutrient contents were calculated as the product of dry masses and the corresponding concentrations.

2.4. Arbuscular Mycorrhizal Colonisation

The sub-sampled roots were cut into about 1 cm long pieces and stained as we described previously [43]. The roots were then dispersed in a Petri dish, and colonisation percent was assessed using the gridline intercept method under an optical microscope for at least 200 intersections per sample [44]. The roots were considered colonised when any of the arbuscular mycorrhizal fungal parts (hyphae, arbuscules, vesicles or spores) were detected.

2.5. Carboxylate Analysis

The procedure for rhizosphere carboxylates was adapted from [45] with the exception of oxalate that was determined as described by [46]. The carboxylates were detected by relating the retention times and absorption spectra of samples with those of working standards (acetic, citric, *cis*-aconitic, fumaric, malic, maleic, malonic, and *trans*-aconitic acids). The total carboxylates represent the sum of those detected in the rhizosphere soil.

2.6. Statistical Analyses

In both experiments, the effect of main factors (grass species and treatments) and their interaction on different measured variables were analysed by two-way ANOVA in Genstat 19 (VSN International Ltd., Rothamsted, Harpenden, UK), with log-transformation of data where required to ensure homogeneity of variances. The complete data set was presented when the two-way interaction was significant at $p \leq 0.05$. Otherwise, the significant main effects were presented by pooling the data across the grass species or treatments. Means are presented with standard errors, separated by Tukey's honest significance difference (HSD) when there were significant main effects or interaction.

3. Results

3.1. Plant Growth

The three species differed in experiment 1, with veldt grass producing significantly higher shoot dry biomass, and tall wheatgrass having significantly greater root biomass than the other two species (Table 1).

Table 1. Shoot and root dry weight (dw), shoot P and K concentrations and contents, and arbuscular mycorrhizal (AM) colonisation in three temperate pasture grasses as influenced by the significant main effects of species and K rates in experiment 1. Means \pm SE. Means within the columns followed by different letters differ significantly. Two-way ANOVA showed significant main effects of species and K rates ($p \leq 0.05$), but no significant interaction. The data on individual species were averaged across four K rates ($n = 16$), and the data on K rates were averaged across the three species ($n = 12$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant.

Species	Shoot Concentration (g kg ⁻¹ dw)				Shoot Content (mg plant ⁻¹)		AM Colonisation (%)
	Shoot dw (g plant ⁻¹)	Root dw (g plant ⁻¹)	P	K	P	K	
Tall fescue	0.71 \pm 0.01 b	0.28 \pm 0.01 b	4.30 \pm 0.09 a	38.50 \pm 0.7 a	3.06 \pm 0.1 b	28.22 \pm 0.9 a	12.27 \pm 0.8 b
Veldt grass	0.78 \pm 0.02 a	0.30 \pm 0.01 b	4.53 \pm 0.09 a	31.17 \pm 0.7 b	3.47 \pm 0.1 a	24.18 \pm 0.9 b	8.85 \pm 0.8 c
Tall wheatgrass	0.70 \pm 0.02 b	0.35 \pm 0.01 a	3.92 \pm 0.09 b	37.23 \pm 0.7 a	2.74 \pm 0.1 b	25.90 \pm 0.9 ab	16.70 \pm 0.8 a
<i>p</i> value	0.001 ***	0.03 *	0.001 ***	0.001 ***	0.001 ***	0.02 *	0.001 ***
K rates							
K0	0.69 \pm 0.02 b	0.28 \pm 0.01 b	4.31 \pm 0.1 ab	12.15 \pm 0.8 d	2.96	8.10 \pm 1.0 c	18.72 \pm 0.9 a
K40	0.73 \pm 0.01 b	0.33 \pm 0.01 a	4.13 \pm 0.1 ab	32.04 \pm 0.8 c	2.99	23.10 \pm 1.0 b	11.15 \pm 0.9 b
K80	0.80 \pm 0.02 a	0.33 \pm 0.01 a	4.03 \pm 0.1 b	45.16 \pm 0.8 b	3.30	36.56 \pm 1.1 a	10.81 \pm 1.0 b
K120	0.71 \pm 0.02 b	0.28 \pm 0.01 b	4.53 \pm 0.1 a	53.19 \pm 0.8 a	3.11	36.61 \pm 1.0 a	9.75 \pm 1.0 b
<i>p</i> value	0.001 ***	0.04 *	0.01 **	0.001 ***	0.22ns	0.001 ***	0.001 ***

Note—Critical shoot K concentration for tall fescue is 24–28 g kg⁻¹ dry weight [47].

Different treatments also had a significant effect on shoot and root production. In experiment 1, the K80 treatment produced higher shoot biomass than the other K rates, whereas K40 and K80 treatments produced significantly higher root weight than the K0 and K120 treatments (Table 1).

In experiment 2, shoot dry weight of tall fescue was significantly higher than that of tall wheatgrass in both growth periods (Figure 1A). The same trend was observed in root production at 60 DAS (Figure 1B), but there was no difference at 30 DAS (data not shown).

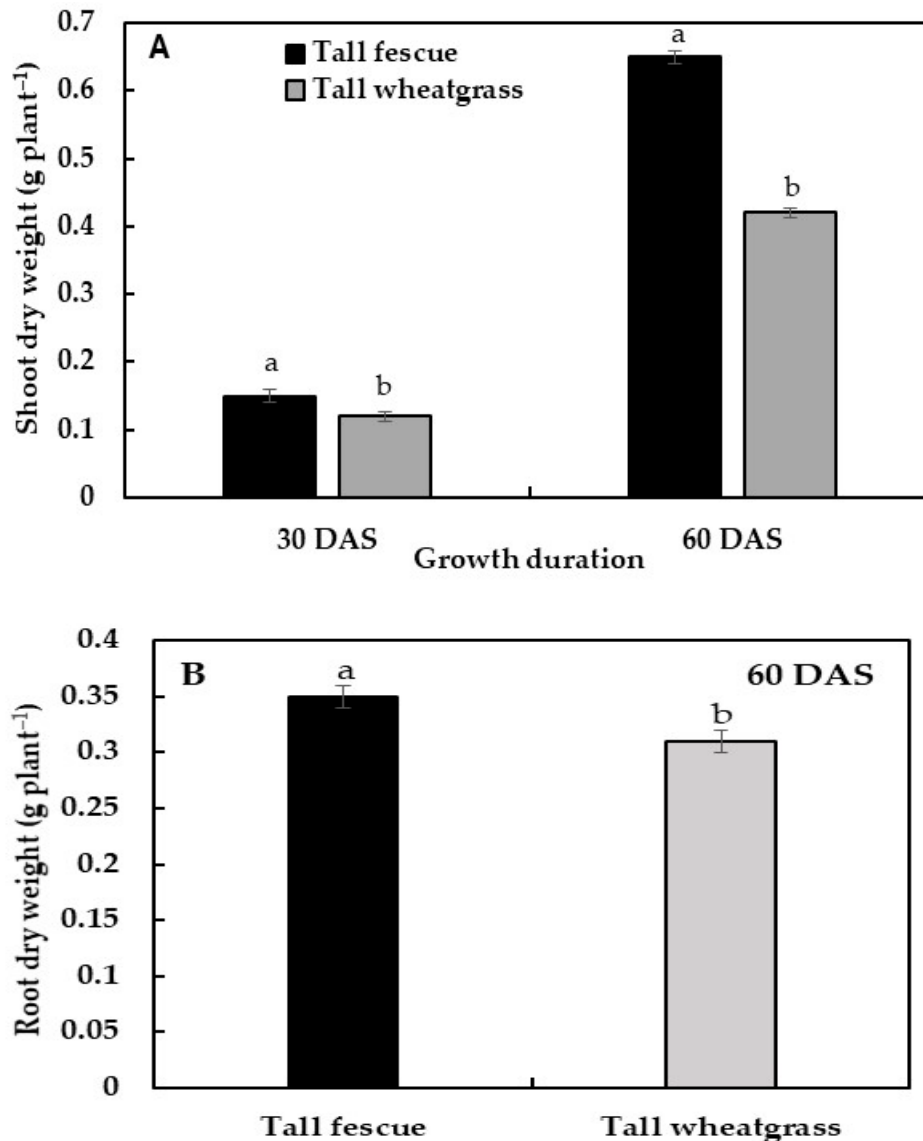


Figure 1. Dry weights of shoots (A) and roots (B) in experiment 2 (mean \pm SE). Means followed by different letters indicate significant differences for a given growth duration. Two-way ANOVA showed significant main effects of species (except for root dry weight in 30 DAS) and treatment, but the interaction was not significant. The data on individual species were averaged across the four fertilisation treatments ($n = 16$). DAS, days after sowing.

In experiment 2, plants receiving +N+K had the highest shoot growth in both growth periods (Figure 2A). Nitrogen application alone (+N-K) stimulated shoot production at both harvests compared to -N-K and -N+K (Figure 2A). Regarding root growth, plants in the -N+K treatment produced significantly greater biomass than +N-K at 30 DAS, whereas at 60 DAS the only difference was the +N+K treatment producing the highest root growth (Figure 2B).

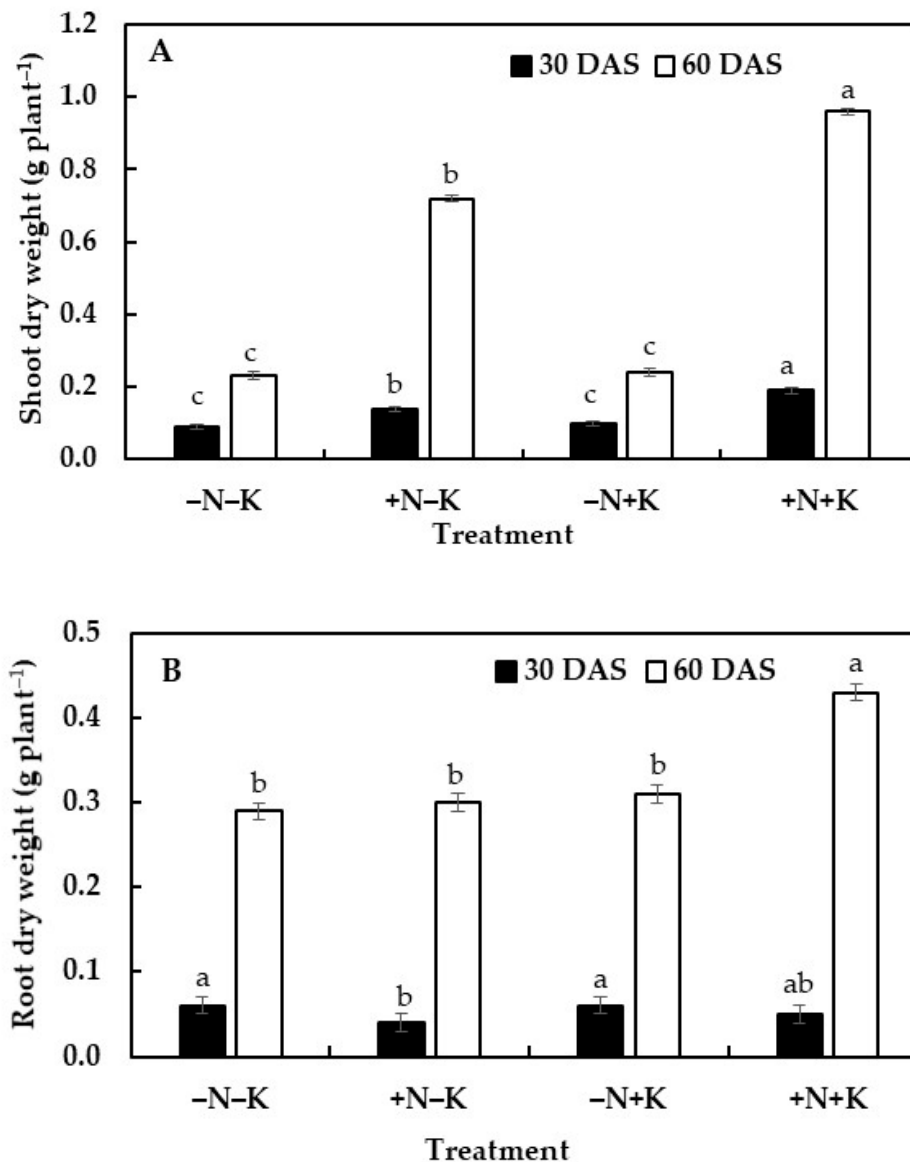


Figure 2. Dry weights of shoots (A) and roots (B) in two temperate perennial pasture grasses (tall fescue and tall wheatgrass) in experiment 2 (mean \pm SE). Means followed by different letters indicate significant differences for a given growth duration. Two-way ANOVA showed significant main effects of treatment and species, but the interaction was not significant. The data on individual treatments were averaged across the two species ($n = 8$). DAS, days after sowing.

3.2. Nutrient Accumulation

In experiment 1, pasture species differed in shoot K concentration and content, with tall fescue having the significantly higher concentration and content than the veldt grass (Table 1). Conversely, veldt grass had significantly higher shoot P concentration than tall wheatgrass, and shoot P content than both tall fescue and tall wheatgrass. The K fertilisation rates also had a significant influence on K concentration with a saturable increase noted with increasing K rates. In addition, higher shoot K content was observed in the K80 and K120 treatments than at K0 and K40 (Table 1).

In experiment 2, species differed in shoot N and K concentrations at 60 DAS and shoot N and K contents at both harvests. Tall fescue had higher N and K content than tall wheatgrass in both growth periods (Table 2). At 60 DAS, tall fescue also had higher N concentration than tall wheatgrass, but the reverse was recorded for K concentration.

Table 2. Shoot N, P and K concentrations and contents in two temperate pasture grasses as influenced by the main effects of species and treatments in experiment 2. Means \pm SE. Means within the columns followed by different letters differ significantly. Two-way ANOVA showed significant main effects of species and treatments, but the interaction was not significant. The data on individual species were averaged across four different treatments ($n = 16$), and the data on individual treatments were averaged across the two species ($n = 8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. DAS, days after sowing; dw, dry weight; ns, not significant.

Species	30 DAS						60 DAS					
	Shoot Concentration (g kg ⁻¹ dw)			Shoot Content (mg plant ⁻¹)			Shoot Concentration (g kg ⁻¹ dw)			Shoot Content (mg plant ⁻¹)		
	N	P	K	N	P	K	N	P	K	N	P	K
Tall fescue	54.3	5.4	39.8	8.5 \pm 0.3 a	0.8 \pm 0.04 a	5.9 \pm 0.3 a	36.9 \pm 0.6 a	3.6 \pm 0.4 b	23.3 \pm 0.4 b	24.0 \pm 0.5 a	2.2 \pm 0.1 a	15.9 \pm 0.2 a
Tall wheatgrass	57.2	5.3	39.1	6.6 \pm 0.4 b	0.6 \pm 0.04 b	4.6 \pm 0.2 b	28.8 \pm 0.5 b	5.0 \pm 0.4 a	29.4 \pm 0.5 a	19.0 \pm 0.5 b	1.8 \pm 0.1 b	13.3 \pm 0.2 b
<i>p</i> value	0.5 ns	0.9 ns	0.4 ns	0.01 **	0.001 ***	0.003 **	0.01 **	0.01 **	0.001 ***	0.001 ***	0.002 **	0.001 ***
Treatment												
-N-K	42.2 \pm 1.5 b	5.2	25.5 \pm 1.0 b	4.1 \pm 0.5 b	0.5 \pm 0.05 c	2.5 \pm 0.3 c	17.4 \pm 0.7c	5.6 \pm 0.6 a	16.2 \pm 0.6c	3.9 \pm 0.7 c	1.2 \pm 0.1 b	3.7 \pm 0.3 c
+N-K	69.9 \pm 1.5 a	5.5	23.5 \pm 1.0b	9.7 \pm 0.5 a	0.8 \pm 0.05 ab	3.1 \pm 0.3 c	51.8 \pm 0.8a	3.7 \pm 0.5 ab	6.7 \pm 0.7 d	36.5 \pm 0.8 b	2.7 \pm 0.16 a	4.6 \pm 0.3 c
-N+K	44.5 \pm 1.7 b	5.2	53.7 \pm 1.0 a	4.8 \pm 0.6 b	0.5 \pm 0.05 bc	5.7 \pm 0.3 b	18.3 \pm 0.7c	4.6 \pm 0.5 ab	38.3 \pm 0.6 b	4.3 \pm 0.7 c	1.1 \pm 0.1 b	9.4 \pm 0.3 b
+N+K	66.4 \pm 1.5 a	5.6	54.9 \pm 1.2 a	11.6 \pm 0.5 a	1.0 \pm 0.06 a	9.8 \pm 0.4 a	43.9 \pm 0.8b	3.1 \pm 0.6 b	44.0 \pm 0.7 a	41.2 \pm 0.8 a	2.9 \pm 0.1 a	40.6 \pm 0.3 a
<i>p</i> value	0.001 ***	0.1 ns	0.001 ***	0.001 ***	0.01 **	0.001 ***	0.001 ***	0.04 *	0.001 ***	0.001 ***	0.001 ***	0.001 ***

The shoot N and K accumulation was also governed by the treatment and plant age. At 30 DAS, the application of only N or K resulted in similar concentration and content as in the +N+K treatment (except K content), but significantly higher than in the treatments not receiving a respective nutrient (Table 2). However, at 60 DAS, the +N+K treatment resulted in the highest shoot N content, as well as shoot K concentration and content (Table 2).

3.3. Rhizosphere Carboxylates

Acetate, citrate, malate, oxalate and *trans*-aconitate were the major carboxylates exuded with the exception of acetate and oxalate (and *trans*-aconitate in case of tall fescue) in experiment 2. In experiment 1, tall fescue released significantly ($p \leq 0.05$) more acetate than tall wheatgrass, whereas the reverse was true for malate exudation (Table 3). Regarding citrate, tall wheatgrass released significantly higher amount than veldt grass. Veldt grass, on the other hand, had higher total carboxylate concentration than tall fescue. Some of the exudates were highly species-specific, such as oxalate in veldt grass and *trans*-aconitate in tall wheatgrass (Table 3).

Table 3. Rhizosphere carboxylates in three temperate pasture grasses as influenced by the significant main effects of species and K rates in experiment 1. Means \pm SE. Means within the columns followed by different letters differ significantly. Two-way ANOVA showed significant main effects of species and K rates ($p \leq 0.05$), but the interaction was not significant. The data on individual species were averaged across four different K rates ($n = 16$), and the data on K rates were averaged across the three species ($n = 12$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant.

Species	Carboxylate Concentration ($\mu\text{mol g}^{-1}$ dry root)					
	Acetate	Citrate	Malate	Oxalate	<i>Trans</i> -Aconitate	Total
Tall fescue	3.41 \pm 0.6 a	0.64 \pm 0.2 ab	2.15 \pm 0.8 b	0.00 \pm 0.3 b	0.00 \pm 0.1 b	6.20 \pm 0.9 b
Veldt grass	1.42 \pm 0.6 ab	0.46 \pm 0.2 b	4.64 \pm 0.8 ab	3.08 \pm 0.3 a	0.00 \pm 0.1 b	9.60 \pm 0.9 a
Tall wheatgrass	0.00 \pm 0.6 b	1.10 \pm 0.2 a	5.34 \pm 0.9 a	0.00 \pm 0.3 b	1.74 \pm 0.1 a	8.18 \pm 0.9 ab
<i>p</i> value	0.002 **	0.03 *	0.03 *	0.001 ***	0.001 ***	0.04 *
K rates						
K0	0.13 \pm 0.6 b	0.48	4.09 \pm 1.01 a	1.04	0.50	6.24 \pm 1.1 b
K40	2.86 \pm 0.6 a	0.81	5.69 \pm 1.01 a	0.70	0.72	10.78 \pm 1.1 a
K80	0.96 \pm 0.6 ab	0.92	4.50 \pm 1.01 a	1.11	0.59	8.08 \pm 1.1 ab
K120	2.49 \pm 0.7 ab	0.69	1.88 \pm 1.06 b	1.26	0.50	6.82 \pm 1.2 ab
<i>p</i> value	0.02 *	0.39 ns	0.05 *	0.65 ns	0.59 ns	0.05 *

The K rates also had a significant impact on the carboxylate exudations. At K40, exudation of acetate and malate was significantly higher ($p \leq 0.05$) than in the K0 treatment, and also exudation of malate compared with the K120 treatment (Table 3). The K40 treatment also had significantly higher total carboxylates than the K0 treatment.

The two species did not differ in root exudations at 30 DAS in experiment 2 except for *trans*-aconitate, which was exuded only by tall wheatgrass (Table 4) as also observed in experiment 1 (Table 3). However, the treatments had a significant impact on the exudation of other carboxylates at 30 DAS. The highest exudation of individual and total carboxylates was recorded in the -N-K treatment (Table 4). In contrast, treatments receiving either N (+N-K) or N with K (+N+K) released only some citrate and malate having the lowest total carboxylates (Table 4). Application of K alone (-N+K) appeared to have favoured to some extent root exudation at 30 DAS.

Table 4. Rhizosphere carboxylates in two temperate pasture grasses as influenced by the significant main effects of species and treatments at 30 DAS in experiment 2. Means \pm SE. Means within the columns followed by different letters differ significantly. Two-way ANOVA showed the significant main effects of species and treatments ($p \leq 0.05$), but the interaction was not significant. The data on individual species were averaged across four different treatments ($n = 16$), and the data on individual treatment were averaged across the two species ($n = 8$). *** $p < 0.001$. ns, not significant.

Carboxylate Concentration ($\mu\text{mol g}^{-1}$ dry root)				
Species	Citrate	Malate	Trans-Aconitate	Total
Tall fescue	1.48	4.71	0.00 \pm 0.4 b	6.19
Tall wheatgrass	1.14	3.67	3.50 \pm 0.3 a	8.31
<i>p</i> value	0.15 ns	0.19 ns	0.001 ***	0.09 ns
Treatments				
-N-K	3.58 \pm 0.2 a	15.53 \pm 0.6 a	5.12 \pm 0.5 a	24.43 \pm 1.3 a
+N-K	0.00 \pm 0.2 c	0.00 \pm 0.6 b	0.20 \pm 0.5 b	0.20 \pm 1.3 c
-N+K	1.67 \pm 0.3 b	1.25 \pm 0.7 b	1.25 \pm 0.5 b	4.17 \pm 1.4 b
+N+K	0.00 \pm 0.2 c	0.00 \pm 0.6 b	0.35 \pm 0.5 b	0.35 \pm 1.3 c
<i>p</i> value	0.001 ***	0.001 ***	0.001 ***	0.001 ***

At the later growth stage (60 DAS), there was the significant interaction between species and treatments in the individual as well as total carboxylates. In the +N+K treatment, tall fescue released significantly higher amount of citrate than in any other treatment (also higher than tall wheatgrass at +N+K) (Figure 3). Malate exudation followed a similar pattern, with tall fescue releasing higher amount in the +N+K treatment than the other nutrient treatments or all tall wheatgrass treatments. In contrast, *trans*-aconitate was confined only to tall wheatgrass, with -N-K plants releasing a significantly higher amount than in the other treatments (Figure 3). Tall fescue exuded higher total carboxylates in the +N+K than any other treatments, except for tall wheatgrass receiving -N-K (Figure 3).

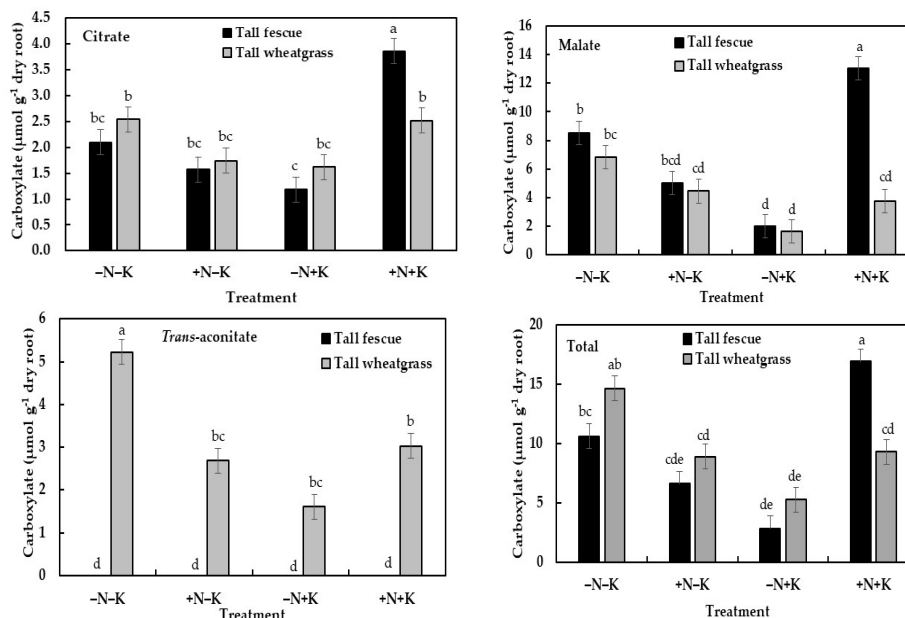


Figure 3. Effect of N and K applications on rhizosphere carboxylate (citrate, malate, *trans*-aconitate, total carboxylates) exudation in two temperate perennial pasture grasses (tall fescue and tall wheatgrass), at 60 DAS in experiment 2 (means \pm SE, $n = 4$). Means denoted by different letters indicate significant differences. Two-way ANOVA showed significant interaction ($p \leq 0.05$) between species and fertilisation treatment.

3.4. Arbuscular Mycorrhizal Colonisation

In experiment 1, tall wheatgrass had significantly higher AM colonisation than the other two species, and the added K treatments had lower colonisation than the control (K0) treatment (Table 1).

In experiment 2, tall wheatgrass had a higher AM colonisation than tall fescue at 30 DAS (Figure 4A). As observed in experiment 1, plants treated with K only (−N+K) had low AM colonisation in experiment 2 at 30 DAS, though significantly different from the N only (+N−K) treatment (Figure 4B). However, at 60 DAS, K supplied plants (with or without N) had significantly lower AM colonisation than in the −N−K treatment. The AM colonisation was constant in the −N−K treatment during plant growth, but there was a declining trend in other treatments as plants matured.

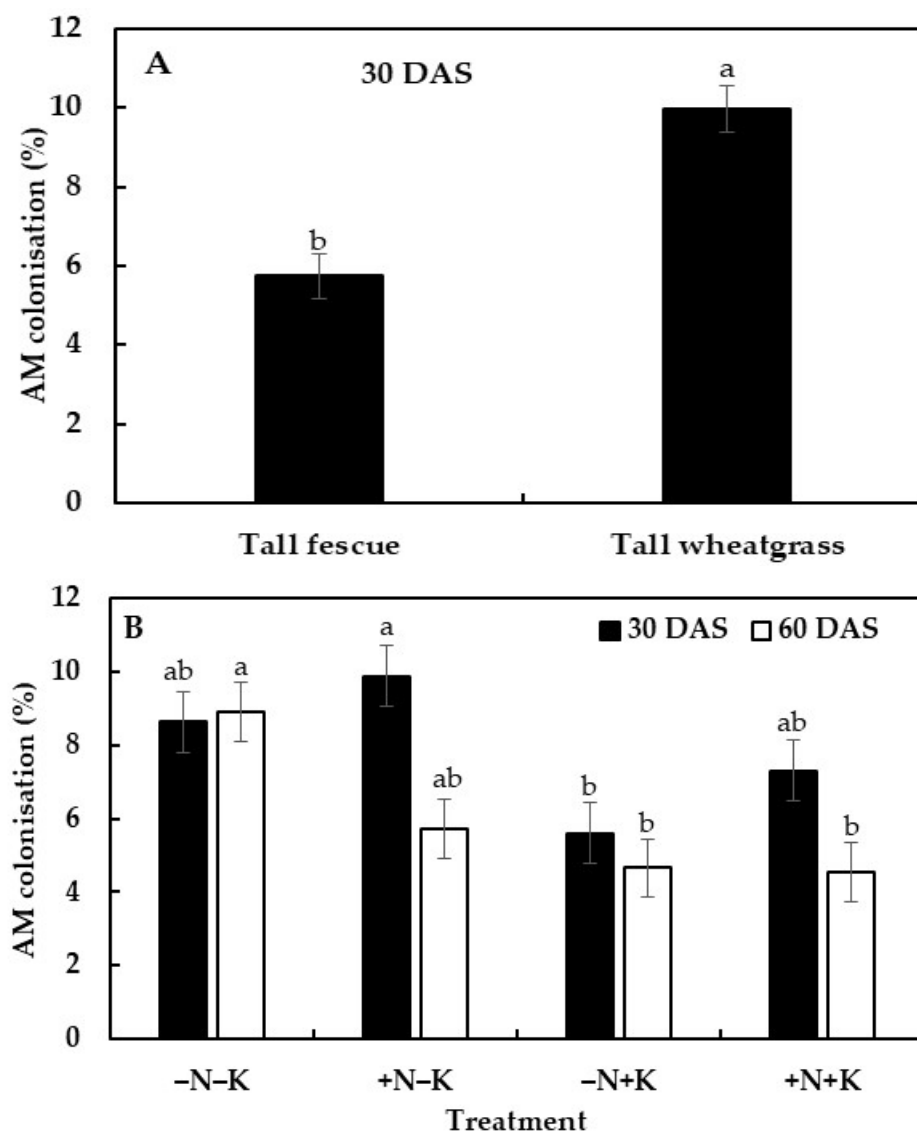


Figure 4. Arbuscular mycorrhizal (AM) colonisation in (A) two temperate perennial pasture grasses at 30 DAS, and as influenced by (B) different treatments at 30 DAS and 60 DAS in experiment 2 (mean \pm SE). Means followed by different letters indicate significant differences for a given growth duration. Two-way ANOVA showed the significant main effect of species (except at 60 DAS) and fertilization treatments in both growth periods, but the interaction was not significant. In (A), the data of individual species were averaged across four different treatments ($n = 16$), and in (B) the data of individual treatments were averaged across two different species ($n = 8$). DAS, days after sowing.

4. Discussion

A component in sustainable Australian farming system is the screening of pasture species for high productivity and wide adaptability [48]. Our studies reported here showed there could be differences in shoot yield among the temperate pasture grass species. The most productive shoot growth was by veldt grass in experiment 1 and tall fescue in experiment 2 (without veldt grass). The better performance of veldt grass when compared with four other pasture grasses (cocksfoot, phalaris (*Phalaris aquatica* L.), tall wheatgrass and tall fescue) supplied with the rock mineral fertiliser and microbial consortium inoculant was also reported in our previous study [32]. Veldt grass is considered to be hardy, having colonised the unproductive sites in the wheatbelt of Western Australia and other parts of Australia [49]. Similarly, tall fescue has a wide adaption to a diverse range of soils and climate, maintaining yield [50].

Across the grass species, highest shoot growth was found in the K80 treatment, and that of roots in the K40 treatment in experiment 1. However, in absolute terms, the yield differences among the treatments (even between K0 and K80) were not substantial (Table 1), and might have little practical relevance. Despite soil being categorised as low K, (Colwell soil test $<50 \text{ mg K kg}^{-1}$ soil for cereals [10] and pasture grass [6]), indicating K fertilizer application, our findings suggested that external K fertilisation may not always be necessary if soil had a considerable amount of native K. In annual ryegrass, the yield response to added K was reported in only 6 out of 100 experiments due to pasture grass roots exploring a large volume of soil and accessing native K effectively [6]. In our study, relatively high shoot K concentration (even in the K0 treatment) suggested that grasses used native soil K. Pasture grasses have long and thin roots [51,52], conducive to good K acquisition efficiency [20], and have relatively high internal K utilisation, even with a low amount of K extracted from soil [2]. Effective K utilisation is important for K efficiency in other *Poaceae* species too [37].

Even though K application alone had little effect on shoot and root growth, the K impact was substantial when applied in conjunction with N as evidenced in experiment 2. The combined application of N and K resulted in a significant increase in shoot production in both harvests compared to other treatments, including the one receiving N only. Our study recorded increased N and K shoot contents in the N and K combination (+N+K) treatment. Previous studies have also reported similar positive N-K interactions. Potassium greatly improved ammonium utilisation and led to yield increase when combined with ammonium-nitrate fertilisation [53]. Lack of K fertilisation limited N supply to plants, leading to a decrease in the N concentration in the xylem sap [54]. In maize, high yield was attributed to increased N absorption as mediated by K [55]. Similarly, K facilitated translocation of nitrogenous compounds from different plant parts to grains in wheat, resulting in high grain yield [56]. However, inherent soil nutrient supply, crop species and agronomic practices can potentially confound these significant N-K interactions [29].

Rhizosphere carboxylate release is often species-dependent because of differential organic anion preferences in maintaining their ionic balance [30]. Our study reported a significant difference between species in both experiments in the individual carboxylates, with some being highly species-specific. Malate and oxalate were the predominant carboxylates in veldt grass, and malate and *trans*-aconitate in tall wheatgrass (Table 3). Oxalate is considered to be a prime compound responsible for enhancing acquisition of nutrients such as P [27], and its occurrence only in veldt grass was coupled with high shoot P content and yield (Table 1). However, shoot K content was not elevated despite the general understanding that oxalate also improves K release from soil minerals. This could have been due to the short-term nature of the experiments reported here because the effect of carboxylates, including oxalate, was more pronounced in the long-term [22].

Carboxylate exudation changes with the plant developmental stage [33], soil type [13,15], nutritional status and plant species [12], making comparison among studies difficult. Within these confounding circumstances, our study noted that K rates had a significant influence only on exudation of acetate and malate. Smith [30] recorded the highest amounts of citrate and malate in the treatment without any added K in a green panic grass. Similar elevated exudation of these compounds along

with aconitate was reported at low K supply in maize [25]. Conversely, our results showed significantly lower acetate and total carboxylate concentrations in the rhizosphere in the K0 than the K40 treatment (Table 3). These discrepancies could be due to species difference and aforementioned confounding factors [12,33]. Carboxylate production is prevented by high shoot P concentration [40,57,58], which was above the critical value in all the K treatments in the current study (Tables 1 and 2). Investigation on how the exudation patterns are influenced by K rates under low P supply would be interesting to assess whether P is the primary determinant, or K itself has a specific role in carboxylate exudation.

Our study suggested that N fertilisation might be more important than K fertilisation regarding the carboxylates exuded, with an impact likely to be greater in the early growth stage. In the current study, N application resulted in no citrate and malate release in the early growth stage (30 DAS) irrespective of K fertilisation (Table 4). In contrast, citrate and malate were exuded in substantial amounts in the -N-K treatment and to a lesser extent in the -N+K treatment. However, in the later growth stage (60 DAS), these compounds were exuded in the +N treatments, with the highest amounts by tall fescue in the +N+K treatment (Figure 3). Our observation of citrate and malate as the major exudates in the later growth stage in experiment 2 closely resembled the data of [40], whereby citrate and malate were also the dominant rhizosphere carboxylates in other pasture grasses, phalaris and cocksfoot.

In the study presented here, AM colonisation was low (<20%) and similar to the observation made in the conventional farming system [59]. Our results indicated that grass species would not be dependent on AM for nutrient acquisition and hence yield production because the highest yielding species (veldt grass in experiment 1 and tall fescue in experiment 2) tended to have lowest AM colonisation (Table 1 and Figure 4A). Grasses, in general, are considered poorly dependent or non-dependent on AM [60] because nutrient uptake was not improved despite AM presence. Instead, fine roots and root hairs of grasses facilitate access to nutrients [61].

The literature on AM and K interaction is scanty, despite some reports suggesting positive association [62,63]. Our study indicated that AM colonisation might be decreased by K fertilisation in accordance with the previous studies [64,65]. However, when N and K were applied together, the colonisation effect was inconclusive and complex in both growth periods in our study, even though previous reports suggested N application did not significantly increase AM colonisation [59,66].

The inter-relationship between AM colonisation and rhizosphere carboxylate exudation has been considered in the past studies. About 52% decrease in carboxylate release was reported in the AM colonised plants as compared to non-colonised ones [42]. Similarly, in certain *Kennedia* species, AM inoculation resulted in up to 50% reduction in carboxylate exudation, likely due to high shoot P in the inoculated plants [58]. In contrast, our studies found no significant positive relationship between AM colonisation and total carboxylate exudation in both experiments ($r^2 = 0.21$ in experiment 1 and $r^2 = 0.10$ in experiment 2). Even though the role of P cannot be ruled out completely, the exuded amounts in our experiments (<20 $\mu\text{mol g}^{-1}$ dry root) were lower than those reported in *Kennedia* species [58], whereby the reduction was more pronounced in the species releasing high quantities (>80 $\mu\text{mol g}^{-1}$ dry root) of carboxylates.

5. Conclusions

Temperate perennial grass species can grow well in low-K soil, and may not necessarily depend on external K fertilisation or mycorrhizal symbiosis for nutrient acquisition. Different rates of K applications did not result in significant changes to carboxylate (citrate, malate and oxalate) exudation, except for decreased malate at the highest K rate (120 mg kg^{-1} soil). In the early growth stage, citrate and malate exudation was higher in the -N-K treatment, and the N application either with or without K suppressed exudation. However, in the later growth stage, application of both N and K was associated with the highest exudation of malate and citrate, particularly by tall fescue.

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and editing, S.T., Z.R., K.H.M.S. and Z.M.S.; supervision, Z.R., K.H.M.S. and Z.M.S. All authors have read and agreed to the published version of the manuscript.

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