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# Adaptive shoot and root responses collectively enhance growth at optimum temperature and limited phosphorus supply of three herbaceous legume species

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• *Background and Aims* Studies on the effects of sub- and/or supraoptimal temperatures on growth and phosphorus (P) nutrition of perennial herbaceous species at growth-limiting P availability are few, and the impacts of temperature on rhizosphere carboxylate dynamics are not known for any species.

• *Methods* The effect of three day/night temperature regimes (low, 20/13 °C; medium, 27/20 °C; and high, 32/25 °C) on growth and P nutrition of *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis* was determined.

• *Key Results* The highest temperature was optimal for growth of *C. cinereum*, while the lowest temperature was optimal for *K. nigricans* and *L. australis*. At optimum temperatures, the relative growth rate (RGR), root length, root length per leaf area, total P content, P productivity and water-use efficiency were higher for all species, and rhizosphere carboxylate content was higher for *K. nigricans* and *L. australis*. *Cullen cinereum*, with a slower RGR, had long (higher root length per leaf area) and thin roots to enhance P uptake by exploring a greater volume of soil at its optimum temperature, while *K. nigricans* and *L. australis*, with faster RGRs, had only long roots (higher root length per leaf area) as a morphological adaptation, but had a higher content of carboxylates in their rhizospheres at the optimum temperature. Irrespective of the species, the amount of P taken up by a plant was mainly determined by root length, rather than by P uptake rate per unit root surface area. Phosphorus productivity was correlated with RGR and plant biomass.

• *Conclusions* All three species exhibited adaptive shoot and root traits to enhance growth at their optimum temperatures at growth-limiting P supply. The species with a slower RGR (i.e. *C. cinereum*) showed only morphological root adaptations, while *K. nigricans* and *L. australis*, with faster RGRs, had both morphological and physiological (i.e. root carboxylate dynamics) root adaptations.

**Key words:** Australian native legumes, carboxylates, climate change, growth, perennial pastures, phosphorus, photosynthesis, root morphology, temperature, water-use efficiency.

## INTRODUCTION

Phosphorus (P) is frequently a limiting nutrient for crop production (Vance *et al.*, 2003; Simpson *et al.*, 2011). Demand for P in agriculture is increasing, and global P reserves are expected to be depleted in 50–100 years (Cordell *et al.*, 2009). Even though soil can hold large amounts of P, the plant-available fraction of P in soil is very low. The concentration of P in soil solution is partly determined by the solubility of soil phosphates (e.g. Ca-P, Al-P and Fe-P) (Elrashidi and Larsen, 1978). Calcium phosphates dominate in neutral–alkaline soils, and increase in solubility with decreasing pH. Conversely, P is bound to Fe and Al oxides in acid soils, and becomes increasingly soluble with increasing pH (Lindsay, 1979; Bolan *et al.*, 1987). One approach to address the limited P supply in natural and agricultural soils is to identify plants that can access sparingly soluble soil P efficiently (Lambers *et al.*, 2006), but ultimately all P that is removed through cropping will have to be replaced (Simpson *et al.*, 2011).

Plants have evolved numerous mechanisms to utilize relatively immobile, and often poorly available, P (e.g. $FePO_4$ in acidic soils) which allows them to acclimate to P deficiency (Vance *et al.*, 2003; Raghothama and Karthikeyan, 2005). Typical mechanisms by which they enhance P acquisition under low P availability include altered root physiology (Neumann and Martinoia, 2002), such as by enhanced exudation of carboxylates (e.g. malate and citrate) and phosphohydrolases (Richardson *et al.*, 2000; Wouterlood *et al.*, 2005), and altered root morphology, such as by increased root development, higher root:shoot ratios, finer roots, longer and more root hairs and the formation of arbuscular mycorrhizas, all of which facilitate exploration of a greater soil volume (Raghothama, 1999; Smith and Smith, 2011). Depending on the extent of these mechanisms, nutrient availability in the rhizosphere may change and affect plant nutrient uptake (Raynaud *et al.*, 2008). Apart from these mechanisms that enhance P uptake, plants also have adaptations efficiently to use P which is taken up. Phosphorus productivity ($P_{prod}$), i.e. the rate of biomass production per unit of P in the plant, is a

more important determinant of plant P use efficiency than the total amount of P taken up by a plant (Ericsson and Ingestad, 1988; Ryser *et al.*, 1997).

Temperature is a major factor determining the length of the growing season, net primary productivity and plant community composition and/or structure (Van Cleve *et al.*, 1981; Harte and Shaw, 1995; Thompson and Naeem, 1996). Moreover, under predicted climate change scenarios, such as increasing temperature, dramatic changes in plant growth and nutrient use responses are expected (Sinclair, 1992). Our knowledge of the impact of temperature, above the optimum range, on root growth and P dynamics is very limited, and contradictory results are reported in the literature. For example, for ponderosa pine seedlings (*Pinus ponderosa* Laws.), high temperature did not increase P uptake (Delucia *et al.*, 1997), while an increase in P uptake was found for muskmelons (*Cucumis melo* L.) (Stoltzfus *et al.*, 1998). Temperatures below the plant's optimum range usually result in reduced overall growth and increased relative investment of biomass in roots (Clarkson *et al.*, 1988). At low soil temperature, P uptake usually decreases more than the uptake of other nutrients (Bravo and Uribe, 1981; Engels *et al.*, 1992). This may be mainly due to reduced root growth, because at low temperatures P uptake is dominated by root production, rather than by chemical availability in soil or physiological uptake capacity (Mackay and Barber, 1984). To date, the way that temperatures either below or above the optimum range affect rhizosphere carboxylate concentration and composition, and thus impact on plant P uptake and growth, is not known for any plant species. When considering shoot responses, photosynthetic rate ($A$), stomatal conductance ($g_s$) and water use-efficiency (WUE) are affected by temperature (Comstock and Ehleringer, 1992; Lambers *et al.*, 2008; Wertin *et al.*, 2011). Moreover, a plant's WUE depends on both $A$ and $g_s$, and therefore WUE (i.e. $A/g_s$) is a better indicator of a plant's physiological adaptability to change in response to changes in environmental temperature than $A$ or $g_s$ alone.

Australian native perennial legumes are likely to have evolved in P-impoverished environments (Beadle, 1966; Handreck, 1997), and are now considered to have potential for development as pasture legumes (Dear *et al.*, 2007; Pang *et al.*, 2010; Suriyagoda *et al.*, 2010b, 2011). Incorporation of native species into Australian agricultural systems may result in more P-efficient agricultural systems, greater plant diversity in agroecosystems and, presumably, wider adaptation to diverse climatic and soil conditions (Cocks, 2001; Suriyagoda *et al.*, 2010a, 2011; Bennett *et al.*, 2011; Lambers *et al.*, 2011). However, the responses of these species to the range of temperatures found under Australian farming conditions are not known. Therefore, the objective of the present study was to investigate the relative change in root morphology, growth and rhizosphere carboxylate dynamics (both concentration and composition) of Australian native perennial legumes with pasture potential when grown below or above their optimal temperature at a low soil P supply. We used relatively insoluble ('plant-unavailable') $FePO_4$ as the source of P; therefore, plants would need to respond morphologically and/or physiologically to take up this sparingly soluble source of P. Moreover, under predicted climate change scenarios, an increase in temperature during the growing season is likely.

However, the ability of relatively insoluble P sources such as $FePO_4$ to supply P for plant uptake at elevated temperatures is largely unknown. We hypothesized that (1) at sub- or supraoptimal temperatures, plants would produce relatively less root biomass (dry weight), have shorter and thicker roots (i.e. low root surface area per unit root biomass), and have a lower root mass ratio (RMR; i.e. the ratio of root dry weight to total dry weight) than at optimal temperature; (2) tissue P concentration, $P_{prod}$ and amount of rhizosphere carboxylates would be higher at optimal temperatures than at sub- or supraoptimal temperatures; (3) a reduction in total plant P content (mg P per plant) at sub- or supraoptimal temperatures would be mainly due to a reduced uptake rate (mg P $cm^{-2}$ root $d^{-1}$), rather than a reduced root length (i.e. morphological adaptation), because physiological adaptations are less costly than morphological adaptations; and (4) WUE would be lower at sub- or supraoptimal temperatures than that at optimal temperatures, and this would be partly due to a low photosynthetic rate ($A$).

## MATERIALS AND METHODS

### Selection of species and accessions

*Cullen cinereum* (Lindl.) J.W.Grimes accession Fortescue was collected from the Fortescue River Valley, Western Australia (21°29'S, 116°14'E) where the mean monthly minimum and maximum temperatures are 20 and 32 °C, respectively. Moreover, in an ecogeographical survey to find the adaptability of a range of *Cullen* spp., Bennett *et al.* (2011) found that *C. cinereum* is highly adapted to warmer regions of Australia. In contrast, *Kennedia nigricans* Lindl. accession NS19026 was collected from the Fitzgerald River National Park, Western Australia (33°75'S, 119°75'E), where the mean monthly minimum and maximum temperatures are 11 and 20 °C, respectively. *Lotus australis* accession SA33610 was collected from Sellicks Beach, South Australia (35°55'S, 138°62'E), where the mean monthly minimum and maximum temperatures are 12 and 21 °C, respectively. Thus, both *K. nigricans* accession NS19026 and *Lotus australis* Andrews accession SA33610 naturally grow in cooler habitats than does *C. cinereum* accession Fortescue.

### Growth conditions

*Cullen cinereum* accession Fortescue, *Kennedia nigricans* accession NS19026 and *Lotus australis* accession SA33610 were grown in 45 cm tall, 10 cm diameter vertically split pots in a growth room. There were three temperature treatments: 20/13, 27/20 and 32/25 °C (day/night). Daylength was 12 h with 240 µmol $m^{-2}$ $s^{-1}$ intensity. Daily average relative humidity was 20–30 %. Eight replicate pots of each species and four replicate control pots without plants were established with 5·8 kg per pot of thoroughly washed, steam-sterilized dried river sand. Basic nutrient concentrations in sand are given in Table 1. Ammonium and nitrate-N concentrations were determined as described by Searle (1984), available P and potassium (K) were measured using the Colwell test (Colwell, 1965; Rayment and Higginson, 1992), available sulfur (S) according to Blair *et al.* (1991), organic carbon according to Walkley

TABLE 1. *Chemical characteristics of the river sand used in the experiment*

| | Mean ± s.e. |
|---|---|
| $NH_4$-N (mg kg$^{-1}$) | 1 ± 0·01 |
| $NO_3$-N (mg kg$^{-1}$) | 1 ± 0·01 |
| Bicarbonate-extractable K (mg kg$^{-1}$) | 17 ± 1·2 |
| Bicarbonate-extractable P (mg kg$^{-1}$) | 2 ± 0·01 |
| Available S (mg kg$^{-1}$) | 4·6 ± 0·34 |
| Organic carbon (g kg$^{-1}$) | 0·006 ± 0·001 |
| Conductivity (dS m$^{-1}$) | 0·018 ± 0·003 |
| pH ($CaCl_2$) | 6·1 ± 0·12 |
| pH ($H_2O$) | 6·7 ± 0·09 |

TABLE 2. *Number of days required for plants of each species to reach the first and second harvest (i.e. when they had produced three and ten fully expanded leaves on the main stem, respectively) at low, medium and high temperatures*

| Species | Low (20/13 °C) | | Medium (27/20 °C) | | High (32/25 °C) | |
|---|---|---|---|---|---|---|
| | First harvest | Second harvest | First harvest | Second harvest | First harvest | Second harvest |
| *Cullen cinereum* | 58 | 142 | 24 | 110 | 23 | 92 |
| *Kennedia nigricans* | 38 | 82 | 24 | 86 | 43 | 98 |
| *Lotus australis* | 48 | 80 | 36 | 86 | 33 | 87 |

and Black (1934), with the remainder of the analyses using protocols outlined in Rayment and Higginson (1992). All essential nutrients, excluding P, were provided by amending the sand with 126·6 mg kg$^{-1}$ Ca(NO$_3$)$_2$·4H$_2$O, 42·8 mg kg$^{-1}$ NH$_4$NO$_3$, 178 mg kg$^{-1}$ K$_2$SO$_4$, 101 mg kg$^{-1}$ MgSO$_4$·7H$_2$O, 11 mg kg$^{-1}$ CaCl$_2$·2H$_2$O, 12 mg kg$^{-1}$ MnSO$_4$·H$_2$O, 8·8 mg kg$^{-1}$ ZnSO$_4$·7H$_2$O, 1·96 mg kg$^{-1}$ CuSO$_4$·5H$_2$O, 0·68 mg kg$^{-1}$ H$_3$BO$_3$, 1·01 mg kg$^{-1}$ NaMoO$_4$·2H$_2$O and 32·9 mg kg$^{-1}$ FeNaEDTA. P was supplied with 60 mg P kg$^{-1}$ dry soil using FePO$_4$. At 32/25 °C only, an additional four pots for each species were established with 60 mg P kg$^{-1}$ dry soil of KH$_2$PO$_4$ as the P source while other nutrients and management were unchanged. This allowed us to confirm that the plants supplied with FePO$_4$ were P limited at the higher temperatures; this has already been established at lower temperatures (Lambers *et al.*, 2002; Pearse *et al.*, 2007; Shane *et al.*, 2008). Seeds of each species were mechanically scarified and allowed to imbibe, before being sown in seedling trays at staggered times according to their pre-determined germination times. The experiment was carried out in controlled-environment chambers at the University of Western Australia, Perth. Pots were re-randomized every day. Three seedlings were planted in each pot and thinned to one plant at 1 week. An additional 100 mL of a 2 mM NH$_4$NO$_3$ solution was twice added to each pot; when plants had produced five and eight fully expanded main stem leaves. Four replicates of each species were harvested twice; when plants had produced three and ten fully expanded leaves on their main stem. KH$_2$PO$_4$-treated plants in the 32/25 °C treatment were harvested when plants had produced ten fully expanded leaves on their main stem. Therefore, days to harvest differed among species and temperature treatments (Table 2). Control (i.e. plant-free) pots were harvested when the second harvest was made for each temperature treatment. Pots were maintained at field capacity. Any drained water from the bottom of each pot was collected into buckets and topped up regularly.

### Physiological measurements

The photosynthetic rate (*A*) and $g_s$ were measured on the youngest fully expanded leaf of all plants, using a portable gas-exchange system (LI6400 portable, LiCor Inc., Lincoln, NE, USA) equipped with a light source (6400-02B LED, LiCor). Measurements were taken 1 week before the final harvest. Photosynthetic photon flux density at the leaf surface

was maintained at 1500 $\mu$mol m$^{-2}$ s$^{-1}$ during the measurement of *A*. Leaf temperature and ambient humidity of the incoming air to the leaf chamber was left at that of the growth room environment. *A* was measured when the ambient CO$_2$ concentration of the incoming gas stream was 380 $\mu$mol mol$^{-1}$, a value close to that during plant growth.

### Plant analyses

Due to the fact that leaf appearance and expansion rates of the three species under the three temperature treatments differed, time between the start of the experiment and harvest varied. At each harvest, shoots were severed at the base of the stem, and leaf area (both green and dead leaves) of each plant determined with a LiCor LI-3000 Portable Area Meter, which was equipped with a LI-3050A Transparent Belt Conveyer Accessory (LiCor). Root systems were gently removed from the bulk soil, shaken slightly to remove the excess soil and the remaining soil was defined as rhizosphere soil (Veneklaas *et al.*, 2003). Root systems were gently dipped in a 200 mL vial of 50 mL of a 0·2 mM CaCl$_2$ solution and carefully washed until as much rhizosphere soil as possible was removed. Care was taken to minimize root damage. A sub-sample of the rhizosphere extract was then filtered using a 0·2 $\mu$m syringe filter into a 1 mL HPLC vial. The vial was acidified with one drop of concentrated phosphoric acid, placed on dry ice and transferred to a −20 °C freezer until HPLC analysis. Detailed methodology of the carboxylate analysis is given in Suriyagoda *et al.* (2010b). The root systems were then washed more thoroughly to remove any residual soil. The length, diameter and area of the collected roots were measured using the commercial software package WinRHIZO 4·1 (Regent Instruments Inc., Quebec, Canada, 2000). Root, stem and leaf samples of each plant were then dried at 60 °C for 1 week and weighed. The RMR was calculated as the ratio between root dry weight and total dry weight of a plant. As the root, stem and leaf dry weight of each plant was very small, those components were combined and ground together in a steel ball mill. For analysis of tissue P concentration, approx. 100 mg of sub-sample was taken and digested in nitric/perchloric acid and analysed using the molybdate and malachite green method (Motomizu *et al.*, 1983). Data from

the first and second harvest were used to calculate the mean P uptake rate (PUR) and mean relative growth rate (RGR) for each treatment combination. The mean PUR (mg P $cm^{-2}$ root $d^{-1}$) for each treatment combination was calculated by taking the difference between the total amounts of P present in plant tissues (i.e. leaves, stems and roots) at the second and first harvests and dividing that value by the difference between the average root areas at the two harvests and the time gap between the two harvests. The mean RGR (mg $g^{-1}$ $d^{-1}$) of each treatment combination during the harvest period was calculated as the slope of the least squares linear regression fit of the log-transformed dry weight data against time (Hoffmann and Poorter, 2002). The ability of plants to produce dry matter per unit of time and unit of P is called the phosphorus productivity ($P_{prod}$) (Ericsson and Ingestad, 1988). It can be calculated using the formula

$$P_{prod} = (e^{RGR/2400} - 1) \times 1000/P_{final}$$

where $P_{final}$ is the final P concentration (mg P $g^{-1}$ d. wt). Phosphorus productivity is then expressed as g d. wt $g^{-1}$ P in plants $h^{-1}$.

### Statistical analyses

Data were subjected to two-way analyses of variance (ANOVA) in SAS/STAT software Version 9·1 (SAS Institute Inc., Cary, NC, USA) to examine the impact of species and temperature and their interactions on response variables at the second harvest. No transformations were needed to meet ANOVA assumptions. Comparisons between means were made using Tukey's Honest Significant Difference procedure. Means are presented with standard errors (s.e.) and significance is expressed at $P < 0.05$. When estimating net PUR, RGR and $P_{prod}$, means of each treatment combination were used for each harvest and, therefore, standard errors could not be calculated.

### RESULTS

Irrespective of the temperature treatment, *C. cinereum* had the lowest root, stem and leaf dry weight (i.e. total dry weight) among the three species. Total dry weight of *C. cinereum* increased from low to medium growth temperature, while that of *K. nigricans* and *L. australis* was reduced from low to high temperature, with the greatest reduction being for *L. australis* (Fig. 1A, Table 3). The dry weight of leaves was the greatest and that of stems was the lowest, across all species and temperatures (Fig. 1A). Total dry weight of $KH_2PO_4$-treated plants (high temperature only) was more than twice that of the $FePO_4$-treated plants for *C. cinereum* and *L. australis*, but was only 40 % higher for *K. nigricans*, the species with the highest dry weight (Fig. 1A). The RMR (the ratio between root dry weight and total plant dry weight) of *C. cinereum* and *L. australis* was 0·35 and 0·34, respectively, and it was not affected by the temperature (Fig. 1B, Table 3). However, for *K. nigricans*, the RMR at low temperature was 0·33 and it decreased to 0·25 at medium and high temperatures. The RGR showed a similar response to dry weight, i.e. the RGR of *C. cinereum* increased from low to high
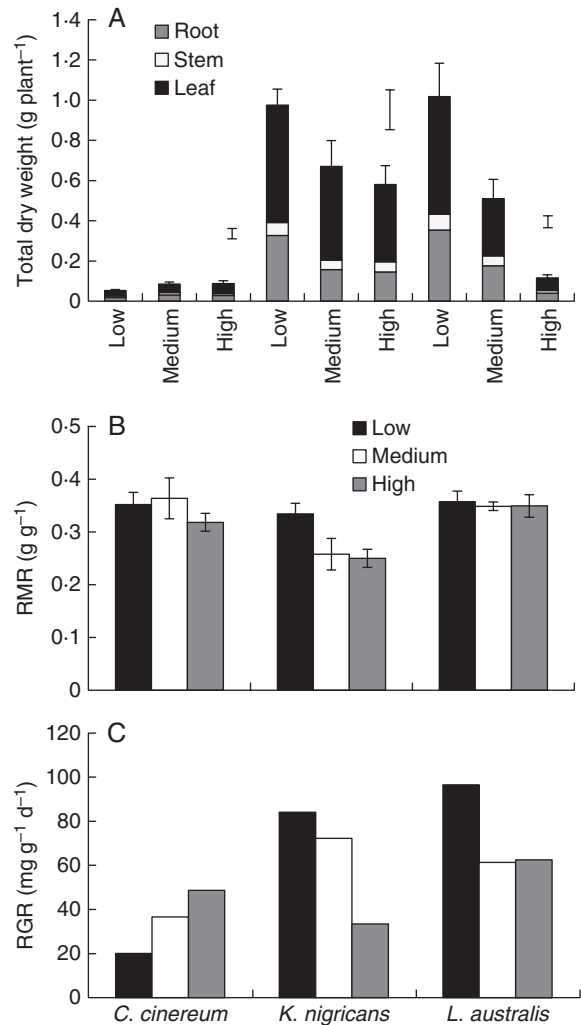


FIG. 1. (A) Total dry weight (partitioned into root, stem and leaf) at the second harvest, (B) root mass ratio (RMR) at the second harvest (i.e. with ten fully expanded main stem leaves) and (C) relative growth rate (RGR) between the first and second harvests of *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis* when grown at low, medium or high temperature with addition of 60 mg $kg^{-1}$ of P as $FePO_4$ (mean ± s.e., $n = 4$). Th error bars above the high temperature treatment in (A) are for the dry weight of plants that received 60 mg $kg^{-1}$ of P as $KH_2PO_4$; in (C), RGR was calculated using the log-transformed treatment means at the first and second harvest and therefore the s.e. could not be calculated.

temperature, while that of *K. nigricans* and *L. australis* decreased (Fig. 1C).

Similar to the dry weight response, root length of *C. cinereum* increased from low to medium temperature, while that of *K. nigricans* and *L. australis* decreased from low to high temperature, with the greatest reduction being for *L. australis* (Fig. 2A, Table 3). *Cullen cinereum* had the lowest root length among the three species, especially at the low and medium temperatures. The root diameter of *C. cinereum* and *L. australis* was reduced from low to high temperature, while that of *K. nigricans* was unaffected (Fig. 2B, Table 3). Comparing species, the root diameter of *C. cinereum* was lower than that of *K. nigricans* and *L. australis*, especially at the medium and high temperatures.

TABLE 3. *Significance of different sources of variability [species (S), temperature (T) and their interaction effects] for the FePO$_4$-treated plants*

| Variable | Source of variability | | | |
|---|---|---|---|---|
| | S | T | S × T | $R^2$ |
| Total dry weight | *** | *** | * | 0·84 |
| Leaf dry weight | *** | ** | NS | 0·78 |
| Stem dry weight | *** | *** | NS | 0·77 |
| Root dry weight | *** | *** | *** | 0·91 |
| Root mass ratio | *** | * | * | 0·60 |
| Root length | *** | *** | ** | 0·85 |
| Root diameter | *** | NS | * | 0·75 |
| Specific root length | ** | NS | * | 0·66 |
| Root length to leaf area ratio | * | NS | ** | 0·59 |
| Plant P concentration | ** | NS | * | 0·78 |
| Total P (plant$^{-1}$) | *** | *** | * | 0·84 |
| Carboxylate content | *** | *** | *** | 0·78 |
| $A$ | *** | * | *** | 0·74 |
| $g_s$ | ** | *** | * | 0·47 |
| WUE | *** | *** | *** | 0·75 |

NS, no significant difference; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
$R^2$ for the full model is given.

Specific root length (SRL, cm g$^{-1}$ root d. wt) of *C. cinereum* increased from low to high temperature, while that of *K. nigricans* and *L. australis* was not responsive (Fig. 2C, Table 3). The root length to leaf area ratio of *C. cinereum* increased from low to high temperature, while that of *K. nigricans* and *L. australis* decreased (Fig. 2D, Table 3).

For *L. australis*, tissue P concentration increased from low to high temperature, while that of *C. cinereum* and *K. nigricans* was unchanged (Fig. 3A, Table 3). When comparing the P concentration of plants treated with KH$_2$PO$_4$ with that of plants treated with FePO$_4$ at high temperature, the P concentration of plants treated with KH$_2$PO$_4$ was 60, 90 and 25 % higher for *C. cinereum*, *K. nigricans* and *L. australis*, respectively (Fig. 3A). Similar to the dry weight and root length response, plant total P for *C. cinereum* increased from low to high temperature, while that of *K. nigricans* and *L. australis* decreased, with the greatest reduction for *L. australis* (Fig. 3B, Table 3). When comparing species, total P taken up was lower for *C. cinereum* than for *K. nigricans* and *L. australis*. For *C. cinereum*, P$_{prod}$ increased from low to high temperature, while that of *K. nigricans* and *L. australis* decreased (Fig. 3C). The change in P$_{prod}$ with temperature was greatest for *L. australis*. The PUR of *C. cinereum* increased and that of *K. nigricans* decreased from low to high temperature (Fig. 3D). However, the PUR of *L. australis* increased only up to the medium temperature.

Across temperature treatments and species, root length was strongly correlated with total P content in a plant ($r = 0.96$, $P < 0.0001$; Fig. 4A) while PUR (mg P cm$^{-2}$ root area d$^{-1}$) was not correlated ($r = 0.23$, $P > 0.05$; Fig. 4B).

Rhizosphere carboxylates (μmol g$^{-1}$ root d. wt) and composition varied depending on species and temperature (Fig. 5, Table 3). *Cullen cinereum* had the lowest amount of carboxylates, irrespective of the temperature treatment. For all the species, rhizosphere carboxylates decreased greatly at
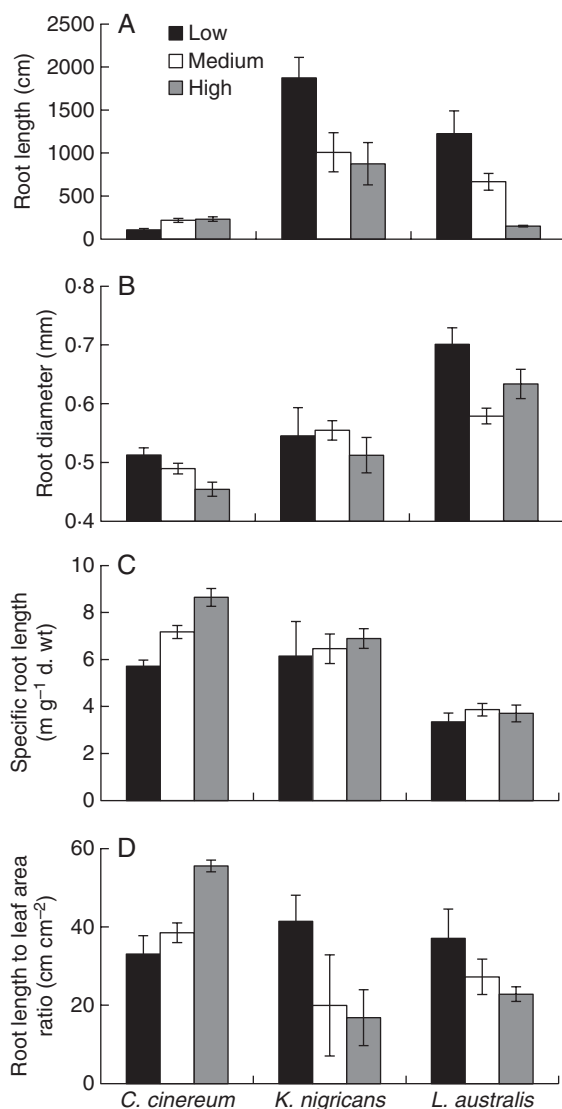


FIG. 2. (A) Root length, (B) root diameter, (C) specific root length and (D) root length to leaf area ratio of *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis* at the second harvest (i.e. with ten fully expanded main stem leaves) when grown at low, medium or high temperature with addition of 60 mg kg$^{-1}$ of P as FePO$_4$ (mean ± s.e., $n = 4$).

high temperature (i.e. approx. 0, 23 and 32 % of the low temperature treatment for *C. cinereum*, *K. nigricans* and *L. australis*, respectively). Citrate was the main constituent in *K. nigricans* and *L. australis*. At the high temperature for *K. nigricans* and *L. australis*, citrate made up >90 % of carboxylates, while at the medium and low temperatures it made up only 67–80 % of total carboxylates. Very low concentrations (i.e. <0·2 μmol g$^{-1}$ root d. wt) of fumaric, maleic, cis-aconitic and trans-aconitic acids were also detected. Similar results were found at the first harvest (data not shown). Carboxylates in the bulk soil were also measured, where very low concentrations of maleic and fumaric acids (i.e. <0·0002 μmol g$^{-1}$ soil d. wt) were present at medium and high temperature treatments, but they were not detected at low temperature treatment.
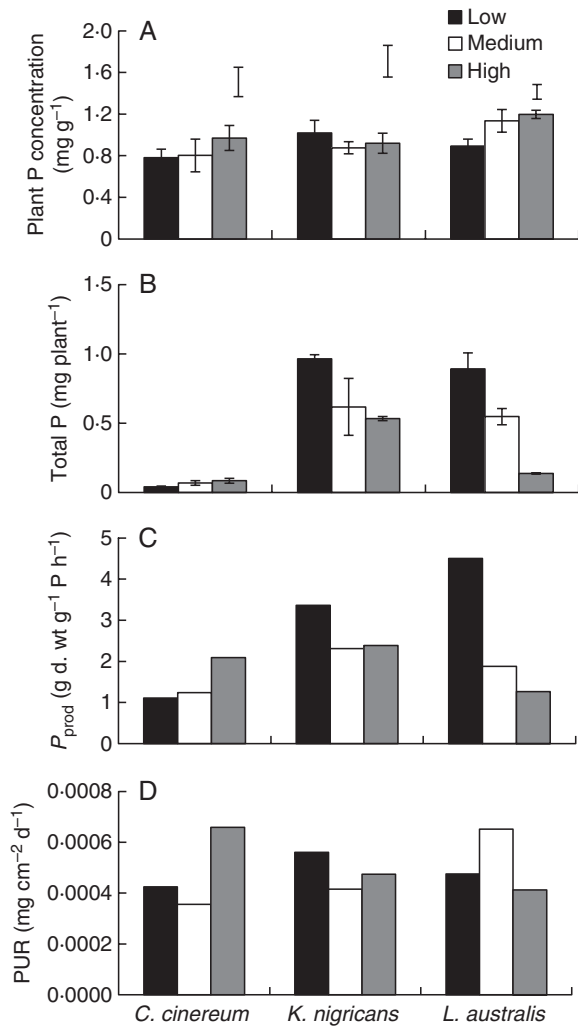
FIG. 3. (A) Plant P concentration at the second harvest, (B) total plant P at the second harvest (i.e. with ten fully expanded main stem leaves), (C) phosphorus productivity (i.e. $P_{prod}$), and (D) phosphorus uptake rate (PUR) of *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis* when grown at low, medium or high temperature with addition of $60 \, mg \, kg^{-1}$ of P as $FePO_4$ (mean $\pm$ s.e., $n = 4$). The error bars above the high temperature treatment in (A) are for P concentration of plants that received $60 \, mg \, kg^{-1}$ of P as $KH_2PO_4$; $P_{prod}$ (C) and PUR (D) were calculated using treatment means at the first and second harvest and therefore the s.e. could not be calculated.

For all species, *A* increased from low to medium temperature, and at high temperature *A* was either similar to or lower than that at medium temperature (Fig. 6A, Table 3). The $g_s$ of *C. cinereum* was reduced with increasing temperature, while for *K. nigricans* and *L. australis* it increased (Fig. 6B, Table 3). The WUE of *C. cinereum* increased, while that of *K. nigricans* and *L. australis* decreased, as temperature increased from low to medium (Fig. 6C).

## DISCUSSION

*Cullen cinereum* plants increased dry weight, root length and RGR with increasing temperature. Therefore, the low temperature treatment (i.e. 20/13 °C) was sub-optimal for growth of *C. cinereum*. In contrast, dry weight, root length and RGR of

*K. nigricans* and *L. australis* decreased from low to high temperature, indicating that the low temperature (i.e. 20/13 °C) was in the optimum range for their growth, and both medium and high temperatures (i.e. 27/20 °C and 32/25 °C) were supraoptimal. These findings are consistent with the mean environmental temperatures of the collection locations of the three species. Therefore, we would expect P nutrition and related processes such as root morphology and physiology of these species to vary among low, medium and high temperature treatments.

### Cullen cinereum, *with a lower RGR, had a higher degree of root morphological adaptation than* K. nigricans *and* L. australis

The first hypothesis that plants would produce less root dry weight and have shorter and thicker roots (i.e. low root surface area per root dry weight) with a lower RMR at sub- or supraoptimal temperatures compared with optimal temperatures was partially supported. As hypothesized, for all species, root dry weight, root length and root length to leaf area ratio were higher at optimum temperatures [i.e. medium to high temperatures (27/20 and 32/25 °C, respectively) for *C. cinereum* and low temperature (20/13 °C) for *K. nigricans* and *L. australis*] than at sub- or supraoptimal temperatures. Similar results have been reported for root growth in deciduous and evergreen temperate tree species (Alvarez-Uria and Körner, 2007). As for other adaptations, root diameter was lower and SRL (cm $g^{-1}$ root d. wt) was higher at optimum temperature only for *C. cinereum*, thus only partially supporting our first hypothesis. These responses can be considered to reflect a more efficient investment of resources for greater soil exploration which would contribute to faster nutrient and water absorption at optimum temperatures. This is particularly important when mycorrhization is very poor (Körner, 1998), as well as when rhizosphere carboxylate content is very low, as we discuss below. *Kennedia nigricans* responded differently to *C. cinereum*, as its RMR was reduced at supraoptimal temperatures, thus supporting the first hypothesis. Therefore, the three species exhibited different root morphological responses to temperature treatments (i.e. *C. cinereum* was the most responsive while *L. australis* was the least responsive).

### $P_{prod}$ *for all species, and carboxylate content for* K. nigricans *and* L. australis *was highest at optimal temperatures*

In addition to morphological root responses, plants may have physiological adaptations to enable greater P uptake at optimum temperatures. Therefore, the second hypothesis that tissue P concentration, P productivity ($P_{prod}$) and rhizosphere carboxylate content would be higher at optimal growth temperatures than at sub- or supraoptimal temperatures was tested. Increased tissue P concentration for *L. australis* in the high temperature treatment (32/25 °C) (i.e. supraoptimal temperature) and unchanged tissue P concentration for *C. cinereum* and *K. nigricans* with temperature treatment are not in agreement with hypothesis 2. In the present experiment, tissue P concentration of $FePO_4$-treated plants ranged from 0·8 to 1·2 mg P $g^{-1}$ d. wt, while tissue P concentration of plants treated with $60 \, mg \, kg^{-1}$ of $KH_2PO_4$ ranged from 1·5 to 1·7 mg P $g^{-1}$ d. wt. Thus the generally low growth and low
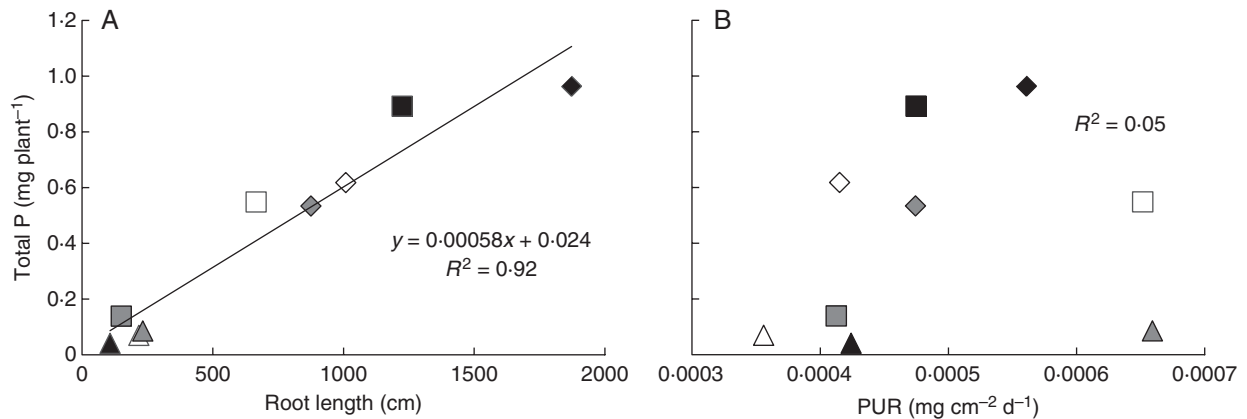
FIG. 4. (A) Relationship between total P content per plant and root length, and (B) total P content per plant and P uptake rate (PUR) for plants grown with addition of 60 mg kg$^{-1}$ of P as FePO$_4$ at the second harvest. Black, white and grey symbols are for low, medium and high temperature treatments, and triangles, diamonds and squares are for *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis*, respectively.
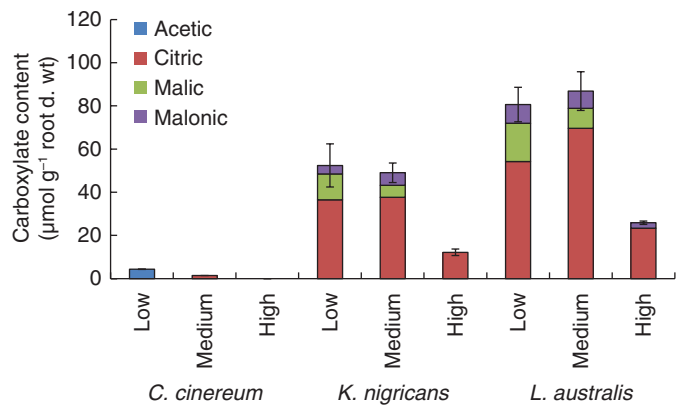


FIG. 5. Amount of rhizosphere carboxylates for *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis* when grown at low, medium or high temperature at the second harvest (i.e. with ten fully expanded main stem leaves) with addition of 60 mg kg$^{-1}$ of P as FePO$_4$ (mean ± s.e., $n = 4$).

tissue P concentration across all the species, even after a supply of 60 mg kg$^{-1}$ Fe(PO$_4$), is likely to be due to the limitation of P supply through FePO$_4$. However, the $P_{prod}$ was higher at optimal temperatures for all the species, supporting the second hypothesis, and a similar response was observed for dry weight and RGR (Fig. 1). Therefore, across species, $P_{prod}$ was highly correlated with dry weight ($r > 0.90$) and RGR ($r > 0.97$), indicating that P was efficiently used for growth at optimal temperatures by all the species, more so than at sub- and supraoptimal temperatures. Ryser *et al.* (1997) also reported a similar correlation between $P_{prod}$ and RGR for several other plant species in a single temperature treatment.

Carboxylate content was higher at the optimal, low temperature, and reduced at the supraoptimal, high temperature, for *K. nigricans* and *L. australis*, supporting hypothesis 2. Increased plant growth and P demand at optimal temperatures might have enhanced the exudation of more carboxylates. On the other hand, increased rhizosphere carboxylate content at optimal temperatures might have favoured a higher PUR and $P_{prod}$ for *L. australis* and *K. nigricans*. This relationship
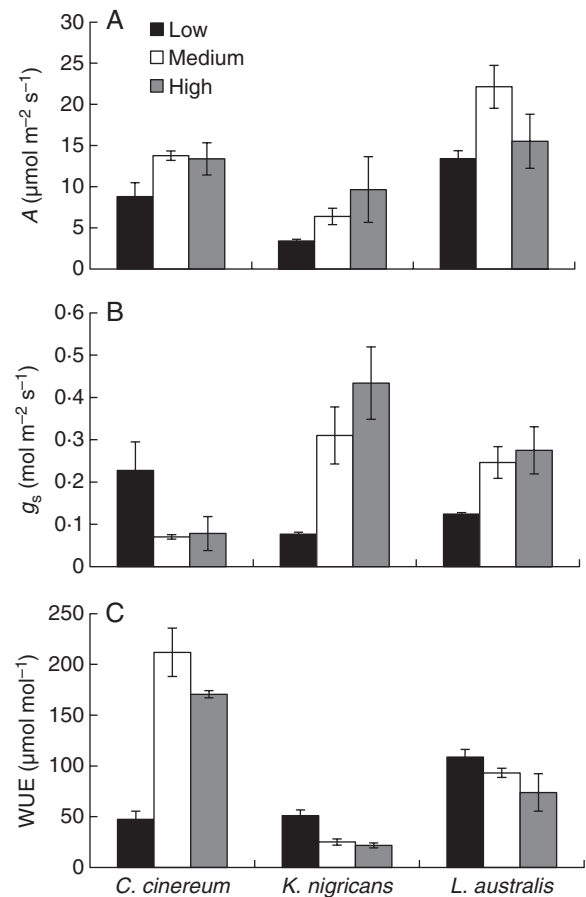


FIG. 6. (A) Photosynthetic rate ($A$), (B) stomatal conductance ($g_s$) and (C) photosynthetic water use efficiency (WUE; $A/g_s$) of *Cullen cinereum*, *Kennedia nigricans* and *Lotus australis* 1 week before the second harvest when grown at low, medium or high temperature with addition of 60 mg kg$^{-1}$ of P as FePO$_4$ (i.e. with ten fully expanded main stem leaves) (mean ± s.e., $n = 4$).

warrants further study. Carboxylate composition also changed at supraoptimal temperatures for *K. nigricans* and *L. australis*, with the contribution of malic acid reduced.

However, results for *C. cinereum* were not in agreement with our hypothesis, although the amount of carboxylates present was very much lower than for *K. nigricans* and *L. australis*.

Therefore, the three species exhibited different responses when acquiring P from the P-limited soil: *C. cinereum* had extensive root morphological adaptations [i.e. long (higher root length per leaf area), thin roots with higher SRL], while physiological adaptation (i.e. high content of carboxylates in the rhizosphere) was prominent in *K. nigricans* and *L. australis*, at their optimum range of temperatures. Lack of root physiological adaptations for *C. cinereum* might be due to its lower RGR and thus lower P demand than that of *K. nigricans* and *L. australis*. Moreover, Clarkson *et al.* (1988) suggested that the degree to which soil temperature might alter nutrient uptake capacity is largely determined by the degree to which growth and RMR are affected (i.e. if changes in soil temperature lead to an increased nutrient-absorbing root surface relative to shoot size, then there will be a downregulation of ion influx, and vice versa). Since root morphological and physiological characteristics are inter-related, they must be studied in conjunction with other compensatory factors, whose responses might counterbalance each other.

The amount of carboxylates in the bulk soil, as expected, was very low compared with that in the rhizosphere, across all temperatures and species. The amount present was higher at the high temperature treatment. However, overall, it seems that the impact of soil biota on increasing soil P availability in bulk soil via carboxylate exudation was negligible under our experimental conditions.

### *Plant P status was highly correlated with root length, rather than with PUR or root diameter*

Hypothesis 3, that the reduction in the total amount of P taken up at sub- or supraoptimal temperature compared with that at optimal temperature was mainly due to reduced uptake rate per unit root surface area, rather than reduced root length (i.e. morphological adaptation), was not supported by our results. In this study, P supply through $FePO_4$ was very low and thus P availability was limited for plants without a capacity to mobilize P. For all species, across temperature treatments, root length and total plant P were highly correlated. However, root diameter showed no correlation with the total amount of P in plant tissues ($r = 0.13$, $P > 0.05$).

Only for *C. cinereum* and *K. nigricans* was PUR (mg P $cm^{-2}$ root area $d^{-1}$) higher at optimal temperatures, but it had a weak correlation with the total amount of P in plant tissues. Therefore, for all species across temperature treatments, plant P status was highly correlated with root length, rather than with PUR or root diameter. This result is in agreement with the results of Mackay and Barber (1984), who found that plant P uptake is mainly determined by root production, rather than by physiological uptake capacity. Our results further validate this across a range of temperatures from optimal to sub- or supraoptimal. Moreover, Ercoli *et al.* (1996) highlighted that temperature and P supply independently weaken growth at sub-optimal temperatures. This is supported by our results as tissue P concentration was not reduced at sub- or supraoptimal temperatures, even though the growth and amount of P in plant tissues were reduced.

Hypothesis 4, that WUE would be lower at sub- or supraoptimal temperatures than that at optimal temperatures and reduced WUE would be partly due to a low photosynthetic rate (*A*), was only partly supported. As hypothesized, *A* (per unit leaf area) was higher at optimal temperature only for *C. cinereum*. A similar response was observed for *Quercus rubra* L. seedlings (Wertin *et al.*, 2011). However, $g_s$ was lower and, therefore, WUE was higher at optimal temperatures for all the species in the present experiment. In a comparison of *Triticum aestivum* L. cultivars at a single temperature, Boogaard *et al.* (1997) found that differences in WUE at the leaf level were related to variation in $g_s$, rather than in the *A*, which supports our findings. Therefore, WUE is a better indicator of a plant's physiological adaptability to environmental temperature than *A* or $g_s$ alone (Comstock and Ehleringer, 1992; Lambers *et al.*, 2008). This was achieved by a reduction in $g_s$ for all the species and, for *C. cinereum* only, an increase in *A*, at optimal temperatures.

In the high temperature treatment, the dry weight of $KH_2PO_4$-treated plants was more than twice that of $FePO_4$-treated plants for *C. cinereum* and *L. australis*, but was only 40 % higher for *K. nigricans*. The high temperature treatment was the optimal temperature for *C. cinereum* and, therefore, a faster growth rate would be expected in the presence of an abundant readily available source of P from $KH_2PO_4$. For *K. nigricans* and *L. australis*, the high temperature treatment was supraoptimal, so for these two species we would expect a smaller growth increase due to $KH_2PO_4$. This was indeed observed for *K. nigricans*, but for *L. australis* the increase in growth was still higher than we would expect. This might be due to a species-specific characteristic where *L. australis* has fewer adaptations to allow it to utilize poorly soluble P from $FePO_4$ and thus showed a greater response to addition of $KH_2PO_4$. Moreover, the differential growth responses can be explained using RGR values of the three species in the high temperature treatment, where *C. cinereum* and *L. australis* showed a higher RGR and *K. nigricans* a lower RGR. Therefore, for species with a higher RGR a higher increase in growth rate in the presence of $KH_2PO_4$ can be expected.

### *Concluding remarks*

The highest temperature in the present experiment (i.e. 32/25 °C) was the optimum for growth of *C. cinereum*, while the low temperature (i.e. 20/13 °C) was the optimum for *K. nigricans* and *L. australis*. At optimum temperatures, when RGR was highest, $P_{prod}$ was also the highest for all the species. RGR and $P_{prod}$ were highly correlated across temperature treatments. All species exhibited multiple adaptive shoot and root responses to achieve a greater dry weight at their growth-limiting P supply and optimum temperatures. For shoots, all the species responded similarly by reducing $g_s$ and increasing WUE at optimum temperatures. However, root responses differed among species. *Cullen cinereum* had several morphological adaptations, including long, thin roots to explore a greater volume of soil, at optimum temperatures. *Kennedia nigricans* and *L. australis* only exhibited long

roots as morphological adaptations, but had a higher content of carboxylates in the rhizosphere as an additional adaptation, which would have enhanced the mobilization of P from FePO$_4$ at optimum temperatures. The amount of P taken up by a plant was mainly determined by root length, rather than by PUR. The present results aid understanding of plant P dynamics and root adaptations when plants are grown in different regions with contrasting temperatures, seasons and under predicted climate change scenarios.

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