



Using activated charcoal to remove substances interfering with the colorimetric assay of inorganic phosphate in plant extracts

Roberta L. C. Dayrell · Gregory R. Cawthray · Hans Lambers · Kosala Ranathunge

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Abstract

Aim Organic substances in leaves of several south-west Australian native species interfere with sensitive colorimetric assays and prevent quantification of inorganic phosphate concentration ([Pi]). We aimed to develop a reproducible routine procedure for treating leaf extracts with activated charcoal (AC) to remove interfering substances, allowing the determination of [Pi] by the malachite green spectrophotometric assay. **Methods** Leaf extracts of native plants from south-west Australia in 1% (v/v) acetic acid were treated with 10 mg mL⁻¹ acid-washed AC for removal of interfering substances. Standard solutions (0 to 18 μ M Pi) with and without AC treatment were compared to quantify Pi loss. A spiking and recovery test was performed to validate the AC treatment.

Results Leaf extracts treated with AC exhibited distinguishable absorbance peaks for the malachite green-orthophosphate complex between 630 and 650 nm, as opposed to untreated samples. The Pi-adsorption by AC represented a relatively larger fraction of [Pi] in solutions at 0–4 μ M Pi range and stabilised at higher [Pi] when maximum adsorption capacity of AC reached at 11.7 μ g Pi g⁻¹AC. The Pi recovery after AC treatment in spiked samples ranged between 100 and 111%.

Conclusion The AC treatment successfully removed interfering substances from samples but caused Pi loss. Thus, quantification of [Pi] in AC-treated extracts requires sample [Pi] \geq 6 μ M Pi and the use of AC-treated standards. The error of the AC treatment was minor compared with environmental variability of leaf [Pi]. The AC treatment was a reproducible time- and cost-effective method to remove interfering substances from leaf extracts.

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R. L. C. Dayrell · G. R. Cawthray · H. Lambers · K. Ranathunge
School of Biological Sciences, University of Western Australia, 35 Stirling Highway, Perth, WA 6009, Australia

R. L. C. Dayrell (✉)
Institute of Plant Sciences, University of Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany
e-mail: dayrell.rlc@gmail.com

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Introduction

Plants in their vegetative stage accumulate phosphorus (P) as inorganic phosphate (Pi) when soil P supply exceeds their requirements for growth (Bollons and Barraclough 1997; White and Hammond 2008). The

variability of Pi concentration ([Pi]) in plant organs reflects differences in P uptake and demand which makes [Pi] a sensitive indicator of plant P status (Bollons and Barraclough 1999; Veneklaas et al. 2012). For this reason, leaf [Pi] is used to understand how P availability influences species distribution and to identify strategies for P-use and -acquisition in plants (Zohlen and Tyler 2004; Yan et al. 2019). Plants that evolved in severely P-impoverished landscapes are able to mobilise sorbed P in soils, then use and recycle P very efficiently in metabolism and growth (Denton et al. 2007; Hayes et al. 2014, 2021), and knowledge about their strategies and requirements can guide us toward the development of more sustainable P-efficient cropping systems (Lambers et al. 2011).

Species that have evolved to function in soils with an extremely low P availability, typically exhibit low leaf [P] (Wright et al. 2004; Lambers et al. 2012; Guilherme Pereira et al. 2018) and consequently, low leaf [Pi]. Hence, the determination of leaf [Pi] in these plants requires sampling and extraction procedures that maximise precision and accuracy: an extraction protocol that causes minimal enzyme-mediated hydrolysis of organic P and conversion into Pi, as well as sensitive quantification methods. These requirements can be achieved by the procedures reported by Yan et al. (2019) which involve immediately freezing samples in liquid nitrogen upon collection followed by storage at -80°C until freeze-drying, Pi extraction with an ice-cold 1% (v/v) acetic acid solution, and Pi quantification with a colorimetric assay (Ames 1966).

Here, we report an issue with the quantification of leaf Pi in samples of some native species inhabiting severely nutrient-impoverished soils of the South-west Australian Floristic Region exhibiting low leaf [P] when following the procedures described by Yan et al. (2019). The presence of organic substances of unknown nature interferes with the colour formation of the molybdate and malachite green method (Motomizu et al. 1983; Figs. 1A, 2) with precipitates also being an issue. This problem cannot be resolved by sample dilution (Fig. S1), or with the use of two other quantification methods that are less sensitive, and thus not ideal to quantify leaf [Pi] of species with low leaf [P]: the molybdenum blue-based method (Ames 1966), used by Yan et al. (2019), and the molybdenum yellow-based method (Heinonen and Lahti 1981).

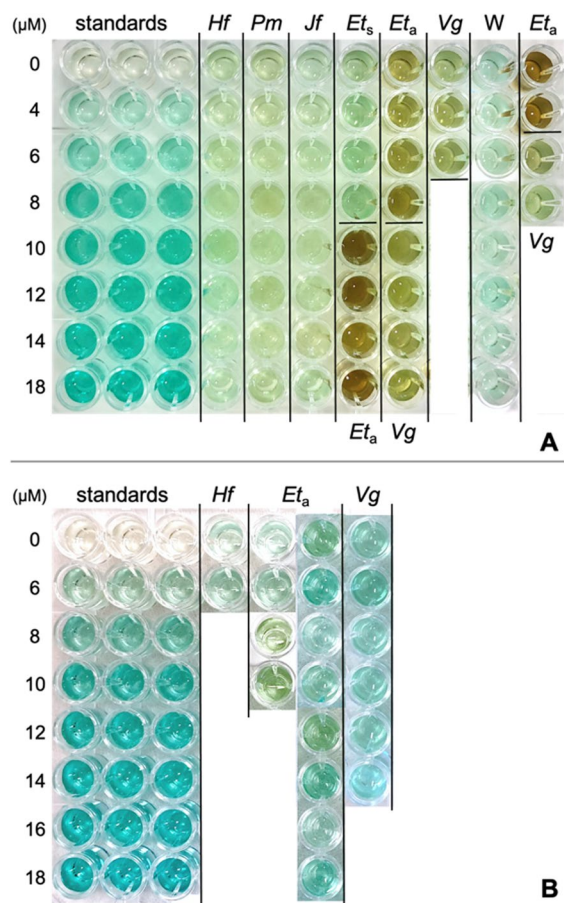
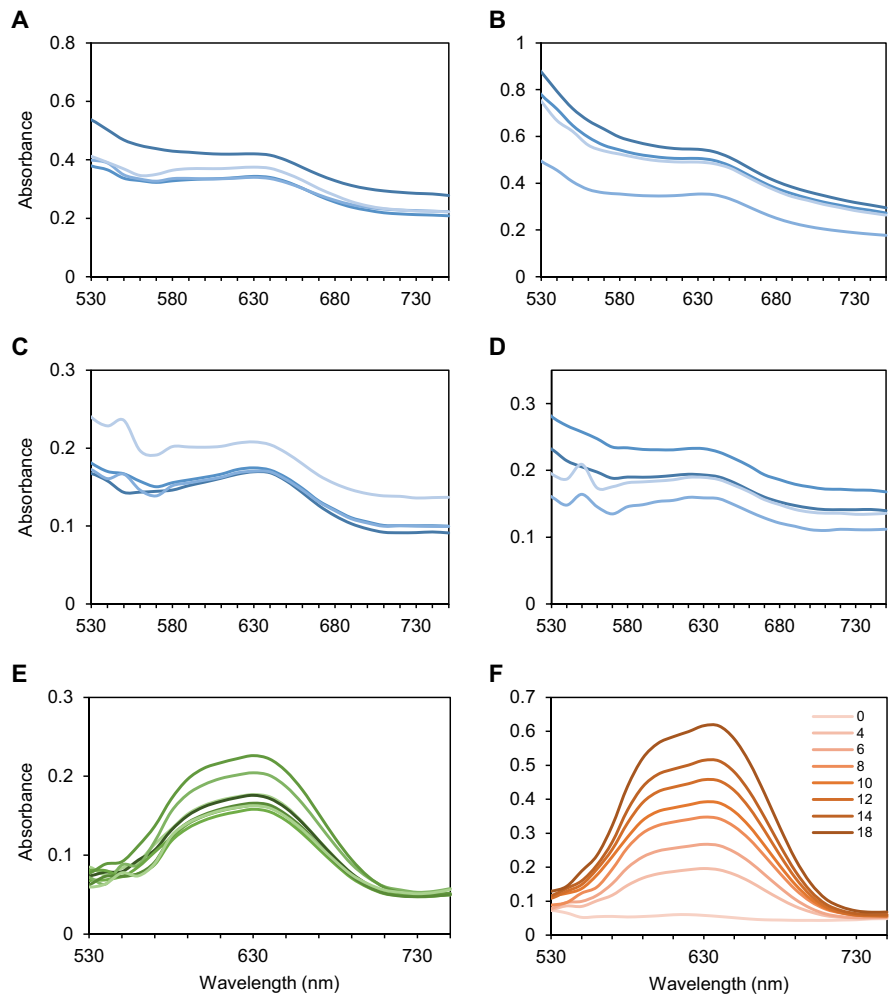


Fig. 1 Photograph of 96-well plates containing 180 μL of either standards or biological samples mixed with 60 μL of malachite green colour reagent. Standards, in triplicate, containing 0–18 μM of inorganic phosphate (Pi) are shown in the first three columns. A) Samples with different levels of colour interference represented by different intensity of a brownish colour before cleaning with activated charcoal (AC). B) Samples without colour interference after treatment with AC. *Hakea flabellifolia* (Hf), *Petrophile macrostachya* (Pm), *Jacksonia floribunda* (Jf), *Eucalyptus todtiana* seedlings (Et_s), *E. todtiana* adult plants (Et_a), *Verticordia grandis* (Vg), wheat (W; *Triticum aestivum* cv. Wyalkatchem)

Activated charcoal (AC) has long been recognised as one of the most versatile adsorbents for removal of organic and inorganic substances from solutions, exhibiting a wide variety of applications (Hassler 1941; Halhouli et al. 1995). Our aim was to develop a routine procedure with high reproducibility for treating 1% (v/v) acetic acid leaf extracts with acid-washed AC to remove interfering substances, allowing the determination of Pi by a sensitive colorimetric

Fig. 2 Absorbance spectra (530–740 nm) of the malachite green-orthophosphate complex. Inorganic phosphate (Pi) for reactions was extracted from the youngest fully-expanded leaves of native plant species from southwest Australia (blue curves) – (A) *Verticordia grandis*, (B) *Eucalyptus tottiana*, (C) *Melaleuca leuropoma*, (D) *Banksia candolleana*; and also, from (E) wheat (*Triticum aestivum* cv. Wyalkatchem; green curves). (F) Standard solutions (orange curves) were prepared with 0–18 μM of potassium dihydrogen phosphate (KH_2PO_4). Individual lines with different shades of the abovementioned colours in individual graphs represent leaf extracts from four (A–D) and eight (E) individual plants. Dilution factors for leaf extracts were 20 (A–D) and 500 (E)



method. We also quantified expected Pi loss during the cleansing process and show how this can be corrected for.

Methods

Leaf sampling and pi extraction

We collected leaf samples in Badgingarra National Park (Cadda Road, 30°23'S, 115°24'E), southwest Australia, c. 180 km north of Perth. The area is located within the Southwest Australian Floristic Region, where soil P availability is particularly low (McArthur et al. 2004; Kooyman et al. 2017). We sampled 8 species that belong to the three most common plant families in the area: Fabaceae (*Daviesia*

chapmanii, *Jacksonia floribunda*), Myrtaceae (*Eucalyptus tottiana*, *Verticordia grandis*, *Melaleuca leuropoma*), Proteaceae (*Banksia candolleana*, *Hakea flabellifolia*, *Petrophile macrostachya*). The samples exhibited different levels of colour interference (Fig. 1), and here we mainly focus on the species in which these interference levels were high; i.e. an absorbance peak of the malachite green-orthophosphate complex could not be clearly distinguished: *V. grandis*, *E. tottiana*, *M. leuropoma* and *B. candolleana* (Fig. 2).

Youngest fully expanded leaves were collected from four mature individuals and petioles were removed, following the same standardised leaf-collecting procedures as those for P measurement (Pérez-Harguindeguy et al. 2013). Leaf blades were wrapped in aluminium foil, and immediately frozen

by immersing in liquid nitrogen. All leaf samples remained immersed in liquid nitrogen while being transported to the laboratory and then stored at $-80\text{ }^{\circ}\text{C}$ until they were freeze-dried for seven days (VirTis Benchtop “K”, New York, USA), then ground to a fine powder in a ball-mill grinder (GenoGrinder 2010, SPEX SamplePrep, Metuchen, NJ, USA) using polypropylene co-polymer vials and yttrium-stabilised zirconium ceramic beads (Inframat Advanced Materials, Connecticut, USA).

Leaf Pi was extracted by disrupting a $22.0 (\pm 2.0)$ mg subsample of dried ground leaf material in 0.5 mL of ice-cold 1% (v/v) acetic acid by mechanical shaking (Precellys 24 Tissue Homogenizer, Bertin Instruments, Montigny-le-Bretonneux, France) at 1500 rpm for 15 s at room temperature, repeated twice more with a 5-min cooling break (samples were kept on ice in the dark) between shakings. The homogenate was centrifuged at $21,000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$ and the supernatant transferred to a clean tube on ice; this process was repeated until the extracts were absolutely clear and free of any debris. Leaf extracts were diluted six times before treatment with AC.

Treatment with activated charcoal (AC)

Overall, the AC treatment has five steps (illustrative flowchart in Supplementary material 2). Acid-washed $18.0 (\pm 0.1)$ mg of AC (washed with hydrochloric acid, Sigma-Aldrich, C9157, Castle Hill NSW, Australia) was transferred to 2 mL Eppendorf tubes and kept on ice. Precision was very important in this step, because it was not straightforward to correct for variation in weight of AC. A diluted cold leaf extract or standard solution of 1.8 mL was transferred to a cold 2 mL Eppendorf tube with AC to achieve a final [AC] of 10 mg mL^{-1} , then mixed by quick vortexing after which samples were immediately placed back on ice for $5\text{--}15\text{ min}$. Samples and standards were again quickly vortexed before filtering with 3 mL syringes (Terumo Philippines Corporation, Laguna, Philippines) and syringe filters (33 mm ; $0.45\text{ }\mu\text{m}$; Sarstedt Australia Pty Ltd., Adelaide, Australia) into clean pre-cooled tubes on ice. The cleansing worked best when using a final [AC] of 10 mg mL^{-1} with sample volumes of $1.5\text{--}2.0\text{ mL}$: smaller volumes led to larger Pi loss due to adsorption onto AC, while cleansing may not eliminate interferences completely with larger volumes (data not shown). The [Pi] of

filtered samples and standards were determined colorimetrically using the molybdate and malachite green method (Motomizu et al. 1983). Absorbance of samples and standards were measured using a Multiskan Spectrum 1500 plate reader (Thermo Scientific, Massachusetts, USA). For both standards and samples, $240\text{ }\mu\text{L}$ ($180\text{ }\mu\text{L}$ sample or standard, and $60\text{ }\mu\text{L}$ colour reagent) was added to a well of a Greiner flat bottom 96-well plate, polystyrene (Greiner Bio-One GmbH, Frickenhausen, Germany). The total volume of a well was $392\text{ }\mu\text{L}$, and absorbance was measured at 630 nm .

Standard curves

We compared standard curves for solutions containing 0 to $18\text{ }\mu\text{M}$ Pi with AC and without AC (control) treatment to assess whether a significant amount of Pi is lost in the cleansing process. Potassium dihydrogen orthophosphate (KH_2PO_4) was oven dried at $60\text{ }^{\circ}\text{C}$ to constant mass and allowed to cool in a desiccator. The dried salt (1.3609 g) was dissolved in 100 mL of 1% (v/v) acetic acid to prepare a 100 mM Pi stock solution. This solution was diluted to prepare a series of solutions ranging from 2 to $18\text{ }\mu\text{M}$ Pi with 1% (v/v) acetic acid to make the standard curves. The use of 1% acetic acid as solvent to prepare all standard solutions, as well as to extract Pi from plant samples, ensured matrix matching of standards with sample extracts, as the concentration of acid in the final assay is important with respect to colour development (Motomizu et al. 1983; Van Veldhoven and Mannaerts 1987), for high-accuracy analysis. The Pi quantification of standard was determined colorimetrically using the molybdate and malachite green method (Motomizu et al. 1983) the same as described above for the leaf samples.

Spiking and recovery

To test Pi recovery after AC treatment, we used samples with a strong presence of interfering substances (*B. candolleana*, *E. todtiana* and *V. grandis*), as well as seedlings of *D. chapmanii*, which showed no signs of interferences in colour formation. A $200\text{ }\mu\text{L}$ spike of $30\text{ }\mu\text{M}$ KH_2PO_4 solution was added to $1960\text{ }\mu\text{L}$ of diluted leaf extract (six-times dilution), thus a calculated standard addition of $0.186\text{ }\mu\text{g}$ Pi representing an increase of $2.8\text{ }\mu\text{M}$ Pi in the final solution.

This level of spike was made to give an expected increase in calculated concentration of 134 (± 5)% for *B. candolleana*, 45 (± 1)% for *E. todtiana*, 30 (± 1)% for *V. grandis* and 24 (± 0.4)% for *D. chapmanii* (mean \pm SE). A calibration curve with AC-treated standards was used to quantify [Pi] in the samples. Recovery for spiked samples was calculated by subtracting added Pi from spiked leaf extracts and dividing the result by samples with no spike; a value of 1 would indicate 100% recovery. Total Pi quantification of these samples was the same as described above.

Statistical models

To assess the linear interval of calibration curves with AC-treated standards, we modelled the AC standard curve (0 to 18 μ M) with a four-parameter log-logistic (LL.4) regression. LL.4 was then compared with linear models using stepwise exclusion of the lowest [Pi] from the calculation until there was an overlap between LL.4 and the linear model (<https://github.com/rdayrell/AC.treatment.git>). We used the 'drm' function with the self-starting 'LL.4' function within the 'drc' package (Ritz et al. 2015) to model the relationship between absorbance and final [Pi] in standard solutions after AC treatment in the interval from 0 to 18 μ M. Control calibration curves and calibration curves of AC-treated standards after stepwise exclusion of lowest [Pi] were calculated with a linear regression model (lm) function. We used non-linear least squares (nls) function with the self-starting asymptotic regression (SSasypm) function within the R 'stats' package to model the effect of AC treatment on final [Pi] of standard solutions. Nonlinear models were validated by visual inspection of diagnostic graphs (Ritz and Streibig 2009). Statistical models were calculated with the R software platform (R Development Core Team 2021).

Results

Treatment with AC successfully removed the colour of interfering substances from plant extracts (Fig. 1B), thereby allowing clear visualisation of an absorbance peak of the malachite green-orthophosphate complex between 630 and 650 nm (Fig. 3). As expected, the standard solutions treated with AC provided a different calibration curve than that of control

standards (Fig. 4A). The Pi loss after AC treatment was considerable at the lower range of 0–4 μ M Pi, but stabilised and reached a plateau at higher concentrations (Fig. S2). The maximum adsorption capacity of the AC used in this study was 11.7 μ g Pi g⁻¹ AC (Table S1). The LL.4 model overlapped with the linear model calculated using the interval of 4–18 μ M Pi (Figs. 4B, S3). Inorganic P recovery after AC treatment ranged between 100 and 111%, representing a difference between 0 to 7 μ g Pi g⁻¹ dry weight (DW) between expected and observed values in spiked samples (Table 1).

Discussion

The Pi concentration in plant organs is a sensitive indicator of plant P status (Bollons and Barraclough 1999; Veneklaas et al. 2012) and can be used to understand species distribution and indicate different strategies of P-use and -acquisition in co-occurring plants (Zohlen and Tyler 2004; Yan et al. 2019). However, leaf [Pi] of some species inhabiting severely P-impooverished soils cannot be quantified by the well-established molybdate and malachite green colorimetric method (Motomizu et al. 1983). This is because of the presence of unknown organic substances in the 1% (v/v) acetic acid leaf extracts which interfere with the colour formation, and an absorbance peak of the malachite green-orthophosphate complex cannot be clearly distinguished. Here, we describe a reproducible procedure for treating leaf extracts with AC to remove interfering substances and enable Pi determination by colorimetric assays.

Similar issues with interfering substances have also been observed in leaf [Pi] assays of eastern Australian woody species of Myrtaceae, Proteaceae, Fabaceae and Meliaceae (Y. Tsujii, personal communication) using the 1% (v/v) acetic acid extraction with the molybdenum blue-based method (Ames 1966). This colour interference has also been detected in another Pi-extraction method, i.e. trichloroacetic acid (TCA) extraction with the molybdenum blue colorimetric assay (Kedrowski 1983), in leaves from Bornean rain forest trees belonging to Fagaceae and Sapotaceae (Y. Tsujii, personal communication). This implies that the AC treatment can be valuable for studies not only in Western Australia but also in other regions as well as for studies using other Pi determination methods.

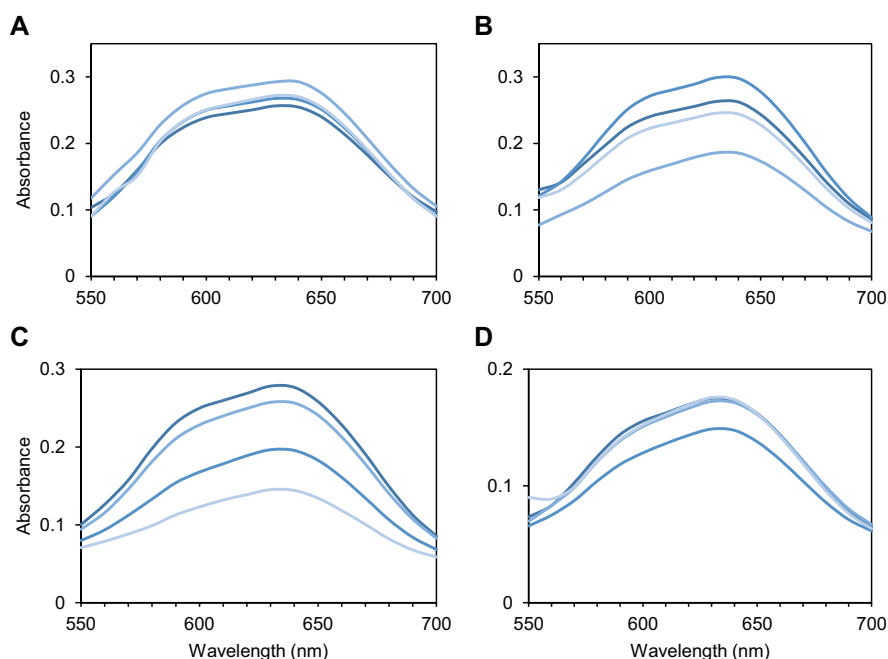


Fig. 3 Absorbance spectra for the malachite green-orthophosphate complex of samples treated with activated charcoal. Inorganic phosphate (Pi) for reactions was extracted from the youngest fully-expanded leaves of A-D native plant species from southwest Australia – (A) *Verticordia grandis*, (B) *Eucalyptus tottiana*, (C) *Melaleuca leuropoma* and (D) *Banksia candolleana*. Dilution factors for leaf extracts were 6–10 times

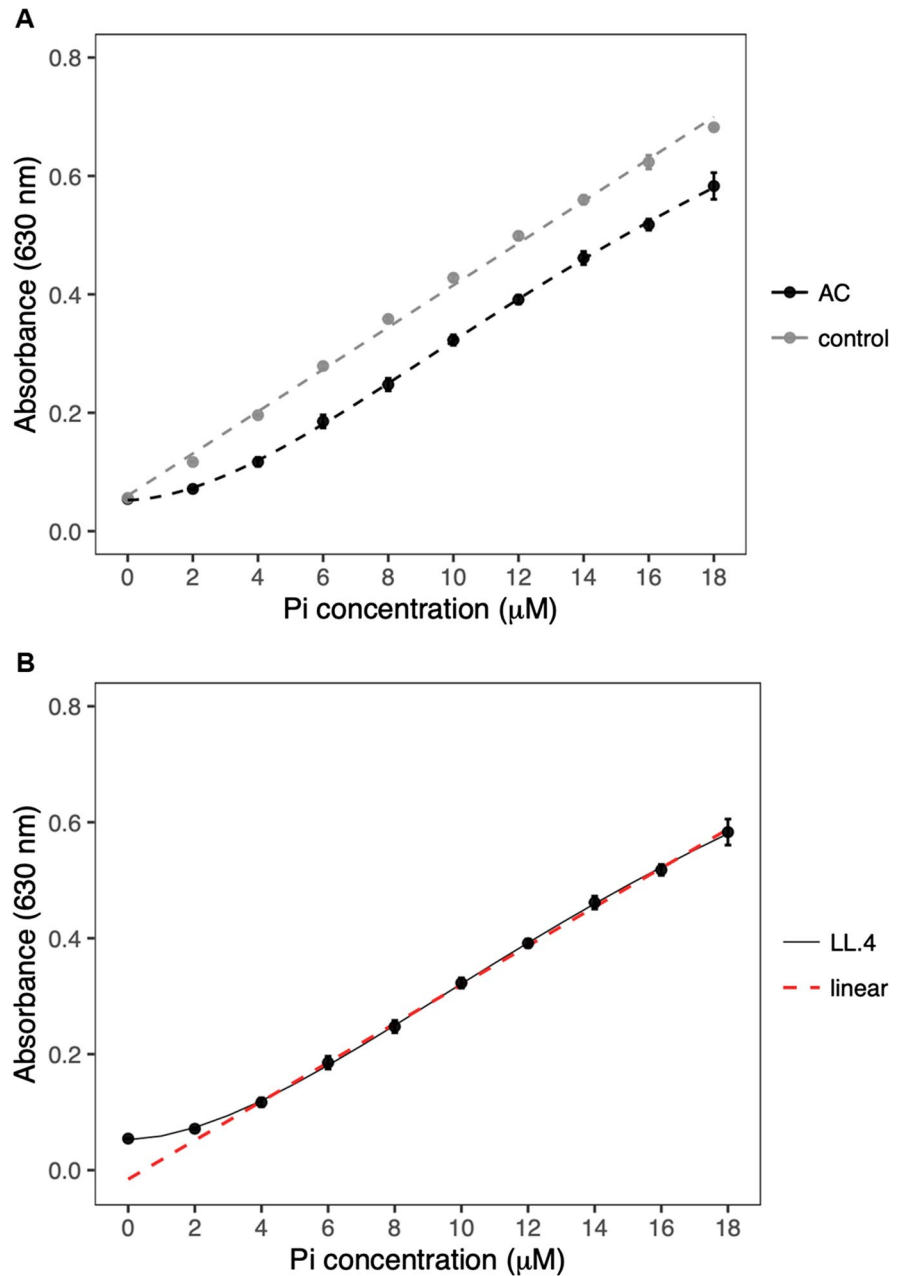
(A), 8 times (B) and 4 times (C and D). Individual lines with different shades of blue in individual graphs represent leaf extracts from four individual plants for each species. Note that the leaf extract dilutions were less than those in Fig. 2. Activated charcoal concentration used for sample cleansing was 10 mg mL^{-1}

After AC treatment, leaf extracts exhibited clear distinguishable absorbance peaks for the malachite green-orthophosphate complex between 630 and 650 nm. This demonstrates AC treatment removes substances interfering with the colour formation from 1% (v/v) acetic acid leaf extracts and allows accurate quantification of sample Pi. The AC treatment resulted in consistent Pi loss across replicates with the same initial [Pi] which means the AC treatment had good reproducibility. However, the comparison of standard curves with and without AC treatment showed that the loss of Pi was not negligible and therefore, [Pi] of AC-treated samples must be calculated using AC-treated standards to account for the Pi loss. The magnitude of the Pi loss varied according to the [Pi] in the AC-treated solution: it represented a relatively larger fraction ($\geq 60\%$) of [Pi] in the solutions at lower concentrations of 0–4 μM Pi and stabilised at higher concentrations (6–18 μM Pi) when the maximum adsorption capacity of AC was reached at $11.7 \mu\text{g Pi g}^{-1}$ AC. Thus,

to increase accuracy, it is recommended that AC treatment should be applied to solutions in which the proportion of Pi adsorbed onto the AC is less than [Pi] that remains in solution, ($[\text{Pi}] \geq 6 \mu\text{M}$).

Another consequence of the Pi adsorption by AC is that fitting a linear regression departing from $[\text{Pi}] = 0 \mu\text{M}$ to calculate the calibration curve (Motomizu et al. 1983) resulted in a poor fit, especially for the points with the lowest Pi concentrations. To address this issue, we recommend checking the linearity of the calibration curve with AC-treated standards through the stepwise exclusion of low [Pi] from the linear regression as described in the methods (R scripts also provided). The LL.4 model overlapped with the linear model calculated using the interval of 4–18 μM Pi, showing that the relationship between absorbance and [Pi] can be described by a linear model in this interval. We tested 18 μM as the upper limit of linearity with the AC treatment using a plate reader in which the light path length was 6.1 mm. This limit would be lower for

Fig. 4 Standard curves for solutions containing 0 to 18 μM inorganic phosphate (Pi) with activated charcoal (AC) and without AC (control) measured at 630 nm. Symbols and error bars show the mean and standard deviation of five replicates. A) Standard curves for solutions with AC treatment and without treatment (control). Differences between curves is due to the Pi loss caused by AC treatment (Fig. S2). B) Comparison between the log-logistic regression with 4 parameters (LL.4) and linear regression (linear) excluding points with the lowest Pi concentrations (0 and 2 μM). The linear regression line shows that there is a linear relationship between absorbance and Pi concentration in the assay in the interval between 4 and 18 μM Pi. Control: $y = 0.0355x + 0.0601$; $R^2 = 0.9973$. AC (4–18 μM Pi): $y = 0.0335x - 0.0156$; $R^2 = 0.9992$. Standard solutions were prepared with oven-dried potassium dihydrogen phosphate (KH_2PO_4)



a spectrophotometer with different specification that resulted in longer light path or if more solution was added to a 96-well when measuring with a plate reader (we used 240 μL per well, whereas the total volume of a well was 392 μL).

The AC concentration of 10 mg mL^{-1} was effective in removing interfering substances while causing a relatively high Pi loss only in solutions with low initial [Pi]; it was therefore considered appropriate for

the treatment. We used acid-washed AC to avoid P contamination, and this pre-treatment may also have affected the Pi adsorption capacity of the AC. This means that AC from different suppliers may differ in the acidity strength, which may lead to different standard curves from those presented here. It is also important to point out that changes in volume interfere with the outcome of the AC treatment even when all ratios are maintained, possibly due to the filtering

Table 1 Recovery of inorganic phosphate (Pi) in leaf extracts with activated charcoal (AC) treatment. The Pi concentration is shown as $\mu\text{g Pi g}^{-1}$ dry weight. Samples were assayed by adding 1960 μL of diluted leaf extract and 200 μL of a 30 μM spike potassium dihydrogen phosphate (KH_2PO_4) solution with a calculated standard addition of 0.186 μg (equivalent to 8.45 $\mu\text{g Pi g}^{-1}$ dry weight) representing an increase of 2.8 μM Pi in the final solution. Values for spiked samples were obtained by subtracting added Pi from spiked leaf extracts.

Species	Pi concentration ($\mu\text{g g}^{-1}$)	Difference in calculated Pi concentration ($\mu\text{g g}^{-1}$)	Recovery (%)
<i>Banksia candolleana</i>	17	2	111
<i>Eucalyptus todtiana</i> (1)	54	0	100
<i>E. todtiana</i> (2)	53	4	107
<i>Verticordia grandis</i>	79	7	109
<i>Daviesia chapmanii</i> (seedlings)	315	1	101

(1), (2) indicate two different individual plants

process. We observed the optimum sample volume to be 1.5–2.0 mL with the materials used. Smaller volumes led to a larger Pi loss resulting in standard curves with lower R^2 values (<0.99), and with greater effect at lower [Pi]. The AC treatment failed to eliminate all the interference in some of the samples with volumes >2 mL. This variation may be caused by using different volumes and materials and reinforces the need to use calibration curves with AC-treated standards and also to check if the 4–18 μM Pi interval remains an adequate range in which sample [Pi] can be determined.

Leaf [Pi] usually ranges from 50 $\mu\text{g Pi g}^{-1}$ DW in P-impooverished habitats (Yan et al. 2019) up to 10 mg Pi g^{-1} DW (Veneklaas et al. 2012). We tested Pi recovery after AC treatment, and differences in [Pi] caused by the AC treatment were within the range of 0 to 7 $\mu\text{g Pi g}^{-1}$ DW for the tested samples (Table 1) and, thus, could represent an error of up to 14%. The spike and recovery test showed that the recovery of Pi after AC treatment was generally within a range of 100 to 109% between expected and observed values in spiked samples for the sampled species. The difference was similar in absolute value (2 $\mu\text{g Pi g}^{-1}$ DW) but larger in percentual value (111%) for *B. candolleana* due to its remarkably low leaf [Pi] (17 $\mu\text{g Pi g}^{-1}$ DW). Technical replicates can be used for samples with extremely low leaf [Pi] to increase precision and accuracy if needed. Nevertheless, the variability of leaf [Pi]

‘Difference in calculated Pi concentration’ was calculated by subtracting values of samples without spike from spiked Pi. Recoveries (%) for spiked samples were calculated by dividing spiked Pi value by samples without spike. Dilution factors for leaf extracts were six times for all species prior to AC treatment. Leaf extracts of *Daviesia chapmanii* seedlings were further diluted three more times after AC treatment because a six-times dilution resulted in absorbances that surpassed the upper limit of linearity in the malachite green assay for these samples

caused by environmental factors is much greater than this error of the AC treatment. For instance, leaf [Pi] in *Hakea prostrata* and *Melaleuca systena* vary from 150 and 250 $\mu\text{g Pi g}^{-1}$ DW, respectively, in less P-impooverished soils to 50 $\mu\text{g Pi g}^{-1}$ DW in extremely P-impooverished soils (Yan et al. 2019). Therefore, the magnitude of error in the AC treatment was within an acceptable range, allowing for leaf [Pi] to continue reflecting differences in P uptake and indicate plant P status (Bollons and Barraclough 1999; Veneklaas et al. 2012).

Conclusion

Treatment with AC removed colour from interfering substances from plant extracts, thereby enabling the determination of leaf [Pi] by the sensitive malachite green colorimetric assay. The treatment led to some Pi loss, which was corrected for by the calibration curves with AC-treated standards and using appropriate sample dilutions in which Pi-adsorption by AC did not represent a large fraction of the [Pi] in solution. The AC treatment requires only basic laboratory equipment (analytical balance and vortex) and low-cost materials; one kilogram of AC would treat more than 55,000 samples. The proposed method is also time-effective as it represents only one additional step to the existing method in which (1) Pi extraction, (2)

AC treatment, and (3) sample reading can be carried out on the same day. Therefore, the AC treatment is a relatively fast, cost-effective, precise and good reproducible method to effectively remove substances from leaf extracts and enabling the determination of Pi by sensitive colorimetric methods.

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Availability of data and material <https://github.com/rdayrell/AC.treatment.git>

Code availability <https://github.com/rdayrell/AC.treatment.git>

Author's contributions RLCD conducted laboratory work. All authors contributed to data analysis and interpretation. RLCD led the writing of the manuscript. GRC, HL and KR provided critical feedback to drafting of the manuscript.

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Declarations

Conflicts of interest/competing interests Authors declare to have no conflict of interest.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals Not applicable.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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